Regulation and functional studies of the cysteine protease legumain in bone cell biology

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Oslo, June 2020

Tatjana Bosnjak

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*, **Bosnjak T***, Solberg R*, Johansen HT* (*contributed equally). *Mammalian legumain - A lysosomal cysteine protease with extracellular functions?* Biochimie, 166, 77-83; 2019

- Paper II
  Bosnjak T, Solberg R, Hemati PD, Jafari A, Kassem M, Johansen HT. *Lansoprazole inhibits the cysteine protease legumain by binding to the active site.* Basic & Clinical Pharmacology & Toxicology, 125 (2), 89-99; 2019

- Paper III
  Bosnjak T, Solberg R, Arnekleiv GL, Jafari A, Hesselson D, Bassatne A, Fuleihan GE-H, Kassem M and Johansen HT. *Regulatory effects of vitamin D_3 on the cysteine protease legumain: Relevance to bone biology.* Submitted

- Paper IV
  Bosnjak T, Solberg R, Jafari A, Poreba M, Hesselson D, Haug KBF, Øvstebø R., Drag M, Kassem M, Johansen HT, Lunde NN. *Legumain enhances fibronectin production and both proteins are present in bone cell exosomes.* Manuscript

Editorial
Tanus-Santos JE, Pinheiro LC. *Proton pump inhibitors: New mechanisms of action*. Basic & Clinical Pharmacology & Toxicology, 125 (2), 87-88; 2019

ABSTRACT

The cysteine protease legumain is involved in several pathologies, i.e. osteoporosis, cancer, cardiovascular and neurodegenerative diseases. Legumain is considered to be mainly an intracellular lysosomal enzyme, but in recent years the presence and role of legumain in the extracellular environment is becoming elucidated. High legumain expression could be beneficial or detrimental depending on the enzyme location and the pathology. Therefore, pharmacological targeting of legumain needs to be carefully considered.

The aim of this thesis was to summarize and investigate the presence and role of extracellular legumain (**paper I** and **IV**) and to characterize degradation of the extracellular matrix protein fibronectin by legumain (**paper IV**), with a focus on bone biology. Furthermore, regulation of legumain by drugs or hormones with known or suspected effects on bone homeostasis was studied (**paper II-III**).

Effect of the proton pump inhibitor lansoprazole on legumain was studied for the first time. Lansoprazole inhibited legumain in several cell types and downregulated legumain secretion during osteoblast (OB) differentiation. The results indicated that lansoprazole binds covalently to the SH-group in the enzyme active site (**paper II**).

Also, for the first time, the interplay between vitamin D_3 (VD_3) and legumain during OB differentiation was studied (**paper III**). Legumain downregulated vitamin D receptor expression and generated a specific vitamin D-binding protein cleavage product of approximately 45 kDa, while VD_3 upregulated legumain expression, activity, and secretion in the early phase (7 days) of OB differentiation. Elderly patients had a negative correlation between baseline levels of legumain and $25OHD_3$ levels following 12 months treatment with VD_3.

Furthermore, the putative role of legumain in extracellular environment was additionally elucidated by legumain detection in bone cell exosomes. Additionally, fibronectin production and degradation were enhanced by legumain (**paper IV**).

In conclusion, the results presented in this thesis contribute to new knowledge on regulation and functional roles of the cysteine protease legumain, especially in bone biology.

ABBREVIATIONS

AD	Alzheimer's disease
ADC	Adipocyte
AEP	Asparaginyl endopeptidase
ALP	Alkaline phosphatase
AP	Activation peptide
APC	Antigen presenting cell
APP	Amyloid precursor protein
ATCC	American Type Culture Collection
CNS	Central nervous system
CTF	C-terminal fragment
CVDs	Cardiovascular diseases
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ENS	Enteric nervous system
EPD	Eukaryotic Promoter Database
EV	Extracellular vesicles
FDA	Food and Drug Administration
FN	Fibronectin
GAGs	Glycosaminoglycans
hBMSC	Human bone marrow-derived stromal (mesenchymal) stem cells
IB	Immunoblotting
ILV	Intraluminal vesicles
KO	Knock-out
LSAM	Legumain stabilization and activity modulation domain
M1	Pro-inflammatory macrophages
M2	Anti-inflammatory and tissue-remodelling macrophages
M38L	Monoclonal legumain over-expressing HEK293 cells
M4C	Monoclonal cystatin E/M over-expressing HEK293 cells
mAb	Monoclonal antibody
MMP	Matrix metalloprotease
mRNA	Messenger RNA
MSC	Mesenchymal stem cells
MV	Microvesicles
MVB	Multivesicular bodies
NDGs	Neurodegenerative diseases
N-terminal	Amino-terminal
OB	Osteoblast
OC	Osteoclast
OPG	Osteoprotegerin
pAb	Polyclonal antibody
PD	Parkinson's disease
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PPIs	Proton pump inhibitors
PTH	Parathyroid hormone
RANK	Receptor activator of NF-κB

RANKL	Receptor activator of NF-κB ligand
RUNX2	Runt-related transcription factor 2
TERT	Telomerase reverse transcriptase
TGF-β1	Tumour growth factor-beta 1
THP-1	Human monocytic cell line
TNF-α	Tumour necrosis factor-α
TRAP	Tartrate-resistant acid phosphatase
VBDP	Vitamin D-binding protein
VD_3	Vitamin D_3
VDR	Vitamin D receptor

INTRODUCTION

Proteolytic enzymes

Proteins are one of the most stable biological polymers. Peptide bonds connecting amino acids can endure boiling acid, but are helpless against cleavage by a specific proteolytic enzyme [1]. Proteolytic enzymes (proteases, peptidases or proteinases) are enzymes responsible for hydrolysis of peptide bonds. Proteolysis is necessary in all life forms, and proteases are found in viruses, bacteria, parasites, plants and vertebrates. Approximately 2 % of all functional genes in the human genome encode for proteases and many proteases are currently used or under investigation as drug targets [2].

Proteolysis is an irreversible process driven by nucleophilic attacks on peptide bonds. Depending on the site of the proteolytic reaction, proteases are divided into exo- and endopeptidases. Exopeptidases hydrolyse peptide bonds at the C- or N-terminal ends of a polypeptide chain, liberating a single amino acid, dipeptide or tripeptide. Endopeptidases cleave proteins within a polypeptide chain. Proteases have numerous functions in human biology. Besides their classical nonspecific roles in protein degradation such as food digestion, proteases are also important in tissue remodelling, protein-protein interactions, cellular signal transduction, stem cell differentiation, wound healing, immunity, bone formation, autophagy and apoptosis [3].

The MEROPS database classifies proteases according to their catalytic mechanisms, evolutionary relationship and amino acid sequence homologies (Fig. 1). Depending on the chemical mechanism of catalysis, MEROPS organizes proteases into aspartic, asparagine, cysteine, glutamic, metallo, serine and threonine classes [2]. Based on the evolutionary relationship between families, proteases are further organized into clans defined by their globular three-dimensional (3D) folding. The criterion for belonging to the same protease family is a significant similarity in amino acid sequence. The focus of this thesis is the cysteine proteases (Fig. 1).

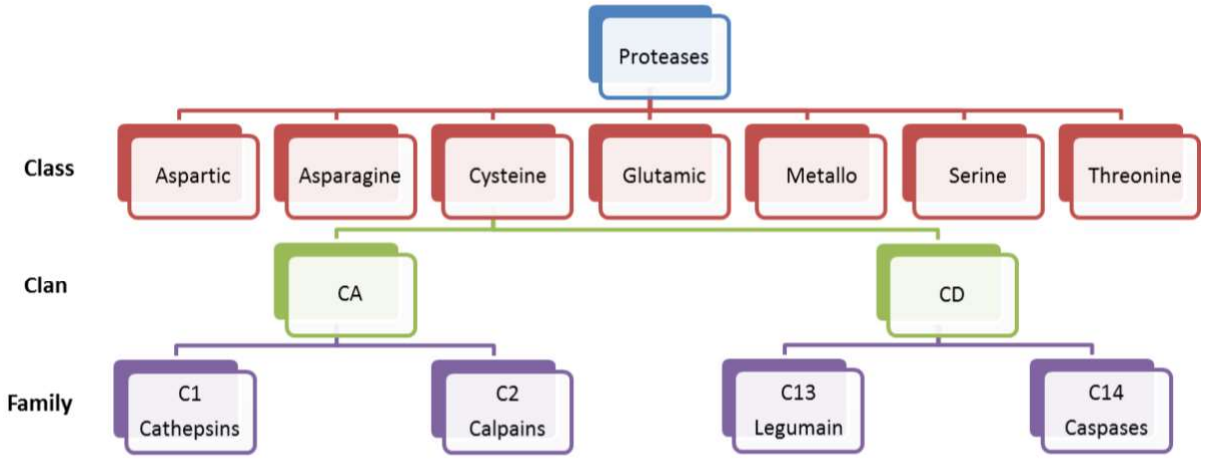

Figure 1. Classification of mammalian proteases according to MEROPS. Proteases are organized based on their chemical mechanism of catalysis, evolutionary relationship and amino acid sequence homologies. The proteases are divided into seven classes based on their catalytic type: aspartic, asparagine, cysteine, glutamic, metallo, serine and threonine (red boxes). Classes are further divided into clans (green boxes, exemplified by clan CA and CD of cysteine proteases) based on a postulated common evolutionary ancestor which is reflected in similarity in 3D structures and the arrangement of amino acids in the catalytic part of the polypeptide chain. Each clan is further classified into families (purple boxes) according to similarities in amino acid sequences. Used as an inspiration for the drawing [2].

Cysteine proteases

The active site of cysteine proteases contains a cysteine residue which serves as a nucleophile and is responsible for the hydrolytic cleavage of peptide bonds [4]. Cysteine proteases are synthesized as inactive zymogens, in order to prevent unwanted protein cleavage. Zymogens contain a prodomain which covers the active site of the enzyme. Removal of the prodomain accompanied by subsequent enzyme activation can be accomplished either by autoactivation at acidic pH, a reductive environment, hydrophobic interactions and disruption of salt bridges, conformational changes, calcium ions or by proteolytic cleavage [5-11].

Cysteine proteases are divided into 11 clans and over 70 families [2]. Apart from their fundamental role in protein catabolism and processing, cysteine proteases have various other functions [12, 13]. Papain was the first characterized cysteine protease, identified in 1937 in the latex of the tropical papaya fruit and classified to clan CA, family C1. The mammalian analogues of papain are the cysteine cathepsins (cathepsin B, C, F, H, K, L, O, S, V, W and X) primarily localized in the lysosomes and the calpains present in the cytosol (Fig. 1). Cysteine

proteases studied in this thesis are primarily legumain and to a certain extent cathepsin B, K and L.

Legumain

The main focus of this thesis is the lysosomal cysteine protease legumain. Legumain was first discovered in mature seeds of beans (legumes) and blood fluke (*Schistoma mansoni*) in the early 1990s [14-17]. In 1997, legumain was for the first time reported in mammals [18]. Due to the strict and unique specificity towards asparagine residues, mammalian legumain is also known as asparaginyl endopeptidase (AEP) [2]. Legumain has a conserved His^{148}-Gly-spacer-Ala-Cys^{189} motif and was thus classified as a member of clan CD together with the caspases (family C14) and included in family C13 (Fig. 1). In addition to the shared catalytic motif, legumain has other evolutionary relationships with the caspases, including approximately 15% sequence homology and a strict specificity for one particular amino acid in the substrate P1 position [19]. In addition to cleaving carboxy-terminally to asparagine (Asn), legumain acquires caspase-like activity and cleaves also after aspartate (Asp) residues at pH<5 [20, 21]. However, there are immense differences between legumain and the caspases since legumain is confined to the lysosomes as active monomers at acidic pH [22], while the caspases are found in the cytoplasm and can be activated as dimers at neutral pH [23]. Mammalian legumain has optimal activity towards asparagine residues at pH 5.8 [18].

The human legumain gene (*LGMN*) is localized to chromosome 14q32.1 encoding a protein of 433 amino acids, expressed as an inactive zymogen (prolegumain, 56 kDa; Fig. 2) consisting of a catalytic- and a prodomain [18, 22, 24]. The prodomain comprises an activation peptide (AP, Lys^{287}-Asn^{323}) and a C-terminal domain, named legumain stabilization and activity modulation (LSAM) domain (Asp^{324}-Tyr^{433}) [22]. Since the surface of the catalytic domain is negatively charged and the prodomain is positively charged, the interaction between the two is predominantly electrostatic [22]. Auto-catalytic activation of legumain in acidic environment (pH < 5.5) triggers cleavage after Asn^{323} (β-site), partially releasing the AP, whereas the C-terminal LSAM remains bound to the protease through electrostatic forces [22], thus rendering a 47 kDa intermediate. Further pH decrease (pH < 4.5) triggers subsequent N-terminal cleavage after Asp^{25}, yielding a 46 kDa active intermediate [25]. A second cleavage at Lys-Arg-Lys^{289} site (KRK^{289} motif; α-site) by a yet unknown protease is necessary for the complete dissociation of AP and LSAM, resulting in the mature active 36 kDa legumain [22,

25, 26]. Mature legumain is a monomer in solution, while prolegumain forms a homodimer [27].

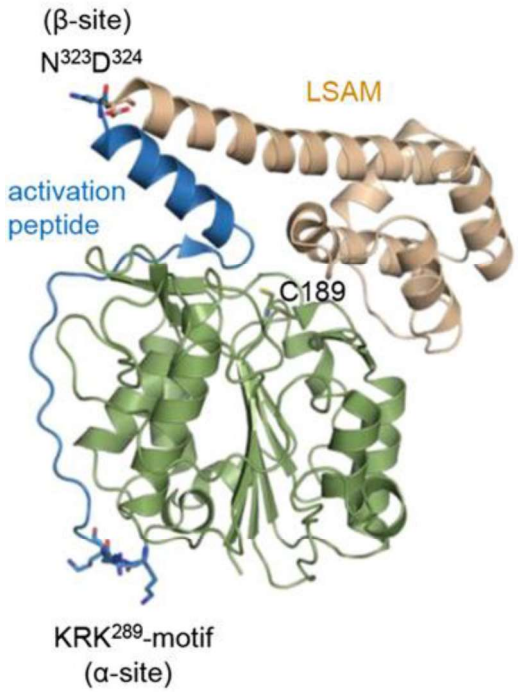

Figure 2. Crystal structure of human prolegumain. Prolegumain consists of a catalytic domain (green), an activation peptide (blue), and a legumain stabilization and activity modulation (LSAM) domain (wheat). Cleavage at the α- (KRK^{289}) and β-sites (N^{323}-D^{324}) releases the AP and LSAM, thus rendering accessibility to the cysteine in the active site (C^{189}). Figure adopted from [22] and journal copyright permission is granted for educational purposes (i.e. in a book that is not for sale).

Legumain is localized mainly in the endo-lysosomal compartments [28] where it is active due to acidic pH and a reductive environment [29]. However, translocations of both mature (36 kDa) and prolegumain (56 kDa) to the cell nuclei, cytoplasm and extracellular environment have been observed [30, 31]. Also, the intermediate active legumain form (46 kDa) has been reported to be present on the surface of tumour-associated macrophages in the tumour stroma [32]. The presence of prolegumain (56 kDa) has been reported in body fluids like plasma, serum and cerebrospinal fluid [30, 31, 33-36]. Mature legumain requires acidic (pH<6) and reducing environment in order to be stable and active, whereas prolegumain is stable at neutral pH [26-28]. The electrostatic interaction between the negatively charged

catalytic domain and the positively charged LSAM of prolegumain explains the stability at neutral pH [27]. Nevertheless, secreted prolegumain is assumed to be activated in acidic extracellular microenvironments during pathological or inflammatory conditions. Interactions with integrins or glycosaminoglycans (GAGs) present in the extracellular matrix could stabilize legumain extracellularly [22, 27, 37, 38]. Legumain binds to the $\alpha v\beta_3$ integrin receptor on cell surfaces via an Arg-Gly-Asp^{120} (RGD) motif [22, 39], resulting in increased stability, catalytic activity and shift in the pH optimum from pH 5.8 to 6.0 [22]. Naturally occurring polysaccharides (i.e. polyanionic GAGs) can increase legumain stability and enhance autoactivation through ionic interactions [37, 40]. Extracellular prolegumain can be internalized by cells and subsequently processed and activated [41]. Glycoproteomic studies have revealed two N-glycosylation sites in prolegumain [42] and the carbohydrates are shown to be of the hybrid or high mannose type and necessary for internalization and correct processing to mature active legumain [43].

