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Printed by: Ridderprint BV | www.ridderprint.nl

The research described in this dissertation was performed at the Laboratory of Experimental Medicine and Pediatrics, Division of Gastroenterology and Hepatology at the University of Antwerp. This work was supported financially by the University Research Fund Doctoral Projects (BOF-DOCPRO) No. DOCPRO4 2014/ID 2964, the Research Foundation Flanders (FWO) No. G034113N and the Research Foundation Flanders-Strategic Basic Research (FWO-SBO) No. S001017N.

Faculty of Medicine & Health Sciences

THE ROLE OF PROTEASES IN THE PATHOGENESIS OF VISCERAL HYPERSENSITIVITY IN INFLAMMATORY BOWEL DISEASE AND IRRITABLE BOWEL SYNDROME.

DE ROL VAN PROTEASEN IN DE PATHOGENESE VAN VISCERALE HYPERSENSITIVITEIT BIJ INFLAMMATOIRE DARMZIEKTEN EN HET PRIKKELBAREDARMSYNDROOM

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Antwerpen, 2018

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“LET THE BEAUTY OF WHAT YOU LOVE BE WHAT YOU DO”

· RUMI

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ABBREVIATIONS AND UNITS OF MEASURE

ABBREVIATIONS

A	**5-ASA**	5-aminosalicylic acid
	5-HT_3	serotonin
	AA	acetic acid
	ACS	acute coronary syndrome
	AEC	aminoethyl carbazole
	AF	atrial fibrillation
	AUC	area under the curve
	AWR	abdominal withdrawal reflex
B	**BBIC**	Bowman Birk inhibitor concentrate
	BHR	bronchial hyperresponsiveness
C	**C1r**	Complement 1r
	C1s	Complement 1s
	CARD15	caspase activation recruitment domain 15
	CatG	cathepsin G
	CD	Crohn's disease
	cDNA	copy DNA
	ceA	corticosterone pellets in the central nucleus of the amygdala
	c-IAP2	inhibitor of apoptosis protein 2
	CRD	colorectal distension
	CRF-1	corticotropin releasing factor-1
	CTx	chemotherapy
D	**DRG**	dorsal root ganglia
	DSS	dextran sodium sulfate
E	**EAR**	early asthmatic response
	EC	ethical committee
	ECC	enterochromaffin cells
	EDTA	ethylenediaminetetraacetic acid

	EMG	electromyographic
F	**FBD**	functional bowel disorder
	FDA	food and drug administration
	fMRI	functional magnetic resonance imaging
	FODMAP	fermentable oligo-, di- and monosaccharides and polyols
G	**GEE**	generalized estimating equations
	GI	gastrointestinal
	GP	glycoprotein
	GPCR	G-protein coupled receptor
H	**H&E**	hematoxylin & eosin
	H1	histamine 1 receptor
	H4	histamine 4 receptor
	HNE	4-hydroxynonenal
	HPA	hypothalamic-pituitary-adrenal
I	**IACUC**	institutional animal care and use committee
	IBD	inflammatory bowel disease
	IBS	irritable bowel syndrome
	IBS-C	irritable bowel syndrome - constipation
	IBS-D	irritable bowel syndrome - diarrhea
	IBS-M	irritable bowel syndrome - mixed
	IBS-U	irritable bowel syndrome - undefined
	IC_{50}	half maximally inhibitory concentration
	ICH	intracranial hemorrhage
	IFN-γ	interferon-γ
	IL	interleukin
	i.p.	Intraperitoneal
J	**JAK**	Janus activated kinase
K	**KLK**	kallikrein
	KO	knock-out
L	**LAR**	late asthmatic response

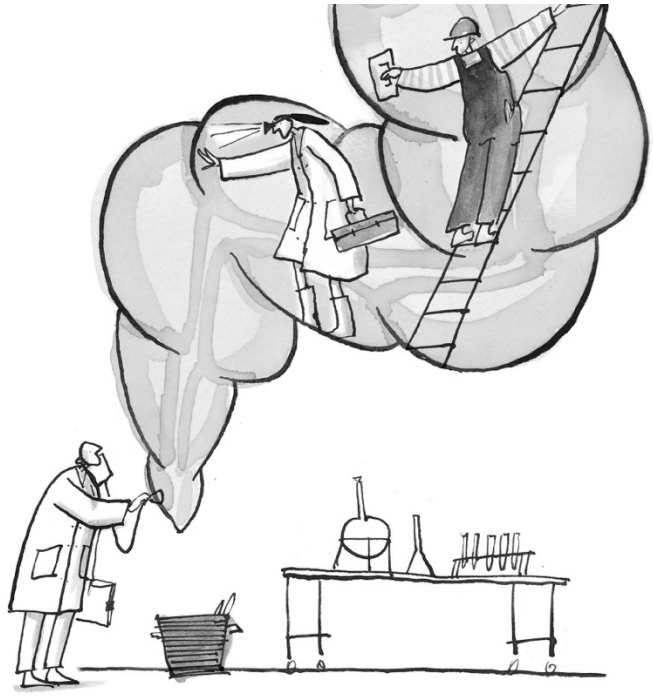
	LMMP	longitudinal muscle myenteric plexus
	LSD	least significant difference
M	**MC**	mast cell
	MMP	matrix metalloprotease
	MPO	myeloperoxidase
	mRNA	messenger ribonucleic acid
	MS	maternal separation
N	**NaCl**	sodium chloride
P	**PA**	protease activity
	PAF	platelet activating factor
	PAR	protease-activated receptor
	PGE2	prostaglandin E2
	PI	protease inhibitor
	PI-IBS	post-infectious IBS
	POC	postoperative complications
Q	**qPCR**	quantitative polymerase chain reaction
R	**RCT**	randomized controlled trial
	RORγT	RAR-related orphan receptor γT
	RTx	radiotherapy
S	**SBTI**	soybean trypsin inhibitor
	SEM	standard error of the mean
	SIRS	systemic inflammatory respiratory syndrome
	SLPI	secretory leukocyte protease inhibitor
	S-N-K	student-newman-keuls
	Sx	surgery
T	**TCA**	trichloroacetic acid
	TIMP	tissue inhibitor of metalloproteases
	TNBS	2,4,6-trinitrobenzene sulphonic acid
	TNF-α	tumor necrosis factor-α
	tPA	tissue plasminogen activator

	TRPA	transient receptor potential ankyrin
	TRPM	transient receptor potential melastatin
	TRPV	transient receptor potential vanilloid
U	**UC**	ulcerative colitis
	uPA	urokinase plasminogen activator
V	**VAS**	visual analog scale
	VHS	visceral hypersensitivity
	VMR	visceromotor response
	VTE	venous thromboembolism

UNITS OF MEASURE

Δ	delta
%	percent
^{o}C	Celsius
^{o}F	Fahrenheit
µl	microliter
µm	micrometer
µmol	micromole
cm	centimeter
g	gram
g	gravity
G	gauge
h	hour
kg	kilogram
M	molar
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mmHg	millimeter of mercury
nm	nanometer
P	pressure
pH	potential of hydrogen
s	second
V	volume

CHAPTER 1 - INTRODUCTION



Parts are adapted from:
Ceuleers H, Van Spaendonk H, Hanning N, Heirbaut J, Lambeir A-M, Joossens J, Augustyns K, De Man JG, De Meester I, De Winter BY. Visceral hypersensitivity in inflammatory bowel disease and irritable bowel syndrome: The role of proteases. World J Gastroenterol 2016 December 21; 22(47): 10275-10286.

Proteases are enzymes catalyzing the hydrolysis of peptide bonds. They are present at high levels in the gastrointestinal tract and they execute a large variety of physiological and pathophysiological functions. Proteases can be classified into several classes. Serine, cysteine, aspartic, metallo and threonine proteases represent the mammalian protease families. In particular the class of serine proteases, which represent over one third of all known proteolytic enzymes, will play a significant role in this thesis. In the last decades, proteases have been suggested to play a key role in the pathogenesis of visceral hypersensitivity, which is a major factor contributing to abdominal pain in patients with inflammatory bowel disease (IBD) and/or irritable bowel syndrome (IBS). In the first paragraphs of this chapter, the gastrointestinal disorders IBD and IBS will be described in more detail (1.1 and 1.2 respectively). In paragraph 1.3 the concept of visceral pain, a major symptom in both IBD and IBS patients, will be clearly explained. In the next paragraph, proteases will be defined and their classification, sources and mechanism of action will be discussed (1.4). Subsequently, the role of proteases will be addressed during IBD and IBS (1.5 and 1.6 respectively). Finally, an overview is provided of the available studies linking proteases to visceral hypersensitivity, focusing on protease-activated receptors (1.7) and protease inhibitors (1.8) as possible therapeutic treatment strategies. In a final paragraph, an overall conclusion of this chapter will be provided (1.9).

1.1 Inflammatory bowel disease (IBD)

Inflammatory bowel disease are chronic gastrointestinal diseases characterized by acute flares of inflammation with intermittent periods of remission. The best described conditions are Crohn's disease (CD) and ulcerative colitis (UC) (Abraham *et al.* 2009). CD is characterized by a transmural inflammation and capable of affecting any region of the intestine, often discontinuously. Moreover, CD can be associated with intestinal granulomas, strictures and fistula. On the other hand, in UC the inflammation is typically restricted to the mucosa and involves the rectum and may affect parts of the colon as well (Abraham *et al.* 2009).

UC patients typically present with bloody diarrhea, passage of mucus or pus and abdominal cramping during bowel movements. For CD, typical features include the discontinuous involvement of various portions of the gastrointestinal tract and the development of complications including strictures, abscesses, or fistulas. IBD patients can also suffer from extra-intestinal manifestations affecting the eyes, the skin, the musculoskeletal and the hepatobiliary system (Baumgart *et al.* 2007).

The incidence and prevalence of IBD is traditionally following a north-west/south-east gradient with the highest rates in the more industrialized countries of Europe (Scandinavia and the United Kingdom) and Northern America while the lowest rates are observed in southern and eastern Europe, though, the manifestation of IBD is also increasing in countries with previously lower incidence rates such as Asia and Eastern Europe. In Europe, approximately 1.1 million are diagnosed with CD and 1.5 million people suffer from UC, for a combined total of 2.6 million IBD patients or 0.3% of the total European population (Burisch *et al.* 2013). Moreover, with its increasing prevalence over time and in different regions around the world, IBD must be seen as an emerging global disease (Molodecky *et al.* 2012).

Although the etiology is not fully understood yet, it is postulated that IBD results from an inappropriate inflammatory response to intestinal microbial flora in genetically susceptible persons (Abraham *et al.* 2009). The importance of genetics in the pathogenesis of IBD has been highlighted by the multiple genome-wide association studies that have been executed in recent years. Initial screening efforts identified NOD2 (also known as CARD15 or IBD1) as a predisposing mutation for CD. Afterwards, additional studies have been successful in identifying more genes (such as IBD5, IL23R, ATG16L1) contributing to IBD susceptibility (Xavier *et al.* 2007). Another risk factor includes an altered gut microbiome composition in IBD patients compared to controls. Evidence of this assumption can be put forward by an amelioration of IBD symptoms after a treatment with antibiotics (Sutherland *et al.* 1991) or probiotics (healthy bacteria) (Gionchetti *et al.* 2003). Moreover, adherent-invasive bacterial strains of *Escherichia coli* have been found to be present at a higher percentage in IBD patients

compared to healthy controls, thereby possibly leading to an epithelial invasion in this first group (Darfeuille-Michaud *et al.* 1998). Finally, the induction of spontaneous chronic colitis in IL-10 deficient mice seems to be bacteria-driven, since germ-free lines were not susceptible to the induction of colitis (Sellon *et al.* 1998). A disrupted epithelial barrier leading to an increased permeability encompasses another important risk factor for IBD. This hypothesis is supported by the detection of an altered permeability in first degree relatives of CD patients (Peeters *et al.* 1997) as well as a downregulation of some tight junctions in human IBD biopsies (Gassler *et al.* 2001). Finally, some environmental factors such as a western lifestyle and smoking (risk factor for CD, protective for UC) are associated with IBD (Xavier *et al.* 2007).

Regarding the treatment of IBD, the classical current clinical practice follows the 'step-up' approach, starting with a less potent therapy with fewer side effects and the subsequent addition of more powerful medication with often more side effects when results are insufficient. The first step of the 'step-up' approach includes the anti-inflammatory drugs e.g. 5-aminosalicylates (5-ASA) and corticosteroid. The second step encompasses the immunosuppressive drugs such as the thiopurines, methotrexate and the calcineurin inhibitors. A third step includes the biologicals such as the anti-TNF-α agents and anti-α4β7-integrin antibodies. Surgery is a fourth and last option. In recent years, researchers have come up with a reversal of the treatment paradigm from a 'step-up' to a 'top-down' approach for CD, implicating the use of highly effective but potentially more toxic therapies early in the onset of CD to prevent disease progression and achieve remission (Leitner *et al.* 2016).

An overview of the currently available treatment options for IBD is shown in table 1.1. For a complete outline of the current and future therapeutic approaches for the management of IBD, we would like to refer the reader to a recent review by Neurath (Neurath 2017).

Table 1.1. Overview of the currently available treatment options for IBD patients.

Therapy	Example	Indications
Anti-inflammatory drugs	5-aminosalicylates (5-ASA)	UC – induction and maintenance of remission
	Corticosteroids	UC, CD – induction of remission
Immunosuppressive drugs	Thiopurines (azathioprine, 6-mercaptopurine)	UC, CD – maintenance of remission
	Methotrexate	CD – induction and maintenance of remission
	Calcineurin inhibitors (ciclosporin-A, tacrolimus)	UC – induction of remission
Anti-TNF-α agents	Infliximab, adalimumab, golimumab, certolizumab pegol	UC, CD – induction and maintenance of remission
Anti-α4β7-integrin antibody	Vedolizumab	UC, CD – induction and maintenance of remission

Adapted from (Neurath 2017)

1.2 Irritable bowel syndrome (IBS)

Irritable bowel syndrome (IBS) is a functional bowel disorder (FBD) defined by the presence of abdominal pain and altered bowel habits (i.e. constipation, diarrhea, or a mix of constipation and diarrhea) in the absence of a specific organic pathology (Mearin *et al.* 2016). The diagnosis of IBS is based on the Rome IV criteria: recurrent abdominal pain for at least 1 day per week in the last 3 months associated with two or more of the following criteria: (1) related to defecation, (2) onset associated with a change in frequency of stool, (3) onset associated with a change in form (appearance) of stool. These criteria should be fulfilled for the last 3 months with symptom onset at least 6 months before diagnosis (Mearin *et al.* 2016). The diagnosis of IBS should be made by a clinician based on these 4 key elements: clinical history, physical

examination, and if clinically indicated minimal laboratory tests and/or a colonoscopy (Mearin *et al.* 2016). Minimal laboratory testing and/or a colonoscopy might be necessary to distinguish between disorders mimicking IBS symptoms (e.g. IBD, celiac disease, lactose/fructose intolerance). However, when Rome IV criteria are fulfilled and alarm features are absent, additional diagnostic testing should be minimized. Currently four IBS subtypes are defined based on the predominant stool pattern: IBS with constipation (IBS-C), IBS with diarrhea (IBS-D), mixed IBS (IBS-M) and unclassified IBS (IBS-U) (Mearin *et al.* 2016). IBS has a worldwide prevalence of 11.2%. The prevalence is higher for women than men as well as for individuals aged younger than 50 years (Lovell *et al.* 2012).

While the pathophysiology of IBS is still not fully understood, researchers have proposed a multifactorial theoretical model in recent years. Two main causing factors are postulated being stress and inflammation. Although at the time of diagnosis in most cases no more signs of inflammation are detectable in the blood or by endoscopy, the inflammation might have triggered a pathological mechanism as explained below. In other cases, evidence is shown for a low-grade inflammation in the gastrointestinal wall which cannot be confirmed in all studies. The mechanisms triggered in IBS relate to the interaction between the microbiome, the intestinal permeability, the response of the submucosal immune network and afferent pain signaling cascades. In a healthy state, tight junctions in the intestinal wall prevent luminal gastrointestinal tract material (e.g. bacteria, chemicals, food antigens) from entering the epithelium. The gut flora is essential for maintaining the pH, nourishing epithelial cells and completing the digestion process with the production of intestinal gases as a result. However, in IBS patients these normal processes can be disrupted by several intestinal causes such as inflammation, infections, bile acid malabsorption, alterations in ion channels, certain foods (e.g. gluten) and dissacharidase deficiency. Subsequently, weakened tight junctions lead to an increased intestinal permeability followed by the activation of inflammatory cells (e.g. mast cells, dendritic cells, macrophages) which on their turn produce excessive amounts of inflammatory mediators (e.g. histamine, serotonin, proteases, cytokines). These mediators can then sensitize the enteric nervous system

as well as the upstream signaling to afferent neurons, dorsal root ganglia and the brain thereby leading to abdominal pain. Besides, these mediators can also initiate changes in the neuromuscular function resulting in diarrhea or constipation. These observations suggest a strong association between gastrointestinal permeability, inflammation and abdominal pain, resulting in clinical symptoms persisting even after complete resolution of the inflammation. Furthermore, changes in the intestinal flora ends up in increased production of intestinal gases leading to symptoms such as bloating and altered motility (Ford *et al.* 2017).

The heterogeneity of IBS as a disease makes it more difficult to provide a single treatment plan that would fit all IBS patients (Ford *et al.* 2017). However, all IBS treatments begin by explaining the condition, providing reassurance and educating the patient. Furthermore, as currently no cure is available, treatment will be based on symptom type and severity (Mearin *et al.* 2016). An overview of the currently available treatment options is given in Table 1.2. For a complete overview of the therapeutic options for IBS, we would like to refer the reader to a recent review by Ford *et al.* (Ford *et al.* 2017)

Table 1.2. Overview of the currently available treatment options for IBS patients.

Therapy	Example	Mechanism of action	Indications
Dietary modifications	Soluble fiber (e.g. psyllium)		Global symptoms
	Low FODMAP diet		Global symptoms
	Gluten-free diet		Global symptoms
Antispasmodics	Hyoscine, pinaverium, otilonium	Ca-channel blocker	Global symptoms, abdominal pain, diarrhea
	Peppermint oil	TRPM8-agonist	Global symptoms
Intestinal secretagogues	Lubiprosotone	PGE1 derivative	Global symptoms, abdominal pain (IBS-C)
	Linaclotide	Guanylate cyclase C agonist	Global symptoms, abdominal pain (IBS-C)
Serotonin receptor antagonists	Alosetron, ondansetron	5-HT_3 receptor antagonists	Global symptoms, abdominal pain (IBS-D)
Antidiarrheics	Loperamide	μ-opioid receptor agonist	Diarrhea
	Eluxadoline	μ- and κ-opioid receptor agonist, δ-opioid receptor antagonist	Global symptoms (IBS-D)
Antibiotics	Rifaximin		Global symptoms, abdominal pain (IBS-D)
Probiotics	*Bifidobacterium infantis 35624*	Bacteria beneficial to the host	Global symptoms, abdominal pain
Antidepressants	Amitryptiline	Tricyclic antidepressant	Global symptoms, abdominal pain
Psychological therapy	Hypnotherapy, cognitive behavioral therapy		Global symptoms, abdominal pain

Adapted from (Ford *et al.* 2017)

1.3 Visceral hypersensitivity

Abdominal pain is a key feature of two major gastrointestinal disorders: IBD and IBS. Although IBD and IBS are regarded as two different diseases, they seem to be related: the prevalence of IBS-like symptoms in IBD patients with an active disease and in IBD patients in remission amounts to 44% and 35%, respectively (De Schepper *et al.* 2008, Halpin *et al.* 2012). Besides being highly prevalent, these disorders have a considerable impact through their chronic character, as well as a negative influence on the quality of life and an important socio-economic impact (Hungin *et al.* 2003, Spiller 2007). Furthermore, the current treatment options are mostly focusing on the reduction of inflammation for IBD or the motility disturbances for IBS. Only a few therapies aim directly at diminishing the abdominal pain. Remarkably, only 24% of the IBS patients report complete relief of abdominal pain after treatment which is mostly only a symptomatic treatment of the most explicit motility-related symptom (Hungin *et al.* 2003). Therefore, further research in this area is of utmost importance. However, the search for new treatment targets is hampered due to the incomplete understanding of the pathogenesis of visceral pain.

Visceral pain - pain originating from internal organs such as the gut - is typically vague, diffuse, poorly localized and often associated with referred pain (Sikandar *et al.* 2012). Visceral hypersensitivity, or an increased pain perception in the bowel, is an important factor underlying the abdominal pain in IBD and IBS (Barbara *et al.* 2011). Hypersensitivity refers to the increased sensation of stimuli: both allodynia (pain evoked by stimuli that are normally not painful) and hyperalgesia (increased response to a painful stimulus) are present (Camilleri *et al.* 2001). The concept of hypersensitivity is clarified in Figure 1.1.

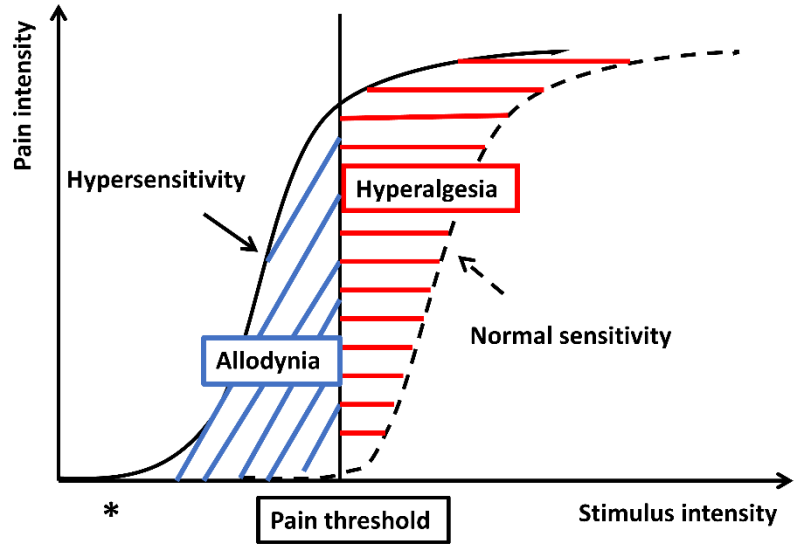


Figure 1.1. This graph explains the concept of visceral hypersensitivity.

The pain intensity (y-axis) is shown in function of the stimulus intensity (x-axis). The dashed black curve represents the normal sensitivity. Stimuli are experienced as painful after exceeding the pain threshold. As the stimulus intensity increases, the pain intensity will increase as well. The continuous black line represents a state of hypersensitivity with a decreased pain threshold (asterisk). Normally non-painful stimuli are experienced as painful (allodynia, light grey shading) and painful stimuli are experienced as more intense (hyperalgesia, dark grey shading).

In both basic and clinical research, visceral hypersensitivity is commonly investigated by colorectal distensions using a barostat (Keszthelyi *et al.* 2012). The barostat pneumatically inflates a rectally inserted balloon at a fixed pressure, resulting in phasic colorectal distensions. Compared to healthy individuals, patients suffering from visceral hypersensitivity report discomfort at lower colonic distension pressures (lower pain threshold) and increased pain in response to standardized stimuli (Camilleri *et al.* 2001).

Currently, the pathophysiology of visceral hypersensitivity has not been fully elucidated yet, but several mechanisms, such as inflammation, psychosocial factors and/or sensorimotor alterations, are thought to be involved. The latter can be located both at the central and/or peripheral level along the anatomical afferent information pathway from the viscera towards the brain. Moreover, an important role for

peripheral as well as central sensitization of the afferent visceral neuron pathways is proposed (de Carvalho Rocha *et al.* 2014).

Visceral hypersensitivity thus occurs via a disturbance of the sensitization pathways that might be located at different levels. For a complete overview of the neuroanatomy of lower gastrointestinal pain disorders, we refer the interested readers to a review by Vermeulen *et al.* (Vermeulen *et al.* 2014). At the peripheral level, chemical, mechanical and thermal information is registered by primary afferent neurons. The cell bodies of these neurons are located in the dorsal root ganglia (DRGs) (Blackshaw *et al.* 2007). Centrally, they connect with secondary afferent neurons in the dorsal horn of the spinal cord or in the brain stem. From there, the signal is transduced to different cerebral areas involved in the somatosensory sensation of (pain) signals arising from the bowel. Within the central nervous system, signals of peripheral afferent nerves are modulated via descending pathways, resulting in facilitation or inhibition of the impulse conduction (Anand *et al.* 2007).

During gastrointestinal inflammation, the continuous release of inflammatory mediators can give rise to the sensitization of peripheral afferent nerves, thus contributing to the development of visceral hypersensitivity. Besides being an important well-known trigger for IBD, (microscopic) inflammation has been demonstrated to play a role in IBS as well. As already mentioned, an increased prevalence of IBS-like symptoms is present in IBD patients. Moreover, a disease state denominated as post-infectious IBS has been described; it is seen in 3%-35% of the patients that experienced an acute gastroenteritis related to water contamination (Marshall *et al.* 2010). These observations suggest a strong association between gastrointestinal inflammation on the one hand and the onset of visceral hypersensitivity on the other hand, in which case the hypersensitivity persists even after complete resolution of the inflammation.

At the peripheral level, epithelial cells and immune cells, e.g., mast cells, T-cells and neutrophils, are activated. Upon activation, they release excessive amounts of

mediators such as histamine, serotonin, several cytokines and proteases. These mediators, in turn, can sensitize peripheral afferent neurons, thus contributing to visceral hypersensitivity (De Schepper *et al.* 2008). The hypothesis stated in this paragraph is a simplified representation of the pathogenesis of visceral hypersensitivity clarified in figure 1.2. The focus was on inflammation as a trigger for IBS, without emphasizing the role of the microbiome, infections, food and the brain in sufficient detail.

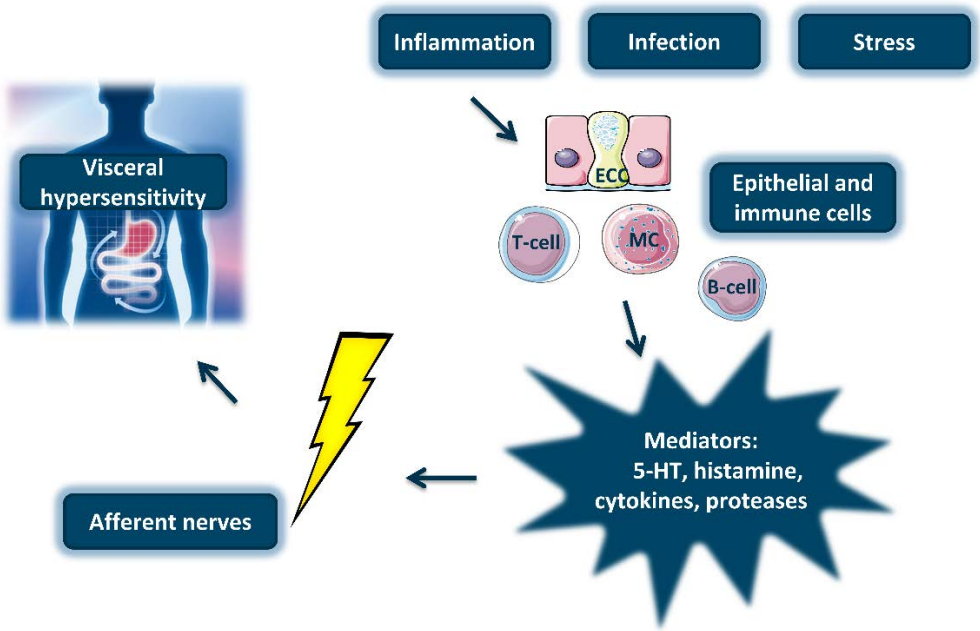


Figure 1.2. Hypothetical scheme of the processes involved in the pathogenesis of visceral hypersensitivity within gastrointestinal wall.

In a state of inflammation, infection or stress epithelial and immune cells (e.g. ECC, T-cells, B-cells, MCs) are activated. Upon their activation, excessive amounts of mediators (e.g. 5-HT, histamine, cytokines, proteases) will be released. These mediators on their turn will sensitize afferent nerves and eventually lead to visceral hypersensitivity. This scheme represents a simplification of the pathogenesis of visceral hypersensitivity focusing on local mechanisms in the gastrointestinal wall without taking into account the microbiome, food constituents or the spinal cord and brain. Image constructed using the Servier Image Bank. 5-HT; serotonin, ECC; enterochromaffin cells, MC; mast cell.

The mast cell is thought to fulfill an important task in the development of abdominal pain in IBS patients (De Winter *et al.* 2012), since an association was found between mast cell infiltration in the bowel wall and the frequency and severity of abdominal

pain (Barbara *et al.* 2004). The mast cell is an important immune cell that can be activated by cytokines, antigens and neuropeptides. This activation is followed by a degranulation of the cell, releasing vasoactive and pro-inflammatory mediators (Akbar *et al.* 2009). Histamine, an important mast cell mediator, has already been shown to play an important role in visceral hypersensitivity: we demonstrated a role for histamine in a rat model for post-inflammatory visceral hypersensitivity, mediated by histamine H1 and H4 receptors (Deiteren *et al.* 2014). Also in humans, a recent clinical trial with the H1 receptor-antagonist ebastine showed promising results in IBS patients resulting in a phase II trial (www.clinicaltrials.gov) (Wouters *et al.* 2016). Apart from histamine, mast cells release many other mediators such as cytokines, growth factors, leukotrienes, prostaglandins, serotonin and several proteases (Zhang et al. 2016). In this PhD dissertation, we will focus on the role of this last group of mediators, the proteases, and their role in visceral hypersensitivity.

1.4 Animal models to study visceral hypersensitivity

As described earlier in the introduction in paragraph 1.3, there are several known triggers for IBS and visceral hypersensitivity. These different triggers were also used in the development of animal models to assess visceral hypersensitivity in IBS. An overview of the animal models is given in Table 1.3 with their most important trigger and their advantages and disadvantages.

Table 1.3. Animal models to study visceral hypersensitivity

Trigger	Animal model	Advantages	Disadvantages
Genetic/ Spontaneous	Knockout mice (e.g. CRF-1)	Investigate the role of a specific gene in VHS	Interpretation of data in a genetic model is complicated
	Wistar Kyoto rat	No additonal intervention necessary	Only rat strain exhibiting spontaneous VHS (limited translational relevance)
Early-life stress	Maternal separation	Investigate relationship between early-life stress and VHS development together with hyperreactivity of the HPA axis	VHS depending on duration of separation, use of varying rat strains, removal of pups (difference in weaning weight), interaction with personnel for separation, high cost animal housing
	Odor-attachment learning	Linking early-life stress to VHS in women (female predominance)	Interaction with personnel for conditioning, high cost animal housing
	Limited nesting	Relevant to investigate neglect and abuse in presence of the mother, no removal of pups needed, little interaction with personnel	High cost animal housing
	Neonatal colonic irritation: mechanical (noxious CRD) or chemical (mustard oil, 0.5% AA)	Relevant to investigate repeated physical/sexual abuse or colonic inflammation during childhood	Interaction with personnel for manipulation, high cost animal housing
Stress-induced	ceA implants	Relevant to IBS patients showing increased activation of amygdala, abdominal pain and anxiety	Surgery needed, measurement of visceral pain in anesthetized animals
	Restraint stress	Robust, reproducible method, resembles IBS related to stress (similar cortisol level)	Translational relevance to clinical stressors, habituation to stressor

Trigger	Animal model	Advantages	Disadvantages
Stress-induced	Water avoidance stress	Mimic psychological stressor, resembles IBS related to stress (similar cortisol level)	Translational relevance to clinical stressors, may engage fear neurocircuitry affecting data interpretation, habituation to stressor
	Variable stress (protocol with random set of stressors)	No habituation to stressor	Translational relevance to clinical stressors
Inflammation	Non-inflammatory colonic irritation: acetic acid (<1%), butyrate (six enemas)	No inflammation or histological damage	Possibly no direct translation of acute sensitization in animals to chronic pain in patients
	Acute inflammatory colonic irritation: capsaicin, mustard oil, DSS, TNBS, zymosan	Pain behavior 20-30 min after irritant administration (capsaicin, mustard oil),	Increased risk for colon perforation when assessing VHS, selection mouse strain (TNBS), nature/severity of inflammatory stimulus important
	Post-inflammatory: AA, DSS, TNBS	Mimic 30-50% of the IBD patients that develop IBS symptoms (VHS), translational relevance (TNBS)	Recovery from colitis does not guarantee presence of VHS, nature/severity of inflammatory stimulus important
Infection	*Campylobacter jejuni, Citrobacter rodentium Trichinella spiralis*	Mimic 3-36% of patients that develop IBS symptoms after an acute gastroenteritis, long-term immune modulation of smooth muscle and enteric nervous system result in persistent altered gut motility and VHS	Protect researchers from pathogens, Majority human post-infectious VHS after *Salmonella, E. coli, Shigella* but no animal models with these bacterial strains.

AA: acetic acid; ceA: corticosterone pellets in the central nucleus of the amygdala; CRD: colorectal distension; CRF-1: corticotropin releasing factor; DSS; dextran sodium sulfate; HPA: hypothalamic-pituitary-adrenal; MS: maternal separation; TNBS: trinitrobenzene sulphonic acid; VHS: visceral hypersensitivity; WAS: water avoidance stress. References: (Larauche *et al.* 2011, Greenwood-Van Meerveld *et al.* 2015, Moloney *et al.* 2015).

1.5 The assessment of visceral pain in animal models

There are several techniques used to assess visceral pain in rodents. The most frequently used method comprises the measurement of the visceromotor response (VMR) to a colorectal distension (CRD) quantified by electromyographic (EMG) electrodes. The reflex abdominal muscle contractions in response to a graded colonic balloon distension protocol, are quantified by the EMG electrodes that are implanted in the external oblique abdominal muscle (Greenwood-Van Meerveld *et al.* 2015). A second possible approach to assess visceral pain is the visual evaluation of the abdominal withdrawal reflex (AWR) in response to a colorectal distension (CRD) (O'Mahony *et al.* 2012, Greenwood-Van Meerveld *et al.* 2015). A third way to measure visceral pain is through the intracolonic instillation of algesic substances (e.g. capsaicin, mustard oil). These substances initiate nociceptive-related behavior (e.g. perianal licking, abdominal retraction/compression, hindlimb stretching, grimace scale) that can be quantified (Laird *et al.* 2001, Greenwood-Van Meerveld *et al.* 2015). A fourth manner to assess visceral pain and to cover the involvement of the brain-gut axis is functional brain imaging (fMRI) in response to visceral stimulation (Wang *et al.* 2008, Johnson *et al.* 2010). An overview of the techniques with their advantages and disadvantages is given in Table 1.4.

Table 1.4. Methods used to assess visceral sensitivity in animal models.

Method	Advantages	Disadvantages
EMG assessment of VMR to CRD	Objective assessment of nociceptive reflexes, measurement in freely moving animals possible, possibility to measure compliance, similarity with method used in patients	Increased animal stress due to new laboratory environment/restraining, need for anesthesia in most animals to insert the balloon into the colorectum
Visual evaluation of AWR to CRD	Nociceptive behavior induced by a brief distension, no surgery required	Subjective behavioral measure, larger sample size required
Quantification of nociception-related behavior to colonic instillation of algesic substances	Spontaneous evoked nociception-related behavior	Inflammatory stimulus (more relevant model for inflammatory pain)
Functional brain imaging (fMRI) in response to CRD	Cover multidimensional nature of visceral pain (brain-gut axis)	Analysis often limited to specific brain regions, use of sedated animals

AWR: abdominal withdrawal reflexes; CRD: colorectal distension; EMG: electromyographic; fMRI: functional magnetic resonance imaging; VMR: visceromotor response. References: (Wang *et al.* 2008, Johnson *et al.* 2010, O'Mahony *et al.* 2012, Greenwood-Van Meerveld *et al.* 2015)

1.6 Proteases

1.6.1 Definitions, classification

Proteases are enzymes catalyzing the cleavage of peptides and/or proteins, thereby releasing amino acids or peptides. They represent up to 2% of the human genome and are present at particularly high levels in the gastrointestinal tract (Vergnolle 2004). Proteases execute a large variety of physiological functions. They are vital for processes such as blood coagulation, cell growth and migration, tissue arrangement, activation of zymogens, protein catabolism and the release of hormones and pharmacologically active peptides from precursor proteins. Apart from that, they are involved in

pathological processes such as inflammation and tumor growth and metastasis (Rao *et al.* 1998, Antalis *et al.* 2007).

Based on the position of the peptide bond that can be cleaved, proteases are subdivided into two major groups: exopeptidases and endopeptidases. Exopeptidases catalyze the cleavage of the terminal or the penultimate peptide bond of the protein, releasing a single amino acid or a dipeptide, respectively (Barrett 1980, Hooper 2002). Since peptide chains have both an amino- and a carboxy-terminus, exopeptidases can be further divided into amino- and carboxypeptidases (Hooper 2002). Unlike exopeptidases, endopeptidases catalyze the cleavage of nonterminal peptide bonds within the molecule, thus releasing larger peptides instead of single amino acids or dipeptides (Barrett 1980, Hooper 2002). The concept of exo- and endopeptidases is represented in a simplified way in Figure 1.3.

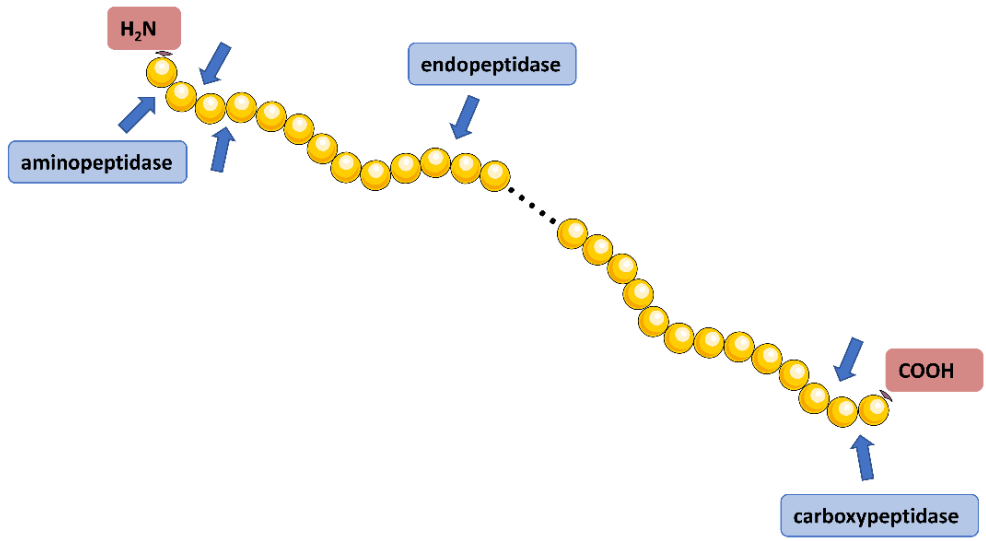


Figure 1.3. Simplified representation of the concept of endo- and exopeptidases.

Endopeptidases cleave internal peptide bonds. Exopeptidases cleave terminal peptide bonds; they can be subdivided into amino- and carboxypeptidases according to the position of the cleavage of the peptide bond. Aminopeptidases cleave at amino (NH2) terminal bonds, while carboxypeptidases cleave at carboxy (COOH) terminal bonds. Image constructed using the Servier Image Bank. COOH; carboxy, NH2; amino.

Due to their huge diversity in action and structure, proteases can be classified in other ways as well (Rao *et al.* 1998). For example, it is possible to classify the enzymes according to their evolutionary relatedness with reference to structure, the type of reaction catalyzed or the pH optimum of the enzyme (Rao *et al.* 1998). However, they are usually categorized according to their catalytic type, based upon the presence of different nucleophiles in the molecular structure of the enzyme (Barrett 1980, Powers *et al.* 1993, Hooper 2002, Rawlings *et al.* 2011). The major advantage of using a catalytic type based classification is that proteases of the same catalytic type usually respond to the same protease inhibitors (Rawlings *et al.* 2011). Historically, four major groups could be distinguished in this classification, based upon the functional group present at the active site: serine, cysteine, aspartic and metalloproteases (Barrett 1980, Powers *et al.* 1993). More recently, threonine, glutamate and asparagine proteases have been added as classes. However, glutamate and asparagine proteases have not been found in humans or other mammals so far (Rawlings *et al.* 2011). The seven catalytic classes are depicted in Figure 1.4.

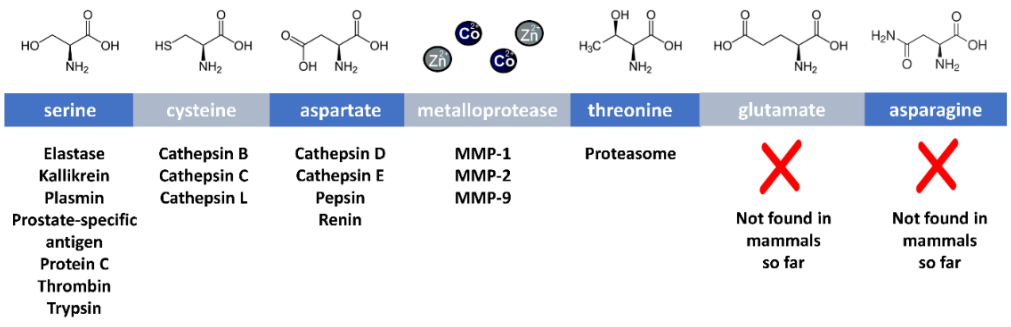


Figure 1.4. This scheme represents the classification of proteases based on the chemical structure of their active site.

