Effects of antiresorptive agents on inflammation and bone regeneration in different osseous sites

- experimental and clinical studies

Carina Cardemil

Department of Biomaterials
Institute of Clinical Sciences
Sahlgrenska Academy at University of Gothenburg

UNIVERSITY OF GOTHENBURG
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carina.cardemil@biomaterials.gu.se

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ABSTRACT

The biological mechanisms involved in bone regeneration in osteoporotic bone and the effect of antiresorptive drugs in relation to surgically inserted biomaterials are not fully understood. Improved osseointegration of titanium implants but also adverse effects of antiresorptive therapies, such as osteonecrotic jaw have been described in the literature. The aims of this research project were, firstly, to investigate and to understand the biological events determining bone regeneration and implant integration, after administration of antiresorptive agents; secondly, to determine the cellular and molecular patterns of bone regeneration at implants and synthetic bone substitutes under osteoporotic conditions and, thirdly, to determine how different skeletal sites are affected. The present research included a study of jawbone morphology and gene expression in patients treated with systemic bisphosphonates. When compared to controls, higher gene expression levels of IL-1β was observed in bisphosphonate treated patients with osteonecrosis while bisphosphonate treated patients without necrosis showed lower expression levels of caspase 8, an apoptosis marker involved in the immune response. In ovariectomised rats, zoledronic acid resulted in site-specific differences in the rate of osseointegration and also of gene expression involved in bone healing and regeneration. Strontium-doped calcium phosphate inserted in the rat femur induced lower expression of osteoclastic markers compared to hydroxyapatite and higher bone formation in the periphery of the defects. Whereas major structural changes were demonstrated in the long bones of the ovariectomised rat, less structural alterations were shown in the mandible. However, ovariectomy resulted in lower expression of genes coding for bone formation and angiogenesis in the mandible. In conclusion, the present study shows that the mandible is differently affected by experimentally induced estrogen deficiency than the long bones. Bisphosphonates, administered systemically to estrogen deficient animals, impair osseointegration in the mandible, at least partly related to a downregulation of genes important for the osteogenic process. These observations may have implications for understanding the mechanisms involved in the deranged bone healing observed in the jawbone of bisphosphonate treated patients.

Keywords: antiresorptive agents, ovariectomised rat, osteoporosis, skeletal site differences, osteonecrosis of the jaw, osseointegration, bone substitute, inflammation, bone regeneration, gene expression, histomorphometry, Micro-CT.

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SAMMANFATTNING PÅ SVENSKA

De biologiska mekanismerna som är inblandade i inflammation och benläkning kring biomaterial i samband med benskörhet (osteoporos) och behandling med antiresorptiva läkemedel är inte helt klarlagda. Antiresorptiva läkemedel används för att behandla osteoporos men även andra sjukdomar i skelettet. Positiva effekter av antiresorptiva läkemedel på inläkning av titanimplantat har beskrivits i litteraturen, men det förekommer också kända biverkningar såsom käkbensnekros. Ett mål med denna avhandling har varit att undersöka hur benläkning och inläkning av implantat påverkas vid osteoporotiska förhållanden efter att antiresorptiva läkemedel administrerats. Ett annat mål har varit att undersöka cellulära och molekylära processer vid inläkning av implantat och syntetiska benersättningsmedel i samband med osteoporotiska förhållanden samt hur hur olika lokalisationer i skelettet påverkas av osteoporos på strukturell och molekylär nivå. Studierna omfattar analyser av vävnadsprover från käkben hos patienter som behandlats med antiresorptiva läkemedel. I en experimentell modell där osteoporos-liknande förhållanden utvecklas i ben pga bristande nivåer av östrogen, har benstruktur och genexpression studerats i olika typer av ben i samband med benväxt och inläkning av implantat. En kombination av analytiska tekniker har använts: genuttryck, proteinanalys, histologi, histomorfometri, och mikro-CT. Analys av käkben från bisfosfonatbehandlade patienter visade på inflammatoriska infiltrat i vävnaden och nedreglerade markörer för programmerad celldöd. I en experimentell modell för osteoporos på råtta behandlad med antiresorptiva läkemedel observerades skillnader i inläkning av titanimplantat mellan käkben och långa rörben. Bensubstitut innehållande strontium, ett ämne som uppvisat antiresorptiva egenskaper, resulterade i lägre markörer för benresorption och förändrad distribution av nybildat ben jämfört med hydroxylapatit. Utvärdering av den experimentella modell av osteoporos som använts visade markanta skillnader mellan långa rörben och käkben vad avser strukturella förändringar och genuttryck av markörer för inflammation och benläkning. Sammanfattningsvis visar resultaten att antiresorptiva läkemedel, men även brist på östrogen, resulterar i olika reaktioner i skelettet på cellulär och vävnadsnivå, beroende på lokalisation. Kombinationen av de använda analysredskapen har ökat förståelsen för benläkning och inläkning av implantat vid osteoporotiska förhållanden i samband med användandet av anti-osteoporotiska läkemedel. Vidare har analyser på moleylär och vävnadsnivå ökat kunskapen om mekanismer kring bristande läkning i käkbenet efter behandling med antiresorptiva läkemedel.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Cardemil C, Omar O, Norlindh B, Larsson Wexell C, Thomsen P. *The effects of a systemic single dose of zoledronic acid on post-implantation bone remodelling and inflammation in an ovariectomised rat model.* Biomaterials. 2013; 34: 1546-1561.

II. Cardemil C[#], Elgali I[#], Norlindh B, Xia W, Emanuelsson L, Omar O, Thomsen P. *Strontium-Doped Calcium Phosphate and Hydroxyapatite Granules Promote Different Inflammatory and Bone Remodelling Responses In Normal and Ovariectomised Rats.* PLoS One. 2013; 8: e84932. [#] Equal contribution.

III. Cardemil C, Thomsen P, Larsson Wexell C. *Jaw bone samples from bisphosphonate-treated patients: a pilot cohort study.* Submitted.

IV. Cardemil C, Granéli C, Palmquist A, Windahl SH, Emanuelsson L, Norlindh B, Larsson Wexell C, Omar O, Thomsen P. *Molecular and structural differences in bone remodelling and inflammation in different bone types of the mature OVX rat model*. In manuscript.

CONTENT

ABBREVIATIONS

Acetyl-Coa	Acetyl coenzyme A
Aln	Alendronate
ALP	Alkaline phosphatase
Apppi	Triphosphoric acid I-adenosin-5′-yl ester 3-(3-methylbut-3-enyl) ester
ATP	Adenosine triphosphate
BA	Bone area
BCP	Biphasic calcium phosphate
BIC	Bone-to-implant contact
BMD	Bone mineral density
BMP	Bone morphogenetic protein
BMSC	Bone marrow stromal cell
BMU	Basic multicellular unit
BP	Bisphosphonate
BS/BV	Specific bone surface
BV/TV	Bone volume fraction
CALR	Calcitonin receptor
CATK	Cathepsin K
cDNA	Complementary DNA
COL	Collagen
Dkk-1	Dickkopf WNT signaling pathway inhibitor 1
DNA	Deoxyribonucleic acid
DPBS	Dulbecco´s phosphate buffered saline
DXA	Dual-energy X-ray absorptiometry
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERα	Estrogen receptor α
ERβ	Estrogen receptor β
FGF	Fibroblast growth factor
FRAX	Fracture Risk Assessment Tool
GH	Growth hormone
GTP	Guanosine triphosphate
HA	Hydroxyapatite
HMG-COA	3-hydroxy-3-methylglutaryl-coenzyme A

HRT	Hormone replacement therapy
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
IGF	Insulin-like growth factor
IL	Interleukin
IPP	Isopentenyl pyrophosphate
LEP	Leptin
LRP-5	Low-density lipoprotein receptor-related protein 5
M-CSF	Macrophage colony-stimulating factor
Micro-CT	Micro-computed tomography
MMPs	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
N-BPs	Nitrogen-containing bisphosphonates
NaCl	Sodium chloride
OC	Osteocalcin
OCP	Octacalcium phosphate
ONJ	Osteonecrosis of the jaw
OPG	Osteoprotegerin
OVX	Ovariectomy
PMNs	Polymorphonuclear neutrophils
PTH	Parathyroid hormone
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
Ris	Risedronate
ROI	Region of interest
RUNX2	Runt-related transcription factor 2
SCP	Strontium-doped calcium phosphate
SEM	Scanning electron microscope
SERMs	Selective estrogen-receptor modulators
SNS	Sympathetic nervous system
Tb.Sp	Trabeular separation
Tb.Th	Trabecular thickness
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TRAIL	TNF-related apoptosis-inducing ligand

TRAP	Tartrate-resistant acid phosphatase
VEGFA	Vascular endothelial growth factor A
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
Wnt	Wingless-related integration site
XRD	X-ray diffraction
ZOL	Zoledronic acid
α-TCP	α-tricalcium phosphate
β-TCP	β-tricalcium phosphate

1. INTRODUCTION

The skeleton supports and protects the organs of the body, stores minerals, produces blood cells, allows movement and also produces endocrine hormones. The human skeleton consists of the axial skeleton (skull, vertebrae, rib cage) and the appendicular skeleton (upper and lower limbs). The skeleton and its bones work in constant coordination with endocrine organs, hormones, muscles and the nervous system. When the homeostasis of the bone metabolism is disturbed, enhanced bone resorption may result in excessive bone loss and pathologic conditions such as osteoporosis. Other systemic conditions where an extensive bone loss can be observed are rheumatoid arthritis, Paget's disease and tumour-induced bone disease. In general, these conditions are treated with antiresorptive agents whereof the most extensively used are the bisphosphonates. The use of antiresorptive agents has enabled great advances in the treatment of a variety of skeletal diseases. Although some antiresorptive drugs have also been shown to improve osseointegration of implants, adverse effects such as osteonecrosis of the jaw (ONJ) and atypical femoral fractures are areas of concern. Thus, there is a need to further elucidate the delicate mechanisms involved in bone healing and implant integration in osteoporotic conditions when treated with antiresorptive agents.

1.1 Bone

1.1.1 Bone structure

There are two different forms of bone tissue, the cortical or compact bone, forming a dense outer shell on most bones and the trabecular or cancellous bone[1]. In an adult, 80% of the weight of the skeleton consists of cortical bone, which has a porosity of 5 – 10%[2]. The cortical bone has a major role in the supportive function of the skeleton, while the trabecular bone is more metabolically active[1]. In cortical bone, lamellae are aligned in cylindrical osteons consisting of a large number of layers surrounding a Haversian canal, containing a central blood vessel and nerves[3,4] (Figure 1). The cortical bone is surrounded by a connective tissue called the periosteum, whereas the inner surface of bone is covered by the endosteum[2]. The longitudinal Haversian canals are interconnected by Volkmann's canals which are oblique vessels, communicating with periosteal vessels[3]. Cancellous bone has a porosity of 50 - 90%, and also contains lamellar bone but without osteons[2]. Irregular bone trabeculae form a porous network surrounded by blood vessels and bone marrow[3]. Bone marrow is also found in the central part of cortical bone and

contains many different haematopoietic and non-haematopoietic cell types[5]. Bone is composed of bone cells and extracellular matrix (ECM), of which the ECM consists of mineralised matrix, organic matrix, lipids and water[2]. The main part of the mineralised matrix is in the form of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)[2]. The organic matrix is secreted by osteoblasts and consists of up to 90% of type I collagen[2]. Other components in the organic matrix are proteoglycans, growth factors and glycoproteins such as osteonectin, osteopontin, and bone sialoprotein[2,6]. When the osteoblasts have secreted organic matrix, mineralisation occurs after 10 – 15 days[2].

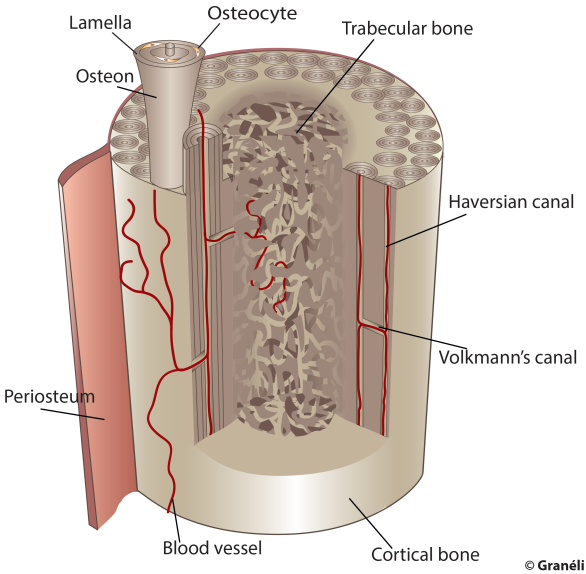

Figure 1. Bone structure. The cortical bone architecture is composed of circumferential systems of lamellae called osteons. In the interior part, bone marrow and trabecular bone forms the medullary cavity. Illustration: Cecilia Granéli.

1.1.2 Bone cells

There are three different types of bone cells, the osteoblasts, osteocytes and the osteoclasts. In total, these cells make up around 10% of the total bone volume[2].

Osteoblasts

Osteoblasts and other connective tissue cells are derived from mesenchymal stem cells (MSCs). The MSCs are promoted towards osteoprogenitor cells by bone morphogenetic proteins (BMPs) and pro-osteogenic pathways such as the Wnt pathway[7]. The osteoblasts are the bone building cells, producing and secreting proteins, thus forming the bone matrix. One of the main proteins is type I collagen, but they also produce osteocalcin (OC), osteonectin, osteopontin, bone sialoprotein and several other minor matrix proteins[8]. Osteoblasts account for 4 - 6 % of the bone cells and are estimated to have a lifespan of three months in human bone[7,9]. By a close cross-talk with osteocytes and osteoclasts, the osteoblast cells regulate bone mass and more recently, osteoblasts have also been demonstrated to have endocrine functions[7]. At the end of a bone formation cycle, osteoblasts undergo transformation into either osteocytes or lining cells. Lining cells are located on top of a thin layer of unmineralised collagen matrix covering the bone surface[8]. The flat and elongated lining cells secrete collagenase to remove the collagen matrix so osteoclasts can attach to bone[8].

Osteocytes

Osteocytes account for more than 95% of all the bone cells and have been estimated to have a mean half-life of 25 years in human bone, although it is probably less due to a constant bone turnover of 4% to 10% per year[9]. During bone formation, some osteoblasts become entrapped in the newly produced osteoid matrix and the subsequent mineralisation process causes them to become embedded within the mineralised matrix. The cells, which have a size of 10 μm – 20 μm in human bone, are located in lacunae and have dendritic extensions into canaliculi, channels which provide connections to other osteocytes within the bone matrix or on the bone surface[9]. Through the interconnected network of fluid containing canaliculi, the osteocytes have an ability to detect mechanical pressure and load[8]. This mechanosensory capacity can induce bone repair following microdamage, bone augmentation or reduction[8]. Additionally, osteocytes can also detect variations in the levels of estrogen and glucocorticoids, via the fluid in the canaliculi[8]. By modulating secretion and expression of a variety of molecules such as insulin-like growth factor, OC, and sclerostin, the osteocytes are able to respond to the various types of stimuli and also regulate skeletal

homeostasis[9,10]. Apoptosis of osteocytes appears to be necessary to initiate the bone remodelling process in response to fatigue microdamage and it has been shown that levels of pro-apoptotic molecules are elevated in osteocytes close to microcracks[9,11].

Osteoclasts

Osteoclasts are responsible for bone resorption and originate from the haematopoietic stem cells by fusion of mononucleated cells[12]. The cells are highly motile, yet they are only found close to the surface of mineralised bone and are never encountered in the circulatory system[12]. The osteoclasts are large multinucleated cells with a diameter of 50 – 100 μm and approximately five to eight nuclei in each cell[8,13]. The most characteristic feature is the finger-shaped extensions of the ruffled border membrane, where bone resorption takes place. The ruffled border is surrounded by the sealing zone membrane, which attaches the cell to the mineralised matrix of bone[8,13]. The unique ability to dissolve mineral is made possible by creating an acidic environment in the resorption lacunae by the action of proton pumps and chloride channels[13]. The secretion of hydrochloric acid into the resorption lacuna initiates the dissolution of hydroxyapatite and is followed by the secretion of proteolytic enzymes such as matrix metalloproteinases (MMPs) and cathepsins K, B and L, which degrade the protein components, mainly collagen[8,13]. Tartrate-resistant acid phosphatase (TRAP) is also present in high amounts and TRAP is often used as a cellular marker for osteoclasts[13]. The osteoclast–specific isoform TRAcP5b, correlates with resorption activity and can be used as a serum marker in clinical evaluations[13]. Degradation products such as calcium, phosphate and bicarbonate ions are removed from the resorption lacuna by transportation through the cells for secretion[13].

1.1.3 Bone development

Skeletal development is orchestrated through different genes coordinating the distribution and proliferation of cells from the three different embryonic lineages[14]. The cranial neural crest cells form the craniofacial skeleton, the paraxial mesoderm (somites) forms the axial skeleton and the lateral plate mesodermal cells produce the appendicular skeleton[14]. Cells in these embryonic lineages migrate to the different sites of skeletal development in the embryo and eventually differentiate into chondrocytes or osteoblasts[14].

