EDITORIAL
10267 Laparoscopic liver resection for posterosuperior tumors using caudal approach and postural changes: A new technical approach
Morise Z

REVIEW
10275 Visceral hypersensitivity in inflammatory bowel diseases and irritable bowel syndrome: The role of proteases

10287 Protocol for laparoscopic cholecystectomy: Is it rocket science?

MINIREVIEWS
10304 Fecal microbial transplant for the treatment of pediatric inflammatory bowel disease
Wang AY, Popov J, Pai N

10316 Evolving management of metaplasia and dysplasia in Barrett’s epithelium
Evans RPT, Mourad MM, Fisher SG, Bramhall SR

ORIGINAL ARTICLE
Basic Study
10325 Aging related methylation influences the gene expression of key control genes in colorectal cancer and adenoma

10341 Anti-viral role of toll like receptor 4 in hepatitis B virus infection: An in vitro study

10353 Vitamin D differentially regulates Salmonella-induced intestine epithelial autophagy and interleukin-1β expression
Huang FC

10364 ANGPTL2 expression in gastric cancer tissues and cells and its biological behavior
Sheng WZ, Chen YS, Tu CT, He J, Zhang B, Gao WD
Retrospective Cohort Study

10371 Dysphagia after vertical sleeve gastrectomy: Evaluation of risk factors and assessment of endoscopic intervention

Nath A, Yewale S, Tran T, Brebbia JS, Shope TR, Koch TR

10380 Factors associated with surgery in patients with intra-abdominal fistulizing Crohn’s disease


10388 Clinical features of acute hepatitis E super-infections on chronic hepatitis B

Chen C, Zhang SY, Zhang DD, Li XY, Zhang YL, Li WX, Yan JJ, Wang M, Xun JN, Lu C, Ling Y, Huang YX, Chen L

Retrospective Study

10398 High-flow nasal oxygen availability for sedation decreases the use of general anesthesia during endoscopic retrograde cholangiopancreatography and endoscopic ultrasound

Schumann R, Natov NS, Rocos-Martinez KA, Finkelman MD, Phan TV, Hegde SR, Knapp RM

10406 Prognostic value of pre-treatment F-18-FDG PET-CT in patients with hepatocellular carcinoma undergoing radioembolization


10415 Role of original and modified Frey’s procedures in chronic pancreatitis

Tan CL, Zhang H, Yang M, Li SJ, Liu XB, Li KZ

Clinical Trials Study

10424 Laparoscopic and endoscopic co-operative surgery for nonampullary duodenal tumors


Observational Study

10432 Short-term effectiveness of golimumab for ulcerative colitis: Observational multicenter study


10440 Relationship between ghrelin, Helicobacter pylori and gastric mucosal atrophy in hemodialysis patients


Prospective Study

10450 Economic burden of irritable bowel syndrome in China

Zhang F, Xiang W, Li CY, Li SC
CASE REPORT

10461 Complete spontaneous regression of giant focal nodular hyperplasia of the liver: Magnetic resonance imaging evaluation with hepatobiliary contrast media
   Mamone G, Caruso S, Cortis K, Miraglia R

10465 Hepatosplenic T-cell lymphoma in a 47-year-old Crohn’s disease patient on thiopurine monotherapy
   van de Meeberg MM, Derikx LAAP, Sinnige HAM, Nooijen P, Schipper L, Nissen LHC

10471 Efficacy of additional treatment with azathioprine in a patient with prednisolone-dependent gastric sarcoidosis
Visceral hypersensitivity in inflammatory bowel diseases and irritable bowel syndrome: The role of proteases

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Abstract
Proteases, enzymes catalyzing the hydrolysis of peptide bonds, are present at high concentrations in the gastrointestinal tract. Besides their well-known role in the digestive process, they also function as signaling molecules through the activation of protease-activated receptors (PARs). Based on their chemical mechanism for catalysis, 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 will play a significant role in this review. 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 diseases and/or irritable bowel syndrome. So far, only a few preclinical animal studies have investigated the effect of protease inhibitors specifically on visceral sensitivity while their effect on inflammation is described in more detail. In our accompanying review we describe their effect on gastrointestinal permeability. On account of their promising results in the field of visceral hypersensitivity, further research is warranted. The aim of this review is to give an overview on the concept of visceral hyper-sensitivity as well as on the physiological and pathophysiological functions of proteases herein.
Key words: Proteases; Proteinase-activated receptors; Protease inhibitors; Visceral hypersensitivity; Visceral pain; Irritable bowel syndrome; Inflammatory bowel diseases