For each clan, the chemical structure of the core residue in their active site is shown on top and a few examples with medical relevance of proteases belonging to that family are displayed below. MMP; matrix metalloprotease.

1.6.2 Sources

As already mentioned earlier, proteases are present at particular high levels in the gastrointestinal (GI) tract and their origin can be both intracellular in the GI tissues as well as extracellular upon secretion into the lumen.

The pancreas comprises an important source of proteases (e.g. trypsin) involved in the digestion process. Other important suppliers of proteases include intestinal microbiota, mast cells (e.g. tryptase, chymase), epithelium (e.g. trypsin) and macrophages (e.g. MMP-12, caspase, cathepsin B and L). Specifically in a state of inflammation, inflammatory cells such as neutrophils release excessive amounts of proteases (e.g. elastase, proteinase-3, cathepsin G) (Vergnolle 2016).

Next to proteases, endogenous protease inhibitors are present in order to maintain the protease homeostasis in the GI tract. The origin of these inhibitors ranges from the liver to the epithelium and inflammatory cells. Just like proteases, protease inhibitors are also classified into different families. The serpins (e.g. serpin A1, A3, A4) inhibit serine proteases such as trypsin, tryptase, chymase and KLKs. The chelonianin family includes: elafin and secretory leukocyte protease inhibitor (SLPI) targeting e.g. elastase and proteinase-3. The tissue inhibitors of metalloproteases (TIMPs) (e.g. TIMP-1, -2, -3) encompass a last family of endogenous protease inhibitors inhibiting different MMPs (Vergnolle 2016).

1.6.3 Mechanism of action

Proteases can act as signaling molecules through the proteolytic cleavage of different mediators/receptors thereby inducing intracellular signals and regulating different GI cell functions. The different mechanisms of action include: protease-activated receptor (PAR) activation, apoptosis, tight junction degradation, inflammatory mediator processing, immunoglobulin cleavage, mucus cleavage and matrix remodeling.

In the following paragraph we will focus on the most studied mechanism of action; PAR activation. PARs belong to a family of cell-surface signaling proteins called G protein-

coupled receptors (GPCRs) and consist of seven transmembrane domains with three intracellular and three extracellular protein loops (Cenac *et al.* 2003, Vergnolle 2004), as visualized in Figure 1.5.

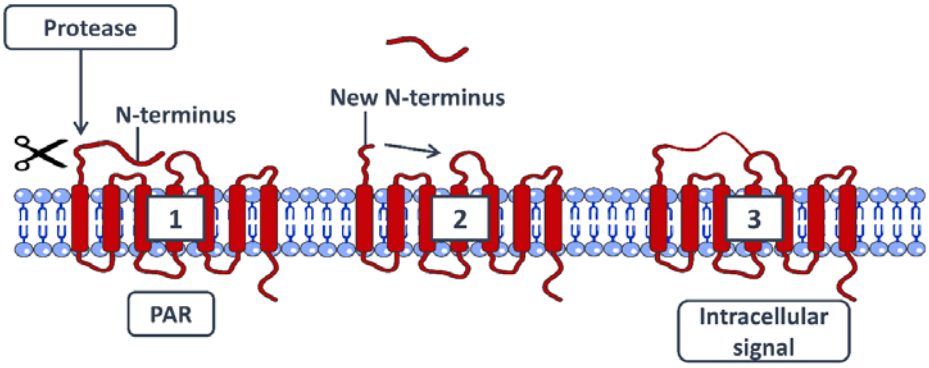


Figure 1.5. **Schematic representation of the activation of a protease-activated receptor (PAR).**
A protease cleaves the N-terminal domain (1), releasing a new N-terminus (2). The new N-terminus binds to the receptor as a tethered ligand, providing an intracellular signal (3). N-terminus; amino-terminus; PAR; protease-activated receptor. Image constructed using the Servier Image Bank.

So far, four PARs have been described: PAR1, PAR2, PAR3 and PAR4 (Cenac 2013). PARs can transduce signals by canonical activation. The activation process starts with a protease recognizing one of the extracellular domains of the receptor, situated on the N-terminus (i.e. the canonical site of the PAR receptor). Some proteases, such as thrombin, subsequently bind to this domain. For other proteases, e.g. trypsin or tryptase, it is not required to establish a stable bond in order to cleave the receptor. When the receptor has been activated, the N-terminal domain will be cleaved by proteolysis at the recognition site, thus exposing a new N-terminal sequence. This sequence acts as a tethered ligand that binds domains situated in the second extracellular loop of the receptor, thus initiating common signaling pathways, such as the G-protein- and/or β-arrestin-dependent pathways (e.g. PAR2 activation by trypsin) (Vergnolle 2004, Zhao *et al.* 2014).

Apart from canonical activation, PAR signaling pathways can be initiated in several other ways. Biased agonism is a second possibility; proteases cleave at sites distinct

from the canonical sites, thereby activating unique and biased signaling pathways (e.g. PAR2 activation by activated protein C) (Zhao *et al.* 2014). Proteases are also able to induce the opposite effect; this third manner is called proteolytic disarming. In that case, proteases can remove or destroy tethered ligands leading to the termination of PAR activation (e.g. PAR2 disarming by cathepsin-G) (Cenac 2013, Zhao *et al.* 2014). A fourth manner is the non-tethered ligand activation, suggesting that the formation of tethered ligands is not essential for PAR activation (e.g. PAR2 activation by elastase). Finally, it is possible to activate PARs via so-called PAR-activating peptides (PAR-APs), synthetic peptides corresponding to the first five or six amino acids of the tethered ligand sequence. This form of artificial activation bypasses the proteolytic cleaving process. PAR-APs are capable of inducing common as well as biased signaling pathways (e.g. PAR2 activation by SLIGKV-NH2) (Hollenberg *et al.* 2002, Zhao *et al.* 2014). Since these peptides are specific for a single receptor, they are very important pharmacological tools to investigate the physiology and physiopathology of PARs (Cenac 2013). The different mechanisms of PAR activation are depicted in figure 1.6.

Currently, thrombin is regarded as the main activator for PAR1, PAR3 and PAR4, as is the serine protease trypsin for PAR2 and PAR4. Although some proteases can activate different PARs, every protease has a preference for one specific receptor. For example, thrombin has the highest potency for PAR1, a lower potency for PAR3, and even weaker for PAR4 (Vergnolle 2004). For an overview of the characteristics of the four PARs, their activating proteases and agonists/antagonists, we refer to a detailed review by Vergnolle *et al.* (Vergnolle 2005).

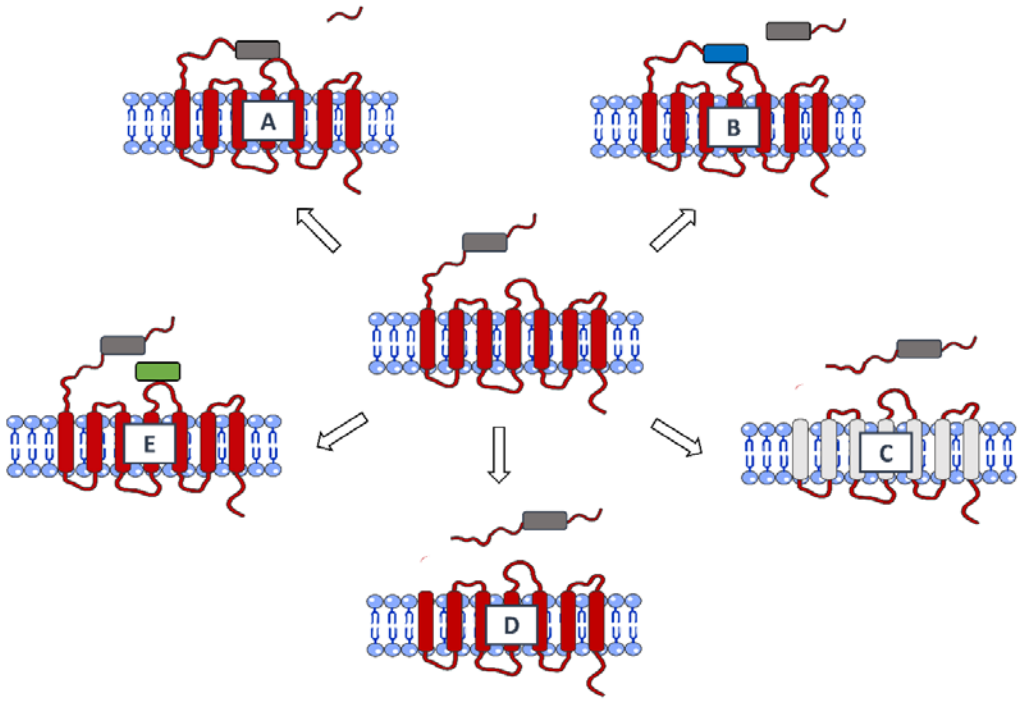


Figure 1.6. Schematic representation of the different activation mechanisms of a protease-activated receptor (PAR).

Canonical activation. Proteases (e.g. trypsin) cleave PAR at a canonical cleavage sites, exposing a tethered ligand domain, binding to the second extracellular loop thereby activating PAR signaling pathways. (B) Biased agonism. Proteases (e.g. activated protein C) cleave at sites distinct from the canonical site, thereby activating unique and biased signaling pathways (C) Proteolytic disarming. Proteases (e.g. cathepsin G) can remove or destroy tethered ligands leading to the termination of PAR activation. (D) Non-tethered ligand activation. Proteases (e.g. elastase) can cleave and activate PARs without revealing tethered ligands. (E) PAR-activating peptides, synthetic peptides corresponding to the first five or six amino acids of the tethered ligand sequence, can artificially activate PARs. Image constructed using the Servier Image Bank. Adapted from (Zhao *et al.* 2014)

1.6.4 Species differences

Regarding the translation of findings from preclinical research to clinical human studies, it is important to take into account species differences regarding proteases and PARs. A genomic analysis has provided insight into the species differences of proteases and protease inhibitors between rats, mice and humans. Several protease families have evolved differently in these different species, thereby possibly explaining some functional differences between these species. A complete overview can be found in a review by Puente and Lopez-Otin (Puente *et al.* 2004).

1.7 Proteases in IBD

As already discussed in the previous paragraph, the gastrointestinal tract is subjected to a massive number of proteases. In physiological conditions, the proteolytic balance is tightly regulated at two different levels, specifically at the level of a physical barrier i.e. tight control of the intestinal barrier function (1) and at a molecular level i.e. maintaining the proteolytic homeostasis through endogenous protease inhibitors (2). However, during pathophysiological conditions such as IBD, the gastrointestinal tissues are exposed to an enormous amount of proteases leading to a disruption of the proteolytic balance thus contributing to inflammation (Motta *et al.* 2011).

Protease expression levels have been measured in samples from both IBD animal models as well as human IBD patients. Hereafter the results of these studies will be discussed, arranged by the respective class of proteases. Firstly, the most abundant class of proteases, namely the serine proteases (e.g. elastase, mast cell proteases, kallikreins and trypsins) will be addressed. Elastase levels are found to be significantly increased in fecal (Langhorst *et al.* 2008) and plasma (Gouni-Berthold *et al.* 1999) samples from IBD patients. Regarding mast cell proteases, elevated expression levels of tryptase (Raithel *et al.* 2001) and chymase (Andoh *et al.* 2006) have been found in colonic biopsy samples from IBD patients compared to controls. Moreover, an upregulation of the kallikrein-kinin system has been observed in IBD patients (Stadnicki *et al.* 2003). Furthermore, a significantly increased expression of granzyme A has been found in colon samples of both infectious colitis mice (Hansen *et al.* 2005) and IBD patients (Muller *et al.* 1998). Another member of the granzyme family, granzyme B, has an elevated mRNA expression in mucosal lesions of CD patients (Jenkins *et al.* 2000).

Cathepsin D, belonging to the class of aspartate proteases, was found to be increasingly expressed in the colonic mucosa of both DSS-colitis mice and IBD patients (Hausmann *et al.* 2004).

The class of cysteine proteases (e.g. caspase, cathepsin-B, cathepsin-L) covers a third class of proteases. An important role for caspase in IBD is first of all demonstrated by an association between caspase activation recruitment domain 15 (CARD15) mutations and CD (Hugot *et al.* 2001). Moreover, several research groups have confirmed an upregulated expression of caspase-1 (McAlindon *et al.* 1998, Seidelin *et al.* 2006) and caspase-5 (Seidelin *et al.* 2006) in colonic biopsy specimens from UC patients compared to controls. Additionally, an increased expression of both cathepsin-B and cathepsin-L was found in the colonic mucosa of IBD patients and the addition of a cathepsin-B and -L inhibitor ameliorated DSS-colitis in mice (Menzel *et al.* 2006).

The metalloproteases are a class of proteases that have been reported to be increased in IBD by several research groups. The mRNA expression of matrix metalloprotease (MMP)-1, MMP-2, MMP-3, MMP-12 and MMP-14 has been found to be significantly higher in inflamed colon samples from IBD patients compared to controls (von Lampe *et al.* 2000, Pender *et al.* 2006). Moreover, proteolytic MMP activity was detected to be increased in inflamed colonic epithelial tissues from IBS patients (Pedersen *et al.* 2009).

As stated earlier in this paragraph, endogenous protease inhibitors or antiproteases play an important role in the maintenance of the proteolytic homeostasis and therefore their expression in IBD is altered.

Regarding the family of serine proteases, there are 2 families of antiproteases: serpins and chelonianins (Motta *et al.* 2011). The studies related to the expression of serpinA1 or α1-antitrypsin (targeting chymase, tryptase, trypsin, elastase, proteinase-3, thrombin and KLK) in IBD are rather conflicting: some studies link α1-antitrypsin deficiency to IBD (Yang *et al.* 2000) while others were unable to find a correlation between α1-antitrypsin and UC (Becker *et al.* 1999). Another member of the serpin family, serpinA3 or α1-antichymotrypsin (targeting chymase, chymotrypsin and CatG) was found to be upregulated in fecal samples of IBD patients (Huet *et al.* 1990).

SerpinA4 or kallikstatin (targeting KLK) was demonstrated to be downregulated in plasma of IBD patients (Stadnicki *et al.* 2003).

The family of chelonian inhibitors includes the secretory leukocyte protease inhibitor (SLPI) and elafin (Motta *et al.* 2011). SLPI (targeting tryptase, chymase, trypsin, chymotrypsin, elastase and CatG) and elafin (targeting elastase and proteinase-3) were found to be increasingly expressed in the colon tissue of UC patients but no differences were found in CD compared to controls (Schmid *et al.* 2007). Moreover, elafin was demonstrated to prevent intestinal inflammation in a TNBS- and DSS-induced mouse model for colitis (Motta *et al.* 2011).

The inhibitor of apoptosis protein-2 (c-IAP2) (targeting caspase-9) belongs to the family of cysteine proteases and was found to upregulated in UC patients (Seidelin *et al.* 2006). A last family comprise the tissue inhibitors of metalloproteases (TIMPs). Serum levels of TIMP-1 and TIMP-2 were significantly increased in both UC and CD patients (Lakatos *et al.* 2012), while TIMP-3 was downregulated in the intestine of CD patients (Monteleone *et al.* 2012).

An overview of the published genetic evidence supporting the association of protease/antiprotease genes with IBD was given in a systematic review by Cleynen *et al.* (Cleynen *et al.* 2011). Remarkably, in total 85 protease/antiprotease encoding genes were retained for CD based on accumulated evidence, while only 18 genes were retained for UC with a considerably lower amount of evidence (Cleynen *et al.* 2011).

The abovementioned studies provided us with a lot of information concerning the levels of proteases in IBD vs control tissues. However, an increased protease expression at the mRNA or protein level does not reflect the exact function of proteases in a disease state. As described earlier, the proteolytic balance is dependent on both proteases as well as endogenous protease inhibitors. Therefore, the proteolytic activity, determined by the resulting effect of both proteases and antiproteases, might be a better measure and give us more insight into the function of proteases in a disease state such as IBD (Vergnolle 2016).

So far, only a few studies have determined protease activity profiles in IBD. An increased elastase activity has been observed in the colon samples of DSS- and TNBS-induced colitis in mice (Motta *et al.* 2011). Moreover, a study from the same research group also provided evidence for an elevated elastolytic activity in human colon samples from IBD patients (Motta *et al.* 2012). Besides serine protease expression levels, also an increased trypsin-like activity has been found in colonic samples from both IBD mice (Motta *et al.* 2011) and patients (Cenac *et al.* 2007). Recently, an increased MMP activity has been found in colon tissue from UC patients (de Bruyn *et al.* 2014). Finally, total proteolytic activity was also elevated in stool samples from UC patients (Annahazi *et al.* 2009).

1.8 Proteases in IBS

So far, the expression and activity of proteases in the colon and feces of IBS patients have not been investigated as extensively as in IBD.

Concerning the class of serine proteases, a higher expression of tryptase (Barbara *et al.* 2004, Cenac *et al.* 2007, Bian *et al.* 2009, Buhner *et al.* 2009, Zhao *et al.* 2012, Liang *et al.* 2016) and trypsin (Cenac *et al.* 2007, Zhao *et al.* 2012) could be detected in colon samples from IBS patients compared to healthy controls. However, no changes in tryptase expression (protein level) could be detected in fecal samples of IBS patients (Lettesjo *et al.* 2006, Roka *et al.* 2007).

Considering the class of cysteine proteases, calpain-8 (Swan *et al.* 2013) and proteases from the proteasome (Coeffier *et al.* 2010) have been demonstrated to be upregulated in the colon of IBS patients.

Similar to studies in IBD, only few research groups have examined proteolytic activities in samples from IBS patients.

Serine protease activity was increased in IBS patients compared to healthy controls in both colon and feces in several studies (Cenac *et al.* 2007, Roka *et al.* 2007, Gecse *et al.* 2008, Annahazi *et al.* 2009). Likewise, an elevated serine protease activity was

observed in colonic samples in a post-infectious IBS mouse model (Ibeakanma *et al.* 2011).

Cysteine protease activity was elevated in fecal samples of IBS-C patients and correlated with the abdominal pain score (Annahazi *et al.* 2013), although this alteration could not be observed in colonic samples of post-infectious IBS mice (Ibeakanma *et al.* 2011).

In the previous paragraph, we already stated the importance of the measurement of proteolytic activities. Recently, concerns have been raised about the tests used to determine the protease activity. Many of these tools suffer from a lack of selectivity for individual proteases and most of them detect several enzymes, such as the generally used azocasein assay. For example, when determining tryptase activity, mostly trypsin-like activity is quantified due to the lack of specificity of the substrates used (Edgington-Mitchell 2015). Therefore, with regard to a detailed study of the role of proteases in e.g. visceral hypersensitivity, the improvement of chemical tools to assess the activity of specific proteases is of utmost importance (Edgington-Mitchell 2015).

Taken together all the studies described above, an important role for serine proteases in IBS cannot be denied.

However, the origin of those serine proteases was unclear for a long time. Recently, it was demonstrated that the origin of the most abundant fecal serine proteases in IBS-D patients is mainly human (Tooth *et al.* 2014).

1.9 Protease-activated receptors in IBD and IBS – focus on visceral pain

Proteases present in the gastrointestinal tract during IBD and IBS could act through the activation of the protease-activated receptors (PARs), as already mentioned before. There is widespread empirical evidence that the activation of these PARs can affect gut physiological functions such as inflammation, motility, intestinal permeability and sensory functions (Vergnolle 2005). In the following paragraph, an overview is given of

the research focusing on the role of PARs in sensory dysfunction leading to visceral pain in IBD and IBS.

Proteases are thought to influence visceral sensitivity through protease-activated receptors (PARs). Remarkably, after the activation of these receptors, the effects on pain are not the same for all PARs. When PAR1 and PAR4 are activated, antinociceptive effects are observed, while the activation of PAR2 induces pronociceptive effects. An overview of the studies described in the following paragraph is shown in Table 1.5.

The antinociceptive properties of PAR1 activation are demonstrated in different animal models showing a decrease in carrageenan-induced visceral hyperalgesia in rats and capsaicin-evoked visceral pain in mice after an intraplantar administration with the PAR1-agonists TFFLR-NH2 and thrombin (Kawabata *et al.* 2002, Kawao *et al.* 2004). Furthermore, a decreased PAR1-expression was found in colon samples of IBS-D patients (Bian *et al.* 2009).

In mice, PAR4 activation seems to inhibit visceral hypersensitivity as well (Annahazi *et al.* 2009, Auge *et al.* 2009). In parallel, PAR4 expression is lowered in the colon of IBS patients (Han *et al.* 2012, Zhao *et al.* 2012). On the contrary, PAR4 expression was increased in colonic biopsies from UC patients (Dabek *et al.* 2009). The PAR4-agonist AYPGKF-NH2 was able to reduce visceral hypersensitivity after an intracolonic administration in sub-inflammatory doses, while higher doses showed pro-inflammatory effects in mice (Auge *et al.* 2009). Thus, the difference in PAR4 expression levels in IBS vs IBD patients could be explained by these findings in mice.

Table 1.5. Preclinical studies investigating the effects of PAR-targeting molecules on visceral hypersensitivity.

PAR	Agonist/antagonist	Species *(hypersensitivity model)*	Study type	Effect	Reference
PAR1	Agonist (thrombin, TFLLR-NH2)	Rat *(carrageenan)*	In vivo	↓ hyperalgesia	(Kawabata *et al.* 2002)
PAR1	Agonist (TFLLR-NH2)	Mice *(capsaicin)*	In vivo	↓ hyperalgesia	(Kawao *et al.* 2004)
PAR2	Agonist (SLIGRL-NH2)	Mice *(PAR2-agonist)*	In vivo	↑ hyperalgesia	(Kawabata *et al.* 2001)
PAR2	Agonist (SLIGRL-NH2, trypsin)	Rat *(PAR2-agonist)*	In vivo	↑ hyperalgesia	(Coelho *et al.* 2002)
PAR2	Agonist (SL-NH2, trypsin, typtase)	Guinea pig submucosal neurons *(PAR2-agonist)*	Ex vivo	↑ neuron excitability	(Reed *et al.* 2003)
PAR2	Agonist (SLIGRL-NH2, Tc-NH2, trypsin, tryptase)	Mice, rat *(PAR2-agonist)*	KO	↑ hyperalgesia, absent in KO	(Vergnolle *et al.* 2001)
PAR2	Agonist (2-furoyl-LIGRL-NH2)	Mice *(capsaicin)*	KO	↑ hyperalgesia, absent in KO	(Kawabata *et al.* 2006)
PAR2	Antagonist (ENMD-1068)	Mice *(IBS-supernatant)*	KO	↓ hypersensitivity, absent in KO	(Cenac *et al.* 2007)
PAR2	/	Mice DRG *(IBS-D supernatant)*	KO	↑ neuron excitability, absent in KO	(Valdez-Morales *et al.* 2013)
PAR4	Agonist (PAR4-AP, Cat-G)	Mice *(IBS-D supernatant)*	In vivo	↓ hypersensitivity	(Annahazi *et al.* 2009)
PAR4	Agonist (AYPGKF-NH2)	Mice *(PAR2-agonist, TRPV4-agonist)*	In vivo	↓ hypersensitivity	(Auge *et al.* 2009)

DRG: Dorsal root ganglia; IBS: Irritable bowel syndrome; KO: Knock-out; PAR: Protease-activated receptor; TRPV: Transient receptor potential vanilloid channels.

In sharp contrast to PAR1 and PAR4, the activation of PAR2 results in a pronociceptive effect. This was firstly demonstrated by Kawabata *et al.* and Coelho *et al.*, who confirmed the presence of visceral hypersensitivity in rats after the administration (intracolonic/intraplantar) of the PAR2-activating peptide SLIGRL-NH2 or trypsin (Kawabata *et al.* 2001, Coelho *et al.* 2002). An increased Fos-expression (Coelho *et al.* 2002) and the presence of PAR2 mRNA in the dorsal root ganglia (DRG) (Kawabata *et al.* 2001) confirmed these results. An ex-vivo study reconfirmed these observations: the application of several PAR2-agonists, such as trypsin, mast cell tryptase and SL-NH2, induced hyperexcitability of submucosal neurons in the ileum of guinea pigs (Reed *et al.* 2003). The next step in this research included the use of experimental knock-out (KO) models. Similar to the studies described above, visceral hyperalgesia was observed in wild-type (WT) mice after the administration (intracolonic/intraplantar) of PAR2-activating peptides such as 2-furoyl-LIGRL-NH2 and trypsin. However, these effects were reduced in PAR2-KO mice (Vergnolle *et al.* 2001, Kawabata *et al.* 2006). The effects described above were confirmed using IBS-patient supernatant, which is a well-known alternative stimulus for visceral pain in experimental animal models. Murine sensory neurons in culture were sensitized after the addition of IBS patient supernatant, while this effect was absent in neurons from the KO mice lacking PAR2. Furthermore, this supernatant caused visceral hypersensitivity in WT mice, but not in mice treated with a PAR2-antagonist or in PAR2-KO mice (Cenac *et al.* 2007). Also, the IBS-D supernatant was able to enhance the neuronal excitability of colonic DRGs in WT but not in PAR2-KO mice, again demonstrating the importance of PAR2 (Valdez-Morales *et al.* 2013). Another study demonstrated an increased visceral hypersensitivity in mice that received intracolonically fecal supernatants of IBS-D patients which could be suppressed after a pre-incubation with a PAR2-antagonist (Wang *et al.* 2015).

Finally, these findings are fortified by increased PAR2 expression levels found in colonic biopsy samples of both UC (Kim *et al.* 2003) and IBS patients (Liang *et al.* 2016).

Based on these literature data we can conclude that the effects of proteases on visceral pain following PAR activation is dependent on the type of receptor involved: PAR1 and PAR4 evoke antinociceptive effects while the activation of PAR2 results in pronociception.

1.10 Protease inhibitors as a new therapeutic strategy for visceral pain

In the last decades, proteases have come into the picture as a new target for drug development. An overview of the protease inhibitors approved for clinical use in diseases such as hypertension, thrombosis, AIDS, cancer and pancreatitis can be found in a Nature review by Turk *et al.* (Turk 2006). Concerning visceral hypersensitivity, serine proteases are thought to be an important class (Vergnolle 2016). Some examples of serine protease inhibitors that were already tested in clinical trials are summarized in Table 1.6, thereby emphasizing the widespread indications.

So far research groups in the field of visceral hypersensitivity have mainly focused on PAR-knockout experiments, while protease inhibitors have been investigated to a lesser extent. In this paragraph, an overview of the studies exploring the effects of protease inhibitors in visceral hypersensitivity, is given. All protease inhibitors, with their respective targets, are listed in Table 1.7.

Table 1.6. Serine protease inhibitors: examples of clinical applications in different organ systems

Category	Indication	Serine protease inhibitor *(target)*	Status	Reference
Cardiovascular	ACS	Bivalirudin *(thrombin)*	Approved	(Mavrakanas *et al.* 2015)
	AF	Rivaroxaban *(factor Xa)*	Approved	(Reddy *et al.* 2014)
		Edoxaban *(factor Xa)*	Approved	(McCormack 2015)
	VTE	Dabigatran *(thrombin)*	Approved	(Fanola 2015)
Dermatology	Herpes zoster	Argatroban *(thrombin)*	Clinical – phase II	(Fujii *et al.* 2001)
	Oral leukoplakia	BBIC *(broad specificity)*	Clinical – phase II	(Armstrong *et al.* 2013)
Hematology	Heparin-induced thrombo-cytopenia	Argatroban *(thrombin)*	Approved	(Scully *et al.* 2016)
		Fondaparinux *(factor Xa)*	Approved	
Oncology	Pancreatic cancer: CTx	Upamostat *(uPA)*	Clinical – phase II	(Heinemann *et al.* 2013)
		Nafamostat mesylate *(broad specificity)*	Clinical – phase II	(Uwagawa *et al.* 2013)
	Lung cancer: RTx	Ulinastatin *(broad specificity)*	Clinical – phase unknown	(Bao *et al.* 2015)
	Colorectal cancer: CTx	Talabostat *(fibroblast activating protein)*	Clinical – phase II	(Narra *et al.* 2007)
	Esophegal cancer: Sx	Ulinastatin *(broad specificity)*	Clinical – phase unknown	(Zhang *et al.* 2013)
Pneumology	Asthma	APC 366 *(mast cell tryptase)*	Clinical – phase II	(Krishna *et al.* 2001)
	α1 antitrypsin deficiency	α1 antitrypsin *(broad specificity)*	Approved	(Strange *et al.* 2015)
Endocrinology	Cystic fibrosis	α1 antitrypsin *(broad specificity)*	Clinical – phase II	(Twigg *et al.* 2015)
	Diabetes			(Thomas *et al.* 2016)
		Gliptins *(DPP-IV)*	Approved	
Surgery	N/A	Aprotinin *(broad specificity)*	Approved	(Royston 2015)
		Gabexate mesylate (*broad specificity)*	Clinical – Phase III	(Ono *et al.* 1999)
		Nafamostat mesylate *(broad specificity)*	Clinical – Phase IV	(Inagaki *et al.* 1999)
		Sivelestat *(neutrophil elastase)*	Clinical – Phase unknown	(Ito *et al.* 2014)

Literature search in PubMed (last updated Sept 19 2016) with MeSH terms *serine protease inhibitor – dpp-4 inhibitor - clinical trials – English – human.* ACS: Acute coronary syndrome; A: Atrial fibrillation; BBIC: Bowman Birk inhibitor concentrate; BHR: Bronchial hyperresponsiveness; cf.: compared to; CTx,: Chemotherapy; EAR: Early asthmatic response; GP: glycoprotein IIb/IIIa inhibitor; ICH: Intracranial hemorrhage; LAR: Late asthmatic response; PA: Protease activity; PI: Protease inhibitor; POC: Postoperative complications; RTx: Radiotherapy; SIRS: Systemic inflammatory respiratory syndrome; Sx: Surgery; uPA: Urokinase plasminogen activator; VTE: Venous thromboembolism.

Nafamostat mesylate or FUT-175 is a broad specificity serine protease inhibitor. In mice, visceral hypersensitivity induced by the intracolonic infusion of IBS-D fecal supernatants, could be suppressed when the supernatant was pre-incubated with nafamostat mesylate (Wang *et al.* 2015). Similar results were observed by the group of Cenac *et al.*, who used a similar, but slightly different experimental design. They used the supernatant of colonic and rectal biopsies of IBS patients instead of fecal samples and apart from a decrease in visceral hypersensitivity, they also observed less sensitization of murine neurons assessed by Ca^{2+}-imaging after a pre-incubation with FUT-175 (Cenac *et al.* 2007). On the contrary, colonic mucosal mediators from IBS patient biopsies enhanced contractions in guinea pig longitudinal muscle myenteric plexus (LMMP) preparations in vitro, but no significant effect was observed after the addition of FUT-175 (Balestra *et al.* 2012).

Camostat mesylate, another serine protease inhibitor with structural properties similar to nafamostat mesylate showed analogous results. Intragastric pre-treatment with camostat mesylate decreased hypersensitivity in rats with visceral hypersensitivity induced by acute restraint stress as well as spinal c-Fos expression (an indirect marker of neuronal activity) and fecal protease activity (Zhao *et al.* 2011, Zhao *et al.* 2014).

Also in an acute TNBS colitis model, which is a preclinical model for IBD, positive results were observed on visceral hypersensitivity after treatment with different protease inhibitors. Moussa *et al.* found a decrease in visceral sensitivity, fecal protease activity and PAR2 expression in acute TNBS colitis rats orally treated for 15 days with a fermented soy germ extract, containing phytoestrogens (isoflavones) and serine protease inhibitors (Bowman-Birk inhibitor). However, the effects on visceral sensitivity were completely reversed by simultaneous treatment with an estrogen receptor antagonist, suggesting that the effects were mostly attributed to the phytoestrogens (Moussa *et al.* 2012). The same group also demonstrated the positive effect of phytoestrogens on hypersensitivity in another animal model: this time, stress-induced hypersensitivity in female rats could be prevented by a treatment with either estradiol benzoate or fermented soy germ extract. Again, this time the positive effects

were abolished after concomitant administration of an estrogen receptor antagonist (Moussa *et al.* 2013).

In sharp contrast to the hypersensitivity seen in mice after an intracolonic infusion with fecal IBS-D supernatant, fecal supernatant of UC patients evoked hyposensitivity to colorectal distension. In IBS-D, PAR2 is stimulated due to the increased fecal serine protease activity, resulting in hypersensitivity. In addition, the activation of PAR4, by adding Cathepsin G (CatG) to the supernatant, reversed this effect. Hyposensitivity could be observed after the infusion of a UC supernatant most likely because PAR4 (activated by CatG) is predominantly activated. However, after the inhibition of PAR4 or CatG, hypersensitivity appeared and the addition of the serine protease inhibitors aprotinin/SBTI normalized sensitivity (Annahazi *et al.* 2009). Based on these studies by Annaházi *et al.*, the importance of the equilibrium between the activation of PAR2 and PAR4 in visceral sensitivity was clearly shown.

While all of the abovementioned studies investigated the effects of serine protease inhibitors on visceral pain, a study by Annaházi *et al.* looked at cysteine protease inhibitors as a possible therapeutic strategy. Repeated colonic application of fecal supernatants from IBS-C patients (with an increased cysteine protease activity, cfr. Proteases in IBS showed increased visceral sensitivity in mice, whereas pre-incubation with the cysteine protease inhibitor E64 abolished this effect (Annahazi *et al.* 2013).

A recent study by Sessenwein *et al.* observed a decreased excitability of mouse DRG neurons after the addition of commensal gastrointestinal bacteria from a healthy human donor. This change in excitability was prevented after a 2-hour pre-incubation with the serine protease inhibitor FUT-175, but not with a cysteine protease inhibitor (E64), aspartyl protease inhibitor (pepstatin), aminopeptidase inhibitor (bestatin) or metalloprotease inhibitor (EDTA) (Sessenwein *et al.* 2017). This last study clearly highlights the serine proteases as an important class in visceral pain.

To summarize this paragraph, the limited amount of data available regarding protease inhibitors and visceral pain show promising results. However, only a few broad

specificity inhibitors were investigated and in a majority of the studies, a preventive treatment scheme was used. Thus, this topic needs to be explored in more detail.

Table 1.7. Protease inhibitors investigated in experimental visceral hypersensitivity models.

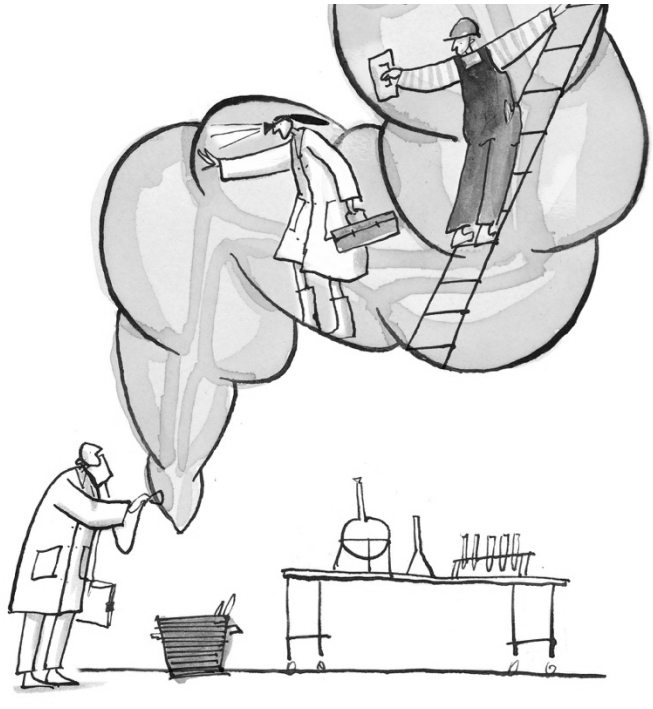
Inhibitor name	Protease class	Target(s)	References
Aprotinin	Serine proteases	Trypsin, chymotrypsin, plasmin, KLK	(Annahazi *et al.* 2009)
Bestatin	Aminopeptidases	Aminopeptidase B, N, leucine aminopeptidase	(Sessenwein *et al.* 2017)
Bowman-Birk inhibitor	Serine proteases	Trypsin, chymotrypsin	(Moussa *et al.* 2012, Moussa *et al.* 2013)
Camostat mesylate (FOY-305)	Serine proteases	Trypsin, matriptase, prostasin, plasmin, tPA, uPA, Xa, IXa, thrombin, tissue factor, complement factors, tryptase, HNE, KLK	(Zhao *et al.* 2011, Zhao *et al.* 2014)
Cathepsin-G inhibitor	Serine proteases	Cathepsin G	(Annahazi *et al.* 2009)
EDTA	Metalloproteases	Metalloproteases	(Sessenwein *et al.* 2017)
E64	Cysteine proteases	Cathepsin B, H, K, L, S, calpain, papain	(Annahazi *et al.* 2013, Sessenwein *et al.* 2017)
Nafamostat mesilate (FUT-175)	Serine proteases	Tryptase, trypsin, C1r, C1s, thrombin, KLK, plasmin	(Cenac *et al.* 2007, Balestra *et al.* 2012, Wang *et al.* 2015, Sessenwein *et al.* 2017)
Pepstatin A	Aspartate proteases	Cathepsin D, bacterial aspartic proteases, HIV proteases, pepsin, renin	(Sessenwein *et al.* 2017)
Soybean trypsin inhibitor (SBTI)	Serine proteases	Trypsin, chymotrypsin, plasmin, KLK, Xa	(Annahazi *et al.* 2009)

C1r: Complement component 1r; C1s: Complement component 1s; HNE: 4-hydoxynonenal; KLK: Kallikrein; tPA: Tissue plasminogen activator; uPA: Urokinase plasminogen activator. Adapted from http://wolfson.huji.ac.il/purification/PDF/Protease_Inhibitors/GBIOSC_ProteaseInhibHandbook.pdf

1.11 Conclusion

The available literature clearly indicates a role for proteases in the pathophysiology of visceral hypersensitivity during IBD and IBS. Although considerable research has been devoted to (ant)agonism of PARs, less attention has been paid to direct protease inhibition as a possible treatment strategy. Therefore, unraveling the exact type of proteases involved in visceral hypersensitivity and assessing the efficacy of direct serine protease inhibition as a possible therapeutic strategy towards visceral pain might be of great interest.

CHAPTER 2 - AIMS



Abdominal pain is a key feature of two major gastrointestinal disorders, inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). As discussed in **Chapter 1**, proteases have been suggested to play a key role in the pathogenesis of visceral hypersensitivity, known as the mechanism underlying abdominal pain in patients with IBD and IBS. While the few preclinical animal studies targeting proteases as a possible therapeutic strategy demonstrated positive results, their role in visceral sensitivity is not fully elucidated yet. Therefore, the general aim of this PhD thesis is to **elucidate the role of proteases in the pathogenesis of visceral hypersensitivity in IBD and IBS, with the emphasis on protease inhibition as a possible therapeutic strategy for visceral pain in IBD and IBS patients.**

All animal models used were based on inflammation, as this is known to be an important trigger for both IBD and IBS. The 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis animal model is frequently used to study the pathophysiology of both IBD and IBS. In the acute inflammatory phase, these animals mimic several features of human IBD. After the resolution of colitis, in the post-inflammatory phase, this model is frequently used to study post-inflammatory IBS. Moreover, we implemented another animal model of IBS using a different species and stimulus: neonatal acetic-acid induced IBS in mice. The visceromotor response (VMR) to colorectal distension (CRD), currently the golden standard, was used to study visceral sensitivity *in vivo* in the different animal models. The therapeutic potential of protease inhibition for visceral pain in IBD and IBS was assessed using different protease inhibitors. Therefore, several new serine protease inhibitors (UAMC-00050, UAMC-01162) developed at the Laboratory of Medicinal Chemistry (University of Antwerp) under the supervision of dr. Jurgen Joossens, prof. Koen Augustyns and prof. Pieter Van der Veken were used. Moreover, the measurement of proteolytic activity was carried out in the Laboratory of Medical Biochemistry under the supervision of prof. Ingrid De Meester and prof. Anne-Marie Lambeir. The marketed and commercially available nafamostat mesylate was used as a positive control. A detailed description of the abovementioned methodological aspects as well as all other experimental techniques used can be found in **Chapter 3**.

The serine protease pathway has been suggested to be involved in IBD and IBS, but its exact role is not fully elucidated yet. Moreover, serine protease inhibition has been put forward as a possible therapeutic strategy for visceral pain in IBD and IBS patients. However, a few considerations should be taken into account. Firstly, large spectrum protease inhibitors could possibly lead to severe side effects thereby indicating the need for protease inhibitors with a well-known inhibitor profile. Nafamostat mesylate is a commercially available broad-spectrum serine protease inhibitor which however also inhibits proteases involved in blood coagulation making it not suitable as a possible treatment strategy for IBD or IBS. Therefore, two serine protease inhibitors (UAMC-00050 and UAMC-01162) with a well-defined multitarget inhibition profile and an irreversible binding character towards some targets were developed by the Laboratory of Medicinal Chemistry (University of Antwerp).

To date, the profiles of proteolytic activity in tissues of IBD/IBS patients are still poorly characterized, making it difficult to define the best molecular targets for therapeutic interventions.

In **Chapter 4** we investigated the effects of nafamostat mesylate and UAMC-00050 on visceral sensitivity in an acute TNBS-colitis rat model for IBD. Furthermore, we determined the expression of a panel of serine proteases in colon samples of IBD animals at the protein/mRNA level.

