Alendronate alters the activities of osteoblasts and fibroblasts - aggravated by high dosages and combination therapy

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List of papers

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Abbreviations

ADR	Adverse drug reaction
ALN	Alendronate
ALP	Alkaline phosphatase
BMD	Bone mineral density
BMU	Bone multicellular unit
BRONJ	Bisphosphonate related osteonecrosis of the jaw
BP	Bisphosphonate
BRU	Bone remodeling unit
BRC	Bone remodeling compartment
CBCT	Cone beam computed tomography
DXA	Dual x-ray absorptiometry
FRAX	Fracture risk assessment tool
G-CSF	Granulocyte colony-stimulating factor
GERD	Gastroesophageal reflux disease
GI	Gastrointestinal
HGF	Human gingival fibroblast
IFN-g	Interferon-gamma
IL	Interleukin
MCP-1	Monocyte chemoattractant protein-1
mRNA	Messenger ribonucleic acid
MRONJ	Medication related osteonecrosis of the jaw
MTT	Assay for measuring cell metabolic activity
NHO	Normal human osteoblast
OC	Osteocalcin
OM	Osteomyelitis
OME	Omeprazole
OPG	Osteoprotegerin
OPN	Osteopontin
PDL	Periodontal ligament
PPI	Proton pump inhibitor
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear factor kappa-B
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
TNF-a	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor

Introduction

The human skeleton is a tissue undergoing constant remodeling. In an adult, a complete remodeling takes 10 years, with approximately 3-4 million bone remodeling units (BRUs) initiated each year [1]. As stated by Langdahl et al. in 2016, the remodeling undergoes four phases: the activation phase with the recruitment of osteoclasts; the resorption phase, when the bone is resorbed; the reversal phase, with the apoptosis of osteoclasts and recruitment of osteoblasts and the formation phase where the osteoblasts lay down new organic bone matrix [2]. The well-regulated interaction between osteoclasts and osteoblasts is crucial for the modulation of a healthy bone metabolism. If this balance is tipped in favor of the osteoclasts, there will be a net loss of bone mineral density (BMD), which could result in osteoporosis [3]. One of the more frequently used drugs to counteract this, is the nitrogenous containing bisphosphonate (BP) alendronate (ALN) [4]. This per oral drug was intended to downregulate and reduce the osteoclasts and their catabolic effect on bone, seeking to counteract conditions like osteoporosis that decreases the BMD [5].

All drugs have potentially side effects. These can be minor, with no need for action to be taken, or more substantial and referred to as an adverse drug reaction (ADR). ADRs have further traditionally been divided into two groups, where type A reactions are pharmacologically predictable and dose-dependent and type B reactions that are idiosyncratic and unpredictable [6]. The terms “side effect” and “adverse reaction” are often used synonymously when concerning drugs. The latter is however becoming more common, and though there are several interpretations, the almost 50 year old definition by WHO is still viewed as generally acceptable: *“a response to a drug that is noxious and unintended and occurs at doses normally used in man for the prophylaxis, diagnosis or therapy of disease, or for modification of physiological function”* [7].

ALN, like all BPs, is a systemic drug intended for long term use, and thus needs to be monitored closely for ADRs [6]. Side effects caused by BPs can manifest in several tissues of the body, with the risk of development increasing over time. In the jaws and oral cavity relevant tissues include bone, gingiva, mucosa and periodontal ligament (PDL) [8, 9]. An ADR affiliated with long term use of ALN, is medication-related osteonecrosis of the jaw (MRONJ) [10]. Several theories exist on the pathogenesis of this destructive condition, where the jawbone becomes necrotic. Key mechanisms often suggested are reduced angiogenesis, altered bone remodeling, BP cytotoxicity and inflammation or infection [11]. As with many maladies, this puzzle consists of several pieces, and it has not been completed yet. The intention of this project was to increase

the knowledge of ALN induced cellular and molecular responses in relevant affected tissues. Osteoporosis can strike at any age, but is far more prevalent in older adults. In this demographic group polypharmacy is common [12], and thus we wanted to explore if the combination of ALN and other pertinent medication could hold parts of the answer to the development of MRONJ.

Bone

Bone is a tissue composed of both flexible and rigid components, exerting functions in the body like movement, support and protection of soft tissues, calcium and phosphate storage and production of blood cells [13]. The 206 bones in an adult body are divided into four main groups: long, short, flat and irregular [14]. Some of them are tiny, like the 3.4 mm long stapes bone behind the eardrum [15], while others, like the femur, average at 42 cm for females and 46 cm for males [16]. They are all living tissue, growing and changing like the rest of the body.

Looking closer at a cross section of a long bone of the human body, like the femur, it is macroscopically constructed of several components, as illustrated in Figure 1. The outer most layer is the periosteum, which envelopes the rigid mineralized connective tissue that lies underneath. First described by Duhamel in 1739, its function was partially compared to the cambium of trees [17]. A century later, the French surgeon Louis Ollier published two volumes entitled "*Traite Experimental et clinique de la regeneration des os*", where he stated that the integrity of the periosteum is crucial to insure successful healing of bone [18]. Today, we know that the periosteum consists of two main layers: an outer fibrous layer comprised largely of collagen with scattered fibroblasts, and an inner osteogenic layer, aptly named the cambium. The outer layer contains most of the vascularization that makes the periosteum a major supplier of blood to bone and even some of the skeletal muscle. Underneath, and in direct contact with the hard surface of the cortical bone, the cambium layer contains several cells instrumental for the osteogenesis, including mesenchymal progenitor cells, osteoblasts and chondrocytes [19]. Throughout life, the periosteum has been demonstrated to play a central role in bone growth, remodeling and repair [20].

The components of the rigid part of bone is largely type I collagen interwoven in layers with bound mineral crystals of hydroxyapatite. Past the periosteal membrane lays the smooth and hard cortical bone. Its main task is to provide the bone with resistance to compression, and though the composition of bone is site dependable, the cortical part of it constitutes about 80 % of the total bone mass in the human body [21]. The minerals play an important part in hardness

of the bone, but the main difference between the cortical bone and the next layer, the trabecular bone, is the porosity. The cortical bone has a porosity of 10 to 15 %, whereas the trabecular bone porosity is in the range of 40 to 95 % [22]. The compact cortical bone serves as a protective shell for all bones, with its bulk located along the diaphysis of long bones. In the magnified part of Figure 1, we can appreciate the longitudinal osteons of the cortical bone and their central Haversian canals. These canals grow in a concentric lamellar pattern, housing nerves and one or two capillaries, tasked to nourish the adjacent undifferentiated lining cells, resting mature osteoblasts, active osteoblasts and osteocytes that comprise the cellular part of the osteon [23]. The Volkmann canals offer additional vascularization in a transversal pattern that also run in to the medial trabecular bone.

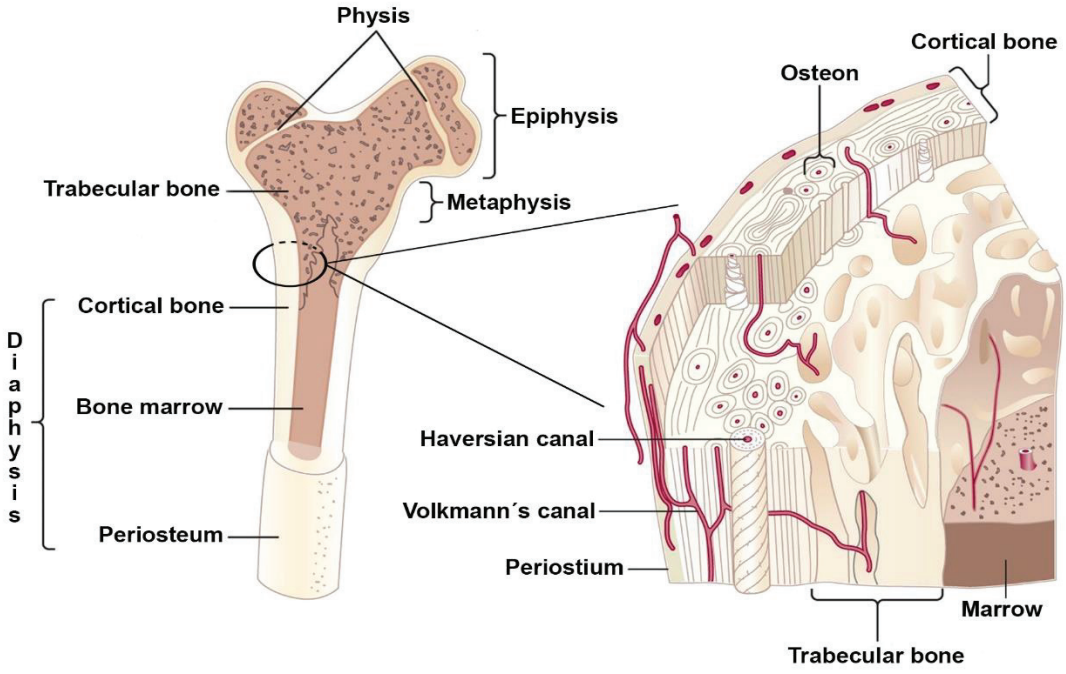

Figure 1: Illustration of bone macrostructure. Modified after Merriam-Webster, Inc. 2012.

In contrast to the dense structure of the cortical bone, trabecular bone is constructed by a mesh of rods and plates called trabeculae, from the Latin for “little beams”. These trabeculae form along the stress lines of the bone to further strengthen it without adding as much weight as cortical bone would [24]. As previously mentioned, the main components of bone are collagen and hydroxyapatite, regardless of what bone or part of the bone that is in question. However, trabecular bone has been demonstrated to have a lower calcium content and tissue density, and

a significantly higher rate of bone remodeling compared to cortical bone [24]. Regarding the cellular component of the trabeculae, it largely consists of osteocytes that are embedded in lacunae between the mineralized collagen fibrils of the laminae [25]. Most of the trabecular bone is found in the vertebrae and at the epiphysis and metaphysis of the long bones. In addition, it is also the medial part of bones such as the pelvis, skull and ribs [26]. Whereas mechanical loads are transferred to the cortical bone in the appendicular skeleton, the trabecular bone is the load bearing structure in the vertebrae. As the trabecular bone is more prone to fractures with reduced BMD conditions such as osteoporosis, this explains part of the pathogenesis of low energy vertebral fractures in osteoporotic patients [27].

The marrow makes up the fourth major component in bone. This gelatinous core of some of the larger bones of the body, like the femur and hip, and its main function is to produce blood and immune cells [28]. It is recognized as one of the largest organs of the human body, and consists of adipose tissue and hematopoietic islands surrounded by scarce trabecular bone with vascular sinuses [29]. As the marrow does little as a load bearing or rigid part of the bone, it is easy to marginalize it when addressing the different aspects of the functional skeleton. However, it houses progenitor and hematopoietic stem cells and is capable of producing up to 5 billion blood and immune cells every day [30]. The bone cells all have their origin in the bone marrow, and their lineage can be traced back to ether hematopoietic or mesenchymal stem cells.

Bone cells

Though bone is a rigid tissue, it is also highly dynamic with a varying degree of remodeling in order to heal fractures, adapt to mechanical stress and to serve as a pool for calcium and phosphate homeostasis [13]. To conduct the intricate task of bone remodeling, there are four cell types directly involved, constituting the aforementioned BRU: osteoblasts, osteoclasts, osteocytes and bone lining cells. The notion of the BRU is a development of the basic multicellular unit (BMU), first suggested by the late Harold M. Frost in 1963 [31]. The actions of the BRU are regulated through the interaction between the different cells by autocrine and paracrine signaling, including growth factors, cytokines and chemokines [2].

Osteoblasts

Derived from mesenchymal stem cells (MSCs), the *osteoblasts* were given their descriptive name in the early 20th century [32]. They are cuboidal cells found along the bone surface that

make up less than 4-6 % of the total resident bone cells [13], and are likely best known for laying down osteoid, the unmineralized, organic portion of the bone matrix which eventually matures and becomes new bone [33]. To initiate this process, osteoprogenitor cells are recruited from the MSCs through regulation of specific genes and synthesis of proteins such as bone morphogenetic proteins (BMPs), runt-related transcription factor 2 (RUNX2), osterix (OSX) and parts of the Wingless-type (Wnt) pathway [34]. Osteoblasts show signs of complete maturation by secretion of bone matrix proteins such as osteocalcin (OC), bone sialoprotein (BSP) I/II, and collagen type I [35]. Fully differentiated osteoblasts are shown in Figure 2. This process takes approximately 100 days, and the cells are now characterized by polarization, an abundant rough endoplasmic reticulum and a prominent Golgi apparatus [1, 34]. The osteoblasts are capable of secreting a multitude of factors. In addition to the direct role in the osteogenesis by osteoid production, this process is also affected indirectly by factors secreted by the osteoblasts, including macrophage colony-stimulating factor (M-CSF), osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), granulocyte colony-stimulating factor (G-CSF), osteopontin (OPN) and sclerostin [36, 37]. Moreover, the osteoblasts are one of the major sources of vascular endothelial growth factor (VEGF) in bone, a crucial angiogenic factor that stimulates proliferation, migration and survival of endothelial cells [38]. Osteoblasts are also the main contributors of interleukin-6 (IL-6) in bone [39], a pro-inflammatory cytokine important in the initial phases of bone healing as well as having several roles in a healthy immune system under normal conditions.