Legumain substrates

Cleavage by legumain results either in activation or inactivation of substrates and presently known substrates are listed in Table 1. Due to its AEP activity, legumain has a role in maturation and processing of cathepsin B, H and L [44], cleavage of vitamin D-binding protein (VDBP) and fibronectin (FN) [45-47]. Ligase activity of plant legumain was reported already in 1994 [48] and in 2015 also confirmed for mammalian legumain [49]. The endogenous legumain inhibitors, cystatin C and E/M, have been shown to be both cleaved at acidic pH ($\leq$4) and religated at pH $\geq$6 by legumain [22, 27, 49]. The ligase activity of legumain is not restricted to cystatins, as legumain may also religate itself during auto-processing [27, 49] which adds to the complexity of this protease (Table 1).

Table 1. Known legumain substrates

AEP substrates	Ligase substrates	References
Acetoacetyl-CoA synthetase		[50]
Alpha-1-macroglobulin		[51]
α-synuclein		[52, 53]
Amyloid precursor protein (APP)		[54]
Annexin A2		[45, 55]
Beta-amyloid protein 40		[51]
Betaine-homocysteine S-methyltransferase 1		[56]
BetV1		[57]
Cathepsin B, H, L, S		[44, 58]
Cystatin C, E/M	Cystatin C, E/M	[49]
Fibronectin		[46]
Invariant chain chaperone (Ii)		[59]
Myelin basic protein		[60, 61]
Progelatinase A (proMMP-2)		[62]
Prolegumain	Prolegumain	[20, 21, 25,
Prothymosin α		[63]
Serotransferrin		[51]
Serum albumin		[51]
SET (also known as PHAPII, TAF-Iβ, I2		[64]
TAR DNA-binding protein 43		[65]
Tau		[66-70]
Toll-like receptor (TLR) 3, 7 and 9		[71-74]
Tetanus toxin C-terminal fragment		[75, 76]
Vitamin D-binding protein (VDBP)		[45]

Tissue expression and functional roles of legumain

Legumain is ubiquitously expressed, but highly abundant in kidneys [18, 28]. Being widespread throughout the body, legumain has multiple functions at multiple locations, both intra- and extracellularly. Legumain deficient mice have significantly reduced body weight, but are normally born and fertile [77, 78]. Nevertheless, abnormal lysosomes in the kidney proximal tubular cells (PTCs) cause accumulation of macromolecules in the endolysosomal system of legumain deficient mice [77]. Thus, legumain deficient mice developed lysosomal storage disease in the kidney PTCs. Studies indicate that the accumulated molecules include cathepsin B, H and L [79]. Legumain is necessary for normal kidney function and mice lacking legumain develop hyperplasia of PTCs, interstitial fibrosis and other kidney anomalies [79]. Moreover, legumain deficient mice have several indications of hemophagocytic lymphohistiocytosis including hepatosplenomegaly, fever and severe anaemia [44]. How the absence of legumain triggers these conditions is not known. However, it has been shown that legumain deficiency

activates the STAT3-dependent signalling pathway leading to transcription activation and increased expression of multiple proteases [80].

Legumain contributes to the processing of macromolecules absorbed by PTCs, such as VDBP [45, 55]. VDBP is an abundant plasma protein and functions as a vitamin D_3 transporter for 25-hydroxyvitamin D_3 ($25OHD_3$). After filtration in the kidney glomeruli the VDBP-$25OHD_3$ complex is endocytosed by PTCs where legumain is highly expressed [45]. Cleavage of VDPB by legumain and other lysosomal proteases has to occur in order to release $25OHD_3$ for further hydroxylation to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the active vitamin D_3 form). Also, legumain has an important role in ECM remodelling through FN degradation in PTCs [46] and processing and activation of pro-matrix metalloproteinase 2 (pro-MMP2) which is one of the main ECM-degrading enzymes [62, 81]. Processing of cathepsin B, H and L from single-chain to the two-chain forms is impaired in legumain deficient mice [58, 77].

Cysteine cathepsins

Cysteine cathepsins (Fig. 1) are one of the most investigated groups of proteolytic enzymes. The zymogens of cysteine cathepsins are monomeric proteins with molecular weights of approximately 30-50 kDa. Typically, cathepsins are endopeptidases, with the exception of cathepsins B, C, H and X which demonstrate exopeptidase activity as well. Cysteine cathepsins cleave their substrates after basic and hydrophobic amino acid residues, which deems cathepsins as not very specific enzymes. Cathepsins are ubiquitously expressed in human tissues; except for cathepsins K, W and S, which have a more specific tissue distribution indicating tissue specific functions [82, 83]. For example, cathepsin K is the most potent mammalian collagenase and is highly expressed in osteoclasts, epithelial cells and synovial fibroblasts [84]. Due to its specific localization, cathepsin K plays a key role in bone remodelling [84-86]. In order to be optimally active, cathepsins require reducing and acidic conditions as found in the endolysosomal compartments. Except for cathepsin S, all other cathepsins are unstable and inactive at neutral pH. Cathepsins are predominantly lysosomal enzymes, and mainly responsible for intracellular protein degradation. However, cathepsins have also been found to be highly active in the extracellular environment and the cytosol, indicating that pH is not the only important factor for proteolytic activity of cysteine cathepsins [87, 88]. Furthermore, cancer cells and tumour-associated cells have shown increased levels of cathepsins in, and increased secretion from tumours [83, 89, 90].

Endogenous cysteine protease inhibitors

Protease activities could be extremely harmful and dangerous if not controlled. Cystatins are endogenous inhibitors of cysteine proteases. Cystatins are non-selective, reversible inhibitors further divided into three families, dependent on structure and localization [2, 91]. Legumain is only inhibited by some members of the type II family, including cystatin C, E/M, and F [92]. Cystatin C is ubiquitously expressed with the highest concentration found in seminal plasma and cerebrospinal fluid [93]. Also, cystatin C is used as a marker for glomerular filtration rate since it is produced by all nucleated cells and has a stable blood concentration [94]. Cystatin E/M is expressed in a variety of human tissues, including skin, heart, brain, placenta, lung, liver, pancreas, spleen, thymus, prostate, ovaries, small intestine and peripheral blood cells [95, 96]. Cystatin F has a more specialized tissue distribution being found primarily in immune cells [91]. Among the cystatins, cystatin E/M has the highest affinity for legumain [92] and is expressed and secreted in two molecular forms, a 14 kDa unglycosylated and a 17 kDa glycosylated form [97]. The N-linked carbohydrates on cystatin E/M are complex mannose-linked [43]. It has been shown that reduced or absent expression of cystatin E/M leads to tumour progression and metastasis in various types of cancers including breast, lung, skin and oral cancers [97-102]. Cystatin C is believed to be involved in pathological conditions such as Alzheimer's disease, cancers, rheumatoid arthritis and atherosclerosis [103-108].

Cysteine proteases in bone remodelling

The skeleton is a metabolically active organ that undergoes continuous remodelling throughout life and every 7-10 years the skeleton is completely renewed. Bone remodelling serves as a response to constant mechanical pressure and micro-damages by replacing the damaged or old bone. Bones serve as a Ca^{2+} reservoir, which is released or incorporated by the bone remodelling mechanism to obtain systemic Ca^{2+} homeostasis [109]. In order to maintain bone homeostasis, an equilibrium between bone resorption and bone formation is essential. The remodelling entails several consecutive phases; the activation phase, where pre-osteoclasts are activated; resorption phase, where osteoclasts degrade the bone; the reversal phase, where cytokines released from the degraded bone matrix recruit pre-osteoblasts to the bone surface; and the

formation phase, where osteoblasts produce and mineralize bone matrix until the resorbed bone is entirely replaced (Fig. 3) [110].

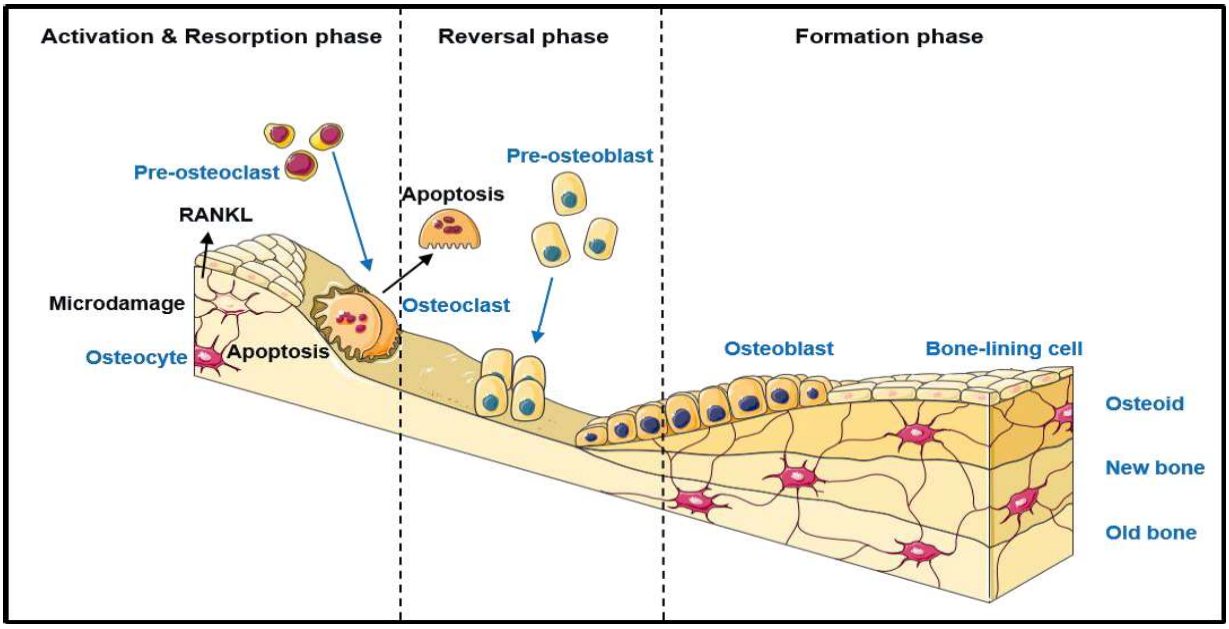

Figure 3. Schematic illustration of bone remodelling. Remodelling starts with osteocyte apoptosis and RANKL-mediated pre-osteoclast recruitment (activation phase), followed by osteoclastogenesis. The osteoclasts resorb bone (resorption phase) allowing the release of factors usually stored in the bone matrix, which recruit osteoblasts in the reabsorbed area (reversal phase). Once recruited, osteoblasts produce new bone matrix thus forming osteoid. After the osteoid formation, osteoblasts promote inorganic salt incorporation leading to bone mineralization (formation phase). RANKL, receptor activator of NF-κB ligand. The figure is drawn by the author.

Bone is composed of various cell types and extracellular matrix (ECM). The major structural component of the bone is an organic matrix of collagen and non-collagenous proteins, termed osteoid. Bone matrix further consist of an inorganic component of calcium-containing hydroxyapatite mineral salts [$(Ca_5(PO_4)_3$]. Bone cells are divided into two main categories: bone resorbing osteoclasts (OCs) and bone forming osteoblasts (OBs). OBs are formed from stem cells in the bone marrow stroma (BMSC) also known as skeletal or mesenchymal stem cells (MSC; Fig.4). OBs form new bone tissue through secretion of collagenous (collagen I and III) and non-collagenous proteins (FN, sulphated GAGs, elastin, osteocalcin, osteonectin and proteoglycans) [111], and subsequent ECM mineralization [112]. Beyond bone tissue maintenance, OBs have been shown to have endocrine roles regulating other functions in the body by production of fibroblast growth factor 23 (FGF23) and osteocalcin [113]. FGF23 regulates serum phosphate and $1,25(OH)_2D_3$ levels, whereas osteocalcin induces insulin

secretion and subsequent glucose utilization [114]. Furthermore, OBs support haematopoiesis through secretion of growth factors and cytokines [115]. OBs control activation of OCs through secretion of TNF superfamily members, i.e. receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG; Fig.4) [116]. Interaction between OBs and the immune system through secretion of immunomodulatory factors has also been described [117].

OCs originate from mononuclear myeloid hematopoietic stem cells (Fig. 4). OCs are large, multinucleated cells rich in tartrate-resistant acid phosphatase (TRAP) [118], with the unique capacity to degrade inorganic and organic matrices of bone. OCs achieve resorption by forming close contact with the bone surface, leading to enlargement of OC plasma membrane into a ruffled border, followed by secretion of protons and proteases into the isolated microenvironment thus creating resorptive pits [119]. Hydroxyapatite mineral salts are dissolved by the acidic pH in the resorptive pits whereas collagenous and non-collagenous matrix proteins are degraded by secreted proteases i.e. cathepsin K, L and matrix metalloproteinases (MMPs) [120-123].

Bone remodelling is regulated both locally and systemically. Major systemic positive regulators are calcitonin, vitamin D_3, sex hormones, thyroid hormone and growth hormone, while negative regulators are parathyroid hormone (PTH) and glucocorticoids. Low Ca^{2+} levels stimulate bone resorption in order to release Ca^{2+} and normalize extracellular Ca^{2+} concentration [109]. Many mediators are involved in bone remodelling, such as tumour growth factor-beta 1 (TGF-β1), prostaglandins, insulin-like growth factors (IGFs) and bone morphogenetic proteins (BMPs). TGF-β1 regulates both OB and OC maturation. TGF-β1 stimulates OB proliferation [124], inhibits OB apoptosis [125], and recruits OB precursors or OBs to the specific bone site through chemotaxis [126]. In addition, TGF-β1 enhances the production of ECM proteins by OBs in the early stages of OB differentiation, i.e. FN [127]. FN is known to be important for OB survival and differentiation through interactions via the $\alpha_5\beta_1$ integrin receptor [128-131]. Also, FN inhibits OC differentiation [132].

When it comes to local regulation of bone remodelling, the RANK/RANKL/OPG system tightly couples OC and OB activity (Fig. 4) [133]. RANKL/RANK signalling controls OC development, activation and survival in normal bone modelling and remodelling, as well as in a variety of pathologic conditions characterized by increased bone turnover. OPG prevents RANKL binding to RANK and therefore inhibits OC formation and activation. High OC activity leads to one of the most common chronic diseases – osteoporosis. Thus,

pharmacological targeting and inhibition of OCs is the main focus of osteoporotic therapy. Around 200 million people suffer from this disease today, and the rates are expected to increase by 300% worldwide in the next 10 years [134].

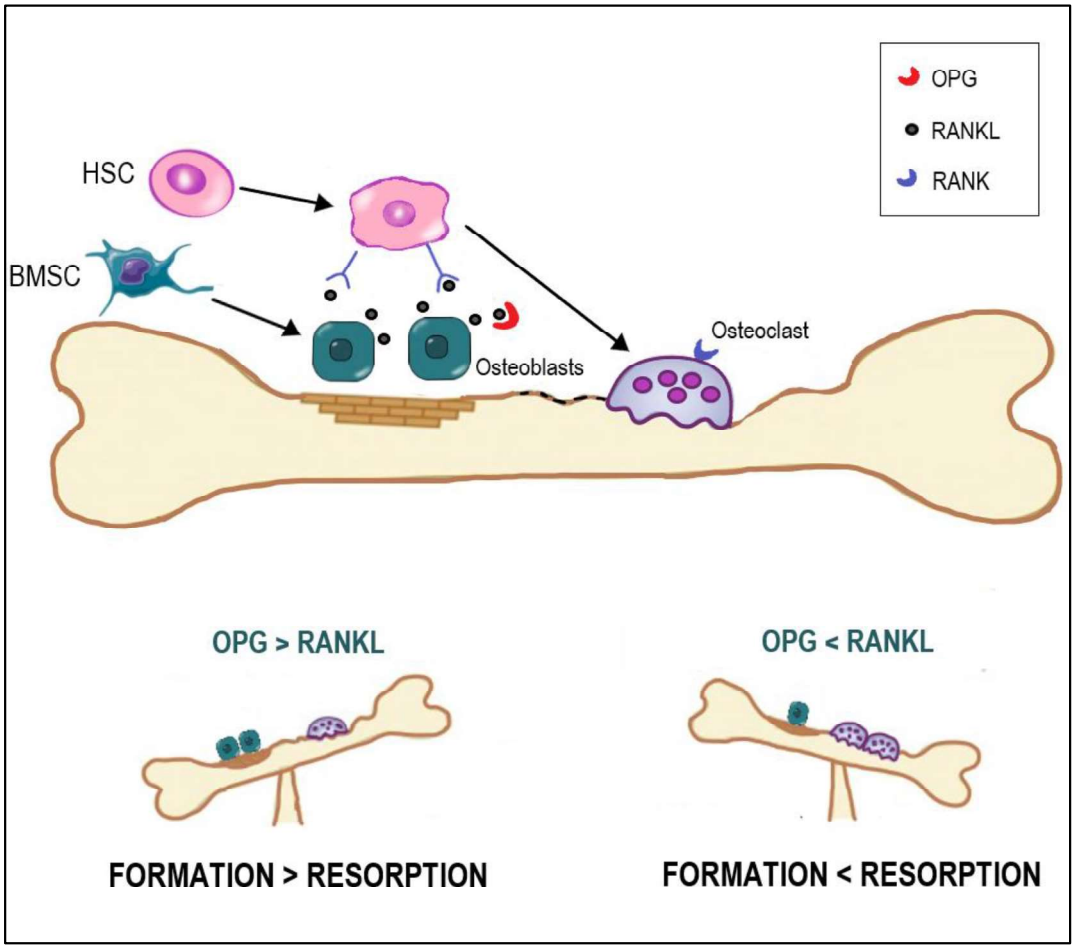

Figure 4. Schematic illustration of the RANK/RANKL/OPG system in bone. Osteoclasts are derived from mononuclear precursors originating from myeloid hematopoietic stem cells (HSC). Bone marrow stromal cells (BMSC) are osteoblast progenitors. Osteoblasts regulate osteoclastogenesis by secreting members of the TNF superfamily, RANKL and OPG. RANKL binds to RANK receptor on osteoprogenitor cells and stimulates osteoclastogenesis and bone resorption. OPG is a decoy receptor for RANKL and thereby protects bone from excessive resorption by binding to RANKL and preventing it from binding to RANK. Thus, the relative concentration of RANKL and OPG in bone is a determinant of bone mass and strength. RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin. The figure is drawn by the author.