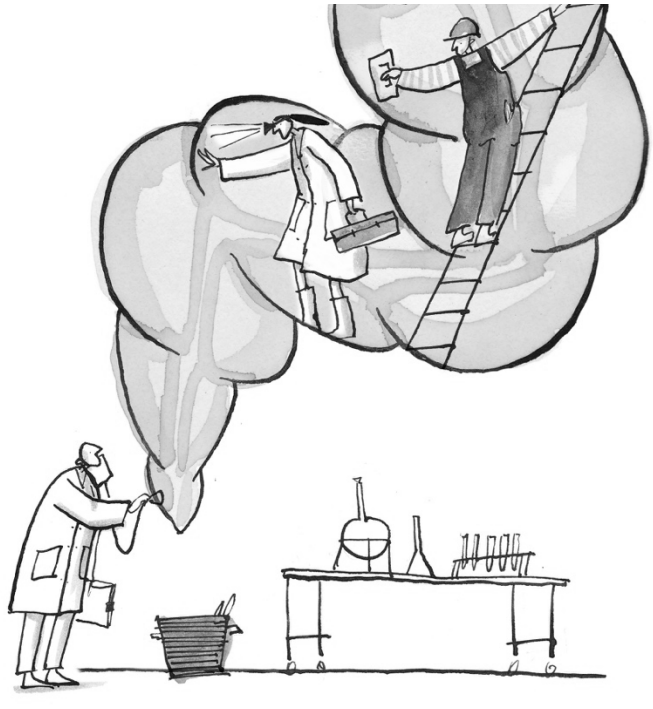
In **Chapter 5**, we investigated the *in vivo* curative effect of two newly developed serine protease inhibitors on visceral hypersensitivity in a post-TNBS-colitis rat model for IBS. We compared the effects of UAMC-00050 and UAMC-01162 to those of the commercially available serine protease inhibitor nafamostat mesylate. Furthermore, we attempted to unravel the type of serine proteases contributing to visceral hypersensitivity to define the optimal inhibition profile for serine protease inhibitors targeting abdominal pain in IBS patients.

In **Chapter 6** we investigated the effect of the newly developed compound UAMC-00050 by using a different species and model to study visceral pain in IBS. Therefore,

we investigated the effect of UAMC-00050 in a neonatal acetic acid-induced mouse model for visceral hypersensitivity in close collaboration with the Center for Neurogastroenterology at the Johns Hopkins University, Baltimore, MD, USA under the supervision of prof. P.J. Pasricha. Again, nafamostat mesylate was used as a positive control.

Finally, the overall results are summarized and discussed in **Chapter 7**, within the broader context of the role of serine proteases in visceral hypersensitivity in IBD and IBS. Additionally, we discuss the clinical relevance of our data and the potential of serine protease inhibition as a therapeutic strategy towards visceral pain in IBD and IBS patients. Finally, we elaborate on future research questions resulting from our current findings.

CHAPTER 3 – MATERIALS & METHODS



3.1 Animals

Male Sprague-Dawley rats (200-225g; Charles River, Italy) were used based on previous studies (Deiteren *et al.* 2014, Deiteren *et al.* 2014) and housed at constant room temperature (22 ± 2°C) and humidity (60%) with two rats per cage. Rats had unlimited access to water and food and were kept on a 12h:12h day-night cycle. All experiments were approved by the Ethical Committee for Animal Experiments of the University of Antwerp (EC nr. 2014-41).

Several studies have shown that female sex hormones modulate sensory signaling in response to colorectal distension (Ji *et al.* 2003, Myers *et al.* 2011) Indeed, female rats display enhanced VMRs compared to their male counterparts (Knuesel *et al.* 2016) and this sexual dimorphism could underlie the increased prevalence of IBS in the female population (Saito *et al.* 2002, Chaloner *et al.* 2013). In addition, VMRs to CRD fluctuate with the estrous cycle phase and were reported to be significantly higher during the proestrus compared to the metestrus and estrus (Ji *et al.* 2008). Therefore, to exclude cycle-dependent effects on VMRs, the estrous cycle phase should be determined in all experiments performed in female rats as previously published (Hubscher *et al.* 2005). In order to exclude this extra factor we decided to use only male rats.

C57BL/6 mice (8-12 weeks old, male and female) were originally purchased from Jackson Laboratories, USA. Breeding is maintained at the Johns Hopkins University, Baltimore, MD, USA. Mice are housed with 6 animals per cage at constant room temperature (23°C) and humidity (45%). They had unlimited access to water and food and were kept on a 12h:12h day-night cycle. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Johns Hopkins University (M016M107).

3.2 TNBS-colitis animal model

The 2,4,6-trinitrobenzenesulphonic acid (TNBS)-induced colitis animal model is frequently used to study the pathophysiology of both IBD and IBS. In the acute

inflammatory phase (maximal on day 3), these animals mimic several features of human IBD. After the resolution of colitis, in the post-inflammatory phase (from day 10 onwards), this model is frequently used to study IBS.

2,4,6-trinitrobenzenesulphonic acid (TNBS) colitis was induced at day 0 by a TNBS-enema containing 4 mg TNBS in 50% ethanol as previously described (Deiteren *et al.* 2015). After an overnight fast and under ketamine (35 mg/kg i.p.; Ketalar®) and xylazine (5 mg/kg i.p.; Rompun®) anesthesia, 0.25 ml of the TNBS solution was administered intrarectally using a flexible catheter (18G, length 4.5 cm). Control animals received 0.25 ml 0.9% NaCl intrarectally. The animals were kept in tail-up position during 1 min and were then allowed to recover in a Trendelenburg position in a temperature-controlled cage (28°C) up until 1h until they regained consciousness. No post-operative analgesia was administered as approved by the Ethical Committee for Animal Experiments of the University of Antwerp (EC nr. 2014-41). Subsequently, animals were brought back to their cages with free access to food and water. Further experiments were performed on day 3 (acute colitis, IBD) or from day 10-18 (post-colitis, IBS).

TNBS is dissolved in ethanol and subsequently intracolonically administered. Ethanol is a crucial component in this model since it temporarily breaks through the mucosal barrier allowing TNBS to penetrate into the lamina propria and the deeper colon layers. There, TNBS acts as a chemical hapten by binding to tissue proteins and eliciting a Th1-cell mediated immunological response. Subsequently, excessive amounts of pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-12 (IL-12), platelet-activating factor (PAF) and prostaglandin E2 (PGE2) are produced, resulting in acute colitis (te Velde *et al.* 2006, Qin *et al.* 2011). The acute transmural inflammation is maximal from 3 days to 1 week after instillation and revolved within 2 weeks. The TNBS model was fully characterized in our laboratory and we would like to refer to the original article by Vermeulen *et al.* for a complete overview of the endoscopic findings and other inflammatory parameters at the different time points (Vermeulen *et al.* 2011).

Acute TNBS-colitis, macroscopically characterized by a transmural colitis and demonstrating clinical features such as severe sometimes bloody diarrhea and body weight loss, shares some characteristic properties with human CD (Antoniou *et al.* 2016). Next to colonic inflammation, acute TNBS-colitis rats also display some other important IBD symptoms: delayed gastric emptying (De Schepper *et al.* 2008), increased intestinal permeability and visceral hypersensitivity (Moussa *et al.* 2012). Thus, this animal model is a good model to study visceral pain in IBD.

As stated earlier in the introduction, stress and inflammation/infection are two important triggers for IBS symptoms. Our choice was to focus on inflammation (e.g. acute gastroenteritis) as a trigger for IBS symptoms. Clinically, this subset of patients is named 'post-infectious IBS' (PI-IBS). There are two different types of reliable animal models mimicking PI-IBS: the post-infectious type is based on a bacterial intestinal infection and the post-inflammatory type is based on a chemical agent-induced inflammation. The TNBS-induced post-inflammatory animal model is a well-studied and frequently used animal model mimicking IBS symptoms (Qin *et al.* 2011).

After recovery from the initial mucosal damage, several IBS features can be identified: visceral hypersensitivity, motility dysfunction and alterations in intestinal permeability. Moreover, an increased concentration of inflammatory cells (e.g. mast cells, enterochromaffin cells) and mediators (e.g. 5-HT) is present in the colon (Menozzi *et al.* 2006, Qin *et al.* 2012). Taken together, this animal model clearly shows a lot of advantages when studying the pathophysiology of IBS. The biggest concern of researchers has been the standardization of the protocols (Qin *et al.* 2011, Qin *et al.* 2012). Here above, we described the protocol that has been standardized and frequently used in our lab (Deiteren *et al.* 2014).

3.3 Acetic-acid induced colitis animal model

Early life events such as psychological stress, infections or dietary intolerance are suggested to be associated with long-term changes in the gastrointestinal tract nervous system leading to the generation and maintenance of visceral hypersensitivity

in IBS patients. This theory has also been used for the generation of IBS animal models where neonatal animals are exposed to different initiating events (e.g. maternal separation or colonic inflammation) (Winston *et al.* 2007).

Mild chemical irritation of the colon using a relatively innocuous stimulus was established using diluted acetic acid (0.5%) in the neonatal period without causing a major inflammatory response. This resulted in long-lasting visceral hypersensitivity in their adulthood (8-12 weeks) in the absence of histological evidence of inflammation (Winston *et al.* 2007). The intracolonic administration of acetic acid in a much higher concentration (4%) in adult animals results in severe inflammation as well as the sensitization of pelvic afferent nerves, but it is unknown whether this sensitization is due to the presence of inflammation or a direct effect of acetic acid itself (Winston *et al.* 2007).

At postnatal day 10, B57BL/6 mice were infused with a 0.5% acetic acid (AA) solution in 0.9% NaCl, as previously described (Winston *et al.* 2007). Under awake, unanesthetized conditions, mice were administered 20µL of the AA solution into the colon 1 cm from the anus using a 100µL syringe (SGE, Australia) attached to a flexible polyethylene tubing (Intramedic Clay Adams, PE-10, 2 cm length) lubricated with glycerol. Control animals received an equal volume of 0.9% NaCl. Mice were weaned at the age of 21 days and used to assess visceral sensitivity at 8-12 weeks old.

3.4 Inflammatory parameters

3.4.1 In vivo colonoscopy

Colonoscopy was performed with a pediatric endoscope (Olympus GIF-N30, Olympus Europe GmbH) as previously described (Vermeulen *et al.* 2011). Under ketamine/xylazine anesthesia (35/5 mg/kg, i.p.), the lubricated tip of the endoscope was introduced into the colon and advanced under endoscopic view until the hepatic flexure was reached (± 10 cm proximal to the anus), as shown in figure 3.1. During withdrawal of the endoscope, intestinal inflammation was evaluated using a

standardized scoring system, as shown in table 3.1. The total score (0-19) was given by summation of the subscores for the degree of inflammation, extent of disease, stenosis, edema and bleeding (Vermeulen *et al.* 2011). While there is certainly a risk of perforation in severe acute TNBS colitis, in our model a mild to moderate colitis was induced by TNBS colitis. We had no casualties or perforations during our experiments in the rats.

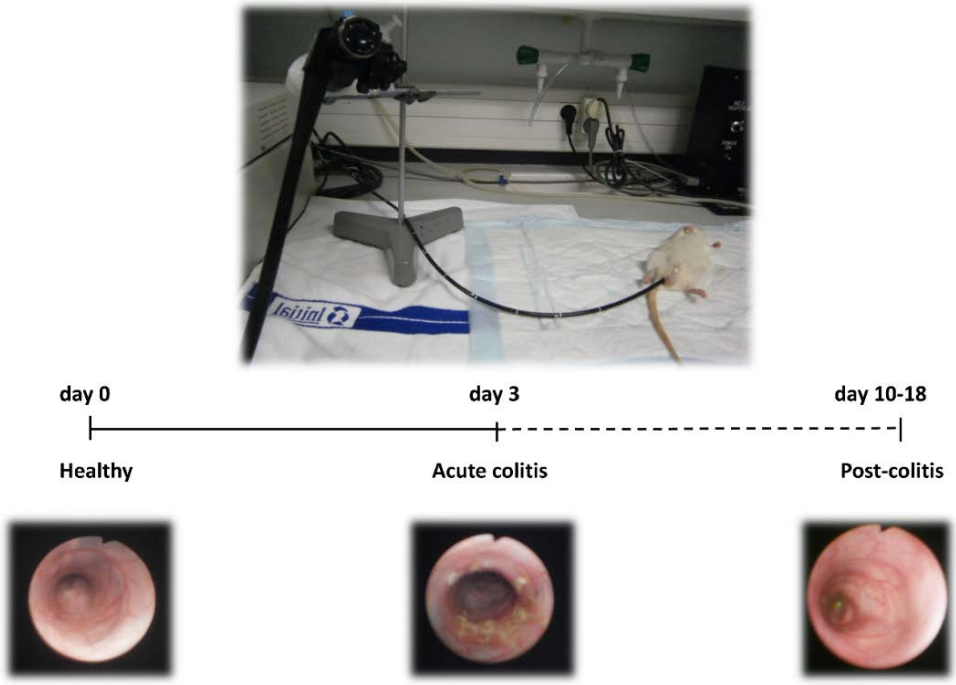


Figure 3.1. In vivo colonoscopy.

The picture on top shows the set-up of a colonoscopy experiment performed with pediatric endoscope (Olympus GIF-N30, Olympus Europe GmbH. The timeline below demonstrates the follow-up of colitis using in vivo colonoscopy with representative images of the colon at different time point: day 0 shows a healthy colon with an intact mucosa and visible blood vessels, day 3 shows a mild colitis with ulcerations and day 10-18 shows a healed colonic mucosa in the post-colitis phase. Based on (Vermeulen *et al.* 2011)

Table 3.1. Colonoscopic scoring system.

Item	Colonoscopic finding	Score
Inflammation	Normal aspect of the mucosa	0
	Hyperemia	1
	Ulceration occupying max ¼ of the luminal circumference	2
	Ulceration occupying max ¼- ½ of the luminal circumference	3
	Ulceration occupying >½ but not the full luminal circumference	4
	Circular ulcer	5
	Cylindric ulcer	6
Extent of the disease	Length 0-10 cm	0-10
Stenosis	Absent	0
	Present	1
Edema	Absent	0
	Present	1
Bleeding	Absent	0
	Present	1
Total score		**0-19**

Adapted from (Vermeulen *et al.* 2011)

3.4.2 Post-mortem inflammatory markers

Macroscopy

At the end of the experiments, the rat was sacrificed and the colon was isolated and rinsed with ice-cold Krebs solution. The colon was opened longitudinally along the mesenteric border, pinned out on a Petri dish and inspected with a binocular (Zeiss Jena, Jena, Germany). Subsequently, the colon was macroscopically evaluated using a validated scoring system (total score 0-10), as shown in table 3.2 (Vermeulen *et al.* 2011).

Table 3.2. Macroscopic scoring system

Macroscopic finding	Score
Normal aspect of the mucosa	0
Localized hyperemia, no ulceration(s)	1
Ulceration(s) without hyperemia or bowel wall thickening (inflammation)	2
Ulceration(s) with hyperemia or bowel wall thickening (inflammation) at 1 site	3
Ulceration(s) with hyperemia or bowel wall thickening (inflammation) at 2 or more sites	4
Major sites of damage extending >1 cm along the length of the colon	5
Major sites of damage extending >2 cm along the length of the colon	6
Increase score by 1 for every additional cm involved	7-10
Total score	**0-10**

Adapted from (Vermeulen *et al.* 2011)

Microscopy

For microscopic evaluation, a colonic segment of approximately 1 cm² was fixed in 4% formaldehyde for 24h and subsequently preserved in a 60% isopropanol solution. Afterwards, the colon was embedded in paraffin and cross sections of 5µm were stained with hematoxylin & eosin (H&E). The microscopic slides were visualized under a light microscope (Olympus BX40, Olympus Europe GmbH) and a histological score of 0-10 was given using a previously published scoring system, as shown in table 3.3 (Vermeulen *et al.* 2011).

Table 3.3. Microscopic scoring system

Microscopic finding	Normal	Minimal	Mild	Maximal
Inflammatory infiltrate	0	1	2	3
Number of infiltrated layers	0	1	2	3
Mucosal damage	0	1	2	3
Mucosal edema	Absent = 0; Present = 1			
Total score				**0-10**

Adapted from (Vermeulen *et al.* 2011)

Myeloperoxidase activity

Myeloperoxidase (MPO) is the most abundant proinflammatory enzyme present in the azurophilic granules of neutrophilic granulocytes. This enzyme catalyzes the reaction

of hydrogen peroxide and chloride anions to produce hypochlorous acid (Pulli *et al.* 2013). The colonic MPO activity, frequently used as a marker of inflammation, was measured using a previously published method (Vermeulen *et al.* 2011). For the assessment of the MPO activity, a representative colon segment (± 1 cm^2) was preserved at -80°C until the day of the analysis. A potassium phosphate buffer (pH 6) with 0.5% hexadecyltrimethylammonium bromide was added to the colon sample in a ratio of 100 ml/5 g tissue. Subsequently, the tissue was homogenized during 30s and underwent 2 sonication and freeze-thaw cycles. The suspension was centrifuged at 15000g during 15 min at 4°C. Afterwards, 0.1 ml of the supernatant was added to 2.9 ml o-dianisidine solution (16.7 mg o-dianisidine in 1 ml 0.9% NaCl, 98 ml potassium phosphate buffer pH 6 and 1 ml 0.05% hydrogen peroxide solution). In the presence of MPO, o-dianisidine is oxidized, followed by a color change (Pulli *et al.* 2013). This change in absorption is measured spectrophotometrically at 460 nm during 60 s using an ATi Unicam UV2 UV/VIS spectrophotometer. The MPO activity is expressed as unit/g tissue and 1 unit is defined as the quantity needed to transform 1 µmol H_2O_2 to H_2O within 1min at 25°C.

3.5 Visceromotor response to colorectal distension

Both in preclinical as well as in clinical research, visceral sensitivity is mostly studied by assessing sensory responses to colorectal balloon distension, the golden standard technique in patients (Keszthelyi *et al.* 2012). The visceromotor response (VMR) is a nociceptive reflex with a contraction of the abdominal musculature as a result of a visceral stimulus, e.g. a colorectal balloon distension (Ness *et al.* 1988). For the quantification of these contractions, patients will be asked to indicate their pain perception on a visual analog scale (VAS). Unfortunately, this is practically impossible in animal models but can be overcome by the implantation of electromyographic (EMG) electrodes in the abdominal musculature. A stimulus that is perceived as more intense results in a greater contraction of the abdominal muscles and thus an increased EMG signal.

3.5.1 VMR in rats

The VMR to colorectal distension (CRD) was objectively assessed by EMG electrodes that were implanted into the abdominal musculature 3 days prior to the VMR measurement, using a previously published method (Vermeulen *et al.* 2013, Deiteren *et al.* 2014, Deiteren *et al.* 2015). Under ketamine (35 mg/kg) and xylazine (5 mg/kg) anesthesia, two Teflon-coated electrodes (Cooner wire G-32) were implanted into the external oblique abdominal muscle via an incision of ± 2 cm proximal to the right inguinal ligament. The electrodes were tunneled subcutaneously and exteriorized between the shoulder blades. The loose ends were protected with parafilm and a metal cap. Rats were allowed to recover in a temperature-controlled cage (28°C) up until 1h until they regained consciousness. No post-operative analgesia was administered as approved by the Ethical Committee for Animal Experiments of the University of Antwerp (EC nr. 2014-41). Subsequently, animals were brought back to their cages with free access to food and water.

Three days later, the VMR registration was executed in awake rats. Hereby, the animals were placed into a restraining glove. A lubricated balloon (length 4 cm) was introduced into the colorectum, approximately 0.5 cm of the anal sphincter, fixed to the tail and connected to a barostat (Distender Series II Barostat, G&J Electronics, Canada). Subsequently, the electrodes were attached to a data-acquisition system to record the EMG abdominal muscle activity. During a standardized distension protocol, phasic balloon distensions are induced through a pressure-controlled air inflation. The abdominal EMG signal is optimized during three test distensions (30 mmHg, 20s, 60s interval), followed by the completion of the experimental protocol (10-20-30-40-60 mmHg, 20s, 240s interval). The EMG signal is registered, amplified (Neurolog, Digitimer Ltd, UK) and digitalized (CED 1401, Cambridge Electronic Design, UK). The EMG recordings were analyzed using Spike2 V5.15 (Cambridge Electronic Design, UK). A representative trace is shown in figure 3.2. The VMR was quantified using the area under the curve (AUC) of the EMG signal during the distension (20s), corrected for the EMG signal before the distension (20s), as shown in figure 3.3.

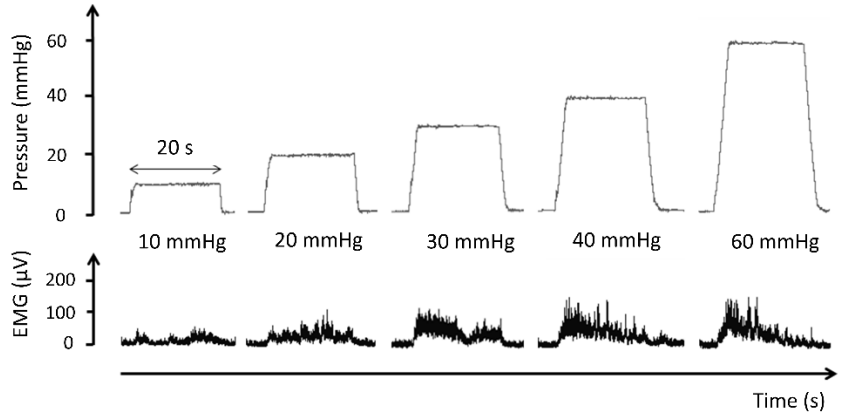


Figure 3.2. Representative EMG recording of a control rat.

The upper panel shows the graded and pressure-controlled distensions. The lower panel shows the corresponding EMG signals. EMG, electromyographic.

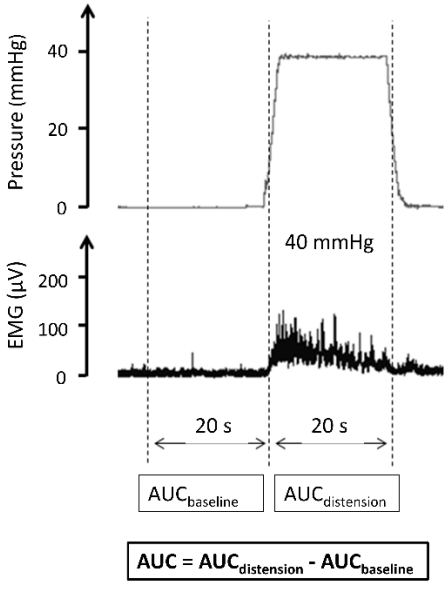


Figure 3.3. Quantification of the EMG signal.

The upper panel shows a distension at 40 mmHg. The lower panel shows the corresponding EMG signal. The absolute AUC is determined by subtracting the AUC measured during 20 s of distension ($AUC_{distension}$) from the AUC measured 20s before distension ($AUC_{baseline}$). AUC, area under the curve; EMG, electromyographic.

3.5.2 VMR in mice

The VMR procedure in mice is similar to that in rats, however a few modifications need to be considered. Similarly, VMR to CRD was evaluated by EMG electrodes that were

implanted into the abdominal musculature 5 days prior to VMR assessment. Under isoflurane anesthesia (4% induction, 1.5% maintenance), a small incision of ± 1 cm was made superior to the right inguinal ligament thereby exposing the external oblique abdominal muscle. Subsequently, two electrode wires (Cooner Wire AS631, 8 cm length) were sutured into the musculature with a small space between them. The electrodes were subcutaneously tunneled, exteriorized between the shoulder blades and protected with parafilm. Mice were allowed to recover from surgery for 5 days before the VMR assessment.

At the day of the VMR registration, mice were anesthetized using isoflurane anesthesia (4% induction, 1.5% maintenance). The implanted electrodes were elongated using another electrode wire (AE Medical Corporation Myo/wire temporary cardiac pacing wire nr. 021-001) and a ground electrode was attached to the tail base. A balloon (2 cm length) lubricated with glycerol was introduced into the colorectum and fixed to the tail base using parafilm. Afterwards, the mouse was put into a restrainer and allowed to recover from anesthesia for 30 min. The balloon was connected to a sphygmomanometer (Labtron Equipment Ltd, Fleet, UK) and the electrode wires were connected to a data-acquisition system to record the EMG activity. Balloon distensions were induced using the sphygmomanometer and with at the following pressures protocol: 15-30-50-70 mmHg, during 10s, with the EMG response to each pressure measured two times. The EMG signal is registered, amplified (Iso-Dam 8A, World Precision Instruments, Sarasota, FL, USA) and digitized (CED Micro 1401 mk II, Cambridge Electronic Design, Cambridge, UK). Moreover, audiological monitoring of the animals was performed during the VMR assessment (audio monitor, Grass Instruments Astro-Med Inc., West Warwick, RI, USA). Finally, the EMG recordings were visualized (TDS 2022B Two Channel Digital Storage Oscilloscope 200 MHz 2GS/s, Tektronix, Beaverton, OR, USA) and analyzed using Spike2 (Cambridge Electronic Design, Cambridge, UK). The VMR signal was quantified as the modulus during distension (10s), corrected for the modulus before distension (10s, baseline).

3.6 Colonic compliance

Colonic compliance can be defined as the degree of elasticity of an organ as a result of a force executed on that organ. The compliance is the ratio of a volume change (ΔV) relative to a pressure change (ΔP). The greater the change in pressure due to a change in volume, the lower the compliance, and thus the lower the elasticity of the colon. Ideally, the colonic compliance is high and thus the colon highly elastic. A decrease in compliance can contribute to an increase in visceral sensitivity (Bharucha *et al.* 2001, Camilleri *et al.* 2001). On one hand, TNBS can induce a reduction in colonic compliance, leading to a higher VMR response. On the other hand, the active compound can decease the VMR response by directly executing an effect on the nociceptive pathways or by changing the colonic compliance. To exclude a direct effect of the compounds on colonic compliance resulting in an effect on visceral sensitivity, all VMR measurements in rats were supplemented with a compliance measurement, using a previously published method (Vermeulen *et al.* 2013, Deiteren *et al.* 2014, Deiteren *et al.* 2015).

Under pentobarbital anesthesia (45 mg/kg, i.p. Nembutal®), a lubricated balloon (length 4 cm) was introduced into the colorectum of the rat approximately 0.5 cm of the anal sphincter and fixed to the tail. Subsequently, the balloon was filled with increasing volumes of water (0-0.5-1.0-1.5-2.0 ml, 80s interval). The corresponding pressure in the colon of the rat was measured and the resulting pressure-volume curves display colonic compliance.

3.7 Quantitative RT-PCR

Rats were sacrificed and distal colonic segments of approximately 50 mg were collected. Dorsal root ganglia (DRGs) T13-L2 (splanchnic colonic afferents) and L6-S1 (pelvic colonic afferents) were harvested bilaterally. Briefly, the spinal column was dissected, cut down the mid-line and after removal of the spinal cord and meninges, DRGs of interest were extracted as previously described (Deiteren *et al.* 2015, Sleigh *et al.* 2016). Samples were snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from colon or DRG (Isolate II RNA Mini Kit, Bioline, London, UK) and RNA

was converted to cDNA by reverse transcription (SensiFAST cDNA Synthesis Kit, Bioline, London, UK). A Taqman gene expression assay (ThermoFisher, Waltham, MA, USA, list of primers in table 3.4) was executed on an ABIPrism 7300 sequent detector system (Applied Biosystems, Foster City CA, USA) in a 25µl reaction volume containing 2µl cDNA, 12.5µl Taqman Universal PCR Master Mix (ThermoFisher, Waltham, MA, USA), 1.25µl Taqman assay probe and 9.25µl RNase-free H_2O. Gene expression assays were executed following the MIQE guidelines (Bustin *et al.* 2009). GAPDH and β-actin were reference genes. The parameters for PCR amplification were 50°C for 2min, 95°C for 10min followed by 40 cycles of 95°C for 15s and 60°C for 1min (Bustin *et al.* 2009). The outcome values were analyzed using qBASE^{PLUS} software (Biogazelle N.V., Zwijnaarde, Belgium).

Table 3.4. Taqman primers used for qPCR analysis of colonic and DRG samples

Protein	Gene ID
Tryptase αβ1	Rn00570928_m1
Matriptase	Rn00586242_m1
Cathepsin G	Rn01489144_g1
Urokinase plasminogen activator	Rn00565261_m1
Kallikrein 2	Rn00820615_m1
Kallikrein 4	Rn01498534_g1
Kallikrein 8	Rn01476995_m1
Trypsin 1 (PRSS1)	Rn04223942_s1
Trypsin 2 (PRSS2)	Rn01773459_g1
Trypsin 3 (PRSS3)	Rn00597545_m1
PAR2	Rn00588089_m1
PAR4	Rn00587480_m1
TRPA1	Rn01473803_m1
TRPV1	Rn00583117_m1
TRPV4	Rn00576745_m1
GAPDH	Rn01775763_g1
β-actin	Rn00667869_m1

3.8 Immunohistochemistry for mast cell tryptase

Colonic samples were fixed (4% formaldehyde), embedded in paraffin and cut into 5 µm sections. Sections were pretreated with trypsin (37°C, 10 min) and citrate buffer pH6 (0.154g citric acid.H_2O and 1.235g trisodiumcitrate.$5H_2O$ in 500 ml distilled water) (microwave, 10 min). Slides were overnight incubated in a moist chamber with mouse mast cell tryptase monoclonal antibody (1:10.000). After Tris-saline buffer wash, slides were incubated (30 min) with biotinylated goat anti-mouse IgG antibody (1:200) and rat serum (1:20) and incubated (60 min) with a Vectastain® avidin-biotin complex. Slides were washed, developed in an aminoethyl carbazole (AEC) solution (20mg AEC in 24 ml DMSO, 200 ml acetate buffer (1.64g sodium acetate in 1L distilled water titrated until pH5 with acetic acid) with 40µL hydrogen peroxide (10 min), counterstained with hematoxylin (2 min) and covered. Colon sections were screened (100x magnification) and the total number of tryptase positive cells in the mucosa was quantified using ImageJ 1.51 J8 (National Institutes of Health, USA) and expressed per mm^2.

3.9 Immunohistochemistry for PAR2, PAR4 and TRPA1

Distal colon samples were fixed in 4% paraformaldehyde and further processed for cryo-embedding. Cryo-sections (12µm) were thaw-mounted on poly-L-lysine-coated slides and dried at room temperature for 2 h. Subsequently, these sections were permeabilized and blocked in 0,01M phosphate-buffered saline (PBS) containing 0,3% Triton X-100 and 10% normal horse serum for one hour at room temperature, after which they were incubated with primary antibodies for 16 h at 4°C. All antibodies were diluted in 0,01M PBS containing 0,3% Triton X-100 and 1% bovine serum albumin. The following primary antibodies were used: rabbit anti-PAR4 (1/200, Alomone labs), rabbit anti-PAR2 (1/100, Santa Cruz), rabbit anti-TRPA1 (1/1000, Abcam) and goat anti-CGRP (1/3000, Abcam). After several washing steps with 0,01M PBS, visualization was done using CY3-conjugated donkey anti-rabbit immunoglobulins (1/1000, Jackson ImmunoResearch) or CY5-conjugated donkey anti-goat immunoglobulins (1/1000, Jackson ImmunoResearch). Specificity was confirmed using negative controls and

isotype controls. High-resolution images were obtained on a Leica TCS SP8 confocal laser scanning microscope and images were processed using the ImageJ software. The abovementioned experiments were carried out in collaboration with the Laboratory of Histology and Cell Biology at the University of Antwerp under the supervision of prof. J-P Timmermans.

3.10 Total protease activity in fecal supernatants – azocasein assay

Fecal supernatants (10 µl) were incubated with 70 µl reaction buffer (0.15 M NaCl, 20 mM Tris-HCl, pH 8.3) and 70 µl azocasein (0.5%) for 20 min at 40°C. Subsequently, the reaction was stopped by adding 70 µl trichloroacetic acid (10%). The samples were then centrifuged at 4000 rpm during 2 min and the absorption of the supernatant was measured on the spectrophotometer at 340 nm. The principle of the azocasein assay is graphically displayed in figure 3.4. Enzymatic activities of the supernatants were normalized to protein content, assessed with Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL, USA).

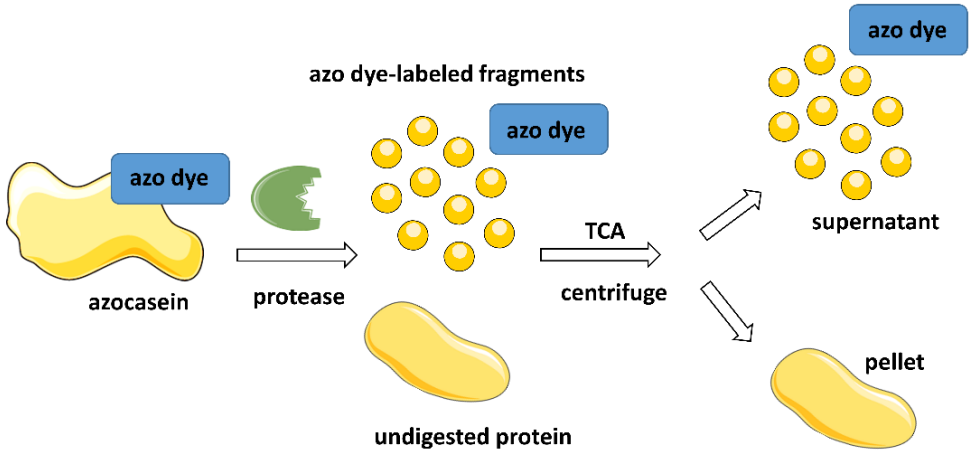


Figure 3.4. Principle of the azocasein assay.

Protease activity results in the cleavage of azocasein into smaller fragments. The reaction is stopped by the addition of TCA. After centrifugation, the undigested protein forms a pellet and the absorption of the supernatant is measured on the spectrophotometer at 340 nm. TCA; trichloroacetic acid.

3.11 Proteolytic activities

To assess the activity of serine proteases in post-inflammatory visceral hypersensitivity, trypsin-like, chymotrypsin-like, neutrophil elastase, pancreas elastase and kallikrein activities were determined in colonic and fecal samples. Distal colon samples of approximately 50 mg were taken upon sacrifice, rinsed with Krebs solution, snap-frozen within 5 min and stored at -80 °C until further processing. The preparation started by crushing the colonic samples on dry ice using liquid nitrogen to avoid loss of activity due to temperature increase. Afterwards the samples were dissolved in lysis buffer (composition depending on assessed activity) for 15 min and centrifuged (4°C, 5 min, 12000 x g). The supernatant was collected and used immediately for the activity measurement. Lysis buffer for trypsin-like activity contained 1% octylglucoside (Roth, Germany) and 0,05% heparin (Sigma Life Science, Germany) in 50 mM Tris-HCl pH 7,4. The buffers for the other enzyme activities were all the same (1% octylglucoside in 50 mM Tris-HCl pH 7,4) except for the addition of 0.1% heparin in the buffer for chymotrypsin-like activity.

Boc-Gln-Ala-Arg-AMC (75 µM) and n-Tosyl-Gly-Pro-Arg-AMC (100 µM) (both Bachem) were used to explore the trypsin-like activity. Colonic supernatant was placed in a cold microtiterplate and preheated (37°C) substrate solutions in 50 mM Tris-HCl pH 8,0 were added to the samples to start the incubation. Fluorescence was measured kinetically for 20 minutes at 37°C on Tecan Infinite F200 Pro. The activities (U L-1) were transformed to specific activities (U g-1) using the protein concentration in the samples as determined by Bradford analysis. Chymotrypsin-like, neutrophil elastase, pancreas elastase and kallikrein activities were measured using Suc-Ala-Ala-Pro-Phe-AMC (0,45 mM), Suc-Ala-Ala-Pro-Val-AMC (0,45 mM), Suc-Ala-Ala-Ala-AMC (0,45 mM) and H-Pro-Phe-Arg-AMC (0,45 mM) (all Bachem) in 50 mM Tris-HCl pH 8,0 respectively. The protocol for these activity experiments was identical to the one for trypsin-like activity except for a 5 min pre-incubation of the samples.

Experiments to determine proteolytic activities in the fecal samples were performed in the same way except for the lysis buffer, which did not contain heparin or

octylglucoside, and lysis time, which was 10 min instead of 15 min. The abovementioned experiments were carried out in collaboration with the Laboratory of Medical Biochemistry at the University of Antwerp under the supervision of prof. I De Meester.

3.12 Statistical analysis

All data are presented as mean ± SEM. The number of animals (n) was calculated using a power analysis and determined to be 10-12 animals per group. Based on previous experience, we included the following parameters: a 30% effect of the studied compound (μ0=1000, μ1=700), a standard deviation of σ =325, a significance level of α=0,05 and a power of 1-β=0,80. For each group the exact number of animals per group is indicated as n = x. The statistical analysis was performed using SPSS (version 24.0, IBM). Results (VMR and compliance) were analyzed using a Generalized Estimating Equations (GEE) test with Least Significant Difference (LSD) post-hoc test. Inflammatory parameters and immunohistochemistry results were analyzed using a Two-way ANOVA with Student-Newman-Keuls (S-N-K) post-hoc test, as appropriate. qPCR results were analyzed using unpaired Student's t-test or Two-way ANOVA with S-N-K post-hoc test. Proteolytic activities were analyzed using a Mann-Whitney U test. A p-value <0.05 was considered statistically significant. Representative graphs were made using GraphPad Prism 7.0.

3.13 Active compounds and other reagents

3.13.1 Active compounds

Nafamostat mesylate, also named FUT-175 (Selleckchem via Bio-connect, Huissen, The Netherlands), is a broad-spectrum serine protease inhibitor, commercially available in Japan for the treatment of acute pancreatitis and disseminated intravascular coagulation (Isozaki *et al.* 2006).

UAMC-00050 and UAMC-01162 are serine protease inhibitors with a well-defined multi-target inhibition profile, developed by the Medicinal Chemistry Laboratory of the

University of Antwerp (Joossens *et al.* 2007). These compounds were firstly patented under WO2007045496 (Augustyns *et al.* 2007) and recently patented for their use in PAR-related diseases under WO2017198753 (inventors: Augustyns K, Joossens J, Van Der Veken P, Cos P, Joossens C, De Winter BY, Ceuleers H, Van Spaendonk H) (Augustyns *et al.* 2017).

These compounds slightly differ in both structural properties as well as in inhibitory potency; UAMC-00050 (*bis*(acetamidophenyl)guanidinophenylethylphosphonate) is a more potent inhibitor compared to UAMC-01162 with a highly similar structure, namely diphenyl guanidinophenylethylphosphonate. The inhibition profiles (displayed as IC_{50} values) of the three serine protease inhibitors used in the present study are presented in table 3.5 and described in detail previously (van Soom *et al.* 2015).

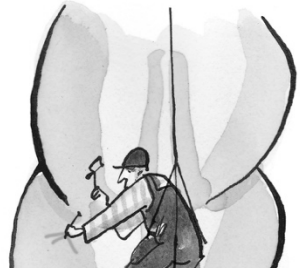
Table 3.5. Inhibitory profiles for the serine protease inhibitors UAMC-00050, UAMC-01162 and nafamostat mesylate

Proteases	UAMC-00050 IC_{50} (µM)	UAMC-01162 IC_{50} (µM)	Nafamostat mesylate Est. IC_{50} (µM)
uPA	0.0042	0.0031	< 0.001
tPA	7	23	> 2.5
plasmin	0.9	13	± 0.05
thrombin	0.39	17	± 0.5
FXa	100	± 250	± 2
FXIIa	1.61	> 2.5	± 0.1
matriptase	0.0025	0.083	< 0.001
tryptase	0.028	0.093	< 0.001
cathepsin G	0.12	0.33	± 0.25
HNE	> 2.5	> 2.5	> 2.5
plasma kallikrein	> 2.5	> 2.5	± 0.005
KLK1	> 2.5	> 2.5	± 1
KLK2	0.11	3	± 0.1
KLK4	0.0017	0.009	± 0.005
KLK8	0.0016	0.028	± 0.015
AChE	> 20	> 10	> 2.5

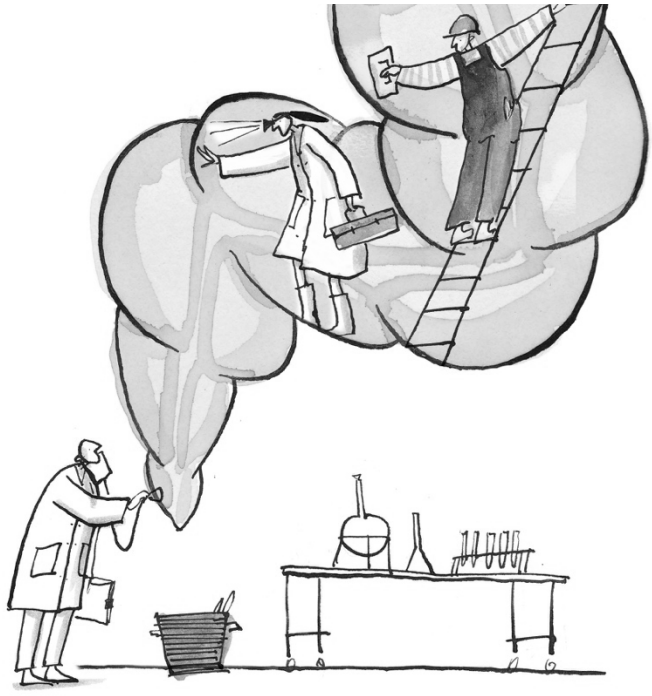
Data are presented as mean. Est.; estimated, FXa; factor Xa, FXIIa; factor XIIa, HNE; human neutrophil elastase, IC_{50}; half maximal inhibitory concentration, KLK; kallikrein, tPA; tissue plasminogen activator, uPA; urokinase plasminogen activator.