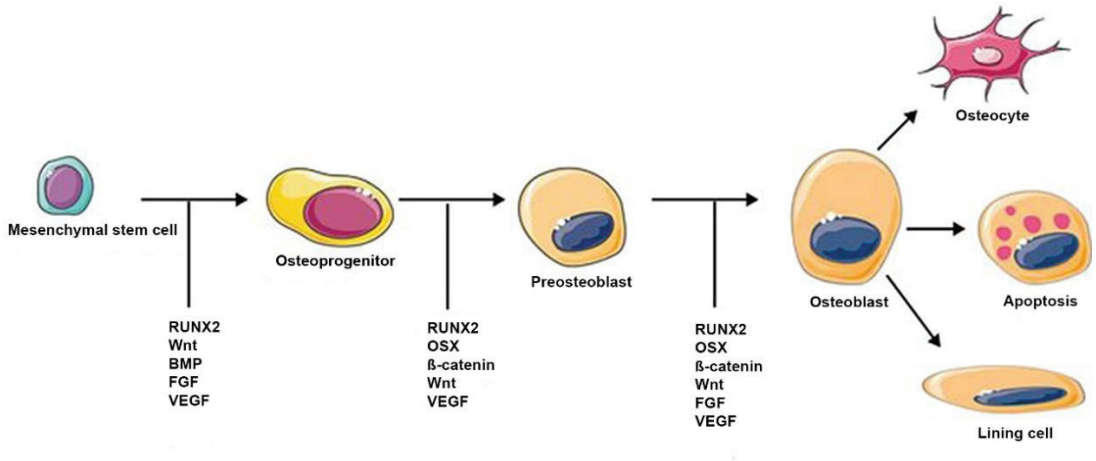

Figure 2: A simplified illustration of the differentiation from mesenchymal stem cell, via pre-osteoblast, to osteoblast. Influenced by factors: runt-related transcription factor-2 (RUNX2), wingless-type (Wnt) bone morphogenetic protein (BMP), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), osterix (OSX). Modified after Arboleya et al. 2013 [40].

Osteoclasts

Serving as the osteoblast's counterpart, the *osteoclasts'* primary function is to resorb bone. Whereas the osteoblasts descend from the MSCs, the multinuclear osteoclasts have their origin in the mononuclear cells of the hematopoietic stem cell lineage. The differentiation has a duration of about 14 days and is activated by factors like RANKL, secreted by osteoblasts, osteocytes, bone lining cells and stromal cells, and by M-CSF secreted by osteoblasts, osteocytes and osteoprogenitor mesenchymal cells [1, 41]. The receptor for M-CSF, c-FMS, is expressed by osteoclast progenitors. When M-CSF binds to c-FMS, these cells will progress to express RANK, the receptor for RANKL. In turn, RANKL secreted by osteoblasts and osteocytes binds to RANK and acts as an instigator to the differentiation of the osteoclasts [42]. This mechanism, demonstrated in Figure 3, is also partly regulated by the secretion of OPG from osteoblasts and osteogenic stromal cells. OPG is a soluble member of the tumor necrosis factor receptor (TNFR) superfamily and its binding to RANKL prevents its interaction with RANK, thus reducing the number of mature osteoclasts and protecting the bone from excessive resorption [43]. Furthermore, there are several membrane-bound mediators of cell-to-cell communication between the osteoclasts and the osteoblasts, as well as factors derived from the matrix, all influencing the up- and downregulation of the osteoclast and osteoblast activity [37]. This crosstalk between osteoclasts and osteoblasts is discussed further in the section of *Cellular interactions and bone metabolism*. Osteoclasts become polarized cells as well and develop traits that make it possible for them to resorb bone. The center of the area responsible for the resorption is the ruffled border, surrounded by the sealing zone. This action is triggered by the osteoclasts coming in direct contact with extracellular mineralized matrix [44]. The resorption lacuna is acidified by an H^+-ATPase from the ruffled border, causing a dissolution of hydroxyapatite crystals. Finally, the products of this process are absorbed through the ruffled border and the resorption is complete [45].

Osteocytes

The osteocytes are longest living bone cell, with some still being alive after 50 years [46]. Totaling 90-95 % of all bone cells, the osteocytes outnumber the other cells involved in the bone metabolism by far [47]. They originate from the MSCs lineage by way of osteoblast differentiation, an intricate process involving changes in cell morphology, proliferation, cytoplasmic volume, polarization and cell organelles, with the most recognizable new trait being the dendritic development [48]. When the cycle of laying down new bone is complete,

the osteoblasts that become osteocytes are imbedded into the bone matrix. Franz-Odendaal et al. described the osteocytes as “buried alive”, which is an apt label to put on these cells [49]. The osteocytes were previously thought to be more or less inactive after incorporation, but have lately emerged as one of the major regulatory cells of the bone, both locally and in an endocrine capacity [50]. In addition, it was suggested in a recent in vitro study that the ostecytes’ state of being buried could be reversible, as the cells dedifferentiated back to osteoblasts when switched from 3D to 2D cellular microenvironments [51].

After being encased within the mineralized bone matrix, the star-like osteocytes are reduced in size and the synthesis and secretion of proteins is decreased. Podoplanin, or E11/gp38, has been found highly expressed in embedding osteocytes though, as well as dentine matrix protein 1 (DMP1) and sclerostin [13, 50]. The body of the osteocyte is now covered in bone fluid to insure its survival and viability [52]. The bone fluid also aids in presenting the osteocyte to factors circulating in the bloodstream. A lacuna canalicular system (LCS) is established by the osteocytes by extending its processes to the surface of the bone, connecting with osteoclasts, osteoblasts, bone lining cells, bone marrow cells, other osteocytes and blood vessels [53]. Through cell-to-cell communication by interstitial fluid in the LCS, the interconnected osteocytes work as mechanoreceptors, detecting changes in pressure and strain, known as mechanotransduction [54]. Changes in the flow of canalicular fluid in the LCS, electrical potential generated by the fluid flow or deformation of the bone matrix are processes that the osteocyte senses and triggers it to produce a response with an anabolic effect on the bone [47]. Several proteins have been found to be involved in the contractile movements of the osteocyte, including CD44, actin, fimbrin and vimentin [55]. Furthermore, sclerostin produced by the osteocytes has recently been suggested as principal in the effect caused by mechanotransduction, as mechanical loading reduces the sclerostin output from the osteocytes, thus favoring bone formation [56].

The balance between RANKL and OPG is heavily influenced by the osteocytes. Even though both factors are secreted by these matrix-embedded cells, it has been suggested that the osteocytes support the osteoclastogenesis more so than the osteoblasts [57]. Other proteins expressed by the osteocytes are also involved in regulation of the osteogenesis and mineralization, such as COL-1, OC, OPN and alkaline phosphatase (ALP) [55]. As a whole, the osteocytes play a key role in conducting bone formation, repair and remodeling by regulation of the osteoblast and osteoclast activities and survival [58]. As a final act of the

osteocyte, this is further emphasized by the fact that its apoptosis serves as a chemotactic signal for the osteoclasts to start bone resorption[59].

Bone lining cells

The *bone lining cell* is another variant from the osteoblast lineage, often described as quiescent osteoblasts. They are flat and elongated in appearance and are distributed along the bone surfaces [13]. Compared to the osteoblasts, bone lining cells have a more moderately developed rough endoplasmic reticulum and Golgi apparatus. Though some of their activity is still a conundrum, bone lining cells are known to prevent the contact between osteoclasts and bone that is not to be resorbed. Everts et al. suggest that they aid the osteoclasts task of resorption by removing non-mineralized collagen from a potential site of resorption with matrix metalloproteinases. They then proceed to digest the collagen left in the Howship's lacunae prior to the osteoid being laid down by the osteoblasts [60]. In addition, the bone lining cells play a part in regulating the osteoclast differentiation by synthesizing RANKL and OPG [61]. Exposure to multiple factors, including parathyroid hormone (PTH), fibroblast growth factor 2 (FGF2), sclerostin inhibition and mechanical loading may activate quiescent lining cells into osteoblasts. In fact, it has recently been suggested by Matic et al. that the lining cells may be a major source of osteoblasts during adulthood [62]. The same study also addresses the inhibiting effect of glucocorticoids on activation of the lining cells, resulting in a decline in numbers of osteoblasts and a subsequent reduced BMD. An overview of the different cells involved in the bone remodeling is shown in Figure 3.

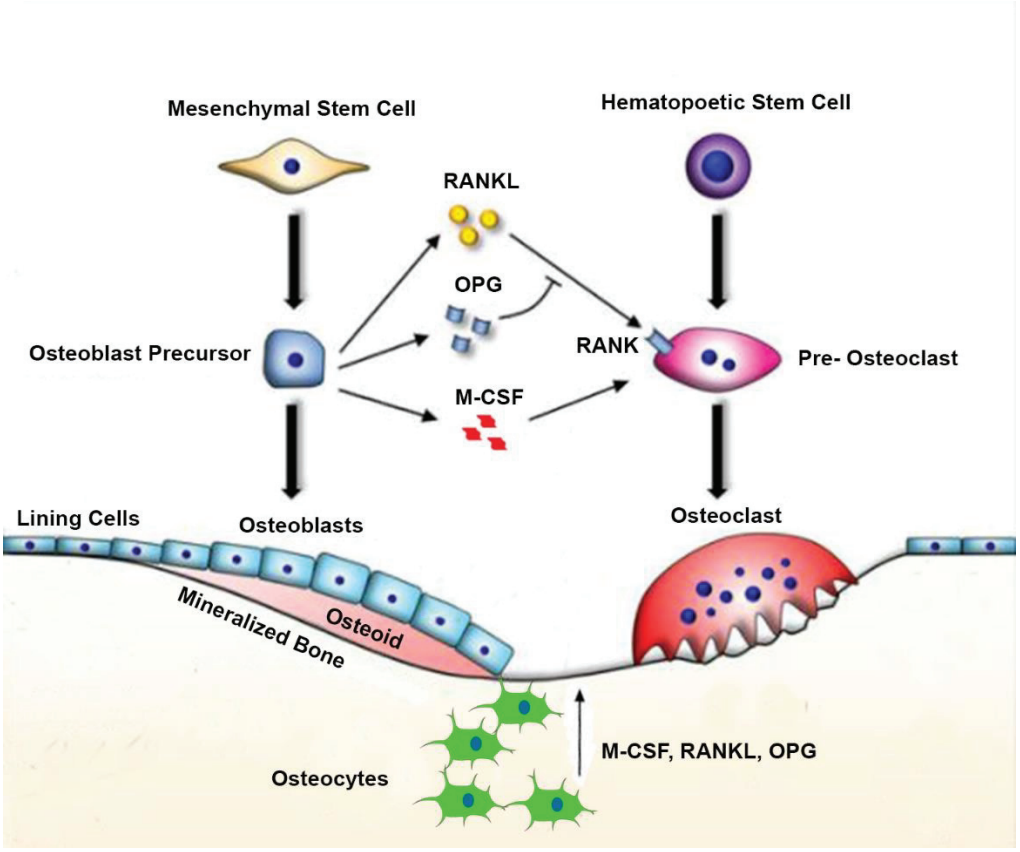

Figure 3: An illustration of the cell types involved in bone remodeling, including the regulatory action on the osteoclasts by OPG, RANKL and M-CSF. Modified after Yorgan et al. 2014 [63].

Cellular interactions and bone metabolism

The homeostasis of bone is largely a result of the bone cells´ intricate crosstalk. To enable direct communication between neighboring cells, Gap junctions connect the cytoplasm of two cells. These specialized intercellular membrane channels consist of connexin (Cx) protein monomers and are found bridging the space between a multitude of human cell-types [64]. Cx monomers assemble in groups of six to form hemichannels that are anchored in the plasma membrane. Hemichannels from two different cells then line up to establish the gap junction, allowing the passage of sub kilo dalton molecules such as vitamins, ions, amino acids and nuclotides [65]. Bone cells express several gap junction proteins, with Cx43 being the most abundant. Recently, several studies have demonstrated Cx43 as an essential component in regulation of bone metabolism [66, 67].

There are several other mechanisms in place to ensure adequate cell-to-cell communication between bone cells, both soluble and membrane bound. The impact on the osteoclastogenesis

by RANKL, OPG and M-CSF expressed by the osteoblasts and osteocytes has been mentioned previously. In addition, the osteoblasts express other soluble factors such as WNT5A and WNT16 that promote up and down regulation of the RANKL-induced osteoclastogenesis, respectively [37]. The signaling affecting osteogenesis goes both ways, exemplified with the supportive impact of the osteoclast secreted soluble factors complement component 3 (C3) and sphingosine 1 phosphate (S1P) on the osteoblastogenesis [68, 69]. Interestingly, the binding of S1P to the osteoblast also upregulates the expression of RANKL, thus promoting the osteoclastogenesis as well. Lately, as S1P has been shown to mutually promote osteolysis and osteogenesis, its receptors have been recognized as promising drug targets for treating diseases affecting the BMD [70].

Membrane bound signaling, which occurs during bone remodeling, is accomplished by the interaction between a cell-surface molecule and its respective receptor on a neighboring cell. This direct contact allows for bidirectional communication, facilitating a well-regulated cellular behavior. In the active phase of bone remodeling, there are several membrane bound interactions between the osteoclasts and the osteoblasts. One example is the ephrin signaling, a coupling that promotes bone formation regardless of direction of the activation [71]. The osteoclasts express ephrin B2 (EFNB2), which binds to its corresponding receptor EPHB4 on the surface of the osteoblasts. Activation of the EPHB4 receptor by EFNB2 supports osteoblast differentiation and survival, while the reversed signaling triggers the EFNB2 and suppresses osteoclast differentiation [72].

If the bone cells work in harmony, the result is a well-balanced bone metabolism with a resorption phase of 2-4 weeks and a formation phase of 4-6 months [73]. As elaborated on in this section, the bone homeostasis is maintained by the bone cells and their intracellular interaction and communication. However, the activity of these cells are influenced by a myriad of systemic factors such as PTH, vitamin D, estrogen, calcitonin, glucocorticoids, growth hormones, cytokines, chemokines and growth factors [74]. With age, there are changes in the secretion of said hormones and other relevant factors controlling the metabolic activities. After reaching peak bone mass in early adulthood, age related loss of bone starts as early as the third decade [75]. According to a longitudinal study by Riggs et al., middle-life women experience a substantial loss of cortical bone, while this does not happen to men until the age of 70-75. They also found that loss of trabecular bone started in early adult life and continued through life regardless of gender. Women underwent 37 % and men 42 % of their lifetime trabecular

bone loss before the age of 50. The lifetime loss of cortical bone for the same age was 6 % and 15 %, respectively [76].