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Core tip: 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. In the last decade, it became clear that proteases fulfill an important role in visceral pain, a major symptom in patients with inflammatory bowel diseases and/or irritable bowel syndrome. These review articles aim at providing an overview of the diverse roles of proteases in both health and disease states related to the gastrointestinal functions, with the emphasis on visceral pain in this review.

INTRODUCTION

Abdominal pain is a key feature of two major gastrointestinal disorders, inflammatory bowel diseases (IBD) and irritable bowel syndrome (IBS). On the one hand, IBD, such as ulcerative colitis (UC) and Crohn’s disease (CD), are characterized by acute flares of inflammation followed by periods of remission[1]. On the other hand, IBS is a functional disorder defined by the presence of altered bowel habits and abdominal pain, in the absence of an organic cause[2]. The diagnosis of IBS is based on the Rome IV criteria: recurrent abdominal pain for at least 3 d/mo in the last 3 mo associated with two or more of the following symptoms: (1) improvement with defecation; (2) onset associated with a change in frequency of stool; and (3) onset associated with a change in form (appearance) of stool[2,3]. 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 unsubtyped IBS (IBS-U)[2,3]. Both IBD and IBS have an increasing prevalence; IBD affects up to 1.5 million people in the United States of America and 2.2 million Europeans[4], whereas IBS has a worldwide prevalence of 11.2%[5]. 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[6,7]. 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[8,9]. 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 symptoms[10]. 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 hypersensitivity, or an increased pain perception in the bowel, is an important factor underlying the abdominal pain in IBS[10]. Visceral hypersensitivity occurs via a disturbance of the sensitization pathways that might be located at different levels. 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)[11]. 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[12]. For a complete overview of the neuroanatomy of lower gastrointestinal pain disorders, we like to refer the interested readers to a review by Vermeulen et al[13].

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[14]. 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, inflammatory cells, e.g., mast cells, T-cells and neutrophils, are activated.
Upon activation, they release excessive amounts of inflammatory mediators such as histamine, serotonin, several cytokines and proteases. These mediators, in turn, can sensitize peripheral afferent neurons, thus contributing to visceral hypersensitivity. The role of most of these inflammatory mediators in visceral hypersensitivity has been investigated extensively\(^7\). However, the role of proteases is not fully elucidated yet. Therefore, the aim of this review is to focus on proteases and their physiological role as well as their role in the pathophysiology of visceral hypersensitivity. In an accompanying paper by Van Spaendonk et al\(^{15}\), we discussed the role of proteases in intestinal permeability.

**VISCERAL HYPERSENSITIVITY**

Irritable bowel syndrome (IBS) is a functional bowel disorder characterized by altered bowel habits and abdominal pain. This visceral pain - pain originating from internal organs such as the gut - is typically vague, diffuse, poorly localized and often associated with referred pain\(^{16}\). Visceral hypersensitivity is the main mechanism underlying this abdominal pain\(^{17}\). 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\(^{18}\). In both basic and clinical research, visceral hypersensitivity is commonly investigated by colorectal balloon distensions using a barostat\(^{19}\). Compared to healthy patients, patients suffering from visceral hypersensitivity report discomfort at lower colonic distension pressures (lower pain threshold) and increased pain in response to standardized stimuli\(^{19}\). 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\(^{20}\).

The mast cell is thought to fulfill an important task in the development of abdominal pain in IBS patients\(^{21}\), since an association was found between mast cell infiltration in the bowel wall and the frequency and severity of abdominal pain\(^{22}\). 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\(^{27}\). 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\(^{23}\). 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)\(^{24}\). Apart from histamine, mast cells release many other mediators such as cytokines, growth factors, leukotrienes, prostaglandins, serotonin and several proteases\(^{25}\). In this review, we will focus on the role of this last group of mediators, the proteases, and their role in visceral hypersensitivity. In the accompanying paper the role in intestinal permeability is addressed.

