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MARKERS OF BONE TURNOVER IN PRECLINICAL DEVELOPMENT OF DRUGS FOR SKELETAL DISEASES

by

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To my family

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ABSTRACT

Skeletal tissue is constantly remodeled in a process where osteoclasts resorb old bone and osteoblasts form new bone. Balance in bone remodeling is related to age, gender and genetic factors, but also many skeletal diseases, such as osteoporosis and cancer-induced bone metastasis, cause imbalance in bone turnover and lead to decreased bone mass and increased fracture risk. Biochemical markers of bone turnover are surrogates for bone metabolism and may be used as indicators of the balance between bone resorption and formation. They are released during the remodeling process and can be conveniently and reliably measured from blood or urine by immunoassays. Most commonly used bone formation markers include N-terminal propeptides of type I collagen (PINP) and osteocalcin, whereas tartrate-resistant acid phosphatase isoform 5b (TRACP 5b) and C-terminal cross-linked telopeptide of type I collagen (CTX) are common resorption markers. Of these, PINP has been, until recently, the only marker not commercially available for preclinical use. To date, widespread use of bone markers is still limited due to their unclear biological significance, variability, and insufficient evidence of their prognostic value to reflect long term changes.

In this study, the feasibility of bone markers as predictors of drug efficacy in preclinical osteoporosis models was elucidated. A non-radioactive PINP immunoassay for preclinical use was characterized and validated. The levels of PINP, N-terminal mid-fragment of osteocalcin, TRACP 5b and CTX were studied in preclinical osteoporosis models and the results were compared with the results obtained by traditional analysis methods such as histology, densitometry and microscopy. Changes in all bone markers at early timepoints correlated strongly with the changes observed in bone mass and bone quality parameters at the end of the study. TRACP 5b correlated strongly with the osteoclast number and CTX correlated with the osteoclast activity in both *in vitro* and *in vivo* studies. The concept “resorption index” was applied to the relation of CTX/TRACP 5b to describe the mean osteoclast activity. The index showed more substantial changes than either of the markers alone in the preclinical osteoporosis models used in this study. PINP was strongly associated with bone formation whereas osteocalcin was associated with both bone formation and resorption.

These results provide novel insight into the feasibility of PINP, osteocalcin, TRACP 5b and CTX as predictors of drug efficacy in preclinical osteoporosis models. The results support clinical findings which indicate that short-term changes of these markers reflect long-term responses in bone mass and quality. Furthermore, this information may be useful when considering cost-efficient and clinically predictive drug screening and development assays for mining new drug candidates for skeletal diseases.

Keywords: bone turnover markers, PINP, osteocalcin, TRACP 5b, CTX, osteoporosis, skeletal diseases, drug development, immunoassay

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YHTEENVETO

Luun uudismuodostusta tapahtuu koko elämän ajan. Tässä prosessissa osteoklastit hajottavat vanhaa luuta ja osteoblastit muodostavat uutta luuta. Tasapainoon vaikuttaa ikä, sukupuoli ja perinnöllisyys, mutta myös monissa luustosairauksissa, kuten osteoporoosissa ja syövän luustometastaaseissa, tämä tasapaino on järkkynyt johtaen vähentyneeseen luun määrään ja lisääntyneeseen murtumaherkkyyteen. Luun biokemialliset merkkiaineet kertovat luun aineenvaihdunnasta eli hajotuksen ja muodostuksen välisestä tasapainosta. Merkkiaineita vapautuu luun uudismuodostuksessa ja niitä voidaan helposti ja luotettavasti mitata seerumista tai virtsasta immunomääritysmenetelmillä. Yleisesti käytettyjä luun muodostuksen merkkiaineita ovat tyypin I kollageenin aminoterminaalinen propeptidi (PINP) ja osteokalsiini, sekä luun hajotuksen merkkiaineita tartraatti-resistentti hapan fosfataasi alatyyppi 5b (TRACP 5b) ja tyypin I kollageenin karboksiterminaalinen telopeptidi (CTX). Näistä PINP on ainoa merkkiaine, jolle ei ole aiemmin ollut saatavilla prekliiniseen käyttöön soveltuvaa kaupallista immunomääritysmenetelmää. Tällä hetkellä biokemiallisten merkkiaineiden laajamittainen käyttö on vielä rajoittunutta, koska niihin liittyy paljon biologista ja analyyttistä variaatiota eikä niiden merkitsevyydestä ja käyttökelpoisuudesta pitkän aikavälin muutosta ennustavana tekijänä ole riittävästi näyttöä.

Tämän tutkimuksen tavoitteena oli selvittää luuston biokemiallisten merkkiaineiden soveltuvuutta lääkemolekyylien tehokkuuden ennustajina prekliinisissä osteoporoositutkimusmalleissa. Tutkimuksessa karakterisoitiin ja validoitiin PINP:lle prekliiniseen käyttöön kehitetty immunomääritysmenetelmä. PINP:n, osteokalsiinin N-terminaalisen keskifragmentin, TRACP 5b:n ja CTX:n tasoja tutkittiin prekliinisissä osteoporoosimalleissa, ja saatuja tuloksia verrattiin perinteisillä menetelmillä kuten histologialla, tiheysmittauksilla ja mikroskopialla saatuihin tuloksiin. Kaikkien tutkittujen luuston merkkiaineiden alkuvaiheen muutosten havaittiin korreloivan kokeen lopussa nähtyihin luuston rakenteellisiin muutoksiin. TRACP 5b korreloi osteoklastien lukumäärään ja CTX osteoklastien aktiivisuuteen sekä *in vitro* että *in vivo* kokeissa. CTX/TRACP 5b suhteelle luotiin termi resorptio-indeksi, joka kuvaa osteoklastien keskimääräistä aktiivisuutta. Indeksi antoi tarkempaa tietoa kuin kumpikaan merkkiaine erikseen käytetyissä prekliinisissä osteoporoosimalleissa. PINP:n havaittiin korreloivan vahvasti luun muodostukseen, kun taas osteokalsiini kuvasi sekä luun muodostusta että hajotusta.

Tämän tutkimuksen tulokset antavat uutta tietoa luun biokemiallisten merkkiaineiden soveltuvuudesta lääkemolekyylien tehokkuuden ennustajina prekliinisissä osteoporoosimalleissa. Havainnot tukevat kliinisiä tutkimuksia, joissa merkkiaineiden on havaittu korreloivan myöhemmin nähtävien luuston rakenteellisten muutosten kanssa. Tutkimuksessa saatu tieto auttaa suunniteltaessa kliinisen ennustettavuuden kannalta parempia määritysmenetelmiä, joiden avulla voidaan nopeammin ja tehokkaammin löytää uusia toimivia lääkemolekyylejä luustosairauksien hoitoon.

Avainsanat: Luun biokemialliset merkkiaineet, PINP, osteokalsiini, TRACP 5b, CTX, osteoporoosi, luustosairaudet, lääkekehitys, immunomääritysmenetelmä

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ABBREVIATIONS

ADME	absorption, distribution, metabolism and excretion
ALP	alkaline phosphatase
ANOVA	analysis of variance
BALP/BAP	bone-specific alkaline phosphatase
BFR/BS	bone formation rate/bone surface
BMC	bone mineral content
BMD	bone mineral density
BMP	bone morphogenetic protein
BMU	basic multicellular unit
BSP	bone sialoprotein
BV/TV	bone volume/tissue volume
CAII	carbonic anhydrase II
Cat K	cathepsin K
c-Fms	M-CSF receptor
CLIA	chemiluminescent immunoassay
ClC7	chloride channel 7
CRP	c-reactive protein
CTR	calcitonin receptor
CTX	C-terminal cross-linked telopeptide of type I collagen
CV	coefficient of variation
DABA	dual action bone agent
DC-STAMP	dendrocyte expressed seven transmembrane protein
DHT	5α-dihydrotestosterone
Dkk	Dickkopf protein family
DMP1	dentin matrix protein 1
DPD	deoxypyridinoline
DXA,DEXA	dual energy X-ray absorptiometry
E64	proteinase inhibitor E 64
E_2	17β-estradiol
ECM	extracellular matrix
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMA (EMEA)	European Medicines Agency
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FACIT	fibril-associated collagens with interrupted triple helices
FDA	US Food and Drug Administration
FESEM	field emission scanning electron microscopy
FGF	fibroblastic growth factor
FSD	functional secretory domain
GAG	glycosaminoglycan
GCT	giant cell tumor
GHS-R1a	ghreline hormone receptor
GIOP/GIO	glucocorticoid-induced osteoporosis
Gla	γ-carboxyglutamic acid
HA	hydroxyapatite
HRT	hormone replacement therapy

HTS	high throughput screening
ICAM	intercellular adhesion molecule
ICTP	C-terminal telopeptide of type I collagen
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
Ig	immunoglobulin
IGF	insulin-like growth factor
IM	immobilization
IOF	International Osteoporosis Foundation
IRMA	immunoradiometric assay
JNK	c-Jun N-terminal kinase
LDL	low-density lipoprotein
Lrp	lipoprotein receptor related protein
MAP	mitogen-activated protein
M-CSF	macrophage-colony stimulating factor
MEPE	matrix extracellular phosphoglycoprotein
MGP	matrix Gla protein
MGT	Masson-Goldner trichrome stain
MiTF	microphthalmia-associated transcription factor
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NFATc1	nuclear factor of activated T cells
N.Oc/B.Pm	number of osteoclasts/bone perimeter
N.Oc/T.Ar	number of osteoclasts/tissue area
NTX	N-terminal cross-linked telopeptide of type I collagen
OA	osteoarthritis
OP	osteoporosis
OC	osteocalcin, bone Gla protein
OC-STAMP	osteoclast stimulatory transmembrane protein
1,25 $(OH)_2D$	calcitriol, dihydroxyvitamin D
OHP	urine hydroxyproline
ONJ	osteonecrosis of the jaw
OPG	osteoprotegerin
OPN	osteopontin
OSX	osterix
OVX	ovariectomy
ORX	orchiectomy
PBMC	peripheral blood mononuclear cells
PEG	polyethylene glycol
PGE2	Prostaglandin E2
PICP	C-terminal propeptides of type I collagen
PINP	N-terminal propeptides of type I collagen
pQCT	peripheral quantitative computed tomography
PTH	parathyroid hormone
PTHrp	PTH-related peptide
PYD	pyridinoline
RA	rheumatoid arthritis
RANK	receptor activator of nuclear factor kappa B
RANKL	ligand for receptor activator of nuclear factor kappa B
RGD	Arg-Gly-Asp tripeptide
RIA	radioimmunoassay
ROS	reactive oxygen species

Runx2/Cbfa1	Runt-related transcription factor 2
SD	standard deviation
SERM	selective estrogen receptor modulators
SIBLING	small integrin-binding ligand, N-linked glycoprotein
Tb.N	trabecular number
Tb.Th	trabecular thickness
Tb.Sp	trabecular separation
TbBMC	trabecular bone mineral content
TbBMD	trabecular bone mineral density
TIMP	tissue inhibitors of metalloproteinases
TMB ONE	3,3',5,5'-tetrametylbenzidine hydrogen peroxide
TNF-α	tumor necrosis factor α
TGF-β	transforming growth factor β
TRACP	tartrate-resistant acid phosphatase, formerly TRAP
V-ATPase	vacuolar-type proton adenosine triphosphatase
Wnt	wingless and Int1 proteins
WHO	World Health Organization
αvβ3	vitronectin receptor

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I–IV. In addition, unpublished results are included. The original publications have been reproduced with the permission of the copyright holders.

I Rissanen JP, Suominen MI, Peng Z, Morko J, Rasi S, Risteli J, Halleen JM (2008) Short-term changes in serum PINP predict long-term changes in trabecular bone in the rat ovariectomy model. Calcif Tissue Int 82:155-161.

II Rissanen JP, Suominen MI, Peng Z, Halleen JM (2008) Secreted tartrate-resistant acid phosphatase 5b is a marker of osteoclast number in human osteoclast cultures and the rat ovariectomy model. Calcif Tissue Int 82:108-115.

III Rissanen JP, Ylipahkala H, Fagerlund KM, Long C, Väänänen HK, Halleen JM. (2009) Improved methods for testing antiresorptive compounds in human osteoclast cultures. J Bone Miner Metab. 27:105-9.

IV Rissanen JP, Halleen JM (2010) Models and screening assays for drug development in osteoporosis. Expert Opin Drug Discov 5:1163-1174.

1. INTRODUCTION

Drug discovery is a complex, expensive and time consuming process. The average cost of bringing a new drug into market has risen rapidly and, at present, the whole process can take over ten years and cost over one billion euros. Thus, pharmaceutical companies are putting an increasing emphasis on strategies which focus on early stage preclinical development in order to reduce clinical phase compound attrition. One approach reducing compound failure in the later stages of drug development and rationalizing the overall cost of drug development is to focus on the use and development of biochemical markers. Biomarkers have been hypothesized to have the potential to bridge *in vitro*, *in vivo* and clinical studies and concomitantly improve the interpretation of the study outcome.

Biomarkers are often defined as biological characteristics that can be objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention (Naylor et al. 2003). In many diseases, effective treatment is available for only a small percentage of patients. Biomarkers have the ability to enhance the diagnosis and prognosis of a disease and its therapy. Moreover, biomarkers enable the physician to design a personalized treatment scheme which is individually tailored for the patient's needs. Biomarkers are associated with a wide range of techniques and applications, but typically they are non-invasive, relatively inexpensive and rapid tools yielding both qualitative and quantitative data, and they can be conveniently measured from blood or urine. However, widespread use of markers has been limited due to reported discrepancies that are mainly due to unclear biological function, variability and insufficient evidence of their prognostic value.

As a consequence of increasing knowledge of bone biology and development of assay technologies, several biochemical markers of bone turnover have been introduced in the past decades. Markers reflecting bone turnover include bone formation and resorption markers. They have already been used for diagnosis and monitoring therapies in skeletal diseases, such as osteoporosis and cancer bone metastases. Osteoporosis is the most common skeletal disease causing millions of fractures annually. In Western countries, approximately 30% of postmenopausal women suffer from this disease. In osteoporosis, bone loss is gradual and it is often referred to as a silent disease. It can progress for years without warning, symptoms or incident. Osteoporotic fractures are a significant cause of morbidity in the elderly and, even before any fractures occur it affects the quality of life. As the life expectancy increases, the number of osteoporotic patients is also expected to increase, which will further cause additional cost and burden to our health care system. Currently, there are many treatments available for osteoporosis prevention and treatment. Traditional target sites for osteoporosis drugs include targets for promoting osteoblast function (anabolic therapies) or inhibiting osteoclast function (anti-catabolic or anti-resorptive therapies). However, most of the existing therapies will only slow down further progress of the disease but will not recover the amount of bone mass back to the normal level. As a result, early diagnosis and development of more advanced therapies would be crucial for the patient and the society.

Markers of bone turnover are released from bone matrix or bone cells. Most of the bone resorption markers are derived from the most abundant protein in bone matrix, type I collagen. In addition to bone formation or resorption, markers can reflect the different stages of bone cell cycle, such as differentiation or activity of osteoblast and osteoclast. N-

terminal propeptides of type I collagen (PINP) are derived from collagen biosynthesis during bone formation. Osteocalcin is the most abundant non-collagenous protein in bone. It is produced by osteoblasts as an intact form during bone formation. Fragments of osteocalcin are released also during bone resorption and, therefore, whether osteocalcin is a marker of bone formation or bone resorption depends on what forms of osteocalcin the assay method detects. Tartrate-resistant acid phosphatase isoform 5b (TRACP 5b) is a glycoprotein specifically expressed in osteoclasts and secreted into the circulation. TRACP 5b is secreted from non-resorbing and resorbing osteoclasts and therefore it can be considered as a marker of osteoclast number. C-terminal cross-linked telopeptide of type I collagen (CTX) is a peptide derived from the cross-link region of collagen. This degradation product is produced by osteoclastic enzymes during bone resorption.

Markers of bone turnover have been demonstrated to reflect long-term changes of bone mass and quality in clinical studies. In preclinical studies, PINP and osteocalcin have been demonstrated to reflect bone formation and rate of bone turnover whereas TRACP 5b and CTX have been demonstrated to indicate the number of osteoclasts and osteoclast activity, respectively. At present, measurements of bone mineral density (BMD) and histomorphometry are the golden standard analyses in diagnostics and bone quality assessment in clinical and preclinical bone studies. Markers may not become overall substitutes for these analyses, but they might provide additional information on the rate of bone loss as they reflect total skeletal metabolism. This study was designed to further clarify the roles of PINP, osteocalcin, TRACP 5b and CTX in preclinical osteoporosis models. In addition, particular emphasis was on the utility of these markers as prognostic markers in drug efficacy studies.

2. REVIEW OF LITERATURE

2.1 The skeleton

Bone is the main component of an adult human skeleton. Bones are composed of bone tissue, which is a highly specialized form of calcified connective tissue. Bones are connected to other bones and to muscles with ligaments and tendons, or other specialized forms of connective tissue. The human skeleton comprises of 206 bones, excluding the sesamoid bones. The skeleton is divided into the axial and appendicular skeleton. There are 80 bones in the axial and 126 bones in the appendicular skeleton. The axial skeleton consists of bones along the central axis of the body, such as skull, vertebra and ribs, whereas the appendicular skeleton consists of long bones such as femur, tibia and humerus. The skeleton has several fundamental functions. It serves as a protection for vital organs, gives structural framework to the body providing body shape and permits movement by providing levers for the muscles. Classically bone was considered to be a structural organ. Today, it is well known that bone is metabolically active tissue serving as a mineral reservoir and regulator for calcium and phosphate homeostasis, environment for hematopoiesis, and a depository for growth factors and cytokines that can be released from bone in the resorption process and exerted locally and systemically. A less well known function of bone is regulating the acid-base balance (Arnett 2003). Bone functions also as an endocrine organ as it is a regulator of a number of other metabolic processes independent of mineral metabolism (Guntur et al. 2012).

The skeletons of humans and rats share many similarities (Figure 1). As all vertebrates, rats have a skeleton made of bones and connected with joints and cartilage. A rat skeleton has 223 bones that can be categorized into the axial and the appendicular skeleton similar to those of the human skeleton. In addition, the main functions of the rat and human skeleton are the same. The main differences between human and rat skeletons are related to the smaller skeletal size and faster bone turnover rate in rats. Moreover, rat bones grow in length through almost their entire lifespan which is in contrast to closure of the human epiphyses early in life. At the anatomical level rats have dramatically different cranial shape, relatively bigger ribcage, a long and narrow pelvis and they lack spinal curvature. The difference in long bone anatomy between humans and rats is related to locomotion. As quadrupeds, rats have dual axes of orientation and their functional anatomy is a consequence of movement in all four limbs. In contrast, the femora of humans are angled and the tibae have thicker proximal surfaces as a result of bearing greater weight. The lumbar curvature in humans is a critical component in the ability to achieve upright and bipedal locomotion (Robinson et al. 1973). Rat bones have also greater density and thickness relative to size and they are less porous in cross section than human bones. Despite the differences at the macroscopic level, rats and humans have closely related bone structure and physiology. Due to this reason rats have commonly been used in preclinical bone studies. In particular, rats have been used to evaluate safety and efficacy of new osteoporosis drugs as the ovariectomy-induced osteopenia in the rat reflects skeletal responses similar to that in the post-menopausal woman.

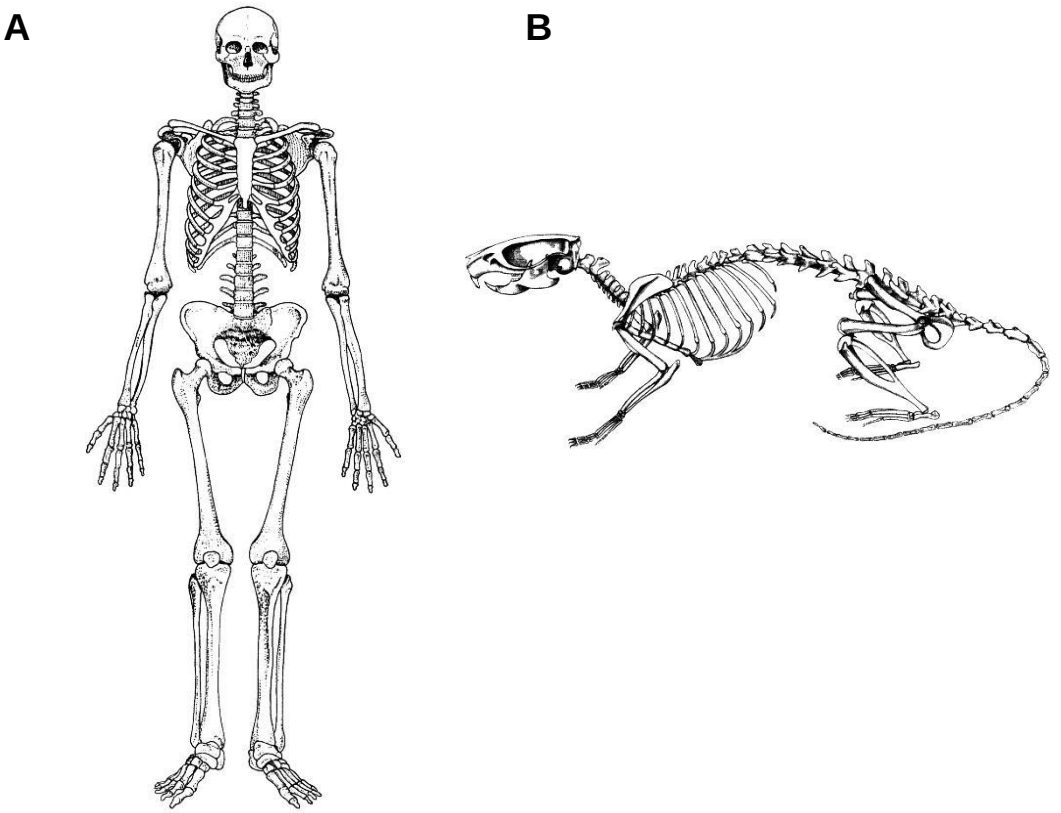

Figure 1. A schematic representation of the human (A) and rat (B) skeleton. Anterior view of adult human skeleton and lateral view of rat skeleton. Modified from Merriam-Webster visual dictionary at: http://visual.merriam-webster.com

2.2 Bone structure and development

Bone is anatomically classified into four different types based on their shape: flat bones, short bones, long bones and irregular bones, of which flat bones and long bones are the most common type of bones. Flat bones and long bones have two distinct developmental origins: endochondral and intramembranous ossification. Endochondral ossification is the more common type of bone formation, giving rise to long bones that comprise the bones in appendicular skeleton whereas intramembranous ossification forms the flat bones orientated in the axial skeleton. Both ossification processes involve condensation and aggregation of mesenchymal stem cells and eventually the formation of calcified bone. In endochondral ossification, condensed mesenchymal cells differentiate to prechondroblasts and form an anlage for the future bone. Hyaline cartilage anlage is later replaced with bone, except on the articular surfaces (Horton 1990). In intramembranous ossification, condensed mesenchymal stem cells differentiate into osteoblasts which form the basis of ossification center in the fibrous connective tissue membrane. Osteoblasts deposit organic bone matrix also known as osteoid within the fibrous membranes. Osteoid is subsequently

mineralized and newly calcified bone matrix is formed. This irregularly oriented woven bone is eventually remodeled and replaced to orientated lamellar bone by osteoclasts and osteoblasts. In contrast, rats have lesser organized bone structure resembling rather woven bone than lamellar bone.

The interior of all types of bones is composed of bone marrow which is surrounded by two morphologically different types of bone tissue, trabecular or cancellous or spongy bone and cortical or compact bone (Figure 2). Trabecular and cortical bone are normally formed in a lamellar pattern, in which collagen fibrils and minerals are deposited in an organized manner. Lamellar bone has significant elastic properties and strength as a result of the alternating orientations of collagen fibrils. At the microscopic level, both trabecular and cortical bone are composed of the same structural units referred to as osteons, which are composed of stacks of lamellae. In cortical bone, osteons are commonly known as Haversian systems, whereas in trabecular bone, osteons are referred to as packets. Blood vessels are located at the center of the Haversian systems referred to as the Haversian canals. However, divergent osteon morphologies have also been reported (Cooper et al. 2011). Although both morphological types of bone tissue result from the same bone cell activities and the same matrix elements, they have distinct roles in the skeletal system. The ratio of cortical and trabecular bone within different bones varies and it is dependent on the function and the anatomical type of the bones. Cortical bone is generally orientated at the bone surface while the interior of bones is composed of trabecular bone. The extracellular matrix (ECM) of porous trabecular bone is loosely mineralized and organized, whereas ECM of dense cortical bone is highly mineralized and organized. Therefore, cortical bone comprises about 80% of the bone mass and trabecular bone only 20% (Eriksen et al. 1994). Despite the low mass, trabecular bars and plates interspersed in the bone marrow compartment provide a large surface for active metabolism. Since trabecular bone has greater surface area than cortical bone, its remodeling rate is higher as well. In general, trabecular bone has an active role in bone metabolism, whereas cortical bone mainly provides a protective and mechanical function. Although trabecular bone is less dense and stiff, it is significantly more ductile than cortical bone. Therefore, trabecular bone has a vital role in the overall mechanical strength of the bone and particularly in its elastic properties.

Flat bones are composed of thin layers of compact bone enclosing trabecular bone between them. The large surface of flat bones provides extensive surfaces for muscle attachments and mechanical protection. Long bones consist of a cylindrical hollow shaft in the middle called diaphysis linked to a rounded end portion called epiphysis and a flared cone-shaped junctional region between them called metaphysis. Epiphysis and metaphysis are composed primarily of trabecular bone while diaphysis is made of cortical bone. The platelike structure between epiphysis and metaphysis called the growth plate is the site where majority of the longitudinal growth of a long bone occurs. Appositional growth occurs at the outer rim of cartilage called perichondrium. Metaphyseal trabecular bone has two spongious regions referred to as the primary and secondary spongiosa which have distinct distribution of bone cells and bone tissue. The primary spongiosa is a region of high turnover which produces woven bone. When woven bone is remodeled to lamellar bone it is referred to as the secondary spongiosa where turnover is relatively low. The rate of rodent bone formation in the primary spongiosa is much faster than in humans (Kimmel et al. 1980). The metaphyseal region is the site where bone loss is most evident. It is therefore the typical site for BMD measurements and histological evaluations in clinical practice and animal studies.

The outer surface of bone is connected to surrounding tissues with a dense layer of connective tissue called periosteum. The endosteum is covering internal surface of bone and it lines the marrow cavity. Bone growth in diameter occurs by bone formation beneath the periosteum. Osteoclasts in the endosteum continue to resorb bone until appropriate thickness is achieved. With aging, bone resorption often exceeds bone formation particularly on the endosteal surface, leading to an expansion of the marrow cavity. For the first two decades, bones grow and the elongation of long bones ends when the growth plates fuse with metaphyses. In contrast to humans, rodents have active growth plate throughout their lives (Kronenberg 2003). The amount of bone tissue present at the end of skeletal growth and maturation has been determined as peak bone mass. It is considered to be one of the key determinants for future bone loss (Riggs and Melton 1986). Thus, although osteoporosis usually appears in advanced age, its roots are traced back into adolescence and the growth phase of bones. The peak bone mass of a young healthy adult is also used as reference when assessing BMD in clinical practice.

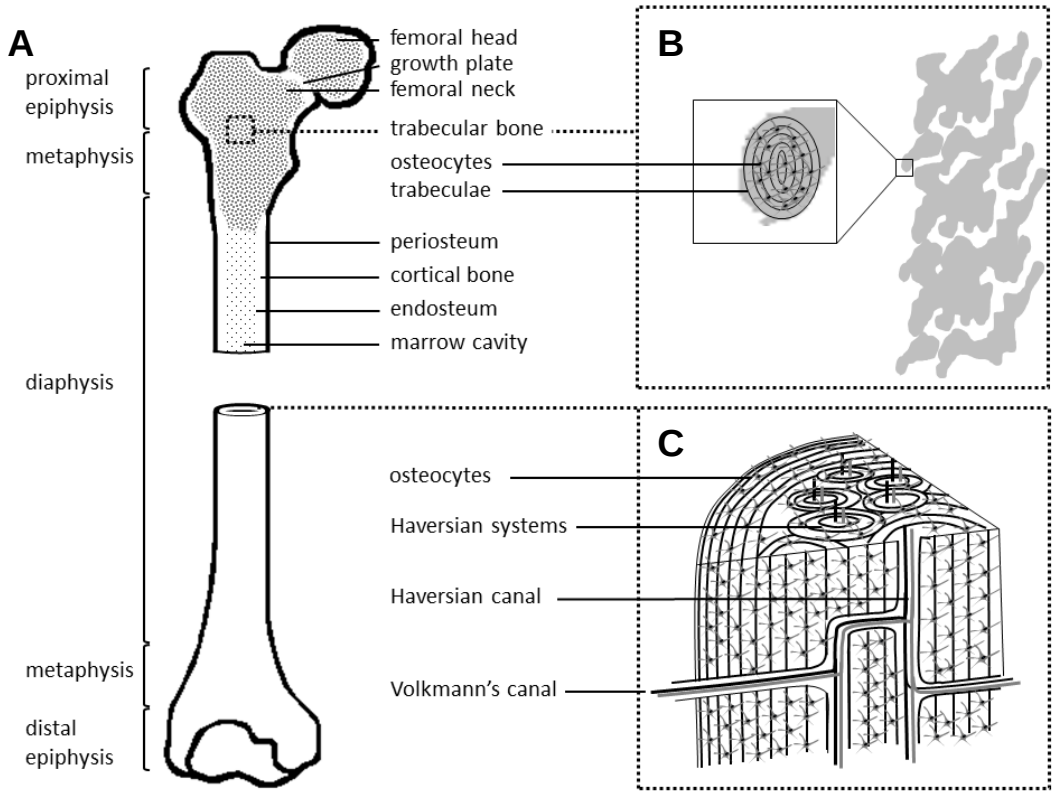

Figure 2. A schematic representation of the human femur (A) showing in detail the structure of trabecular bone (B) and cortical bone (C). Haversian canals are lengthwise the central canal of the Haversian system (osteon) enclosing blood vessels and nerves, while Volkmann's canals are the respective transverse canals of compact bone. Proximal; closer to the midline of the body, distal; further from the midline of the body.

