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"Proteases: Pivot Points in Functional Proteomics"

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Abstract

Proteases drive the life cycle of all proteins, ensuring the transportation and activation of newly minted, would-be proteins into their functional form while recycling spent or unneeded proteins. Far from their image as engines of protein digestion, proteases play fundamental roles in basic physiology and regulation at multiple levels of systems biology. Proteases are intimately associated with disease and modulation of proteolytic activity is the presumed target for successful therapeutics. "Proteases: Pivot Points in Functional Proteomics" examines the crucial roles of proteolysis across a wide range of physiological processes and diseases. The existing and potential impacts of proteolysis-related activity on drug and biomarker development are presented in detail. All told the decisive roles of proteases in four major categories comprising 23 separate sub-categories are addressed. Within this construct, 15 sets of subject-specific, tabulated data are presented that include identification of proteases, protease inhibitors, substrates and their actions. Said data are derived from and confirmed by over 300 references. Cross comparison of datasets indicates that proteases, their inhibitors/promoters and substrates intersect over a range of physiological processes and diseases, both chronic and pathogenic. Indeed, "Proteases: Pivot Points …" closes by dramatizing this very point through association of (pro)Thrombin and Fibrin(ogen) with: hemostasis, innate immunity, cardiovascular and metabolic disease, cancer, neurodegeneration and bacterial self-defense.

Keywords

Protease; Peptidase; Proteolysis; Protease Inhibitor; Protease Promoter; Digestion; Hemostasis; Complement System; Immune Regulation; Signaling; Cell Migration; Cell Proliferation; Programmed Cell Death; Protein Secretion; DNA Replication; DNA Repair; DNA Processing; Intranuclear Proteolysis; Transmembrane Proteolysis; Intramembrane Proteolysis; Cytosolic Proteolysis; Epigenetics; Inflammation; Cardiovascular Disease; Metabolic Disease; Stroke; Cancer; Neurodegenerative Disease; Autoimmune Disease; Infectious Organisms; Drug Target; Drug Development; Biomarker Development; Precision Medicine

INTRODUCTION

Proteases: More than Just Protein Digestion

One or more proteolytic events initiate the active life for many proteins and proteolytic events terminate the relatively short life of all proteins. Roughly a third of proteins, as translated, include peptides that influence proteins' lifespan (initiator methionine), govern their transport (signal peptide), direct them to the appropriate organelle (transit peptide) and produce their active form (propeptide). These processes are merely those proteolytic events that begin the active life of proteins. Over the course of lifetimes that may be as brief as 11 minutes or as long as four months, additional proteolytic events remake individual proteins – one example being the much studied A4-Human amyloid precursor transmembrane protein whose 13 proteolytic products include a family of pathology-related, β amyloid peptides. Having served their temporary purposes, all proteins are once again substrates for proteases with digestion proceeding to amino acids recycled as raw materials for ongoing expression of new proteins. In the light of these varied and continuous processes, proteolytic activity literally reshapes the proteome. Of course, this brief discourse is but the tip of the iceberg. Proteolytic enzymes – and their promoters and inhibitors – are essential actors in nearly all biological processes. This chapter addresses many of those processes especially in relation to disease and development of new therapeutics. Truly, proteases give definition to the Functional Proteome - one protein at a time.

In the 1830s, parallel studies in Europe and the US demonstrated that digestion of proteins in food required a compound in the gastric mucosa, in addition to gastric juice hydrochloric acid [1]. The discovery of pepsin triggered the formulation of popular therapeutic preparations for dyspepsia, and intensified pepsin purification efforts. However, it would take nearly a century before crystallization of pure pepsin was achieved. Northrop, Kunitz and Herriott published the earliest characterization of classical digestive proteases such as pepsin, chymotrypsin, trypsin, pancreatic carboxypeptidases, and their zymogens as pure compounds [2]. The protease field has come a long way since the discovery of pepsin. Rapidly developing methods for protein purification, sequencing, structure-function analysis, X-ray crystallography and synthetic substrate development have since then accelerated the identification of countless mammalian, plant, fungal and bacterial proteolytic enzymes [3], and the number of identified sequences is growing exponentially. As of September 2017, the MEROPS database of proteolytic enzymes (https://www.ebi.ac.uk/merops/) listed more than one million sequences [4].

The complete ensemble of human proteases, known as the protease degradome, currently consists of 588 proteases, organized into five classes: aspartic, cysteine, metallo-, serine and threonine proteases [5]. They represent >2% of the human genome, and a database is maintained by the lab of López-Otín (http://degradome.uniovi.es) [6]. Protease systems have gained considerable recognition since the realization that they are important regulators of countless biological mechanisms, and not simply part of a machinery for non-specific protein digestion. Blood coagulation, fibrinolysis, complement activation, peptide hormone processing, protein secretion and degradation, DNA replication and repair, cell signaling and proliferation, and programmed cell death are just a few of these processes in which

physiological proteases target specific substrates. The peptidase field is rapidly evolving, and extensive volumes have already been dedicated to cataloguing and documenting its recently discovered and previously known members [7]. Here we focus on novel aspects of the intimate connection between disease and proteolysis, and the potential of mechanism-based drug targeting.

Physiological and Regulatory Roles

Physiological protease activity is strictly controlled to avoid indiscriminate and unwanted protein degradation [8]. Proteases may be present as inactive zymogens requiring a proteolytic activation step followed by a conformational rearrangement to form the active site (hemostatic protease zymogens); complexed with an inhibitory propeptide or N-terminal domain that blocks the active site (matrix metalloproteinases or MMPs); or in a low-reactive state requiring allosteric activation (factor VII and tissue factor), proteolysis (single chain tPA), or di/multimerization (caspase-8 and −9, proteasome proteases. Active proteases may be pH-controlled, and examples of this are lysosomal cathepsins and gastric pepsin. Many proteases are transcriptionally regulated and only expressed in specific cells and tissues, sometimes temporally restricted, whereas housekeeping proteases are expressed constitutively. Physiological triggers such as inflammation may temporarily upregulate zymogen expression, as seen for the precursors of cathepsins and MMPs.

Because many proteases are associated with other proteins and act within networks of effectors, ligands and receptors, studying their biological functions has become increasingly complex. *In vitro* delineation of structural and kinetic properties of a protease is required for defining its mechanisms of substrate and inhibitor specificity, but its catalytic efficiency is often profoundly affected *in vivo* by complex macromolecular interactions. Protease activity may be up- or downregulated by physiological feedback mechanisms, processes at the genomic level, and stressors in the molecular environment. Depending on the molecular environment and the binding partners, proteases may catalyze reactions that result in opposite physiological processes. Thrombin, the central protease in hemostasis, is a good example of this. Uncomplexed thrombin is procoagulant, and cleaves fibrinogen to fibrin to form a thrombus. However, when bound to thrombomodulin, thrombin activates the anticoagulant protein C. The proteases of the contact activation system in the intrinsic coagulation pathway aid in sustaining clotting once the extrinsic pathway is activated, but they also activate plasminogen in the fibrinolytic pathway.

Protease networks are regulated by intrinsic inhibitors of a protein or polypeptide nature. Serine proteases feature predominantly in coagulation, fibrinolysis, and the complement system, and also play roles in digestion, late stage apoptosis, development, fertilization and membrane-associated signaling. They are typically inhibited by serpins (serine proteinase inhibitors) in irreversible covalent complexes, or by polypeptide inhibitors of the Kunitz, Kazal or elafin types, with a protease binding loop in a conserved canonical backbone conformation, complementary to the protease active site. Inhibitory serpins belong to a superfamily of ubiquitous proteins with a typical fold that includes a metastable reactive center loop (RCL) for baiting their target proteases, and a core consisting of 3 β-sheets and 8 or more α-helices [9]. Upon cleavage of the RCL, the protease stays attached, and the

resulting acyl-enzyme undergoes a dramatic conformational rearrangement during which the cleaved RCL inserts as an additional strand in β-sheet A, and the attached protease translocates to the distal end of the serpin. Deformation of the active site prohibits completion of hydrolysis, with a stable covalent complex as the end product. Kunitz, Kazal and elafin-like inhibitors act by a different mechanism of tight binding in a substrate-like mode, followed by slow and reversible cleavage of the reactive bond [10]. The cleaved inhibitor stays bound to the protease but typically loses inhibitory potency by several orders of magnitude.

Analysis of known cleavage events has shown that regulation of the proteome *in vivo* occurs through an interconnected protease-inhibitor web [11], with protein protease inhibitors being protease substrates themselves. Many inhibitors target groups of related proteases rather than single enzymes, and proteolytic inactivation of one inhibitor may represent a key on/off switch for an entire protease subnetwork. Multiple regulatory mechanisms of proteolysis have been identified, such as the presence of PEST (proline, glutamic acid, serine, threonine) sequences in intracellular proteins targeted for rapid degradation; KFERQ motifs (lysine, phenylalanine, glutamic acid, arginine, glutamine) guiding cytosolic proteins to the endosome or lysosome for degradation; and RxxLxxIxN destruction box motifs that tag proteins for degradation by the ubiquitin-proteasome system [12]. Whereas the protease/substrate/inhibitor interconnectedness adds many levels of complexity, it also provides more opportunities for design of new therapeutic approaches.

Proteases and Disease

Protease functionality often depends on the concerted action of a catalytic domain and specific non-enzymatic domains and modules, either incorporated in the protein or as separate entities associated with the catalytic domain. Anion-binding sites, kringle and apple domains, epidermal growth factor (EGF) and fibronectin domains, thrombospondin repeats and transmembrane domains have diverse functions such as localization; recognition of substrates, inhibitors and effectors; and interactions with various ligands, cofactors and other proteins. Many of these domains are evolutionary conserved, and are present in diverse proteases either as single units (e.g. EGF domains), or as repeats (kringle and apple domains, thrombospondin domains). Not surprisingly, defects in the catalytic domain or any of the regulatory domains results in a physiologically dysfunctional protease. Intrinsic protease dysregulation is a hallmark of many pathologies such as inflammation, cancer, hemostatic and autoimmune diseases, and neurodegeneration [13,14]. A selection of recent findings with regard to abnormal protease activity in these pathologies is discussed below. The López-Otín degradome database currently lists 132 hereditary diseases due to protease mutations, and many other pathologies are associated with post-translational and epigenetic changes in protease activity. Although much progress has recently been made in inflammation-related protease research, many processes are still unclear with respect to upregulation of protease activity as a cause or consequence. However there is ample consensus about the importance of proteases as attractive drug targets in many disease states. Effector ligands and substrates may provide additional targets. Protease up- or downregulation may be used as a diagnostic, and proteolytic generation of signal peptides may provide a previously underappreciated arsenal of biomarkers in various disease states.

REGULATION OF PHYSIOLOGICAL PROCESSES

Hemostasis

If you are familiar with only one proteolytically regulated process in the human body, it is likely hemostasis. Featured prominently in documentaries about European royalty and in nightly medication commercials, the formation and degradation of blood clots has been intensely studied. It is a model system for illustrating the complexity of proteolysis: multiple pathways acting both in concert and in opposition to each other, dozens of actors, extensive regulation at each step, and even individual actors performing opposing roles under different conditions. Each step is an opportunity for both dysfunction and intervention, and both affects and is affected by processes in cells throughout the body.

In primary hemostasis, vascular injury exposes the highly thrombogenic subendothelium, and platelets are recruited to the site of injury, where they are activated and form a platelet plug. In the “secondary hemostasis” cascade model of coagulation, proposed more than 50 years ago, vascular injury triggers a stepwise amplification of clotting factor activity. This culminates in the formation of thrombin, the central coagulation protease, that cleaves fibrinogen to fibrin [15]. In the extrinsic pathway, plasma factor VIIa forms a highly reactive complex with tissue factor exposed during injury, and sequential activation of factor X and prothrombin leads to clot formation. The intrinsic or contact activation pathway consists of prekallikrein, and factors XII and IX. Stepwise activation of factors XII and XI generates factor IXa that sustains formation of factor Xa. Except for factor XIIIa, a transaminase that crosslinks fibrin, all the hemostatic enzymes are serine proteases. Intrinsic serine protease inhibitors (antithrombin, heparin cofactor II) and the Kunitz inhibitor TFPI provide regulatory control. Phospholipid surfaces and the non-enzymatic cofactors VIII and V are required for activation of factor X and prothrombin, respectively. This “waterfall” mechanism did not explain why factor XII-deficient patients do not have a bleeding tendency, and a new “hemostatic network” mechanism was discovered in which thrombin activates factor XI to sustain hemostasis [16,17]. Whereas the contact activation proteases are not critical in normal hemostasis, animal studies have shown that they are important contributors to formation of pathologic intravascular thrombi [18], and may be suitable targets for therapeutic inhibitors.

When the blood clot has served its purpose, endothelial cells release tPA that converts plasminogen to plasmin on the fibrin surface. Both tPA and plasminogen bind to fibrin through their kringle structures (binding domains specific to blood clotting factors), resulting in significant enhancement of the rate of plasmin formation. Plasmin degrades fibrin which exposes additional carboxy-terminal lysines for interaction with the kringles on tPA, plasminogen and plasmin, ultimately resulting in accelerated fibrin degradation. In a regulatory process, carboxypeptidase U, also known as thrombin-activatable fibrinolysis inhibitor (TAFIa), removes carboxy-terminal lysine residues and stabilizes fibrin thrombi. TAFI proved to be a poor substrate for thrombin in the absence of thrombomodulin, and a riveting chronicle of the lengthy process of its discovery, purification and characterization again demonstrates the gratifying aspect of understanding the molecular basis of a biochemical process [19]. The serpins plasminogen activator inhibitor-1 (PAI-1) and α2-

antiplasmin (α2-AP), respectively targeting tPA and plasmin, provide additional regulation of fibrin degradation. Plasmin bound to fibrin is protected from α2-AP, whereas TAFI decreases this protection by eliminating plasmin binding lysine residues on fibrin.

Platelets can both promote and counteract fibrinolysis. Activated platelets localize plasminogen and its physiological activators tPA and urokinase via the GPIIb/IIIa (integrin αIIbβ3) complex. Thrombospondin, released from platelet granules and exposed on the platelet surface, also binds plasminogen and enhances its activation. Hence activated platelets provide an alternative surface for promoting fibrinolysis. As part of a regulatory mechanism, platelets secrete two anti-fibrinolytic serpins, PAI-1 and α2-AP, and as a result platelet-rich thrombi resist fibrinolysis.

Thrombin may act both as a procoagulant and as an anticoagulant enzyme, and has been called a “Janus-headed” protease [20]. Crystal structures of α-thrombin bound to numerous effectors have aided in identifying extended recognition sites on its surface. By combining these discrete functional surface regions thrombin interacts with various substrates and ligands in a selective and specific manner. Binding of Na^+ causes thrombin to adopt a ‘fast’ conformation which rapidly cleaves procoagulant substrates. In the Na^+-free state, thrombin bound to thrombomodulin preferentially initiates the protein C anticoagulant pathway in which factors V and VIII are proteolytically inactivated [21,22].

The Complement System and Immune Regulation

The plasma complement system regulates the innate immune defense by opsonization and elimination of pathogens, cell debris and host cells that have undergone alterations [23–25]. Activation of the complement system occurs via three pathways: in the classical pathway, the recognition protein C1q binds to antigen-antibody complexes, the C1 complex is activated, and a series of serine protease activation reactions leads to formation of C3- and ultimately C5-convertase; in the lectin pathway, mannose-binding lectin binds to mannose on the surface of pathogens as a trigger to formation of C3/C5-convertase; in the alternative pathway, continuous low level activation of C3 and binding to the pathogen leads to C3/C5-convertase. C3-convertases cleave C3 into the anaphylatoxin C3a and the opsonin C3b which is deposited on the pathogen surface and facilitates targeting by macrophages. C5-convertases generate the pro-inflammatory anaphylatoxins C5a and C5b, and a membrane attack complex (MAC) is formed by C5b–C9 assembly. This complex forms a pore in the membrane that kills the pathogen or the targeted cell. Anaphylatoxins C3a and C5a promote chemotaxis of immune cells. The complement serine proteases involved in these complicated interrelated processes include C1r, C1s, MASPs 1–3 [26], C2 and Factors B, D and I, all with restricted specificity. The plasma serpin, C1-inhibitor, covalently inactivates C1r, C1s, and MASPs 1 and 2. C2 and Factor B activity is controlled by the Regulation of Complement Activation (or RCA) proteins, and no endogenous inhibitors are known for Factors I and D. Complement deficiency causes increased susceptibility to infection, and clearance impairment of immune complexes and apoptotic cells results in the development of systemic lupus erythematosus (SLE). However, excessive complement activation is also associated with autoimmune diseases such as SLE, rheumatoid arthritis and certain cancers. The monoclonal antibody Eculizumab inhibits C5, and was recently approved for the

treatment of complement hyperactivation in paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome. It may ultimately prove useful in the treatment of SLE as well [27].

Human cytotoxic T lymphocytes and natural killer cells secrete five types of granzymes (A, B, H, K, M), serine proteases that aid in the neutralization of virus-infected and tumor cells. Only granzyme B and M have known intracellular inhibitors, serpinB9 (PI-9) and serpinB4 (SCCA2), respectively [28]. Increased PI-9 expression may be an immune evasion mechanism used by lung cancer cells for protection from granzyme B-mediated cytotoxicity [29].

Immune regulation is tightly associated with proteolytic processes in the gut. In immune diseases of the gut, cytokines upregulate protease activity, resulting in inflammation and exacerbated immune response [30]. In inflammatory bowel disease, MMPs, neutrophil elastase and cathepsins are typically overexpressed in the gut epithelium and basement membrane. The nature of the gut microbiome is equally important for maintaining immune homeostasis, and commensal and pathogenic bacteria produce a wide range of proteases that differentially affect the integrity of the intestinal mucosa.

The serpin α1-antitrypsin (now renamed α1-proteinase inhibitor or α1-PI), produced in the liver, protects the lungs from inflammatory neutrophil elastase damage, and it is also an acute-phase protein that reduces pro-inflammatory cytokine production, inhibits apoptosis, blocks leukocyte degranulation and migration, and modulates local and systemic inflammatory responses [31]. In monocytes, α1-antitrypsin increases intracellular cAMP, regulates CD14 expression, and suppresses NF-κB nuclear translocation. These functions may be related to the inhibitory activity of antitrypsin, protein-protein interactions, or both. Preclinical use of antitrypsin in autoimmunity and transplantation models showed that it is capable of preventing or reversing autoimmune disease and graft loss.

Proteolytic Processing

The lysosome and the ubiquitin-proteasome are the two major intracellular proteolytic systems keeping the protein pool in balance. Originally considered strictly degradative, these systems have revealed regulatory functions beyond catabolism, and their molecular defects are associated with various disease states. Lysosomes contain cathepsins B, D and L in addition to lipases, nucleases, glycosidases, phospholipases, phosphatases, and sulfatases that are active in an acidic milieu [32]. Lysosomes regulate autophagy during nutrient starvation, and participate in development and differentiation, induction of cathepsin-dependent cell death and degradation of apoptotic cells. Cancer cell lysosomes have a higher membrane permeability (“leaky”) and express more cathepsin than those of normal cells, and this property may be exploited in cancer treatment. Agents such as terahydrocannabinol and chloroquine may disrupt the lysosome and trigger killing of the cancer cells.

The ubiquitin-proteasome, an intracellular high molecular weight protease complex located in the nucleus and cytosol, selectively degrades proteins tagged with ubiquitin at lysine residues [33]. Its “central pore” contains several inward facing protease active sites, with caspase-, trypsin- and chymotrypsin-like specificity. This multi-protein construct is capped

by one or two activator complexes that conformationally regulate access of protein substrates to the pore. Whereas the physiological function of the proteasome was originally thought to be restricted to intracellular protein catabolism, new functions have been discovered with respect to regulation of the cell cycle progression, gene expression and responses to cellular stress [34]. Protein ubiquitination is reversible, and more than 100 potentially regulatory deubiquitinase (DUB) genes have been identified, mainly cysteine and metalloproteinases (MMPs). DUBs rescue proteins from degradation and reverse ubiquitination-induced signaling. The immunoproteasome, containing specific subunits with increased chymotrypsin- and trypsin-like activities, and decreased caspase-like activity, participates in production of peptide epitopes for cytotoxic T lymphocytes. In the thymoproteasome, chymotryptic activity is attenuated but the caspase- and trypsin-like activities are conserved. Its peptide products are MHC class I ligands with moderate avidity, which supports positive selection of CD8+ T cells [35]. Foreign peptides, generated during the breakdown of virus and cancer cell proteins, bind MHC class I molecules on the cell surface, and the cells are recognized by cytotoxic T cells as potentially dangerous and are destroyed. The proteasome discards misfolded proteins, and proteasome defects may contribute to the pathogenesis of neurodegenerative diseases such as Parkinson's, Huntington's, Alzheimer's, and ALS. A decline in proteasome activity is also a hallmark of aging cells.

The gastrointestinal tract contains the highest concentrations of endogenous and exogenous proteases. The intestinal mucosa is constantly exposed to low level protease activity, from bacteria in the lumen, immune and mesenchymal cells in the basement membrane, and epithelial cells at the brush border membrane. Protease activity is tightly controlled, as the mucosal barrier is thin and susceptible to proteolysis. The intestinal epithelium is at the interface of digestive, absorptive and secretory functions, and signaling processes to the mucosal immune, vascular and nervous systems. Endogenous growth factors, cytokines and extracellular matrix (ECM) proteins that modulate these functions, are digestive protease substrates [36]. The biochemistry of digestive protein processing has been studied for more than 80 years since the first crystallographic studies were published, and the functions of pancreatic trypsin, chymotrypsin and elastase and their inhibitors are well known. Pancreatic trypsinogen is activated to trypsin by membrane-bound enterokinase in the small intestine. Trypsin activates pancreatic chymotrypsinogen, procarboxypeptidases, proelastases, and prolipases. Turnover of intestinal epithelium is rapid, requiring tight control of gut protease activity under normal physiological conditions. Pancreatic PRSS3/mesotrypsin, discovered in the late 1970s, is an atypical trypsin with an evolutionary mutation that renders the protease resistant to inactivation by the physiological Kazal inhibitor, pancreatic secretory trypsin inhibitor (SPINK1), and endows it with specific digestive trypsin inhibitor-degrading properties [37]. In pancreatitis, trypsin is activated in the pancreas, causing tissue destruction and inflammation. Mesotrypsin is upregulated in certain cancers, and SPINK1 deficiency is associated with hereditary pancreatitis.

Tissue Remodeling, Signaling, Cell Migration and Proliferation

Zinc proteases feature prominently in these biological processes. They can be subdivided according to the structure of their catalytic sites and their domain organization [38]. The

human ADAM family (a disintegrin and metalloprotease) currently counts 13 proteolytically active transmembrane and secreted members. ADAMs are largely tissue-specific and play roles in fertilization, proliferation, migration and cell adhesion. Transmembrane ADAMs act as sheddases, *i.e.* proteases that cause extracellular shedding of adjacent transmembrane proteins by proteolytic cleavage at the membrane. Examples of such activated proteins are TNF-α and the ErbB family of receptor tyrosine kinases; and EGF receptor ligands such as TGF-α, heparin-binding EGF-like growth factor, betacellulin, epiregulin and amphiregulin. ADAM-mediated shedding is often followed by RIPping, or **R**egulated **I**ntramembrane **P**roteolysis, in which the intracellular portion of these transmembrane proteins is cleaved off by aspartyl proteases, S2P-metalloproteases and rhomboid serine proteases. The released intracellular domain participates in signaling to the nucleus to modify gene expression. Processing of amyloid precursor protein (APP) and Notch signaling are typical examples of RIPping. The 19 known human ADAM-TS proteases have a similar architecture to the ADAMs, except for the presence of thrombospondin repeats instead of a transmembrane domain, which makes them extracellular. They process procollagen and von Willebrand factor, and cleave extracellular matrix aggrecan, versican, brevican and neurocan. Matrix metalloproteinases (MMPs) typically have three common domains: the N-terminal propeptide that keeps the protease in an inactive form, the catalytic domain containing the Zn^{2+} ion, and a C-terminal hemopexin-like β-propeller domain for protein-protein interactions. MMPs are not only instrumental in matrix remodeling and tissue maintenance, but also as regulators of signaling pathways [39].

MMPs, originally thought to degrade extracellular matrix proteins in a rather indiscriminate fashion, were later shown to have specific physiological roles in shedding, activation and inactivation of proteins such as growth factors and cytokines. They cleave their substrates using a HExxHxxGxxH motif which contains three zinc-binding histidines and a glutamate that acts as a general base/acid during catalysis. To date, there are 23 known human MMPs, organized in four classes according to their substrate specificity: collagenases (MMP-1, −8, and −13), gelatinases (MMP-2 and −9), stromelysins (MMP-3, −10, and −11) and a group containing matrilysin (MMP-7), metallo-elastase (MMP-12), enamelysin (MMP-20), matrilysin-2 (MMP-26), and epilysin (MMP-28). Pro-MMPs are activated by proteolytic removal of the N-terminal pro-domain that keeps the zymogen inactive by using a cysteine switch to bind the catalytic zinc ion. MMPs participate in multiple processes that involve tissue remodeling, *e.g.* embryo implantation, wound healing, cell proliferation, bone ossification and blood vessel remodeling; signaling by all 54 human chemokines; and innate immune defense [39]. MMP activity is regulated by endogenous, tight-binding tissue inhibitors of metalloproteinases (TIMPs). Abnormal MMP expression and activity have been observed in cancer, cardiac remodeling and aneurysm formation, impaired wound healing, neurodegeneration, and after UV radiation exposure of the aging skin and the cornea [40]. The family of endogenous tissue-inhibitors of metalloproteinases (TIMPs) consists of 4 proteins that target protease activity of MMPs, ADAMs and ADAM-TSs. They also affect cell growth and differentiation, cell migration, anti-angiogenesis, anti- and pro-apoptosis, and synaptic plasticity in biological processes different from protease inhibition [41].

Protease signaling is a relatively new concept, and in contrast with other types like receptor or kinase signaling the process is irreversible [8]. The major immediate results of protease signaling are target protein activation or inactivation, exposure of cryptic sites, shedding of transmembrane proteins, and receptor agonist/antagonist interconversion. These processes may initiate downstream signaling, resulting in a wide variety of physiological or pathological responses. Selection of a physiological substrate is facilitated by protease – substrate colocalization; substrate specificity, as determined by the complementarity of the protease active site to the reaction transition state; interactions immediately distal from the substrate-binding pocket; and interactions with protease exosites remote from the active site.

Protease-activated receptors (PARs) are prototypical examples of protease signaling. These four G protein–coupled receptors are activated irreversibly by extracellular proteases, by cleavage of the N-terminal ectodomain and exposure of a tethered peptide ligand. Transmembrane signaling is initiated by binding of this tethered peptide to the body of the receptor [42]. PAR1, PAR3 and PAR4 are activated by thrombin, and signaling occurs during tissue injury, hemostasis and inflammation. Signaling is regulated by rapid internalization of spent receptors. PAR1 and PAR4 cleavage on platelets causes robust platelet activation. Thrombin has a higher affinity for PAR1, and the PAR1 antagonist vorapaxar was approved in 2014 as antiplatelet drug. However, major bleeding side effects prompted the development of PAR4 antagonists which are currently in clinical testing. PAR4 signaling promotes vascular disease and cardiac post-infarction remodeling, and these antagonists are promising candidates for safer antithrombotic and anti-inflammatory therapy [43]. PAR1 on endothelial cells is productively cleaved by activated protein C (APC) in the presence of the endothelial protein C receptor. This triggers expression of monocyte chemo-attractant protein-1, acting as a protective component during sepsis. PAR2 is activated by trypsin, tryptase, the coagulation factors VIIa and Xa, and matriptase. PAR2 signaling is thought to regulate epithelial growth and function. Thrombin-mediated PAR activation has been implicated in vascular smooth muscle cell migration and proliferation as causative processes in restenosis after stent placement [44], and in tumor metastasis, where a simultaneous requirement for PARs and fibrinogen was found [45]. Thrombosis and cancer have since long been recognized as interconnected pathologies, and in this light argatroban, the tight-binding competitive inhibitor of active thrombin, has been re-evaluated as a clinically useful antiproliferative and antimetastatic agent [46].

Programmed cell death

For obvious reasons, the process of apoptosis must be both highly regulated and a model of organized efficiency once initiated. It is governed by a cascade of caspases which, though they sometimes have other functions, thoroughly dismantle the innards of a cell so that it may be phagocytosed by immune cells without releasing cytosolic components into the extracellular space. Caspases are cysteine aspartate proteases involved in cell death, cellular remodeling, stem cell fate determination, spermatogenesis, and red blood cell differentiation. Their sets of substrates with regard to apoptosis are well-defined, and cooperative cleavage of these substrate sets triggers apoptosis. With regard to apoptosis, their functions fall into one of two categories: Initiator (caspases 2, 8, 9, and 10) or Executioner (caspases 3, 6, and 7). Caspases 1, 4, 5 and 12L are considered inflammatory [47]. Activation of the Initiator

caspases can be induced intrinsically, by release of cyctochrome c into the cytosol by mitochondria, or extrinsically by ligand binding to Death Receptors. When cyctochrome c is released, it binds to adaptor protein APAF-1, inducing it to form the apoptosome oligomer, which then binds to the caspase activation domain (CARD) of pro-caspase 9, inducing oligomerization of the procaspase. This induces autoproteolysis of caspase 9 to activate it [48,49]. This process can be suppressed by the presence of the β transcription variant of caspase 9 which lacks a catalytic domain [50,51]. Caspase 9 then activates procaspases 3 and 7 by cleaving them at a L-G-H-D-(cut)-X sequence [52,53]. Caspase 9 can be down-regulated by phosphorylation, or inhibited by proteins in the Inhibitor of Apoptosis (IAP) family [54]. Activated Caspase 3 is known to inhibit the function of IAPs [55], ensuring that once the cascade is initiated it progresses rapidly, and to activate caspases 6, 7, and 9, which further accelerates the process. Extrinsic activation is initiated by binding of Death Factors, such as FasL, to Death Receptors, such as FasR. The conformation change in the receptor's cytoplasmic Death Domain (DD) induces a change in the bound adaptor protein FADD which recruits pro-caspase 8 to bind at its Death Effector Domain (DED, proving gallows humor is irresistible even at the cellular level), activating caspase 8 [56]. Caspase 8 activates caspases 3, 4, 6, 7, 9, and 10. Caspase 10 then activates Caspases 3, 4, 6, 7, 8, and 9. Caspase 6 has a limited capacity to autoproteolyze and activate itself [57], and is known to target both Huntington and Amyloid Precursor Protein (APP), linking it to neurodegenerative diseases [58]. In apoptosis it is responsible for dis-inhibition of the immune system by cleaving interleukin-10 and interleukin-1 receptor-associated kinase 3 (IRAK3) [59]. The Executioner Caspases, as a group, are responsible for cleavage of over 600 other proteins [60]. Caspase 3 can also be activated by Granzyme B, allowing T lymphocytes and natural killer cells to initiate apoptosis in target cells. The substrate landscape in non-apoptotic events may be much broader [61], as suggested by recent global proteomics studies. The "forward" approach involves triggering endogenous caspases to identify native substrates in intact cells, whereas in the "reverse" approach exogenous caspases are added to cell lysates. Isolated cleavage products are digested and identified by tandem mass spectrometry. The forward method allows identification of substrates in intact cells, rather than which caspase performs the cleavage. In the reverse method, specific caspases are tested for their ability of cleaving substrates, but in a cell lysate with destroyed organelles, endogenous proteases may contribute to substrate cleavage, requiring the need for strict controls. Moreover, proteolysis in organelle membranes may be missed due to removal of insoluble material before analysis. A combination of current methods has yielded several hundred potential native substrates for caspases, and measuring rates and extent of substrate cleavage allows distinguishing functional from bystander targets [62]. Eight human endogenous inhibitors of apoptosis (IAPs) have been identified, and their inhibitory activity is neutralized by the mitochondrial protein Smac/DIABLO. Development of Smac/DIABLO-like peptidomimetics has been proposed as a potential therapeutic approach in cancer treatment. Because of the roles of caspases in inflammation, caspase inhibitors may also prove beneficial in treating sepsis [63]. In addition to caspases, other proteases are also associated with apoptosis, *e.g.* calpains, cathepsins, granzymes and the proteasome. These are regulated by their respective endogenous inhibitors: calpastatins, cystatins, the serpin PI-9, and various macromolecular proteins.