**PROTEASES**

Proteases are enzymes catalyzing the hydrolysis 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\(^{26}\). 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\(^{27,28}\).

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\(^{29,30}\). Since peptide chains have both an amino- and a carboxy-terminus, exopeptidases can be further divided into amino- and carboxypeptidases\(^{30}\). Amino-opeptidases could release a single amino acid, a dipeptide or a tripeptide from the N-terminus, while carboxypeptidases can liberate an amino acid or a dipeptide from the C-terminus\(^{31}\). Unlike exopeptidases, endopeptidases catalyze the cleavage of nonterminal peptide bonds within the molecule, thus releasing larger peptides instead of single amino acids, dipeptides or tripeptides\(^{29,30}\). The concept of exo- and endopeptidases is represented in a simplified way in Figure 1.

Due to their huge diversity in action and structure, proteases can be classified in other ways as well\(^{28}\). 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\(^{28}\). However, they are usually categorized according to their catalytic type, based upon the presence of different nucleophiles in the molecular structure of the enzyme\(^{29-33}\). 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\(^{33}\). Historically, four major groups

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**Reference:**

Ceuleers H et al. Proteases and visceral hypersensitivity.
glutamate and asparagine proteases have not been found in humans or other mammals so far. The seven catalytic classes are depicted in Figure 2. In the last decades, proteases have come into the picture as a new target for drug development. An overview of could be distinguished in this classification, based upon the functional group present at the active site: serine, cysteine, aspartic and metalloproteases. 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. The seven catalytic classes are depicted in Figure 2. 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 can be found in a Nature review by Turk. Concerning visceral hypersensitivity, serine proteases are thought to be an important class. Some examples of serine protease inhibitors that were already tested in clinical trials are summarized in Table 1, thereby emphasizing the widespread indications. Proteases can also act as signaling molecules; they regulate cell functions by modulating protease-activated receptors (PARs). 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, as visualized in Figure 3. So far, four PARs have been described: PAR1, PAR2, PAR3 and PAR4. PARs have been found in various cell types throughout the whole gastrointestinal tract. Remarkably, a great overlap can be detected in the cell types expressing PAR1 and PAR2. PAR1 and PAR2 have already been described in enterocytes, neurons, fibroblasts, mast cells, smooth muscle cells, endothelium and immune cells. On the other hand, PAR3 has only been identified in enterocytes, neurons, endothelium and immune cells. Furthermore, PAR4 has been identified in stomach and small intestine but the exact cell types still need to be defined. For a complete overview of the location of the different PARs in the gastrointestinal tract, we would like to refer to a review by Vergnolle.

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., PAR activation by trypsin). The activation process is visualized in Figure 3.

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., PAR activation by activated protein C). 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., PAR disarming by cathepsin-G). A fourth manner is the non-tethered ligand activation, suggesting that the formation of tethered ligands is not essential for PAR activation (e.g., PAR activation by elastase). Finally, it is possible to activate PARs via so-called PAR-activating

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Table 1  Serine protease inhibitors (mammalian): examples of clinical applications in different organ systems