2.3 Bone matrix

Bone tissue, as other connective tissues, is characterized by relatively large ECM content compared with the amount of cells. The bone tissue is composed primarily of organic and inorganic matrixes, which together provide its unique mechanical, homeostatic and protective functions. Bone ECM is first deposited as an organic bone referred as osteoid which is later mineralized. Distribution of organic and inorganic matrixes by weight varies with age, turnover state, anatomic location, diet, health and medications, but it is typically 50-70% mineral, 20-40% organic matrix, 5-10% water, and less than 3% lipids. The cellular content is less than 1% of the bone mass (Robey and Boskey 2008). The organic matrix contains numerous proteins and proteoglycans. Of these, type I collagen is the major component in bone matrix. It constitutes up to 85-90% of the bone total protein content. Non-collagenous proteins constitute the remaining 10-15% of the protein content. The most important non-collagenous proteins are classified as proteoglycans, glycosylated proteins, glycosylated proteins with cell attachment activities and γ-carboxylated (Gla) proteins (Robey and Boskey 2008). The organic matrix provides elasticity and flexibility for bones and determines structural base for deposition of inorganic matrix (Marks and Odgren, 2002). The inorganic matrix is predominantly calcium and phosphate in the form of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, which is responsible for the rigidity of bone. 99% of the body calcium, 85% of the phosphate and 50% of the magnesium are stored in the skeleton (Breslau 1996).

2.3.1 Organic matrix

Type I collagen

Collagens are the most abundant proteins in the body. Today, at least 28 different types of collagens have been identified (Gordon and Hahn 2010). Collagen in bone is the building block of the fiber network where minerals are deposited. Collagens are a large family of trimeric extracellular matrix molecules that provide structural integrity. The basis of the collagen molecule is three parallel alpha chains which form the triple helical part of the molecule. Each alpha chain is coiled in a left-handed helix while the whole triple helix is right-handed. The chains are composed of repeating peptide triplets of glycine-X-Y motifs, where X and Y are often proline or hydroxyproline. This unique triplet structure is essential for the stability of the triple helix (Rossert et al. 2002).

Type I collagen is the most abundant protein in bone. Type I collagen is also expressed in most of the other connective tissues, especially in tendons, ligaments and dermis (Myllyharju and Kivirikko 2001). In bone, type I collagen is produced by osteoblasts and it forms covalent cross-links, which differ from those that are found in other connective tissues. Measuring these bone-derived cross-links is widely used in assessment of bone turnover. Type I collagen is a heterodimer of two α1 and one α2 chains (Kimura 1983). Large triple helical middle is approximately 300 nm in length and 1.5 nm in diameter flanked by short non-helical telopeptide regions at each end of the molecule. Cross-striated fibrils give the tissues their mechanical strength.

Several phases can be identified in the synthesis of type I collagen: Initially, α1 and α2 chains form intrachain and interchain disulphide bonds. Formation of the triple helix begins at the C-terminus end and continues towards the N-terminal end (Kielty and Grant 2002).

After intracellular post-translational modifications, the triplehelical procollagen molecule is secreted into the extracellular space. In the extracellular space, propeptidases then convert the procollagen molecule into collagen by cleaving the propeptides and eventually incorporate it into stable, cross-linked collagen fibrils. At this stage of collagen synthesis, N-terminal (PINP) and C-terminal (PICP) propeptides are released into the circulation and they can be used to assess bone formation rate (Risteli et al. 1997). After the cleavage of propeptides, mature collagen molecules begin to aggregate and form collagen fibrils. Fibrils form larger fibrils by growing longitudinally and laterally. Finally, interchain covalent bonds are formed between collagen molecules. Between two collagen molecules, empty 40 nM gaps serve as sites for mineralization.

Many macromolecules such as proteoclycans and integrins bind to type I collagen and thus regulate their synthesis or function. Collagen fibrils in bone contain also traceable amounts of other type of collagens, such as types III, V, XII and FACIT collagens (fibril-associated collagens with interrupted triple helices). FACIT is a group of nonfibrillar collagens that are important for the stability and organization of ECM (Robins and Brady 2008). Type V collagen is located in the core of collagen fibril and types III and XII on the surface. These other types of collagen are present in certain stages of the bone formation process and they may participate in regulating the collagen fibril diameter. Type II collagen is not present in bone but it is the most abundant collagen in cartilage. Measurement of cartilage-derived cross-links of type II collagen is used in assessment of cartilage metabolism.

Non-collagenous proteins

Proteoglycans are macromolecules containing acidic polysaccharide side chains called glycosaminoglycans (GAGs) covalently attached to a linear core protein. In cartilage, proteoglycans provide elastic properties for the tissue. It is assumed that they play a similar role in bone. In bone, two small chondroitin sulphate proteoglycans, biglycan and decorin, are highly enriched. They belong to a family of small proteoglycans known as SLRPs (small leucine-rich proteoglycans). They bind to type I collagen but they also interact with several important growth factors, including transforming growth factor beta. Therefore, it is assumed that they play a part in modulating the activity of growth factors and matrix proteins (Hildebrand et al. 1994). Biglycan deficient knock-out mice displayed a phenotype of decreased bone mass and reduced growth rate resulting in delayed peak bone mass, which is why it is suggested to be a determinant of bone mass (Xu et al. 1998). Decorin deficient knock-out mice had skin fragility (Danielson et al. 1997) and biglycan and decorin deficient double knock-out mice showed severe collagen related abnormalities in bone and skin (Ameye et al. 2002).

Glycosylated proteins (non-RGD containing glycoproteins) have more protein and fewer carbohydrates in their structure than proteoglycans. Most of the glycosylated proteins that are present in bone have been identified also in the cartilaginous ECM (Robey and Boskey 2008). Many glycoproteins are phosphorylated or sulfated and they can be further classified into groups of RGD or non-RGD containing glycoproteins based on their capacity to mediate cell attachment. RGD containing glycoproteins have RGD tripeptide sequence (Arg-Gly-Asp) that binds to the cell-surface integrin molecules (Ruoslahti et al. 1996) and thus facilitates cell adhesion.

Non-RGD containing glycoproteins in bone include alkaline phosphatase, osteonectin and tetranectin. The non-RGD containing glycoproteins of bone matrix have various functions in bone metabolism. They control osteoblast proliferation and activity via regulating the mineralization. The main glycosylated proteins that are present in bone are alkaline phosphatase and osteonectin. Alkaline phosphatase (ALP) is an enzyme that is responsible for dephosphorylation and, as the name suggests, it is most effective in an alkaline environment. Several isoforms of ALP have been identified, including nonspecific bone, germ-cell, intestinal, kidney, liver and placental ALP. The major source of the total ALP in human serum is liver and bone. ALP produced by both of these is encoded by the same genes (Moss 1982) and therefore it is often difficult to distinguish these two isoforms. However, new assay methods have shown improved diagnostic accuracy for bone specific isoform of alkaline phosphatase (BAP or BALP). ALP was one of the first key players in the osteogenesis that was recognized (Moss 1982). Despite the long history, its role in bone metabolism has remained partly unknown. ALP is found on the surface of osteoblasts and within the mineralized matrix (Väänänen et al. 1987). In bone, ALP is derived from osteoblasts and other cells of osteoblast lineage, and it has a role in bone mineralization. ALP may increase the local concentration of inorganic phosphate, destroy local inhibitors of matrix mineralization, transport phosphate and act as a calcium carrier (Kanis 1994, Golub 1996). Deletion of ALP in mice results in abnormal pattern of mineralization, which confirms the significant role of ALP in bone mineralization. As ALP is expressed in osteoblasts and their precursors, it is an important histochemical marker for osteoblasts.

Osteonectin (also referred as SPARC) binds to collagens, hydroxyapatite and several growth factors. It has been suggested to nucleate mineral deposition into the collagen fibril network (Termine et al. 1981). Osteonectin deficient mice exhibit decreased bone remodeling leading to osteopenia in older animals. The bone formation rate in these mice is decreased nearly 50% in both trabecular and cortical compartments, but interestingly, osteopenia is observed primarily in trabecular bone (Delany et al. 2000). Tetranectin has been identified in woven bone and it is also assumed to be involved in mineralization (Wewer et al. 1994).

Glycosylated proteins with cell attachment activities (RGD containing glycoproteins)
Bone matrix contains several RGD containing glycoproteins, including bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE), osteopontin (OPN), fibrillin, fibronectin, thrombospondins and vitronectin from which the first four belong to the SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein) family (Robey and Boskey 2006). Of these, sialoproteins osteopontin and bone sialoprotein are the most abundant and best known sialoproteins in bone. OPN and BSP are known to play a role in osteoclast attachment to bone. They both bind to calcium and especially hydroxyapatite with extremely high affinity. In addition to cell attachment, OPN has at least three other roles in bone. It regulates osteoclast differentiation and activity, matrix mineralization and, possibly, also angiogenesis. In bone, osteoblasts, osteocytes and osteoclasts express OPN (Noda et al. 2002). Despite their hypothetically vital role in bone, OPN deficient mice do not have severe skeletal abnormalities. Their osteoclast number is increased at the metaphyseal trabecular bone, but, despite this observation, their osteoclasts are resistant to ovariectomy induced bone resorption. This is most probably due to a compensatory mechanism where impaired resorption capacity of the osteoclasts results in an increase in their number (Yoshitake et al. 1999). BSP is the main sialoprotein in bone. 50% of it is composed of carbohydrate, of which approximately 12% is sialic acid. BSP knock-out mice exhibited diminished bone growth and impaired mineralization (Malaval et al. 2008). DMP1 and MEPE are both regulators of mineralization. DMP1

deficient mice display severe osteomalacia, whereas MEPE deficient mice exhibit increased bone mass due to increased number and activity of osteoblasts (Gowen et al. 2003). Fibronectin, thrombospondins, vitronectin and fibrillin are mainly involved in cell attachment functions and they mostly interact with matrix proteins, such as collagens, and thus regulate the matrix formation.

γ-carboxylated (Gla) proteins contain γ-carboxyglutamic acid (Gla) residues that enable their calcium-binding properties. They are also referred to as vitamin K-dependent proteins or γ-carboxylated proteins. Calcium binding capacity requires vitamin K-dependent gamma-carboxylation of three glutamic acid residues. The most important Gla proteins identified in bone include osteocalcin (OC, bone Gla protein) and matrix Gla protein (MGP). Of these, osteocalcin is the most common non-collagenous protein in bone. It is also among the most common proteins in the body (Hauschka et al. 1989). Osteocalcin has several roles in bone metabolism. Its intact form is secreted by osteoblasts during bone formation, but fragments of osteocalcin are also detected during bone resorption. As it is produced by osteoblasts, it is often referred to as a marker of bone formation. Osteocalcin has high affinity to bone mineral and it has been speculated to regulate mineralization (Romberg et al. 1986). Osteocalcin also has a role that is not specific to bones. It functions as a hormone in the body, causing the release of insulin by beta cells in the pancreas (Lee et al. 2007). Osteocalcin-deficient mice have increased bone mass and impaired mineral maturation (Ducy et al. 1996). MGP is an important inhibitor of calcification process. MGP knock-out mice die within two months as a result of calcification in arteries which leads to blood-vessel rupture (Luo et al. 1997).

In addition to the proteins discussed above, bone contains several other non-collagenous and non-proteoglycan proteins. These are mainly growth factors, enzymes and their inhibitors. Their role is reviewed in more detail in the chapters about bone cells and bone remodeling.

2.3.2 Inorganic matrix

70% of bone is composed of the inorganic mineral hydroxyapatite (HA). It is analog for the geological form of hydroxyapatite. HA is predominantly crystalline, although it may be present in amorphous forms (Termine et al. 1967). In contrast to the geological form, the crystals in bone are rod or platelet shaped and extremely small; about 10 – 40Å wide and thick and 200 - 400Å long. The size and distribution of crystals influences the mechanical strength of the bone (Boskey 2003). Bone mineral is not entirely stoichiometric HA as it contains several substituted ions, such as acid phosphate, calcium carbonate, calcium fluoride, calcium hydroxide, magnesium, sodium, potassium, chlorine and citrate. Of these, the proportion of carbonate ions in the HA lattice is 2-8%, and thus it has been suggested to play a significant role in the biochemistry of hard tissues (Zapanta-LeGeros 1965). It has been further demonstrated that osteoclasts adhere and resorb better carbonate substituted HA than pure HA (Nakamura et al. 2013). In addition, some heavy metals, like cadmium, or cations, such as strontium and radium, can incorporate into bone and substitute calcium in the crystal. This phenomenon has been utilized when developing new bone seeking drugs for skeletal diseases. Hydroxyapatite is often referred to as carbonate substituted poorly crystalline apatite (Glimcher 1984). These small imperfect crystals are more soluble than geological apatite. As a result, hydroxyapatite is easily dissolved in an acidic environment

enabling bone to function as a reservoir for vital ions, such as calcium, phosphate and magnesium.

Deposition of hydroxyapatite by osteoblasts is dependent on the orientation of collagen fibrils. Hydroxyapatite crystals are always deposited parallel to the axis of the collagen fibril. The initial deposition of minerals is followed immediately after collagenous matrix has been synthesized and laid down. As bone matures, crystals aggregate and become larger. The size of the crystals is dependent on the presence of impurities and the composition and orientation of the organic matrix. There are several factors that have been associated with the regulation of mineralization process. Type I collagen and SIBLING proteins have been shown to promote hydroxyapatite formation (Qin et al. 2004). Important regulators are also phosphoprotein kinases and alkaline phosphatase, which enhance the mineralization rate. After rapid primary mineralization of osteoid tissue secondary mineralization occurs. This rather slow mineralization process eventually leads to higher bone density and is likely to last for several months or even years (Boskey 2006).

2.4 Differentiation and function of bone cells

Four different types of bone cells are responsible for formation, maintenance and resorption of bone tissue; osteoblasts, osteocytes, bone-lining cells and osteoclasts. First three of these originate from mesenchymal cells and are responsible for bone formation and maintenance of bone tissue while osteoclasts originate from hematopoietic mononuclear cells and are responsible for bone resorption (Kahn and Simmons 1975). Various hormones, vitamins, growth factors, matrix proteins and transcription factors regulate the differentiation and activity of these cells (Boyle et al. 2003). Bone cells also form intracellular network via gap junctions, which is assumed to play a central role in signaling between cells (Noble et al. 2000).

2.4.1 Osteoblasts

Osteoblasts synthesize the majority of the proteins of bone matrix and are responsible for mineralization. Osteoblasts also play a vital role in regulation of osteoclast differentiation and activity. Osteoblasts, osteocytes and bone-lining cells are derived from the same lineage of mesenchymal multipotent stem cells that also give rise to adipocytes, chondroblasts, fibroblasts and myoblasts. Marrow stroma is an important source of these mesenchymal cells. The first step in osteogenic lineage is the formation of osteoprogenitor cells. They are proliferative cells present at sites near the bone surface such as endosteum and periosteum. Osteoblasts are the first type of mature cells in this lineage. They are cuboidal shaped mononuclear cells (Figure 3) found as clusters along the bone surface of recently resorbed bone. At the cellular level, osteoblasts show typical secretory characteristics with abundant cytoplasmic organelles, including well-developed rough endoplasmic reticulum, a large Golgi complex, vesicles and vacuoles containing fibrillar structures which are assumed to represent pro-collagen and proteoglycans. During the differentiation from osteoprogenitor cells to a mature osteoblast, three different phases can be distinguished: proliferation, matrix formation and mineralization (Dworetzky et al. 1990). There are distinct subpopulations within the osteoblast lineage. Osteoblasts at the axial skeleton respond differently in regulatory signals than osteoblasts at the appendicular

skeleton (Aubin 2006). In addition, the cellular and the tissue environment affects the function of osteoblasts.

Osteogenic pathways hedgehog (Hh) and wingless and Int1 proteins (Wnt) influence the transcription of many genes (Hooper & Scott 2005). Hh signaling promotes early stages of osteoblast differentiation by activating the expression of Runt-related transcription factor 2 (Runx2, also known as Cbfa1), type I collagen and alkaline phosphatase (Hu et al. 2004). Runx2 alone is not capable to induce osteoblastogenesis. Osterix (Osx), a downstream factor for Runx2, has been demonstrated to play a vital role in the later stages of osteoblast differentiation. Runx2 and Osterix are assumed to be key regulators for osteoblast differentiation (Lian et al. 2004) and mutations in these genes exhibit dramatic phenotypes. Runx2 deficient mice lack osteoblasts and thus bone, and show severe abnormalities in cartilage (Komori et al. 1997), whereas overexpression of the Runx2 protein has been linked to the development of bone cancer (Perinpanayagam et al. 2004). Osterix deficient mice also lack osteoblasts and exhibit defective bone formation (Nakashima et al. 2002). In addition to these regulatory functions, Runx2 activated osteogenesis is mediated through several target genes, including OPN, osteocalcin and BSP (Ducy et al. 1997).

The Wnt proteins are secreted signaling molecules, which are important in osteoblast maturation, skeletal acquisition, and maintenance (Day et al. 2005). Wnt/β -catenin pathway (also referred to as the canonical pathway) integrates signals from other pathways, such as fibroblast growth factor (FGF), transforming growth factor β (TGF-β), and bone morphogenetic protein (BMP) pathways. The canonical pathway is controlled by several other factors. Dkk1 and Dkk2, the members of the Dickkopf (Dkk) protein family, together with their receptors Kremen1/2, inhibit Wnt/β-catenin signaling through internalization of low-density lipoprotein receptor related protein 5/6 (Lrp 5/6) from the cell surface (Li et al. 2002). Mutations on the proteins involved in this pathway, such as Lrp5/6, lead to abnormalities in bone. Both canonical and non-canonical pathways are involved in coordinating osteoblast functions. The non-canonical Wnt pathway includes the planar cell polarity pathway and the Wnt/Ca^{2+} pathway which regulate the intracellular cytoskeleton and calcium content, respectively. The mechanisms on how non-canonical Wnt pathways are activated are not well understood. However, recent findings suggest that Wnt16, which has been demonstrated to signal via the non-canonical pathway, has a vital impact on bone mass and its strength as well as osteoporotic fracture risk (Zheng et al. 2012). The canonical Wnt/ β -catenin pathway stimulates also Runx2 expression in the early stages of osteoblastogenesis, and, in the later stages, it downregulates receptor activator of nuclear factor kappa-B ligand (RANKL, also known as ODF or TRANCE) expression (Spencer et al. 2006). Therefore, mature osteoblasts do not synthesize RANKL, but instead they synthesize osteoprotegerin (OPG, also known as OCIF), an inhibitor of RANKL (Simonet et al. 1997). The inhibition of Wnt signaling and β -catenin prevents maturation of osteoblasts (Hu et al. 2004). Sclerostin and Notch signaling pathways are among the best known inhibitors of Wnt/ β -catenin signaling.

Several other systemic and local factors have been shown to regulate osteoblast differentiation and maturation. Particularly estrogen, glucocorticoids, vitamin D, parathyroid hormone (PTH) and PTH-related peptide (PTHrP) play a major role in regulation of osteoblast differentiation. In addition to these systemic regulators, osteoblast maturation requires local factors, such as BMPs, FGFs, TGF-β and insulin-like growth factors (IGFs) (Ducy et al. 1997, Schinke and Karsenty 1999). These growth factors are stored in bone

matrix, released when needed and exerted locally or systemically. Thus, calcified bone matrix acts as a reservoir for growth factors, similarly as for calcium and phosphates. BMPs include the TGF-β superfamily, and their vital function is to facilitate mesenchymal cells into the osteoblastic lineage (Canalis et al. 2003). Several multifunctional BMPs have been identified (Reddi et al. 2009). In osteoblasts, they regulate growth, differentiation and apoptosis. At least BMPs 2-7 have been shown to induce bone formation mostly by upregulating Runx2 expression (reviewed by Yamaguchi et al. 2000) and thereby inducing osteoblastogenesis. Mature osteoblasts, as all other osteoblast lineage cells, express significantly ALP activity on their plasma membrane. This histochemical feature has been traditionally used as a marker of differentiation for this lineage.

Interaction between cells among the osteoblast-lineage and cell-matrix interaction among matrix proteins are also important for osteoblast differentiation and function. Osteoblast lineage cells are connected through gap junctions. Thus, gap junctions are engaged to respond to various physiological signals. During matrix formation, osteoblasts produce type I collagen, alkaline phosphatase and BSP. Newly formed bone matrix is not immediately calcified. Osteoid, uncalcified organic matrix is deposited first. The quantity of osteoid is associated with the bone-forming activity of osteoblasts. During matrix mineralization, the osteoblasts express osteocalcin, OPN and collagenase. At this stage the formed osteoid is mineralized with inorganic hydroxyapatite. Regulation of bone formation is discussed in more detail in the chapter about bone remodeling.

Toward the end of the bone formation period, osteoblasts become isolated and embedded into bone lacunae, and they can become a lining cell, an osteocyte or can undergo programmed cell death via apoptosis (reviewed by Manolagas 2000).

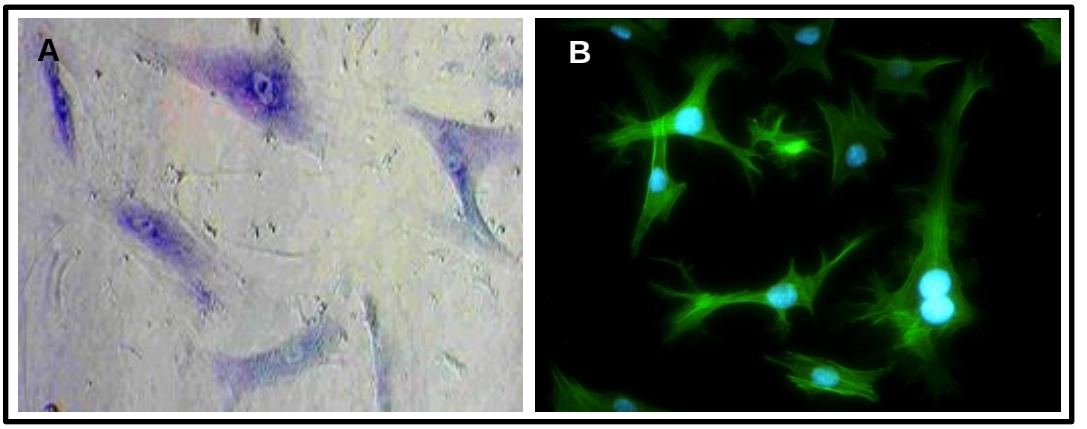

Figure 3. Photomicrographs of osteoblasts and osteocytes. Toluidine blue stained human primary osteoblasts (A) and fluorescence microscopic image of mouse primary osteocytes (B). Overlay image of actin filaments (green) and nuclei (blue). Unpublished data.

2.4.2 Osteocytes and lining cells

Previously, the majority of bone cell studies have focused on mature osteoblasts and osteoclasts and the role of osteocytes and bone lining cells has largely been left unexplored. Today, increasing interest has been directed towards these two types of bone cells. Osteocytes are the final stage of cell differentiation in the osteoblast lineage. They are osteoblasts which have become embedded in bone matrix, after which they are referred to as osteocytes. Approximately every tenth osteoblast undergoes differentiation into an osteocyte (Noble 2003). Osteocytes are the most common bone cells found throughout the skeleton. Their lifespan is much longer than that of other bone cells as they have the potential to live for decades in healthy bone. Osteocytes are stellate-shaped cells (Figure 3) which lack most of their cytoplasmic organelles. Despite this, embedded osteocytes have active cytoplasmic signaling through a dendritic-like network throughout the bone matrix. With this network, osteocytes can transmit signals over relatively long distances, analogous to the nervous system. Osteocytes signal through the small canals called canaliculi, which form a communication network between mechanical and biochemical signals. The canalicular network is also crucial for the survival of osteocytes, because it is a metabolically and electronically active route (Lian et al. 2004). This large network suggests an important role for osteocytes in bone metabolism.

Osteocytes have been suggested to be multifunctional cells that are even orchestrators of the whole of bone remodeling, as they can regulate bone formation and resorption in response to mechanical and hormonal stimuli (Bonewald 2011). Osteocytes are a source of bone remodeling growth factors and signaling molecules. As the osteoblast differentiate into an osteocyte, alkaline phosphatase expression is reduced, and osteocalcin expression is elevated (Mikuni-Takagaki et al. 1995). In addition, osteocytes produce DMP1, nitric oxide (NO), OPN, osteonectin, and sclerostin, modulators of bone formation. Of these, sclerostin is an inhibitor of bone formation while the others are inductors. Sclerostin is encoded by SOST gene (Poole et al. 2005). It inhibits the Wnt signaling pathway and, as a result of this, it antagonizes the activity of BMPs, which are cytokines that induce bone and cartilage formation. Osteocytes are the only cells that express sclerostin, which makes them an attractive target for drug development. β-catenin is another key regulator in the Wnt signaling pathway. Osteocyte Wnt/ β-catenin signaling is crucial in bone development and for normal bone homeostasis. Overexpression of β-catenin is associated with many cancers (Morin 1999). Osteocytes are a significant source of RANKL, a cytokine that supports osteoclastogenesis. Furthermore, under certain conditions osteocytes can also express osteoclast markers, including acid phosphatase and cathepsin K (Qing et al. 2012). In addition, osteocytes are the main source of phosphate regulator FGF-23 that targets the kidney and other organs. This demonstrates that osteocytes act also as endocrine cells by involving systemic regulation of phosphate homeostasis. Recent studies support the assumption that the osteocyte lacunar network is a storehouse for various rapidly released regulatory factors, even superior to bone matrix, which requires bone degradation by osteoclasts. If this assumption is elucidated in the future, preserving the function of the osteocyte network becomes an imperative and prominent target for drug development (Bonewald 2011).

Many studies have demonstrated the mechanical load-related responses in osteocytes, supporting their role as mechanotransducers in bone (Pead et al. 1988). Osteocytes are known to transduce mechanical stress into signals by sensing the fluid shear stress of extracellular fluids in the canalicular network. As a result of the mechanical stimulus,

osteocytes release factors that regulate bone remodeling in a similar manner as described above. As an example, mechanical unloading of osteocytes augments osteoclastogenesis by releasing RANKL. On the other hand, mechanical loading protects osteocytes against apoptosis. Loss of bone strength and quality with advancing age is associated with an increase in the prevalence of apoptotic osteocytes. In addition, osteocyte apoptosis induced by dexamethasone, a common side effect of glucocorticoids in bone, can be prevented by mechanical loading and estrogens (Gu et al. 2005a). Immobilization and reactive oxygen species (ROS) have been shown to induce hypoxia in osteocytes, which also leads to osteocyte apoptosis. The progressive-free radical damage is associated with normal aging and therefore the levels of ROS increase in bone with age and sex steroid deficiency. Osteocytes are enriched in proteins and mechanisms that are resistant to hypoxia and thus protect osteocytes from apoptosis (Almeida et al. 2007). With aging, osteocytes eventually die by apoptosis or necrosis. As a consequence, they leave behind empty lacunae that are frequently resorbed by osteoclasts (Bonewald 2011).

Instead of differentiating into osteocytes, certain resting osteoblasts at quiescent bone surfaces take flattened shape, after which they are referred to as bone lining cells. Their role is still poorly understood. It is speculated that they are not involved in modeling or remodeling phases since only little osteoid is seen under them. They have only few remaining cytoplasmic organelles and they are connected to each other and also to osteoblasts and osteocytes through gap junctions (Miller et al. 1989). They are capable of differentiating into osteogenic cells and thus serve a reservoir of osteoprogenitor cells. One suggested role for bone lining cells is the regulation of bone remodeling (Everts et al. 2002). Bone lining cells also participate in bone resorption by expressing RANKL and intercellular adhesion molecule 1 (ICAM-1), which are cytokines that promote osteoclast differentiation (Tanaka et al. 2000). Moreover, bone lining cells could migrate into resorption lacunae and remove the bone matrix leftovers by osteoclasts (Everts et al. 2002).

2.4.3 Osteoclasts

Osteoclasts, originally identified by Albert Kölliker already in 1873, are large multinucleate cells of hematopoietic origin responsible for bone resorption. An increase in the number or activity of osteoclasts induces osteoporosis. On the other hand, decrease in their number or activity leads to osteosclerosis or osteopetrosis, demonstrating that osteoclasts play a vital role in the bone turnover balance. Although some other types of cells may contribute the bone resorption, osteoclasts are the only cells capable of effective bone resorption. Osteoclasts are distinguished from other bone cells by morphology and function (Väänänen et al. 2000). At the morphological level, osteoclasts are relatively large cells containing several nuclei. They have well-developed Golgi apparatus around nuclei and numerous mitochondria and endoplasmic reticulum. They also contain various vesicles, lysosomes and vacuoles. These cytoskeletal structures are needed in the production of lysosomal enzymes required in bone resorption. Mature bone-resorbing osteoclasts are highly polar cells having their plasma membrane classified into three distinct regions: the basolateral membrane, the ruffled border and the sealing zone, which all have their unique roles in the resorption process. These membrane domains disappear once the resorption process is ceased. At the functional level osteoclasts have two unique features. They have ability to degrade both organic and inorganic bone matrix. Osteoclasts can create a highly acidic environment by enabling dissolution of the hydroxyapatite, whereas organic matrix is degraded by enzymes. Osteoclasts are in close contact with the osteoblast-lineage cells. It

has been assumed that cell-cell interaction between osteoclast precursors and osteoblast-lineage cells is vital for osteoclast differentiation (Takahashi et al. 1988b).