Mechanically induced damage to bone has various dimensions, from almost unnoticeable microdamage to complete fractures. As with other tissues of the body, the cells in bone detect mechanical alterations and try to adjust their functions to accommodate this. This mechanism is known as mechanotransduction, as previously mentioned, but was first proposed by Frost as the *mechanostat theory* [77]. With injuries that result in fractures, the BRUs will increase in numbers and pace. The process is less evident when it is a response to normal activity and may take years to come apparent. The German surgeon Julius Wolff summarized this in what has been known as *Wolff's law*. Though it is judged a bit imprecise by todays scientific standard, it is known to be at the core of bone mechanobiology [78]. An excellent example of this is found in a study by Jones et al., where they noted that the cortical thickness of the dominant arm of professional tennis players increased by 34.9 % in men and 28.4 % in women when compared with the contralateral bone [79]. With decreased stress on the mechanoreceptors, such as after a serious accident requiring a longer period of resting, the effect is the opposite resulting in a net loss of bone [80].

Bone as an endocrine organ

Bone cells have in recent years been recognized to not only exert autocrine and paracrine effects, but also to have endocrine capabilities [81]. By definition, this capacity is achieved by distant regulation of functions through the secretion of proteins and hormones [82]. Revisiting the secretory actions of the osteoblasts, OC is a protein that is specific for the mature osteoblast. When its carboxylated form is released from bone, OC affects bone remodeling by promoting osteoclast activity, though the exact mechanisms are yet to be fully elucidated [83]. In its uncarboxylated form (unOC), it is released to the bloodstream and acts as a hormone. As such, it has been proven to have an endocrine effect on several physiological processes, including the energy metabolism of the body through regulation of glucose uptake and insulin signal transduction [84, 85]. It has also been suggested that insulin increases the levels of unOC by binding to insulin receptors on the osteoblasts, upregulating the decarboxylation of OC, thus indicating an increased bone remodeling by means of a regulating loop between OC and insulin [86]. Moreover, unOC has been associated with regulation of factors affecting the liver metabolism [87], thermogenesis through brown adipocytes [88] and development and function of the brain [89].

With the increasing focus on the endocrine functions of bone cells, FGF23 is another peptide that has gotten attention. After birth, it is secreted by osteoblasts and osteocytes, and affects the kidneys, parathyroid, bone, heart and possibly other organs [90]. Though the physiological and pathophysiological effect on bone structure and metabolism of FGF23 is not fully understood, the peptide has been recognized as an important regulator of phosphate recycling and calcitriol ($1,25(OH)_2D$) synthetization in the kidneys [91]. Mutations to the FGF23 gene or the genes coding for its receptors (FGRs) could lead to high serum levels of FGF23, potentially resulting in chronic hypophosphatemia, a major cause for diseases characterized by compromised mineralization of the bone matrix, such as rickets and osteomalacia [92].

In addition to its well-established direct impact on the osteoclastogenesis, the RANKL/OPG balance influences the energy metabolism, since OPG promotes proliferation of islet β-cells by hindering of the RANKL/RANK interaction [93]. Furthermore, OPG knockout mice show a marked increase in OC expression and insulin sensitivity, indicating another route of impact on bone resorption and glucose metabolism [94]. High levels of circulating RANKL has been associated with an increased risk of developing vascular disease, as RANKL promotes vascular calcification [95]. It has also been suggested that OPG induces angiogenesis via regulation of extracellular signal regulated kinase (ERK) and the non-receptor tyrosine kinase Src [96].

Another protein expressed by bone cells that influences regulation of insulin levels is sclerostin. As a product of the SOST gene and primarily secreted by mature osteocytes, it has recently been negatively associated with insulin sensitivity in several studies on obese patients [97, 98]. The aforementioned studies suggest that this is achieved through the inhibitory effect of sclerostin on the Wnt/β-catenin pathway. Furthermore, Urano et al. concluded some years earlier that levels of circulating sclerostin correlated to fat mass, low-density lipoprotein cholesterol and homocysteine, thus indicating that prolonged high systemic levels of sclerostin increases the risk of conditions like diabetes, dementia and cardiovascular disease [99]. Sclerostin regulation of the same signaling pathway also affects the bone mass. Since sclerostin is a Wnt antagonist, it prevents binding of Wnt ligands to its co-receptors LRP5 and LRP6. This inhibits the canonical Wnt signaling, resulting in a reduction of osteogenic differentiation and osteoblastic survival and maturation [100].

There are numerous other factors secreted by bone cells that demonstrate bone as an endocrine organ, including osteopontin, bone morphogenetic protein (BMP), lipocalin 2 (LCN2), neuropeptide Y (NPY) and leptin [86]. In addition, there are factors like cytokines affecting inflammation and angiogenesis. Some have proposed these to be hormones as well, and though

the differentiation is blurred they are generally viewed as a separate entity [101]. As discussed, many of the bone-derived factors that act as hormones have an effect on the energy metabolism. The mechanisms are however complex and the interaction with bone metabolism and its regulation is yet to be fully elucidated.

Gingiva

When addressing changes in bone metabolism, it is important to explore changes in proximate tissues. In the jaws, some of the more rapid changes are located near the tooth bearing section of the alveolar ridge [102]. Thus, the gingiva is affected by these alterations and undergoes constant remodeling and adaptation. The gingiva is a combination of epithelial and connective tissue, located around the cervical part of the teeth and is covering the coronal part of the alveolar ridge and interdental bony septa [103]. As described by Lang and Löe in 1972, the primary function of keratinized gingiva is to protect and maintain the periodontal health and as such the collagenous connection between the teeth and alveolar bone [104], illustrated in Figure 4. The necessary thickness and width of keratinized tissue has since then been a matter of controversy [105]. What is not disputed, is the highly dynamic nature of the gingiva. This is accomplished through complex mechanisms of resorption and initial healing or modulation, followed by the fibroblasts creating new extracellular matrix (ECM) and collagen structures [106].

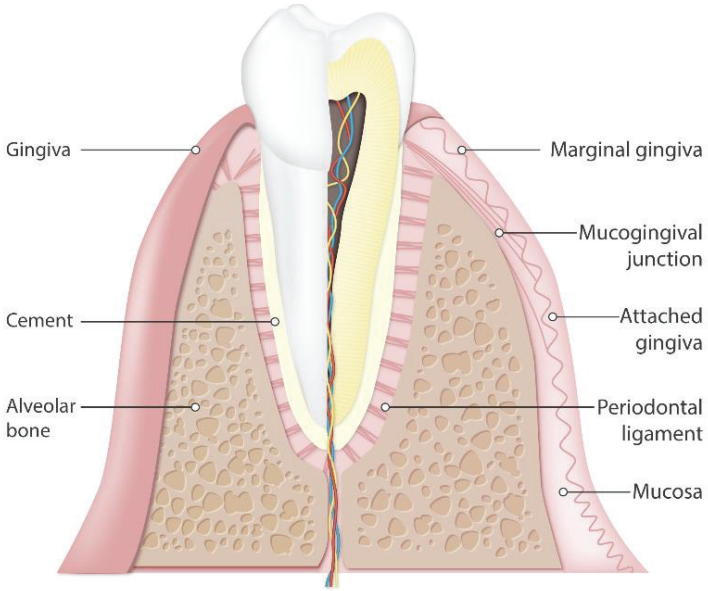

Figure 4: The components of a tooth-bearing section of the alveolar ridge.

If there is injury to soft tissue, like mucosa and gingiva, readily available *fibroblasts* are recruited and activated. Traumatic changes in the microenvironment of these tissues is recognized through an integrin-ECM binding. As a response, this mechanoperception could stimulate a differentiation of fibroblasts to myofibroblasts [107]. The myofibroblasts infiltrate and degrade tissue if needed, and subsequently lay down ECM components such as collagen I-IV, hyaluronic acid (HA), glycoproteins and proteoglycans [108]. In normal soft tissue, the fibroblasts would be protected by the cross-linked structure of the ECM. Disrupting this shielding is believed to be a part of instigating the fibroblast activation. Increased levels of chemo attractants like IL-1b and tumor necrosis factor-alpha (TNF-a) call for a fibroblast migration, while an abundant release of transforming growth factor-b (TGF-b) from the macrophages initiates the differentiation of fibroblasts to myofibroblasts [109]. At the end of the healing phase, the fibroblasts and myofibroblasts cause contraction of the wound and return to normal numbers by way of apoptosis [110]. To conclude, fibroblasts are essential for wound healing and soft tissue modulation.

Osteoporosis

Over 99 % of the calcium in a human body is located in mineralized tissues like teeth and bones. A healthy human adult body contains approximately 1 kg of calcium, which is mainly present as calcium phosphate [111]. Through absorption, secretion and excretion, the calcium is kept under tight homeostatic control regulated by osteotropic hormones like calcitonin and PTH [112]. A sufficient uptake of calcium is imperative to insure a normal growth and function of the skeleton. It is absorbed and stored at a rate of 150mg per day during skeletal growth and stays stable during the adult life until about 50 years of age when the net balance becomes negative for both sexes [113]. This change in calcium homeostasis is associated with a reduced of BMD and increased risk of fractures.

Osteoporosis is a condition where loss of bone mass and quality results in a compromised strength of the bone, consequently predisposing an individual for low-energy fractures [114]. To define what constitutes as osteoporosis, BMD, T-score and the Fracture Risk Assessment Tool (FRAX) are key terms. The BMD is based on findings with dual x-ray absorptiometry (DXA), most commonly of the femoral neck. T-score is a statistical measurement expressing the BMD as a standard deviation to the BMD of a 30-year-old healthy person. FRAX is an algorithm that predicts the 10-year incidence of hip and major osteoporotic fractures, launched by The University of Sheffield in 2008. The National Bone Health Alliance (NBHA) and the

Clinical Diagnosis of Osteoporosis Working Group [115] stated in a report from 2014 that: "postmenopausal women and men aged 50 years or older are diagnosed with osteoporosis if they have: T-score ≤ −2.5 at the spine or hip; low-trauma hip fracture with or without BMD assessment, osteopenia by BMD with a low-trauma vertebral, proximal humerus, pelvis, or in some cases distal forearm fracture, and FRAX risk estimates above the country specific threshold" [116]. In addition, a T-score between −1 and −2.5 qualifies for the diagnosis osteopenia, a condition where the BMD is below average, but still not osteoporosis. Osteopenia is associated with osteoporosis and warrants precautions and measures to be taken to halt lowering the T-score further [117].

On a cellular level, there are several processes in play that contribute to the bone loss. As the estrogen levels drop after menopause, T-cell activation is stimulated which can result in an increased secretion of cytokines that promote osteoclast activity and impede the osteoblasts [118]. Specifically, there is an increase in the production of pro-inflammatory cytokines like, IL-1, IL-6 and TNF-a. Both TNFs and IL-1 are known to activate the osteoclasts via the osteoblasts. In addition, the increased levels of TNF-a will stimulate to a higher output of RANKL and M-CSF which contributes to an even further escalating osteoclast activation and osteoclastogenesis [119]. At menopause there is an increase in the bone remodeling, doubling one year post menopause and tripling 13 years later. This surge in bone renewal causes an excess of weakened sites, resulting in structural failure that surpasses the ability to repair [120]. The microarchitecture of the bone is also affected. Where the trabeculae in healthy bone has a flattened and plate like shape, it is thinned and with at sticklike appearance if the bone is osteoporotic. This leaves the remaining scaffold of the trabecular bone less sturdy, resulting in an even further compromised mechanical strength [121]. As a consequence of the role increased remodeling plays in the development of osteoporosis, reviewing markers of bone resorption like N-terminal telopeptide (NTX) or C-telopeptides of type I collagen (CTX) has been suggested as a tool in predicting decreasing BMD. So far this has proven to be accurate, but difficult to put into clinical use on individual patients [122].

In 2010, an estimated 22 million women and 5.5 million men suffered from osteoporosis in the EU. The number of fragility fractures recorded in the EU the same year were 3.5 million in total and estimated to reach as high as 4.5 million by the year 2025 [123]. Typical fractures related to osteoporosis are ribs, pelvis, proximal humerus, distal femur, hip, distal radius and vertebral compression fractures. The latter three constituting the three largest groups [124]. Approximately 143.000 deaths after fractures were recorded in the EU in 2010, regardless of

the BMD. The costs related to osteoporosis at that time was estimated to €37 billion. Pharmacological prevention accounted for 5 % of that, the rest was related to treatment and care of fractures [125]. The Norwegian population has an undesirable pole position in this matter, with amongst the lowest BMD and the highest incidence of hip fractures in the world [126].

Osteoporosis is divided into two categories, based on the pathogenesis. If the bone loss is primarily age-related or postmenopausal, it is noted as primary. The main culprit is the estrogen deficiency in women, and for men this is combined with a reduced level of testosterone. Typically, the first phase of accelerated bone loss will only concern the trabecular bone. This is followed by a phase of even, but slower loss of cortical and trabecular bone [127]. The other category of osteoporosis is secondary osteoporosis, which is the case if the reduction in BMD is caused or exacerbated by other disorders or medication [128]. Relevant conditions are endocrine disorders, gastrointestinal disease, genetic disorders, premature menopause, multiple myeloma and liver disease, and medications that are associated with increased risk of developing secondary osteoporosis include breast and prostate cancer drugs, proton pump inhibitors (PPIs), blood pressure drugs, heparin and diuretics [128]. But the by far largest group of drugs contributing to a reduced BMD are the glucocorticoids. Though a common denominator for drug induced osteoporosis is long term use, glucocorticoids cause a rapid loss of BMD already after the first few months of use [129].