<table>
<thead>
<tr>
<th>Category</th>
<th>Indication</th>
<th>Serine protease inhibitor (target)</th>
<th>Status</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiovascular</td>
<td>ACS</td>
<td>Bivalirudin (thrombin)</td>
<td>Approved [73]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AF</td>
<td>Rivaroxaban (factor Xa)</td>
<td>Approved [74]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>VTE</td>
<td>Edoxaban (factor Xa)</td>
<td>Approved [75]</td>
<td></td>
</tr>
<tr>
<td>Dermatology</td>
<td>Herpes zoster</td>
<td>Argatroban (thrombin)</td>
<td>Clinical-phase</td>
<td>[77]</td>
</tr>
<tr>
<td></td>
<td>Oral leukoplakia</td>
<td>BBIC (broad specificity)</td>
<td>Clinical-phase</td>
<td>[78]</td>
</tr>
<tr>
<td></td>
<td>Thrombocytopenia</td>
<td>Fondaparinux (factor Xa)</td>
<td>Approved</td>
<td>[79]</td>
</tr>
<tr>
<td>Oncology</td>
<td>Pancreatic cancer CTx</td>
<td>Upamostat (αPAs)</td>
<td>Clinical-phase</td>
<td>[80]</td>
</tr>
<tr>
<td></td>
<td>Lung cancer: RTx</td>
<td>Nafamostat mesilate (broad specificity)</td>
<td>Clinical-phase</td>
<td>[81]</td>
</tr>
<tr>
<td></td>
<td>Colorectal cancer: CTx</td>
<td>Talabostat (fibroblast activating protein)</td>
<td>Clinical-phase</td>
<td>[83]</td>
</tr>
<tr>
<td></td>
<td>Esophageal cancer: Sx</td>
<td>Ulinastatin (broad specificity)</td>
<td>Clinical-phase unknown</td>
<td>[84]</td>
</tr>
<tr>
<td>Pneumology</td>
<td>Asthma</td>
<td>APC 366 (mast cell tryptase)</td>
<td>Clinical-phase</td>
<td>[85]</td>
</tr>
<tr>
<td></td>
<td>α1 antitrypsin deficiency</td>
<td>α1 antitrypsin (broad specificity)</td>
<td>Approved</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td>Cystic fibrosis</td>
<td>α1 antitrypsin (broad specificity)</td>
<td>Clinical-phase</td>
<td>[87]</td>
</tr>
<tr>
<td>Endocrinology</td>
<td>Diabetes</td>
<td>Gabexate mesilate (broad specificity)</td>
<td>Clinical-phase III</td>
<td>[90]</td>
</tr>
<tr>
<td></td>
<td>NA</td>
<td>Aprotinin (broad specificity)</td>
<td>Approved</td>
<td>[89]</td>
</tr>
<tr>
<td></td>
<td>Nafamostat mesilate (broad specificity)</td>
<td>Clinical-phase</td>
<td>[91]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sivelastat (neutrophil elastase)</td>
<td>Clinical-phase unknown</td>
<td>[92]</td>
<td></td>
</tr>
</tbody>
</table>

1DPP-IV is a non-classical serine protease belonging to family 59: prolly oligopeptidase (novel class). 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; DPP-IV: Dipeptidylpeptidase IV; 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.
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-NH239,40). Since these peptides are specific for a single receptor, they are very important pharmacological tools to investigate the physiology and pathophysiology of PARs27.

Currently, thrombin is regarded as the main activator for PARs, PAR2 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 PAR4.26. For an overview of the characteristics of the four PARs, their activating proteases and agonists/antagonists, we refer to a detailed and excellent review by Vergnolle.38.

Table 2 Preclinical studies investigating the effects of protease-activated receptor-targeting molecules on visceral hypersensitivity