Osteoclasts differentiate from monocyte/macrophage lineage cells, which serve as precursors for osteoclasts. These CD14 positive mononuclear precursor cells circulate in the blood and start to proliferate and fuse into multinucleated osteoclasts at endosteal bone surfaces (Massey et al. 1999). Mature multinucleated osteoclasts capable of bone resorption are formed by the fusion of the mononuclear osteoclast progenitors (Scheven et al. 1986). Interestingly, recent data shows that osteoclasts can also form from mature dendritic cells through a process called transdifferentiation (Alnaeeli and Teng 2009). The role of this alternative pathway of osteoclast differentiation needs to be further elucidated. During the differentiation, multiple regulatory factors are needed. The most important cytokines known to regulate osteoclastogenesis are RANKL, its endogenous inhibitor OPG and macrophage colony stimulating factor (M-CSF), all produced by osteoblasts or stromal cells. M-CSF is required for proliferation and differentiation of osteoclast precursors and survival of mature osteoclasts. Its primary role is suggested to provide survival signals during osteoclastogenesis (Boyle et al. 2003). However, M-CSF alone is not enough to induce osteoclast differentiation. RANKL, a member of the tumor necrosis factor (TNF) family, participates in differentiation and maturation of osteoclasts via activating RANK receptor expressed on the plasma membrane of the osteoclast progenitors and mature osteoclasts (Lacey et al. 1998, Yasuda et al. 1998). RANKL signaling is mediated by TNF-receptor-associated factor 6 (TRAF 6) that binds to the cytoplasmic domain of RANK, resulting in the activation of nuclear factor NF-κB. This activation cascade is mediated by mitogen-activated protein (MAP) kinases, including c-Jun N-terminal kinase (JNK), p38 MAP kinase, and extracellular signal-regulated kinase (ERK) (Boyle et al. 2003). It is further demonstrated that JNK1-activated c-Jun signaling acts in concert with nuclear factor of activated T cells (NFATc1) and is essential to RANKL regulated osteoclast differentiation (Ikeda et al. 2004). NFATc1 is suggested to be involved in regulation of β3 integrin expression in co-operation with PU.1 during osteoclast differentiation (Crotti et al. 2008). PU.1 is an essential transcription factor at early phases of osteoclastogenesis since disruption of PU.1 gene results not only in lack of osteoclasts but also lack of macrophages (Tondravi et al. 1997). Moreover, stimulation of p38 MAP kinase results in the downstream activation of the microphthalmia-associated transcription factor (MITF), which regulates the expression of the genes that encode TRACP and cathepsin K (Mansky et al. 2002, Hodgkinson et al. 1993), and indicates the significance of p38 in these signaling cascades. Proto-oncogene c-Fos is also an essential transcription factor in osteoclastogenesis by stimulating proliferation of osteoclast progenitors (Grigoriadis et al. 1994). OPG is structurally analogous to RANK and functions as a “decoy” receptor for RANKL, thus inhibiting osteoclastogenesis (Simonet et al. 1997). The significant roles of RANKL and M-CSF were demonstrated by mouse knock-out studies where RANKL (Kong et al., 1999) or RANK (Dougall et al. 1999) deficient mice show osteopetrosis due to severe defect in osteoclastogenesis. Mice lacking M-CSF (Yoshida et al., 1990) or c-Fms (Dai et al. 2002), the receptor for M-CSF exhibit a similar phenotype. In contrast, OPG deficient mice exhibit severe osteoporosis due to the increased number of osteoclasts (Mizuno et al. 1998).

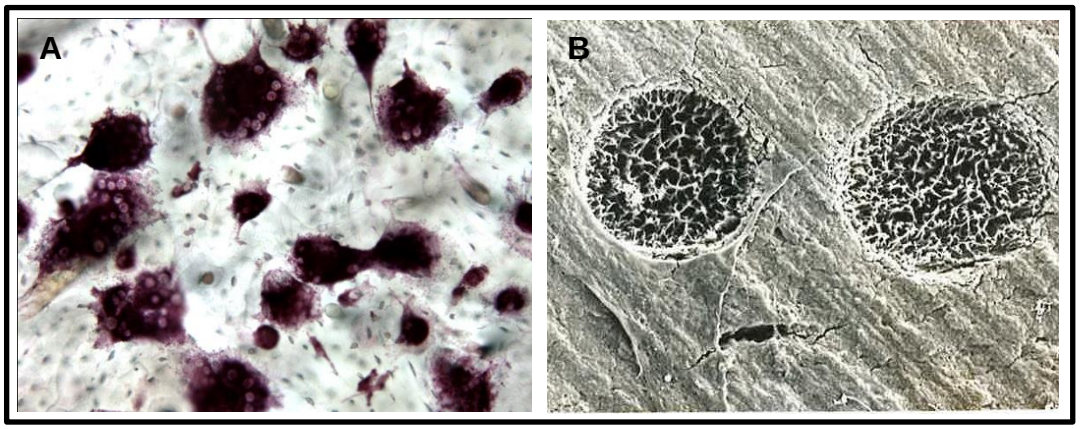

Figure 4. Photomicrographs of osteoclasts and resorption pits. TRACP stained human bone marrow derived osteoclasts on bovine bone surface (A) and FESEM electronmicroscopic image of resorption pits by human osteoclasts on bovine bone surface (B). Unpublished data.

Majority of the positive and negative regulators of osteoclastogenesis regulate RANK/RANKL pathway. Prostaglandin E2 (PGE2), 1,25-(OH)2 D3 (vitamin D), interleukins (IL) 1 and 11, TNF-a, PTH and PTH related protein (PTHrP) upregulate the expression of RANKL in osteoblast-lineage cells. Most of these are negatively regulated by estrogen. In contrast, estrogen stimulates synthesis of antiresorptive factors including OPG and TGF-β. Despite these detailed observations in signaling, the detailed mechanism by which these numerous factors coordinate osteoclast differentiation is still poorly understood. At the final stage of differentiation, the genes encoding proteins essential for inorganic and organic bone degradation are expressed (reviewed by Teitelbaum 2000).

When osteoclasts resorb bone (Figure 4), they anchor themselves on the surface of bone and form an isolated and acidified extracellular microenvironment between themselves and the bone matrix. Attachment to the bone results in significant polarity in osteoclasts via formation of distinct regions in their plasma membranes; a sealing zone, a ruffled border, and a basolateral membrane which, furthermore, has two separate regions, a functional secretory domain (FSD) and a basolateral domain (Väänänen et al. 2000). The ruffled border and sealing zone membrane domains are in direct contact with bone matrix whereas plasma membrane and FSD are in direct contact with other cells (Mulari et al. 2003). The sealing zone forms a tight attachment underneath the osteoclast, between the plasma membrane and the bone surface enclosing the resorption site from its surroundings. This resorption site underneath the osteoclast is referred to as the resorption lacuna (reviewed by Väänänen and Horton 1995). The most prominent morphological feature of resorbing osteoclast is the formation of actin ring at the site of the sealing zone when the cell becomes in contact with bone (Lakkakorpi et al. 1989). Cytoplasm at the sealing zone contains bundles of F-actin filaments which are organized as a ring structure during the resorption phase. Actin rings can be visualized by phalloidine staining as a marker of active bone resorbing osteoclasts. The ruffled border serves as targeted membrane for bidirectional trafficking pathway. Hydrochloric acid and proteolytic enzymes needed in the bone degradation and also bone degradation products from the resorption lacuna are transcytosed through the ruffled border. By secreting protons through the ruffled

border membrane, osteoclasts generate low pH at the resorption lacuna, which is required for dissolution of inorganic hydroxyapatite crystals. The organic matrix degraded by proteolytic enzymes is removed from the resorption lacuna through the ruffled border and further to the FSD via the transcytotic vesicular pathway. FSD appears on the uppermost region of the osteoclast and it serves as a transportation route for the matrix degradation products to the extracellular matrix (Nesbitt and Horton 1997, Salo et al. 1997, Väänänen and Zhao 2008). The transport through FSD is partly regulated by Ras-related small GTPases of the rab-protein family (Mulari et al. 2003, Sun et al. 2005). These unique plasma membrane domains allow osteoclasts to prolong resorption process and remove a large amount of matrix degradation products.

Several molecules are essential in the bone-resorbing phase of multinucleated osteoclasts. DC-STAMP (dendrocyte expressed seven transmembrane protein) and OC-STAMP (osteoclast stimulatory transmembrane protein) are transmembrane proteins critical for the cell-cell fusion of osteoclasts (Miyamoto et al. 2012). At least four different integrins are expressed in osteoclasts: $\alpha v\beta 3$, $\alpha v\beta$ 5, $\alpha v2\beta 1$ and $\alpha v\beta 1$ (Nesbitt et al. 1993). Integrins are a large family of cell-surface receptors that mediate cell-matrix and cell-cell interactions. In osteoclasts, they promote attachment to bone among several other vital functions. $\alpha v\beta 3$ integrin is highly expressed during osteoclast differentiation and, in the mature osteoclasts, it is localized in the ruffled border and in the basolateral membrane (Lakkakorpi et al. 1991, Mulari et al. 2003). It binds to a variety of ECM proteins containing RGD-sequence including BSP, OPN and vitronectin. Recent data suggests that upon αvβ3 occupancy, activated c-Src links both RANK and αvβ3 and permits the complex to organize the osteoclast cytoskeleton. Thus, the activated RANK possesses two distinct signaling pathways; one promotes osteoclast differentiation, and the other, in concert with c-Src-mediated linkage to αvβ3 integrin, organizes the cell's cytoskeleton (Izawa et al. 2012). Recent data also suggests that the αvβ3 integrin may serve as a biosensor that regulates apoptosis (Zhao et al. 2005).

The vacuolar-type H(+)-ATPase (V-ATPase), carbonic anhydrase II (CAII) and chloride channel 7 (ClC7) enable dissolution of the mineral crystals. Their expression in osteoclasts is upregulated towards the maturation. However, it has been demonstrated that CA II plays a key role already in the osteoclast differentiation (Lehenkari et al. 1998). Protons are produced as a result of cytoplasmic CA II enzyme activity. CA II catalyzes rapid conversion of water (H_2O) and carbon dioxide (CO_2) to bicarbonate (H_2CO_3), which dissociates to protons (H+) and bicarbonate anions ($HCO3^-$). Leftover cytoplasmic bicarbonate is removed via the chloride-bicarbonate exchanger located in the basolateral membrane (Hall and Chambers 1989). Proton transport through the ruffled border is mediated by V-ATPase proton pump, resulting in the acidification of the resorption lacuna. Corresponding secretion of counter Cl^- -ions to the resorption lacuna to maintain electroneutrality is mediated through the ClC7 channel (Schlesinger et al. 1997). V-ATPase is a macromolecular complex containing at least 14 subunits. The significant importance of V-ATPases and CA II are demonstrated by *in vitro* studies and knockout studies in mice, which exhibit osteopetrotic phenotype owing to impaired osteoclastic function (Laitala et al. 1994, Laitala-Leinonen and Väänänen 1999, Lee et al. 1999). Furthermore, mutations of V-ATPase a3 subunit encoding gene in humans cause infantile malignant osteopetrosis (Kornak et al. 2000).

The acidification is enhanced by stimulators of bone resorption, such as PTH and prostaglandin E2 (Anderson et al. 1985, Anderson et al. 1986). In contrast, calcitonin

inhibits acidification. The mineral matrix is resorbed to a deeper extent than the organic matrix, as the resorption lacunas are covered with asymmetrical packages of collagen fibrils after the resorption phase ceases (Ren et al. 2005). Bone resorption is highly dependent on the intracellular and extracellular pH. Maximum stimulation of bone resorption by cultured osteoclasts has been observed at an extracellular pH of about 6.9 (Arnett 2003). The reduction in blood pH enhances urinary calcium loss and bone resorption (Krieger et al. 2004). Bone loss is increased as the reduction in pH increases osteoclast differentiation and activity, and, concomitantly, decreases collagen synthesis and mineralization of osteoblasts (Arnett et al. 1994, Lehenkari et al. 1998, Arnett 2003). Excess acidosis leads to hypoxia associated bone loss due stimulation of osteoclastogenesis (Arnett et al. 2003, Kato et al. 2011). As a result of the dissolution process, calcium and phosphate are released into the circulation from the matrix (Mundy 1996, Boyle et al. 2003). Dissolution of minerals is followed by secretion of proteases able to degrade the type I collagen and other components of organic matrix.

TRACP 5b expression is significantly increased during osteoclast differentiation and, due to this phenomenon, it is also used as a histological marker of osteoclasts. Nevertheless, TRACP is also expressed in mature bone resorbing osteoclasts as well as in other members of the monocyte/macrophage lineage cells. TRACP functions mainly as an acid phosphatase. However, it is also capable of producing ROS. The ROS producing activity of TRACP has been demonstrated to facilitate collagen fragmentation and it may thus have a role in the finalization of matrix degradation (Väänänen and Zhao 2008). In addition to TRACP, mature osteoclasts express several other enzymes and proteases essential in the resorption process. The most abundant protease enzyme expressed in osteoclasts during the resorption process is cathepsin K (Gowen et al. 1999). Cathepsin K is also expressed in breast cancer cells, where it contributes to tumor invasiveness. Cathepsin K is a lysosomal cysteine protease having the ability to degrade elastin, collagen, and gelatin thus breaking down bone and cartilage. Cathepsin K is a major enzyme responsible in the degradation of organic bone matrix. Overexpression of cathepsin K increases bone resorption and leads to accelerated turnover in trabecular bone and to increased cortical bone porosity (Morko et al. 2005, Kiviranta et al. 2001), whereas cathepsin K deficient mice exhibit osteopetrotic phenotype due to a deficit in matrix degradation but not demineralization (Gowen et al 1999). Moreover, patients who have a mutation in the gene encoding cathepsin K display pycnodystosis, a disease characterized with abnormally dense bones (Johnson et al. 1996).

In addition to cathepsin K, other enzymes of osteoclastic origin, such as other cathepsins and matrix metalloproteinases (MMPs), contribute to the resorption process (Väänänen and Zhao 2008, Everts et al. 2006). MMPs are zinc binding endopeptidases that are capable of degrading the majority of organic matrix components in skeletal tissue. To date, at least 23 MMPs have been identified. Of the MMP family, MMP-13 and MMP-9 are the most essential in bone degradation. MMP-13 is synthesized and secreted by osteoblast-lineage cells and is localized at resorption lacunae. MMP-13 plays an important role in the type I collagen degradation of bone matrix, acting in co-operation with cathepsin K and MMP-9 produced by osteoclasts (Nakamura et al. 2004). In addition to collagen degradation, MMP-13 is suggested to have several other roles including modulating osteoclast differentiation and activity (reviewed by Delaissé et al. 2003). MMP-13 production is upregulated within the microenvironment of inflammatory bone in breast tumor cells. Thus, it is hypothesized that MMP-13 might be involved in the network of complex interactions between bone cells and tumor, promoting not only osteoclast resorption activity, but also osteoclast differentiation (Pivetta et al. 2011). After the initial

degradation of collagens by collagenases, denatured collagen fragments are further degraded by gelatinases. The major gelatinase in bone is MMP-9 (gelatinase-B). It regulates bone resorption and remodeling by various mechanisms. It has been suggested to promote invasion of osteoclasts, as its main substrate is type IV collagen which is found primarily in the basal lamina. However, its main function is suggested to be collagen degradation (Okada et al. 1995). Studies with gelatinases MMP-9 and MMP-2 deficient mice revealed that that these MMPs influence trabecular architecture but not volume, and thus is actively involved in maintaining bone integrity and quality (Nyman et al. 2011). MMPs are inhibited and regulated by specific endogenous tissue inhibitors of metalloproteinases (TIMPs), while cystatins modulate the functions and expression of cathepsins. Cathepsin K and MMPs degrade type I collagen at the acidic pH at distinct sites. This degradation results in various collagen fragments that can be used for assessment of bone turnover.

Regulation of bone resorption is discussed in more detail in the chapter about bone remodeling. When the resorption process is complete, the osteoclasts either start another resorption cycle or undergo apoptosis (Väänänen and Zhao 2008). Absence of trophic factors, such as M-CSF and RANKL, directs osteoclast to apoptotic pathway. In addition, thyroid C-cells secrete calcitonin, a polypeptide hormone that binds to the calcitonin receptor (CTR) expressed by active osteoclasts, leading to a rapid termination of resorption activity (Lian and Stein 2006).

2.5 Bone remodeling and its regulation

When new bone is formed and added to an existing structure, such as in appositional growth, it is referred to as bone modeling (construction). Bone modeling takes place during development and growth. In modeling, bone formation and bone resorption are uncoupled, and active osteoblasts and osteoclasts are at the distinct sites of bone. The eighteenth century English surgeon John Hunter observed that when new bone is formed, old bone is resorbed. This lifelong process, where bone formation and bone resorption are coupled, is now known as bone remodeling (reconstruction or bone turnover). Modeling is the predominant form until cessation of growth after which the body strives to maintain the obtained peak bone mass by remodeling. Remodeling also plays a role in bone growth in optimizing strength and minimizing the mass of the growing structure. The purpose of modeling and remodeling during growth is to achieve the skeleton´s peak strength. Bone remodeling is not harmful for the bones unless it becomes excessive and untargeted. Each bone constantly undergoes modeling during its lifespan. Remodeling is crucial for bones in adapting to altering biomechanical forces, removing woven or old bone as well as microdamaged bone, and replacing it with new, mechanically stronger bone. In addition, it is essential in regulating homeostasis of calcium, phosphate and other minerals. In remodeling, active osteoblasts and osteoclasts are sequentially at the same sites of bone forming temporary anatomic structures called basic multicellular units (BMUs)(Frost 1969). The BMU arises in a particular place at a particular time and it consists of osteoblasts, osteoclasts, connective tissue, blood vessels and nerves (Parfitt 1994). Remodeling takes place mostly at endosteal surfaces of bone and, to a much lesser extent, in periosteal surfaces. Remodeling is observed in trabecular and endocortical bone and intracortical walls of the Haversian system, but the rate of the remodeling process varies in these components, as there are diversities in bone shape, size, function and spatial distribution of its mass. Age is the main determinant for the remodeling rate, it being fastest during

growth and slowing down towards advanced age. Before menopause, the remodeling rate is slow, after which it is increased leading to negative BMU balance. In addition, there are gender and racial differences that affect the remodeling rate. It is estimated that the human skeleton has continuously millions of BMUs (reviewed by Riggs et al. 2005) and the whole skeleton is entirely regenerated every 10 years (Parfitt 1994, reviewed by Manolagas 2000). A new BMU unit will typically arise after mechanical stress, exposure to some cytokines, microdamage to the bone, or systemic need for minerals (Parfitt 2002). Each BMU undergoes phases where bone is first resorbed and then followed by bone formation in the same location. This cycle is referred to as a bone remodeling cycle (Figure 5). The remodeling cycle is composed of consecutive but distinct phases. Activation and recruitment of osteoclasts precedes bone resorption, which is followed by reversal phase where osteoblast precursors appear on the bone surface and mature, which, in turn, is followed by bone formation and mineralization until the resorbed bone is completely replaced. The final phase, referred to as quiescence, is the resting state of the bone surface.

2.5.1 Cellular regulation

The key cellular players in targeted bone remodeling are the osteoblasts and the osteoclasts. However, recent data suggests that osteocytes and their apoptosis in particular have a pivotal role in cellular level regulation of bone remodeling (Bonewald 2011, Noble et al. 1997, Pead et al. 1988). Osteocyte apoptosis is likely to be one of the earliest signals for the need of remodeling. Osteocyte apoptosis is induced in numerous ways. Microcracks sever osteocyte canalicular network resulting in osteocyte death (Hazenberg et al. 2006). Moreover, estrogen deficiency and glucocorticoid therapy are known causes of osteocyte apoptosis (Gu et al. 2005a and 2005b). It is assumed that the need for remodeling is likely to be signaled by osteocyte apoptosis via their canalicular network and processes connected by gap junctions to osteoblasts and osteoblast lining cells located at the endosteal surfaces where the remodeling takes place. Thus, osteocytes signal the location and size of the damage (Seeman 2006). Osteocyte death has been shown to precede osteoclast differentiation and thus initiate a remodeling cycle. This is further demonstrated by *in vitro* studies where scratching induced apoptosis of osteocyte-like MLO-Y4 cells results in the formation of osteoclast-like cells (Kurata et al. 2006). Enhanced osteoclastogenesis is most likely due to osteocyte mediated signaling to other cells of osteoblast lineage, which express inducing factors of osteoclastogenesis, such as RANKL. Therefore, the prevention of osteocyte death is an attractive therapeutic target.

2.5.2 Molecular regulation

Several systemic factors such as estrogens, androgens and calciotropic hormones regulate bone remodeling in concert with local factors, such as growth factors, prostaglandins and inorganic ions, by interacting at cellular, tissue and organ levels. Estrogen and androgens are the key systemic regulators of bone remodeling. The physiological role of estrogens and androgens is the maintenance of normal bone remodeling. In human skeleton, the most pivotal systemic hormone in maintaining normal bone remodeling is estrogen (Väänänen et al. 1996, Pacifici et al. 1991). This has been further demonstrated with numerous preclinical studies (reviewed by Kalu et al. 1991, Khosla et al. 2012). Estrogen deficiency leads to a rapid increase in bone remodeling

where resorption exceeds formation resulting in decreased bone mass. Also men with defects either in the synthesis of estrogen from testosterone or in the estrogen receptor display similar bone loss (Bilezikian et al. 1998). Estrogen acts in bone by several mechanisms which are still partly unknown. All bone cells, osteoblasts, osteocytes and osteoclasts express both estrogen receptors ERα and ERβ, but their distributions within bone are not consistent; ERα seems to be the predominantly expressed in cortical bone, whereas ERβ seems to be the predominant form in trabecular bone (Bord et al. 2001). Estrogen is assumed to participate in the regulation of the production and activity of local factors that regulate osteoblast and osteoclast precursors (Pacifici et al. 1991). At the cellular level, estrogen is shown to increase osteoblast differentiation and function, but decrease osteoclast differentiation and activity, and increase osteoclast apoptosis, thus decreasing osteoclast lifespan. Estrogen promoted osteoclast apoptosis is likely to be at least partly mediated by osteoblast TGF-β production (Hughes et al. 1996). It has been further demonstrated that estrogen negatively regulates NF-kB and M-CSF-induced osteoclastogenesis (Shevde et al. 2000). Despite the partly speculative conclusions of estrogen´s role in bone remodeling, it is evident that estrogen regulates both bone formation and resorption in a direction that will resist bone loss, decrease remodeling and increase bone mass.

Testosterone is the most abundant androgen in the male body. Testosterone can be converted into 5α-dihydrotestosterone (DHT) or catalyzed by the aromatase enzyme into estradiol. DHT and testosterone bind with androgen receptors whereas estradiol binds with estrogen receptors. Both of these receptors are highly expressed in bone tissue (reviewed by Riggs et al. 2002). Androgen receptors are expressed in both osteoblasts and osteoclasts (reviewed by Vanderschueren et al. 2004). It was long believed that androgens, particularly testosterone, play an analogous dominant role to estrogen in the male bone metabolism. However, as new data has been discovered over the years, the dominant role of androgens in male bone physiology has been questioned and, in contrast, it has been suggested that estrogens and other metabolites of testosterone have an important role in male skeletal development (Leder et al. 2003). Based on case reports, men with genetic mutations in their estrogen receptor-α gene or in the aromatase gene have elevated biochemical markers of bone turnover and reduced BMD due to the fact that they are unable to either respond to or synthesize estrogen (Smith et al. 1994, Morishima et al. 1995, Carani et al. 1997). Furthermore, BMD increased dramatically in men with aromatase deficiency after estrogen administration (Carani et al. 1997, Bilezikian et al. 1998). Cross-sectional observational studies in elderly men have demonstrated that estrogen correlates better with BMD than testosterone (Khosla et al. 1998). However, despite the predominant role of estrogen in both male and female bone remodeling, androgens have been demonstrated to have direct effects on bone cells through androgen receptors (Bellido et al. 1995). Testosterone, DHT and nonaromatizable androgens increase the proliferation of osteoblast cells in cultures, and induce osteoblast differentiation (Kasperk et al. 1989). In addition to enhanced osteoblast proliferation, androgens, like estrogens, prevent osteoblast apoptosis. Induced osteoblast apoptosis can be prevented with DHT (Kousteni et al. 2001). These observations are further supported by animal studies where orchidectomy of mice increases osteoblast apoptosis *in vivo* (Kousteni et al. 2002). Furthermore, both orchidectomized growing rats and orchidectomized aged rats exhibited reduced trabecular bone volume and other trabecular bone parameters and the condition was reversed by testosterone administration indicating the significance of testosterone and its metabolites in bone remodeling (Wakley et al. 1991, Vanderschueren 2000).

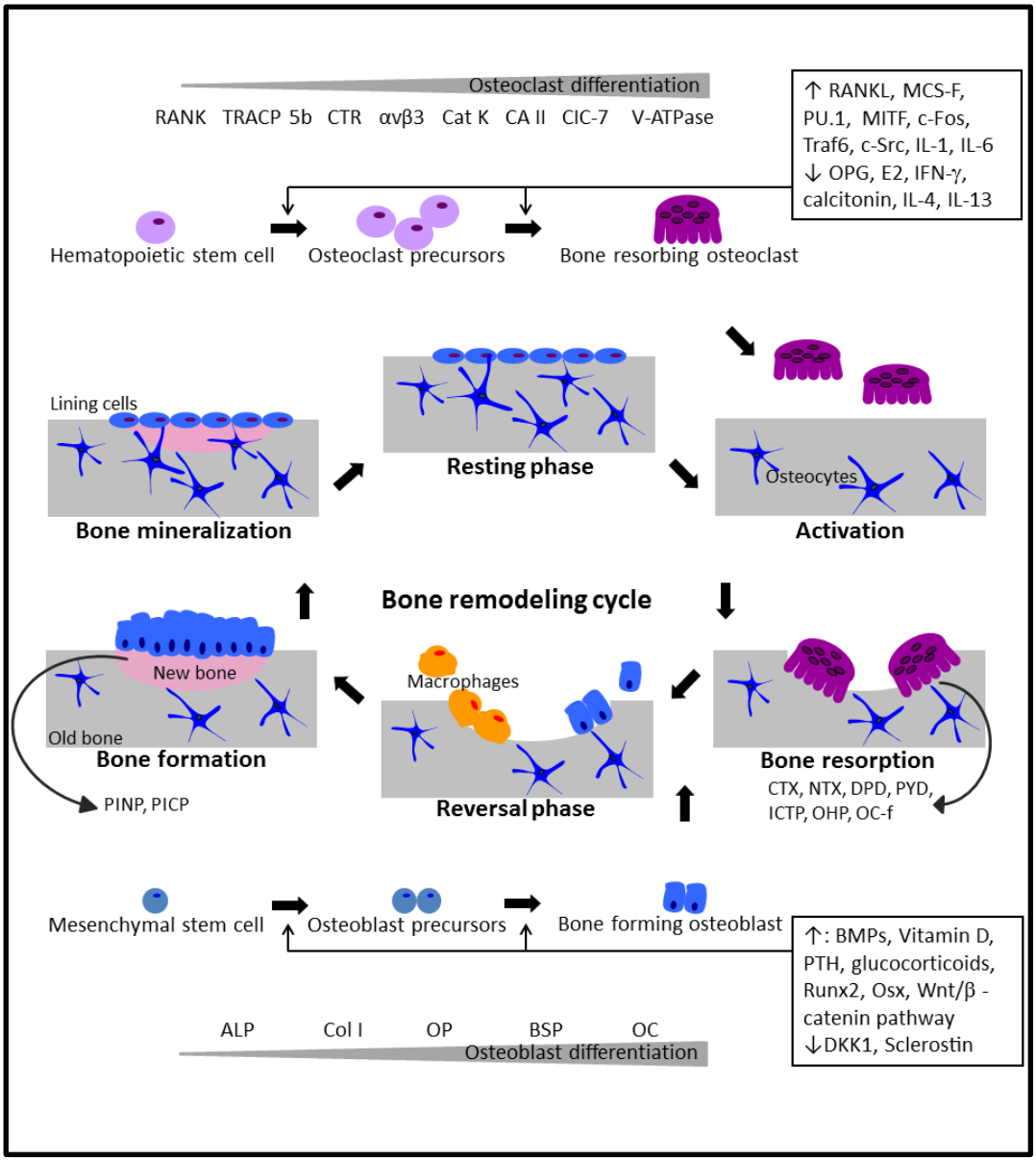

Figure 5. Schematic model illustrating bone remodeling cycle and differentiation of bone cells. Remodeling consists of the following sequential, tightly regulated events: 1) resting phase, 2) activation and bone resorption phase, 3) reversal phase, 4) bone formation and mineralization phase. Osteoclasts are of hematopoietic origin and osteoblasts of mesenchymal origin. The major factors regulating bone cells' differentiation and activity are shown. In addition, the major bone-derived markers resulting in osteoblast or osteoclast activity are shown. Abbreviations and detailed functions of bone cells, regulatory factors and bone markers are reviewed in the text.

The most important calcium-regulating hormones are mineral homeostasis regulators parathyroid hormone (PTH) and 1,25-dihydroxy vitamin D and calcitonin, which inhibits bone resorption. PTH is secreted when the level of calcium in the blood falls below the amount needed by the body's cells. It promotes bone degradation, and thus calcium is released into the blood by osteoclasts. PTH has biphasic effects on bone formation by causing an acute inhibition of collagen synthesis with high concentrations, but, on the contrary, it increases bone formation with prolonged intermittent administration. The latter is the property for which it has been explored and developed as an anabolic therapeutic agent (Dempster et al. 1993). In contrast, vitamin D plays a vital role in limiting withdrawals of calcium from bone by promoting calcium and phosphate absorption from food in the intestines. Glucocorticoids are required for differentiation of bone cells, but their greatest postnatal effect is to inhibit bone formation (Advani et al. 1997). Growth hormone acts systemically but is regulated locally via insulin-like growth factor (IGF) production, which can further stimulate bone formation and resorption (Rosen et al. 1998). In addition, thyroid hormones are able to stimulate bone resorption and formation and are essential for maintenance of normal bone remodeling (Kawaguchi et al. 1994).

One interesting recently observed regulator of bone remodeling is leptin, a hormone produced by adipocytes which normally plays a key role in regulating energy intake, but it also regulates bone metabolism through the central nervous system (Takeda et al. 2002). In addition to its effects through the brain and central nervous system, leptin may act directly on bone cells in order to regulate bone remodeling. These observations provide a novel link between energy and bone metabolism and growing evidence of adipose tissue participation in the regulation of bone mass. Furthermore, elucidating the actions of leptin on the skeleton may provide a link between disorders of these metabolisms, such as obesity and osteoporosis. It has been suggested that also another energy homeostasis regulator, ghrelin hormone, participates in bone remodeling as its receptor GHS-R1a was recently identified in osteoblasts (Fukushima et al. 2005). As the bone and bone cells contain a large number of growth factors and cytokines, several local factors can affect bone remodeling. The major local factors that regulate bone turnover are osteoclast differentiation and activity mediators RANKL, OPG, M-CSF, cathepsin K and MMPs, and osteoblast differentiation and activity mediators such as BMP, TGF-β, FGF and IGF. The effect of these mediators on bone cells is discussed in more detail in the chapter about bone cells.

2.6 Skeletal diseases

A healthy skeletal system with strong and functional bones provides a base for overall health and quality of life. Strong and elastic bones support the body; they protect vital organs from injury and are the framework for muscles which allow movement. Moreover, bones are a reservoir for life-supporting minerals. Many factors and conditions affect the skeletal system and may lead to a skeletal disease, which eventually makes bones weak and prone to fractures. The skeletal system is affected by e.g. other disorders, medical conditions, degenerative wear and tear, genetic anomalies and infectious agents. Commonly known diseases of the skeletal system are osteoporosis, bone metastases and osteoarthritis. Of these, osteoporosis is the most prevalent skeletal disease, particularly among the elderly. It is the second largest healthcare problem after cardiovascular disease (WHO 2004).