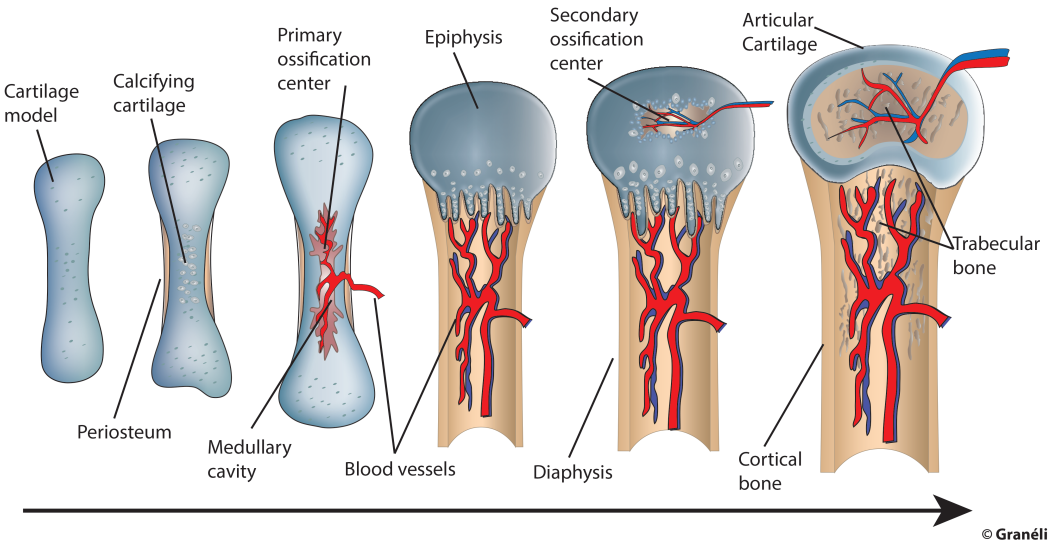

Figure 2. Schematic drawing of endochondral bone formation. Printed with permission from the author[15].

Endochondral bone formation

Bone is formed from cartilage models that expand in size by chondrocyte proliferation and deposition of the cartilage matrix (Figure 2). The most central chondrocytes mature into hypertrophic cells that produce extracellular matrix and secrete angiogenic factors[14]. The cartilage models are then invaded by sprouting blood vessels bringing osteoblasts, osteoclasts and hematopoietic cells, thus forming primary ossification centres. Subsequently, the hypertrophic chondrocytes shrink and collapse to finally undergo apoptosis[14,16]. When the chondrocytes are eliminated, they are replaced by blood vessels and primary bone trabeculae produced by osteoblasts, thus forming bone marrow[16]. Around the middle part of the cartilage called the diaphysis, a collar of compact bone is formed by osteoblasts differentiated in the perichondrium, which is a fibrous tissue surrounding the developing bone[14,17]. Secondary ossification centres are formed at the epiphyses, leaving a plate of cartilage between the epiphyses and the metaphyses called the growth plate. In the growth plate chondrocyte proliferation, hypertrophy and apoptosis result in longitudinal bone growth[14,16]. The activity in the growth plate is regulated by systemic and local factors such as genetic, endocrine and hormonal influence[16].

Intramembranous bone formation

The neural crest progenitor cells derived from the ectoderm undergo epithelial-mesenchymal transition and migrate to give rise to different

tissues[18]. Cells originating from the neural crest migrate and differentiate into osteoblasts and chondrocytes to finally give rise to the majority of cranial bones and cartilage[18]. Crest cells migrate into the first branchial arch and give rise to the maxilla, mandible, and parts of the middle ear ossicles and the temporal bone, whereas the hyoid bone and other parts of the temporal bone and the middle ear are derived from cells in the second branchial arch[14,19].

A vast number of genes are involved in controlling the process of craniofacial bone development and dysregulation during this process may result in congenital craniofacial disorders[18]. While bones in the cranial base are formed by endochondral ossification, the calvaria and the mandible undergo intramembranous ossification, where mesenchymal cells are directly differentiated into osteoblast progenitors[18]. Small capillaries invade the sites of initial ossification and following further proliferation and differentiation of the cells they start to produce a fibrous matrix[18,20]. Ossification centres are formed and the deposited bone matrix goes on to mineralise and form flat bones[21]. Bone spicules are formed by the differentiated osteoblasts to further develop and fuse to form trabeculae, a process associated with an extensive internal and external vascularisation[20]. Woven bone is formed when the trabeculae become interconnected, creating a bone lattice, which becomes filled when the ossification progresses. Osteoblasts aligned along the surface of the woven bone deposit new matrix, forming lamellar bone[4,20].

1.1.4 Bone metabolism

Bone is constantly remodelled to allow bone growth, bone healing and to uphold the homeostasis of calcium and phosphate[22]. The bone remodelling process is carried out by osteoclasts and osteoblasts. The coupling between these two cell types is regulated by local and systemic factors and imbalance in the bone homeostasis can lead to pathological conditions such as osteopenia, osteoporosis, and osteopetrosis, depending on which cell activity is favoured[23].

Bone and the immune system

The term osteoimmunology was first used in 2000 by Arron and Choi[24] to describe the research field of the interactions between bone and the immune system. Bone regulation by hematopoietic and immune cells serves many functions during normal bone development and during inflammatory conditions by producing local or circulating cytokines[25]. The receptor activator of nuclear factor κB ligand (RANKL) is a member of the TNF (tumour necrosis factor) family and has a crucial role in the differentiation of osteoclast precursor cells to fully activated multinucleated osteoclasts[26]. The

macrophage colony-stimulating factor (M-CSF) also has a role in influencing hematopoietic stem cells to differentiate into macrophages and osteoclasts[26]. RANKL is expressed by osteoblasts and bone marrow stromal cells (BMSC), but also T- and B-lymphocytes. When RANKL binds to its receptor, RANK, located on the surface of the osteoclast, differentiation, proliferation, activation and survival of the osteoclasts is promoted, resulting in enhanced bone resorption[26]. Osteoprotegerin (OPG) is a naturally occurring antagonist of RANKL and has potent inhibitory effects on osteoclastogenesis and bone resorption since it acts as a decoy receptor to RANKL and blocks the RANKL/RANK interaction[8]. Runt-related transcription factor 2 (RUNX2) is an essential factor for osteoblast differentiation and RUNX2 has also been shown to promote osteoclast differentiation by inducing RANKL while inhibiting OPG[27]. A number of cytokines are involved in the regulation of bone cells under inflammatory conditions[28]. Among them are TNF-α, which stimulates osteoclast formation and bone resorption in vivo, interleukin-1 (IL-1) which is a potent stimulator of bone resorption acting on the osteoclast via enhanced RANKL production and activity; and finally interleukin-6 (IL-6), which is produced by osteoblastic cells and bone marrow stem cells and regulate development of mature osteoclasts and also stimulate the production of RANKL and OPG[28]. Additionally, colony-stimulating factors, chemokines and a large number of interleukins produced by T-cells and macrophages are also involved in the interplay of bone and the immune system[28,29].

When bone cells die, they go into apoptosis, a programmed cell death with organised degradation of cellular organelles. This is a process common to several regenerating tissues, and the same growth factors and cytokines that stimulate osteoclast and osteoblast development can also influence their apoptosis[8]. Except for its anti-osteoclastogenic property, OPG is also a receptor for the cytotoxic TNF-related apoptosis-inducing ligand (TRAIL) to which it binds and inhibits TRAIL-mediated apoptosis in lymphocytes and also regulates antigen presentation and T-cell activation[8]. Apoptosis is activated by two signaling pathways; the intrinsic pathway activated by the tumour suppressor gene p53 in response to DNA damage or severe cell stress and the extrinsic pathway activated by pro-apoptotic ligands which bind to receptors on the cell membrane[30]. Both pathways activate caspases, which are proteases that degrade intracellular proteins leading to cell apoptosis. There may also be cross talk between the two pathways[30].

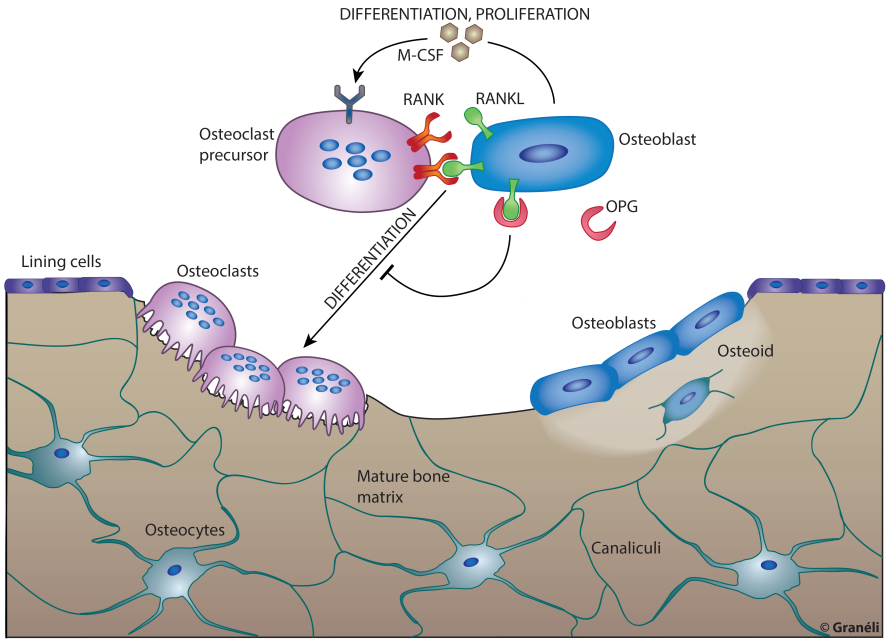

Figure 3. Schematic drawing of bone remodelling in a basic multicellular unit. M-CSF and RANKL, produced by osteoblasts, recruit and differentiate osteoclast precursors into bone resorbing osteoclasts. Illustration: Cecilia Graneli.

Bone remodelling

The process of bone remodelling (Figure 3) takes place in a basic multicellular unit (BMU), which consists of bone resorbing osteoclasts, the bone forming osteoblasts, osteocytes within the bone matrix, bone lining cells on the bone surface, and the capillary blood supply[23]. In human bone, the lifespan of osteoclasts and osteoblasts is about 2 weeks and 3 months, respectively, thus much shorter than the lifespan of the BMU which is 6 - 9 months[8]. In the initiation phase, osteoclast precursors are recruited and differentiate through M-CSF and RANKL produced by osteoblasts and osteocytes, after which bone resorption is initiated[22,23]. This is followed by a reversal period where osteoclasts undergo apoptosis[23]. During the resorption process, growth factors transforming growth factor beta (TGF-β) and insulin growth factor 1 (IGF-1) are released from the bone matrix, which subsequently recruit mesenchymal osteoblast progenitors that differentiate into mature osteoblasts to form osteoid[22]. The recruitment and differentiation of osteoblasts can also be initiated by cytokines produced by osteoclasts [22]. Cell-to-cell contact may also mediate bidirectional signalling via cytokines[22].

During the bone formation phase, some osteoblasts become osteocytes when they are embedded in the matrix[22]. The bone formation and mineralisation phase is the final stage in the bone remodelling process and is called the termination phase[23]. The bone remodelling process is shorter in cortical bone than in cancellous bone, where the length of the process is about 200 days in human iliac bone[23].

Systemic regulation of bone metabolism

Several endocrine pathways control bone metabolism and regulate mineral and glucose homeostasis[17]. Among the factors controlling mineral homeostasis are the parathyroid hormone (PTH), vitamin D hormone ($1,25(OH)_2D$) and the fibroblast growth factor 23 (FGF23) produced by osteocytes. The regulation of energy metabolism involves leptin (LEP), the sympathetic nervous system (SNS), OC and insulin[17].

Vitamin D is formed in the skin when exposed to sunlight, where the previtamin D_3 is converted to vitamin D_3 by body heat, which subsequently is converted to 25-hydroxyvitamin (25(OH)D) in the liver. 25(OH)D is then converted to the metabolically active vitamin D hormone $1,25(OH)_2D$ in the kidney[31]. When the calcium-sensing receptor in the parathyroid gland detects a decreased serum level of calcium, the release of PTH is stimulated. PTH subsequently stimulates osteoclastic bone resorption, renal reabsorption of calcium and renal production of $1,25(OH)_2D$ to increase intestinal calcium absorption, resulting in increased serum levels of calcium[17]. When serum levels of phosphate and $1,25(OH)_2D$ are elevated, the production of FGF23 in bone is stimulated which inhibits PTH and $1,25(OH)_2D$ production, thus the intestinal absorption of $1,25(OH)_2D$ is also inhibited. In addition, renal phosphate excretion is stimulated[17].

Leptin is a peptide hormone produced by adipocytes, and is believed to have a regulating effect on bone mass, although the precise role of LEP in bone is still controversial[24]. There are two main hypotheses of how LEP regulates bone; an indirect suppression of bone formation through the hypothalamus by increasing SNS signalling through supressed serotonin synthesis, and a direct positive effect through increased osteoblast proliferation and differentiation[17,32]. Additionally, the increased SNS signalling increases the production of OC from osteoblasts and osteocytes, which subsequently stimulates pancreatic β-cells to increase their production of insulin that further stimulates osteoblasts and their production of OC[17]. Insulin signalling in osteoblasts also promotes bone resorption by decreasing the expression of OPG, thus stimulating osteoclastogenesis[24]. Further, OC also stimulates

adipocytes to increase the production of adiponectin, an insulin-sensitising hormone[17].

Estrogen and androgens have a potent influence on skeletal growth and are also involved in skeletal homeostasis[33]. Estrogen prevents bone loss by increasing osteblastic expression of OPG and by decreasing the expression of RANKL and TNF-α[34]. Estrogen affects longitudinal bone growth, since estrogen in low levels enhances skeletal growth while high levels result in fusion of growth plates[34]. Estrogens are also important regulators of growth hormone (GH) and insulin-like growth factor 1 (IGF-1). Further, it has been suggested that estrogen induces precursor cells to differentiate into osteoblasts at the expense of adipocyte differentiation thus preventing osteoblast apoptosis[34,35]. Estrogen receptor α (ERα) is the most important mediator of estrogenic effects in bone[34]. The sex-steroid receptors have different roles in trabecular versus cortical bone, and the response to changes in growth factors, hormones and mechanical load is also different in periosteal versus endosteal surfaces of long bones[33]. Direct effects of estrogen on osteoclasts, and direct or indirect effects on B lymphocytes, mediated by ERα result in decreased trabecular bone resorption[33]. Cortical bone mass is protected by estrogens via ERα in osteoblast progenitors by indirectly attenuating bone resorption at the endocortical surface while the androgen receptor in osteoblasts is necessary for maintenance of trabecular bone in males[33]. Activation of the low-density lipoprotein receptor-related protein 5 (LRP-5)-Wnt-β-catenin signalling pathway is required for the physiological response of bone to mechanical loading and ERα has been shown to potentiate Wnt signalling in osteoblast progenitors[33]. A decreased responsiveness of osteoblasts to mechanical stimulation may be the result of loss of estrogen at menopause due to a downregulation of ERα expression[33].

Bone repair

The process of wound healing in dental extraction sites has been thoroughly described by Amler et al. (1960)[36]. After tooth extraction, a clot is formed by blood cells and fibrin, a protein involved in blood haemostasis. Within the next 4 - 5 days, the clot is replaced by granulation tissue containing erythrocytes, leucocytes and endothelial cells. In the third stage, the granulation tissue is replaced by connective tissue during a period of 14 - 16 days. Bone formation begins after seven days with osteoid formation in the base and the periphery of the extraction socket and the socket is filled with trabecular bone after 38 days, soon after the epithelial closure at 24 – 35 days[36,37].

During fracture repair, reduction and fixation of the bone fragments are vital for achieving optimal fracture healing. Fracture healing can be divided into three phases: inflammation, repair and remodelling[38]. When blood vessels rupture, vasodilatation and exudation of plasma and leucocytes occur while the bone at the ends of the fracture goes into necrosis. The fracture gap is filled with fibrin and a haematoma is formed which is characterised by low pH and hypoxia. The haematoma contains pro-inflammatory and anti-inflammatory cytokines and multiple leucocytes[38]. Polymorphonuclear neutrophils (PMNs) are the first cells to invade the callus after which macrophages, T-cells and B-cells follow. Dead cells and debris attract PMNs, which during their short lifespan secrete chemokines such as IL-6, which attract macrophages and lymphocytes into the callus. Other proinflammatory cytokines released in the inflammatory phase are IL-1, TNF, RANKL, M-CSF-1, members of the TGF-β superfamily and BMPs[38]. As a result of hypoxic conditions, angiogenic factors such as vascular endothelial growth factor (VEGF) are released followed by the migration of endothelial cells from the periosteal vessels to form new blood vessels in the haematoma. Fibroblasts produce new collagen, the haematoma is replaced by granulation tissue, and the differentiation of MSCs into osteoblasts is promoted[38]. Resident macrophages are believed to be pivotal for intramembranous bone formation while the inflammatory macrophages recruited to the site influence endochondral ossification[38].

There are four types of bone healing: endochondral bone repair, primary bone repair, direct bone repair, distraction osteogenesis[4]. Endochondral bone repair takes place when there is a low grade of stability. A soft callus is formed initially which is then transformed into a bone callus[4]. Periosteal precursor cells differentiate into osteoblasts, which initiate intramembranous bone formation followed by further callus growth by chondrocytes forming cartilage, surrounded by connective and granulation tissue[38]. After 10 - 14 days, the chondrocytes become hypertrophic and undergo apoptosis. The cartilage becomes hypervascularised and the recruited MSCs and monocytes differentiate into osteoblasts and osteoclasts, respectively. Following resorption of the calcified cartilage, new woven bone with a trabecular structure is formed and when bone bridges are present, the connective and the granulation tissues are replaced through intramembranous bone formation[38]. After the fracture gap is filled by new bone, osteoclasts begin to resorb periosteal callus and woven bone is remodelled to lamellar bone in the cortical fracture gap after which the resorption and remodelling continue in the medullary callus[38].

Primary bone repair occurs in the cortex when there is direct contact and rigid stability. Osteoclasts resorb bone on both sides of the gap with cutting cones, thereby enabling blood vessels to grow into the callus, followed by precursor cells that differentiate into osteoblasts which synthesise lamellar bone in which no remodelling is needed[4]. Direct bone repair is mediated without cartilage by the vessels and mesenchymal cells derived from the marrow, which differentiate and synthesise woven and lamellar bone, with remodelling along the long axis of the bone. This type of bone repair takes place when the interfragmentary gap is >0.1 mm and there is rigid fixation[4]. Distraction osteogenesis is mediated by the periosteum, endosteum and bone marrow, in which woven and lamellar bone is produced along the widening gap[4].

