The cysteine protease legumain – from cell biology to cardiovascular disease

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LIST OF PUBLICATIONS

This thesis is based on the following publications which will be referred to by their roman numeral in the text:

Paper I

Lunde NN, Haugen MH, Bodin Larsen KB, Damgaard I, Pettersen SJ, Kasem R, Rut W, Drag M, Poreba M, Johansen HT, Solberg R.

Glycosylation is important for legumain localization and processing to active forms but not for cystatin E/M inhibitory functions

Biochimie, 10.1016/j.biochi.2017.05.009

Paper II

Lunde NN, Holm S, Dahl TB, Elyouncha I, Sporsheim B, Gregersen I, Abbas A, Skjelland M, Espevik T, Solberg R, Johansen HT, Halvorsen B.

Increased levels of legumain in plasma and plaques from patients with carotid atherosclerosis

Atherosclerosis, 10.1016/j.atherosclerosis.2016.11.026

Paper III

Lunde NN[#*], Gregersen I[#], Ueland T, Shetelig C, Holm S, Kong XY, Michelsen AE, Otterdal K, Yndestad A, Broch K, Gullestad L, Nyman TA, Bendz B, Eritsland J, Hoffmann P, Skagen K, Gonçalves I, Nilsson J, Grenegård M, Poreba M, Drag M, Sporsheim B, Espevik T, Skjelland M, Johansen HT, Solberg R, Aukrust P[#], Björkbacka H[#], Andersen GØ[#], Halvorsen B.

Upregulation of the cysteine protease legumain in acute cardiovascular events – potential role in platelet-monocyte/macrophage interactions

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Submitted

ABSTRACT

The cysteine protease legumain is reported to be involved in the pathologies of several inflammatory conditions. The subcellular localization of legumain is thought to be mainly lysosomal. However, translocation to other cell compartments as well as secretion to the extracellular environment and appearance in serum has been reported. Being widespread in the body, legumain is suggested to have multiple functions, both intra- and extracellularly. Dysregulation of legumain is suggested to be of consequence for proteolytic degradation and activation of several proteins. Furthermore, high legumain expression is correlated with cancer progression and malignancy as well as atherosclerotic plaque instability, and pharmacological intervention targeting legumain could be beneficial.

The aim of this thesis was to investigate posttranscriptional glycosylation, localization and regulation of legumain in inflammatory conditions (**paper I-III**), with a focus on atherosclerosis and cardiovascular disease (CVD). Atherosclerosis is a persistent inflammatory process, whereas alteration in N-linked glycosylation, a major posttranslational modification, is associated with inflammation and malignant conditions.

The glycosylation of legumain and cystatin E/M were investigated for the first time. The sugar moieties on legumain were shown to be of high or hybrid type, whereas cystatin E/M is complex mannose-linked. The results suggest that glycosylation of prolegumain is necessary for correct processing to active forms and internalization, whereas the inhibitory property of cystatin E/M is independent of the glycosylation status (**paper I**).

Also, for the first time, legumain was quantified in plasma from patients with carotid atherosclerosis (**paper II**). Patients had increased levels of legumain in plasma and plaques, with the highest level in patients with symptomatic disease. Within the atherosclerotic lesion, legumain was co-localized with macrophages, and *in vitro*, pro-inflammatory M1 macrophages secreted higher levels of legumain after stimulation with cholesterol crystals. Together with the proposed role of legumain in matrix degradation, these findings suggest a role of legumain as a potential biomarker for atherosclerotic disease.

At present, data on regulation of legumain during acute cardiovascular events and in patients with CVD are scarce. The results presented in this thesis show elevated circulating levels of legumain in two large patient populations with stable or acute CVD (**paper III**). High levels of

legumain in acute event patients correlated with improved outcome. Macrophages, platelets and alterations in glycosylation promote extracellular localization of legumain (**paper I-III**). Furthermore, depending of glycosylation status, legumain is internalized and processed by cells (**paper I**) to mediate anti-inflammatory effects through platelet and macrophage interactions to modulate plaque stability (**paper III**). Also, legumain is present in thrombus material obtained at the site of vascular occlusion in STEMI patients and patients with acute ischemic stroke (**paper III**).

In conclusion, the results presented in this thesis contribute to new knowledge on regulation of the cysteine protease legumain, especially during atherosclerosis. The studies indicate a shift of paradigm from the classical malign and pro-inflammatory association of this protease to a novel player in modulation of plaque stability and potentially mediating anti-inflammatory effects, and/or potentially as a new biomarker in CVD.

ABBREVIATIONS

ABP	Activity-based probe
ACP	Asparaginyl carboxypeptidase
AEP	Asparaginyl endopeptidase
AP	Activation peptide
APC	Antigen presenting cell
APP	Amyloid precursor protein
Asn/N	Asparagine
Asp/D	Aspartate
ATCC	American Type Culture Collection
C-terminal	Carboxy-terminal
C4S	Chondroitin-4-sulfate
Cath	Cathepsin
CC	Cholesterol crystal
CD	Cluster of differentiation
CVD	Cardiovascular disease
E64	Trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
GAG	Glycosaminoglycan
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
hCE	Human cystatin E/M
HCT116	Human colorectal cancer cell line
HEK293	Human embryonic kidney 293 cells
HS	Heparan sulfate
IB	Immunoblot
IC_{50}	Half maximal inhibitory concentration
IF	Immunofluorescence
IL	Interleukin
IFN	Interferon
K_i	Inhibition constant
KO	Knock-out
LDL	Low-density lipoprotein
LEL	Legumain exosite loop
LPS	Lipopolysaccharide
LSAM	Legumain stabilization and activity modulation domain
M1	Pro-inflammatory macrophages
M2	Anti-inflammatory and tissue remodeling macrophages
M38L	Monoclonal legumain over-expressing HEK293 cells
M4C	Monoclonal cystatin E/M over-expressing HEK293 cells
mAb	Monoclonal antibody
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
MI	Myocardial infarction

MMP	Matrix metalloprotease
mRNA	Messenger RNA
N-terminal	Amino-terminal
NSTEMI	Non-ST-elevated MI
NTF	N-terminal fragment
oxLDL	Oxidized low-density lipoprotein
pAb	Polyclonal antibody
PAR	Protease-activated receptor
PBMC	Peripheral blood mononuclear cells
PCI	Percutaneous coronary intervention
PMA	Phorbol 12-myristate 13-acetate
PRL	Platelet releasate
PRP	Platelet-rich plasma
PTC	Proximal tubular cells
PTM	Posttranslational modification
qRT-PCR	Quantitative real-time polymerase chain reaction
RGD	Arginine-glycine-aspartate
RNA	Ribonucleic acid
SMC	Smooth muscle cell
SP	Signal peptide
STEMI	ST-elevated MI
SW620	Human colorectal cancer cell line
T2D	Type-2 diabetes mellitus
TAM	Tumor-associated macrophage
TGN	*Trans*-Golgi-Network
THP-1	Human monocytic cell line
TLR	Toll-like receptor
TNF	Tumor necrosis factor

INTRODUCTION

Proteolytic enzymes

Proteolytic enzymes (also known as proteases, peptidases or proteinases) are important in mammalian physiology as they participate in numerous biological processes, including embryonic development, differentiation, cell proliferation, apoptosis, tissue remodeling, wound healing, cell migration and angiogenesis [1, 2]. These enzymes constitute 2 % of all functional genes [3], and it is estimated that 14 % of the 500 known human proteases are under investigation as drug targets [3].

Proteolysis is cleavage of polypeptides by irreversible hydrolysis (nucleophilic attack) of peptide bonds. Endopeptidases cleave internally in the polypeptide chain, whereas exopeptidases cleave at the C- or N-terminal end, resulting in the release of either a single amino acid or a dipeptide. The MEROPS database classifies mammalian proteases into five classes based on their proteolytic mechanism; aspartic, cysteine, metallo, serine and threonine proteases [3] (**Figure 1**). This thesis will be focusing on the cysteine proteases.

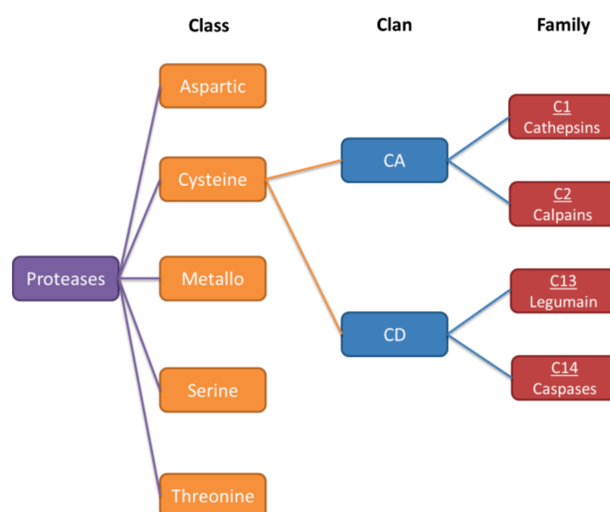

Figure 1. Classification of mammalian proteases according to the MEROPS database. Proteases are organized based on their catalytic mechanisms and structural similarities. The proteases are first divided into classes based on their catalytic type, called aspartic, cysteine, metallo, serine or threonine (orange boxes). These protease classes are further divided in clans (blue boxes, exemplified by clan CA and CD of cysteine proteases) based on common evolutionary origins such as similar three-dimensional protein structures or the order of amino acids comprising the catalytic part of the polypeptide chains. Finally, each clan is further divided into one or more protease families (red boxes) based on high similarity in the amino acid sequences. Modified from [3].

Cysteine proteases

Cysteine proteases (with a cysteine in the active site) are divided into 11 different clans and over 60 families, and found in all living organisms, emphasizing their functional importance [3]. The first identified cysteine protease was found in latex of the papaya fruit, was designated papain and classified to clan CA, family C1. The mammalian analogs of papain are the lysosomal cysteine cathepsins (cathepsin B, C, F, H, K, L, O, S, V, W and X) [3, 4]. The levels of several cathepsins are elevated in cancer cells and tumor-associated cells, and increased secretion from tumors has been shown [5-7]. Also, cathepsins B, K, L and S are implicated in cardiovascular disease [8-13]. In addition, cathepsin B, L and S play important roles in the major histocompatibility complex (MHC) class II-mediated antigen processing and presentation [14-16]. The main focus in this thesis is another lysosomal cysteine protease, legumain, which belongs to family C13, and is described in detail below.

Legumain

The first reports of legumain were in beans (presence in legumes, thus termed legumain) [17-19] and blood fluke (*Schistosoma mansoni*) [20, 21]. In 1997, Chen and co-workers described the mammalian legumain with the presence of a conserved His^{148}-Gly-spacer-Ala-Cys^{189} motif, which is highly conserved for the clan CD members [22]. Legumain is closely related to caspases (family C14) with approximately 15 % sequence homology and sharing the strict specificity for one particular amino acid at the P1 position. While caspases prefer aspartate (Asp), legumain cleaves only carboxy-terminally to asparagine (Asn), thus synonymously termed asparaginyl endopeptidase (AEP). However, at acidic pH, legumain also acquires activity towards cleavage at Asp, becoming a caspase-like enzyme [23-25]. A striking difference between legumain and the caspases is the cellular location (lysosomal versus cytosolic) and the pH-dependence (active at acidic versus neutral pH). The functional human legumain gene (*LGMN*) is localized on chromosome 14 at locus 14q32.12, and the gene is encoding a protein of 433 amino acids [22, 26]. In addition, a pseudogene of legumain has been reported with unknown function in humans [3].

Mammalian legumain is expressed as an inactive proenzyme of 56 kDa consisting of a catalytic domain and a C-terminal prodomain (**Figure 2A**). The crystal structure of prolegumain was revealed in 2013 by the Brandstetter group [27] (**Figure 2B**). This structure confirmed the postulated structural similarity and evolutionary relationship with the caspases [23, 28]. The

prodomain comprises an activation peptide (AP) and a positively charged legumain stabilization and activity modulation (LSAM) domain, positioned on the top of the active site, restricting substrate access, explaining the enzymatically inactivity of prolegumain. The AP blocks access on the non-primed and S1' site, while the LSAM domain stabilizes the highly negatively charged catalytic domain by electrostatic interactions [27].

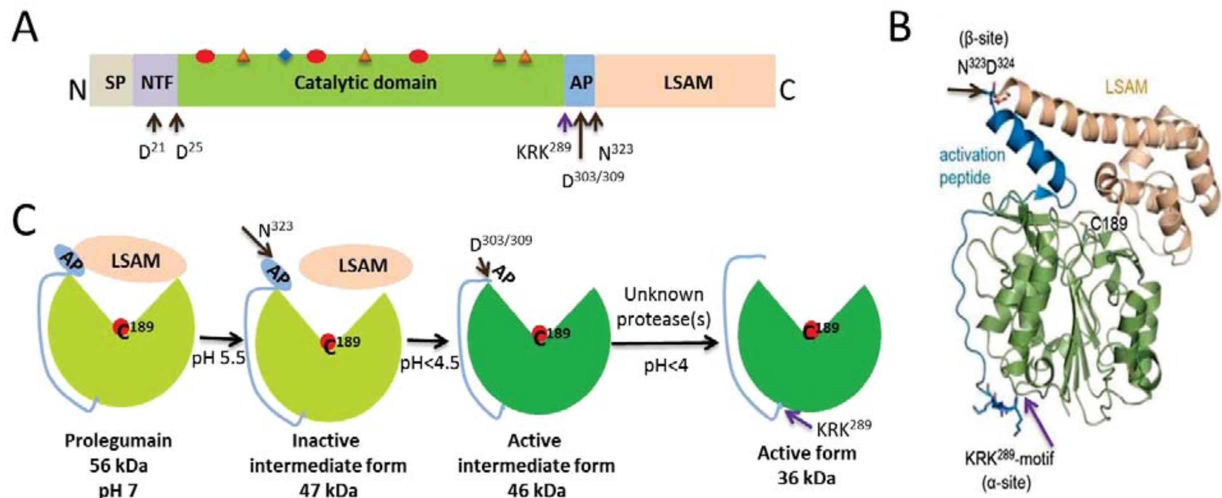

Figure 2. The 1D-diagram, crystal structure and the proposed multistep activation of human legumain. **A**. Prolegumain consists of a signal peptide (SP, grey), N-terminal fragment (NTF, light purple), catalytic domain (green), activation peptide (AP, blue), legumain stabilization and activity modulation (LSAM, wheat) domain. Brown arrows indicate autocatalytic processing sites while purple arrow indicates non-autocatalytic processing. Red circles indicate catalytic residues (Asn^{42}, His^{148} and Cys^{189}), orange triangle indicates possible glycosylation sites and the RGD^{120} motif is labeled by a blue diamond. Figure modified from [29]. **B**. The crystal structure of prolegumain with the main domains from A. Cleavages at Lys-Arg-Lys^{289} (KRK^{289}, α-site, purple arrow) and Asn^{323}-Asp (N^{323}-D, β-site, brown arrow) are essential for legumain activity. The catalytic cysteine residue (C^{189}) is protected by the LSAM. Figure modified from [27]. **C**. Inactive prolegumain (light green catalytic domain with AP and LSAM) is synthesized at neutral pH. The proenzyme undergoes autocatalytic processing (brown arrows) at the C-terminal Asn^{323} (N^{323}, pH ≤ 5.5) and Asp$^{303/309}$ (D$^{303/309}$, pH ≤ 4.5) sites, and N-terminally after Asp^{21} (D^{21}) and Asp^{25} (D^{25}) (not shown). Another *in trans* processing occurs after the KRK^{289} motif (purple arrow) by unknown protease(s). Release of the LSAM domain is mandatory to gain AEP-activity (dark green).