2.6.1 Osteoporosis

Pathogenesis and prevalence
In the 1830s, the French pathologist and surgeon Jean Georges Chrétien Frédéric Martin Lobstein (1777-1835) observed that some patients' bones had larger than normal holes, and he coined the term osteoporosis, meaning porous bone, to describe such deteriorated human bone (reviewed by Schapira et al. 1992). At that time, this observation was considered an unavoidable aspect of aging. Initially, osteoporosis was linked with the postmenopausal state by endocrinologist Fuller Albright (Forbes 1991).

Osteoporosis is a systemic metabolic skeletal disease characterized by low bone mass and deterioration in the bone microarchitecture. It is a progressive disease which will eventually lead to bone fragility and to an increased risk of fractures. Osteoporosis is referred to as a silent disease because its progression is gradual. It can progress for years without any warning, symptoms or incident. Osteoporosis is often diagnosed when an insignificant fall results in a fracture. Osteoporotic fractures typically occur in the hip, spine or wrist. They contain high percentages of trabecular bone, which is more vulnerable because of the higher turnover rate. Osteoporosis is not a life-threatening disease, although it causes an increased mortality rate due to complications of fractures and it can dramatically reduce quality of life even before fractures occur by causing loss of mobility which leads to hospitalization. It is estimated that over 200 million people worldwide have osteoporosis. In addition, over 40% of postmenopausal Caucasian women are estimated to have osteoporosis (Rachner et al. 2011). Prevalence of osteoporosis rises dramatically with aging in all geographic areas. It rises from 5% among women of 50 years of age to 50% at the age of 85 years, whereas among men, the comparable numbers are 2.4% and 20%, respectively (Kanis et al. 2000). Furthermore, there are annually 9 million osteoporotic fractures, of which 1.6 million occur at the hip, 1.7 million at the forearm and 1.4 million at the vertebra. Over half of all the fractures occurred in Europe or America, while most of the remaining fractures occurred in the Western Pacific region and Southeast Asia (Johnell et al. 2006). Osteoporosis is a growing public health concern as life expectancy increases. By the year 2050, the incidence of hip fracture is estimated to rise from 1.6 million to over 6 million. Particularly in many Asian countries, prevalence of osteoporosis is rapidly increasing due to socio-economic development and aging (Mithal et al. 2009).

Male osteoporosis has received much lesser attention in epidemiological investigations than postmenopausal osteoporosis; as such, prevalence rates are more difficult to estimate. However, men with advanced age suffer osteoporosis similar to women despite the lack of menopause and rapid hormone deficiency. With both genders, bone loss often starts already during the fourth decade. Trabecular bone loss is more severe and starts earlier than the cortical bone loss. Among women, trabecular connectivity is dramatically reduced and individual trabeculi even completely resorbed, whereas among men, the trabeculi become thinner (Aaron et al. 1987). During a normal lifetime, women lose typically half of the trabecular bone mass and one third of the cortical bone mass, while men lose one third of trabecular bone mass and one fifth of the cortical bone. After menopause, BMD in the lumbar spine and at the hip is decreased at the annual rate of 1.6% and 2%, respectively (Finkelstein et al. 2008).

Risk factors
Age and gender are the best predictors of osteoporosis, but also early menopause, genetic history of maternal hip fracture, smoking, low calcium intake, a fracture after 40 years of age, low body weight, number of primary diseases or their treatment increase the probability of fractures. In addition, poor muscle strength, balance or vision increases the probability to fall and is prone to cause fractures (Lane 2006). Undergoing glucocorticoid therapy has a well-known adverse effect on bone resulting in a decrease in bone mass. Glucocorticoid therapy leads to suppression in bone formation, which is a key feature in the pathophysiological mechanisms of glucocorticoid-induced osteoporosis (GIO or GIOP) (Canalis et al. 2005). Glucocorticoids also favor osteoclastogenesis, and as a consequence, increase bone resorption (Takuma et al. 2003). Immobilization and several diseases such as inflammatory bowel disease (IBD), osteolytic bone metastases, rheumatic disease, chronic liver or renal disease, primary hyperparathyreosis and organ transplantation predispose the body to increased bone loss and osteoporosis. Of these, immobilization, primary diseases or medication induced bone loss is referred to as secondary osteoporosis, while estrogen and androgen deficiency induced bone loss due to aging is referred to as primary osteoporosis. Glucocorticoids are the most common cause of secondary osteoporosis (Mazziotti et al. 2006).

Diagnosis
Current standard in osteoporosis diagnosis is the measurement of BMD from lumbar spine or femoral neck. WHO has defined the three stages of bone loss based on the severity of an individual´s bone loss according to BMD (referred to as T-score). A diagnostic criterion for osteoporosis is a T-score that is 2.5 times below the standard deviation (SD) of the mean value of young adult women's peak bone mass, whereas osteopenia, a condition of low bone mass, is diagnosed when the T-score falls between 1 and 2.5 times SD below the mean value. Severe osteoporosis is diagnosed when, in addition to osteoporotic T-score, the patient has a fragility fracture present (WHO 1994). FRAX is a novel diagnostic tool used to evaluate the probability of bone fracture risk. FRAX integrates clinical risk factors and BMD at the femoral neck to calculate the 10-year probability of osteoporotic fracture (WHO 2011). Currently, bone turnover markers are not routinely used in osteoporosis diagnostics. However, they can be used for monitoring treatment and also in the assessment of fracture risk. In the future, as new bone markers are developed, it might be possible to use them to also diagnose osteoporosis.

2.6.2 Other skeletal diseases

Cancer bone metastases
Despite the recent advances, cancer remains one of the most serious health problems in the developed world. Bone metastases are a consequence of several metastatic cancers, such as breast, prostate, lung or even blood cancer. During the tumor growth, new blood vessels are formed. Cancer cells can travel through these blood vessels to other tissue and metastasize. Particularly the metaphysis region of long bones and spine are common sites for osteolytic or osteoblastic metastases due to their high bone turnover rate in trabecular bone. The osteolytic or osteoblastic phenotype is caused by the action of tumor-produced factors to stimulate either osteoclast or osteoblast differentiation and/or activity, thus disrupting normal bone remodeling. As a consequence, increased bone resorption or formation activity in the bone's microenvironment provide growth factors for the tumor cells, which further fuel tumor growth in the bone. This cycle is referred to as a vicious

cycle of bone metastases (Guise 2002). TGF-β and PTHrP are one of the key cytokines that regulate tumor growth and also bone remodeling. Breast and prostate cancer are the most common origins of bone metastases and they can exhibit osteolytic or osteoblastic phenotype, while multiple myeloma is cancer arising from blood cells having osteolytic phenotype. Bone cancer is a primary tumor that starts growing inside a bone, as opposed to metastatic bone cancers. Osteosarcoma is a type of bone cancer that involves the growth of primary tumors in the rapidly growing regions of bones, including metaphyseal regions of long bones. It mostly occurs before adolescence, with the average age of diagnosis being only 15 years.

Other bone diseases
Paget's disease is a chronic disorder that affects mainly people in advanced age, causing skeletal abnormalities and fractures. The excessive and untargeted resorption and formation results in abnormal bone that is sparse, enlarged, brittle, and prone to fractures. Paget's disease typically affects only few bones, as opposed to osteoporosis. Osteogenesis imperfecta, also referred to as brittle bone disease, is an inherited genetic anomaly that causes brittle bones, loose joints, blue sclera and frequent fractures in children. Osteonecrosis is a disease where insufficient blood supply to large joints, such as hips, knees, elbows and shoulders leads to necrosis of the bone tissues. This can occur after fractures, trauma, injury, radiation therapy, bone dislocations or because of certain antiresorptive medications. Osteomalacia is a disease that eventually leads to fragile bone tissue. This disease, also referred to as rickets, is often originated by prolonged vitamin D deficiency and is common in children in certain developing countries. Osteopetrosis is a rare inherited disorder wherein bone resorption capacity is altered due to osteoclast dysfunction. This leads to hardening of bones and an increased BMD. In contrast to osteoporosis, osteosclerosis is an elevation of bone density. Various underlying diseases including myelofibrosis, hemangioma and prostate cancer can cause osteosclerosis.

Osteoarthritis and rheumatoid arthritis
Arthritis is a group of several inflammatory diseases that has detrimental effects on joints and their surrounding structures. Most common forms of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA). OA is the most common joint disorder and it occurs in the majority of people by the age of 65. Some of the features of OA are common with RA. They both are joint diseases characterized by motional pain, swelling of joints, stiffness and limited joint function. In addition, both of them may result in bone loss. OA is a degenerative joint disease caused by the breakdown of joint cartilage. OA allows bone to grate on bone which erodes the cartilage. The cause of OA is often unknown. However, it is mainly related to aging and wear and tear on a joint. Other risk factors include obesity, female gender and bone deformities. RA is an auto-immune disease, which causes inflammation of the synovial membrane and deformity of the joint. Eventually it may lead to bone loss. In time, if treatment is not started promptly, the joint shape deformates and the bones no longer line up correctly.

2.6.3 Treatment of osteoporosis

In Western countries, approximately 30% of postmenopausal women suffer from osteoporosis. As the life expectancy increases, the number of osteoporotic patients is also expected to increase, which will further cause additional cost and burden to our health care system. Despite its long history, osteoporosis is still a challenge to medicine. Currently, due

to the recent improvements in the understanding of the molecular level regulation of bone remodeling, novel treatments are available for prevention and treatment of osteoporosis. Traditional target sites for osteoporosis drugs include targets for promoting osteoblast function (anabolic therapies) or targets for inhibiting osteoclast function (anti-catabolic or anti-resorptive therapies). Of these, antiresorptives are currently the major class of prescribed therapies in osteoporosis. At the cellular level, these effects can be obtained either by affecting the differentiation or activity of osteoblasts or osteoclasts. Most of the current treatments have shown reductions in the vertebral and non-vertebral fracture risk, which is the key objective in osteoporosis therapy. However, most of the existing therapies will only slow down the further progress of the disease but will not recover the amount of bone mass back to the normal level. Therapies also have adverse effects, which can occasionally be even of greater magnitude than the reported beneficial effects on the bone. In principle, there are two options to how osteoporosis is prevented or treated: decreasing bone resorption by anti-resorptive drugs or increasing bone formation by anabolic drugs. Secondary osteoporosis can often be prevented and treated in the same manner as primary osteoporosis. However, an underlying disease and medication set their own requirements for the management of secondary osteoporosis.

Current medication

Pharmacological treatment of bone loss began in the 1970s with estrogen treatment, as osteoporosis was recognized as a consequence of estrogen deficiency. Estrogen and its derivatives were the mainstay of postmenopausal osteoporosis prevention and treatment over the following decades after the 70s. Today, hormone replacement therapy (HRT), including estrogen or estrogen combined with progestin, is considered only in the case of women who have multiple menopausal symptoms and low risk of breast cancer, as its probability is increased after estrogen treatment. Calcitonin has also been used for several decades in the treatment of osteoporosis, as it acutely blocks osteoclast activity. Today, calcitonin is used only for relatively small proportion of patients because recent evidence shows that long-term use of calcitonin is associated with an increased risk of cancer (EMA 2012). The effects of estrogen, androgens and calcitonin are reviewed in more detailed in the chapter about bone remodeling as they are endogenous bone remodeling regulators.

At present, when osteoporosis is diagnosed, calcium and vitamin D supplementation is initiated practically for all patients. Often, along with medication, lifestyle change is needed as continuous physical activity and exercising slows down the bone loss. Thus, it is vital to maintain physical activity throughout life. Selective estrogen receptor modulators (SERMs) are compounds acting through estrogen receptor and mimicking actions of estrogen in bone. They have been developed to avoid adverse effects of estrogen and to concomitantly preserve its beneficial bone effects. As the name implies, their action is different in different tissues, which grants the possibility to selectively act as an agonist or an antagonist in various tissues. Best known SERM is raloxifene, which can be used to treat the osteoporosis of post-menopausal women with the history of breast cancer. However, similarly to estrogen, it increases a risk of thrombosis. Moreover, muscle cramps and hot flushes are common adverse effects associated with SERMs.

A recombinant PTH fragment 1-34 has been developed as an anabolic treatment with the name teriparatide for severe osteoporosis. The role of PTH in bone remodeling regulation is reviewed in more detail in the chapter about bone cells and bone turnover. Currently, teriparatide is the only anabolic therapy available for osteoporosis, although clinical studies with sclerostin antibody have shown promising results. It has been suggested that also strontium ranelate, a calcium mimetic, consisting strontium ions (Sr^{2+}) and ranelic acid,

would possess the capability to induce bone formation and even concomitantly reduce bone resorption. Strontium ranelate is therefore hypothesized to be the only dual action bone agent (DABA). However, some of the clinical studies were in controversy and not able to confirm this observation. Due to observed adverse effects, which are increased risk of myocardia and venous thromboembolism, the use of strontium ranelate is currently restricted to treatment of severe osteoporosis in postmenopausal women who have a high risk of fractures.

The most widely prescribed osteoporosis therapies are bisphosphonates such as alendronate, ibandronate, risedronate and zoledronic acid (Table 1). These antiresorptive compounds have been used in osteoporosis treatment for decades and are therefore considered as the gold-standard treatment for osteoporosis (reviewed by Russell et al. 1999, Cosman 2009). Bisphosphonates are stable derivatives of inorganic pyrophosphate and they contain two phosphonate (PO_3) groups covalently linked to carbon (C). The bisphosphonate group inhibits the activation of enzymes that utilize pyrophosphate, as it mimics pyrophosphate's structure. Bisphosphonates have a high affinity for the hydroxyapatite in bone and, due to this phenomenon, they are incorporated into sites where bone resorption occurs and are eventually resorbed together with bone degradation products inside the osteoclasts. Bisphosphonates act inside the osteoclasts by disrupting the production and attachment of regulatory molecules to the cell membranes and, as a result, drive osteoclasts to apoptosis. There are concerns that long-term bisphosphonate treatment can result in oversuppression of bone turnover, which leads to impaired ability to repair normal microfractures present in bone. It is assumed that these microfractures start to propagate, resulting in atypical fractures, which tend to heal poorly. Bisphosphonate-related osteonecrosis of the jaw (ONJ) is a rare condition that can arise when the jaw bone fails to heal after a minor injury, such as a dental procedure (Rizzoli et al. 2008).

Denosumab is a novel antiresorptive drug recently approved for treatment of osteoporosis and bone metastases (Goessl et al. 2012). It is a fully human antibody against RANKL that prevents its binding to RANK, and thus inhibits osteoclast development. By regulating the RANK/RANKL pathway, denosumab action mimics natural action of endogenous OPG. It has been hypothesized that oversuppression of bone remodeling and bone resorption may contribute to the pathogenesis of atypical femoral fractures and ONJ. Therefore, it is possible that also denosumab is associated with such pathological conditions. More common side effects of denosumab are hypocalcemia and various infections.

Some of the osteoporosis drugs can also be used for treatment of bone metastasis. These drugs either inhibit osteoclast function or they may also have direct effects on tumor cells. Both denosumab and zoledronic acid have been demonstrated to have direct effects on tumor cells. Of these, Denosumab seemed to prevent fractures better than zoledronic acid in breast and prostate cancer patients with bone metastasis (Henry et al. 2011). Denosumab is also used to increase bone strength in breast cancer patients who are being treated with aromatase inhibitors (Drooger et al. 2013).

Recent expert opinions highlight the combination of two different mechanisms of action drugs, such as teriparatide and denosumab, which might be more beneficial than treating osteoporosis with either drug alone (Tsai et al. 2013).

Table 1. The most commonly prescribed drugs for the treatment of postmenopausal osteoporosis approved by the European Medicines Agency (EMA).

Active compound/ Trade name*	Common side effects (seen in 1-10% of the patients)	Trade name authorisation holder**	Year of approval
Alendronate Fosavance	esophageal irritation, dyspepsia, diarrhoea	Merck Sharp & Dohme Ltd.	2005
Ibandronate Bonviva	esophageal irritation, arthralgia, rash	Roche Registration Ltd.	2004
Zoledronic acid Aclasta	fever, myalgia, flu-like symptoms, arthralgia, headache	Novartis Europharm Limited	2005
Raloxifene Evista	venous thromboembolism, vasodilation, nausea, cramps	Daiichi Sankyo Europe GmbH	1998
Strontium ranelate Protelos	headache, inflammation, nausea, myocardial infarction, venous thromboembolism	Les Laboratoires Servier	2004
Teriparatide Forsteo	hypercalcaemia, nausea, pain in the arms or legs	Eli Lilly Nederland B.V.	2003
PTH (1–84) Preotact	hypercalcaemia, hypercalciuria, nausea	Nycomed Danmark ApS	2006
Denosumab Prolia	infections, sciatica, rash, constipation	Amgen Europe B.V.	2010

*One example out of the several approved trade names; **In some cases not the company who originally developed the drug.

2.6.4 New developments in the treatment of osteoporosis

The development of a new osteoporosis drug is dependent not only on its efficacy and safety, but also on the cost and healthcare infrastructure. Since osteoporosis is not a life-threatening disease and is often asymptomatic, it sets special requirements for osteoporosis drugs; only minimal adverse effects are acceptable and at the same time it must exhibit significant efficacy. In the face of increasing competition against new product launches in the osteoporosis market, current players reconsider their strategies for their leading brands, which include new product formulations, repacking and investigation of new indications. These activities are expected to soften the impact of genericization and maximize peak sales before the expiry of the patent. There are several drug candidates in phase I or later stage of development, indicating that drug development companies recognize the commercial potential of osteoporosis. In the future, as the life expectancy increases, the number of osteoporotic patients is expected to rise even further. Currently, the development of new drugs is temporarily stagnant, because two expected block buster drugs denosumab and zoledronic acid have entered the markets. The pipeline trend in osteoporosis drug development shows a clustering of bisphosphonates and other antiresorptives in late-stage phase studies, indicating that this class of drugs is reaching maturation. The use of anabolic therapy is still limited with only one product class available, teriparatide and its derivatives. However, it is estimated that in the short- to mid-term, a large number of new anabolic drug approvals can be expected from this pipeline.

Recent evidence shows that osteocytes play an important role in maintaining bone quality and are therefore important and interesting target cells for drug development. Particularly sclerostin, inhibitor of canonical Wnt/β-catenin signaling, secreted by osteocytes has been studied extensively. Romosozumab (also known as AMG785), a humanized monoclonal antibody against sclerostin, has been demonstrated to increase osteoblastic bone formation and thus to have an osteoanabolic effect with a favorable safety profile. Sclerostin inhibition increased bone formation in human and rat studies without the increase in bone resorption that is seen in the teriparatide therapy in preclinical and clinical studies (Rachner et al. 2011, Liu et al. 2012a). This further suggests uniqueness of Romosozumab in uncoupling formation and resorption. It is currently at the phase II trials. Odanacatib, a selective inhibitor of cathepsin K, has been shown to significantly suppress bone resorption and to increase lumbar spine and total-hip BMD, while also being well-tolerated (Bone et al. 2010). Adverse effects in the odanacatib treated group did not differ statistically from the placebo group in the phase studies. Thus it seems to avert from skin-related adverse effects, which can be seen with various cathepsin inhibitors, as the cathepsins are widely expressed in the skin. Odanacatib is expected to enter the retail market at 2013 being the first cathepsin K inhibitor in the osteoporosis market. ED71 is an active vitamin D analogue that increases bone mass in osteoporotic patients under vitamin D supplementation (Matsumoto et al. 2005). It is currently in the phase III trials. Clinical studies designed to determine the efficacy of MMP inhibitors, such as MMP-9 and MMP-2 inhibitors, against a variety of tumors have been disappointing, but not unexpectedly considering all the numerous and diverse functions of the various MMPs in the human body. Also in skeletal tissue, various MMPs have diverse roles. MMP-13 is known to stimulate osteoclastogenesis and osteoclast activation in bone metastases (Pivetta et al. 2011). Currently, specific MMP-13 inhibitor has shown promising therapeutic potential in both primary breast cancer and cancer-induced bone osteolysis and it could be suggested as a new anti-resorptive or cancer therapeutic agent in the future (Shah et al. 2012). BMPs are strong anabolic compounds that are currently used clinically for fracture healing. They are not approved for osteoporosis treatment because of the short half-life and lack of ability to administer them systemically (Khosla et al. 2008).

Recent insights into bone metabolism and its regulation have led to a better understanding of molecular mechanisms of bone remodeling. Understanding these molecular interactions and mechanisms not only shows how current osteoporosis drug therapies exert their effects on bone remodeling, but also identifies novel targets for potential drug treatments in the future. One of the future challenges is to identify those groups of patients and individual patients who are expected to have the highest benefit from a given drug therapy or regimen. These individually tailored therapies are referred to as personalized medication which is one of the ongoing megatrends in drug development.

2.7 Models and screening assays in drug development of osteoporosis

The purpose of the preclinical evaluation of a new osteoporosis drug compound falls into two categories – those relating to ADME (absorption, distribution, metabolism, excretion) and safety properties of a compound and those relating specifically to efficacy and ability to regulate bone metabolism. The results of preclinical *in vitro* and *in vivo* studies determine whether a compound should be tested in clinical studies. The preclinical toxicological

studies and clinical studies are beyond the scope of this thesis work and will not be discussed further. The World health organization (WHO 1998) and regulatory guidelines (FDA 1994, EMA 2006) have set specific aims for the preclinical efficacy tests: Significant relationship between the novel compound on bone mass and strength should be demonstrated with particular emphasis on demonstrating that the compound is able to increase bone strength in concert with preservation of normal bone architecture. Moreover, the mechanism of the action of the compound should be elucidated and thus provide its rationale to human use. Animal efficacy studies should also demonstrate the effects of long term exposure to the compound on bone quality as well as the effects of the treatment on fracture healing. Today, a majority of the new experimental drugs still fail at the clinical phases despite the promising results at the preclinical phases. Therefore, great emphasis is placed on the better predictivity of preclinical *in vitro* assays and animal models.

2.7.1 Biochemical and cellular assays

Although pathology of osteoporosis becomes manifest in bone tissue that has several complex signaling pathways and regulators, some of the pathways can be modeled with novel well-defined biochemical and cellular assays. A large variety of biochemical assays, such as enzymatic assays, receptor-ligand binding assays and protein-protein interaction assays are widely used in drug discovery. These early-phase research tools are utilized to demonstrate the desired biochemical effect of a drug candidate. High throughput screening (HTS) assays are automated large scale format assays used often as a starting point of the drug screening process and they enable the finding of lead candidates from libraries containing hundreds of thousands of compounds. In osteoporosis drug development, one such approach is using enzymatic HTS assays to find compounds that inhibit key bone resorption enzymes thus suggesting their potential as anti-catabolic drugs for osteoporosis. Corresponding analysis methods could be utilized for anabolic compounds. However, these enzymatic assays are not able to demonstrate the efficacy of the drug compound on their target cells or organs. The efficacy of the most promising compounds to inhibit bone resorption or stimulate bone formation should, therefore, be confirmed in *in vitro* bone cell cultures. Furthermore, the efficacy of the lead compound on bone tissue should be confirmed in animal models of osteoporosis. A large variety of different cell culture assays are available for osteoporosis drug discovery to assist on the decision-making whether or not performing any further studies and, most importantly, if an expensive and large regulatory *in vivo* study is worth performing. *In vitro* bone cell cultures are typically used for further screening of positive hits resulted by HTS but also for complementing *in vivo* studies. Bone cell cultures themselves can also be used for screening purposes when performed in miniaturized forms, such as 96-well plates, allowing the testing of numerous compounds in a relatively short period of time. Novel 3D *in vitro* co-culture models have been developed recently to investigate cellular interactions. This is a very extensively studied approach when attempting to mimic the vicious cycle of bone metastases with cancer and bone cells (Curtin et al. 2012). Also bone remodeling involving osteoclasts, osteoblasts and possibly also osteocytes has been studied with co-culture models (Heinemann et al. 2011, Nakagawa et al. 2004, Mbalaviele et al. 1999). However, co-cultures have not yet provided such new outcomes that cannot be obtained from a separate osteoclast or osteoblast cultures. Despite the novel technologies and theories, cell culture models lack the central control of bone turnover, feedback mechanisms and numerous other signaling pathways. Therefore, findings provided by *in vitro* assays are considered rather as preliminary and suggestive than conclusive, and the results should be further confirmed with animal studies.

Osteoclast assays

Osteoclasts are typically obtained by isolating osteoclasts directly from bone *in vitro* or by inducing their formation from a source of macrophages or hematopoietic cells, such as the spleen, bone marrow and human peripheral blood mononuclear cells (hPBMC) (reviewed by Kartsogiannis et al. 2004). The generally accepted criteria for identifying osteoclasts are multinuclearity, TRACP positivity, the ability to resorb calcified matrices and expression of calcitonin receptors (Takahashi et al. 1988a). Direct isolation of osteoclasts from bone involves mechanical disaggregation of osteoclasts. With this method, mature osteoclasts can be isolated with a scalpel from the long bones of neonatal rodents, rabbits or chicks. Traditionally, bone resorption studies have been performed with isolated murine osteoclasts cultured on the top of bone or dentine slices (Boyde et al. 1984, Chambers et al. 1984). Until recently, giant cell tumors (GCTs) have been the only useful source of human osteoclasts. GCTs (also referred to as osteoclastomas) are rare primary tumors of the skeleton, containing osteoclasts and causing extensive osteolysis (reviewed by Kartsogiannis et al. 2004). Recent advancements in cellular and molecular regulation of osteoclasts differentiation, specifically the recognition the importance of the RANK/RANKL/OPG and M-CSF/c-Fms systems, have provided insight into the mechanisms of osteoclast differentiation and initiation of bone resorption. Furthermore, these advances have enabled the use of preosteoclastic cells of hematopoietic origin for various purposes. They are typically utilized for examining osteoclast differentiation and activity under these osteoclastogenic inductors supplemented in the culture medium. The major advancement in hematopoietic precursor cell-derived osteoclast cultures is the lack of stromal cells. It enables the study of pure osteoclast differentiation and activity and makes possible to obtain sufficient numbers of human osteoclasts. The most important and practical approach to obtain human osteoclasts today is the use of human PBMCs, which, cultured in the presence of soluble RANKL and M-CSF, form multinuclear bone resorbing osteoclasts (Fujikawa et al. 1996). However, PBMC are a heterogeneous group of various cells, including lymphocytes, subsets of monocytes and other blood cells. Therefore, it is vital to purify the PBMC cell pool with advanced techniques, such as magnetic bead sorting or density gradient centrifugation. A highly enriched subpopulation of osteoclast-forming PBMC can be obtained by selecting the CD34 or CD14 as markers for isolation. These markers are strongly expressed in monocytes, which is the osteoclast precursor cell in peripheral blood (Sørensen et al. 2007a, Nicholson et al. 2000, Pierelli et al. 1997). A frequently used osteoclast cell line, RAW264.7, is a mouse osteoclast-like myeloma cell line which can differentiate into osteoclasts in the presence of RANKL (Hsu et al. 1999). Unlike spleen or bone marrow derived hematopoietic progenitor cells, RAW264.7 cells do not require M-CSF alongside RANKL. In the past, RAW264.7 cells were a substitute to hematopoietic cells in the osteoclastogenesis studies (reviewed by Kartsogiannis et al. 2004).

Traditionally, osteoclasts in bone resorption studies are cultured on the surface of native bone, such as human and bovine bone, dentin or synthetic bone substitutes such as hydroxyapatite slices. It has been demonstrated that synthetic bone substitutes are more slowly resorbed by human osteoclasts than bovine bone (Perrotti et al. 2009, Keller et al. 2012). In addition, we and others have demonstrated that many of the properties of bovine bone are equal to human bone and, thus, bovine bone slices are good substrates in human osteoclast cultures (Lakkakorpi et al. 1989, a congress abstract by Rissanen et al. 2005, Keller et al. 2012). This is an important observation because of the limited availability of human bone. Furthermore, it has been demonstrated that human osteoclasts prefer to resorb old bovine bone rather than young bone (Henriksen 2007b). In all osteoclast assays, the differentiation and activity of osteoclasts have been traditionally quantitated by

visualizing the formed osteoclasts or resorption pits under a microscope (Selander et al. 1994). However, this approach is laborious and time consuming. A more rapid and convenient approach might be using bone turnover markers secreted into the culture medium during the culture period or at the end of the study or during the study. Particularly TRACP 5b and CTX have been proposed for these purposes (Alatalo et al. 2000, Karlsdahl 2003, Henriksen 2007a).

Osteoblast assays

Osteoblast cultures have been used for various purposes. They have been utilized to investigate the mechanisms of bone formation or to study the cellular basis of various bone diseases. Furthermore, osteoblast cultures have been used to screen potential therapeutic agents and biomaterials that affect bone formation. In addition, their capability to regulate bone resorption has been studied. Development of various human and animal derived osteoblastic cell lines has made it possible to study osteoblast differentiation and bone formation, different signaling pathways and drug pharmacokinetics in greater detail. Cell lines are an interminable and homogenous source of osteoblastic cells and allow the study of particular stages of osteoblast phenotype. The most commonly used cells lines are pre-osteoblastic cell lines MC3T3-E1 and UMR 201 derived from rodent calvarias, human osteosarcoma cell lines SaOs-2 and MG-63 and rat osteosarcoma cell lines UMR 106 and ROS17/2 (reviewed by Kartsogiannis et al. 2004). Cell lines established from pluripotent mesenchymal stem cells provide vital information on the signaling pathways and mechanisms regulating differentiation of osteoblasts, chondrocytes, adipocytes and myocytes. One interesting pluripotent preosteoblastic cell line is the mouse clonal cell line KS483, which is the subclone of the KS-4 immortalized cell line isolated from mouse calvaria (Yamashita et al. 1990). KS483 cells form ALP positive mineralized bone nodules already within three weeks of the culture period. They can be differentiated to all cell types of mesenchymal origin depending on the culture conditions. Importantly, their proliferation is dramatically enhanced after treatment with various BMPs and estrogen (Van der Horst 2002, Dang et al. 2002), while most other osteoblastic cells or even the primary osteoblasts respond poorly to estrogen treatment. Of the various cell lines, the KS483 based culture systems are particularly useful in the testing of novel anabolic compounds, whereas MC3T3-E1 represents a reliable option to primary human osteoblasts, as they share similar growth rate to human osteoblast cells. Despite the advantages, the cell line osteoblasts do not entirely reflect the behavior of primary cells (Czekanska et al. 2012). The majority of osteoblast-like cell lines do not form mineralized bone nodules in cultures, with the exception of KS483, MC3T3-E1 and few others.