Drugs used to treat osteoporosis

A reduced BMD does not automatically warrant for actions to be taken. As a BMD higher than −1.0 SD is considered by WHO to be a normal variation, patients falling into this category only need to be monitored and optionally given activity and nutritional advice. Usually, a fracture risk assessment is not indicated [130]. If a patient has osteopenia, with a BMD between −1.0 and −2.5 SD, it is imperative to evaluate additional risk factors for osteoporosis and fractures before choosing which therapeutic step to take. A history of low energy fractures, low bodyweight, smoking and use of corticosteroid therapy should be considered as substantially disadvantageous. It has been suggested that the focus should be on patients in the lower end of the osteopenic range, instead of using −2.5 SD as an absolute threshold. This would include a greater portion of the population at risk, but also lead to a larger percentage of patients with a low risk of fracture to be evaluated and treated [131]. Vitamin D deficiency may also contribute to bone loss in postmenopausal women and should be assessed before starting therapy directed

at osteoporosis. A vitamin D insufficiency (levels under 20 ng/ml) argues for the use of a supplementary [131]. When the BMD is reduced to below -2.5 SD, the patient has per definition osteoporosis. Alongside prior fractures, the BMD is suggested to be the best predictor for the risk of future fractures [132]. Still, how to proceed and what treatment to choose, is again based on an individual evaluation. The treatment of conditions causing the BMD to decrease, is defined as antiresorptive. There are several drugs fitting into this description, but the most common are calcium, vitamin D, denosumab, hormone therapy, strontium ranelate and bisphosphonates [133]. Through different mechanisms, these medications affect the bone metabolism to either prevent or reverse further loss of bone. In this thesis, the focus will be on the bisphosphonates.

Bisphosphonates

Bisphosphonates, also known as diphosphonates, is a group of drugs to treat skeletal disorders like osteoporosis, Paget's disease, bone metastases and multiple myeloma [4]. Though the first bisphosphonates were chemically synthesized in the middle of the 1800s, it was not until 1969 that its modulating effect on bone metabolism was described in a scientific paper [134]. Bisphosphonates are synthetic analogs of naturally occurring inorganic pyrophosphates, which can be detected in blood and urine as they are a byproduct of many of the body's reactions [135]. Pyrophosphates are recognized by two phosphate-groups connected by an oxygen atom (P-O-P). Structurally, bisphosphonates differ from their chemical relative by replacing the oxygen atom with a carbon atom (P-C-P), leaving the molecule resistant to chemical and enzymatic hydrolysis [135]. The two molecule structures are illustrated in Figure 5. Today, there are several types of bisphosphonates, with varying potency, affinity for hydroxyapatite and routes of administration. The first two bisphosphonates that were produced, etidronate and clodronate, differ from today's bisphosphonates in that they are missing a hydroxyl group and an amino group. These new additions make modern bisphosphonates bind stronger to the hydroxyapatite and increases the potency up to 10.000-fold compared to the older ones. As an example of this, ALN, with its less than 1 % bioavailability, is effective in prevention of fractures and a declining BMD related to osteoporosis with a weekly per oral administration of 70 mg. Barrionuevo et al. reported that using ALN for two to three years lowered the risk of vertebral, non-vertebral and hip fractures with 43 %, 16 % and 22 %, respectively [136].

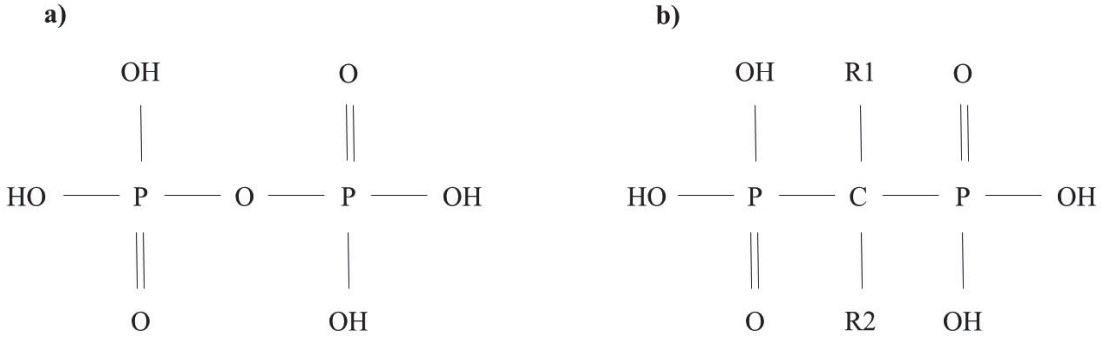

Figure 5: The molecule structures for pyrophosphate (a) and bisphosphate (b). R1 and R2 represents sidechains that could be replaced by a hydroxyl (-OH) or an amino (-NH3) group to increase the affinity for calcium [137].

Bisphosphonates are recognized as highly potent inhibitors of bone resorption through their suppression of hydroxyapatite breakdown [138]. The early bisphosphonates reduced the life span and inhibited functions of mammalian osteoclasts by incorporating into newly formed non-hydrolysable analogues of adenosine triphosphate (ATP). With the more potent amino-bisphosphonates, said suppression is achieved by way of binding to and inhibiting activity of farnesyl pyrophosphate synthase, a key regulatory enzyme in the mevalonate pathway. This causes proteins promoting cell survival and traits like the ruffled border to be inhibited, eventually culminating in apoptosis of the osteoclasts, as shown in Figure 6 [139]. The bisphosphonates are released from bone during the phase of resorption, subsequently internalizing in the osteoclasts by endocytosis [140]. Drake et al. suggests that this is a central part of the explanation for the selective effect on the osteoclasts, recalling the low bioavailability combined with the substantial affinity for bone [141]. Furthermore, in vitro studies have found that amino-bisphosphonates cause a dose-dependent change in the gene expression of RANKL and OPG [142]. The clinical effect concerning RANKL and OPG is however uncertain, with Stuss et al. observing no significant change in serum levels after 6 months of treatment with ibandronate [143].

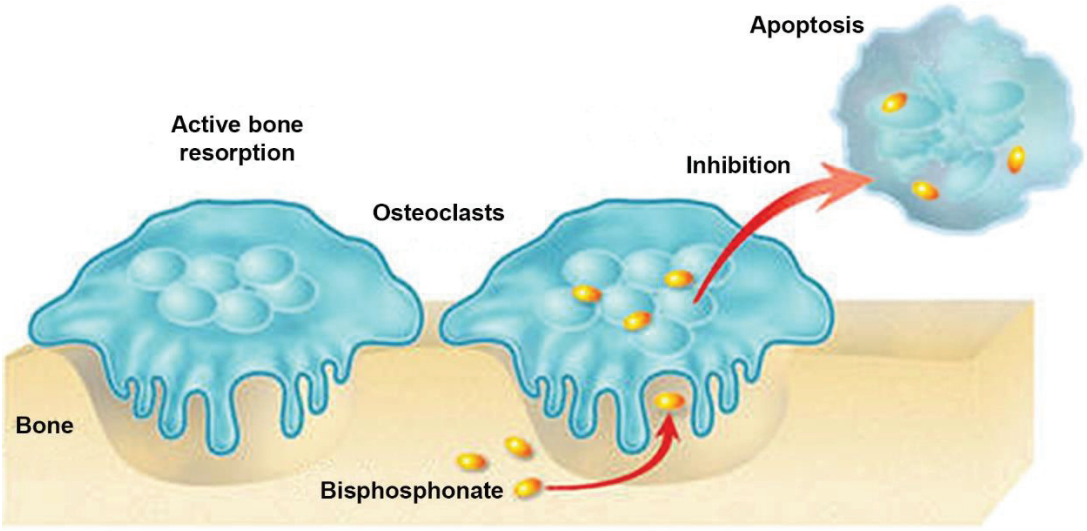

Figure 6: The osteoclast becomes apoptotic after internalization of BP. Modified after Lindsay et al. 2002 [144].

Adverse effects related to the use of bisphosphonates

One of the more common short term adverse effects of per oral BP therapy, is upper gastrointestinal (GI) discomfort [145]. This is mainly caused by damage to the GI mucosa by the BPs. As a consequence of ALN only dissolving at low pH, esophageal discomfort is more prevalent among patients with a pre-existing esophageal reflux [146]. To counteract these symptoms, a PPI is often prescribed, as the main indication for PPI usage is treatment and prevention of gastroesophageal reflux disease (GERD) and peptic ulcers. This group of drugs inhibits the H,K-ATPase, a protein located in the parietal cell and responsible for secretion of gastric acid and if overstimulated could cause said conditions [147]. Unfortunately, continuous use of PPIs, like omeprazole (OME) and esomeprazole, has been associated with a reduced BMD and an increased risk of fractures [148]. This is likely due to OME causing a malabsorption of calcium [149], though in vitro studies have also stated that PPIs have an inhibitory effect on human osteoclastic and osteoblastic cells [150]. A study on mice also found that PPIs cause reduced levels of BMPs and RANKL, causing a delay in the healing of fractures [151]. Moreover, it has been found that concurrent prolonged use of PPIs and ALN has a blunting effect on the antiresorptive properties of ALN and that such a concomitant treatment increases the risk of fractures, suggesting a drug interaction [152, 153].

With medications intended for long term use, like bone modulating drugs, it is important to monitor for ADRs. As mentioned, one of the more common side effects associated with oral

BPs, such as ALN, is upper GI symptoms. If the route of BP administration is intravenous, muscle pain, flu-like and febrile symptoms are not uncommon [154]. A less prevalent, but more severe ADR, is atypical femur fracture. This is a diagnosis with specific clinical and radiographical criteria, though broadly defined as a subtrochanteric fracture owing to a less flexible femur as a result of chronic use of BPs [155]. Lastly, and the ADR in focus in this project, is the MRONJ.

Medication related osteonecrosis of the jaw

Osteonecrosis of the jaw (ONJ) as an ADR caused by BPs was first described by Marx in 2003 [156]. The condition has since then been explored and described in thousands of studies, with no unambiguous explanation to the pathogenesis as of yet. Even the name has been the subject of several changes, as drugs other than BPs have come to be associated with the development of ONJ. After a long period of being known as bisphosphonate-related osteonecrosis of the jaw (BRONJ), it is now trending towards a broader description; MRONJ. This is a result of new non-BP antiresorptive and antiangiogenic drugs on the market being reported to induce osteonecrosis of the jaw, like denosumab [157]. The condition is anything but novel, though, BRONJ has been viewed as synonymous with "phossy jaw", an osteolytic disease prevalent amongst workers in matchmaking factories using phosporous, with cases dating back as far as 1858 [158].

As defined by the American Association of Oral and Maxillofacial Surgeons (AAOMS), MRONJ is a condition requiring the following [11]:

- Current or previous treatment with antiresorptive or antiangiogenic agents
- Exposed bone or bone that can be probed through an intraoral or extraoral fistula in the maxillofacial region that has persisted for longer than 8 weeks
- No history of radiation therapy to the jaws or obvious metastatic disease to the jaws

Typically, MRONJ presents itself as a persistent, non-healing lesion after tooth extraction or intraoral surgery [10]. The lesion may cause both local and systemic symptoms or be discovered at random at a dental checkup. Treatment options ranges from non-surgical (observation, pain and infection management) to surgical (including large resections combined with systemic antibiotics). If left unchecked, the condition can develop and cause pain and impairment of oral

functions. The AAOMS has defined recommendations for staging and treatment, as illustrated in Table 1.

Table 1: An overview of staging and treatment of MRONJ. Adapted from Ruggiero et al. 2014 [11].

Stage	Treatment strategy
At risk: previous treatment with oral or intravenous bisphosphonates, no apparent necrotic bone.	No treatment indicated.
Stage 0: no clinical evidence of necrotic bone. Symptoms, nonspecific clinical findings and radiographic changes.	Systemic management, including use of pain medication and antibiotics.
Stage 1: exposed and necrotic bone or fistulas that probes to bone in patients who are asymptomatic and have no evidence of infection.	Antibacterial mouth rinse and clinical follow-up on a quarterly basis. Patient education and review of indications for continued bisphosphonate therapy.
Stage 2: exposed and necrotic bone or fistulas that probes to bone associated with infection as evidenced by pain and erythema in the region of exposed bone with or without purulent drainage.	Antibacterial mouth rinse, pain control and symptomatic treatment with oral antibiotics. Debridement to relieve soft tissue irritation and infection control.
Stage 3: exposed and necrotic bone or a fistula that probes to bone in patients with pain, infection, and ≥ 1 of the following: exposed and necrotic bone extending beyond the region of alveolar bone resulting in pathologic fracture, extraoral fistula, oral antral or oral nasal communication, or osteolysis extending to inferior border of the mandible or sinus floor.	Antibacterial mouth rinse, pain control and symptomatic treatment with oral antibiotics. Surgical debridement or resection for longer-term palliation of infection and pain.

The incidence of MRONJ varies, with several affecting factors, including treatment indication, route and frequency of administration and type of antiresorptive agent used. Recent studies have found that the usage of ALN following today's protocol with 70 mg/week yields a MRONJ incidence of >0.01 % [159]. With infusions of the amino-bisphosphonate zoledronate as treatment of osteoporosis the correlative number is >0.09 % [160], while if used as part of treatment of bone metastasis from solid tumors it is 1.4 % [161]. Denosumab is one of the new and promising non-BP antiresorptive agents [162]. It is a human monoclonal antibody and works as an antiresorptive agent by binding to RANKL, which prevents RANKL from binding to RANK, and thus reducing osteoclast activity and osteoclastogenesis [163]. Unfortunately, denosumab has also been associated with MRONJ development, with studies reporting a cumulative incidence as high as 5.7 % in cancer treatment [161].