When entering the endoplasmic reticulum (ER), the signal peptide is cleaved. This is followed by an autocatalytic processing of prolegumain at both termini at pH below 5.5 in the endolysosomal compartments, resulting in an inactive 47 kDa and an active 46 kDa intermediate variant [30, 31] (**Figure 2C**). In mammals, the C-terminal cleavage after Asn^{323} was proven to be critical for

activation, while the N-terminal processing after Asp[21] and Asp[25] does not seem to be essential for protease activity [25]. Under the maturation process, autoproteolytic cleavage of the LSAM domain opens access to the active site [28]. Zhao and co-workers discovered an intermediate autoproteolytic maturation stage at approximately pH 4.5 [28]. The partially activated legumain could be reversed to its proform in a pH close to neutral or further irreversibly processed to a fully activated mature 36 kDa form at pH below 3.5 [28]. Processing to the mature form *in vivo* is not autocatalytic and dependent on other as yet unknown protease(s) [30]. At the intermediate stages, legumain could rapidly shift between active and proform to achieve either proteolytic activity in acidic pH or stability in a more neutral pH [32]. Interestingly, the enzymatic (AEP) activity of the 36 kDa and the 46 kDa forms towards low molecular substrates seems to be identical [31]. Active legumain exists as a monomer in solution, whereas prolegumain forms a homodimer [27].

Legumain is predominantly localized to the late endosomes and lysosomes [33] where it is active due to the acidic pH and a reducing redox-potential (**Figure 3**). The activity and stability of legumain is highly regulated by its environment [22]. Legumain is stable in its proform at pH 7, however the mature form is unstable and rapidly inactivated at pH >6 [25, 29, 31]. Nevertheless, active legumain has been identified in the nucleus of colorectal cancer cells [34]. Posttranslational conjugation of ubiquitin monomers to legumain by the ubiquitination enzyme TRAF6 can promote intracellular stability [35]. In addition, ubiquitinated legumain results in a ternary complex formation with heat shock protein HSP90α, which increases intracellular legumain stability [35]. The proenzyme has also been reported to be extensively secreted and present extracellularly [36], as well as associated with matrixes and cell surfaces [37]. Interestingly, matrix components like endogenous polyanionic glycosaminoglycans (GAGs) and alginates can accelerate the autocatalytic activation of prolegumain through ionic interactions in a concentration-, pH- and time-dependent manner [38, 39]. Moreover, legumain can bind to the $\alpha_v\beta_3$ integrin receptor on cell membranes due to an Arg-Gly-Asp[120] (RGD) motif [27, 40]. Upon binding to this receptor, the pH optimum of legumain activity is increased, indicating that legumain could also be active extracellularly (**Figure 3**). Also, complex formation with endogenous cystatin inhibitors (cystatins E/M and C, see below) can stabilize active legumain at neutral pH [41, 42]. LSAM can also function as a direct stabilizer of the catalytic domain by physically protection in a neutral pH environment [27]. Recently, legumain has been found in

exosomes derived from M2 macrophages [43]. Extracellular legumain is internalized by cells and subsequently processed and activated [44].

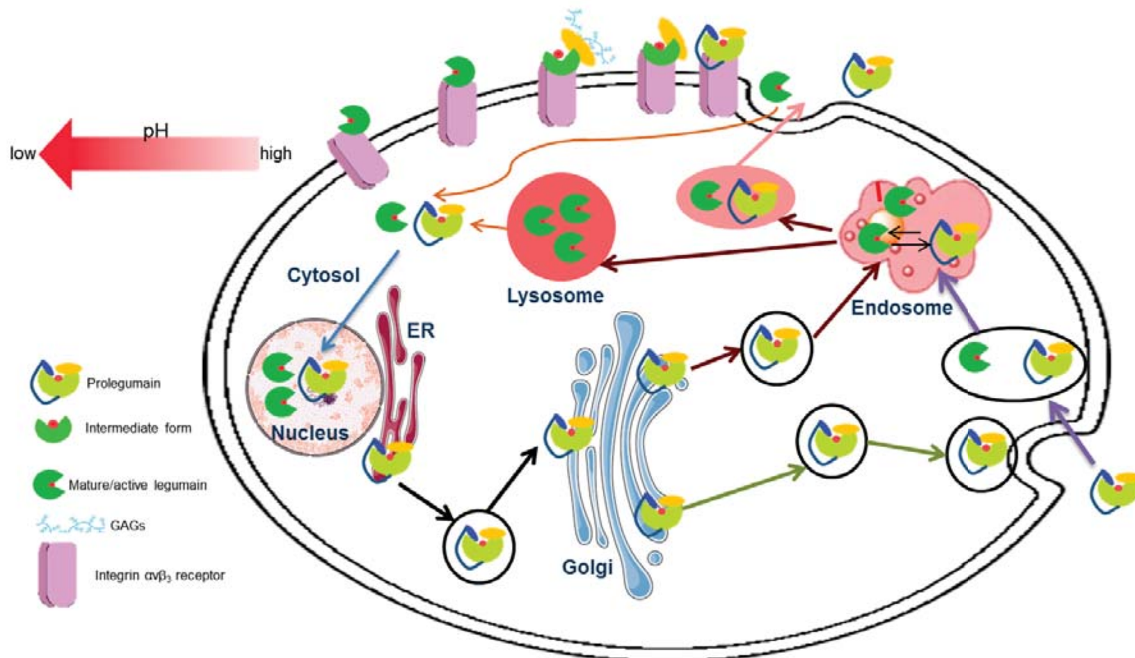

Figure 3. Intra- and extracellular trafficking of legumain. After synthesis, inactive prolegumain is targeted via endoplasmic reticulum (ER) and Golgi to the endolysosomal system, where it is processed and activated at acidic pH (black and brown arrows). Alternatively, prolegumain is secreted, directly via Golgi (green path) or indirectly via the endosomal system (brown and pink path). Glycosaminoglycans (GAGs) and integrin receptors might stabilize active legumain extracellularly. Extracellular legumain may internalize via endocytosis (purple path) or directly via translocation to the cytosol (orange path). In pathological conditions where the lysosomes are altered, i.e. Alzheimer's disease, legumain may enter the cytosol directly from leaky lysosomes. Additionally, legumain may enter the nucleus (blue path).

Endogenous regulators of legumain

In the late 60's, the cystatins were first discovered in chicken egg white [45], and later 12 functional human cystatins have been described [46]. The role of cystatins is to protect cells and tissues from harmful proteolytic activity [47]. Loss of cystatin functions results in uncontrolled activity of cysteine proteases and may lead to a variety of disorders, including chronic inflammations [48] and tumor malignancies [49]. Thus, the interplay between proteases and their endogenous inhibitors is of major importance.

Cystatins are non-selective, reversible cysteine protease inhibitors and are classified into three subfamilies [3]: Family 1 including the primarily intracellular cystatin A and B (stefin A and B),

family 2 including cystatins C, D, E/M, F, G, S, SN and SA, whereas family 3 includes L- and H-kininogen present in plasma.

The family 2 cystatins are mainly secretory proteins. Some of the family 2 cystatins are ubiquitous in protease inhibition and tissue distribution, like cystatin C, while others have more specialized biological functions, such as cystatin F and D [46, 50]. Cystatin C, E/M and F harbor, in addition to their papain-binding site, a legumain binding site [51]. Cystatin E/M has the highest inhibitory activity against legumain (K_i= 10-13 pM) [42], and is further addressed below.

Cystatin E/M

The gene encoding cystatin E/M (*CST6*) is located on chromosome 11 and is expressed in most human tissues, with the highest expression in uterus and liver [52]. The inhibitor can be N-glycosylated (see below) [52]. Cystatin E/M has a legumain binding site localized to the reactive center loop different from the papain binding site [29, 51] (**Figure 4**). Furthermore, the interaction with legumain requires an additional legumain exosite loop and complex formation leads to conformational stabilization of the pH-sensitive legumain at near neutral pH [42]. Moreover, cystatin E/M forms an irreversible homodimer via domain swapping under destabilizing conditions (low pH and heating) and is the thermodynamically preferred conformation [42]. The dimeric human cystatin E/M has two symmetric and functional legumain binding sites, however simultaneous binding to both sites is sterically impossible [42] (**Figure 4**). Dimeric cystatin E/M further converts to fibrils but is still functional as a legumain inhibitor.

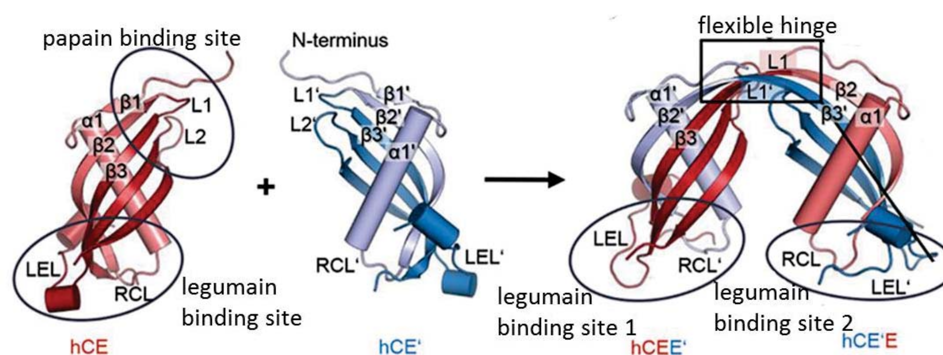

Figure 4. The human cystatin E/M (hCE) forms a dimer via domain swapping and interaction with legumain. Crystal structure of monomeric hCE with exposed papain- and legumain-binding sites. The regions of two monomers (hCE, red and hCE′, blue) that undergo domain swapping are shown in light colors. The dimer is composed of two symmetric subunits, hCEE′ and hCE′E. RCL, reactive center loop; LEL, legumain exosite loop. Figure adopted from [42].

In breast cancer, cystatin E/M has been described as a candidate tumor suppressor [53-55] as epigenetic silencing of cystatin E/M occurs during breast cancer progression [56]. In tumor cells, legumain and cathepsin L appear to be regulated by cystatin E/M. *In vitro*, cystatin E/M suppresses legumain activity and invasion of human melanoma cells [57]. In cystatin E/M deficient mice, unrestricted legumain activity is involved in disturbed epidermal cornification [58], abnormal stratum corneum and hair follicle formation, as well as to a severe disturbance of skin barrier function [59].

Activities of legumain and interplay with substrates

Processing of a protein substrate by legumain could either result in activation or inactivation of substrate functions. Legumain is highly specific for hydrolysis of the peptide bonds at the C-terminal side (P1 position) of Asn [22]. However, at low pH, legumain has also substrate specificity towards carboxy-terminal cleavage after Asp [24, 25], thus acquiring caspase-like properties. Legumain is involved in the proteolytic maturation of cathepsins B, H and L in the endo/lysosomes due to its AEP-activity [60] (**Table 1**). In addition, legumain is postulated to have asparaginyl carboxypeptidase activity which remains stable at neutral pH due to selective proteolytic removal of the AP domain, whereas LSAM is still bound to the catalytic domain by electrostatic interactions [27]. Such pH-dependent endo- and carboxypeptidase activities is also observed for cathepsin B [61].

The notion that legumain in addition to being a protease also harbors ligase activity was postulated in 1993 by Ishii [62]. The ligase reaction is independent of the catalytic cysteine (Cys^{189}) but exploits an endogenous energy reservoir that results from the conversion of a conserved aspartate to a metastable aspartimide [63]. Cystatin E/M and C have been shown not only to inhibit legumain but also to be both cleaved and ligated by legumain [27, 32, 41] (**Table 1**). The cleavage of cystatin E/M was shown to be favorable at acidic pH with complete cleavage at pH ≤ 4, while ligation was most efficient at pH ≥ 6 [41]. Thus, the dual protease-ligase activities of legumain are controlled by the environmental pH. Legumain could possibly act as a ligase in the extracellular or cytosolic neutral milieu [63]. The ligase activity of legumain is not limited to cystatins, as legumain may also religate itself during autoprocessing [63], shown for both human [41] and mouse legumain [32]. Known legumain substrates are listed in **Table 1**.