3.13.2. Other chemicals and reagents

Mouse mast cell tryptase monoclonal antibody, rabbit anti-TRPA1 and goat anti-CGRP were purchased from Abcam (Cambridge, UK). Eosin and 100% ethanol were purchased from Acros Organics (Geel, Belgium). Isoflurane (Forane®) was purchased from Baxter (Deerfield, IL, USA). Xylazine (Rompun®) was purchased from Bayer (Bayer, Leverkusen, Germany). 0.9% Sodium chloride solution was purchased from Braun (Diegem, Belgium). Pentobarbital 60 mg/ml (Nembutal®) was purchased from Ceva (Brussels, Belgium). Acetic acid, citric acid.H_2O, dimethyl sulfoxide (DMSO), formaldehyde, hematoxylin, isopropanol, potassium dihydrogen phosphate, potassium hydrogen phosphate, sodium acetate, sodium chloride, trisodiumcitrate.$5H_2O$ were purchased from Merck (Darmstadt, Germany). Ketamine (Ketalar®) was purchased from Pfizer (Puurs, Belgium). Aminoethyl carbazole (AEC), azocasein, heparin, hexadecyltrimethylammonium bromide (HTAB), hydrogen peroxide, o-dianisidine hydrochloride, trichloroacetic acid (TCA) 2,4,6-trinitrobenzenesulphonic acid (TNBS) and trypsin were purchased from Sigma-Aldrich (Steinheim, Germany). Goat anti-mouse IgG antibody, rat serum and Vectastain® avidin-biotin complex were obtained from Vector Laboratories (Vector Laboratories, CA, USA). The Krebs-Ringer solution has the following composition: 118.3 mM NaCl, 4.7 mM KCl, 1,2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 2 mM $NaCHO_3$, 0.026 mM CaEDTA and 11.1 mM glucose. Rabbit anti-PAR4 was purchased from Alomone Labs (Jerusalem, Israel). Rabbit anti-PAR2 was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). CY3-conjugated donkey anti-rabbit immunoglobulins and CY5-conjugated donkey anti-goat immunoglobulins were obtained from Jackson ImmunoResearch (Ely, UK). Octylglucoside was purchased from Roth (Wiesbaden, Germany). Boc-Gln-Ala-Arg-AMC, n-Tosyl-Gly-Pro-Arg-AMC, Suc-Ala-Ala-Pro-Phe-AMC, Suc-Ala-Ala-Pro-Val-AMC, Suc-Ala-Ala-Ala-AMC, H-Pro-Phe-Arg-AMC were obtained from Bachem (Bubendorf, Switzerlan



CHAPTER 4 – THE ROLE OF SERINE PROTEASES IN AN ACUTE TNBS-COLITIS RAT MODEL FOR VISCERAL HYPERSENSITIVITY



Adapted from: Ceuleers H, De Man JG, Joossens J, Van Der Veken P, Augustyns K, Francque S, De Meester I, De Winter BY. The serine protease inhibitors nafamostat mesylate and the newly developed UAMC-00050 decrease visceral hypersensitivity in a rat model for acute colitis. Will be submitted to Neurogastroenterology & Motility.

4.1 Abstract

Background and aims

Serine proteases are believed to play a key role in the origin of abdominal pain in IBD patients, but up until now their exact role is insufficiently elaborated. Therefore, the aim of this study was (1) to investigate the efficacy of a pharmacological intervention using the serine protease inhibitors nafamostat mesylate and the newly developed UAMC-00050 and (2) to examine colonic mRNA/protein levels of a panel of serine proteases in an experimental rat model for IBD.

Methods

On day 0, rats were intrarectally instilled with 2,4,6-trinitrobenzenesulphonic acid (colitis) or 0.9% NaCl (control). All experiments were performed on day 3. Visceral sensitivity was quantified by visceromotor responses to colorectal distension, 30 min after an intraperitoneal injection with the serine protease inhibitors nafamostat, UAMC-00050 or vehicle. Colonic samples were used to quantify the mRNA expression of a panel of serine proteases and mast cell tryptase by immunohistochemistry. Fecal protease activity was determined using an azocasein assay.

Results

Acute TNBS-colitis rats displayed visceral hypersensitivity. Nafamostat and UAMC-0050 significantly reduced VMRs in colitis rats. Nafamostat also decreased visceral sensitivity in controls. A small albeit not significant increase in tryptase expression was observed in colon samples of acute colitis rats both at the mRNA and protein level, while matriptase was significantly downregulated at the mRNA level. Fecal protease activity was significantly increased in acute colitis animals.

Conclusions

These data highlight that serine proteases could be put forward as a possible target in the search for novel treatments for abdominal pain in IBD patients. Both nafamostat and UAMC-00050 showed positive results, however, UAMC-00050 seems to have more

potential as a further potential drug, since nafamostat also affected visceral sensitivity in control animals.

4.2 Introduction

Inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC), are characterized by acute flares of inflammation and intermittent periods of remission (Podolsky 2002). The prevalence of IBD is increasing at strong pace in the Western world but also in developing countries. Due to the chronic character of the disease and the absence of a complete cure, IBD has an enormous impact on the quality of life of patients (Burisch *et al.* 2013, Ananthakrishnan 2015).

Visceral hypersensitivity, the key factor underlying abdominal pain, is a main symptom in 50-70% of the IBD patients, highly impacting their general wellbeing (Srinath *et al.* 2012). Not only during acute flares of inflammation, but also in periods of remission, 33-57% of the patients report chronic abdominal pain. Although visceral pain is thus a common and cumbersome symptom in IBD patients, the current treatment for IBD focusses on the anti-inflammatory and immunosuppressive properties as described in Chapter 1.1 (De Schepper *et al.* 2008). Nowadays, to alleviate this pain, analgesics such as antispasmodics, narcotic opiates and antidepressants are used. However, their effects are minimal and many side effects are observed (Srinath *et al.* 2012), thereby emphasizing the need for new treatments specifically tackling visceral pain and its pathogenesis.

The exact mechanism behind the arousal of abdominal pain in IBD is currently unknown, but gastrointestinal inflammation has been suggested as an important trigger. Inflammatory cells (e.g. mast cells, T-/B-cells) release excessive amounts of mediators (e.g. histamine, serotonin, cytokines, proteases) on their turn sensitizing the sensory pathways, leading to changes in afferent neuron signaling, spinal cord and central nervous system pain processing, eventually resulting in visceral hypersensitivity (Bielefeldt *et al.* 2009). Several research groups have been investigating the role of selective inflammatory mediators in visceral hypersensitivity, but only in recent years

the role of proteases is being investigated to a larger extent (Ceuleers *et al.* 2016, Vergnolle 2016).

We focus our study on serine proteases. Numerous research groups have reported increased serine protease expression and activity in plasma, fecal and colonic biopsy samples from IBD mice and IBD patients compared to healthy controls (Gouni-Berthold *et al.* 1999, Jenkins *et al.* 2000, Raithel *et al.* 2001, Stadnicki *et al.* 2003, Hansen *et al.* 2005, Andoh *et al.* 2006, Cenac *et al.* 2007, Langhorst *et al.* 2008, Motta *et al.* 2011, Motta *et al.* 2012). So far, research groups in the field of visceral hypersensitivity have mainly focused on the effects of protease-activated receptor (PAR) agonists/antagonists in preclinical animal models, while direct protease inhibition has been investigated to a lesser extent. Annaházi *et al.* demonstrated a normalization of the visceral sensitivity in mice after a 30 min *in vitro* pre-incubation of UC patient fecal supernatants with the serine protease inhibitors aprotinin/SBTI (Annahazi *et al.* 2009). Besides, one other study showed a decrease in acute restraint stress-induced visceral hypersensitivity in rats after an *in vivo* intragastric pre-treatment with camostat mesylate (Zhao *et al.* 2011). The lack of studies clearly points towards the need of more extensive research, if one wants to consider direct serine protease inhibition as a possible treatment strategy for visceral pain in IBD patients.

Therefore, we aimed at (1) examining the efficacy on visceral pain of treatment with the serine protease inhibitors nafamostat mesylate and the newly developed UAMC-00050 and (2) quantifying the intestinal expression levels of a panel of serine proteases in an experimental acute colitis rat model for IBD.

4.3 Material and methods

All techniques are described in detail in chapter 3. Briefly we report on the techniques used in this chapter underneath.

4.3.1 Animals

Male Sprague-Dawley rats (200-225 g; Charles River, Italy) were used in all experiments. Rats were housed at a constant room temperature (22 ± 2°C) and humidity (60%) with two rats in each cage. The animals had unlimited access to water and food and were kept on a 12h:12h day-night cycle.

4.3.2 Induction of TNBS-colitis

After an overnight fast, 0.25 ml of a TNBS-enema (4 mg TNBS, 50% ethanol) was administered intrarectally under ketamine (35 mg/kg i.p) and xylazine (5 mg/kg i.p.) anesthesia. Control animals received 0.25 ml 0.9% NaCl intrarectally. The animals were kept in a tail-up position during 1 min and were then allowed to recover in a Trendelenburg position.

4.3.3 Experimental design

An overview of the experimental course is shown in figure 4.1. On day 0, a mild colitis was induced using a TNBS-enema. The control group received an intrarectal administration with 0.9% NaCl. On day 3, experiments were performed after confirming the presence of acute inflammation using colonoscopy. Rats were administered nafamostat (10 mg/kg), UAMC-00050 (1-5 mg/kg) or vehicle intraperitoneally (i.p.) 30 min before the start of the VMR experiment. The dosage of nafamostat mesilate was based on a previous study demonstrating a significant decrease in inflammation in acute DSS-colitis mice after a 6-day treatment with 20 mg/kg nafamostat p.o, taking into account a dose conversion from mice to rats (Cho *et al.* 2011). The dosage of UAMC-00050 was based on a previous study showing a dose-dependent antimetastatic effect after an 18-day treatment daily with 1 mg/kg of the UAMC compound in a rat mammary tumor model (Joossens *et al.* 2007).

Afterwards, the colonic compliance was evaluated and finally the animals were sacrificed and the inflammatory parameters (colonoscopy, macroscopy, microscopy and myeloperoxidase activity (MPO)) were scored. Colonic samples were taken from another group of control and acute colitis animals for further qPCR and immunohistochemistry experiments (n=8/group). Fecal samples were obtained to assess protease activity using an azocasein assay (n=12/group).

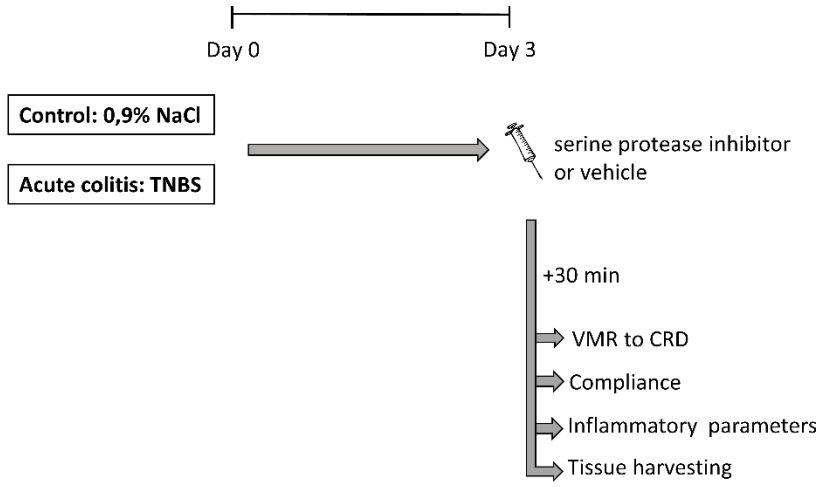


Figure 4.1. Overview of the experimental design.

On day 0, rats had an intrarectal administration with TNBS (colitis) or saline (control) and the implantation of EMG electrodes. Further experiments were conducted at day 3. A compound (nafamostat or UAMC-00050) or vehicle was injected i.p. 30 min prior to the VMR experiment. Afterwards, the colonic compliance and the inflammatory status were determined. EMG; electromyographic; TNBS; 2,4,6-trinitrobenzenesulfonic acid; VMR; visceromotor response.

4.3.4 Visceromotor response

The VMR is a validated, objective method, frequently used in our lab, to quantify visceral sensitivity and described in detail in Chapter 3 (Vermeulen *et al.* 2013, Deiteren *et al.* 2014, Deiteren *et al.* 2015). In short, a lubricated balloon was introduced into the colorectum of the conscious rat and subsequently inflated with increasing pressures according to the following protocol: 10-20-30-40-60 mmHg, 20 s, 4 min interval. The resulting abdominal contractions are measured using electromyographic (EMG) electrodes that were implanted 3 days before. The magnitude of the VMR signal was

quantified at each distension pressure using the area under the curve (AUC) of the EMG signal during the distension (20 s) corrected for the EMG signal before the distension (20 s).

4.3.5 Colonic compliance

The colonic compliance, the resistance of the colon against deformation, was evaluated to check whether the serine protease inhibitors execute their positive effects on visceral sensitivity through a change in viscoelastic properties of the colon as described in more detail in Chapter 3. Under pentobarbital anesthesia (45 mg/kg, i.p.), a lubricated balloon was introduced into the colorectum of the rat and filled with increasing volumes of water (0-0.5-1.0-1.5-2.0 ml, 80s interval). The corresponding pressure in the colon of the rat was measured and the resulting pressure-volume curves display the colonic compliance.

4.3.6 Inflammatory parameters

Colonoscopy

Under ketamine/xylazine anesthesia (35/5 mg/kg, i.p.), an *in vivo* colonoscopy was performed with a pediatric endoscope (Olympus GIF-N30, Olympus Europe GmbH), according to a previously published method(Vermeulen *et al.* 2011). The lubricated tip of the endoscope was introduced into the colon and advanced under endoscopic view until the hepatic flexure (± 10 cm proximal to the anus) was reached. During withdrawal, the intestinal inflammation was evaluated using a standardized scoring system (total score 0-19) (Vermeulen *et al.* 2011).

Post-mortem inflammatory markers

At the end of the experiments, the rat was sacrificed and the colon was excised, rinsed with Krebs solution, opened along the mesenteric border and macroscopically evaluated using a validated scoring system (total score 0-10) (Vermeulen *et al.* 2011).

For microscopic evaluation, a colonic segment of approximately 1 cm^2 was fixed in 4% formaldehyde for 24h and embedded in paraffin for hematoxylin & eosin staining. The

histological sections were given a microscopic score using a previously published scoring system (total score 0-10) (Vermeulen *et al.* 2011).

Finally, colonic myeloperoxidase (MPO) activity was determined as previously published (Vermeulen *et al.* 2011). The MPO activity is defined as the amount required to convert 1 µmol H_2O_2 to H_2O within 1min at 25°C and is expressed as unit/g tissue (Pulli *et al.* 2013).

4.3.7 Quantitative RT-PCR

The mRNA expression levels of a panel of serine proteases was determined in colon samples by means of qPCR, as described in more detail in Chapter 3. Distal colonic segments were harvested from control and acute colitis rats, snap-frozen in liquid nitrogen and stored at -80°C. Briefly, total RNA was extracted from colon and converted to cDNA by reverse transcription followed by a Taqman gene expression assay. GAPDH and β-actin were used as reference genes and the outcome values were analyzed using qBASEPLUS software (Biogazelle N.V., Zwijnaarde, Belgium).

4.3.8 Immunohistochemistry

Colonic samples were fixed in 4% formaldehyde, embedded in paraffin and sections of 5 µm were cut. In order to measure the number of tryptase positive cells, sections were incubated with a mouse mast cell tryptase antibody as described in detail in Chapter 3. The colon sections were screened for mast cell tryptase positivity at 100x magnification and for each layer (mucosa-submucosa-muscularis externa), the total number of tryptase positive cells was quantified using ImageJ 1.51 J8 (National Institutes of Health, USA) expressed per mm^2.

4.3.9 Total protease activity in fecal supernatants

Fecal supernatants (10 µl) were incubated with 70 µl reaction buffer (0.15 M NaCl, 20 mM Tris-HCl, pH 8.3) and 70 µl azocasein (0.5%) for 20 min at 40°C. Subsequently, the reaction was stopped by adding 70 µl trichloroacetic acid (10%). The samples were then centrifuged at 4000 rpm during 2 min and the absorption of the supernatant was

measured on the spectrophotometer at 340 nm. Enzymatic activities of the supernatants were normalized to protein content, assessed with Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Rockford, IL, USA).

4.3.10 Statistical analysis

All data are presented as mean ± SEM. The statistical analysis was performed using SPSS Statistics version 24.0 (IBM). VMR and colonic compliance data were analyzed using a Generalized Estimating Equations (GEE) test with Least Significant Difference (LSD) post-hoc test. Inflammatory parameters and immunohistochemistry results were analyzed using a Two-way ANOVA with Student-Newman-Keuls (S-N-K) post-hoc test. qPCR results were analyzed using unpaired Student's t-test or Two-way ANOVA with S-N-K post-hoc test. A p-value <0.05 was considered statistically significant. The graphs were made with GraphPad Prism 6.0.

4.4 Results

4.4.1 Nafamostat mesylate decreases visceral hypersensitivity during acute TNBS-colitis

Vehicle-treated acute colitis rats showed significantly higher VMRs to colorectal distension compared to vehicle-treated controls at 10-30-40-60 mmHg (figure 4.2A), indicating the presence of acute inflammatory visceral hypersensitivity, characterized by visceral allodynia (10-30 mmHg) and hyperalgesia (40-60 mmHg). A single administration of 10 mg/kg nafamostat i.p. could reduce visceral sensitivity both in TNBS (figure 4.2A) and control (figure 4.2B) rats. In acute TNBS-colitis rats, significance could be reached at 10-40-60 mmHg (figure 4.2A). In control animals, however, a significant decrease could only be seen at 40-60 mmHg (figure 4.2B).

Before sacrificing the animals, colonic compliance was measured to verify whether the anti-nociceptive effect of nafamostat was the direct result of a change in elastic properties of the colon. The intracolonic pressure was significantly increased in acute TNBS-colitis rats compared to controls, indicating a reduced compliance in the first

group (figure 4.2C). However, there was no significant effect of the factor "drug" (nafamostat) on colonic compliance and there was no significant interaction between the factors "group" and "drug".

Acute colitis was present 3 days after an intrarectal administration of TNBS in rats. This was demonstrated by a higher colonoscopic score in the TNBS group compared to the controls (table 4.1). Furthermore, the same increase was observed in the post-mortem inflammatory markers (macroscopy, microscopy and MPO activity), as can be seen in table 4.1.

In a dose of 10 mg/kg i.p., nafamostat significantly decreased the MPO activity, however no significant differences could be observed for colonoscopic, macroscopic and microscopic scores (table 4.1).

Table 4.1. Inflammatory parameters of all animals included in the nafamostat experiment.

Group	Drug	N	Colonoscopy	Macroscopy	Microscopy	MPO activity
Control	Vehicle	7	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.1 ± 0.1
	10 mg/kg	7	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.6 ± 0.2
Acute colitis	Vehicle	7	10.0 ± 0.9*	6.4 ± 0.7*	6.4 ± 0.2*	26.3 ± 6.1*
	10 mg/kg	7	7.7 ± 1.2*	5.6 ± 0.8*	4.7 ± 1.3*	11.9 ± 3.6*#

Data are presented as mean ± SEM. Two-way ANOVA followed by S-N-K post-hoc test. Significant interaction between the factor 'group' and 'drug' for MPO activity [#p<0.05; significantly different from 'acute colitis + vehicle']; significant effect of the factor 'group'; *p<0.05; no significant effect of the factor 'drug'. MPO; myeloperoxidase; N; number.

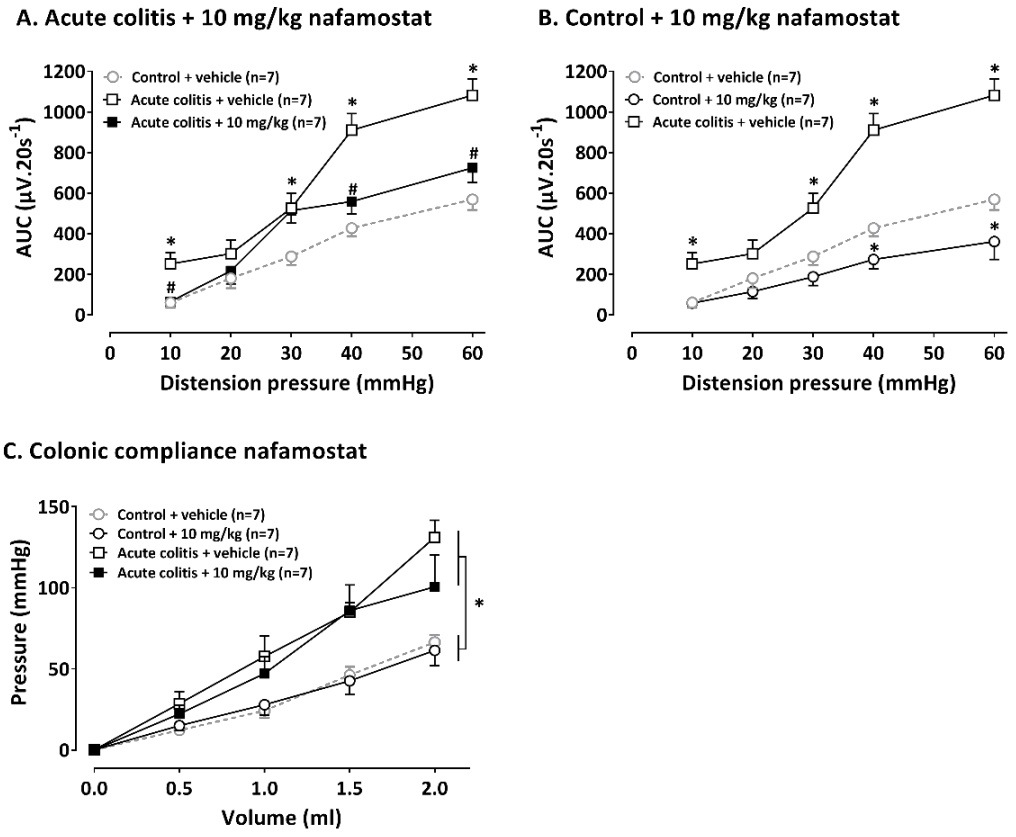


Figure 4.2. The effect of nafamostat (10mg/kg) and its vehicle (water for injection) on VMRs in acute colitis (squares) and control (bullets) rats.

The statistical analysis was performed on the complete dataset, but separate graphs were made for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; n=7; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from acute colitis + vehicle.

4.4.2 UAMC-00050 decreases visceral hypersensitivity during acute TNBS-colitis

The VMRs of the vehicle-treated acute colitis rats were significantly increased compared to the controls at 10-60 mmHg, indicating the presence of acute inflammatory visceral hypersensitivity. A single administration of 1 mg/kg UAMC-00050 i.p. had no significant effect on the VMRs of the acute colitis rats (figure 4.3A). On the other hand, after administering 5 mg/kg UAMC-00050 i.p. in animals with acute colitis, a significant decrease of the VMRs at 40-60 mmHg could be detected (figure

4.3B). In a dose of 5 mg/kg i.p, UAMC-00050 had no significant effect on the sensitivity of control animals (figure 4.3C).

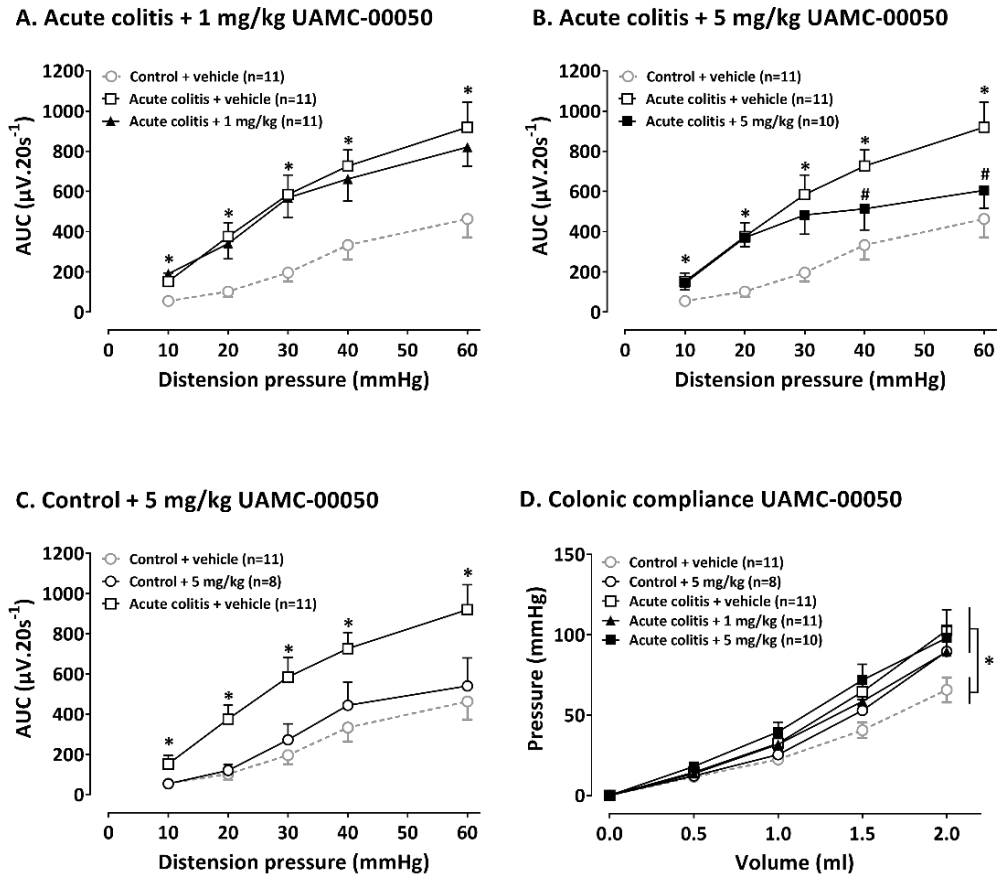


Figure 4.3. The effect of UAMC-00050 (1-5 mg/kg) and its vehicle (5% DMSO) on VMRs in acute colitis (squares) and control (bullets) rats.

The statistical analysis was performed on the complete dataset, but separate graphs were made for each dose for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; n=7; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from acute colitis + vehicle.

Finally, the colonic compliance was measured to check whether the anti-nociceptive effect of UAMC-00050 resulted from a change in elastic properties of the colon. The intracolonic pressure was significantly higher in acute colitis animals compared to the controls, indicating a reduced compliance after the induction of colitis (figure 4.3D). However, there was no significant effect of the factor “drug” (UAMC-00050) on colonic

compliance and there was no significant interaction between the factors “group” and “drug” (figure 4.3D).

On day 3, the colonoscopic score of the rats with acute colitis was significantly increased compared to the control animals, indicating the presence of colitis in this first group (table 4.2). The inflammatory status of the animals was confirmed by the other post-mortem markers (macroscopy, histology and MPO activity) (table 4.2).

In the highest dose (5 mg/kg i.p.), UAMC-00050 had no significant effect on the inflammatory parameters. However, the administration of 1 mg/kg UAMC-00050 significantly increased the macroscopic and microscopic score as well as the MPO activity, suggesting a pro-inflammatory effect of UAMC-00050 in this dose in an acute inflammatory setting (table 4.2).

Table 4.2. Inflammatory parameters of all animals included in the UAMC-00050 experiment

Group	Drug	N	Colonoscopy	Macroscopy	Microscopy	MPO activity
Control	Vehicle	8	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.1 ± 0.1
	5 mg/kg	8	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.3	0.6 ± 0.2
Acute colitis	Vehicle	11	7.2 ± 0.9*	5.4 ± 0.6*	5.9 ± 0.7*	10.2 ± 3.4*
	1 mg/kg	11	9.1 ± 1.1*	7.6 ± 0.7*#	7.3 ± 0.5*#	31.9 ± 4.2*#
	5 mg/kg	10	7.7 ± 1.2*	6.3 ± 0.8*	4.9 ± 0.7*	10.4 ± 3.2*

Data are presented as mean ± SEM. Two-way ANOVA followed by S-N-K post-hoc test. No significant interaction between the factor ‘group’ and ‘drug’; significant effect of the factor ‘group’; *p<0.05; significant effect of the factor ‘drug’; #p<0.05. MPO; myeloperoxidase; N; number.

4.4.3 Serine protease mRNA expression levels are altered during acute TNBS-colitis

The mRNA expression level of a selected panel of serine proteases was determined in colonic samples from acute TNBS-colitis vs control rats. The relative mRNA expression of Tpsab1 (tryptase) was elevated, however not significantly, in the colon of acute colitis rats compared to controls as opposed to the significant downregulation of St14 (matriptase) in acute colitis rats compared to controls. The expression of Plau (urokinase plasminogen activator) and KLK8 (kallikrein 8) were comparable between

control and acute colitis groups (table 4.3). Ctsg (cathepsin G), KLK2 (kallikrein 2) and KLK4 (kallikrein 4) were below detection limit.

Table 4.3. Relative mRNA expression of serine proteases in colon samples

Gene	Control	Acute colitis
Tryptase αβ1	1.25 ± 0.28	2.45 ± 1.03[a]
Matriptase	1.04 ± 0.10	0.58 ± 0.06*
Cathepsin G	<LOD	<LOD
uPA	1.05 ± 0.12	1.05 ± 0.17
Kallikrein 2	<LOD	<LOD
Kallikrein 4	<LOD	<LOD
Kallikrein 8	1.16 ± 0.27	1.05 ± 0.17
	n=8	n=8

Data are expressed as relative mRNA expression and presented as mean ± sem for n=8. Unpaired T-test. Significant effect of the factor 'group'; *p<0.05; [a]p=0.28. LOD; limit of detection.

4.4.4 Mast cell tryptase is increased during acute TNBS-colitis

Mast cell tryptase was quantified in the colon of the rats using immunohistochemistry. In the colon of control rats, the following distribution of mast cell tryptase could be observed: mucosa > submucosa > muscularis externa (figure 4.4B). In acute TNBS-colitis animals, the dispersal of tryptase positive cells was similar. However, the total number of mast cell tryptase positive cells was significantly increased in acute colitis rats compared to control animals (figure 4.4B).

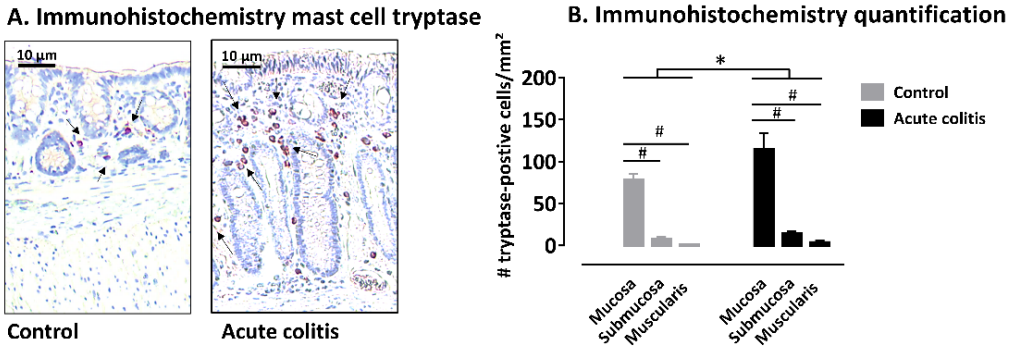


Figure 4.4. Immunohistochemistry with mast cell tryptase antibody in rat colon.

A. Representative images of the colonic mucosa of a control and an acute colitis animal with mast cell tryptase (purple-red). B. The number of tryptase-positive mast cells per mm² in the mucosa, submucosa and muscularis externa in control (grey bars) and acute colitis (black bars) animals. Two-way ANOVA followed by S-N-K post-hoc test; n=8; *p<0.05; significant effect of the factor 'group'; #p<0.05; significant effect of the factor 'layer'; no significant interaction between the factors 'group' and 'layer'.

4.4.5 Fecal protease activity is increased during acute TNBS-colitis

Total protease activity was assessed by means of an azocasein assay in fecal samples of control vs acute colitis rats. Fecal protease activity was significantly increased in acute colitis rat samples compared to controls (Figure 4.5).

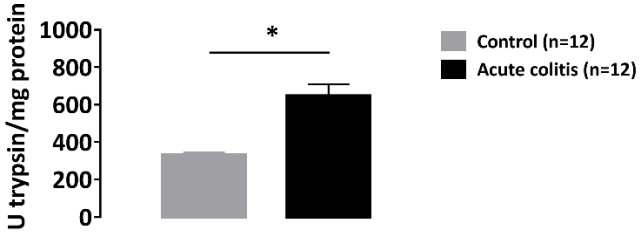


Figure 4.5. Fecal protease activity.

The protease activity expressed as mean U trypsin/mg protein ± SEM in fecal samples of control (grey bar) and acute colitis (black bar) animals. Unpaired Student's T-test; n=12; p<0.05; significant effect of the factor 'group'.

4.5 Discussion

We demonstrated in this chapter a significant increase in visceral hypersensitivity in the rats with an acute TNBS-colitis used as a model for IBD flares. Nafamostat mesylate and the newly developed UAMC-00050 could decrease visceral hypersensitivity in this preclinical rat model for IBD. Furthermore, we hypothesize a role for serine proteases in this animal model by showing a significant downregulation of matriptase and a trend towards an upregulated tryptase expression at the mRNA level in the colon of acute colitis rats. Moreover, we found a significantly increased number of mast cell tryptase positive cells and an increased fecal protease activity during acute TNBS-colitis.

In a first part of this study we tried to prove the efficacy of direct serine protease inhibition as a possible new pharmacological treatment strategy for visceral pain in IBD. To this end, we investigated the effects of the serine protease inhibitors nafamostat mesylate (marketed in Japan) and UAMC-00050 (newly developed by the Laboratory of Medicinal Chemistry at the University of Antwerp) in a rat model for visceral hypersensitivity, in an acute inflammatory setting.

The involvement of serine proteases in visceral hypersensitivity was already proven earlier by several research groups. An important difference however is that these studies considered the effects of the protease-activated receptors (PARs) and their agonists or antagonists (Coelho *et al.* 2002, Auge *et al.* 2009, Cattaruzza *et al.* 2014). However, none of these compounds were tested in clinical trials to the best of our knowledge. Therefore, our strategy was to intervene earlier in the serine protease pathway, more specifically by directly inhibiting the serine proteases.

The broad-spectrum serine protease inhibitor nafamostat mesylate (10 mg/kg i.p.) was able to reduce visceral hypersensitivity in acute TNBS-colitis animals, reaching significance at both low (10 mmHg) and high (40-60 mmHg) pressures. We confirmed that these positive effects of nafamostat mesylate were not a direct effect of a change in elastic properties of the colon. Notably, 10 mg/kg nafamostat mesylate also lowered visceral sensitivity in control animals, albeit only at the highest pressures (40-60

mmHg). Concerning the inflammatory parameters, nafamostat significantly reduced MPO activity, without significantly influencing the other inflammation markers. In literature evidence is present that nafamostat mesylate, when clinically used as an anticoagulant, selectively suppressed MPO elevation during hemodialysis (Inose *et al.* 1997). Moreover, we previously demonstrated a significant anti-inflammatory effect (shown by a decreased colonoscopic and macroscopic score) of nafamostat mesylate after a 2-week treatment with a daily dose of 10 mg/kg i.p. in a chronic murine transfer colitis model (Van Spaendonk *et al.* 2017). We therefore hypothesize that a single i.p. treatment with nafamostat 30 min before the experiment might be too short to allow us to observe a clear effect on the other inflammatory parameters (colonoscopy, macroscopy and microscopy). Furthermore, we cannot exclude that this effect of nafamostat on MPO affects visceral pain directly.

Our results are in line with previous studies investigating the effect of nafamostat (or FUT-175) on visceral hypersensitivity, with the important difference that IBS animal models were used instead of a model for IBD. In mice, visceral hypersensitivity induced by the intracolonic infusion of IBS-D fecal supernatants, could be suppressed when the supernatant was pre-incubated with nafamostat mesylate (Wang *et al.* 2015). Similar results were observed by the group of Cenac *et al.*, after pre-incubating the supernatant of colonic and rectal biopsies of IBS patients with nafamostat (Cenac *et al.* 2007). Moreover, research groups investigating the effects of nafamostat mesylate in experimental animal models for IBD have been focusing on the effects on inflammation. A decrease in inflammation has been shown in both DSS-colitis mice (Cho *et al.* 2011) as well as in TNBS-colitis rats (Isozaki *et al.* 2006) after a 6-day oral and a 6-day intrarectal treatment with nafamostat respectively. Therefore, to our knowledge, we are the first to consider the effects of nafamostat on visceral pain in an acute animal model for IBD.

The newly developed serine protease inhibitor, UAMC-00050, significantly decreased visceral hyperalgesia in a dose-dependent way, without influencing the allodynia. It was confirmed that the anti-nociceptive effect of 5 mg/kg UAMC-00050 was not due

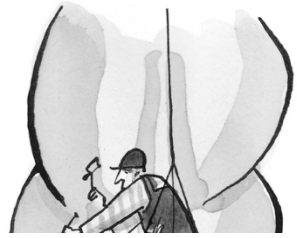
to a change in colonic compliance of the animals. The effect of this new compound on the inflammatory markers was remarkable as the lowest dose seemed to have pro-inflammatory effects that were absent in the higher dose.

An interesting finding of our study is that UAMC-00050 reduced visceral hyperalgesia in the acute colitis model, without affecting the associated visceral allodynia. These findings are completely in line with a study from Moussa *et al*. In this study, female Wistar rats received a daily oral administration with a fermented soy germ (FSG) extract containing isoflavones and a Bowman-Birk inhibitor (trypsin-like serine protease inhibitor) for 15 days. On day 15, acute TNBS-colitis was induced and 5 days later, visceral sensitivity was objectified. The vehicle-treated acute colitis rats presented increased VMRs at 30-45-60 mmHg, demonstrating the presence of visceral hypersensitivity. The rats treated with FSG, showed a significant reduction in VMR, but this effect was only seen at the higher pressures (45-60 mmHg) (Moussa *et al.* 2012). Moreover, our results are also in accordance with previous findings of Coelho *et al.*, demonstrating that the intrarectal administration of a PAR_2-agonist (SLIGRL-NH_2) induces visceral hyperalgesia without the associated allodynia in male Wistar rats (Coelho *et al.* 2002). These two studies both suggest the release of serine proteases at noxious colorectal distensions and therefore mainly playing a role in visceral hyperalgesia and not in allodynia in an acute inflammatory setting. In contrast, Augé *et al.* demonstrated a state of visceral hypersensitivity, characterized by visceral allodynia as well as hyperalgesia, after the intracolonic administration of a PAR2-agonist (SLIGRL-NH_2) in male C57Bl6 mice, thereby emphasizing the role of proteases in both allodynia and hyperalgesia (Auge *et al.* 2009). In contrast to our acute colitis rats, displaying a state of hypersensitivity, Annaházi *et al.* found that mice presented hyposensitivity after the infusion of colonic supernatant from UC patients. Additionally, after the administration of a PAR4-antagonist the mice were hypersensitive, while a mixture of aprotinin and soybean trypsin inhibitor (SBTI) could restore the sensitivity to normal levels (Annahazi *et al.* 2009).