The first attempts to isolate human osteoblasts from adult human bone took place already several decades ago (Bard et al. 1972). Human primary osteoblasts are typically isolated from the trabecular bone of long bones, the mandible and the iliac crest. As the accessibility of human osteoblast cells is limited, cells isolated from other species, such as rodents, can provide an alternative option for *in vitro* research models. In fact, rodent bone marrow osteoblast cultures have already been used for a quite a long time in the *in vitro* models to study bone formation and novel anabolic therapies (Luben 1976, Qu et al 1998). Currently, there are many new developments in this field, and, due to emerging information, it is now possible to differentiate human cord blood-derived pluripotent stem cells into osteoblasts and other terminal cells of mesenchymal origin, such as chondrogenic and adipogenic lineage cells, as well as into cardiomyocytes, skeletal muscle and neural precursors (Kögler et al. 2004). Their use provides more relevant results and boosts the clinical advantages into the translational process. Specifically in the areas of medical and pharmacological research, such as when developing for therapeutic

agents for osteoporosis, studies with cells derived from human donors with heterogeneous genetic background and with diagnosed illnesses are beneficial. Interesting additional advantage of these cells is their capability to form bone and cartilage tissues when implanted *in vivo*. Therefore, they can be used as seed cells in bone tissue engineering (Zheng et al. 2013). Despite of the several advantages of using human osteoblast cultures, it has not been demonstrated that they reflect clinical responses to several anabolic treatments, such as estrogen or PTH, which can be seen in human patients. However, it has not been confirmed whether or not these agents could even have direct anabolic effects on osteoblasts. In contrast, their anabolic responses are most probably mediated by a complex signaling network where osteoblastic bone formation is the final step.

The defining characteristic for all osteoblast cultures is their ability to produce a mineralized collagenous bone matrix. Another characteristic feature is their high ALP activity. Therefore, ALP activity is routinely used in *in vitro* experiments as a relative marker of osteoblast differentiation. In osteoblast differentiation and activity, three stages can be characterized: cell proliferation, matrix maturation, and matrix mineralization. The speed of precursor cells differentiating into mature osteoblasts determines the rate of bone formation. Matrix maturation and mineralization are usually enhanced along the culture period when osteoblasts grow in the presence of specific osteogenic factors. Ascorbic acid (vitamin C) and β-glycerophosphate supplementation is an essential prerequisite for matrix mineralization. Vitamin C is required for collagen synthesis whereas β-glycerophosphate serves as a source of phosphate ions. Once matrix mineralization is completed, calcium deposition can be visualized using adequate staining methods, such as Von Kossa or Alizarin red staining. Correspondingly to osteoclast assays, measurements of bone turnover markers secreted into the culture medium during the culture period or at the end of the study might provide more rapid and convenient approach for assessment of osteoblast differentiation and activity. Particularly the analysis of ALP, intact osteocalcin, and type I collagen levels as an index of osteoblast differentiation and organic matrix maturation and quantification of formed bone nodules by calcium and/or phosphorus measurement might be useful techniques to characterize osteoblast differentiation and activity *in vitro*. The assessment of the mineralization process of osteoblasts has been particularly used as a model for testing the effects of novel drug treatments as well as a mechanical loading on osteoblast differentiation and bone formation.

Osteocyte cultures and co-culture models

The location of osteocytes deep within the bone matrix is ideal for their function as regulators of several pathways and mechanosensors. However, this location also makes their observation and isolation difficult. By applying repeated enzymatic digestion and decalcification it has been demonstrated that primary osteocytes can be isolated from rat or mouse calvarial cortical bone (Gu et al. 2005a). The isolated primary osteocytes exhibited the classic osteocyte-like phenotype with long dendritic cell processes projecting away from the cell body and expression of several osteocyte specific genes, including osteocalcin, PHEX, DMP1 and sclerostin, as well as low expression levels of ALP. The responses of these primary osteocytes to glucocorticoid induced apoptosis and its protection by estrogen were similar to that what is seen in human GIOP patients. More advanced techniques integrate isolation with fluorescence-activated cell sorting (FACS), which may result in more homogenous and highly enriched population of mouse primary osteocytes (Stern et al. 2012).

To facilitate the study of osteocytes *in vitro*, immortalized osteocyte-like cell lines have been generated. One of these cell lines is well characterized MLO-Y4 cells which represent the phenotype of early osteocytes (Kato et al. 1997, Bonewald 1999). MLO-Y4 cells have been used to study the response of osteocytes to fluid flow and mechanical stimulus as well as in studies of communication between osteocytes and other bone cells and their responses to novel drug compounds. The known difference between the immortalized MLO-Y4 cell line and primary osteocytes is the undetectable expression of DMP1 and sclerostin in MLO-Y4 cells, which makes the use of MLO-Y4 cells less attractive in drug discovery as osteocytes are known to express these genes and, in the future, sclerostin, in particular, has been suggested to have high potential in the anabolic therapy in the treatment of osteoporosis. Another cell line, MLO-A5 that has the characteristics of a postosteoblastic and preosteocytic phenotype, has been used for osteocyte studies as it expresses several markers of osteocytes in the formation of cell processes (Barragan-Adjemian et al. 2006). However, these cells also express low levels of sclerostin and high levels of the markers of late osteoblast phenotype, such as ALP.

The role of osteocytes was detracted for decades as they were characterized by cells with reduced synthetic activity and a lack of capability of mitotic division with unknown function in bone metabolism. Currently, the role of osteocytes as multifunctional bone cells has been recognized and great emphasis is placed towards different strategies to develop novel osteocyte cell culture assays. Some studies show advanced 3D co-culture methods with other bone cells, while others have speculated even further with the systemic central regulation of bone remodeling by generating co-culture models of osteocytes and neurons (Boggs et al. 2011).

2.7.2 Experimental animal models of osteoporosis

Several animal species, including rats, mice, other rodents, dogs, rabbits and primates, have been used as animal models in osteoporosis studies (reviewed by Lelovas et al. 2008). Different approaches with different species have been developed for studies of primary and secondary osteoporosis. However, before new osteoporosis drug candidates can enter into clinical phases, their efficacy and safety must be demonstrated in specific preclinical models. The regulatory guidelines of the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA, previously EMEA) recommend that efficacy evaluation of a new osteoporosis drug should be performed with two different animal models, including the ovariectomized (OVX) rat model followed by the non-rodent vertebrate model (FDA 1994, EMA 2006). Due to this regulatory requirement, the rat is the most extensively studied species in osteoporosis studies. OVX primates are most often the second non-rodent animal species required by guidelines. In preclinical studies, the term osteoporosis is not typically used, because official age and gender matched T-score references are available only on human osteoporosis. In contrast, the term osteopenia, low bone mass, is utilized to describe decreased bone mass and architecture. Osteopenia in rats can be induced in numerous methods. The removal of organs producing gonadal steroids (gonadectomy) is the most typical procedure for inducing osteopenia in rats.

In the human skeleton, the transition from modeling to remodeling occurs approximately after the first two decades of life. In contrast, rats have significantly faster metabolism having respective transition taking place at the age of 3 months in the trabecular bone of the lumbar vertebrae, and at 6-9 months in the trabecular bone of proximal tibia. Furthermore, in the endocortical bone of the lumbar vertebrae this gradual transition

occurs at 3-6 months and in the endocortical bone of proximal tibia at 9-12 months (Erben 1996, Jee et al. 2001). These transition times are critical when designing optimal setup for the efficacy study. In theory, the clinically predictive model, age and bone site should be such that the predominant activity is remodeling. However, anabolic compounds are often tested with aged animals having low bone turnover and antiresorptive compounds are tested with younger animals having high bone turnover despite the prevailing state between modeling and remodeling. The major drawback of the rat skeleton is that as the epiphyses do not fuse, most of its long bones maintain their capability for longitudinal growth throughout their lifespan (Jee et al. 2001). As the average lifespan of most commonly used strains, Wistar and Sprague Dawley rats, is approximately 2-3 years, the epiphyseal growth plates of male rat long bones remain open even for 30 months, which reduces the exploitation of the male rats in osteoporosis studies. In female rat lumbar vertebra, the plates are open for 21 months and in proximal tibia, epiphysis growth stops at the age of 15 months. In female rats, elongation of long bones is remarkably ceased already at 10 months of age which can be considered as their peak bone mass age. At this age, the periosteal expansion at long bone diaphysis is already stopped (Jee et al. 2001). In intact female rats, age-related decrease in cortical BMD starts in the trabecular bone of the proximal tibia at the age of 12 months and in the lumbar vertebrae at the age of 15 months (Fukuda et al. 2004). Rats lack Haversian system remodeling in the cortical bone and are, in that sense, a poor animal model for studying cortical bone. In humans and larger animals, such as primates and rabbits, Haversian system remodeling causes cortical porosity which is an important predictor of osteoporosis as high porosity drastically decreases bone strength. However, endocortical and trabecular bone loss are the primary causes of postmenopausal osteoporosis in human, while Haversian system remodeling remains in lesser predominant role. Therefore, the lack of Haversian system remodeling in rats has no remarkable effect on the applicability of the rat model. Another limitation of rat models is the absence of impaired osteoblast function during the late stages of estrogen deficiency (Wronski et al. 1991). Despite all the disadvantages and caveats that rat has, it can largely be considered an applicable model for osteoporosis research. Particularly the OVX rat model bears a strong correlation to human postmenopausal osteopenia.

In animal models of osteoporosis, the study design plays a vital role. There is no such animal model that exactly mimics the various conditions of the human bone pathophysiology. Therefore, one must know the applicability and limitations of the different animal models. All experimental animal studies should include a reference compound used in clinical care to demonstrate the function of the experimental model but also the potential of the novel test compound. The methods used in the studies for the evaluation of bone metabolism, mass and architecture are basically the same as those used for humans. Several invasive or non-invasive techniques recommended by the guidelines should be used for evaluations during the study and at the end of the study. These techniques include static and dynamic histomorphometry, BMD measurements, bone mechanical testing, bone ash weight measurement and bone turnover marker measurements.

Histomorphometric analysis can provide a better understanding of bone dynamics and improve diagnostic and therapeutic interventions. Embedding undecalcified bone samples in plastic and their sectioning are the most widely used techniques for bone histomorphometric analysis (Baron et al. 1983, Parfitt et al. 1987, Eriksen et al. 1994). With a suitable staining method, for example Masson-Goldner Trichrome, the undecalcified sections can provide qualitative and quantitative information about the bone structure and components. In double fluorescent labeled samples, the kinetic variables show more information about the bone metabolic processes. BMD is a reproducible, accurate and

non-invasive way of diagnosing low bone mass in humans and in animals. Bone mineral density and cross-sectional dimensions of bone can be measured using several techniques. The most widely used techniques are peripheral quantitative computed tomography (pQCT) for the detailed trabecular and cortical bone analyses of long bones and dual energy x-ray absorptiometry (DEXA), for the analyses of the lumbar spine and the hip. Bone mechanical strength is considered as one of the most important parameters related to fracture risk in humans but also in preclinical animal studies (Peng et al. 1994). In animal models, mechanical testing of bone is performed for *ex vivo* harvested bone samples by applying force until the bone breaks. Three point bending test for long bones provides information on cortical bone properties, while the cantilever bending test for the femoral neck is used to measure trabecular bone properties (Ruhmann et al. 2006). This is a particularly important measurement as the femoral neck is a typical site where fractures occur in humans. In trabecular bone, the architecture of trabeculae seems to be a more important determinant for strength than the material properties (Currey 2003). The compression test can also be utilized to test trabecular bone from disc or flat shaped bones such as vertebral bodies. When bones are burned in high temperature, the remaining ash contains the inorganic matrix of bone as the organic matrix is absent. Thus, ash weight determination shows the overall amount of inorganic bone matrix. In addition, it is possible to further analyze the detailed mineral content of the ash, the ratio of calcium and phosphorus. Bone turnover markers are discussed in more detail in the chapters about biochemical markers of bone turnover and materials and methods.

2.7.3 Ethics in animal studies

When selecting an appropriate animal model for animal studies, the correspondences in pathophysiologic responses between the animal species and human is often the main determinant, and financial issues and ease of use are often other determinants of the animal species selection. The general public is accustomed to the use of rats and mice in medical research. In osteoporosis studies the use of rodents cannot be avoided since the complete bone remodeling cycle occurs only in vertebrae. With bone cell cultures it is possible to mimic parts of the remodeling cycle, but these results are not predictive for the systemic effect evaluation. Currently, great emphasis is placed on the development of novel co-culture systems of osteoclasts, osteoblasts and osteocytes which, in the future, might provide a better outcome and thereby enable reducing the use of animals. Experimentation with all animal species should always adhere to the 3R principles of ethical animal use: replacement, reduction, and refinement (The National Centre for the Replacement, Refinement and Reduction of Animals in Research, NC3Rs). The 3Rs principles are an ethical framework for using animals humanely while conducting scientific experiments; replacement aims to increase the use of non-animal methods, reduction promotes methods which reduce the number of animals used and refinement focuses on methods which improve animal welfare. Experiments involving non-human primates are controversial. Primates are used because they share structural and functional features with humans. In osteoporosis research, non-human primates demonstrate many advantages over rat and other animal models as their organ systems most closely resemble the human systems. Primate study is even recommended to be the final test before clinical trials after rats and possibly some larger animals have been tested. However, the use of primates raises difficult philosophical debate regarding the benefits and moral costs. Furthermore, in osteoporosis research, it is speculated that there is no such new information forthcoming from primate studies that would significantly improve the interpretation of the results obtained from well-designed rat OVX studies (Jee et al. 2001).

2.7.4 Ovariectomy (OVX) and orchiectomy (ORX) rat models

Surgical removal of ovaries is referred to as ovariectomy (OVX) and it leads to dramatic decrease in the estrogen production in female rats. Similarly, the removal of testis, referred as orchiectomy (ORX), leads to decreased androgen levels in male rats. Following these surgical gonadectomy operations trabecular bone turnover increases, leading to rapid bone loss (Figure 6). Estrogen and androgen treatments completely block the activation of bone turnover and bone loss (Thompson et al. 1995, Borst et al. 2007). Osteopenia, similar to surgical gonadectomy, can also be induced with pharmaceutical drugs which are routinely used in humans for the treatment of breast cancer or endometriosis. These drugs include estrogen receptor antagonists, gonadotropin releasing hormone agonists and aromatase inhibitors which are all associated with accelerated bone loss. The effects of these drugs are reversible, in contrast to surgically induced osteopenia (Gasser et al. 2006). The OVX rat model is well characterized and demonstrated to mimic postmenopausal bone loss in trabecular bone (reviewed by Kalu et al. 1991, Jee et. al 2001). However, this correlation is competent only when examined over relatively short periods of time. When the study duration extends to one year as suggested by the regulatory guidelines, the bone turnover in trabecular bone returns to the value of sham control animals, although the trabecular bone volume remains to be lower. Therefore, bone turnover is a particularly challenging parameter when evaluating the efficacy of potential therapeutic agents in estrogen deficient conditions in long-lasting animal studies (Thompson et al. 1995). On the other hand, as older animals more accurately reflect human pathophysiology, the very aged rat model (30-month old) might also be cost-effective and a predictive animal model (Gaumet et al. 1996). After ovariectomy, bone turnover increases rapidly within a few days and bone resorption exceeds formation leading to bone loss. Remodeling reaches a steady state within a few weeks. Not all trabecular bone sites exhibit similar bone loss and neither do all trabecular bone sites lose bone at the same rate (Jee et al. 2001). Statistically significant bone loss is seen in the trabecular bone of proximal tibia after two weeks, (Wronski et al. 1988), in the femoral neck after 1 month (Wronski et al. 1989a) and in the lumbar vertebral body after 2 months (Wronski et al. 1989b). In contrast, OVX induced bone loss does not occur in epiphyseal trabecular bone of long bones, the metaphyseal trabecular bone of distal tibial or the trabecular bone of caudal vertebra (Li et al. 1996, Miyakoshi et al. 1999). In cortical bone of long bone diaphysis, OVX enhances periosteal bone growth (Turner et al. 1987). On the other hand, the endosteum of diaphysis in the ovariectomized rat exhibits increased bone resorption leading to an enlargement of the medullary cavity (Turner et al. 1987, Danielsen et al. 1993). This leads to the fact that bone lost at the endosteum is being replaced on the periosteum. As a result of these exclusive changes, cortical bones display very slow changes in the OVX model (reviewed by Kalu et al. 1991). OVX also induces significant weight gain, and the resulted increased body weight has a tendency to increase bone mechanical loading thereby increasing bone mass. However, this protective effect is of a lesser magnitude than the actual OVX induced bone loss (Peng et al. 1997). OVX leads also to muscle atrophy. Muscle mass or strength measurements have been demonstrated to be early predictors of bone changes because of a strong correlation between different measurements of bone strength and muscle strength (Burr 1997).

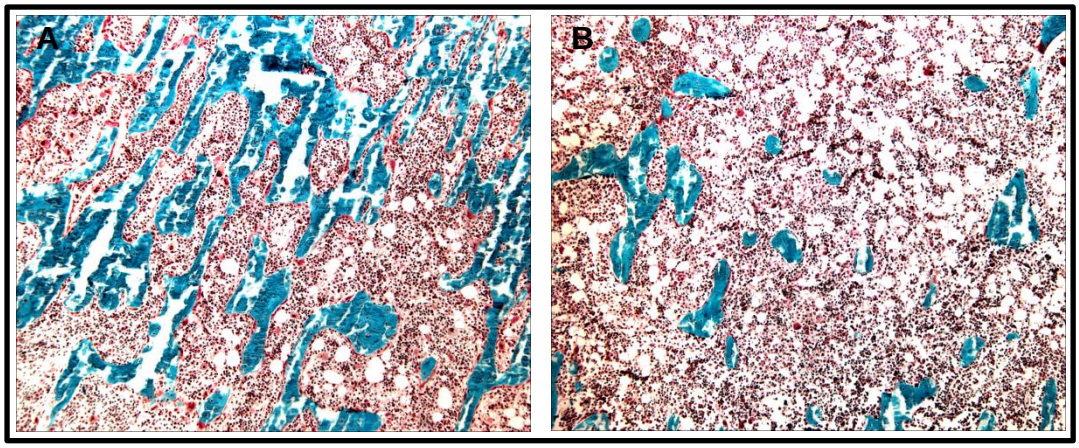

Figure 6. A photomicrograph of rat trabecular bone at proximal tibia metaphysis. Normal bone of a healthy rat (A) and osteopenic bone of a OVX rat at 8 weeks after operation (B). Masson-Goldner Trichrome staining showing trabecular bone (blue) and bone marrow (red). Unpublished data.

Regulatory rat OVX model

If the results of the preceding preclinical studies still support the efficacy of the drug candidate, it can finally be tested further in regulatory studies, first in the rat OVX model and then in a primate OVX model (Brommage et al. 1999) or with other large mammalian species according to the EMA and the FDA guidelines. The regulatory studies are designed in co-operation with the drug approval authorities. In general, study design of a regulatory rat OVX study should reflect clinical indication in humans. Specific attention should be paid to issues such as prevention versus treatment schedule, continuous versus intermittent dosing and duration of a dosing. All these parameters are strongly related to the OVX model but also to the physico-chemical properties of the experimental drug candidate. The new experimental drug therapy should be tested at a clinical dose and a dose at least 5 times higher than the clinical dose. Duration of the study should be comparable to 4 years of human exposure. This designates the duration of rat studies from 12 to 16 months. High costs, long time frame and ethical issues of primate use in the regulatory preclinical efficacy studies are the reasons why these studies are regarded as outdated and undesirable. In contrast, more predictive, faster and lower cost structure animal models and *in vitro* assays play a crucial role in the future osteoporosis research.

2.7.5 Secondary osteoporosis models

Immobilization induced osteoporosis

Respectively to gonadectomy-induced primary osteoporosis models, secondary osteoporosis can be induced to rats and other laboratory animals by mimicking the pathophysiology of human diseases. Immobilization (IM, also referred to as disuse-induced osteoporosis) and glucocorticoid-induced osteoporosis (GIOP) are the main forms of secondary osteoporosis. A number of animal models of IM-induced osteoporosis have been developed to provide a basis for understanding bone loss induced by IM. Originally, it was studied with a tail suspension model (Zhang et al. 1995), but it is no longer an ethically accepted model as it makes the movement of animals highly limited. The relevant models

for studying disuse-induced osteoporosis include surgical models, where IM is induced by nerve, tendon or spinal cord resection (Zeng et al. 1996, Thompson et al. 1988, Okumura et al. 1987). IM can be induced also reversibly by conservative methods such as immobilization of hind limb to plantar flexion with a plaster cast (Fleisch et al. 1969) and a model where neurotoxin botulinum toxin is injected intramuscularly into the hind leg causing temporarily paralysis of muscles and disuse of the leg (Grimston et al. 2007). In all IM-induced osteoporosis models, bone loss occurs at the site of immobilization, most typically in one of the hind legs. IM leads to muscle atrophy and radical changes in bone turnover. It causes simultaneous increase in bone resorption and a decrease in bone formation (Izawa et al. 1981). This is followed by a low bone turnover state which leads eventually to bone loss and reduced mechanical properties that mimic the clinical situation of osteoporosis caused by immobilization (Peng et al. 1994). Immobilization leads to relatively rapid bone loss which is faster in the trabecular than in the cortical bone. The statistically significant bone loss in the trabecular bone of proximal and distal tibia is observed already at 2 and 4 weeks after the initiation of immobilization (Li et al. 1990). In the diaphysis of long bones, marrow cavity enlargement and significant thinning of cortical bone is seen after 6 weeks in tibial diaphysis and after 3 weeks in femoral diaphysis, respectively (Li et al. 1991). In the IM model, unloading results in cessation of periosteal bone formation while endosteal resorption is a continuous process. Both gonadectomy and immobilization increase the endocortical resorption and thereby the endocortical perimeter, leading to the expansion of the marrow cavity. Because of concomitantly suppressed periosteal bone formation with increased endocortical resorption, bone loss and decrease in mechanical properties is more severe after immobilization than after ovariectomy (Bagi et al. 1993). One of the advantages of the immobilization model is that, as it has overall a low bone turnover rate, it is suitable for the anabolic compound testing, whereas the rapid increase in bone turnover immediately after immobilization enables its utilization also for antiresorptive testing.

Combining ovariectomy with IM might pair the advantages of both methods, as, in humans, ovariectomy is often followed by a fracture treated with plaster cast. Most importantly, this combination model allows eliciting bone loss at the distal tibia which remains unchanged after OVX operation alone. Based on animal studies, it is hypothesized that estrogen deficiency of postmenopausal women, together with reduced physical activity or hypokinesis, are key factors in the pathogenesis of postmenopausal osteoporosis (Miyagawa 2011). Combining these experimental animal models might also reduce the time when bone loss becomes significant, particularly in cortical bone (Okumura et al. 1987). However, it is not clear if this reduction in timeline is relevant and consistent with human pathophysiology.

Glucocorticoid induced osteoporosis

GIOP can be induced in intact mice by glucocorticoid treatment such as dexamethasone or prednisolone (Yao et al. 2008). The model is commonly used to determine the effects of novel drug candidates in prevention or treatment of GIOP or discovering new glucocorticoids with less severe side effects in the bone. The biology behind the GIOP model is partly unknown. GIOP studies have yielded inconsistent results where some studies have been unable to observe bone loss (Shen et al. 1997), and other studies show bone loss after glucocorticoid administration (Nitta et al. 1999). The evidence demonstrating that the rat is capable of mimicking the glucocorticoid induced bone loss observed in humans is not available. Studies with mice have resulted a similar phenotype; significant decrease in bone formation but not prominent bone loss (Yao et al. 2008). These observations might be partly explained by the study set up in which the time period

is too short for the development of bone loss, but sufficient enough to observe the cessation in bone formation. In well characterized and largely cited GIOP studies by Manolagas and Weinstein, mice that received glucocorticoid for 7 days displayed rapid increase in bone resorption and after 4 weeks displayed a decrease in BMD, numbers of bone cells, mineral appositional rate and bone formation rates and a dramatic reduction in the trabecular bone mass. Furthermore, glucocorticoid administration caused a 3-fold increase in osteoblast apoptosis in vertebral trabecular bone. In the metaphyseal cortical bone, none of the osteocytes of control animals exhibited apoptotic features, whereas 28% of the osteocytes were apoptotic after prednisolone treatment (Weinstein et al. 1998, Manolagas and Weinstein 1999). In humans, glucocorticoid therapy is often continued despite the underlying progression of osteoporosis due to some other primary disease that requires glucocorticoid medication. Also aged people, having already been diagnosed with osteoporosis, are often prescribed glucocorticoids for a variety of inflammatory conditions. Therefore, a combination model of OVX and GIOP might provide a more relevant and advanced model than either of the models alone. OVX rats treated with dexamethasone displayed slightly a pronounced effect on osteoporotic phenotype, similarly to humans (Heiss et al. 2012). Prednisolone treatment in OVX rabbits resulted in a significant bone loss in trabecular bone, but not in cortical bone when compared to the OVX group (Liu et al. 2012b). In mice, yet to date, no comparison studies exist to demonstrate a significant pronouncing effect of these methods.

2.7.6 Other animal models of osteopenia

Other experimental methods inducing osteopenia in rats include a low-calcium diet for immature rats and alcohol abuse (Seto et al. 1999). In the past, dogs have been used for osteoporosis studies because of their extensive basic multicellular unit (BMU) bone remodeling (Nakamura et al. 1992). However, reasons similar to primates, i.e. ethical issues, costs and lack of forthcoming information superior to rodent models, dogs are not used for bone studies nowadays. Today, sheep is a more common choice for the second animal species stated in guidelines.

Mice are largely used for bone studies despite the regulatory requirement of the rat model. Mice have many advantages over the rat and other animal species commonly used in bone studies. Mice have a similar skeletal development to that seen in humans, wide accessibility and relatively low costs and defined genetics. In addition, mice are extensively studied animals with well documented results and established protocols. Currently, numerous transgenic mouse models have been used to study bone loss and remodeling. Their genome is well characterized and numerous strains of mouse mutants with altered bone phenotype are easily available. Therefore, mice are always the logical starting point for the genome studies. For example, a Senescence-accelerated mouse (SAM) has been developed as a model for age-related osteopenia (Okamoto et al. 1995). The successful development of several transgenic animal models has led to remarkable breakthroughs in the drug development of osteoporosis. These knock-out and overexpression models have increased our knowledge of the role of specific proteins in bone metabolism. One such example is the recently approved antiresorptive therapy, denosumab, the development of which was remarkably boosted by the findings obtained in the RANK, RANKL and OPG deficient mice (Kong et al. 1999, Dougall et al. 1999, Mizuno et al. 1998). Mouse OVX and ORX models can also be utilized to bone studies. In general, what is mentioned above of the rat OVX and ORX models can be applied to the mouse OVX and ORX models. The time periods of trabecular and cortical bone changes are similar to that of the rats. The

OVX or ORX mouse models can be particularly applicable as an initial *in vivo* screening of new drug compounds, as lesser amount of drug is needed compared to the rat studies. In contrast, the small size of mice causes technical challenges for executing the surgical OVX and ORX operations reliably.

2.8 Biomarkers

Although the term biochemical marker is relatively new, they have been used in pre-clinical and clinical research for a long time. Already 6000 years ago, human urine was analyzed for diagnostic and predictive purposes (Armstrong et al. 2007). For example, urinalysis was performed by one of the earliest civilizations, an ancient Sumer, who recognized that urine characteristics were associated with different diseases (Connor et al. 2001). At that time, traditions and myths were more determinative for the management of therapy than the outcome of the visual urine analysis. More advanced methods were developed in ancient Egypt, where pharaohs used wheat and barley seeds for assessing pregnancy and even gender of unborn infant from urine. This theory was tested in 1963 and it showed 70% predictive value (Halim et al. 2011), which is a relatively poor outcome for today's biomarkers but an amazingly modern method thousands of years ago. Over the times, assays have become more sophisticated and during the last decades, the importance of biomarkers in preclinical and clinical studies has increased exponentially. The number of articles containing biochemical markers or biomarkers as a keyword has increased rapidly over the past two decades (Hagen 2012). To date, over 600 000 scientific articles contain biomarker as a keyword (Pubmed search) and several thousands have focused purely on a new biomarker discovery (Table 2).

Table 2: Overview of the relationship between publications focused on discovery of different potential biomarkers and patenting of biomarkers. Modified from Drucker et al. 2013.

Year	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Publications	625	1087	1478	1920	2661	3270	4168	5033	6208	7720
Patents	50	74	95	180	238	320	396	593	471	407

The classical definition of a biomarker is: "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" (Biomarker Definitions Working Group 2002). Thus, by definition biomarkers are objective, quantifiable characteristics of biological processes. Biomarkers typically denote molecular biomarkers which are referred to as alterations of a nucleic acid, metabolite or protein levels. These indicators can be measured from blood, urine, other body fluids or tissues. Biomarkers are categorized as markers of 1) natural history (type 0), indicating disease progression; 2) drug activity (type 1), indicating response to drug therapy; or 3) surrogates (type 2), which are intended to substitute the clinical end points. Biomarkers can also be categorized as pharmacodynamic, prognostic or predictive biomarkers. Technically biomarkers can be further sub-classified into categories by characteristics and measurement technology such as imaging or molecular biomarkers.

Currently one of the best known biomarkers are glucose for diabetes disease, low-density lipoprotein (LDL) cholesterol for coronary and vascular disease risk assessment and c-reactive protein (CRP) for inflammation diseases. In addition, over a hundred different biomarkers with regulatory approval are today used in the field of laboratory diagnostics. The most common tests are fully automated for both human and animal testing. However, most biomarkers are still under investigation and thus they have the potential to become a diagnostic practice. Moreover, biomarkers still have several technical and scientific limitations. Reasons for these pitfalls are manifold, including challenges in validation, analysis methods, sample handling, study design or execution. In addition, biomarkers are often assayed by research-grade tests and with unstandardized techniques, which leads to challenges in result interpretation. Despite of these disadvantages, approaches to address these limitations are currently extensively studied and thus biomarkers have the potential to have a significant impact on the economics and time lines in drug development and patient management in several fields of medical research. Particularly drug development companies are increasingly using strategies that are based on biomarkers to improve their drug development processes (Figure 7).