Table 1: Known mammalian AEP- and ligase substrates of legumain

AEP-substrates	Ligase-substrates	Reference
Acetoacetyl-CoA synthetase		[64]
Alpha-1-macroglobulin		[65]
α-synuclein		[66, 67]
Amyloid precursor protein (APP)		[68]
Annexin A2		[69]
Beta-amyloid protein 40		[65]
Betaine-homocysteine S-methyltransferase 1		[70]
BetV1		[71]
Cathepsin B, H, L, S		[60, 72]
Cystatin C, E/M	Cystatin C, E/M	[41]
Fibronectin		[69, 73]
Invariant chain chaperone (li)		[74]
Myelin basic protein		[75, 76]
Progelatinase A (proMMP-2)		[77]
Prolegumain	Prolegumain	[24, 25, 30, 31]
Prothymosin α		[78]
Serotransferrin		[65]
Serum albumin		[65]
SET (also known as PHAPII, TAF-Iβ, I2 PP2A)		[79]
TAR DNA-binding protein 43		[80]
Tau		[81-85]
Toll-like receptor (TLR) 3, 7 and 9		[86-90]
Tetanus toxin C-terminal fragment		[91, 92]
Vitamin D binding protein		[69]

Posttranslational modifications of legumain and cystatin E/M

Posttranslational modification (PTM) is referred to the covalent attachment of a chemical group (i.e. phosphate, glycan, ubiquitin) to amino acids subsequent to protein translation [93]. Some PTMs are very rare, whereas others are common. The nature of some of PTMs makes them reversible whereas others are permanent, and some occur spontaneously while others require enzymatic reactions. The majority of membrane bound and secreted proteins, as well as many intracellular proteins, are glycosylated [94]. Protein glycosylation is the covalent attachment of glycan moieties to polypeptides [93] and is complex due to a variety in character and heterogeneity of the attached carbohydrate groups [95]. Glycoproteins are ubiquitous in the

extracellular milieu and are typically dysregulated in cancers [96]. Since glycoproteins are likely to enter the blood, they are attractive candidates as cancer biomarkers.

N-glycosylation is a major PTM known to influence protein transport, localization, conformation, stability and activation [97-101]. However, studies have revealed that approximately one third of the potential N-glycosylation sites are actually glycosylated [102]. Both legumain and cystatin E/M are endogenously glycosylated with four and one putative N-glycosylation site(s), respectively [22, 46], but the type of carbohydrates attached is not known. Cystatin E/M is expressed and secreted as two molecular forms, one 14 kDa unglycosylated and one 17 kDa glycosylated form [57]. By use of glycoproteomics, two glycosylation sites at N^{91} and N^{167} have been confirmed in prolegumain [103].

Functional roles of legumain

Legumain is ubiquitously expressed in mammals, with highest expression in the kidneys, placenta, spleen, liver and testis [33]. Legumain knock out (KO) mice are born viable and fertile, with no obvious abnormality, although reduced body weight was observed at birth [104]. Interestingly, processing of the lysosomal cathepsins B, H, and L from single-chain to two-chain forms was completely defected in legumain deficient mice [72] (**Table 1**). Among its functional roles, legumain has pivotal role in the endosomal/lysosomal degradation system and deficiency caused an accumulation of macromolecules in enlarged lysosomes (hyperplasia) [104].

Legumain is required for normal kidney physiology and homeostasis [105]. Normally, legumain is abundant in late endosomes and lysosomes of renal proximal tubular cells (PTCs). However, mice lacking legumain accumulate a discrete set of proteins in the endosomes and lysosomes of PTC, indicating a defect in normal catabolism of proteins captured from the kidney filtrate, and develop progressive kidney pathology [105]. Also, legumain controls extracellular matrix remodeling through degradation of fibronectin in mouse renal PTCs [73] (**Table 1**). Purified legumain from bovine kidneys has been shown to inactivate and degrade annexin A2 (**Table 1**), which is abundant in the receptor-recycling compartments of endosomes/lysosomes [69]. This was also shown *in vivo* as knockdown of legumain decreases cleavage of annexin A2 in mouse kidney [106].

Cysteine proteases are thought to have important roles in bone homeostasis. The C-terminal fragment of legumain has previously been identified as an osteoclast inhibitory factor (OIP-2) *in*

vitro, and a possible inhibitor of bone resorption *in vivo* [107]. Lately, legumain has been reported to inhibit osteoblast differentiation through degradation of fibronectin and vertebral mineralization in zebrafish was shown to be increased by legumain deficiency [108]. In addition, postmenopausal women showed decreased plasma legumain levels with aging.

The involvement of legumain in neuronal apoptosis has been shown in neurodegenerative diseases such as stroke, ischemia, amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Parkinson's disease (PD) and Alzheimer's disease (AD) [83, 84]. In AD, activation of legumain (also known as δ-secretase) caused Tau hyper-phosphorylation and neuronal death [81] (**Table 1**), contributing to the age-dependent pathology [68]. Recently, it was shown that legumain cleaves amyloid precursor protein (APP; **Table 1**) at two sites (N^{373} and N^{585}). The secreted fragment (APP^{1-373}) is neurotoxic, accelerating the generation of amyloid β which aggregates to amyloid plaques [109]. Also, legumain is highly activated in human brains with PD, and overexpression of legumain cleaves α-synuclein in an age-dependent manner and mediates PD pathology [66] (**Table 1**).

Legumain is present in antigen presenting cells (APCs), such as B-cells, macrophages and dendritic cells. In APCs, legumain can process proteins for MHC class II presentation and initiate removal of the invariant chain chaperone of MHC-II (**Table 1**), hence plays a role in both peptide generation and MHC-II activation [74, 91, 110]. Thus, legumain is involved in the generation of tolerance by destructing self-proteins and in addition mount immune responses towards foreign antigens [111]. However, Maehr *et al*. postulated that legumain is not essential for MHC-II antigen presentation as cathepsins are sufficient to carry out protein processing, but legumain is required for processing of cathepsin L [72]. Legumain also assist in proteolytic maturation of the toll-like receptors (TLRs) 3, 7 and 9 [86-90] (**Table 1**), which is essential for immune signaling. In induced regulatory T cells, blocking legumain imparts regulatory function of these cells [112]. Legumain is required for normal Th1 induction in human and mouse $CD4^+$ T cells, through processing of cathepsin L and subsequently cathepsin L-mediated complement C3 activation [113]. Also, legumain can process prothymosin α (**Table 1**) into active α1-thymosin [78], which is implicated in human $CD4^+$ T cell activation and modulation of cytokine production [114].

In addition, high levels of legumain have been reported secreted in the tumor microenvironment [115] and is increased in serum of breast cancer patients compared to controls [35], as well as in

unstable atherosclerotic plaques [116, 117]. The remaining of this introduction will focus on legumain in cardiovascular disease.

Legumain in cardiovascular disease

Cardiovascular diseases (CVDs) are leading causes of death world-wide. A major cause of CVD is atherosclerosis [118], a pathological process characterized by non-resolving inflammation in the wall of large and medium-sized arteries [119]. Lipid accumulation and persistent inflammation in the vessel wall are the hallmarks of atherosclerosis, driven by the interplay between lipids and immune cells [118]. This progressive inflammatory process results in the development and growth of complex atherosclerotic lesions known as plaques. As the lesion develops, the vessel lumen is narrowed and the plaque can rupture, leading to acute events like myocardial infarction (MI) and ischemic stroke [120, 121].

The vessel wall endothelium is the primary site of the earliest changes preceding the formation of atherosclerotic lesions [122, 123]. Risk factors such as hypercholesterolemia, hypertension, diabetes and smoking, cause damages to the endothelium and result in endothelial dysfunction [124]. The consequences of this injury are increased lipid permeability, macrophage recruitment and formation of foam cells, as well as recruitment of T-lymphocytes and platelets [125]. Endothelial cells, platelets and inflammatory cells release various mediators (**Figure 5**), such as growth factors and cytokines that induce effects on other cell types, such as smooth muscle cells (SMCs).

Macrophages have a key role in regulation of the atherosclerotic-related inflammation, and different phenotypes of macrophages have various effects on initiation, progression and maintenance or resolution of the inflammatory status [126]. Both pro-inflammatory M1 and pro-resolving M2 macrophages are present in the atherosclerotic lesion, and the balance between macrophage subtypes is of importance for the fate of the plaque and the occurrence of adverse events [127, 128]. Also, within the atherosclerotic lesion, the interaction between macrophages and oxidized LDL (oxLDL), and subsequently the development into lipid-loaded foam cells is a key event in atherogenesis (**Figure 5**) [128, 129]. Foam cells are crucial players, contributing to lipid accumulation, inflammation, matrix degradation and thrombus formation [118, 130, 131].

Traditionally, platelets are known to play important roles in hemostasis, thrombosis, and wound healing. However, increasing evidences suggest that activated platelets may promote

atherosclerosis and other inflammatory disorders [132-137] by releasing substances and interacting with adjacent cells such as monocytes/macrophages [138, 139]. The platelet components are stored in three types of compartments, the α-granules (large adhesive and healing proteins, for example fibrinogen), dense granules (small non-protein molecules, such as adenosine diphosphate and serotonin) and lysosomes (hydrolases and acidic proteases), which are released upon platelet activation [140, 141]. Each granule population has specific properties to facilitate recruitment, activation and aggregation of platelets [140, 141]. Activation of platelets is also associated with binding of fibrinogen to its major receptor (integrin α2Iiβ3), which is essential for platelet bridging and subsequent platelet aggregation [142].

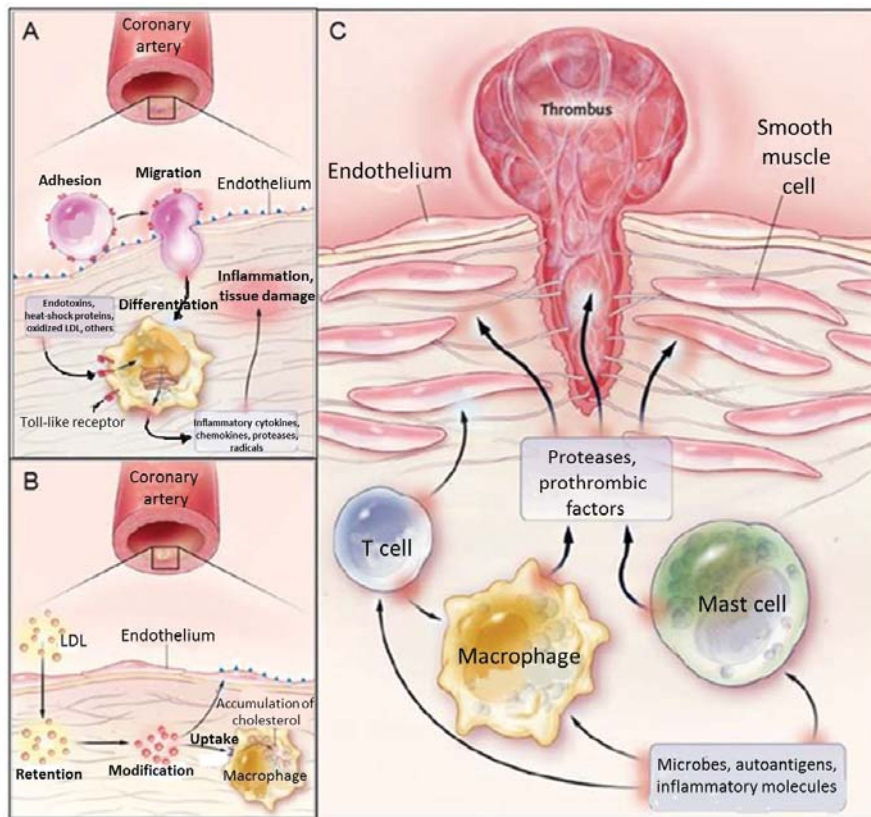

Figure 5. Roles of macrophages, LDL infiltration and atherosclerotic lesion in the artery wall. A. Monocytes are recruited through the vessel wall by adhesion to the activated endothelium, which release inflammatory mediators resulting in inflammation and tissue damage. After migration, monocytes differentiate to macrophages and subsequently secret inflammatory mediators. **B**. Modified LDL particles are taken up by scavenger receptors on the macrophages, and trigger conversion of macrophages to foam cells. **C**. Various inflammatory stimuli activate T cells, macrophages and mast cells, leading to further secretion of inflammatory mediators, including proteases and prothrombotic factors that directly participate in the formation of a thrombus at the site of plaque rupture. Figure adopted from [130].

Both pro-inflammatory mediators and the inflammation itself stimulate secretion of matrix metalloproteinases (MMPs) and promote lesion formation [143]. The biological roles of MMPs have been associated with degradation and turnover of components of the extracellular matrix (ECM) [144]. ECM components such as elastin, collagen, fibronectin, laminin and proteoglycans are essential components of both aorta and heart, providing structural and biochemical support of the surrounding cells and regulating the intercellular communication [145]. Changes in synthesis or degradation of ECM components can trigger the development of atherosclerotic lesions and plaque rupture. Moreover, pharmacological inhibition of MMPs or genetic deficiency showed incomplete suppression of cardiovascular remodeling in experimental animal disease models [146-148], indicating the presence and role of other proteases, such as cysteine proteases [8, 149-151]. However, this needs to be further investigated.

The functional role of legumain in CVD, especially atherosclerosis is not extensively studied. It has previously been shown that legumain is highly upregulated in unstable compared to stable carotid plaques [117, 152]. Legumain is among 18 genes associated with plaque rupturing and may represent a novel target for treatment of unstable plaques or a diagnostic marker of plaque instability [152]. Furthermore, Wang *et al*. have shown downregulation of legumain mRNA in monocytes from patients with atherosclerosis treated with atorvastatin [153]. Moreover, both increased legumain expression, activity and secretion was inhibited by atorvastatin during monocyte-to-macrophage differentiation [36]. Previously, it was shown in a small number of patients that unstable regions contained more than twice the amount and activity of legumain compared with stable regions of the same plaque [116]. Recently, legumain was shown to suppress oxLDL induced macrophage apoptosis through enhancement of the autophagy pathway [154]. Furthermore, in stroke, legumain was concluded not to be essential for the functional deficit after middle cerebral artery occlusion but may be involved in mechanisms of immune cell invasion [155]. In addition, legumain may promote ECM degradation due to ability to activate proMMP-2 [77], or by processing the cathepsins [104, 105] or by direct proteolysis of ECM components like fibronectin [73]. Thus, the involvement of legumain in CVD needs to be further investigated.

