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The interplay between genetic risk factors and proteolytic dysregulation in the pathophysiology of inflammatory bowel disease

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of inflammatory bowel disease (IBD). Previous studies reported increased levels of proteolytic activity in stool and tissue samples from IBD patients, while the reestablishment of the proteolytic balance abrogates the development of experimental colitis. Furthermore, recent data suggest that IBD occurs in genetically predisposed individuals who develop an abnormal immune response to intestinal microbes once exposed to environmental triggers. In this review, we highlight the role of proteases in IBD pathophysiology, we showcase how the main cellular pathways associated with IBD influences proteolytic unbalance and how functional proteomics are allowing the unambiguous identification of dysregulated proteases in IBD, paving the way to the development of new protease inhibitors as a new potential treatment.

Key words: proteases, NOD2, autophagy, endoplasmic reticulum stress, Crohn's disease, ulcerative colitis, inflammatory bowel disease

1. Introduction

Inflammatory Bowel Diseases (IBD) are chronic idiopathic inflammation of the gut including two main forms: Crohn's disease (CD) and ulcerative colitis (UC). IBD are multifactorial diseases with a complex aetiology, involving an intricate interaction between environmental and genetic factors. Indeed, CD and UC occur in genetically predisposed individuals whom immune response to intestinal microbes is abnormal after being exposed to specific environmental triggers.¹

The gastrointestinal (GI) tract is a complex interface that provides a physical barrier against luminal environment while performing the absorption of dietary nutrients and the exclusion of harmful compounds. The ability to control the passage of molecules throughout the mucosa is defined as the intestinal barrier function (IBF). The human gut mucosa is composed by; a well-integrated epithelial monolayer, a physical thick layer of mucus, secretory immunoglobulins A (IgA) and antimicrobial peptides (AMP) and finally, the immune system.² Many human diseases including inflammation, metabolic, infectious and neurologic dysfunctions are linked to a deficiency of the IBF.

Over the past 20 years, an important body of literature has pointed out the pivotal role played by proteases in GI homeostasis, such as in IBF, while their dysregulation promotes the development of the inflammatory process.^{3,4} Indeed, since proteolysis is an irreversible process, the proteolytic activity needs to be tightly regulated to avoid tissue damage: First, most proteases are synthesized as inactive zymogens. Once active, protease activity is finely tuned by endogenous inhibitors. Dysregulation in proteolytic balance has been reported in gastrointestinal diseases including IBD or Irritable Bowel Syndrome (IBS).⁵

The study of proteases is embedded with many challenges, since expression levels do not necessarily correlate with activity, changes in proteolytic activity is somehow invisible to traditional genomic and proteomic assays. From a biochemical point of view, these enzymes present a high degree of redundancy in structure and affinity to enzymatic substrates and inhibitors, abrogating unambiguous identification by standard biochemical assays. Nevertheless, functional proteomics, based on activity-based probe targeting active proteases succeeded at identifying dysregulated proteases secreted by the colonic mucosa of a small cohort of IBD patients.⁶ In this review, we present and discuss recent developments about the role of host and microbiota-derived proteases in the regulation of IBF and the control of inflammation with relevance to IBD, how genetic pathways associated with IBD pathophysiology correlate with proteolytic dysregulation and how functional proteomics could be used to identify overactive proteases associated to well-defined predisposing genetic signatures in IBD.

2. Proteases and their inhibitors

The human degradome consists of 588 genes encoding proteases, which represents about 2% of the protein-coding genome. Proteases are phylogenetically classified within five classes with major distinct catalytic properties: as serine-, metallo-, cysteine-, aspartate- and threonine proteases (Figure 1). Serine, metallo and cysteine proteases are the predominant protease classes, thus likely regulating a broader array of biological functions,⁷ including in the GI tract. In fact, the GI tract is the organ containing the highest amount of proteases, including endogenous enzymes from IECs (*e.g.* enterocytes, goblets and Paneth cells), resident and migrating immune cells (*e.g.* macrophages, dendritic cells, mast cells, neutrophils) and stroma cells (*e.g.* fibroblasts),⁵ as well as exogenous enzymes derived from the microbiota (*e.g.* bacteria, yeast and helminths).⁸

2.1. Serine proteases (Table 1)

Serine proteases hydrolyze peptide bonds by using a serine as the nucleophile at the active site and represent the second most abundant class of proteases in the human degradome, comprising 184 enzymes from 19 families.⁷ Ubiquitously expressed in the human body, enzymes pertaining to this class are predominantly secreted to the extracellular milieu and they play major roles in diverse biological processes such as digestion, blood coagulation, apoptosis and host defence.⁹ In mammals, this class of proteases comprises three subgroups with distinct substrate preferences; chymotrypsin-, trypsin- and elastase-like serine proteases.

Trypsin-like serine proteases are abundantly expressed and secreted as inactive zymogens (trypsinogens) by the pancreas. The human pancreatic juice contains three different isoforms of trypsinogens: trypsinogen-1 (cationic), trypsinogen-2 (anionic) and trypsinogen-3 (mesotrypsin). Trypsinogens-1 and -2 are the most abundant isoforms, responsible for protein degradation associated with physiological digestion and are inhibited by endogenous serpins and inhibitors of alimentary origin. On the other hand, human trypsin-3 is expressed and secreted by the pancreas in a lower proportion and is resistant to endogenous and alimentary inhibitors of proteases.¹⁰ To date, no endogenous inhibitor of trypsin-3 has been identified. These enzymes are potentially implicated in many other physiological processes beyond digestion. For instance, trypsin-2 and -3 has been shown to be expressed by small intestinal Paneth cells, wherein they promote the proteolytic maturation of human defensin-5 and -6 (HD-5 and -6), thereby being central in host defence.^{11,12} Additionally, previous studies reported an upregulation of trypsin-like activity in the mucosa of irritable bowel syndrome (IBS) patients.¹³ We then identified trypsin-3 as the trypsin isoform upregulated in IBS, which is secreted by the basolateral side of IECs and promotes sensory neuron activation and visceral hypersensitivity.¹³ Nevertheless, the expression and activity status of these enzymes in IBD remain elusive. The human degradome contains 15 families of protease inhibitors such as Serpins, which regulate the proteolytic activity of several trypsin-like serine proteases important in inflammation.¹⁴