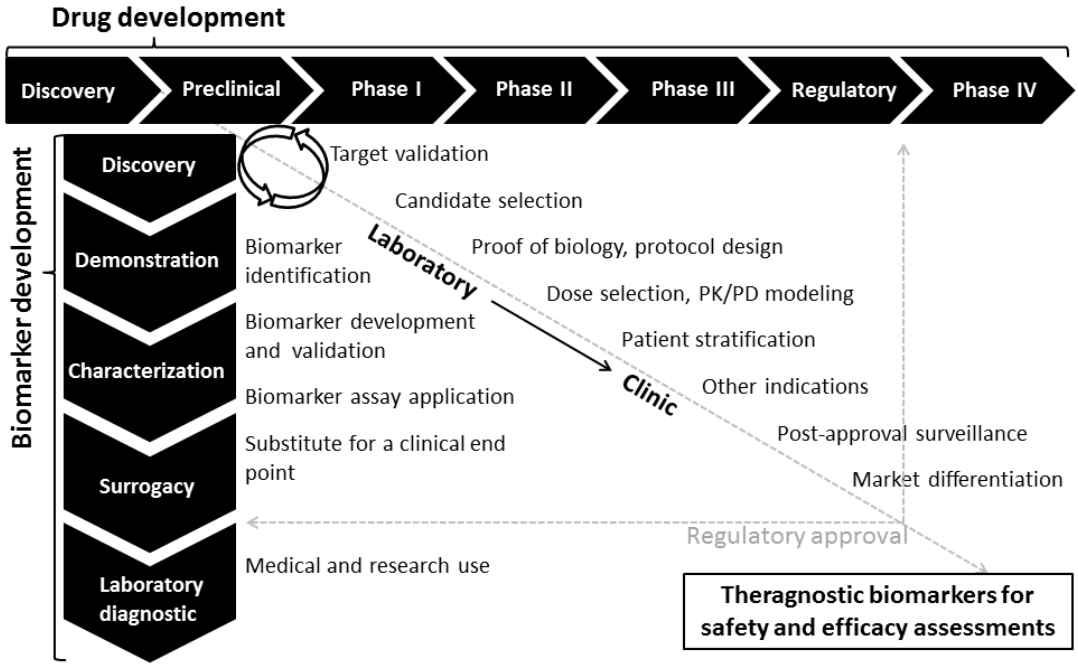

Figure 7. A Schematic drawing indicating intertwined processes of biomarker development and drug development. Several efficacy and safety biomarkers can be developed and applied to numerous steps in drug development processes. The ultimate goal of biomarker development is theragnostic (or theranostic) biomarker, which employs predictive use of biomarkers combining therapeutics with diagnostics. Modified from Amir-Aslani et al. 2010, Lee et al 2007.

The development of biomarker involves sequential phases, similar to drug development. Identification of a new biomarker leads to validation studies and eventually to regulatory approval and commercialization for laboratory diagnostics. As with drug development,

several go- versus no-go-decision making steps are related to the development pathway. The decision to proceed with biomarker development should be based on balancing between the assay robustness, feasibility, strength of science and resources with the burden of proper sample collection. In addition, potential impression on drug development should be considered (Dancey et al. 2010).

Biomarkers can be detected by several established technologies, including immunoassays, mass spectrometry, liquid chromatography and microscopic quantification. Of these, immunoassays are the most convenient as they are highly sensitive, specific and selective technology to interrogate such markers. In addition, immunoassay represents the most commonly used relatively inexpensive technique, thus providing a good applicability in most of the laboratories to numerous of compounds that are of interest. Over the years, the launching of various new immunoassays has risen rapidly, as new knowledge has been discovered in the fields of various diseases, application technology and immunochemistry. Immunoassays are used in a large variety of medical research, ranging from HTS of early drug discovery to surrogate endpoint in the clinic. Immunoassays are a diverse group of analytical techniques, but most immunoassays are typically based on the same strategy in which the antigen specific antibodies incorporated in the measurement plate are used to capture the antigen present in a biological sample added to the plate.

2.9 Biochemical markers of bone turnover

In the normal skeleton of vertebrates, the bone formation markers follow the same pattern as the bone resorption markers, because these two crucial processes that regulate bone mass are tightly coupled. The coupling is maintained in bone multicellular units in a process called bone turnover or remodeling. The levels of bone turnover markers are high during childhood and adolescence, lower towards adulthood, and increase rapidly after menopause. Various drug therapies and diseases affect bone remodeling and the secretion of bone markers. They decrease after anti-resorptive drugs or hormone replacement therapy which is followed by a decrease in bone formation markers. Formation and resorption tend to follow the same pattern, but in some diseases, including osteoporosis and bone metastasis, there are remarkable delays in the coupling, which leads to pathological bone remodeling and eventually to abnormal bone mass and architecture.

Bone turnover markers are released during the remodeling process and their levels can be conveniently and reliably measured from blood or urine by immunoassays. Traditionally, bone turnover markers have been categorized into markers of bone formation and markers of bone resorption (Table 3). They originate either from the bone cells or from the bone matrix during resorption. Therefore, bone turnover markers reflect bone cell differentiation or activity and can be further classified as markers that reflect (1) osteoclast differentiation referring to osteoclast number, (2) osteoclast activity referring to bone resorption, (3) osteoblast differentiation referring to osteoblast number, and (4) osteoblast activity referring to bone formation. However, the classification of a marker is not always distinct. Some markers have several distinct fragments that reflect different phases of the bone remodeling cycle. For example, certain forms and fragments of osteocalcin reflect both formation and resorption.

Table 3. Commonly used markers of bone formation and resorption and their commercially available measurement technology (modified from Seibel 2005, Sørensen et al. 2007b and Vasikaran et al. 2011).

Marker	Origin	Bone cell process	Specimen	Methods
Bone formation markers				
ALP	several tissues	osteoblast differentiation	serum	Colorimetry
BALP	bone	osteoblast differentiation	serum	IRMA, EIA
OC	bone, platelets	bone formation	serum	RIA, IRMA ELISA
PICP	tissues containing type I collagen	bone formation	serum	RIA, ELISA
PINP	tissues containing type I collagen	bone formation	serum	RIA, ELISA
Bone resorption markers				
αCTX	tissues containing type I collagen	bone resorption (new bone)	urine, serum	ELISA, RIA
βCTX	tissues containing type I collagen	bone resorption (old bone)	serum	ELISA, RIA
BSP	bone, dentin, hypertrophic cartilage	bone resorption	serum	ELISA, RIA
Cat K	primarily osteoclasts	osteoclast differentiation	plasma, serum	ELISA
DPD	bone, dentin	bone resorption	urine, serum	HPLC, ELISA
ICTP	bone, skin	bone resorption (new bone)	serum	RIA
NTX	tissues containing type I collagen	bone resorption	urine, serum	ELISA, CLIA, RIA
OHP	bone, cartilage, skin, soft tissue	bone resorption	urine	Colorimetry, HPLC
OC-f	bone	bone resorption	urine	ELISA
PYD	bone, cartilage, tendon, blood vessels	bone resorption	urine, serum	HPLC, ELISA
TRACP 5b	osteoclasts	osteoclast differentiation	plasma, serum	Colorimetry, ELISA, RIA

ALP, total alkaline phosphatase; BALP, bone specific alkaline phosphatase; OC, intact osteocalcin; PICP, C-terminal propeptide of type I collagen; PINP, N-terminal propeptide of type I collagen; CTX, C-terminal crosslinked telopeptide of type I collagen; BSP, bone sialoprotein; Cat K, cathepsin K; DPD, deoxypyridinoline; ICTP, C-terminal crosslinked telopeptide of type I collagen; NTX, N-terminal crosslinked telopeptide of type I collagen; OHP, hydroxyproline; OC-f, osteocalcin fragments; PYD, pyridinoline; TRACP 5b, tartrate-resistant acid phosphatase 5b; CLIA, chemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; RIA, radioimmunoassay.

In skeletal biology, applications of bone turnover markers today and in the future may be summarized as being either diagnostic of osteoporosis and other bone diseases, prognostic of these diseases or a therapeutic outcome of existing or novel anabolic or antiresorptive drug compounds. Bone markers are not yet used to diagnose osteoporosis. Instead, bone markers are mainly applied in monitoring the efficacy of the treatment of osteoporosis in both clinical and preclinical studies (Cremers et al. 2006, Sørensen et al. 2007b). It has been demonstrated in clinical studies that short-term changes in bone turnover markers can predict long-term changes in BMD and future fracture risk (Garnero et al. 1999, Gerdhem et al. 2004).

There are several bone turnover markers that can be measured in preclinical efficacy studies. Today, majority of these are commercially available, including C-terminal crosslinked telopeptides of type I collagen (CTX), Tartrate-resistant acid phosphatase isoform 5b (TRACP 5b), procollagen I N-terminal propeptide (PINP) and osteocalcin, all of which can be conveniently measured in the same serum or culture media samples. Of these, PINP and CTX are widely used and recommended by various experts and guidelines to be included as reference markers for bone formation and bone resorption. In the following chapters, these four markers will be discussed in more detail. In addition, the most commonly used bone formation and resorption markers will be introduced.

2.9.1 Markers of bone formation

Alkaline phosphatase (ALP)
In addition to previously mentioned PINP and osteocalcin, there are several other markers that reflect differentiation and activity of osteoblasts. ALP belongs to a large family of proteins which are attached to the extracellular surface of cells and it is expressed in several other tissues besides bone. The most common sources of ALP at the organ level are bone and liver. Despite the fact that they are encoded by the same tissue non-specific gene, there exist tissue specific differences between the carbohydrate side chains of the molecule (Weiss et al. 1988). This phenomenon has facilitated the development of bone specific form of ALP (BALP). Until the initiation of this thesis work, only human BALP immunoassay has been developed and there exist no immunoassay method for the determination of rodent bone-specific ALP. If available, this would allow reliable detection of ALP secreted into the culture medium or serum in preclinical *in vitro* and *in vivo* rodent based assays. Without such assays, the detection of ALP is challenging, because the amount of total ALP activity in the culture medium, which can be measured by indirect robust measurement, is low and other factors in the medium interfere with the measurements. In bone, ALP is expressed on the surface of active osteoblasts, and, therefore, it has been used as a histochemical marker for osteoblasts (Henrichsen 1956). Immunoassays utilizing BALP display 10-20% cross-reactivity with the liver isoform (Garnero et al. 1993) which sets limitations regarding the interpretation of BALP results. On the other hand, BALP has good applicability in various measurements as it has no significant circadian variations. BALP has a half-life of approximately 40 hours before being cleared in the liver (Crofton 1982). BALP levels are affected by several factors including gender, hormonal status, age, and underlying diseases, such as osteoporosis or fractures. BALP is demonstrated to be an adequate marker in clinical practice for assessments of several metabolic bone diseases, such as osteoporosis, Paget disease and chronic kidney disease (reviewed by Naylor 2012).

Propeptides of type I collagen (PINP and PICP)

In addition to PINP, other propeptides of type I collagen are produced during the bone formation. Along the collagen synthesis C-terminal propeptides (PICP) are cleaved off and released into circulation. As with PINP, PICP is released in a stoichiometric relationship to collagen molecules during their synthesis. Therefore, it can be considered as a measure of recently synthesized type I collagen. Type I collagen is a major component also in other connective tissues which might contribute to the PINP and PICP levels in the serum. However, in bone, the collagen turnover rate is faster than in other connective tissues, and therefore the major changes in PINP and PICP concentrations are assumed to reflect changes in collagen synthesis in bone (Cremers et al. 2008). PICP displays good correlation in overall bone formation level (Eriksen et al. 1993), but only minor increase after menopause, and it has poor correlation with spinal BMD. It is therefore not routinely used in the assessments of osteoblast function, but instead it has been demonstrated to significantly reflect children's growth (Trivedi et al. 1991). Both propeptides have a circadian rhythm of peak values in the early morning. Age has a profound effect on the levels of circulating PINP and PICP. Thus, PINP and PICP levels are higher in children than in adults (Crofton et al. 2004). PINP has a half-life of 1 minute while PICP has a half-life of 6-8 minutes (Smedsrød 1990). Immunoassays for human PICP and PINP have been developed and are currently available (Taubman et al. 1974, Melkko et al. 1990, Melkko et al. 1996). In addition, separate assays have been developed for the two antigens present in human serum, intact trimeric PINP and monomeric form. The intact PINP assay measures the trimeric form and the total assay measures both forms (Koivula et al. 2012). In humans, PINP circulates as different forms including intact trimeric form, a smaller monomeric form and several fragments. The intact form is cleared in the liver. However, the other smaller forms are cleared by the kidneys. Therefore, serum levels of total PINP can be significantly increased in patients with chronic kidney disease (Brandt et al. 1999). Until this thesis work, no commercial immunoassay method for measuring rodent PINP has been available, though some mass spectrometry-based methods have been described (Han et al. 2007).

Osteocalcin (OC)

Originally Gla residues were found in bovine prothrombin and assumed to be required for the binding of prothrombin to calcium phosphate in the presence of Ca^{2+} (Gitel et al. 1973). After association to bone tissue, the protein was abbreviated to bone Gla protein (BGP, BGLAP), or osteocalcin (OC). Osteocalcin is a small hydroxyapatite-binding protein synthesized by mature osteoblasts, osteocytes, odontoblasts and hypertrophic chondrocytes. It has three calcium binding vitamin K-dependent Gla residues responsible for the hydroxyapatite binding properties of the protein (Hauschka et al. 1989). Vitamin K is essential for the γ-carboxylation of glutamic acid residues which are needed for the mineral binding ability of the osteocalcin molecule. In other words, undercarboxylated osteocalcin reflects vitamin K insufficiency. After its release from osteoblasts, osteocalcin is primarily incorporated into the extracellular matrix of bone, but a small fraction is released into the circulation. The intact osteocalcin is relatively rapidly degraded into large N-terminal mid fragment 1-43, decreasing the applicability of the intact osteocalcin measurement. N-mid osteocalcin is thought to be generated by proteolysis in the circulation but also during sample processing and storage, particularly if stored at room temperature. The role of the N-terminal fragment is partly unclear. The majority of circulating osteocalcin is catabolized in renal degradation and filtration. The half-life in plasma is approximately 20 minutes in humans. Circulating osteocalcin follows a circadian rhythm peaking late at night followed by decline towards the noon (Gundberg et al. 1985). Intact osteocalcin is suggested to represent about one third of the total osteocalcin in serum, N-mid fragment another third

and the remainder third is composed of various fragments (Garnero et al. 1994). Several commercial immunoassays have been developed for human and rodent osteocalcin. The newer assays detect both the intact molecule and its N-terminal mid fragment. As intact osteocalcin is markedly unstable *in vitro*, it causes additional complexity to osteocalcin measurements (Garnero et al. 1994, Käkönen et al. 2000). Another concern is the antibodies used to detect various fragments of osteocalcin. Their specificity and cross reactivity to other fragments might partly explain the observed assay variability. In addition, osteocalcin is sensitive to hemolysis (Tracy et al. 1990). Despite these limitations, osteocalcin in serum reflects osteoblast activity and is considered as a sensitive and specific marker of bone formation (Ivaska 2005). Furthermore, some osteocalcin fragments are also released from bone matrix during the resorption process (Ivaska et al. 2004).

2.9.2 Markers of bone resorption

Cross-linked telopeptides of type I collagen (CTX, ICTP and NTX)
Bone resorption markers of collagen breakdown products include telopeptides of N-terminal (NTX) and C-terminal (CTX and ICTP) end of type I collagen. Bone resorption by osteoclasts is overwhelmingly a cathepsin K-driven process and MMPs play an important but minor role in the resorption process. Only cathepsin K produces CTX and NTX fragments whereas only MMPs produce ICTP fragments. Thus, the type I collagen fragments ICTP, NTX and CTX reveal distinct enzymatic pathways of bone collagen degradation (Atley et al. 2000, Garnero et al. 2003). Serum and urine CTX and NTX levels are markedly increased in patients with postmenopausal osteoporosis, whereas ICTP seems to be more useful for assessment of pathological bone resorption as seen in bone metastases. CTX exists in an isomerized β-CTX and non-isomerized α-CTX forms. The native α-CTX undergoes spontaneous β-isomerization which is associated with protein ageing. The β-CTX contains a β-aspartyl peptide bond, which is assumed to result from the ageing of ECM proteins (Bonde et al. 1997). The equilibrium of the isomerization process is reached after about 150 days after the bone is mineralized, and thus β-CTX reflects the resorption of rather old bone (Fledelius et al. 1997). In contrast, the α-CTX is more abundant than the β form in healthy individuals or in various disease stages in which newly formed type I collagen is present and thereby it represents the resorption of relatively young bone (Bonde et al. 1997). Measurement of both forms and calculating the ratio of α-CTX/β-CTX as an index of bone turnover might be useful and provide additional information (Garnero et al. 1997). There are commercial assays available for urinary α-CTX (Bonde et al. 1996) and β-CTX (Bonde et al. 1994), serum β-CTX (Bonde et al. 1997) and ICTP (Risteli et al. 1993) measurements. Currently, serum β-CTX is the most extensively used crosslink assay and will therefore be exclusively referred to as CTX later in this thesis work.

CTX shows significant diurnal variation and the effect of food intake, which highlights the need for fasting before CTX measurements (Qvist et al. 2002). The half-life of serum CTX is estimated to be about 1 hour. Levels of serum CTX are highest in newborn children and then significantly decrease after 1 year of age. A second peak is observed around teenage (Crofton et al. 2002). Urinary NTX is likely to reflect the degradation of both isomerized and non-isomerized type I collagen. However, CTX is today, in Europe, the preferred assay over NTX by experts and guidelines, because it tends to have slightly more sensitive responses in pathological skeletal processes than urine NTX (Takahashi et al. 2002). Nevertheless, both markers are useful in reflecting bone resorption and currently in worldwide use along with pyridinium crosslinks.

Tartrate-Resistant Acid Phosphatase 5b TRACP 5b

TRACP belongs to the family of the acid phosphatases, of which five different isoforms are known. In an acidic environment, TRACP is able to catalyze the hydrolysis of several phosphate esters, including α-naphtyl phosphate, phosphotyrosine and 4-nitrophenyl phosphate, but also to a lesser extent with β-glycerophosphate. Because of its abundant expression in osteoclasts, it has been widely used as a histochemical marker of osteoclasts. The two forms of TRACP enzyme that circulate in human blood are TRACP 5a and TRACP 5b. Of these, TRACP 5a is derived from macrophages whereas TRACP 5b is derived from osteoclasts. TRACP has been localized in transcytotic vesicles and intracellular vacuoles of osteoclasts (Halleen et al. 1999). As these vesicles contain also endocytosed bone matrix components, TRACP 5b was originally suggested as a marker of bone resorption. Later, it was observed that TRACP 5b is secreted into the circulation by both non-resorbing and resorbing osteoclasts (Alatalo et al. 2000, Karsdal et al. 2003), and therefore TRACP 5b is recently considered as a specific and sensitive marker of osteoclast number. TRACP 5b shows relatively low circadian variation, negligible effect after food intake and a favorable signal-to-noise ratio. Thus, the half-life of TRACP is relatively long compared to other bone markers. TRACP 5b has been reported to be stable up to 2 days at room temperature but longer storage should be kept at -70°C or lower (Haleen et al. 2006). Several immunoassays for rodents and human have been developed for the determination of circulating TRACP 5b (Halleen et al. 2000, Janckila et al. 2004, Ohashi et al. 2007).

Hydroxyproline (OHP), pyridinoline (PYD) and deoxypyridinoline (DPD)

In addition to previously mentioned TRACP 5b and CTX, there are several other markers that reflect the differentiation and activity of osteoclasts. Urine hydroxyproline (OHP) is derived from collagen degradation but also from collagen synthesis. However, it lacks bone specificity and therefore its usage as bone resorption or bone turnover marker is limited. When osteoclasts resorb bone in the acidic environment with proteases, they degrade the collagen fibrils incorporated in the bone matrix into cross-linked collagen fragments. Pyridinoline (PYD) and deoxypyridinoline (DPD) are intramolecular and intermolecular crosslinks of mature type I collagen needed for stabilization of collagen fibrils. They are formed by the enzymatic action of lysyl oxidase at the final stages of fibril formation and thus unaffected by newly synthesized collagen formation (Eyre et al. 1980). During the collagen fragmentation in bone resorption process, these crosslinks are released into the circulation and are not affected by diet or further metabolism making them applicable for assessment of bone resorption. DPD is a predominant form in bone and dentin, whereas PYD is expressed in several other tissues including cartilage, ligaments and vessels (Eastell et al. 1997). Human and rodent derived forms of PYD and PDP can be measured by novel immunoassays. The levels of pyridinium cross links are age and gender specific, being higher in healthy adults and adolescents than in the elderly. PYD and DPD are stable in urine samples for several weeks at room temperature. Pyridinium cross links show both seasonal and diurnal variation.

Osteocalcin fragments (f-OC)

Most of the osteocalcin that is in the human blood circulation is intact; therefore, the total amount of osteocalcin in the serum can be mainly considered as a marker of bone formation. However, recent studies have demonstrated that fragments of osteocalcin are released from the bone matrix during bone resorption (Ivaska et al. 2004). A part of osteocalcin is shown to escape the proteolytic degradation and is released both as intact molecules and fragments. In humans, immunoreactive fragments of osteocalcin have been reported in urine. Thus, urinary osteocalcin has been shown to be an encouraging new

marker for the prediction of forthcoming fractures, especially vertebral fractures (Ivaska et al. 2005). Furthermore, immunoassays have been developed for urinary osteocalcin fragments measurement (Ivaska et al. 2005). Although circulating osteocalcin is largely used as an index of bone formation, urinary osteocalcin has a promising role as a bone metabolism marker. High correlation of osteocalcin and CTX in several *in vivo* and *in vitro* studies further supports this novel role (Ivaska et al. 2004).

Cathepsin K (Cat K)
Cathepsin K is located in intracellular vesicles, granules and vacuoles of osteoclasts and is secreted into a resorption lacuna in the bone resorption process. Bone degradation products and Cat K are transported from the resorption lacuna through the cells and released into the circulation. Unlike other cathepsins, it has unique capability to degrade type I collagen. Due to this feature, Cat K itself is suggested as a marker of bone resorption. Commercial kits are available for Cat K detection in serum. However, there is only limited data available on its clinical use. In addition, concentration of circulating Cat K is very low. A pilot clinical study suggested that cathepsin K might be a useful marker of bone resorption (Meier et al. 2006), while others have suggested that Cat K serum concentrations do not appear to reflect the activity of osteoclasts and it should rather be considered as a potential marker of osteoclast number (Karsdal et al. 2003. Cat K is highly expressed in both resorbing and non-resorbing osteoclasts correspondingly to TRACP 5b, and therefore it does not reflect purely the resorption activity despite its vital role in the bone resorption process. Cat K decreases with age in both women and men, and it correlates with BMD and non-traumatic fracture history (Holzer et al. 2005).

Bone sialoprotein (BSP)
BSP is a phosphorylated glycoprotein produced by active osteoblasts and odontoblasts, but it has been also found in osteoclast-like and malignant cells (Seibel 2005). BSP is considered to play an essential role in cell-matrix-adhesion processes of mineralized tissues. Serum BSP levels are reported to be increased in postmenopausal osteoporosis and malignant bone diseases and decreased by antiresorptive treatment. Therefore, it is assumed that serum BSP levels reflect mainly processes related to bone resorption (Seibel et al. 1996). BSP is a relatively stable marker, but, overall, quite little is known about the kinetics and metabolism of BSP. In addition, until recently, a commercial kit for BSP measurements has not been available. These features have limited its widespread use for the assessment of bone metabolism.

2.10 Applications and limitations of bone turnover markers

Traditionally, routine assessment and golden standard analyses of the skeleton has consisted of histology and imaging techniques including radiography, magnetic resonance imaging (MRI), computed tomography (CT), and bone mineral densitometry (BMD) measurements. Although these techniques provide accurate and sensitive information, their applicability is limited by the fact that they can only provide the net effect of past skeletal activities at the tissue level. One of the key advantages of the use of bone markers is that they provide nearly real-time information of skeletal activities at the cellular level of all types of bone cells. In addition, the use of bone turnover markers has several other advantages in preclinical and clinical drug development studies as well as in management

of osteoporosis and fracture risk assessment. Thus, scientific rationale behind the use of bone markers has been well described in the previous chapters of this thesis work and several other publications.

The use of bone markers has also limitations and caveats. Most of the limitations are related to non-specific variability, which is designated by the preanalytical and analytical variability, often neglected issues in the preclinical and clinical use of bone markers. Accuracy, assay precision, standardization and quality controls are the main factors of analytical variability. The analytical variability is often low in both manual and automated assays and, thereby, preanalytical variability is a more essential source of variation. Among the preanalytical factors influencing the measurements, both technical and biological variability influence the marker levels. Specimen characteristics, sample handling, patient or animal age, gender, diurnal or circadian variation, diet, growth, and renal and hepatic function all have been shown to affect marker results (Seibel et al. 2001). Of these, circadian variability is one of the key variables. Most of the bone turnover markers display significant circadian rhythm where marker levels are highest at night and lowest in the afternoon (Blumsohn et al. 1994). The resorption markers in general, and particularly CTX and DPD, are sensitive for the circadian variation. The circadian rhythm is usually related to stability, synthesis and half-life of markers. In general, a more stable marker has lower circadian rhythm. Some markers are also associated with significant intra-individual variability. Moreover, some markers of bone turnover are sensitive to temperature changes. Thermodegradation should always be taken into account when measuring the intact osteocalcin molecule as it is rapidly degraded into fragments at room temperature. Similarly the activity of TRACP 5b declines rapidly if kept at room temperature. The mode of sample collection, serum or urine, is a vital factor that affects marker concentrations. The variability is, in general, lower for serum markers than for urine markers. Variation between laboratories is mostly a result of the use of non-standardized in-house assays. Furthermore, the use of not only different techniques but also different calibrators within the same assay in the assessment of same markers has raised the need for standardization of bone marker measurement. Results of bone turnover marker immunoassays cannot therefore be exchanged between laboratories without careful evaluation, normalization of data and cross-calibration. Currently, there is no general consensus on which bone markers and which methods should be used in routine practice (Vasikaran et al. 2011).

The variability caused by the factors described above is relatively easy to minimize since most of these factors are already known factors. However, there are other factors which affect to applicability of bone markers and which are more challenging to overcome than technical variables. One is that some markers, like osteocalcin, reflect both bone formation and bone resorption. Thereby, whether osteocalcin is a marker of bone formation or bone resorption depends on what forms of osteocalcin the assay method detects and, thus, the appropriate assay has to be carefully selected. Another challenge for the interpretation of the assay results is that even though changes in bone markers may predict quite well the tendency where a disease or treatment is developing, these changes are not disease specific in most cases and cannot thereby distinguish different skeletal diseases. Thus, they reflect alterations in bone metabolism independent of the underlying cause. For example, all collagen-derived bone markers are mainly related to type I collagen, which is not bone specific despite its abundance in bone and is widely distributed in several other tissues. In addition, bone turnover markers always reflect systemic levels of skeletal turnover, thus being unable to provide distinct information on trabecular and cortical bone remodeling. Often baseline levels of bone turnover markers are not informative, as their

reference interval is large. Therefore, most of the bone markers, if not all, require sequential follow up measurements to determine the direction of a change. As a conclusion, researcher must be highly knowledgeable about all the variables mentioned above and the details of assay principle to ensure integrity of results. Therefore, questions about the information needed to select the appropriate marker or to decide whether the use of bone markers is likely to be helpful in certain cases should be addressed by experienced researchers.

2.11 Bone turnover markers in preclinical and clinical studies

2.11.1 PINP

PINP is a specific and sensitive bone turnover marker for monitoring anabolic treatment (Chen et al. 2005, Finkelstein et al. 2006). Because of the good clinical performance of PINP, it has also been shown to be a useful tool in monitoring antiresorptive osteoporosis treatment (Finkelstein et al. 2006, Nenonen et al. 2005). Serum PINP has been utilized to follow-up the treatment of osteoporosis as well as indicating the progression of Paget's disease and osteoblastic bone metastases. Due to its dynamic response to treatment, PINP is also recommended as a reference marker for bone formation in studies concerning fracture risk assessment and treatment response by the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (reviewed by Naylor 2012).

PINP has been successfully used in few human osteoblast cultures to demonstrate bone formation (de la Piedra et al. 2008). Until the initiation of this thesis work, it has been possible to determine PINP only in humans (Melkko et al. 1996). Therefore, the use of PINP in drug development has been limited to clinical studies. PINP reflects several therapies and disease stages in bone pathology. In healthy individuals, no significant differences were observed between the results obtained for intact and total PINP. In contrast, in breast carcinoma, slight differences between these assays were observed (Marin et al. 2011). However, the roles of these two assays are not fully understood. Administration of several glucocorticoids represses type I collagen synthesis which can be demonstrated in a decreasing concentration of PICP, but most probably also with PINP as these two markers are almost identical (Koivula et al. 2012). Reduction in PINP and also in BALP and OC after raloxifene treatment was highly predictive of the 3-year vertebral fracture risk (Reginster et al. 2004). Modest, but statistically significant early decrease has also been observed in PINP levels after strontium ranelate treatment, whereas BALP levels were unchanged. Correspondingly, PINP levels were increased of greater magnitude compared to BALP levels after teriparatide treatment demonstrating the sensitivity of PINP as bone formation marker over BALP, and also the applicability of PINP for the follow up of various anabolic treatments (Recker et al. 2009). However, after alendronate treatment reduction in bone formation markers levels were most consistent for BALP, and to a lesser extent for PINP (Bauer et al. 2004). PINP has also been associated with the spreading and prognosis of breast and prostate cancer due to fibroproliferative reaction induced by malignant epithelium (Jensen et al. 2002).

2.11.2 Osteocalcin

Osteocalcin is one of the few osteoblast specific proteins (Hauschka et al. 1989). Intact osteocalcin is secreted into the blood circulation during bone formation and is thus a marker of bone formation, whereas osteocalcin fragments are released from the bone matrix during bone resorption. Thereby, whether osteocalcin is a marker of bone formation or bone resorption depends on what forms of osteocalcin the assay method detects. These various molecular forms are often differently detected by various assays, causing a challenge for the interpretation of results. Due to these appearances, it is generally referred to as a marker of bone metabolism.