1.2 Biomaterials in bone

In 1999, the term biomaterial was defined as a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body[39]. However, the development of new medical technologies such as drug and gene delivery systems, tissue engineering, cell therapies, organ printing, nanotechnology-based diagnostic systems and microelectronic devices have been added to the implantable medical devices including metals, ceramics, synthetic polymers, biopolymers and nanoparticles among others[39]. These new types of medical technologies and substances may lead to a change in what is considered a biomaterial.

1.2.1 Titanium implants

Osseointegration is defined as a structural and functional connection between ordered, living bone and the surface of a load-carrying implant[40]. Albrektsson et al[41] have described six factors important for osseointegration: implant material, implant design, implant finish, status of the bone, surgical technique and implant loading conditions. Titanium implants have been successfully used in dental rehabilitation for nearly 50 years. Implant surface properties influence biological performance of implants. When titanium is exposed to oxygen, a thin surface oxide is formed, titanium dioxide TiO_2, which is chemically stable and corrosion-resistant[42]. Organic molecules adsorbed onto the surface influence wetting properties of the implant surface, which subsequently affects protein adsorption. Surface modifications, using different chemical and phase compositions, surface topography and coatings are used to enhance the biological performance of implants[42]. Bone regeneration around an implant has been compared to direct bone repair in fracture healing, although there is one fundamental difference, bone unites to

an implant surface which is a foreign material, instead of bridging bone to bone[43]. The implant itself acts as an osteoconductive substrate and the surface properties influence initial protein adsorption, platelet adhesion and haemostasis, complement activation, inflammation and the osteogenic cell response[42]. Histological studies have shown that after insertion of an implant, red blood cells and macrophages are present at the implant surface after three days, followed by multinuclear giant cells at seven days and mineralised tissue at the implant surface from day 14 onwards[44]. However, the onset and duration of specific events may vary between different animal species and models. In both the early healing period during the first week, but also at 28 days after insertion of titanium implants in rat tibia, increased expression of pro-inflammatory cytokines TNF-α and IL-1β has been observed on machined surfaces[45]. Increased expression levels of RUNX2, OC, TRAP, and cathepsin K (CATK) indicating active remodelling, have also been observed at oxidised surfaces[45].

1.2.2 Bone substitutes

Bone augmentation can be achieved by several different harvested grafts: autografts transferred within an individual, allografts transferred to another individual, xenografts which are transferred between different species and synthetic bone graft substitutes. Autografts are both osteoconductive as bone is formed around the resorbing graft, and osteoinductive due to the release of proteins which stimulate osteoblasts or pre-osteoblasts to from new bone[46]. Allografts, xenografts and synthetic bone graft substitutes are used to replace autografts to avoid donor site morbidity or when bone supply is limited[47]. To avoid immunological risks, allogenic bone grafts can be freeze-dried, or freeze-dried and demineralised, while xenografts are deproteinised[46]. Synthetic substitutes include polymers, bioactive glass ceramics, calcium sulphate and also calcium phosphate ceramics such as hydroxyapatite, β-tricalcium phosphate (β-TCP) or biphasic calcium phosphate (BCP)[46,48]. Calcium phosphates have different rates of solubility *in vitro*, which may reflect the degradation *in vivo*[48]. One main characteristic of the calcium phosphates is the porosity and tentatively the ideal pore size would be similar to that of trabecular bone. Macroporosity accounts for 50% of the porosity and provides a scaffold for bone-cell colonisation, while the microporosity, which can be controlled by the sintering process, allows body fluid circulation[48]. Ionic substitution in calcium phosphates has attracted attention due to its possible biological relevance and several different ions such as strontium, magnesium, silicon, zinc and manganese have been explored[49]. However, only a minor part has been evaluated *in vivo* and only in a few

cases, the *in vitro* or *in vivo* biological response can be ascribed to the presence of the foreign ion[49].

1.3 Osteoporosis

Osteoporosis is a systemic skeletal disease characterised by low bone mass and deterioration of bone microarchitecture leading to impaired bone strength, increasing bone fragility and fracture risk[50].

Epidemiology

A recent publication has estimated the prevalence of osteoporosis according to the WHO criteria in nine industrialised countries (USA, Canada, UK, France, Germany, Italy, Spain, Australia and Japan) at the ages of 50 or above, showing a prevalence ranging from 1 - 8% in men and 9 - 38 % in women, resulting in a total of 49 million affected individuals[51]. In Sweden, 2.5% of the male population and 6.3% of the females are affected at the age of 50 and the numbers increase to 16.6% of the males and 47.2% of the female population at 80 years of age[52]. Age is an important risk factor for osteoporotic fractures and the remaining lifetime probability of a fracture in the forearm, hip, spine or humerus is 22.4% in men and 46.4% in women at the age of 50[53]. The most common osteoporotic fractures are hip fractures, vertebral compression fractures, fractures of the distal radius, fractures of the pelvis, proximal humerus, distal femur and ribs[54], where the hip, vertebrae, and distal radius are the three major ones[55].

Diagnosis and assessment of fracture risk

Osteoporosis is defined as bone mineral density (BMD) 2.5 standard deviations or more below the average value of young, adult Caucasian women[54]. The term osteopenia is used for low bone mass and denotes a T-score between -1 and -2.5[54]. The T-score is used when comparing bone density values of an individual to sex-matched young healthy adults, while the Z-score is used to compare bone density values of an individual with a sex-matched and age-matched healthy population[56]. Bone mineral density (g/cm^3 or g/cm^2) is generally evaluated by dual energy x-ray absorptiometry (DXA) in the clinic and the femoral neck is the standard measurement site. DXA provides a two-dimensional area value and not a volumetric value and values can therefore be influenced by bone size and not only the true density[57,58]. Another disadvantage is that DXA does not distinguish cortical from trabecular bone[26]. However while new methods have been introduced, the DXA scan remains the gold standard method to assess BMD[56]. A thorough medical examination including patient history, radiograph of the spine and blood and serum analysis are routinely performed in the diagnostic

procedure[54]. A country-specific computer based algorithm to calculate fracture probability from risk factors and patient characteristics, the fracture risk assessment tool (FRAX), is used as a diagnostic tool worldwide[59].

1.3.1 Osteoporosis and pathogenesis

Osteoporosis is mainly caused by an imbalance in bone remodelling with excessive bone resorption and/or decreased bone formation[56]. The disease can also be a result of a disturbance in the accumulation of bone mass in childhood or early adulthood caused by genetic, hormonal or environmental factors, leading to failure to achieve peak bone mass[56]. Excessive exercise and anorexia nervosa causing estrogen deficiency in premenopausal women may contribute to bone loss and reduced peak bone mass[50]. However, age-related bone loss commences in both men and women immediately after peak bone mass is achieved, and is considered to represent a significant part of the trabecular bone loss throughout life[33]. Postmenopausal and age-related bone loss are referred to as primary osteoporosis, while secondary osteoporosis is caused by other medical conditions or medications[60].

Primary osteoporosis

Bone loss in postmenopausal osteoporosis is characterised by an accelerated early phase lasting for a decade or less, mainly involving cancellous bone, followed by a late continuous slow phase with a proportional bone loss in cortical and cancellous bone[61]. Both phases of postmenopausal osteoporosis are caused by estrogen deficiency and in men both estrogen and testosterone levels decline with age[61]. As testosterone can be aromatised into estrogen, the decrease in androgen also causes reduced serum levels of estrogen, a mechanism that partly explains the bone loss caused by androgen deficiency[62,63]. Osteoporosis in men is considered under-diagnosed and under-treated, although more than a third of new osteoporotic fractures occurring worldwide are in men[64]. Osteoporotic fractures occur in men at an average age approximately 5 - 10 years later in life than women and the morbidity and mortality rates after a hip fracture are higher among men compared to women[64].

Secondary osteoporosis

Medical conditions known to cause osteoporosis are multiple myeloma, hypogonadism, endocrine disorders, gastrointestinal disease, cystic fibrosis, genetic disorders, premature menopause, chronic liver disease and alcoholism[56,60]. Among the medical drugs causing osteoporosis, glucocorticoids are considered to be the most common and long-term use of glucocorticoids is associated with an increased rate of fracture[60]. Clinical risk

factors include maternal family history of hip fracture, low body mass index, immobilisation, smoking, loss of height and previous fragility fracture[50,54,60]. Premenopausal women who have received non-surgical breast cancer treatment are also at risk of developing osteoporosis due to adverse effects of the therapies on the skeleton[65]. A side effect of chemotherapy is primary ovarian failure resulting in decreased levels of estrogen and early menopause leading to osteopenia[65]. Aromatase inhibitors such as Tamoxifen, used as adjuvant hormonal treatment can potentially cause a decrease in BMD by acting as an estrogen antagonist[65]. Men treated for prostate cancer with hormone ablation are at risk of increased bone remodelling and loss of BMD due to a decrease of testosterone to castrate levels, resulting in impaired conversion of testosterone into estrogen[63].

Osteoporosis - inflammation and cellular mechanisms

Bone loss in osteoporosis is caused by an imbalance in bone remodelling which is partly a result of an increased number of active osteoclasts. Postmenopausal bone loss has been suggested to be a result of loss of the direct effect of estrogens on osteoclasts[33]. However, a series of mechanisms is involved in the development of the condition. Estrogen is an inhibitor of IL-1β, TNF-α and IL-6 and as the estrogen levels decline at menopause, the levels of these pro-inflammatory cytokines are increased[66-68]. Estrogen deficiency increases the monocytic production of IL-1 and TNF, which activate the osteoclast via the osteoblasts[66]. TGF-β increases the number of T-cells secreting TNF which subsequently stimulates the production of M-CSF and RANKL, resulting in the stimulation of osteoclastogenesis and osteoclast activation[68]. As RANKL and IL-1 prevent osteoclast apoptosis, the number of bone-resorbing cells is increased further[68]. With increasing age, structural changes in the hydroxyapatite and also micropetrosis (mineralisation of the osteocyte lacunae and canaliculae) have been observed[33]. Increased cortical porosity is suggested to be a result of increased apoptosis of the osteocytes in cortical bone, upregulating RANKL release by surrounding osteocytes[33]. Loss of estrogen also affects osteoblast progenitor cells through decreased ERα expression resulting in a lower response to mechanical stimulation[33]. Other factors suggested to be involved in the pathophysiology of age-related bone loss by reducing osteoblast generation and causing osteoblast and osteocyte apoptosis are oxidative stress, increasing endogenous levels of glucocorticoids, increased sensitivity of bone cells to glucocorticoids, and increasing the number of adipocytes in the bone marrow[69].

Treatment of osteoporosis

Drugs used to treat osteoporosis are divided into antiresorptive therapies that decrease bone resorption, and anabolic agents that enhance bone formation[26]. The antiresorptive drugs include calcium, vitamin D, bisphosphonates, estrogen, selective estrogen-receptor modulators (SERMs), calcitonin, strontium ranelate and denosumab[26,68]. The only clearly anabolic drugs in use today are the intact parathyroid hormone (PTH 1-84) and its N-terminal fragment, teriparatide (PTH 1-34) which when given in intermittent doses have been shown to reduce fractures among postmenopausal women[26,68]. New anabolic agents targeting the inhibitors of Wnt signalling, sclerostin and Dkk-1, using antibodies are presently being evaluated in clinical trials[26]. Another new class of drugs under development is the calcium-sensing receptor antagonists, calcilytics, which increase the release of endogenous PTH in short pulses, resulting in a bone anabolic effect[26].

Osteoporosis in different bones

It is well known that the axial and appendicular skeleton is affected by osteoporotic changes. An accelerated loss of trabecular bone is observed in the spine, while osteoporotic changes in long bones result in increased cortical porosity and a thinner cortex due to an increased inner diameter[33]. However, periosteal bone apposition is increased after menopause, possibly as a protective mechanism preserving bone strength[33].

In the craniofacial skeleton, evaluations of BMD using DXA scan have demonstrated correlations between BMD of the mandible and the lumbar spine, the femoral neck and the forearm[70]. Furthermore, a moderate to strong correlation between BMD of the skull (including the mandible) and total BMD of the body has been observed[71]. A recent study has reported correlations between maxillary BMD (CT scan) and BMD of hip and spine (DXA)[72]. In the same study a significantly lower BMD was observed in the maxilla of osteoporotic women compared to healthy individuals[72]. However, the authors concluded that in the possible implant sites, i.e. the incisor to the premolar region, the correlation with hip and spine was weak[72]. Additionally, others have reported that maxillary and mandibular BMD values do not correlate with the BMD of the femoral neck[73].

1.3.2 Osteoporosis and biomaterials

A decreased capacity for fracture healing in osteoporotic bone has been described in a large number of animal studies, however, there are only a few human studies supporting delayed bone repair in osteoporotic individuals[74-76]. *In vitro* studies performed on osteoblasts from surgically induced estrogen

deficient rats and surgically induced estrogen deficient sheep, cultured on bioactive glasses have shown decreased cell viability and OC levels when compared to osteoblasts from intact animals[77]. *In vivo*, a decreased osseointegration rate for bioactive glasses, ZrO_2 and hydroxyapatite has been observed in surgically induced estrogen deficient rats compared to intact animals[77]. Estrogen deficiency has also been shown to have a negative effect on osseointegration of titanium implants in studies performed on rats[78-81]. However, no differences in dental implant survival between osteoporotic patients and healthy individuals have been observed[82,83]. These observations have been confirmed by histological findings showing no differences in bone-to-implant contact between implants retrieved from patients with osteoporosis and individuals without osteoporosis[84].

1.3.3 Animal models of osteoporosis

Animal species used to study osteoporosis are mouse, rat, ferrets, guinea pigs, rabbits, minipigs, sheep, cats, dogs and primates[85,86]. However, the rat is one of the most frequently used in osteoporosis research[86]. A vast number of different techniques have been used to achieve osteoporotic conditions, such as ovariectomy (OVX), orchidectomy, hypophysectomy, use of glucocorticosteroids, special diets and limb immobilisation[87].

1.3.4 The ovariectomised rat

The OVX rat is frequently used as a model for postmenopausal osteoporosis. An unbalanced increased bone remodelling is observed after OVX with bone resorption exceeding bone formation. OVX in a 3 months old rat results in rapid progressive bone loss in the proximal tibia in the first 100 days, followed by stabilisation and a late phase of slow bone loss after 270 days[88]. The bone loss is evident at two weeks after OVX and absolute after one month[88] (Figure 4). Following OVX, there is an increase in serum alkaline phosphatase (ALP) and serum OC[89] and the rats gain weight resulting in increased mechanical loading and subsequently also in increased bone mass, thus protecting the animals from loss of bone strength[90]. As the OVX rat does not develop fractures, the term osteopenia is used when evaluating the effect of OVX in rats, and it should be considered as a model of postmenopausal bone loss[85,89]. The expected lifespan of a rat is 3 – 4 years and it is reproductively mature at three months; a time point when bone growth has slowed down considerably. It has been observed that proximal epiphysis closes at between 6 – 18 months of age. The rat does not have a menopause but it becomes chronically anovulatory at 19 months of age[89]. Although the main part of bone loss in rats following OVX occurs in the trabecular bone, BMU based bone remodelling occurs in both cortical and trabecular bone[86,89].

Since rats younger than eight months lack Haversian systems, intracortical bone remodelling appears to be age-related although bone remodelling in younger rats can be activated, resulting in cortical porosity and concentric lamellae having characteristics of Haversian systems[89]. Trabecular bone remodelling activities similar to human trabecular bone remodelling, have been demonstrated in rat, with typical BMUs in a number of locations in the rat skeleton, including alveolar bone and the periosteal surface of the mandible[89,91]. In the rat mandible, OVX results in increased bone turnover[92], and reduced bone area fraction and stiffness[93]. Studies on the effects of OVX in rats have shown increased bone resorption in the alveolar ridge after tooth extraction[94,95] and structural changes in mandibular alveolar bone[96]. When the effect of OVX on rat mandible was compared to proximal tibia, OVX resulted in a reduction of bone volume fraction of 4.9% while the tibia displayed 82% reduction, a difference which is believed to be explained by mechanical loading of the alveolar process during mastication[97].

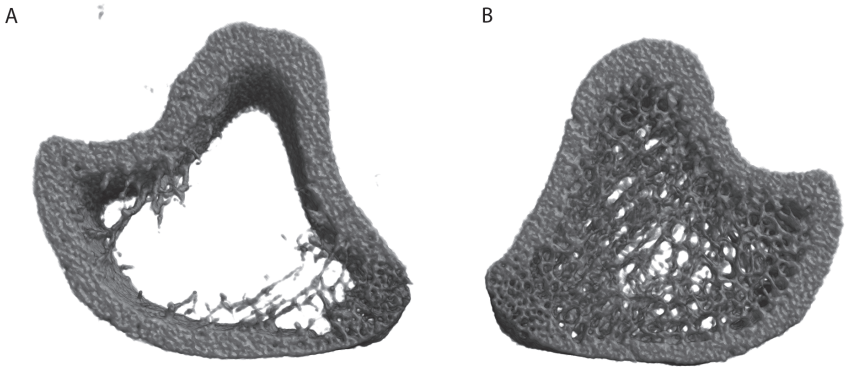

Figure 4. A. Micro-computed tomography scan of left proximal tibia in OVX rat (one month after ovariectomy). B. Right tibia of intact rat.

1.4 Antiresorptive agents

The antiresorptive agents include bisphosphonates, strontium ranelate, calcium, vitamin D, hormone therapy, selective estrogen-receptor modulators (SERMs), calcitonin, denosumab, odanacatib, and saracatinib.