As a whole, the main cellular sources of proteases in the human body seem to be epithelial cells, immune cells and commensal microbiota. Intestinal epithelial cells are an important source of proteases that help to regulate and to maintain intestinal barrier integrity: Trypsin, besides acting as a digestive enzyme, is also able to induce inflammation and pain;¹⁵ Chymotrypsin, elastase and matriptase-1 promote tight-junction integrity.^{16–18} (Table 1) Both resident and infiltrated immune cells are able to release a large spectrum of proteases, especially under inflammation. Under an inflammatory trigger, immune cells secrete their proteases within granules. Most leukocyte derived serine proteases, such as neutrophil elastase, proteinase 3, cathepsin-G, mast cell-restricted tryptase and chymase have a primary role in host defence.¹⁹ Otherwise, under proteolytic unbalance, these proteases can mediate severe tissue damage. Other serine proteases participate in process classically associated with other classes, e.g. granzymes can induce cellular apoptosis²⁰ while plasminogen activator regulates the extracellular matrix.²¹ (Table 1)

2.2. *Cysteine proteases*

In humans, this class of proteases has a predominant intracellular localization and is represented by 164 enzymes from 28 families. Cysteine protease zymogen becomes active by trans-activation by other enzymes or by auto-activation under the influence of acidic pH and redox status.²² Besides their fundamental functions in catabolism and protein processing, cysteine proteases are implicated in signalling pathways for programmed cell death and inflammation. In the context of GI physiology and disease, the best-characterised cysteine proteases belong to the families C14 (caspases), C02 (calpains), C54 (autophagins) and C12 (ubiquitin C-terminal hydrolases), mostly localized intracellularly. Additionally, although the family C01 (cathepsins) is also primarily intracellular, increasing reports indicate that these enzymes also localize extracellularly, such as cathepsins B, K and L. Caspase 8 is involved in apoptosis and inflammation, both activating pro-apoptotic genes and the NF- κ B signalling pathway.²³ Cysteine proteases affect the intestinal mucosa integrity through inflammation and cell death signalling pathways.²⁴ Cathepsins play an important role in degrading intracellular proteins, and maintaining the intestinal epithelium turnover and homeostasis.²⁵ Altered activity of caspases or cathepsins is associated with IBD.²⁶

2.3. *Matrix metalloproteases (MMPs)*

This class of metal-dependent (mainly zinc) peptidases consists of 192 enzymes from 29 families in humans. Historically, MMPs have been traditionally associated with the degradation and turnover of extracellular matrix (ECM) components. Additionally, some enzymes from this family have been designated according to their predominant subcellular localization, such as membrane-type (MT)-MMPs. These overly simplified classifications not only fall short into making appropriate reference to this extensive class of peptidases, but also belittle the broader

spectrum of MMPs endogenous substrates, a misconception that impacted the translational potential of these enzymes.²⁷

Indeed, the role of MMPs in tissue homeostasis and disease encompasses many other processes beyond ECM turn over, such as the processing of soluble mediators like chemokines, cytokines and growth factors.²⁷ MMPs are secreted as inactive zymogens by different cell types including mesenchymal cells, T cells, neutrophils and IECs.²⁸ As it will be detailed ahead, dysregulation of MMP activity leads to the development of several pathologies. Endogenous inhibitors of MMPs, known as tissue inhibitors of metalloproteinases (TIMPs), belong to the family I35 and consists of four members: TIMP1-4.²⁹ Dysfunction or altered expression of TIMPs is associated with inflammation and tissue damage. TIMPs are tissue-specific, constitutively or inductively expressed and are able to regulate the metabolism of several cytokines and growth factors.³⁰

2.4. Aspartic proteases

Aspartic proteases represent the smallest class of the human degradome, with only 21 members subdivided within 4 families. These enzymes use an activated water molecule bound to one or more aspartate residues for catalysis of their peptide substrates. All known aspartic proteases are inhibited by pepstatin, originally isolated from *Actinomyces* spp. The family A1 contains the classical aspartic proteases (*e.g.* renin, pepsin A, pepsin C, cathepsin D, cathepsin E, BACE-1, BACE-2 and napsin A), while family A22 comprises the presenilins. Aspartic proteases are described to be involved in several diseases such as IBD.^{26,31} Thus, dietary Sphingomyelin induces cathepsin D-mediated apoptosis in IECs and increases the colitis severity in mouse model.³²

3. Protease signaling

Proteases are not merely degradative enzymes, but are implicated in multiple signalling pathways such as the cleavage and activation the Protease-Activated Receptors (PARs). PARs are seven-transmembrane domain receptors that belongs to the superfamily of G protein-coupled receptors (GPCRs). Four different PARs have been discovered: PAR1-4³³ and they are ubiquitously expressed in human tissues (vascular, immune cells, IECs, nervous systems, etc). Similarly to rodhopsin and some hormone receptors, PAR agonists are embedded within the receptor structure. In this regard, PAR activation mechanism is unique since it is mediated by proteolysis Among proteases capable of activating PARs, cysteine-proteases, MMPs and a wide array of serine-proteases have been described.³⁴