AIMS OF THE THESIS

The overall aim of this thesis is to expand our knowledge concerning localization and regulation of the cysteine protease legumain and especially the role in CVD, by using human cell models and analyses of patient samples. This thesis also aims to exploit legumain as a biomarker or a target for pharmacological interventions.

Specific objectives are as follows:

- To characterize and elucidate the importance of glycosylation for functions, localizations and interplay of legumain and the endogenous legumain inhibitor cystatin E/M (**paper I**)
- To investigate legumain in cardiovascular disease (**paper II** and **III**)
- To investigate legumain in platelets and in the platelet-monocyte/macrophage interactions (**paper III**)

SUMMARY OF PAPERS

Paper I: ***Glycosylation is important for legumain localization and processing to active forms but not for cystatin E/M inhibitory functions***

Legumain and its inhibitor cystatin E/M are both endogenously glycosylated. However, little is known about the nature of the carbohydrate groups and whether these affect the functions of the protease or inhibitor. In this study, the carbohydrates on legumain were shown to be of the hybrid or high mannose type, whereas cystatin E/M was characterized as complex mannose-linked. While glycosylated prolegumain was able to autoactivate, the unglycosylated form was not. Glycosaminoglycans (GAGs) enhanced autoactivation of glycosylated prolegumain, whereas unglycosylated prolegumain did not autoactivate either in absence or presence of GAGs. Glycosylated prolegumain was internalized and processed to the mature active form, but no internalization was observed of unglycosylated prolegumain. A Cy5-labelled legumain selective activity-based probe (MP-L09) was synthesized and shown to be a novel tool to study intracellular legumain. Also, internalization of mature legumain (36 kDa) was visualized both alone and complexed with MP-L09. Contrary to the importance of legumain glycosylation, both glycosylated and unglycosylated cystatin E/M showed similar capacity to inhibit legumain. In conclusion, glycosylation of prolegumain is necessary for correct processing to active forms and internalization, whereas the inhibitory property of cystatin E/M is independent of the glycosylation status.

Paper II: ***Increased levels of legumain in plasma and plaques from patients with carotid atherosclerosis***

The cysteine protease legumain has previously been shown to be upregulated in unstable atherosclerotic plaques. This study aimed to further elucidate legumain in atherosclerosis, by examining legumain in a large cohort of plasma and carotid plaques from patients (n=254) with carotid stenosis versus control (n=91). Also, legumain secretion from monocyte-derived macrophages treated with atherogenic lipids during macrophage polarization was studied. In this study, we reported for the first time quantification of legumain in plasma from patients with carotid stenosis. Both symptomatic and asymptomatic patients had significantly higher levels of plasma legumain compared to healthy controls, but there was no difference between the patient groups and symptomatology. As a consequence, legumain plasma levels can potentially be used

as a biomarker for carotid stenosis but not to predict clinical outcome. Plaque analysis showed that expression of mature legumain (36 kDa) was increased in symptomatic (symptoms<2 months) compared to asymptomatic patients, whereas legumain mRNA was increased in patients compared to healthy controls. Within the plaques, legumain co-localized with macrophages and areas rich in foam-like cells. In addition, polarization of macrophages showed that legumain secretion was significantly higher from M1 than M2 macrophages, and especially from M1 macrophages treated with cholesterol crystals. In conclusion, legumain is increased in both plasma and plaques of patients with carotid stenosis and might be a new biomarker of atherosclerosis.

Paper III: ***Upregulation of the cysteine protease legumain in acute cardiovascular events – potential role in platelet-monocyte/macrophage interactions***

The cysteine protease legumain is postulated to participate in extracellular matrix degradation, and we have previously found enhanced legumain expression in symptomatic carotid plaques. To this end, however, there are no data on the regulation and/or role of legumain in acute cardiovascular events. In the *SUMMIT Malmö* cohort (134 subjects with type 2 diabetes [T2D] and cardiovascular disease [CVD], 134 subjects with T2D and no CVD, 71 with CVD and no T2D, and 64 healthy controls without T2D or CVD) circulating legumain was associated with the presence of CVD in non-diabetics patients, with no relation to outcome. In a STEMI population (n=272), patients had significantly higher serum legumain before and immediately after percutaneous coronary intervention compared with healthy controls (n=67), and interestingly high levels were associated with improved outcome. Furthermore, legumain was co-localized with macrophages in the same region as platelets in unstable carotid plaques and in samples from both coronary and intracerebral thrombi obtained during acute cardiovascular events. *In vitro*, legumain was found present and released from platelets upon activation, and was markedly enhanced in THP-1 monocytes exposed to releasate from activated platelets. Surprisingly, legumain enhanced expression of interleukin (IL) 10 and the M2 marker CD163 and reduced expression of MCP-1 in peripheral blood mononuclear cells from healthy individuals. Our data demonstrate for the first time that legumain is upregulated during acute cardiovascular events, and present at the site of thrombus formation. Our findings suggest that legumain could be a novel player in modulation of plaque stability operating in the interaction between platelets and monocytes/macrophages, potentially mediating anti-inflammatory effects.

DISCUSSION

Methodological considerations

Cellular models

In this work, *in vitro* cell models of various origins have been used, i.e. commercially available or gene manipulated cell lines as well as primary cell cultures isolated from whole blood (monocytes). There are several advantages of using cell lines instead of primary cells. Cell lines *in vitro* are able to live with less influence of physiological factors and interplay with other cell types compared to primary cells. *In vitro* cell models are valuable in basic research, although effects observed in cell lines may be difficult to extrapolate to normal cells and tissues *in vivo*. In addition, ethics, costs and less variability, favors the use of cell lines. However, immortalized cells from cancerous cell lines are often dissimilar in behavior and response compared to cells *in vivo*, which makes it difficult to compare data obtained *in vitro* versus *in vivo* [156]. Primary cells are closer to the *in vivo* settings as they are harvested directly from animal or human origins. However, the major disadvantages of primary cell cultures are the short life span and the changes in phenotype during cultivation, in addition to difficulties in gene manipulation [157].

In **paper I**, we used legumain or cystatin E/M monoclonal overexpressing cell lines previously established in our laboratory [44]. M38L is the human embryonic kidney (HEK) 293 cells stably transfected with human legumain cDNA (*LGMN*), whereas M4C is HEK293 cells stably transfected with human cystatin E/M cDNA [44]. In addition, HEK293 and HCT116 (human colorectal cancer) cell lines commercially available from American Type Culture Collection (ATCC) were used.

THP-1 (ATCC TIB-202) is a widely used monocyte cell line suitable for studies of monocyte and macrophage functions [158-160], inflammation and atherosclerosis, and was used to study interactions of monocytes/macrophages with platelets in **paper III**. However, there are significant differences in mRNA and protein expressions in THP-1 cells compared to primary monocytes, and results are not easily translated to *in vivo* settings [159, 160]. For example, studies have shown that THP-1 cells produce less cytokines after lipopolysaccharide (LPS) stimulation compared with peripheral blood mononuclear cells (PBMCs)-derived monocytes [161].

Clinical samples

Clinical materials are the cornerstone of translational research which can contribute to the development of better diagnostics and treatment of diseases, such as CVD. With regards to ethical issues, the overall goal is to limit the burden on study participants by thorough use of valuable clinical materials. To ensure this, a good study protocol and detailed characterization of the subjects are mandatory. Three patient cohorts were included in this thesis: Patients with carotid atherosclerosis (n=254, **paper II**), patients with CVD and/or T2D (n=403, **paper III**) and patients with STEMI (n=272, **paper III**). All studies were approved by the local ethical review boards and conducted according to the Declaration of Helsinki. All subjects enrolled gave written informed consent.

The patients are well characterized clinically and enrolled based on consensus criteria for the respective diagnosis (**paper II** and **III**). Patients with concomitant inflammatory diseases, malignancies or overt liver and kidney disease were excluded due to other factors that could affect their inflammatory status. However, there is a possibility that patients with latent infections or other undiagnosed conditions have been enrolled. For comparison, apparently healthy, well-characterized age and sex matched controls were recruited based on absence of a disease history, clinical evaluation and standard biochemical tests. The healthy donors used for some *in vitro* experiments were recruited locally.

The following clinical samples were used in this thesis and will be discussed below: Blood (including plasma, serum, primary monocytes, platelet-rich plasma and platelets), atherosclerotic plaques and thrombi materials.

Primary monocytes

PBMCs were isolated from buffy coats obtained from the Blood Bank at Ullevål, Oslo University Hospital, and were used in **paper II** and **III**. Buffy coat is the fraction of anticoagulated blood harvested after centrifugation that mostly contains white blood cells and platelets. This composition gives an opportunity to study the interactions between immune cells without the interference from other cells like the endothelium. Primary monocytes were further extracted from PBMCs by plastic adherence, where the adherence of monocytes to plastic surfaces of culture plates was utilized. Thorough washing procedures were employed in order to reduce lymphocyte-contamination.

Differentiation of macrophages

In the literature, there are numerous methods for differentiating monocytes to macrophages. Macrophages can also exist in intermediate polarization states and switch phenotype in response to stimuli, for example the macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) polarizes monocytes to M2 and M1 macrophages, respectively [162, 163]. In **paper II**, differentiation of primary monocytes towards macrophages was performed using M-CSF for 7 days before stimulation with either interferon (IFN)γ and LPS or interleukin (IL) 4 for M1 or M2 polarization, respectively [163, 164]. Verification of macrophage polarization by this protocol has been done previously using mRNA expression of markers like tumor necrosis factor (TNF) and IL-6 for M1 and CD163 and peroxisome proliferator-activated receptor γ for M2 [164]. Primary monocytes have previously been shown not to secrete legumain, whereas M-CSF-differentiation of macrophages resulted in substantial secretion of prolegumain to the conditioned media [36]. Also, differentiation of THP-1 monocytes to macrophages by phorbol 12-myristate 13-acetate (PMA) has shown a 500-fold increase in legumain expression [36]. TNF-α is involved in acute systemic inflammation, is an established pro-atherosclerotic factor [165], and was used to stimulate monocytes in **paper III**.

Blood sampling, sample storage and legumain in serum *versus* plasma

In this thesis, both plasma and serum have been used to measure circulating legumain levels (**paper II** and **III**). Plasma is the supernatant fluid obtained after centrifugation of anti-coagulated blood, while serum preparation involves blood clotting which can contribute to release of inflammatory mediators [166]. This might explain the discrepancy in legumain concentrations measured in plasma and serum samples from the same donor (**Figure 6**). In general, legumain levels were higher in serum versus plasma samples, as shown in **Figure 6A**. Whereas plasma only contains free circulating legumain, serum samples contained the sum of circulating legumain and legumain released from blood components during coagulation. As shown in **paper III**, platelets contain and release legumain upon activation. Also, other factors related to blood collection and sample preparation may have influenced the legumain levels in plasma/serum. For example, contamination of collection tubes, buffers and media with endotoxins may activate blood cells and induce inflammatory responses [167].

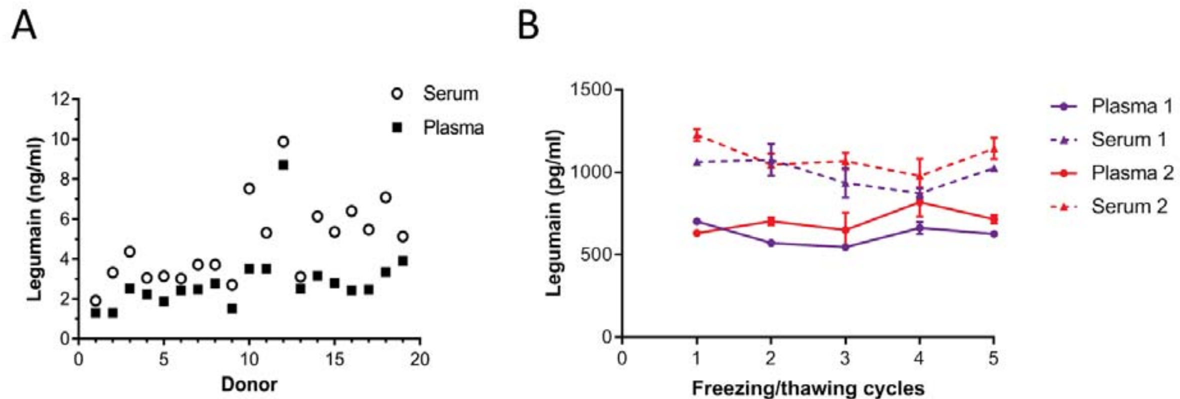

Figure 6. Legumain concentration in serum and plasma from healthy donors and effect of repeated freezing and thawing cycles. A. Legumain measured by enzyme-linked immunosorbent assay (ELISA) in serum or plasma of healthy donors (n=19). **B**. Plasma and serum samples from two healthy donors were subjected to multiple cycles of freezing and thawing before legumain was analyzed by ELISA. Data are presented as mean ± SEM, n=1.

Furthermore, plasma and serum samples should be handled carefully and frozen at -80°C as soon as possible, to avoid *ex vivo* degradation of proteins. Biological samples are valuable and used in several studies, degradation during long-time storage and during repeated freezing and thawing might occur. Repeated freezing and thawing cycles (five) did not seem to affect legumain levels in serum and plasma from two individual donors (**Figure 6B**), but should be kept at a minimum as it might affect the stability of other mediators [168, 169].