Osteocalcin measurement has been applied largely to various preclinical bone studies. It has been widely used in various human and rodent osteoblast cultures to demonstrate bone formation (Ma et al. 2013, Ivaska et al. 2004). In the isolated cell culture systems, all osteocalcin is derived from osteoblasts, which facilitates the assay selection and interpretation of the results. In rat OVX studies, osteocalcin has been used to assess bone formation after treatment of anabolic agents, including teriparatide and strontium ranelate (Ma et al. 2011). Several animal studies have demonstrated the correlation between osteocalcin and CTX and other bone resorption markers corresponding to human skeletal metabolism, which highlights the significance of the assay selection between osteoblast derived osteocalcin reflecting bone formation and bone derived fragments of osteocalcin reflecting bone resorption. *In vitro* studies with osteoclasts have further demonstrated that osteocalcin fragments in urine are highly correlated to osteoclast-mediated pit formation (Cloos et al. 2004). High levels of the undercarboxylated form of osteocalcin have been shown to reflect hip fracture risk in elderly women (Vergnaud et al. 1997). In addition, the rate of γ-carboxylation is shown to be associated with fracture risk (Luukinen et al. 2000). As with BALP and PINP, levels of osteocalcin are decreased after glucocorticoid treatment (Dovio et al. 2004). Moreover, changes in osteocalcin levels together with other bone formation markers, including PINP and BALP, were highly informative and associated with the risk of fracture in the clinical study with treatment of alendronate, zoledronic acid and raloxifene (Bjarnason et al. 2001, Bauer et al. 2004, Delmas et al. 2009, Reginster et al. 2004). After fracture, osteocalcin levels are elevated at least 3 months (Obrant 1990). Both urinary and serum osteocalcin have been shown to be applicable markers for monitoring short-term effects of alendronate therapy in postmenopausal women (Ivaska et al. 2005). However, urinary osteocalcin responded to therapy more rapidly than serum osteocalcin, indicating its potential as a resorption marker. Based on recent studies, the undercarboxylated form of osteocalcin is shown to be active in mice glucose metabolism and, thus, osteocalcin has been suggested to be a marker or mediator also in human glucose metabolism (Booth et al. 2013).

2.11.3 TRACP 5b

In normal conditions with healthy adults, majority of the serum TRACP amount is of the TRACP 5a form, and thus assays that detect total TRACP provide only an estimate of the inactive TRACP 5a levels. TRACP 5b has higher specific activity than the 5a form, which results in that both isoforms represent about half of the circulating TRACP activity. Therefore, the importance of being aware of these features is highlighted when designing marker studies with TRACP-measurements. Three distinct TRACP 5b activity specific immunoassays have been developed. Of these, one is using a selective pH (Halleen et al.

2000) and the two others are using selective substrates (Janckila et al. 2004, Ohashi et al. 2007). TRACP 5b is a particularly interesting and promising bone marker because its activity originates exclusively from osteoclasts, it has a relatively low diurnal and negligible circadian variability and it is not accumulated in the bloodstream in renal or hepatic failure (Halleen et al. 2001).

TRACP 5b has been used for various purposes in several preclinical studies. It has been applied in assessment of increased bone resorption due to osteopetrosis in animal studies (Bargman et al. 2012), but also with human patients suffering from osteopetrosis (Alatalo et al. 2004). Studies in the rat ORX model (Alatalo et al. 2003) and in patients with renal bone disease (Chu et al. 2003) demonstrate a strong and highly significant correlation with the histologically determined number of osteoclast compared with serum TRACP 5b levels. Furthermore, *in vitro* studies have demonstrated that TRACP 5b is released from the osteoclasts independently of whether the cells are seeded on plastic where osteoclasts are inactive or on bone slices where they are active (Alatalo et al. 2000, Karsdal et al. 2003). In addition, cathepsin K inhibitors inhibit bone resorption without affecting osteoclast number. Thereby, levels of TRACP 5b do not change with cathepsin K inhibition whereas NTX and CTX are suppressed by these agents (Eastell et al. 2011). Taken together, these studies indicate that TRACP 5b is secreted from nonresorbing and resorbing osteoclasts, and, thus, it reflects the number of the osteoclasts instead of their activity (Halleen et al. 2000, Alatalo et al. 2004). It has been assumed that by combining markers such as the CTX and TRACP 5b ratio could facilitate the interpretation of the changes of bone resorption (Henriksen et al. 2007). Typically, TRACP 5b levels are increased in patients with bone diseases and decreased during various antiresorptive treatments (Halleen et al. 2006). TRACP 5b has been reported to reflect changes in bone turnover after estrogen replacement therapy (Halleen et al. 2000). TRACP 5b could be implemented also to fracture risk assessments. Elevated levels of TRACP 5b, urinary osteocalcin and CTX are demonstrated to be associated with increased fracture risk for up to several years in elderly women (Ivaska et al. 2010).

2.11.4 CTX

CTX measurement has been applied in *in vitro* culture systems where osteoclasts are cultured on bovine bone slices to demonstrate changes in bone resorption (Neutzsky-Wulff et al. 2010). Commercially available immunoassay recognizes both human and bovine CTX fragment due to their high homologies. It has been demonstrated that measuring the amount of CTX released into the culture medium is a fast and convenient method of volumetrical quantitating bone resorption, and is an accurate indicator of the total resorbed volume, including both the depth and area of the formed resorption pits (Christgau et al. 1998).

The levels of serum and urine CTX or NTX are markedly increased in postmenopausal osteoporosis. In contrast to ICTP, their values decrease rapidly with antiresorptive treatment (Eastell et al. 2011, Ensrud et al. 2004). Serum ICTP is shown to be a sensitive marker in pathological bone resorption, other than osteoporosis, including metastatic bone disease and myeloma. High serum CTX levels, as with other resorption markers, have been associated with increased fracture risk for more than a decade (Garnero et al. 2000).
Both type I collagen telopeptides, NTX and CTX, are widely and equally used bone resorption markers. In some studies, serum or urinary CTX was demonstrated to reflect more specifically bone resorption than urinary NTX (Garnero et al. 2000). Serum CTX was

also demonstrated to respond rapidly to alendronate therapy induced reduction in bone turnover, although with lesser extent than formation marker ALP (Bauer et al. 2004). In contrast, the changes in CTX and TRACP 5b levels were more consistent than changes in PINP and BALP levels after denosumab therapy. Of the resorption markers, TRACP 5b did not decrease to the same extent than CTX in the denosumab study (Eastell et al. 2011). Postmenopausal women with high levels of urinary and serum CTX were at higher risk for future fracture (Garnero et al. 2000). Interestingly, in that study BALP had even better correlation to fracture risk than any of the resorption markers. Furthermore, increased bone turnover, as assessed by serum CTX levels together with BMD measurements, has been demonstrated to predict risk of osteoporotic fractures better than either of the technologies alone (Garnero et al. 1996). The IOF has recommended that the serum CTX level should be used as the reference marker for bone resorption.

3. AIMS OF THE STUDY

When the present study was initiated, bone markers had been used for years in clinical practice for diagnosis, prognosis and therapy monitoring. Short term changes in bone turnover markers had been demonstrated to reflect long term responses in bone mass and quality in clinical practice (Ravn et al. 2003). PINP, osteocalcin, TRACP 5b and CTX had particularly been used for monitoring various osteoporosis therapies and predicting fracture risk. Of these, PINP immunoassay was the only marker that had not been commercially available for rat and mouse. However, despite the relative good commercial availability of markers, widespread use of bone markers had been limited partly due to their unclear biological significance (Brady et al. 2013), variability (Blumsohn et al. 1997), and insufficient evidence of their endpoint predictivity. Overall, there has not been general consensus on which bone markers should be used in routine practice (Vasikaran et al. 2011).

BMD measurements and histomorphometrical analyses have been considered to be the golden standards of bone quality assessment in preclinical *in vivo* animal studies, whereas microscopical imaging has been the standard analysis in preclinical *in vitro* cell cultures. These analyses are informative but, on the other hand, laborious or they don't offer timely response needed for monitoring and endpoint predicting. Respectively to clinical practice, there have not been standardized routines on which bone markers should be used in preclinical studies.

This study was initiated to broaden our understanding on the role of PINP, osteocalcin, TRACP 5b and CTX as predictive biomarkers in commonly used preclinical osteoporosis models. The specific aims of the study were as follows:

1. To design and perform controlled *in vitro* and *in vivo* studies with preclinical osteoporosis models including human osteoclast bone resorption assay and rat ovariectomy model

2. To characterize and validate PINP immunoassay for preclinical use

3. To measure the levels of PINP, osteocalcin, TRACP 5b and CTX at various timepoints of *in vitro* and *in vivo* studies

4. To determine structural changes at tissue level *ex vivo* by histomorphometry, BMD analyses and microscopical imaging

5. To evaluate the association of marker levels at various timepoints with structural changes in tissue level at the end of the study and thereby elucidate the prognostic potential of bone turnover markers in preclinical osteoporosis models

6. To evaluate the observed preclinical findings with published clinical findings and to provide contribution and suggestion on optimal bone marker selection when using bone markers as predictors of drug efficacy in osteoporosis studies

4. MATERIALS AND METHODS

4.1 Human osteoclast assay

Osteoclast differentiation assay
In vitro experiments were performed with human bone marrow derived osteoclast cells. Human CD34+ osteoclast precursor cells (Lonza Group Ltd, Switzerland) were cultured on bovine bone slices (IDS Ltd, UK) for 7 days. The bone slices were placed in 96-well tissue culture plates containing αMEM culture medium supplemented with 33 ng/ml M-CSF and 66 ng/ml RANKL (Lonza and PeproTech EC, UK). The cells were seeded on top of the bone slices. The cultures were stopped after differentiation period at day 7, and the cells were fixed with 3% PFA (paraformaldehyde). TRACP 5b activity was measured from the culture medium obtained at day 7 with a commercial ELISA assay (BoneTRAP assay; IDS Ltd, UK). Osteoprotegerin (OPG; PeproTech EC, UK) and alendronate (Merck Research Labs, USA) were added into the cultures at the beginning of the study at day 0. OPG was added directly into the culture medium containing the osteoclast precursor cells whereas alendronate was coated onto the bone slices by incubating the bone slices in solutions containing appropriate concentrations of alendronate for 45 min before adding the cells. Eight replicates were included in each study group and cultures with vehicle and baseline (without inhibitors) were added into each experiment.

Osteoclast activity assay
After completion of osteoclast differentiation at day 7, the culture medium was removed and new αMEM culture medium containing M-CSF and RANKL was added into the wells. The pH was adjusted to 6.8 to favor resorption and the formed mature osteoclasts were cultured for an additional 3-day period, allowing them to resorb bone. At day 10, the cultures were stopped with 3% PFA fixation and the cells were detached from the bone slices by mechanical scraping with a soft brush. TRACP 5b activity was measured from the culture medium obtained at day 7 and CTX with a commercial ELISA assay (CrossLaps for Culture assay; IDS Ltd) from the culture medium obtained at day 10. The cysteine protease inhibitor E64 (Sigma-Aldrich, USA) and the cathepsin K inhibitor ORG-29762 (Organon Laboratories, Scotland) were added into the cultures at day 7, after completion of the osteoclast differentiation period. CTX values obtained after the resorption period at day 10 were divided by the TRACP 5b values obtained in the beginning of the resorption period (at day 7, before adding the test compounds). Eight replicates were included in each study group and cultures with vehicle and without resorption inhibitors were added into each experiment and named as baseline cultures.

Determination of osteoclast number and resorbed area by microscopy
To visualize osteoclasts formed at day 7, the PFA fixed cells were stained for TRACP with leukocyte acid phosphatase kit (Sigma-Aldrich) used to demonstrate acid phosphatases in tissue preparations and Hoechst 33258 (Sigma-Aldrich) to visualize nuclei of formed multinuclear TRACP positive osteoclasts. The number of multinuclear TRACP-positive osteoclasts was calculated under a microscope. Apoptotic osteoclasts were identified as osteoclasts with damaged nuclei and abnormal morphology based on the Hoechst staining, and osteoclast apoptosis was determined as the percentage of apoptotic osteoclasts from all osteoclasts. The resorbed area was determined from cultures stopped at day 10. The cells were mechanically detached from the surface of bone slices and the

formed resorption pits were visualized using TRITC-conjugated wheat germ agglutinin lectin (Sigma-Aldrich). Cell analyses were determined with Leica DM 4000 microscope.

4.2 Experimental osteoporosis models

4.2.1 Animal experimentation

***In vivo* experiments**
Three different animal experiments were performed, all under animal experiment license 1336/03 granted by the State Provincial Office of Western Finland. The first study was carried out to study the effects of estrogen withdrawal on osteoclast number and osteoclast activity in the rat ovariectomy (OVX) model. The second animal study was carried out to characterize rat specific PINP assay. The third animal study was carried out to validate the novel PINP assay and to study if PINP can predict long-term changes in trabecular bone parameters. Three-month old female Sprague-Dawley rats (Harlan Nederland) underwent OVX in all experiments. The animals were randomized to groups according to body weight. The surgeries were performed on day 0 under general anesthesia and analgesia by removing both ovaries using the dorsal approach. After the in-life phase, uterine weight was determined in order to insure that the operations and dosing were performed successfully. Left tibiae were harvested and used for bone histomorphometric analysis.

Estrogen withdrawal study
The first study was continued for 8 weeks after OVX. The following study groups were included (n = 12 in each group): 1) sham operated rats receiving vehicle; 2) OVX rats receiving vehicle; 3) OVX rats receiving E2 (17β-estradiol, Sigma, USA). The animals were randomized to groups according to body weight. E2 was dissolved in polyethylene glycol (PEG400) and administered daily by subcutaneous injections at a dose of 10 μg/kg/day. Treatment was started on day 1 and continued daily for 8 weeks. Blood samples for marker measurements were taken before the operations at day 0 and at 2 and 8 weeks.

PINP assay characterization study
A pilot 2-week study was performed first with the following study groups (n = 8): 1) sham-operated control group and 2) OVX group. Blood samples for marker measurements were taken before the operations at day 0 and at days 2, 4, 7, 10, and 14.

PINP assay validation study
The pilot characterization study was followed by an extensive 8-week study including the following study groups (n = 12): (1) sham-operated control group receiving vehicle, (2) OVX group receiving vehicle, and (3) OVX group receiving 17b-estradiol (E2; Sigma, St. Louis, MO). E2 was dissolved in polyethylene glycol (PEG400) and administered by s.c. injection at a dose of 10 μg/kg/ day. Treatment was started at day 1 and continued daily for 8 weeks. Blood samples for marker measurements were taken before the operations at day 0 and at 2 and 8 weeks.

4.2.2 Bone parameter measurements

pQCT Measurements
Mineral density and cross-sectional dimensions of trabecular bone were measured from the metaphysis of the left tibia using peripheral quantitative computed tomography (pQCT). The pQCT measurements in 8 weeks studies were performed *in vivo* before the operations and at the end of the studies using Norland Stratec XCT Research SA+ equipment and Stratec software version 6.0 (Norland Stratec Medizintechnik, Birkenfeld, Germany). The site of the CT scan was at the proximal end of tibia 0.5–2 mm below the growth plate. The following parameters were analyzed:

Trabecular BMD (mg/cm^3) = Volumetric mineral density of the marrow cavity
Trabecular BMC (mg/mm) = Mineral content of the marrow cavity within 1 mm slice

Histomorphometric analyses
Left tibiae were embedded in plastic and undecalcified 5 μm longitudinal sections were stained with Masson-Goldner Trichrome stain. Histomorphometric analysis was performed on the metaphysis of the left proximal tibia harvested at 8 weeks using OsteoMeasure software version 2.2 (Osteometrics, USA), following the suggestions of the American Society for Bone and Mineral Research (Parfitt et al. 1987). Detailed trabecular bone analysis was performed on an area from 0.5 to 2 mm below the growth plate. For dynamic histomorphometry, tetracycline labeling (20 mg/kg in 0.9% NaCl) was performed 9 days before termination and calcein labeling (20 mg/kg in 0.9% NaCl) 5 days before termination. The following parameters were analyzed:

BV/TV (%) = Bone volume as a percentage of tissue volume, relative amount of bone in terms of the whole tissue volume
Tb.N (mm^{-1}) = Trabecular number, number of individual trabeculae
Tb.Th (μm) = Trabecular thickness, average thickness of the individual trabeculae
Tb.Sp (μm) = Trabecular separation, average distance between two individual trabeculae
N.Oc/T.Ar (mm^{-2}) = Number of osteoclasts/tissue area, number of osteoclasts relative to the whole tissue area
N.Oc/B.Pm (mm^{-1}) = Number of osteoclasts/bone perimeter, number of osteoclasts relative to the external perimeter of the bone
BFR/BS (μm^3/μm^2/year) = Bone formation rate/bone surface, bone formation rate measured by double labeling expressed per unit of bone surface

4.3 Immunoassays

Immunoassays are biochemical assays that measure the concentration of a certain antigen of interest in a solution through the use of antibodies. Labels in immunoassays allow for detection of antigens and are typically enzymes. Other commonly used labels to detect antigens from the solution are radioactive isotopes referred to as radioimmunoassay (RIA) or emission of light produced by chemical reaction referred to as chemiluminescence-immunoassay (CLIA). Immunoassays which utilize enzymes are referred to as enzyme immunoassays. Several different formats have been utilized in enzyme immunoassay applications. Antigen immobilization on the bottom of microtiter wells can be accomplished by direct adsorption, indirectly via a capture antibody or with two antibodies when the

assay technology is referred to as sandwich assay. The most commonly known forms of enzyme immunoassays are enzyme immunoassay (EIA) and enzyme-linked immunosorbent assays (ELISA) (Figure 8).

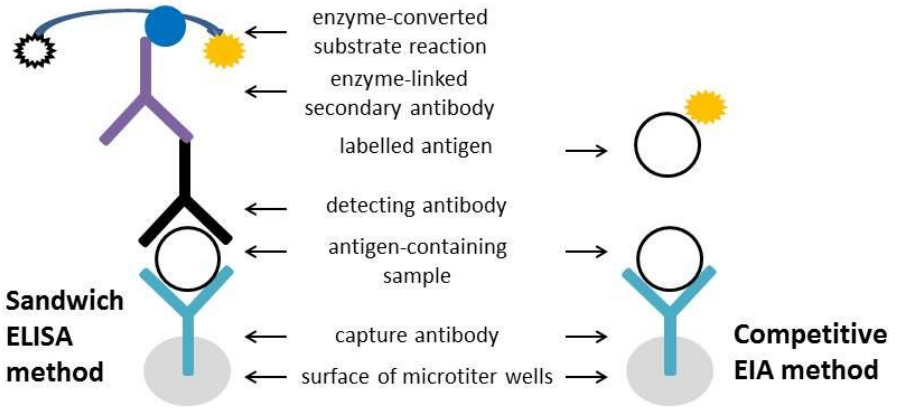

Figure 8. Schematic presentation of ELISA and EIA assay A) The sandwich ELISA method occupies an attached capture antibody and a labeled free detection antibody; B) The competitive EIA method is based on competitive binding between the analyte and a labeled antigen.

4.3.1 PINP immunoassay characterization and validation

Antibody Development
A polyclonal PINP antiserum was developed by IDS Ltd in rabbits using a synthetic peptide, pQEDIPEVS (molecular weight 897.93, obtained from NeoMPS, France), corresponding to the first amino acids of the rat PINP a1 chain, as described previously (Hale et al. 2007). The peptide was conjugated to keyhole limpet hemocyanin and injected intradermally with Freund's adjuvant into two New Zealand white rabbits. Boosters were given at 3-week intervals until a good titer was reached.

Rat PINP Immunoassay
A nonradioactive competitive enzymeimmunoassay (EIA) was developed by IDS Ltd using the polyclonal antiserum described above. The synthetic peptide pQEDIPEVS was biotinylated at the carboxy terminus and used as a tracer in the immunoassay. Nonbiotinylated peptide was used as a standard. Fifty-microliter aliquots of standards or serum samples (5 μL sample + 45 μL buffer) were incubated with 50 μL of tracer solution (10 μg/L) and 50 μL of diluted anti-PINP antiserum in anti-rabbit immunoglobulin G (IgG)–coated microtiter wells (IDS, Boldon, UK) for 1 hour at room temperature. The wells were washed four times with 10 mM Tris-buffered saline, and 150 μL of horseradish peroxidase–avidin D conjugate (Vector Laboratories, CA) was added. After 30-minute incubation at room temperature, the wells were washed and 150 μL of TMB ONE (3,3',5,5'-tetramethylbenzidine hydrogen peroxide; Kem-En-Tec Diagnostics, Denmark) was added. The reaction was stopped after 15-minute incubation with 50 μL of 0.5 M HCl. Absorbance

was measured at 450 nm, and the final PINP concentration of samples was determined from the standard curve. The results are shown as nanograms per milliliter, where 1.0 ng of peptide corresponds to 1.1 pmol of PINP. Characterization of the PINP Immunoassay Intra- and interassay coefficients of variation (CVs) were calculated from a panel of rat serum samples at three different PINP levels (n = 10 in each level). Sensitivity was determined as the value 2 standard deviations above the zero level. Dilution linearity was determined by serial dilutions of rat serum samples. Recovery was determined by spiking rat serum samples with known amounts (2, 6, and 20 ng/mL) of the synthetic peptide pQEDIPEVS. Finally, the newly developed immunoassay was commercialized (Rat/Mouse PINP EIA, IDS Ltd).

4.3.2 Bone turnover marker measurements

Blood for marker measurements was collected from the lateral tail vein after overnight fasting, and serum was separated by centrifugation and stored at -70°C until the analyses. PINP (Rat/Mouse PINP EIA assay, IDS Ltd), N-terminal mid-fragment of osteocalcin (Rat-MidTM osteocalcin ELISA assay, IDS Ltd), serum TRACP 5b (RatTRAP ELISA assay, IDS Ltd) and C-terminal crosslinked telopeptides of type I collagen (CTX, RatLaps ELISA assay, IDS Ltd) were measured from the serum samples according to the manufacturer's instructions. All samples were assayed in duplicate and a standard curve was generated from which concentrations were extrapolated. Relative marker values were calculated for each individual animal at each time point by dividing the values obtained from each animal with the values obtained from the same animal before the operations. In the estrogen withdrawal study CTX and TRACP 5b were measured before the operations at day 0 and at 2 and 8 weeks. PINP, CTX, and osteocalcin were measured at days 0, 2, 4, 7, 10, and 14 in the PINP development study and at day 0, at 2 and 8 weeks in the PINP validation study.

4.4 Statistical analyses

Statistical analyses were carried out using ORIGIN 8.5 program (OriginLab, USA) or SPSS 14.0 for Windows software (SPSS, USA). All statistical analyses were performed as two-sided tests. Normal distribution and homogeneity of variance were checked before further analyses. If these assumptions were violated, either log transformation or other appropriate transformation (e.g. square root, reciprocal) was applied. If the assumptions were fulfilled as such or after transformation, one-way analysis of variance (ANOVA) was used to study whether the values obtained in the different groups were statistically different (with $p<0.05$). If a statistically significant difference was observed, the results of the other treatment groups were compared separately with the results of the baseline group in the *in vitro* studies and the results of the sham-operated control group and OVX group receiving E2 were compared separately with the results of the OVX group using Dunnet's test. If the assumptions were not fulfilled even after the transformations described above, rank-transformation was applied and the nonparametric Kruskal-Wallis test was used instead of ANOVA. If a statistically significant difference was observed in the Kruskal-Wallis test, the nonparametric Mann-Whitney test was used instead of Dunnet's test.

5. RESULTS

5.1 Human osteoclast cultures

5.1.1 TRACP 5b and CTX in human osteoclast cultures (II, III)

The correlation of secreted TRACP 5b with osteoclast number and secreted CTX with the resorbing activity of osteoclasts was studied in human osteoclast cultures. During a 7 day culture period, the human bone marrow-derived CD34+ osteoclast precursor cells formed TRACP-positive multinuclear osteoclasts. At day 7, more than 90% of the CD34+ monocytes originally present in the wells were differentiated into mature osteoclasts. The number of the formed mature osteoclasts at day 7 showed a strong correlation with secreted TRACP 5b activity (Table 4). After a subsequent 3 day resorption period (days 7-10), the formed mature osteoclasts resorbed large and numerous resorption pits. The total resorbed area showed a strong correlation with the amount of CTX released into the culture medium at day 10 (Table 4). Reassuringly, this study suggests that TRACP 5b is a marker of the osteoclast number while CTX secreted into culture medium reflects the total resorbed volume in human osteoclast cultures (Table 5, II: Figure 1).

Based on the obtained results, two improved methods for testing the effects of antiresorptive compounds on human osteoclasts were accomplished. In the first method, referred to as osteoclast differentiation assay, the cultures were stopped after the differentiation period at day 7, and secreted TRACP 5b activity was quantitated from the culture medium as an index of the number of formed mature osteoclasts. In the second improved method, referred to as osteoclast activity assay, the culture medium was replaced with new medium after the differentiation period at day 7, and the cultures were incubated for three additional days, allowing the formed mature osteoclasts to resorb bone. Secreted CTX was quantitated from the culture medium at day 10 as an index of total resorbed volume and osteoclast activity. In order to avoid biological variability in the number of osteoclasts formed in different wells, no matter how carefully the osteoclast precursor cells were originally pipetted into each well, TRACP 5b levels were quantitated from the culture medium obtained at day 7 to demonstrate the number of osteoclasts formed in each well before adding the test compounds. By using biomarkers, it was possible to perform this measurement without stopping the cultures. In contrast, traditional quantification techniques would have required stopping the cultures, fixing the cells, and visualizing the osteoclasts by microscopy. The term resorption index was applied to the relation of CTX/TRACP 5b to describe mean osteoclast activity.

5.1.2 The effects of antiresorptive compounds in *in vitro* studies (III)

In the osteoclast differentiation assay, the effects of two inhibitors of osteoclast differentiation, OPG and alendronate, on osteoclast number, osteoclast apoptosis, and TRACP 5b secretion were studied. OPG significantly decreased osteoclast number and

secretion of TRACP 5b in a concentration-dependent manner without affecting osteoclast apoptosis, whereas alendronate decreased osteoclast number and secretion of TRACP 5b and, at the same time, increased osteoclast apoptosis in a concentration-dependent manner (III: Figure 1). An interesting observation was that, although the highest doses of OPG decreased the number of osteoclasts close to zero as determined by microscopy, the medium TRACP 5b values remained at a much higher level, approximately 40% from the baseline level. Despite this low correspondence at negligible levels of osteoclast number, the pattern of changes in osteoclast number and TRACP 5b activity after OPG treatment is very similar and shows significant correlation.

In the osteoclast activity assay, the effects of two inhibitors of osteoclast activity, cysteine protease inhibitor E64 and cathepsin K inhibitor ORG-29762 were studied. Both E64 and ORG-29762 inhibited significantly and dose-dependently the release of CTX into the culture medium. When the CTX values were normalized by the TRACP 5b values obtained at day 7, the resulting resorption index showed a more balanced dose-dependent and statistically significant decrease in both compounds. The decrease was more substantial than either with CTX or TRACP 5b alone (III: Figure 2).

5.2 Rat OVX model

5.2.1 TRACP 5b and CTX after estrogen withdrawal (II)

The correlation of serum TRACP 5b and CTX to bone parameters were studied in the rat OVX model. *Ex vivo* parameters were assessed by traditional methods including uterine weight measurement and trabecular bone parameter determination by pQCT and histomorphometry. Body weights were increased and uterine weight decreased by OVX, demonstrating that the operations were performed successfully. Bone parameters Tb.BMD, Tb.BMC, BV/TV, Tb.Th and Tb.N were decreased and Tb.Sp increased by OVX, indicating that OVX caused significant trabecular bone loss. The histomorphometrically determined number of osteoclasts normalized with the amount of bone (N.Oc/B.Pm) was increased while the absolute number of osteoclasts (N.Oc/T.Ar) in trabecular bone was decreased. Serum TRACP 5b values were decreased and the pattern of changes in TRACP 5b values at 2 weeks and at 8 weeks was similar to the pattern of changes in N.Oc/T.Ar. Thus, TRACP 5b correlated strongly with N.Oc/T.Ar demonstrating the potency of TRACP 5b in predicting long-term changes of osteoclast number at the trabecular bone (Table 4). All effects caused by OVX were prevented by E2 treatment.

Serum TRACP 5b values were decreased, but CTX values were increased by OVX at both 2 and 8 weeks after the operations. As a result of opposing results of TRACP 5b and CTX, their ratio, the resorption index, showed more substantial changes than either CTX or TRACP 5b alone (II: Figure 3). These results demonstrate that the resorption index is particularly useful in situations where osteoclast activity is increased and osteoclast number is decreased, such as in the rat OVX model. Based on the TRACP 5b results, osteoclast number was decreased in the skeleton by approximately 60% at 8 weeks after OVX. At the same time, based on the CTX results, total activity of osteoclasts in the skeleton was increased by approximately 50%. From these values it can be calculated that mean osteoclast activity showed approximately 3.75-fold increase at 8 weeks after OVX. This demonstrates that estrogen withdrawal generates high activity in osteoclasts.

5.2.2 Characterization and validation of the PINP Immunoassay (I)

The developed nonradioactive rat PINP immunoassay had an intra-assay CV of 2.8%, interassay CV of 7.5%, analytical sensitivity of<1.0 ng/mL, dilution linearity of 95%, and recovery of 107%. The synthetic peptide pQEDIPEVS that was used as antigen in the development of the polyclonal antibody used in the assay is found in an identical form in the mouse PINP sequence but not in the human PINP sequence. Therefore, the assay had a cross-reactivity of 100% with mouse PINP but<1% with human PINP. In the 3-month-old female Sprague-Dawley rats used in this study, the mean PINP value obtained before the operation was 31.5 ng/mL, which corresponds to 34.7 nM PINP.

In the 8-week rat OVX study, uterine weight and static bone parameters determined by pQCT and histomorphometry were decreased by OVX correspondingly to the previous OVX study, where TRACP 5b and CTX were studied. In addition, all the effects caused by OVX were prevented by E2 treatment (I: Figure 2). Bone formation rate/bone surface (BFR/BS) determined by dynamic histomorphometry was substantially increased by OVX, which was prevented by E2 treatment. PINP, osteocalcin and CTX increased after the first week of OVX (I: Figure 3). All marker levels were elevated at 2 weeks after OVX, and the elevation was prevented by E2 treatment. Osteocalcin and CTX values stayed elevated at 8 weeks after OVX. PINP values were decreased back to sham level at 8 weeks, although the histomorphometrically determined BFR was strongly elevated. However, in dynamic histomorphometry, BFR was calculated as a relative value compared with BS. BV/TV values indicate that substantial bone loss occurred at 8 weeks after OVX. Therefore, we calculated a ‘‘relative PINP value’’ for each animal by dividing the PINP value by the BV/TV value obtained from the same animal. These relative PINP values showed a profile very similar to BFR/BS at 8 weeks (Table 4, I: Figure 4). PINP, osteocalcin and CTX correlated strongly with each other and with the trabecular bone parameters. Changes in PINP showed stronger correlations with the trabecular bone parameters than with the changes in osteocalcin or CTX.

Table 4. Correlations of short-term changes in marker levels with long-term changes in tissue levels in *in vitro* human osteoclast assay and *in vivo* rat OVX model used in this thesis work. The most relevant correlations observed in the studies are shown.