In the secluded microenvironment of bones, the intercellular communication is complex and not fully elucidated. Regulation of bone remodelling is only partially explained by the roles of growth factors, cytokines and hormones. In recent years, attention has been drawn to exosomes [135-137], which are cell-secreted nanovesicles with a diameter size of 40-120 nm

[138]. Exosomes belong to the extracellular vesicles (EV), together with microvesicles (MV) and apoptotic bodies [138]. MVs are formed by outward budding from the plasma membrane, and cells undergoing apoptosis release apoptotic bodies [138]. Late endosomal compartments known as multivesicular bodies (MVB) lead to exosome generation. Inward budding of endosomal membranes results in the formation of intraluminal vesicles (ILV) within MVB [139]. Majority of ILV are exocytosed into the extracellular environment after fusion with the plasma membrane and are referred to as exosomes [139]. Exosomes are carriers of many different proteins, lipids and RNAs [136]. In the context of bone physiology, TGF-β1, RANKL, TRAP, OPG and multiple miRNAs have been identified in the OB exosome cargo [136]. Exosomes have the ability to transfer their cargo, leading to reprogramming and modified gene expression in the recipient cells [135]. An overview of exosome communication between bone cells is described in Fig. 5.

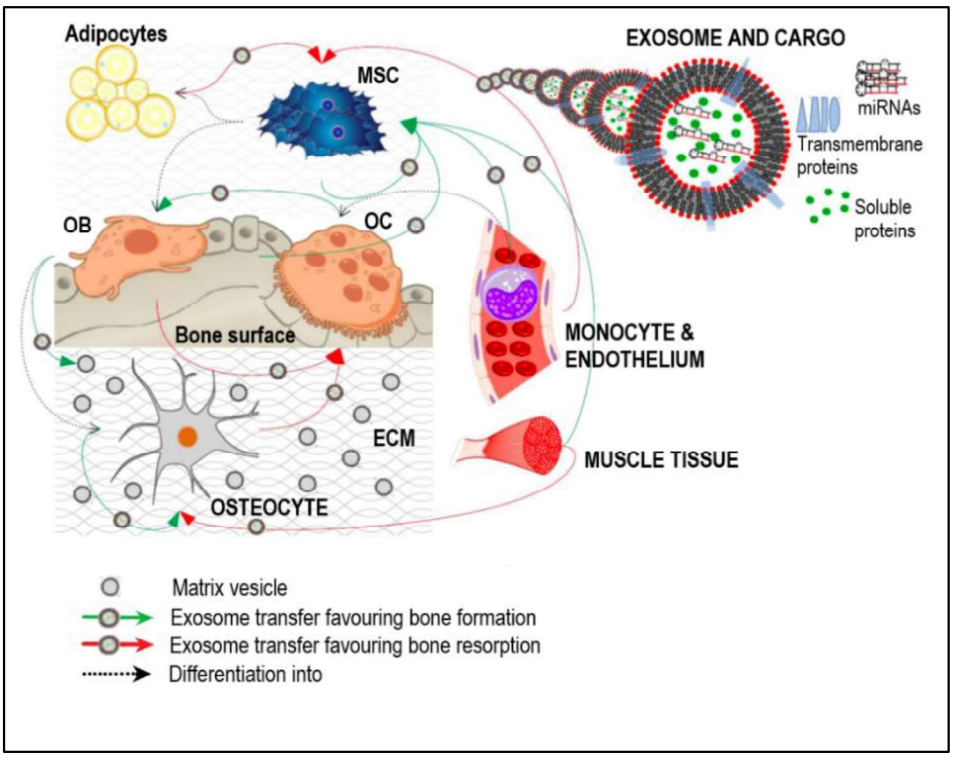

Figure 5. The role of exosomes in the processes of bone remodelling. In the secluded environment of bones, exosomes are mediating cell-to-cell communication between osteoblasts (OB), osteoclasts (OC), osteocytes and their precursors. Exosomes are also released by adipocytes, myoblasts, and the endothelium. Exosomal cargo represents a variety of proteins and miRNAs (upper right), which support either bone formation (green arrows) or bone resorption (red arrows) depending on the type of secreting or receiving cell. MSC, mesenchymal stem cells; ECM, extracellular matrix. Figure modified from [135] and the copyright permission from the journal is granted.

Activity of proteolytic enzymes is crucial for bone remodelling and for mediating bone cell activities [123, 140, 141]. Two protease families are primarily involved in bone resorption by OCs, the cysteine proteases and MMPs. A great number of studies have identified cathepsin K as the main enzyme in bone resorption [142]. OCs from mice deficient in cathepsin K can dissolve inorganic matrix but the degradation of protein matrix is impaired [122]. Mice deficient in cathepsin B or L also have impaired bone resorption [143, 144]. Cathepsin K deficient mice compensate by increased secretion of MMPs in the resorption area [142]. On the contrary, in cathepsin L deficient mice, MMPs are not used by the OCs for the resorption of bone matrix. This might imply that cathepsin L plays a role in bone matrix resorption by activating MMPs [140]. However, involvement of MMPs in bone degradation is shown to be site-specific, as OCs in long bones do not use MMPs whereas OCs in flat bones do [145]. Nonetheless, MMP-2, -9, -13, -14 or -16 deficient mice have a clearly weakened bone phenotype manifesting in osteopenia, osteoporosis and shorter bones [121]. Furthermore, cystatin C has been shown to decrease bone resorption by acting as an inhibitor of extracellular proteases and OC differentiation through interference with RANK signalling [146, 147]. Also, serum cystatin C levels are negatively correlated with a higher prevalence of osteoporosis [148].

Studies of legumain in bone remodelling are relatively limited. Legumain has been found in the OB secretome and identified as a potential regulator of OB differentiation [149]. Accordingly, our research group has reported inhibition of OB differentiation by legumain through degradation of FN, as well as increased levels of legumain in the bone microenvironment of osteoporotic patients [47]. Legumain has been identified in the OC secretome as well [150]. Similarly, the C-terminal fragment (17 kDa) of legumain has been reported as the osteoclast inhibitory factor 2 (OIP-2) *in vitro* [151] and legumain was recently shown to inhibit osteoclastogenesis, partially through modulation of cathepsin L activity [152]. Furthermore, legumain has been reported in exosomes secreted from M2 macrophages and pancreatic cancer cells leading to ECM degradation [153, 154]. Legumain was identified in a proteomic characterization of extracellular vesicles from OCs but presence of legumain in exosomes from OBs has not been studied [155]. Hence, the involvement of legumain in bone biology needs to be further studied.

Drugs affecting bone homeostasis

Osteoporosis is a condition with reduced bone mass and change in bone microstructure which leads to reduced strength and increased risk of fracture. Typical osteoporotic fractures are a major health care problem and an economical burden [1]. Studies have shown that 50% of women and 25 % of men over the age of 50 years will suffer an osteoporotic fracture during their lifetime and Scandinavia has the highest reported incidence of osteoporotic fractures worldwide [156, 157]. Usually, Ca^{2+} and vitamin D_3 supplements are used as a prophylactic treatment of osteoporosis, but also as a part of combination therapy. Osteoporotic therapy mostly consists of drugs that inhibit bone resorption, i.e. bisphosphonates, raloxifene, oestrogen, tibolone, and denosumab. Testosterone and parathyroid hormone (PTH) are the only approved bone anabolic drugs. However, testosterone is indicated in men with osteoporosis caused by hypogonadism and PTH treatment is limited to 2 years, thus establishing a need for development of novel anabolic therapies [158]. In practice, treatment with bisphosphonates (alendronate, etidronate, ibandronate, risedronate and zoledronate) predominates in combination with Ca^{2+} and vitamin D_3 supplements. Osteoporosis can develop as a side effect of other drugs, for example systemic use of glucocorticoids [159]. Lately, another drug class has been associated with increased risk of osteoporosis and fractures, namely the proton pump inhibitors (PPIs) [160]. Since proteolytic enzymes are important for degradation of bone matrix and proteases might also act as mediators between bone cells, it would be interesting to study how legumain is regulated by drugs or hormones with known or suspected effects on bone homeostasis.

Proton pump inhibitors (PPIs)

Proton pump inhibitors (PPIs) are potent drugs used to reduce gastric acid secretion by inhibiting the parietal proton pump (H^+K^+-ATPase). PPIs are among the most widely used medications worldwide due to their efficiency in the treatment of acid peptic disorders [161]. Omeprazole was the first PPI introduced to the market in 1989, followed by pantoprazole, lansoprazole, rabeprazole, esomeprazole and dexlansoprazole. All PPIs share the same core structures including benzimidazole and pyridine. PPIs are taken orally, absorbed by the intestines and, because of their lipophilic nature, easily move across cell membranes [162]. Since PPIs are weak bases with a pKa of ~4.0 (omeprazole, lansoprazole and pantoprazole) or 5.0 (rabeprazole), it is predictable that PPIs accumulate in the acidic space of the secretory

canaliculus of the stimulated parietal cell [163]. Mean plasma concentration for therapeutic oral dosing of lansoprazole is 4.8 μM [164] whereas tissue concentration might reach millimolar levels due to accumulation [165]. PPIs achieve irreversible inhibition of the H^+K^+-ATPase through covalent binding to the thiol groups in the active site (Fig. 6).

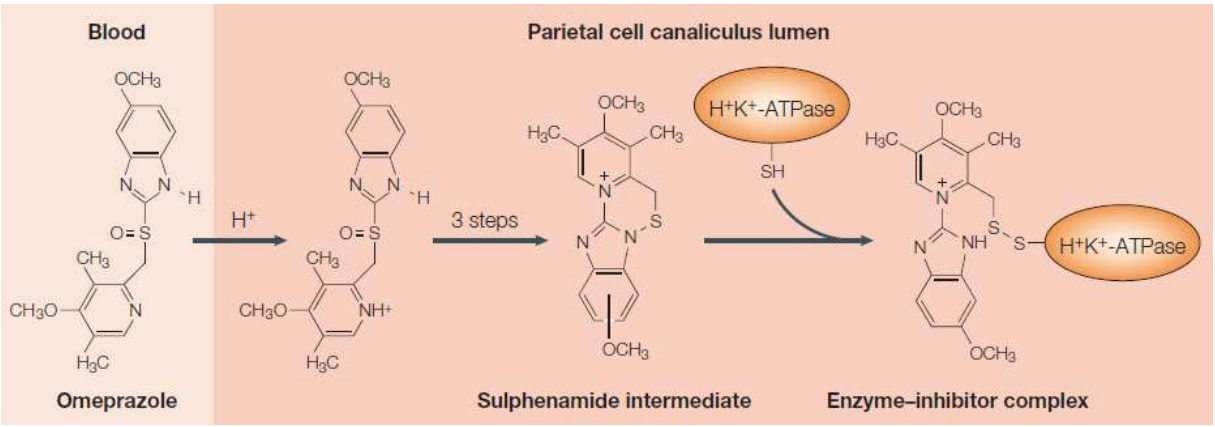

Figure 6. Mechanism of proton pump inhibition by omeprazole. Omeprazole is a prodrug that is transformed into its active forms in acidic environments. Omeprazole accumulates in the acidic secretory canaliculi of the gastric parietal cell, where it is transformed by protons into the active –sulphonamide form. Sulphenamide inhibits the proton pump by covalently binding to sulfhydryl groups of cysteine residues (Cys^{819}) in the extracellular domain of the H^+K^+-ATPase and thus, inhibiting it. Figure adopted from [166] and the copyright permission from the journal is granted.

When used for a brief period, PPIs cause only mild side effects, but long-term exposure has been associated with osteoporosis and bone fractures [160, 167-171]. Accordingly, companies producing PPIs in the USA are required by the Food and Drug Administration (FDA) to label the drugs with a warning about possible hip, wrist and spine fractures when used in high doses or for an extended period of time (>1 year) [172]. The World Health Organization VigiBase database of suspected adverse reactions (AR) contains a long list of AR for PPIs regarding skeletal tissue disorders, i.e. osteoarthritis, osteoporosis, bone pain, osteonecrosis, fractures, increased blood alkaline phosphatase (ALP) and decreased blood Ca^{2+} levels [173]. Increased serum ALP is associated with metabolic bone diseases and is used in diagnosis of rickets and osteomalacia [174, 175].

Statistics on PPI usage in Norway shows that the number of users has doubled since 2008 (Fig. 7A). Prevalence of usage is highest for pantoprazole, followed by esomeprazole,

omeprazole and lansoprazole. On average, each patient received 220 daily doses (DDDs) per year, which indicates chronic use and could lead to adverse effects (data extracted from the Norwegian Prescription Database (http://www.norpd.no/) at the Norwegian Institute of Public Health). The prevalence of PPI use increases with age (Fig. 7B). For all age groups, the proportion of women using PPIs is slightly higher than in men, except for the youngest and oldest age group (Fig. 7B).

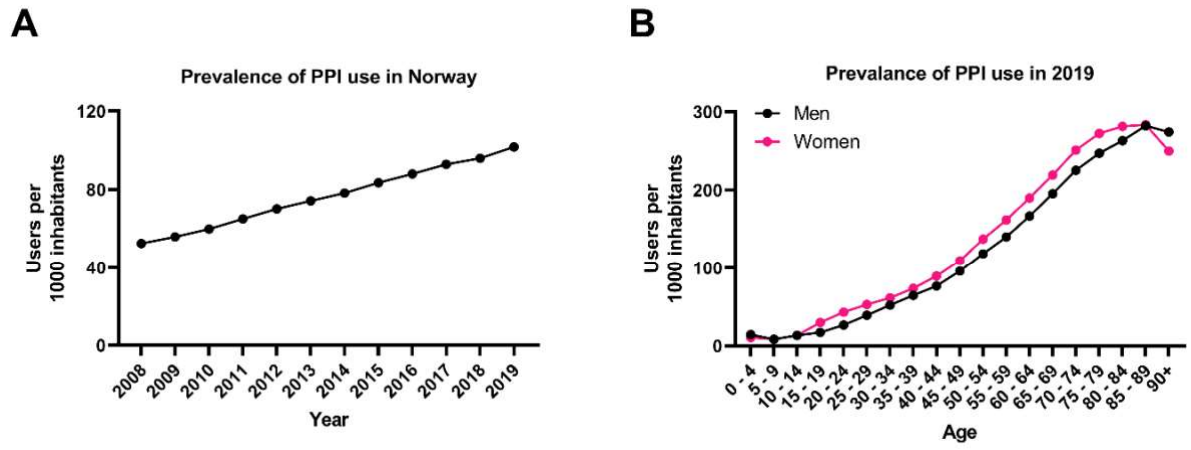

Figure 7. The use of proton pump inhibitors (PPIs) in Norway. (A) Prevalence of PPIs (ATC group A02BC) used during the period from 2008 to 2019. (B) One-year prevalence (per 1000) of PPIs used by age groups and according to sex in 2019. Data are presented as users per 1000 inhabitants in each age group. Data collected from the Norwegian Prescription Database (http://www.norpd.no/).