Isolation of platelet-rich plasma and platelets

To ensure intact, non-activated platelets which at a later stage were able to be activated, sodium citrate was used as the anticoagulant in **paper III**. Citrate binds calcium ions (Ca^{2+}) to avoid plasma coagulation and allows platelet activation and aggregation [170]. Platelet-rich plasma (PRP) used in **paper III** was obtained as previously described [138, 171], although there are several methods to obtain PRP [172]. The methods may differ in the isolation procedure (1-step or 2-step centrifugation), the speed of centrifugation, and the type of collection tube system and operation. The specific composition of PRP varies not only from person to person but also when the isolation process is repeated in the same individual [173], and growth factor and catabolic cytokine concentrations are influenced by the cellular composition of PRP [174]. Platelet isolation contains multiple washing steps, which results in some degree of platelet activation and artefactual errors in many applications. Also, to prevent activation of platelets during isolation, strong mechanical forces (i.e. fast pipetting, vigorous shaking) must be avoided.

Tissue analysis of atherosclerotic plaques and thrombi materials

The expression of legumain was examined in both atherosclerotic plaques and in thrombus materials (**paper II** and **III**). The composition of an atherosclerotic plaque is heterogeneous, and both intra- and inter-patient variations are present [175]. Plaque samples from **paper II** and **III** were retrieved from patients undergoing carotid endarterectomy surgery at Oslo University Hospital, Rikshospitalet. Thrombus materials were obtained at the site of plaque rupture in patients with STEMI and in intracranial thrombi from patients with acute ischemic stroke (**paper III**). Samples were rapidly frozen in liquid nitrogen. The material used for RNA and protein extraction is not homogenous from the same part of the plaque.

Methods used to detect and quantify legumain

Cell biology methods like immunoblotting (IB), enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF) and immunohistochemistry were used in this thesis (**paper I-III**). Common for these methods is the use antibodies to identify and quantify proteins of interest. IB and ELISA were used in all papers and are discussed in more detail below.

By IB, the legumain polyclonal antibody (pAb; AF2199, R&D Systems) detects the proform (56 kDa), the intermediate (47/46 kDa) and mature (36 kDa) forms. It is important to notice the discrepancy in legumain detection between cell lines. In the lysates of legumain overexpressing cell line (M38L), all three legumain forms are detected (**paper I**). However, in control and cancerous cell lines (HEK293 and HCT116) with a lower expression of legumain, the pro- and mature form are detected. Also, a previously undescribed 25 kDa active legumain form was detected by the same pAb in cell lysates, as well as both unglycosylated and deglycosylated legumain (**paper I**). Although pAbs were used to quantify the expression of several proteins in **paper II**, it is important to underscore that IB is a semi-quantitative method [176, 177]. In our effort to limit blot-to-blot variations, each blot in **paper II** contained one sample from the same patient and the band intensity was correlated with this patient sample in addition to a housekeeping protein (β-actin).

In recent years, ELISA has been considered as the standard quantitative method for measurement of several proteins in diseases, such as cytokines [178], cathepsin L and K [179, 180], and serum levels of cystatin C have clinical value in estimation of glomerulus filtration rate [181, 182]. A commercially available human legumain ELISA-duokit (DY4769) from R&D Systems,

containing a biotinylated legumain pAb for detection, was used to measure the total legumain concentrations in plasma or serum obtained from patients with carotid atherosclerosis, STEMI and CVD patients, healthy controls, and in conditioned media from primary cells and cell lines (**paper I-III**). According to the manufacturer, this kit measures total legumain (i.e. pro and mature recombinant human legumain), and also recombinant legumain complexed to recombinant human cystatin [183]. The quality of the measurement is dependent on the specificity and selectivity of the antibody. In ELISA, sample analysis is limited by a narrow dynamic range of the standard curve. In most cases, plasma and serum samples were diluted 1:5, with exceptions of some patient samples with high legumain levels which required 1:10 dilution. Quality assessment of the legumain kit in our hands was performed to ensure reproducibility and comparability between samples in various plates. The average coefficient of inter-variation for the legumain standard was 7 %, and 16 % in plasma, while the intra-variation was 5 %.

The manufacturer of ELISA-duokit also supplies a kit (DY2199) to measure human prolegumain only as this kit does not detect the mature legumain [184]. A pilot was initiated to compare the two kits and to investigate whether plasma contained pro- and/or mature legumain. Seven plasma samples from healthy donors were used in this pilot (**Figure 7**). Four out of the seven donors had equal amount of total legumain and prolegumain concentrations, whereas three donors had higher total legumain indicating that the mature form might be present in plasma. The fraction of the measured mature legumain varied in the samples, ranging from minor to more than half of the total legumain amount. Also, the legumain levels between donors varied and need to be investigated.

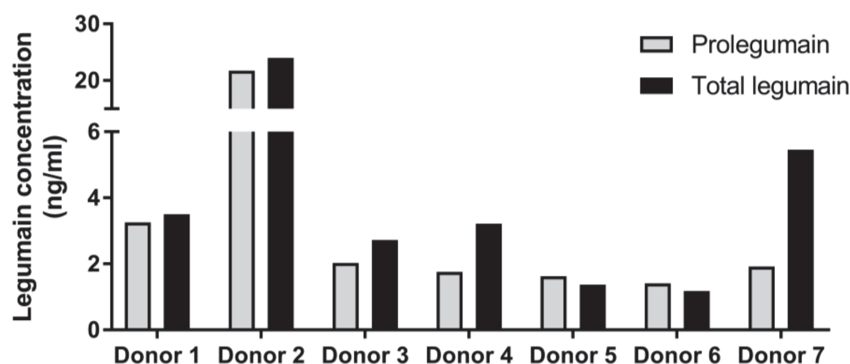

Figure 7. Concentration of prolegumain versus total legumain in plasma. The concentration of prolegumain (grey bars) and total legumain (black bars) measured in plasma from healthy donors (n=7) using two different ELISA-kits (DY2199 and DY4769, respectively, from R&D Systems).

Activity-based probes and inhibitors of legumain

It is the activity of a protease that mediates a biological effect. Thus, small-molecular substrates, inhibitors and activity-based probes (ABP) can be used to interrogate this activity. Since legumain is the only well-characterized asparaginyl endopeptidase, the peptide substrate Z-Ala-Ala-Asn-AMC is suitable for detection of legumain activity *in vitro* [22, 185] and used in **paper I**. In the recent years, multiple attempts have been made to understand the functional roles of proteases *in vivo* by using ABPs [186-189]. Several ABPs for non-invasive optical imaging have been published [190, 191] and may be used in surgery to eradicate the tumor and tumor-associated surrounding tissues [192].

Legumain ABPs have also been constructed, containing Asp in the P1 position for stability, and are useful tools to investigate binding to and localization of legumain [193-196]. By introducing bulky groups (fluorophore and fluorescence quencher), one of these probes (LE-28) was shown to be legumain selective *in vivo* by directing the probe to the lysosomes [193]. The design of legumain-selective probes with Asp in the P1 position is highly challenging as most of these peptides are also recognized by caspases [197]. One approach to overcome caspase-specificity is to use a Counter Selection Substrate Library (CoSeSuL) introducing both natural and unnatural amino acids in P4-P1. We have recently published the synthesis of fluorogenic substrates, inhibitors and APBs (MP-L0X) that selectively targeted legumain [196]. These probes contain a biotin tag on the N-terminus, an aminohexanoic linker (6-ahx) and acyloxymethylketone (AOMK) as the warhead, exemplified by MP-L01used in this thesis (**Figure 8A**). The MP-L0X probes selectively bind to and irreversible inhibit active legumain (**paper I** and **III**) [196].

Immunofluorescence and immunoblotting were used to detect the interaction between legumain and the legumain-selective ABPs (**paper I** and **III**). MP-L01 is biotin-labelled, thus streptavidin is used for probe detection but unfortunately streptavidin also interacts with endogenously biotinylated proteins [196]. For this reason, MP-L01 is not optimal for IF microscopy. A new Cy5-labelled legumain-selective ABP (MP-L09) was synthesized and detected using near-infrared scanning at 700 nm (**paper I** and **Figure 8B**). Cy5 is a bright, far-red-fluorescent tag with excitation ideally suited for the 633 nm or 647 nm laser lines, but can also be used in direct eye visualization [198]. A significant advantage to use long wavelength dyes such as Cy5 compared to other fluorophores is the low autofluorescence of biological matrixes in this region of the spectrum [198]. Internalization of MP-L09 was confirmed by fluorescent microscopy and

shown to co-localize with legumain in vesicles (**paper I**), and MP-L09 might be a novel tool for further *in vitro* or *in vivo* studies to track active legumain in biological models.

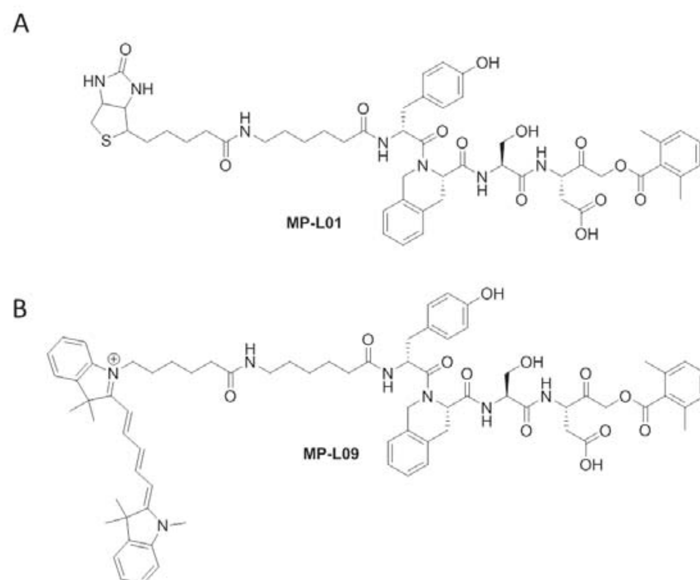

Figure 8. The structure of the legumain-selective activity-based probes used in this thesis. A. Biotin-labelled MP-L01 (biotin-6ahx-D-Tyr-Tic-Ser-Asp-AOMK). **B**. Cy5-labelled MP-L09 (Cy5-6ahx-D-Tyr-Tic-Ser-Asp-AOMK). Figure from Marcin Poreba.

One of the major disadvantages of recently published legumain ABPs [193, 196] in biological systems is low cell permeability. Methylation in P1 position or a more bulky group in P4 did not seem to increase permeability of MP-L01 [196]. However, probe prebound to a protease, such as MP-L01-legumain complex, seemed to facilitate internalization as shown in **paper I**, where the legumain-probe complex is internalized at a 10 times lower concentration than the probe alone. This could represent a transporter role of proteases across the cell membrane to facilitate cellular uptake and consequently increase cellular effect of drugs, but needs further investigations.

Another approach to facilitate membrane permeability is to utilize small molecular inhibitors. Unlike the other cysteine proteases, legumain is not affected by the pan-inhibitor E64 [22]. Over the years, several small molecular legumain inhibitors have been synthesized [58, 82, 195, 199-208]. Recently, one of these inhibitors, called compound 11, was shown to be a potent and selective inhibitor of legumain (IC_{50}~700 nM). This inhibitor displays promising results as the compound was shown to be non-toxic, cell permeable and able to cross the blood brain barrier, both *in vitro* and in a mouse Alzheimer’s disease model [82]. Whether any of these promising compounds are able to be utilized in drug therapy requires further evaluations.

General discussion

Posttranslational glycosylation of legumain and cystatin E/M

Two N-glycosylation sites have been confirmed in prolegumain by glycoproteomics [103], although legumain has four potential glycosylation sites [22]. Cystatin E/M is expressed and secreted as a 14 kDa unglycosylated and a 17 kDa glycosylated form [52]. Glycosylation is an enzymatic process that attaches glycans (polysaccharides) to proteins. However, the term glycan may also be used to refer to the carbohydrate portion of a glycoconjugate, such as a glycoprotein, proteoglycan or glycolipid, even though the carbohydrate is only an oligosaccharide [209]. There are five classes of glycosylation, where N-linked glycans are most abundant in eukaryote cells and are extremely important in proper protein folding and targeting of degradative lysosomal enzymes [99, 100]. To identify the glycosylation type of legumain and cystatin E/M, PNGase F and Endo H were used in **paper I**. By PNGase F, both legumain and cystatin E/M were confirmed to be N-glycosylated, as indicated by others [22, 103]. PNGase F is the most effective enzymatic method for removing almost all N-linked oligosaccharides from glycoproteins, due to its function as an amidase (cleaves between the innermost N-acetylglucosamine (GlcNAc) and asparagine residues) [210-212]. Cleavage with PNGase F converts the asparagine residue to an aspartic residue. On the other hand, Endo H is a glycosidase which cleaves within the core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins, generating a truncated sugar molecule with one GlcNAc residue remaining on the asparagine [213]. Thus, complex glycans are not affected by Endo H. The carbohydrates on legumain was cleaved after incubation with Endo H, suggesting hybrid or high mannose, whereas cystatin E/M was not affected by Endo H, indicating complex mannose (**paper I**). As an alternative, oligosaccharides can be characterized by mass spectrometry to determine the molecular weight of the deglycosylated protein of interest and to identify the glycosylation site(s) [214].

Glycoproteomics is a novel sub-proteomic approach that elucidates levels of expression of glycoproteins that are present in a given sample. This technique has been fundamental in the discovery of cancer biomarkers and diagnosis of human diseases [215-218]. By the use of this technique, Zarif *et al*. found that the glycosylation phenotype was significantly different among PBMCs, M1 and M2 cells. Several proteins were enriched on M2 macrophages compared to M1, including legumain, cathepsin L1, integrin α3, macrophage mannose receptor 1 (CD206), and sodium/hydrogen exchanger 7 [96].

The use of tunicamycin to block N-linked glycosylation is well studied in various cells [219-225] and was used in this thesis (**paper I**). Given the key role of N-glycosylation in protein folding and quality control in the ER, it is not surprising that tunicamycin may be cytotoxic to cells [226, 227]. In many cells, the drug can initiate apoptosis and unfolded protein response due to alterations in glycosylation of various cell-surface receptors and signaling molecules by inducing ER stress at concentrations between 2.5-5 μg/ml [228, 229]. However, cell viability was not significantly influenced by the concentrations of tunicamycin used in our experiments (**paper I**).