1.4.1 Bisphosphonates

Bisphosphonates are powerful antiresorptives routinely used as primary therapy in different skeletal conditions such as osteoporosis, Pagets's disease, multiple myeloma, bone metastases from solid tumours (breast, prostate, lung and renal cancer) and hypercalcaemia of malignancy[98-100]. Alendronate has been shown to reduce the risk of vertebral fractures in osteopenic women with 44% while zoledronic acid decreased the risk of vertebral fractures by 70% over a three-year period[101]. The mortality following hip fractures have decreased by 28% with zoledronic acid[56]. In cancer treatment, bisphosphonates effectively relieve skeletal pain, reduce skeletal complications, and the incidence of hypercalcaemia[102].

Chemical composition and pharmacodynamics

Bisphosphonates are non-hydrolysable, chemically stable synthetic analogues of inorganic pyrophosphate. Pyrophosphates have a strong affinity for calcium phosphate and belong to the polyphosphate group of compounds frequently used in the industry for their capability to inhibit precipitation of calcium carbonate[102,103]. Pyrophosphates are found in body fluids and inhibit ectopic calcification by regulating mineral deposition and dissolution[103] and can be hydrolysed by enzymes. Developed for medical use in the 1960s, the chemical structure of bisphosphonates is characterised by a P-C-P structure, distinguishing them from pyrophosphate, which contains an oxygen atom instead of the carbon[103]. The P-C-P structure is responsible for the high affinity to bone; and by varying the side chains bisphosphonates are able to acquire different chemical and biological characteristics[103,104]. There are two main groups of bisphosphonates: the early bisphosphonates (etidronate, clodronate) and the bisphosphonates currently clinical use (pamidronate, alendronate, risedronate, zoledronic acid), which have a hydroxyl group attached to the central carbon atom enhancing the binding to hydroxyapatite and a nitrogen or amino group that increases the potency 10 to 10,000 fold compared to etidronate[102,104].

In contrast to pyrophosphates, the bisphosphonates are resistant to hydrolysis and have a strong affinity to calcium and metal ions. They bind strongly to calcium phosphate and inhibit crystal formation, aggregation and dissolution[103]. Only $< 1 - 2.5\%$ of orally administered bisphosphonate is

absorbed from the gut, of which 50% is taken up by the skeleton, binding strongly to hydroxyapatite while the rest is eliminated from the plasma through renal excretion[105-107]. Pharmacokinetic studies of zoledronic acid have shown that only 39 – 46% of intravenously administered drug is released into urine at 24 h after administration, suggesting that a majority of the drug is taken up by bone[108]. As bone turnover slows down during bisphosphonate treatment bisphosphonates are deposited in the skeleton. The half-life of the drugs is believed to be several years and may be over ten years for some bisphosphonates[103].

Mechanism of action and effects on bone cells

The main effect and primary biological action of bisphosphonates is to suppress bone resorption through inhibition of osteoclast recruitment, lowered activity and reduced lifespan of the osteoclast[109]. Bisphosphonates are adsorbed onto the mineral at bone surfaces and are then released during resorption to finally become internalised in the osteoclasts through endocytosis[110].

The non-nitrogen containing bisphosphonates are metabolically incorporated into non-hydrolysable analogues of adenosine triphosphate (ATP), which have detrimental effects on osteoclast function and induce osteoclast apoptosis by inhibiting intracellular metabolic enzymes[110]. Far more potent are nitrogen-containing bisphosphonates. These act by inhibiting the farnesyl pyrophosphate synthase, an enzyme in the mevalonate pathway[111] (Figure 5). The mevalonate pathway is responsible for cholesterol production and the synthesis of isoprenoid lipids, which are important for prenylation of small GTPases, proteins important for internal signalling in osteoclasts, regulating osteoclast function and cell survival by affecting vesicular transport, cytoskeletal organisation and membrane ruffling[110-113]. The inhibition of farnesyl pyrophosphate synthase also results in the accumulation of the metabolite isopentenyl diphosphate (IPP), that activates γδ T-cells, leading to the release of TNF-α and initiation of an acute phase response occurring when nitrogen-containing bisphosphonates are injected for the first time[110]. When IPP accumulates in the cells, another ATP analog ApppI is produced, which through intracellular actions causes osteoclast apoptosis[110,114,115].

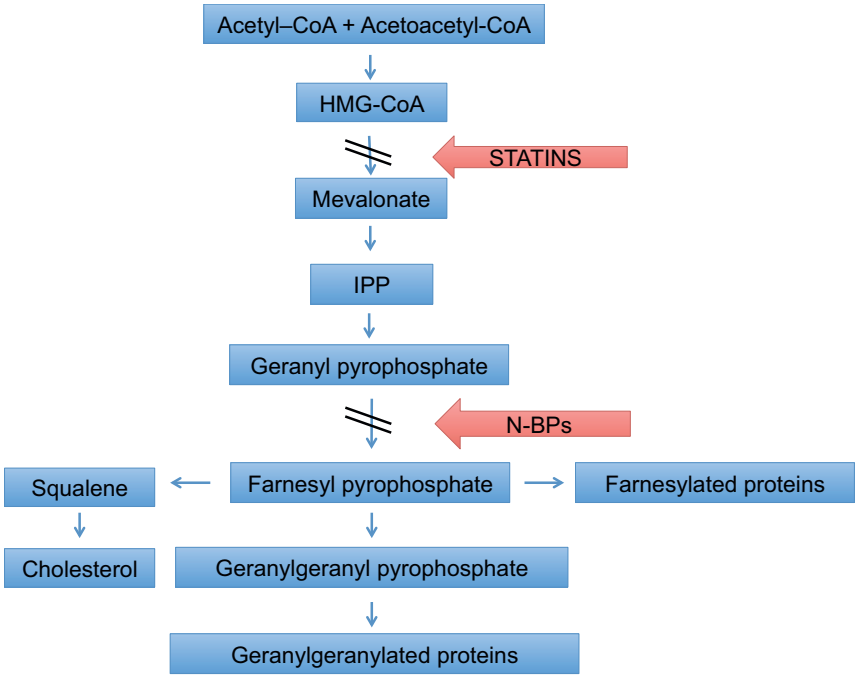

Figure 5. Nitrogen-containing bisphosphonates inhibit prenylation of small GTP-binding proteins (Rho, Rac, Rab), important for osteoclast morphology and intracellular mechanisms.

Weinstein and co-workers investigated transiliac bone biopsies from postmenopausal women with long-term oral bisphosphonate treatment and reported an increased number of osteoclasts, giant osteoclast formation with pyknotic nuclei adjacent to superficial resorption cavities, and hypernucleated, detached osteoclasts undergoing apoptosis[116]. *In vitro*, nitrogen-containing bisphosphonates suppress osteoclastic resorption by a direct action on the early osteoclast precursors at the bone surface[117]. Bisphosphonates have also been shown to prevent osteoblast and osteocyte apoptosis *in vitro* and *in vivo*[118,119]. Several studies have shown that osteoblast proliferation and differentiation are stimulated by the presence of bisphosphonates in low concentrations whilst larger concentrations seem to have an inhibitory effect[120].

In vitro nitrogen containing bisphosphonates have been shown to increase OPG gene expression in human osteoblasts[121]. However, when the effect of nitrogen-containing pamidronate was investigated on rat osteosarcoma cells, RANKL expression was downregulated, but no effect on OPG and ALP was

observed[122]. Other *in vivo* studies on the effects of bisphosphonates on osteoblasts have shown increased bone mineralisation suggesting a positive effect on bone formation[120].

Bisphosphonates and inflammation

Most of the original studies investigating the effects of bisphosphonates on osteoclasts were performed on macrophages, showing inhibition of proliferation and cell death by apoptosis depending on type of bisphosphonate and concentration[123-125]. The two main groups of bisphosphonates seem to have completely different effects on cytokine production by macrophages or macrophage-like cells; while non-nitrogen-containing bisphosphonates have a predominantly anti-inflammatory effect, reducing IL-1β, IL-6, TNF-α and nitric oxide (NO) production, nitrogen-containing bisphosphonates have pro-inflammatory effects by enhancing IL-1beta, TNF-α and IL-6 release[126-130].

Systemic and local effects on bone healing

Systemic delivery of bisphosphonates has been shown to reduce the negative effect of OVX and improve osseointegration of titanium implants in several experimental studies[131-136]. Systemic treatment with zoledronate and alendronate has also prevented resorption of bone allografts in rats[137,138]. In a model of fracture repair, single systemic infusions of bisphosphonates improved bone mineral content, bone volume and strength of healing fractures, while no significant effect was observed upon local application[139,140]. In experimental studies, systemic bisphosphonate treatment resulted in significantly impaired alveolar bone repair as well as decreased angiogenesis after extraction of teeth[141,142].

Local application of bisphosphonates has also been shown to improve bone healing around implants in experimental and clinical studies. Bioactive glass soaked in alendronate and inserted in a rat mandible defect showed significantly higher bone formation compared to saline-treated control group[143]. Further, titanium implants coated with bisphosphonates have shown improved implant fixation in both OVX and intact rats[144,145]. A recent clinical study showed improved implant fixation of dental implants coated with pamidronate and ibandronate inserted in the maxilla, showing less marginal bone loss and increased resonance frequency values compared to control implants[146].

Effects at different skeletal localisations

Concerns over ONJ, an adverse effect of bisphosphonate treatment, and an increasing number of reports of atypical femoral fractures in patients with

long-term bisphosphonate treatment, have initiated several studies on site-specific bone response to bisphosphonate treatment.

In vitro, human BMSCs from the mandible have been observed to be more susceptible to pamidronate compared to BMSCs from the iliac crest[147]. *In vivo*, increased protein levels of RANKL and OPG in the tibia and decreased levels of RANKL levels in the mandible have been observed after bisphosphonate injections[148]. Moreover, the uptake and release of bisphosphonates in the mandible and appendicular bones is similar, but lower than in axial bones[149]. Bisphosphonates have previously been demonstrated to decrease the rate of mandibular bone loss[150]. Further, experimental studies have shown reduced bone formation rates at the endocortical surface of long bones and the endosteal surface of the mandible, following bisphosphonate treatment, but no significant effects on periosteal bone formation have been observed[151,152]. Additionally, experimental studies of fracture repair in tibia and mandible have shown that bisphosphonate injections to result in a more profound effect in the mandible compared to tibia, reducing osteoclast number, delaying callus formation and remodelling of cartilage and bone[153].

Adverse effects of bisphosphonates

Reported adverse effects of bisphosphonates include ONJ, (which will be discussed later in this thesis), atypical femoral fractures, gastrointestinal discomfort, oesophageal cancer, atrial fibrillation, acute inflammatory response, ocular inflammation and severe musculoskeletal pain[102,154].

Gastointestinal discomfort is a common adverse event in patients treated with oral bisphosphonates and is one of the most common reasons for patients discontinuing the drugs[154]. Oesophageal cancer has been reported in patients treated with bisphosphonates, although no evidence of increased risk has been observed in large cohort studies[154]. A transient acute-phase response is a common side effect of nitrogen-containing bisphosphonates when they are injected for the first time, causing fever, pain, nausea and fatigue[155,156]. Following intravenous injection, nitrogen-containing bisphosphonates are taken up by monocytes circulating in peripheral blood resulting in the activation of γδ T-cells which cause a release of TNF-α, thus initiating the acute phase response[110,156]. There is still a lack of evidence if bisphosphonate treatment increases the risk of atrial fibrillation due to conflicting outcomes in large cohort studies[154]. Ocular inflammatory conditions have also been reported after administration of intravenous pamidronate with an incidence of < 1%[154]. An increased incidence of musculoskeletal pain has been reported both in patients treated with orally administered bisphosphonates and with infusions[154]. A large number of atypical femoral fractures located in the

subtrochanteric region or diaphysis of the femur have been reported in patients on bisphosphonate use[157]. The atypical femoral fractures have several features in common with stress fractures as they are often associated with no or minor trauma and may be incomplete[154]. It is concluded that there is a significant association between atypical femoral fractures and bisphosphonate use, and although the absolute risk of atypical femoral fractures in bisphosphonate treated patients is low, the risk increases with long-term use[157]. Reduced bone remodelling and accumulation of microdamage resulting in a change in the mechanical properties of bone are believed to cause the condition[158]. A recent experimental study showed decreased osteocyte lacunae density and reduced osteonal areas in alendronate treated dogs, while the total number of osteons remained unchanged[158]. However, histomorphometric evaluation of bone biopsies obtained from bisphosphonate treated patients have shown that the frequency of microcracks does not differ from the control group[159]. The risk of adverse effects has led to a discussion of when cessation of bisphosphonate treatment in osteoporotic patients is appropriate. Five years of treatment is believed to be sufficient, although an estimation of risks has to be made on an individual basis and patients should be followed with BMD measurements[154].

1.4.2 Strontium ranelate

Strontium ranelate in the treatment of osteoporosis is recommended when patients are intolerant to bisphosphonates or when there are contraindications to bisphosphonate use[56]. Strontium ranelate has been used to treat osteoporosis since 2004 is frequently described to have a dual mode of action (i) increasing bone formation, and (ii) decreasing bone resorption[160]. However, the mechanism of action of strontium ranelate is still unclear. It was shown to increase the BMD (lumbar spine and femoral neck) and reduce the risk of vertebral fractures by 49% in the first year of treatment in a large phase III trial[161]. Another phase III study of 5,091 postmenopausal women with osteoporosis showed that strontium ranelate significantly reduced the risk of all nonvertebral fractures, and also hip fractures in a high-risk group over a 3-year period[162].

Chemical composition and pharmacodynamics

Strontium ranelate, consists of two atoms of stable non-radioactive strontium combined with organic ranelic acid[160]. Strontium (Sr), an alkaline earth metal, is mainly absorbed through the gastrointestinal tract and excreted mainly in urine[163]. As strontium shares some chemical and physical characteristics with calcium, absorbed strontium is deposited in bone tissue (>99.1%). Strontium ions compete with calcium ions which bind more easily

to the hydroxyapatite crystals because of the smaller size of the calcium ion in comparison to strontium[163]. At high concentrations of strontium, calcium ions are replaced by strontium ions, which subsequently decreases bone calcium content, leading to distortion of the crystal lattice, impaired crystal growth and an increased dissolution of mineralised bone[163]. The result is hypomineralisation and a lower BMD, which is in contrast to observations made from administration of strontium in low doses, where experimental studies have demonstrated increased bone formation rates and higher bone density[163]. Additionally, strontium in low doses has been shown to remineralise bone lesions in patients with metastatic cancer[163]. Strontium incorporation into bone reaches a plateau level after continuous administration although the level of strontium varies depending on the anatomical site and bone structure, for instance higher amounts of strontium are found in cancellous bone than in cortical bone and the concentration of strontium is higher in newly formed bone compared to old bone[164].

Mechanism of action

Preclinical studies have indicated strontium to have a dual effect, increasing bone formation and reducing bone resorption[165]. However, this proposed mechanism of action is under debate. Recently, histomorphometric analysis of transiliac bone biopsies from osteoporotic women treated with strontium ranelate or alendronate showed a significant decrease in the bone formation parameters after 6 and 12 months of treatment in both alendronate and strontium ranelate treated groups[166]. However, the strontium ranelate groups had significantly higher values of bone formation parameters than the alendronate treated samples, whereas decreased bone resorption, was only observed in the alendronate groups[166]. This lack of evidence of an anabolic effect of strontium ranelate in the clinical situation was recently discussed in conjunction with the interpretation of increased BMD values of patients treated with strontium ranelate[167]. As strontium replaces some of the calcium in bone, x-ray absorption by the tissue is increased which leads to higher BMD values not reflecting a true increase of bone tissue[167]. A suggested mechanism explaining the beneficial effects of strontium on the BMD and fracture prevention is that strontium has a physical effect on bone strength[167].

Strontium effects on bone cells

In several animal studies strontium ranelate has been shown to increase bone formation, bone mass and bone strength without altering bone stiffness[160,165]. In OVX rats, treatment with strontium ranelate has resulted in reduced bone resorption and maintained bone formation[168]. Systemic strontium ranelate has also proved to promote fracture healing in OVX rats[169]. One suggested mechanism of action is that strontium ranelate promotes bone formation by

enhanced pre-osteoblast replication, osteoblast differentiation, collagen type I synthesis and bone matrix mineralisation via the calcium-sensing receptor[170]. In addition, strontium ranelate is believed to increase OPG and decrease RANKL, thus inhibiting osteoclast differentiation and activity[170]. *In vitro,* strontium ranelate has been shown to inhibit osteoclast activity and decrease the number of mature osteoclasts[171]. Additonally, stimulation of osteoblastic differentiation, maturation and function resulting in increased expression of ALP, OC and bone sialoprotein has been observed[171,172]. Moreover, promoted osteocyte differentiation, increased OPG/RANKL ratio, and inhibited osteoblast-induced osteoclastogenesis has been observed *in vitro*[172]. Studies of osteoblastic cells in strontium chloride, ($SrCl_2$) and conditioned media have shown an increased OPG expression and OPG protein levels[173]. $SrCl_2$ increased bone formation and promoted the antiresorptive effects in proximal tibia of OVX rats[173].

Strontium and implants

Systemic administration of strontium ranelate has been shown to increase pull-out and push-out strength of titanium implants, increase bone-to-implant contact and improve bone microarchitecture in the vicinity of the implant in rat long bones[174]. In contrast, a rat study where stainless steel implants were inserted in rat tibia, revealed that strontium ranelate did not result in any significant difference on pull-out force, while animals treated with bisphosphonates showed a doubled pull-out force[175]. When strontium is administered locally the effect appears to be weak. Experimental studies on strontium-doped bone substitutes mixed with allograft inserted around titanium alloy implants resulted in increased volume of new bone and a larger volume of remaining allograft compared to controls, although no improvement of mechanical fixation of the screw was observed[176]. Further, titanium implants coated with Sr-substituted hydroxyapatite did not show any improvement in implant fixation when compared to implants coated with stoichiometric hydroxyapatite[177].