<table>
<thead>
<tr>
<th>PAR</th>
<th>Agonist/antagonist</th>
<th>Species (hypersensitivity model)</th>
<th>Study type</th>
<th>Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAR-1</td>
<td>Agonist (thrombin, TFFLR-NH2)</td>
<td>Rat (carrageenan)</td>
<td>In vivo</td>
<td>↓ hyperalgesia</td>
<td>[56]</td>
</tr>
<tr>
<td>PAR-1</td>
<td>Agonist (TFFLR-NH2)</td>
<td>Mice (capsaicin)</td>
<td>In vivo</td>
<td>↓ hyperalgesia</td>
<td>[55]</td>
</tr>
<tr>
<td>PAR-2</td>
<td>Agonist (SLIGRL-NH2)</td>
<td>Mice (PAR2-agonist)</td>
<td>In vivo</td>
<td>↑ hyperalgesia</td>
<td>[59]</td>
</tr>
<tr>
<td>PAR-2</td>
<td>Agonist (SLIGRL-NH2, trypsin)</td>
<td>Rat (PAR2-agonist)</td>
<td>In vivo</td>
<td>↑ hyperalgesia</td>
<td>[60]</td>
</tr>
<tr>
<td>PAR-2</td>
<td>Agonist (SL-NH2, trypsin, typtase)</td>
<td>Guinea pig submucosal neurons (PAR2-agonist)</td>
<td>Ex vivo</td>
<td>↑ neuron excitability</td>
<td>[61]</td>
</tr>
<tr>
<td>PAR-2</td>
<td>Agonist (SLIGRL-NH2, Tc-NH2, trypsin, typtase)</td>
<td>Mice, rat (PAR2-agonist)</td>
<td>KO</td>
<td>↑ hyperalgesia, absent in KO</td>
<td>[62]</td>
</tr>
<tr>
<td>PAR-2</td>
<td>Agonist (2-furoyl-LIGRL-NH2)</td>
<td>Mice (capsaicin)</td>
<td>KO</td>
<td>↑ hyperalgesia, absent in KO</td>
<td>[63]</td>
</tr>
<tr>
<td>PAR-2</td>
<td>Antagonist (ENMD-1068)</td>
<td>Mice (IBS-supernatant)</td>
<td>KO</td>
<td>↓ hypersensitivity, absent in KO</td>
<td>[41]</td>
</tr>
<tr>
<td>PAR-2</td>
<td>/</td>
<td>Mice DRG (IBS-D supernatant)</td>
<td>KO</td>
<td>↑ neuron excitability, absent in KO</td>
<td>[64]</td>
</tr>
<tr>
<td>PAR-4</td>
<td>Agonist (PAR-4-AP, Cat-G)</td>
<td>Mice (IBS-D supernatant)</td>
<td>In vivo</td>
<td>↓ hypersensitivity</td>
<td>[49]</td>
</tr>
<tr>
<td>PAR-4</td>
<td>Agonist (AYPGKF-NH2)</td>
<td>Mice (PAR2-agonist, TRPV4-agonist)</td>
<td>In vivo</td>
<td>↓ hypersensitivity</td>
<td>[57]</td>
</tr>
</tbody>
</table>

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

PROTEASES, PROTEASE-ACTIVATED RECEPTORS AND PROTEASE INHIBITORS IN VISCERAL HYPERSENSITIVITY

Proteases

So far, the expression and activity of proteases in the colon and feces of IBS patients have not been investigated intensively. A higher expression of the serine proteases trypsin25,41-45 and trypsin25,41 as well as the cysteine protease calpain-846 was described in colonic biopsy samples of IBS patients compared to healthy controls. However, no changes in trypsin expression (protein level) could be detected in fecal samples of IBS patients47,48. Serine protease activity was increased in IBS patients compared to healthy controls in both colon and feces in several studies41,48-50. Likewise, an elevated serine protease activity was observed in colonic samples in a post-infectious IBS mouse model51. 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, the Spiller group published a paper demonstrating the human (and not bacterial) origin of the most abundant fecal serine proteases in IBS-D patients52. Cysteine protease activity was elevated in fecal samples of IBS-C patients53, although this alteration could not be observed in colonic samples of post-infectious IBS mice51. 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. For example, when determining trypsin-like activity, mostly trypsin-like activity is quantified due to the lack of specificity of the substrates used54. 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 importance54.

Protease-activated receptors

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 2. 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 PAR-agonists TFFLR-NH2 and thrombin55,56. In mice, PAR1 activation seems to inhibit visceral hypersensitivity as well59,57. In parallel, PAR6 expression is lowered in the colon of IBS
patients[42,58]. The PAR-agonist AYPGKF-NH$_2$ was able to reduce visceral hypersensitivity after an intracolonic administration in sub-inflammatory doses, while higher doses showed pro-inflammatory effects in mice[57].