Proteases bind to and cleave at a specific site of the extracellular N-terminal domain of PARs to unmask a new N-terminus tethered ligand. The newly generated N-terminal domain binds to the second extracellular loop and triggers intracellular signalling.³⁵ A different tethered ligand sequence is therefore generated for each PAR. As a general feature, activation of PARs by proteolysis is an irreversible process. After proteolytic activation, the receptor cannot be recycled and undergoes endocytosis via a clathrin-mediated process, where most of them are degraded into lysosomes.³⁶ It is important to note, that the cleavage and activation of the receptor by proteases is affinity-dependent. Indeed, PAR1 and PAR3 contains a hirudin-like domain, which gives thrombin high affinity to cleave the receptors. On the other hand, PAR4 lacks the hirudin-like domain, thereby requiring higher levels of thrombin to be activated.^{37,38} Despite thrombin cleaves and activates both receptors, PAR1 is 30 times more sensitive to thrombin than PAR4, and this differs on the intracellular downstream effects: with PAR1, the cytosolic rise of calcium is faster and shorter, while with PAR4 the increase is slower but prolonged.³⁹ Trypsin and trypsin play a major role cleaving PAR2 and signaling downstream effects. Trypsins are generally digestive enzymes, found on the digestive tract and in the lumen. Thus, PAR2 is present in both sides of the polarized IECs, apically and basolaterally, and are abundant in the gut.^{40,41} Trypsin also express affinity towards PAR4. Depending on the location, the activation of PAR will lead to different intracellular effects. PAR2 is also present in the dorsal root ganglia (sensory neurons) triggering inflammation and pain by nociceptive receptors. Activation of neuronal PAR2 by trypsin leads to a local inflammation and the transmission of neuropeptides triggering pain.^{42,43}

Activation of PARs present in epithelial cells leads to changes in paracellular permeability. Cenac et al. have shown that in mouse model, PAR2 activation by trypsin, trypsin and chymase, all serine proteases, promote increased colonic permeability displaying inflammation and disruption on the intestinal physiology.⁴⁴ Other studies have observed an alteration on the intestinal barrier function and increased permeability using PAR2 agonists.^{45,46} Investigation into the mechanism of action exhibited the involvement of calmodulin and MLCK in the PAR2-mediated modification of intestinal permeability. PAR2 agonist and calmodulin increase MLC phosphorylation which leads to epithelial cell cytoskeleton contraction and enhanced mucosal permeability. ML-7, an MLCK inhibitor, abolished disruption of TJ composition and function.^{47,48} Another study revealed that

213 activation of ERK1/2 by tryptase in cultured colonocytes also phosphorylates MLCK, leading to
214 epithelial cells disruption.⁴⁹

215 The localization of the receptor, at the basal or apical side, on IECs suggest a different intracellular
216 signaling pathway. Activation of basal PAR2 - by infiltrated or resident immune cells' proteases
217 or proteases coming from the bloodstream - leads to re-distribution of ZO-1, tight junction
218 formation and cytoskeleton reorganization.⁵⁰ While activation of apical PAR2 – by bacterial
219 proteases or digestive enzymes – favors the translocation of bacteria, activating the immune
220 response.⁵⁰

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4. Inflammatory bowel diseases and proteases

Although most mechanisms remain elusive, proteases are implicated in multiple pathophysiological processes underlying IBD onset and chronicity. Likewise, proteolytic cleavage increases the bioactivity of pro-inflammatory mediators such as CXCL8 and CXCL5, cleaved by proteinase-3 and cathepsin G, respectively. Proteases also contribute to the activation of programmed cell death such as caspases and autophagins and the increase of paracellular permeability.⁵ To date, genetic studies have associated 75 genes coding for proteases and 7 genes coding for protease inhibitors involved in CD pathology, and 14 proteases and 4 protease inhibitors genes associated to UC.⁵¹

4.1. Serine proteases (Figure 2)

Matriptase-1, membrane-type serine protease-1, widely expressed in epithelial cells of the GI tract is essential in the formation and integrity of intestinal barrier. The loss of matriptase-1 leads to leakage of intestinal barrier along with increased TEER and over-expression of claudin-2.¹⁶ Accordingly, matriptase-hypomorphic mice develop severe colitis to DSS. Moreover, gene expression of matriptase-1 is downregulated in colonic epithelium of CD and UC patients.⁵² Matriptase-1 highlights the complexity underlying proteolytic balance in homeostasis, not only as a product of an interplay between protease activation and regulatory inhibitors, but also of the repertoire of active proteases within the tissue environment.

Colonic tissues from UC and CD patients display high elastolytic activity correlated with increased levels of trypsin, and elastase.³⁴ Interestingly, *in situ* zymography revealed that the proteolytic activity is predominantly associated with IECs.¹⁷ Trypsin and chymase, released by mast cells, constitute another source of proteases contributing to IBD pathogenesis.⁵³ Kallikreins and Granzymes are also upregulated in IBD.⁵⁴ Granzymes promote cell death by activating several apoptosis mediators such as caspase-3, -8. The signalling pathways of these proteases have not been defined in the context of IBD. However, it is known that these proteases can cleave PARs. In particular, a link between PAR2 and Granzymes have been made in the context of colon infection.⁵⁵ PAR1-signalling is also involved in epithelial barrier dysfunction. *In vivo* and *in vitro* experiments have shown that PAR1 activators such as thrombin, increases paracellular permeability through the induction of apoptosis and ZO-1 degradation.⁵⁶