Osteoblasts from OVX rats cultured in contact with Sr-substituted hydroxyapatite resulted in increased levels of Col1, ALP, and decreased levels of IL-6 when compared to stoichiometric hydroxyapatite, indicating increased proliferation[178]. Sr-substituted bioactive glasses have been shown to enhance the metabolic activity of osteoblasts and to inhibit osteoclast activity[179]. However, a deterioration of implant fixation has also been reported for Sr-substituted bioactive glass-coated titanium implants, indicating that the delivery of strontium to the material-tissue interface remains a challenge[177].

Adverse effects of strontium ranelate

Reported adverse effects of strontium ranelate treatment include thromboembolic disease, drug rash with eosinophilia systemic syndrome, abdominal discomfort and memory loss[26,101]. In a study following patients treated for postmenopausal osteoporosis with strontium ranelate, the drug was well tolerated over ten years, with low incidence of venous thromboembolism and neurological disorders[180]. In a study of five years treatment with strontium ranelate in women over 80 years of age, adverse events showing statistically significant differences between treatment and placebo included headaches, deep venous thromboembolic events, seizures and seizure disorders[181].

1.4.3 Other antiresorptive agents

Calcium intake from food or supplements has been shown to act as a weak antiresorptive, slowing down bone turnover and postmenopausal bone loss, by suppressing the age-related increase in PTH levels and bone resorption[60,182]. However several large randomised controlled trials and recent meta-analyses have failed to show that calcium supplements with or without vitamin D prevents fractures[182]. Calcium supplements have been shown to increase cardiovascular events, kidney stones and gastrointestinal symptoms. Since the risk is not balanced by the benefits, calcium supplements are currently regarded as having only a minor role in osteoporosis treatment although dietary intake has not been associated with negative effects[182].

The use of vitamin D supplements for the prevention and management of osteoporosis has for many years been recommended as a baseline treatment together with calcium supplements[26]. Recently, the use of vitamin D supplementation has been questioned due to poor evidence of fracture prevention. In a systematic review and meta-analysis on the effects of vitamin D supplements on the BMD, the results showed a small benefit at the femoral neck, while no effect was observed in the lumbar spine, total hip, trochanter, total body scans or forearm[183]. No obvious negative effects of vitamin D supplements used to prevent or treat osteoporosis have been reported. However, a randomised controlled trial of oral high-dose vitamin D therapy has reported an increased risk of falls and fractures[184].

Hormone replacement therapy (HRT) with estrogen alone or with a combination of estrogen and progesterone was the first choice of treatment before the introduction of bisphosphonates. Large trials of healthy postmenopausal women showed increases in the BMD and substantial

reduction in vertebral, non-vertebral and hip fractures[56,101]. Although HRT was effective in fracture prevention and also reduced the risk of colon cancer[56], HRT has been shown to increase the risk of stroke, breast cancer and thromboembolism, and is considered to have a limited role in the treatment of postmenopausal osteoporosis[101].

Calcitonin is a peptide hormone produced by the thyroid gland, which regulates calcium levels. Synthetic calcitonin has been used as an antiresorptive drug for many years. The drug exerts its effect by binding to specific proteins on osteoclasts and inhibits osteoclast activity via intracellular second messengers[185]. It has been shown to increase BMD but as the effects of calcitonin on bone resorption and BMD are modest, the role of calcitonin in osteoporosis treatment is still unclear[68,185,186]. The effect of calcitonin on BMD was evaluated in postmenopausal women who were administered the drug in the form of oral tablets or nasal spray. When compared to placebo, 80% of the women in each treatment group experienced adverse events and nearly half of the subjects in each treatment group had gastrointestinal complications[186].

Selective estrogen receptor modulators (SERMs) are substances that have the possibility to bind to estrogen receptors and exert an agonist or antagonist effect depending on the targeted tissue[68]. Raloxifene, which is the only SERM approved for prevention and treatment of osteoporosis, has been shown to reduce vertebral fractures[68]. Raloxifene also reduces the risk of breast cancer in the individuals receiving the drug, though it is only approved for the treatment of osteoporosis. Currently the role of SERMs in clinical use is limited and they are mainly used to treat mild osteoporosis or as an alternative treatment for patients intolerant to bisphosphonates[56,68,101]. SERMs have been shown to increase the risk of thromboembolism and also to cause or exacerbate vasomotorsymptoms such as hot flushes associated with menopause[56,68].

Newer therapies

Denosumab is a human monoclonal antibody that binds to RANKL, thus preventing it to bind to its receptor RANK, thereby reducing osteoclastogenesis and arresting osteoclast activity[56,101]. Clinical trials have shown a rapid decrease in bone resorption markers, an increase in BMD and a reduced risk of vertebral fractures, hip fractures and non-vertebral fractures by 68%, 40% and 20%, respectively[187,188]. When the effects of denosumab and alendronate on BMD and biochemical markers of bone turnover in postmenopausal women were compared in a large randomized, blinded phase III trial, denosumab showed significantly greater increases in BMD and

significantly greater reduction of bone turnover markers compared to alendronate[189]. Adverse events were similar in both treatment groups[189]. Further, in large trials on patients with advanced prostate or breast cancer, denosumab has been superior to zoledronic acid in preventing tumour-related bone lesions or delaying the time until the first skeletal-related event[187]. Since denosumab is an anti-RANKL antibody, the activation of its receptor RANK is prevented and while RANK is mainly expressed by osteoclasts and its precursors, it is also expressed by endothelial cells, smooth muscle cells, T-and B-lymphocytes, dendritic cells and a variety of malignant cells[187]. In a clinical phase III study a higher incidence of eczema and cellulitis including erysipelas was reported in women treated with denosumab compared to placebo[188]. However, no other extraskeletal effects of denosumab have been observed in clinical trials[187]. ONJ is a rare complication of denosumab treatment and the incidence has been reported to be at a similar level as with bisphosphonate treatment[190,191].

Odanacatib, an inhibitor of CATK, suppresses osteoclast activity without enhancing osteoclast apoptosis thus leaving the osteoclast-osteoblast signalling intact, which results in maintained bone formation[26]. Odanacatib has been shown to decrease bone resorption markers in serum and increase BMD in lumbar spine, total hip and the femoral neck[26,192]. A recent meta-analysis of trials comparing odanacatib to placebo indicates a reduction of all fractures. However, this remains to be confirmed[192]. A phase III study investigating the effect of odanacatib on fractures in postmenopausal women was stopped in 2012 and the results are pending[192]. Odanacatib is generally well tolerated and has shown adverse reactions at the same level as placebos during phase I and phase II studies[26,192].

Saracatinib is an inhibitor of Src kinase, an enzyme involved in osteoclast activation. Saracatinib has been shown to decrease bone resorption markers in serum and urine in healthy men, while bone formation markers were similar to placebo[26]. In a phase I trial evaluating the effect of saracatinib on bone turnover in patients with advanced malignancies, a decrease in bone resorption markers was observed in the urine and serum[193]. Moreover, saracatinib has been well tolerated with no significant adverse events[193,194].

1.5 Osteonecrosis of the jaw

ONJ has been described in literature for more than a decade as an adverse effect of bisphosphonate treatment[195,196] (Figure 6). A similar condition called "phossy jaw" has been described, affecting factory workers exposed to yellow phosphorous in the match making industry during the late 1800s[197]. A number of abbreviations have been used to refer to the condition, for instance BRONJ (bisphosphonate related osteonecrosis of the jaw), and ARONJ (antiresorptive agent-induced osteonecrosis of the jaw), although the most frequently used has been ONJ (osteonecrosis of the jaw). Recently, a change of nomenclature to MRONJ (medication-related osteonecrosis of the jaw) has been suggested due to an increased number of drugs being associated with ONJ[190,191]. Other medical therapies linked to ONJ are the antiresorptive drug denosumab, the antiangiogenic cancer drugs bevacizumab, sunitinib, sorafenib, and the immunosuppressant sirolimus used to prevent rejection after organ transplantation[190,191]. To define the condition the following criteria should be met: the patient is currently or has previously been treated with antiresorptive or antiangiogenic drugs, the presence of exposed bone in the maxillofacial region persisting more than eight weeks, and no history of radiotherapy to the region or metastatic disease to the jaw bone[190,191].

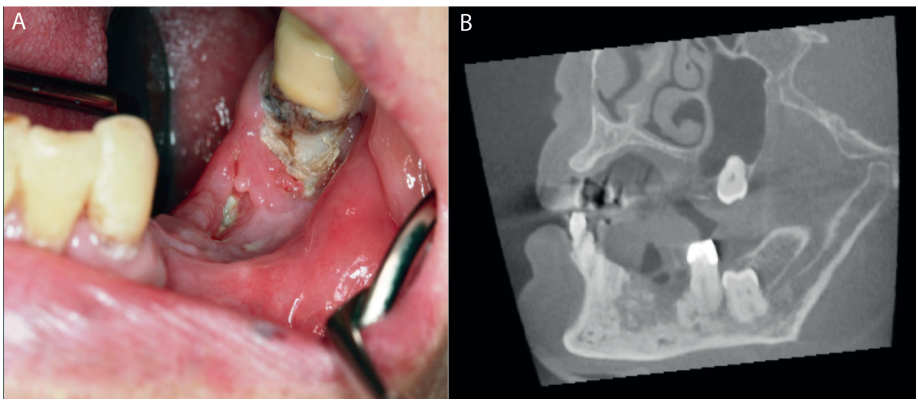

Figure 6. (A). ONJ patient with osteoporosis treated with alendronate for >3 years. A small area of exposed bone is observed in the region of the first molar and close to the second molar on the left side of the mandible. (B). A cone-beam computed tomography scan revealing that the lesion extends beyond the alveolar bone.

1.5.1 Epidemiology and risk factors

The incidence of ONJ among cancer patients treated with zoledronic acid ranges from 0.7% to 6.7%[190,191]. In a recent meta-analysis, the overall incidence of ONJ among cancer patients treated with denosumab was 1.7%[198]. In osteoporotic patients, the incidence has been reported to be between 0.004% and 0.2% when treated with oral bisphosphonates and when treated with injections of zoledronic acid or denosumab the incidence ranges from 0.017% to 0.04%[190,191]. The incidence of ONJ appears not to differ between denosumab and intravenous bisphosphonate treatment in women treated for breast cancer and bone metastases[199,200]. If prophylactic measures such as dental examination and appropriate dental treatment are undertaken before commencement of antiresorptive drug treatment, the risk of ONJ is reduced[201,202]. Discontinuation of antiresorptive drug treatment has been discussed to prevent and also to treat ONJ, however, there are no data supporting this strategy[190,191]. Among the recognised risk factors for development of ONJ are dentoalveolar surgery, concurrent treatment with glucocorticoids or antiangiogenic agents, the use of more potent bisphosphonates such as zoledronic and prolonged treatment with bisphosphonates[203,204]. The mandible is affected by ONJ more frequently than the maxilla[203]. An increased sensitivity to bisphosphonates affecting the risk of developing ONJ has been discussed after several observations of genetic polymorphisms associated with ONJ in cancer patients[205-207].

1.5.2 ONJ and pathogenesis

Although ONJ has been reported since 2003, the pathogenesis is still under debate and a vast number of theories have been presented to explain the condition. When the condition was first reported, similarities with osteopetrosis, a hereditary skeletal disorder with defective osteoclast resorption, were discussed. As the clinical characteristics include dense, avascular bone, it was suggested that a combination of reduced bone remodelling and the involution of small capillaries caused by bisphosphonate treatment, resulted in acellular, avascular necrotic bone failing to heal when exposed[208].

Dysregulated bone remodeling

In healthy conditions, alveolar bone appears to have a higher remodelling rate compared to other skeletal locations and it has been suggested that the downregulation of bone remodelling and suppression of bone resorption is an important factor in the pathophysiology of ONJ[209,210]. However, the uptake of bisphosphonates appears to be similar in the jaw as in long bones or vertebrae[211]. An accumulation of nonviable osteocytes as a result of the

suppressed bone remodelling is also believed to play a part in the development of necrotic bone areas[209,212]. The effect of bisphosphonates on osteoclasts is believed to impair the osteoblast function by disrupting the cross-talk between the two cell types, resulting in reduced osteoclast recruitment and bone matrix deposition[212]. When bone areas with apoptotic osteocytes are not remodelled, microcracks and areas of sclerotic bone are developed, compromising vascularisation. Subsequently the risk of infection is increased and the healing capacity of alveolar bone is reduced[212]. Investigations of bone samples from ONJ patients using scanning electron microscopy have shown a high number of microcracks[213]. As mechanical stress induces microcracks in bisphosphonate treated bone, the impaired bone cells are not able to adequately repair this bone damage, suggested to facilitate deep bacterial invasion after exposure of bone, for example at dental extraction sites[213].

Toxic effects on osteoblasts, osteocytes and soft tissue

An additional hypothesis explaining the development of ONJ is the accumulation of non-viable osteocytes due to toxic effects of bisphosphonates[209]. The anti-apoptotic effects of bisphosphonates on osteoblasts and osteocytes have been observed both *in vitro* and *in vivo*[118,119]. In contrast, bisphosphonate administration has been shown to inhibit osteoblast growth and function and also to increase osteoblast apoptosis *in vitro*[214,215]. ONJ attributable to the toxic effect of bisphosphonates on the oral mucosa has also been discussed in the literature[216] since inhibitory effects of bisphosphonates on oral fibroblast and keratinocytes have been reported by numerous *in vitro* studies[214,217-221]. *In vivo,* bisphosphonates have been shown to delay soft tissue closure after tooth extractions[222]. Decreased levels of transforming growth factor β1 (TGF-β1), an important factor for fibroblast differentiation and proliferation have been observed in human soft tissue samples from patients diagnosed with ONJ[223]. Furthermore, increased mucosal healing time after tooth extraction has been observed in bisphosphonate treated patients[224]. Interestingly, densoumab which also causes ONJ appears to have few if any extraskeletal effects[187].

Negative effects on angiogenesis and blood flow

Negative effects of bisphosphonates on angiogenesis have also been implicated in ONJ. Observations of reduced blood flow and decreased superficial vascular network in bone have been made after bisphosphonate injections[225,226]. Furthermore, clinical studies have shown downregulated VEGF levels in serum of cancer patients after bisphosphonate treatment[227,228]. Histological and ultrastructural examinations of bone samples from ONJ patients have shown obliterated vessels and few irregular

vessels[229,230] while other studies have not observed such findings[231,232]. Nevertheless, as ONJ is now widely considered a side effect of several antiangiogenic drugs, the hypothesis of angiogenesis and blood flow playing a key role in the development of ONJ is strengthened. However, denosumab is not reported to affect angiogenesis[233].

Bacterial infection and biofilm

Bacterial infection and biofilm formation have also been suggested to be involved in the pathophysiology of ONJ. In bone specimens from ONJ patients, biofilms have been identified containing the bacterial species Fusobacterium, Bacillus, Actinomyces, Staphylococcus, Streptococcus, Selenomonas, Treponemes and also Candida embedded in a matrix of extracellular polymeric substances[234]. Despite the effect of bisphosphonates on the recruitment and function of osteoclasts, extensive resorption has been observed in bone samples from ONJ samples[229,235,236]. Bone resorption caused by bacteria liberating acids and proteases, stimulating bone degradation and inhibiting bone matrix synthesis has been suggested to cause bone destruction and sequestration. Furthermore, the biofilm contributes to the resistance against conventional treatment, including the use of antibiotics[234]. Recently, it has been suggested that the condition should be regarded as chronic osteomyelitis, caused by mixed-species pathogenic oral biofilms[237,238].

Dysregulation of the immune response

An alternate hypothesis regarding ONJ pathogenesis involves the detrimental effects of bisphosphonates on macrophages[239]. With repeated bisphosphonate administration bone remodelling sites are reduced in number and skeletal binding of bisphosphonates may decrease, allowing monocytes and macrophages to be exposed to bisphosphonates for longer periods, thus affecting cellular activity and cell numbers in a negative way[239]. Denosumab may also affect monocyte migration and function but also cell numbers and survival owing to the blockade of the RANK-RANKL interaction. Additionally, sunitinib inhibits M-CSF thus affecting the monocyte-macrophage development and supporting the hypothesis of negative side-effects of antiresorptive and antiangiogenic drugs on macrophages having a role in the pathogenesis of ONJ[239]. A possible involvement of vitamin D deficiency in the pathophysiology of ONJ has also been proposed, since vitamin D is required for specific immune responses to be activated in macrophages[239]. Local tissue acidosis has also emerged as a hypothesis explaining the mechanism leading to ONJ, as it is known that bone-bound bisphosphonates are released at acidic pH[113,240]. When an oral infection is present or when dentoalveolar surgery is performed, the local tissue becomes

acidic and induces release of bisphosphonates, which in turn exert detrimental effects on osteoclasts, mucosal cells, angiogenesis and immune cells which may result in ONJ[240].

1.5.3 Clinical manifestations and treatment

ONJ is characterised by exposed necrotic bone with or without signs of infection in the surrounding soft tissue. The American Association of Oral and Maxillofacial Surgeons (AAOMS) has presented a staging system which is commonly used to assess the severity of the condition, prognosis and facilitates the choice of treatment and data collection[190,191] (Table 1). However, it was recently suggested that the subgroup non-exposed ONJ should be included in the classification system, since these cases could develop extraoral fistulas, paraesthesia and other symptoms indicating an extensive lesion, thus being different from stage 0[241].