Protein Secretion

Many current cardiovascular biomarkers are secreted proteins, generated by cleavage of their pro-proteins at the endoplasmatic reticulum (ER). Upon release of the mature protein, the signal peptide is proteolytically separated from the ER by signal peptidase, an intramembrane aspartic protease. The long-held belief that signal peptides are invariably recycled or degraded by ubiquitin-proteasome related factors has been challenged during recent years, and several were shown to remain intact after cleavage in the ER [64]. These signal peptides have biological functions of their own, and play roles in regulation of immunity, trafficking and other processes. In type I diabetes, a signal peptide fragment from pre-proinsulin, presented at the surface of the pancreatic β-cells, acts as antigen and flags the cell for destruction by cytotoxic T cells. Identifying agents to control β-cell destruction may be a new therapeutic strategy. Similarly, a signal peptide fragment from pre-procalcitonin, highly abundant in several lung cancers and medullary thyroid cancers, is an epitope for T-killer cells. This knowledge may aid in the development of treatments of these cancers. Rapidly increasing plasma levels of N-terminal adducted signal peptide fragments from A-, B- and C-type natriuretic peptides are characteristic of ST-elevated myocardial infarction. The nature of the N-terminal adducts may be useful in assay design and disease assessment, and development of fast biomarker assays for these signal peptide fragments may ultimately be beneficial in clinical decision making.

DNA replication, repair and processing

DNA damage hinders replication, and may lead to strand breaks, genomic instability, aging and cancer [65]. DNA-topoisomerase 1 crosslinks (DPC) are bulky lesions that trap otherwise transient covalent DNA-protein intermediates, and inhibit movement of polymerases and helicase, causing stalling of the replication fork. In yeast models, the protease Wss1 was identified as effector of DPC repair. BLAST searches revealed a conserved family of DPC proteases, with Spartan being the human member of this class [66]. Spartan was recently characterized as a DNA replication-coupled metalloprotease for DPC repair and restoration of genomic stability [67,68]. Mutations have been associated with premature aging and early onset hepatocellular carcinoma, suggesting Spartan as a tumor suppressor, and DPC repair as a protective anti-tumor mechanism. Double strand DNA breaks are repaired by the DNA damage response, in pathways that are tightly controlled by ubiquitinylation and deubiquitinylation events. The latter are catalyzed by deubiquitinases (DUBs) that can positively or negatively affect the damage response. The proteasomal de-ubiquitinating enzyme POH1 promotes double-strand DNA break repair [69]. Various DUBs are also associated with transcriptional and epigenetic control of gene expression, DNA damage repair pathways and cell cycle checkpoint control, often deregulated in tumor cells [70]. These DUBs may be potential targets for therapeutic inhibition, and they are currently the subject of small molecule screening. DNA processing is also under indirect control of proteases. DNA fragmentation and chromatin condensation are final processes in apoptosis. The DNase CAD, catalyzing these reactions, is normally under tight control of its ligand ICAD, that acts as a chaperone and inhibitor when bound to CAD. This prevents spontaneous activation of CAD in non-apoptotic cells. ICAD cleavage by caspase-3 during the apoptotic execution phase liberates active CAD that enters the nucleus to fragment DNA and catalyze chromatin condensation [71]. Human Lon protease

binds to mitochondrial single-stranded DNA sequences with a propensity for forming G-quadruplexes [72]. The precise role of human Lon protease is not yet clear, but it has been suggested that DNA-bound Lon may process proteins involved in mitochondrial DNA and RNA metabolism.

Intranuclear, Cytosolic, Transmembrane and Intramembrane Proteolysis

In addition to Spartan and the proteasome involved in DNA processing, other proteases are known to have intranuclear functions. Interleukin-1β-converting enzyme (ICE), also known as caspase-1, plays a role in the inflammatory immune response. The N-terminal prodomain of its precursor, pro-caspase-1, possesses a nuclear localization signal. Tumor necrosis factor induces translocation of pro-caspase-1 from the cytosol to the nucleus where it is activated by proteolytic removal of the intact prodomain [73]. Cell transfection studies showed that the prodomain alone is capable of triggering apoptosis, which suggests separate nuclear roles for the prodomain and active caspase-1. Caspase-3 also effects nuclear changes in apoptotic cells. Its inactive precursor is cleaved in the cytoplasm by initiator caspases in response to death signals, and also by cytosolic MMP-2 and −9. The active caspase-3 dimerizes and translocates to the nucleus by an active transport system. Caspase-7 is only found in the cytoplasm, suggesting that translocation is caspase-3 specific and not the result of simple diffusion after the nuclear-cytoplasmic barrier is disrupted [74]. The extracellular properties of MMPs as tissue remodeling proteases are well documented but less is known about their intracellular functions. Various MMPs have been detected in the intranuclear space, and they are mostly associated with pathological processes [75,76]. Oxygen-glucose deprivation in ischemic stroke induces an intrinsic, caspase-independent apoptotic pathway in neurons, characterized by elevated intranuclear MMP-2 and −9 activity that targets nuclear DNA repair proteins. Similarly, MMP-2 in the nuclei of stressed cardiac myocytes can induce apoptosis. Intranuclear MMP-3 in osteoarthritis and certain cancers upregulates connective tissue growth factor, a mediator of cell migration, proliferation, and pathological fibrosis. During viral infections, macrophage-secreted MMP-12 translocates to the nucleus of infected cells, and enhances transcription of IκBα, a protein that promotes antiviral interferon-α (IFN-α) secretion. Extracellular MMP-12 degrades excess secreted IFN-α, thus limiting its systemic toxicity. It is becoming increasingly clear that the same protease may exert significantly divergent functions, depending on its micro-environment, substrates and effectors. Understanding these different mechanisms of interaction on a molecular level is ultimately the key to successful design of therapeutics.

Promotion of angiogenesis is regulated by binding of single chain urokinase-type plasminogen activator (scuPA) to its receptor on the endothelial cell surface, and subsequent transport of the protease to the nucleus [77]. scuPA de-represses transcription of the VEGF receptor 1 (VEGFR1) and 2 (VEGFR2) genes by interfering with the proline-rich homeodomain protein that represses the activity of vegfr1 and vegfr2 gene promoters. The VEGF growth factors are known targets for control of pathologic angiogenesis in macular degeneration, and the discovery of the scuPA-mediated pathway may offer additional avenues for therapeutic intervention.

Transmembrane proteases may be anchored to the membrane by a C-terminal domain (Type I), an N-terminal domain with cytoplasmic extension (Type II), or by glycosylphosphatidylinositol (GPI) [78]. Their catalytic domains are extracellular. Among the zinc-dependent proteases are MMP-14, 15, −16, −24, ADAM-10 and −17, meprins α and β (Type I), MMP-23 (Type II) and MMP-17 and −25 (GPI). The Type I zinc proteases act as sheddases upon proteolytic removal of their N-terminal propeptide. The majority of transmembrane serine proteases are classified as Type II subfamilies: hepsin/TMPRSS (transmembrane protease/serine), matriptase, corin, and HAT/DESC (human airway trypsin-like protease/differentially expressed in squamous cell carcinoma). Corin in cardiomyocytes activates the atrial natriuretic factor (ANF), a cardiac hormone that regulates blood pressure and cardiac function by promoting natriuresis, diuresis, and vasodilation. Tryptase γ1 is the only known Type I serine protease, and prostasin and testisin are GPI-anchored. Prostasin plays a role in epithelial sodium channel regulation, and testisin regulates germ cell maturation. All these proteases are involved in physiological development, but also in pathological processes of inflammation and cancer. They activate peptide hormones, growth and differentiation factors, receptors, enzymes, adhesion molecules, and viral coat proteins. Matriptases 1 and 2, and prostasin are expressed in human epithelium, and inhibited by their cognate Kunitz-type inhibitors, membrane-anchored hepatocyte growth factor activator inhibitors (HAI) 1 and 2 [79]. Matriptase overexpression elicits signaling via the PAR-2, and promotes fibroblast activation, proliferation, and migration in idiopathic pulmonary fibrosis [80]. Matriptase proteolytically activates hepatocyte growth factor (HGF) that binds to its receptor c-Met, a receptor tyrosine kinase. This activates critical signaling pathways in organ development. Abnormal c-Met signaling is associated with cell proliferation, migration and invasion, and progression of lung, breast, ovary, kidney, colon, thyroid, liver, and gastric carcinomas. There is also some matriptase crosstalk with the hemostatic system with regard to epithelial defense and repair after injury and infection. Exposure of membrane-anchored tissue factor (TF) in damaged vascular endothelium to factor VIIa triggers the extrinsic coagulation pathway and formation of factor Xa. The TF:fVIIa complex and factor Xa activate epithelial pro-matriptase to matriptase which cleaves the PAR2 receptor. This induces enhancement of the epithelial barrier function [81].

Intramembrane proteases (IMPs), a fairly recently discovered class, are embedded in lipid bilayers and their catalytic site is formed by residues in different transmembrane helices [82]. The four IMP families are metallo-, serine, aspartate and glutamate proteases, and they are found in the Golgi apparatus, endosomes and lysosomes, the plasma membrane, endoplasmatic reticulum, and the inner mitochondrial membrane. IMPs cleave their substrates with a fairly high specificity, given the fact that of the ~2,500 identified single-pass transmembrane proteins only a limited number are identified as IMP substrates. The function of IMPs is diverse, ranging from transcription factor signaling, mitochondrial remodeling and protein maturation to regulation of immunity, and quorum sensing and parasite-host interactions in pathogens. Many IMP defects are associated with pathogenesis.

Site-2 protease (S2P) is the only member of the metalloproteinase IMP group, and mutations cause ichthyosis follicularis and osteogenesis imperfecta. The HIV inhibitor Nelfinavir was found to inhibit S2P in castration-resistant prostate cancer cell lines, however due to the multitude of other Nelfinavir targets and the less than impressive effects on PC-3 cancer

cells this drug may not be specific enough for specific use in prostate cancer. Five human rhomboid serine IMPs are known, but no function or substrates have been identified for rhomboid 1 and 3. Epidermal growth factor and thrombomodulin are rhomboid 2 substrates, and the protease is thought to control cell migration and proliferation [83]. Lowered expression impairs wound healing, and overexpression may be linked to tumor metastasis. Rhomboid 4 upregulation is associated with poor outcome in colorectal cancer, but there is no clear consensus on the molecular mechanism of this process. Rhomboid 4 cleaves amyloid precursor protein (APP) within its ectodomain and reduces formation of Aβ38, 40 and 42 peptides. This pathway may be an alternative to the pathological processing of APP by γ-secretase in Alzheimer's disease. The role of the mitochondrial rhomboid protease PARL in Parkinson's disease is controversial: some studies attribute a protective function to PARL in inducing removal of defective mitochondria through autophagy, whereas others suggest that PARL knockdown is responsible for this process. A potential link of low level PARL activity with type 2 diabetes was first seen in obese sand rats with diet-induced diabetes. Normal PARL levels and insulin sensitivity were restored when the rats were put on an exercise regimen. Similarly, in humans with type 2 diabetes, PARL mRNA and mitochondrial DNA are reduced in skeletal muscle. PARL was recently identified as a pro-apoptotic protease because it cleaves mitochondrial Smac/DIABLO [84]. The processed protein is released into the cytosol and binds an apoptosis inhibitor, thereby triggering the caspase cascade. The most studied member of the aspartate IMPs is the γ-secretase complex, with presenilin as the intramembrane catalytic subunit. Presenilin and APP mutations are linked to familial Alzheimer's disease (AD), and γ-secretase was considered as an anti-AD drug target. However, severe side effects limit the use of γ-secretase inhibitors. Processing of Notch by γ-secretase releases the intracellular Notch domain for intranuclear modulation of gene expression. Alterations of this pathway are linked to several types of cancer [85], and γ-secretase inhibitors are now in clinical trials as potential anti-cancer drugs. The multifunctionality of these proteases illustrates the caveats in developing drugs that indiscriminately target protease activity without taking specific molecular mechanisms and the protease micro-environment into account.

PROTEASES AND DISEASE

Epigenetics and Disease

Many inheritable diseases are directly related to DNA modification, however epigenetic processes are equally prominent in the disease state, and they are essential contributors to normal physiological development. Environmental factors, diet, aging and diseases such as cancer may contribute to positive or negative changes in gene expression that are passed onto daughter cells: DNA (hydroxyl)methylation, covalent histone modification and chromatin remodeling, arrangement of the chromatin-histone nucleosomes along the DNA sequence, gene-activating transcription factor activity of new gene products, and downregulation of messenger RNA by non-coding microRNA. Formation of prion structures in transmissible spongiform encephalopathies is also considered an epigenetic phenomenon.

Proteases play regulatory roles in epigenetic mechanisms of altered gene expression. Various non-specific and N-tail specific histone proteases are thought to assist in fertilization,

histone turnover, gene de-repression, histone removal during spermatogenesis, and reversal of N-tail methylation [86]. In N-tail clipped histone H3, lysines for acetylation have been removed, which may result in transcriptionally inactive chromatin. This process may be in part responsible for age-related declining gene expression. Cathepsin L, susceptible to stefin B inhibition, was identified as the histone H3-cleaving protease during stem cell differentiation in mice, however the corresponding H3-clipping protease in human embryonic stem cells is refractory to specific cathepsin L inhibitors and remains to be identified [87].

Inflammation as an Over-Arching Symptom of Disease

A comprehensive, recent overview of protease activity in inflammation is given by Deraison *et al*. [88]. The host inflammatory response is accompanied by release of proteases from neutrophil granulocytes, macrophages and mast cells. These proteases form a first line of defense in bacterial infections but, if left uncontrolled, are also damaging to host tissues. Human neutrophil elastase, present in six isoforms, and the related cathepsin G and proteinase-3 are serine proteases localizing to neutrophil extracellular traps (NETs) in a defense mechanism against bacterial invasion. They cleave collagen-IV and elastin, and excessive secretion may cause idiopathic pulmonary fibrosis, rheumatoid arthritis and adult respiratory distress syndrome. Alpha1-proteinase inhibitor (α1-PI) and leukocyte elastase inhibitor are their major endogenous irreversible serine proteinase inhibitors (serpins). Historically α1-PI was identified as a trypsin inhibitor and later found to be more specific for elastase inactivation. Smoking-induced oxidative inactivation or mutation-induced misfolding of α1-PI causes emphysema and cystic fibrosis, and may be treated with α1-PI augmentation therapy [89], as gene therapy for α1-PI deficiency is still in the Clinical Phase II stage. Increased risk for lung cancer has also been observed in α1-PI-deficient patients who never smoked [90]. Proteinase-3 generates antimicrobial peptides by cleaving cathelicidin in neutrophils, but it is also predominant in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, a severe multisystem autoimmune disease with poor prognosis [91].

Tryptase (6 isoforms), chymase, granzyme B and carboxypeptidase A, released by mast cells, degrade extracellular matrix components. An imbalance between tryptase and its endogenous inhibitors is characteristic in rheumatoid arthritis [92]. Experimental tryptase inhibition in an *in vivo* model alleviated some, but not all symptoms, suggesting the necessity for a multi-drug approach. Cell surface proteoglycan-bound chymase is partially protected from endogenous inactivation by α1-PI and α1-antichymotrypsin, and inhibitory caging by α2-macroglobulin. Mast cell infiltration of atherosclerotic plaque aggravates the local inflammatory status by causing smooth muscle cell (SMC) apoptosis in a chymase-dependent process: fibronectin cleavage by chymase unmasks pro-apoptotic epitopes and disrupts the p-FAK–dependent cell-survival signaling cascade, leading to SMC cell death [93]. Mast cell tryptase and chymase activity is also associated with several other pathological processes in atherosclerosis, abdominal aortic aneurysm (AAA) formation and metabolic disease, as discussed below [94].

Macrophages release matrix metalloproteinases (MMPs), cysteine proteinases (caspases and cathepsin L), and cathepsin D, an aspartyl protease also present in lysosomes. Their proteolytic power is a part of the diverse arsenal of mechanisms macrophages utilize in vessel wall-localized pro-inflammatory processes associated with the development of vasculitis, a fast-developing pathology, and atherosclerosis, a disease developing over decades [95].

Cathepsin B, a lysosomal cysteine proteinase, preferentially activates mesotrypsinogen in pancreatic acinar cells. Increased mesotrypsin (trypsin-3) activity as a result from overexpressed cathepsin B lowers protective SPINK1 levels and initiates apoptosis by activating caspase-3 [96]. Both processes contribute to the development of human pancreatitis. Crystallization studies identified diminazene analogues as small molecule inhibitors of mesotrypsin, and these structures may form the basis for developing selective, tight-binding drugs [97]. In irritable bowel syndrome (IBS) the intestinal epithelium overproduces mesotrypsin, which increases intestinal epithelium permeability, signals to human submucosal enteric neurons and induces visceral hypersensitivity by a protease-activated receptor-2-dependent mechanism [98]. Mesotrypsin may be a suitable biomarker for IBS as well as a target for novel, specific drugs.

Inflammation and complement activation are interconnected, and the inflammatory environment in degenerative diseases, cancer, transplant rejection and exposure to chronic external stimuli is characterized by excessive activation or insufficient control of complement activation [99]. The physiological response of complement involves self-recognition of normal cells; immune recognition and clearance of diseased cells, apoptotic cell debris and immune complexes; elimination and danger signaling of pathogens; and tolerance to transplants and biomaterials. Excessive complement activation triggers inflammatory reactions seen in danger signaling and attack of “self” cells resulting in autoimmune disease, pathogen infection, and tissue/biomaterial rejection. The proteases and other components of the complement system may be attractive candidates for novel anti-inflammatory drug intervention, however the complexity and extensive cross-talk of this network poses significant challenges in the development of specific inhibitors devoid of off-target side effects.

Cardiovascular and Metabolic Diseases, and Stroke

Due to the interconnectedness of cardiovascular and metabolic disease, a new term, cardiometabolic disease has been coined, and recent studies underscore the critical roles of MMPs, calpains, cathepsins and caspases in disease progression [100]. Physiological hemostasis is a delicate balance between cascading serine protease zymogen activation, positive and negative feedback mechanisms, protease inhibition and thrombus dissolution. Many clotting and bleeding disorders are the result of gene defects affecting the expression or function of protease zymogens, or the substrates, cofactors and inhibitors of the active proteases. In the extrinsic pathway, deficiency of factors VII, IX (hemophilia B) and X causes bleeding. The G20210A mutation in the 3’ untranslated region of the prothrombin gene stabilizes the precursor mRNA which results in increased prothrombin levels and venous thrombosis risk. Up to 8% of the Caucasian population is heterozygous for this

mutation. In the contact activation pathway, deficiency of factor XI causes mild to moderate bleeding, whereas factor XII and prekallikrein deficiencies are generally asymptomatic. This is consistent with a fairly recently discovered role for thrombin, generated through the extrinsic pathway, as a factor XI activator in the absence of factor XII [16]. Pathogen infections may also trigger hypercoagulability, as seen in coagulase-positive *Staphylococcus aureus* infections. Staphylocoagulase is not a protease but activates host prothrombin conformationally, resulting in deposition of fibrin/bacterial vegetations that can embolize to the lungs, brain and other parts of the body [101].

The proteases of the contact pathway play a role in inflammatory and immune processes as well as in maintaining hemostasis [102]. Kallikrein cleaves high molecular weight kininogen to the pro-inflammatory bradykinin. This nonapeptide is a vasodilator, increases vascular permeability and contributes to inflammatory pain by binding to bradykinin receptors. Elevated bradykinin levels are seen in rheumatoid arthritis and IBS. The serpin, C1-inhibitor is the main physiological inhibitor of factor XIIa and kallikrein. An overactive contact pathway can be the result of decreased or dysfunctional C1-inhibitor, or a factor XII mutation leading to a more active fXIIa form. The pathological manifestation is hereditary angioedema, with sometimes life-threatening swelling in the upper respiratory tract or the intestinal mucosa. Abnormal activation of factor XII by β-amyloid triggers inflammation in Alzheimer's patients [103]. These findings suggest that drug targeting of the contact pathway proteases and regulators may prove beneficial in a variety of pathologies.

In anticoagulant protein C deficiency, insufficient proteolytic inactivation of factors V and VIII weakens this negative regulatory feedback, causing thrombophilia. Protein S is an obligatory cofactor in this reaction, and its deficiency also results in thrombophilia even at normal levels of functional protein C [104]. Patients with the Arg506Gln factor V Leiden mutation at one of the cleavage sites for activated protein C have a higher risk for developing venous thromboembolism due to decreased proteolytic factor V processing [105]. About 40–50% of inherited thrombophilia cases are due to factor V Leiden, and 4 – 10% of the Caucasian population is heterozygous. The heterozygosity has been suggested as a potentially protective, evolutionarily conserved factor against excessive blood loss during child birth, and was shown to protect from post-cardiac surgery hemorrhage [106].

Venous thrombosis can also originate from functional or expression defects of antithrombin, the endogenous serpin that irreversibly inactivates thrombin, and factors Xa, IXa and XIa in reactions that are dramatically accelerated by heparin. The antithrombin mutation database currently lists 127 different mutations [107], and major functional impairments are due to missense mutations in the reactive site, the heparin-binding site and serpin-protease contact regions important for serpin folding and stable covalent complex formation. Heparin cofactor II (HCII) is an equally potent, but highly specific thrombin inhibitor, in the presence of cell surface dermatan and heparan sulfate, and small oversulfated molecules that do not affect the antithrombin – thrombin interaction [108–111]. HCII deficiency is associated with arterial thrombosis, development of atherosclerosis and in-stent restenosis [44,112]. As 60% of HCII is extravascular, it may control thrombin's signaling properties, and other extravascular serine proteases may be yet unidentified HCII targets. A few serpin-related bleeding disorders are known: α1-PI Pittsburgh has a Met358Arg mutation in its reactive

site, which shifts its specificity from elastase to thrombin, thereby impairing normal blood clotting; and congenital α2-antiplasmin deficiency results in premature lysis of hemostatic plugs by excess plasmin. Selective inhibition of the anticoagulant activated protein C by mutating the reactive site-flanking residues of α1-PI Pittsburgh to lysines has been shown successful in normalizing bleeding in a hemophilia B mouse model, and may show promise as a novel hemophilia drug [113].

Hyperfibrinolysis by uncontrolled plasminogen activation is characterized by excessive bleeding mimicking hemophilia. In the congenital disease α2-AP or PAI-1 are deficient, and acquired hyperfibrinolysis may occur in liver disease, trauma, or during surgery. Treatment with tranexamic acid, ε-aminocaproic acid or other lysine analogs inhibits plasminogen activation by tPA on the surface of the fibrin clot by occupying the lysine binding sites on plasminogen. This displaces plasminogen from the fibrin surface and inhibits plasmin formation. Tranexamic acid may be anti-inflammatory by inhibiting plasmin-dependent activation of complement, monocytes and neutrophils. The lysine analogs also block conformational plasminogen activation by the bacterial non-enzymatic cofactor streptokinase (SK), a fibrinolytic that has been displaced by tPA in the US but is still used in many European and non-Western countries. SK has a C-terminal lysine residue that binds to plasmin(ogen) kringles, thereby increasing the affinity of the plasmin(ogen) complexes with SK and the rate of plasminogen activation [114–116].

Many cardiovascular and metabolic pathologies have an inflammatory component throughout the development of the disease, and extravascular infiltration of circulatory hemostatic proteases upon tissue damage contributes to inflammation. Cellular proteases produced by white blood cells also feature prominently in inflammation. Macrophages transform into cholesterol- and lipid-laden foam cells in the atherosclerotic vascular wall. Monocytes, neutrophils, lymphocytes, and mast cells in particular play a role in foam cell formation in the arterial intima [94]. Mast cell chymase converts angiotensin-I in vascular cells to the potent pro-inflammatory angiotensin II that upregulates expression of redox-sensitive cytokines, chemokines, and growth factors implicated in the formation of atherosclerotic lesions [117]. Elevated angiotensin II causes arterial hypertension, and has been implicated in vascular proliferation, aortic valve disease, myocardial infarction, heart failure, and abdominal aortic aneurysm (AAA). Mice with angiotensin II–induced hypertension develop arterial vascular inflammation, dependent on thrombin-triggered activation of factor XI bound to platelets via its receptor glycoprotein Iba [118]. Patients with uncontrolled arterial hypertension also exhibit factor XI-dependent, amplified platelet-localized thrombin generation which may serve as an inflammatory marker of high blood pressure. Blocking factor XIa activity in combination with inhibition of the renin-angiotensin system may show promise in treating hypertension and associated vascular inflammation. Inhibition of the renin-angiotensin system in animal models and humans also diminishes plaque formation, and may provide an avenue for treatment and prevention of atherosclerosis.

Chymase and tryptase degrade ApoE and HDL3, thereby decreasing cholesterol efflux from foam cells and impairing cholesterol reverse transport. Chymase induces SMC apoptosis, inhibits SMC growth and collagen synthesis, and degrades endothelin-1, leading to impaired

vasodilation. Chymase activates pro-MMP-9 whereas tryptase activates pro-MMP-1, −2 and −3, all involved in the development of atherosclerosis and abdominal aortic aneurysm. Extracellular matrix degradation by elevated MMPs facilitates chemokine- and angiogenic factor-triggered migration of leukocytes and endothelial cells, which accompanies neovascularization and growth of the atherosclerotic lesion, and eventually facilitates plaque rupture [119]. Chymase-activated TGF-β1 disrupts endothelial function and also contributes to intima thickening. Elevated plasma chymase and tryptase levels were detected in patients with acute myocardial infarction (MI) or unstable angina pectoris but not in stable angina, indicating a correlation with plaque instability. The mast cell inflammatory cytokines IL-6, TNF-α, and IFN-γ induce smooth muscle cell and endothelial cell expression of chymase and tryptase, and their plasma levels correlate directly with the AAA expansion rate. Elevated levels of matrix MMPs are associated with the development of AAA, and high plasma MMP-1 and −9 concentrations are indicative of poor outcomes after aneurysm rupture [120]. Anti-angiogenic drugs showed adverse effects in clinical trials with cancer patients suffering from atherosclerosis, and drugs targeting proteases may be an alternative to help combat atherosclerosis. Caspase-mediated apoptosis occurs in atherosclerosis, and both beneficial and harmful caspase effects have been reported. In a population study, apoptotic markers of 4284 subjects were measured, and at the mean 19-year follow-up, 381 patients presented with adverse cardiovascular events. Elevated caspase-8 at baseline was strongly correlated with their incidence [121]. Macrophage apoptosis in atherosclerosis may have both pro- and antiatherogenic effects, and more studies are needed to elucidate these complex mechanisms.

Plasma chymase is elevated in type 2 diabetes and pre-diabetes, and clinical trials currently evaluate chymase and tryptase as drug targets for small molecule inhibitors. Chymase-generated angiotensin II contributes to islet disorganization and high risk for cardiovascular events in diabetic patients. Urinary extracellular vesicles of patients with diabetic nephropathy contain elevated levels of MMP-9, proteinase-3, kallikrein 13, and cathepsins A, C, D, L and X/Z/P compared to controls [122], and these proteases derive from neutrophils and monocytes, recruited to the glomerular endothelial cells. The profiles may have prognostic and diagnostic value in the assessment of kidney damage in type 1 and 2 diabetes. Proteinase-3 cleaves insulin-like growth factor 1 and promotes glomerular inflammation. These combined findings illustrate how endothelial dysfunction and inflammation may be predictors of diabetic nephropathy.

A strong association exists between calpain-10 and type 2 diabetes, and blocking calpain activation prevents diabetes-associated cardiac injury. Mast cells and macrophages produce cathepsins, among which L and K are associated with obesity. In mouse models of obesity, L and K knock-out mice or wild-type mice given L- and K-selective small molecule inhibitors were significantly leaner than control mice and had improved glucose sensitivity [94]. Cathepsin K is a marker of adiposity, and recent findings report cathepsin S and D association with human obesity. Maternal diabetes may cause embryonic neural tube defects, characterized by elevated levels of caspase-3, −6 and −8. The mechanism involves proteolytic activation of the effector caspases-3 and −6 by initiator caspase-8 [123]. Caspase-3 is also a major effector of insulin-producing pancreatic β-cell apoptosis in type 1 diabetes [124].