Low pH in the stomach (pH<4) serves to accumulate and activate PPIs locally. The concentration of PPIs at the site of the proton pump is about 1000 times higher than in the blood [176]. Proton pumps are not found only in the stomach, they are also present in the lysosome membrane of almost every cell. Furthermore, proton pumps are found on the cell membranes of tumour cells and OCs, as well as in cells of the renal collecting ducts. Although proton pumps present in the above mentioned locations are not identical to the parietal H^+K^+ ATP-ases, they are similar in function and termed vacuolar H^+-ATP-ases (V-ATP-ases) [177]. PPIs can bind to thiol-containing peptides even at a moderately acidic pH (pH 5.0) [178], suggesting that PPIs could also target proteins present in less acidic environments. Accordingly, concerns have been raised regarding possible off-target effects of PPIs in other acidic compartments besides the stomach. Considering the mechanism of action, PPIs could directly inhibit cysteine proteases

by binding to the thiol group of the cysteine in the active site. Another possibility is an accumulation of PPIs in lysosomes (pH 3.5), subsequent activation and lysosomal proton pump inhibition leading to higher lysosomal pH and subsequent destabilization and inactivation of cysteine proteases.

Vitamin D_3

Vitamin D (VD) is a steroid hormone produced in the skin from 7-dehydrocholesterol by ultraviolet (UV) radiation, forming pre-D_3 [179]. Pre-D_3 is transported from the skin by binding to VDBP in the circulation. Additional sources of VD are food and supplements where VD can be in the form of D_2 or D_3, which differ in their side chains impacting both their affinity for VDBP and subsequent metabolism. The liver metabolizes pre-D_3 to 25OHD_3 by CYP27A1 (mitochondrial) and CYP2R1 (microsomal) enzymes. The main circulating form of VD_3 is 25OHD_3. Kidney PTCs metabolize 25OHD_3 to 1,25$(OH)_2D_3$ by the enzyme CYP27B1, but other cells such as keratinocytes, cells of the parathyroid gland, intestinal epithelial cells, macrophages, and various bone cells and chondrocytes also contain this enzyme [180]. The main active form of VD_3 is 1,25$(OH)_2D_3$. Production of 1,25$(OH)_2D_3$ is tightly controlled, both in the kidney and in other tissues. PTH stimulates 1,25$(OH)_2D_3$ production, while Ca^{2+}, phosphate, and FGF23 inhibit 1,25$(OH)_2D_3$ production in the kidneys. Extrarenal production of 1,25$(OH)_2D_3$ is stimulated predominantly by cytokines such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-1β (IL-1β) [180, 181]. As a negative feedback mechanism to avoid VD_3 toxicity, 1,25$(OH)_2D_3$ regulates itself by decreasing production or stimulating degradation through the induction of CYP24A1, a 24-hydroxylase [182]. Hydroxylation of 25OHD_3 and 1,25$(OH)_2D_3$ by this enzyme forms the inactive metabolites 24,25$(OH)_2D_3$ and 1,24,25$(OH)_3D_3$, respectively. The VD_3 metabolites are transported in the blood bound to either VDBP or albumin, while very small amounts circulate unbound [183].

Both 25OHD_3 and 1,25$(OH)_2D_3$ bind to the nuclear vitamin D receptor (VDR), although 25OHD_3 has 50-600-fold lower affinity than 1,25$(OH)_2D_3$ [184]. VDR belongs to a large family of nuclear hormone receptors including the receptors for glucocorticoids, mineralocorticoids, sex hormones, thyroid hormone, and vitamin A metabolites. VDR is a transcription factor that mediates the biological activities of VD_3 through regulation of target genes [185, 186]. In addition to regulating gene expression, a number of non-genomic effects have been described for 1,25$(OH)_2D_3$, including the immediate stimulation of Ca^{2+} transport across the plasma

membrane thus regulating systemic Ca^{2+} homeostasis [187]. The central role of VD_3 in systemic Ca^{2+} homeostasis indirectly affects bones by stimulating bone resorption during hypocalcaemia and bone formation during hypercalcemia.

Rickets is caused by VD_3 deficiency, VDR mutations (hereditary vitamin D resistant rickets), or deficient production of $1,25(OH)_2D_3$ due to CYP27B1 mutations (pseudo-vitamin D deficiency) [175, 188, 189]. In addition, VD_3 deficiency leads to hypocalcaemia [190], hypophosphatemia [191], and hyperparathyroidism [192], resulting in weaker bones. VDR and CYP27B1 are found in all cell types of the skeleton such as chondrocytes, OB, osteocytes and OC [181, 193-201], corroborating that $1,25(OH)_2D_3$ and $25OHD_3$ are of critical importance in bone physiology. Therefore, VD_3 is a standard regime in osteoporosis treatment and is recommended for daily use in the months of low sun exposure. Supplementation with at least 700 IU of VD_3 is necessary to improve physical condition, bone density and prevention of fractures [202].

Being a major regulator of gene expression and therefore cellular protein production, VD_3 is important for normal cell function. VD_3 regulates several cysteine proteases and inhibitors in different cell types thereby maintaining cellular homeostasis. The active metabolite, $1,25(OH)_2D_3$, was shown to induce the expression of cystatin A in keratinocytes, cystatin D in colon cancer and cystatin E/M in squamous cell carcinoma [203]. Induction of cystatin D and E/M in cancer cells is thought to partially contribute to the VD_3 antitumor effect. In 1996, VD_3 was first reported to induce cathepsin B activity in breast cancer cells and as a result contributing to cancer cell apoptosis [203]. On the other hand, VD_3 has been shown to inhibit cathepsin L in breast cancer cells thus lowering cancer malignancy [203]. Single previously known connection between VD_3 and legumain was VDBP cleavage by legumain [45]. Regulation of cysteine proteases by VD_3 was not previously assessed in bone cells. Therefore, studying the relationship between legumain and VD_3 in OBs was of particular interest.

AIMS OF THE THESIS

The overall aim of this thesis is to elucidate new molecular mechanisms for legumain regulation and function, especially in bone-forming cells (osteoblasts).

Specific objectives are as follows:

- To summarize the presence and importance of extracellular legumain (**paper I**)
- To investigate and elucidate whether the PPI lansoprazole affects cysteine protease activities (**paper II**)
- To investigate a possible interplay between vitamin D_3 and legumain during osteoblast differentiation (**paper III**)
- To characterize degradation of the bone extracellular matrix protein fibronectin by legumain (**paper IV**)
- To investigate the presence of legumain and fibronectin in exosomes from bone cells (**paper IV**)

SUMMARY OF THE PAPERS

Paper I: ***Mammalian legumain – a lysosomal cysteine protease with extracellular functions?***

This review gives an overview of secreted and extracellular legumain from various normal cells and during diseases. Legumain is ubiquitously expressed and has mainly been considered to be present and functional in acidic lysosomes. Various observations over the last years have shown that legumain is extensively secreted from various cells and tissues, and an increasing number of reports show that legumain has extracellular roles as well. Herein, we present the current status on extracellular presence and functions of legumain and address new findings in relation to specific pathologies.

Paper II: ***Lansoprazole inhibits the cysteine protease legumain by binding to the active site***

Proton pump inhibitors (PPIs) are prodrugs used in the therapy of gastrointestinal diseases. Activated by acidic pH, PPIs inhibit the secretion of gastric acid by forming covalent disulphide bonds with the SH-groups of the parietal proton pump (H^+/K^+-ATPase). While short-term use of PPIs has mild side effects, chronic use has been associated with numerous adverse effects, including bone fractures. Considering the mechanism of prodrug activation, PPIs could also be active in acidic micro-environments such as in lysosomes, tumour microenvironments and bone resorption sites. We show both a pH- and dose-dependent inhibition of the cysteine proteases legumain and cathepsin B by lansoprazole. Furthermore, lansoprazole blocked binding of the legumain-selective activity-based probe MP-L01 indicating a direct interaction of lansoprazole with the SH-group in the enzyme active site. Lansoprazole was also shown to inhibit legumain and cathepsin B in various cell line models like HEK293 cells, monoclonal legumain over-expressing HEK293 cells (M38L) and RAW264.7 macrophages, but not in human bone marrow-derived skeletal (mesenchymal) stem cells (hBMSC). During osteoblast differentiation from hBMSC cells, lansoprazole inhibited legumain secretion, alkaline phosphatase activity, but had no effects on *in vitro* mineralization. In conclusion, lansoprazole achieves a direct covalent inhibition of legumain and cathepsin B by forming disulphide bonds with the SH-group in the protease active site. Such inhibition of cysteine proteases could explain some of the off-target effects of PPIs.

Paper III: ***Regulatory effects of vitamin D_3 on the cysteine protease legumain: Relevance to bone biology***

Vitamin D_3 (VD_3) has a significant role in the regulation of bone homeostasis. We have previously reported that legumain has an inhibiting effect on the differentiation of human bone marrow skeletal stem cells (hBMSC) to osteoblasts (OB). In this study, we have showed that both $1,25(OH)_2D_3$ and $25OHD_3$ significantly increase legumain expression, secretion and activity in the first 7 days of OB differentiation. Additionally, we reveal a legumain-specific vitamin D-binding protein (VDBP) cleavage product of approximately 45 kDa. Also, legumain deficiency *in vivo* lead to an enhanced expression of the VD_3 receptor (VDR). On the other hand, legumain overexpression *in vitro* downregulated VDR expression, thus there is a reciprocal regulation of legumain and VD_3. Serum legumain and VDBP were measured in an osteoporosis prevention trial of a large cohort of elderly patients receiving high (3,750 IU) or low (600 IU) dose vitamin D_3. Baseline legumain was negatively correlated with 12 months serum levels of $25OHD_3$ in both treatment groups, whereas there was no correlation between legumain and VDBP levels.

Paper IV: ***Legumain enhances fibronectin production and both proteins are present in bone cell exosomes***

Fibronectin is an important component of the extracellular matrix in bones and critical for OB maturation, survival and matrix mineralization. In this study we have extended previous reports of legumain degradation of FN by showing that FN can be internalized by cells and degraded intracellularly giving a legumain-specific cleavage product of approximately 100 kDa. Inhibition of legumain by the legumain-selective probe MP-L01 or cystatin E/M reduced fibronectin degradation. Culturing of hBMSC with TGF-β1 induced the expression of both FN and the mature form of legumain. Surprisingly, we also observed a significant increase in FN expression after culturing and internalization of prolegumain, which was processed to mature legumain. Also, expression of FN was almost completely abolished in kidney homogenates from legumain deficient mice or kidneys from mice treated with MP-L01. Both FN and prolegumain were present in exosomes from early osteoblasts and osteoclasts, whereas mature legumain only was present in the osteoclasts.

DISCUSSION

Methodological considerations

Cellular models

In this work, different *in vitro* cell line models have been used, i.e. both commercially available or gene manipulated. There are several advantages in using cell lines compared to primary cells. Cell lines have lower cost, are easier to work with and can be cultured for longer periods of time. Additionally, experiments with cell lines are more reproducible, although effects observed in cell lines are difficult to extrapolate *in vivo*. On the other side, the ability to be expanded and grown in many passages could lead to changes in genotype and phenotype of cells. Primary human cells more closely represent *in vivo* conditions and a limited culture period ensures genome and phenotype stability. However, primary cells have limited accessibility, long isolation procedure and heterogeneous phenotype sensitive to donor-related factors. Also, primary cells are more sensitive to handling and require special culture media (including growth additives and serum restrictions). Also, ethical regulations when it comes to the use of human (or animal) primary cells need to be considered. Primary mouse/rat/fish cells are also used in research, with a possibility to control the selection of donor animals, but disadvantages are genomic and interspecies differences when extrapolating data to human biology.

Legumain or cystatin E/M overexpressing cell lines have previously been established in our laboratory [41]. M38L cells are monoclonal human embryonic kidney (HEK) 293 cells stably transfected with human legumain cDNA (*LGMN*), whereas M4C cells are monoclonal HEK293 cells stably transfected with human cystatin E/M cDNA [41]. In addition, commercially available HEK293 and RAW264.7 (murine macrophages) cell lines from American Type Culture Collection (ATCC) were used. Also, primary human bone marrow stromal cells (hBMSC) stably transfected with the cDNA for the catalytic subunit of human telomerase (TERT) were used [204]. Human BMSC are described in more detail below.

Human bone marrow derived stromal (mesenchymal) stem cells

Human bone marrow is composed of hematopoietic tissue and the surrounding stroma. In the stroma, a specific cell type known as bone marrow-derived stromal (mesenchymal) stem cells (BMSC) is present [205]. Human BMSC are widely used in research and are one of the best

characterized stem cells. Due to their immunomodulatory and self-renewal properties, hBMSC are used in clinical treatments as well, including tissue-engineering, regenerative medicine and autoimmune diseases [206, 207]. Human BMSC are multipotent non-hematopoietic stem cells capable of differentiation into mesodermal lineages such as osteoblasts (OBs), chondrocytes and adipocytes (ADCs) [208]. Also, the ability of hBMSC to differentiate to myocytes, hepatocytes, cardiogenic and neuronal cells has been reported [209, 210]. The cell phenotype is determined by various methods. For example, OB studied in this thesis are recognized by upregulation of alkaline phosphatase (ALP) activity and mineral deposition.

The disadvantage of primary hBMSC culturing is a limited expansion *in vitro* and impaired proliferation rate with growth arrest after around 24-40 population doublings (PD) depending on the donors age [211, 212]. The cellular senescence might be caused by factors such as DNA damage, protein accumulation, mitochondrial changes and progressive telomere shortening [213-215]. Human BMSC lack telomerase activity as a result of absence of expression of the telomerase reverse transcriptase (TERT) gene leading to telomere shortening [216]. In an ongoing collaboration with professor Moustapha Kassem and associate professor Abbas Jafari at Odense University Hospital, and Danish Stem Cell Center (DanStem), Copenhagen, Denmark, we have received primary hBMSC stably transfected with the catalytic subunit of TERT (hBMSC-TERT) [204]. Human BMSC-TERT cultures were established from a healthy male donor (age 33) [204]. Overexpression of TERT led to extension of the life span of hBMSC to approximately 250 PD while maintaining stem cell characteristics such as full potential for differentiation and preserved genetic and epigenetic profiles [217].

In vitro OB differentiation occurs over a three to four week period [218]. The final stage of OB differentiation begins after two weeks of culturing and is marked by mineral deposition [219]. In **paper II and III,** hBMSC-TERT were differentiated towards OBs for up to 21 days and mineral deposition could not be detected before 14 days of OB differentiation. In the literature, there are several methods for differentiating hBMSC to OBs. In this thesis, OB differentiation was performed using OB induction media consisting of MEM, ascorbic acid (50 μg/ml), dexamethasone (10 nM), vitamin D_3 (10 nM) and a phosphatase substrate (β-glycerol phosphate, 10 mM). The culture medium was changed every 3-4 days and the cells were harvested on day 3, 7, 14 and 21 of differentiation. There are several studies that do not use vitamin D_3 during OB differentiation, most likely because vitamin D_3 has been shown to downregulate OB proliferation [220]. However, vitamin D_3 has also been reported to enhance OB maturation and mineralization [220-222]. Furthermore, verification of OB phenotype

resulting from the employed differentiation protocol has been done previously using mRNA expression of OB markers like runt-related transcription factor 2 (RUNX2), ALP, osteocalcin and osteopontin, along with ALP activity and mineralization level assessment [223]. Early OBs have previously been shown to express and secrete legumain, whereas complete differentiation to mature OBs resulted in substantial downregulation of cellular legumain expression and secretion to the conditioned medium [149].

Osteoblast differentiation markers

Deciphering OB biology may help treating diseases such as osteoporosis or bone metastases, therefore studies on differentiation of hBMSC to OBs is extensively performed in research laboratories. As mentioned, hBMSC can differentiate into several cell lineages and the key molecular switch for OB differentiation is the RUNX2. A few endocrine factors fine-tune this process, i.e. vitamin D_3 [224]. Once RUNX2 is activated, the cells are considered as preosteoblasts and undergo OB differentiation characterized by expression of different markers (Fig. 8). Interestingly, *in silico* analysis using the Eukaryotic Promoter Database (EPD; [225]) revealed the presence of nine potential RUNX2 binding sites in the legumain gene promoter at the following positions relative to the transcriptional start site: nucleotide -225, -906, 87, 97, 144, 361, 456, 736, and 863, respectively ($p = 0.001$; data not published).