Pathogenesis of MRONJ

Though MRONJ caused by bisphosphonates is far from a new condition, the pathogenesis is still not settled. Studies have concluded that there is likely no single answer, but rather a sum of several mechanisms triggering the onset [164]. An important trait of the BPs is the reduced bone resorption as a result of inhibited differentiation and increased apoptosis of the osteoclasts [165]. Due to the disrupted crosstalk with the osteoblasts, bone formation and further osteoclast recruitment is also suppressed [166]. The jaw bones are exposed to a high level of mechanical activity, and if previously treated with BPs, these bones are prone to extensive microcracking [167]. This phenomenon is one of two types of microdamage to the trabeculae and can be viewed as partial fractures with a length of 30-100 μm. The other type is perforation, which is a complete fracture of the bone trabeculae as a result of osteoclast activity [168]. Both conditions result in reduced mechanical strength and a higher risk of infection caused by deeper bacterial invasion in the bone [167]. The anomalies are illustrated in Figure 7. In a study on rats, Kim et al. found microcracks to be significantly associated with the development of MRONJ [169].

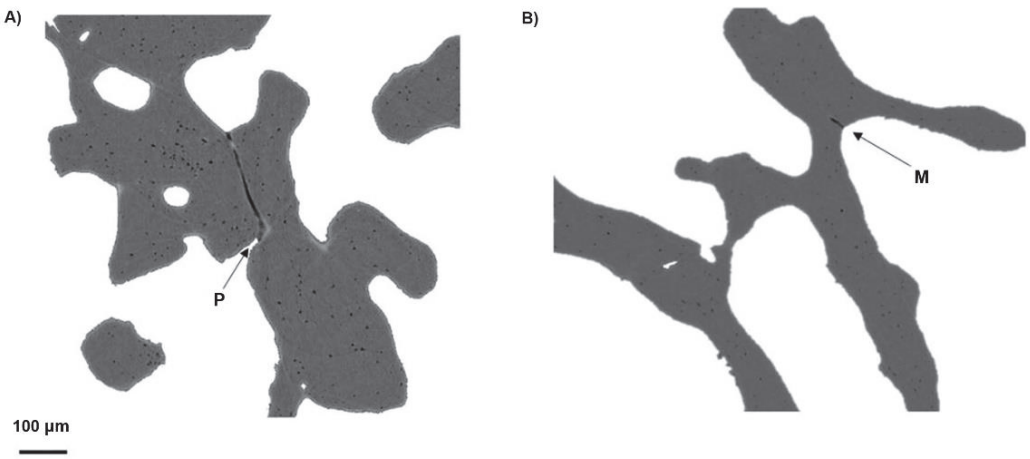

Figure 7: Microcracks or damage of trabecular bone: perforation (P) and microcrack (M). 2D micro-CT image by Ma et al. 2017 [168].

MRONJ and osteomyelitis (OM) of the jaw can be difficult to distinguish clinically and histologically, and has previously been viewed as synonymous [170]. OM is an infection or inflammation of bone, and in the jaws it is predominantly caused by an exacerbation of a persisting odontogenic infection [171]. Besides the clinical signs of inflammation and infection, like swelling and pain, the two conditions also have radiographical similarities. In a study by Gaêta-Araujo et al. the only features found with CBCT to be unique for MRONJ when compared to OM, were sclerotic areas and bone sequestra [172]. Bacterial infections and biofilms have been found in MRONJ, containing *Staphylococcus, Fusobacterium, Selenomonas, Bacillus, Streptococcus, Treponemes, Candida and Actinomyces* [173]. This could play a part in the development of MRONJ, as microorganisms provoke an inflammatory response, causing thrombosis and thus creates an anaerobic environment [174]. Moreover, in vitro studies have shown that BPs cause a rise in secretion of the pro-inflammatory cytokine IL-6 from both osteoblasts and fibroblasts [175]. The inflammation and disruption of normal bone healing is driven further by the lipopolysaccharides (LPS), as the fibroblasts respond to LPS by secreting more IL-6 and RANKL [176]. Recalling that treatment with BPs increases the amount of microcracks, Hoefert et al. hypothesizes that this would allow microorganisms to penetrate deeper into the bone [167]. The presence of biofilm also contributes to making the condition harder to treat with antibiotics [173]. De Caulaer et al. boldly states that "BRONJ is a bisphosphonate-induced *Actinomyces* osteomyelitis of the jaw" [177].

Insufficient mucosal wound closure has been associated with an increased risk of developing MRONJ after tooth extractions [178], illustrated under in Image 1. Thus, it is imperative to

minimize the chance of a delayed healing or dehiscence. Several studies have found that BPs have an inhibitory effect on both fibroblasts and keratinocytes, two cell types paramount in wound healing [179, 180]. A study on mice concluded with BPs having a negative impact on soft tissue closure after tooth extractions, with a delayed healing of the extraction sockets [181]. This is in accordance with findings by Migliorati et al., as they noted a prolonged mucosal healing post extraction in patients previously treated with BPs [182]. In vitro studies have also recorded that BPs have a negative effect on cell vitality and proliferation in human osteoblasts [175]. The literature is however contrasting, with studies suggesting that BPs have an anti-apoptotic effect on osteoblasts and osteocytes. As stated by Plotkin et al., this could to a certain degree explain the increase in trabecular thickness and the disproportionate anti-fracture efficacy when considering the relatively modest increase in BMD by BP [183].

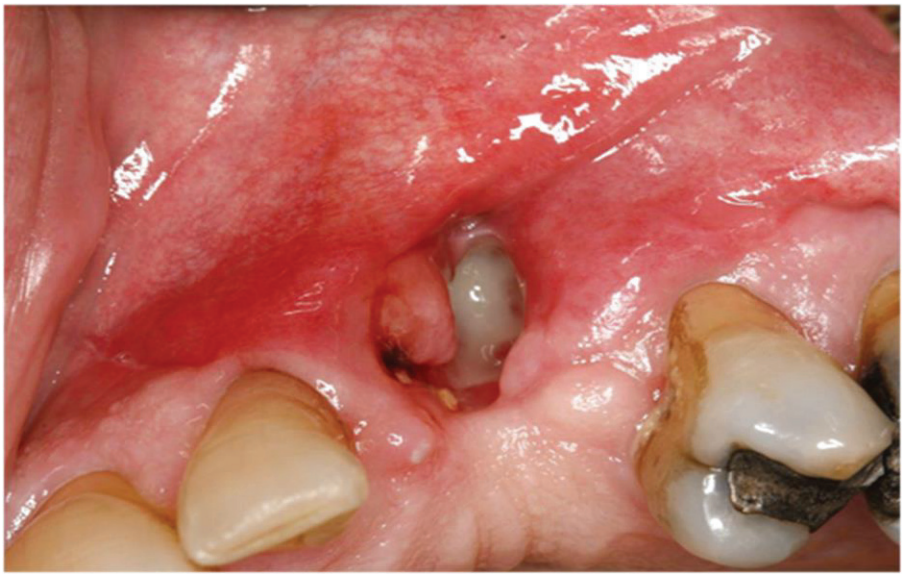

Image 1: MRONJ following insufficient wound closure after tooth extraction on elderly patient treated with zoledronate due to myelomatosis. Photo: Tormod B. Krüger.

Vascularization is essential in bone repair and remodeling. One of the leading theories in the pathophysiology of MRONJ has been the negative effect that BPs exert on angiogenesis [184]. In vitro studies have found that BPs can up-regulate cellular apoptosis in vascular endothelial cells, as well as inhibiting the cell activities of proliferation and migration [185]. Furthermore, BPs have been found to dose-dependently reduce the levels of pro-angiogenic factors secreted by osteoblasts, such as VEGF and angiopoietin [186]. These are factors which affect endothelial

cell growth, migration and vessel formation in several tissues, and play an important role in the regulation of vascular growth in the skeleton [187]. In addition, a study on mice has shown reduced blood flow and decreased superficial vascular network after BP injections [188]. Observations from clinical studies have reported reduced levels of VEGF in serum of cancer patients following BP treatment [189]. The postulation of reduced angiogenesis playing a part in MRONJ development is even further strengthened by studies showing histological findings of diminished vessels in bone samples from MRONJ patients [190].

Aims of the research

BPs are intended to increase the BMD by inhibiting the osteoclasts. Adverse effects associated with the use of BPs, like MRONJ, suggests that other cells involved in repair and metabolism of bone may be affected by this group of antiresorptive drugs. The main aim of this study was to investigate the cellular response of human osteoblasts and fibroblasts following exposure to BPs. ALN was selected as it is one of the most frequently used BPs to counter osteoporosis. A secondary aim was to evaluate the effect of a combined exposure to ALN and a commonly used PPI, OME, on said cell types.

The following research questions were addressed:

1. Does ALN affect growth and function of human osteoblasts? (Paper I, II and III)
2. Can a therapeutically relevant concentration of ALN for in vitro studies on human osteoblasts be identified? (Paper II)
3. Do combination therapies involving ALN affect the regeneration potential of human osteoblasts in vitro? Combination of ALN and OME as model system (Paper III)
4. Do combination therapies involving ALN affect the regeneration potential of human gingival fibroblasts in vitro? Combination of ALN and OME as model system (Paper III)

Methodological considerations

Study design

The initial concept of this study was to evaluate the cellular response from human osteoblasts after exposure to different concentrations of a BP. ALN was an obvious choice, as it is one of the most frequently administered BPs when treating osteoporosis [191]. We also wanted to investigate the effect of this antiresorptive drug on other cells involved in remodeling and repair of bones in the jaw, resulting in human gingival fibroblasts being added to the protocol. Lastly, as there have been studies suggesting that combining ALN with other drugs could alter the clinical effect of the BP, we wanted to explore this in an in vitro setting [192]. Recalling that PPIs have a blunting effect on the anti-fracture properties of ALN, as well as PPIs being a highly relevant drug for patients using BPs, OME was selected for the co-treatment [152]. An in vitro design of cell cultures with multiple replicas seemed most appropriate. With this approach, parameters like cell viability, proliferation and multianalyte profiling of secreted proteins could be explored using the same basic technique for acquiring the needed number of cells [193]. This presented us with certain challenges, as discussed in the following sections, but provided a cost effective and easily monitored method of producing highly repeatable and consistent results.

Cell cultures

To achieve results applicable to what is being investigated and relatable to in vivo conditions, it is essential to use relevant cell lines. In this study, we wanted to explore the cellular response of certain drugs on cells involved in remodeling and repair of bone and soft tissue. Both primary human osteoblasts (Paper I, II and III) and primary human gingival fibroblasts (Paper III) were selected and cultured separately. The experiments were not repeated, but there were three biological replicas for controls and each tested factor.

The human osteoblasts were harvested from the femur of a 10 and 22 year old male (Paper I) and from the tibia of a one day old donor (Paper II and III). It would have been preferable to use cells from a relevant site, like the mandible, as well as from multiple donors of the same age and sex for more reliable results [194, 195]. We used two separate donors for Paper I, but as a result of a call for more cost and time effective studies, the work for Paper II and III was conducted using multiple biological replicas at each time point. Alternatives to primary human osteoblasts would typically be MG-63 osteoblast-like cells (osteosarcoma) or a murine osteoblast-like cell line (MC3T3-E1) [196]. These permanent cell lines are less fragile when

culturing and have a more rapid proliferation than the primary human osteoblasts, making them easier to work with. However, even though these cells express many characteristics of human osteoblasts, their cellular response is less relatable to in vivo conditions compared to human cells [197]. Primary human fibroblasts are more robust than osteoblast when cultured and thus a need for less demanding alternatives has not been present [198]. In addition to all considerations taken when performing in vitro work like this, it is also important to take note of the culturing history of the cells, like the passage number, as this could have significant impact on the expression of the cells [193].

Viability

Lactate dehydrogenase (LDH) is an omnipresent cytoplasmic enzyme that can be found in the cells of a vast diversity of organisms. Its function is to convert lactate to pyruvate, as it converts NAD^+ to NADH. The reaction is also reversed by LDH when needed to. The conversion of pyruvate to lactate happens as a result of glycolysis under conditions deprived of oxygen, while the opposite triggers a transformation back to pyruvate through the lactate acid cycle (Cori cycle) in the liver [199]. If there is damage to or lysis of the cell membrane, LDH is released into the surroundings, which in case of in vitro work would be the cell culture medium. Thus, measuring the LDH activity in the medium in vitro will reveal cytotoxicity or increased cell death [200]. As mentioned, we used multiple biological replicas in our work, and each parameter tested was compared to unexposed cells as control. By doing so, there was no need for an exact cell count, and we got a fast, cost effective and reliable quantitative monitoring of the cytotoxicity without the need of running separate cell studies.

Proliferation

We used two separate methods to assess cell proliferation, incorporation of [^{3}H]-thymidine and monitoring the level of tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The most frequently used method for strict evaluation of proliferation, and also used in Paper I, II and III, is the incorporation of [^{3}H]-thymidine into new strands of DNA [201]. This is a reliable and inexpensive analysis, but there are certain obvious drawbacks. [^{3}H]-thymidine is a radioactive isotope, and as such its own protocols for handling and a need for special equipment. The fluids used for scintillation are also toxic, adding further demand for attention to safety. In the later years, non-radioactive labelling of DNA with bromodeoxyuridine

(BrdU) has gotten traction and could be a viable alternative to the radiolabeled, tritiated thymidine. The method does however have its challenges, with multiple steps of operation and being excessively time consuming. Recently, an analog of BrdU, 5-ethynyl-2-deoxyuridine (EdU), has proven to be a faster and highly sensitive alternative [202].