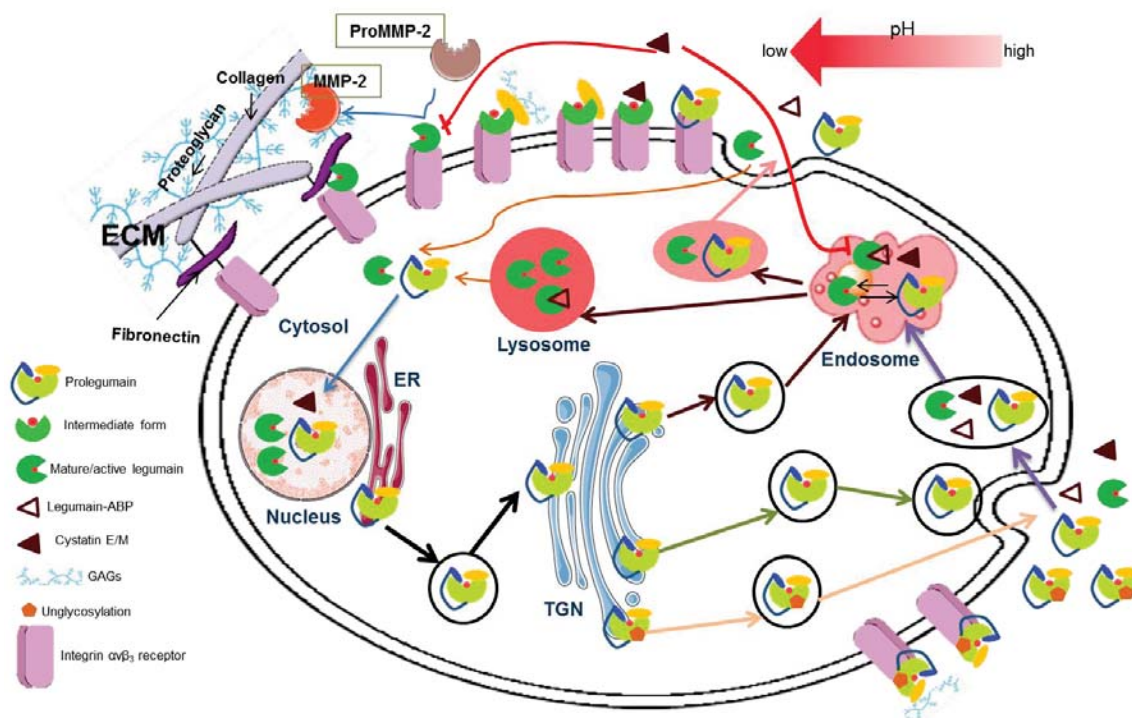

Figure 9. Cellular localizations of legumain and its inhibitors. Legumain is synthesized as inactive prolegumain in ER. Prolegumain is transported via TGN to the acidic endosomes/lysosomes to be activated (black and brown arrows) or directly secreted after posttranslational modifications in TGN (green arrows). Unglycosylated prolegumain is also secreted (light orange arrows, **paper I**). Secreted prolegumain can interact with GAGs to be activated (**paper I**) and/or the $\alpha_v\beta_3$ integrin receptor to be stabilized extracellularly. Secreted prolegumain may also be stabilized by interaction with cystatin E/M. Once activated, legumain can cleave ECM components (fibronectin) directly or indirectly by activation of proMMP-2 to active MMP-2. Extracellular prolegumain can internalize and be transported to the endo-lysosomal system for activation (purple arrows), whereas unglycosylated prolegumain is not internalized (**paper I**). Inhibitors of legumain, cystatins and ABPs, are also able to internalize (**paper I**). In addition, extracellular prolegumain can translocate to the cytosol (orange path) and further to the nucleus (blue path). ABP, activity-based probe; ECM, extracellular matrix; ER, endoplasmic reticulum; GAGs, glycosaminoglycans; MMP, matrix metalloprotease; TGN, trans-Golgi Network.

Importance of glycosylation for cellular localization of legumain

To proteolytic cleave substrates, prolegumain must be activated. Maturation and stability of legumain, the presence of endogenous inhibitors and/or GAGs, environmental conditions (especially pH and redox potential) and subcellular localization are factors that regulate the legumain activity [27, 29, 34, 113] (**Figure 9**). In **paper I**, very low levels of legumain were observed in the cytosolic fraction of both untreated and tunicamycin-treated cells after cell fractionation, showing both glycosylated and unglycosylated legumain in the cytosol. Cytosolic legumain has previously been reported, both as the zymogen and as the fully activated form [34, 230]. Also, prolegumain was observed in the nuclear fraction, whereas unglycosylated prolegumain was not, indicating the importance of glycans for translocation to the nucleus (**paper I**). Active legumain have recently been observed in the nucleus and suggested to participate in histone degradation, and postulated to have a role in DNA replication [34]. Other lysosomal cysteine proteases have also been detected in the nucleus [231-233]. The subcellular localization of legumain seems to be altered in diseases such as cancer, as legumain can move from the lysosomes to the cytosol, nucleus, cell surface and extracellular space [34, 230] (**Figure 9**). In some pathological conditions, lysosomal leakage of cathepsins to the cytosol have been observed, where the cathepsins are involved in apoptosis [187, 234].

It is poorly understood whether (or how) lysosomal cysteine proteases, especially legumain, is active extracellularly or in the cytosol in nearly neutral environments, since legumain has a catalytic optimum at approximately pH 6 and the stability is poor above 6 [22]. Inhibition of glycosylation by using tunicamycin increased the secretion of prolegumain (**paper I** and **Figure 9**), indicating that the cellular level and secretion of prolegumain is dependent on the glycosylation status. Other studies have suggested that reduced acidification of the lysosomal compartment forces prolegumain into a secretory pathway [44, 235]. Extensive secretion from macrophages, especially pro-inflammatory M1 macrophages stimulated with cholesterol crystals (CC), was shown in **paper II**. Furthermore, higher levels of legumain were measured in plasma and serum samples from patients with carotid atherosclerosis (**paper II**) and in acute and stable CVD patients (**paper III**) compared to controls. Also, in **paper III**, legumain was shown for the first time to be present in and rapidly secreted from activated platelets. Higher levels of serum legumain have also been detected in breast cancer patients compared to controls, and increased

circulating legumain was suggested as a biomarker for metastatic breast cancer diagnosis [35]. Legumain in human plasma and/or serum will be further discussed below.

In **paper I**, we showed that glycosylation is necessary for autoactivation of prolegumain as unglycosylated prolegumain was not able to autoactivate, neither in presence or absence of GAGs. Autoactivation of prolegumain is initiated by cleavage at Asn^{323} at pH ≤5.5 [22]. Previously, it was shown in our laboratory that secreted prolegumain is able to autoactivate after acidification at pH 4 but is inhibited by cystatin E/M [44]. GAGs are able to facilitate prolegumain autoactivation in a dose-, time- and pH-dependent manner [38], and have been suggested to interact with and pull the LSAM domain away, resulting in exposure of the active site [38, 39]. Since endogenous GAGs are located both intra- and extracellularly, they can probably facilitate prolegumain autoactivation in both compartments. However, alterations in conformation and/or charge could explain why unglycosylated prolegumain was not autoactivated and unable to interact with GAGs. Heparan sulfate (HS) and chondroitin 4-sulfate (C4S) used in **paper I** were the selected GAGs based on findings by Berven *et al*. [38]. The anticoagulant heparin displays close structural relationship to HS [236], and is routinely administrated to patients prior to percutaneous coronary intervention (PCI) [237]. This drug has previously been shown to influence circulating levels of several cytokines [238, 239]. To study whether heparin influenced the level of circulating legumain, a small population of patients (n=9) with suspected stable coronary artery disease was administrated with 5000 IU heparin. Interestingly, a decline in legumain levels was observed when comparing with levels prior to heparin injection, indicating that the effect of heparin underestimated the level of circulating legumain in patients undergoing PCI (**paper III**). A possible explanation might be the effect of heparin in activation of anti-thrombin and thus inhibition of platelet activation by thrombin [240] and subsequent release of legumain, as **paper III** shows that platelets contain and release legumain.

Legumain can be stabilized by other proteins to maintain activity in a neutral environment. Complex formation with cystatin E/M or C have been shown to stabilize legumain at near neutral pH [42] (**Figure 9**). Also, legumain interacts with the cell surface integrin receptor $\alpha_v\beta_3$, increasing the pH optimum of legumain activity from pH 5.5 to pH 6, indicating that legumain also could be active in a slightly less acidic environment than the lysosomes (pH 4.5-5) [27]. Extracellular activation of prolegumain is highly possible, perhaps by conformational

stabilization by the $\alpha_v\beta_3$ integrin receptor. Interestingly, an intermediate form of legumain has been reported to have ligase capacity to reform prolegumain by ligation of the LSAM in order to resist denaturation in non-acidic compartments [32, 41].

Furthermore, the slightly acidic pH in inflammatory microenvironments [241] and in the resorptive pits between osteoclasts and bone matrix [242], represent possible areas for extracellular activation of prolegumain. Macrophages incubated with oxLDL are able to reduce the environmental pH to 5.5 during inflammation [243]. Other lysosomal cysteine proteases, like cathepsins B and L have been shown to be active for a prolonged time under non-favorable conditions [97]. Interestingly, extracellular cathepsins can degrade two-thirds of insoluble elastin, and intracellular cathepsins degrade the rest [244]. Thus, continuous secretion of active legumain, although short-lived, might enable ECM degradation. This suggests an extracellular role of legumain.

Internalization and vesicular localization of purified mature legumain in HEK293 cells were for the first time shown in **paper I** (**Figure 9**). However, mature legumain added to the cell culture medium was most likely inactive due to near neutral pH (7.4). Internalization of extracellular prolegumain has previously been shown by Smith *et al*. [44], although the internalization mechanism of prolegumain was not investigated. In our experiments, cell treatment with the endocytosis inhibitor Dyngo-4a indicated that the mechanism for internalization of extracellular legumain is not by clathrin-mediated endocytosis (**paper I**). Off-target effects of Dyngo-4a by inhibiting vacuolar H^+-ATPases [245], which increases the pH in lysosomes, also makes it difficult to conclude the exact internalization mechanism of legumain in our experimental setup.

Also, we have previously shown internalization of the novel legumain-selective ABP MP-L01 in M38L cells [196]. In **paper I**, we extended these findings by showing internalization of MP-L01 in HEK293 cells and vesicular co-localization with legumain (**Figure 9**). This was also shown for a new Cy5-labelled legumain-selective MP-L09 probe using immunofluorescence microscopy, as the probe was observed to internalize and co-localize with vesicular legumain. Interestingly, preincubation of cells with Dyngo-4a reduced internalization of the probe alone, but increased internalization of the preformed probe-legumain complex (**paper I**). This indicates different internalization mechanisms of free probe compared to the probe-legumain complex. Micropinocytosis could be a possible uptake mechanism for the ABP as postulated for the internalization of LE-28 [193] and needs to be further addressed. Moreover, the preformed

legumain-probe complex was internalized by living cells at approximately 10 times lower concentration than the probe alone (**paper I**). A previously undescribed 25 kDa legumain form was also detected after internalization of mature legumain, both alone and complexed with the probe, and must be a further processed form of the mature active legumain (36 kDa).

Although the endogenous legumain inhibitor cystatin E/M is mainly described as a secretory protein, internalization of both glycosylated and unglycosylated cystatin E/M was also shown in **paper I** (**Figure 9**), and is supported by others [44, 246]. Interestingly, although both forms are synthesized by cells, the unglycosylated form (14 kDa) was predominantly internalized but the mechanism is not known. Additional studies using chemical and/or genetic endocytosis inhibitors [247] are required to elucidate the internalization mechanism of legumain, probes and cystatin E/M.

Recently, N-glycosylation was shown to be associated with inflammation [248-250] and IgG-glycosylation was recently demonstrated as a potential biomarker for inflammation and metabolic health [249]. Furthermore, glycans play a critical role in interactions between cells [251]. Also, abnormal glycosylation leads to cellular overload of dysfunctional proteins and is involved in the pathologies of Parkinson's disease [252]. Although unglycosylation does not normally occur and we chemically modified glycosylation of legumain in **paper I**, this study highlights the importance for glycosylation of transport and processing of legumain during altered PTM in pathological conditions.

Importance of legumain in cardiovascular disease

Legumain is expressed by monocytes and macrophages [36, 111, 153] (**paper II**). Monocytes originate from stem cells in the bone marrow and migrate to various tissues, and develop into tissue-specific macrophages. The "classical" activated macrophages induced by IFNγ secreted by T helper (Th) 1 cells are entitled M1 (pro-inflammatory), while M2 (anti-inflammatory) are the "alternatively" activated macrophage induced by the Th2 cytokines IL-4 and IL-13. When polarizing THP-1 monocytes using IFNγ and LPS or IL-4, respectively, higher secretion of legumain was detected from THP-1 differentiated M1 compared to M2 macrophages (**Figure 10A**). In contrast, M2 macrophages displayed higher cellular legumain activity compared to M1 macrophages (**Figure 10B**). Since M1 had a 9-fold higher legumain secretion than M2 (**paper II**

and **Figure 10**) or resting macrophages, this could indicate that M1 macrophages are an important cellular source of circulating legumain.