In this thesis, ALP activity and mineralization were used as markers of OB differentiation. ALP is a tissue-specific metallo-isoenzyme and four ALP isoenzymes are present in human intestine, placenta, mammary glands and bones/liver/kidneys, respectively [226]. Bone-specific ALP is synthesized by and bound to the cell membrane of OB via glycosylphosphatidylinositol (GPI) anchors [227]. There are multiple reasons why ALP activity is used as a marker for OB. Firstly, nearly 100 years ago (1923), ALP was for the first time associated with the bone mineralization process [228] and recent proteomic studies have shown that in bone extracellular vesicles (EV), ALP is the most abundant enzyme [229].

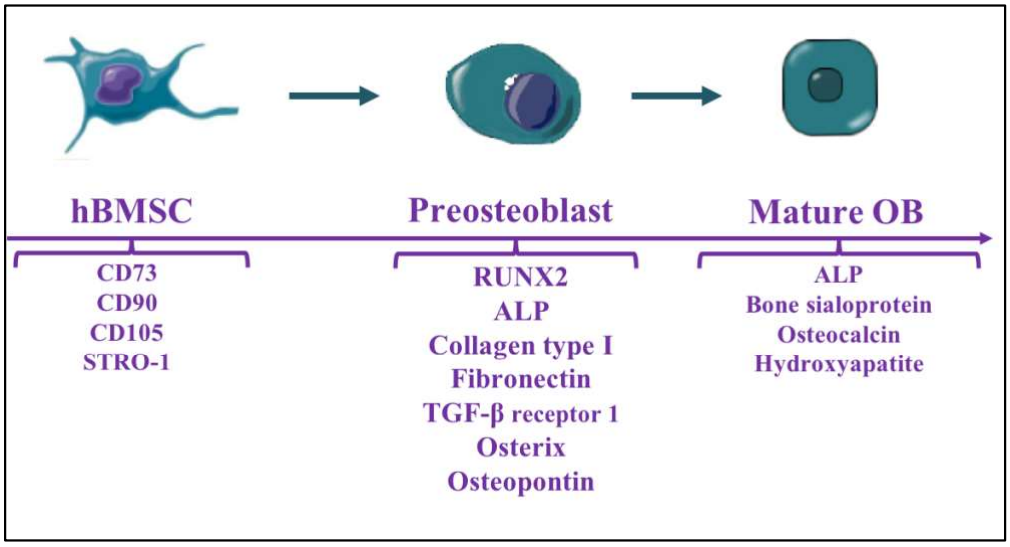

Figure 8. The process of osteoblast maturation and the stage-dependent differentiation markers. Human bone marrow-derived stromal (mesenchymal) stem cells (hBMSC) express CD73, CD90, CD105 and STRO-1 on the cell surface. Once runt-related transcription factor 2 (RUNX2) is activated, hBMSC are defined as preosteoblasts and the expression of alkaline phosphatase (ALP) and collagen type I is upregulated. As preosteoblasts continue to proliferate and differentiate, expression of fibronectin, TGF-β receptor 1, osterix and osteopontin increases. Mature osteoblasts (OB) have a distinctive cuboidal shape and upregulated expression of bone sialoprotein and osteocalcin, which promotes deposition of mineral salts (hydroxyapatite). The figure is drawn by the author.

Also, ALP is concentrated on the EV membrane budding from the OB and is involved in the ECM mineralization process i.e. deposition of hydroxyapatite [227]. Mutations in the ALP gene lead to a genetic disease known as hypophosphatasia which is characterized by defect in bone mineralization [230]. ALP activity increases in early OB and continues to rise until OB mature ([219, 231] **paper II** and **III)**. Lastly, monitoring of ALP activity levels is a reliable and convenient method and therefore there are countless studies using ALP as a marker of OB differentiation. Different methods of measuring ALP activity exist, from which our laboratory has used p-nitrophenyl phosphate (pNPP) as a colorimetric substrate. Hydrolysis of pNPP by ALP is followed by formation of a yellow product of p-nitrophenol (pNP), which can be measured by absorbance at 405 nm. The advantages of this method are speed, low cost, easily optimized for automation, and minimal instrumentation. Also, pNP is stable, provides high sensitivity and exhibits linear relationship between absorbance and concentration [232]. On the other hand, colorimetric methods are not as sensitive as fluorescence-based assays; however,

for the evaluation of *in vitro* OB differentiation, detection of very low pNP concentrations is not necessary [232].

Calcium-chelating compounds such as Alizarin Red have been used effectively for the detection of bone mineralization [233]. However, this method includes fixation of cells, thus preventing additional measurements. In this thesis, the chelating-agent BoneTag™ was used for measuring mineralization during OB differentiation. The advantage of BoneTag™ is a direct detection in living cells without the need of fixation. BoneTag™ is a tetracycline derivate conjugated to a near-infrared (NIR) dye (IRDye 800CW), thus extending fluorescence signal detection to the NIR region of the spectrum (800 nm). Another advantage of BoneTag™ is that detection of fluorescence in the NIR region improves depth of penetration due to low auto-fluorescence, translating to low background interference. BoneTag™ efficacy in measuring mineralization had previously been examined in MC3T3 cells (osteoblasts) in a cell-based assay where MC3T3 cells showed increase in fluorescence when incubated with BoneTag™ [234]. Incubation of BoneTag™ with A431 (epidermoid carcinoma) cells, showed low non-specific binding [234]. BoneTag™ was used to analyse OB differentiation in **paper II** and **III** and can also be used to visualize bone anatomy and structures *in vivo* [234, 235].

Methods used to detect and quantify proteins

In this thesis both non-specific and specific methods have been used to detect proteins. The amount of total proteins was quantified by the Bradford assay [236], while the exact amount or forms of a single protein was determined by enzyme-linked immunosorbent assay (ELISA) or immunoblotting (IB). IB and ELISA were used in **paper II-IV**. One central common factor for the above-mentioned methods is the use of antibodies. In the present work, well characterized commercial antibodies for legumain, cathepsin B, K and L, cystatin E/M and C, fibronectin (FN), VDBP and VDR **(paper II-IV**) were used [30, 35, 41, 43, 97].

In IB analyses, a legumain polyclonal antibody (pAb, AF2199, R&D Systems) detected both the proform (56 kDa), the intermediate (46/47 kDa) and mature (36 kDa) forms (**paper II-IV**). Additionally, the antibody detected a newly characterized active legumain form (28 kDa) ([43], **paper III-IV**). The legumain pAb AF2199 was chosen because it is well characterized, thoroughly assessed and validated to be specific and sensitive towards legumain [33, 43, 237]. Although AF2199 is primarily reactive towards human legumain, we have shown that the

antibody also detects mouse legumain (**paper III and IV**). Additionally, pAb are more cost-effective than monoclonal antibodies (mAb). There are several methods to consider when detecting binding of primary antibodies on immunoblots. A general principle is the use of a secondary antibody conjugated to e.g. horseradish peroxidase or fluorophores followed by colorimetric, chemiluminescent or fluorescence detection. We have utilized near-infrared fluorescence detection (700/800 nm) because of several advantages compared to other methods. For fluorescence detection, no further chemical reactions are necessary, and blots are visualized after binding of the secondary antibody to the primary antibody and a washing procedure [238]. Another key advantage is the option to utilize primary antibodies from different species and different wavelengths for secondary antibodies, thus allowing probing of multiple targets simultaneously.

For exosome isolation, conditioned culture medium is ideally obtained in the absence of foetal calf serum (FBS), to avoid co-isolation of exogenous exosomes. However, serum depletion can affect cells negatively. In this study, we have cultured cells for the last 48 hours in 2% exosome-depleted FBS and visual inspection showed no changes in the cellular morphology. Although we have used exosome-depleted FBS, non-conditioned culture medium was used as a negative control. The presence of exosomes from bone cells was demonstrated by morphological and molecular characterization. The morphological characterization was performed by Nanoparticle Tracking Analysis (NTA) and molecular characterization by IB. The composition of the exosomal bilayer membrane varies depending on the parent cell [135, 136, 239]. Generally, exosomes contain protein members of the transmembrane 4 superfamily (CD9, CD63 and CD81) [239]. Herein, an antibody against the transmembrane protein CD81 was used as an exosome marker but was only recognized in OCs (**paper IV**). CD9 antibody was tested as well but was only detected in exosomes isolated from a colorectal adenocarcinoma cell line (SW480, ATCC® CCL-228™, which was used as a positive control for CD9 (data not shown). To identify OB exosomes, flotillin-1 or -2 could be used as markers, as previously shown [136, 240]. However, since exosomes from the cell sources investigated herein (hBMSC-TERT, OBs, OCs and RAW264.7) were isolated simultaneously and OC exosomes were verified, it is tempting to speculate that the exosome isolation method also isolated OB exosomes. Nevertheless, the exosomes are not sufficiently characterized using only NTA and IB, according to the guidelines from the International Society for Extracellular Vesicles (ISEV) [241]. Additional analysis comprised of scanning or transmission electron microscopy and high-resolution flow cytometry for vesicle verification would be beneficial [241-243].

ELISA was described for the first time in 1971 [244] and in recent years has gained status as the standard quantitative method for detecting proteins in body fluids or *in vitro* cell cultures. A commercially available human legumain ELISA kit (DY4769, R&D Systems) containing a legumain mAb for capture and a biotinylated legumain pAb (AF2199) for detection. The kit was used to measure total legumain (i.e. pro- and mature forms) concentrations in serum obtained from patients **(paper III)** and in conditioned media from *in vitro* cell cultures **(paper II-IV)**. This ELISA kit is previously characterized for both inter- and intra-variable legumain detection [33, 237].

Statistical perspectives

Statistical tests are used to analyse quantitative data generated in a research study. There are several aspects to consider when choosing a statistical test. For example, number of samples and data distribution has to be taken into the consideration. Predominantly used in this thesis are Mann-Whitney and Kruskal-Wallis tests. Majority of data generated in **papers II-IV** were unpaired and nonparametric and the data did not follow Gaussian distribution. The Mann-Whitney test was chosen when comparing nonparametric distributions of two unpaired groups [245]. For comparison of three or more groups, Kruskal-Wallis test was used. By selecting these nonparametric tests, we have avoided the assumption that our data were sampled from a Gaussian distribution. Furthermore, review of the literature revealed that studies with similar type of data characteristics have used the same type of tests [198]. However, nonparametric tests have less power and parametric tests should be chosen when applicable. Another aspect to consider is a post hoc test following Kruskal-Wallis analysis. In this thesis, multiple comparison tests were used post hoc, thus allowing for identification of groups differing from the control.

General discussion

Dysregulation of protease activity leads to conditions such as cancer, atherosclerosis, osteoporosis and neurodegenerative diseases. Of ~ 500 currently known drug targets, around 200 are enzymes and over 60 % of these enzymes are proteases [246]. There are many successful examples of proteases as drug targets, such as inhibitors of angiotensin-converting enzyme, human immunodeficiency virus protease, dipeptidyl peptidase 4, thrombin and factor Xa [247-249]. However, it is hard to predict the effects of systemic inhibition of a particular protease since most proteases are ubiquitously expressed, regulate multiple functions and have numerous substrates. To predict the possible effects of inhibiting a protease requires detailed knowledge of the functions of the enzyme both in health and disease. Faced with an incomplete picture of *in vivo* functions we are left with the task of generating hypotheses about outcome when proteases are inhibited by drugs. The development of MMP inhibitors to treat cancer serve as an example of how insufficient knowledge about *in vivo* functions of proteases resulted in negative outcomes [250]. Another example is the inhibition of cathepsin K in the treatment of osteoporosis which seemed very promising in preclinical studies showing reduction of bone resorption and maintained bone formation [251-253]. However, the cathepsin K inhibitor odanacatib was cancelled in phase III clinical trials due to increased risk of cardiovascular events, especially stroke [254].

In the rest of the discussion I will explore how new knowledge generated in this thesis could contribute to existing information in order to better understand the consequences of pharmacological targeting legumain activity and/or expression.

Bone biology

Osteoporosis is characterized by reduced bone mass and abnormal microarchitecture, leading to bone fragility and risk of fractures [255]. Human BMSC mediate fracture healing by differentiating into OBs and are therefore utilized in regenerative medicine through cell transplantation. However, a major disadvantage of this approach is poor engraftment of hBMSC in damaged bone tissue thus providing only transitory effects [256]. Around 15% of fractures never heal, due to compromised functions of endogenous hBMSC followed by loss of OB efficacy [257, 258]. Non-healing fractures lead to additional surgical procedures and hospitalization, presenting a major burden on the patient's quality of life and the healthcare

system [257]. Usually, non-healing fractures are treated by bone graft implantation thus providing hBMSC and growth factors necessary to enhance bone regeneration [259]. However, bone grafting requires a second surgery to harvest autologous bone material and leads to donor-site morbidity, such as pain and discomfort [260]. As a result, novel treatment approaches are required to treat both healing and non-healing fractures.

An alternative approach to regenerative medicine is to stimulate OB differentiation of the *in vivo* resident hBMSC. Human BMSC secrete large numbers of bone tissue regeneration factors thus stimulating OB formation [261]. Targeting regulatory factors in the hBMSC secretome could improve OB differentiation leading to enhanced bone formation and bone regeneration. Therefore, local pharmacological targeting of proteases involved in bone remodelling could be beneficial. In this regard, targeting of legumain in the OB microenvironment might be advantageous for bone formation. Also, legumain is the only human protease known to cleave substrates at the amino acid asparagine, representing a non-redundant activity [2]. Apart from being present in the OB secretome and overexpressed in hBMSC from osteoporotic or obese patients [47, 149], legumain has been reported as a mediator enhancing hBMSC differentiation to ADCs [47]. Moreover, additional *in silico* analysis of the legumain gene promoter using EPD [225] revealed four binding sites for peroxisome proliferator-activated receptor gamma (PPAR-γ), a master switch for hBMSC differentiation towards ADCs (nucleotide: -837, -636, -474, 973; p= 0.001; unpublished) and hBMSC cultured in OB induction media containing prolegumain for 14 and 21 days showed accumulation of lipid droplets, demonstrated by Oil Red O-staining (Fig. 9). In fact, *in vitro* transdifferentiation studies from ADCs to OBs and vice versa is possible and have shown that the cells keep their plasticity [262]. Therefore, by inhibiting legumain, ADCs might be reprogrammed to OBs and thus avoid the negative effect of legumain on OB differentiation.

Involvement of legumain in the bone microenvironment is further elucidated in **paper IV** where legumain is detected in exosomes isolated from both OBs and OCs, primarily present as the proform. Legumain expression was upregulated by TGF-β1 (25 ng/ml) in early OBs and surprisingly, uptake and processing of prolegumain increased the expression of FN. In healthy subjects, levels of TGF-β1 have been reported to be approximately 15 ng/ml, whereas postmenopausal osteoporotic females had elevated TGF-β1 serum levels (23.8 ng/ml) [263] and enhanced legumain expression in hBMSC [47]. In light of these reports and the findings in **paper IV**, a possible explanation for high legumain expression in hBMSC of osteoporotic patients could be caused by increased TGF-β1 levels.