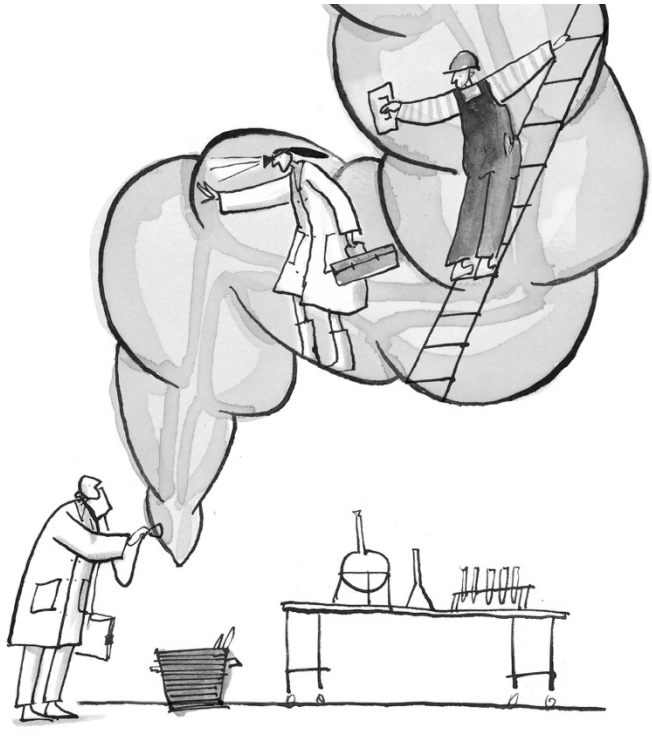
In a second part of this study, we confirmed a role for serine proteases in the pathogenesis of visceral hypersensitivity during acute TNBS-colitis. Firstly, we investigated the mRNA expression levels of a panel of serine proteases in colonic samples of acute colitis rats compared to controls. Consistent with previous studies, this study demonstrated a significant downregulation of matriptase in acute TNBS-colitis animals compared to controls. Decreased matriptase mRNA expression levels were observed in DSS-colitis mice, an experimental animal model for IBD (Buzza *et al.* 2017), as well as in inflamed colonic tissue from Crohn and ulcerative colitis patients (Netzel-Arnett *et al.* 2012). On the contrary, the expression of tryptase tended to be elevated in acute colitis rats, however no significant results could be detected. Looking at these results in detail, not all rats show a similar response to the drug. Secondly, we studied the presence of tryptase positive mast cells in the colon of the rats and found a significant higher number of positive mast cells in the acute TNBS-colitis animals compared to the controls, demonstrating an important role for these cells in an acute inflammatory setting. This observation is in accordance with the recent literature. As such, increased levels of intestinal mast cell tryptase are observed in both experimental rat and mice models for IBD (Hamilton *et al.* 2011, Lohman *et al.* 2012). Furthermore, several clinical studies also revealed higher amounts of tryptase in colonic biopsies from IBD patients (Raithel *et al.* 2001, Cenac *et al.* 2007, Peterson *et al.* 2007). Thirdly, we assessed the total fecal protease activity and found a significant increase in acute colitis animals compared to controls. Similar differences in fecal protease activity have been documented in an acute TNBS-colitis animal model for IBD (Moussa *et al.* 2012), in addition to UC patient samples (Roka *et al.* 2007, Annahazi *et al.* 2009).

We can conclude that serine proteases, besides many other mediators, seem to be involved in visceral hypersensitivity during acute inflammation. Nafamostat mesylate (broad specificity and marketed in Japan for the treatment of acute pancreatitis) as well as the newly developed UAMC-00050 (narrower spectrum) could decrease visceral hypersensitivity in an acute TNBS-colitis rat model. However, interesting differences between the effects of both compounds were observed. Firstly,

nafamostat mesylate had an antinociceptive effect at almost all pressures while UAMC-00050 clearly only showed efficacy at higher pressures. Secondly, nafamostat mesylate also decreased visceral sensitivity in control animals, while UAMC-00050 had no effect on the pain responses in control rats, thus demonstrating a non-disease specificity of nafamostat mesylate. The effects of the serine protease inhibitors on inflammatory markers are much more difficult to explain and one needs to consider methodological issues, and /or different balances between proteases and anti-proteases at different moments of inflammation. The conclusion of this study is the clear involvement of serine proteases in visceral hypersensitivity in an acute inflammatory setting suggesting serine proteases as new therapeutic targets in the management of abdominal pain in IBD.



CHAPTER 5 - THE ROLE OF SERINE PROTEASES IN A POST-INFLAMMATORY RAT MODEL FOR VISCERAL HYPERSENSITIVITY



Adapted from: Ceuleers H, Hanning N, Heirbaut J, Van Remoortel S, De bruyn M, Joossens J, van der Veken P, Lambeir A-M, Francque SM, De Man JG, Timmermans J-P, Augustyns K, De Meester I, De Winter BY. Study of the nature, origin and mechanism of action of serine proteases on visceral hypersensitivity in a post-inflammatory model for irritable bowel syndrome. British Journal of Pharmacology. 2018. In press.

5.1 Abstract

Background and aims

Serine proteases are suggested as important players in visceral pain. We investigated their effect by using newly developed serine protease inhibitors with a well-characterized inhibitory profile in a post-inflammatory IBS rat model.

Methods

Colitis rats received intrarectal TNBS and controls 0.9% NaCl. Colonoscopies were performed on day 3, to confirm colitis, and later on until mucosal healing. Visceral hypersensitivity was quantified by visceromotor responses (VMR) to colorectal distension, 30 min after intraperitoneal injection of the serine protease inhibitors nafamostat, UAMC-00050 or UAMC-01162. Serine proteases, protease-activated receptors (PARs) and transient receptor potential channels (TRPs) were quantified via qPCR and immunohistochemistry. Proteolytic activity was characterized using fluorogenic substrates.

Results

VMR was significantly elevated in post-colitis rats. Nafamostat normalized VMRs at the lowest dose tested. UAMC-00050 and UAMC-01162 significantly decreased VMR dose-dependently. mRNA expression of tryptase-αβ-1, PAR4 and tryptase immunoreactivity were significantly increased in the colon of post-colitis animals. Accordingly, trypsin-like activity was significantly increased in the colon but not in the feces. PAR2 and TRPA1 immunoreactivity colocalized with CGRP-positive nerve fibers in control and post-colitis animals.

Conclusions

The increase in serine protease expression and activity together with the increased expression of downstream molecules at the colonic and DRG level and in CGRP positive sensory nerve fibers point to a role for serine proteases in post-inflammatory visceral hypersensitivity. The results of this study call for further investigation of serine protease inhibitors as an interesting treatment strategy for IBS related visceral pain.

5.2 Introduction

Irritable bowel syndrome (IBS) is a functional gastrointestinal disorder, characterized by chronic abdominal pain and altered defecation patterns, in the absence of any organic cause (Chey, Kurlander & Eswaran, 2015). Visceral hypersensitivity is a main factor underlying abdominal pain in IBS patients (Barbara et al., 2011) and an important contributor to gastrointestinal symptom generation in IBS patients, thus making it a relevant treatment target (Simren, Tornblom, Palsson & Whitehead, 2017). Remarkably, only 24% of the IBS patients report complete relief of abdominal pain after treatment, which is usually only a symptomatic treatment of the most explicit motility-related symptom (Hungin, Whorwell, Tack & Mearin, 2003). Therefore, further research towards new treatments for abdominal pain in IBS patients is of utmost importance.

Gastrointestinal inflammation is considered as an important trigger for the onset of visceral hypersensitivity (De Schepper, De Man, Moreels, Pelckmans & De Winter, 2008). Upon activation of inflammatory cells (e.g. mast cells, T-/B-cells) at the peripheral level, excessive amounts of mediators (e.g. histamine, serotonin, cytokines, proteases, bradykinin, ATP) are released, thereby sensitizing peripheral afferent neurons contributing to visceral hypersensitivity. Considerable research efforts are devoted to the role of various inflammatory mediators in visceral hypersensitivity but the role of proteases only starts to be understood (Ceuleers et al., 2016; Vergnolle, 2016).

Especially the class of serine proteases attracts attention in visceral nociception research because elevated serine protease expression and/or activity is repeatedly found in colonic and fecal samples of IBS patients (Annahazi et al., 2009; Barbara et al., 2004; Buhner et al., 2009; Cenac et al., 2007; Gecse et al., 2008; Roka et al., 2007; Tooth et al., 2014). Despite suggestions of serine protease inhibitors as a possible treatment option for visceral pain, only few preclinical animal studies actually investigated the effect of serine protease inhibitors on visceral sensitivity (Ceuleers et al., 2016; Vergnolle, 2016). A clear role for PARs in visceral hypersensitivity is shown in

preclinical in vitro and in vivo studies but only few animal studies have been conducted evaluating serine protease inhibitors, thereby focusing on proteases rather than PARs. It is shown that supernatant from colonic biopsies from IBS patients loses its capacity to induce visceral hypersensitivity in mice if the supernatant is pretreated in vitro with nafamostat, a broad-spectrum serine protease inhibitor (Cenac et al., 2007; Wang et al., 2015). However, proof of an in vivo effect remains scarce. To our knowledge, the only evidence that in vivo serine protease inhibition reduces visceral pain was provided by Zhao et al., showing that intragastric pre-treatment with camostat, structurally related to nafamostat, reduced pain in a rat model of stress-induced visceral hypersensitivity (Zhao et al., 2011). This highlights the importance of more extensive research on serine protease inhibitors as a possible treatment option for visceral pain.

Therefore, we aimed at investigating the in vivo symptomatic effect of two newly developed serine protease inhibitors (UAMC-00050 and UAMC-01162; patent WO2007045496 (Joossens et al., 2007)) in a post-inflammatory rat model for IBS. We compared the effects of UAMC-00050 and UAMC-01162 to those of nafamostat and attempted to unravel the type of serine proteases contributing to visceral hypersensitivity to define the optimal inhibition profile for serine protease inhibitors targeting abdominal pain in IBS patients.

5.3 Materials and methods

5.3.1 Animals

Male Sprague-Dawley rats (200-225g) were housed at constant room temperature (22 ± 2°C) and humidity (60%) with two rats per cage. Rats had unlimited access to water and food and were kept on a 12h:12h day-night cycle.

5.3.2 Induction of TNBS-colitis

After an overnight fast and under ketamine (35 mg/kg i.p) and xylazine (5 mg/kg i.p.) anesthesia, rats were intrarectally instilled with 0.25 ml of a trinitrobenzene sulphonic acid (TNBS) solution containing 4 mg TNBS in 50% ethanol. Control animals received a

0.25 ml enema with 0.9% NaCl, under ketamine (35 mg/kg i.p.) and xylazine (5 mg/kg i.p.) anesthesia. The animals were kept in a tail-up position during 1 min and were then allowed to recover in a Trendelenburg position in a temperature-controlled cage (28°C) up until 1h until they regained consciousness. No post-operative analgesia was administered as approved by the Ethical Committee for Animal Experiments of the University of Antwerp (EC nr. 2014-41). Subsequently, animals were brought back to their cages with free access to food and water.

5.3.3 Experimental design

The experimental course is shown in figure 5.1. A mild colitis was induced on day 0 by a TNBS-enema. Controls received an intrarectal administration with 0.9% NaCl. Colitis was verified by colonoscopy on day 3. From day 10 onwards, colonoscopy was performed every 4 days to follow up the healing of the colonic mucosa. Functional experiments (VMR) were performed three days after complete resolution of colitis. The compounds (0.01-10 mg/kg nafamostat mesilate, 0.01-1 mg/kg UAMC-00050, 1-2.5 mg/kg UAMC-01162) or vehicle (sterile water for nafamostat, 5% DMSO for UAMC-00050 and UAMC-01162) were injected intraperitoneally (i.p.) 30 min before the start of the VMR experiment (n=7-10/group). Group sizes are unequal due to experimental loss (electrode failure, anesthesia, reamining colonic inflammation). The highest dosage of nafamostat mesilate was based on a previous study (dose conversion from mice to rats was taken into account) demonstrating a significant decrease in inflammation in acute DSS-colitis mice after a 6-day treatment with 20 mg/kg nafamostat p.o (Cho *et al.* 2011). The dosages of the newly developed compounds were based on a previous study showing a dose-dependent antimetastatic effect after an 18-day treatment daily with 0.1 mg/kg and 1 mg/kg of the UAMC compound in a rat mammary tumor model (Joossens *et al.* 2007). The active compounds are described in detail in Chapter 3. Colonic compliance was evaluated before animals were sacrificed (exsanguination under 45 mg/kg i.p pentobarbital anesthesia) to asses colonic inflammatory parameters (colonoscopy, macroscopy, microscopy and myeloperoxidase activity (MPO)). Colonic samples were taken for

immunohistochemistry, qPCR and proteolytic activity experiments. DRG samples were obtained from another group of control and post-colitis IBS rats (n=12/group). All animals were randomised for treatment and data analysis was carried out by a person blinded to this study.

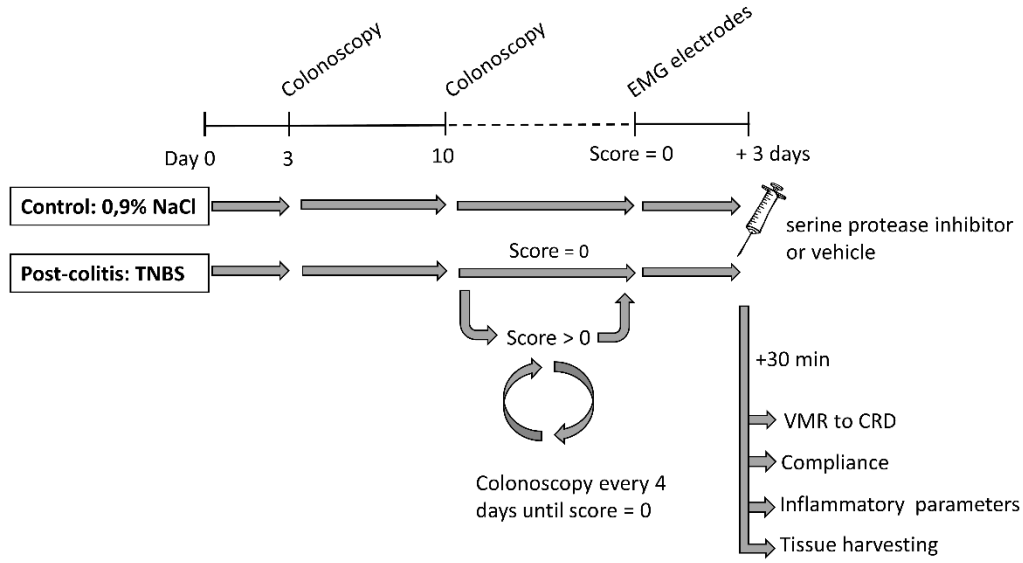


Figure 5.1. Overview of the experimental design.

On day 0, rats received an intrarectal administration with TNBS (colitis) or 0.9% NaCl (control). The severity of colitis and the mucosal healing were monitored *in vivo* using colonoscopy: on day 3 to confirm the presence of colitis and from day 10 onwards every 4 days until complete mucosal healing. Hereafter, EMG electrodes were implanted and 3 days later all experiments were conducted after a single i.p. injection with serine protease inhibitor/vehicle. EMG; electromyographic; TNBS; 2,4,6-trinitrobenzenesulfonic acid; VMR; visceromotor response.

5.3.4 Visceromotor response

The visceromotor response (VMR) to a colorectal balloon distension is a validated and objective method to quantify visceral sensitivity in rats, as described in more detail in Chapter 3 (Vermeulen *et al.* 2013, Deiteren *et al.* 2014, Deiteren *et al.* 2014, Deiteren *et al.* 2015). Briefly, a balloon was introduced into the colorectum of the rat and inflated with increasing pressures according to a barostat-controlled distension protocol (10-20-30-40-60 mmHg, 20 s, 4 min interval). The resulting abdominal contractions were measured using electromyographic (EMG) electrodes that were implanted into the abdominal muscle 3 days before. The VMR was quantified by

calculating the area under the curve (AUC) of the EMG signal during colorectal distension (20 s) corrected for the EMG signal before the distension (20 s).

5.3.5 Colonic compliance

Colonic compliance was assessed to study possible changes in the viscoelastic properties of the colon due to the administration of the serine protease inhibitors. Briefly, under pentobarbital anesthesia (45 mg/kg), a balloon was introduced into the colorectum of the rat and filled with increasing volumes of water (0-0.5-1.0-1.5-2.0 ml, 80 s interval). The corresponding pressure in the colon of the rat was measured and the resulting pressure-volume curves display colonic compliance.

5.3.6 Inflammatory parameters

Colonoscopy

Colonoscopy was performed with a pediatric endoscope (Olympus GIF-N30, Olympus Europe GmbH), as described in Chapter 3. Under ketamine/xylazine anesthesia (35/5 mg/kg, i.p.), the lubricated tip of the endoscope was introduced into the colon and advanced under endoscopic view until the hepatic flexure was reached (± 10 cm proximal to the anus). During withdrawal of the endoscope, intestinal inflammation was evaluated using a standardized scoring system (total score 0-19) (Vermeulen *et al.* 2011).

Post-mortem inflammatory markers

At the end of the experiments, the rat was sacrificed and inflammation was evaluated as explained in Chapter 3. The colon was isolated, rinsed with Krebs solution, opened longitudinally and macroscopically evaluated using a validated scoring system (total score 0-10) (Vermeulen *et al.* 2011).

For microscopic evaluation, a colonic segment of approximately 1 cm^2 was fixed in 4% formaldehyde, embedded in paraffin and stained with hematoxylin & eosin. A microscopic score of 0-10 was given using a previously published scoring system (Vermeulen *et al.* 2011). Finally, colonic myeloperoxidase (MPO) activity was measured

as previously published (Vermeulen *et al.* 2011). The MPO activity is defined as the quantity needed to transform 1 µmol H_2O_2 to H_2O within 1min at 25°C and is expressed as unit/g tissue (Pulli *et al.* 2013).

5.3.7 Quantitative RT-PCR

As described in detail in Chapter 3, mRNA expression levels of different key mediators involved in the serine protease pathway were quantified in distal colonic samples and dorsal root ganglia (DRGs) T13-L2 (splanchnic colonic afferents) and L6-S1 (pelvic colonic afferents). Concisely, total RNA was extracted from colon/DRG and converted to cDNA followed by a Taqman gene expression assay. GAPDH and β-actin were used as reference genes and the outcome values were analyzed using qBASE^{PLUS} software (Biogazelle N.V., Zwijnaarde, Belgium).

5.3.8 Immunohistochemistry for mast cell tryptase

Colonic samples were fixed in 4% formaldehyde, embedded in paraffin and cut into 5 µm sections. In order to quantify the number of tryptase positive cells, sections were incubated with a mouse mast cell tryptase antibody as described in detail in Chapter 3. Colon sections were screened (100x magnification) and the total number of tryptase positive cells in the mucosa was quantified using ImageJ 1.51 J8 (National Institutes of Health, USA) and expressed per mm^2.

5.3.9 Immunohistochemistry for PAR2, PAR4 and TRPA1

Distal colon samples were fixed in 4% paraformaldehyde and further processed for cryo-embedding. Cryo-sections (12µm) were incubated with primary antibodies (rabbit anti-PAR4, rabbit anti-PAR2, rabbit anti-TRPA1 and goat anti-CGRP) for 16 hours at 4°C and visualized using CY3-conjugated donkey anti-rabbit or CY5-conjugated donkey anti-goat immunoglobulins as described in detail in Chapter 3. High-resolution images were obtained on a Leica TCS SP8 confocal laser scanning microscope and images were processed using the ImageJ software.

5.3.10 Total protease activity in fecal supernatants

Fecal supernatants were incubated with reaction buffer and azocasein (0.5%), as described in detail in Chapter 3. Subsequently, the reaction was stopped by adding trichloroacetic acid (10%). The samples were then centrifuged and the absorption of the supernatant was measured on the spectrophotometer at 340 nm. Enzymatic activities of the supernatants were normalized to protein content.

5.3.11 Proteolytic activities

To assess the activity of serine proteases, trypsin-like, chymotrypsin-like, neutrophil elastase, pancreas elastase and kallikrein activities were determined in colonic and fecal samples, as described in detail in Chapter 3. Distal colon and fecal samples were taken upon sacrifice, snap-frozen and stored at -80 °C until further processing. In short, samples were crushed on dry ice, dissolved in lysis buffer, centrifuged and the supernatant was collected for the activity measurements. Trypsin-like activity was assessed with the following substrates: Boc-Gln-Ala-Arg-AMC and n-Tosyl-Gly-Pro-Arg-AMC. Chymotrypsin-like, neutrophil elastase, pancreas elastase and kallikrein activities were measured using Suc-Ala-Ala-Pro-Phe-AMC, Suc-Ala-Ala-Pro-Val-AMC, Suc-Ala-Ala-Ala-AMC and H-Pro-Phe-Arg-AMC. Fluorescence was measured kinetically (20 min, 37°C). The activities (U L-1) were transformed to specific activities (U g-1) using the protein concentration in the samples as determined by Bradford analysis.

5.3.12 Statistical analysis

All data are presented as mean ± SEM. VMR and compliance data were analyzed using a Generalized Estimating Equations (GEE) test with Least Significant Difference (LSD) post-hoc test. Inflammatory parameters and immunohistochemistry results were analyzed using a Two-way ANOVA with Student-Newman-Keuls (S-N-K) post-hoc test, as appropriate. qPCR results were analyzed using unpaired Student's t-test or Two-way ANOVA with S-N-K post-hoc test. Proteolytic activities were analyzed using a Mann-Whitney U test. The statistical analysis was performed using SPSS Statistics (version 24.0, IBM). A p-value <0.05 was considered statistically significant.

5.4 Results

5.4.1 Nafamostat mesylate decreases visceral hypersensitivity in a post-inflammatory setting

TNBS rats developed colitis on day 3, as demonstrated by the significantly increased colonoscopic scores compared to controls (table 5.1). At the day of the VMR, the post-inflammatory status of the animals was confirmed by colonoscopy, macroscopy, microscopy and colonic MPO activity.

Table 5.1. Inflammatory parameters of nafamostat mesylate in post-colitis

Group	Drug	N	Colonoscopy (0-19)		Macroscopy	Microscopy	MPO activity
			Day 3	Day VMR			
Control	Vehicle	8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.9 ± 0.3
	0.1 mg/kg	8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.4	0.8 ± 0.3
Post-colitis	Vehicle	8	5.8 ± 0.8*	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.3	1.4 ± 0.4
	0.01 mg/kg	8	6.6 ± 0.7*	0.0 ± 0.0	0.0 ± 0.0	1.0 ± 0.5	1.5 ± 0.5
	0.1 mg/kg	8	7.0 ± 0.5*	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	1.7 ± 0.4
	1 mg/kg	8	5.0 ± 0.4*	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.4	1.4 ± 0.5
	10 mg/kg	7	6.0 ± 0.7*	0.0 ± 0.0	0.1 ± 0.1	0.7 ± 0.7	2.1 ± 0.5

Data are presented as mean ± SEM. Two-way ANOVA followed by S-N-K post-hoc test. No significant interaction, significant effect of the factor 'group' for colonoscopy at day 3; *$p<0.05$; no significant effect of the factor 'drug'. MPO; myeloperoxidase; N; number; VMR; visceromotor response.

Compared to control rats, vehicle-treated post-colitis rats showed significantly increased VMRs for all distension pressures (20-60mmHg), indicating post-inflammatory visceral hypersensitivity (figure 2A). A single i.p. administration of nafamostat (0.01-10 mg/kg) 30 min before the VMR experiment, attenuated visceral sensitivity. In a dose of 0.01 mg/kg, VMRs were significantly decreased at 20-60 mmHg (figure 5.2A), whereas a dose of 0.1 mg/kg 1 mg/kg significantly lowered VMRs at 30-40-60 mmHg (figure 5.2B) and a dose of 1 mg/kg only affected VMRs at 20 and 30 mmHg (figure 5.2C). In the highest dose (10 mg/kg), no significant effect of nafamostat was observed in post-colitis rats (figure 5.2D). In control animals, the most effective dose of 0.1 mg/kg had no effect on visceral sensitivity (figure 5.2E).

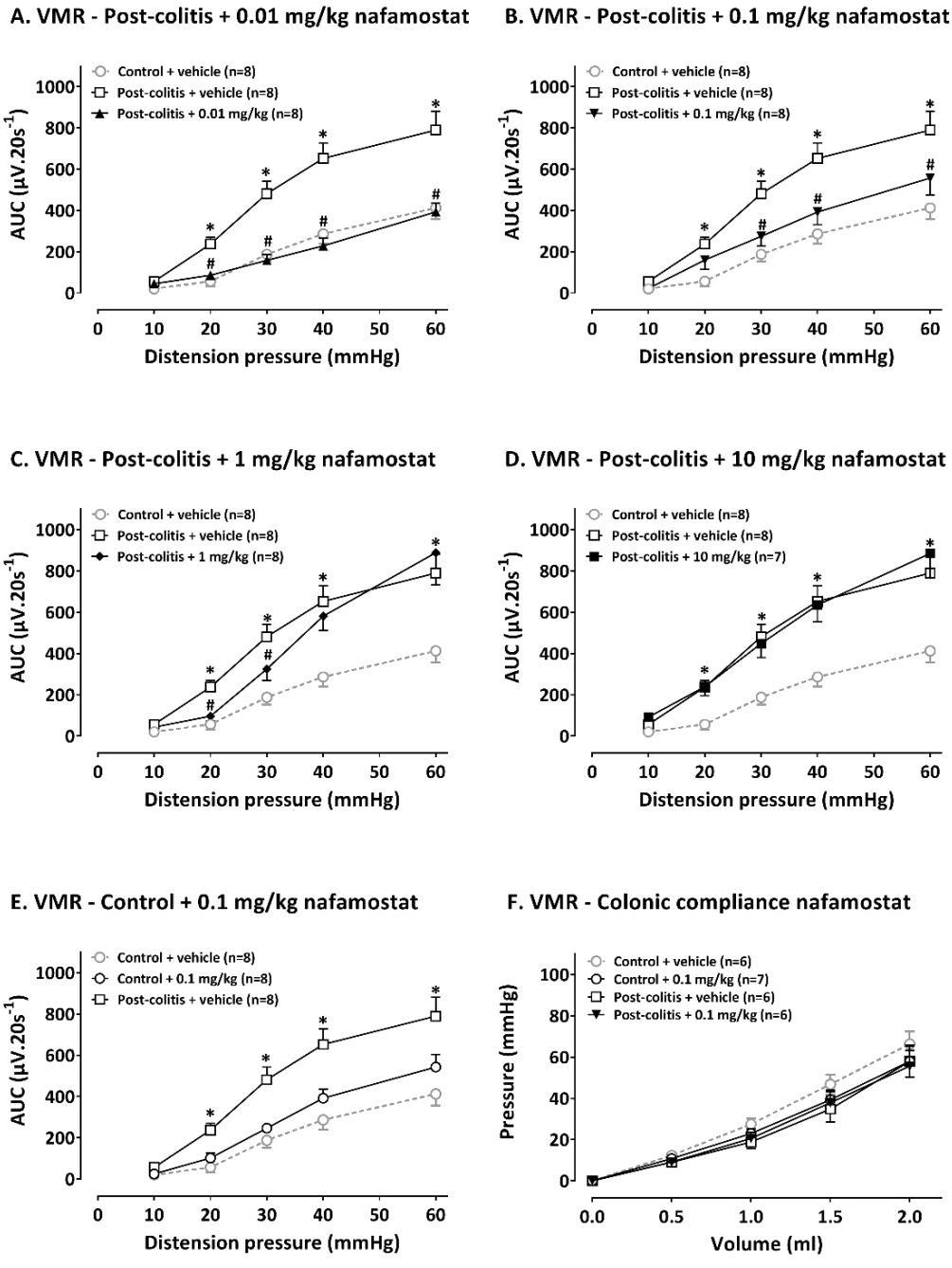


Figure 5.2. The effect of nafamostat mesylate (0.01-10 mg/kg) and its vehicle (water for injection) on VMRs and colonic compliance in post-colitis and control rats.

The statistical analysis was performed on the complete dataset, but separate graphs were made for each dose for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from post-colitis + vehicle.

Nafamostat (0.1 mg/kg) had no effect on colonic compliance (figure 5.2F). Moreover, nafamostat treatment did not affect the inflammatory parameters compared to vehicle-treated controls (table 5.1).

5.4.2 UAMC-00050 decreases visceral hypersensitivity in a post-inflammatory setting

On day 3, all TNBS rats displayed significantly higher colonoscopic inflammatory scores compared to controls, indicating the presence of a mild colitis (table 5.2). At the day of the VMR, the post-inflammatory status of all animals was confirmed by colonoscopy and post-mortem inflammatory markers.

Table 5.2. Inflammatory parameters of UAMC-00050 in post-colitis

Group	Drug	N	Colonoscopy (0-19)		Macroscopy	Microscopy	MPO activity
			Day 3	Day VMR			
Control	Vehicle	7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.9 ± 0.2
	1 mg/kg	6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.1
Post-colitis	Vehicle	8	5.4 ± 0.7*	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.2	1.6 ± 0.5
	0.01 mg/kg	7	7.0 ± 0.9*	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.2 ± 0.5
	0.1 mg/kg	6	5.3 ± 0.9*	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	1.8 ± 0.9
	1 mg/kg	9	4.3 ± 0.6*	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	1.2 ± 0.6

Data are presented as mean ± SEM. Two-way ANOVA followed by S-N-K post-hoc test. No significant interaction, significant effect of the factor 'group' for colonoscopy at day 3; *$p<0.05$; no significant effect of the factor 'drug'. MPO; myeloperoxidase; N; number; VMR; visceromotor response.

Rats in the post-colitis group displayed significantly higher VMRs compared to controls, confirming visceral hypersensitivity (figure 5.3A). A single i.p. administration with UAMC-00050 decreased VMRs in a dose-dependent manner in post-colitis animals: 0.01 mg/kg had no effect (figure 5.3A) whereas 0.1 mg/kg UAMC-00050 significantly decreased VMRs at 30-40-60 mmHg (figure 5.3B) and 1 mg/kg UAMC-00050 completely restored sensitivity to normal values (figure 5.3C). The most effective dose of 1 mg/kg UAMC-00050 had no effect on VMRs in control animals (figure 5.3D) and had no effect on colonic compliance (figure 5.3E). UAMC-00050 (0.01-1 mg/kg) had no effect on the inflammatory markers (table 5.2).

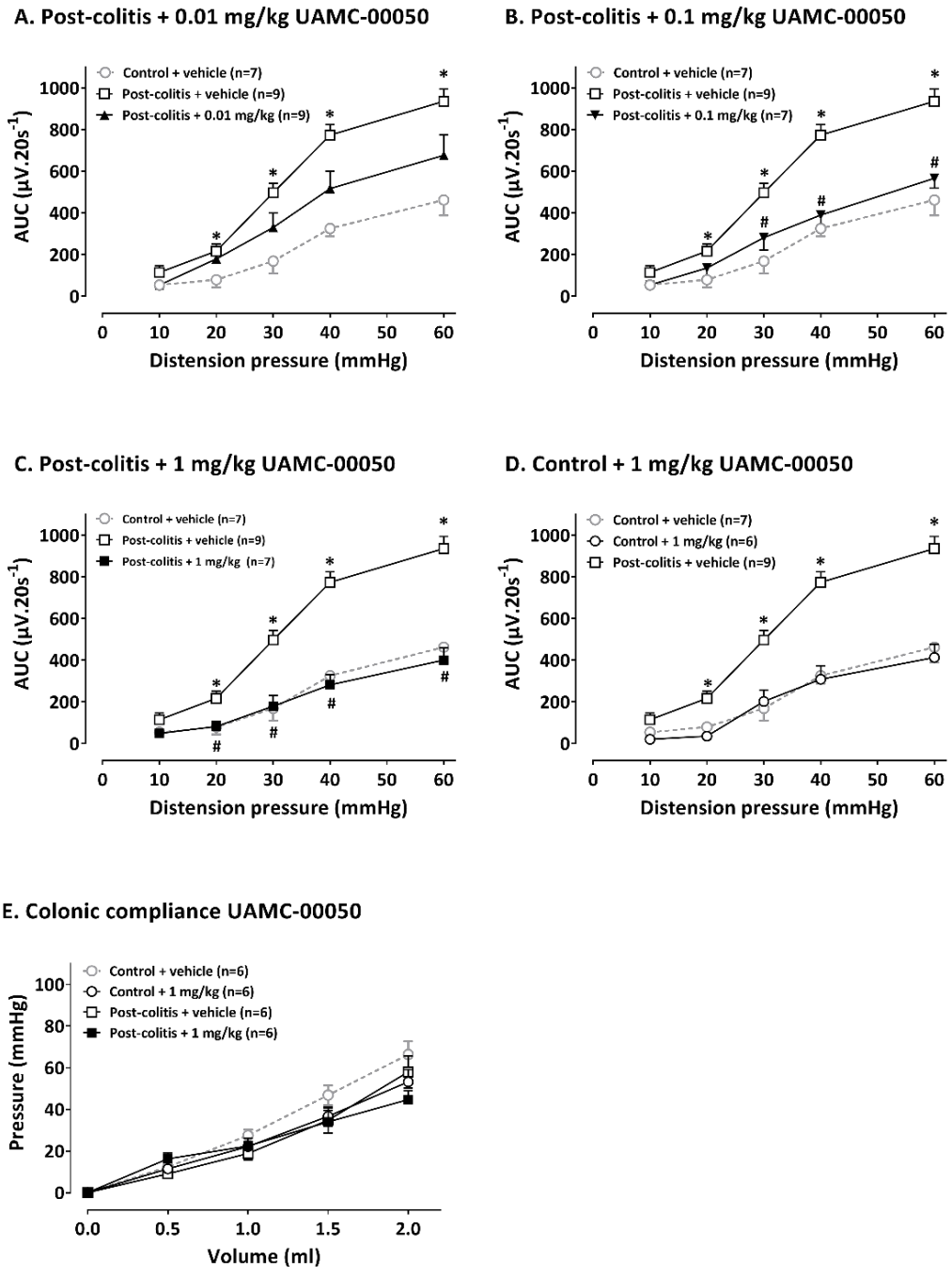


Figure 5.3. The effect of UAMC-00050 (0.01-1 mg/kg) and its vehicle (5% DMSO) on VMRs and colonic compliance in post-colitis and control rats.

The statistical analysis was performed on the complete dataset, but separate graphs were made for each dose for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from post-colitis + vehicle.

5.4.3 UAMC-01162 decreases visceral hypersensitivity in a post-inflammatory setting

A mild colitis was present in all TNBS animals, as confirmed by the significant higher colonoscopic inflammatory score at day 3 compared to controls (table 5.3). At the end of the experiments, the post-inflammatory status of all animals was confirmed.

Table 5.3. Inflammatory parameters of UAMC-01162 in post-colitis

Group	Drug	N	Colonoscopy (0-19)		Macroscopy	Microscopy	MPO activity
			Day 3	Day VMR			
Control	Vehicle	10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.9 ± 0.3
	2.5 mg/kg	10	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.6 ± 0.2
Post-colitis	Vehicle	10	6.5 ± 0.9*	0.0 ± 0.0	0.0 ± 0.0	0.6 ± 0.2	0.9 ± 0.4
	1 mg/kg	10	6.4 ± 0.8*	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	1.3 ± 0.2
	2.5 mg/kg	10	7.9 ± 0.9*	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	1.0 ± 0.2

Data are presented as mean ± SEM. Two-way ANOVA followed by S-N-K post-hoc test. No significant interaction, significant effect of the factor 'group' for colonoscopy at day 3; *$p<0.05$; no significant effect of the factor 'drug'. MPO; myeloperoxidase; N; number; VMR; visceromotor response.

Vehicle-treated post-colitis rats displayed significantly higher VMRs compared to controls, demonstrating visceral hypersensitivity (figure 5.4A). A single i.p. injection of 1 mg/kg UAMC-01162 in post-colitis animals had no effect on VMR (figure 5.4A), whereas 2.5 mg/kg UAMC-01162 completely reversed visceral hypersensitivity (figure 5.4B). In control animals, no changes in visceral sensitivity were observed after a single i.p. injection with 2.5 mg/kg UAMC-01162 (figure 5.4C). UAMC-01162 did not alter colonic compliance (figure 5.4D) and had no effect on colonic inflammatory markers (table 5.3).

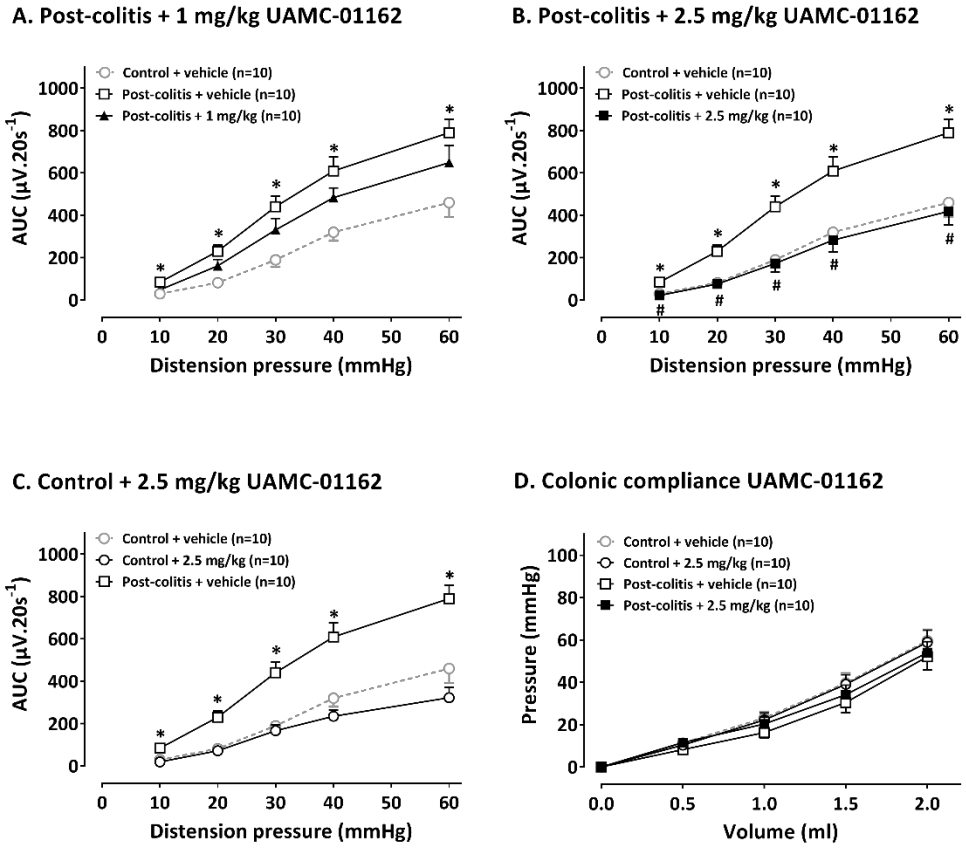


Figure 5.4. The effect of UAMC-01162 (1-2.5 mg/kg) and its vehicle (5% DMSO) on VMRs and colonic compliance in post-colitis and control rats.

The statistical analysis was performed on the complete dataset, but separate graphs were made for each dose for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; *p<0.05; **p<0.01; ***p<0.001; significantly different from control + vehicle. #p<0.05; ##p<0.01; ###p<0.001; significantly different from post-colitis + vehicle.

5.4.4 mRNA quantification of serine proteases, protease-activated receptors and transient receptor potential ion channels

The selection of serine proteases for qPCR analysis was based on the inhibition profiles of the respective inhibitors. The relative mRNA expression of Tpsab1 (tryptase) was increased in the colon of post-colitis rats, while the expression of Plau (urokinase plasminogen activator), St14 (matriptase) and KLK8 (kallikrein 8) were comparable between control and post-colitis groups. KLK2 (kallikrein 2), KLK4 (kallikrein 4) and Ctsg (cathepsin G) could not be detected (table 5.4, 5.5, 5.6). Nafamostat, UAMC-00050 or

UAMC-01162 did not affect serine protease expression at the mRNA level (table 5.4, 5.5, 5.6).

Table 5.4. Relative mRNA expression of serine proteases in colon samples –nafamostat

Gene	Control		Post-colitis	
	Vehicle	NFM 0.1 mg/kg	Vehicle	NFM 0.1 mg/kg
Tryptase αβ1	1.30 ± 0.37	1.32 ± 0.45	4.65 ± 1.07*	4.26 ± 1.41*
Matriptase	1.07 ± 0.15	1.25 ± 0.17	1.00 ± 0.16	1.05 ± 0.15
Cathepsin G	<LOD	<LOD	<LOD	<LOD
uPA	1.12 ± 0.21	1.77 ± 0.33	1.43 ± 0.31	1.07 ± 0.23
Kallikrein 2	<LOD	<LOD	<LOD	<LOD
Kallikrein 4	<LOD	<LOD	<LOD	<LOD
Kallikrein 8	1.21 ± 0.24	2.02 ± 0.57	1.18 ± 0.18	1.67 ± 0.63
	n=8	n=8	n=8	n=8

Data are expressed as relative mRNA expression and presented as mean ± sem for n=8. Two-way ANOVA. Significant effect of the factor 'group' *p<0.05 for tryptase αβ1. No significant effect of the factor 'drug'. No significant interaction. LOD; limit of detection.

Table 5.5. Relative mRNA expression of serine proteases in colon samples –UAMC-00050

Gene	Control		Post-colitis	
	Vehicle	UAMC-00050	Vehicle	UAMC -00050
Tryptase αβ1	1.30 ± 0.37	3.14 ± 1.19	4.65 ± 1.07*	2.63 ± 0.44
Matriptase	1.07 ± 0.15	0.88 ± 0.09	1.00 ± 0.16	1.00 ± 0.13
Cathepsin G	<LOD	<LOD	<LOD	<LOD
uPA	1.12 ± 0.21	1.43 ± 0.31	1.43 ± 0.31	1.23 ± 0.19
Kallikrein 2	<LOD	<LOD	<LOD	<LOD
Kallikrein 4	<LOD	<LOD	<LOD	<LOD
Kallikrein 8	1.21 ± 0.24	1.50 ± 0.37	1.18 ± 0.18	1.28 ± 0.21
	n=8	n=6	n=8	n=7

Data are expressed as relative mRNA expression and presented as mean ± sem for n=6-8. Two-way ANOVA followed by One-way ANOVA + LSD post-hoc test if applicable. No significant effect of the factor 'group'. No significant effect of the factor 'drug'. Significant interaction for tryptase αβ1. *p<0.05 significantly different from "control + vehicle". LOD; limit of detection.