Table 1. *Classification of different stages of ONJ.*

Stage	Criteria by AAOMS Medication-Related Osteonecrosis of the Jaw – 2014 Update[190]
At risk	No apparent necrotic bone in patients treated with oral or IV antiresorptive or antiangiogenic drugs
0	No clinical evidence of necrotic bone, but non-specific symptoms or clinical and radiographic findings
1	Exposed and necrotic bone, or fistulae to the bone in asymptomatic patients with no evidence of infection
2	Exposed and necrotic bone or fistulae to the bone with evidence of infection in the region. These patients often have symptoms.
3	Exposed and necrotic bone or fistulae to the bone, with evidence of infection and one or more of the following: exposed and necrotic bone beyond the alveolar bone, pathologic fracture, extra-oral fistula, oral-antral communication, oral-nasal communication or osteolysis extending to the inferior border of the mandible or sinus floor

In the lower stages of ONJ, patients may be asymptomatic, having radiographic signs of osteonecrosis alone, or non-specific symptoms such as neurosensory disturbance, loose teeth or sequestration. In the case of infection, patients often develop symptoms such as pain, fistulas and in severe cases even pathological fractures and the condition has been found to severely affect the quality of life of patients with cancer[190,191,242]. Current treatment strategies include conservative treatment such as patient education, antibacterial mouth rinses, the use of pain medications, and antibiotic therapy when needed. When conservative treatment fails, debridement or resection is performed[190,191]. In the most severe cases, resection followed by reconstruction with osseous free flaps may be considered[243,244].

2 AIM

The main aim of this thesis has been to investigate the biological events determining bone regeneration and inflammation in osteoporotic conditions at separate skeletal localisations, after administration of antiresorptive agents, thereby advancing the understanding of site-specific differences in bone response.

2.1 Specific aims of the included studies

- To investigate bone remodelling and inflammation after a single systemic dose of zoledronic acid followed by implant installation at different locations in a rat model of osteopenia.

- To study and compare the healing process in femur defects implanted with hydroxyapatite or strontium-doped calcium phosphate granules, in non-ovariectomised and ovariectomised rats.

- To explore and characterise jawbone from patients exposed to bisphosphonates on the cellular and tissue level.

- To study differences in bone structure and cellular response to estrogen deficiency at disparate bone sites in the mature ovariectomised rat model.

3 MATERIALS AND METHODS

3.1 Patients

3.1.1 Patient selection

All patients participating in the study (paper III) were referred to the Department of Oral and Maxillofacial Surgery, Sahlgrenska University Hospital, Mölndal and were included in a consecutive order after obtaining informed consent. In Group I, the inclusion criteria were a medical history of bisphosphonate treatment and exposed necrotic bone in the oral cavity persisting more than eight weeks, diagnosed as bisphosphonate related osteonecrotic jaw. In Group II, the inclusion criteria were a medical history of bisphosphonate treatment, not displaying any clinical or radiological signs of bone necrosis. Patients with intravenous bisphosphonate treatment were only included if surgery was considered necessary (pain, infection etc.). If the patients had been treated with oral bisphosphonates, the patients were included only if the treatment exceeded two years. Exclusion criteria were radiation therapy to the head and neck region. In Group II, patients with intravenous bisphosphonate treatment were excluded if surgery was elective.

Five patients were included in Group I and another 5 in Group II (Table 2). The patients in group II and 10 control patients without any previous bisphosphonate treatment were referred for surgical removal of teeth or dental implant rehabilitation. The control patients were chosen to match sex, age and the site of biopsy compared to the patients treated with bisphosphonates.

Table 2. *Patients included in the study. BP (Bisphosphonate), ONJ (osteonecrotic jaw).*

Group	Mean age	Female/Male	Maxilla/Mandible	BP
I (BP + ONJ) n=5	76.6	4/1	1/4	Aln, ZOL
II (BP) n=5	74.4	5/0	2/3	Aln, Ris
Control n=10	70.4	9/1	3/7	-

3.1.2 Bone sampling

After clinical and radiological examination the patients were planned for surgery under local anaesthesia with lidocaine hydrochloride (20 mg/ml with 12.5 µg/ml epinephrine, Xylocain Dental Adrenalin, Dentsply Limited, Addlestone, UK) or prilocaine hydrochloride (30 mg/ml with 0,54 µg/ml felypressin, Citanest Dental Octapressin, Dentsply Limited, Addlestone, UK).

Two bone samples from the alveolar process in the maxilla or mandible were collected from each patient for gene expression analysis and histology using a 4 mm diameter trephine (Figure 7). In Group I, the bone specimens were collected from the perinecrotic area. In Group II and the corresponding control group, collection of bone samples was performed in close proximity to the area of tooth removal or implant installation. Bone samples for gene expression analysis were inserted in tubes containing RNAlater® (Qiagen GmbH, Hilden, Germany) and stored at -80 °C until analysis. Bone specimens for histology were immersed in 4% paraformaldehyde and stored in 4-6 °C until further preparation. After intervention in the patients treated with bisphosphonates, an incision of the periosteal layer of the mucosa was performed to enable soft tissue coverage without tension. Wounds were sutured with Vicryl 4-0 (Ethicon®; Johnson & Johnson, St-Stevens-Woluwe, Belgium). Follow up of the patients was planned on an individual basis.

Figure 7. Sample from the molar area in the mandible of a patient treated with alendronate (without ONJ).

3.2 Biomaterials

3.2.1 Titanium alloy implants

Titanium alloy (Ti-6Al-7Nb) self-drilling, screw-shaped implants of 1.5 mm diameter and 3 mm length (MatrixMIDFACE, Synthes, GmbH, Oberdorf, Switzerland) were used in paper I. The implants were produced, sterilised and packaged by the manufacturer.

3.2.2 Strontium-doped calcium phosphate and hydroxyapatite granules

In study II, calcium phosphate granules were prepared using analytical grade calcium chloride, strontium nitrate, sodium chloride, magnesium chloride, potassium chloride, sodium phosphate dibasic and potassium phosphate monobasic. Following preparation, the granules were moulded into strontium-doped calcium phosphate and hydroxyapatite granules.

Strontium-doped calcium phosphate granules were synthesised by dissolving strontium nitrate in phosphate buffered saline containing 0.9mM and 10mM concentrations of calcium and phosphates, respectively. The solution was transferred to a glass bottle and kept at 100 °C for 24 h and thereafter centrifuged. The precipitate was separated from the solution, washed with ethanol and dried. Hydroxyapatite granules were prepared by mixing calcium nitrate solution and ammonium phosphate dibasic. After adjusting the pH to 11 from the initial 7.4, the solution was stored in a polytetrafluoroethylene (PTFE, Teflon) container and heated at 150 °C for 24 h, followed by separation of the precipitate from the solution. The precipitate was washed with ethanol and dried. To produce the granules, Teflon moulds with holes of 1.5 x 1.5 mm diameter and height were used. The strontium-doped calcium phosphate spheres and the hydroxyapatite particles were mixed with water. To adjust the viscosity of the strontium-doped calcium phosphate and the hydroxyapatite particles, calcium phosphate cement, containing β-tricalcium phosphate (β-TCP) and monocalcium phosphate, was added and the pastes were inserted into the moulds at 100% humidity at 37 °C for two days. To improve their mechanical strength, the granules were then calcined at 600 °C for one hour and autoclaved at 125 °C.

To characterise the different granules, X-ray diffraction (XRD), a technique used to reveal long-range order (crystallinity/crystal structure), chemical composition and physical properties of materials, was performed using a Siemens 5000 Diffractometer with Cu-Kα radiation. The strontium

substitution level and the amount of different ions in the granules were evaluated by inductively coupled plasma atomic emission spectroscopy (ICP-AES). The granule porosity was analysed by micro-computed tomography. A field-emisson scanning electron microscope (FESEM, LEO 1550) was used at 5 kV accelerating voltage to perform the morphological analysis. To test the degradation of the strontium-doped calcium phosphate and the hydroxyapatite particles an *in vitro* dissolution test was performed in, Dulbecco´s phosphate buffered saline (DPBS), a solution containing calcium and magnesium and an inorganic composition close to body fluid The granules were soaked in the solution and placed on a shaker for different time points (3, 7, 14 and 28 days) followed by evaluation of weight loss of the granules and ICP-AES analysis of the released strontium, calcium, phosphate and magnesium over time.

A pre-test was performed to estimate the amount and weight of granules to be used in the *in vivo* experiment. Defects were created in solid polyurethane foams (Sawbones®, Pacific Research Laboratories, Vashon, USA) using a trephine with a diameter of 2.3 mm.

3.3 Antiresorptive drugs

3.3.1 Zoledronic acid

In paper I, a group of rats was given an intravenous injection of zoledronic acid (Aclasta®, Novartis, International, Basel, Switzerland) administered into the jugular vein, in a dose of 100 µg/kg, equivalent to the human dose given once yearly when treating osteoporosis.

3.4 *In vivo* studies

3.4.1 Animal model

Female Sprague Dawley rats were used in papers I, II and IV. The rats were ovariectomised by the supplier at 12 weeks of age and received one week later together with intact control rats. All animals were allowed acclimatisation and free movement with food and water *ad libitum* for the following three weeks.

3.4.2 Surgical procedure

In the experimental studies included in this thesis, animal surgery was performed under anaesthesia using isoflurane inhalation. The implantation

procedure in papers I, II was preceded by disinfecting (5 mg/mL clorhexidine in 70% ethanol), shaving the area of surgery and injection of a local anaesthetic (Xylocaine Dental Adrenalin, Dentsply Limited, Addlestone, UK). Wounds were sutured in layers using Maxon 3-0 (Covidien, Mansfield, USA) and Vicryl 4-0 (Ethicon®; Johnson & Johnson, St-Stevens-Woluwe, Belgium) (paper I) or Vicryl 4-0 (Ethicon®; Johnson & Johnson, St-Stevens-Woluwe, Belgium) and Monocryl 4-0 (Ethicon®; Johnson & Johnson, St-Stevens-Woluwe, Belgium) (paper II). Postoperative analgesic was given subcutaneously (Temgesic 0,03 mg/kg, Reckitt & Colman, Hull, UK). In papers I, II and IV, blood was collected from the jugular vein and inserted in tubes before being centrifuged at 1000 G for 10 minutes. Serum samples were stored at -80 °C until analysis. Blood collection and retrieval of bone samples for gene expression analysis were performed before the animals were sacrificed using an overdose of barbiturate (Mebumal, ACO Läkemedel AB, Solna, Sweden). Before bone sampling, the area was disinfected (5 mg/mL clorhexidine in 70% ethanol) and shaved, followed by an injection of a local anaesthetic (Xylocain Dental Adrenalin, Dentsply Limited, Addlestone, UK). The bone samples were preserved in tubes containing RNAlater® (Qiagen GmbH, Hilden, Germany) and stored at -80 °C until analysis. Bone blocks for histology and histomorphometry were collected post-mortem and immediately immersed in formalin to be stored in 4-6 °C until further preparation.

In paper I, four weeks after OVX, serum and bone specimens were harvested from four OVX and four intact animals to serve as baseline controls. The remaining 56 OVX rats were intravenously injected with zoledronic acid (100 µg/kg) or normal saline (0.9% w/v of NaCl) (1mg/kg). After yet another four weeks, titanium alloy implants were inserted in the proximal tibia and in the posterior part of the mandible. After a skin and periosteal incision in the medial aspect of the tibia, a flap was raised and a self-drilling screw was inserted in the metaphysis under generous irrigation with 0.9% w/v of NaCl. A submandibular incision was made through the skin followed by dissection through the muscles. After incising the periosteum, the lateral aspect of the mandibular corpus and ramus was exposed. The oblique line was identified and a titanium alloy screw was inserted lateral to the last molar, followed by suturing. One side of the rat was used for gene expression analysis and the contralateral side for histology. A 5 mm diameter threphine-drill was used for sampling the peri-implant bone for gene expression analysis. Bone blocks for histology were harvested using a dental disc on a low-speed drill.

In paper II, a longitudinal incision was made along the distal aspect of the femur. After raising a skin and periosteal flap, a defect was created using a

2.3 mm diameter trephine-drill under profuse irrigation with 0.9% w/v of NaCl. The bone samples were preserved in tubes containing RNAlater® for gene expression analysis, serving as a baseline control in a group of eight rats. In each of the sixty-four rats, the defects were filled with strontium-doped calcium phosphate granules in one of the femurs and hydroxyapatite granules in the contralateral femur defect. For the gene expression analysis, 2.3 mm diameter trephine-drill was used to collect the filled defects and bone blocks for histology were harvested using a dental disc.

In paper IV, skin incisions were performed in the submandibular area of the rats, followed by dissection through the muscles and periosteal incision to access the bone. Longitudinal incisions through skin and periosteum were made in the lateral aspect of the distal femur and on the medial aspects of the proximal tibia. Bone samples were collected from the posterior mandible, above the oblique line, laterally to the last molar and from the metaphysis of tibia and femur. A 2.5 mm diameter trephine-drill was used to collect bone for gene expression analysis and a dental disc was used to harvest bone blocks for histology and micro-CT analysis. After careful dissection, the uterus of each rat was collected and weighed.

3.5 Gene expression analysis

Through gene regulation, cells can control structure and function when exposed to different environments or stimuli by inducing synthesis of gene products such as proteins. In this thesis, bone samples were analysed using reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) to measure the gene expression level of cytokines involved in inflammation, bone resorption, osteogenesis, angiogenesis and apoptosis. In paper IV, an additional analysis of estrogen receptors was performed.

Bone samples were homogenised with the TissueLyser® instrument using a 5 mm stainless steel bead (Qiagen GmbH, Hilden, Germany) and phase separated with TriZol® solution (Qiagen GmbH, Hilden, Germany) during centrifugation. Total RNA was isolated using RNA Tissue Kit SII on the QuickGene extraction robot (Fujifilm Life Science, Tokyo, Japan) or RNeasy® mini kit Qiagen GmbH, Hilden, Germany) (paper IV) and treated with an RNase free DNase Set (Qiagen GmbH, Hilden, Germany) to reduce genomic DNA contamination. The RNA quantity was evaluated by using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, USA) and the quality of the RNA was measured with an Experion™ RNA StdSens Analysis Kit (Bio-Rad Laboratories, Inc.,

Hercules, USA). Primer design was performed with Primer3 based software (papers I – III). The reverse transcription PCR reactions were performed using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, USA) (papers I – III) or the High-Capacity cDNA Reverse Transcription Kit (Life technologies, Carlsbad, United States) with random primers (paper IV). qPCR reactions were performed on the LightCycler480 Instrument (Roche Diagnostics Corporation, Indianapolis, USA) with iQTM SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, USA) (papers I – III) or with the TaqMan Fast PCR master mix with 1× assay-on-demand mixes of primers and TaqMan MGB probes (Life technologies, Carlsbad, USA) (paper IV). The quantities of the target genes were normalized using the mean of Cq values of the reference genes and the relative levels were calculated using GenEx software (MultiD Analyses AB, Göteborg, Sweden) (papers I – III) or manually (paper IV) by the delta delta Cq method ($2^{-\Delta\Delta Cq}$).

3.6 Protein analysis

3.6.1 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) is a highly sensitive *in vitro* diagnostic tool. In general, a plate surface is coated with an antigen to detect a specific protein. A specific antibody, binding to the antigen is applied and the unbound components are rinsed off the plate. An enzyme-linked secondary antibody binding to the initial antibody is then added and the plate is rinsed again to remove unbound secondary antibodies. The enzyme conjugated to the secondary antibody creates a colour change when substrate is added. The change in colour is measured by a spectrometer.

In paper I, II and IV, commercially available ELISA kits were used according to the manufacturers' instructions to evaluate the serum level of IL-1β, TNF-α, IL-6, OC, OPG and TRAP.

3.7 Histology

Bone samples removed *en bloc* and immersed in formalin were dehydrated by a series of alcohol and embedded in acrylic resin, (LR White, London Resin Company Ltd, Berkshire, UK). Undecalcified ground sections (10 - 20 μm thick) were prepared by sawing and grinding (Exakt Apparatebau GmbH & Co, Norderstedt, Germany) and stained with 1% toluidine blue. The sections were analysed in an optical microscope (Nikon Eclipse E600). The collected bone samples were evaluated for bone structure, formation of

new bone, vascularisation, surrounding soft tissue and cells. In paper II, the degradation and distribution of the two different types of granules were evaluated.

3.7.1 Histomorphometry

In paper I, the osseointegration of the titanium alloy screws was evaluated. Analysis of the relative proportion of bone within the threads was performed by measuring the percentage of the area within the threads filled with bone in relation to the total area (bone area; BA%). The bone along the surface in direct contact with the implant in relation to total implant surface was also measured (bone-to-implant contact; BIC%).

In paper II, the percentage of the area within the defects filled with newly formed bone or with granules was calculated in relation to the total area. The relative proportion of newly formed bone or granules in the peripheral and central areas of the defect were also measured by using a software grid, dividing the total defect area into central and peripheral zones. Measurements of newly formed bone and granules were made in each zone and the relative proportion of bone and granules was calculated in the respective region (central or peripheral) and in the total defect area.

3.8 Micro-computed tomography

The micro-computed tomography (micro-CT) is a high-resolution imaging technique enabling studies of morphology and microstructure of bone and other tissue in 3D. The working principle of the micro-CT technique is that the object under investigation is mounted between an x-ray source and a detector and a series of 2D projection images are acquired at different rotation steps[245]. The contrast in the images is attained through the difference of x-ray absorption in different materials. A back projection algorithm allows a reconstruction of the 3D volume based on the multiple 2D projection views.

In paper IV, resin embedded bone samples were scanned in a micro-computed tomography scanner (Skyscan 1172, Bruker-microCT, Kontich, Belgium). For density calibration, hydroxyapatite phantoms (0.25 and 0.75 mg/cm^3) were used, allowing values to be converted into mineral density. The source was set at 70 kV and 141 µA, with a rotation step size of 0.7° for tomographic rotation of 180° and frame averaging of 5. The image pixel size of 13.98 µm was chosen with an exposure time of 250 µs. Aluminium (Al) and copper (Cu) filters were used to minimise artefacts. The images were

reconstructed using Skyscan software (NRecon, Bruker-microCT, Kontich, Belgium) and calibrated with the hydroxyapatite phantoms (CTAn, Bruker-microCT, Kontich, Belgium) to adjust attenuation values. The dataset was aligned followed by selection of a region of interest (ROI). The area between the roots of the last molar of the mandible was chosen and delineated by freehand drawing. On the lateral aspect of the distal femur and the medial side of proximal tibia, the ROI (2.5 mm in diameter and 3 mm in depth) was selected at a 5 mm distance from the joint space. The image slices of cortical bone were identified and excluded. 3D morphometric parameters were calculated for the selected ROIs; bone mineral density (BMD), bone volume fraction (BV/TV), specific bone surface (BS/BV), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) using Skyscan software (CTAn, Bruker-microCT, Kontich, Belgium).