In ischemic stroke, arterial blockage can be caused by a thrombus formed within the brain, or an embolus formed elsewhere in the body. Hemorrhagic stroke, less frequent but frequently more severe, is the result of a ruptured blood vessel. In patients on anticoagulants or fibrinolytics, ischemic stroke may develop a hemorrhagic component, known as hemorrhagic transformation. Factor VII activating protease (FSAP) is a plasma serine protease that activates pro-urokinase (pro-uPA) rather than factor VII. The FSAP-Marburg I polymorphism (1704G > A), which reduces FSAP activity, increases stroke risk and mortality but seems to lower the risk of developing carotid restenosis in atherosclerotic patients [125]. Ischemic stroke triggers uncontrolled MMP-2 and MMP-9 activity, associated with disruption of the blood-brain barrier and onset of edema, and MMP-9 is also elevated in hemorrhagic transformation [126]. Expression of the endogenous tissue inhibitor of metalloproteinase-1 (TIMP-1) is observed in conjunction with elevated MMP-9, as a protective response to tissue injury. Neutrophils, rather than resident brain cells are the main source of pro-MMP-9 following stroke, and upon degranulation, the proenzyme is proteolytically activated in the extracellular space. MMP-2 and TIMPs are ubiquitously expressed in tissues of the central nervous system [127]. These findings suggest that focusing on endothelial cells, pericytes, astrocytes, and infiltrating leukocytes, rather than neurons, may prove to be more successful in identifying new therapeutic targets. Understanding the relationship between MMP-9 and neutrophils may help elucidate mechanisms involved in disruption of the blood-brain barrier, and lead to more successful therapeutic approaches.

Cancer

The long-held concept that somatic mutations are the causal event in the majority of cancers has recently come under scrutiny [128,129]. Priming the cellular micro-environment for development of cancer is characterized by a sequence of events that precede the transformation of a normal cell into a cancer cell, and somatic mutations are actually later events in the development of many cancers. Chronic inflammation and fibrosis have been identified as two of these events. Hemostatic proteases have recently been recognized to contribute to inflammatory processes in cancer. Disruption of the endothelial barrier during tissue damage allows hemostatic zymogens to be activated. Not only do these proteases contribute to extravascular coagulation and fibrinolysis, they also trigger signaling through cell-surface activation of PAR receptors, binding to uPAR and LRP-1, and activation of MMPs [130]. Inflammation also triggers the release of TGF-β that potently induces MMP-2 and MMP-9 expression. In turn, MMP-2, −9 and −14 proteolytically activate latent TGF-β in the ECM. Transmembrane MMP-14 and several members of the ADAM family are localized on invadopodia of migrating cells. The involvement of MMPs in extracellular matrix remodeling facilitates tumor invasion, and MMPs also figure prominently in cancer-related signaling. Whereas many MMPs are recognized as pro-tumorigenic, some may negatively affect cancer progression, depending on the micro-environment of the cell [131]. The MMP and ADAM inhibitor Marimastat showed no broad therapeutic anti-cancer potential due to lack of specificity, however it inhibited ADAM-17, highly expressed in renal cell carcinoma [132]. This inhibition downregulated Notch pathway-mediated cell proliferation and invasion more effectively than γ-secretase inhibition. Hence, Marimastat may have therapeutic potential in renal cell cancer. Tissue inhibitors of metalloproteinases

(TIMPs) are differentially expressed in cancer: high TIMP1 expression is associated with fibrotic processes and poor outcome, and TIMP3 silencing indicates advanced disease [133]. TIMPs figure prominently in other pathologies such as cardiovascular disease and sepsis, and fibrosis as measured by TIMP1 levels was recently shown to predict all-cause mortality in the AGES-Reykjavik Study [134].

Epigenetic processes are increasingly recognized as essential in carcinogenesis. During epithelial-mesenchymal transition (EMT) in cancer initiation, progression and metastasis, epigenetic mechanisms such as DNA methylation and histone modifications regulate EMT-related genes [135]. The transformation of epithelial cells into migratory fibroblasts and mesenchymal cells is a hallmark of metastasis, and various protease activities are associated with this process. In human gastric cancer cells, thrombin-catalyzed activation of PAR-1 is thought to trigger EMT [136], and in breast cancer, induction of the SUMO-specific protease 7 long variant promotes gene expression favoring cell proliferation and EMT [137]. Mammalian intracellular high-temperature requirement A (HtrA) serine proteases contain a chymotrypsin-like domain and play a role in protein quality control. Epigenetic silencing of the *HTRA1* gene in cancer cells may be caused by histone deacetylase targeting of the promoter, or transcription repression of the methylated promoter by binding of methyl-CpG-binding domain protein 2 (MBD2) [138]. Silencing of tumor suppressor microRNAs via protease-activated PAR and Nf-κB signaling, and of caspase-8 expression by DNA methylation are yet a few other epigenetic mechanisms associated with the development of certain cancers [139,131].

Altered expression of secreted lysosomal cysteine cathepsins has been associated with a variety of cancers, and several studies correlate either overexpression or gene knock-out with progression of malignancy, depending on the type of cathepsin, and the nature and localization of the cancer. Overexpression of tumor tissue cathepsins B and L is detected in ovarian cancer but not in benign tumors and control tissue, and plasma cathepsin L is elevated in patients with malignant tumors. These proteases may be useful biomarkers [140]. Native and mutated forms of the aspartic protease cathepsin D precursor feature prominently in metastatic breast cancer. This tumor marker is present at higher levels in invasive ductal carcinomas, lymph node metastases and hormone-receptor negative cancers than in lobular cancers and nodal positive carcinomas [141].

About 90% of the cancers originate in the epithelium. Epithelial cells express mesotrypsin and matriptase, and upregulation of these proteases is observed in epithelial cancers. Increased mesotrypsin activity is an indicator of poor prognosis in breast, prostate, pancreatic and many other cancers [142]. Mesotrypsin is unusual in that it is not inhibited by Kunitz and Kazal-like trypsin inhibitors, but rather recognizes these proteins as substrates. It exhibits specificity for Arg/Lys-Ser/Met bonds, targets thrombin substrates such as the PAR1, 3 and 4 receptors, and is not inhibited by α1-antitrypsin (Met-Ser reactive bond) but by α1-antitrypsin Pittsburgh (Arg-Ser). Engineering a triple mutant M17G/I18F/F34V of the human amyloid precursor protein Kunitz protease inhibitor domain (APPI) created a selective, tight-binding inhibitor with an inhibition constant (K_i) of 89 pM, active in cell-based models of mesotrypsin-dependent prostate cancer cell invasiveness [143]. The crystal structure of the APPI M17G/I18F/F34V/mesotrypsin complex shows unique active site

features that may be critical in driving metastasis, given the observation that other trypsins do not contribute to the invasive prostate cancer phenotype. This structural information may facilitate development of therapeutic peptide inhibitors, complementary to the mesotrypsin active site. Matriptase is the best known member of the serine proteases with a type II N-terminal transmembrane domain, and its endogenous inhibitors are the Kunitz-type hepatocyte growth factor activator inhibitor types 1 and 2 (HAI-1 and 2). In normal tissue, matriptase proteolytic activity is tightly regulated by excess HAI-1 and 2, whereas in cancer tissue this balance is tilted heavily toward excess matriptase. Transgenic expression of epidermis matriptase in a squamous cell carcinoma mouse model causes tumor formation that is inhibited by HAI-1 or HAI-1 co-expression [144], strongly suggesting matriptase proteolytic activity as an essential trigger for malignancy. In highly aggressive inflammatory breast cancer (IBC), matriptase proteolytically activates pro-hepatocyte growth factor (pro-HGF). Binding of HGF to the receptor tyrosine kinase, c-Met, activates signaling pathways leading to cell proliferation, migration, morphogenesis, and invasion [145]. Both matriptase and c-Met are membrane-bound in IBC cells, and upregulated in cancer cells of IBC patients. Proliferation and invasion of IBC cells is halted by silencing with RNAi or treatment with synthetic matriptase inhibitors, illustrating their potential merit in IBC therapy.

Excessive proteasome activity occurs in certain blood cancers. Degradation of pro-apoptotic factors such as p53 impairs programmed cell death in cancer cells, and proteasome inhibition was proposed as a potential cancer treatment. The proteasome inhibitors bortezomib, carfilzomib and ixazomib were approved by the FDA for the treatment of multiple myeloma, and are currently in clinical trials of blood, lung and breast cancers. These potent inhibitors of the β5 peptidase activity of the 26S proteasome have only modest activity against β1 and β2 peptidases, which appears to limit their usefulness to multiple myeloma [146]. The β5 peptidase inhibitors have not been successful in treatment of solid tumors, and in recent studies triple negative breast cancer cell lines only responded to bortezomib or carfilzomib after CRISPR gene editing to inactivate β2 [147]. Development of dual β2/β5 inhibitors, while conceptually attractive, may prove daunting, and combination therapy with the β5 and known β2 inhibitors is a more realistic approach.

Balanced complement-associated inflammation can be of advantage in potentiation of immunotherapy, whereas an imbalance may sustain tumor cell proliferation, migration, invasiveness and metastasis [148]. Genetic and epigenetic changes mark tumor cells as non-self, and the innate immune cells assist in clearing opsonized tumor cells through concerted actions of anti-tumor monoclonal antibodies (mAb) and complement cytotoxicity. Rituximab and ofatumumab, FDA-approved chimeric anti-CD20 mAbs were developed for the treatment of B cell lymphomas and chronic lymphocytic leukemia. Their targeting of tumor antigens elicits complement-dependent phagocytosis. Binding of C1q to the Fc portion of the mAbs results in formation of the proteolytically active C1 complex that initiates the cascade. Other tumor-specific mAbs recognize CD38 and CD52 as epitopes highly expressed on B cell- or T cell-derived tumors, and they are well-studied in terms of their ability to elicit complement-dependent cytotoxicity. However, some solid tumors downregulate complement cytotoxicity and opsonization by overexpressing or sequestering surface proteins, thus limiting the efficacy of therapeutic antibodies.

The connection between complement activation, chronic inflammation and cancer is becoming increasingly evident [149]. Complement factors and their active cleavage products themselves contribute to mitogenic signaling cascades and growth factor production (C3a, C5a and the membrane attack complex MAC), angiogenesis (C3, C3a, C5a, MAC), protection from antigrowth signals and apoptosis (C3a, C4, C5a, MAC), cellular invasion and migration through the extracellular matrix (C1q, C1s, factor B, C3, C3a, C3d, C5, C5a, C9), proliferation (C3, C3a, C4, C5a, MAC), and suppression of antitumor immunity (C5a). From these observations it is clear that complement itself can promote cancer, under the "right" circumstances. The physiological role of the MAC complex is to disrupt the cell membrane and cause lysis, however sub-threshold MAC activity does not kill the cell but activates the cell cycle and triggers proliferation. Hence inhibition of complement may become an emerging strategy for the fight against cancer.

Excessive complement activation in colorectal, breast, pancreatic, lung, prostate, esophageal cancer, lymphoma and leukemia has suggested the use of C3 activation fragments as diagnostic or prognostic biomarkers. However, C3 is abundant in plasma, and mass spectrometry quantitation may not be straightforward. Intra-tumoral C3 expression in ovarian cancer has been linked with disease prognosis, and a C3 fragment is found in prostatic fluid from cancer patients. Prostate-specific antigen (PSA) cleaves C3 and C5, and may act pro-tumorigenic by proteolysis of complement proteins.

Human tissue kallikreins (hK) are secreted serine proteases that are differentially expressed in many endocrine cancers. The *KLK3*, *8*, *10, 13* and *14* genes are thought to encode tumor suppressor proteins, illustrating the recently recognized concept that protease upregulation does not always reflect tumor progression [131]. With this in mind, defining target protease specificity is crucial when developing protease inhibitors as potential anti-cancer drugs. hK3 or prostate-specific antigen (PSA) is the best known biomarker for screening, diagnosis, and monitoring of prostate cancer. PSA is also elevated in benign prostatic hyperplasia, and its extent of complex formation with the serpin, α1-antichymotrypsin, differentiates between both pathologies. Tissues and plasma of prostate cancer patients contain higher levels of the complex than those of patients without cancer, and a level of 25% or more of free PSA activity is generally a good indicator of benign hyperplasia [150]. Many other kallikreins may also be suitable cancer biomarkers. hK5 proteolytically activates PAR-2, leading to Nf-κB activation and downregulation of tumor suppressor microRNAs in oral squamous cell carcinoma [139]. Plasma kallikrein is capable of activating the complement system, linking inflammatory responses to many cancer-related processes [151].

A very strong correlation has also been observed between cancer and hemostasis, and cancer patients invariably exhibit hypercoagulability, contributing to mortality and morbidity [152]. This prothrombotic state is attributed to the ability of tumor cells to activate coagulation, by producing procoagulant factors and inflammatory cytokines; interacting with monocytes, platelets, neutrophils and vascular cells; and triggering acute phase reactants and necrosis. Various anticoagulant therapies with heparins, vitamin K antagonists, or direct oral anticoagulants have proven to be beneficial in the treatment of cancer patients [153].

Neurodegenerative Diseases

Patients with Alzheimer's disease (AD), representing up to 70% of the dementia cases, have brain tissue containing amyloid plaque composed of toxic Aβ peptides, and neurofibrillary tangles composed of tau protein. Tau is localized in neuronal axons, promotes tubulin polymerization and stabilizes microtubules. Amyloid β A4 precursor protein (APP) is a highly conserved synaptic integral membrane protein, thought to regulate synapse formation, neural plasticity and maintenance of homeostasis in the central nervous system. Normal proteolytic processing of APP occurs via cleavage by α-secretase to release extracellular APPsα, followed by cleavage by intramembrane γ-secretase, with release of an extracellular p3 fragment, and an intracellular AICD fragment [154]. APP processing is different in AD: sequential cleavage by β- and γ-secretase releases the extracellular APPsβ, $Aβ_{1–40}$ and $Aβ_{1–42}$ peptides, and intracellular AICD. Heterogeneous proteolytic degradation yields several other extracellular species, ranging from 37 to 49 residues. $Aβ_{1–40}$ and $Aβ_{1–42}$ are considered neurotoxic, and form plaques in the brain. The APPsβ fragment oligomerizes and mediates death receptor signaling. The $Aβ_{1–42}$ fragment in cerebrospinal fluid is routinely used as a biomarker, combined with the measurement of total and hyperphosphorylated tau protein. This combination assay can diagnose AD in an early stage, and provide a prognosis of disease progression [155]. N-truncated Aβ peptides with cyclized terminal glutamate residues figure prominently in amyloid deposits, and are particularly useful additional biomarkers. However, no clear correlation could be made between plasma Aβ fragment concentrations and AD, dementia and various stages of cognitive decline, perhaps in part due to the limited sensitivity of the current analytical methods [156]. Due to observed discrepancies between AD dementia and amyloid deposition, some groups have suggested that either soluble oligomeric Aβ peptides may be more toxic, or that tau neurofibrillary tangles may be the pathogenic species [157].

APP is also produced in platelets, and cleaved by platelet α-secretase to release soluble APPsα in the circulation upon platelet activation [158]. APP isoforms of 130 kDa (intact) and 106–110 kDa (cleaved) can be detected by immunoblotting of platelet lysates. Patients with AD and mild cognitive impairment (MCI) have a significantly lower ratio of intact vs. cleaved APP than healthy controls. The decrease in ratio parallels cognitive decline and may predict conversion from MCI to AD. The metalloprotease ADAM10 in the brain processes APP through an alternative, non-amyloidogenic pathway [159], and platelets have the same proteolytic machinery as neurons for processing APP. The increase in the amyloidogenic pathway in AD patients is reflected by a decrease of platelet α-secretase and ADAM10 activity, and an increase of platelet β-secretase activity. These observations suggest that platelet biomarker assays for AD may be feasible, and raised the question if Aβ peptides generated by platelets can enter the brain and contribute to neuronal deficit. A recent elegant *in vivo* study showed indeed that Aβ peptides, originating from transgenic AD mice in prolonged parabiosis with healthy wild-type mice, accumulated in the brain of the healthy mice [160]. This finding strongly suggests a biological connection between altered platelet and neuronal protease expression in AD patients.

Clinical trials have focused on the development of β- and γ-secretase inhibitors, and antibodies targeting Aβ peptides with the goal of peptide removal as a means of reducing

plaque formation. To date the results have been disappointing, except for clinical trials of the monoclonal Aducanumab, directed against aggregated and soluble Aβ peptides [161]. In contrast, the monoclonal Solanezumab, targeting soluble monomeric Aβ, failed in 3 consecutive trials, the latest one Expedition3, which was halted in January 2018 [162]. Small molecule inhibitors of β-secretase were also put to the test: negative results halted the Verubecestat Epoch trial in February 2017, but two trials studying the compound JNJ-54861911 are set to run until 2023. The 2010 phase III failure of Semagacestat, a γ-secretase inhibitor, was due to side effects of blocking Notch signaling, however some questions remain about the design of the clinical trial, and potential optimization of the drug dosage [163]. Although chronic but partial lowering of γ-secretase activity in heterozygous knock-out mice does not cause a diseased phenotype and might have been tolerated in humans, the trial design opted for short peaks of complete γ-secretase inhibition in the brain alternated with periods of normal activity. This proved to wreak havoc on the the ultradian oscillation of Notch signaling, as corroborated by severe Notch phenotypes in complete γ-secretase KO mice. Plasma concentrations of the drug were ~360-fold higher than the IC_{50} for γ-secretase inhibition in cell culture, and the side effects on the skin, gastrointestinal system and weight loss due to very high intermittent drug dosage might also have contributed to poor performance in cognitive tests. With these caveats, there are still unexplored options for the development of γ-secretase inhibitors as drugs for cognitive decline.

Other proteases have been implicated in neurogenerative diseases. Several MMPs cleave APP *in vitro*, raising the question whether they also do so *in vivo*, and if there is a correlation with circulating MMPs and their inhibitors in AD. Expression of MMP-9 and the tissue inhibitors (TIMPs) was found to be elevated in postmortem AD brain tissue [164]. Significantly higher levels of MMP-9, but not of MMP-2 or the TIMPs were found in the plasma of AD patients, suggesting that MMP-9 may contribute to AD. Caspase-6 is found in non-apoptotic brain tissue of Huntington's disease and AD patients, indicating a function other than its executioner role [58]. Caspase-6 is implicated in axonal degeneration and neuronal loss in both diseases, and it cleaves tau, CREB-binding protein (CBP) which regulates transcription in cortical neurons, and NF-κB. Hence selective caspase-6 inhibitors may have therapeutic potential. CREB is indispensable for synaptic plasticity, and its impaired activation contributes to AD [165]. CREB is a substrate of the neutral, cytosolic cysteine protease calpain, and inhibition of this protease restores synaptic plasticity in a mouse model of familial AD. Calpain also cleaves tau protein, and upregulation or decreased degradation of the endogenous calpain inhibitor calpastatin has been a therapeutic goal in AD. Defective mitochondrial proteases can cause neuronal cell death and axonal dysfunction [166], and the human proteases m-AAA, the serine protease HTRA2 (high temperature requirement) and the rhomboid protease PARL have been identified in with neurodegenerative processes. Two human m-AAA isoenzymes are differentially involved in neurodevelopment and protection against neurodegeneration, by preventing accumulation of misfolded polypeptides, and regulating mitochondrial protein synthesis, transport and proteolytic control of gatekeeping functions to prevent Ca^{2+} overload in the neuron [167]. Mutations in m-AAA cause hereditary spastic paraplegia and spinocerebellar ataxia. HTRA2 and PARL increase the susceptibility of neurons to apoptotic cell death. HTRA2 is involved

in caspase-dependent apoptosis and in Parkinson's disease [168], but the role of PARL is still controversial. Post-translational modification of proteins by small ubiquitin-related modifier (SUMO) can be reversed by SUMO-specific protease 2 (SENP2), and accumulation of SUMO-conjugated proteins is observed in patients with neurodegeneration. A knock-out mouse model confirmed that disruption of this mitochondrial protease causes neuronal cell death [169]. Finally, the link between neurodegeneration and dysregulated complement activity has been firmly established. Acute brain injury triggers uncontrolled complement activation, flooding of the injury site with inflammatory anaphylatoxins and phagocytes, and blood brain barrier (BBB) damage [170]. However, normal complement function plays a role in brain development (wiring), and brain homeostasis and repair during adulthood. Therapeutic approaches of complement modulation will therefore depend on its acute, subacute and chronic nature of activation, and will require selective targeting of complement components.

Autoimmune Diseases

Upregulation and activation of pro-inflammatory cytokines and chemokines, uncontrolled endogenous protease activity, inflammation and antibodies/T lymphocytes against "self" antigens are hallmarks of autoimmune diseases [171]. Chemokines recruit leukocytes to release MMP-9 that generates peptides with immunodominant epitopes. These epitopes are presented to autoreactive T lymphocytes and stimulate B cells to produce autoantibodies. The "Remnant Epitopes Generate Autoimmunity" (REGA) model, based on cytokine, chemokine and protease action, has been validated for multiple sclerosis, rheumatoid arthritis and diabetes. According to this model, potential strategies for disease treatment may involve the use of anti-inflammatory cytokines, and the inhibition of pro-inflammatory and protease-inducing cytokines and chemokines. MMP-9 cleavage of collagen in rheumatoid arthritis, and of insulin in autoimmune pancreatitis was found to generate remnant epitopes. Inflammasomes are large macromolecular complexes involved in activation of pro-caspase-1. Caspase-1 proteolytically activates the precursors of the pro-inflammatory cytokines IL-1β, IL-18 and IL33, and has been implicated in various autoimmune diseases. IL-1β blockade in autoimmune diseases can be accomplished with IL-1 receptor antagonists, neutralizing monoclonal antibodies, and the injectable IL-1β inhibitor Rilonacept [172]. Clinical trials of Pralnacasan, an oral caspase-1 inhibitor for treatment of rheumatoid arthritis were halted in 2003 after liver toxicity was observed in animal studies.

B cells contribute to autoimmune diseases by secretion of autoantibodies, presentation of autoantigen, and inflammatory cytokine secretion. Antibody therapy with Rituximab targets CD20 on the B cell surface, triggering cell death, and is used for B cell depletion to treat rheumatoid arthritis, idiopathic thrombocytopenic purpura, pemphigus vulgaris and myasthenia gravis. The recent discovery that the intramembrane signal peptide peptidase-like protease SPPL2A promotes B cell differentiation by cleavage of CD74 suggested that SPPL2A may be a suitable target for inhibition in the treatment of autoimmune diseases [82]. Major histocompatibility complex (MHC) class II-mediated priming of T and B lymphocytes occurs in systemic lupus erythematosus (SLE) and lupus nephritis. The cysteine protease cathepsin S degrades CD74 during MHC II assembly with antigenic peptide in antigen-presenting cells, and cathepsin S inhibition might be therapeutic in SLE

[173]. In some cases, deficiency of protease activity is associated with autoimmune disease. The plasma of thrombotic thrombocytopenic purpura (TTP) patients contains unusually large forms of von Willebrand factor multimers. Most TTP cases arise from autoantibody-mediated inhibition or accelerated clearance of ADAMTS13 [174]. Highly similar anti-ADAMTS13 autoantibodies were found in unrelated TTP patients, suggesting that this autoimmune response is antigen-driven.

Proteases, Inhibitors and Cofactors in Infectious Organisms

Infectious organisms employ their own arsenal of proteases for propagation and virulence, such as HIV protease [175], *Trypanosoma cruzi* cruzipain [176], *Porphyromonas gingivalis* gingipains [177], and *Bacillus anthracis* lethal factor [178]. Several bacterial, viral, protozoan and fungal proteases trigger inflammation by activating the intrinsic coagulation pathway [102], or act as procoagulants by non-canonical, direct activation of prothrombin [179]. Large panels of small-molecule inhibitors of the proteasome in pathogenic organisms are currently being screened for potential therapeutic benefit and minimal toxicity toward the cellular machinery of the host [180]. Bacterial infections are associated with increased thrombotic risk, however this correlation is not restricted to pathogenic bacteria. Subtilisin, produced by the non-virulent bacterium *Bacillus subtilis*, can cleave prothrombin to an active thrombin-like species that converts fibrinogen to fibrin [181]. Deregulation of the intestinal microbiota is typical in IBS, and excess protease production by commensal enteric bacteria has been proposed to promote adherence to and invasion of intestinal epithelial cells, activate protease-activated receptors (PARs), disrupt the intestinal barrier, and facilitate bacterial interaction with immune cells, leading to inflammation [182].

Pathogens may use mechanisms other than direct proteolytic activity to enhance their virulence or facilitate dissemination and propagation. The streptococcal and staphylococcal cofactors streptokinase (SK) and staphylocoagulase (SC) are not enzymes themselves, and respectively bind and activate host plasminogen and prothrombin in a non-proteolytic fashion by inserting their N-termini into the zymogen activation pocket. This triggers a conformational change that forms the active site in the zymogen [183,101]. The cofactor complexes with the zymogens as well as the active proteases are very tight, and refractive to endogenous serpins that inactivate plasmin and thrombin, thus adding to the bacterial virulence. The prothrombin•SC and thrombin•SC complexes cleave host fibrinogen to form fibrin barriers, protecting the pathogen from the host immune system. Upon activation of free host plasminogen to plasmin by the plasminogen•SK complex [183], the tighter binding plasmin•SK complex is formed, and degrades the host extracellular matrix to facilitate pathogen invasion and dissemination. Numerous streptococcal strains also increase their invasiveness by recruiting host plasminogen and plasmin to bacterial cell wall M-proteins [184]. Von Willebrand factor-binding protein (VWbp) is another conformational prothrombin activator secreted by *S. aureus*, and belongs to the family of staphylococcal and streptococcal homologs named zymogen activator and adhesion proteins (ZAAPs), based on the SC structure [185]. Staphylokinase (SAK) bears no sequence similarity to SK, but shares a similar domain fold. It does not activate plasminogen conformationally, but forms a tight plasmin•SAK complex that cleaves plasminogen as a substrate [186]. The skizzle (SkzL) protein, secreted by *Streptococcus agalactiae*, has moderate sequence identity to SK and

SAK [187]. SkzL binds host plasminogen, and enhances its activation by the plasminogen activators uPA and single chain tPA, and plasma clot lysis by these plasminogen activators. *S. agalactiae* pathogenesis likely includes SkzL to enhance bacterial spreading through fibrinolytic enhancement. These are prime examples of pathogens exerting virulence by hijacking the host coagulation and fibrinolytic systems [188].

The human host proteases ADAM-TS7, carboxypeptidase E, dipeptidyl peptidase 3, macrophage stimulating 1 protease and neurotrypsin are required for influenza A virus replication, and are under control of eight host miRNAs regulating gene expression during virus replication [189]. These host genes and microRNAs may provide new therapeutic targets. The ubiquitin-specific protease 14 (USP14), a deubiquitinating enzyme, prevents degradation of prion protein by rescuing it from the proteasome, and may be a suitable target in the development of therapeutic strategies for prion diseases [190].

Porphyromonas gingivalis is prevalent in periodontitis, a risk factor for oral and gastric tract tumors, and also lung cancer, as recently identified in a follow-up of the Atherosclerosis Risk in Communities (ARIC) study [191]. *P. gingivalis* gingipains are cysteine proteases associated with this type of chronic inflammation, and they are the only bacterial proteases that degrade SPINK6, a Kazal-type inhibitor of various human kallikreins in skin and oral epithelium. Loss of this proteolytic control has been suggested as a link between periodontal disease and tumor development [177].

PROTEOLYSIS-RELATED PROCESSES AS DRUG TARGETS

Overexpressed or Impaired Endogenous Proteolytic Activity

As of 2010, an estimated 5–10% of all drugs under development were targeted toward proteases [192], many of them small molecules designed to block the protease active site. Among past and present commercially successful protease inhibitors are blood pressure regulators (e.g. captopril and aliskiren) which respectively inhibit the metalloprotease angiotensin-converting enzyme (ACE), and the aspartic protease renin, by competitive binding to the protease active site; dipeptidyl peptidase-4 inhibitors (e.g. sitagliptin) to combat type 2 diabetes; the threonine protease inhibitor bortezomib as a cancer drug directed against the proteasome; the direct oral anticoagulants (DOACs), thrombin and factor Xa inhibitors (argatroban, dabigatran, apixaban, rivaroxaban, edoxaban) that bind tightly and reversibly to the protease active site; tight-binding hirudin-based thrombin inhibitors (lepirudin, desirudin, bivalirudin) for patients with heparin sensitivity.

Several endogenous protease, cofactor and inhibitor deficiencies are treated by augmentation therapy. Hemophilia A is the deficiency of factor VIII, the essential cofactor of factor IXa to activate factor X; and hemophilia B patients lack functional factor IX. Both deficiencies prevent the formation of the intrinsic Xase complex that is responsible for generation of the majority of active factor Xa, with as end-result the impairment of clot formation. Intravenous replacement with plasma-derived or recombinant fVIII and factor IX requires frequent injections, although preparations with longer half-life are being developed. Gene therapy for hemophilia B, based on *in vivo* gene transfer with adeno-associated viral (AAV) vectors to the liver has been in clinical trials for 16 years, with partial success due to cellular

immune responses [193]. However, as of December 2017 the results of two small cohort studies are promising: 52 weeks after infusion of a single intravenous dose of an AAV5 vector encoding factor VIII, no cellular immune response, liver toxicity or inhibitory antibodies were observed in a clinical trial for treatment of severe hemophilia A [194]; and in a small-scale hemophilia B patient study, a high level of expression of functional factor IX was seen after a single injection of an AAV vector containing the hyperfunctional factor IX Padua gene, targeting the liver [195]. Deficiency of factor XI, also known as hemophilia C, is a rare bleeding disorder, often seen in Ashkenazi Jewish populations, and does not cause bleeding in the joints. Tranexamic acid is administered to control traumatic bleeding incidents and during dental procedures, whereas fresh frozen plasma or recombinant factor XI may be used during surgery.

In sepsis, the systemic response by the host to pathogenic invasion triggers activation of inflammatory and coagulation pathways and inhibition of fibrinolysis. In this regard, administration of recombinant human activated protein C (drotrecogin alpha activated, DAA) as an anticoagulant was deemed a useful strategy, and in 2001 it became the first biologic approved for treatment of severe sepsis [196]. Although a first trial indicated reduction in mortality, later trials failed to confirm these findings, and DAA was withdrawn in 2011. Observational trials consistently showed a benefit while randomized trials did not. The difficulties associated with obtaining reproducibility in these trials may be attributed to a variety of reasons: differences in acute illness of patient subgroups, perhaps as a result of conscious or subconscious patient selection; midway amendment through the first trial, changing inclusion/exclusion criteria, the type of placebo and the drug formulation, the combination of which favored the use of DAA and led to early termination; and differences in the timing and appropriateness of antibiotic administration and fluid resuscitation. New drug development for targeting severe sepsis will undoubtedly benefit from targeting pathophysiologic pathways characterized by specific biomarkers rather than heterogeneous patient populations grouped by clinical phenotypes, and DAA may yet be found beneficial for well-defined target groups.