In situ hybridization showcased a decreased mRNA expression of Elafin in IECs of both UC and CD patients, suggesting a potential differential regulation of protease inhibitors (i.e. Elafin) in IECs and in immune cells for instance.¹⁷ In several murine models of IBD, the elafin delivered by transgenic probiotic bacteria induces a potent anti-inflammatory effect.⁵⁷ Neutrophil elastase (NE), proteinase-3 and cathepsin G, a chymotrypsin-like serine protease, are increased in IBD. NE induces inflammation upregulating CXCL8 via TLR-4 downstream signalling pathway.⁵⁸ Cathepsin G, present during inflammation within the submucosa, is able to activate PAR4. Studies have demonstrated the pro-inflammatory role of PAR4 activation,⁵⁹ and a potential role for this

receptor in IBD.⁶⁰ UC patients display increased colonic levels of cathepsin G and PAR4, both are related to increase paracellular permeability.⁶¹

Most of the proteases cleaving and activating PARs belong to the class of serine proteases, notably from family S01. Thus, the expression of PAR1, 2 and 4 is up-regulated in the colonic mucosa of IBD patients and inhibition of each of these receptors is a protective element against the development of colitis in animal models of IBD.⁶¹ Activation of PAR2 in IECs, by trypsin, tryptase and chymase, leads to changes in colonic paracellular permeability, favouring the development of inflammation.⁴⁴ These results have been supported by other studies which have shown an alteration on the IBF function and increased permeability using PAR2 agonists.⁴⁵ Hyun et al. demonstrated that deficiency mice of PAR2 reduce colitis severity induced by dextran sodium sulfate (DSS) and trinitrobenzene sulphonic acid (TNBS). Moreover, PAR2^{+/+} chimeric mice with PAR2-deficiency expressing bone marrow-derived cells attenuated colitis from TNBS.⁶² Investigation into the mechanism of action exhibited the involvement of calmodulin and MLCK in the PAR2-mediated modification of intestinal permeability.⁴⁸

4.2. Cysteine proteases (Figure 3)

Inappropriate production (up-regulation) of caspases or autophagins have been detected especially in UC patients, leading to activation of apoptosis and autophagy. Caspase-1 and -5 form the inflammasome complex, promoting IL1- β and IL-18 maturation, both pro-inflammatory cytokines involved in the pathogenesis of IBD.⁶³ Caspase-8 is involved in both apoptotic and inflammatory signalling pathway, activating respectively pro-apoptotic proteins and NF- κ B.²³ Conditional knockout mice for caspase-8 in IECs, a protease involved in apoptosis, necrosis and cell death, have shown increased cell death of epithelial cells in general and Paneth and Goblets cells in particular, developing spontaneous inflammation in the terminal ileum and high susceptibility to colitis.⁶⁴ Cathepsin K is highly secreted by goblet cells in the intestinal mucus, acting as an antimicrobial peptide shaping bacterial communities and modulating inflammation. Furthermore, cathepsin K-deficient mice treated with DSS develop severe colitis, while administration of recombinant cathepsin K improves it.³ Cathepsin L has been found increased in colorectal adenocarcinoma accelerating cell proliferation.²⁵ Another study observed increased cathepsin B and L in intestinal macrophages from inflamed mucosa of IBD patients associated with tissue degradation. Inhibition of these cysteine proteases improved colitis.²⁶

4.3. MMPs (Figure 4)

Beside their role in ECM turnover, MMPs exert their proteolytic action on a wide range of molecules, including cytokines, growth factors, surface receptors, cytoskeleton proteins and sealing proteins, leading to their activation. UC patients display an excessive production of MMP-7 related to severe inflammation levels.⁶⁵ IBD patients also have increased levels of MMP-9, which in healthy mucosa is usually undetectable. Deficient-MMP-9 mice exhibit resistance to

experimental gut inflammation.⁶⁶ Intestinal mucosa protein extracts from IBD patients exhibit increased expression of MMP-1, -2, -3, -12, -13 and -14 compared to healthy gut. In the context of IBD, like in many other chronic inflammatory diseases, ADAM17, the TNF- α converting enzyme, represent the most discerning example of how proteases can mediate pathophysiological signalling via their convertase activity. ADAM17 not only promotes the activation of TNF- α , but also the processing of its receptor (TNFRs), along with many other substrates of relevance to inflammation and cancer.⁶⁷ Likewise, CD patients have increased TNF- α levels, which plays a crucial role on increasing intestinal permeability.⁶⁸ In addition, a disruption of the balance between MMPs and TIMPs (MMPs inhibitors) is linked with inflammation and tissue damage. TIMP-1, a MMPs endogenous tissue inhibitors, is increased in UC biopsies, whereas TIMP-2 mRNA expression remains unaltered.^{69,70} Due to the implication of MMPs on cancer development such as invasion and metastasis, many studies focused the research on MMPs inhibitors. However, their clinical trials failed due to the severe side effects observed in patients, and the use of these inhibitor for acute or chronic inflammation is still under study.⁷¹

4.4. Aspartic proteases

Although less studied than major group of proteases, aspartic proteases have also been described to be involved in the development of intestinal inflammation and in IBD.^{26,31} For instance, cathepsin D is up-regulated in macrophages from colonic IBD patients³¹ and in DSS-induced colitis, whereas its inhibition reduces colitis severity.²⁶ Similarly, dietary Sphingomyelin has been reported to increase the expression of cathepsin D, triggering apoptosis in IECs and increasing colitis severity in mice model.³² An increased expression and activity of cathepsin E, involved in antigen presentation of bacterial peptides in dendritic cells, has been described in the gastric epithelium of patients suffering from *Helicobacter pylori* infections.⁷² More recently, deficiency of cathepsin E has been reported to decrease the motility of dendritic cells,⁷³ although its role in IBD remains to be investigated.