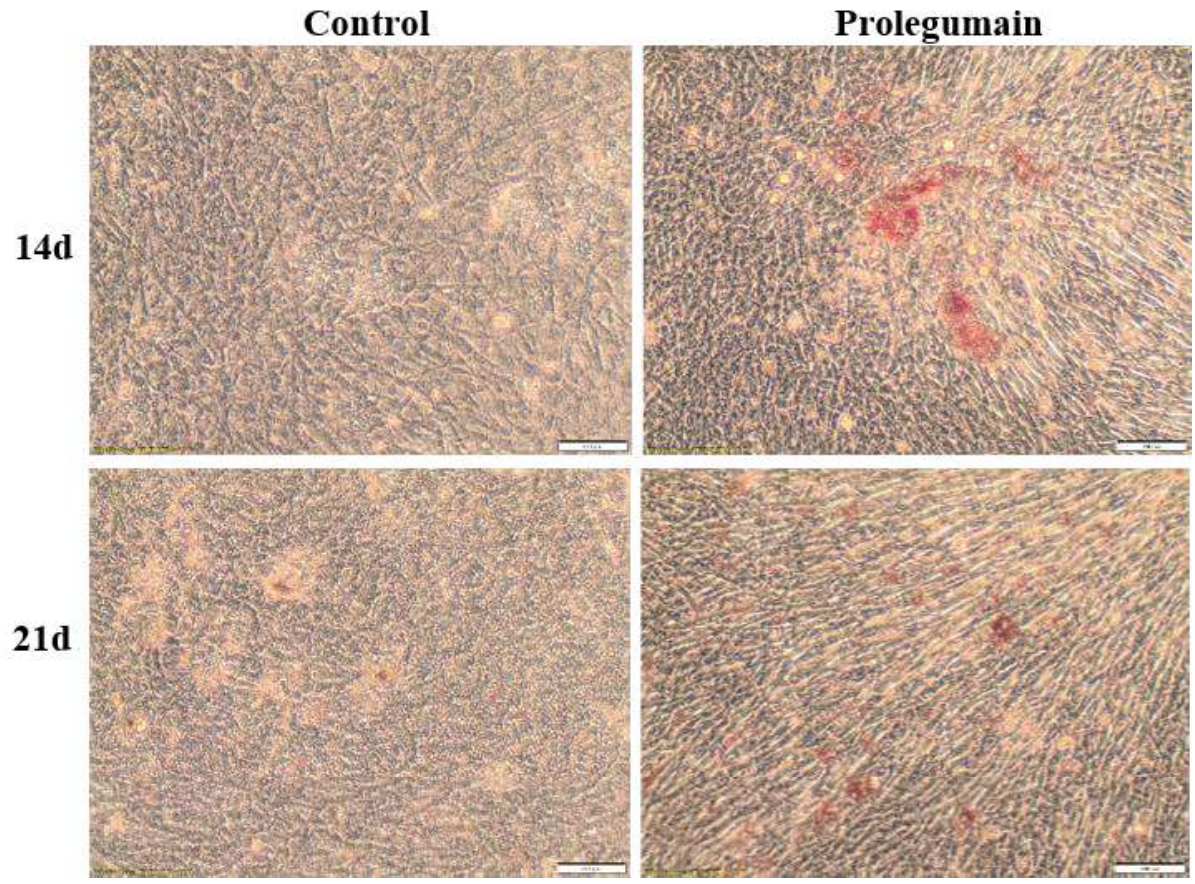

Figure 9. Legumain induces lipid droplet accumulation in osteoblasts. Human BMSC-TERT were cultured in osteoblast induction media with conditioned media (1:1) from HEK293 (control) or M38L (containing prolegumain) cells for 14 and 21 days. Light microscopy pictures from one representative experiment is shown (n=3). Cultured cells were stained with Oil Red O. 4x magnification.

In **paper III** we reported that both $1,25(OH)_2D_3$ and $25OHD_3$ significantly induce legumain protein expression, secretion and activity during the commitment phase (7 days) of OB differentiation, but not in the late phase. The mechanism behind the effect seen in **paper III**, might be explained by the fact the VD_3 downregulates RUNX2 [264]. Specifically, $1,25(OH)_2D_3$ (10 nM) has been reported to inhibit OB differentiation and induce OB to ADC transdifferentiation in the early stage of OB differentiation (6 days) [264]. This is in line with the results in **paper III**, where the effect of VD_3 was not seen beyond 7 days of differentiation. Additionally, the authors reported an upregulation of PPAR-γ at the same time [264]. Since the promotor for legumain has binding sites for both master regulators for OBs (RUNX2) and ADCs (PPAR-γ), it is plausible that this is the mechanism VD_3 employs when upregulating legumain (**paper III**). It is common knowledge that VD_3 is beneficial for bone strength [265], and our results seem paradoxical as legumain has negative effect on OB differentiation [47].

However, VD_3 is also known to regulate OB mineralization and differentiation through upregulation of another inhibitor of OB differentiation, namely activin A [266, 267]. Also, the mechanism of OB recruitment under remodelling of adult bone is still unclear. It is known that bone formation depends on a certain OB density on the bone surface [268]. To accomplish the necessary number of OB progenitor cells at the bone surface, a temporary raise in OB differentiation inhibitors might serve as a mechanism for increased OB recruitment, thus increasing cell number and postponing differentiation. This could be a logical explanation for the transient upregulation of legumain by VD_3 during the initial phase of OB differentiation but needs to be explored.

In **paper II** we report legumain inhibition by lansoprazole in OC progenitors (RAW 264.7 macrophages). Similar experiments in OCs showed that acute treatment with lansoprazole significantly downregulates legumain expression but did not affect cathepsin K expression (Fig. 10). In general, cathepsin K expression was higher in OCs than in unstimulated RAW264.7 macrophages (data not shown). Lansoprazole slightly downregulated legumain activity in OCs, with no effect on legumain secretion (data not shown). Legumain mRNA expression and secretion were shown to be downregulated in mature OCs, suggesting that the decrease in legumain enhances OC formation [150, 269]. Therefore, downregulation of legumain expression in OCs by lansoprazole could lead to enhanced OC formation and activity. Interestingly, treatment with lansoprazole influenced OC morphology. OCs stimulated with lansoprazole (10 μM) appeared to enhance the cell size and number of nuclei (Fig. 10), and large OCs have been reported to resorb 2.5 times more of apatite-collagen complexes than small OCs [270]. Likewise, it was previously reported that lansoprazole (< 2.5 μM) enhances OC maturation and bone resorption *in vitro* and induces an osteomalacia-like condition *in vivo* [271]. The authors examined both acute and chronic use of lansoprazole where rats were given the same dose of lansoprazole for 4 or 12 weeks, respectively [271]. Acute (4 weeks) lansoprazole administration lead to increased OC parameters, e.g. eroded surface and OC size. Chronic (12 weeks) administration decreased trabecular thickness and increased osteoid thickness resulting in osteomalacia-like condition [271].

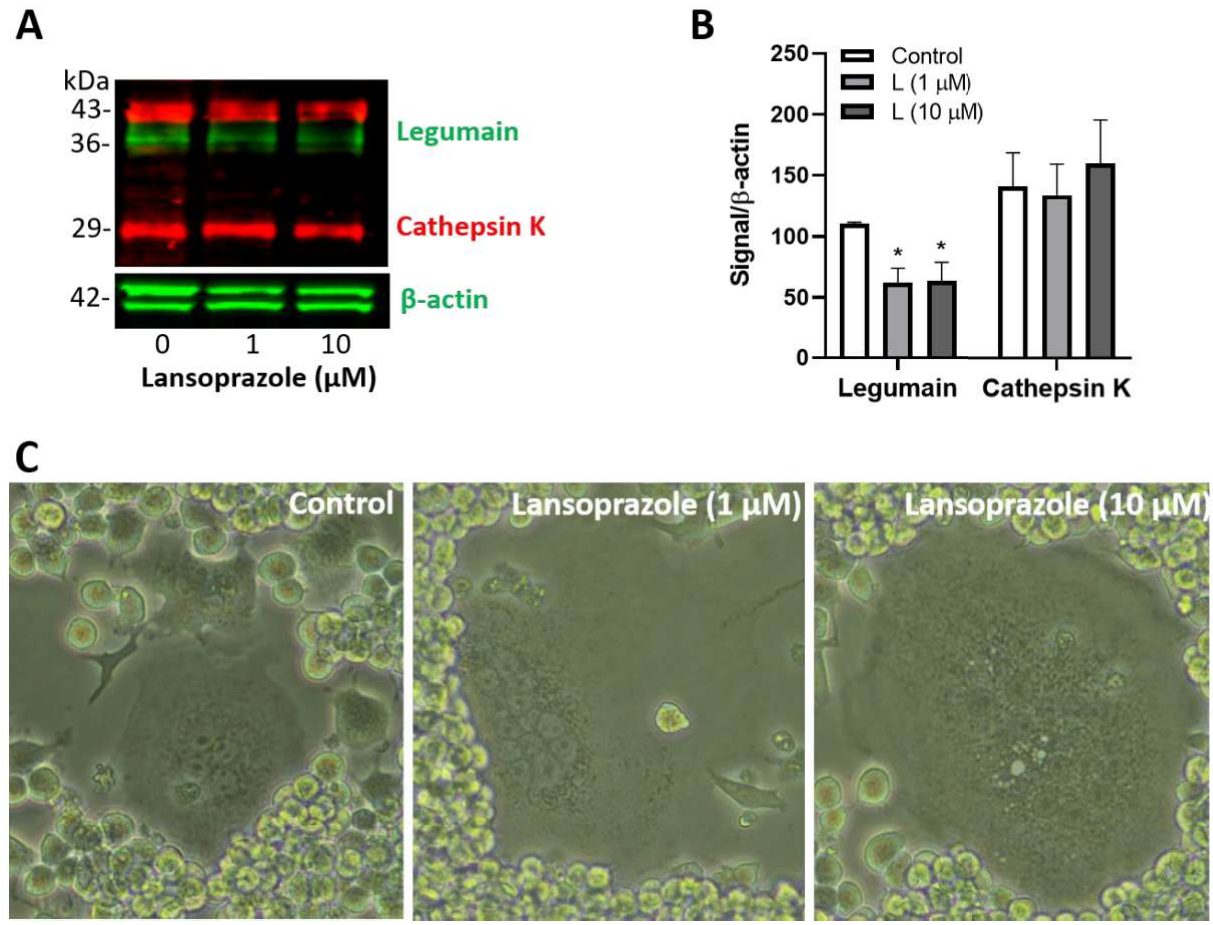

Figure 10. Lansoprazole downregulates legumain expression in osteoclasts and affects cellular morphology. RAW264.7 macrophages were induced towards osteoclast differentiation with RANKL (35 ng/ml) for 6 days, with or without lansoprazole (1 or 10 μM) for the last 48 h. **A.** One representative immunoblot of cell lysates is shown (n = 3). β-actin was used as housekeeping control. **B.** Quantification of immunoblot as shown in A for mature legumain (36 kDa) and cathepsin K (29 kDa). Analysed by Kruskal-Wallis test; *p<0.05 compared to control. **C.** Light microscopy pictures of osteoclast morphology after 6 days of differentiation from one representative experiment (n=3). 20x magnification. L, lansoprazole.

In contrast, in a rat model of femoral fracture, lansoprazole upregulated RUNX2 and thus stimulated OB maturation resulting in induced bone regeneration [271]. An important detail to consider is that the mechanisms of bone remodelling under normal physiological condition or during fracture healing are regulated differently since fractures trigger an inflammatory response. Inflammatory mediators, such as interleukin-1 (IL-1), IL-6, IL-11, IL-18 and tumour necrosis factor-α (TNF-α), are significantly elevated within the first few days [272]. Blood vessel damage activates platelets which release TGF-β1, platelet-derived growth factor and legumain [63, 237, 273]. Human BMSC are recruited and differentiate into bone forming OBs.

Enhanced presence of OB progenitors and TGF-β1 could explain why lansoprazole stimulate bone formation in injured bone tissue. In **paper II** legumain activity during OB differentiation was not affected by lansoprazole, however, legumain secretion was impaired. Upregulation of RUNX2 in OB progenitors by lansoprazole [271] could possibly downregulate legumain in the OB secretome since analysis of the legumain gene promoter showed four binding sites for RUNX2 (see above). Additionally, in **paper IV** we show that TGF-β1 downregulates legumain secretion in OBs, and decreased legumain secretion to the OB microenvironment could explain improved bone formation in the rat model of femoral fracture treated by lansoprazole [271].

Whether legumain targeting is a promising strategy in bone regeneration should be tested *in vivo* by comparing bone homeostasis and microarchitecture.

Cardiovascular diseases

CVDs are major causes of death and account for approximately 31% of all deaths worldwide [274]. Elevated expression and activity of legumain and cathepsins has been described in CVDs. Cathepsin B, K, L and S levels are increased in atherosclerotic arteries [275-279]. Carotid plaques harvested from symptomatic patients have increased expression of mature legumain (36 kDa; [33]) that is shown to be active (personal communication from Ngoc Nguyen Lunde). The presence of mature and active legumain in carotid plaques is especially fascinating since pH in plaques is 7.55 (±0.32) and legumain is a proenzyme processed and activated in acidic pH (<6) [21, 280]. In **paper I**, we highlight that the fate of atherosclerotic plaques is decided by the balance between pro-inflammatory M1 and anti-inflammatory M2 macrophages and that legumain found in plaques most likely originates from M1 pro-inflammatory macrophages involved in plaque progression [33, 281]. These findings indicate that legumain could initiate plaque progression and could be used as a biomarker for early atherosclerosis. On the other hand, by studying circulating levels of legumain from patients and legumain released from platelets, legumain was found in thrombus material retrieved from ruptured plaques and shown to be upregulated during acute cardiovascular events and associated with improved outcome [237]. Furthermore, stimulation of primary monocytes from healthy individuals with macrophage colony stimulating factor (M-CSF) and prolegumain lead to M2 polarization, increased secretion of the anti-inflammatory mediators CD163 and IL-10, and downregulation of the pro-inflammatory MCP-1 [237]. Also, FN is one of the first ECM proteins deposited at atherosclerosis sites and was proposed as a promoter of atherosclerotic lesion development

[282]. Correspondingly, atherosclerotic mice deficient in hepatocyte-derived plasma FN had significantly smaller and fewer atherosclerotic plaques [282]. Thus, legumain could contribute to resolution of atherosclerotic plaques through FN degradation, which is characterized in **paper IV**. Besides, in a study of unilateral ureteral obstruction mouse model legumain was reported as a mediator of M2 anti-inflammatory effects in interstitial renal fibrosis [153]. Therefore, in light of recent findings, legumain is considered to be a beneficial agent in atherosclerosis progression, mediating interaction between macrophages and platelets.

Standard treatment of atherosclerosis is focused on reduction of risk factors and the use of PPIs is associated with increased risk of CVDs [283]. One of the proposed mechanisms for PPI-induced cardiovascular risk is impaired activity of the dimethylarginine dimethylaminohydrolase which leads to enhanced platelet reactivity and thrombosis [283]. Furthermore, in human microvascular endothelial cells esomeprazole (10 μM) inhibited the activity of lysosomal enzymes cathepsin B and acid phosphatase thus leading to impaired proteostasis and endothelial senescence [284]. Similarly, we show direct inhibition of legumain in RAW264.7 macrophages by lansoprazole (**paper II**). Due to legumain involvement in anti-inflammatory action of M2 macrophages [237], treating atherosclerotic patients with PPIs may negatively modify the therapeutic outcome as a result of legumain inhibition. Further *in vivo* studies and randomized clinical trials are needed to elucidate the effect of PPIs in atherosclerotic patients.

Although VD_3 plays a major role in bone health and Ca^{2+} metabolism, VD_3 deficiency has many non-skeletal consequences. Current studies associate VD_3 deficiency with higher risk of CVDs, development of atherosclerosis and coronary heart disease [285]. In a case-control study of 18 225 men, low serum levels of $25OHD_3$ were associated with higher risk of myocardial infarction. The mechanism behind VD_3 involvement in CVD pathology is not fully elucidated. VD_3 has been demonstrated as a strong inhibitor of foam cell formation and production of inflammatory cytokines, and thus attenuating atherogenesis [286]. In **paper III** we showed a cell type-specific regulation of legumain by VD_3. Patients receiving $1,25(OH)_2D_3$ for 12 months had no change in legumain serum levels. However, baseline legumain was negatively correlated with $25OHD_3$ serum levels after 12 months of treatment (**paper III**). Since emerging evidence portrayed legumain as a positive factor in CVD pathology [237], one could hypothesize that VD_3 might enhance legumain secretion in M2 macrophages and in that way contribute to the improvement of cardiovascular health. Indeed, preliminary studies on THP-1-

macrophages show increased legumain secretion by $1,25(OH)_2D_3$ in a dose-dependent manner (performed by master student Guro L. Arnekleiv, unpublished; Fig. 11).

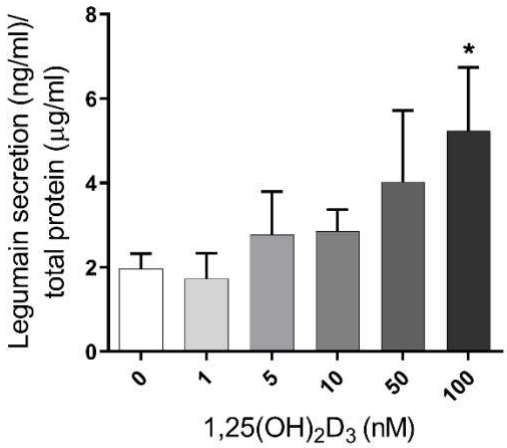

Figure 11. Legumain secretion is upregulated by $1,25(OH)_2D_3$ in THP-1-macrophages. Human acute monocytic leukaemia cells (THP-1; ATCC) were stimulated with phorbol 12-myristate 13-acetate (PMA; 62 ng/ml) for 6 hours prior to the addition of $1,25(OH)_2D_3$ (0-100 nM). Cell media was collected after 3 days and legumain secretion (ng/ml) was measured by ELISA versus cellular total protein concentrations (µg/ml) and presented as mean ± SEM (n=3). Analysed by Spearman's correlation test, *$p<0.05$.

Despite the increasing knowledge of VD_3 and atherosclerosis, the literature at present is inconclusive and further efforts are required to design and execute quality studies that would assess this very interesting and important context.