Dysfunctional or poorly expressed serpins, inhibitors of serine proteases, cause a variety of severe diseases. COPD, emphysema, cystic fibrosis, liver disease and panniculitis due to functional α1-PI deficiency and accumulation of inhibitor polymers are alleviated by intravenous administration of plasma-derived α1-PI. Experimental approaches include aerosolized formulations of plasma or recombinant inhibitor, and direct delivery to the lung is expected to circumvent short half-life issues plaguing the intravenous formulation. However, no clinical trial reports are available to date. Intravenous recombinant α1-PI formulations are in the experimental stage, and conjugation with polyethylene glycol may delay rapid renal clearance [89]. It has been recognized that α1-PI inhibits proteases other than elastase and trypsin, namely proteinase-3, kallikreins 7 and 14, matriptase, caspase-3 and the metallopeptidase ADAM17 [197]. This opens up new avenues for modulating the activities of these proteases in disease states. Recombinant human antithrombin (Atryn) is purified from the milk of transgenic goats, and used to avoid peri-operative and peri-partum clotting complications in patients with hereditary antithrombin deficiency. It is not indicated for treatment of thromboembolic events in these patients. Its glycosylation profile differs

from that of plasma-derived antithrombin, with increased heparin affinity as a result. The modification ensures efficient inhibition of elevated thrombin and factor Xa.

C1-inhibitor is a serpin targeting C1 esterase of the complement system, and it is also the physiological inhibitor of kallikrein, and factors XIIa and XIa of the contact activation pathway of coagulation. Both inherited and acquired C1-inhibitor deficiency can lead to life-threatening angioedema [198]. Inherited, heterozygous deficiency results in lack of transcription, translation or secretion, or in expression of mutated, dysfunctional inhibitor. Acquired deficiency is the result of inhibitor depletion due to autoantibody formation or accelerated consumption in lymphoproliferative diseases. Elevated kallikrein activity causes unregulated cleavage of high molecular weight kininogen and release of bradykinin, the mediator of angioedema. Acute attacks of angioedema are treated with C1-inhibitor concentrate from plasma, recombinant inhibitor, and the kallikrein inhibitor ecallantide. Prophylactic treatment with the antifibrinolytic agents ε-aminocaproic acid and tranexamic acid regulate the fibrinolytic system which is continuously activated in autoimmune angioedema. Aprotinin (Trasylol), or bovine pancreatic trypsin inhibitor, is a Kunitz-type inhibitor of kallikrein and plasmin. It was used to treat laryngeal edema until its temporary withdrawal from the market in 2007 due to reports of increased death risk as a bleeding preventative during cardiac surgery. As aprotinin was derived from bovine lung tissue, concerns for allergic reactions and bovine spongiform encephalopathy prompted its discontinuation in Italy. In 2012 the European Medicines Agency proposed to lift the ban, and aprotinin is currently marketed by Nordic.

The proteases of the complement system have increasingly been recognized as potentially attractive points of interference for mitigation of inflammatory diseases. Recent developments in complement therapeutics focus on the proteases of the initiation pathways, with C1-inhibitor targeting C1r/s and mannan-binding lectin serine protease (MASP) (*e.g.* Cinryze, Berinert, Cetor, Ruconest), and antibodies targeting C1q, C1s, C2, MASP-2 and −3 (ANX-005, TNT009, OMS721, CLG561, NM9401) [199].

Protease Inhibitors as Drugs: Some Caveats

Undesired properties or side effects of many of these therapies underscore the need for continued mechanism-based drug design. In 2014 the renin inhibitor aliskiren was placed on the list of drugs to avoid, due to severe side effects in patients with diabetes and kidney impairment [200]. Hirudin derivatives are an attractive alternative for treatment of patients with heparin hypersensitivity or thrombocytopenia, however they have a short half-life. Hirudins are cleared through the kidneys, and dose adjustment is required in patients with renal impairment. Some small molecule drugs have limited bioavailability and solubility, and their efficiency may be mitigated by resistance mutations in the target proteases. Fast acting DOACs are at least as effective as warfarin, with reduced risk for intracranial bleeding, and are prescribed for stroke prevention in atrial fibrillation, thromboprophylaxis in hip or knee replacement surgery, and for treatment and secondary prevention of venous thromboembolic disease [201]. Unlike with vitamin K antagonists, no routine monitoring of coagulation is needed. A major disadvantage is the lack of antidotes for direct factor Xa inhibitors in case of traumatic bleeding. Activated prothrombin complex concentrates and

recombinant activated factor VIIa have been proposed to reverse DOAC action, and the monoclonal antibody idarucizumab was approved by the FDA in October 2015 as a specific antidote against dabigatran.

MMP-dependent degradation of extracellular matrix proteins is associated with angiogenesis and metastasis in cancer, and MMP inhibitors were proposed as suitable anticancer drugs. The zinc ion in MMPs was the first target, but small molecule peptidomimetic inhibitors based on zinc-targeting warheads (*e.g.* batimastat) had limited selectivity, failed to distinguish between different MMP classes also involved in the Notch-, Wnt- and NFκB-signaling pathway, and were fraught with off-target side effects [202,203]. A novel class of small molecules blocking the hydrophobic S1′ specificity pocket, exosites and other MMP domains yielded reasonably specific inhibitors for several MMPs, however their efficacy as second generation drugs has not yet been demonstrated. None of the 50some clinical trials with MMP inhibitors were successful, due to off-target toxicity or absence of efficacy. A recent study reports effective allosteric prevention of pro-MMP-9 activation in a mouse neuroinflammatory model by a small, highly selective heterocyclic chemical inhibitor [204]. This orally administered compound does not prevent activation of the structurally related pro-MMP-2, and does not inhibit catalytically active MMP-1, −2, −3, −9 or −14. Although these findings are encouraging for future drug development, the efficacy of this compound needs to be tested in other models, relevant to cancer, fibrosis and neurodegeneration.

Indiscriminate targeting of overexpressed MMPs in certain cancers may not always yield desired results [205,206]. Pancreatic ductal adenocarcinoma cells (PDAC) overexpress MMP-9, thought to play a role in invasion and metastasis. However, systemic knock-out of MMP-9 in a PDAC mouse model caused increased interleukin-6 (IL-6) expression, and induced invasive growth and STAT3 activation in PDAC cells via IL-6 receptor signaling. The model system, animal genetic background and other experimental conditions may influence the effect of MMP activity, resulting in tumor-promoting, -inhibitory or null effect, as seen in various mouse models of breast cancer. In the light of many controversial experimental results, systemic MMP inhibitors should be used with caution, and information obtained from clinical studies associating specific MMPs with diseases profiles will be critical for identifying the proper MMPs as therapeutic targets. The metalloprotease ADAM10 has been associated with various disease states [159]. In the brain, it cleaves APP via a non-amyloidogenic pathway, with formation of the neuroprotective soluble ectodomain, and decrease of the toxic Aβ fragment. It may also slow down progression of fibrosis in chronic liver inflammation. However, it acts as a sheddase for the cellular prion protein, potentially promoting spreading, and increased activity is seen in synaptic dysfunction linked to Huntington’s disease. Respectively, ADAM10 inhibition would be therapeutic or detrimental in these pathologies. ADAM10 is upregulated in various cancers, atherosclerosis and various autoimmune diseases, suggesting a potential benefit of inhibition. The broad substrate specificity of ADAM10 and its similarity with ADAM17, its systemic presence, and its divergent effects in various diseases pose significant problems in targeting ADAM10, either for upregulation or inhibition. Ideally, drugs would be needed that regulate ADAM10 activity in a tissue and substrate specific manner. Focusing on the interaction of ADAM10 with specific substrates or regulatory partner proteins may provide some promise.

Efforts to develop γ-secretase inhibitors for treating Alzheimer's disease turned out to miss the mark, mainly due to the fact that the mechanism of APP processing by γ-secretase was not fully understood. Mutations in its presenilin domain were originally interpreted to enhance γ-secretase activity, however the γ-secretase inhibitor Semagacestat showed worsening of familial Alzheimer's patients in a phase III clinical trial that was halted in 2010. In addition to issues with the design and dosage, this result was in part also explained by a recent *in vivo* study showing that the presenilin-1 mutations inactivate rather than enhance γ-secretase activity, impair hippocampal memory and synaptic function, and cause neurodegeneration [207]. γ-Secretase inhibitors have since then been repurposed as potential cancer therapeutics due to their inhibition of the Notch signaling pathway, upregulated in many cancers. However the panel of known inhibitors shows a wide range of activities toward cleavage of various other γ-secretase substrates, and off-target interference is likely to cause major side effects, thus limiting the long-term clinical usefulness of these inhibitors [208].

Osteoclasts express the cysteine protease cathepsin K, which degrades type I collagen in bone. Selective inhibition of cathepsin K increases bone mass, improves bone strength, decreases bone resorption and contributes to bone formation [209]. The small molecule inhibitors relacatib, balicatib and odanacatib were initially tested clinically as potential drugs for treatment of postmenopausal osteoporosis, with odanacatib ultimately making it through Phase II and III trials. The phase III trial was halted early after reports of positive efficacy and safety, however a more thorough analysis discovered an increased risk of atrial fibrillation and stroke [210]. Odanacib development was discontinued in 2016, after more than 12 years of research.

In the treatment of ischemic stroke, an active protease, rather than an inhibitor, is used as a therapeutic. Tissue-type plasminogen activator (tPA) was approved in 1996 for clot dissolution in ischemic stroke, however its use is restricted to the first 3 hours post-stroke, and carries an established risk for bleeding. One study attributed the increased blood-brain barrier permeability to tPA-catalyzed activation of platelet-derived growth factor-CC, with Mac-1 integrin and LRP1 acting as co-factors in this reaction [211]. Another study suggested increased MMP-9 activity as a potential cause for increased bleeding risk [212], while yet another report pointed toward a connection of tPA-induced bleeding with hyperglycemia [213].

These are but a few examples of disappointing outcomes, or interference of significant off-target and other side effects in protease-related drug development. A recurring theme is that of insufficient knowledge of the underlying biochemical mechanisms and of the interconnectedness of protease and inhibitor activity in the proteome network. With the increasing availability of large online platforms and databases such as the National Center for Biotechnology Information, RCSB Protein Databank, UniProt and MEROPS, discovery of such interconnectedness should prove increasingly less challenging.

Targeting Exogenous Proteolytic Activity

As antibiotic-resistant infections are on the rise, targeting proteases and protease-related processes in pathogenic bacteria may offer novel avenues for drug development. Proteases

and protease cofactors that function as virulence factors are obviously first choice targets. Progress has been made in developing small molecule compounds that inhibit the expression of SK in Group A streptococci [214]. In addition, the proteolytic complexes Lon, ClpXP, HtrA, the proteasome, and signal peptidases are good candidates for disruption of bacterial mechanisms necessary for survival and pathogenicity [178]. Caseinolytic proteases (ClpPs) are conserved multimeric complexes that are conformationally activated by binding of Hsp100 ATPases. This binding aligns the catalytic triad of the proteases, and energy provided by ATP is used to unfold protein substrates for entry into the pore and subsequent degradation in an energy-dependent manner. Several mechanisms for ClpP deregulation have been proposed: (a) inhibitors such as phenyl esters and β-lactones can directly interact with the catalytic residues and halt protein degradation; (b) blocking of ATPase binding and uncoupling of ClpP and Hsp100 ATPase activity by acyldepsipeptides (ADEPs) and macrocyclic peptides may result in continuous ClpP activation and promiscuous protein degradation. However, many of these compounds are not 100% efficient due to limitations of stability, solubility, resorption and half-life. Small molecule inhibitors have limited specificity and may not distinguish between bacterial protease complexes and their human orthologs. Recently developed ADEP derivatives are active against methicillin-, vancomycin- and penicillin-resistant pathogens, and work well in combination therapy. The natural oligopeptide compounds cyclomarin A, ecumicin and lassomycin target Mycobacterium tuberculosis but do not kill commensal members of the human microbiota. Encouraging *in vitro* and *in vivo* results have also been reported for small molecule inhibitors of *B. anthracis* lethal factor, and the cysteine protease domains of *Clostridium difficile* toxins. Specific interference with proteases required for bacterial viability offers an attractive possibility for developing a novel class of therapeutics, less prone to trigger resistance.

Parasitic cysteine proteases may be attractive targets for treatment of Chagas disease, African sleeping sickness and leishmaniasis. Recent progress was made in the design of more efficient peptide inhibitors of cruzipain, by modifying the electrophilic warhead group that forms a covalent bond with the catalytic cysteine [215]. Classical nitrile group warheads are metabolically stable, polar and small, but proved to be less potent than oxime and aldehyde warheads. Substitutions at the P_1 and P_3 residues alter the inhibitory potency and provide a means of modulating specificity. Ideally, a successful drug specifically recognizes the parasitic proteases over the host caspases, calpains, and cathepsins by forming specific non-covalent interactions adjacent to the active site, but obtaining this level of selectivity is challenging. Covalent binding is often irreversible, and one drawback of permanent attachment to peptide fragments after protein degradation is the immunogenicity of these fragments.

Currently ten FDA-approved HIV protease inhibitors are approved, and nine available, with structures that were thought to mimic the substrate transition state [216]. HIV protease inhibitors show off-target interference with proteases required for maturation of SREBP-1, a transcription factor that regulates gene expression in lipogenesis, with as result lipodystrophy syndrome; and blocking of glucose transporter-4 with as result insulin resistance; inhibition of the proteasome, resulting in metabolic complications, increased ER stress and autophagy; and caspase-dependent apoptosis, the discovery of which triggered

interest in HIV protease inhibitors as potential anticancer drugs. The emergence of HIV-1 strains that are resistant to the current protease inhibitor drugs prompted the design of novel compounds with broad-spectrum activity against these variants [217,218]. Small non-peptide molecules with substituted pyrrolidines, piperidines and thiazolidines as P2-P3 ligands for binding to the S2-S3 specificity site, and flexible macrocyclic P1'-P2' tethers were good candidates, with inhibition constants (K_i) and IC_{50} values in the nanomolar range. Incorporation of heteroatoms in the macrocyclic skeleton yielded inhibitors with picomolar K_i and nanomolar IC50 antiviral activity. Biological evaluation, structure-activity relationships, and X-ray studies of the protease-inhibitor complexes validated the design approach, illustrating the power of structure-based molecular design. Peptide ketoamide-based NS3/4A serine protease inhibitors (boceprevir and telaprevir) are used for treating genotype 1 hepatitis C (HCV) [219]. In August 2017 Abbvie released Mavyret, a combination drug of glecaprevir/pibrentasvir targeting NS3/4A serine protease activity and the NS5A replication complex, and suitable for treatment of all HCV genotypes. However, complications may arise in HCV/hepatitis B (HBV) co-infected patients who completed treatment with HCV direct-acting antivirals and were not receiving HBV antiviral therapy. Fulminant hepatitis, hepatic failure, hepatitis flare, HBV reactivation and death have been reported.

THE FUTURE OF PROTEOLYSIS-RELATED DRUG AND DIAGNOSTIC DEVELOPMENT

Active Site Targeting, Exosite and Effector Binding Sites

Active site targeting is a main component in many established approaches of drug development for controlling protease activity. However, small molecule inhibitors that are limited to interference with the conserved catalytic machinery of an entire class of proteases may have severe limitations due to their broad spectrum activity, resulting in off-target protease inhibition. This was illustrated by the failure of many small molecule and zinc-targeting MMP inhibitors in clinical trials. Early irreversible inhibitors used active site targeting of nucleophilic proteases with electrophilic alkylating agents such as diazo- or halo-ketone warheads, however the necessity of attaching a sizeable peptide to the warhead for specific protease binding proved impractical as large libraries of peptide warheads were required to identify effective inhibitors. The development of sitagliptin, an inhibitor of dipeptidyl peptidase 4 (DPP4) in type 2 diabetes is a success story after a few consecutive setbacks [220]. DPP-4 inhibitors threo- and allo-isoleucyl thiazolidide initially showed significant animal toxicity due to off-target reaction with DPP8 and DPP9. Alpha amino compounds related to isoleucyl thiazolidide proved non-selective, but structure-activity screening identified a highly selective but rapidly metabolized beta-amino acid piperazine series. Bicyclic derivatization resulted in triazolopiperazine compounds that had suitable preclinical pharmacokinetic properties. Optimization led to the discovery of the highly selective sitagliptin.

Pitfalls in inhibitor design based on substrate interaction with the active site are illustrated by the development of transition state analogs against HIV protease. Enzyme transition states are very short-lived, on the femto- to picosecond timescale, but binding of transition

state analogs converts these to a stable thermodynamic state. Kinetic isotope effects and computational chemistry identify which chemical steps are involved in transition state binding. Typically, these analogs can bind up to millions of times tighter than substrates, making them attractive compounds in drug development [221]. The HIV-1 protease – substrate complex has three transition states with partial bonds in the reaction coordinate, and two intermediates with equilibrated bonds. The high-energy intermediates bind tightly to the protease, as do inhibitors designed as mimics of these intermediates. Ten FDA-approved competitive HIV-1 protease inhibitors, with Saquinavir as first and prototypical drug, were originally considered transition state analogs because they have an sp3 center to mimic the geometry of the transition state, but they were later actually found to be intermediate mimics. Ile84Val and Leu90Met and several other mutations in HIV protease were symptoms of emerging drug resistance toward these inhibitors [222], prompting investigators to take a closer look at the protease transition states. The crystal structures of the transition states of both native and protease-inhibitor-resistant HIV-1 proteases showed that they are chemically and structurally identical, which means that resistance is due to changes outside the true transition state [223]. Mimicking specific chemical features of the true transition state may solve this resistance problem.

The active site of proteases forms a groove that accommodates several substrate residues adjacent to the scissile P_1-P_1′ bond. Substrates exhibit complementarity with the protease residues S_4-S_3-S_2-S_1-S_1′-S_2′-S_3′-S_4′ in the binding site, causing a favorable binding interaction. The architecture of the S_1 or specificity pocket often defines the nature of substrate cleavage, *e.g.* hydrophobic and aromatic P_1 substrate residues for chymotrypsin-like proteases, basic P_1 residues for trypsin-like proteases, small aliphatic P_1 residues for elastase-like proteases, and hydrophobic bonds for aspartate proteases. Cysteine proteases prefer bulky nonpolar residues at the P_2 position. The MMP substrate specificity is more involved, with the S_1′ pocket selectively accommodating the substrate residue immediately after the scissile bond [224]. MMP-1 and −7 have small S_1′ pockets preferring small hydrophobic residues, whereas MMP-2, −3, −8, −9 and −13 have large pockets and bind a diverse array of amino acids. Additional allosteric, exosite and effector-binding site interactions are expected to contribute significantly to exclusive selection of the target protease. Structural conservation of the specificity site throughout a protease family with diverse catalytic properties and biological targets may pose a problem in designing specific drugs, and a promising alternative was developed by targeting zymogen activation rather than the active protease [204]. A highly selective compound that allosterically inhibits MMP-9 activation by binding to a pocket near the zymogen cleavage site may be a first viable drug candidate.

Identification of protease subsite preferences can be achieved by positional scanning to identify the best fit. Peptides spanning the active site cleft carry a fluorophore and an internal quencher, and preferential cleavage of peptide libraries is determined from fluorescence yields upon proteolytic removal of the quencher [192]. High-throughput screening (HTS) and fragment-based screening do not require previous knowledge of substrate specificity and may yield rapid results, but need appropriate filtering by functional activity to eliminate nonselective reactions. Fragment-based screening using NMR, mass spectrometry or differential scanning fluorimetry identifies moderate to weak binders that

can be optimized into more potent inhibitors. The use of X-ray crystallographic structural information coupled with *in silico* drug design is getting a boost from rapidly developing high-throughput X-ray crystallization and structure determination, using TRAP screens with the most successful crystallization conditions [225]. High-throughput crystallographic screening of brominated fragment libraries, based on anomalous scattering to localize bromine, successfully identified targets for HIV protease, and detected novel binding sites in the surface-exposed active site glycine-rich β-hairpin flap region and the exosite region [226].

The importance of exosite interactions in protease inhibition is illustrated by the thrombin inhibitor, hirudin, a polypeptide produced by the salivary glands of medicinal leeches such as *Hirudo medicinalis*. Hematophagous animals have a need for a natural anticoagulant to prevent their food from clotting. The crystal structure of the hirudin – thrombin complex shows a globular N-terminal domain making contact with the active site, and a 17-residue extended C-terminal chain wrapping around thrombin exosite I, the anion-binding site (ABE I) that binds the thrombin substrate, fibrinogen [227]. For this reason hirudins are sometimes referred to as bivalent direct thrombin inhibitors [228]. This dual interaction confers tight binding, and the classification as a slow tight-binding inhibitor indicates that the off-rate for inhibitor dissociation is extremely slow. Several recombinant hirudins are on the market. They have a short half-life, but may be preferred to heparin as an anticoagulant in patients with heparin-induced thrombocytopenia. Thrombin exosites also figure prominently in its irreversible inactivation by the serpins antithrombin and heparin cofactor II (HCII). Thrombin ABE I binds fibrinogen, and exosite II (ABE II) binds heparin. Because heparin accelerates the irreversible inactivation of thrombin by endogenous serpins, it is sometimes called an indirect anticoagulant. A long heparin template binds both thrombin via ABE II and antithrombin or HCII via their heparin-binding site. This approximation causes a dramatic increase in the thrombin inactivation rate. HCII provides an extra interaction by binding of its N-terminus to thrombin ABE I. Fragment screening against caspase-7 identified two small molecule non-competitive inhibitors with potential for drug development [229]. X-ray crystallography showed allosteric binding at the caspase dimer interface, more than 17 Å removed from the active site. This recent finding illustrates that allosteric control is yet another approach toward drug development.

Lysosomal cysteine cathepsins are required for normal lipid metabolism and cholesterol homeostasis, proper function of mitochondria and clearance of apoptotic cells [230]. A deregulated lipid metabolism, vascular inflammation, arterial remodeling, neovascularization, autophagy and necrosis are all hallmarks of atherosclerosis, and these processes are linked to upregulated cysteine cathepsin activity. Because vascular lesions in atherosclerotic patients may remain silent until the disease is well advanced, suitable biomarkers for these dysregulated pathways would be useful. Detection of cysteine cathepsins in macrophages allows distinguishing between stable and unstable lesions in excised carotid plaques, and this information from the plaque micro-environment may be harnessed in the development of molecular imaging. With this non-invasive technique, localized cleavage or covalent retention of specific protease substrates and inhibitors carrying fluorophores, reporter groups or contrast agents can be detected. Substrate-based probes change their spectral properties upon cleavage, and the commercial self-quenched

poly-lysine cathepsin probe Prosense has shown usefulness in preclinical cardiovascular imaging studies for detection of vascular inflammation, macrophage concentration and cathepsin activity. Newer, lipidated cathepsin substrates show promise due to their improved homing properties. Quenched activity-based probes (qABPs) for cysteine cathepsin activity have the quencher QSY21 attached to a near-infrared fluorophore Cy5-labeled acyloxymethyl-ketone analog. The cell-permeable probe covalently modifies the target cathepsin, resulting in loss of the quenching group, and formation of a fluorescently labeled target protease [231]. This type of probe has a low nonspecific fluorescent background and has been successfully applied for detecting cysteine cathepsin activity in tumor cells, and in preclinical models of atherosclerosis. Reduced light penetrance is a major issue, necessitating further development of multi-modal ABPs for PET-CT application [232]. This imaging approach has already been used successfully to localize idiopathic pulmonary fibrosis in patients, and may be adapted to detect rupture-prone atherosclerotic lesions.

Indirect, Mechanism-Based Targeting

Cytokines and chemokines are activated by uncontrolled protease action in many immune pathologies, and these proteins and their receptors may be potential therapeutic targets themselves. Cytokine storms typically occur as a reaction of a healthy immune system against new, highly pathogenic invaders, and are thought to be responsible for many deaths in influenza pandemics. Cleavage of the influenza hemagglutinin by host trypsin-like proteases is required for infectivity, and IL-1β was identified as the major cytokine that upregulates host trypsin expression and triggers formation of more IL-1β [233]. An anti-IL-1β antibody successfully suppressed upregulation of pro-inflammatory cytokines and trypsin in a mouse model, and antibodies against IL-1β and its receptor have been proposed as potential therapeutics. Drug targeting of non-protease components of the complement network involve the activation and amplification pathways, with peptides (AMY-101, APL-1 and −2), proteases (CB2782) and protein inhibitors (AMY-201, Mirococept) targeting C3 and C3b; and inhibitors of the terminal pathways, with antibodies (eculizumab), a tick saliva protein (Coversin) and aptamers (Zimura), binding to C5 and preventing C5 convertase activity [199]. The complement component C5a, generated in the terminal pathway of complement activation, is a chemokine, and attracts leukocytes to the inflammatory focus. Patients with paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome exhibit uncontrolled complement activation, and the recombinant tick protein prevents release of C5a and formation of C5b–9, the membrane attack complex (MAC). The natural function of the saliva protein in *Ornithodoros moubata* is suppression of the host immune response when the tick is feeding. The therapeutic protein is also effective in patients with C5 polymorphisms who are resistant to eculizumab.

Protease-activated receptors have long been considered potential drug targets, and the therapeutic applications of PAR-1 and PAR-4 antagonists are discussed in a previous section. In preclinical models, a PAR-2 antagonist inhibited tumor growth and the formation of new blood vessels in cancer, and inflammation in rheumatoid arthritis and acute inflammation models, perhaps indicating a potential link between inflammation and cancer [234,235]. Strategies for developing suitable PAR antagonists include modified peptidomimetics such as trans-cinnamoyl-YPGKF-NH_2 that bind but not activate the

receptor; low molecular weight heterocyclic structures *e.g.* 1-benzyl-3-(ethoxycarbonylphenyl)-indazole; N-terminal palmitate-modified oligopeptides (pepducins) that anchor the peptide to the cell membrane; and specific function-blocking monoclonal antibodies [236]. The majority of these efforts is ongoing, and focused on PAR-4 antagonists as novel anti-platelet agents.

Drugging the Undruggable – a Familiar Theme, with some Fancy Targeting

Designing substrates, inhibitors and activity-based probes with specificity toward a single protease is a major challenge when the protease shares a similar catalytic mechanism and substrate specificity with other proteases in the same family, but is functionally completely different [237]. The main approaches utilized to date for optimizing complementarity with the protease specificity sites include positional scanning synthetic combinatorial libraries with coumarin-derived reporters (PS-SCL) [238], phage display, hybrid combinatorial substrate libraries using unnatural amino acids that allow more thorough scanning of the active site (HyCoSuL), counter selection substrate libraries (CoSeSuL), internally quenched fluorescent substrate or fluorescence resonance energy transfer libraries (IQF or FRET), proteomics and exopeptidase fingerprinting. HyCoSuL screening of the P_3 and P_4 positions respectively with methionine sulfone and 2-amino-6-benzyloxyhexanoic acid allowed distinguishing between neutrophil elastase and proteinase-3 for the first time, and tracking neutrophil elastase activity in neutrophil traps [239]. Whereas PS-SCL, HyCoSuL and CoSeSuL allow determination of protease preferences only in prime active site pockets, IQF can be used to refine complementarity to both prime and non-prime pockets. The advantage of phage display is its ability to generate large and diverse substrate arrays, up to 10^{10} peptides, and enrich for specificity after each cycle, which is not feasible with chemical synthesis. The label-free nature of this method requires individual kinetic analysis and reporter group labeling of positive hits. Multiplex substrate profiling with liquid chromatography – tandem mass spectrometry sequencing proved successful for distinguishing neutral serine protease activity in human neutrophil extracellular traps toward a large and diverse tetradecapeptide panel, and ranking granzyme B substrate efficiency, using label-free quantitation of precursor-ion abundance [240]. The above techniques have also allowed differentiation between various diverse members of the granzyme, kallikrein, caspase, metalloproteinase, exopeptidase, deubiquitinating and desumoylating protease families. A novel approach of profiling protease specificity is currently being developed, combining yeast endoplasmatic reticulum (ER) sequestration with next-generation sequencing (YESS-NGS) [241]. A substrate library, targeted to the ER, is exposed to ER proteases as it transports through the secretory pathway, and cleaved/uncleaved substrates localize to the cell surface. FACS analysis of cells labeled with fluorophore-conjugated antibodies specifically detects substrate cleavage. Characterization of proteolytic processing in secretory pathways may be useful to detect changes in the secretome in various disease states.

Recent, targeted “intractable” protein degradation methodology uses heterobifunctional chemistry for simultaneous binding of proteins inside cells, which tags them for degradation by the cell’s own ubiquitin-proteasome system. The small molecule drug candidate uses a protein-binding domain linked to a ubiquitin ligase-binding domain, with the goal of

eliminating pathological or defective intracellular proteins. This approach is currently developed by Kymera. A variant of this method is targeted proteolysis of endogenous proteins via the affinity-directed protein missile (AdPROM) system [242], that harbors the von Hippel-Lindau (VHL) protein, the substrate receptor of the Cullin2 (CUL2) E3 ligase complex, tethered to polypeptide binders that selectively bind and recruit endogenous target proteins to the CUL2-E3 ligase complex for ubiquitination and proteasomal degradation. Synthetic monobodies and a camelid-derived VHH nanobody were used in a feasibility model to target the tyrosine phosphatase SHP2 and the inflammasome protein ASC for degradation. This method has advantages over CRISPR/Cas9-mediated gene knockouts that are irreversible and may not always be feasible, and over RNA interference that requires prolonged treatment and may be incomplete. Both methods may have off-target effects. A possible breakthrough in nanomedicine may be the construction of a “DNA vault”, a DNA origami nanodevice that locks up a single enzyme molecule, and that can be opened and closed by DNA locks to regulate access to substrate [243]. In a proof-of-principle model, chymotrypsin was covalently anchored to an open DNA vault, and after closure a FITC-casein substrate was added, together with the opening key or a control key. Enzymatic activity was detected predominantly in the reaction containing the opening key. This technology can be refined to program natural enzymes to operate as signal amplifiers for diagnostics applications and as delivery vehicles for therapeutic applications.