Renin expression is also up-regulated in IBD.⁷⁴ In experimental colitis model, using rectal instillation of 2,4,6-trinitrobenzene sulphonic acid, an inhibitor of the renin-angiotensin system has been reported to reduce colitis severity.⁷⁵ Thus, although no study has reported a role for others aspartic proteases including pepsin A and C, BACE-1 and -2 and napsin A in IBD or in intestinal inflammation, cathepsin D and E as well as the renin-angiotensin system are clearly involved in the regulation of the inflammatory process.

5. IBD pathways and proteolytic unbalance (Figures 5 and 6)

Although IBD is an idiopathic disease, over the ten past years, genetic studies have identified more than 170 loci of susceptibility for developing IBD. The strongest associations pointed out four main pathways altered in IBD: bacterial sensing (*NOD2*, CD),⁷⁶ autophagy (*ATG16L1*, *IRGM*, CD),⁷⁷ Th17 Profile (*IL23* receptor, CD)⁷⁸ and ER-Stress (*XBPI*, UC)⁷⁹ pathways.

5.1. Autophagy

Autophagy is a self-degradative process regulating the source of energy during nutrient starvation, besides the required basal level to maintain tissue homeostasis. CD patients, homozygous for the *ATG16L1* variant (T300A), present “loss-of-function” autophagy due to the impairment of autophagosome formation associated with an altered capacity to clear cytoplasmic content such as bacteria.⁷⁷

Autophagy signaling pathway, induced by rapamycin stimulation or nutrient starvation (NS), decreased the release of serine protease by Caco-2 cell line (Figure 5).⁸⁰ In addition, a large spectrum of mRNA encoding for serine proteases is also downregulated (e.g. *PRSSI*, -2, -3, *matriptase-1* and the serine protease inhibitor *elafin*).⁸⁰ These results clearly disproved the hypothesis originally proposed that autophagy could lead to increased serine protease release by IECs. However, NS increased both cysteine proteolytic activity, and permeability (Figure 6).⁸⁰ Cysteine inhibitor E64 normalized paracellular permeability under NS condition.⁸⁰ These results suggested that increased cysteine activity might be the responsible for permeability increase. Thus, although autophagy induction is not responsible for trypsin activity increase, it does modulate cysteine proteases balance. Nevertheless, the cysteine proteases dysregulated as well as the mechanisms underlying it remain to be investigated.

5.2. Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is an essential subcellular organelle responsible for the synthesis and maturation of proteins that traffic through the secretory pathway. Failure of protein folding due to changes in the environment such as inflammation leads to a dysfunction within ER, termed as ER stress.⁸¹ IBD genetic studies have identified susceptible risk alleles, such as *XBPI* and *ARG2* (Anterior Gradient 2), both involved in ER-stress signalling cascade.⁷⁹ Previous studies showed that genetic deletion of components involved in ER-stress signalling are linked to spontaneous intestinal inflammation and/or enhanced sensitivity to DSS-induced colitis.⁸²

Likewise, Caco-2 and HT29-MTX cell lines stimulated with tunicamycin or thapsigargin, two well-known ER-stress inducers, increased the release of trypsin-like activity at the apical side (Figure 5) without modification of cysteine proteolytic activity (Figure 6).¹⁵ Moreover, our results showed upregulation of *PRSSI*, -2 and *elastase 2* after 6 hours of ER-stress stimulation on Caco-2

cells,¹⁵ whereas mRNA levels of *PRSS3* increased 24 hours after induction in Caco2 cells, and was significantly up-regulated as soon as 6h after ER stress induction in HT29-MTX cells. Together these results showed that ER stress profoundly altered proteolytic homeostasis in IECs, not only at the granule secretion level, but also at the transcriptional level.

5.3. *NOD2 pathway*

NOD2 (Nucleotide-binding oligomerization domain 2) recognizes the muramyl dipeptide (MDP), widely expressed among both Gram-negative and -positive bacteria. About half of the patients with CD carry at least one polymorphism on *NOD2* gene.⁷⁶ Although the mechanism by which NOD2 mutation influences the development of CD is poorly understood, CD-associated NOD2 polymorphisms lead to a loss of function of MDP-downstream pathways, promoting alterations of the intestinal barrier function, gut microbiota and inflammation.^{83,84} For instance, the level of mature HD-5 in ileostomy fluid from CD patients is lower than in controls, a phenomenon exacerbated in patients with homozygous/compound heterozygous mutations in NOD2 and seemingly due to a dysregulation in trypsin-2 and/or -3 activity.⁸⁵ Nevertheless, the consequences of NOD2 loss of function on proteolytic homeostasis remain largely elusive.

5.4. *Th17 (IL23R) pathway*

Infiltration of immune cells and the secretion of cytokines and chemokines are central processes on the development of IBD. Interleukin 23 (IL-23) is a proinflammatory cytokine released during IBD inflammation crucial for the maintenance and maturation of Th17.⁸⁶ Th17 is a subset of helper T cells identified to produce IL-17; a strong proinflammatory cytokine concerned in several inflammatory pathologies including spontaneous arthritis autoimmune encephalomyelitis or IBD.⁸⁶⁻⁸⁸ In some publications, authors have shown an association between active IL-17 and MMPs upregulation; Biancheri et al, have identified that IL-17 was overexpressed in strictured tissues of CD, and that one upregulated MMP-3, -12 and TIMP-1, a tissue inhibitor of metalloproteinase, resulting in intestinal fibrosis.⁸⁹ Mycetoma, a progressive granulomatous inflammation, is also characterized by a large inflammation, where IL-17 play an important role and its levels correlated to MMP-9 levels.⁹⁰ In the pathogenesis of tuberculosis, another study associated the levels of IL-17 and MMP-3, leading to pulmonary tissue damage.⁹¹