Table 5.6. Relative mRNA expression of serine proteases in colon samples –UAMC-01162

Gene	Control		Post-colitis	
	Vehicle	UAMC-01162	Vehicle	UAMC-01162
Tryptase αβ1	1.29 ± 0.39	1.11 ± 0.37	2.27 ± 1.00	0.75 ± 0.22
Matriptase	1.05 ± 0.10	0.65 ± 0.11*	0.71 ± 0.11*	0.64 ± 0.07*
Cathepsin G	<LOD	<LOD	<LOD	<LOD
uPA	1.09 ± 0.16	1.09 ± 0.20	1.08 ± 0.16	1.07 ± 0.12
Kallikrein 2	<LOD	<LOD	<LOD	<LOD
Kallikrein 4	<LOD	<LOD	<LOD	<LOD
Kallikrein 8	1.19 ± 0.21	1.09 ± 0.12	0.91 ± 0.20	0.88 ± 0.16
	n=10	n=10	n=10	n=10

Data are expressed as relative mRNA expression and presented as mean ± sem for n=10. Two-way ANOVA followed by One-way ANOVA + LSD post-hoc test if applicable. No significant effect of the factor 'group'. No significant effect of the factor 'drug'. Significant interaction for matriptase *p<0.05 significantly different from "control + vehicle". LOD; limit of detection.

Recently, the implication of the serine protease trypsin has been shown in visceral hypersensitivity (Rolland-Fourcade *et al.* 2017). We therefore also investigated the colonic mRNA expression levels of the three trypsin isoforms: PRSS1 (trypsin-1 precursor), PRSS2 (trypsin-2 precursor), PRSS3 (trypsin-3 precursor). The mRNA expression level of PRSS1 was comparable in control and post-colitis rats, while PRSS3 was significantly upregulated in the post-colitis group (figure 5.5). PRSS2 was below the limit of detection.

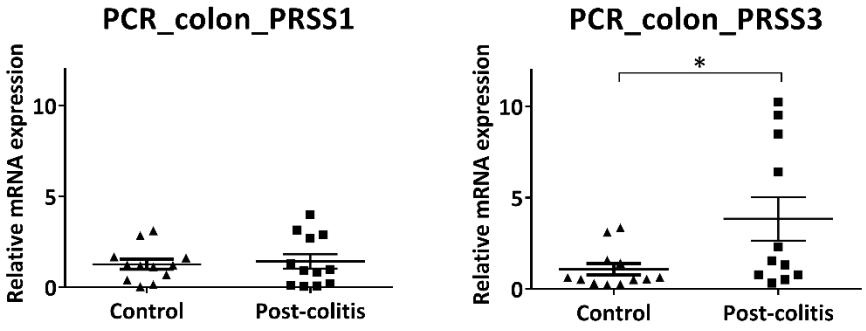


Figure 5.5. Relative mRNA expression of PRSS in colonic samples.

Data are expressed as relative mRNA expression and presented as mean ± sem for n=12 per group. Independent samples T-test. *p<0.05 significant effect of the factor "group".

We also assessed colonic mRNA expression of receptors involved in the serine protease signaling cascade i.e. protease-activated receptors (PAR) and transient receptor potential ion channels (TRP)(Cenac 2013). mRNA expression of PAR4 was significantly upregulated in colonic samples of post-colitis animals, while TRPV4 tended to be increased but this upregulation did not reach significance (p=0.074). No significant differences were detected for PAR2, TRPA1 and TRPV1 in the colon of control versus post-colitis animals (table 5.7).

Table 5.7. Relative mRNA expression of PARs and TRPs in colonic, DRG T13-L2 and DRG L6-S1 samples.

Gene	Colon		DRG T13-L2		DRG L6-S1	
	Control	Post-colitis	Control	Post-colitis	Control	Post-colitis
PAR2	1.05 ± 0.11	0.90 ± 0.14	1.07 ± 0.11	1.31 ± 0.08	1.07 ± 0.13	0.75 ± 0.04*
PAR4	0.94 ± 0.15	1.87 ± 0.30*	1.03 ± 0.08	1.60 ± 0.17*	1.05 ± 0.12	1.21 ± 0.25
TRPA1	1.09 ± 0.19	1.27 ± 0.28	1.04 ± 0.08	1.40 ± 0.12*	1.10 ± 0.15	0.81 ± 0.09
TRPV1	1.03 ± 0.10	0.81 ± 0.15	1.04 ± 0.08	1.19 ± 0.07	1.07 ± 0.12	1.49 ± 0.20
TRPV4	1.04 ± 0.11	1.73 ± 0.36[a]	1.10 ± 0.18	1.48 ± 0.17	1.05 ± 0.11	0.88 ± 0.10
	n=8	n=8	n=12	n=12	n=12	n=12

Data are expressed as relative mRNA expression and presented as mean ± sem for n=8-12 per group. Independent samples T-test. *p<0.05 significant effect of the factor "group"; [a]p=0.074. DRG; dorsal root ganglia; PAR; protease-activated receptor; TRPA; transient receptor potential cation channel.

qPCR experiments on DRGs were executed at the level of colonic splanchnic afferent nerves (T13-L2) and colonic pelvic afferent nerves (L6-S1). qPCR on DRG level T13-L2 revealed a clear trend towards upregulation of all PARs and TRPs in the post-colitis group, showing significant results for PAR4 and TRPA1. At DRG level L6-S1, a trend towards a lower mRNA expression of PARs and TRPs was seen in the post-colitis animals, showing significant results for PAR2 (table 5.7).

5.4.5 Immunohistochemistry for mast cell tryptase

The total number of mast cell tryptase positive cells per mm^2, quantified by immunohistochemistry in colonic mucosa, was significantly increased in post-colitis rats compared to control animals (figure 5.6). Similar to the mRNA results, treatment

with serine protease inhibitors did not affect mast cell tryptase expression at the protein level (figure 5.6).

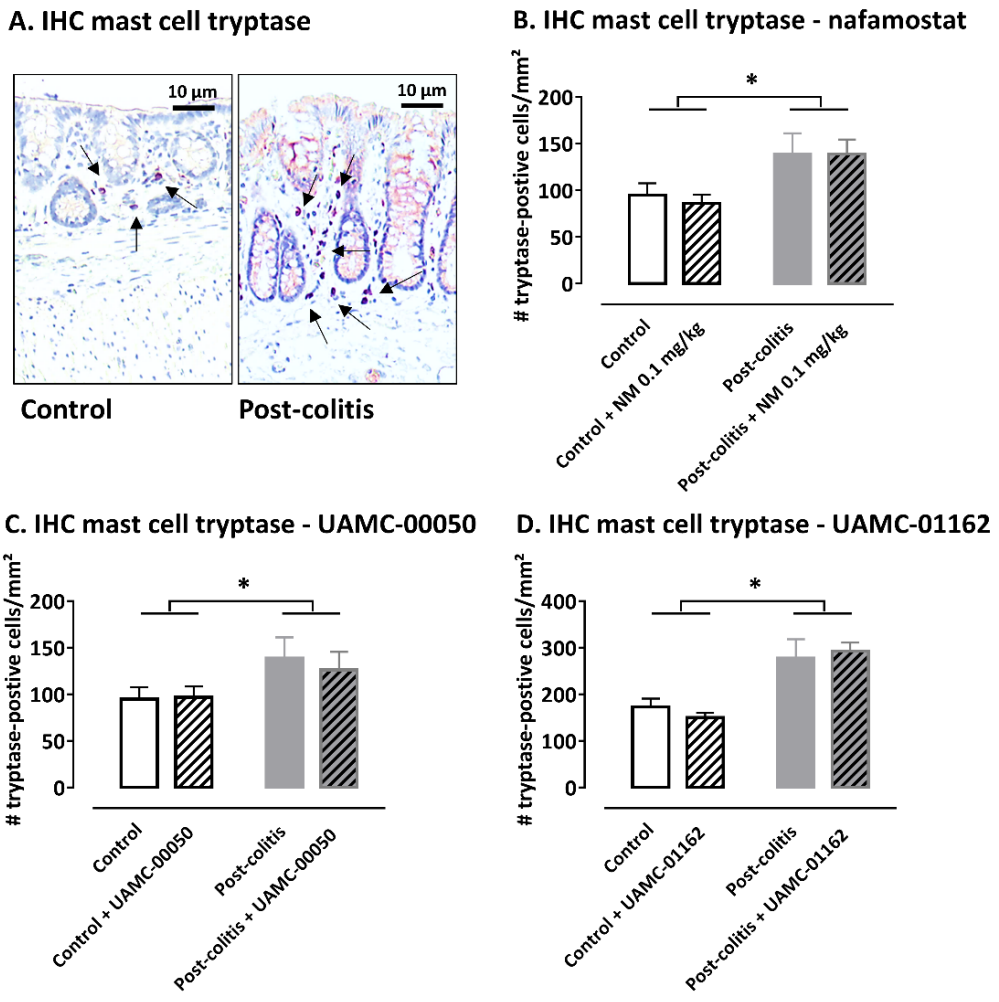


Figure 5.6. Immunohistochemistry with mast cell tryptase antibody in rat colon.

A. Representative images of the colonic mucosa of a control and a post-colitis animal with mast cell tryptase (arrow). B, C, D. The number of tryptase-positive mast cells per mm² in the colonic mucosa of control and post-colitis rats with or without treatment with a serine protease inhibitor. Two-way ANOVA; n=6-8; *p<0.05; significant effect of the factor 'group'; no significant effect of the factor 'drug'; no significant interaction between the factors 'group' and 'drug'.

5.4.6 Immunohistochemistry for PAR2, PAR4 and TRPA1

To investigate the potential involvement of the protease-PAR-TRP axis, we determined the expression of PAR2, PAR4 and TRPA1 in nerve fibers in the lamina propria of the distal colon. CGRP-immunoreactive sensory nerve fibers showed a clear co-expression

of PAR2 (figure 5.7 A-B-C), whereas PAR4 appeared to be absent in these fibers (figure 5.7 D-E-F). TRPA1, which is a downstream target of PAR2 signaling, was also co-expressed with CGRP in lamina propria nerve fibers (figure 5.7 G-H-I).

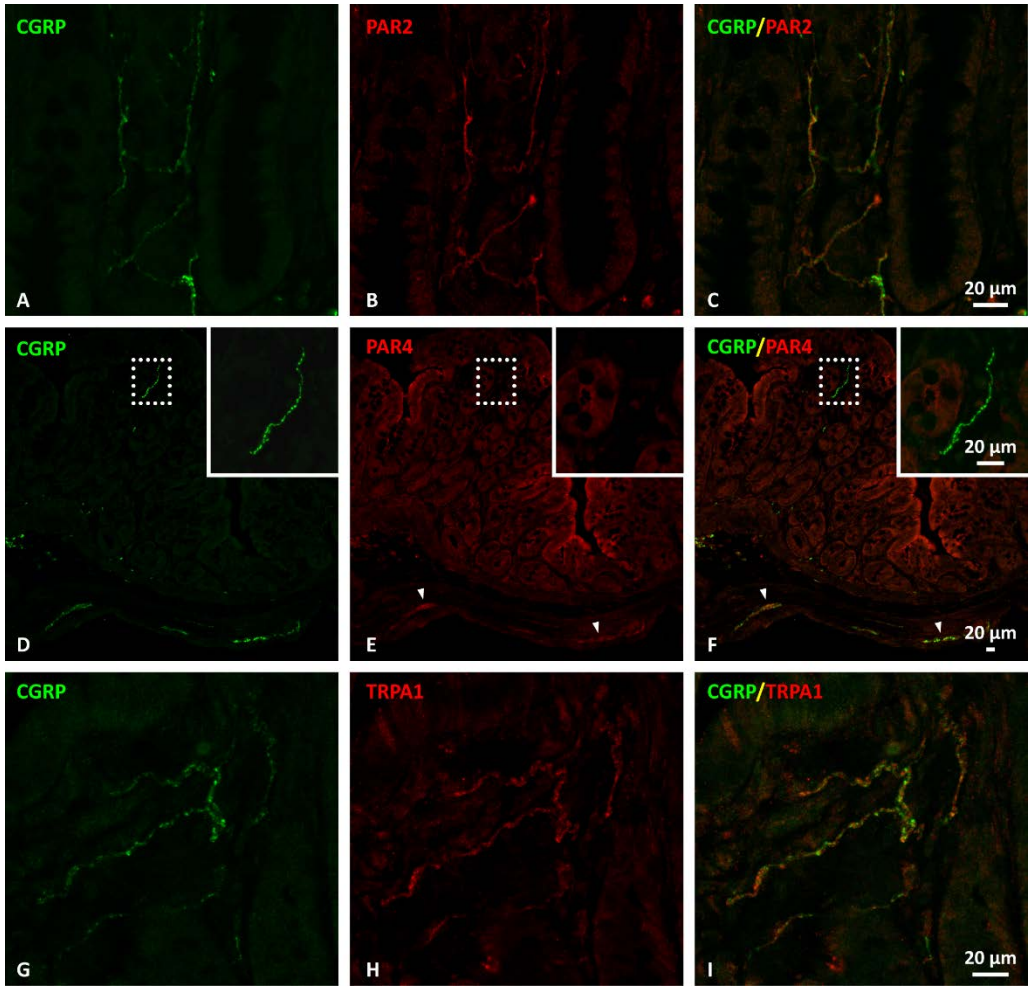


Figure 5.7. Immunohistochemical localization of PAR2, PAR4, TRPA1 in sensory nerve fibers of the distal colon.

A-C. Representative image showing co-localization of PAR2 (red) in CGRP-immunopositive nerve fibers (green). D-F. Representative image showing the presence of PAR4 immunoreactivity (red) in the colonic epithelium and in enteric nerve plexuses (arrowheads), but not in the CGRP-immunoreactive nerve fiber population (inset). G-I. Representative image showing co-localization of TRPA1 (red) in CGRP-immunopositive nerve fibers (green).

5.4.7 Total fecal protease activity was increased during post-colitis

Total protease activity was assessed by means of an azocasein assay in fecal samples of control vs post-colitis rats. Fecal protease activity was significantly increased in post-colitis rat samples compared to controls (Figure 5.8).

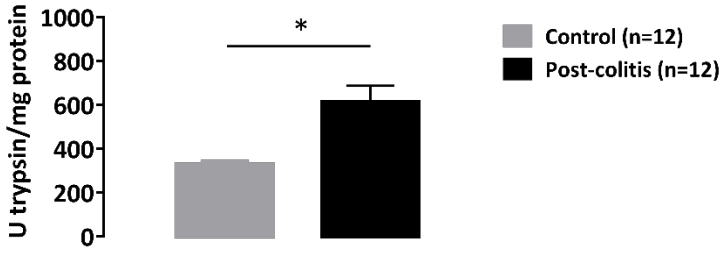


Figure 5.8. Fecal protease activity.

The protease activity expressed as mean U trypsin/mg protein ± SEM in fecal samples of control (grey bar) and post-colitis (black bar) animals. Unpaired Student's T-test; n=12; $p<0.05$; significant effect of the factor 'group'.

5.4.8 Proteolytic activities

In order to assess the proteolytic activities in control vs post-colitis rats, we determined trypsin-like, chymotrypsin-like, neutrophil elastase, pancreas elastase and kallikrein activities in colon and fecal samples. Trypsin-like activity was found to be significantly upregulated in the colon of post-colitis rats compared to controls using both Boc-Gln-Ala-Arg-AMC (figure 5.9 A) and n-Tosyl-Gly-Pro-Arg-AMC (figure 5.9 B) as a substrate. Neutrophil elastase, pancreas elastase, kallikrein and chymotrypsin-like activities were not significantly different in colonic samples from post-colitis vs control animals (figure 5.9 C-F). In fecal samples, no significant differences could be detected between control and post-colitis rats (figure 5.10 A-F).

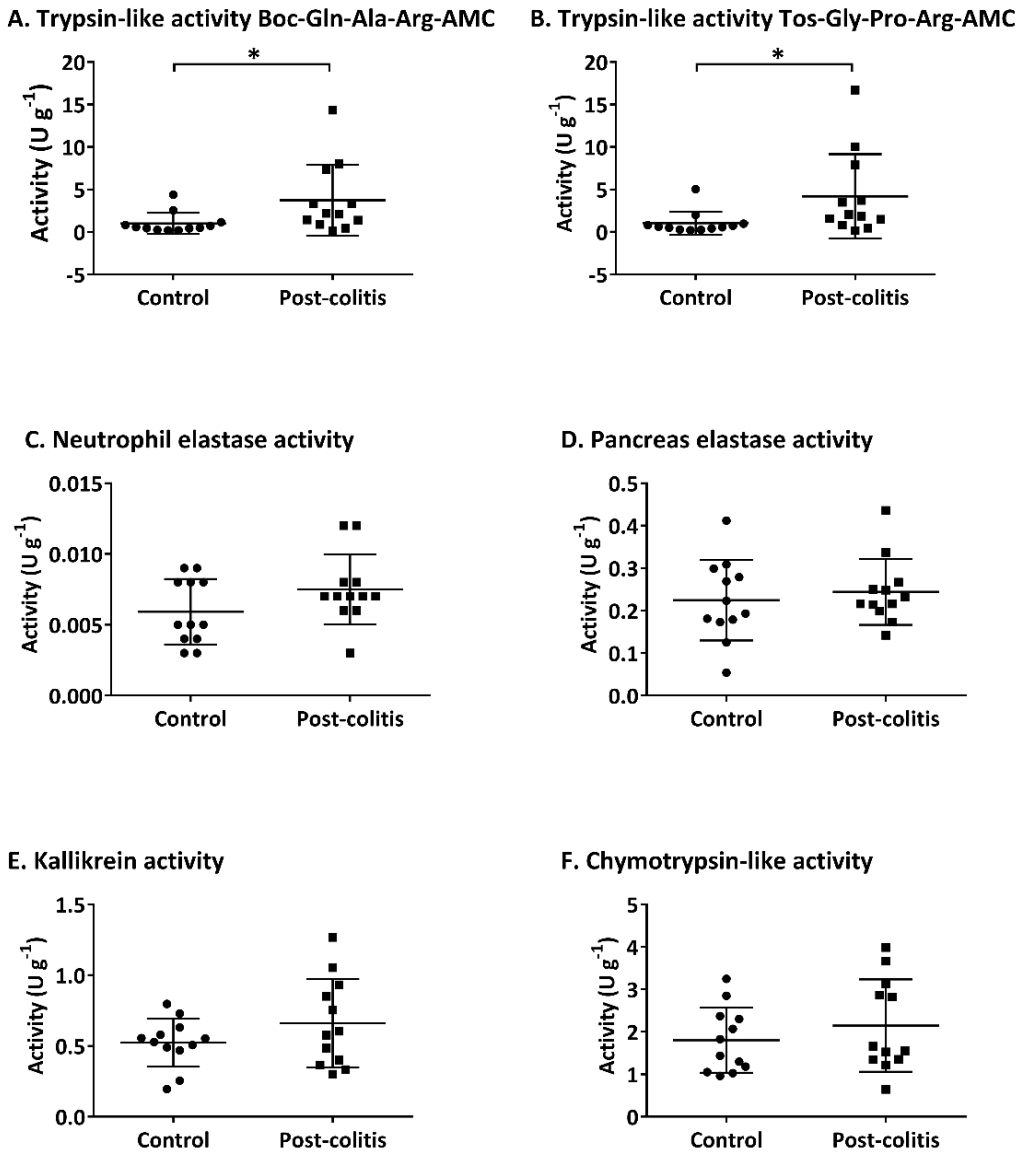


Figure 5.9. Proteolytic activities determined in colon samples.

Trypsin-like activity was measured using Boc-Gln-Ala-arg-AMC and n-Tosyl-Gly-Pro-Arg-AMC. To determine the neutrophil elastase, pancreas elastase, kallikrein and chymotrypsin-like activity, Suc-Ala-Ala-Pro-Val-AMC, Suc-Ala-Ala-Ala-AMC, H-Pro-Phe-Arg-AMC and Suc-Ala-Ala-Pro-Phe-AMC were used respectively. Data are presented as mean ± SEM. Mann Whitney U-test; * $p<0.05$ n=12.

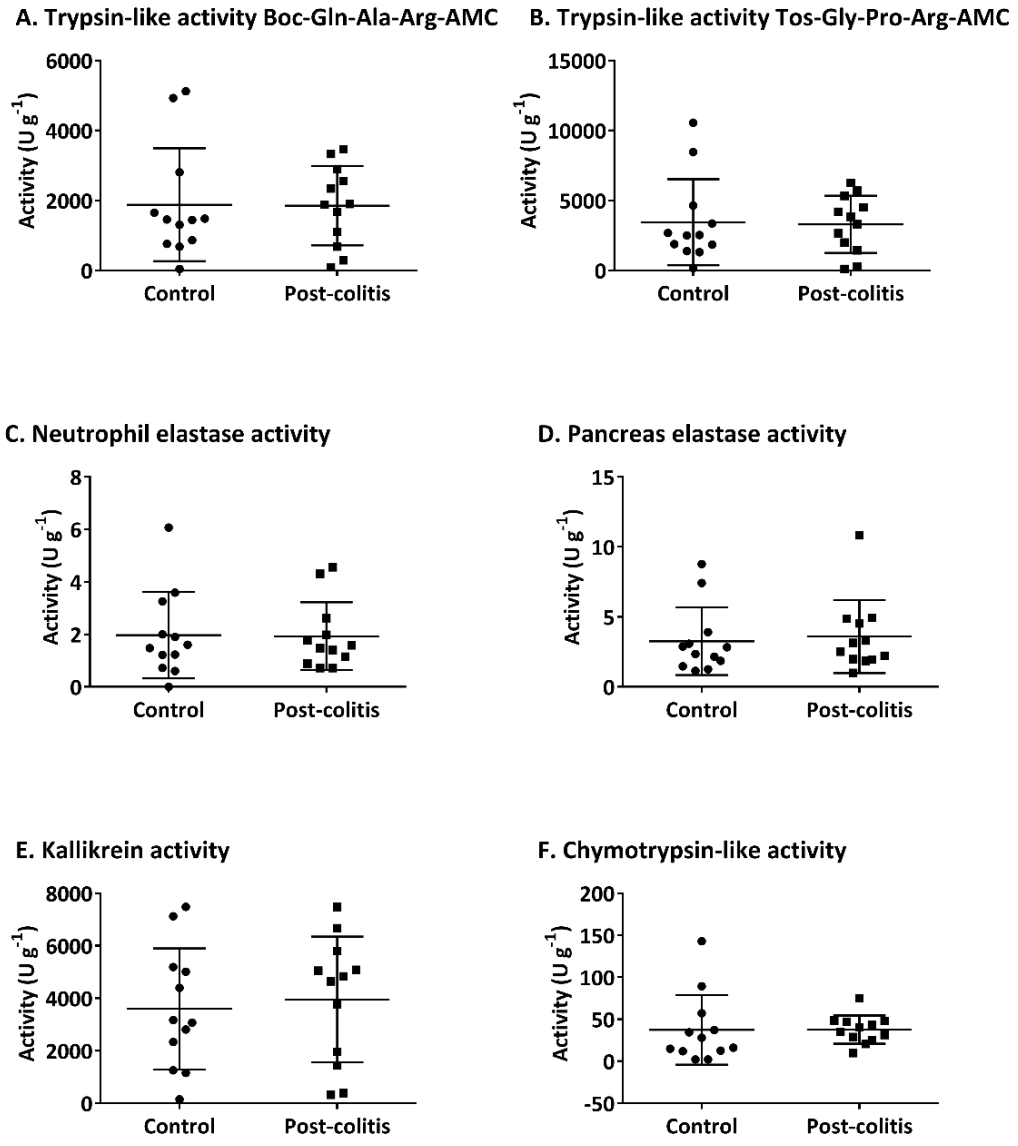


Figure 5.10. Proteolytic activities determined in fecal samples.

Trypsin-like activity was measured using Boc-Gln-Ala-arg-AMC and n-Tosyl-Gly-Pro-Arg-AMC. To determine the neutrophil elastase, pancreas elastase, kallikrein and chymotrypsin-like activity, Suc-Ala-Ala-Pro-Val-AMC, Suc-Ala-Ala-Ala-AMC, H-Pro-Phe-Arg-AMC and Suc-Ala-Ala-Pro-Phe-AMC were used respectively. Data are presented as mean ± SEM. Mann Whitney U-test; n=12.

5.5 Discussion

The aim of this study was to elucidate the effects of serine protease inhibitors on post-inflammatory visceral hypersensitivity and their targets of action. Our results suggest that treatment with serine protease inhibitors reverses the visceral pain response to colonic distension in rats with post-inflammatory visceral hypersensitivity. Nafamostat significantly decreased visceral hypersensitivity, with a complete reversal in the lowest dose tested. Furthermore, both UAMC-00050 and UAMC-01162, two newly developed serine protease inhibitors [patent WO2007045496 (A1)] (Joossens *et al.* 2007) significantly lowered visceral hypersensitivity in a dose-dependent way, completely restoring visceral sensitivity in the highest dose used. The serine protease inhibitors had no effect in healthy controls and did not affect colonic compliance, excluding an effect on viscoelastic properties of the colon.

Next, we tried to further unravel the specific serine proteases involved and their possible mode of action. Firstly, in order to define the serine proteases involved in a state of post-inflammatory visceral hypersensitivity, mRNA gene expression assays were performed. Colon tissue was tested for the expression of a panel of serine proteases, defined by those with an $IC_{50} < 10^{-6}$M for the serine protease inhibitors tested in this study. Our data revealed a 4-fold increased mRNA expression of the serine protease tryptase as well as a significant upregulation of PRSS3 at the colonic level in post-colitis rats, while all other serine proteases tested in our panel were not significantly altered or below the detection limit in colon tissue. We confirmed this result at the protein level, by demonstrating a significantly increased number of mast cell tryptase positive cells in the colonic mucosa of post-colitis rats compared to controls. Our results thereby point towards an important role for the serine protease tryptase in post-inflammatory visceral hypersensitivity. Moreover, we could not show evidence for KLK2, KLK4, KLK8, cathepsin G and uPA at the colonic level.

These serine protease profile determinations could be of great importance towards the development of a new biomarker for IBS (Barbara 2015). Previous studies showed an increased fecal protease activity in IBS-D (serine proteases) and IBS-C (cysteine

proteases) patients compared to healthy controls (Roka *et al.* 2007, Gecse *et al.* 2008, Annahazi *et al.* 2013), highlighting the potential of proteases as a diagnostic tool.

Interestingly, the three serine protease inhibitors did not affect mRNA/protein expression, suggesting an effect on the activity of the enzymes rather than on the expression. It is of great interest to determine the serine protease activity as well as the effect of the serine protease inhibitors hereon, but the currently available tools to study protease activity, such as the azocasein assay, are very unspecific (Edgington-Mitchell 2015). The fluorogenic substrates used here allow to better characterize the proteolytic activities. Trypsin-like activity was found to be significantly increased in the colon of post-colitis rats compared to controls with both substrates, while no significant differences could be detected in fecal samples. However, more specific probes and/or inhibitors to directly assess the activity of individual proteases are essential for a more precise interpretation of the results. Edgington-Mitchell *et al.* recently demonstrated the efficacy of two fluorescent activity based probes detecting serine proteases *in vitro*, but their use was less valuable in two *in vivo* animal models for acute inflammation (pancreatitis and colitis) (Edgington-Mitchell *et al.* 2017). Optimization of these probes for *in vivo* use is of great importance when studying the role of serine proteases in several pathologies.

Remarkably, nafamostat showed its greatest potential at the lowest dose used pointing to a loss of specificity of the compound on visceral hypersensitivity: nafamostat 0.01 mg/kg was more effective than 0.1 mg/kg and 1 mg/kg and no effect was observed in a dose of 10 mg/kg. Amongst all of the examined proteases, the affinity of nafamostat is the highest for human tryptase ($9.53x10^{-11}$M). Thus, when used in a relatively low concentration, nafamostat is an extremely potent and selective inhibitor of human tryptase (Mori *et al.* 2003) suggesting that by using higher concentrations the selectivity for tryptase might get lost, altering the protease profile in a more profound way.

Besides investigating the type of serine proteases involved in visceral hypersensitivity, we also studied the source of the proteases and site of action of the serine protease inhibitors. Sources of proteases in the gastrointestinal tract are diverse and include pancreas, microbiome, epithelial cells, neutrophils, macrophages and mast cells (Vergnolle 2016). Our results provide evidence for the presence of tryptase in mast cells. Recently, the group of Vergnolle demonstrated the release of trypsin-3 by the intestinal epithelium and showed its involvement in visceral hypersensitivity (Rolland-Fourcade *et al.* 2017). This highlights that further study is needed towards the source of proteases implicated in visceral pain.

We hypothesize that the compounds used in our study directly inhibit serine proteases early in the signaling cascade, thereby preventing the proteases from activating PARs which on their turn are incapable of TRP sensitization thereby preventing visceral hypersensitivity. A recent review on TRP channels reports that pro-inflammatory mediators such as serotonin, histamine, bradykinins and proteases can activate various G-protein coupled receptors (GPCR) such as PAR receptors, thereby triggering TRP sensitization in visceral hypersensitivity (Balemans et al. 2017). To investigate this hypothesis a bit further in our model, we assessed the mRNA expression of PARs and TRPs which play a role in visceral hypersensitivity and serine protease downstream signaling pathways, at the colonic level and at DRGs. qPCR analysis revealed a significant upregulation of PAR4 in IBS rats at the colonic level and DRG T13-L2. A significant upregulation of TRPA1 was found in DRG T13-L2, while TRPV4 showed a tendency to increase in the colon of IBS rats. Interestingly no significant upregulations of PARs and/or TRPs were found in DRGs L6-S1, highlighting the importance of thoracolumbar spinal cord (T13-L2) in the processing of visceral pain signals (Traub 2000, Christianson *et al.* 2006). These mRNA data support the involvement of both PARs and TRPs in the downstream pathways of serine proteases involved in post-inflammatory visceral hypersensitivity.

In order to reinforce our hypothesis on the involvement of PAR and TRP receptors in visceral hypersensitivity, immunohistochemical experiments were performed next to

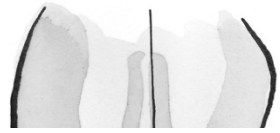
the qPCR experiments. Increased expression of mast cell tryptase was observed both at the mRNA level as well as immunohistochemically in the colon of post-colitis rats compared to control animals. Tryptase is a PAR2 ligand and the involvement of both tryptase and the PAR2 receptor in visceral hypersensitivity has already been reported previously (Vergnolle *et al.* 2001, Cenac *et al.* 2007). We now demonstrate the presence of PAR2-immunopositive nerve fibers in the colon of post-colitis rats, for which double labeling with CGRP confirmed their sensory origin. We thus provide evidence at the protein level of the presence of both tryptase and PAR2 in the colon of post-colitis animals. Furthermore, we could also demonstrate the presence of TRPA1 in CGRP-positive nerve fibers in the lamina propria of the colon. Previously, Cattaruzza and colleagues already demonstrated that TRPA1 receptors mediate PAR2-induced visceral hypersensitivity (Cattaruzza et al. 2010). Thus, our results fit in the general hypothesis mentioned above and previously described in the review by Balemans *et al*. (Balemans *et al.* 2017).

Interestingly, a downregulation of PAR4 was shown in colonic biopsies of IBS-D, IBS-C and post-infectious IBS patients (Han *et al.* 2012, Zhao *et al.* 2012). This discrepancy may be explained by a study of Annahazi *et al.* (Annahazi *et al.* 2012). In a low-grade TNBS-colitis mouse model, PAR4 antagonism increases colorectal hyperalgesia. Their proposed mechanism is an endogenous activation of PAR4 (possibly by Cat-G), inducing a feedback antinociceptive effect. Moreover, PAR4 activation has been shown to result in antinociceptive effects as demonstrated by the inhibition of the excitability of mouse and rat colonic DRGs after the application of a PAR4-activating peptide (AP) (Asfaha *et al.* 2007, Karanjia *et al.* 2009), a decrease in carrageenan-induced inflammatory visceral hypersensitivity after an injection with a PAR4-AP (Asfaha *et al.* 2007) and a reduced visceral hypersensitivity in mice after an intracolonic administration with the PAR4-agonist AYPGKF-NH2 (Auge *et al.* 2009). We therefore hypothesize that the increased mRNA expression of PAR4 in the colon and DRG samples of post-colitis rats might be a consequence of post-inflammatory visceral hypersensitivity, rather than a cause. Besides, we were able to localize PAR4 in epithelial cells as well as in myenteric and submucosal neurons.

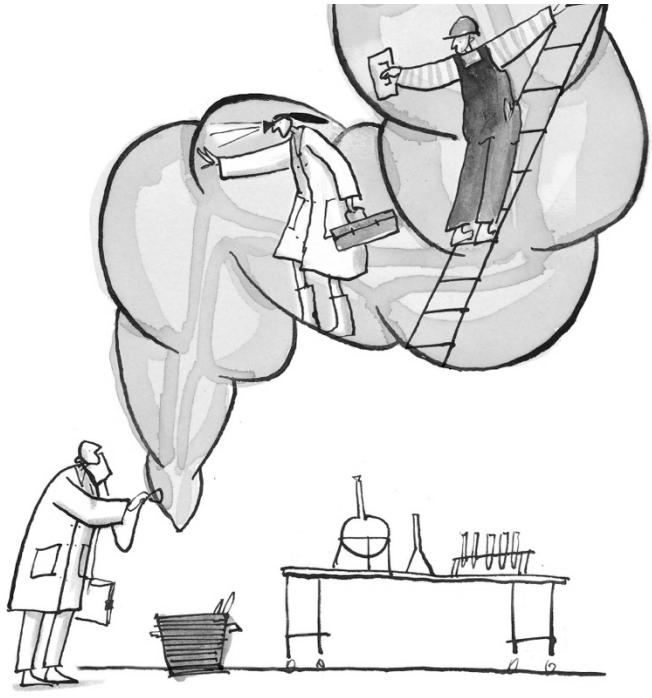
The trend towards increased expression of TRPV4 in colon of post-inflammatory IBS rats is in line with the increased expression of TRPV4 in human colon biopsies in IBD patients (Fichna *et al.* 2012). However, to our knowledge, no such data are available for IBS patients. Also, the upregulation of TRPA1 in DRGs of rats with visceral hypersensitivity following TNBS-induced colitis is in line with the findings from Yang *et al.* (Yang *et al.* 2008). Also in a water-avoidance-stress-induced rat model for IBS, a significant upregulation of TRPA1 was seen in DRGs (Yu *et al.* 2010).

In summary, we investigated the effect of three different serine protease inhibitors in a rat model for post-inflammatory visceral hypersensitivity. After a single administration of either of these compounds, a decrease in visceral pain was proven in post-colitis rats but no effect was observed in healthy controls. Our work differs from previous studies in three key aspects. Firstly, we opted for a direct *in vivo* treatment strategy, with a single intraperitoneal injection of the animals, while preceding studies employed an *in vitro* treatment strategy (Cenac *et al.* 2007, Wang *et al.* 2015). Secondly, we are the first to demonstrate a positive outcome after a symptomatic treatment instead of a preventive treatment in earlier studies (Cenac *et al.* 2007, Zhao *et al.* 2011, Wang *et al.* 2015). Thirdly, the animal models used are different. Previous studies employed intracolonic administration in mice of supernatants from IBS patient (Cenac *et al.* 2007, Wang *et al.* 2015) or acute stress in rats (Zhao *et al.* 2011) to induce visceral hypersensitivity whereas we used an inflammation-triggered post-colitis model based on chemically-induced colitis in rats. We provide further evidence for the role for the serine proteases tryptase and trypsin-3 in this post-inflammatory rat model for IBS, evidenced by elevated mRNA expression and an increased number of mast cell tryptase positive cells. Moreover, the increased tryptase expression at the colonic level of post-colitis rats, increased trypsin-like serine protease activity in the colon of post-colitis rats, the localization of PAR2 and TRPA1 in the sensory nerve fibers in the colon together with the increased TRPA1 mRNA expression at the DRG level in post-colitis rats, suggest their possible involvement in post-inflammatory visceral hypersensitivity.

Our results indicate that serine protease inhibition represents an interesting new treatment strategy for abdominal pain in IBS patients. Regarding the search for new biomarkers in IBS patients, our study points towards serine proteases and more specifically tryptase as a possible candidate.



CHAPTER 6 - VALIDATION OF A NEWLY DEVELOPED SMALL-MOLECULE SERINE PROTEASE INHIBITOR IN A NEONATAL ACETIC ACID MOUSE MODEL FOR VISCERAL HYPERSENSITIVITY



Adapted from: Ceuleers H, Li Q, De Man JG, Liu L, Joossens J, Augustyns K, De Meester I, De Winter BY, Pasricha PJ. Targeting the serine protease pathway in IBS: beneficial effects of a novel small molecule inhibitor in mice. Will be submitted to American Journal of Physiology – Gastrointestinal and Liver physiology.

6.1 Abstract

Background and aims

Serine proteases are enzymes present at high levels in the gastrointestinal tract, playing a role in different (patho-)physiological processes (e.g. inflammation, permeability, visceral pain) through the activation of protease-activated receptors. Serine proteases have been suggested to be involved in IBS, but its exact role is not fully elucidated yet. Previously, we demonstrated a beneficial effect of UAMC-00050 on visceral hypersensitivity in a TNBS post-colitis rat model for IBS. The aim of this study was to validate the beneficial effects of UAMC-00050 on visceral pain by using a different species and a different IBS model. Therefore, we investigated the effect of UAMC-00050 in a neonatal acetic-acid induced mouse model for visceral hypersensitivity. Nafamostat mesylate was used as a positive control.

Methods

Ten-day old C57BL/6 mice pups were colorectally infused with 20 µl 0.5% acetic acid (IBS) or 0.9% NaCl (control). At 12 weeks of age, mice received a single intraperitoneal (i.p) injection with either vehicle (1% DMSO in sterile water), nafamostat mesylate (0.2 mg/kg) or UAMC-00050 (2 mg/kg). All dosages were based on previously proven effective doses in the rat model as described in chapter 5. The animals were randomized and the investigators were blinded to the treatment. Visceral sensitivity was assessed 30 min later, by measuring the visceromotor responses (VMR) to colorectal distension at distension pressures of 15-30-50-70 mmHg, using electromyographic recordings from the external oblique muscle.

Results

The vehicle-treated IBS mice displayed significantly higher VMRs compared to controls, confirming the presence of visceral hypersensitivity. A single i.p. injection with UAMC-00050 (30 min before VMR measurement) significantly decreased VMRs at all distension pressures and even completely restored sensitivity to normal values. Similarly, a single i.p. administration of nafamostat mesylate significantly lowered

VMRs at all distension pressures. Both UAMC-00050 and nafamostat mesylate had no effect on visceral sensitivity in control mice.

Conclusions

Our results demonstrate beneficial effects of two different serine protease inhibitors in a neonatal acetic acid mouse model for IBS. Our results in this model thus confirm a role for serine protease inhibitors as a new therapeutic strategy for visceral pain.

6.2 Introduction

Chronic abdominal pain is a main symptom in functional gastrointestinal disorders such as irritable bowel syndrome (IBS), as described in detail in Chapter 1. Visceral hypersensitivity is considered as an important mechanism underlying the abdominal pain in IBS patients (Barbara *et al.* 2011). However, current treatment strategies for IBS mostly focus on the normalization of the associated motility dysfunction (Hungin *et al.* 2003), thereby indicating the need for research towards new treatment strategies directly tackling visceral hypersensitivity in IBS patients.

Serine proteases are enzymes present at particularly high levels in the gastrointestinal tract. They can affect gut physiological functions such as inflammation, motility, intestinal permeability and sensory functions through the activation of protease-activated receptors (PARs) (Vergnolle 2005).

The implication of serine proteases in IBS has clearly been shown by several research groups demonstrating an increased serine protease expression and/or activity in colonic and fecal samples of IBS patients (Barbara *et al.* 2004, Cenac *et al.* 2007, Roka *et al.* 2007, Gecse *et al.* 2008, Annahazi *et al.* 2009, Buhner *et al.* 2009, Tooth *et al.* 2014). Much research effort has been put into the investigation of PARs influencing sensory dysfunction, thereby leading to visceral pain in IBS. Remarkably, direct serine protease inhibition as a possible new treatment strategy for visceral pain has been studied to a lesser extent (Ceuleers *et al.* 2016, Vergnolle 2016). Indeed, only very few

preclinical animal studies assessed the effect of serine protease inhibitors on visceral hypersensitivity.

Visceral hypersensitivity, induced in mice after a colonic infusion with the supernatant from IBS patient biopsies, is shown to be abolished after *in vitro* pretreatment of the supernatant with the broad-spectrum serine protease inhibitor nafamostat mesylate (Cenac *et al.* 2007, Wang *et al.* 2015). We believe that, up until now, Zhao et al. provided the only proof of a decrease in visceral pain by direct *in vivo* serine protease inhibition, demonstrating reduced pain in a rat model of stress-induced visceral hypersensitivity after an intragastric pre-treatment with camostat, structurally related to nafamostat (Zhao *et al.* 2011). The limited amount of studies available regarding the effects of serine protease inhibitors in visceral hypersensitivity underlines the need for more extensive research on this topic.

In chapter 5, we demonstrated a beneficial *in vivo* effect of UAMC-00050 on visceral hypersensitivity in a post-inflammatory rat model for IBS. The aim of this study was to validate UAMC-00050 by using a different species and a different model to study visceral pain. Therefore, we investigated the effect of UAMC-00050 in a neonatal acetic-acid induced mouse model for visceral hypersensitivity. Nafamostat mesylate was used as a positive control.

6.3 Material and methods

6.3.1 Animals

C57BL/6 mice (8-12 weeks old) were housed with 6 animals per cage at constant room temperature (23°C) and humidity (45%). They had unlimited access to water and food and were kept on a 12h:12h day-night cycle.