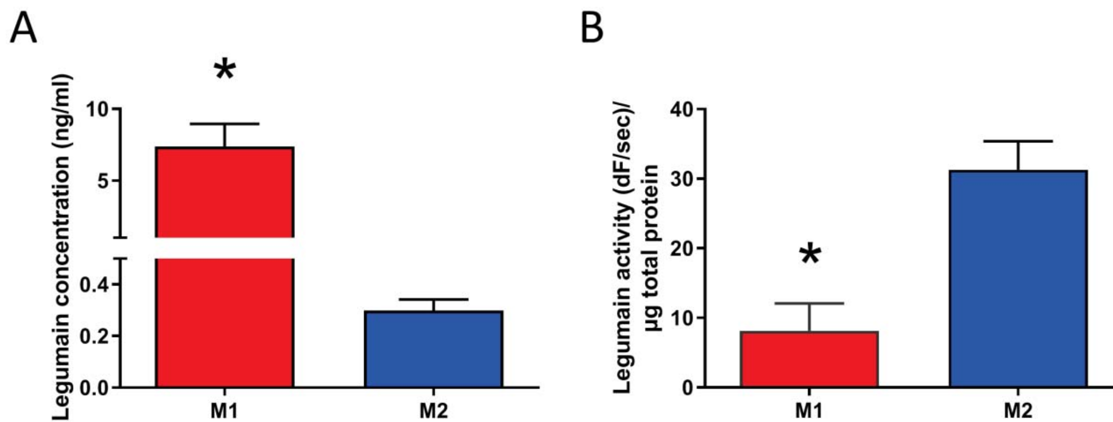

Figure 10. Differences in legumain secretion (A), and legumain activity (B) between THP-1-derived M1 and M2 macrophages. THP-1 monocytes were incubated with PMA (5 ng/ml) for 48 hours before stimulation for 72 hours with either interferon (IFN) γ (20 ng/ml) and lipopolysaccharide (LPS; 1 µg/ml) or interleukin (IL) 4 (20 ng/ml) for M1 or M2 polarization, respectively. **A**. Legumain concentrations in conditioned media (n=3-6). **B**. Legumain activity in cell lysates (n=4). Data are presented as mean ±SEM. *p<0.05 versus M2.

Interestingly, legumain co-localized with macrophages at areas rich in foam-like cells within the atherosclerotic plaques and these cells most likely contain early phases of cholesterol crystals (CC) deposition that may trigger legumain secretion (**paper II**). The interactions between lipids and macrophages are crucial in the development of atherosclerotic plaques. Other studies have also shown that CC appears early within macrophages and can promote pro-inflammatory polarization, resulting in arterial inflammation and destabilization of atherosclerotic plaque [253-256]. Crystallization of cholesterol led to volume expansion and could result in sharp-edged cholesterol crystals with the potential to penetrate biological membranes [257]. This may explain our findings *in vitro* as M1 macrophages stimulated with CC were shown to secrete significantly more legumain compared to cells stimulated with other atherogenic stimuli (**paper II** and **Figure 11**). An alternative mechanism could be that CC stimulates complement activation, as this activation has been shown to induce an inflammatory response and cytokine release [258].

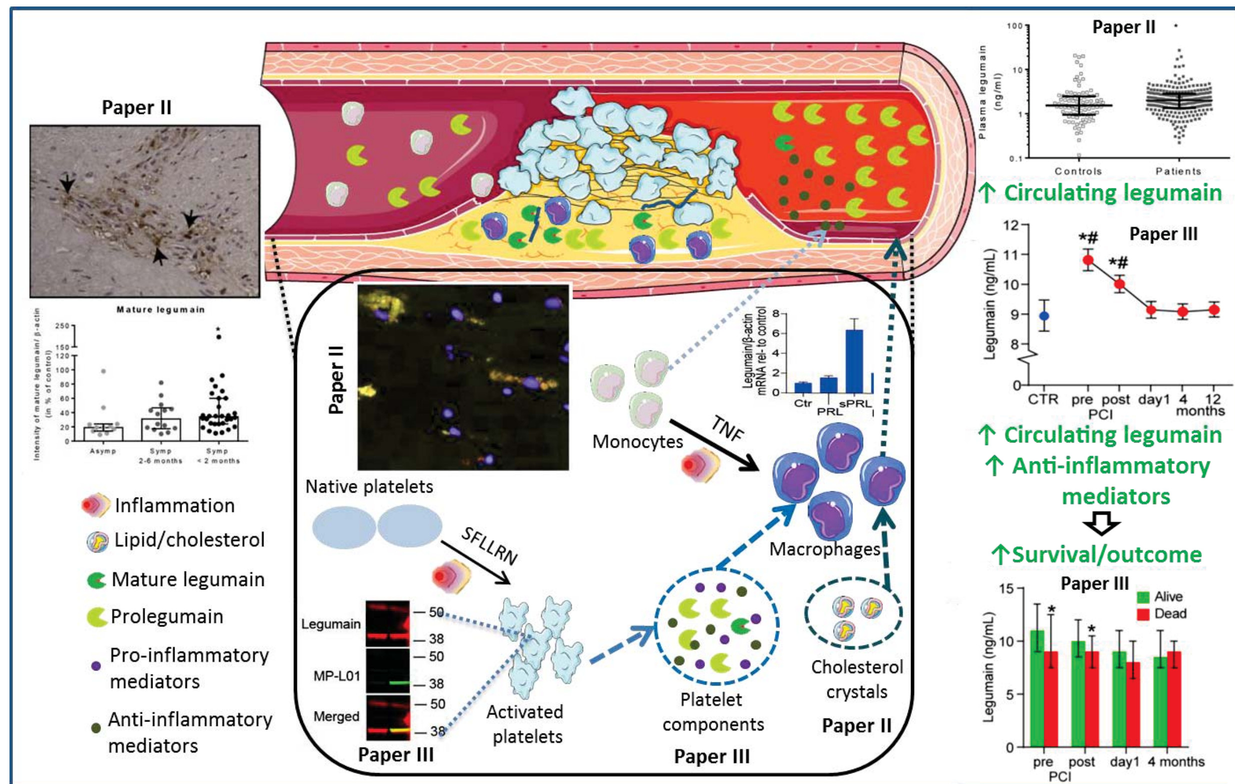

Figure 11. Summary of legumain localizations in the vasculature and interactions with macrophages and platelets. In **paper II**, increased levels of legumain in plaques and plasma were detected, and legumain expression in plaques corresponded with the time since last symptoms. Legumain was shown to co-localize with macrophages, and macrophages stimulated with cholesterol crystals secreted high legumain levels. In **paper III**, we showed that activation of native platelets by SFLLRN, a protease-activated receptor (PAR) 1 agonist. Both prolegumain and active legumain were present in and secreted from activated platelets together with other platelet-derived molecules. When THP-1 pre-activated monocytes were stimulated with platelet components, increased legumain mRNA expression was seen (**paper III**). Also, cholesterol crystal-stimulated macrophages contributed to the increased legumain secretion *in vitro* and may affect circulating legumain measured in patients with carotid atherosclerosis (**paper II**) and patients with acute and stable cardiovascular disease (**paper III**). Interestingly, high circulating legumain in the acute phase (prior to and immediately after PCI) corresponded with increased secretion of anti-inflammatory mediators and survival.

In the carotid plaques, legumain was located near necrotic areas, co-localized with macrophages and was present in areas rich in foam-like cells (**paper II** and **Figure 11**). Enhanced legumain expression has previously been detected in stable and unstable human atherosclerotic plaques [116], and in **paper II** we corroborated and extended these findings by showing increased expression of legumain within carotid plaques from patients with various times since symptoms (**Figure 11**). We found increased expression of both legumain mRNA and mature legumain (36

kDa) in plaques from patients with symptoms the last two months compared to asymptomatic patients. The detection of mature legumain is highly interesting due to the fact that the mean pH measured in carotid plaques is 7.55 (±0.32) [259]. Also, it is of great interest to elucidate whether legumain in plaques is active or not. Preliminary experiments using the legumain-selective ABP MP-L01 showed that legumain in plaques is active (**Figure 12A**). This was also confirmed by activity measurements using the selective legumain substrate Z-Ala-Ala-Asn-AMC (**Figure 12B-C**). Interestingly, the identification of a new 25 kDa active legumain form in M38L cells (**paper I**) was also detected in carotid plaques as this form bound MP-L01 (**Figure 12A**).

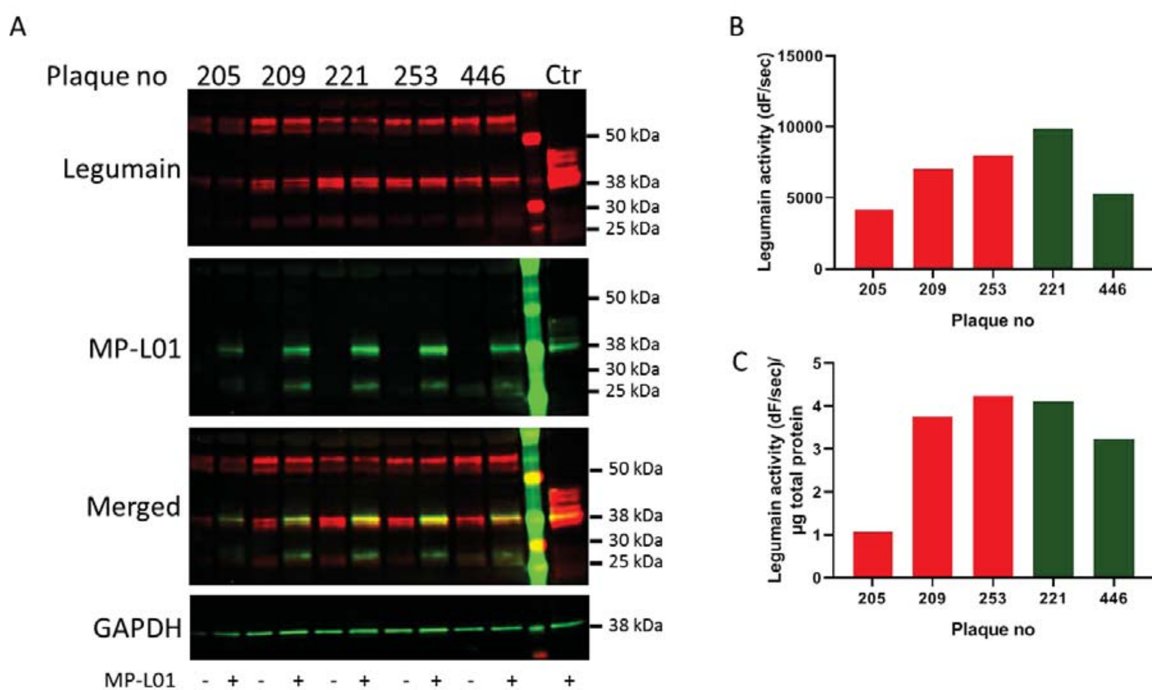

Figure 12. Active legumain is present in carotid plaques. Pulverized tissue from human carotid plaques (n=5) were homogenized by a metal blade homogenizer in ice cold legumain lysis buffer pH 5.8 at a ratio 0.1 ml per 10 mg wet weight tissue. The carotid plaques were from asymptomatic patients (plaque no 221 and 446; B-C, green columns) and from patients with symptoms the last 2 months (plaque no 205, 209 and 253; B-C, red columns). M38L cell lysate was used as a positive control (ctr). **A**. Plaque and cell lysates were incubated with 1 µM MP-L01 (+) or DMSO (-) for 30 min at 30°C before gel electrophoresis and immunoblotting using goat anti-human legumain. The immunoblot shows legumain (red, upper panel), MP-L01 (green, second panel) and merged panels (yellow, third panel). GAPDH was used as housekeeping control (lower panel). **B**. Legumain activity (dF/sec) in carotid plaques measured by cleavage of the peptide substrate Z-Ala-Ala-Asn-AMC. **C**. Legumain activity normalized to µg total protein. (n=1).

Expression of cysteine cathepsin B and L were also measured in all plaques included in **paper II** and semi-quantified (**Figure 13**), and similarly to legumain, higher expression of both cathepsins was detected in patients with symptomatic compared to asymptomatic carotid atherosclerosis. In plaques of symptomatic patients, significantly higher levels were detected for the pro- and single chain form of cathepsin L, as well as the two chain forms of both cathepsin B and L. Some of the cysteine cathepsins have previously been shown to be overexpressed in atherogenesis [8, 260]. However, as far as we know, this is the first time the expressions of legumain, cathepsins B and L have been studied in such details.

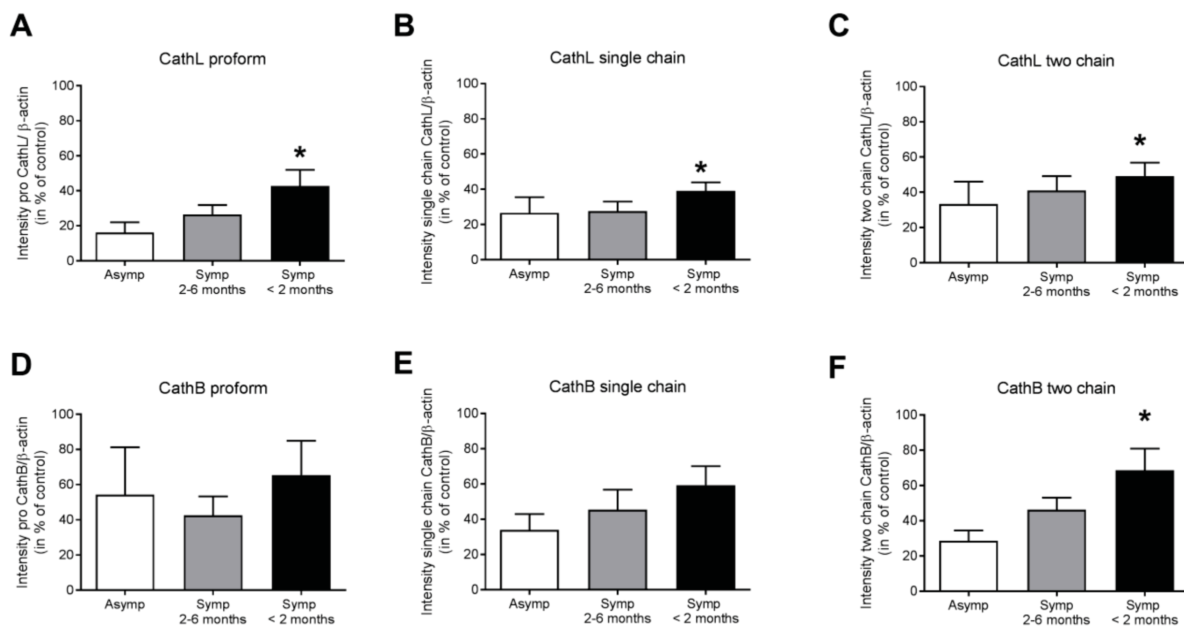

Figure 13. Expression of cysteine cathepsin B and L in carotid plaques. Immunoblotting of plaque lysates (n=53) were performed to detect expressions of pro-cathepsin L (cathL) (**A**), cathL single chain (**B**), cathL two chain (**C**), pro-cathepsin B (cathB) (**D**), cathB single chain (**E**), and cathB two chain (**F**) in asymptomatic patients (Asymp; n=10), patients with symptoms 2-6 months (Symp 2-6; n=13) and symptoms the last 2 months (Symp < 2 months; n=30). Immunobands were quantified by measuring band intensities compared to a housekeeping control (β-actin) and normalized to band intensities of one patient sample (control) used in all immunoblots. Data are presented as mean ± SEM, *p<0.05 versus asymptomatic.