The MTT assay operates through different mechanisms, measuring the mitochondrial activity. This colorimetric assay works by reducing the yellow tetrazolium dye MTT to insoluble, purple formazan by mitochondrial succinate dehydrogenase. The notion being that the samples tested will turn increasingly purple with a rising cell count [203]. Important factors to keep in mind is that cells have different rates of metabolism and mitochondrial activity, and spontaneous reduction of MTT in lipid compartments has been documented, both factors potentially leading to false results and misinterpretations [204].

Protein quantification

To evaluate the cellular response, protein quantification is essential. As with our work, multiple biological replicas and a comparison to control at several relevant time points assures an elucidating monitoring of changes in protein secretion from the affected cells. The method of choice for us was the Luminex 200 multiplex analyzed on the XY-platform, an immunoassay providing a multi-parameter analysis with high sensitivity and specificity when measuring cytokines and chemokines [205]. These bead-based assays rely on labeling of antigens or antibodies with fluorescent markers, the results being recorded later by laserscanning with the aforementioned Luminex XY-platform from 96-well microtiter plates [205]. The technique is capable of running up to 100 analytes per well with as little sample as 25 µl. The Millipore 25-Milliplex Human Cytokine Immunoassay kit (eotaxin, G-CSF, interferon alpha-2 (IFN-a2), IFN-g, , IL-1ra, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1a (MIP-1a), MIP-1b, Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES), soluble IL-2 receptor a (sIL-2Ra), TNF-a, and VEGF) and Millipore Milliplex Human Bone Panel 1B Immunoassay kit (OPG, OC, leptin, OPN, parathyroid hormone, adiponectin and insulin) (Millipore, Billerica, MA, USA) were used in Paper I, while the Millipore 29-Milliplex Human Cytokine Immunoassay kit (epidermal growth factor (EGF), eotaxin, G-CSF, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-a2, IFN-g, IL-1ra, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1a, MIP-1b, TNF-a, TNF-b and VEGF)

and Millipore Milliplex Human Bone Panel Immunoassay kit, HBNMAG-51K-7plex (IL-6, leptin, fibroblast growth factor 23 (FGF-23), TNF-a, OPG, OC and sclerostin) (Millipore, Billerica, MA, USA) were used in Paper II and III.

There are however limitations. A study on non-human primates revealed that several commercial Luminex kits were cross-reactive for certain cytokines, like IL-6 and IL-8. In addition, there have been studies suggesting false positives using Luminex assays for antibodies [206], and discrepancies in cytokine concentrations measured with kits from different suppliers [207]. These errors could to some degree be explained by the presence of autoantibodies and heterophilic antibodies, and operators should strive to eliminate these for more reliable results [205, 208]. Another drawback of the Luminex, though not related to the performance of the method, is the need for dedicated analysis instruments, which generates a high initial cost.

An alternative to Luminex when evaluating the levels of protein secretion, is the Enzyme-Linked Immunosorbent Assay (ELISA). ELISA is based on a different method for identifying analytes, as it uses a solid-phase enzyme immunoassay for detection. Antigens from an analyte to be tested is applied to a dedicated plate, then to be dosed with matching antibodies which will bind to the antigens. As the name of this colorimetric immunoassay suggests, these antibodies are linked to enzymes that will react with a substrate and produce a detectable signal, often a change of color [209]. ELISA is by many considered to be the gold standard in quantitative analysis of hormones and cytokines in biomedical research and clinical laboratory testing, as it is regarded as highly accurate. There are numerous pros and cons between Luminex and ELISA to be discussed, but the main limitation excluding ELISA from our study was its incapability of simultaneous detection of several analytes in one sample [210].

Cell differentiation/gene expression

In addition to potentially changing the secretion of proteins from a cell, exposure to a drug might also affect the cells expression of biomarkers related to differentiation. This was evaluated in Paper I using real-time reverse transcription polymerase chain reaction (RT-PCR) to detect changes in the levels of the following biomarkers: LEP, COL1A1, OC, ALP, and CD44. COL1A1, OC and ALP were selected as they are important markers of differentiation from preosteoblasts to osteoblasts [211]. CD44 is a known indicator of osteocytic differentiation [47], and LEP has been associated with osteoblastic differentiation of human bone marrow stromal cells [212]. Prior to running the RT-PCR, the total RNA was extracted

using the RNeasy mini kit (Sigma, St. Lois, MO, USA) with extreme caution as to avoid contamination with ribonuclease (RNase). As RNase is a nuclease that catalyzes the degradation of RNA into smaller components, any trace of it could cause a shortening of cDNA products and weaken the otherwise excellent sensitivity [213]. Another important factor as part of ensuring an optimal result for this analysis, is the choice of a suitable primer, as this will have an obvious impact on the outcome [214]. As Oligo-dT primers are appropriate for amplification of several target mRNAs from a limited RNA sample, they were well suited for the design of the study in Paper I [215].

Another approach to the evaluation of gene expression would have been an assessment of mRNA, e.g. through isolation and enrichment of the mRNA by the use of magnetic beads like Dynalbeads or other techniques. This method allows for a more precise quantification, as the mRNA has been found to have a yield of 3.1 ± 1.5 % of total RNA by mass [216, 217]. Also, using techniques like an Affymetrix GeneChip probe array to evaluate the expression of a larger number of genes could have provided valuable data [218]. Identification of pathways affecting osteoblasts and bone formation, like the IFN-β/STAT1 signaling pathway, would have further strengthened the conclusions [219].

In accordance with the Minimum Information for Publication of Quantitive Real-Time PCR Experiments (MIQE) guidelines, two housekeeping genes (β-actin and GAPDH) were used as normalized references in Paper I [220]. In contrast to the genes evaluated in this paper, the housekeeping genes are constitutive genes and thus expressed continuously within different cell types as they are maintaining basic cell processes or structure [221]. These genes should not alter their expression as consequence of a pathological development, and as such their mRNA levels will not change as a response to experimental treatment in an in vitro setting. β-actin and GAPDH have previously been evaluated as suitable housekeeping genes for RT-PCR of gene expression in bone-related cells and are commonly used as such [222]. Though others have found that both β-actin and GAPDH have been regulated under similar conditions [223, 224], the expression did not change significantly in our study.

For Paper II and III we chose to focus on evaluation of secreted proteins from the cells and did not include RT-PCR and assessment of gene expression as part of the analysis. This is reasoned with us wanting to address the changes in secretion of the product of gene expression, rather than the genes themselves. Furthermore, eliminating exploration of gene expression from the protocols of the last two papers made them more cost- and time-effective.

Statistics

Throughout the three studies of this project, a central part of the statistics was to review the difference in cellular response of cells exposed to a medication to that of untreated cells. The study design for paper I differed from the other two other studies, having two donors compared to only one in the latter two. As quality control, we ran two biological replicas of each donor for the first paper, while we increased it to three for paper II and III. Neither of the three studies included technical replicates or repeated experiments. Albeit less time- and cost-effective, and not a necessity for these designs, both elements would have strengthened the scientific value of the studies [225]. The statistical significance was evaluated by applying Student's t-testing, a parametric method where the means of two independent groups are compared. P-value was set at 0.05. This was a well suited statistical approach to assess our findings, as the data groups in question passed tests for normality and equality [226]. If these requirements had not been met, the Wilcoxon-Mann-Whitney test could have been a valid option [227]. Software used for running the t-tests, as well as for calculating the standard deviations and creating the figures, was Sigmaplot (Systat Software, San Jose, CA, USA) in versions 11.0 and 13.0 for paper I and version 13.0 and 14.0 for paper II and III.

When running RT-PCR assays, the cycle threshold value (Ct-value), is the number of cycles needed for the fluorescent signal to reach a set limit. This threshold represents the amount of a target nucleic acid in a sample [228]. For paper I, the significance in differences between registered Ct-values from running the RT-PCR was analyzed using the GenEx standard package (http://www.biomcc.com).

Ethical considerations

In modern medicine, animal and ultimately human trials, are often based on laboratory and in vitro research. The usage of commercially available human osteoblasts in our studies tentatively resulted in data that are more relatable to what happens in the human body, than what i.e. murine cell lines would. However, with bone being a complex tissue that interacts with the rest of the body, our findings call for careful interpretation when relating it to in vivo conditions. As elaborated on in a recent paper by Baker et al., considerations regarding the ethics in laboratory research are numerous and intricate [229]. To ensure that in vitro research is sound and that the results are trustworthy for further research to be based upon, there are several aspects that needs to be addressed: good design and protocols, responsible conduction of the work, keeping good

records, data integrity, data transparency and finally a realistic representation of the results [230]. These factors provided a solid ethical frame for the three studies included in this project. But many lessons were still learned in the process, as cell cultures died, some results in the early phases did not add up and protocols needed revision.

Summary of results

High dosage of ALN may have undesirable local effects on bone (Paper I)

Neither 5, 20 nor 100 μM ALN caused a significant change in the viability of primary human osteoblasts. The high concentration of ALN abolished the proliferation of the osteoblasts, whereas the exposure to the two lower concentrations resulted in a 20 % increase. Moreover, 14 days of incubation with 100 μM ALN promoted a significant increase in the levels of the pro-inflammatory factors IL-8 and RANTES, as well as a decline in expression of osteoblast differentiation, ALP and CD44. In conclusion, incubation with 100 μM ALN produced a response from the osteoblasts that indicated an increased risk of inflammation combined with reduced proliferation and differentiation. Translated to an in vivo setting, this might facilitate development of local pathological conditions in bone.

5 μM ALN could be a relevant concentration for in vitro studies on osteoblasts (Paper II)

Compared to ALN at concentrations of 20 and 100 μM, exposure to 5 μM ALN resulted in a significantly less negative impact on primary human osteoblasts. 100 μM ALN was excluded early in the study as it diminished the proliferation of the osteoblasts. Dosing the cells with 20 μM ALN resulted in a decreased metabolic activity and a decline in secretion of angiogenic growth factors. Incubation with 5 μM ALN caused the least change in cellular response from the osteoblasts compared to untreated cells regarding proliferation and secretion of factors of growth, angiogenesis and inflammation. Taken together with current available literature, an ALN concentration of around 5 μM seems to be a relevant dosage for in vitro work on osteoblasts.

The combination of ALN and OME seems to amplify the negative effects of each drug separately on human osteoblasts and human gingival fibroblasts (Paper III)

ALN, OME, or the combination of the two drugs had no effect on the viability of the two cell types. The concomitant exposure caused a significant time dependent decline in proliferation of both osteoblasts and fibroblasts. OME alone and ALN + OME caused an initial decrease in the secretion of the pro-angiogenic factors VEGF, MCP-1 and IL-6 from the osteoblasts. In summary, the combination of ALN and OME appear to exaggerate any negative effects of each separate drug on the two cell lines, resulting in a reduced proliferation and angiogenesis, and a

modulation of pro-inflammatory cytokines. The findings suggest that a concomitant therapy with ALN and OME could induce conditions in periodontal tissue and approximate bone favoring development of osteonecrosis.

Discussion

As a result of an increasing elderly population, osteoporosis and other age-related conditions are becoming more frequent [231]. Consequently, there has been a rise in the use of relevant drugs, like the antiresorptive BPs [232]. Most medical drugs, especially after prolonged use, involve a risk of developing adverse effects. For BPs, the association with both MRONJ and atypical femur fractures have been established for years [155, 156]. However, the pathogenesis of these complications has yet to be fully elucidated. In addition to an apparent link between age and risk of adverse effects, the specific skeletal sites in question suggest that the local bone metabolism plays an important role [233]. Summarily, there is a necessity for a more complete understanding of the molecular mechanisms involved in the metabolism and repair of bone affected by antiresorptive treatment. This thesis explored the cellular response from human osteoblasts and human gingival fibroblasts after exposure to ALN alone, and in combination with the commonly used PPI, OME. The findings were evaluated to see if they could explain parts of the enigma concerning disrupted healing and suboptimal metabolism of bone associated with the use of BPs.

The effect of ALN on osteoblasts (Paper I, II and III)

None of the tested concentrations of ALN proved cytotoxic

Drugs are designed to achieve an intended effect by evoking a cellular response or to counter a cellular output through interaction with cell surface receptors or intracellular enzymes. As such, most drugs target specific cell types or enzymes to limit the risk of ADRs [234]. ALN is intended to have an inhibiting effect on the osteoclasts, consequently improving a reduced BMD [235]. It has however been found that it also affects the osteoblasts in several aspects. Addressing the potential cytotoxicity, some have reported a negative effect on the osteoblast's viability at ALN concentrations higher than 5 μM [236]. This is in contrast with our findings by evaluation of LDH activity in Paper I, II and III, where we noted no significant change in levels after exposure to ALN. Though other studies have concluded on ALN being cytotoxic at similar concentrations [237, 238], this might be a result of applying different methods of evaluation. Assessing the integrity of the cellular membranes, as is the case when measuring the activity of LDH, has been found to be less sensitive in evaluating viability than running MTT-assays that analyze the cellular metabolic activity [239]. Nevertheless, we did not identify

an increase in damage to the cells leading to cell necrosis at any time point after exposure to the ALN concentrations used in our studies.

Higher concentrations of ALN reduced proliferation

Another important marker of a drugs impact on specific cell types, is proliferation. As debated earlier, there are several commercially available methods addressing this. Many of which are capable of evaluating both changes in the proliferation or if the drug in question has a direct cytotoxic effect. This is accomplished with methods based on factors such as cell membrane permeability, cell adherence, enzyme activity, ATP production, co-enzyme production and nucleotide uptake activity [240]. In accordance with the findings of Manzano-Moreno et al. [241], we found that 100 µM ALN affected the proliferation of the osteoblasts adversely (Paper I and II). The resulting abolished proliferation translated to in vivo conditions would mean a complete halt in osteogenesis, thus, such a dosage is unlikely to be physiologically relevant. The less pronounced effect of the lower concentrations further suggests that these are more compatible with prolonged survival of the osteoblasts. As with our findings, the present literature reports diverging results regarding the impact of ALN on the proliferation of osteoblasts and osteoblast-like cells [241, 242]. However, for the lower dosages, these variations are marginal [238, 243]. Making a careful conclusion based on our findings and previous studies, it seems that a low dosage of ALN has a positive effect on proliferation of human osteoblasts if administered for a limited time period.