6.3.2 Acetic-acid induced IBS mouse model

At postnatal day 10, B57BL/6 mice were intracolonically infused with 20µL of a 0.5% acetic acid (AA) solution in 0.9% NaCl, as described in detail in Chapter 3. Control animals received 0.9% NaCl. The infusion of diluted acetic acid (0.5%) in neonatal mice

results in a mild chemical irritation of the colon without causing inflammation as evidenced by the absence of any histological signs of inflammation assessed by H&E staining in both pups and adult mice, no increase in MPO value and no increase in pro-inflammatory cytokines (IL-1α, IL-1β, IL-2, IL-6, TNF-α) at the colonic level (Winston *et al.* 2007). The infusion of acetic acid thereby mimics an early-life stress event which has been described as a possible cause for visceral hypersensitivity in IBS patients. This early-life stress event eventually leads to the presence of visceral hypersensitivity in these mice in their adulthood (age 8-12 weeks) (Winston *et al.* 2007). Mice were weaned at the age of 21 days and used to assess visceral sensitivity at 8-12 weeks old.

6.3.3 Experimental design

The experimental course is shown in figure 6.1. Visceral sensitivity was assessed by means of the visceromotor responses (VMR) to colorectal distension at the age of 8-12 weeks old. Mice received a single intraperitoneal (i.p) injection with either vehicle (1% DMSO in sterile water), nafamostat mesylate (0.2 mg/kg) or UAMC-00050 (2 mg/kg), 30 min prior to the VMR experiment. The active compounds are described in detail in Chapter 3. All dosages were based on the previously proven most effective doses in a TNBS-induced post-inflammatory rat model for IBS as described in Chapter 5. A point of particular interest in this case was the dose conversion between two different animal species, namely from rats to mice. We therefore used a table displaying approximate conversion factors for different species based on the equivalent surface area (Freireich *et al.* 1966). This table indicates a factor x2 when converting dosages expressed as mg/kg from rats to mice leading to the calculation of 0.2 mg/kg (0.1 mg/kg x 2) for nafamostat mesylate and 2 mg/kg (1 mg/kg x 2) for UAMC-00050. Furthermore, we took into account that nafamostat has a 10x higher affinity for most of the proteases compared to UAMC-00050. The animals were randomized and the investigators were blinded to the treatment.

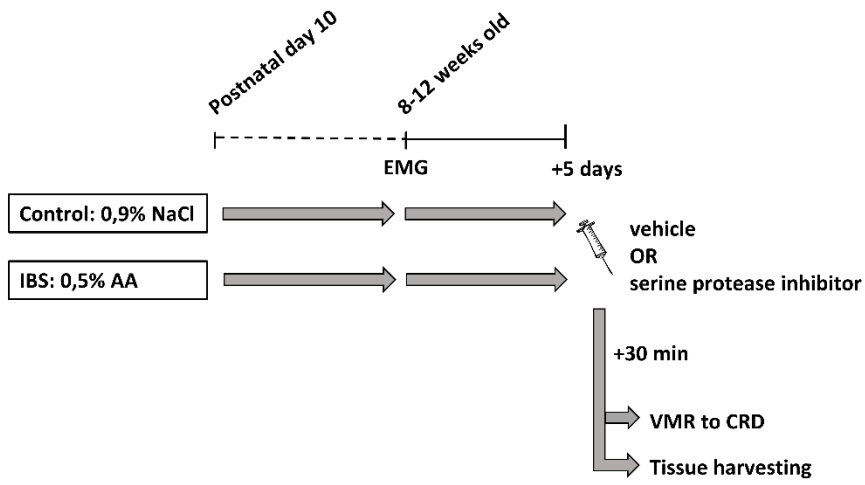


Figure 6.1. Overview of the experimental design.

On postnatal day 10, mice received an intrarectal administration with AA (IBS) or 0.9% NaCl (control). In their adulthood (8-12 weeks old), EMG electrodes were implanted and 5 days later visceral sensitivity was assessed by VMR to CRD after a single i.p. injection with serine protease inhibitor/vehicle. EMG; electromyographic; AA: acetic acid; CRD: colorectal distension; VMR; visceromotor response.

6.3.4 Visceromotor response

Visceral sensitivity was evaluated by measuring the VMR to a colorectal balloon distension (CRD) as extensively described in Chapter 3. In short, VMR to CRD was evaluated by electromyographic (EMG) electrodes that were implanted into the abdominal musculature 5 days prior to VMR assessment. At the day of the VMR registration and under isoflurane anesthesia, a lubricated balloon (2 cm length) was introduced into the colorectum and the mouse was put into a restrainer and allowed to recover from anesthesia for 30 min. Balloon distensions were induced according to the following pressure protocol: 15-30-50-70 mmHg, during 10s. The resulting EMG recordings were registered for each pressure and the VMR signal was quantified as the modulus during distension (10s), corrected for the modulus before distension (10s, baseline).

6.3.5 Statistical analysis

All data are presented as mean ± SEM. VMR data were analyzed using a Generalized Estimating Equations (GEE) test with Least Significant Difference (LSD) post-hoc test.

The statistical analysis was performed using SPSS Statistics (version 24.0, IBM). A p-value <0.05 was considered statistically significant.

6.4 Results

6.4.1 Nafamostat mesylate decreases acetic acid-induced visceral hypersensitivity

The vehicle-treated IBS mice displayed significantly higher VMRs compared to controls at all distension pressures (15-70 mmHg), confirming the presence of visceral hypersensitivity (figure 6.2A). A single i.p. injection with nafamostat mesylate (0.2 mg/kg) 30 min before the VMR measurement significantly lowered VMRs at all distension pressures (figure 6.2A). In a dose of 0.2 mg/kg, nafamostat had no significant effect on visceral sensitivity in control mice (figure 6.2B).

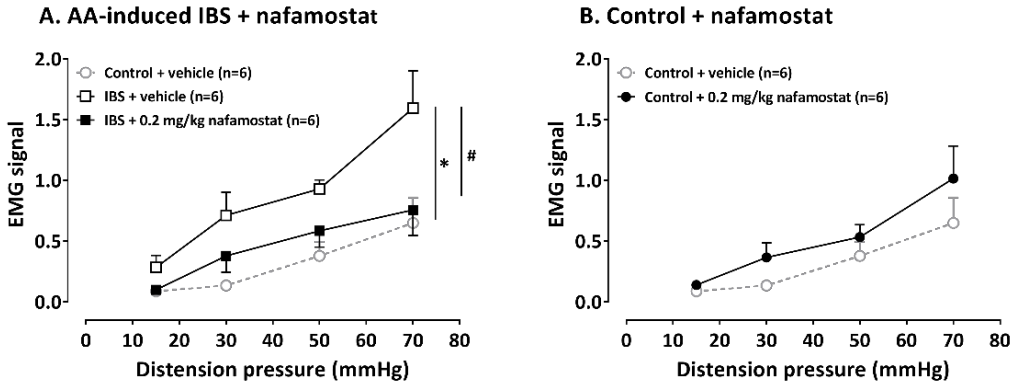


Figure 6.2. The effect of nafamostat mesylate (0.2 mg/kg) and its vehicle (water for injection) on VMRs in AA-induced IBS and control mice.

The statistical analysis was performed on the complete dataset, but separate graphs were made for IBS and control groups for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from IBS + vehicle.

6.4.2 UAMC-00050 decreases acetic acid-induced visceral hypersensitivity

The presence of visceral hypersensitivity in IBS mice was proven by the significantly higher VMRs in this group compared to control mice (Figure 6.3A). A single i.p. administration with 2 mg/kg UAMC-00050 30 min prior to VMR assessment

significantly decreased VMRs at all distension pressures and even completely restored sensitivity to normal values (Figure 6.3A). UAMC-00050 (2 mg/kg i.p.) did not affect visceral sensitivity in control mice (Figure 6.3B).

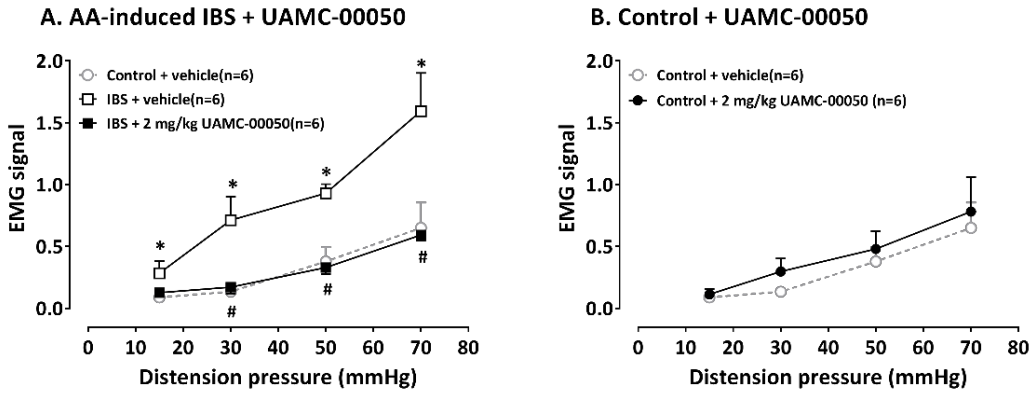


Figure 6.3. The effect of UAMC-00050 (2 mg/kg) and its vehicle (1% DMSO) on VMRs in AA-induced IBS and control mice.

The statistical analysis was performed on the complete dataset, but separate graphs were made for IBS and control groups for the purpose of clarification. Data are presented as mean ± SEM. Generalized Estimating Equations (GEE) + LSD post-hoc test; *p<0.05; significantly different from control + vehicle. #p<0.05; significantly different from IBS + vehicle.

6.5 Discussion

The aim of this study was to validate the effect of UAMC-00050 and nafamostat mesylate on visceral hypersensitivity in a different species and a different model for IBS. We thus used in this chapter the neonatal AA-induced mouse model for IBS. We first confirmed the presence of visceral hypersensitivity in AA-induced IBS mice illustrated by the significantly increased VMRs in vehicle-treated IBS mice compared to vehicle-treated controls. Therefore, next to the post-inflammatory TNBS rat model, this IBS mouse model could be used to test the efficacy of different compounds on visceral hypersensitivity. Secondly, after a single i.p. injection with the newly developed serine protease inhibitor UAMC-00050 in IBS mice, we showed a significant decrease in VMRs at all distension pressures and even a complete restoration to normal sensitivity values. Moreover, a single i.p. administration with the positive control compound, nafamostat mesylate indicated significantly lowered VMRs in IBS

mice as well. Although we did calculate equal concentrations for both compounds based on their protease profiles, we cannot ascertain that nafamostat is less potent than the UAMC-00050 compound based on this single dose study. Both serine protease inhibitors did not affect visceral sensitivity in control animals. These data are also in accordance with the results obtained in the post-inflammatory rat model for IBS as can be seen in paragraph 5.4.2.

This study was designed to validate the effect of the newly developed serine protease inhibitor UAMC-00050 on visceral hypersensitivity during IBS. In chapter 5, we proved a complete restoration of visceral sensitivity equal to normal values after a single i.p. injection with 1 mg/kg UAMC-00050 in a TNBS-induced post-inflammatory rat model for IBS. Moreover, we demonstrated a similar decrease in post-inflammatory visceral hypersensitivity after a single i.p. administration of 0.1 mg/kg nafamostat mesylate, a marketed broad-spectrum serine protease inhibitor.

Regarding the validation of the newly developed small molecule serine protease inhibitor for the indication of visceral pain, a few aspects should be considered.

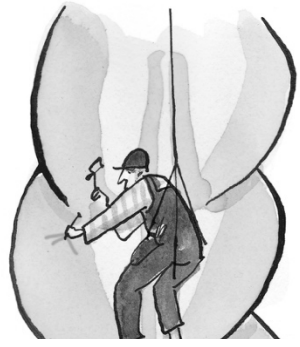
A first aspect is the choice of a preclinical animal model. We thereby opted for a different species as well as a different onset for IBS. Many research has been devoted to the origin of visceral hypersensitivity in IBS patients. An acknowledged hypothesis involves the occurrence of early life events (e.g. stress, infections, dietary allergies) causing permanent changes to the enteric nervous system and the brain-gut axis eventually leading to the origination of visceral hypersensitivity (Winston *et al.* 2007). Researchers have been inspired by this hypothesis for the generation of IBS animal models, specifically the exposure of neonatal animals to different initiating events (e.g. maternal separation, colonic inflammation) (Winston *et al.* 2007). We therefore selected the neonatal acetic-acid induced IBS mouse model. The neonatal animals used were C57BL/6 mice at postnatal day 10 and the initiating event was a mild chemical irritation of the colon by using diluted acetic acid (0.5%). In their adulthood (8-12 weeks old), these mice display visceral hypersensitivity without the presence of any

inflammatory signs (Winston *et al.* 2007). Moreover, this animal model has been validated and frequently used in the Center for Neurogastroenterology at the Johns Hopkins University, Baltimore, MD, USA.

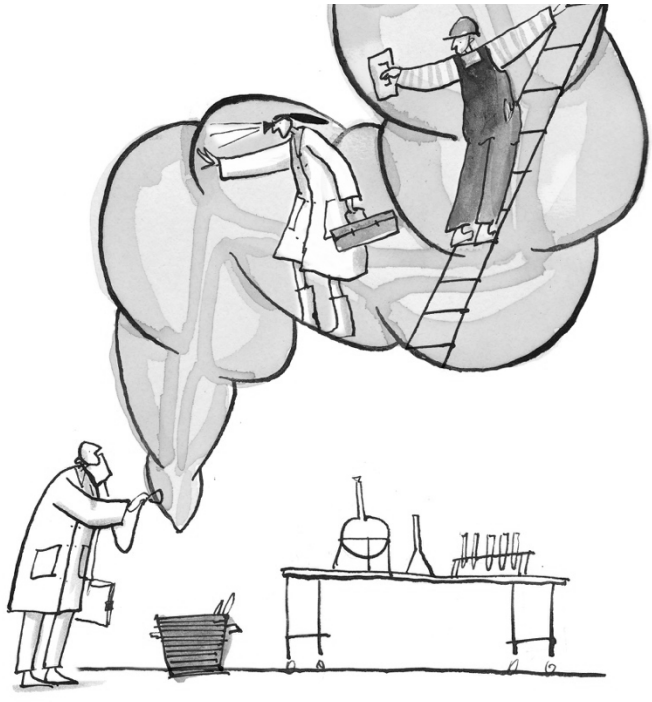
Secondly, in order to be able to validate serine protease inhibitors as a possible treatment strategy for visceral pain in IBS, we wanted to compare the results of our newly developed compound to a positive control. We picked nafamostat mesylate (or FUT-175), a commercially available broad-spectrum serine protease inhibitor, as a positive control. Nafamostat is marketed in Japan for the treatment of acute pancreatitis and disseminated vascular coagulation. Even though this compound has been used as a prototype serine protease inhibitor in several preclinical animal studies investigating its effect on visceral pain, there are important disadvantages as well. Besides inhibiting a whole range of serine proteases, nafamostat also inhibits proteases involved in blood coagulation, thereby making it not suitable as a possible treatment strategy for IBS. Moreover, this also emphasizes the need for new serine protease inhibitors with a well-known inhibition profile and a 10x to 100x lower specificity for proteases involved in the coagulation cascade such as plasmin, thrombin, FXa and FXIIa, in order to minimize side effects when treating disorders such as IBS.

In summary, we showed a significantly reduced visceral hypersensitivity after a single i.p. administration of the newly developed serine protease inhibitor UAMC-00050 and the commercially available serine protease inhibitor nafamostat mesylate in a neonatal acetic acid-induced mouse model for IBS.

Taking together the results of chapter 5 and 6, we demonstrated beneficial effects of two different serine protease inhibitors in two different animal models for visceral pain in IBS and in two different species. Our results provide fundamental evidence for serine protease inhibitors as a new therapeutic strategy for visceral pain in IBS.



CHAPTER 7 – GENERAL DISCUSSION AND FUTURE PERSPECTIVES



7.1 General discussion

Visceral hypersensitivity can be defined as an increased perception of stimuli arising from the intestines and is known as an important mechanism underlying abdominal pain, albeit not the only pathogenic mechanism involved. Abdominal pain is a common symptom of two frequent gastrointestinal disorders: inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) (Barbara *et al.* 2011). Although IBD and IBS are considered as two different diseases, there are potential comparable pathogenic mechanisms inducing abdominal pain. It is for example largely substantiated in the literature that IBD patients, both during active disease as well as in remission, frequently report IBS-like symptoms (De Schepper *et al.* 2008, Halpin *et al.* 2012). Both disorders are regarded as an important healthcare problem because of their chronic character, the increasing prevalence, the considerable impact on the patients' quality of life and the association with a high socio-economic burden (Lovell *et al.* 2012, Ananthakrishnan 2015) Besides, the currently available therapeutic strategies mostly aim at reducing the inflammation for IBD or ameliorating motility disturbances for IBS. This is in sharp contrast with the limited amount of therapies available directly aiming at reducing abdominal pain. Besides, pain treatment in these patients is challenging due to the known side effects of classical analgesics and their potential to even exacerbate symptoms (Norton *et al.* 2017). Hence, additional studies towards the search for new treatment targets in the domain of visceral pain are of great interest but are unfortunately delayed due to the incomplete comprehension of the pathophysiological mechanisms involved.

Regarding the pathophysiology of visceral pain during IBD, gastrointestinal inflammation is often suggested to be an important trigger. We hypothesize that upon activation of inflammatory cells (e.g. mast cells, T/B-cells), excessive amounts of inflammatory mediators (e.g. cytokines, serotonin, histamine, proteases) are released. These mediators thereby initiate the sensitization of peripheral afferent neurons eventually leading to visceral hypersensitivity even persisting after total disappearance of local inflammation (De Schepper *et al.* 2008). Concerning the underlying

mechanisms of visceral hypersensitivity during IBS, low-grade inflammation of the bowel has been postulated. However, a recent study demonstrated long-term sensitization in post-inflammatory IBS patients in the absence of any signs of inflammation but supported the evidence for the implication of mast cells via the histamine 1 receptor (Balemans *et al.* 2017).

The role of some of the abovementioned mediators in the pathophysiology of visceral hypersensitivity have been studied extensively for instance showing the therapeutic potential of anti-histaminergic drugs. However, the role of proteases has been investigated to a lesser extent.

The objective of this dissertation was therefore to clarify the role of proteases in the pathogenesis of visceral hypersensitivity in IBD and IBS, with the emphasis on protease inhibition as a possible new treatment strategy for visceral pain in IBD and IBS patients. We therefore defined three aims: 1) to investigate the potential of serine protease inhibitors in the treatment of visceral pain in an IBD rat model of acute inflammation and a rat model for post-inflammatory IBS; 2) to investigate the effects of the serine protease inhibitors in a different IBS model more precisely the neonatal acetic acid model in mice and 3) to study the proteolytic activities and activated pathways present in colonic tissue during acute and post-inflammatory conditions in an attempt to further unravel the mechanism of action of the serine protease inhibitors.

The implication of the serine protease pathway in the pathophysiology of visceral hypersensitivity has been shown by multiple research groups. Moreover, interference with this pathway via protease-activated receptors (PAR) has been put forward as a possibly interesting treatment option. Multiple *in vitro* and *in vivo* studies have shown beneficial effects of PAR-agonism/-antagonism on visceral pain, however, none of these compounds eventually made it to the clinic. In more recent years, an intervention more upstream of this PAR-activating cascade, namely a direct inhibition of proteases, has been suggested (Vergnolle 2016). Up until now, only few preclinical animal studies demonstrated the *in vitro* effect of serine protease inhibitors on visceral

pain (Cenac *et al.* 2007, Wang *et al.* 2015). Moreover, to our knowledge, the only *in vivo* proof-of-concept was given by Zhao *et al.* demonstrating a reduction in acute stress-induced visceral hypersensitivity in rats after an intragastric pre-treatment with the serine protease inhibitor camostat mesylate (Zhao *et al.* 2011). This underlines the need for more extensive *in vivo* studies towards the effect of serine protease inhibitors on visceral hypersensitivity.

However, caution should be taken when considering a large spectrum serine protease inhibitor as a possible new treatment, since its use could result in serious side effects. A well-known example is the failure of clinical trials investigating large spectrum serine protease inhibitors as a possible new treatment strategy for colorectal cancer (Lopez-Otin *et al.* 2007). Indeed, it was discovered that serine proteases involved in colorectal cancer could be divided into two different groups with opposite functions: one group facilitating oncogenesis and tumor growth and another group encompassing natural tumor suppression agents (Vergnolle 2016). This further highlights the importance of the determination of the serine protease profiles considering the balance between proteases and endogenous protease inhibitors as well as the potential opposite effects of different proteases even from the same class. These findings also point to the need of a careful selection of serine protease inhibitors with a well-known inhibition profile. Nevertheless, the serine protease inhibitor nafamostat mesylate is currently used in Japan for acute pancreatitis and diffuse intravascular clotting syndrome (Mori *et al.* 2003) and its potential on postreperfusion syndrome during liver transplantation and during continuous renal replacement therapy has been shown in clinical trials (www.clinicaltrials.gov; NCT01001403, NCT02478242) showing its therapeutic potential in concisely selected diseases.

Therefore, the **first goal** of this PhD project was to provide evidence for direct **serine protease inhibition** as a possible new treatment strategy for **visceral pain** in IBD and IBS. We therefore introduced two experimental animal models both relying on the chemical TNBS as an inflammatory stimulus to induce colitis. We used the TNBS colitis model in the acute inflammatory phase as a model for IBD and after endoscopically

proven resolution of colitis as a model for post-inflammatory IBS. Visceral sensitivity was assessed *in vivo* by means of the golden standard method in preclinical research studies more precisely the visceromotor responses (VMR) to colorectal distension. Regarding the pharmacological intervention, the commercially available broad-spectrum serine protease inhibitor nafamostat mesylate was used as a proof-of-concept. However, from a translation point of view, this compound would not be suitable as a possible treatment strategy for visceral pain in IBD and IBS patients since it also inhibits proteases implicated in the coagulation cascade. Previously, new serine protease inhibitors with a well-known inhibition profile (UAMC-00050, UAMC-01162) were developed by the Laboratory of Medicinal Chemistry of the University of Antwerp, as described in detail in chapter 3.11. These compounds were firstly patented under WO2007045496 (Augustyns *et al.* 2007) and recently patented for their use in PAR-related diseases under WO2017198753 (Augustyns *et al.* 2017). Now, forces were joined applying these inhibitors in animal models for visceral hypersensitivity in IBD and IBS. When comparing the inhibition profiles of the UAMC-compounds to nafamostat mesylate, all these compounds clearly inhibit a large spectrum of serine proteases. However, a major advantage of the UAMC-compounds as opposed to nafamostat is that they have a 100x lower specificity for proteases implicated in the coagulation cascade such as thrombin, plasmin, FXa and FXIIa. Therefore, by using the UAMC-compounds as a prototype serine protease inhibitor for the treatment of visceral pain, possibly major side effects resulting from the inhibition of proteases implicated in the coagulation cascade, could be avoided. This PhD trajectory has indeed provided evidence that direct serine protease inhibition encompasses a new therapeutic strategy for visceral pain in IBD and IBS.

In more detail, we demonstrated in chapter 4 that both nafamostat mesylate as well as the newly developed UAMC-00050 could decrease visceral hypersensitivity in an acute TNBS-colitis rat model for IBD. Nevertheless, the effect on visceral pain clearly differs between both compounds in two key ways. Firstly, nafamostat decreased visceral pain at both low (allodynia) and high distension pressures (hyperalgesia) while UAMC-00050 was only efficient in decreasing hyperalgesia. This finding is fortified by

a previous study showing that a 2-week treatment of acute TNBS-colitis rats with a fermented soy germ extract containing a Bowman-Birk inhibitor (serine protease inhibitor) resulted in a decrease in visceral hyperalgesia (Moussa *et al.* 2012). Secondly, besides its effect on acute colitis-induced visceral hypersensitivity, nafamostat also affected visceral sensitivity in control animals. This finding might negatively affect the further development of this compound as a treatment for IBD patients. On the contrary, UAMC-00050 did not affect visceral sensitivity in healthy control rats, showing a more profitable profile for further development.

Additionally, we ascertained a positive effect on visceral pain after administering the serine protease inhibitors nafamostat mesylate, UAMC-00050 and UAMC-01162 in a post-inflammatory rat model for IBS in chapter 5. The UAMC-01162 compound structurally slightly differs from the lead compound UAMC-00050 in that is has a diphenyl group instead of the *bis*(acetamidophenyl) group. Our results are completely in line with previous studies demonstrating a decrease in visceral hypersensitivity induced by a colorectal infusion of supernatant from IBS patients in mice after an *in vitro* pre-incubation with the serine protease inhibitor nafamostat (Cenac *et al.* 2007, Wang *et al.* 2015). Next to the abovementioned studies demonstrating an effect of a serine protease inhibitor following a preventive *in vitro* treatment, only one research group showed an *in vivo* effect to the best of our knowledge. Zhao *et al.* showed a reduction in acute restraint stress-induced visceral hypersensitivity in rats after an intragastric pre-treatment with camostat mesylate, structurally related to nafamostat (Zhao *et al.* 2011).
The conclusion of this study is that direct serine protease inhibition might be a new therapeutic target for the treatment of abdominal pain in both IBD and IBS, although their potential in patients needs to be studied in more detail.

A **second aim** of this dissertation was to **validate** our **newly developed serine protease inhibitor** as a new therapeutic strategy for visceral pain in a preclinical animal model using a different species and a different onset for IBS. We therefore opted for a

neonatal acetic acid-induced mouse model for IBS, validated and frequently used in the Center for Neurogastroenterology at the Johns Hopkins University, Baltimore, MD, USA. Similar to the experimental set-up in chapter 4 and 5, we now also used the commercially available serine protease inhibitor nafamostat mesylate as a positive control. The doses of nafamostat mesylate and UAMC-00050 proven most effective in decreasing post-inflammatory visceral hypersensitivity in rats in chapter 5 were converted for their use in mice in chapter 6. From this validation study we concluded that, also in a neonatal acetic acid-induced mouse model for IBS, a positive effect of the serine protease inhibitors nafamostat and the newly developed UAMC-00050 could be observed on visceral hypersensitivity. As such, nafamostat significantly decreased VMRs at all distension pressures without a complete reduction up to normal values, while UAMC-00050 significantly reduced visceral pain resulting in a complete reversal of visceral hypersensitivity in the doses used. These results therefore strengthen the potential of serine protease inhibitors in the treatment of visceral pain in both rats and mice and during both acute or post-inflammatory conditions. Their potential in a translational perspective thus warrants further research.

Additionally, we investigated the **proteases** involved and their mechanism of action in more detail both during **acute inflammation** and in the **post-inflammatory phase**. We thus used molecular biological tools and immunohistochemistry to study the serine proteases in IBD and IBS. In chapter 4 we demonstrated a significant downregulation of matriptase mRNA levels in the colon of acute colitis rats, in accordance to previous findings in DSS-induced colitis in mice, an experimental animal model for IBD (Buzza *et al.* 2017), as well as in IBD patients (Netzel-Arnett *et al.* 2012). Matriptase has previously been shown to be a critical factor in epithelial barrier homeostasis and to protect mice from DSS-induced colitis (Netzel-Arnett *et al.* 2012). Therefore, our finding of downregulation of matriptase during acute TNBS-induced colitis rats is completely in line with these previous studies and points towards an important protective role for matriptase in the development of IBD. Moreover, one should keep this in mind when determining the inhibition profiles for the development of new serine protease inhibitors for visceral pain in IBD patients. Furthermore, consistent

with previous studies, we confirmed a trend towards an upregulated tryptase mRNA expression and a significant increase in the number of mast cell tryptase positive cells in the colon of acute colitis rats. Increased levels of tryptase were previously several times documented during acute experimental colitis (Hamilton *et al.* 2011, Lohman *et al.* 2012) and in IBD patients (Raithel *et al.* 2001, Cenac *et al.* 2007, Peterson *et al.* 2007). Tryptase is the most abundant mast cell mediator that is released upon mast cell degranulation and known to play an important role in inflammation (Payne *et al.* 2004). The mRNA expression levels of urokinase plasminogen activator and KLK8 were comparable between control and acute colitis groups and cathepsin G, KLK2 and KLK4 were below the limit of detection. Moreover, we revealed an elevated fecal protease activity in the acute inflammatory setting. Similar differences in protease activity were found earlier by other research groups in an acute TNBS colitis rat model (Moussa *et al.* 2012) and UC patients (Roka *et al.* 2007, Annahazi *et al.* 2009).

In addition, we proved a significant role for serine proteases in a TNBS-induced rat model for post-inflammatory IBS in chapter 5. Interestingly, the colonic expression profiles of matriptase differed in the post-inflammatory phase versus the acute inflammatory state, whereas it was similar regarding other serine proteases. As such, matriptase mRNA expression levels were not significantly altered in the colon of post-colitis rats, in contrast with our findings in acute colitis rats. To our knowledge, matriptase levels were not investigated earlier in animal models for IBS or samples from IBS patients. In agreement with our findings in acute colitis animals, tryptase expression levels were significantly upregulated in the post-inflammatory rat model for IBS. Similar results were obtained in IBS patients revealing an increased tryptase expression (Barbara *et al.* 2004, Cenac *et al.* 2007, Bian *et al.* 2009, Buhner *et al.* 2009, Zhao *et al.* 2012, Liang *et al.* 2016). The mRNA expression of urokinase plasminogen activator and KLK8 were comparable between control and post-colitis groups and cathepsin G, KLK2 and KLK4 could not be detected in the acute colitis animals. Furthermore, we also detected an increased fecal protease activity in the post-colitis rats, in line with similar data in IBS patients (Cenac *et al.* 2007, Roka *et al.* 2007, Gecse *et al.* 2008, Annahazi *et al.* 2009). Trypsin-like activity, assessed with the fluorogenic

substrates Boc-Gln-Ala-Arg-AMC and Tos-Gly-Pro-Arg-AMC was found to be significantly increased in the colon of post-colitis rats compared to controls, while no significant differences could be detected in fecal samples. The lack of highly specific inhibitors and/or substrates for trypsin and/or tryptase prohibits the measurement of the activity of the individual proteases at the moment.

We also assessed the mRNA expression of receptors involved in the serine protease signaling pathways i.e. PARs and TRPs at both the colonic and the DRG level in control vs post-colitis rats. mRNA expression levels of PAR4 were significantly upregulated in post-colitis animals both at the colonic level and in DRG T13-L2. Remarkably, other studies have shown a downregulation of PAR4 in colonic biopsies of IBS-D, IBS-C and post-infectious IBS patients (Han *et al.* 2012, Zhao *et al.* 2012). This discrepancy may be explained by a study of Annaházi *et al.*, demonstrating that PAR4-antagonism induces an increase in colorectal hyperalgesia in TNBS-colitis mice (Annahazi *et al.* 2012). Their proposed mechanism is an endogenous activation of PAR4 (possibly by Cat-G), activating a feedback antinociceptive effect. On the contrary, no significant differences could be detected for PAR2. Taking a look at the mRNA expression levels of the TRP channels, a tendency toward an increased TRPV4 expression in the colon of post-colitis rats was found, which is in line with the increased expression of TRPV4 in human colon biopsies in IBD patients (Fichna *et al.* 2012). However, to our knowledge, no such data are available for IBS patients. Moreover, a significant upregulation of TRPA1 was found in DRG T13-L2 of post-colitis animals as reported earlier by others in both a TNBS-induced rat model for IBS (Yang *et al.* 2008) and a water-avoidance-stress-induced rat model for IBS (Yu *et al.* 2010). TRPV1 mRNA expression levels were not significantly altered in post-colitis rats compared to controls. These mRNA studies demonstrate the importance of both PARs and TRPs during post-inflammatory visceral hypersensitivity. In order to investigate the involvement of the protease-PAR-TRP axis in visceral hypersensitivity, immunohistochemical experiments were performed next to the qPCR experiments. PAR2 and TRPA1 immunoreactivity colocalized with CGRP-positive nerve fibers in the colon of control and post-colitis animals, while PAR4

seemed to be absent in these fibers. As elegantly described in a recent review on TRP channels, various G-protein coupled receptors (GPCR) can be activated by pro-inflammatory mediators such as serotonin, histamine, bradykinins and proteases triggering TRP sensitization in visceral hypersensitivity (Balemans *et al.* 2017). Our results fit in with this general hypothesis. However, in order to establish the involvement of these receptors in the mechanism of action of the serine protease inhibitors, more functional studies such as an *in vitro* Ca^{2+}imaging of DRGs with or without the addition of a serine protease inhibitor and PAR or TRP (ant)agonists or an *in vivo* VMR assessment in post-colitis rats with e.g. a PAR4-antagonist would have to be carried out.

Taken together, we can conclude from our data that serine proteases play a crucial role both during experimental IBD and IBS, while the exact type of proteases involved might differ between these disorders but favoring tryptase in both conditions, whereas matriptase seems more important in the acute inflammatory setting. These findings might have important implications for the further development of specific serine protease-targeted inhibitors that might differ between acute and post-inflammatory conditions.

To summarize the conclusions from chapters 4, 5 and 6 and looking back at the initial aims of this PhD project, we can conclude that serine proteases indeed play an important role in the pathogenesis of visceral hypersensitivity in two major gastrointestinal disorders IBD and IBS. From our results, mainly tryptase and matriptase showed interesting and disease-specific profiles. Most importantly, we demonstrated an amelioration of visceral hypersensitivity after a pharmacological intervention with novel serine protease inhibitors in a TNBS-induced animal model for IBD and IBS. Finally, we were able to validate our results in a neonatal acetic acid-induced animal model for IBS. Thus, we could finally conclude that our results clearly point towards an important role for serine protease inhibitors as a new treatment strategy for visceral pain in patients with IBD and IBS.

7.2 Future perspectives

In this PhD thesis, we provided evidence for the contribution of serine proteases to the pathophysiology of visceral hypersensitivity both during IBD and IBS. Moreover, we revealed direct serine protease inhibition as a possible new treatment strategy for visceral pain during IBD and IBS. However, there are still several questions that need to be addressed in future research before these serine protease inhibitors could make it to the clinic as a new therapeutic strategy for visceral pain in IBD and IBS patients.

A **first** question comprises the unravelling of the exact mechanism of action by which these serine protease inhibitors can decrease visceral hypersensitivity. Our hypothesis regarding a possible mechanism of action includes the involvement of both protease-activated receptors (PARs) and transient potential receptor channels (TRPs). The TRP channels (TRPV1, TRPV4, TRPA1) are a family of nociceptors situated on peripheral afferent neurons. Their involvement in the arousal of the sensitization of these neurons and thus visceral hypersensitivity has already been demonstrated by several research groups including ours (Vermeulen *et al.* 2013). In order to investigate this hypothesis, we could make use of an *in vitro* assay, specifically Ca^{2+} imaging of the dorsal root ganglia (DRG) containing the cell bodies of the peripheral afferent neurons. The addition of specific PAR and TRP-agonists/antagonists would then give us some more information regarding the involvement of the respective receptor(s) in the mechanism of action of the serine protease inhibitors. Additionally, we could visualize several neuronal markers (e.g. cFos, NMDA), PARs (PAR2, PAR4) and TRPs (TRPV1, TRPV4, TRPA1) in the DRGs by means of immunohistochemistry in order to find out possible differences between control and IBD/IBS animals.

A **second** future research question includes the discovery of the exact signal transduction pathways involved in the effects of the serine proteases in visceral hypersensitivity. Currently some preliminary experiments are carried out using the innovative PamGene® technology, in collaboration with Prof. Wim Vanden Berghe and dr. Claudina Perez Novo of the Laboratory of Protein science, Proteomics & Epigenetic Signalling (PPES) at the University of Antwerp. The recently developed PamGene®

technology allows us to identify new signal transduction pathways in a simple and fast way. This technique is based on the parallel measurement of multiple kinase activities by profiling peptide phosphorylation changes using a pharmacology-on-chip approach. The PamChip®, a peptide microarray, is loaded with 144 Serine/Threonine kinases (STK assay) peptide substrates. Fluorescently labelled anti-phospho-antibodies are used to detect the phosphorylation intensities. The eventual analysis shows clusters of kinases that can subsequently be linked to the exact pathways involved. In a first set of experiments we would like to investigate whether any differences could be observed in the kinase pattern in colon samples of control vs post-colitis rats. In a second set of experiments, the kinase clusters of colonic samples from untreated post-colitis rats could be compared to those from post-colitis rats treated with a serine protease inhibitor. Preliminary results show the feasibility of this approach (n =3) in the colon of post-colitis rats, indicating the MAPK pathways as important targets differentially affected in the colon of control versus post-colitis rats.

A **third** question implicates the measurement of the activity of specific serine proteases such as tryptase in samples from IBD and IBS preclinical animal models and eventually in patient samples. As discussed earlier, it would be of great interest to determine more specific serine protease activity as well as the possible effects of the serine protease inhibitors hereon. Unfortunately, the currently available tools to study protease activity (e.g. azocasein assay) are very unspecific (Edgington-Mitchell 2015). More selective substrates (e.g. trypsin-like proteases) exist, although no specific substrate, nor a specific tryptase inhibitor is available. Moreover, tryptase is very unstable, which implies that rigorous pre-analytical procedures are needed for the measurement of its activity. Therefore, researchers in the field of proteases eagerly await the development of more specific probes in order to assess the activity of individual proteases. Recently, in their attempt to develop more specific probes, Edgington-Mitchell *et al.* showed the *in vitro* efficacy of two fluorescent activity based probes but they were unable to prove their value in an *in vivo* animal model for acute colitis and pancreatitis (Edgington-Mitchell *et al.* 2017). Therefore, the search for new probes and the optimization process for *in vivo* use continues. We are currently

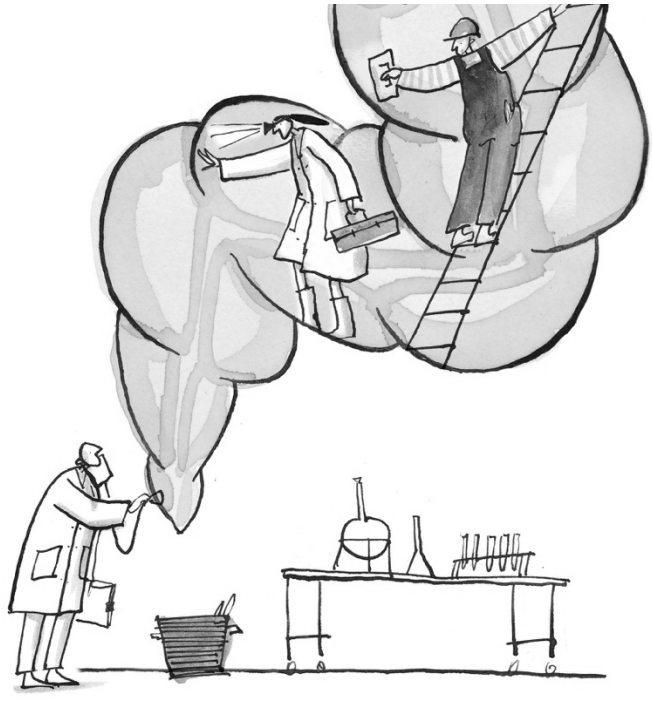
working together with the Laboratory of Medical Biochemistry of the University of Antwerp to assess more specific serine protease activity in colonic and/or fecal samples from our IBD/IBS animals.

A **fourth** future perspective includes the investigation of serine protease inhibitors in main symptoms of functional gastrointestinal diseases, beyond visceral hypersensitivity. Besides the effects of the serine protease inhibitors on visceral pain, it would be interesting to investigate whether these serine protease inhibitors directly affect other important contributors to the pathophysiology of both IBD and IBS such as inflammation and intestinal permeability.

A **fifth** important question remains the source of the serine proteases involved in visceral hypersensitivity. Sources of proteases in the gastrointestinal tract are diverse and include the pancreas, the microbiome, epithelial cells, neutrophils, macrophages and mast cells (Vergnolle 2016). We provided evidence for the presence of tryptase in mast cells in rat colon. Recently, the group of Vergnolle showed the release of trypsin-3 by the intestinal epithelium and showed its involvement in visceral hypersensitivity (Rolland-Fourcade et al. 2017). This emphasizes the importance of further research towards the source of serine proteases implicated in visceral pain.

In conclusion, we demonstrated an important role for serine proteases in the pathophysiology of visceral hypersensitivity during IBD and IBS as well as a possible role for direct serine protease inhibition as a new treatment strategy for visceral pain in both acute IBD and post-inflammatory and/or neonatal IBS models. We can therefore suggest their therapeutic potential in patients with IBS and IBD. However, from a translational point of view, future research needs to focus on the mechanism of action of these compounds, assess their effects on intestinal permeability, the pharmacokinetic profiles and the measurement of more specific serine protease activity.

CHAPTER 8 - SUMMARY



Visceral hypersensitivity, a mechanism underlying abdominal pain, is a major symptom in inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS). Despite their chronic character, increasing prevalence, negative impact on the quality of life and enormous socio-economic burden, a curative treatment is still eagerly awaited by patients and physicians up until today. Moreover, the currently available therapeutic strategies mostly focus on the decrease of inflammation for IBD and the amelioration of an altered motility function for IBS meaning that the abdominal pain might be an undertreated symptom as also appraised in literature. Additionally, recent research demonstrated an association between visceral hypersensitivity and gastrointestinal symptom severity in functional gastrointestinal disorders, thereby highlighting abdominal pain as a relevant treatment target.