Marker	Tissue parameter	R-value/ significance	Study	Reference
PINP	BFR/BS	0.84***	Rat OVX model	I
TRACP 5b	N.Oc/T.Ar	0.84***	Rat OVX model	II
TRACP 5b	Osteoclast number/ bone slice	0.79***	Human osteoclast assay	II, III
CTX	Resorbed area/ bone slice	0.87***	Human osteoclast assay	III

BFR/BS, Bone formation rate/bone surface; N.Oc/T.Ar, Number of osteoclasts/tissue area

Table 5. Summary of the cellular functions that PINP, osteocalcin, TRACP 5b and CTX reflect in preclinical osteoporosis studies used in this thesis work.

Markers of bone formation	Function	Study	Reference
PINP	Osteoblast activity	Mouse osteoblast assay, Rat OVX model	Unpublished, I
N-Mid-osteocalcin	Osteoblast activity	Rat OVX model	I
Markers of bone resorption			
TRACP 5b	Osteoclast number	Human osteoclast assay, Rat OVX model	III II
CTX	Osteoclast activity	Human osteoclast assay, Rat OVX model	III I, II
N-Mid-osteocalcin	Osteoclast activity	Rat OVX model	I

5.3 Experimental osteoporosis models (IV)

Based on the literature review and supplemented with our data and findings, we generated suggestion on optimal strategy to perform preliminary efficacy studies from early discovery to regulatory studies (Table 6). The first step in these strategies is to find the compounds affecting their desired target. Various assays can be implemented for these purposes, including enzymatic assays, receptor-ligand binding assays, protein-protein interaction assays and biochemical high throughput screening (HTS) models. HTS assays are automated large scale format assays used often as a starting point for the drug screening process by enabling the finding of the lead candidates from libraries including hundreds of thousands of compounds. The positive hits should be tested further in *in vitro* bone cell cultures, including osteoclast and osteoblast differentiation and activity assays relevant for the target. Such assays include human CD34 positive osteoclast assay and mouse KS483 osteoblast assay.

Table 6. A proposed strategy for performing preclinical efficacy studies in bone cell cultures and rat OVX model for compounds aimed at treatment of postmenopausal osteoporosis. Modified from IV: Table 2.

Step	Model	Study type	Compounds	Doses	Replicates
1	Bone cell cultures	Screening	5000	1	4
2	Bone cell cultures	Screening	500	1	4
3	Bone cell cultures	Preliminary	50	2	6
4	Bone cell cultures	Extensive	10	7	8
5	Rat OVX	Preliminary	3	5	8
6	Rat OVX	Extensive	1	3	12
7	Rat OVX	Regulatory	1	3	25
8*	Rat ORX	Extensive	1	3	12
9*	Immobilization	Extensive	1	3	12
10*	GIO	Extensive	1	3	12

*Optional studies for indications beyond postmenopausal osteoporosis. GIO: Glucocorticoid-induced osteoporosis; HTS: High throughput screening; ORX: Orchidectomy; OVX: Ovariectomy.

Biochemical marker measurements were shown to be highly applicable and useful in these assays and they exhibited significant prognostic value (I, II and III). TRACP 5b and CTX markers were demonstrated to reflect osteoclast number and activity in the osteoclast cell cultures, thus saving a substantial amount of time and workload compared with the conventional microscopic techniques. Similarly ALP, PINP and calcium measurements had corresponding benefits in the osteoblast cell cultures (data not shown). Ideally, extensive *in vitro* studies would provide EC50 values for the lead compounds, which would further assist optimal dose response assessments for *in vivo* osteoporosis studies. From a methodological perspective, it is important to use highly validated and optimized assay systems that are repeatable and reliable. Every cell culture plate should always contain a baseline group with vehicle and a control group with an internal reference compound, which should preferably be a clinically used treatment for osteoporosis. Prognostic value of PINP, osteocalcin, TRACP 5b and CTX was also studied in the rat OVX models. Correspondingly to *in vitro* assays, the markers exhibited significant prognostic value and were highly applicable and useful in these models. In the OVX studies, the bone turnover markers provided rapid and reliable confirmation of the efficacy of the compounds. In addition, PINP, osteocalcin, TRACP 5b and CTX were found to reflect cellular stages of differentiation and activity of osteoclasts and osteoblasts. The studied markers were demonstrated to predict long-term changes in trabecular bone parameters thus having the potential to provide more convenient and rapid analysis methods over the golden standard analysis methods, such as histomorphometry and bone mineral density measurements.

Table 7. Optimal study set ups for testing anti-resorptive and anabolic compounds in the 2-month extensive rat OVX model. Modified from IV: Table 1.

Step	Anti-resorptive	Anabolic
Age of animals	3 months	6 months
Start of dosing	Immediately after the operation	1-2 months after the operation
Dosing period	6-8 weeks	6-8 weeks
Bone turnover markers (PINP, osteocalcin, TRACP 5b and CTX)	Before the operations At 2 weeks At the end of the study	Before the start of dosing At 2 weeks At the end of the study
pQCT measurements (*in vivo*)	Before the operations At the end of the study	Before the operations Before the start of dosing At the end of the study
Histomorphometry (static and dynamic)	At the end of the study	At the end of the study
Ash weight	At the end of the study	At the end of the study
Biomechanical testing	At the end of the study	At the end of the study

OVX: Ovariectomy; pQCT: Peripheral quantitative computed tomography.

A convenient way for fast testing of several compounds in several doses is to perform two-week preliminary studies including only bone turnover marker measurements as end point values. The short-term animal studies are then followed by more extensive animal studies, and finally a regulatory study is performed according to the guidelines of regulatory authorities. The mode of action of the compounds, anabolic or anti-catabolic, should be considered carefully when designing optimal studies (Table 7) and clinically relevant reference compounds should be included in all the studies to obtain reliable data with

prognostic value. By following this strategy, it is possible to perform cost-effective preclinical efficacy studies for new osteoporosis drug candidates and obtain possible negative results as early as possible with minimum costs.

The optimal age of OVX rats for anabolic studies is from 6 months onwards because by that age their bone modeling is ceased and the predominant form is remodeling in the trabecular bone. Their overall bone turnover is low, enabling follow-up of the increase in bone turnover caused by the anabolic compounds. In contrast, compounds with antiresorptive potential are preferably tested with younger, growing rats despite the fact that they have still high levels of modeling in all bones, but instead, they have high bone turnover and maximal amount of trabecular bone left. Thus, the optimal animal age of OVX rats for antiresorptive studies is 3-6 months, enabling follow-up of the decrease in bone turnover caused by the anti-resorptive compounds. Anabolic compounds should be optimally tested in the treatment setting which enables follow-up of the treatment of established osteoporosis, whereas antiresorptive compounds should be tested in preventive setting which enables efficacy monitoring of the compounds in preventing bone loss. In both the preventive and the treatment studies, the dosing period can be limited to only 6 - 8 weeks, which is much lower than the 12 - 16 months indicated in the regulatory guidelines for a regulatory study.

6. DISCUSSION

6.1 Bone turnover markers in preclinical osteoporosis models

Traditionally, osteoblast and osteoclast function in preclinical models has been studied with laborious techniques, such as static and dynamic histomorphometric indices, BMD measurements, mechanical strength tests and microscopical analyses. These analyses are informative, but on the other hand strenuous approaches. In addition, some of them do not offer timely response needed for monitoring and endpoint predicting. A more practical approach is to use biochemical markers of bone turnover secreted into the serum or culture medium during the in-life phase or at the end of the study. Bone turnover markers are surrogates for bone metabolism, and significantly correlate with the golden standard methods saving a considerable amount of time, costs and workload. Bone markers are powerful tools in monitoring the treatment efficacy of anabolic or antiresorptive drugs in postmenopausal osteoporosis. Bone markers are also useful in various drug development processes. They are able to provide critically important information for making key decisions in costly drug development processes, such as whether or not to continue the development of a certain drug. The usage of bone turnover markers has greatly enhanced efficacy predictions in preclinical and clinical studies, drug safety and biomarker validation (Garnero et al. 1999, Gerdhem et al. 2004, Henriksen et al. 2010, Sørensen et al. 2007b). Today, several bone turnover markers are commercially available for rats, mice and human studies, including PINP, osteocalcin, TRACP 5b and CTX that can all be conveniently measured from the same serum, urine or culture media samples by utilizing immunoassays.

In this study, the feasibility of bone markers as predictors of drug efficacy in preclinical osteoporosis models was elucidated. A non-radioactive PINP immunoassay was characterized and validated for preclinical use. The levels of PINP, N-terminal mid-fragment of osteocalcin, TRACP 5b and CTX were studied in preclinical osteoporosis models and the results were compared with results obtained by traditional analysis methods such as histomorphometry, densitometry and microscopy. The observations supported earlier findings that TRACP 5b correlates strongly with the osteoclast number and CTX correlates strongly with the osteoclast activity in both *in vitro* and *in vivo* studies. PINP was strongly associated with bone formation whereas osteocalcin was associated with both bone formation and resorption in the *in vivo* studies. Changes in all bone markers at early timepoints correlated strongly with the changes in bone mass and quality parameters at the end of the study (Table 4). The results support the clinical findings that short term changes of these markers reflect long term responses in bone mass and quality (Table 5). Utilizing these markers in preclinical and clinical studies allows detection of metabolic change long before these alterations are detected with BMD, histomorphometry or other golden standard analysis methods.

6.2 CTX, TRACP5b and resorption index

Earlier studies have demonstrated that serum TRACP 5b is a useful marker in monitoring alendronate treatment because of its low analytical and biological variability (Hannon et al. 2004, Nenonen et al. 2005). Serum TRACP 5b values have been shown to decrease by approximately 40% at 3 months after the start of alendronate treatment, and the values stay at this reduced level until at least 12 months after the start of the treatment (Nenonen et al. 2005). In the osteoclast studies, it was demonstrated that alendronate decreases the number of osteoclasts, which is caused partly by the induction of osteoclast apoptosis. Despite the observed induction of osteoclast apoptosis, alendronate also inhibited secretion of TRACP 5b from osteoclasts. The result reveals that TRACP 5b is not released from apoptotic osteoclasts as an active enzyme. This is an important observation, because the release of active TRACP 5b from apoptotic osteoclasts would interfere with the use of secreted TRACP 5b activity as a marker of the number of living osteoclasts. In the osteoclast differentiation studies, an interesting observation was that TRACP 5b levels did not decrease to zero level even though only negligible amounts of osteoclasts were left on the bone slices after treatment with OPG. An explanation for this captivating scientific phenomenon remains to be elucidated. However, a considerably more important observation was that the pattern of changes in the osteoclast number and TRACP 5b activity was very similar after OPG treatment, and that the increase in osteoclast number from the zero level correlates strongly with the increase in TRACP 5b activity from the zero level.

In the rat OVX model, TRACP 5b correlated strongly with the histomorphometrically determined absolute number of osteoclasts (N.Oc/T.Ar), demonstrating the potency of TRACP 5b in predicting long-term changes of osteoclast number at the trabecular bone. At the same time, osteoclast number normalized with the amount of bone (N.Oc/B.Pm) was increased, indicating that bone resorption was still increased, leading to further bone loss. Although N.Oc/T.Ar and serum TRACP 5b showed a similar pattern of changes in OVX studies, it should be noted that N.Oc/T.Ar was measured from a specific site in the tibial metaphyseal trabecular bone, while the serum TRACP 5b values describe the alterations of TRACP 5b levels in the whole skeleton. This limitation is typical to all bone markers; they are not able to identify the site of bone remodeling. Despite this limitation, N.Oc/T.Ar in the tibial metaphysis and serum TRACP 5b values appear to be closely related in the rat OVX model.

The resorption index appeared to be an extremely valuable parameter in the rat OVX model, where osteoclast activity is increased while osteoclast number is decreased. Another useful application for this index is in testing the effects of antiresorptive compounds on the resorbing activity of osteoclasts in *in vitro* osteoclast activity assays. The resorption index showed more substantial changes in the osteoclast activity assay than either of the markers alone in studies with cysteine protease inhibitor E64 and cathepsin K inhibitor ORG-29762. In rat OVX studies, the resorption index revealed that mean osteoclast activity showed an approximately 3.75-fold increase at 8 weeks after OVX, demonstrating that estrogen deficiency generates high activity in osteoclasts. This is in contrast to previous findings obtained from *in vitro* osteoclast cultures where estrogen suppresses osteoclast differentiation (Shevde et al. 2000, Srivastava et al. 2001, Ramalho et al. 2002) and induces osteoclast apoptosis (Kameda et al. 1997) without affecting the resorbing activity of osteoclasts (Sørensen et al. 2006). These contradictory findings can be partly explained by the fact that the effects of estrogen on osteoclasts are mediated by

other cell types and other complex pathways that are not present in *in vitro* cultures, where only direct effects on osteoclasts can be seen.

Despite the advantages in preclinical studies, the resorption index is probably not a very valuable diagnostic tool for osteoporosis, because the bone loss leading to osteoporosis is usually a slow process in humans and, therefore, the absolute number of osteoclasts is increased rather than decreased. This is supported by the findings that serum TRACP 5b values are increased in postmenopausal women and in bone diseases (Halleen et al. 2006). Furthermore, the most commonly used antiresorptive treatments, including estrogen, SERMs, and bisphosphonates, decrease serum TRACP 5b values, indicating that the treatments affect both osteoclast number and osteoclast activity in an analogous manner. Therefore, because both the number and activity of osteoclasts are increased similarly in bone diseases and decreased similarly by the currently used antiresorptive treatments, the resorption index is probably not a useful parameter for these clinical applications. At the same time, these facts demonstrate that, although serum TRACP 5b cannot be considered as a marker of bone resorption in the rat OVX model, it can be considered as a useful marker of bone resorption in bone diseases and for monitoring currently used antiresorptive treatments in humans. Moreover, the reason why TRACP 5b levels are decreased, whereas levels of CTX and most other resorption markers are increased after OVX in rats, is most probably related to the fact that TRACP 5b is secreted from osteoclasts, while the other markers are typically derived from bone. Therefore, as the parameter N.Oc/T.Ar demonstrated, the absolute number of osteoclasts decreases after OVX as a result of rapid bone loss in trabecular bone, which is the most abundant site of osteoclasts. This phenomenon is the most obvious 8 weeks after OVX when bone loss is evident, but it can be observed after only 2 weeks. The significant and rapid trabecular bone loss in rats has been demonstrated in proximal tibia and lumbar vertebra as early as at 2 weeks after OVX (Yamaura et al. 1996).

Recent data suggests that osteoclasts might have two completely separate functions: to resorb bone and to maintain the balance between bone resorption and bone formation (Henriksen et al. 2007, Karsdal et al. 2007). The role in bone remodeling regulation is associated with the number of osteoclasts. Therefore, drugs that would decrease the resorption index by decreasing osteoclast activity without affecting osteoclast number could theoretically be able to uncouple bone turnover by decreasing bone resorption without affecting bone formation. Thus, analysis of both CTX and TRACP 5b would be extremely useful in clinical studies of new antiresorptive osteoporosis drug candidates, facilitating the demonstration of the drug candidates' effects on both osteoclast activity and osteoclast number.

6.3 PINP and osteocalcin

In the rat OVX studies aimed to validate the PINP assay, CTX, osteocalcin, and PINP were elevated at 2 weeks after OVX. Of these, CTX and osteocalcin were elevated also at 8 weeks after OVX, while PINP had returned to sham level. Thus, osteocalcin correlated better with CTX than with PINP after 8 weeks. This suggests that at least part of the N-terminal mid-fragment of osteocalcin in rat serum may be derived from bone resorption rather than from bone formation. An important observation is that the short-term changes (at 0-2 weeks) in PINP levels correlated substantially better with long-term changes (at 8 weeks) in trabecular bone parameters than the changes of osteocalcin and CTX levels. An

earlier study has demonstrated partly controversial results by using in-house PINP radioimmunoassay (Hale et al. 2007). In that study, PINP values were not significantly different between sham and OVX. However, they measured PINP only at the late-phases of the study, 47 days after OVX. This observation is in line with our results demonstrating that PINP values were similar in sham and OVX groups at 8 weeks (56 days). In contrast, Hale and coworkers focused on monitoring changes in PINP levels after treatment with PTH, and they were able to demonstrate that PINP values are increased rapidly after PTH treatment in both intact and ovariectomized young rats. Our recent unpublished data confirms this observation by demonstrating a rapid and highly significant elevation in PINP levels already at 2 weeks after OVX in aged rats.

The observation that PINP is decreased to sham level at 8 weeks after OVX leads to reasonable conclusion that ‘‘absolute’’ bone formation is decreased to normal level at this time point. Conventional analysis methods, including pQCT analysis and histomorphometry, demonstrated that a substantial amount of trabecular bone is lost at 8 weeks after OVX. When the PINP values obtained at 8 weeks were normalized with the amount of bone left by dividing the PINP values with the BV/TV values, it was observed that this index changed in a similar pattern and similar order of magnitude to BFR/BS. Thus, this index describes the ‘‘relative to bone amount” PINP values. Furthermore, these observations suggest that PINP is an accurate marker of bone formation, comparable with parameters of dynamic histomorphometry. The fact that the PINP values were decreased to sham level at 8 weeks after OVX while CTX and osteocalcin were still elevated leads to the conclusion that bone resorption would be more elevated than bone formation at 8 weeks, causing further bone loss in the rat OVX model.

6.4 Preclinical development of drugs for osteoporosis

Osteoporosis affects millions of people worldwide and the number of patients suffering from this disease is increasing due to the ageing of the population. The prevalence of osteoporosis is increasing more rapidly in the developing countries as they gradually adopt the Western lifestyle that predisposes humans to osteoporosis. This will inevitably lead to growth in the size of the osteoporosis market. Osteoporosis has high incidence and high mortality rate among severe osteoporosis patients. It causes long-term implications, financial burden and a decrease in the quality of life. Therefore, early diagnosis would be crucial for the patient and for society. Currently, there are many options available for the prevention and treatment of osteoporosis. However, most of the existing therapies will only decelerate further progress of the disease but will not recover the amount of bone mass back to the normal level. Furthermore, some therapies are limited by side effects and inadequate long-term compliance. Two novel antiresorptive drugs have recently received approval for the prevention and treatment of osteoporosis; Denosumab, an antibody inhibiting RANK/RANKL pathway, and zoledronic acid, a potent stabile bisphosphonate. Despite the efficiency of these drugs, antiresorptive drugs show a clustering in late-stage phase studies, indicating that the pipeline of this class of drugs is reaching maturation. There are increasing efforts to better understand the complex nature of coupled bone remodeling and to develop more efficient and safer drugs. Several new therapies for the management of osteoporosis are completing clinical phase II and phase III trials that have distinct mechanisms of action compared with current therapies (Rachner et al. 2011). In

the future, anabolic therapies, combination therapies, and compounds that manage uncoupling hold the greatest potential in the prevention and treatment of osteoporosis and are assumed to have an advantage over conventional antiresorptives. Sclerostin antibody and Dkk-1 neutralizing antibody, both antagonists of Wnt inhibitors, together with novel calcilytics are promising new developments in the field of anabolic therapies. Overall, the most prominent new therapies to enter the osteoporosis therapy market are antiresorptive cathepsin K inhibitor Odanacatib, undergoing phase III trials, and anabolic sclerostin antibody AMG 785, undergoing phase II trials. In addition to safety and efficacy, forthcoming osteoporosis therapies should depict affordable costs and compatibility with drugs prescribed for other medical conditions.

Future challenges include development of individualized osteoporosis therapies in accordance with the patient's genotype. Osteoporotic patients carrying a predisposing variant in any of the vital genes involved in bone turnover regulation, such as ERα, ERβ, RANK, RANKL, OPG, LRP5 or SOST gene, would benefit from the use of drugs specifically developed to consider these variants. In this regard, drugs based on humanized antibodies have become prevalent in a variety of medical fields and thus personalized therapies are already taken into consideration in several other diseases. Cancer, in particular, is a highly dispersed disease having numerous genetic variants. Designing cancer therapy has long included histological examination of tumor specimens from individual patients followed by therapies tailored to the individual patient. Thus, personalized medicine has been in practice long before the term was originally coined. The implications of the osteoporosis genetics on personalized medicine will also require updating the measurement technologies of preclinical models, particularly at the molecular level. Moreover, theragnostics, which combines therapeutics with diagnostics, employs predictive use of biomarkers and may therefore advance the personalized medication field to a great extent. Apart from the focus on personalized therapy, there is a need for new preclinical assays and models that better reflect the clinical responses. In the foreseeable future, we will hopefully be able to choose the most effective and efficient drugs for prevention and treatment of osteoporosis in accordance with the patient's genetic variants. Biomarkers may also play a vital role in shifting the emphasis of drug development towards personalized medicine.

The development of a new osteoporosis drug, as is the case with all drugs, is a long and expensive process. The drug development process typically costs up to USD 1 billion and the development of a drug for osteoporosis is not an exception. One reason for the high costs is that total development costs of a new drug include the cost of preceding failed compounds. Progress in osteoporosis drug development has been decelerated over the past few years mostly by the market entry of several effective drugs, but also by a lack of predictive preclinical efficacy models that reliably, rapidly and cost efficiently predict clinical activity of novel compounds. In order to enter the markets, or even a clinical phase, new osteoporosis drugs have to be tested in preclinical efficacy models. There are no completely satisfactory animal models for human osteoporosis, but a number of useful models exist (Turner 2001). Humans and rats share a surprising number of similar characteristics including physical, psychological and behavioral characteristics. The changes in bone metabolism in rats and humans following estrogen deficiency are very similar. The similarities between the human and rat skeleton in pathophysiologic responses, combined with the financial advantages, have made the rat a valuable and most commonly used model in osteoporosis research (reviewed by Lelovas et al. 2008).

From preclinical to clinical practice, several therapeutic compounds have been studied in the rat OVX model, some of which have already been translated into clinical practice.

Regulatory efficacy studies are well characterized in the guidelines of the FDA and EMA. According to the guidelines, the regulatory studies should be performed in the rat OVX model and in a model of a larger animal species, preferably in a primate OVX model. However, these preclinical efficacy studies performed according to the regulatory guidelines are large, long and expensive and sometimes even disinformative as they do not reflect all clinical stages of osteoporosis (Thompson et al. 1995). In addition, existing guidelines only cover primary osteoporosis, and are thus largely inadequate in the assessment of a secondary osteoporosis. Moreover, guidelines give recommendations only to *in vivo* testing excluding the earlier phases such as HTS and *in vitro* studies. Prior to large, long and expensive regulatory studies, drug development companies should undergo cost-effective preliminary efficacy studies to assist in identifying the lead compound and determining its optimal doses as well as to assure the overall relevance of performing the regulatory studies. Selection and development of a new research model should be based on clinical predictability, while also keeping in mind the 3Rs of ethical animal use: replacement, reduction and refinement. The selection of appropriate animal models of osteoporosis based on the similarity to human pathophysiology carries a considerable potential to ensure a higher predictability of preclinical results. A carefully chosen, appropriate and well-designed experimental *in vitro* and *in vivo* osteoporosis preclinical model minimizes the future risk of a failure of the novel drug compound in clinical studies. However, despite the clear guidance on the regulatory preclinical efficacy studies, there is a need for guidance and recommendations on how to perform the preliminary studies prior to the regulatory studies. In this thesis work, optimal strategies are suggested to perform such preliminary efficacy studies for compounds affecting directly osteoclasts or osteoblasts (summarized in Tables 6 and 7).

We reviewed various preclinical assays and models that can be used in the different stages of preclinical efficacy testing of new osteoporosis drug candidates. Based on the literature review supplemented with our own findings, we generated a suggestion on an optimal strategy to perform preliminary efficacy studies for compounds aimed at the treatment of postmenopausal osteoporosis, which directly affects osteoclasts or osteoblasts. The proposed strategy focuses on performing preclinical efficacy studies in bone cell cultures and the rat OVX model. The primary focus was to demonstrate the utility of these models in go- versus no-go-decision making in whether the actual regulatory preclinical study should be performed or not. These models demonstrate a step-by-step and cost-efficient pathway on how to select the most potential compound from a large amount of compounds on a rational basis, from the early discovery phase up to the regulatory efficacy study. The described pathway provide different approaches for testing anti-catabolic and anabolic compounds from *in vitro* bone cell culture systems up to osteoporosis animal models. Clinically relevant reference compounds should be included in all studies to obtain reliable data with prognostic value. The efficacy of new osteoporosis drug candidates can be first demonstrated using HTS and other enzymatic biochemical assays, and then by *in vitro* bone cell cultures, followed by short-term animal studies. The *in vivo* efficacy is further confirmed by more extensive animal studies having similar measurement parameters to the regulatory study. Finally, a regulatory study according to the guidelines of regulatory authorities is performed to demonstrate the detailed preclinical efficacy and dose response with rats and secondly with non-rodents. These final stage preclinical efficacy studies are typically performed concomitantly with early phase clinical studies. Additionally, strategies for preclinical efficacy testing of secondary osteoporosis as

well as male osteoporosis were provided. Importantly, bone turnover markers were implemented in all assays and models as it has been demonstrated by others and in this work that the usage of bone turnover markers reflects responses in bone mass and quality. Bone turnover markers alone or combined with other markers and traditional analysis methods have the potential to significantly impact the economics and the time lines of drug development and improve patient management in skeletal diseases. In particular, bone markers provide support when considering cost-efficient and clinically predictive drug screening and development assays. Markers are also considered as reliable and reproducible tools. Furthermore, from the ethical point of view, the utilization of bone markers decreases the amount of unnecessary long and extensive animal studies. Despite the several advantages, bone markers, as with all markers, have also limitations which delineate their use. Majority of these limitations are related to biological or analytical variability. Reasons for the pitfalls in new biomarker development are manifold, including challenges in validation, analysis methods, sample handling, study design or execution. However, approaches to addressing these pitfalls and limitations are abundantly described in medical literature, thus underscoring the need of new biomarkers.

In early phases of new osteoporosis drug development, it is cost-efficient to use only bone markers, similar to bone cell studies or short-term preliminary animal studies. However, there is no single bone turnover marker that should be chosen over others in preclinical or clinical practice. Therefore, it is important to select a large panel of various bone markers that reflect different stages of skeletal metabolism. It is also important to acknowledge confounding factors and limitations when interpreting bone turnover marker assay results. The conventional approaches such as histomorphometry and BMD measurements are still needed in the later phases of drug development studies. The most successful concept seems to be combining several analysis methods with several bone marker measurements, thus obtaining all available information to assist interpretation of the study results.

6.5 Future aspects in the use of bone markers

In this study, markers PINP, Osteocalcin, TRACP 5b and CTX were demonstrated to be powerful tools for various drug development processes and assessment of bone metabolism in preclinical skeletal disease models. In particular, combining these markers with each other or with golden standard measurements improved interpretation of the results compared to the markers alone. The correlation of the short term change in marker levels with the long term change in tissue levels facilitates not only the validation of marker measurement method, but also provides novel information on the treatment or on the underlying disease. In this study, histological parameters N.Oc/T.Ar and BFR/BS were found to correlate with early timepoint changes in TRACP 5b and PINP levels, and thus confirmed the use of TRACP 5b as an indicator of osteoclast number and PINP as an indicator of osteoblastic collagen synthesis. Presumably, there are several similar correlations yet unidentified between markers and golden standard methods as well as between markers themselves. Particularly, the development of commercially available rodent specific BALP measurement kit would provide novel and more accurate information on the rodent bone metabolism. It would allow calculation of bone formation per osteoblast, thus generating bone formation index corresponding to the resorption index generated in this study. Although rodent BALP would most probably provide useful information on rodent osteoblast differentiation, this hypothesis has yet to be proven.

In this study, four markers of bone turnover were used. Either alone or in combination with each other they provided novel and predictive information on the skeletal metabolism. In the future, once multiplex technology and robotics have paved the way for new immunoassay technologies, the ratio and combination of multiple markers might provide new, yet unidentified possibilities for the use of bone turnover markers. Although estrogen deficiency in women and androgen deficiency in men are significant factors in the pathogenesis of osteoporosis among the elderly in particular, animal studies have demonstrated that daily physical activity is an independent determinant of the mechanical properties of bone (Miyagawa et al 2011). Recent studies also suggest that obesity, Alzheimer's disease and inflammatory diseases might be related to pathogenesis of osteoporosis. Therefore, molecular level studies of crosstalk between skeletal metabolism and other diseases remain to be elaborated on.

7. SUMMARY AND CONCLUSIONS

Osteoporosis is an attractive therapy area for pharmaceutical companies because of its increasing prevalence rate and unmet clinical needs. Preclinical evaluation is an essential component in the development of new therapies for osteoporosis. The strategies for enabling an early assessment of the efficacy of these novel therapies would be essential for the patient, industry and society. Most preclinical assays and models are simplified systems that mimic human pathophysiology and are designed to address specific simplified questions. The capacity of these systems to predict the clinical response is limited. In addition, they include mostly laborious, expensive and non-dynamic analysis methods. Thus, there is a need for more predictive preclinical models which would also be rapid, affordable and ethical. In this study, bone turnover markers were demonstrated to have the potential to serve as early predictors in various preclinical efficacy studies, thereby providing useful tools for drug discovery in osteoporosis.

Based on the results obtained in this thesis work, the following conclusions can be made:

1) Preclinical osteoporosis models are valuable tools in drug development processes, as they provide essential information on the cellular mechanisms of the underlying disease and on the efficacy of novel drug compounds in the prevention or treatment of the disease in strictly controlled and simple systems.

2) An optimal strategy for performing preclinical efficacy studies in bone cell cultures and the rat OVX model was proposed for drug candidates aimed at treatment of postmenopausal osteoporosis. The optimal strategy enables the finding of lead anabolic or antiresorptive candidates from libraries including hundreds of thousands of compounds.

3) Biochemical markers of bone turnover such as PINP, osteocalcin, TRACP 5b and CTX are extremely useful measurements in the preclinical *in vitro* bone cell cultures and animal models, saving a substantial amount of time and workload compared with the conventional analysis techniques. They also allow performing predictive short-term animal studies for rapid and reliable confirmation of the beneficial bone effects of the novel drug compounds *in vivo*.

4) TRACP 5b correlated strongly with the osteoclast number and CTX with the osteoclast activity in both *in vitro* and *in vivo* studies. The term “resorption index” was applied to describe the relation of CTX/TRACP 5b. The index showed more substantial changes than either of the measurements, CTX or TRACP 5b, alone in the used preclinical osteoporosis models.

5) The high elevation of resorption index by OVX demonstrates that estrogen withdrawal generates high osteoclast activity in the rat OVX model.

6) Short-term changes in PINP were shown to predict long-term changes in the trabecular bone parameters, suggesting that PINP is a reliable marker of bone formation, whereas osteocalcin is associated with both bone formation and resorption in the rat OVX model. Changes in PINP levels showed stronger correlations with the trabecular bone parameters than with the changes in osteocalcin or CTX.

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