3.9 Ethical approvals

3.9.1 Human bone samples

Human bone sample collection was approved by the Regional Ethical Review Board, Gothenburg (Dnr 424-08). All patients were informed and gave their written consent to participate in the study.

3.9.2 Animal studies

Animal experiments were approved by the University of Gothenburg Local Ethics Committee for Laboratory Animals (Dnr 64-2008, paper I; Dnr 279-2011, paper II; Dnr 227/2011 paper IV)

3.10 Statistics

Non-parametric tests, Kruskal-Wallis one-way analysis of variance was used followed by Wilcoxon signed-rank test or Mann-Whitney test to evaluate the statistical significance between groups.

4 RESULTS

4.1 Paper I

In the first study of this thesis, inflammation and bone remodelling was studied in ovariectomised (OVX) rats after a single systemic dose of zoledronic acid (ZOL) followed by implant installation. The effect of the injected ZOL was evaluated against injected saline serving as control. Four weeks after ZOL administration titanium alloy-screws were inserted in the tibia and the mandible. Serum markers of bone turnover and the peri-implant bone were evaluated at four different time points.

To evaluate the experimental model, OVX rats were compared to intact rats four weeks after ovariectomy. The OVX rats showed increased levels of IL-1β and OC in serum and upregulated gene expression levels of Col1a1, ALP and OC in bone samples from the tibia. OVX resulted in an upregulated expression level of CATK in mandibular bone and upregulated expression of caspase 8 at both skeletal sites.

After implant insertion in the tibia and the mandible, histology revealed organised tissue close to the implant surface and islands of osteoid in both the ZOL and saline group at day 3. At day 14, a higher amount of mineralised bone in direct contact with the tibial implants was observed in the group having received ZOL compared to the saline group. Further, histomorphometry revealed higher bone-to-implant contact in the ZOL group. In contrast, in the mandible histological findings revealed less interfacial bone in the ZOL group compared to the saline group, which was also observed in the bone-to-implant measurements. 28 days after implant installation, histology revealed active bone resorption and new osteoid, especially in the tibia, however no histomorphometric differences were found between the ZOL or saline groups. Lower levels of serum markers for bone formation and bone resorption were observed in the rats injected with ZOL. After implant insertion serum levels of IL-1β levels were higher at day 3 in the ZOL group compared to the saline group. Gene expression analysis revealed that in bone samples from the tibia, ZOL resulted in an upregulation of Col1a1 at 3 days following implant installation, downregulated expression of OPG and TRAP at day 28 and an upregulated of CATK at the early time points, 0 and 3 days. In the mandible, ZOL resulted in lower levels of IL-6 at 3 days after implant insertion and downregulated expression of TNF-α, IL-1β, Col1a1, ALP, VEGFA, caspase 3, caspase 8 and p53 at day 28 post-implantation.

4.2 Paper II

In the second study, the healing process in femur defects implanted with hydroxyapatite (HA) and strontium-doped calcium phosphate (SCP) granules was evaluated in both OVX and intact rats at 6 and 28 days after implantation.

X-ray diffraction of confirmed the HA and SCP particles and granules to be composed of only calcium phosphate and apatite phases. While SEM showed no differences in granule size or shape between the two materials, HA granules were condensed more densely than the SCP granules. I*n vitro,* a gradual increase of the degradation rates of the two granules was observed, while the % weight loss was more pronounced in the SCP granules which also retained their shape to a lesser extent than the HA granules. The release analysis revealed a small increase in the concentration of Ca ions over time for both materials. A slight initial increase in the concentration of P ions was observed in both types of granules, followed by a decrease. Moreover, the SCP granules had a slight initial increase in the release of Sr ions that levelled out and decreased.

The gene expression analysis in the baseline bone samples revealed a downregulation of ALP, CATK, VEGFA and caspase 3 in OVX rats compared to intact animals. In the HA group, OVX resulted in downregulated bone formation markers at 6 days after implantation and an upregulated expression of IL-6 and CATK at day 28. In the SCP group there were no significant differences between OVX and intact rats. The OVX rats showed increased expression levels of TNF-α and CATK in the HA group at 6 and 28 days, respectively, after implantation. The intact rats showed an increased expression level of caspase 3 at 6 days and the calcitonin receptor (CALR) after 6 and 28 days in the HA group. Bone samples from intact rats also revealed an increased level of IL-6 in the SCP group at 28 days after implantation. In serum, OVX resulted in higher levels of IL-β at day 6 after implantation whereas lover levels of TRAP were demonstrated after 6 and 28 days in comparison with intact rats. In both intact and OVX rats, the serum level of OC decreased after insertion of the materials from day 6 to day 28. Histology showed that the HA granules had a distinct shape at day 28 after implantation while the SCP granules were dissolved and scattered in the defect area. Histomorphometry revealed a comparable bone formation between the two materials but the relative bone area was larger in the periphery of the defects filled with SCP granules compared to defects filled with HA. In contrast, HA granules promoted a larger relative bone area in the central part of the defect than SCP granules.

4.3 Paper III

In the third study, jawbone samples from patients treated with bisphosphonates with ONJ (ONJ) and without ONJ (BP) were evaluated at the cellular and tissue level and compared to a control group. Clinically, no signs of inflammation were present in the soft tissue surrounding the exposed necrotic bone in the ONJ patients at the time of bone sampling. When the bone samples were trephined from the jaw of the ONJ and BP groups, there was an overall clinical impression of a harder and denser bone compared to controls. Histology showed no signs of obliteration of the vessels in bone samples from the peri-necrotic area of the ONJ patients. In the bone samples from the BP and ONJ groups, inflammatory infiltrates in the bone marrow space, osteoclasts and multinuclear giant cells were present. In the two latter groups, few signs of bone formation, or the presence of osteoblasts could be observed, while bone surfaces had a ragged appearance in several samples (Figure 8). In the bone samples from the control group, a larger amount of bone cells and osteoid were observed and the bone marrow space contained vessels and numerous fat cells. In the bone samples from the ONJ group, gene expression of IL-β was upregulated compared to the control group. The apoptosis marker caspase 8 was downregulated in the bone samples from the BP group compared to controls. When comparing markers of bone formation, bone resorption, and angiogenesis, no significant differences between the groups were observed. The RANKL/OPG ratio did not differ significantly between the groups.

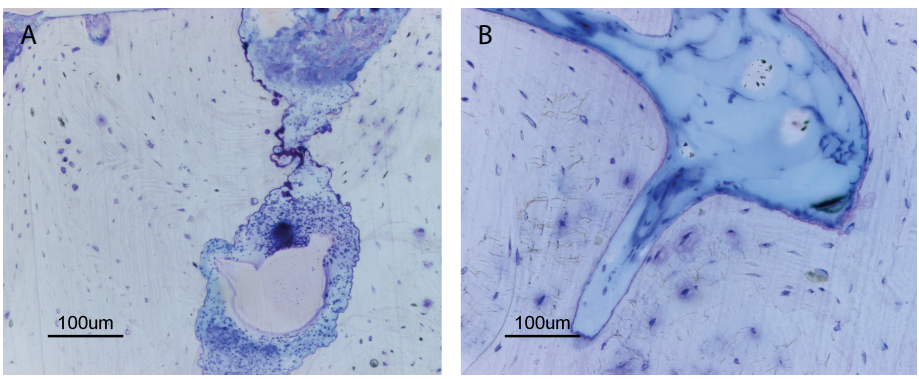

Figure 8. (A). Bone sample from the perinecrotic area in the mandible of a patient treated with alendronate. (B). Bone sample from the mandible of a patient in the control group.

4.4 Paper IV

In the final study of this thesis, bone structure and molecular response to estrogen deficiency caused by ovariectomy were investigated in intact and ovariectomised rats at different skeletal sites and at two different time points, 4 and 8 weeks.

OVX resulted in increased rat weight and a lower uterine weight both at the early and late time point. The temporal increase in body weight between 4 and 8 weeks was significant in the OVX rats. In rat serum, OVX resulted in a decrease of IL-1β, an increase of OC at 4 weeks and a decrease of TRAP at 8 weeks. Micro-CT showed a decrease of BMD and relative bone volume in the femur and the tibia at 4 and 8 weeks after OVX. OVX also resulted in an increased relative bone surface in the femur and the tibia in the investigated region of interest. In the OVX rats, an increased trabecular separation was observed after four weeks in the tibia and after eight weeks in the femur. Trabecular thickness appeared to decrease in the OVX rats after 4 weeks in the femur and after 8 weeks in the mandible, and was the only investigated structural parameter showing significant changes in the mandible. Histology showed numerous bone trabeculae surrounded by fat cells and bone marrow cells in the tibia and the femur. Additionally, osteoblast seams, osteoid and newly formed bone were observed in both the intact and OVX rats. However, bone trabeculae appeared to be fewer and thinner in the long bones of the OVX rats. The mandibular bone was characterised by cortical bone, blood vessels and few bone trabeculae in the alveolar bone. Osteoclast-like cells, areas of bone resorption and bone formation were present in both the intact and OVX bone samples. The gene expression levels of TNF-α, IL-1β, IL-6, Col1a1, OC, RANKL and LEP were higher in the femur for the OVX group when compared to intact animals. However, CATK was downregulated in the femur and the tibia at the early time point for the OVX rats. After OVX, a downregulation of the sclerostin gene SOST in femur was seen at 4 weeks and additionally there was an upregulated expression level of LEP in the tibia. OVX also resulted in lower expression of genes coding for RUNX2, OC and VEGF in the rat mandible, while the expression of SOST was upregulated. Generally, the mandible had higher gene expression levels of Col1a1, OC, RUNX2, CALR, CATK, RANKL, OPG, VEGF and SOST when compared to the femur and the tibia.

5 DISCUSSION

With an increasing elderly population, osteoporosis and the use of antiresorptive drugs are becoming more frequent. A longer life expectancy may also lead to a higher probability of requiring biomaterials to treat fractures or to reconstruct tissue. A large number of investigations have been carried out on the possibility to enhance implant fixation and osseointegration in osteoporotic bone using systemic or local administration of antiresorptive agents[132,135,246-248]. However, almost all medical drugs have adverse effects and bisphosphonates, the most commonly used antiresorptives, are known to be associated with an increased risk of ONJ and atypical femoral fractures[154]. Why these negative side effects are observed at specific skeletal locations remains unclear. Moreover, the molecular mechanisms involved in the bone healing process at these sites following treatment with antiresorptive agents are incompletely understood. An important factor that may have an influence on differences in bone response is how osteoporosis in itself affects bone tissue on a structural and molecular level at separate skeletal sites. While some experimental studies have shown that mandibular bone appears to be less sensitive to estrogen deficiency compared to long bones[93,97], micro-CT studies have shown disparate results[249,250]. Additionally, clinical studies have reported correlations in BMD between the craniofacial bones and the axial skeleton[70,71]. This thesis investigated factors involved in bone healing and osseointegration in compromised bone and explored molecular and tissue response to antiresorptive agents.

5.1 Methodological considerations

To investigate bone tissue response to estrogen deficiency and treatment with antiresorptive agents a combination of experimental models, including a clinical study of human bone biopsies, has been employed in this thesis. Further, several analytical tools and techniques were employed to determine biological events in compromised bone.

Reproductively mature rats were ovariectomised to serve as a model for postmenopausal bone loss. The ovariectomised model is well established as it replicates the initial increased bone turnover, which results in ostepenia in the peri-menopausal and post-menopausal periods in women, and is one of the most frequently used animal for studying osteoporosis[86]. In a young rat, bone loss following OVX is usually progressive and bone loss is seen primarily in cancellous bone, with substantial bone loss in proximal tibia,

while cortical bone is relatively unaffected by OVX[88]. However, rat bone lacks Haversian systems and therefore it is considered to be a poor model to study the effect of OVX on the osteonal structure in cortical bone[88,89,251]. In addition, the rat does not develop osteoporotic fractures and has a continuous growth until at least 6 months of age[88,89]. Nevertheless, the advantages of using the reproductively mature rat are availability, low cost, and that the effect of OVX is apparent within a short period of time, with bone loss characteristics similar to those in the aged rat[89].

One limitation of the *in vivo* studies (presented in this thesis) is that sham operated rats were not used. Sham operations are intended to ensure that the acquired data reflects the experiment itself and is not an effect of the ovariectomy, in order to exclude the effects of anaesthesia, the surgical trauma and pre- and post-operative care. However, the animals used were allowed to heal for four weeks after the ovariectomy until the commencement of the actual experiments, and by that time the effects of anaesthesia, surgical trauma, and pre- and post-operative care should no longer interfere with the results of the studies. Quality criteria for animal studies have been discussed in the literature and it has been suggested that sham and/or vehicle treatment matched in age, sex and strain to the experimental group may be regarded as appropriate controls[252].

In paper I, a self-drilling screw was used to evaluate osseointegration after a single dose of zoledronic acid in OVX rats. The main purpose of using a self-drilling screw was to induce slightly exaggerated surgical trauma at the time of implant installation, since traumatic injury to bone is a known risk factor in defective healing in human bisphosphonate-treated jawbone. Self-drilling screws have been shown to be associated with a greater amount of bone damage compared to self-tapping screws in an ultrastructural study where microfractures were observed at the bone-implant interface of the self-drilling screws[253].

The human bone biopsies investigated in paper III are limited in number and within the separate groups samples were obtained from both maxillary and mandibular bone. Moreover, the individuals from whom the biopsies were collected had distinctly different ways of bisphosphonate administration, different primary diagnoses, disparate co-morbidities and medication histories. Although interpretation of the results should be restricted and made with caution, the results of our study indicate a need to further explore the role of the immune system in the development of ONJ.

5.2 Bone response to bisphosphonate treatment

The effect of bisphosphonate treatment in OVX rats was investigated with serum analysis of inflammatory, bone formation and bone resorption markers. Further, gene expression analysis was used to study changes in peri-implant bone and a similar panel of markers were used in the clinical study of bisphosphonate treated patients with and without ONJ.

In paper I, a single injection zoledronic acid did not affect the levels of the bone formation marker OC or the pro-inflammatory marker IL-1β four weeks after the injecion, while the level of TRAP was downregulated and remained consistent thereafter, indicating a strong negative effect on bone resorption. Decreased serum levels of OC have previously been reported in a clinical study of bisphosphonate treated women[254]. The 100 μg/kg dose of zoledronic acid used in our study is equivalent to the 5 mg human dose given once yearly to treat osteoporosis[255] and in a previous study on the effects of OVX in rats, OC and TRAP levels were strongly reduced by the same dose[255].

The gene expression profile in rat bone changed four weeks after administration of zoledronic acid, showing an increased level of TNF-α in the tibia directly after implant installation. These results are in line with the serum analysis showing that the inflammatory response was significantly higher 3 days after implant insertion in the zoledronic group, although the effect was transient.

This is also in accordance with previously reported pro-inflammatory effects of nitrogen-containing bisphosphonates *in vitro*[126-130]. IL-6, which promotes osteoclastogenesis through RANKL induction on mesenchymal cells[29], showed a substantial increase at day three after insertion of the implant in the tibia and the mandible, in both the zoledronic acid group and in the rats given saline. However, in the mandible, the peak of IL-6 expression at day 3 after implant installation was significantly decreased in the zoledronic acid group. Bisphosphonates have been shown to increase the production of OPG and to decrease the production of RANKL and IL-6 in osteoblast and osteoblast-like cells, which subsequently inhibits osteoclastic bone resorption[121,256,257]. Zoledronic acid causing inhibition of IL-6 secretion has been observed in previous *in vitro* studies [258,259] and some clinical studies have revealed decreased serum levels of IL-6 after bisphosphonate treatment[256,260]. Mandibular rat bone also had a significant decrease of TNF-α and IL-1β eight weeks after the injection of zoledronic acid, i.e. four weeks after implant installation. It is therefore concluded that the rat mandible had a

different inflammatory gene expression profile after bisphosphonate treatment compared to the tibia. A difference in constitutive expression of pro-inflammatory genes between femur and tibia has previously been reported[261]. In the human bone samples examined in paper III, there was a significantly higher gene expression level of IL-1β in the group of patients with ONJ, compared to the control group. Neither did any of the other inflammatory markers show a significant change between the groups, nor did the angiogenic marker VEGFA.

In the tibia, there was an increased gene expression level of VEGFA three days after implant insertion in the zoledronic group. Interestingly, a continuous decrease of the expressed level of VEGFA was observed in the mandible of the zoledronic acid treated rats, resulting in a significantly lower level of the VEGFA at 28 days compared to saline treated rats. The increased gene expression level of VEGFA observed in the tibia following implant installation, is in accordance with the release of angiogenic factors occurring early in the inflammatory phase during bone healing[38] which appeared to be enhanced by zoledronic acid. At the same time as serum levels of IL-1β were found to be elevated in the zoledronic group and the gene expression levels of IL-6, Col1a1 and ALP were increased in bone samples from the tibia in both groups.

The decreased expression of VEGFA in the mandibular peri-implant bone samples is an interesting observation in this model, since there are clinical reports of downregulated serum levels of VEGF following bisphosphonate treatment[227,228], and negative effects of bisphosphonates on angiogenesis have been implicated in the development of ONJ[190]. Other studies investigating the effect of systemic zoledronic acid on protein or gene expression levels in rats[262,263] found no influence on VEGF, while studies in mice showed a reduced number of blood vessels in tooth extraction sockets compared to control animals[222]. Our findings together with previous reports indicate that VEGFA is negatively affected by zoledronic acid. However, contrasting results obtained from the serum in the clinical scenario and the gene expression from different locations in the skeleton in an OVX rat emphasises the need to clarify the effect by further studies.