Inflammation has been put forward as an important trigger for both IBD and IBS. The function of most of the inflammatory mediators (e.g. serotonin, histamine, cytokines) in the pathophysiology of visceral hypersensitivity has been studied to a large extent while the role of proteases has been investigated to a lesser extent. The available literature clearly points towards an implication of the protease pathway in visceral hypersensitivity during IBD and IBS. Most of the studies have been focusing on the role of protease-activated receptors (PARs) as well as PAR-agonism/antagonism as a potential new treatment strategy for visceral pain, but none of the newly developed PAR compounds made it to the clinic so far. Another possible treatment strategy for interfering with the protease pathway, direct protease inhibition, has only gained attention in the last few years.

Therefore, the aims of this PhD thesis were (1) to provide evidence for direct serine protease inhibition as a possible new treatment strategy for visceral pain in an TNBS-induced colitis rat model for IBD and a rat model for post-inflammatory IBS, (2) to validate a newly developed serine protease inhibitor for the indication of visceral hypersensitivity using a different preclinical animal model for IBS, namely the neonatal acetic acid-induced mouse model and (3) to start the research on the proteases and pathways involved in colonic tissue of rats during acute and post-inflammatory

conditions in an attempt to further unravel the mechanism of action of the serine protease inhibitors.

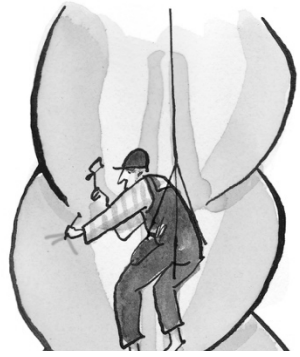
First, we investigated the effect of serine protease inhibitors on visceral hypersensitivity during IBD and IBS. In the acute TNBS-colitis rat model for IBD, we demonstrated a decreased visceral hypersensitivity after the administration of two different serine protease inhibitors: nafamostat mesylate (commercially available) and UAMC-00050 (newly developed by the Laboratory of Medicinal Chemistry of the University of Antwerp). However, some minor differences between these compounds were noticeable. Nafamostat mesylate decreased visceral pain at low distension pressures (allodynia) and higher pressures (hyperalgesia), but also affected visceral sensitivity in control animals. On the other hand, UAMC-00050 only decreased visceral hyperalgesia without affecting allodynia, but had no significant effect on visceral sensitivity in healthy controls. In the post-inflammatory rat model for IBS, we demonstrated a significant decrease in visceral hypersensitivity after a single i.p. administration of nafamostat mesylate with a complete reversal in the lowest dose tested. Furthermore, the newly developed serine protease inhibitors UAMC-00050 and UAMC-01162 significantly lowered visceral hypersensitivity in the IBS model in a dose-dependent way, completely restoring visceral sensitivity in the highest dose used.

Next, we validated the newly developed serine protease inhibitor UAMC-00050 in an animal model for visceral hypersensitivity using a different species and a different onset for IBS. Indeed, we were able to find a positive effect of the serine protease inhibitors nafamostat and the newly developed UAMC-00050 on visceral pain in a neonatal acetic acid-induced mouse model for IBS.

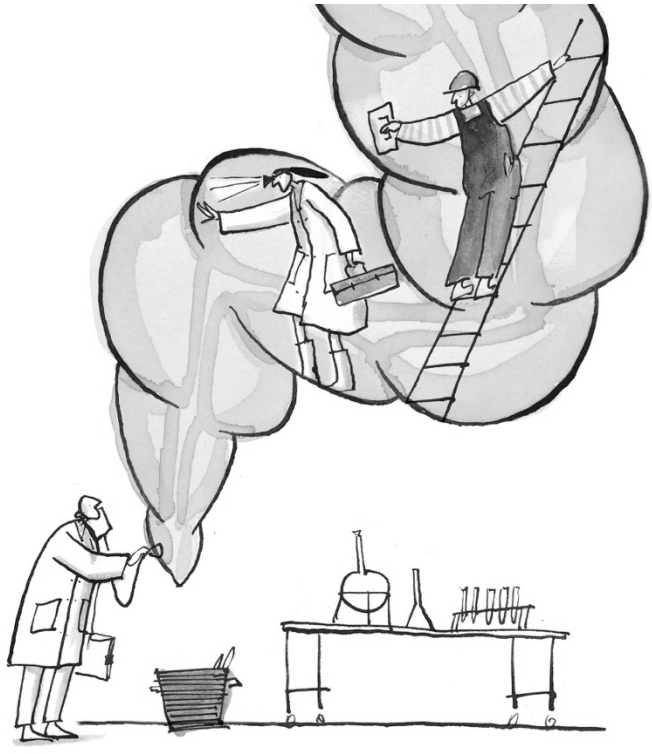
Finally, we studied the protease expression profiles involved in visceral hypersensitivity in an acute colitis rat model for IBD and a post-inflammatory rat model IBS. We confirmed a role for serine proteases in visceral hypersensitivity in an acute colitis rat model for IBD. In colon samples of rats with acute TNBS colitis, matriptase mRNA expression was significantly downregulated during acute inflammation, while tryptase

mRNA levels were elevated and mast cell tryptase positive cells significantly increased. Moreover, fecal protease activity determined by an azocasein assay was significantly increased in acute colitis rats compared to controls. Similarly, we also proved a function for serine proteases in a TNBS post-inflammatory rat model for IBS. Interestingly, the colonic expression profiles differed in the post-inflammatory phase versus the acute inflammatory state. As such, matriptase mRNA expression levels were not significantly altered in post-colitis rats compared to controls. In line with our finding in the acute TNBS-colitis rat model, tryptase expression was significantly upregulated in post-colitis rats at the mRNA and protein level. Furthermore, we also discovered an augmented fecal protease activity in the post-colitis rats. Accordingly, trypsin-like activity was significantly increased in the colon but not in the feces. To conclude, we provided evidence for a role for serine proteases in visceral hypersensitivity both during IBD and IBS. In order to examine the downstream signaling pathways involved in serine protease signaling, we assessed the involvement of PAR and TRP receptors with a preliminary qPCR analysis. mRNA expression of PAR4 was significantly increased in the colon and DRG T13-L2 of post-colitis rats. TRPA1 was significantly upregulated in DRG T13-L2, while a tendency towards an increased TRPV4 expression was found at the colonic level of post-inflammatory IBS rats. Both PAR2 and TRPV1 were not significantly altered in colonic or DRG samples from post-colitis rats compared to controls. Furthermore, PAR2 and TRPA1 immunoreactivity colocalized with CGRP-positive nerve fibers in control and post-colitis animals.

To conclude, we demonstrated a decrease in visceral hypersensitivity after the administration of serine protease inhibitors in an acute TNBS colitis-induced rat model for IBD and a post-inflammatory IBS rat model. Furthermore, we validated these results in an acetic acid-induced mouse model for IBS. Additionally, we examined the protease profiles involved in in the pathophysiology of visceral hypersensitivity during IBD and IBS, pointing towards an important role for matriptase in IBD and for tryptase in IBD and IBS. Considering the abovementioned results, we believe that direct serine protease inhibition might encompass an interesting new treatment strategy for visceral pain in IBD and IBS patients.



CHAPTER 9 - SAMENVATTING



Viscerale hypersensitiviteit, een mechanisme onderliggend aan abdominale pijn of buikpijn, is een belangrijk symptoom bij inflammatoire darmziekten (*inflammatory bowel disease,* IBD) en het prikkelbaredarmsyndroom (*irritable bowel syndrome,* IBS). Ondanks het chronisch karakter, de stijgende prevalentie, de negatieve invloed op de levenskwaliteit en de enorme socio-economische impact van deze ziektebeelden wachten artsen en patiënten tot op de dag van vandaag op een curatieve behandeling. Bovendien focussen de huidige beschikbare therapeutische strategieëen voornamelijk op het verminderen van de inflammatie voor IBD en het verbeteren van een gewijzigde motiliteit voor IBS, wat zou kunnen wijzen op een onderbehandeling van de abdominale pijn. Daarenboven toonde recent onderzoek een verband aan tussen viscerale hypersensitiviteit enerzijds en de ernst van de gastro-intestinale symptomen in functionele gastro-intestinale aandoeningen anderzijds, wat de relevantie van abdominale pijn als potentieel aangrijpingspunt benadrukt.

Inflammatie wordt vooropgesteld als een belangrijke trigger voor IBD en IBS. De functie van de meeste inflammatoire mediatoren (bv. serotonine, histamine, cytokines) in de pathofysiologie van viscerale hypersensitiviteit werd reeds uitgebreid bestudeerd, maar de rol van proteasen werd minder uitvoerig onderzocht. De beschikbare literatuur verwijst duidelijk naar een rol van de protease pathway in viscerale hypersensitiviteit tijdens IBD en IBS. Tot nu toe hebben de meeste studies zich geconcentreerd op de rol van *protease-activated receptors* (PARs) en PAR-agonisten/antagonisten als een potentiële nieuwe behandelingsstrategie voor viscerale pijn, nochtans werd tot op heden geen enkel van deze nieuw ontwikkelde PAR verbindingen op de markt gebracht. Een andere mogelijke behandelingsstrategie om te kunnen interfereren met de protease pathway bestaat uit de directe inhibitie van proteasen.

De doelen van deze PhD thesis bestonden eruit (1) om bewijs te levereren voor directe serine protease inhibite als een mogelijke nieuwe behandelingsstrategie voor viscerale pijn in een TNBS-geïnduceerd colitis rat model voor IBD en een rat model voor post-inflammatoire IBS, (2) om een nieuw ontwikkelde serine protease inhibitor te valideren

voor de behandeling van viscerale hypersensitiviteit gebruikmakend van een tweede preklinisch model voor IBS, namelijk het neonataal azijnzuur-geïnduceerd muismodel en (3) om te starten met het onderzoek naar de betrokken proteasen en pathways tijdens acute colitis en in de post-inflammatoire fase in een poging om het werkingsmechanisme van de serine protease inhibitors verder te ontrafelen.

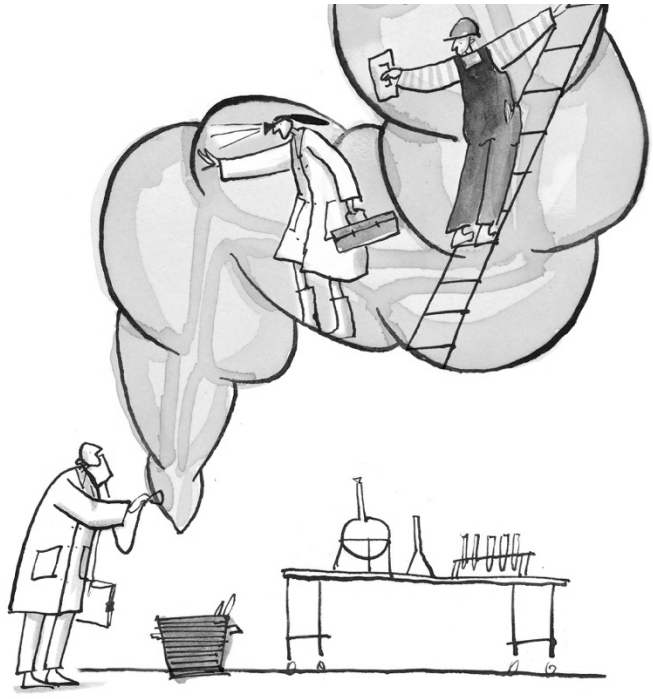
Eerst onderzochten we het effect van de serine protease inhibitoren op de viscerale hypersensitviteit tijdens IBD en IBS. In het acute TNBS-colitis ratmodel voor IBD, toonden we een verminderde viscerale hypersensitiviteit na de toediening van twee verschillende serine protease inhibitoren: nafamostat mesylate (commercieel beschikbaar) en UAMC-00050 (nieuw ontwikkeld door het Laboratorium voor Medicinale Chemie van de Universiteit Antwerpen). Er waren echter enkele kleine verschillen tussen deze verbindingen merkbaar. Nafamostat mesylate verminderde de viscerale pijn bij lage distensiedruk (allodynie) en hogere drukken (hyperalgesie), maar beïnvloedde ook de viscerale hypersensitiviteit bij controledieren. Aan de andere kant verminderde UAMC-00050 enkel de viscerale hyperalgesie zonder de allodynie te beïnvloeden, en deze compound had geen significant effect op de viscerale sensitiviteit bij gezonde controles. In het post-inflammatoire rat model voor IBS toonden we een significante afname in viscerale hypersensitiviteit na een eenmalige i.p. toediening van nafamostat mesylate met een volledige omkering in de laagst geteste dosis. Bovendien verminderden de nieuw ontwikkelde serine protease inhibitoren UAMC-00050 en UAMC-01162 de viscerale hypersensitiviteit in het IBS model aanzienlijk op een dosisafhankelijke manier, waarbij de viscerale sensitiviteit volledig werd hersteld in de hoogst geteste dosis.

Vervolgens valideerden we de nieuw ontwikkelde serine protease inhibitor UAMC-00050 in een diermodel voor viscerale hypersensitiviteit gebruikmakend van een ander species en een tweede IBS model met een andere manier van inductie van viscerale hyersensitiviteit. Ook in dit neonataal azijnzuur geïnduceerd muismodel voor IBS konden we een positief effect van de serine protease inhibitoren nafamostat en de nieuw ontwikkelde UAMC-00050 op viscerale pijn aantonen.

Ten slotte hebben we de protease expressieprofielen bestudeerd die betrokken zijn bij viscerale hypersensitiviteit in een acuut colitis rat model voor IBD en een post-inflammatoir rat model voor IBS. We bevestigden een rol voor serine proteasen in viscerale hypersensitiviteit in een acuut colitis rat model voor IBD: in het colon van ratten met acute TNBS-colitis was de matriptase mRNA expressie significant verlaagd tijdens acute inflammatie, terwijl het mRNA niveau van tryptase verhoogd was en het aantal tryptase-positieve mestcellen significant toenam. Bovendien was de fecale protease-activiteit bepaald door een azocaseïne assay significant verhoogd bij ratten met acute colitis vergeleken met controles. Eveneens hebben we evidentie voor serine proteasen in een post-inflammatoir rat model voor IBS aangetoond. Een interessante bevinding was het verschil tussen de expressieprofielen van het colon in de post-inflammatoire fase versus de acute inflammatoire fase. Zo waren de matriptase mRNA expressieniveaus niet significant veranderd bij post-colitis ratten in vergelijking met controles. In overeenstemming met onze bevindingen in het acute TNBS-colitis ratmodel, was de expressie van tryptase significant verhoogd bij post-colitis ratten op het mRNA- en eiwitniveau. Bovendien ontdekten we ook een verhoogde fecale protease activiteit in de post-colitis ratten. Daarnaast was de trypsin-like activiteit eveneens verhoogd in het colon maar niet in de feces van de post-colitis ratten. Samengevat, hebben we bewijs geleverd voor een rol voor serine proteasen in viscerale hypersensitiviteit, zowel tijdens IBD als IBS. Om de downstream signaalroutes die betrokken zijn bij de serine protease signalering te onderzoeken, hebben we de betrokkenheid van PAR- en TRP-receptoren beoordeeld met behulp van een qPCR analyse. De mRNA expressie van PAR4 was significant verhoogd in het colon en DRG T13-L2 van post-colitis ratten. TRPA1 was significant opgereguleerd in DRG T13-L2, terwijl een trend tot een verhoogde TRPV4-expressie werd gevonden op het colonniveau van post-inflammatoire IBS ratten. Zowel PAR2 als TRPV1 waren niet significant gewijzigd in colon- of DRG-stalen van post-colitis ratten vergeleken met controles. Tenslotte werden PAR2 en TRPA1 immunoreactiviteit gecolokaliseerd met CGRP-positieve zenuwvezels in controle- en post-colitisdieren.

Samengevat kunnen we stellen dat de viscerale hypersensitiviteit verminderd was na de toediening van serine protease inhibitoren in een acuut TNBS colitis-geïnduceerd ratmodel voor IBD en een post-inflammatoir rat- en muismodel voor IBS. Daarnaast onderzochten we de protease profielen betrokken bij de pathofysiologie van viscerale hypersensitiviteit tijdens IBD en IBS, duidend op een belangrijke rol voor matriptase bij IBD en voor tryptase bij IBD en IBS. Uit de bovengenoemde resultaten besluiten we dat de directe inhibitie van serine proteasen een interessante nieuwe behandelingsstrategie kan zijn voor viscerale pijn bij patiënten met IBD en IBS.

CHAPTER 10 – REFERENCE LIST



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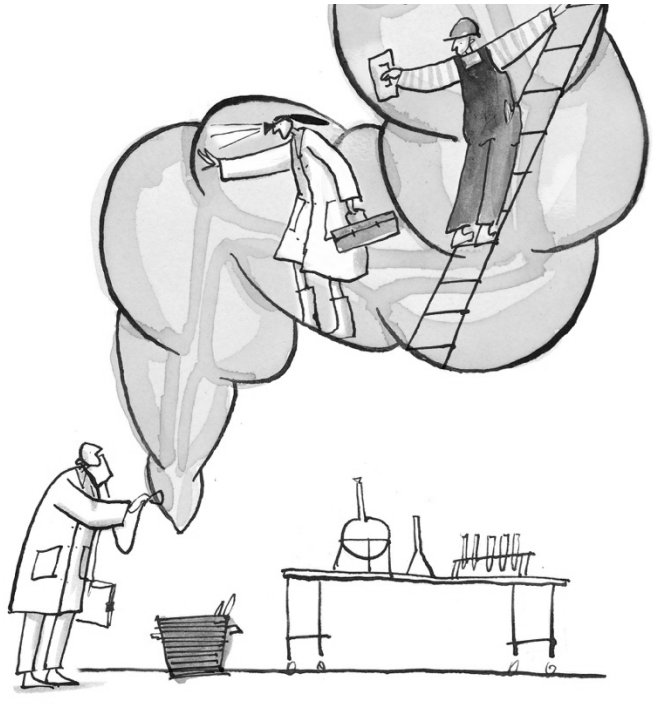
SCIENTIFIC CURRICULUM VITAE



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Education

2012-2014	Master of Science in Drug Development: Pharmacist, University of Antwerp, Belgium, Distinction.
2009-2012	Bachelor of Science in Pharmaceutical Sciences, University of Antwerp, Belgium, Distinction.
2003-2009	Secondary School, ASO, Latin-Sciences, Koninklijk Lyceum Antwerpen, Belgium.

Training and post-graduate education

Jan-Mar/2017	Writing Academic Papers in English, Linguapolis, University of Antwerp, Belgium.
Jan-Feb/201	Statistics, Analysis of Variance, Ghent University, Belgium.
Oct/2014	Laboratory Animal Sciences (FELASA C certificate), University of Antwerp, Belgium.

Professional experience

Oct/2014-present	PhD researcher, Laboratory of Experimental Medicine & Pediatrics (LEMP), Gastroenterology, University of Antwerp, Belgium. The role of proteases in visceral hypersensitivity during inflammatory bowel disease and irritable bowel syndrome. Supervisors: prof. dr. Benedicte De Winter, prof. dr. Ingrid De Meester.
March/2017-March/2018	Visiting PhD researcher, Center for Neurogastroenterology, Johns Hopkins University, Baltimore, MD, USA. Supervisor: prof. dr. P.J. Pasricha.
Jul-Dec/2013	Pharmacy Internship, Sollie Apothekers, Antwerpen, Belgium.

Publications

a. Peer-reviewed articles – international journals

Deiteren A, van der Linden L, de Wit A, Ceuleers H, Buckinx R, Timmermans JP, Moreels TG, Pelckmans PA, De Man JG, De Winter BY. P2X3 receptors mediate visceral hypersensitivity during acute chemically-induced colitis and in the post-inflammatory phase via different mechanisms of sensitization. PLoS One. 2015 Apr 17;10(4): e0123810.

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b. Abstracts

Ceuleers H, Deiteren A, Joossens J, Augustyns K, Van Der Veken P, Pelckmans PA, De Man JG, De Winter BY. Evaluation of a new serine protease inhibitor on visceral pain in a rat model of acute colitis. Acta Gastroenterologica Belgica 2015;78(1):B09.

Ceuleers H, Deiteren A, De Man JG, De Schepper H, Joossens J, Francque S, De Meester I, De Winter BY. Visceral hypersensitivity in the rat: role of proteases. 5th Research Day Faculty of Medicine & Health sciences University of Antwerp Abstract book, April 24 2015, p10.

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Segaert E, Ceuleers H, De Man JG, Francque S, Joossens J, De Meester I, Augustyns K, De Winter BY. Study of the effect of a newly developed serine protease inhibitor on visceral hypersensitivity in a post-inflammatory rat model. Antwerp Medical Student's Congress Abstract Book 2015. 1st price.

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c. Patent

WO/2017/198753

Title: Bis(acetamidophenyl) guanidinophenylethylphosphonates for use in the prevention and/or treatment of PAR-related diseases.

Inventors: Augustyns K, Joossens J, Van Der Veken P, Cos P, Joossens C, De Winter BY, Ceuleers H, Van Spaendonk H.

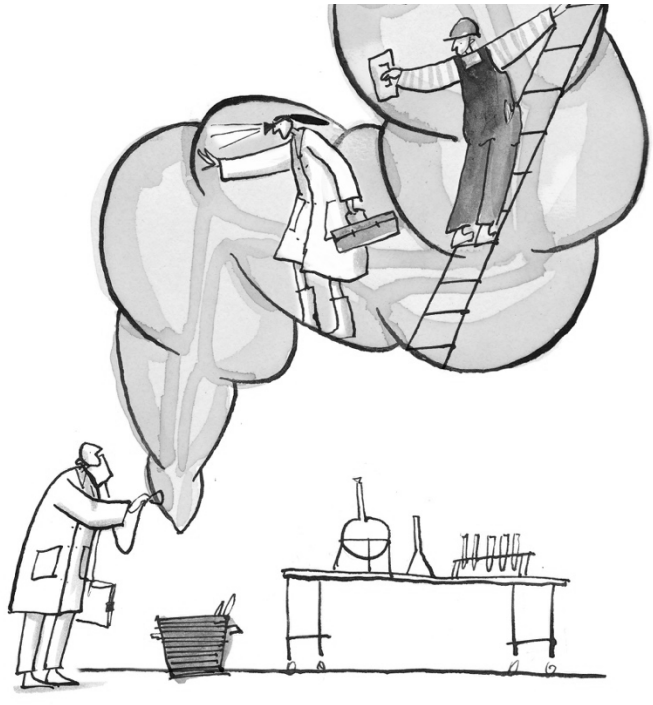
Awards and grants

May/2017	Certificate of Recognition as an Early Career Investigator *Digestive Disease Week 2017, Chicago, USA.*
Jun/2015	Best Young Investigator Presentation Award *ESNM Meeting NeuroGASTRO 2015, Istanbul, Turkey.* Best Oral Presentation
April/2015	*5th Research Day of the Faculty of Medicine & Health Sciences, University of Antwerp, Belgium*

Student supervision and training

2015-present	Nikita Hanning & Jelena Heirbaut (Medicine) *Effect of serine proteases on post-inflammatory visceral pain.*
2014-2016	Laura van der Linden & Anouk de Wit (Medicine) *Effect of a P2X3 receptor antagonist in a rat model for visceral pain.*
March-Aug/2015	Evelyn Segaert (Pharmaceutical sciences) *Effect of a new serine protease inhibitor and acetaminophen in a rat model for post-inflammatory visceral hypersensitivity.*

ACKNOWLEDGEMENTS - DANKWOORD



Toen ik hier iets meer dan vier jaar geleden binnenwandelde op T2 als masterproefstudente had ik zelfs in mijn stoutste dromen niet durven denken dat ik hier vandaag op mijn eigen doctoraatsverdediging zou staan. Ze zeggen wel eens dat een doctoraat een rollercoaster is van A tot Z – én dat is het ook! Doctoreren is een enorme verrijking, niet alleen op professioneel, maar ook op persoonlijk vlak. Tijdens die vier jaar ben ik mezelf regelmatig tegengekomen, waarbij ik alleen maar kan zeggen dat ik er uiteindelijk sterker door geworden ben. Als ik vandaag opnieuw de vraag zou krijgen of ik wil doctoreren, zou ik meteen opnieuw volmondig 'ja' antwoorden! Maar een doctoraat schrijf je natuurlijk niet alleen. Ik wil van deze gelegenheid dan ook gebruik maken om alle collega's, familie en vrienden te bedanken die er uiteindelijk voor gezorgd hebben dat ik hier vandaag sta.

Prof. Benedicte De Winter, als promotor stond u steeds voor mij klaar. Bedankt om mij vier jaar geleden de kans te geven om aan een doctoraat te beginnen en het mee tot een goed einde te brengen! Bedankt, dat ik telkens uw bureau mocht komen binnenvallen om mijn nieuwste resultaten te komen tonen. Bedankt, om op de meest onmogelijke momenten (lees: in het midden van de nacht) nog een abstract, een paper of een presentatie van mij te bekijken. Bedankt, voor de gezellige babbels bij een cocktailtje op congres in Istanbul en Cork. Bedankt, om mij de kans te geven om een jaar onderzoek te doen aan de Johns Hopkins University in Baltimore, MD, USA. Bedankt, om een luisterend oor te zijn en ook steeds rekening te houden met mijn persoonlijke situatie. Bedankt, om dat research-beestje destijds in mij wakker te maken. Dankzij u heb ik de onderzoekskriebel nu volledig te pakken. U bent een straffe madam! Een gezin combineren met een meer-dan-fulltime job en zo'n enorme passie voor het onderzoek, dat is iets waar ik alleen maar heel hard naar kan opkijken! Benedicte, bedankt voor alles!

Ing. Joris De Man, als begeleider was u onmisbaar tijdens dit doctoraat. Voor alle technische problemen, het bespreken van resultaten, het nalezen van een paper, een oneindige voorraad aan materiaal en nog zoveel meer: één adres – T.205, ofwel Joris' bureau. Bedankt om me tijdens de afgelopen vier jaar telkens met raad en daad bij te

staan. Met uw jarenlange ervaring bent u een gevestigde waarde binnen het gastro-labo. Wat een geluk dat u er de laatste maanden was om wat rust te brengen in de rechte lijn naar de finish. Dat had ik écht nodig! Joris, bedankt voor alles!

Prof. Ingrid De Meester, als co-promotor van dit doctoraat wil ik u graag bedanken voor het nalezen van abstracts, manuscripten en natuurlijk deze thesis. De vele meetings over de metingen van de proteolytische activiteit hebben dan uiteindelijk toch zijn vruchten afgeworpen. Uw biochemische kijk op dit doctoraatsproject heeft bijgedragen tot de kwaliteit van dit werk.

Prof. Heiko De Schepper, als begeleider van dit doctoraat wil ik u graag bedanken voor uw klinische kijk op dit doctoraatsproject en het bekijken van menig abstract en manuscript.

I would also like to thank the members of the jury, prof. Niall Hyland and prof. Guy Boeckxstaens for the critical evaluation of this thesis. I thank the members of my doctoral committee, prof. Guy Hans and prof. Louis Maes, for monitoring my progress during the past years.

I am especially grateful to prof. Jay Pasricha for giving me the opportunity to join his lab at the Center for Neurogastroenterology of the Johns Hopkins University in Baltimore, MD, USA during the last year of my PhD. This allowed me to gain experience in the acetic-acid induced mouse model for IBS and EMG electrode implantation and VMR to CRD recordings in mice. Furthermore, I was trained in some other experimental techniques such as the Ussing Chambers and Ca^{2+}-imaging in DRG neurons and I had the chance to collaborate with experts in the field of neurogastroenterology on a daily basis. The skills and expertise I gained during this year were a great added value to this PhD thesis. Thank you for all the time and effort you invested in me as well as the fruitful collaboration.

De gastro-bureau, T.2.27, de plek waar ik de afgelopen vier jaar ontelbaar veel uren gesleten heb met het motto: “You don’t have to be crazy to work here, we’ll train you”.

Annemie, bedankt om mij gedurende mijn masterproef te introduceren in de wondere wereld van de viscerale pijn en mij de nodige technieken met veel geduld aan te leren die uiteindelijk zorgden voor de *kickstart* van mijn doctoraat. Bedankt om mijn doctoraat van dichtbij te blijven opvolgen en voor het uitwisselen van onze buitenlandse ervaringen. Hanne, nadat we afstudeerden als apotheker startten we hier vier jaar geleden samen aan ons doctoraat en legden we het volledige traject samen af. Dat schept automatisch een band. Bedankt om mijn partner-in-crime te zijn tijdens de doctoraatsopleiding. Bedankt voor je wetenschappelijke input, voor de vele hilarische momenten in onze bureau, en de jaarlijkse DDW congressen. Ik wens je heel veel succes met het afronden van je doctoraat! Sara, bedankt om mij steeds bij te staan met jouw experimentele skills wanneer ik weer eens een nieuwe techniek wou uitproberen. Je bent een pittige madam met een kritische kijk op de wetenschap en een enorm doorzettingsvermogen. Ik wens je nog veel succes met je jaren als assistent en nadien met je carrière als gastro-enterologe. Wilco, bedankt om me telkens weer met goede raad en daad bij te staan wanneer ik weer eens een vraag of een probleem had. Bedankt voor jouw oneindig positivisme, de vele hilarische momenten, en onze wetenschappelijke discussies in de late uurtjes. Je werd naast een goede collega ook een goede vriend. Onze band werd het laatste jaar alleen nog maar versterkt doordat we tegelijkertijd onderzoek deden in de USA. Jij een jaartje postdoc in Boston en ik tijdens het laatste jaar van mijn doctoraat in Baltimore. Bedankt voor de soms urenlange facetime-gesprekken, voor het topweekend in Boston en jouw fijne bezoekjes aan ons in DC. Ik wens je alle succes toe in de kliniek en je verdere carrière als hepatoloog én researcher. Hanne, Sara en Wilco, bedankt voor de fijne jaren, jullie waren top-collega's. Ik hoop dat we ons T.2.27 whatsapp-groepje nog lang in leven houden en ik kijk al uit naar onze volgende 'Komen Eten'! Philip, bedankt voor die ontelbare keren dat je weer eens IT-helpdesk voor mij speelde. Ik betwijfel ten zeerste of mijn doctoraat er vandaag zou gelegen hebben zonder jouw hulp. Je bent een ongelofelijk harde werker en een hele loyale collega. Het organiseren van de LEMP-PhD drinks en BBQ's is ook jouw verdienste. Ik hoop stiekem toch dat ik bij de volgende editie nog een uitnodiging in mijn postvak krijg. Ik wens je alle succes toe met het

verdere verloop van jouw doctoraat! Denise, bedankt voor jouw dagelijkse portie kurkdroge humor. Toen je hier binnenwandelde als masterproefstudent wisten we het eigenlijk al, die moeten we bijhouden! Je voert experimenten uit aan de lopende band en schrijft een abstract aan een record-tempo. Het was fijn om je in DC bij ons te mogen ontvangen. Heel veel succes met de rest van je doctoraat! Nikita Hanning, Jelena Heirbaut, Anouk de Wit, Laura van der Linden en Evelyn Segaert, bedankt voor jullie inzet tijdens jullie masterproeven. Nikita, ik draag de 'viscerale pijn'-fakkel met heel veel plezier aan jou over! Ik wens je heel veel succes met je doctoraat en stuur me regelmatig maar een update!

Prof. Koen Augustyns en dr. Jurgen Joossens wil ik graag bedanken voor het aanleveren van de serine protease inhibitoren en hun kritische opmerkingen bij mijn abstracts en manuscripten. Bedankt voor de fijne samenwerking!

Prof. Anne-Marie Lambeir en Michelle de Bruyn wil ik graag bedanken voor het bepalen van de proteolytische activiteit. De weg ernaartoe was zeker niet altijd even gemakkelijk, maar het eindresultaat mag er zijn! Bedankt voor de fijne samenwerking. Michelle, je bent nog maar pas aan je doctoraat gestart, maar met jouw doorzettingsvermogen kijk ik nu al uit naar het eindresultaat! Veel succes!

Prof. Jean-Pierre Timmermans en Samuel Van Remoortel wil ik graag bedanken voor de prachtige immunohistochemische kleuringen. Het is altijd een eer om jullie beeldjes ergens te mogen tonen. Bedankt om onze lastminute wensen telkens weer te vervullen en bedankt voor de aangename samenwerking. Samuel, ik wens jou ook nog veel succes met het afronden van jouw doctoraat. Tot op de LBBB meeting!

Graag wil ik ook mijn collega's van het Laboratorium voor Experimentele Geneeskunde en Pediatrie (LEMP) uitgebreid bedanken. Angelika, Ilse, Lieve, Marleen en Petra, bedankt voor jullie hulp bij de experimenten en in het bijzonder de lastminute PCRs, de MPO bepalingen en het labelen van de ontelbare cryotubes. Ilse, het was ongelofelijk fijn om jou de wondere wereld van de VMR metingen te mogen leren kennen. Jouw leergierigheid en Kempische gezelligheid werkten aanstekelijk. Bedankt

voor al het harde werk! Jessy, bedankt voor de administratieve ondersteuning en de organisatie van de LEMP-vergaderingen. Prof. Sven Francque, bedankt voor het nalezen van mijn abstracts en manuscripten en uw klinische kijk op mijn doctoraatsproject. Prof. Annemieke Smet, bedankt voor jouw wetenschappelijke input en het meedenken naar mogelijke pistes wanneer een experiment niet de beoogde resultaten gaf. Kristien, Annelies, Kevin, An, Tom, Els, Eline, Mikhaïl, Stijn, Marijke, Kristien en Jonas, bedankt voor jullie vragen en kritische opmerkingen tijdens de LEMP-vergaderingen.

Natuurlijk mag de T2-familie ook niet ontbreken! Het leven zoals het is… op T2: een gezellige babbel bij de koffiemachine 's morgens, gesprekken over de meest uiteenlopende onderwerpen tijdens de middaglunch (gaande van het hoogtepunt van het nieuws tot de bespreking van Temptation Island en De Mol), werkvergadering op dinsdagmiddag, T2-café op vrijdagavond, de jaarlijkse labo-uitstap en op tijd en stond eens een feestje (want er valt altijd wel iets te vieren). Het is geen geheim en ex-T2'ers kunnen er ongetwijfeld over meepraten: die speciale T2-vibe ga ik ongelofelijk hard missen! Aan de hele T2-familie, bedankt voor die leuke sfeer en om van T2 zo'n aangename werkomgeving te maken! Jullie zijn toppers!

Prof. Guido De Meyer, prof. Wim Martinet, prof. Pieter-Jan Guns, prof. Dorien Schrijvers, prof. Katrien Lemmens, prof. Gilles De Keulenaer, prof. Vincent Segers wil ik graag bedanken voor de interessante discussies tijdens de wekelijkse werkvergaderingen. Jullie verfrissende kijk op onze resultaten zorgden steevast voor heel wat nieuwe ideeën.

Er zijn ook nog een aantal personen op T2 die ik graag in het bijzonder wil bedanken. Rita, bedankt voor de vele coupes, H&E kleuringen en het oppuntstellen van de tryptase kleuring. Altijd goedgezind, het herkenbare geluid van jouw teenslippers in de gang en de gezellige babbels (met af en toe wat goede raad) bij een koffie: ik ga het enorm missen! Bedankt voor alles! Maya, bedankt voor jouw hulp bij het schrijven van de aanvragen voor de FWO reisbeurs en het IOF-POC project. Je bent een intelligente,

ambitieuze madam en ik ben ervan overtuigd dat we nog veel van jou gaan horen! Lynn, bedankt voor de vele babbels over onze verdere carrièreplannen. Het timmeren aan je academische carrière in combinatie met een gezinsleven, er zijn er niet veel die het je zouden nadoen. You go, girl! Mandy, dat jij je roeping in het onderzoek gevonden had werd me al snel duidelijk. Bedankt voor al die lekkere cupcakes en taarten die je maakte. Ik wens je alle succes toe met je postdoc in Cambridge. Miche, bedankt voor de leuke babbels en veel succes met je verdere carrière bij Histogenex. Hadis, we begonnen samen aan ons doctoraat en ook al had je het geluk niet altijd aan je zijde, je bleef doorzetten. Veel succes met het afronden van je doctoraat. Leni en Zarha, bedankt om mij onder jullie vleugels te nemen toen ik als masterproefstudent hier op T2 kwam binnenwaaien. Lotte en Inn, bedankt voor de administratieve ondersteuning, de wekelijkse nieuwsbrief en de verjaardagskaartjes. Sonja, bedankt om het hier op T2 dag-na-dag zo proper te houden. Daarnaast wil ik ook graag alle T2-collega's en ex-collega's bedanken voor de ongelofelijk leuke tijd hier: Ammar, Annie, Anne-Elise, Arthur, Besa, Bieke, Carole, Cédéric, Chris, Cor, Dorien, Dries, Eline, Farnaz, Griet, Ilse, Isabelle, Lindsey, Min, Marc, Maria, Paul, Paula, Rachid, Sofie, Steffi, Tine.

Graag wil ik ook de medewerkers van het animalarium bedanken voor de goede zorgen voor mijn proefdieren. Experimenten met proefdieren zijn onontbeerlijk in het onderzoek naar nieuwe geneesmiddelen. Ze geven ons de nodige informatie om uiteindelijk de stap te kunnen zetten richting een eerste klinische trial met patiënten. Mijn dank gaat dan ook uit naar 'mijn vriendjes', de ratjes, die de afgelopen vier jaar hun leven lieten voor de wetenschap.

Tot slot wil ik natuurlijk heel graag mijn familie en vrienden bedanken voor hun onvoorwaardelijke steun de afgelopen jaren.

In het bijzonder een dikke merci aan Mattias voor de creatieve interpretatie van de 'prikkelbare darm' en aan Yana voor het pimpen van de lay-out van deze thesis.

Yana, Elisa, Tiffany, Sara, Shanice (*mijn sjoekes)*, Marjan & Sanne (*mijn Gentse madammen)*, Alex & Thomas (*de Brusselse of tegenwoordig eigenlijk Antwerpse boys)*,

Emilie en Birger (*the Belgians in DC)*, Pedro, Matti, Bud, Charly, Posh, Sas, Sarah, Kris, Lennie, Bodsie, Vicky, Dirk, Eva, Freddy, Emilie, Falcon, Annemie, Vinnie, Jan, Filip, Sandrine, Bruno (*de Zurenborg- & Peñiscola-gang)*, ik wil jullie enorm bedanken voor jullie steun, de getoonde interesse in mijn onderzoek, de brunchkes, de etentjes, de vakanties, de weekendjes, de shop-tripkes, de gin-tonics, de fitness- & dansavonden, het oneindige getetter, en nog zoveel meer! Merci om er altijd voor mij te zijn!

Annemie, Luc en Jens. De voordeur in Westmalle stond van in het begin altijd op een kier. Merci dat ik op de meest onmogelijke momenten altijd mocht binnenvallen voor een wandeling met Baron, een onverwachte shop-namiddag en natuurlijk een glaasje witte wijn. Bedankt om telkens weer te vragen naar de updates over mijn onderzoek en om mij af en toe een dikke knuffel te geven wanneer ik het nodig had. Tout court, merci voor jullie onvoorwaardelijke steun!

Mama, papa en Brittje. Zonder jullie had ik hier nu niet gestaan. Zonder jullie steun was ik waarschijnlijk zelfs nooit aan een doctoraat begonnen. Papa, je zal het niet altijd met zoveel woorden zeggen, maar ik weet dat je ongelofelijk trots bent op je dochters. We hadden het er onlangs nog over, die assertiviteit heb ik duidelijk van jou. Mama, bedankt om altijd naar mij te luisteren en mij steeds met goede raad en daad bij te staan. Het was zo fijn om je telkens weer de laatste updates mee te geven over mijn onderzoek en jouw interesse voor de medische wereld nog wat meer aan te wakkeren. Soms denk ik toch dat jij jouw roeping gemist hebt. Brittje, mijn kleine zusje… onze gemeenschappelijke voorliefde voor alles wat met Geneeskunde te maken heeft, zorgde ervoor dat jij ongeveer de enige bent die écht helemaal begrijpt waar mijn doctoraat nu juist rond draait. Ondertussen loop je al rond in je doktersjas in menig Antwerps ziekenhuis tijdens jouw stages Geneeskunde. Volgend jaar is het zover, dan heb je officieel het diploma van arts op zak. Als grote zus ben ik zo ongelofelijk fier op jou! Ik ga onze sister-dates missen! Mama, papa, zusje, bedankt voor jullie eindeloze interesse in mijn doctoraat! Bedankt om altijd voor mij klaar te staan!

Save the best for last! Arne, Nino'tje, bedankt om altijd voor 100% achter mij staan! Bedankt, om naar mijn urenlange verhalen over mijn onderzoek te luisteren. Bedankt, om mijn testpubliek te zijn bij de try-out van elke presentatie. Bedankt, om mij weer tot rust te brengen wanneer ik weer eens in overdrive ga. Bedankt, voor de vele ontspannende avonden, weekendjes en vakanties. Bedankt, om mij mijn dromen te laten najagen en te geloven in mijn kwaliteiten als researcher. Bedankt, om samen met mij onze eigen 'American Dream' waar te maken. Bedankt, om mij te wijzen op dat ene lichtpuntje in de duisternis wanneer ik het niet meer zie. Bedankt, om van mij de gelukkigste vrouw ter wereld te maken, elke dag opnieuw! Nino'tje, ik zie je doodgraag! Team Prutsos & Ninos!