In sharp contrast to PAR$_2$ and PAR$_4$, the activation of PAR$_{3}$ results in a pronociceptive effect. This was firstly demonstrated by Kawabata et al[59] and Coelho et al[60] who confirmed the presence of visceral hypersensitivity in rats after the administration (intracolonic/intraplantar) of the PAR$_3$-activating peptide SLIGRL-NH$_2$ or trypsin. An increased Fos-expression[60] and the presence of PAR$_{3}$ mRNA in the dorsal root ganglia (DRG)[59] confirmed these results. An ex vivo study reconfirmed these observations: the application of several PAR$_3$-agonists, such as trypsin, mast cell trypsin and SL-NH$_2$, induced hyperexcitability of submucosal neurons in the ileum of guinea pigs[61]. 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 PAR$_3$-activating peptides such as 2-furoyl-LIGRL-NH$_2$ and trypsin. However, these effects were reduced in PAR$_3$-KO mice[62,63]. 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 PAR$_3$. Furthermore, this supernatant caused visceral hypersensitivity in WT mice, but not in mice treated with a PAR$_3$-antagonist or in PAR$_3$-KO mice[64]. Also, the IBS-D supernatant was able to enhance the neuronal excitability of colonic DRGs in WT but not in PAR$_3$-KO mice, again demonstrating the importance of PAR$_3$[64]. 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: PAR$_2$ and PAR$_3$ evoke antinociceptive effects while the activation of PAR$_4$ results in pronociception.

**Protease inhibitors**

So far, research groups in the field of visceral hyper-
sensitivity 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 3. Nafamostat mesilate 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 mesilate[65]. Similar results were observed by the group of Cenac et al[41] who used a similar, but slightly different experimental design. They used the supernatant of biopsies of IBS patients instead of fecal samples and apart from a decrease in visceral hypersensitivity, they also observed less sensitization of murine neurons after a pre-incubation with nafamostat mesilate. We recently demonstrated a positive effect of a single intraperitoneal injection of nafamostat mesilate in a trinitrobenzenesulfonic acid (TNBS)-induced rat model for both acute and post-inflammatory visceral hypersensitivity[66,67]. Furthermore, the newly developed serine protease inhibitor benzyl N-1-[(bis(4-acetamidophenoxy)phosphoryl)-2-(4-carbamimidamidophenyl)ethyl-carbamate [UAMC-0050, patent WO2007045496 (A1)] showed anti-nociceptive properties as well, both in an acute and in a post-inflammatory setting[66,67]. Camostat mesilate, another serine protease inhibitor with structural properties similar to nafamostat mesilate showed analogous results. Intragastric pre-treatment with camostat mesilate 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[66,69]. 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[70] found a decrease in visceral sensitivity, fecal protease activity and PAR$_{4}$ expression in acute TNBS colitis rats treated with a fermented soy germ extract, containing

<table>
<thead>
<tr>
<th>Inhibitor name</th>
<th>Target(s)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>Chymotrypsin, elastase, KLK, plasmin, PA, trypsin, urokinase, XIIa</td>
<td>[49]</td>
</tr>
<tr>
<td>Bowman-Birk inhibitor</td>
<td>Chymotrypsin, trypsin</td>
<td>[70,71]</td>
</tr>
<tr>
<td>Camostat mesilate (FOY-305)</td>
<td>Trypsin, matriptase, prostasin, plasmin, tPA, uPA, Xa, Xa, thrombin, tissue factor, complement</td>
<td>[68,69]</td>
</tr>
<tr>
<td>Cathepsin-G inhibitor</td>
<td>Cathepsin G</td>
<td>[49]</td>
</tr>
<tr>
<td>Nafamostat mesilate (FUT-175)</td>
<td>Trypsin, trypsin, C1r, C1s, thrombin, kallikrein, plasmin</td>
<td>[37,65-67]</td>
</tr>
<tr>
<td>UAMC-0050</td>
<td>Trypsin, matriptase, KLK4, KLK8, uPA</td>
<td>[66,67,93]</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor (SBTI)</td>
<td>Trypsin, chymotrypsin, plasmin, kallikrein, Xa</td>
<td>[49]</td>
</tr>
</tbody>
</table>

C1r: Complement component 1r; C1s: Complement component 1s; HNE: 4-hydroxynonenal; KLK: Kallikrein; PA: Plasminogen activator; tPA: Tissue plasminogen activator; uPA: Urokinase plasminogen activator.
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. 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 [71].