In paper I, the increased inflammatory response observed in the tibia, after the bisphosphonate injection was associated with an enhanced osteogenic response at the early time points, with higher expression levels of the bone formation marker Col1a1. In the mandible, the expression levels of osteogenic and inflammatory markers in the zoledronic group were downregulated at 28 days.

These are several reported beneficial effects of bisphosphonates on osteoblast proliferation, differentiation and bone formation[119,120]. In contrast, a study performed on OVX rats has shown that bisphosphonates suppressed a cluster of genes associated with bone formation activity in the femur[264].

The contradictory results could be explained by several factors, such as the different types of bisphosphonates and dosage, the age of the rat model and the different bone sites. In a clinical study of bone biopsies from osteoporotic patients treated with alendronate, bone mineralisation was normal but osteoid thickness, volume, and surface (osteoid surface/bone surface) was markedly decreased with long-term bisphosphonate treatment[265]. In the human study of this thesis (paper III), histomorphometry showed few signs of bone formation but no significant differences in the level of bone formation genes were revealed between the groups. The results of paper I indicate that bone formation may be suppressed by bisphosphonate treatment, which may be explained by an intact coupling of the osteoclast and osteoblast. Since bisphosphonates affect both osteoclast activity as well as viability, the bidirectional communication involving paracrine signalling from osteoclasts to osteoblasts may lead to a negative effect on bone formation[26].

The expression of resorption marker CATK was upregulated at the early time points in the tibia attributable to the injected bisphosphonate, while at later time points OPG and TRAP were downregulated. The downregulation of bone resoption markers is in line with the decreased serum levels of TRAP and with the expected effects of bisphosphonate administration. Elevated serum levels of total RANKL were reported in a study involving postmenopausal women treated with alendronate[266]. However, circulating concentrations of OPG and RANKL could also reflect extra-osseous sources of the two cytokines, thus complicating the interpretation of results[267].

Interestingly, Hansen et al. (2006) have observed increased numbers of osteoclasts expressing CATK and also extracellular expression of CATK in tissue from patients with ONJ and comparing it with controls[268]. This is in parallel with our finding of an initial increase in CATK levels, indicating increased osteoclast activity in bisphosphonate treated bone, although only transiently in our rat study. In our clinical study, however, no differences in bone resorption markers were observed between bisphosphonate treated patients with or without ONJ and controls.

However, in bone samples from the ONJ patients, large osteoclast-like cells were found detached from the bone surface, while in the bisphosphonate treated samples scalloped bone surfaces were observed, indicating active

bone resorption. Raje et al. (2008) investigated the molecular profile of 11 multiple myeloma patients treated with nitrogen-containing bisphosphonates diagnosed with ONJ[269]. Gene expression analysis from peripheral blood mononuclear cells revealed that the genes involved in osteoclast and osteoblast signalling, activation or differentiation, were downregulated in ONJ patients, while no differences were observed in genes involved in angiogenesis[269]. Wehrhan et al. (2010) examined mucoperiostal samples from sites adjacent to exposed necrotic bone from 20 ONJ patients, showing suppressed gene expression levels of RANKL in the surrounding soft tissue[270].

In an *in vitro* study by Koch et al. (2012), human osteoblasts were stimulated by nitrogen-containing bisphosphonates in high concentrations, which resulted in a strong increase in RANKL expression and a moderately enhanced OPG level, resulting in a RANKL/OPG ratio >1[271]. This was interpreted as an osteoblast-mediated stimulation of osteoclastogenesis and differentiation, i.e. an anabolic effect on osteoclast via the osteoblasts[271]. In contrast, the same study also demonstrated expression of OPG exceeding the expression of RANKL when bisphosphonates were administered at lower concentrations[271]. The authors suggest an acceleration of the osteoclast metabolism and enhanced liberation of bisphosphonates due to increased resorption, which eventually results in an apoptotic cell death of the osteoclasts[271]. This could possibly explain the early increase in osteoclast activity with higher expression levels of CATK observed in paper I. In the clinical study, however, the RANKL/OPG ratios were calculated as 0.63,0.83, and 0.60 for the ONJ, the bisphosphonate treated and the control group respectively. Despite the RANKL/OPG ratios being slightly higher for the ONJ and the bisphosphonate treated patients without ONJ groups, the differences were not statistically significant.

In paper I, the only gene expression levels that were affected in the tibia four weeks after administration of zoledronic acid together with CatK and TNFα were the apoptosis markers caspase 3 and caspase 8, all of which were upregulated. In the mandibular bone, the only markers downregulated at the same time point were Col1a1 and OC. Interestingly, caspase 3, caspase 8, and another apoptosis marker p53 were downregulated in the mandibular bone samples at eight weeks after bisphosphonate administration. In paper III, downregulation of caspase 8 in the bone samples from bisphosphonate treated patients without ONJ has been discussed. Caspase 8 has an important role in the immune response against infections and in the activation of T, B and NK cells[272,273]. Osteocyte apoptosis and expression of caspase 3

reportedly increases with the severity of the inflammatory process in bone surrounding ONJ lesions[274].

In the original studies on bisphosphonates performed on macrophages, inhibition of proliferation and cell death by apoptosis was shown to depend on the type and concentration of bisphosphonate used[124,125]. Several studies have reported morphologically changed osteoclasts and lower erosion depths in bone biopsies from the iliac crest in patients treated with bisphosphonates compared to control samples[116,275]. Jobke et al. (2014) postulated that large morphologically changed osteoclasts might represent pre-stages of a prolonged apoptosis, possibly resulting in an increased number of non-resorbing osteoclasts, although maintaining the coupling activity during bone turnover, and thus preserving bone mass and architecture[275]. The findings reported in papers I and III of downregulated apoptosis markers in bisphosphonate treated bone, as well as the detached osteoclasts may be indicative of this delayed apoptosis. Interestingly, there were no indications of such a process in samples taken from rat tibia in paper I, which strengthens the earlier reports of different bone response at separate bone sites[147,149,153]. The mechanisms involved in the development of morphologically changed osteoclasts in bisphosphonate treated patients and how they affect bone remodelling properties at separate bone sites need to be explored further.

5.3 Bone healing and implants/bone substitutes

In paper I, systemically administered zoledronic acid resulted in improved bone-to-implant contact in the tibia 14 days after implant insertion while the reverse was true in the mandible. Improved osseointegration in the tibia was associated with increased expression levels of Col1a1, CatK and VEGFA in the peri-implant bone of the bisphosphonate treated rats. In contrast, the mandibular peri-implant bone samples exhibited downregulated osteogenic genes at the time of implant installation therefore it can be assumed that the conditions were comparatively poor from the very beginning of the healing process. At later time points, gene expression of inflammatory, osteogenic, angiogenic, and apoptosis markers was downregulated in the mandibular bone samples. Taken together, these findings suggest a negative effect of zoledronic acid on implant osseointegration in the rat mandible, at least during the early process of bone healing around an implant. However, it would be interesting to further evaluate the development of bone response up to 56 days or longer.

In two recent studies the effect of locally administered bisphosphonates on implant osseointegration in long bones was evaluated, showing no significant effects in the expression levels of genes coding for bone formation, bone resorption or inflammatory markers, although histomorphometry showed an improved bone-to-implant contact when bisphosphonates were used[276,277]. In contrast, a study evaluating the local application of alendronate or HA in femur defects of OVX rats showed new bone formation originating at the borders developing towards the centre and closing the bone defect when using HA, while no new bone formation was observed centrally in the defect when alendronate was used, thus having an adverse effect on bone repair[278].

In a recent review discussing the effects of systemic bisphosphonate delivery on osseointegration of implants under osteoporotic conditions, Vohra and co-workers[279] identified 12 studies showing that systemic bisphosphonate delivery increases bone volume and bone-to-implant contact significantly, two studies showing no difference in osseointegration between bisphosphonate treated animals and controls and one study showing a negative influence of systemic bisphosphonates on osseointegration[280]. The study showing delayed osseointegration of newly formed bone around a titanium implant after administration of systemic alendronate is one of three studies, together with our study (paper I) where implants were placed in craniofacial bone[280]. Although several studies exist where local or systemic application of bisphosphonates has been implicated in delayed bone healing around implants, a majority of studies have shown positive effects on osseointegration.

The survival rate of dental implants in bisphosphonate users was investigated recently and the overall implant survival is said to range between 95 - 100% in bisphosphonate users and 96.5 - 99.2% in non-users[281] and it was concluded that short-term bisphosphonate use neither increases nor decreases the survival rate of dental implants[281]. There is a need to further explore if dental implant survival is affected in patients with long-term use of bisphosphonates but also to understand the early stages bone healing during osseointegration in bisphosphonate treated bone in different bone types. The dosage, time of treatment, and the mode of drug administration may potentially be important, but also the underlying conditions of compromised bone quality such as osteoporosis, and other systemic factors which influence the bone regenerative capacity.

In paper II, HA or SCP granules were inserted in rat femur in OVX and intact rats, resulting in an overall comparable bone formation of the two materials, although differently distributed in the femur defect and with

different degradation rates of the materials *in vitro* and *in vivo*. SCP granules showed higher amounts of mineralised bone in the periphery of the defects regardless of OVX or not. HA resulted in amounts of bone in the central part of the femur defect and mainly in OVX rats. Faster degradation rate of SCP, both *in vitro* and *in vivo* compared to HA, partly explains the more pronounced bone formation in the central zone of the HA treated defects.

One possible explanation may be that rapid dissolution of SCP granules provided less stable surfaces for bone formation in the central region of the defects, thus favouring a more peripheral bone formation, along the walls of the defect. Similar distribution of new bone was detected in a study comparing β-TCP with higher degradation (showing more peripheral bone formation) and more stable granules consisting of a mixture of octacalcium phosphate (OCP) and α-tricalcium phosphate (α-TCP) displaying more central bone formation[282]. An alternative explanation may be that SCP dissolution results in different concentrations of Sr^{2+} ions, thus affecting bone formation and resorption in different ways, since *in vitro* studies of strontium showing pro-osteoblastic[283] and anti-osteoclastic[284] effects of strontium have used concentrations of Sr^{2+} ions similar to the concentration released *in vitro* in our study, paper II. Earlier studies on biomaterials and strontium have shown increased volumes of new bone[176], and increased gene expression levels of several inflammatory, osteogenic and bone resorption genes including TNF-α, RUNX, OC and RANKL[256], thus strengthening the evidence of the proposed dual mode of action of strontium.

The findings in paper II did not show enhanced bone formation in the SCP group. On the contrary, bone formation was similar to the HA group and the expression of osteogenic genes ALP and Co1a1 was similar for both materials. The anti-ostoclastic effect though, was evident from the lowered osteoclastic markers CR and CatK and also from the fewer number of osteoclast-like cells in the defects filled with SCP compared to HA. This is in agreement with a study on the effect of systemically administered strontium ranelate on OVX rats showing maintained bone formation and decreased bone resorption[285]. Moreover, the effect of strontium on human bone is under debate since a recent clinical longitudinal study with large number of paired biopsy specimens showed decreased bone formation and no decrease in bone resorption in osteoporotic women treated with strontium ranelate[166]. These findings underscore the need to further investigate the effect of strontium *in vivo* and in the clinical scenario.

5.4 Effects of ovariectomy in rats

Serum analysis was used in the experimental animal studies of this thesis to evaluate changes in serum markers of inflammation, bone formation and bone resorption. Increased levels of OC and decreased levels of TRAP were observed four weeks after OVX, in line with earlier reports[89,255]. Serum levels of IL-1β were increased after OVX in papers I and II, while there was an opposite effect in the serum analysis in paper IV. Others have reported significantly increased serum levels of IL-1β in OVX rats[286] and pro-inflammatory cytokines are known to have an important role in osteoporosis by modulating osteoclast differentiation, formation and activation[67]. The discrepancy in the results of the serum analyses is difficult to explain, as similar ELISA kits were used, at least for IL-1β and TRAP.

In paper IV, the uterine wet-weight, which is frequently used to evaluate the effect of OVX[287], showed a significant reduction in OVX rats compared to intact rats at both the studied time points. This was accompanied by an increased body weight, which is also reported as an effect of OVX [85,287]. The increased weight of the OVX rats also increases the mechanical loading of the skeleton which subsequently increases bone mass, explaining why the term osteopenia is preferred over osteoporosis for this experimental model[288].

In long bones, OVX resulted in a significant reduction of BMD and bone volume, while trabecular separation increased (paper IV). These findings confirm earlier reports on structural changes in long bones[250]. In the mandible, OVX resulted in reduced trabecular thickness while no significant effects on BMD, bone volume, or trabecular separation were observed. Microarchitectural differences have been observed following OVX in the maxillary and the mandibular bone, although not until at least three months after OVX[250,289].

OVX also resulted in higher gene expression levels of pro-inflammatory, bone resorption (RANKL) and osteogenic (OC and Col1a1) markers in femur (paper IV). This is in agreement with the findings in the tibia in paper I, where upregulated levels of osteogenic markers were observed four weeks after OVX. Moreover, some interesting differences in the gene expression between the different skeletal sites were detected. Expression of SOST was significantly upregulated in the mandible of OVX rats in comparison to intact rats. In contrast, in the femur, OVX resulted in lower expression levels of SOST, indicating reduced negative effects on bone formation by sclerostin, which is derived from the SOST gene and is secreted by the osteocyte[290]. Increased expression levels of LEP, a hormone secreted by adipocytes with

possible regulatory effects on bone mass[291], was observed in long bones following OVX. In contrast, no effect on LEP was observed in mandibular bone sites and one possible explanation could be that long bones contain more fat cells in the bone marrow at the diaphysis. Ovariectomy resulted in lower expression of genes coding for bone formation (RUNX2 and OC) and VEGF in the rat mandible. The gene expression levels of ERα and ERβ did not reveal any differences between intact and OVX rats (paper IV), contrary to studies on mice where decreased gene expression levels of ERα and increased levels of ERβ have been demonstrated in the femur following OVX[292]. It is possible that differences in estrogen receptors following OVX are not detectable within the time limits of our study.

One of the main findings in paper IV was the different response to OVX in long bones and mandible, as displayed in microarchitectural changes. The differences could be caused by anatomical structure, different mechanical loading conditions, or developmental origin, as craniofacial bones are derived from the neural crest cells while the appendicular skeleton is derived from the mesoderm[293]. Another interesting observation, detected in paper IV, was the downregulation of osteogenic and angiogenic genes in the mandible after OVX, suggesting a negative effect on the bone healing capacity in the jaw bone in osteopenic conditions.

6 SUMMARY AND CONCLUSIONS

- A systemic single dose of zoledronic acid preceding implant installation in OVX rats resulted in site-specific differences with increased pro-inflammatory, osteogenic and angiogenic gene expression in the tibia and decreased pro-inflammatory, osteogenic, angiogenic and apoptosis gene expression in the mandible.

- Zoledronic acid administration also resulted in site-specific differences in the rate of osseointegration, with increased bone-to-implant contact in the tibia and reduced bone-to implant contact in the mandible. Taken together, the morphological and molecular data suggests negative effects of the antiresorptive agent on the healing of bone in the mandible.

- Compared to the control group of patients, gene expression analysis revealed increased IL-1β levels in the peri-necrotic jawbone of ONJ patients and decreased levels of the apoptosis marker caspase 8 in the jawbone of bisphosphonate treated patients without ONJ.

- HA and SCP granules inserted in femur bone defects of Non-OVX and OVX rats resulted in an overall comparable bone formation that did not differ, regardless of OVX or not. Distinctly different distribution of newly formed bone and different inflammatory and bone remodelling responses were demonstrated by histology and gene expression.

- The rat mandible showed only a few structural effects of OVX in comparison to long bones, while decreased expression levels of genes coding for bone formation and angiogenesis were observed in the rat mandible, suggesting a different bone healing capacity in the alveolar bone of the OVX rat mandible compared to long bones.

In conclusion, the present study shows that the mandible is differently affected by experimentally induced estrogen deficiency than the long bones. Bisphosphonates, administered systemically to estrogen deficient animals, impair osseointegration in the mandible, at least partly related to a downregulation of genes important for the osteogenic process. These observations may have implications for understanding the mechanisms involved in the deranged bone healing observed in the jawbone of bisphosphonate treated patients.

7 FUTURE PERSPECTIVES

The findings of this thesis demonstrate strong skeletal site-specific differences in molecular and tissue response to estrogen deficiency and also to systemic treatment with a potent antiresorptive agent. These findings together with the characterisation of jawbone samples emphasise the need to further investigate the biological mechanisms of bone regeneration in osteoporotic bone and the effect of antiresorptive drugs in the jawbone. Therefore it would be of interest to further explore the following:

- If site-specific differences in bone structure and molecular pattern are present in jawbone versus long bones in human bisphosphonate treated bone.
- If the observed negative effect on bone-to implant contact in rat mandible after systemic bisphosphonate treatment is transient or permanent. It is therefore interesting to pursue longer evaluation periods.
- If there are differences in bone response to estrogen deficiency and systemic antiresorptive treatments between the maxilla and the mandible and how these potential differences affect osseointegration and bone healing.
- If the anti-RANKL antibody, denosumab, induces negative effects similar to the potent bisphosphonate in the same rat model, and whether these effects are reversible.
- If there are differences in the structural and molecular pattern in jawbone samples from osteoporotic patients treated with bisphosphonates compared to untreated controls.

The combination of tools used in the studies presented in this thesis has proven to be useful in enhancing the understanding of bone healing in estrogen deficient conditions in combination with antiresorptive treatment and biomaterials. The methods will be useful also for my future studies on the mechanisms involved in defect bone healing in jawbone of bisphosphonate treated patients. Larger, prospective studies in more specific patient groups are needed to gain further knowledge of the enigma that is osteonecrosis of the jaw.

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