Discovery of Protease and Inhibitor Biomarkers for Disease

Conventional shotgun proteomics for biomarker discovery lack sensitivity and selectivity because they focus mainly on quantitative rather than qualitative differences between the normal and the diseased state. These approaches are also ill-suited for detecting pathologic post-translational modifications. Novel MS-based proteomics techniques have the potential of identifying newly generated N- and C-termini that are characteristic in disease-related proteolysis. The TAILS and C-TAILS techniques enrich for protein N- and C-terminal peptides by polymer-based removal of internal peptides generated by tryptic digestion, and improve detection limits by several orders of magnitude [154]. The differential processing of APP protein in Alzheimer’s disease, and of the chemokines CCL7 and SDF1 in arthritis and HIV-associated dementia, respectively, are prime examples of neo-terminus formation. N- and C-terminal peptide removal may activate or inactivate chemokines, turn them into receptor antagonists or change their receptor specificity. As a result, simple quantitation of chemokine expression is not sufficient to define the extent of the pathological inflammatory response, but quantitation of neo-terminus formation. A fraction of Aβ peptides in Alzheimer’s patients is N-truncated, with a cyclized terminal glutamate post-translational modification ($A\beta_{3(Pe)}$), and this species may be a promising biomarker for early detection of Alzheimer’s disease. A search of the TopFIND public database [244] shows that many FDA-approved biomarker proteins have multiple different N- and C-termini, potentially affecting their biological activity and “visibility” in current assays. Specific disease-related protease assays may be confounded by endogenous and exogenous inhibitors, and varying concentrations of cofactors and competing proteases in biological samples. A combination of targeted MS and neo-terminal-directed antibody assays may vastly improve the development of reliable biomarkers.

Novel functional proteomics techniques such as bead-based proteome enrichment combined with the 2D-electrophoresis-based Protein Elution Plate (PEP) also prove extremely useful for rapid, convenient profiling of functional protease activity in physiological and pathological patient samples [245]. This versatile plate reader technology uses specific protease substrates with various degrees of sensitivity and allows functional analysis of the protease landscape beyond the information available from protein abundance measurements.

The pursuit of biomarker development within a Functional Proteomics context is addressed elsewhere in this book ("Making the Case for Functional Proteomics" and "Methods to Monitor the Functional Subproteomes of SERPIN Protease Inhibitors"). Some of the observations of those sections are re-viewed here through the lens of proteolytic activity. Historically, biomarkers that drive drug and/or diagnostic discovery comprise a single entity, be it a metabolite, waste product or protein. In no small part that pattern reflects technology limitations of the time, not a preferred indicator of biological functionality. Further, of the approximately 2,000 in vitro protein assays approved by the US Food and Drug Administration, only seven assays reference protease activity (and some of those are redundant for a given assay). This apparent lack of emphasis also points to limitations in proteolytic assay procedures, especially in media such as body fluids. Finally, as is abundantly apparent from the information presented in this chapter, the activity of a single protease is an unreliable indicator of physiological activity. A wider net must be cast.

For any functionally defined biological process, e.g. apoptosis or pathogen response, multiple proteolytic events occur in (relatively) linear sequences or in (parallel) cascades. Thus, any proposed therapeutic, regardless of selectivity at the level of a single entity, modulates multiple outcomes. And, any diagnostic must reflect that multi-entity process. The odds that a single proteolytic event or single intermediate or end product will define that process are slim indeed. The logic seems inescapable that biomarker development of the future must focus on biological networks, not single entities. Pioneering efforts are occurring as this is written.

An example: one diagnostic in development [246] for cancer "Stroma Liquid Biopsy™" demonstrates a network (or pattern [247])-based analysis of LC/MS data, connecting multiple networks or pathways that rely profoundly on proteolytic activity (coagulation, complement and inflammation). The discovery and development sequence for such biomarker development is addressed elsewhere in this book ("Making the Case …"). Application of the diagnostic in the clinic relies on selection of a test "subproteome" as described in "Methods to Monitor the Functional Subproteomes of SERPIN Protease Inhibitors." It's noted that not only does the "Stroma Liquid Biopsy™" apparently differentiate serum from cancer patients, the pattern on which it is based informs cancer biology. Further, the identified subproteome(s) become a relevant test bed for screening new therapeutics.

The future is integrated discovery of network-based biomarkers that support therapeutic and diagnostic discovery while informing both biology and disease. Detection of protease activity per se in complex media will likely require development of new methods.

A Two-Protein Wrap-Up

This chapter presents, in text and tabulated formats, the diversity of proteases and protease-related activity. Perhaps the most compelling overall observation is the degree of overlap among physiological processes and disease states at the level of individual proteases. This is most readily seen in the tabulated summary of proteases, inhibitors and activity. A thumbnail analysis shows that half of all listed proteases are active in multiple physiological processes and/or associated with multiple disease states. Chief among the multi-process activities are the cathepsins, followed by the caspases and MMPs. The chapter closes with an examination of the multiple associations with physiological processes and disease states of two related proteins: the protease thrombin and the proteolysis product fibrin (gene expression products, prothrombin and fibrinogen).

Textbooks cite the cleavage of fibrinogen by thrombin to form fibrin as a central event in formation of blood clots formed in response to injury. This common response is certainly beneficial. However, other actions and associations of thrombin and fibrin are of a darker or unexpected nature (Figure 2).

In addition to its participation in blood clot formation, thrombin also triggers proteases that dissolve clots [20]. Thus thrombin (and prothrombin) is associated with both thrombosis and uncontrolled bleeding. Elevated fibrin(ogen) levels track CV disease severity [248], and elevated levels of fibrin are found in diabetic patients [249]. In cancer, thrombin activates key pathways and fibrin is found in tumors. Thrombin is often cited as a neurotoxin [250] and is implicated in both Alzheimer’s Disease and peripheral neuropathy. Fibrin is found in AD plaques and may be oligomerized by amyloid beta [251]. Fibrinogen is hijacked by bacteria and through non-enzymatic pathways forms fibrin barriers to evade cellular defenses [101,185]. Finally, thrombin cleaves complement C3 [252], thus igniting the innate immune system independent of any triggers associated with infection. This latter event may explain, in part, thrombin’s apparent and destructive ability to trigger inflammatory and immune responses. Proteolytic events govern the life cycle of all proteins, as was noted in the Introduction to this chapter.

The clear lesson of the contents of this chapter is that proteolysis is central to the functioning of the organism, indeed can reshape the organism. Proteolytic activity is essential to multiple physiological processes and, specific proteases are central to multiple processes. It is not surprising, therefore, that disease is also strongly connected to proteolysis. Indeed, even at the resolution of a single protease, activity is not always to the benefit of the organism. As was demonstrated in the paragraph immediately above, what might be called the side effects of proteolysis can prove quite harmful. Of particular interest is the tantalizing correlation of proteolytic activity and chronic disease.

Within the context of Functional Proteomics, whether the scale is that of reshaping individual proteins or determining the state of the organism, proteolytic events are foundational.

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References

1. Fruton JS (2002) A history of pepsin and related enzymes. Q Rev Biol 77 (2):127–147 [PubMed: 12089768]
2. Northrop JH, Kunitz M, Herriott RM (1938) Crystalline Enzymes. Columbia Univ. Press, New York,
3. Neurath H (1999) Proteolytic enzymes, past and future. Proc Natl Acad Sci U S A 96 (20):10962–10963 [PubMed: 10500108]
4. Rawlings ND, Barrett AJ, Finn R (2016) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 44 (D1):D343–350. doi:10.1093/nar/gkv1118 [PubMed: 26527717]
5. Kappelhoff R, Puente XS, Wilson CH, Seth A, Lopez-Otin C, Overall CM (2017) Overview of transcriptomic analysis of all human proteases, non-proteolytic homologs and inhibitors: Organ, tissue and ovarian cancer cell line expression profiling of the human protease degradome by the CLIP-CHIP DNA microarray. Biochim Biophys Acta 1864 (11 Pt B):2210–2219. doi:10.1016/j.bbamcr.2017.08.004
6. Perez-Silva JG, Espanol Y, Velasco G, Quesada V (2016) The Degradome database: expanding roles of mammalian proteases in life and disease. Nucleic Acids Res 44 (D1):D351–355. doi:10.1093/nar/gkv1201 [PubMed: 26553809]
7. Rawlings ND, Salvesen G (2013) Handbook of proteolytic enzymes. Third edition. / edn. Elsevier/AP, Amsterdam
8. Turk B, Turk D, Turk V (2012) Protease signalling: the cutting edge. EMBO J 31 (7):1630–1643. doi:10.1038/emboj.2012.42 [PubMed: 22367392]
9. Gettins PG, Olson ST (2016) Inhibitory serpins. New insights into their folding, polymerization, regulation and clearance. Biochem J 473 (15):2273–2293. doi:10.1042/BCJ20160014 [PubMed: 27470592]
10. Laskowski M Jr., Kato I (1980) Protein inhibitors of proteinases. Annu Rev Biochem 49:593–626. doi:10.1146/annurev.bi.49.070180.003113 [PubMed: 6996568]
11. Fortelny N, Cox JH, Kappelhoff R, Starr AE, Lange PF, Pavlidis P, Overall CM (2014) Network analyses reveal pervasive functional regulation between proteases in the human protease web. PLoS Biol 12 (5):e1001869. doi:10.1371/journal.pbio.1001869 [PubMed: 24865846]
12. Rechsteiner M, Rogers SW (1996) PEST sequences and regulation by proteolysis. Trends Biochem Sci 21 (7):267–271 [PubMed: 8755249]
13. Lopez-Otin C, Bond JS (2008) Proteases: multifunctional enzymes in life and disease. J Biol Chem 283 (45):30433–30437. doi:10.1074/jbc.R800035200 [PubMed: 18650443]
14. Proteases in human diseases (2017). Springer Singapore, New York, NY
15. Macfarlane RG (1964) An Enzyme Cascade in the Blood Clotting Mechanism, and Its Function as a Biochemical Amplifier. Nature 202:498–499 [PubMed: 14167839]
16. Gailani D, Broze GJ Jr. (1993) Factor XI activation by thrombin and factor XIa. Semin Thromb Hemost 19 (4):396–404. doi:10.1055/s-2007-993291 [PubMed: 8140432]
17. Gailani D, Renne T (2007) Intrinsic pathway of coagulation and arterial thrombosis. Arterioscler Thromb Vasc Biol 27 (12):2507–2513. doi:10.1161/ATVBAHA.107.155952 [PubMed: 17916770]
18. Gailani D, Renne T (2007) The intrinsic pathway of coagulation: a target for treating thromboembolic disease? J Thromb Haemost 5 (6):1106–1112. doi:10.1111/j.1538-7836.2007.02446.x [PubMed: 17388803]
19. Nesheim M, Bajzar L (2005) The discovery of TAFI. J Thromb Haemost 3 (10):2139–2146. doi:10.1111/j.1538-7836.2005.01280.x [PubMed: 16194190]
20. Bode W (2006) The structure of thrombin: a janus-headed proteinase. Semin Thromb Hemost 32 Suppl 1:16–31. doi:10.1055/s-2006-939551 [PubMed: 16673263]

21. Huntington JA (2008) How Na+ activates thrombin--a review of the functional and structural data. Biol Chem 389 (8):1025–1035. doi:10.1515/BC.2008.113 [PubMed: 18979627]
22. Di Cera E (2007) Thrombin as procoagulant and anticoagulant. J Thromb Haemost 5 Suppl 1:196–202. doi:10.1111/j.1538-7836.2007.02485.x [PubMed: 17635727]
23. Trouw LA, Pickering MC, Blom AM (2017) The complement system as a potential therapeutic target in rheumatic disease. Nat Rev Rheumatol 13 (9):538–547. doi:10.1038/nrrheum.2017.125 [PubMed: 28794515]
24. Sim RB, Laich A (2000) Serine proteases of the complement system. Biochem Soc Trans 28 (5): 545–550 [PubMed: 11044372]
25. Cooper NR, Muller-Eberhard HJ (1970) The reaction mechanism of human C5 in immune hemolysis. J Exp Med 132 (4):775–793 [PubMed: 5508377]
26. Dobo J, Szakacs D, Oroszlan G, Kortvely E, Kiss B, Boros E, Szasz R, Zavodszky P, Gal P, Pal G (2016) MASP-3 is the exclusive pro-factor D activator in resting blood: the lectin and the alternative complement pathways are fundamentally linked. Sci Rep-Uk 6 10.1038/srep31877
27. Sciascia S, Radin M, Yazdany J, Tektonidou M, Cecchi I, Roccatello D, Dall'Era M (2017) Expanding the therapeutic options for renal involvement in lupus: eculizumab, available evidence. Rheumatol Int 37 (8):1249–1255. doi:10.1007/s00296-017-3686-5 [PubMed: 28258475]
28. de Koning PJ, Kummer JA, de Poot SA, Quadir R, Broekhuizen R, McGettrick AF, Higgins WJ, Devreese B, Worrall DM, Bovenschen N (2011) Intracellular serine protease inhibitor SERPINB4 inhibits granzyme M-induced cell death. PLoS One 6 (8):e22645. doi:10.1371/journal.pone.0022645 [PubMed: 21857942]
29. Soriano C, Mukaro V, Hodge G, Ahern J, Holmes M, Jersmann H, Moffat D, Meredith D, Jurisevic C, Reynolds PN, Hodge S (2012) Increased proteinase inhibitor-9 (PI-9) and reduced granzyme B in lung cancer: mechanism for immune evasion? Lung Cancer 77 (1):38–45. doi:10.1016/j.lungcan.2012.01.017 [PubMed: 22387007]
30. Biancheri P, Di Sabatino A, Corazza GR, MacDonald TT (2013) Proteases and the gut barrier. Cell Tissue Res 351 (2):269–280. doi:10.1007/s00441-012-1390-z [PubMed: 22427120]
31. Ehlers MR (2014) Immune-modulating effects of alpha-1 antitrypsin. Biol Chem 395 (10):1187–1193. doi:10.1515/hsz-2014-0161 [PubMed: 24854541]
32. Boya P (2012) Lysosomal function and dysfunction: mechanism and disease. Antioxid Redox Signal 17 (5):766–774. doi:10.1089/ars.2011.4405 [PubMed: 22098160]
33. Schmidt M, Finley D (2014) Regulation of proteasome activity in health and disease. Biochim Biophys Acta 1843 (1):13–25. doi:10.1016/j.bbamcr.2013.08.012 [PubMed: 23994620]
34. Goldberg AL (2005) Nobel committee tags ubiquitin for distinction. Neuron 45 (3):339–344. doi:10.1016/j.neuron.2005.01.019 [PubMed: 15694320]
35. Tanaka K (2009) The proteasome: overview of structure and functions. Proc Jpn Acad Ser B Phys Biol Sci 85 (1):12–36
36. Antalis TM, Shea-Donohue T, Vogel SN, Sears C, Fasano A (2007) Mechanisms of disease: protease functions in intestinal mucosal pathobiology. Nat Clin Pract Gastroenterol Hepatol 4 (7): 393–402. doi:10.1038/ncpgasthep0846 [PubMed: 17607295]
37. Alloy AP, Kayode O, Wang RY, Hockla A, Soares AS, Radisky ES (2015) Mesotrypsin Has Evolved Four Unique Residues to Cleave Trypsin Inhibitors as Substrates. Journal of Biological Chemistry 290 (35):21523–21535. doi:10.1074/jbc.M115.662429 [PubMed: 26175157]
38. Giebeler N, Zigrino P (2016) A Disintegrin and Metalloprotease (ADAM): Historical Overview of Their Functions. Toxins (Basel) 8 (4):122. doi:10.3390/toxins8040122 [PubMed: 27120619]
39. Rodriguez D, Morrison CJ, Overall CM (2010) Matrix metalloproteinases: what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochim Biophys Acta 1803 (1):39–54. doi:10.1016/j.bbamcr.2009.09.015 [PubMed: 19800373]
40. Freitas-Rodriguez S, Folgueras AR, Lopez-Otin C (2017) The role of matrix metalloproteinases in aging: Tissue remodeling and beyond. Biochim Biophys Acta 1864 (11 Pt A):2015–2025. doi:10.1016/j.bbamcr.2017.05.007
41. Brew K, Nagase H (2010) The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochim Biophys Acta 1803 (1):55–71. doi:10.1016/j.bbamcr.2010.01.003 [PubMed: 20080133]

42. Coughlin SR (2005) Protease-activated receptors in hemostasis, thrombosis and vascular biology. J Thromb Haemost 3 (8):1800–1814. doi:10.1111/j.1538-7836.2005.01377.x [PubMed: 16102047]
43. Fender AC, Rauch BH, Geisler T, Schror K (2017) Protease-Activated Receptor PAR-4: An Inducible Switch between Thrombosis and Vascular Inflammation? Thromb Haemost 117 (11): 2013–2025. doi:10.1160/TH17-03-0219 [PubMed: 29044290]
44. Takamori N, Azuma H, Kato M, Hashizume S, Aihara K, Akaike M, Tamura K, Matsumoto T (2004) High plasma heparin cofactor II activity is associated with reduced incidence of in-stent restenosis after percutaneous coronary intervention. Circulation 109 (4):481–486. doi: 10.1161/01.CIR.0000109695.39671.37 [PubMed: 14744972]
45. Nierodzik ML, Karpatkin S (2006) Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype. Cancer Cell 10 (5):355–362. doi: 10.1016/j.ccr.2006.10.002 [PubMed: 17097558]
46. Asanuma K, Wakabayashi H, Okamoto T, Asanuma Y, Akita N, Yoshikawa T, Hayashi T, Matsumine A, Uchida A, Sudo A (2013) The thrombin inhibitor, argatroban, inhibits breast cancer metastasis to bone. Breast Cancer 20 (3):241–246. doi:10.1007/s12282-012-0334-5 [PubMed: 22359194]
47. McIlwain DR, Berger T, Mak TW (2013) Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol 5 (4):a008656. doi:10.1101/cshperspect.a008656 [PubMed: 23545416]
48. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91 (4):479–489 [PubMed: 9390557]
49. Zhivotovsky B, Samali A, Gahm A, Orrenius S (1999) Caspases: their intracellular localization and translocation during apoptosis. Cell Death Differ 6 (7):644–651. doi:10.1038/sj.cdd.4400536 [PubMed: 10453075]
50. Li P, Zhou L, Zhao T, Liu X, Zhang P, Liu Y, Zheng X, Li Q (2017) Caspase-9: structure, mechanisms and clinical application. Oncotarget 8 (14):23996–24008. doi:10.18632/oncotarget. 15098 [PubMed: 28177918]
51. Vu NT, Park MA, Shultz JC, Goehe RW, Hoeferlin LA, Shultz MD, Smith SA, Lynch KW, Chalfant CE (2013) hnRNP U enhances caspase-9 splicing and is modulated by AKT-dependent phosphorylation of hnRNP L. J Biol Chem 288 (12):8575–8584. doi:10.1074/jbc.M112.443333 [PubMed: 23396972]
52. Kuida K (2000) Caspase-9. Int J Biochem Cell Biol 32 (2):121–124 [PubMed: 10687948]
53. Blasche S, Mortl M, Steuber H, Siszler G, Nisa S, Schwarz F, Lavrik I, Gronewold TM, Maskos K, Donnenberg MS, Ullmann D, Uetz P, Kogl M (2013) The E. coli effector protein NleF is a caspase inhibitor. PLoS One 8 (3):e58937. doi:10.1371/journal.pone.0058937 [PubMed: 23516580]
54. Li P, Nijhawan D, Wang X (2004) Mitochondrial activation of apoptosis. Cell 116 (2 Suppl):S57–59, [PubMed: 15055583]
55. Denault JB, Eckelman BP, Shin H, Pop C, Salvesen GS (2007) Caspase 3 attenuates XIAP (X-linked inhibitor of apoptosis protein)-mediated inhibition of caspase 9. Biochem J 405 (1):11–19. doi:10.1042/BJ20070288 [PubMed: 17437405]
56. Creagh EM (2014) Caspase crosstalk: integration of apoptotic and innate immune signalling pathways. Trends Immunol 35 (12):631–640. doi:10.1016/j.it.2014.10.004 [PubMed: 25457353]
57. Wang XJ, Cao Q, Liu X, Wang KT, Mi W, Zhang Y, Li LF, LeBlanc AC, Su XD (2010) Crystal structures of human caspase 6 reveal a new mechanism for intramolecular cleavage self-activation. EMBO Rep 11 (11):841–847. doi:10.1038/embor.2010.141 [PubMed: 20890311]
58. Graham RK, Ehrnhoefer DE, Hayden MR (2011) Caspase-6 and neurodegeneration. Trends Neurosci 34 (12):646–656. doi:10.1016/j.tins.2011.09.001 [PubMed: 22018804]
59. Bartel A, Gohler A, Hopf V, Breitbach K (2017) Caspase-6 mediates resistance against Burkholderia pseudomallei infection and influences the expression of detrimental cytokines. PLoS One 12 (7):e0180203. doi:10.1371/journal.pone.0180203 [PubMed: 28686630]
60. Sollberger G, Strittmatter GE, Garstkiewicz M, Sand J, Beer HD (2014) Caspase-1: the inflammasome and beyond. Innate Immun 20 (2):115–125. doi:10.1177/1753425913484374 [PubMed: 23676582]

61. Duclos C, Lavoie C, Denault JB (2017) Caspases rule the intracellular trafficking cartel. FEBS J 284 (10):1394–1420. doi:10.1111/febs.14071 [PubMed: 28371378]
62. Julien O, Wells JA (2017) Caspases and their substrates. Cell Death Differ 24 (8):1380–1389. doi: 10.1038/cdd.2017.44 [PubMed: 28498362]
63. Aziz M, Jacob A, Wang P (2014) Revisiting caspases in sepsis. Cell Death Dis 5:e1526. doi: 10.1038/cddis.2014.488 [PubMed: 25412304]
64. Pemberton CJ (2014) Signal peptides: new markers in cardiovascular disease? Biomark Med 8 (8): 1013–1019. doi:10.2217/bmm.14.64 [PubMed: 25343673]
65. Morocz M, Zsigmond E, Toth R, Enyedi MZ, Pinter L, Haracska L (2017) DNA-dependent protease activity of human Spartan facilitates replication of DNA-protein crosslink-containing DNA. Nucleic Acids Res 45 (6):3172–3188. doi:10.1093/nar/gkw1315 [PubMed: 28053116]
66. Stingele J, Habermann B, Jentsch S (2015) DNA-protein crosslink repair: proteases as DNA repair enzymes. Trends Biochem Sci 40 (2):67–71. doi:10.1016/j.tibs.2014.10.012 [PubMed: 25496645]
67. Vaz B, Popovic M, Newman JA, Fielden J, Aitkenhead H, Halder S, Singh AN, Vendrell I, Fischer R, Torrecilla I, Drobnitzky N, Freire R, Amor DJ, Lockhart PJ, Kessler BM, McKenna GW, Gileadi O, Ramadan K (2016) Metalloprotease SPRTN/DVC1 Orchestrates Replication-Coupled DNA-Protein Crosslink Repair. Mol Cell 64 (4):704–719. doi:10.1016/j.molcel.2016.09.032 [PubMed: 27871366]
68. Maskey RS, Flatten KS, Sieben CJ, Peterson KL, Baker DJ, Nam HJ, Kim MS, Smyrk TC, Kojima Y, Machida Y, Santiago A, van Deursen JM, Kaufmann SH, Machida YJ (2017) Spartan deficiency causes accumulation of Topoisomerase 1 cleavage complexes and tumorigenesis. Nucleic Acids Res 45 (8):4564–4576. doi:10.1093/nar/gkx107 [PubMed: 28199696]
69. Butler LR, Densham RM, Jia J, Garvin AJ, Stone HR, Shah V, Weekes D, Festy F, Beesley J, Morris JR (2012) The proteasomal de-ubiquitinating enzyme POH1 promotes the double-strand DNA break response. EMBO J 31 (19):3918–3934. doi:10.1038/emboj.2012.232 [PubMed: 22909820]
70. Pinto-Fernandez A, Kessler BM (2016) DUBbing Cancer: Deubiquitylating Enzymes Involved in Epigenetics, DNA Damage and the Cell Cycle As Therapeutic Targets. Front Genet 7:133. doi: 10.3389/fgene.2016.00133 [PubMed: 27516771]
71. Enari M, Sakahira H, Yokoyama H, Okawa K, Iwamatsu A, Nagata S (1998) A caspase-activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. Nature 391 (6662):43–50 [PubMed: 9422506]
72. Venkatesh S, Lee J, Singh K, Lee I, Suzuki CK (2012) Multitasking in the mitochondrion by the ATP-dependent Lon protease. Biochim Biophys Acta 1823 (1):56–66. doi:10.1016/j.bbamcr. 2011.11.003 [PubMed: 22119779]
73. Mao PL, Jiang Y, Wee BY, Porter AG (1998) Activation of caspase-1 in the nucleus requires nuclear translocation of pro-caspase-1 mediated by its prodomain. J Biol Chem 273 (37):23621–23624 [PubMed: 9726961]
74. Kamada S, Kikkawa U, Tsujimoto Y, Hunter T (2005) Nuclear translocation of caspase-3 is dependent on its proteolytic activation and recognition of a substrate-like protein(s). J Biol Chem 280 (2):857–860. doi:10.1074/jbc.C400538200 [PubMed: 15569692]
75. Hill JW, Poddar R, Thompson JF, Rosenberg GA, Yang Y (2012) Intranuclear matrix metalloproteinases promote DNA damage and apoptosis induced by oxygen-glucose deprivation in neurons. Neuroscience 220:277–290. doi:10.1016/j.neuroscience.2012.06.019 [PubMed: 22710064]
76. Eguchi T, Calderwood SK, Takigawa M, Kubota S, Kozaki KI (2017) Intracellular MMP3 Promotes HSP Gene Expression in Collaboration With Chromobox Proteins. J Cell Biochem 118 (1):43–51. doi:10.1002/jcb.25607 [PubMed: 27206651]
77. Stepanova V, Jayaraman PS, Zaitsev SV, Lebedeva T, Bdeir K, Kershaw R, Holman KR, Parfyonova YV, Semina EV, Beloglazova IB, Tkachuk VA, Cines DB (2016) Urokinase-type Plasminogen Activator (uPA) Promotes Angiogenesis by Attenuating Proline-rich Homeodomain Protein (PRH) Transcription Factor Activity and De-repressing Vascular Endothelial Growth Factor (VEGF) Receptor Expression. J Biol Chem 291 (29):15029–15045. doi:10.1074/ jbc.M115.678490 [PubMed: 27151212]

78. Antalis TM, Bugge TH, Wu Q (2011) Membrane-anchored serine proteases in health and disease. Prog Mol Biol Transl Sci 99:1–50. doi:10.1016/B978-0-12-385504-6.00001-4 [PubMed: 21238933]

79. Friis S, Sales KU, Schafer JM, Vogel LK, Kataoka H, Bugge TH (2014) The protease inhibitor HAI-2, but not HAI-1, regulates matriptase activation and shedding through prostasin. J Biol Chem 289 (32):22319–22332. doi:10.1074/jbc.M114.574400 [PubMed: 24962579]

80. Bardou O, Menou A, Francois C, Duitman JW, von der Thusen JH, Borie R, Sales KU, Mutze K, Castier Y, Sage E, Liu L, Bugge TH, Fairlie DP, Konigshoff M, Crestani B, Borensztajn KS (2016) Membrane-anchored Serine Protease Matriptase Is a Trigger of Pulmonary Fibrogenesis. Am J Respir Crit Care Med 193 (8):847–860. doi:10.1164/rccm.201502-0299OC [PubMed: 26599507]

81. Le Gall SM, Szabo R, Lee M, Kirchhofer D, Craik CS, Bugge TH, Camerer E (2016) Matriptase activation connects tissue factor-dependent coagulation initiation to epithelial proteolysis and signaling. Blood 127 (25):3260–3269. doi:10.1182/blood-2015-11-683110 [PubMed: 27114461]

82. Verhelst SHL (2017) Intramembrane proteases as drug targets. FEBS J 284 (10):1489–1502. doi: 10.1111/febs.13979 [PubMed: 27889944]

83. Dusterhoft S, Kunzel U, Freeman M (2017) Rhomboid proteases in human disease: Mechanisms and future prospects. Biochim Biophys Acta 1864 (11 Pt B):2200–2209. doi:10.1016/j.bbamcr. 2017.04.016

84. Saita S, Nolte H, Fiedler KU, Kashkar H, Venne AS, Zahedi RP, Kruger M, Langer T (2017) PARL mediates Smac proteolytic maturation in mitochondria to promote apoptosis. Nat Cell Biol 19 (4): 318–328. doi:10.1038/ncb3488 [PubMed: 28288130]

85. Ranganathan P, Weaver KL, Capobianco AJ (2011) Notch signalling in solid tumours: a little bit of everything but not all the time. Nat Rev Cancer 11 (5):338–351. doi:10.1038/nrc3035 [PubMed: 21508972]

86. Chauhan S, Mandal P, Tomar RS (2016) Biochemical Analysis Reveals the Multifactorial Mechanism of Histone H3 Clipping by Chicken Liver Histone H3 Protease. Biochemistry 55 (38): 5464–5482. doi:10.1021/acs.biochem.6b00625 [PubMed: 27586699]

87. Vossaert L, Meert P, Scheerlinck E, Glibert P, Van Roy N, Heindryckx B, De Sutter P, Dhaenens M, Deforce D (2014) Identification of histone H3 clipping activity in human embryonic stem cells. Stem Cell Res 13 (1):123–134. doi:10.1016/j.scr.2014.05.002 [PubMed: 24874291]

88. Deraison C, Bonnart C, Vergnolle N (2018) Proteases In: Cavaillon J-M, Singer M (eds) Inflammation: From Molecular and Cellular Mechanisms to the Clinic. Wiley-VCH, Weinheim, Germany, pp 727–766

89. Chotirmall SH, Al-Alawi M, McEnery T, McElvaney NG (2015) Alpha-1 proteinase inhibitors for the treatment of alpha-1 antitrypsin deficiency: safety, tolerability, and patient outcomes. Ther Clin Risk Manag 11:143–151. doi:10.2147/TCRM.S51474 [PubMed: 25673994]