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, PAR-4 is stimulated due to the increased fecal serine protease activity, resulting in hypersensitivity. In addition, the activation of PAR-3 by adding Cathepsin-G (Cat-G) to the supernatant, reversed this effect. Hyposensitivity could be observed after the infusion of a UC supernatant most likely because PAR-3 (activated by Cat-G) is predominantly activated. However after the inhibition of PAR-4 or Cat-G, hypersensitivity appeared and the addition of the serine protease inhibitors aprotinin/SBTI normalized sensitivity [49]. Based on these studies by Annaházi et al. [49] the importance of the equilibrium between the activation of PAR-2 and PAR-4 in visceral sensitivity was clearly shown.

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 further explored.

CONCLUSION
The pharmacological treatment of gastrointestinal disorders such as IBD and IBS remains a challenge and until today, mainly focuses on symptomatic control. One of the biggest challenges is the management of visceral hypersensitivity, which is seen as the mechanism behind abdominal pain. In the last decades, many possible pharmacological targets have been proposed, but unfortunately an effective, causative treatment is still lacking. Therefore, further elucidating the pathophysiology of visceral hypersensitivity and eventually discovering new possible pharmacological targets is of great importance. Recently, serine proteases have come into the picture as a promising new pharmacological target for visceral pain. Up until now, research has focused mainly on PAR-agonists/antagonists, but none of these compounds made it to the clinic yet. A more recent strategy is the direct inhibition of serine proteases, which shows promising results in a limited number of animal experiments in the field of visceral hypersensitivity. So far, serine protease inhibitors have not been tested in clinical trials for IBS either. However, serine protease inhibitors are well known in the treatment of other diseases, e.g. diabetes and pancreatic cancer. In the domain of gastroenterology, protease inhibitors have already been investigated in animal models, focusing on the effects on intestinal inflammation and permeability. Protease inhibitors were able to ameliorate inflammation as well as permeability, suggesting that proteases may be valuable treatment targets.

The few preclinical studies investigating the effect of protease inhibitors on visceral hypersensitivity show promising results. Therefore it seems necessary that more in-depth research on the therapeutic potential of protease inhibitors in abdominal pain is conducted in the upcoming years. The emphasis should be on the detection and eventually the targeting of specific proteases that might be crucial in visceral hypersensitivity. First of all, as proteases often have overlapping substrate specificities and specific inhibitors are in many cases not available, the methods used to measure the activity of individual proteases should be improved. Furthermore, more and better validated tools are needed to quantify their protein levels. Measuring specific protease activities remains a great challenge. However, investigating the link between certain protease activities and IBS-subtypes would be of great interest and could possibly lead towards the discovery of a new drug target or the development of a new biomarker. Concerning therapeutic options we need to take into account that IBS is not considered to be a life-threatening disease and thus medication needs to be free of substantial side effects. This is certainly an important consideration given the various functions of proteases in the human body, and yet another reason to focus on the specificity of protease inhibitors, thereby not forgetting the potential importance of the equilibrium between proteases and anti-proteases. Direct inhibition of proteases has already been put forward as a possible strategy for the treatment of visceral hypersensitivity in IBS patients. Therefore, we believe that the development of new and more specific protease inhibitors could be of great interest. In the future, not only the safety profile of these compounds but also their route of administration will become a very important subject. Up until now, protease inhibitors are administered via systemic routes, but ideally, the treatment of IBS should focus on the gastrointestinal tract in a further attempt to reduce systemic side-effects. A serine protease inhibitor for local delivery is unfortunately not available at this time and it would be groundbreaking if researchers came up with a solution for this problem. In a recent article from Bermúdez-Humarán, a re-
volutary method using recombinant lactic acid bacteria (recLAB) to deliver serine protease inhibitors (Elafin and Secretory Leukocyte Protease Inhibitor-SLPI) at the mucosal level, is described[22]. However, it should be taken into account that in this case the serine protease inhibitors had a protein structure and therefore recombinant bacteria were able to produce and express them. Other serine protease inhibitors such as nafamostat mesilate are synthetic compounds with an organic chemical structure and thus cannot be produced by recombinant bacteria. Therefore, research groups should come up with new strategies to deliver synthetic compounds at the mucosal level. In our opinion, in the following years, research groups should focus on the development of specific serine protease inhibitors, taking into account the potentiality of delivering that compound at the level of the colonic mucosa after correctly measuring the specific protease profile of the individual patient, allowing individually tailored therapy.

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