Although the mechanism of IL-17 in IBD is uncertain, S Fujino shown that inflamed tissue of active CD and UC patients displayed increased mRNA expression of IL-17.⁹² IL23R is highly expressed in immune cells, such as: Th17, natural killer cells, dendritic cells or monocytes. IL-23 regulates the activation of the receptor IL23R, leading to the production of IL-17. Genome-wide association study has linked the variants Arg381Gln (rs11209026) in the *IL23R* gene region, associated as risk factors for CD.⁹³ Nevertheless, other coding variants of the IL23R gene were found protective against CD. This effect might be explained by the gain-of-function or loss-of function of the receptor.⁹⁴ A Scottish study have found an association between the variant Arg381Gln on the IL23R and the susceptibility to develop IBD in childhood onset.⁹⁵ A recent study have linked the upregulation of PAR1 expression in inflamed mucosa of pediatric CD patients with

the correlated production of IL-17 and IL23 cytokines. In the same study, involving *C. rodentium*-induced colitis in mice, the Th17 response and severity of colitis were strongly attenuated in PAR1 knockout mice.⁹⁶

5.5. Interplay of host genetics, dysbiosis and proteolytic balance

As aforementioned, IBD is driven by dysfunction between host genetics, the microbiota, and immune system. Over the last years, the unifying principles underlying the interplay among these processes started to emerge. Polymorphisms in susceptibility genes such as *ATG16L1* and *NOD2* promote disease through defects in "sensing" protective signals from the microbiome, defining a potentially critical gene-environment aetiology for IBD.^{97,98} These genetic variants influence the gut microbiota of healthy individuals,^{99,100} while the transfer of human faecal samples into *ATG16L1* T300A knock-in mice promotes dysbiosis and activation of Th17 cells before disease onset.¹⁰¹ As a whole, these studies indicate that genetic variants involved in host defence contributes to dysbiosis and immune activation prior to the clinical onset of the disease. Less explored though are the mechanisms by which host genetics and dysbiosis drive the onset and perpetuation of disease, but microbiota-derived proteases are seemingly at play. As a whole, microbiota-derived proteases can participate in IBD pathophysiology by four main mechanisms: by mediating adhesion and invasion to the intestinal epithelium, by activating PARs, by the disruption of the intestinal barrier or by immune cell regulation (for a comprehensive review see ⁸).

Pouchitis is a relatively common medical condition in individuals who receive ileal pouch-anal anastomosis (IPAA) surgery for UC, while it is rare in familial adenomatous polyposis (FAP) patients,¹⁰² suggesting that genetics and microbial dysbiosis associated with IBD may precipitate pouchitis in UC patients.¹⁰³ Conversely, in a case report involving two FAP siblings who underwent IPAA surgery. one sibling who inherited IBD-associated risk alleles for *NOD2* (rs17221417 and rs2076756) from his healthy father developed pouchitis, while his brother and mother, which also had an IPAA for FAP but lacked these variants, remained pouchitis-free. Microbiome sequencing of the terminal ileum and rectum found reduced levels of potentially beneficial bacteria such as *Faecalibacterium prausnitzii*, *Bacteroides*, and *Ruminococcaceae* in the sibling with pouchitis relative to his brother.¹⁰⁴ In a more recent study, UC patients who developed pouchitis exhibited about 5-fold higher faecal protease activity in comparison to pouchitis-free and control individuals. Furthermore, faecal supernatants from pouchitis patients activated PAR2 on Caco-2 monolayers, disrupted TJ proteins, and increased epithelial permeability.¹⁰⁵

With few exceptions, the microbial source of luminal proteases remains largely elusive. *Enterococcus faecalis*, a constituent of the normal intestinal microbiota capable of inducing inflammation in the IL-10-deficient mouse model of colitis,¹⁰⁶ secretes two dominant proteases, gelatinase (GelE) and serine protease (SprE).¹⁰⁷ As discussed above, microbial protease-mediated disruption of the intestinal epithelium is a potential mechanism whereby a dysbiotic enteric microbiota can lead to disease. Indeed, *E. faecalis*-induced intestinal inflammation is associated

with enteric permeability mediated by GeIE degradation of the epithelial junctional protein E-cadherin,¹⁰⁸ as well as by the activation of PAR2 in epithelial cells.¹⁰⁹ On the other hand, while in healthy subjects the major source of luminal proteases seem to be the microbiota,¹¹⁰ antibiotic therapy is often associated with increased pancreatic serine protease activity and constitutes a risk for CD. Of note, in a study in IL10^{-/-} mice, antibiotic therapy recapitulates the increase of pancreatic proteases in the faeces, impaired epithelial barrier integrity and development of colitis, indicating that the commensal microbiota has a role in pancreatic protease neutralization in physiology.¹¹¹ Other microbiota-derived proteases have been described to be implicated in gut inflammation. For instance, elastase from *Pseudomonas aeruginosa* acts as a major virulence factor promoting gut tissue damage.¹¹² In addition, the proteases Hbp and Pic, derived from pathogenic *Escherichia coli*, *S. flexneri* and enteroaggregative *E. coli*, respectively promote the destruction of the mucus layer.¹¹³ (Table 1).