90. Torres-Duran M, Ruano-Ravina A, Parente-Lamelas I, Abal-Arca J, Leiro-Fernandez V, Montero-Martinez C, Pena C, Castro-Anon O, Golpe-Gomez A, Gonzalez-Barcala FJ, Martinez C, Guzman-Taveras R, Provencio M, Mejuto-Marti MJ, Fernandez-Villar A, Barros-Dios JM (2015) Alpha-1 Antitrypsin Deficiency and Lung Cancer Risk: A Case-Control Study in Never-Smokers. J Thorac Oncol 10 (9):1279–1284. doi:10.1097/JTO.0000000000000609 [PubMed: 26287318]

91. Soderberg D, Segelmark M (2016) Neutrophil Extracellular Traps in ANCA-Associated Vasculitis. Front Immunol 7:256. doi:10.3389/fimmu.2016.00256 [PubMed: 27446086]

92. Denadai-Souza A, Ribeiro CM, Rolland C, Thouard A, Deraison C, Scavone C, Gonzalez-Dunia D, Vergnolle N, Avellar MCW (2017) Effect of tryptase inhibition on joint inflammation: a pharmacological and lentivirus-mediated gene transfer study. Arthritis Res Ther 19 10.1186/ s13075-017-1326-9

93. Leskinen MJ, Lindstedt KA, Wang Y, Kovanen PT (2003) Mast cell chymase induces smooth muscle cell apoptosis by a mechanism involving fibronectin degradation and disruption of focal adhesions. Arterioscler Thromb Vasc Biol 23 (2):238–243 [PubMed: 12588765]

94. He A, Shi GP (2013) Mast cell chymase and tryptase as targets for cardiovascular and metabolic diseases. Curr Pharm Des 19 (6):1114–1125 [PubMed: 23016684]

95. Shirai T, Hilhorst M, Harrison DG, Goronzy JJ, Weyand CM (2015) Macrophages in vascular inflammation--From atherosclerosis to vasculitis. Autoimmunity 48 (3):139–151. doi:10.3109/08916934.2015.1027815 [PubMed: 25811915]

96. Sendler M, Maertin S, John D, Persike M, Weiss FU, Kruger B, Wartmann T, Wagh P, Halangk W, Schaschke N, Mayerle J, Lerch MM (2016) Cathepsin B Activity Initiates Apoptosis via Digestive Protease Activation in Pancreatic Acinar Cells and Experimental Pancreatitis. J Biol Chem 291 (28):14717–14731. doi:10.1074/jbc.M116.718999 [PubMed: 27226576]

97. Kayode O, Huang Z, Soares AS, Caulfield TR, Dong Z, Bode AM, Radisky ES (2017) Small molecule inhibitors of mesotrypsin from a structure-based docking screen. PLoS One 12 (5):e0176694. doi:10.1371/journal.pone.0176694 [PubMed: 28463992]

98. Rolland-Fourcade C, Denadai-Souza A, Cirillo C, Lopez C, Jaramillo JO, Desormeaux C, Cenac N, Motta JP, Larauche M, Tache Y, Berghe PV, Neunlist M, Coron E, Kirzin S, Portier G, Bonnet D, Alric L, Vanner S, Deraison C, Vergnolle N (2017) Epithelial expression and function of trypsin-3 in irritable bowel syndrome. Gut 66 (10):1767–1778. doi:10.1136/gutjnl-2016-312094 [PubMed: 28096305]

99. Ricklin D, Lambris JD (2013) Complement in immune and inflammatory disorders: pathophysiological mechanisms. J Immunol 190 (8):3831–3838. doi:10.4049/jimmunol.1203487 [PubMed: 23564577]

100. Hua Y, Nair S (2015) Proteases in cardiometabolic diseases: Pathophysiology, molecular mechanisms and clinical applications. Biochim Biophys Acta 1852 (2):195–208. doi:10.1016/j.bbadis.2014.04.032 [PubMed: 24815358]

101. Friedrich R, Panizzi P, Fuentes-Prior P, Richter K, Verhamme I, Anderson PJ, Kawabata S, Huber R, Bode W, Bock PE (2003) Staphylocoagulase is a prototype for the mechanism of cofactor-induced zymogen activation. Nature 425 (6957):535–539. doi:10.1038/nature01962 [PubMed: 14523451]

102. Weidmann H, Heikaus L, Long AT, Naudin C, Schluter H, Renne T (2017) The plasma contact system, a protease cascade at the nexus of inflammation, coagulation and immunity. Biochim Biophys Acta 1864 (11 Pt B):2118–2127. doi:10.1016/j.bbamcr.2017.07.009

103. Zamolodchikov D, Renne T, Strickland S (2016) The Alzheimer's disease peptide beta-amyloid promotes thrombin generation through activation of coagulation factor XII. J Thromb Haemost 14 (5):995–1007. doi:10.1111/jth.13209 [PubMed: 26613657]

104. Esmon CT, Vigano-D'Angelo S, D'Angelo A, Comp PC (1987) Anticoagulation proteins C and S. Adv Exp Med Biol 214:47–54 [PubMed: 2959034]

105. Bertina RM, Koeleman BP, Koster T, Rosendaal FR, Dirven RJ, de Ronde H, van der Velden PA, Reitsma PH (1994) Mutation in blood coagulation factor V associated with resistance to activated protein C. Nature 369 (6475):64–67. doi:10.1038/369064a0 [PubMed: 8164741]

106. Kujovich JL (1993) Factor V Leiden Thrombophilia. In: Adam MP, Ardinger HH, Pagon RA et al. (eds) GeneReviews(R) Seattle (WA),

107. Lane D Antithrombin mutation database.

108. Verhamme IM, Olson ST, Tollefsen DM, Bock PE (2002) Binding of exosite ligands to human thrombin. Re-evaluation of allosteric linkage between thrombin exosites I and II. J Biol Chem 277 (9):6788–6798. doi:10.1074/jbc.M110257200 [PubMed: 11724802]

109. Sarilla S, Habib SY, Kravtsov DV, Matafonov A, Gailani D, Verhamme IM (2010) Sucrose octasulfate selectively accelerates thrombin inactivation by heparin cofactor II. J Biol Chem 285 (11):8278–8289. doi:10.1074/jbc.M109.005967 [PubMed: 20053992]

110. Raghuraman A, Mosier PD, Desai UR (2010) Understanding Dermatan Sulfate-Heparin Cofactor II Interaction through Virtual Library Screening. ACS Med Chem Lett 1 (6):281–285. doi:10.1021/ml100048y [PubMed: 20835364]

111. Tollefsen DM, Maimone MM, McGuire EA, Peacock ME (1989) Heparin cofactor II activation by dermatan sulfate. Ann N Y Acad Sci 556:116–122 [PubMed: 2660682]

112. Aihara K, Azuma H, Takamori N, Kanagawa Y, Akaike M, Fujimura M, Yoshida T, Hashizume S, Kato M, Yamaguchi H, Kato S, Ikeda Y, Arase T, Kondo A, Matsumoto T (2004) Heparin cofactor II is a novel protective factor against carotid atherosclerosis in elderly individuals.

Circulation 109 (22):2761–2765. doi:10.1161/01.CIR.0000129968.46095.F3 [PubMed: 15148272]

113. Polderdijk SG, Adams TE, Ivanciu L, Camire RM, Baglin TP, Huntington JA (2017) Design and characterization of an APC-specific serpin for the treatment of hemophilia. Blood 129 (1):105–113. doi:10.1182/blood-2016-05-718635 [PubMed: 27789479]
114. Panizzi P, Boxrud PD, Verhamme IM, Bock PE (2006) Binding of the COOH-terminal lysine residue of streptokinase to plasmin(ogen) kringles enhances formation of the streptokinase.plasmin(ogen) catalytic complexes. J Biol Chem 281 (37):26774–26778. doi: 10.1074/jbc.C600171200 [PubMed: 16857686]
115. Verhamme IM, Bock PE (2008) Rapid-reaction kinetic characterization of the pathway of streptokinase-plasmin catalytic complex formation. J Biol Chem 283 (38):26137–26147. doi: 10.1074/jbc.M804038200 [PubMed: 18658146]
116. Verhamme IM, Bock PE (2014) Rapid binding of plasminogen to streptokinase in a catalytic complex reveals a three-step mechanism. J Biol Chem 289 (40):28006–28018. doi:10.1074/jbc.M114.589077 [PubMed: 25138220]
117. Weiss D, Sorescu D, Taylor WR (2001) Angiotensin II and atherosclerosis. Am J Cardiol 87 (8A):25C–32C
118. Kossmann S, Lagrange J, Jackel S, Jurk K, Ehlken M, Schonfelder T, Weihert Y, Knorr M, Brandt M, Xia N, Li H, Daiber A, Oelze M, Reinhardt C, Lackner K, Gruber A, Monia B, Karbach SH, Walter U, Ruggeri ZM, Renne T, Ruf W, Munzel T, Wenzel P (2017) Platelet-localized FXI promotes a vascular coagulation-inflammatory circuit in arterial hypertension. Sci Transl Med 9 (375). doi:10.1126/scitranslmed.aah4923
119. Camare C, Pucelle M, Negre-Salvayre A, Salvayre R (2017) Angiogenesis in the atherosclerotic plaque. Redox Biol 12:18–34. doi:10.1016/j.redox.2017.01.007 [PubMed: 28212521]
120. Wilson WRW, Anderton M, Choke EC, Dawson J, Loftus IM, Thompson MM (2008) Elevated plasma MMP1 and MMP9 are associated with abdominal aortic aneurysm rupture. Eur J Vasc Endovasc 35 (5):580–584. doi:10.1016/j.ejvs.2007.12.004
121. Xue L, Borne Y, Mattisson IY, Wigren M, Melander O, Ohro-Melander M, Bengtsson E, Fredrikson GN, Nilsson J, Engstrom G (2017) FADD, Caspase-3, and Caspase-8 and Incidence of Coronary Events. Arterioscler Thromb Vasc Biol 37 (5):983–989. doi:10.1161/ATVBAHA.117.308995 [PubMed: 28302628]
122. Musante L, Tataruch D, Gu D, Liu X, Forsblom C, Groop PH, Holthofer H (2015) Proteases and protease inhibitors of urinary extracellular vesicles in diabetic nephropathy. J Diabetes Res 2015:289734. doi:10.1155/2015/289734 [PubMed: 25874235]
123. Zhao Z, Yang P, Eckert RL, Reece EA (2009) Caspase-8: a key role in the pathogenesis of diabetic embryopathy. Birth Defects Res B Dev Reprod Toxicol 86 (1):72–77. doi:10.1002/bdrb.20185 [PubMed: 19194987]
124. Augstein P, Bahr J, Wachlin G, Heinke P, Berg S, Salzsieder E, Harrison LC (2004) Cytokines activate caspase-3 in insulinoma cells of diabetes-prone NOD mice directly and via upregulation of Fas. J Autoimmun 23 (4):301–309. doi:10.1016/j.jaut.2004.09.006 [PubMed: 15571924]
125. Trompet S, Pons D, Kanse SM, de Craen AJ, Ikram MA, Verschuren JJ, Zwinderman AH, Doevendans PA, Tio RA, de Winter RJ, Slagboom PE, Westendorp RG, Jukema JW (2011) Factor VII Activating Protease Polymorphism (G534E) Is Associated with Increased Risk for Stroke and Mortality. Stroke Res Treat 2011:424759. doi:10.4061/2011/424759 [PubMed: 21789270]
126. Turner RJ, Sharp FR (2016) Implications of MMP9 for Blood Brain Barrier Disruption and Hemorrhagic Transformation Following Ischemic Stroke. Front Cell Neurosci 10:56. doi: 10.3389/fncel.2016.00056 [PubMed: 26973468]
127. Crocker SJ, Pagenstecher A, Campbell IL (2004) The TIMPs tango with MMPs and more in the central nervous system. J Neurosci Res 75 (1):1–11. doi:10.1002/jnr.10836 [PubMed: 14689443]
128. Brucher BL, Jamall IS (2016) Somatic Mutation Theory - Why it's Wrong for Most Cancers. Cell Physiol Biochem 38 (5):1663–1680. doi:10.1159/000443106 [PubMed: 27160408]
129. Brucher BL, Jamall IS (2014) Epistemology of the origin of cancer: a new paradigm. BMC Cancer 14:331. doi:10.1186/1471-2407-14-331 [PubMed: 24885752]

130. Schuliga M (2015) The inflammatory actions of coagulant and fibrinolytic proteases in disease. Mediators Inflamm 2015:437695. doi:10.1155/2015/437695 [PubMed: 25878399]

131. Fan J, Ning B, Lyon CJ, Hu TY (2017) Circulating Peptidome and Tumor-Resident Proteolysis. Enzymes 42:1–25. doi:10.1016/bs.enz.2017.08.001 [PubMed: 29054266]

132. Guo Z, Jin X, Jia H (2013) Inhibition of ADAM-17 more effectively down-regulates the Notch pathway than that of gamma-secretase in renal carcinoma. J Exp Clin Cancer Res 32:26. doi: 10.1186/1756-9966-32-26 [PubMed: 23659326]

133. Jackson HW, Defamie V, Waterhouse P, Khokha R (2017) TIMPs: versatile extracellular regulators in cancer. Nat Rev Cancer 17 (1):38–53. doi:10.1038/nrc.2016.115 [PubMed: 27932800]

134. LaRocca G, Aspelund T, Greve AM, Eiriksdottir G, Acharya T, Thorgeirsson G, Harris TB, Launer LJ, Gudnason V, Arai AE (2017) Fibrosis as measured by the biomarker, tissue inhibitor metalloproteinase-1, predicts mortality in Age Gene Environment Susceptibility-Reykjavik (AGES-Reykjavik) Study. Eur Heart J 38 (46):3423–3430. doi:10.1093/eurheartj/ehx510 [PubMed: 29020384]

135. Lee JY, Kong G (2016) Roles and epigenetic regulation of epithelial-mesenchymal transition and its transcription factors in cancer initiation and progression. Cell Mol Life Sci 73 (24):4643–4660. doi:10.1007/s00018-016-2313-z [PubMed: 27460000]

136. Otsuki T, Fujimoto D, Hirono Y, Goi T, Yamaguchi A (2014) Thrombin conducts epithelialmesenchymal transition via proteaseactivated receptor1 in human gastric cancer. Int J Oncol 45 (6):2287–2294. doi:10.3892/ijo.2014.2651 [PubMed: 25231630]

137. Bawa-Khalfe T, Lu LS, Zuo Y, Huang C, Dere R, Lin FM, Yeh ET (2012) Differential expression of SUMO-specific protease 7 variants regulates epithelial-mesenchymal transition. Proc Natl Acad Sci U S A 109 (43):17466–17471. doi:10.1073/pnas.1209378109 [PubMed: 23045645]

138. Schmidt N, Irle I, Ripkens K, Lux V, Nelles J, Johannes C, Parry L, Greenow K, Amir S, Campioni M, Baldi A, Oka C, Kawaichi M, Clarke AR, Ehrmann M (2016) Epigenetic silencing of serine protease HTRA1 drives polyploidy. BMC Cancer 16:399. doi:10.1186/s12885-016-2425-8 [PubMed: 27388476]

139. Johnson JJ, Miller DL, Jiang R, Liu Y, Shi Z, Tarwater L, Williams R, Balsara R, Sauter ER, Stack MS (2016) Protease-activated Receptor-2 (PAR-2)-mediated Nf-kappaB Activation Suppresses Inflammation-associated Tumor Suppressor MicroRNAs in Oral Squamous Cell Carcinoma. J Biol Chem 291 (13):6936–6945. doi:10.1074/jbc.M115.692640 [PubMed: 26839311]

140. Zhang W, Wang S, Wang Q, Yang Z, Pan Z, Li L (2014) Overexpression of cysteine cathepsin L is a marker of invasion and metastasis in ovarian cancer. Oncol Rep 31 (3):1334–1342. doi: 10.3892/or.2014.2967 [PubMed: 24402045]

141. Dian D, Heublein S, Wiest I, Barthell L, Friese K, Jeschke U (2014) Significance of the tumor protease cathepsin D for the biology of breast cancer. Histol Histopathol 29 (4):433–438. doi: 10.14670/HH-29.10.433 [PubMed: 24265119]

142. Cohen I, Kayode O, Hockla A, Sankaran B, Radisky DC, Radisky ES, Papo N (2016) Combinatorial protein engineering of proteolytically resistant mesotrypsin inhibitors as candidates for cancer therapy. Biochem J 473 (10):1329–1341. doi:10.1042/BJ20151410 [PubMed: 26957636]

143. Salameh MA, Radisky ES (2013) Biochemical and structural insights into mesotrypsin: an unusual human trypsin. Int J Biochem Mol Biol 4 (3):129–139 [PubMed: 24049668]

144. Tanabe LM, List K (2017) The role of type II transmembrane serine protease-mediated signaling in cancer. FEBS J 284 (10):1421–1436. doi:10.1111/febs.13971 [PubMed: 27870503]

145. Zoratti GL, Tanabe LM, Hyland TE, Duhaime MJ, Colombo E, Leduc R, Marsault E, Johnson MD, Lin CY, Boerner J, Lang JE, List K (2016) Matriptase regulates c-Met mediated proliferation and invasion in inflammatory breast cancer. Oncotarget 7 (36):58162–58173. doi: 10.18632/oncotarget.11262 [PubMed: 27528224]

146. Rolfe M (2017) The Holy Grail: Solid Tumor Efficacy by Proteasome Inhibition. Cell Chem Biol 24 (2):125–126. doi:10.1016/j.chembiol.2017.01.007 [PubMed: 28212756]

147. Weyburne ES, Wilkins OM, Sha Z, Williams DA, Pletnev AA, de Bruin G, Overkleeft HS, Goldberg AL, Cole MD, Kisselev AF (2017) Inhibition of the Proteasome beta2 Site Sensitizes Triple-Negative Breast Cancer Cells to beta5 Inhibitors and Suppresses Nrf1 Activation. Cell Chem Biol 24 (2):218–230. doi:10.1016/j.chembiol.2016.12.016 [PubMed: 28132893]

148. Reis ES, Mastellos DC, Ricklin D, Mantovani A, Lambris JD (2018) Complement in cancer: untangling an intricate relationship. Nat Rev Immunol 18 (1):5–18. doi:10.1038/nri.2017.97 [PubMed: 28920587]

149. Rutkowski MJ, Sughrue ME, Kane AJ, Mills SA, Parsa AT (2010) Cancer and the complement cascade. Mol Cancer Res 8 (11):1453–1465. doi:10.1158/1541-7786.MCR-10-0225 [PubMed: 20870736]

150. Zhu L, Jaamaa S, Af Hallstrom TM, Laiho M, Sankila A, Nordling S, Stenman UH, Koistinen H (2013) PSA forms complexes with alpha1-antichymotrypsin in prostate. Prostate 73 (2):219–226. doi:10.1002/pros.22560 [PubMed: 22806587]

151. DiScipio RG (1982) The activation of the alternative pathway C3 convertase by human plasma kallikrein. Immunology 45 (3):587–595 [PubMed: 6916710]

152. Caine GJ, Stonelake PS, Lip GY, Kehoe ST (2002) The hypercoagulable state of malignancy: pathogenesis and current debate. Neoplasia 4 (6):465–473. doi:10.1038/sj.neo.7900263 [PubMed: 12407439]

153. Amiral J, Seghatchian J (2017) Monitoring of anticoagulant therapy in cancer patients with thrombosis and the usefulness of blood activation markers. Transfus Apher Sci 56 (3):279–286. doi:10.1016/j.transci.2017.05.010 [PubMed: 28552490]

154. Huesgen PF, Lange PF, Overall CM (2014) Ensembles of protein termini and specific proteolytic signatures as candidate biomarkers of disease. Proteomics Clin Appl 8 (5–6):338–350. doi:10.1002/prca.201300104 [PubMed: 24497460]

155. Kang JH, Korecka M, Toledo JB, Trojanowski JQ, Shaw LM (2013) Clinical utility and analytical challenges in measurement of cerebrospinal fluid amyloid-beta(1–42) and tau proteins as Alzheimer disease biomarkers. Clin Chem 59 (6):903–916. doi:10.1373/clinchem.2013.202937 [PubMed: 23519967]

156. Janelidze S, Stomrud E, Palmqvist S, Zetterberg H, van Westen D, Jeromin A, Song L, Hanlon D, Tan Hehir CA, Baker D, Blennow K, Hansson O (2016) Plasma beta-amyloid in Alzheimer's disease and vascular disease. Sci Rep 6:26801. doi:10.1038/srep26801 [PubMed: 27241045]

157. Roher AE, Kokjohn TA, Clarke SG, Sierks MR, Maarouf CL, Serrano GE, Sabbagh MS, Beach TG (2017) APP/Abeta structural diversity and Alzheimer's disease pathogenesis. Neurochem Int 110:1–13. doi:10.1016/j.neuint.2017.08.007 [PubMed: 28811267]

158. Evin G, Li QX (2012) Platelets and Alzheimer's disease: Potential of APP as a biomarker. World J Psychiatry 2 (6):102–113. doi:10.5498/wjp.v2.i6.102 [PubMed: 24175176]

159. Wetzel S, Seipold L, Saftig P (2017) The metalloproteinase ADAM10: A useful therapeutic target? Biochim Biophys Acta 1864 (11 Pt B):2071–2081. doi:10.1016/j.bbamcr.2017.06.005

160. Bu XL, Xiang Y, Jin WS, Wang J, Shen LL, Huang ZL, Zhang K, Liu YH, Zeng F, Liu JH, Sun HL, Zhuang ZQ, Chen SH, Yao XQ, Giunta B, Shan YC, Tan J, Chen XW, Dong ZF, Zhou HD, Zhou XF, Song W, Wang YJ (2017) Blood-derived amyloid-beta protein induces Alzheimer's disease pathologies. Mol Psychiatry. doi:10.1038/mp.2017.204

161. Budd Haeberlein S, O'Gorman J, Chiao P, Bussiere T, von Rosenstiel P, Tian Y, Zhu Y, von Hehn C, Gheuens S, Skordos L, Chen T, Sandrock A (2017) Clinical Development of Aducanumab, an Anti-Abeta Human Monoclonal Antibody Being Investigated for the Treatment of Early Alzheimer's Disease. J Prev Alzheimers Dis 4 (4):255–263. doi:10.14283/jpad.2017.39 [PubMed: 29181491]

162. Honig LS, Vellas B, Woodward M, Boada M, Bullock R, Borrie M, Hager K, Andreasen N, Scarpini E, Liu-Seifert H, Case M, Dean RA, Hake A, Sundell K, Poole Hoffmann V, Carlson C, Khanna R, Mintun M, DeMattos R, Selzler KJ, Siemers E (2018) Trial of Solanezumab for Mild Dementia Due to Alzheimer's Disease. N Engl J Med 378 (4):321–330. doi:10.1056/NEJMoa1705971 [PubMed: 29365294]

163. De Strooper B (2014) Lessons from a failed gamma-secretase Alzheimer trial. Cell 159 (4):721–726. doi:10.1016/j.cell.2014.10.016 [PubMed: 25417150]

164. Lorenzl S, Albers DS, Relkin N, Ngyuen T, Hilgenberg SL, Chirichigno J, Cudkowicz ME, Beal MF (2003) Increased plasma levels of matrix metalloproteinase-9 in patients with Alzheimer's disease. Neurochem Int 43 (3):191–196 [PubMed: 12689599]

165. Siklos M, BenAissa M, Thatcher GR (2015) Cysteine proteases as therapeutic targets: does selectivity matter? A systematic review of calpain and cathepsin inhibitors. Acta Pharm Sin B 5 (6):506–519. doi:10.1016/j.apsb.2015.08.001 [PubMed: 26713267]

166. Martinelli P, Rugarli EI (2010) Emerging roles of mitochondrial proteases in neurodegeneration. Biochim Biophys Acta 1797 (1):1–10. doi:10.1016/j.bbabio.2009.07.013

167. Konig T, Troder SE, Bakka K, Korwitz A, Richter-Dennerlein R, Lampe PA, Patron M, Muhlmeister M, Guerrero-Castillo S, Brandt U, Decker T, Lauria I, Paggio A, Rizzuto R, Rugarli EI, De Stefani D, Langer T (2016) The m-AAA Protease Associated with Neurodegeneration Limits MCU Activity in Mitochondria. Mol Cell 64 (1):148–162. doi:10.1016/j.molcel.2016.08.020 [PubMed: 27642048]

168. Strauss KM, Martins LM, Plun-Favreau H, Marx FP, Kautzmann S, Berg D, Gasser T, Wszolek Z, Muller T, Bornemann A, Wolburg H, Downward J, Riess O, Schulz JB, Kruger R (2005) Loss of function mutations in the gene encoding Omi/HtrA2 in Parkinson's disease. Hum Mol Genet 14 (15):2099–2111. doi:10.1093/hmg/ddi215 [PubMed: 15961413]

169. Fu J, Yu HM, Chiu SY, Mirando AJ, Maruyama EO, Cheng JG, Hsu W (2014) Disruption of SUMO-specific protease 2 induces mitochondria mediated neurodegeneration. PLoS Genet 10 (10):e1004579. doi:10.1371/journal.pgen.1004579 [PubMed: 25299344]

170. Orsini F, De Blasio D, Zangari R, Zanier ER, De Simoni MG (2014) Versatility of the complement system in neuroinflammation, neurodegeneration and brain homeostasis. Front Cell Neurosci 8:380. doi:10.3389/fncel.2014.00380 [PubMed: 25426028]

171. Descamps FJ, Van den Steen PE, Nelissen I, Van Damme J, Opdenakker G (2003) Remnant epitopes generate autoimmunity: from rheumatoid arthritis and multiple sclerosis to diabetes. Adv Exp Med Biol 535:69–77 [PubMed: 14714889]

172. Dinarello CA, Simon A, van der Meer JW (2012) Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat Rev Drug Discov 11 (8):633–652. doi:10.1038/nrd3800 [PubMed: 22850787]

173. Rupanagudi KV, Kulkarni OP, Lichtnekert J, Darisipudi MN, Mulay SR, Schott B, Gruner S, Haap W, Hartmann G, Anders HJ (2015) Cathepsin S inhibition suppresses systemic lupus erythematosus and lupus nephritis because cathepsin S is essential for MHC class II-mediated CD4 T cell and B cell priming. Ann Rheum Dis 74 (2):452–463. doi:10.1136/annrheumdis-2013-203717 [PubMed: 24300027]

174. Schaller M, Vogel M, Kentouche K, Lammle B, Kremer Hovinga JA (2014) The splenic autoimmune response to ADAMTS13 in thrombotic thrombocytopenic purpura contains recurrent antigen-binding CDR3 motifs. Blood 124 (23):3469–3479. doi:10.1182/blood-2014-04-561142 [PubMed: 25261198]

175. Sadiq SK, Noe F, De Fabritiis G (2012) Kinetic characterization of the critical step in HIV-1 protease maturation. Proc Natl Acad Sci U S A 109 (50):20449–20454. doi:10.1073/pnas.1210983109 [PubMed: 23184967]

176. Duschak VG, Couto AS (2009) Cruzipain, the major cysteine protease of Trypanosoma cruzi: a sulfated glycoprotein antigen as relevant candidate for vaccine development and drug target. A review. Curr Med Chem 16 (24):3174–3202 [PubMed: 19689291]

177. Plaza K, Kalinska M, Bochenska O, Meyer-Hoffert U, Wu Z, Fischer J, Falkowski K, Sasiadek L, Bielecka E, Potempa B, Kozik A, Potempa J, Kantyka T (2016) Gingipains of Porphyromonas gingivalis Affect the Stability and Function of Serine Protease Inhibitor of Kazal-type 6 (SPINK6), a Tissue Inhibitor of Human Kallikreins. J Biol Chem 291 (36):18753–18764. doi:10.1074/jbc.M116.722942 [PubMed: 27354280]

178. Culp E, Wright GD (2017) Bacterial proteases, untapped antimicrobial drug targets. J Antibiot (Tokyo) 70 (4):366–377. doi:10.1038/ja.2016.138 [PubMed: 27899793]

179. Chang AK, Kim HY, Park JE, Acharya P, Park IS, Yoon SM, You HJ, Hahm KS, Park JK, Lee JS (2005) Vibrio vulnificus secretes a broad-specificity metalloprotease capable of interfering with blood homeostasis through prothrombin activation and fibrinolysis. J Bacteriol 187 (20):6909–6916. doi:10.1128/JB.187.20.6909-6916.2005 [PubMed: 16199560]