Cancer

Cancer is the leading cause of death in developed countries. One of the hallmarks of cancer is an acidic tumour microenvironment [287]. Acidosis is driving cancer progression, metastasis and resistance to therapy [164] and a pH shift is mediated by ion pumps such as the V-ATP-ases localised on the plasma membrane of cancer cells. Furthermore, increased expression and activity of V-ATP-ases have been reported in metastatic cancers [288]. In recent years, attempts to reposition PPIs to oncology have increased. Numerous studies have shown inhibition of V-ATP-ases by PPIs [289], thus, inhibiting the mechanism crucial to cancer homeostasis in concentrations above 100 µM [290]. In **paper II**, lansoprazole did not inhibit lysosomal pH, most likely due to the low dosage used (10 µM). The mechanism behind the antineoplastic

effect of PPIs is still unclear, but one could suggest that inhibition of legumain (or other cysteine proteases) demonstrated in **paper II** might be part of the explanation. Cysteine proteases are found to be involved in all stages of cancer progression such as tumour initiation, growth, angiogenesis and metastasis. An acidic extracellular microenvironment leads to activation of proteases like legumain, cathepsin B and L, and thereby ECM protein cleavage and damage, resulting in increased metastatic potential [291, 292]. High levels of legumain have been reported in most solid tumour tissues [292]. In **paper I** we highlight the important extracellular roles of legumain in tumours, such as legumain presence on tumour cell-surfaces co-localized with integrins leading to tumour invasion and metastasis [292]. A number of studies suggest that elevated legumain expression leads to worse outcomes in glioblastoma, breast, colorectal and ovarian cancer patients [293-296] and legumain is suggested as a potential target for tumour therapy [62, 297, 298]. In fact, in **paper IV** we demonstrate FN degradation by legumain and tumour cells use degradation and remodelling of ECM by proteases for tumour metastasis as mentioned above [299, 300]. In **paper III** we show an inverse relationship between legumain and VDR both *in vitro* and *in vivo*, and high VDR expression is a positive prognostic factor in cancer [301]. Coupled with the fact that inhibition of legumain suppresses breast cancer cells invasion *in vitro* and inhibits metastasis in a mice breast cancer model [302], targeting legumain locally with PPIs could contribute to the antineoplastic effect of PPIs.

Neurodegenerative diseases

Neurodegenerative diseases (NDGs) are conditions characterized by neuronal degeneration in the central nervous system (CNS). To date, NDGs are incurable and result in progressive degeneration and reduced CNS functions. The prevalence of NDGs is increasing and will become a major challenge for public health in the future due to demographic changes with longer life spans. The most common NDGs are Alzheimer's (AD) and Parkinson's disease (PD) with AD as the most frequent cause of dementia in the Western world [303]. Hallmarks of AD are accumulations of neurotoxic extracellular amyloid-beta (Aβ) and intracellular tau proteins [304, 305], whereas PD is characterized by Lewy bodies composed of α-synuclein (α-Syn) that progressively spread from the enteric nervous system (ENS) to the CNS [306]. Today, as the incidence of NDGs is rising, it is important to highlight the findings of recent studies describing association between onset of NDGs and long-term use of PPIs [307]. PPIs cross the blood-brain-barrier, but the exact mechanism behind the association with NDGs is unknown. Some

authors suggest that chronic use of PPIs leads to a pH shift in microglial lysosomes from acidic to basic and thereby debilitating the degradation of Aβ [308]. In **paper II** we demonstrated that lansoprazole (10 μM) did not affect lysosomal pH. Plasma concentration in patients receiving lansoprazole is only 4.8 μM [309], however, PPIs accumulate in acidic compartments and might achieve much higher local concentrations [165]. Other authors suggest that PPI-NDG association is due to the negative effect of PPIs on vitamin B_{12} absorption and since vitamin B_{12} is important for myelin production, vitamin B_{12} is essential for the nervous system [310, 311].

The pathology of NDGs is elusive, but there have been suggestions that inflammation in the brain caused by activated microglia contributes to NDG progression. Microglia secrete several proteases that are associated with NDG pathology, such as legumain and cathepsins B, D, S and L [312-314]. Cathepsin B is one of the most abundant cysteine cathepsins in the CNS, found in secretory vesicles of neuronal chromaffin cells and identified as a β-secretase that produces the Aβ peptides [315-317]. Cathepsin B is also associated with PD pathology through stimulation of α-Syn aggregate formation [318]. In **paper I**, we highlight the involvement of legumain (also known as δ-secretase) in neuronal apoptosis in NDGs such as stroke, intracerebral thrombi, ischemia, AD, PD, multiple sclerosis and amyotrophic lateral sclerosis [54, 67-69, 237, 319]. Levels of α-Syn and Tau are raised in the gut and brain of PD patients. Legumain has been shown to cleave both α-Syn and Tau, and mediates their fibrillization and transport from the gut to the brain, thereby triggering nigra dopaminergic neuronal loss associated with Lewy bodies and motor dysfunction [52, 320]. Analysis of legumain gene variations in 676 AD patients showed no association with AD [321]. Another plausible explanation on how legumain impacts neurological health might be the negative effect legumain has on VDR expression (**paper III**). VDR gene polymorphism is associated with AD and VDR overexpression suppress amyloid precursor protein (APP) transcription in neuroblastoma cells [322-324]. Furthermore, degradation of FN (**paper IV**) could possibly further contribute to the negative effects of legumain since FN has a neuroprotective role through neurotrophic and anti-inflammatory effects in the brain, thus promoting growth and survival of neurons [325]. Another key point to consider is that legumain and cathepsin B deletion in mice models of AD (APP/PS1 and 5XFAD) led to improvement of memory deficits, substantially reduced deposition of Aβ and less pathophysiological changes in the brain [54, 326]. Therefore, targeting legumain or cathepsin B in the brain of AD patients might be a therapeutic strategy and should be explored.

CONCLUSIONS

This thesis sheds new light on legumain, and especially in relation to bone cell biology. Our main conclusions are:

- Recent research indicates important extracellular functions of legumain (**paper I**)
- Lansoprazole directly inhibits legumain in various cell types by binding to the SH-group in the active site, but not in OB precursor cells (hBMSC-TERT) (**paper II**)
- Lansoprazole downregulates legumain secretion during OB differentiation (**paper II**)
- Both VD_3 forms ($1,25(OH)_2D_3$ and $25OHD_3$) upregulate legumain expression, activity and secretion in the early phase (7 days) of OB differentiation (**paper III**)
- VDBP co-localizes with legumain in mouse kidneys and legumain generates a specific VDBP cleavage product of approximately 45 kDa (**paper III**)
- Legumain downregulates VDR expression (**paper III**)
- Baseline legumain levels are negatively correlated with $25OHD_3$ levels following 12 months treatment with VD_3 in elderly overweight patients (**paper III**)
- Legumain enhances the cellular expression and degradation of fibronectin, whereas legumain deficiency or legumain inhibition diminishes FN expression (**paper IV**)
- TGF-β1 increases legumain (36 kDa) expression and downregulates legumain secretion during OB differentiation (**paper IV**)
- Legumain and FN are both present in bone cell exosomes (**paper IV**)

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Mini-review

Mammalian legumain – A lysosomal cysteine protease with extracellular functions?

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ABSTRACT

The cysteine protease legumain (asparaginyl endopeptidase, AEP) plays important roles in normal physiology but is also associated with several disorders, such as atherosclerosis, osteoporosis, cancer and neurodegenerative diseases. The functional roles of legumain have mainly been associated with the presence in lysosomes where legumain is active and mediates processing of multiple proteins, such as the conversion of single to double chain forms of cysteine cathepsins. However, in recent years, a number of studies point to extracellular roles of legumain in addition to the pivotal roles in the lysosomes. In this review, recent knowledge on novel extracellular functions of this protease will be addressed and new discoveries in relation to the diseases mentioned above will be presented.

1. Introduction

The cysteine protease legumain (EC 3.4.22.34) is ubiquitously expressed in mammals with highest expression in kidneys, spleen, liver, placenta and testis [1]. Legumain displays specificity towards asparagine peptide bonds, thus synonymously termed asparaginyl endopeptidase (AEP) [1]. Later, it was shown that legumain acquires caspase-like properties at low pH (<5) by cleaving after aspartic acid [2]. In cells, legumain is primarily localized to the acidic lysosomal compartments, where activation of the protease takes place. However, legumain is shown to be extensively secreted from various cell types and reported to appear extracellularly, although only the proform has been detected [3,4]. Legumain has been found in extracellular fluids such as serum, plasma and cerebrospinal fluid (CSF) [5–7], and is suggested to have multiple functions both intra- and extracellularly (reviewed in Ref. [8]). In addition, legumain has been found in exosomes [9,10]. Legumain deficient ($LGMN^{-/-}$) mice are born with no distinct anatomical or morphological abnormalities [11]. However, enlarged lysosomes and deficient processing of lysosomal cathepsins and toll-like receptor 9, kidney failure, and characteristic features of hemophagocytic syndrome are observed [12,13].

In vitro differentiation of human primary monocytes indicates that M1 macrophages are an important source of secreted legumain, which is measureable in plasma [7]. It has been suggested that polyubiqutinylation of prolegumain or reduced acidification of the lysosomes promote extracellular secretion, which may regulate whether legumain is retained intracellularly or secreted to the environment [14,15]. Since posttranslational polyubiquitinylation occurs in the cytosol, lysosomal prolegumain is not an obvious substrate. However, the presence of legumain in the cytosol has been described in cancer cells [16,17] and thus, such modification of legumain is possible [14]. In addition to being extensively secreted, legumain has also been reported to be present in the nucleus of colorectal cancer cells and in activated Th1 lymphocytes [17,18], and nuclear transport seems to be dependent on posttranslational glycosylation [16]. In this review we will focus on extracellular legumain and explore the hypothesis that this protein is not only functioning as a lysosomal enzyme but has additional important extracellular functions.

Legumain is synthesized as a glycosylated 56 kDa proform (prolegumain) which is auto-catalytically activated at acidic pH and reducing conditions to a 46 kDa intermediate that is further processed to a 36 kDa mature and a newly discovered 25 kDa active forms [16,19,20]. Endogenous inhibitors of legumain are the family 2 cystatins including cystatin C, E/M and F, with cystatin E/M being the most potent inhibitor [21]. Two molecular forms of cystatin E/M are expressed and secreted, a 14 kDa unglycosylated and a 17 kDa glycosylated form [22]. We have previously shown that secreted

Abbreviations: AEP, asparaginyl endopeptidase; ECM, extracellular matrix; GAG, glycosaminoglycan; hBMSC, human bone marrow skeletal (mesenchymal) stem cells; NDG, Neurodegenerative.

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prolegumain can be internalized and subsequently processed and activated intracellularly [4]. Similarly, secreted cystatin E/M can be internalized and is able to inhibit intracellular legumain [4].

Both legumain and cystatin E/M are endogenously glycosylated, and such posttranslational modification plays an important role in protein trafficking and function [23]. In general, initiation of N-linked glycosylation occurs in the rough endoplasmic reticulum (ER), and involves attachments of high-mannose oligosaccharides to selected asparagine residues in the polypeptide [24]. During transport through ER and Golgi, the linked oligosaccharides are further modified to the formation of three main types of N-linked oligosaccharide structures (i.e. high mannose, hybrid or complex) [25]. Two N-linked glycosylation sites (N91 and N167) have been confirmed in prolegumain, although the protease has four potential N-linked sites [26]. Recently, we showed that the carbohydrates on legumain are of the hybrid or high mannose type, whereas cystatin E/M has complex mannose-linked carbohydrates [16]. N-glycosylation of prolegumain is necessary for cellular internalization and correct processing to active forms, whereas cystatin E/M is independent of the glycosylation status for inhibition of legumain [16]. In addition to function as legumain inhibitors, both cystatin C and E/M are substrates of legumain [27–29] (Table 1). Furthermore, inhibition of posttranslational glycosylation facilitates cell secretion of prolegumain [16].

The prevailing theory for intracellular transport of newly synthesized legumain is through the trans-Golgi network and further into lysosomes through endosomes (Fig. 1; reviewed in Refs. [8,30]). It is assumed that glycosylation is important for transport to the lysosomes, the main compartment for prolegumain autoactivation [15]. A mannose-6-phosphate (M6P) motif has been reported on the N-terminus of legumain [31] and could target legumain to the vesicular pathway depending on the interaction with different M6P-receptors. The cation-dependent M6P-receptor is important for routing prolegumain and precursors of other lysosomal enzymes from Golgi to the late endosomes, while the cation-independent M6P-receptor on the cell surface facilitates re-capturing of secreted proenzymes, followed by clathrin-mediated endocytosis and subsequent trafficking to the lysosomes [32,33]. We have recently shown that internalization of legumain is not through clathrin-mediated endocytosis since blocking of this endocytosis pathway did not inhibit legumain internalization [16]. Thus, the exact internalization mechanism of legumain is still unknown and needs further investigations. However, rerouting from classical transport pathways is often associated with pathological conditions [34]. A previous review addressed myths and common questions regarding localization and trafficking of endo-lysosomal cysteine proteases [30]. Nevertheless, the regulation and mechanisms of legumain trafficking in or out of cells is still poorly understood. Similarly to legumain, both cystatin C and E/M are internalized [4,35] and thus, the interplay between legumain and its inhibitors appears to take place both intra- and extracellularly.

Secreted prolegumain could be activated in acidic extracellular microenvironments during pathological or inflammatory conditions. Prolegumain is stable in neutral pH, whereas mature legumain is unstable and rapidly inactivated at pH > 6 [1,20]. Stabilization of the proenzyme is postulated to be mediated by the positively charged C-terminal domain located on top of the protease catalytic domain [27,36]. Legumain is stabilized in the extracellular environment by interactions with integrins, glycosaminoglycans (GAGs) or cystatins forming amyloid fibril fragments (Fig. 1; [29,36,37]) and such interactions might increase the half-life of the protease. Since the nature of cystatin inhibition is reversible, amyloid fibrils could act as a legumain storage [29]. The legumain structure contains a RGD motif which allows binding to integrin receptors on the cell surface [38]. Legumain binding to the $\alpha_V\beta_3$ integrin results in increased stability, catalytic activity and shift in the pH optimum from pH 5.5 to 6.0 [36]. Similarly, we have previously shown that extracellular matrix (ECM) components, such as the GAGs chondroitin 4-sulfate (C4S) and heparan sulfate (HS), accelerate prolegumain autoactivation in a pH-, concentration- and time-dependent manner [37] and the carbohydrates on legumain seem to play a key role in the interaction between prolegumain and GAGs [16]. Thus, the interplay between legumain and components in the extracellular milieu is of great interest to understand the extracellular roles of this protease.

Legumain has been postulated to have various activities, i.e. asparaginyl endopeptidase (AEP), asparaginyl carboxypeptidase and ligase activities (reviewed in Ref. [8]). The suggested extracellular AEP-substrates are presented in Table 1. Below, we will focus on extracellular legumain (AEP) in pathology, as legumain has been shown to be involved in inflammation, atherosclerosis, bone homeostasis and osteoporosis, cancer and neurodegenerative diseases. In addition, legumain has been suggested as a biomarker of idiopathic pulmonary fibrosis [45], liver fibrosis [46] and pancreatitis [47].

Table 1
Suggested extracellular legumain (AEP) substrates.

Substrates	References
Amyloid precursor protein (APP)	[39]
Cathepsins B, L, S	[40]
Cystatin C	[27]
Cystatin E/M	[28,29]
Fibronectin	[41–43]
Prolegumain	[2,19,20]
ProMMP-2 (progelatinase A)	[44]

2. Extracellular legumain in atherosclerosis

Atherosclerosis is a major cause of cardiovascular disease and responsible for high mortality and morbidity world-wide. In the arteries, lipids and immune cells are central players, driving the persistent inflammatory process which is characteristic for atherosclerosis [48,49]. During inflammation, pro-inflammatory mediators are secreted, promote lesion formation and are responsible for plaque destabilization. Proteases, especially matrix metalloproteases (MMPs), are implicated in atherosclerosis. Various MMPs can degrade vascular ECM, thus leading to the development of atherosclerotic lesions and plaque rupture (reviewed in Refs. [50,51]). In recent years, the role of cysteine cathepsins in atherosclerosis have also started to be elucidated [52,53], whereas legumain has been less studied.