5.6. Single nucleotide polymorphisms directly associated with proteolytic dysregulation

Besides single nucleotide polymorphisms (SNPs) indirectly affecting the regulation of proteases, as aforementioned, SNPs in protease coding genes have been associated as risk factors for IBD. Indeed, 10 SNPs in four genes were significantly associated with CD: CYLD, USP40, APEH and USP3. Among those, CYLD was the most significant gene, with the intronically located rs12324931 presenting the strongest association (p(FDR)=1.74e-17, OR=2.24 (1.83 to 2.74)). Five SNPs in four genes were significantly associated with UC: USP40, APEH, DAG1 and USP3. CYLD, as well as some of the other associated genes, are part of the ubiquitin proteasome system (UPS). Hence, the authors assessed if the IBD-associated adherent-invasive *Escherichia coli* (AIEC) can modulate the UPS functioning. Indeed, infection of IECs with the AIEC LF82 reference strain modulated the UPS turnover by reducing poly-ubiquitin conjugate accumulation, increasing 26S proteasome activities and decreasing protein levels of the NF- κ B regulator CYLD. This resulted in I κ B- α degradation and NF- κ B activation. This activity was very important for the pathogenicity of AIEC since decreased CYLD resulted in increased ability of AIEC LF82 to replicate intracellularly. Therefore, this study revealed that the UPS, and CYLD specifically, is an important contributor to IBD pathogenesis, by a mechanism involving the interaction between genetic and microbial factors.⁵¹

6. Activity-based probe proteomic profiling of proteases in IBD

As a whole, studies aiming at identifying dysregulated proteases in IBD have primarily investigated expression profiles, not taking into account enzymatic activity. Conversely, studies focusing on proteolytic activity remain elusive or ambiguous on the identity of overactive proteases, since most proteases share a non-negligible overlap towards enzymatic substrates and inhibitors. Nonetheless, a recent study based on functional proteomics unveiled for the first time both the identity and activity status of proteases released by the GI mucosa of a small cohort of IBD patients and healthy volunteers. Likewise, by using a serine protease-targeted activity-based probe (ABP) coupled with mass spectral analysis, seven active proteases: cathepsin G, plasma kallikrein, plasmin, tryptase, chymotrypsin-like elastase 3A, thrombin and aminopeptidase B were identified as active. Furthermore, cathepsin G and thrombin were found overactive in colon biopsies from IBD patients compared to healthy volunteers. Interestingly, the genes encoding these proteases, including thrombin, were also expressed within the colonic mucosae, suggesting the landscape of proteases locally expressed and potentially implicated in GI physiology and disease might be broader than previously anticipated.⁶ Indeed, in a follow up study we demonstrated that the intestinal epithelium is the source of thrombin in the colonic mucosa, wherein its expression is directly regulated by the presence of commensal microbiota. On the other hand, the inhibition of luminal thrombin in mice caused transcriptional changes to genes involved in host-microbiota interactions, along with microscopic and macroscopic tissue damage. Further, we demonstrated that luminal thrombin proteolytically regulates the biofilm matrix of reconstituted mucosa-associated human microbiota, while thrombin inhibition impairs the spatial segregation of microbiota biofilms, allowing bacteria to invade the mucus layer and to translocate across the epithelium.¹¹⁴

Although the pathophysiological role of these dysregulated proteases remain to be further investigated, this study provides the proof of concept that proteases can be unambiguously identified in association with their activity status from complex biologic samples such as colonic biopsy supernatants, paving the way for the identification of dysregulated enzymes in other GI disorders including cancer. Therefore, the association of functional proteomic approaches with larger, well-defined genetic cohorts of IBD patients will be instrumental at identifying dysregulated proteases associated to specific pathophysiological pathways.

7. Future perspective treatments to target IBD

Patients with abnormal activity of ER-stress display increased trypsin-like in intestinal epithelial cells. Increased trypsin proteolytic activity is responsible for intestinal barrier alteration, while ER-stress induces inflammation. Thus, we suggest that inhibiting dysregulated trypsin-like enzymes could be beneficial for specific subsets of IBD patients. However, one has to wonder which patients might benefit the most from such treatment, and when would be the best time and in which combination with potential other treatments this approach could be used? Pentasa (Mesalamine) is used to induce remission in mild to moderate active IBD. Ingested orally, Pentasa is slowly released into the body, acting locally in the colon instead of systemically. The mechanisms of action of Mesalamine include the inhibition of lipoxygenase and cyclooxygenase pathways, cytokine inhibition (interleukin-1, interleukin-2, TNF). It also acts as a potent antioxidant and a free-radical scavenger, as well as a Peroxisome proliferator-activated receptor (PPAR)-gamma. Through all these mechanisms, Mesalamine treatment usually achieve a good inhibition of cell recruitment and a fair repair of damaged tissues. However, the effects that Mesalamine treatments have on epithelial homeostasis are most likely indirect. No direct effect for this treatment on epithelial repair or on reinforcement of epithelial barrier have ever been demonstrated. Therefore, there might be some space for a combinatory treatment in mild and moderate IBD patients that would target a fast repair of epithelial functions and integrity. Anti-trypsin treatment might well fit within that space. While classical anti-inflammatory drugs such as Mesalamine would suppress the inflammation and inflammatory cell recruitment, trypsin-like inhibition would promote repair and reconstruct rapidly the damaged mucosa. ER-stress inhibition though would not be a good strategy since ER-stress is an essential cellular mechanism, especially for secretory lineages like Paneth and Goblet cells. Anti-inflammatory drugs have to be taken during active phases, until remission is achieved. But one can imagine that low doses of trypsin-like inhibitors could be taken even in remission phases. Since ER-stress is constitutively active in these patients, trypsin inhibition would lower the pro-inflammatory effects of constitutive up-regulation of ER stress associated with UC and might maintain remissions for a longer period.

In case of autophagy malfunction, the unbalance between cysteine proteases and trypsin-like proteases should be restored. Downregulation of trypsin proteolytic activity might be a protective mechanism for the cells to avoid losing essential components for cell maintenance, while increased cysteine activity alters intestinal barrier. Once again, we propose to specifically inhibit the responsible cysteine-like protease at the same time, that patient is treated with an anti-inflammatory drug.

8. Conclusions

Taken together, the data discussed herein highlights the importance of proteolytic balance for GI homeostasis and give insights into the consequences of proteolytic balance disruption in IBD. Although recent advances, there is still a major need to better characterize the proteases involved in these diseases. Ideally, their identification in well-characterized genetic cohorts of IBD patients will subside the rational design of highly specific inhibitors for defined subsets of patients.