In this thesis, expression of the endogenous inhibitor cystatin E/M is for the first time detected in carotid plaques and shown to be elevated in patients with recent symptoms (**paper II**). Implications of cystatin C, another member of family 2 cystatins, in atherosclerosis has been shown in several studies [261-263]. In **paper II**, we showed elevated expression of cystatin C in

patients with symptoms the last two months compared to asymptomatic patients. This is surprising as cystatin C has previously been shown to be reduced in atherosclerotic lesions and the level of cystatin C in atherosclerotic plaques appear to correlate inversely with disease progression [260]. However, comparison of cystatin C expression in patient plaques (**paper II**) compared to control vessels (i.e. the common iliac artery of organ donors) could be different. In cystatin C and ApoE double-deficient mice ($CysC^{-/-}$ $ApoE^{-/-}$), larger subvalvular plaques were detected compared to control mice ($CysC^{+/+}$ $ApoE^{-/-}$), indicating a protective role of cystatin C in atherogenesis [264]. It is interestingly that both legumain and cystatin E/M were highly upregulated in carotid plaques, especially in patients with symptoms the last 2 months (**paper II**). In contrast, in melanoma cells an inverse correlation between the expression of cystatin E/M and legumain has been observed and over-expression of cystatin E/M significantly inhibited legumain and decreased invasiveness of melanoma cells [265]. These findings suggest an imbalance in the interplay between the protease(s) and inhibitor(s) in various pathologies and in association with ongoing inflammation. To possibly understand this interplay in atherosclerosis, it would be interesting to study legumain and cystatin E/M in an atherosclerotic mouse model.

Importance of legumain in platelets

In **paper III**, we demonstrated for the first time that legumain is present in and rapidly secreted from platelets upon activation (**Figure 11**). In patients with atherosclerotic disorders, platelet-mediated inflammation appears to be operating in spite of the wide use of platelet aggregation-inhibiting drugs, thus new therapeutic tools is needed that more specifically target the pathways in platelet-mediated inflammation [139]. Upon activation, platelets release and express inflammatory mediators which induce an inflammatory response in neighboring leukocytes and endothelial cells [135, 139]. The interaction between platelets and leukocytes/endothelial cells plays a pathogenic role in atherosclerosis as well as in other immune-related disorders [266]. In THP-1 monocytes pre-activated with TNF-α, platelet releasate (sPRL) significantly enhanced the legumain mRNA level, whereas other potent inducers of monocyte activation did not induce any changes (**paper III** and **Figure 11**).

Using a proteomic approach, legumain has been shown to be among approximately 4500 proteins stored in human platelets, but was not released from platelets following thrombin or collagen stimulation [267]. The rapidly secretion of legumain from platelets upon protease-activated receptor (PAR) 1 activation (**paper III**) suggests storage in and release from α-granules

[268]. The incubation time with a platelet activator is of importance when studying platelets. Prolonged incubation with a platelet activator may result in degradation of releasable specific mediators (like legumain) as well as ongoing uptake mechanism by the platelet themselves [269]. In addition, high expression of mature and active legumain (36 kDa) was detected in platelets as this form bound the legumain-selective ABP MP-L01 (**paper III** and **Figure 11**). Moreover, active legumain in platelets might indicate lysosomal storage, as acidic pH is necessary for activity of legumain and other proteases such as cathepsin D [140]. The secreted lysosomal contents of platelets are postulated to have important extracellular functions, like receptor cleavage, fibrinolysis, degradation of ECM components, and remodelling of the vasculature [270]. However, the amount of lysosomal granules is low (1-3 per platelet) compared to α-granules (50-80 per platelet) [271]. Interestingly, legumain was shown to be localized to platelets both in unstable plaques and thrombi materials obtained during acute cardiovascular events (**paper III**). Our findings and the proteomic study strongly indicate legumain as a potential platelet-derived mediator and platelets as a source of circulating legumain. Use of the PAR-1 antagonist vorapaxar indicated a direct involvement of PAR-1 in legumain release from platelets (**paper III**). Human platelets express PAR-1 and 4 in the membrane, which are proteolytic activated by cleavage at the N-terminus to expose a tethered ligand to further interact with the extracellular loops of the receptor resulting in multiple G-protein-dependent and -independent signaling pathways [272]. Thrombin, a serine protease, is the canonical activator of PAR-1, leaving the tethered ligand beginning with SFLLRN [273] which was used to activate platelets in **paper III**. Recently, the lysosomal cathepsin S was shown to be involved in PAR-2 activation [274]. It is highly interesting to investigate if legumain may be involved in PAR activation, especially activation of PAR-1, and thus whether legumain is capable of mediating its own release.

Clinical relevance of legumain

At present, no drugs targeting or utilizing legumain have yet been marketed. Current treatment of atherosclerosis is focused on reduction of risk factors [275]. Statins are used as both primary and secondary prevention [276] and in addition to lowering cholesterol levels by inhibition of the HMG-CoA-reductase, statins reduce inflammation and improve endothelial function by pleiotropic effects [277-279]. Interestingly, monocytes isolated from patients treated with atorvastatin have shown downregulation of legumain mRNA expression [153]. Moreover,

although legumain is highly upregulated during monocyte-to-macrophage differentiation, legumain activity, expression and secretion has been shown to be inhibited by atorvastatin [36]. In addition, simvastatin has been shown to inhibit legumain processing and activity in human primary myotubes [280].

Paper II indicated that plasma legumain might be a usable biomarker in atherosclerosis together with other disease biomarkers. We postulated that legumain could be part of an algorithm to estimate the risk of cardiovascular disease, as suggested for idiopathic pulmonary fibrosis [281]. The potential role and limitations of legumain as a biomarker was discussed in an editorial in the same issue of Atherosclerosis [282] as **paper II**. Legumain has also been suggested as biomarker for liver fibrosis [283] and pancreatitis [284]. A biomarker is defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, or pharmacological responses to a therapeutic intervention" [285]. Biomarkers can indicate health and disease characteristics, and can be used as indicators of disease trait (i.e. risk factors), disease state (i.e. preclinical or clinical) or disease rate (progression of disease) [286]. The relevance of biomarkers of systemic inflammation to progression of atherosclerosis is not well established. Some serum markers, especially markers of inflammation (C-reactive protein [CRP], IL-18) or proteolytic activities (MMPs and cysteine cathepsins) are promising in distinguishing unstable from stable carotid artery stenosis [287, 288]. Legumain might add to the repertoire of such circulating markers. Interestingly, legumain seems to be regulated differently during stable and acute events (**paper III**). The rapid decrease of circulating legumain in association with PCI, with normalization within 24 hours could suggest that legumain is not an optimal biomarker in patients with stable atherosclerotic disease. However, during the acute phase, legumain could potentially give prognostic value. This may explain why we did not detected differences between the subgroups when patients in **paper II** were divided according to time since last symptoms, as the earliest time was one month or less since symptoms. Very recently, serum legumain were also closely associated with the severity of idiopathic pulmonary arterial hypertension [289].

In **paper III**, high legumain levels upon hospitalization were associated with improved outcome during follow-up (**Figure 11**). Although the association is based on study populations that yield relatively small number of events, results from primary monocytes showed a net anti-inflammatory effect of legumain. We observed significantly increased secretion and enhanced mRNA expression of the anti-inflammatory mediators CD163 and IL-10, in addition to

downregulation of the pro-inflammatory MCP-1 (**paper III**). Based on these findings, it is plausible to postulate a resolving function of legumain in atherosclerosis, potentially by polarization of monocytes towards a M2 phenotype and subsequent release of anti-inflammatory mediators (**Figure 11**). Legumain has previously been suggested to play a role in tissue repair [290]. Moreover, in data from an experimental mouse model, legumain was shown to mediate anti-inflammatory and pro-resolving effects of M2 macrophages by attenuating renal interstitial fibrosis in obstructive nephropathy [291].

The novel pro-resolving roles of legumain suggested in **paper III** contradict the previous findings where legumain has been found to be one among 18 genes associated with atherosclerotic plaque rupture [148]. The authors postulated that legumain represents a novel target for treatment of unstable plaques or as a diagnostic marker of plaque instability. Also, degradation of ECM caused by proteolytic enzymes is an important factor in destabilization of atherosclerotic plaques, as shown for MMPs (recently reviewed in [292, 293]). In addition to activation of MMP-2, legumain can directly degrade fibronectin, a major component of ECM [73, 108]. Macrophages can perform phagocytosis of ECM components, transport to and subsequently ECM degradation in the endo-lysosomal compartments [294]. However, the possibility for release of active proteases from macrophages and/or extracellular activation of proteases prompted the opportunity of ECM degradation in a more direct manner. The inflamed arterial wall of an atherosclerotic process can create an acidic milieu required for activation of legumain. Also, activated macrophages possess pumps for protons and lactic acid [243], thus the concept of acidic microenvironments in atherosclerosis is highly possible.

Although plasma contains other legumain form(s) besides prolegumain (**Figure 7**), we assume that circulating legumain is inactive due to the environmental conditions. However, we showed that prolegumain is able to be internalized and processed in cells (**paper I**) to mediate anti-inflammatory effects in monocytes/macrophages (**paper III**). Our findings in **paper II** and **III** suggest that legumain could be a novel player in CVD, operating during modulation of plaque stability and potentially modulating monocytes/macrophages-platelet interactions. These results may contribute to a shift from the postulated pathological role of legumain (i.e. destabilization of plaque) to a pro-resolving function, probably involving secreted legumain.

Due to the findings in **paper III**, pharmacological targeting of legumain is not desired in CVD, in contrast to cancer. Due to high legumain expression in solid tumors and correlation with poor

prognosis, legumain has been suggested as a prognostic marker in various cancers [57, 115, 230, 295-299]. For cancer treatment, several legumain-cleavable prodrugs have successfully been constructed based on a legumain-cleavable peptide linked to a cytotoxic drug (doxorubicin, etoposide, auristatin or colchicine), thus utilizing high legumain expression for cancer drug delivery [115, 300-303].

As mentioned, N-glycosylation is shown to be important for legumain function (**paper I**). Whether the glycosylation pattern of legumain is involved in pathology is still unknown and needs further investigations. Although glycosylation did not affect the inhibitory capacity of cystatin E/M, subcellular localization of the two cystatin E/M forms might give additional information on regulation of legumain. In-depth knowledge on the subcellular localization, processing and activity of legumain by investigating the interplay with intra- and extracellular components such as GAGs, fibronectin, inhibitors and CC, are fundamental for understanding the functional roles of this cysteine protease in cardiovascular disease.

The involvement and interplay of all cell types within the vasculature, including endothelial cells and SMCs and the involvement and regulation of legumain in all these cell types need to be addressed before a conclusion can be drawn concerning a beneficial or detrimental effect of this protease in atherosclerosis. At present, no data on legumain in SMCs or endothelial cells have been published. Regulation of legumain and the effects of pharmacological drugs (such as statins) in all vascular cell types may give additional information on legumain in cardiovascular disease.

Legumain has potential as a biomarker for acute coronary syndromes and carotid atherosclerosis (**paper II** and **III**). Measuring legumain in platelets from patients with CVD could add important information to the role of legumain in platelets, as well as the effect of anti-platelet therapy on circulating legumain. Also, forthcoming studies should more thoroughly examine the functional consequences of enhanced legumain levels during STEMI and other acute cardiovascular events. At the present, we possess several puzzle pieces in a complex picture and it is still challenging to see the whole picture. Further studies, especially studies in mouse atherosclerotic models are required to elucidate the roles of legumain in CVD.

CONCLUSIONS

This thesis sheds new light on the localization and regulation of legumain in cardiovascular disease and the importance of glycosylation for the processing and localization of legumain and function of cystatin E/M. Our main conclusions are:

- The carbohydrates on legumain and cystatin E/M are identified and shown to be of the hybrid or high mannose type and complex mannose-linked, respectively (**paper I**)
- Glycosylation of prolegumain is important for localization and processing, while the inhibitory function of cystatin E/M is independent of glycosylation (**paper I**)
- Circulating legumain is measureable in plasma/serum and increased in patients with carotid atherosclerosis, acute (STEMI) and stable CVD compared to controls (**paper II** and **III**)
- Carotid plaques from patients with recent atherosclerotic symptoms have increased legumain expression, specially of the mature form (36 kDa) (**paper II**)
- High circulating legumain in the acute phase of CVD correlates with improved outcome (**paper III**)
- Legumain is localized with both pro-inflammatory M1 and pro-resolving M2 macrophages, as well as foam cells (**paper II**), and with platelets in (unstable) carotid lesions and thrombi materials (**paper II** and **III**)
- Secretion of legumain is significantly higher from M1 than M2 macrophages (**paper II**)
- Platelets contain active legumain, and legumain is rapidly released upon platelet activation (**paper III**)
- Extracellular legumain stimulates secretion of anti-inflammatory mediators such as CD163 and IL-10, and deceases secretion of pro-inflammatory MCP-1 (**paper III**)

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