Recently, we quantified legumain for the first time in plasma from patients with carotid artery stenosis and concluded that patients with carotid plaques had significantly higher levels of plasma legumain than healthy controls [7]. In addition, patients with recent symptoms had both significantly higher expression of legumain in plaques compared to asymptomatic patients, indicating an upregulation in acute stages of the disease. Our study further confirmed previous data showing increased levels of legumain in unstable compared to stable regions of human carotid plaques [54–56]. As a consequence, legumain plasma levels can potentially be a novel biomarker to identify the presence and characterization of carotid plaques in atherosclerotic disease [7,57]. Very recently, serum legumain was also closely associated with the severity of idiopathic pulmonary arterial hypertension [58].

Monocyte differentiation to macrophages and subsequent uptake of oxidized low-density lipoprotein (oxLDL) to develop foam

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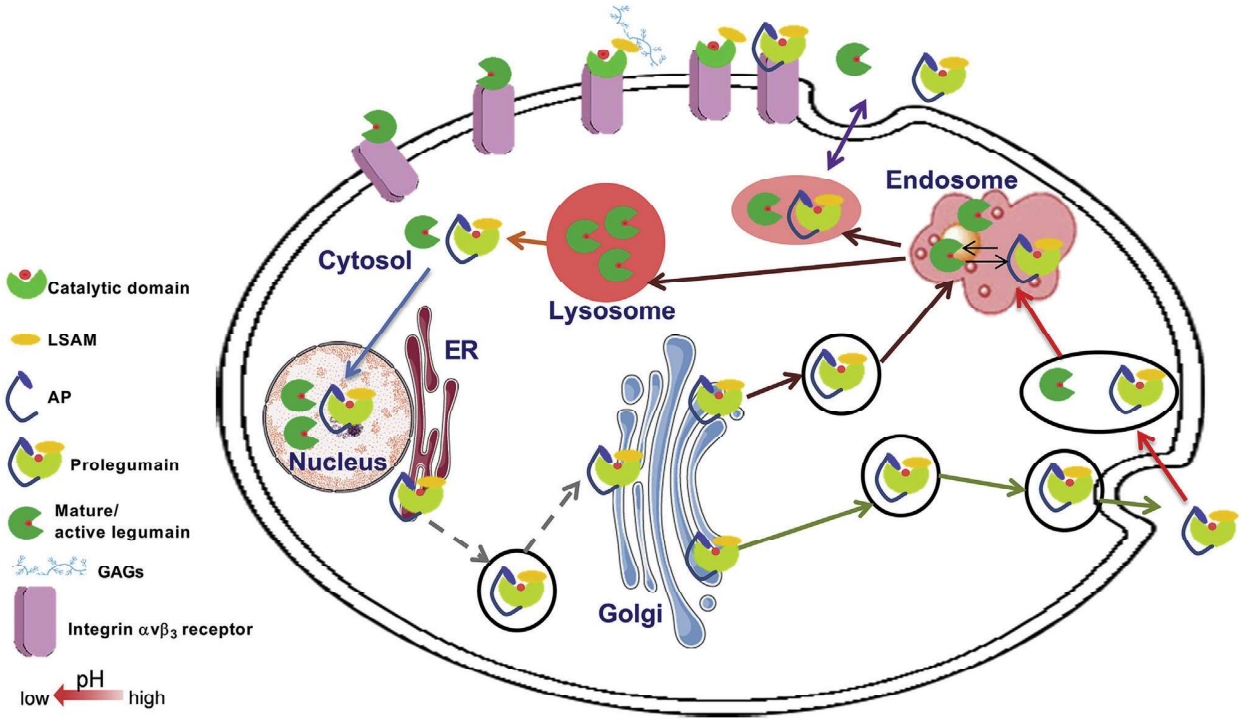

Fig. 1. Proposed intra- and extracellular trafficking of legumain. After synthesis, inactive prolegumain, containing a catalytic domain (green) with a cysteine (red) in the active site, an activation peptide (AP; blue) and a legumain stabilization and activation modulation domain (LSAM; yellow) is targeted via endoplasmic reticulum (ER) and Golgi to the endolysosomal system (dashed grey and brown arrows), where it is processed and activated at acidic pH (red vesicles). Alternatively, prolegumain is secreted, either directly via Golgi (green path) or indirectly via the endosomal system (brown and purple path). Glycosaminoglycans (GAGs) and $\alpha_V\beta_3$ integrin receptors might stabilize active legumain on the cell surface. Extracellular legumain may internalize via bulk endocytosis (red and purple path). In pathological conditions where the lysosomes are altered (i.e. Alzheimer's disease), legumain may enter the cytosol directly from leaky lysosomes (orange arrow) and presence of mature legumain in the cytosol have been reported in cancer cells. Additionally, legumain may enter the nucleus (blue arrow).

cells play a pivotal role in atherosclerosis [59,60]. Macrophages can perform phagocytosis and subsequent lysosomal degradation of ECM components. However, release of active proteases from macrophages and/or extracellular activation of proteases might cause ECM degradation in a more direct manner. The inflamed arterial wall of an atherosclerotic process creates a local acidic milieu required for extracellular activation of legumain and other cysteine proteases. This could further be facilitated by the proton and lactic acid pumps present on macrophages [61]. However, there are so far no direct evidences supporting the presence of active legumain extracellularly.

The origins of circulating legumain in atherosclerosis remain to be elucidated. In an atherosclerotic lesion, smooth muscle cells, endothelial cells and/or immune cells such as invading monocytes and macrophages could be sources of extracellular legumain. Unstimulated human THP-1 monocytes do not secrete legumain, whereas THP-1-differentiated macrophages do, indicating a secretory regulation of legumain during monocyte-to-macrophage differentiation [3]. Both pro-inflammatory M1 and anti-inflammatory M2 macrophages are present in atherosclerotic lesions and the balance between macrophage subtypes is of importance for the fate of the plaques [62,63]. We have recently shown that unstimulated M1 has a 9-fold higher legumain secretion than M2 or resting macrophages, which could indicate that M1 macrophages are a main source of circulating legumain [7]. This suggests that the distribution of macrophage subtypes have a major impact on the secreted level of legumain. In addition, cholesterol crystals (CC) together with cell debris forming the necrotic core are important characteristics of vulnerable plaques. Exposure of primary macrophages to atherogenic lipids, especially CC, increases legumain secretion, although only significantly from the M1 phenotype [7], underscoring the interplay between macrophages and lipids in the secretion of legumain. Furthermore, legumain regulates oxLDL-induced macrophage apoptosis by enhancing the autophagy pathway, which may also influence the vulnerability of atherosclerotic plaques [64]. Interestingly, legumain has very recently been shown to contribute to induction of atherosclerotic vascular remodelling, and chronic infusion of legumain to apolipoprotein E-deficient mice (*ApoE*$^{-/-}$, an established mouse model of atherosclerosis) potentiated the development of atherosclerotic lesions [65]. This study postulated legumain as a novel therapeutic factor for the treatment and prevention of atherosclerosis and vascular remodelling.

Current treatment for atherosclerosis is focusing on reduction of risk factors, such as hypertension, hyperlipidemia and thrombosis. To lower cholesterol levels, statins (HMG-CoA reductase inhibitors) are used as both primary and secondary prevention. In addition, statins reduce inflammation and improve endothelial function in atherosclerotic patients, indicating pleiotropic effects [66]. High concentrations of atorvastatin ($\geq$100 μM) inhibits the secretion of legumain from primary macrophages *in vitro*, whereas 10 μM atorvastatin increases legumain secretion [3]. In addition, statin treatment reduces legumain expression in circulating monocytes [67], as well as legumain expression and activity in human myotubes [68], but the mechanisms are not known.

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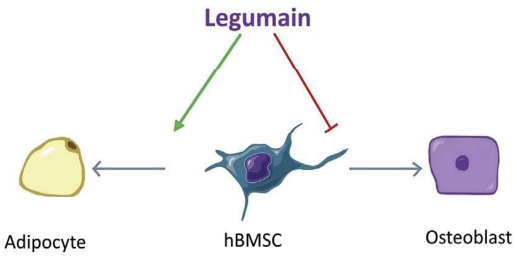

Fig. 2. Legumain inhibits osteoblast but facilitates adipocyte differentiation of human bone marrow skeletal (mesenchymal) stem cells (hBMSC).

3. Extracellular legumain in bone homeostasis and osteoporosis

Bone is a complex tissue composed of bone forming (osteoblasts, OBs) and bone resorptive (osteoclasts, OCs) cells, as well as solid extracellular matrix. It is well known that bone cells secrete proteins with autocrine, paracrine or endocrine functions. Legumain has been identified in the secretome of human bone marrow skeletal (mesenchymal) stem cells (hBMSC) during *ex vivo* OB differentiation [69]. Human BMSCs are multipotent non-hematopoietic stromal cells that can differentiate into OBs or adipocytes (Fig. 2) [70]. Legumain expression is upregulated at the start of OB differentiation from hBMSC (days 1–7) and is subsequently and completely downregulated in mature OBs [43]. Furthermore, we recently showed that legumain inhibits the lineage commitment of hBMSC to OBs, but stimulates adipocyte differentiation (Fig. 2) [43]. *In vivo* bone formation and mineralization of the vertebrate spinal column in zebrafish was shown to be increased by legumain deficiency [43]. Stem cell commitment and bone formation were shown to be mediated through fibronectin degradation by legumain, since intact fibronectin enhanced OB and inhibited adipocyte differentiation [43]. Moreover, serum analysis of postmenopausal women (48–87 years) showed decreased plasma legumain with aging. In addition, increased expression of legumain in bone marrow-derived adipocytes was inversely correlated with adjacent trabecular bone mass in patients with postmenopausal osteoporosis. Thus, altered activity of legumain in the bone microenvironment contributes to decreased bone mass in postmenopausal osteoporosis [43].

Previously, legumain has been detected in the OC secretome and the secretion was downregulated during differentiation towards mature OCs [71,72]. Legumain was reported to regulate bone resorption *in vitro* by a protease-independent function of the C-terminal fragment (17 kDa), described as an osteoclast inhibitory factor (OIP-2) [73]. No further data on OIP-2 has been presented, but legumain was recently shown to inhibit osteoclastogenesis, in part through modulation of cathepsin L activity [74]. Furthermore, expression of the bone resorptive cysteine protease cathepsin K has been reported to increase upon intracellular inhibition of legumain [74]. OCs derived from long or flat bones are different, and OCs in long bones are engaged in cathepsin-mediated bone resorption, whereas OCs in flat bones are more dependent on MMPs [75]. Legumain is known to activate proMMP-2 [44] (Table 1), as well as processing of cysteine cathepsins [11,40] (Table 1), and therefore, legumain could have regulating roles both in long and flat bones.

4. Extracellular legumain in cancer

Several studies have linked legumain to the development, progression and severity of cancer. A meta-analysis has shown that legumain is overexpressed in cancer compared with normal tissue and was higher in late stages (III-IV) than in early stages (I-II) of disease [76]. Moreover, legumain overexpression was correlated with poor prognosis and clinical stage. Although being primarily a lysosomal protease, legumain is secreted by cancer cells to the extracellular space. Tumours generate acidic microenvironment at the tumour border [77,78], where secreted prolegumain could be activated, allowing substrate activation or degradation. In the tumour microenvironment legumain is expressed not only by tumour and surrounding stromal cells but also by tumour-associated macrophages [79,80]. Interestingly, increased legumain serum levels have been detected in breast cancer patients compared to controls, and legumain is suggested as a biomarker for metastatic breast cancer diagnosis [14]. Moreover, it is suggested that legumain may influence the reprogramming of macrophages leading to inflammation-induced pancreatic cancer [47].

The link between legumain and cancer is associated with indirect ECM degradation by legumain activation of proMMP-2 [44] or processing of cathepsins [40], or by direct proteolysis of ECM components like fibronectin [41] (Table 1). This results in solubilisation of ECM which is necessary for invasive growth and metastasis. Also, other ECM components are of great importance for legumain activation and GAGs like C4S and HS could facilitate and accelerate prolegumain autoactivation in the acidic tumour microenvironment [37].

To allow tumour growth, new blood vessels are required to supply nutrients to the cancer cells. The process of angiogenesis is important and at least partly stimulated by paracrine factors secreted from the tumour cells and affecting local endothelial cells [81]. Formation of new vessels requires both generation of new endothelial cells and ECM degradation. Interestingly, legumain has been identified in the secretome of early pro-angiogenic cells [82] but the significance of extracellular legumain in angiogenesis is not known.

The interplay between cysteine proteases and cystatins are important in cancer development and progression. The involvement of cystatin C in malignant diseases is controversial and studies have highlighted that this cysteine protease inhibitor may function either as a tumour suppressor or a tumour promoter (reviewed in Ref. [83]). In a number of studies and in various cancers, cystatin E/M is suggested as a tumour suppressor, including triple negative breast cancer [84,85]. Also, a study of the breast cancer cell secretome established cystatin E/M as a suppressor of breast cancer bone metastasis [86].

Alterations in protein glycosylation occur during inflammation or malignancy [87,88]. Various proteins with altered glycosylation patterns have been studied as potential biomarkers in cancer and chronic inflammatory conditions, and shown to correlate with disease severity [89,90]. Melanoma cells predominantly secrete glycosylated compared to unglycosylated cystatin E/M and we have reported an inverse correlation between the secretion of cystatin C and E/M, as well as an inverse correlation between the expression of cystatin E/M and legumain [22]. Also, over-expression of cystatin E/M in melanoma cells significantly inhibits legumain and decreased invasiveness into matrigel. Collectively, these data suggest that cystatin E/M contributes in inhibiting cancer progression by suppressing legumain activity [22]. We have shown that N-glycosylation is important for legumain localization and function [16] but whether this is important in pathology is not known and needs further investigations.

Since legumain is over-expressed in various tumours and participate in invasive growth and metastasis, this protease is an interesting pharmacological or diagnostic target. Several groups have reported high sensitivity for cancer cell imaging using quenched activity-based probes or nanoprobes for legumain [91,92]. Due to a number of side-effects of traditional chemotherapy, several approaches have been applied to increase targeting or decrease

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toxicity of cancer drugs. Various substances have been developed to target or utilize legumain in cancer by DNA vaccines, azopeptides, small molecule inhibitors and legumain-cleavable prodrugs of cytotoxic drugs (reviewed in Ref. [93]). At present, no drugs targeting or utilizing legumain have yet been marketed in cancer treatment.

5. Extracellular legumain in neurodegenerative diseases

Neurodegenerative diseases (NDG) share a common feature in accumulation of proteins that are processed aberrantly and misfolded. These proteins form neurotoxic aggregates that cause diseases such as Alzheimer's disease (AD) and Parkinson's disease. Clearance of such proteins is important for preventing development of NDG [94], and autophagy or protein degradation in lysosomes are crucial. Legumain has been shown to be involved in autophagy [95] and the presence in lysosomes contributes to degradation of proteins involved in NDG, such as α-synuclein [96], TDP-43 [97], tau [98,99] and amyloid precursor protein (APP) [39].

Neurotoxic proteins can be released from neurons by various mechanisms including exosomes which have been shown to contain α-synuclein, tau and APP/Aβ42. Clearance of extracellular proteins can take place by glial endocytosis but also directly by proteolytic enzymes. It has been shown that brain acidosis caused activation of legumain [100] and since both increased age and AD cause reduced pH [101,102], it was postulated that legumain could play a role in NDG. A study of experimental stroke in mice indicated both increased expression and secretion of legumain after middle cerebral artery occlusion and legumain was detected in CSF [5].

Previously, legumain was renamed δ-secretase to indicate a role in the processing of APP [39] (Table 1). Since then several reports have identified legumain in neurodegenerative diseases like AD, stroke, ischemia, amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) [103]. APP is at present probably the best candidate of an extracellular protein involved in NDG and cleaved by legumain after autoactivation of prolegumain in an acidic environment. Interestingly, the brain pH is reduced during aging which might facilitate legumain activity and thus, legumain could be involved in AD with age [39]. It was shown that legumain cleaves APP at two sites (N373 and N585) and the cleavage at N373 generates a unique 80 kDa fragment shown to be neurotoxic. This study also showed extracellular binding of legumain to APP on the plasma membrane and subsequent internalization. These findings suggest that targeting legumain could be a novel therapeutic strategy in NDG diseases.

6. Perspectives

Further studies are required to understand the molecular mechanisms, regulation and cellular interplay of secreted legumain and cystatins, and whether legumain can be used as a biomarker, pharmacological target or be utilized for drug targeting in the treatment of various diseases. Of special interest is to unequivocally establish whether legumain exists as an active enzyme (AEP) extracellularly and to unravel its local substrates. Furthermore, possible legumain functions not dependent on the enzymatic activity need to be further investigated. Finally, the mechanisms for regulation of legumain expression dependent on endogenous mediators or transcription factors needs to be further elucidated.

Conflict of interest

None.

Author's contribution

All authors have contributed equally and approved the final manuscript.

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