180. Bibo-Verdugo B, Jiang Z, Caffrey CR, O'Donoghue AJ (2017) Targeting proteasomes in infectious organisms to combat disease. FEBS J 284 (10):1503–1517. doi:10.1111/febs.14029 [PubMed: 28122162]
181. Pontarollo G, Acquasaliente L, Peterle D, Frasson R, Artusi I, De Filippis V (2017) Non-canonical proteolytic activation of human prothrombin by subtilisin from Bacillus subtilis may shift the procoagulant-anticoagulant equilibrium toward thrombosis. J Biol Chem 292 (37): 15161–15179. doi:10.1074/jbc.M117.795245 [PubMed: 28684417]
182. Carroll IM, Maharshak N (2013) Enteric bacterial proteases in inflammatory bowel disease-pathophysiology and clinical implications. World J Gastroenterol 19 (43):7531–7543. doi: 10.3748/wjg.v19.i43.7531 [PubMed: 24431894]
183. Boxrud PD, Verhamme IM, Bock PE (2004) Resolution of conformational activation in the kinetic mechanism of plasminogen activation by streptokinase. J Biol Chem 279 (35):36633–36641. doi:10.1074/jbc.M405264200 [PubMed: 15215240]
184. Chandrahas V, Glinton K, Liang Z, Donahue DL, Ploplis VA, Castellino FJ (2015) Direct Host Plasminogen Binding to Bacterial Surface M-protein in Pattern D Strains of Streptococcus pyogenes Is Required for Activation by Its Natural Coinherited SK2b Protein. J Biol Chem 290 (30):18833–18842. doi:10.1074/jbc.M115.655365 [PubMed: 26070561]
185. Panizzi P, Friedrich R, Fuentes-Prior P, Bode W, Bock PE (2004) The staphylocoagulase family of zymogen activator and adhesion proteins. Cell Mol Life Sci 61 (22):2793–2798. doi:10.1007/s00018-004-4285-7 [PubMed: 15558209]
186. Parry MA, Zhang XC, Bode I (2000) Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. Trends Biochem Sci 25 (2):53–59 [PubMed: 10664583]
187. Wiles KG, Panizzi P, Kroh HK, Bock PE (2010) Skizzle is a novel plasminogen- and plasmin-binding protein from Streptococcus agalactiae that targets proteins of human fibrinolysis to promote plasmin generation. J Biol Chem 285 (27):21153–21164. doi:10.1074/jbc.M110.107730 [PubMed: 20435890]
188. Verhamme IM, Panizzi PR, Bock PE (2015) Pathogen activators of plasminogen. J Thromb Haemost 13 Suppl 1:S106–114. doi:10.1111/jth.12939 [PubMed: 26149011]
189. Meliopoulos VA, Andersen LE, Brooks P, Yan X, Bakre A, Coleman JK, Tompkins SM, Tripp RA (2012) MicroRNA regulation of human protease genes essential for influenza virus replication. PLoS One 7 (5):e37169. doi:10.1371/journal.pone.0037169 [PubMed: 22606348]
190. Homma T, Ishibashi D, Nakagaki T, Fuse T, Mori T, Satoh K, Atarashi R, Nishida N (2015) Ubiquitin-specific protease 14 modulates degradation of cellular prion protein. Sci Rep 5:11028. doi:10.1038/srep11028 [PubMed: 26061634]
191. Michaud DS, Lu J, Peacock-Villada AY, Barber JR, Joshu CE, Prizment AE, Beck JD, Offenbacher S, Platz EA (2018) Periodontal Disease Assessed Using Clinical Dental Measurements and Cancer Risk in the ARIC Study. J Natl Cancer Inst. doi:10.1093/jnci/djx278
192. Drag M, Salvesen GS (2010) Emerging principles in protease-based drug discovery. Nat Rev Drug Discov 9 (9):690–701. doi:10.1038/nrd3053 [PubMed: 20811381]
193. Herzog RW (2015) Hemophilia Gene Therapy: Caught Between a Cure and an Immune Response. Mol Ther 23 (9):1411–1412. doi:10.1038/mt.2015.135 [PubMed: 26321180]
194. Rangarajan S, Walsh L, Lester W, Perry D, Madan B, Laffan M, Yu H, Vettermann C, Pierce GF, Wong WY, Pasi KJ (2017) AAV5-Factor VIII Gene Transfer in Severe Hemophilia A. N Engl J Med. doi:10.1056/NEJMoa1708483
195. George LA, Sullivan SK, Giermasz A, Rasko JEJ, Samelson-Jones BJ, Ducore J, Cuker A, Sullivan LM, Majumdar S, Teitel J, McGuinn CE, Ragni MV, Luk AY, Hui D, Wright JF, Chen Y, Liu Y, Wachtel K, Winters A, Tiefenbacher S, Arruda VR, van der Loo JCM, Zelenaia O, Takefman D, Carr ME, Couto LB, Anguela XM, High KA (2017) Hemophilia B Gene Therapy with a High-Specific-Activity Factor IX Variant. N Engl J Med 377 (23):2215–2227. doi: 10.1056/NEJMoa1708538 [PubMed: 29211678]
196. Lai PS, Thompson BT (2013) Why activated protein C was not successful in severe sepsis and septic shock: are we still tilting at windmills? Curr Infect Dis Rep 15 (5):407–412. doi:10.1007/s11908-013-0358-9 [PubMed: 23925482]

197. Janciauskiene SM, Bals R, Koczulla R, Vogelmeier C, Kohnlein T, Welte T (2011) The discovery of alpha1-antitrypsin and its role in health and disease. Respir Med 105 (8):1129–1139. doi: 10.1016/j.rmed.2011.02.002 [PubMed: 21367592]

198. Carugati A, Pappalardo E, Zingale LC, Cicardi M (2001) C1-inhibitor deficiency and angioedema. Mol Immunol 38 (2–3):161–173 [PubMed: 11532278]

199. Ricklin D, Lambris JD (2016) New milestones ahead in complement-targeted therapy. Semin Immunol 28 (3):208–222. doi:10.1016/j.smim.2016.06.001 [PubMed: 27321574]

200. Towards better patient care: drugs to avoid in 2014 (2014). Prescrire Int 23 (150):161–165 [PubMed: 25121155]

201. Adcock DM, Gosselin R (2015) Direct Oral Anticoagulants (DOACs) in the Laboratory: 2015 Review. Thromb Res 136 (1):7–12. doi:10.1016/j.thromres.2015.05.001 [PubMed: 25981138]

202. Tallant C, Marrero A, Gomis-Ruth FX (2010) Matrix metalloproteinases: fold and function of their catalytic domains. Biochim Biophys Acta 1803 (1):20–28. doi:10.1016/j.bbamcr. 2009.04.003 [PubMed: 19374923]

203. Gomis-Ruth FX (2017) Third time lucky? Getting a grip on matrix metalloproteinases. J Biol Chem 292 (43):17975–17976. doi:10.1074/jbc.H117.806075 [PubMed: 29079642]

204. Scannevin RH, Alexander R, Haarlander TM, Burke SL, Singer M, Huo C, Zhang YM, Maguire D, Spurlino J, Deckman I, Carroll KI, Lewandowski F, Devine E, Dzordzorme K, Tounge B, Milligan C, Bayoumy S, Williams R, Schalk-Hihi C, Leonard K, Jackson P, Todd M, Kuo LC, Rhodes KJ (2017) Discovery of a highly selective chemical inhibitor of matrix metalloproteinase-9 (MMP-9) that allosterically inhibits zymogen activation. J Biol Chem 292 (43):17963–17974. doi:10.1074/jbc.M117.806075 [PubMed: 28860188]

205. Grunwald B, Vandooren J, Gerg M, Ahomaa K, Hunger A, Berchtold S, Akbareian S, Schaten S, Knolle P, Edwards DR, Opdenakker G, Kruger A (2016) Systemic Ablation of MMP-9 Triggers Invasive Growth and Metastasis of Pancreatic Cancer via Deregulation of IL6 Expression in the Bone Marrow. Mol Cancer Res 14 (11):1147–1158. doi:10.1158/1541-7786.MCR-16-0180 [PubMed: 27489361]

206. Radisky ES, Raeeszadeh-Sarmazdeh M, Radisky DC (2017) Therapeutic Potential of Matrix Metalloproteinase Inhibition in Breast Cancer. J Cell Biochem 118 (11):3531–3548. doi:10.1002/ jcb.26185 [PubMed: 28585723]

207. Xia D, Watanabe H, Wu B, Lee SH, Li Y, Tsvetkov E, Bolshakov VY, Shen J, Kelleher RJ 3rd (2015) Presenilin-1 knockin mice reveal loss-of-function mechanism for familial Alzheimer's disease. Neuron 85 (5):967–981. doi:10.1016/j.neuron.2015.02.010 [PubMed: 25741723]

208. Ran Y, Hossain F, Pannuti A, Lessard CB, Ladd GZ, Jung JI, Minter LM, Osborne BA, Miele L, Golde TE (2017) gamma-Secretase inhibitors in cancer clinical trials are pharmacologically and functionally distinct. EMBO Mol Med 9 (7):950–966. doi:10.15252/emmm.201607265 [PubMed: 28539479]

209. Duong le T, Leung AT, Langdahl B (2016) Cathepsin K Inhibition: A New Mechanism for the Treatment of Osteoporosis. Calcif Tissue Int 98 (4):381–397. doi:10.1007/s00223-015-0051-0 [PubMed: 26335104]

210. Drake MT, Clarke BL, Oursler MJ, Khosla S (2017) Cathepsin K Inhibitors for Osteoporosis: Biology, Potential Clinical Utility, and Lessons Learned. Endocr Rev 38 (4):325–350. doi: 10.1210/er.2015-1114 [PubMed: 28651365]

211. Su EJ, Cao C, Fredriksson L, Nilsson I, Stefanitsch C, Stevenson TK, Zhao J, Ragsdale M, Sun YY, Yepes M, Kuan CY, Eriksson U, Strickland DK, Lawrence DA, Zhang L (2017) Microglial-mediated PDGF-CC activation increases cerebrovascular permeability during ischemic stroke. Acta Neuropathol 134 (4):585–604. doi:10.1007/s00401-017-1749-z [PubMed: 28725968]

212. Lakhan SE, Kirchgessner A, Tepper D, Leonard A (2013) Matrix metalloproteinases and blood-brain barrier disruption in acute ischemic stroke. Front Neurol 4:32. doi:10.3389/fneur. 2013.00032 [PubMed: 23565108]

213. Hafez S, Coucha M, Bruno A, Fagan SC, Ergul A (2014) Hyperglycemia, acute ischemic stroke, and thrombolytic therapy. Transl Stroke Res 5 (4):442–453. doi:10.1007/s12975-014-0336-z [PubMed: 24619488]

214. Sun H, Xu Y, Sitkiewicz I, Ma Y, Wang X, Yestrepsky BD, Huang Y, Lapadatescu MC, Larsen MJ, Larsen SD, Musser JM, Ginsburg D (2012) Inhibitor of streptokinase gene expression improves survival after group A streptococcus infection in mice. Proc Natl Acad Sci U S A 109 (9):3469–3474. doi:10.1073/pnas.1201031109 [PubMed: 22331877]

215. Silva DG, Ribeiro JFR, De Vita D, Cianni L, Franco CH, Freitas-Junior LH, Moraes CB, Rocha JR, Burtoloso ACB, Kenny PW, Leitao A, Montanari CA (2017) A comparative study of warheads for design of cysteine protease inhibitors. Bioorg Med Chem Lett 27 (22):5031–5035. doi:10.1016/j.bmcl.2017.10.002 [PubMed: 29054358]

216. Lv Z, Chu Y, Wang Y (2015) HIV protease inhibitors: a review of molecular selectivity and toxicity. HIV AIDS (Auckl) 7:95–104. doi:10.2147/HIV.S79956 [PubMed: 25897264]

217. Ghosh AK, Brindisi M, Nyalapatla PR, Takayama J, Ella-Menye JR, Yashchuk S, Agniswamy J, Wang YF, Aoki M, Amano M, Weber IT, Mitsuya H (2017) Design of novel HIV-1 protease inhibitors incorporating isophthalamide-derived P2-P3 ligands: Synthesis, biological evaluation and X-ray structural studies of inhibitor-HIV-1 protease complex. Bioorg Med Chem 25 (19): 5114–5127. doi:10.1016/j.bmc.2017.04.005 [PubMed: 28434781]

218. Ghosh AK, Sean Fyvie W, Brindisi M, Steffey M, Agniswamy J, Wang YF, Aoki M, Amano M, Weber IT, Mitsuya H (2017) Design, synthesis, X-ray studies, and biological evaluation of novel macrocyclic HIV-1 protease inhibitors involving the P1'-P2' ligands. Bioorg Med Chem Lett 27 (21):4925–4931. doi:10.1016/j.bmcl.2017.09.003 [PubMed: 28958624]

219. McCauley JA, Rudd MT (2016) Hepatitis C virus NS3/4a protease inhibitors. Curr Opin Pharmacol 30:84–92. doi:10.1016/j.coph.2016.07.015 [PubMed: 27544488]

220. Thornberry NA, Weber AE (2007) Discovery of JANUVIA (Sitagliptin), a selective dipeptidyl peptidase IV inhibitor for the treatment of type 2 diabetes. Curr Top Med Chem 7 (6):557–568 [PubMed: 17352677]

221. Schramm VL (2013) Transition States, analogues, and drug development. ACS Chem Biol 8 (1): 71–81. doi:10.1021/cb300631k [PubMed: 23259601]

222. Mitsuya H, Maeda K, Das D, Ghosh AK (2008) Development of protease inhibitors and the fight with drug-resistant HIV-1 variants. Adv Pharmacol 56:169–197. doi:10.1016/S1054-3589(07)56006-0 [PubMed: 18086412]

223. Kipp DR, Hirschi JS, Wakata A, Goldstein H, Schramm VL (2012) Transition states of native and drug-resistant HIV-1 protease are the same. Proc Natl Acad Sci U S A 109 (17):6543–6548. doi: 10.1073/pnas.1202808109 [PubMed: 22493227]

224. Overall CM (2002) Molecular determinants of metalloproteinase substrate specificity: matrix metalloproteinase substrate binding domains, modules, and exosites. Mol Biotechnol 22 (1):51–86. doi:10.1385/MB:22:1:051 [PubMed: 12353914]

225. Skarina T, Xu X, Evdokimova E, Savchenko A (2014) High-throughput crystallization screening. Methods Mol Biol 1140:159–168. doi:10.1007/978-1-4939-0354-2_12 [PubMed: 24590716]

226. Tiefenbrunn T, Forli S, Happer M, Gonzalez A, Tsai Y, Soltis M, Elder JH, Olson AJ, Stout CD (2014) Crystallographic fragment-based drug discovery: use of a brominated fragment library targeting HIV protease. Chem Biol Drug Des 83 (2):141–148. doi:10.1111/cbdd.12227 [PubMed: 23998903]

227. Rydel TJ, Tulinsky A, Bode W, Huber R (1991) Refined structure of the hirudin-thrombin complex. J Mol Biol 221 (2):583–601 [PubMed: 1920434]

228. Warkentin TE (2004) Bivalent direct thrombin inhibitors: hirudin and bivalirudin. Best Pract Res Clin Haematol 17 (1):105–125. doi:10.1016/j.beha.2004.02.002 [PubMed: 15171961]

229. Vance NR, Gakhar L, Spies MA (2017) Allosteric Tuning of Caspase-7: A Fragment-Based Drug Discovery Approach. Angew Chem Int Ed Engl 56 (46):14443–14447. doi:10.1002/anie.201706959 [PubMed: 28940929]

230. Weiss-Sadan T, Gotsman I, Blum G (2017) Cysteine proteases in atherosclerosis. FEBS J 284 (10):1455–1472. doi:10.1111/febs.14043 [PubMed: 28207191]

231. Lee S, Xie J, Chen X (2010) Activatable molecular probes for cancer imaging. Curr Top Med Chem 10 (11):1135–1144 [PubMed: 20388112]

232. Ren G, Blum G, Verdoes M, Liu H, Syed S, Edgington LE, Gheysens O, Miao Z, Jiang H, Gambhir SS, Bogyo M, Cheng Z (2011) Non-invasive imaging of cysteine cathepsin activity in

solid tumors using a 64Cu-labeled activity-based probe. PLoS One 6 (11):e28029. doi:10.1371/journal.pone.0028029 [PubMed: 22132198]

233. Indalao IL, Sawabuchi T, Takahashi E, Kido H (2017) IL-1beta is a key cytokine that induces trypsin upregulation in the influenza virus-cytokine-trypsin cycle. Arch Virol 162 (1):201–211. doi:10.1007/s00705-016-3093-3 [PubMed: 27714503]
234. Kelso EB, Lockhart JC, Hembrough T, Dunning L, Plevin R, Hollenberg MD, Sommerhoff CP, McLean JS, Ferrell WR (2006) Therapeutic promise of proteinase-activated receptor-2 antagonism in joint inflammation. J Pharmacol Exp Ther 316 (3):1017–1024. doi:10.1124/jpet.105.093807 [PubMed: 16260582]
235. Vergnolle N (2009) Protease-activated receptors as drug targets in inflammation and pain. Pharmacol Ther 123 (3):292–309. doi:10.1016/j.pharmthera.2009.05.004 [PubMed: 19481569]
236. French SL, Hamilton JR (2016) Protease-activated receptor 4: from structure to function and back again. Br J Pharmacol 173 (20):2952–2965. doi:10.1111/bph.13455 [PubMed: 26844674]
237. Kasperkiewicz P, Poreba M, Groborz K, Drag M (2017) Emerging challenges in the design of selective substrates, inhibitors and activity-based probes for indistinguishable proteases. FEBS J 284 (10):1518–1539. doi:10.1111/febs.14001 [PubMed: 28052575]
238. Harris JL, Backes BJ, Leonetti F, Mahrus S, Ellman JA, Craik CS (2000) Rapid and general profiling of protease specificity by using combinatorial fluorogenic substrate libraries. Proc Natl Acad Sci U S A 97 (14):7754–7759. doi:10.1073/pnas.140132697 [PubMed: 10869434]
239. Kasperkiewicz P, Poreba M, Snipas SJ, Parker H, Winterbourn CC, Salvesen GS, Drag M (2014) Design of ultrasensitive probes for human neutrophil elastase through hybrid combinatorial substrate library profiling. Proc Natl Acad Sci U S A 111 (7):2518–2523. doi:10.1073/pnas.1318548111 [PubMed: 24550277]
240. O'Donoghue AJ, Eroy-Reveles AA, Knudsen GM, Ingram J, Zhou M, Statnekov JB, Greninger AL, Hostetter DR, Qu G, Maltby DA, Anderson MO, Derisi JL, McKerrow JH, Burlingame AL, Craik CS (2012) Global identification of peptidase specificity by multiplex substrate profiling. Nat Methods 9 (11):1095–1100. doi:10.1038/nmeth.2182 [PubMed: 23023596]
241. Li Q, Yi L, Hoi KH, Marek P, Georgiou G, Iverson BL (2017) Profiling Protease Specificity: Combining Yeast ER Sequestration Screening (YESS) with Next Generation Sequencing. ACS Chem Biol 12 (2):510–518. doi:10.1021/acschembio.6b00547 [PubMed: 27977123]
242. Fulcher LJ, Hutchinson LD, Macartney TJ, Turnbull C, Sapkota GP (2017) Targeting endogenous proteins for degradation through the affinity-directed protein missile system. Open Biol 7 (5). doi:10.1098/rsob.170066
243. Grossi G, Dalgaard Ebbesen Jepsen M, Kjems J, Andersen ES (2017) Control of enzyme reactions by a reconfigurable DNA nanovault. Nat Commun 8 (1):992. doi:10.1038/s41467-017-01072-8 [PubMed: 29051565]
244. Lange PF, Huesgen PF, Overall CM (2012) TopFIND 2.0--linking protein termini with proteolytic processing and modifications altering protein function. Nucleic Acids Res 40 (Database issue):D351–361. doi:10.1093/nar/gkr1025 [PubMed: 22102574]
245. Wang X, Davies M, Roy S, Kuruc M (2015) Bead Based Proteome Enrichment Enhances Features of the Protein Elution Plate (PEP) for Functional Proteomic Profiling. Proteomes 3 (4):454–466. doi:10.3390/proteomes3040454 [PubMed: 28248280]
246. Zheng H, Roy S, Soherwardy A, Rahman S, Kuruc M (2017) Stroma Liquid Biopsy - Proteomic Profiles for Cancer Biomarkers.
247. Rifai N, Gillette MA, Carr SA (2006) Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nat Biotechnol 24 (8):971–983. doi:10.1038/nbt1235 [PubMed: 16900146]
248. Koenig W (2003) Fibrin(ogen) in cardiovascular disease: an update. Thromb Haemost 89 (4):601–609 [PubMed: 12669113]
249. Dunn EJ, Ariens RA, Grant PJ (2005) The influence of type 2 diabetes on fibrin structure and function. Diabetologia 48 (6):1198–1206. doi:10.1007/s00125-005-1742-2 [PubMed: 15864538]
250. Grammas P, Martinez JM (2014) Targeting thrombin: an inflammatory neurotoxin in Alzheimer's disease. J Alzheimers Dis 42 Suppl 4:S537–544. doi:10.3233/JAD-141557 [PubMed: 25079808]

251. Ahn HJ, Zamolodchikov D, Cortes-Canteli M, Norris EH, Glickman JF, Strickland S (2010) Alzheimer's disease peptide beta-amyloid interacts with fibrinogen and induces its oligomerization. Proc Natl Acad Sci U S A 107 (50):21812–21817. doi:10.1073/pnas.1010373107 [PubMed: 21098282]

252. Amara U, Rittirsch D, Flierl M, Bruckner U, Klos A, Gebhard F, Lambris JD, Huber-Lang M (2008) Interaction between the coagulation and complement system. Advances in Experimental Medicine & Biology 632:71–79 [PubMed: 19025115]

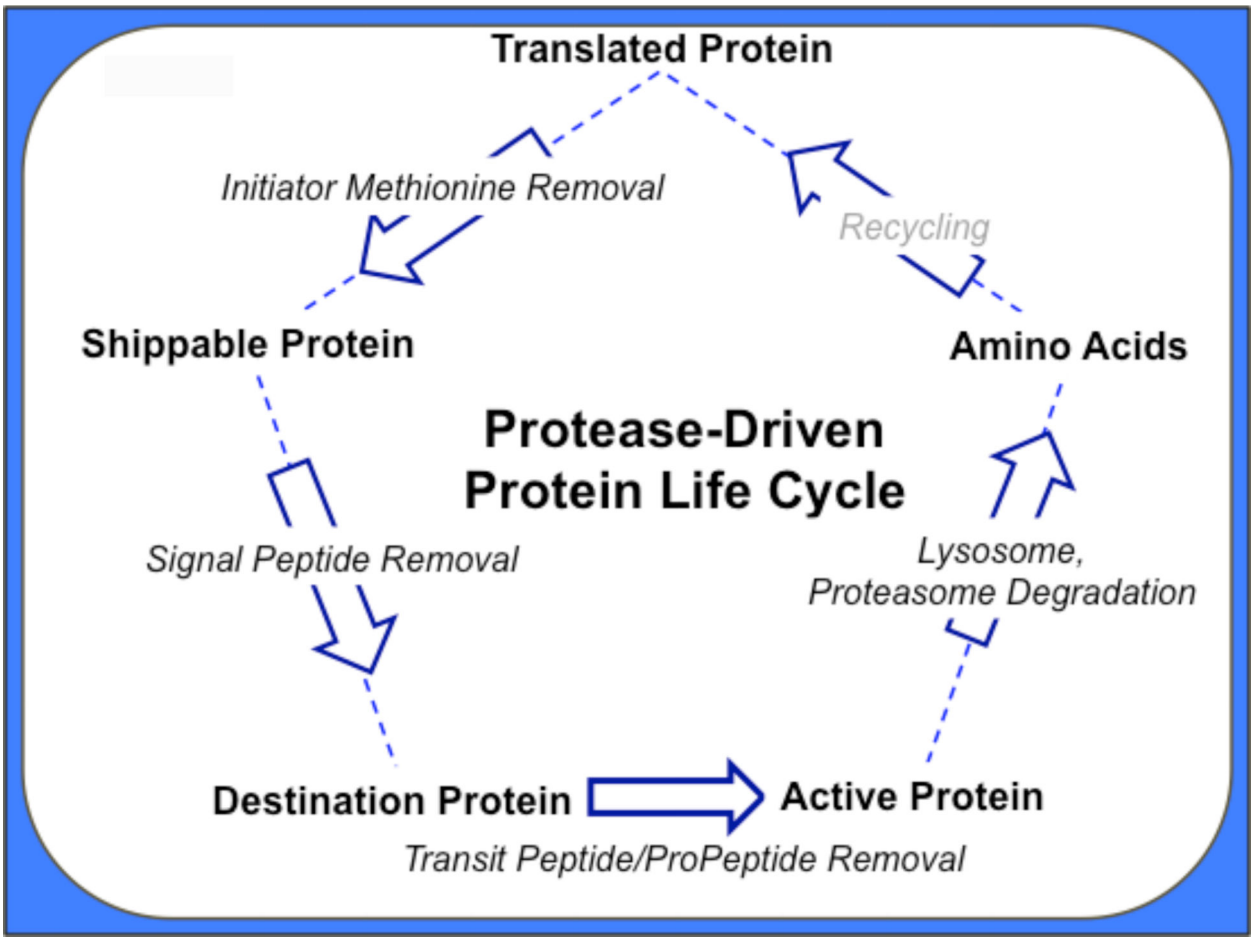

Figure 1:
Tracking the common steps in the “life” of a secreted protein, most are initiated or regulated by proteases as indicated by the arrow labels.

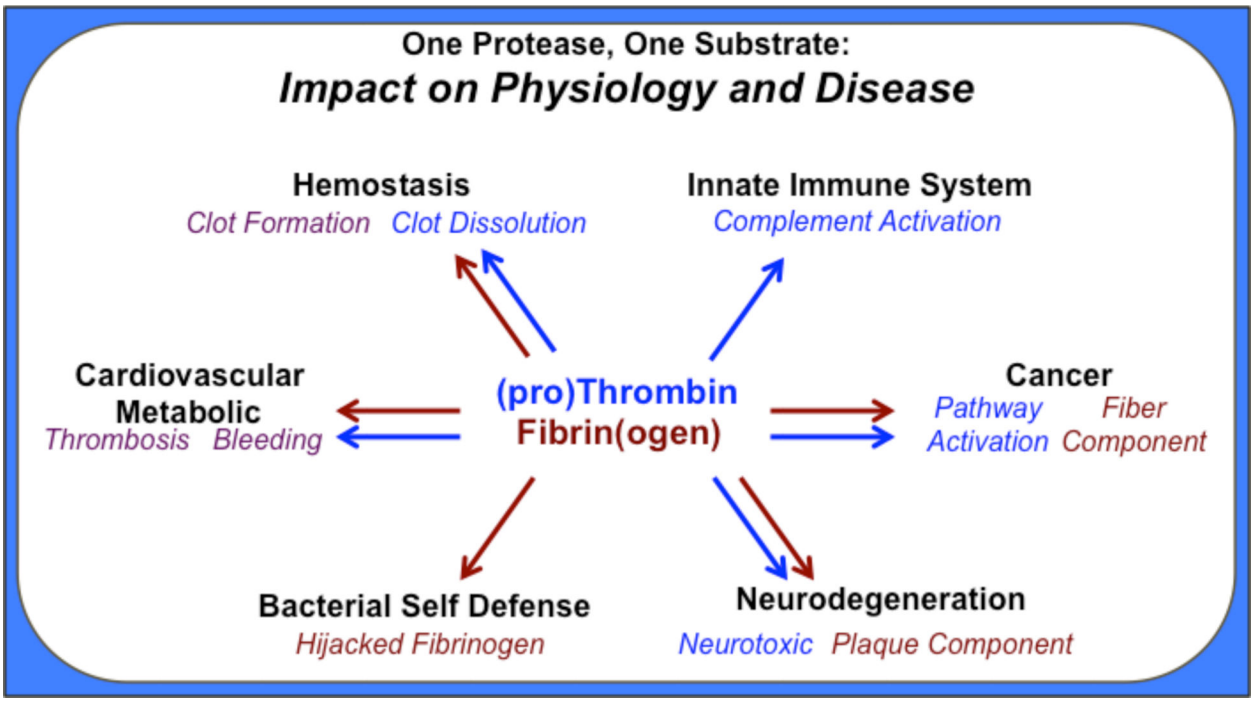

Figure 2:

The dual forms of Thrombin and Fibrin play critical roles in all of the systems and diseases shown in bold, with the specific effects italicized and color-coded to indicate which protein initiates the effect. This suggests a means by which an event, such as an immune response, may affect multiple systems or disease states through Thrombin or Fibrin activation, such as cardiovascular disease or cancer.

Table 1a:

Hemostasis Clot Formation

Protease/Inhibitor	Uniprot	Action
Thrombin (Prothrombin)	P00734	Converts fibrinogen to fibrin
		Activates factors V, VII, VIII, XI, XIII
		Complexes with thrombomodulin
		Thrombin/thrombomodulin activates protein C
Plasma factor VIIa (Coagulation factor VII)	P08709	Complexes with Tissue Factor
		VIIa/TF converts/activates X to XA
		VIIa/TF converts/activates IX to IXa
Tissue factor	P13726	Propagates coagulation protease cascade
		Complexes with phospholipids
		Complexes with circulating factor VII or VIIa
		VIIa/TF converts/activates X to XA
		VIIa/TF converts/activates IX to IXa
Coagulation factor X	P00742	Converts/activates prothrombin to thrombin
		Complexes with phospholipids & calcium
		Activates factor VII to form factor VIIa
Prekallikrein (Plasma kallikrein)	P03952	Reciprocal activation of factor XII
Coagulation factor XII	P00748	Reciprocal activation of Prekallikrein
Coagulation factor IX	P00740	Converts/activates factor X
		Activates factor VII to form factor VIIa
		Activates factor X to form factor Xa
Antithrombin (Antithrombin-III)	P01008	Inhibits thrombin & factors IXa, Xa and XIa
		Activity enhanced by heparin
Heparin cofactor II	P05546	Inhibits thrombin & factors IXa, Xa and XIa
		Inhibits chymotrypsin
TFPI (Tissue factor pathway inhibitor)	P10646	Inhibits factor X (X(a))
		TFPI + Xa inhibits VIIa/tissue factor

Hemostasis Clot Formation: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with hemostasis, specific to clot formation.

Table 1b:

Hemostasis Clot Degradation

Protease/Inhibitor	Uniprot	Action
tPA (Tissue-type plasminogen activator)	P00750	Converts plasminogen to plasmin on the fibrin surface
		Displaces plasmin from fibrin, promoting inhibition by alpha-2-antiplasmin
Plasmin (Plasminogen)	P00747	Dissolves the fibrin of blood clots
carboxypeptidase U (carboxypeptidase B2, thrombin-activatable fibrinolysis inhibitor)	Q96IY4	Removes C-terminal lysine residues from fibrin
		Down-regulates fibrinolysis
		Cleaves complement proteins C3a & C5a
		Activated by thrombin/thrombomodulin complex
Plasminogen activator inhibitor 1	P05121	Bait' for tissue plasminogen activator, urokinase, protein C and matriptase3/TMPRSS7
a2-antiplasmin (Alpha-2-antiplasmin)	P08697	Inhibits plasmin and trypsin
		Inactivates matriptase-3/TMPRSS7 and chymotrypsin
Protein C (Vitamin K-dependent protein C)	P04070	Inactivates factors Va and VIIIa in the presence of calcium ions and phospholipids
		Activated by Thrombin/thrombomodulin complex

Hemostasis Clot Degradation: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with hemostasis, specific to clot degradation.