9. Search strategy and selection criteria

References for this Review were selected from PubMed searches adopting the following criteria: “Proteases”, “IBD genetic polymorphism”, Intestinal epithelial” from 1986 until 2018. Also books concerning an overview of “proteases and their receptors” was used to complete the review, as well as abstracts presented in conferences. Moreover, articles were preferred by the author’s research quality, information relevance and clarity of the content given. Only papers written in English were taken into account.

10. Authorship statement

FB is the guarantor of the article. NST, AD-S and FB performed bibliographic research, wrote and revised the manuscript. NV revised the manuscript. All authors approved the final version of the manuscript.

11. Conflicts of Interest and Funding

NV has licenced a patent to Biose. All other authors declare no conflicts of interest. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale and the Direction Générale de l’Offre de Soins Label Mardi (Rare Digestive Diseases).

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Table 1. Cellular source and function of serine proteases in GI physiology and pathophysiology.

Figure 1. Proteases wheel. Part of a whole representation of the human degradome according to the percentage of each protease class.

Figure 2. Serine proteases in Inflammatory bowel disease. Overexpression or downregulation of serine proteases in IECs and immune cells in IBD context.

Figure 3. Cysteine proteases in Inflammatory bowel disease. Overexpression or downregulation of cysteine proteases in IECs and immune cells in IBD context.

Figure 4. Metalloproteinases in Inflammatory bowel disease. Overexpression or downregulation of Metalloproteinases in IECs and immune cells in IBD context.

Figure 5. Illustration of trypsin proteolytic activity released by intestinal epithelial cell in response to the main inflammatory bowel disease pathways. (A) Basal level of serine proteolytic activity in IECs in physiological condition. (B) No modulation of serine proteases activity upon NOD2 stimulation. (C) Decreased serine proteolytic activity upon autophagy induction in both IECs compartments: apical and basal. (D) Increased serine proteases activity upon ER-stress induction at the apical side of IECs. ^{15,80}

Figure 6. Illustration of cysteine proteolytic activity released by intestinal epithelial cell in response to the main inflammatory bowel disease pathways. (A) Basal level of cysteine proteolytic activity in IECs in physiological condition. (B) No modulation of cysteine proteases activity upon NOD2 stimulation. (C) Increased cysteine proteolytic activity upon autophagy induction at the apical compartments. (D) No modulation of cysteine proteases activity upon ER-stress stimulation. ^{15,80}

Table 1

Serine Proteases		Function examples	Ref
Intestinal Epithelial Cells			
	Trypsin	Protein degradation, degradation of extracellular matrix, inflammation, pain, transepithelial resistance	11
	Chymotrypsin	Cleave proteins, transepithelial resistance	68
	Elastase	Transepithelial resistance, inflammation	31
	Matriptase-1	Regulates intestinal barrier integrity	29
	Thrombin	Mucosal homeostasis	114
Commensal microbiota			
	Subtilisin (Bacillus subtilis)	Extracellular and membrane-bound proteases	69
	Elastase (Pseudomonas aeruginosa)	Major virulence factor that cause major tissue damage during infection	70
	Hbp (Pathogenic E.coli)	Cleave mucins	71
	Pic (S. flexneri and enteroaggregative E.coli)	Cleave mucins	71
Immune cells			
	Granzymes	Induce apoptosis	72
	Neutrophil Elastase (NE)	Induce bacterial elimination, ECM degradation and controls inflammatory response	73
	Proteinase 3 (PR3)	Induce inflammatory response, stimulate bacterial and fungal elimination	73
	Cathepsin-G	Promotes bacterial elimination, ECM degradation and controls inflammatory response	73
	Tryptase	Stimulates extracellular antigens clearance, ECM degradation, controls inflammatory response	73
	Chymase	Stimulates extracellular antigens clearance, ECM degradation, controls inflammatory response	73
	Plasminogen Activator	Regulates coagulation and fibrino(geno)lysis	74

Figure 1

Figure 2

Figure 3

Intestinal Epithelial Cells (IECs)

Macrophage

Eosinophil

Basophil

Neutrophil

Mast cell

NK cell

T cell

B cell

Figure 4

Intestinal Epithelial Cells (IECs)

Macrophage

Eosinophil

Basophil

Neutrophil

Mast cell

NK cell

T cell

B cell

Figure 5

Illustration of trypsin proteolytic activity released by IECs depending on the stimulate pathway

Autophagy 62. Vergnolle N, Solà Tapias N, Deraison C, Blanpied C, Edir A, Gilles D, et al. Autophagy controls proteolytic homeostasis of intestinal mucosa.

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ER-stress 65. Solà Tapias N, Denadai-Souza A, Rolland-Fourcade C, Blanpied C, Dietrich G, Bonnart C, et al. Intestinal epithelial cells under endoplasmic reticulum stress boosts serine proteolytic activity and modulates barrier function. Elsevier B.V. on behalf of European Crohn's and Colitis Organisation; 2017

Figure 6

Illustration of Cysteine proteolytic activity released by IECs depending on the stimulate pathway

Autophagy 62. Vergnolle N, Solà Tapias N, Deraison C, Blanpied C, Edir A, Gilles D, et al. Autophagy controls proteolytic homeostasis of intestinal mucosa. Federation of American Societies for Experimental Biology; 2017

ER-stress 65. Solà Tapias N, Denadai-Souza A, Rolland-Fourcade C, Blanpied C, Dietrich G, Bonnart C, et al. Intestinal epithelial cells under endoplasmic reticulum stress boosts serine proteolytic activity and modulates barrier function. Elsevier B.V. on behalf of European Crohn's and Colitis Organisation; 2017