5 µM ALN had the least negative impact on factors affecting angiogenesis

Osteoblasts are a major source of VEGF in bone, one of the most important factors in vascular growth [244]. VEGF, usually synonymous with VEGF-A, is the most copious of the homodimeric proteins that constitute the VEGF family [245]. VEGF secreted from the osteoblasts have been found to mainly affect cells in close proximity. This paracrine effect involves several different cell types, including vascular endothelial cells, pericytes and osteoclasts [246]. Through binding to the VEGFR2-receptor of the endothelial cells, VEGF stimulate the migration, proliferation and vascular permeability [247]. In the early phases of bone repair, Hu et al. found a significant increase in the level of VEGF around sites of fractures [187], indicating a physiological demand for increased angiogenesis. It has also been reported that VEGF induces neutrophil chemotaxis, subsequently followed by a recruitment of

macrophages and other inflammatory cells to the site of injury [248]. Thus, as macrophages secrete angiogenic factors, VEGF stimulate angiogenesis both directly through endothelial cells and indirectly via macrophages.

In addition to be involved in regulation of inflammation through binding to its chemokine receptor (CCR2) [249], MCP-1 also play a part in the regeneration of the endothelial layer when these activated receptors are expressed in vascular endothelial cells [250]. More recently, angiogenesis induced by MCP-1 has been found to be mediated by VEGF-A [251]. An insufficient vascularization during bone repair could result in failure to heal, non-union of a fracture, or development of inferior bone structure [252]. Previous studies have found that exposure of osteoblastic cells to concentrations of ALN in the range of 0.001-10 μM, has resulted in a dose dependent reversely proportional increase in secretion of VEGF [253]. This somewhat correlates to our findings, with a level of VEGF near that of untreated cells after exposure to 5 μM ALN (Paper II and III) and a decreased secretion after incubation with 20 μM (Paper II). Furthermore, we found that incubation with 20 μM ALN for 14 days induced a significant reduction in angiogenic chemokine MCP-1 compared to control. Interestingly, neither of the concentrations of ALN caused significant changes to the levels of VEGF or MCP-1 at any time point in Paper I. As the methods of evaluation were near identical in Paper II, this could be a question of donor variation [254].

ALN at higher concentrations modulated secretion of inflammatory cytokines

Inflammation plays an important part in the healing and remodeling of bone. As a normal response to bone injury, there is an increase in the levels of pro-inflammatory cytokines like IL-1a, IL-1b, IL-6 and TNF-a [255]. These factors will contribute to an upregulation of osteogenesis, instrumental to achieving an optimal healing of the bone. Numerous studies have attempted to single out certain cytokines to assess their role in fracture healing and bone repair, only to arrive at the conclusion that these factors act in intricate cascades of physiological mechanisms regulated by extensive crosstalk and feedback. However, inflammation could also have a negative effect on bone. In murine models, a chronic high level of TNF-a has been found to induce tissue damage, decreased BMD and a reduced mechanical strength of bone [256]. Furthermore, persisting elevated serum levels of IL-6 in human patients after bone fracture correlates with a weakened loadbearing capacity of the injured bone [257]. In addition to the aforementioned connection between age and risk of adverse drug reactions, higher age has also been associated with a rise in levels of advanced glycation end products (AGEs) in all tissues

of the body [258]. An increased presence of AGEs has been linked with the development of osteoporosis, and also causes the bone to lose some of its biomechanical properties by the formation of covalent cross-links between collagen and other bone proteins [259, 260]. This modification of bone proteins has been suggested to be responsible for changes in the functionality of osteoclasts and osteoblasts, tentatively of pathophysiological importance [261]. To further compromise the osteogenesis, AGEs have been found to stimulate osteoblast apoptosis [262]. Unfortunately, due to its nature of a reduced bone remodeling, using BPs for more than 1 year has been related to accumulation of AGEs [263].

As a consequence of the natural depletion of the immune system by aging, increased levels of oxidative stress mediators and pro-inflammatory cytokines, elderly have a steady state of low-grade inflammation. This condition has aptly been coined by Franceschi et al. as *inflammaging* [264]. In bone, this inflammaging has been suggested to increase the pathogenic factors in osteoporosis [265]. Though previously mentioned as part of the normal response to bone injury and indicated as pro-osteoporotic factors, IL-1, IL-6 and TNF-a have also been associated with inflammaging [266, 267]. Concerning the in vitro findings in this thesis, and in accordance with a previous study by Acil et al., we registered no changes in the secretion of IL-6 from the osteoblasts after 14 days of incubation [238]. It is worth noting, though, that Acils group found a significant increase in secretion of IL-6 from the osteoblasts after exposure to zoledronate at concentrations ranging from 0.15 μM to 2.5 μM. In a study on mice, Morita et al. concluded that the presence of IL-6 increased the risk of developing osteonecrosis [268]. Taken together with Acils findings, this could help explain the increased incidence of MRONJ amongst patients receiving zoledronate compared to patients receiving ALN. In Paper I, the incubation of osteoblasts with ALN at concentrations of 20 and 100 μM induced a dose-dependent increase in levels of the pro-inflammatory cytokines IL-8 and RANTES. Both cytokines are previously described as possible factors in the development of osteolysis and osteonecrosis [269, 270]. We did see a discrepancy in Paper II, where dosing the osteoblasts with 20 μM ALN resulted in a reduced secretion of IL-8. There was no clear explanation for this, and again the question as to the effect of donor variation is raised.

ALN had a dose-dependent negative impact on osteoblast growth and function

As previously elaborated on, BPs are known to affect the OPG/RANKL balance, an important regulator of the osteoclast activity [142]. Interestingly, others have also found that the OPG/RANKL ratio in serum could be possible biomarkers of MRONJ [271]. Though Paper I

and II revealed no significant change to OPG levels after exposure to 20 and 100 μM ALN, we registered a transient increase following incubation with 5 μM ALN (Paper III). To further complicate the relevance of these findings, several studies have demonstrated a vascular structure covering the sites of active bone remodeling, the bone remodeling compartment (BRC) [272]. The outer lining of the BRC is constituted of cells resembling lining cells, readily influenced by osteotropic growth factors and cytokines. The BRC presence on the surface varies proportionally to the rate of bone turnover, with an increased rate resulting in more remodeling compartments. The BRC is separated from the bone marrow, thus leading to a local modulation of the bone remodeling and is not affected by factors from the blood cells in the marrow space. Findings in Paper III also include transiently elevated levels of G-CSF, IFN-g and MCP-1 after exposure to 5 μM ALN. An increase in G-CSF and MCP-1 is suggested to have a stimulatory effect on osteoclastogenesis and has been associated clinically with osteopenia and osteoporosis [273-275]. As opposed to this, the elevated levels of OPG and IFN-g could contribute to a reduced number of osteoclasts and a higher BMD [276, 277]. The collective outcome of changes to the secretion of these factors regarding osteogenesis is not clear. However, we found that ALN reduced the expression of OC and ALP mRNAs (Paper I), known markers of osteoblast differentiation [278]. Taken together with the reduction of OPN (Paper I) having been linked to BRONJ [279], the dose-dependent potential negative impact of ALN on osteoblast growth and function, and bone in general, cannot be ignored.

Therapeutically relevant concentration of ALN for in vitro studies on osteoblasts (Paper II)

There have been numerous in vitro studies assessing the effect of different BPs on human osteoblasts and osteoblast-like cells [280-283]. A common denominator for these studies, is that the effect of the BPs in question is evaluated by using a range of concentrations. Due to the nature of BPs strong affinity to hydroxyapatite, and thus their accumulation in bone, an assessment of what concentration of ALN the osteoblasts are exposed to is complicated. We wanted to investigate if a therapeutically relevant dosage of ALN for in vitro studies on osteoblasts could be found, as to contribute to more precise future studies relatable to the clinic. Contrasting no change compared to control after incubation with 5 μM ALN, the angiogenic factors VEGF and MCP-1 were both negatively affected by 20 μM ALN, indicating a reduced capacity of neovascularization and thus limiting the potential for optimal healing following a bone injury. Furthermore, both VEGF and MCP-1 have been linked with activation of bone

turnover and remodeling [244, 274], underlining the possible negative ramifications on bone quality following a decline in secretion of these factors.

A rise in secretion of sclerostin, as registered after dosing the osteoblasts with 20 μM ALN compared to 5 μM (Paper II), advocates that the higher dosage could facilitate an increased resorption of bone [284]. Sclerostin inhibits the Wnt signaling cascade and consequently has an unfavorable influence on bone formation [285]. The negative effect of this glycoprotein on osteogenesis is so potent, that there are newer antiresorptive agents on the market specifically targeting and inhibiting sclerostin with monoclonal antibodies [284]. However, as there are no studies indicating chronic use of ALN causing elevated serum levels of sclerostin, we carefully conclude that 5 μM ALN is closer to a clinically relevant dosage. It is also worth noting that studies on rats have shown that clinically relevant doses of sclerostin related to antiresorptive treatment, did not induce MRONJ [286]. Our findings of a reduced secretion of G-CSF resulting from cultivation with 20 μM ALN, suggests a decreased osteoclastogenesis. Even though the clinical consequence is uncertain with ALN already being a strong suppressor of the osteoclasts, it does potentially have negative effect on bone metabolism.

The transiently increased levels of the inflammatory cytokines IL-8 and IFN-a2 induced by incubation with 5 μM ALN, is accompanied with progressive decrease after exposure to 20 μM ALN (Paper II). One angle in explaining the decline could be the reduced metabolic activity (Paper I and II). However, this does not fit with other findings in Paper I, recalling that we noted a substantial increase in secretion of IL-8 and RANTES concurrent with an abolished proliferation and a strongly reduced metabolic activity after incubation with 100 μM ALN. Previous literature indicates that the osteoclasts in a resorption lacuna are exposed to substantially higher concentrations of BPs than the osteoblasts [287], and concentrations higher than 10 μM seem to affect the osteoblasts adversely [288]. The latter correlates with our findings described in Paper II and supports our conclusion that a relevant concentration of ALN for in vitro studies on osteoblasts may be about 5 μM. Moreover, recalling the association between the use of BPs and microcracks, it seems logical that compromised bone would result in a release of imbedded BP from the bone, possibly leading to higher local BP concentrations that could have a negative impact on the osteoblasts.

Do combination therapies involving ALN affect the regeneration potential of human osteoblasts and gingival fibroblasts in vitro? Combination of ALN and OME as model system (Paper III)

Unrelated to the use of medication, higher age is associated with poorer healing outcome after fractures [289]. This is partly due to an age-related reduction in serum levels of angiogenic factors, such as VEGF, resulting in an impaired angiogenesis [290]. In a recent study, Kim et al. found that about 30 % of the population aged 65 years or older in developed countries take 5 or more medications on a regular basis [291]. With an increasing part of the population becoming elderly [292], monitoring for adverse drug reactions following the use of concomitant drugs becomes crucial. In addition to evaluation of clinical data, it is important to analyze the molecular mechanisms. Though others have suggested that patients using PPIs have an increased risk of fractures [293], Aasarød et al. recently found that GERD patients naive to PPIs had an inferior BMD and higher CTX levels than age- and sex-matched controls [294]. This questions the established negative notion of PPIs unfavorable impact on bone. However, long term use of PPIs has also been associated with gastric neoplasia, kidney disease, dementia and liver disease [295], thus potentially affecting numerous cell types. Eiken et al. found that the use of PPIs could be associated with treated cases of BRONJ, suggesting an interaction between or additive effect of PPIs and BPs [296]. Even though there is a well-documented dose-dependent negative effect of ALN on human osteoblasts, and an unfavorable effect of PPIs on osteoblasts [150], there has not been any in vitro studies on human osteoblasts assessing the effect of combining ALN and a common PPI like OME.

Reduced proliferation and angiogenesis following the co-treatment

Previous studies have concluded that neither 5 µM ALN nor 1 µM OME has a direct cytotoxic effect on osteoblasts [236, 297]. Our findings suggest that this also applies to the combination of these drugs. While the proliferation of the osteoblasts went through an alternating pattern of decrease and increase after exposure to the drugs separately, the combination induced a steady decline. This indicate a time-dependent deleterious effect, which is difficult to explain in any other way than by synergism. Factors secreted from the osteoblasts affecting the angiogenesis also seem to suffer from an additive effect. The levels of VEGF after incubation with OME and OME in combination with ALN are substantially reduced, and more so with the co-treatment. Though MCP-1 is addressed mainly as a factor related to inflammation and osteoclastogenesis in Paper III, its role as a pro-angiogenic cytokine has been elaborated on earlier in this thesis

[249]. As the level of MCP-1 is reduced after 14 days of exposure succeeding cultivation with both drugs compared to control, and each drug separately, this is another indication that the angiogenesis could be impaired by a synergistic effect of the co-treatment. Remembering that VEGF and MCP-1 also have a stimulatory effect on the remodeling and turnover of bone, the reduction in secretion of both factors as a result of cultivation with ALN and OME simultaneously advocates that the co-treatment has negative impact on bone quality. This notion is further strengthened by the modulatory effect of the combination of drugs on factors regulating the osteoclastogenesis. In addition, combining ALN and OME induced an increase in the secretion of the pro-inflammatory cytokine IL-8 after 7 and 14 days of incubation, compared to control and the single drug treatments. This may indicate that treatments with both drugs over a longer duration, could initiate local inflammatory environments in bone. Interestingly, there was a transitory substantial drop in the level of IL-6 following the co-treatment compared to each drug separately. IL-6 is an important regulator of a functional immune system, and the presence of it is instrumental to insure sufficient angiogenesis and osteogenesis in the early stages of bone repair and healing [298, 299].