Table 2:

The Complement System and Immune Regulation

Protease/Inhibitor	Uniprot	Action
C1R (Complement C1r subcomponent, Classical)	P00736	Cleaves/Activates C1s
C1S (Complement C1s subcomponent, Classical)	P09871	Cleaves/Activates C2 and C4
C2a (Complement C2, C1s cleavage product)	P06681	Combines with C4b to form C3 convertase (classical, lectin)
Factor D (Complement factor D, Bb fragment)	P00746	Cleaves/Activates Complement Factor B
Factor B (Complement factor B, Bb fragment)	P00751	Cleavage Product Bb combines with C3b to form C3 Convertase (Alternative)
C3-convertase (Classical, Lectin: C4bC2a)		Cleaves C3 into the anaphylatoxin C3a and the opsonin C3b
C3-convertase (Alternative: C3bBb)		Cleaves C3 into the anaphylatoxin C3a and the opsonin C3b
C3-convertase (Aqueous: C3:H2O)		Cleaves C3 into the anaphylatoxin C3a and the opsonin C3b
C5-convertase (Classical: Cell Membrane, C4b2b3b)		Cleaves C5 into the anaphylatoxin C5a and the MAC component C5b
C5-convertase (Alternative: Cell Membrane, C3bBbC3b)		Cleaves C5 into the anaphylatoxin C5a and the MAC component C5b
C5-convertase (Classical: Fluid, C4b2boxy3b)		Cleaves C5 into the anaphylatoxin C5a and the MAC component C5b
MASP 1 (Mannan-binding lectin serine protease 1)	P48740	Activates MASP2 or C2 or C3
MASP 2 (Mannan-binding lectin serine protease 2)	O00187	Cleaves/Activates C2 and C4
MASP 3 (Mannan-binding lectin serine protease 3)	P48740*	Cleaves/Activates complement pro-factor D *Alternative Splicing Product
C1-inhibitor (Plasma protease C1 inhibitor)*	P05155	Complexes with/Inactivates C1r, C1s, MASP 1, MASP 2, chymotrypsin, kallikrein, fXIa, FXIIa
		* complement activation, blood coagulation, fibrinolysis and the generation of kinins
Factor I (Complement factor I)	P05156	Cleaves/Inactivates C3b, iC3b and C4b

Complement System and Immune Regulation: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with the complement system and immune regulation.

Table 3:

Proteolytic Processing

Protease/Inhibitor	Uniprot	Action
Cathepsin B	P07858	Intracellular degradation and protein turnover
		Upregulation of Cathepsin D, matrix metalloproteinase, and urokinase
		Implicated in metastasis and immune resistance
Cathepsin D	P07339	Intracellular degradation and protein turnover
		Used by macrophages to degrade bacterial proteins
		Activates ADAM30, implicated in Alzheimer's progression
		Implicated in metastasis in breast cancer
Cathepsin L1	P07711	Intracellular degradation and protein turnover
		Degrades collagen ad elastin
		Degrades alpha-1 protease inhibitor
Deubiquitinase		Cleaves ubiquitin from proteins and other molecules
		Group of approx. 102 cysteine proteases and metalloproteases
Immunoproteasome		Degrades proteins into peptide ligands for Major Histocompatibility Complex (MHC)
		Proteasome with β1i, β2i, and β5i subunits
Thymoproteasome		Degrades proteins into peptide ligands for MHC 1, selective for CD8+ T cells
		Unique to thymic cortex
Trypsin-1	P07477	Degradation of food proteins in small intestine
Trypsin-2	P07478	Degradation of food proteins in small intestine
Mesotrypsin	P35030	Degradation of antitrypsin inhibitors
Chymotrypsinogen B1	P17538	Degradation of food proteins in small intestine
Chymotrypsinogen B2	Q6GPI1	Degradation of food proteins in small intestine
Serine Protease Inhibitor Kazal-type 1 (SPINK1)	P00995	Trypsin inhibitor, in pancreas protects against selfactivated trypsin
		Inhibits calcium binding and NO production in sperm

Proteolytic Processing: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with proteolytic processing.

Table 4:

Tissue remodeling, Signaling, Cell Migration and Proliferation

Protease/Inhibitor	Uniprot	Action
ADAM family (a disintegrin and metalloprotease)		Play roles in fertilization, proliferation, migration and cell adhesion
		Not all are proteases, those which are act as sheddases
ADAM-TS (a disintegrin and metalloproteinase with thrombospondin motifs		Process procollagen and von Willebrand factor, and cleave extracellular matrix aggrecan, versican, brevican and neurocan
MMPs (Matrix metalloproteinases)		All implicated in metastasis except 12, 20, and 28
TIMPs (tissue-inhibitors of metalloproteinases)		Endogenous inhibitors of MMPs
MMP-1	P03956	Cleaves collagens I, II, III, VII, and X
		Mediates neurotoxicity of HIV viral Tat protein
MMP-8	P22894	Degrades fibrillar collagens I, II, and III
MMP-13	P45452	Cleaves collagens I, II, III, IV, XIV, and X
		Degrades fibrillar collagen, fibronectin, tenascin C, and aggrecan
MMP-2	P08253	Degrades extracellular matrix proteins, including collagen I and IV
MMP-9	P14780	Cleaves collagen IV and V and fibronectin
		Implicated in neovascularization in malignant gliomas
MMP-3	P08254	Degrades fibronectin, laminin, gelatins of type I, III, IV, and V; collagens III, IV, X, and IX, and cartilage proteoglycans
		Activates MMPs 1, 7, and 9
MMP-10	P09238	Degrades fibronectin, gelatins of type I, III, IV, and V, collagens III, IV, and V
		Activates procollagenase
MMP-11	P24347	Cleaves alpha 1-proteinase inhibitor, activated intracellularly by furin
MMP-7	P09237	Degrades casein, gelatins of types I, III, IV, and V, and fibronectin
		Activates procollagenase
		Activates MMP2 and MMP9
MMP-12	P39900	Cleaves elastin, implicated in aneurysm formation
MMP-20	O60882	Degrades amelogenin, aggrecan, and cartilage oligomeric matrix protein (COMP)
MMP-26	Q9NRE1	Degrades collagen type IV, fibronectin, fibrinogen, beta-casein, type I gelatin and alpha-1 proteinase inhibitor
		Activates progelatinase B
MMP-28	Q9H239	Degrades casein

Tissue Remodeling, Signaling, Cell Migration, and Proliferation: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with tissue remodeling, signaling, cell migration, and proliferation.

Table 5:

Programmed cell death

Protease/Inhibitor	Uniprot	Action
Caspase-2	P42575	Function uncertain, sequence homology with initiator caspases
Caspase-8	Q14790	Activates caspases 3, 4, 6, 7, 9, and 10
		Activated by death receptors via FADD
Apoptotic protease activating factor 1 (APAF1)	O14727	Forms apoptosome complex
		Activated by binding of cytochrome c and ATP
Caspase-9	P55211	Activates caspase 3
		Activated by the apoptosome complex
		Cleaves poly(ADP-ribose) polymerase (PARP)
		Implicated in activation of Abelson murine leukemia viral oncogene homolog 1 (ABL1)
Caspase-10	Q92851	Activates caspases 3, 4, 6, 7, 8, and 9
		Activated by caspase 8
Caspase-3	P42574	Activates caspases 6, 7, and 9
		Activated by caspases 8 and 9
		Cleaves poly(ADP-ribose) polymerase (PARP)
		Cleaves and activates sterol regulatory element binding proteins (SREBPs)
		Implicated in Huntington's disease
Caspase-6	P55212	Dis-inhibits immune system, cleaves interleukin-10 and interleukin-1 receptor-associated kinase 3
		Cleaves poly(ADP-ribose) polymerase (PARP) and lamins
		Implicated in Huntington's and Alzheimer's
Caspase-7	P55210	Degradation of cellular proteins in apoptosis
		Cleaves poly(ADP-ribose) polymerase (PARP)
		Cleaves and activates sterol regulatory element binding proteins (SREBPs)
Apoptosome		Heptomeric complex of APAF-1, activates caspase 9
FasL	P48023	Tumor necrosis factor ligand, activates death receptors to initiate apoptosis
FasR	P25445	Death receptor, tumor necrosis factor receptor, activates caspase 8

Programmed Cell Death: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with programmed cell death.

Table 6:

Protein Secretion

Protease/Inhibitor	Uniprot	Action
Signal Peptidase		Removes amino terminal signal sequences from secretory pro-proteins
		Group of aspartic proteases
Insulin	P01308	increases cell permeability to monosaccharides, amino acids and fatty acids
		accelerates glycolysis, the pentose phosphate cycle, and glycogen synthesis in liver
Calcitonin	P01258	Promotes rapid incorporation of calcium and phosphate into bone
Atrial natriuretic peptide (ANP)	P01160	Peptide hormone
		regulates of natriuresis, diuresis, and vasodilation
		promotes trophoblast invasion and spiral artery remodeling in uterus during pregnancy
		Binds and stimulates the cGMP production of the NPR1 receptor
		Binds the clearance receptor NPR3
Brain natriuretic peptide (BNP)	P16860	Peptide hormone
		Regulates natriuresis, diuresis, vasorelaxation, and inhibition of renin and aldosterone secretion
		Binds and stimulates the cGMP production of the NPR1 receptor
		Binds the clearance receptor NPR3
Natriuretic peptide precursor C (NPPC)	P23582	Peptide hormone
		Regulates of cartilaginous growth plate chondrocytes proliferation and differentiation
		Binds and stimulates the cGMP production of the NPR2 receptor

Protein Secretion: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with protein secretion.

Table 7:

Intranuclear, Transmembrane, Intramembrane and Cytosolic Proteolysis

Protease/Inhibitor	Uniprot	Action
Matriptase (Suppressor of tumorigenicity 14 protein)	Q9Y5Y6	Degrades extracellular matrix, trypsin-like activity
		Promotes epithelial differentiation and possibly growth
		Implicated in metastasis
Matriptase 2	Q8IU80	Cleaves collagen I, fibronectin and fibrinogen
		Involved in matrix remodeling processes in liver
		Regulates the expression of the iron absorptionregulating hormone hepicidin/HAMP
Prostasin	Q16651	Stimulates epithelial sodium channel (ENaC) activity through activating cleavage of the gamma subunits (SCNN1G)
		Also found in seminal fluid
hepatocyte growth factor (HGF) activator inhibitor type2 (SPINT2)	O43291	Inhibits HGF, possibly inhibits serine proteases generally
		Implicated in suppression of liver cancer
hepatocyte growth factor activator inhibitor type1 (SPINT1)	O43278	Inhibits HGF and matriptase

Intranuclear, Transmembrane, Intramembrane and Cytosolic Proteolysis: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with intranuclear, transmembrane, intramembrane and cytosolic proteolysis.

Table 8:

Inflammation as an Over-Arching Symptom of Disease

Protease/Inhibitor	Uniprot	Action
Neutrophil Elastase	P08246	Broad substrate specificity, chymotrypsin family
		Inflammatory response triggers bacterial and host tissue destruction
		Inhibits C5a-dependent neutrophil enzyme release and chemotaxis
		Proteolysis of collagen-IV and elastin of the extracellular matrix
		Degrades pathogenic outer membrane proteins and virulence factors
Cathepsin G	P08311	Degrades ingested host pathogens
		Breaks down ECM components at inflammatory sites
		Cleaves complement component C3
		Inhibited by Rv3364c (M.tuberculosis protein)
		Indirect suppression of macrophage apoptosis
		Converts angiotensin I to angiotensin II
Proteinase-3 (Myeloblastin)	P24158	Degrades elastin, fibronectin, and collagen (in vitro)
		Target antigen for anti-neutrophil cytoplasmic antibodies (ANCA)
Alpha1-proteinase inhibitor (a1-PI, Alpha-1-antitrypsin)	P01009	Inhibits elastase, plasmin and thrombin
		Irreversibly inhibits trypsin, chymotrypsin and plasminogen activator
		Possible non-protease inhibitor activities:
		Anti- and pro-inflammation
		Anti-apoptosis
Leukocyte Elastase Inhibitor	P30740	Inhibits neutrophil elastase, cathepsin G, proteinase-3, chymase, chymotrypsin, and kallikrein-3
		Potent intracellular inhibitor of granzyme H
Tryptase:		Major neutral protease present in mast cells
		Resistant to endogenous proteinase inhibitors
		Active only as heparin-stabilized tetramers
		Six Isoforms: Tryptase alpha/beta-1(Q15661)
		Tryptase beta-2(P20231), Tryptase delta(Q9BZJ3)
		Tryptase gamma(Q9NRR2), Brain-specific serine protease 4(Q9GZN4)
Chymase (CMA1)	P23946	Major secreted protease of mast cells
		Release may promote inflammatory response
		Converts angiotensin I to angiotensin II
Granzyme B (GZMB)	P10144	Unique to cytolytic T-lymphocytes and natural killer cells
		Activates caspases 3, 7, 9, and 10
		Cleaves/activates BH3 interacting-domain death agonist (BID)
		Cleaves/activates Inhibitor of caspase-activated DNase (ICAD)
		Generate a cytotoxic level of mitochondrial reactive oxygen species
Carboxypeptidase A3 (CPA3)	P15088	Cleaves C-terminal aromatic or aliphatic residue
		Unique to mast cells

Protease/Inhibitor	Uniprot	Action
		Upregulated in sepsis and anaphylaxsis
		Implicated in autoimmune diseases
Alpha 1-antichymotrypsin (SERPINA3)	P01011	Inhibits neutrophil cathepsin G and mast cell chymase
Cathepsin L1	P07711	Intracellular degradation and protein turnover
		Degrades collagen ad elastin
		Degrades alpha-1 protease inhibitor
Cathepsin B	P07858	Intracellular degradation and protein turnover
		Upregulation of Cathepsin D, matrix metalloproteinase, and urokinase
		Implicated in metastasis and immune resistance
Cathepsin D	P07339	Intracellular degradation and protein turnover
		Used by macrophages to degrade bacterial proteins
		Activates ADAM30, implicated in Alzheimer’s progression
		Implicated in metastasis in breast cancer
Trypsin-3 (PRSS3) (mesotrypsinogen)	P35030	Degrades trypsin inhibitors
Serine Protease Inhibitor Kazal-type 1 (SPINK1)	P00995	Trypsin inhibitor, specifically inhibits autoactivated trypsin in the pancreas
		Inhibits calcium binding and NO production in sperm
Caspase-3	P42574	Activates caspases 6, 7, and 9
		Activated by caspases 8 and 9
		Cleaves poly(ADP-ribose) polymerase (PARP)
		Cleaves and activates sterol regulatory element binding proteins (SREBPs)
		Implicated in Huntington's disease

Inflammation as an Over-Arching Symptom of Disease: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with inflammation, specifically as related to symptoms of disease.

Table 9:

Cardiovascular and Metabolic Diseases, and Stroke

Protease/Inhibitor	Uniprot	Action
Factor VII (F7)	P08709	Complexes with Tissue Factor
		VIIa/TF converts/activates X to XA
		VIIa/TF converts/activates IX to IXa
Factor IX (or Christmas factor) (F9)	P00740	Converts/activates factor X
		Activates factor VII to form factor VIIa
		Activates factor X to form factor Xa
Factor X (Stuart–Prower factor) (F10)	P00742	Converts/activates prothrombin to thrombin
		Complexes with phospholipids & calcium
		Activates factor VII to form factor VIIa
Thrombin (Prothrombin)	P00734	Converts fibrinogen to fibrin
		Activates factors V, VII, VIII, XI, XIII
		Complexes with thrombomodulin
		Thrombin/thrombomodulin activates protein C
Factor XI (Plasma thromboplastin antecedent)	P03951	Activates factor IX
		Inhibited by protein Z-dependent protease inhibitor (ZPI)
Coagulation factor XII (Hageman factor)	P00748	Reciprocal activation of Prekallikrein
Prekallikrein (Plasma kallikrein)	P03952	Reciprocal activation of factor XII
C1-inhibitor (Plasma protease C1 inhibitor)*	P05155	Complexes with/Inactivates C1r, C1s, MASP 1, MASP 2, chymotrypsin, kallikrein, fXIa, FXIIa
		* complement activation, blood coagulation, fibrinolysis and the generation of kinins
Protein C (Vitamin Kdependent protein C)	P04070	Inactivates factors Va and VIIIa in the presence of calcium ions and phospholipids
		Activated by Thrombin/thrombomodulin complex
Factor V	P12259	Cofactor required by factor Xa
		Activated by Thrombin
		Degraded by protein C
Factor VIII (FVIII)	P00451	Cofactor required by factor IXa
		Deficiency results in hemophillia A
		High levels implicated in deep vein thrombosis and pulmonary embolism
Antithrombin (Antithrombin-III)	P01008	Inhibits thrombin & factors IXa, Xa and XIa
		Activity enhanced by heparin
Heparin cofactor II	P05546	Inhibits thrombin & factors IXa, Xa and XIa
		Inhibits chymotrypsin
Alpha 2-antiplasmin (or α2-antiplasmin or plasmin inhibitor)	P08697	Inhibits plasmin and trypsin
		Inactivates matriptase-3/TMPRSS7 and chymotrypsin
Plasmin	P00747	Dissolves the fibrin of blood clots

Protease/Inhibitor	Uniprot	Action
tPA (Tissue plasminogen activator)	P00750	Converts plasminogen to plasmin on the fibrin surface
		Displaces plasmin from fibrin, promoting inhibition by alpha-2-antiplasmin
Mast cell chymase	P23946	Major secreted protease of mast cells
		Release may promote inflammatory response
		Converts angiotensin I to angiotensin II
Tryptase {6 isoforms, see previous list table}		Major neutral protease present in mast cells
		Resistant to endogenous proteinase inhibitors
		Active only as heparin-stabilized tetramers
		Six Isoforms: Tryptase alpha/beta-1(Q15661)
		Tryptase beta-2(P20231), Tryptase delta(Q9BZJ3)
		Tryptase gamma(Q9NRR2), Brain-specific serine protease 4(Q9GZN4)
pro-MMP-9 (MMP-9)	P14780	Cleaves collagen IV and V and fibronectin
		Implicated in neovascularization in malignant gliomas
pro-MMP-1 (MMP-1)	P03956	Cleaves collagens I, II, III, VII, and X
		Mediates neurotoxicity of HIV viral Tat protein
pro-MMP-2 (MMP-2)	P08253	Degrades extracellular matrix proteins, including collagen I and IV
pro-MMP-3 (MMP-3)	P08254	Degrades fibronectin, laminin, gelatins of type I, III, IV, and V; collagens III, IV, X, and IX, and cartilage proteoglycans
		Activates MMPs 1, 7, and 9
Proteinase-3 (PRTN3)	P24158	Degrades elastin, fibronectin, and collagen (in vitro)
		Target antigen for anti-neutrophil cytoplasmic antibodies (ANCA)
Kallikrein 13	Q9UKR3	cleaves kininogen to the pro-inflammatory bradykinin
Cathepsin A	P10619	Protects beta-galactosidase and neuraminidase
Cathepsin C (CTSC)	P53634	Activates elastase, cathepsin G, granzymes A and B, neuraminidase, factor XIII, chymase, and tryptase
Cathepsin D	P07339	Intracellular degradation and protein turnover
		Used by macrophages to degrade bacterial proteins
		Activates ADAM30, implicated in Alzheimer’s progression
		Implicated in metastasis in breast cancer
Cathepsin L1	P07711	Intracellular degradation and protein turnover
		Degrades collagen ad elastin
		Degrades alpha-1 protease inhibitor
Cathepsin X/Z/P	Q9UBR2	Lysosomal protease, cleaves C-terminal residue
Calpain-10	Q9HC96	Limited proteolysis of substrates involved in cytoskeletal remodeling and signal transduction
Cathepsin K	P43235	Cleaves elastin, collagen, and gelatin
		Involved in breakdown of bone for remodeling
		Implicated in emphysema
		Activated by inflammatory cytokines
		Degraded by cathepsin S

Protease/Inhibitor	Uniprot	Action
Caspase-3 (CASP3)	P42574	Activates caspases 6, 7, and 9
		Activated by caspases 8 and 9
		Cleaves poly(ADP-ribose) polymerase (PARP)
		Cleaves and activates sterol regulatory element binding proteins (SREBPs)
		Implicated in Huntington's disease
Caspase-6	P55212	Dis-inhibits immune system, cleaves interleukin-10 and interleukin-1 receptor-associated kinase 3
		Cleaves poly(ADP-ribose) polymerase (PARP) and lamins
		Implicated in Huntington's and Alzheimer's
Caspase-8	Q14790	Activates caspases 3, 4, 6, 7, 9, and 10
		Activated by death receptors via FADD
Factor VII activating protease (FSAP)	Q14520	Activates factor VII and pro-urokinase
		May act as tumor suppressor
Tissue inhibitor of metalloproteinase-1 (TIMP-1)	P01033	Irreversibly inhibits MMP 1, 2, 3, 7, 8, 9, 10, 11, 12, 13, and 16
		Activates integrin signaling via CD63 and ITGB1

Cardiovascular and Metabolic Diseases, and Stroke: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with cardiovascular disease, metabolic diseases, and stroke.

Table 10:

Cancer

Protease/Inhibitor	Uniprot	Action
MMP-2	P08253	Degrades extracellular matrix proteins, including collagen I and IV
MMP-9	P14780	Cleaves collagen IV and V and fibronectin
		Implicated in neovascularization in malignant gliomas
MMP-14 (Matrix metalloproteinase-14)	P50281	Degrades extracellular matrix proteins
		Activates progelatinase A and MMP15
		Inhibits angiogenesis via cleavage of ADGRB1
ADAM-17 (ADAM metallopeptidase domain 17)	P78536	Activates tumor necrosis factor alpha
		Activates Notch Pathway
		Sheddase, activates multiple growth factors
		Implicated in tumor resistance to radiotherapy
Tissue inhibitor of metalloproteinase-1 (TIMP-1)	P01033	Irreversibly inhibits MMP 1, 2, 3, 7, 8, 9, 10, 11, 12, 13, and 16
		Activates integrin signaling via CD63 and ITGB1
TIMP-3 (Metalloproteinase inhibitor 3)	P35625	Irreversibly inhibits MMP 1, 2, 3, 7, 9, 13, 14, and 15
Thrombin (Prothrombin)	P00734	Converts fibrinogen to fibrin
		Activates factors V, VII, VIII, XI, XIII
		Complexes with thrombomodulin
		Thrombin/thrombomodulin activates protein C
PAR-1 (Proteinase-activated receptor 1 or coagulation factor II (thrombin) receptor)	P25116	Stimulates phosphoinositide hydrolysis
		Activated by thrombin
		May play a role in vascular development
SUMO (Sentrin-specific protease 7)	Q9BQF6	Removes SUMO (Small Ubiquitin-like Modifier protein) 2 and 3
Serine Protease HTRA1	Q92743	Degrades extracellular matrix
		Degrades insulin-like growth factor receptors and tuberin
Cathepsin L1	P07711	Intracellular degradation and protein turnover
		Degrades collagen ad elastin
		Degrades alpha-1 protease inhibitor
Cathepsin B	P07858	Intracellular degradation and protein turnover
		Upregulation of Cathepsin D, matrix metalloproteinase, and urokinase
		Implicated in metastasis and immune resistance
Cathepsin D	P07339	Intracellular degradation and protein turnover
		Used by macrophages to degrade bacterial proteins
		Activates ADAM30, implicated in Alzheimer’s progression
		Implicated in metastasis in breast cancer
Mesotrypsin (Trypsin-3)	P35030	Degradation of antitrypsin inhibitors

Protease/Inhibitor	**Uniprot**	**Action**
Matriptase (Suppressor of tumorigenicity 14 protein)	Q9Y5Y6	Degrades extracellular matrix, trypsinlike activity
		Promotes epithelial differentiation and possibly growth
		Implicated in metastasis
HAI-1 (hepatocyte growth factor activator inhibitor type1 (SPINT1))	O43278	Inhibits HGF and matriptase
HAI-2 (hepatocyte growth factor activator inhibitor type2 (SPINT2))	O43291	Inhibits HGF, possibly inhibits serine proteases generally
		Implicated in suppression of liver cancer
Proteasome		Group of massive protease complexes, stacked ring structure
		Degrade proteins “tagged” with multiple ubiquitins
		Critical in protein turn-over, apoptosis, and adaptive immune response
Kallikrein-3 (hK3)	P07288	Liquifies seminal fluid, degrades cervical mucus
		Elevated levels associated with prostate cancer
Kallikrein-5 (hK5)	Q9Y337	Degrades extracellular matrix proteins in epithelium, leads to cell shedding
Gingipains (RgpA, RgpB and Kgp)		
rgpA (Gingipain R1) <human>	P28784	Bacterial thiol proteases
		Degrade host tissue proteins and cytokines
SPINK6 (Serine protease inhibitor Kazal-type 6)	Q6UWN8	Inhibits KLK4, KLK5, KLK6, KLK7, KLK12, KLK13 and KLK14

Cancer: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with cancer.

Table 11:

Neurodegenerative Diseases

Protease/Inhibitor	Uniprot	Action
Amyloid β A4 precursor protein (APP)	P05067	Cell surface receptor involved in neuronal growth, adhesion, and motility
		Upregulated in neuronal repair
		Proteolysis generates amyloid β peptides (Abeta) from 37 to 49 residues in length, Abeta 40 and Abeta 42 implicated in Alzheimer's disease
α-secretase		Group of ADAM family sheddases which cleave the amyloid precursor protein (APP)
gamma secretase		Cleaves single-pass transmembrane proteins, including APP
		Transmembrane complex of presenilin-1 (PSEN1) (P49768), nicastrin (Q92542), anterior pharynxdefective 1 (APH-1) (Q5TB21), and presenilin enhancer 2 (PNE-2) (Q9NZ42)
Beta-secretase 1 (BACE1)	P56817	Transmembrane aspartic-acid protease important for myelin sheath formation
		Cleaves APP to form Abeta 40 and Abeta 42
		Several BACE1 inhibitors currently being tested as Alzheimer's treatments
ADAM10	O14672	Primary alpha secretase
MMP-9	P14780	Cleaves collagen IV and V and fibronectin
		Implicated in neovascularization in malignant gliomas
TIMPs (tissue-inhibitors of metalloproteinases)		Endogenous inhibitors of MMPs
Caspase-6	P55212	Dis-inhibits immune system, cleaves interleukin-10 and interleukin-1 receptor-associated kinase 3
		Cleaves poly(ADP-ribose) polymerase (PARP) and lamins
		Implicated in Huntington's and Alzheimer's
Calpain (family)		Family of calcium-dependent, non-lysosomal cysteine proteases
		Excessive activity implicated in cytoskeletal degradation and altered calcium homeostasis in Alzheimer's disease
Calpastatin	P20810	Endogenous calpain inhibitor
m-AAA (Mitochondrial AAA proteases)		Group of ATP-dependent mitochondrial proteases
HTRA2 (high temperature requirement)	O43464	Mitochondrial serine protease, initiates cell death by binding IAPs
		Implicated in Parkinson's disease
Presenilins-associated rhomboid-like protein (PARL)	Q9H300	Antiapoptotic, activates optic atrophy 1 (OPA1) which prevents release of cyctochrome C into cytosol
		Mutation controversially implicated in Parkinson's disease
SUMO-specific protease 2 (SENP2)	Q9HC62	Processes SUMO1, SUMO2, and SUMO3 into mature proteins
		Deconjugates SUMO1, SUMO2, and SUMO3 from target proteins

Neurodegenerative Diseases: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, a critical disease-related protease target, and cofactors associated with neurodegenerative diseases.

Table 12:

Autoimmune Diseases

Protease/Inhibitor	Uniprot	Action
MMP-9	P14780	Cleaves collagen IV and V and fibronectin
		Implicated in neovascularization in malignant gliomas
Caspase-1 (Interleukin-1 converting enzyme) CASP1	P29466	Activates interleukin 1β and interleukin 18, initiating inflammation
		Activates gasdermin D, initiating lytic cell death
		Activated by incorporation into inflammasone complex, initiated by NOD-like receptors or AIM-1 like receptors
		Inhibited by CARD only proteins (COPs), COPs prevent formation of inflammasome
SPPL2A (Signal peptide peptidase-like 2A)	Q8TCT8	Cleaves type II membrane signal peptides, such as tumor necrosis factor alpha (TNF), the Fas antigen ligand (FASLG), and Cluster of Differentiation 74 (CD74)
		Initiation of innate immune response through CD74 activation implicated in autoimmune diseases
Cathepsin S	P25774	Cleaves proteins into peptides for presentation as antigens in macrophages, B-lymphocytes, microglia, and dendritic cells
ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13)	Q76LX8	Degrades von Willebrand factor, adversely affects clot formation

Autoimmune Diseases: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with autoimmune diseases.

Table 13:

Proteases, Inhibitors and Cofactors in Infectious Organisms

Protease/Inhibitor	Uniprot	Action
HIV protease (HIV-1)	P04585	Cleaves viral polyprotein into individual proteins, including itself
		Critical for viral replication, prominent drug target
Trypanosoma cruzi cruzipain	P25779	cysteine protease expressed by *Trypanosoma cruzi,* vital to the parasitic protozoa's life cycle
Porphyromonas gingivalis gingipains	P28784	Bacterial thiol proteases
		Degrade host tissue proteins and cytokines
Bacillus anthracis lethal factor		Anthrax protein which degrades mitogen-activated protein kinase kinase, disrupting function of mitogen-activated protein kinases (MAPKs)
Subtilisin		Nonspecific bacterial protease known to activate thrombin
staphylocoagulase (1 & 2)	P07767 & P17855	Activates thrombin through binding, not a protease
streptokinase		Activates plasmin
Von Willebrand factorbinding protein	A0A1D4Z3F9	Staphylococcus protein promotes clot formation
Staphylokinase	P68802	Staphylococcus protein which activates plasmin
skizzle (SkzL)	Q8DZH4	Streptococcus protein, enhances activation of plasminogen
ADAMTS7	Q9UKP4	Degrades cartilage oligomeric matrix protein (COMP)
		Implicated in cancer, arthritis, and coronary artery disease
		Required for influenza A virus replication
carboxypeptidase E	P16870	Cleaves C-terminal arginine of lysine residue
		Processes most neuropeptides and peptide hormones
		Required for influenza A virus replication
dipeptidyl peptidase 3	Q9NY33	Degrades angiotensin, Leu-enkephalin, and Metenkephalin
		Implicated in ovarian cancer
		Required for influenza A virus replication
macrophage stimulating 1 protease (macrophage stimulating protein)	P26927	Unknown, sequence homology with hepatocyte growth factor
		Required for influenza A virus replication
neurotrypsin	PRSS12	Cleaves agrin
		Multidomain serine protease expressed in the nervous system
		Required for influenza A virus replication
ubiquitin-specific protease 14	P54578	Proteasome-associated deubiquitinase, prevents ubiquitin digestion
		Prevents degradation of prion protein

Proteases, Inhibitors and Cofactors in Infectious Organisms: Activities and uniprot ID codes (where applicable) of proteases, protease inhibitors, and cofactors associated with infectious organisms.