To further explore the effect of co-treatment, cultivated human gingival fibroblasts were also dosed with 5 μM ALN and/or 1 μM OME. However, relevant literature addressing the effect on gingival fibroblasts of OME alone or in combination with ALN could not be found. Not having previous studies or results to compare with proved to be challenging when discussing our findings, but it also underlines the need for additional focus on addressing the in vitro effect of co-treatments involving BPs. As with the osteoblasts, neither the single nor the combined administration of the drugs had an impact on the cell viability of the fibroblasts. This correlates with previous studies regarding ALN [300]. Whereas dosing the fibroblasts with ALN and OME separately only generated marginal changes to the proliferation, the co-treatment resulted in a time-dependent significant decrease. These findings somewhat fit with previous in vitro studies, where cultured human fibroblasts have been incubated with ALN [236]. However, Yuan et al. found that assessing the effect of different BPs on gingival fibroblasts by wound healing assays revealed a reduction in cell numbers and a delayed wound healing [301]. Thus, the decline in proliferation following the combination of drugs suggests that exposing fibroblasts to ALN and OME simultaneously could impair important functions of these cells, i.e. healing of soft tissue [106].

MRONJ is in most cases related to dental or surgical treatment that compromises the oral mucosa or gingiva and the underlying bone [302]. A reduced ability of soft tissue healing could

increase the risk of developing MRONJ and complicate the potential subsequent surgical treatment. All though the chondrocytes and osteoblasts are the major contributors of VEGF in relation to angiogenesis in bone, this growth factor is also secreted by fibroblasts [303]. We found that, succeeding an initial reduction, the co-treatment produced a more than two-fold increase in the secretion of VEGF from the fibroblasts after 14 days of incubation. This agrees with the findings of Yuan et al. [301], but the clinical impact of this is uncertain, considering the substantial reduction of VEGF and MCP-1 from the osteoblasts after cultivation with OME alone and in combination with ALN. Furthermore, the initial drop in secretion of IFN-g after exposure to the co-treatment could be interpreted as influencing the angiogenesis in a positive manner [304], but the level rebounds to an equivalent increase at day 3 followed by a decline to near control values, resulting in an influence on the vascularization that is difficult to interpret from a clinical perspective. This applies to the effect on the osteoclastogenesis as well, as an increased secretion of IFN-g has been associated with osteoclast stimulation [305]. The levels of pro-inflammatory cytokines in the fibroblast cell cultures did not seem to be affected by exposure to ALN, OME or the co-treatment. Regarding the fibroblasts dosed with ALN alone, these findings contrasted the elevated secretion of IL-1b, IL-6 and IL-8 found by others under similar in vitro conditions [301].

Co-treatment with ALN and OME could impair healing of bone and soft tissue

The rate of bone remodeling varies depending on factors like age, sex, site and type of bone [254, 306]. A study by Marolt et al. also state that the primary human alveolar osteoblasts have significantly higher rates of proliferation than osteoblasts from long bones [307]. These trait differences could help explain some of the discrepancies we found and suggest that using cells originating from sites related to the mechanism under investigation would make the results more valuable. Even though the osteoblasts are regarded to be less exposed to BPs than the osteoclasts under normal conditions, the local concentrations of BPs might increase as result of injury or altered rate of remodeling. As we have debated in this thesis, higher concentrations of BPs could have a negative impact on the osteoblasts, possibly resulting in a suboptimal bone quality with a reduced ability of healing. This also applies to a range of other cells, including fibroblasts, as stated by Tanaka et al. [308]. Moreover, the intraoral mucosa is in very close proximity of the underlying bone, mostly without buffering tissues like fat and muscle. Recalling that ALN also impairs healing of soft tissue dose-dependently, conditions increasing the concentrations of BPs in the periosteal area might result in exposed bone. Our findings

regarding co-treatment with OME indicate that concomitant use of BPs and a PPI could further aggravate the situation, illustrated in Figure 8.

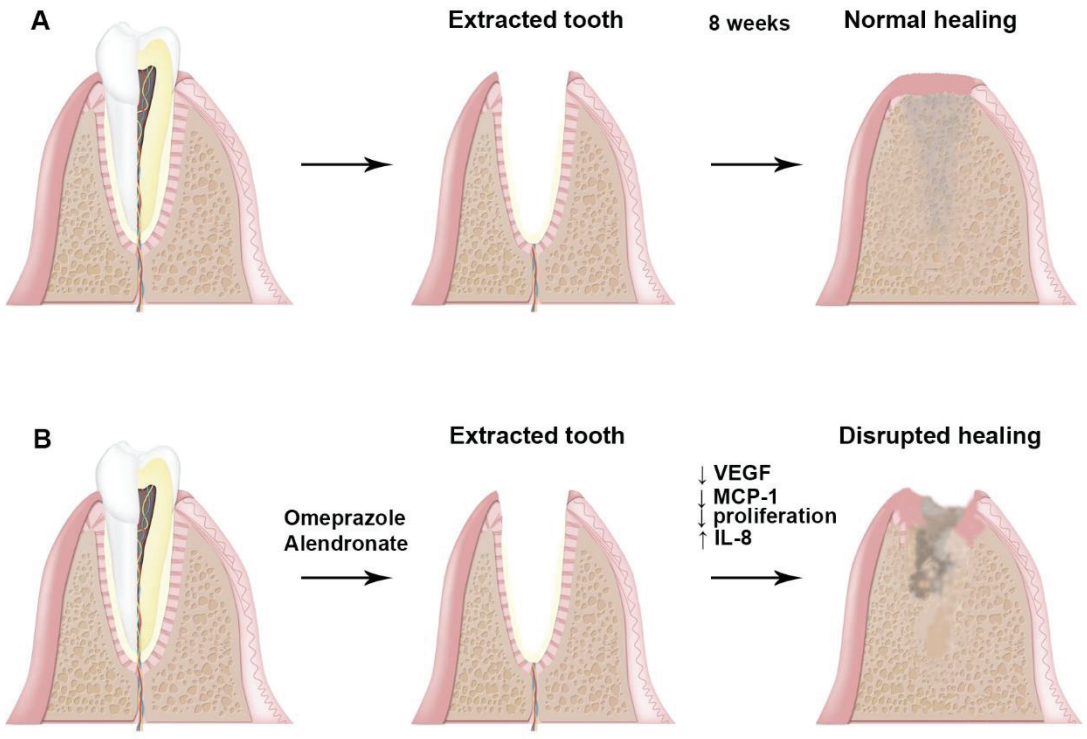

Figure 8: Healing of bone and soft tissue after tooth extraction on a patient not receiving bone modulating medication (A) and the putative disruptive impact on healing following co-treatment with alendronate and omeprazole (B).

As studies have shown a correlation between the presence of oral bacteria, exposure to antiresorptive agents and the risk of developing MRONJ [173, 268], this could increase the risk of developing MRONJ and would be interesting to investigate further in an in vitro setting. It is, however, a limiting factor that we have only tested one concentration of OME, based primarily on the findings of Costa-Rodrigues et al. [150]. Previous studies on co-cultures of osteoblasts and osteoclasts have reviewed the cellular responses to different BPs and found that the cells reacted differently when together compared to single-cell cultures [309]. Combining bone cells from relevant cites and cells from soft tissue, i.e. fibroblasts, would have strengthened our studies from a clinical perspective. Incubating these co-cultures with different oral bacteria, could provide information that is more relatable to the clinic when assessing the effect of medical treatments and possible adverse drug reactions like MRONJ.

Conclusions and future perspectives

In this thesis, we found that exposing osteoblasts to ALN induced a dose-dependent response regarding proliferation and factors involved in angiogenesis, inflammation and osteogenesis. Though none of the tested concentrations proved cytotoxic to the cells, it became evident in that the higher concentrations affected the osteoblasts adversely. More specifically, growth and function were influenced through a reduction in proliferation, secretion of angiogenic factors and markers of osteoblast differentiation combined with an increase in levels of pro-inflammatory cytokines associated with MRONJ. For subsequent in vitro studies on osteoblasts, we wanted to explore if a therapeutically relevant concentration of ALN when working with osteoblasts could be identified. 5 μM is suggested in previous literature to be a plausible concentration of ALN for the osteoblasts in an in vivo setting. This concentration did not affect the osteoblast viability in vitro, and only induced marginal changes to proliferation and secretion of factors regulating angiogenesis and bone homeostasis. With an applicable concentration identified, the last study sought to evaluate the in vitro effect of co-treatment with ALN and OME on cells involved in healing of bone and soft tissues. Dosing osteoblasts and gingival fibroblasts with a combination of 5 μM ALN and 1 μM OME proved to have a synergistically time-dependent diminishing effect on the proliferation. The co-treatment also produced results indicating a negative influence on the angiogenesis in bone and a modulation of the pro-inflammatory cytokines, more so than with each drug separately. Overall, the combination of the drugs appeared to have a definite unfavorable impact, which could translate to an impaired healing of bone and soft tissue in a clinical setting.

As elaborated on, the exact BP concentration that the different cell types involved in bone- and wound healing are exposed to is uncertain. Factors such as individual bone metabolism, site variances, age and varying affinity of different BPs to bone clouds a predictive outcome. Putative clinical implications of our in vitro findings could be that a higher concentrations of BPs, i.e. following trauma to bone, sets the scene for suboptimal healing of both bone and soft tissue. Combining BPs with other medications, like omeprazole, might further antagonize the situation. In conclusion, even when treated with dosages of BPs that are viewed as safe, co-treatment and local conditions in bone could have an impact on bone formation, healing and remodeling. Patients receiving treatment as discussed in this thesis, should be monitored closely when recovering from trauma or surgery. This should be stressed particularly when bones with a high degree of remodeling are involved, i.e. the jaws, as they are more prone to relevant pathological conditions such as MRONJ.

To further investigate interactions between BPs and other relevant medications, we have used the same design of co-treatment and assessed the effects of ALN in combination with simvastatin, gabapentin, levothyroxine, metoprolol and fluoxetine. The findings from these analyzes were planned to converge into one study, together with our results on ALN and OME, but the large amount of data proved to be difficult to effectively integrate into one manuscript. We aim to return to this project of mapping the impact of co-treatments with ALN and hope to publish the results in peer reviewed journals. It would also be interesting to compare this with output from co-cultures following the same protocols, ideally with osteoblasts from the jaw. It is important to identify cellular and molecular mechanisms triggered by different drug treatments, but one should strive to avoid animal testing [310].

Switching from 2D to 3D cell spheroids would make for a setting more relatable to in vivo conditions and enable testing of additional parameters, like the effect of treatments on mechanical properties [311]. This 3D culture method has recently been used successfully with both human osteoblasts and human PDL-fibroblasts [312, 313]. The spheroids can also consist of co-cultures of two or more cell types, and be applied in assessing the effect of drugs, both single drugs and drug combinations [314].

In a clinical aspect, we have started a project reviewing the oral health of osteoporotic patients receiving antiresorptive treatment. This is an ethically approved study where 160 patients are to be included, and parameters like BMD, drug use, relevant bloodwork, facial x-ray and quality of life will be reviewed in addition to a thorough clinical examination. The registration of data is complete and will hopefully culminate in several manuscripts adding new and valuable information to field.

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ARTICLE

Targeting a therapeutically relevant concentration of alendronate for in vitro studies on osteoblasts

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ABSTRACT

Objective: Bisphosphonates like alendronate mainly exert their effects on osteoclasts. However, osteoblasts are also affected, but exposed to a much lower concentration *in vivo* than the osteoclasts. Given that the effects are dose-dependent, the intention of the study was to identify a therapeutically relevant concentration of alendronate for *in vitro* studies on osteoblasts.

Materials and methods: Primary human osteoblasts were incubated with alendronate (5, 20 and 100 μM) for 1, 3, 7 and 14 days. Proliferation and viability were assessed, and the effects on cellular growth and function were evaluated by multianalyte profiling of selected proteins in cell culture media using the Luminex 200™.

Results: The viability was not affected by any of the dosages. Exposure to 5 μM alendronate had a neutral effect on osteoblast proliferation, and on secretion of osteogenic and inflammatory markers, while enhancing synthesis of a marker of angiogenesis. 20 μM alendronate induced a decline in proliferation and affected angiogenic and osteogenic biomarkers adversely. 100 μM alendronate reduced proliferation dramatically, and this dosage was excluded from further experiments.

Conclusion: A concentration of 5 μM alendronate exerted effects on human osteoblasts that may translate to those observed *in vivo* and could therefore be relevant for *in vitro* studies.

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Introduction

The bisphosphonate (BP) alendronate (ALN) is the most widely used drug in treatment of osteoporosis [1]. BPs have demonstrated to be efficacious by increasing bone mineral density (BMD) and reducing fracture rates [2]. These effects are mainly attributed to inhibition of bone resorption mediated by the osteoclasts [3]. However, the effect on BMD cannot completely explain the substantial reduction in fracture incidence in patients treated with BPs. There is a body of evidence that BPs also interact with the osteoblasts, and a stimulatory effect on osteoblast proliferation and maturation has been shown *in vitro* [4]. Moreover, both *in vitro* and *in vivo* studies have demonstrated that BPs are capable of preventing osteoblast and osteocyte apoptosis [5].

A multitude of *in vitro* studies have been performed to explore the mechanisms of action of BPs on osteoblasts and other cell types and how these potentially may explain positive or adverse effects observed *in vivo* [6,7]. These effects are dose-dependent, and it is challenging to identify a concentration that reflect the *in vivo* conditions. To approach this, it is essential to understand bone metabolism and the behaviour of BPs in the body, here exemplified by ALN. The BP is rapidly eliminated from the circulation, binds with high affinity to hydroxyapatite and tends to concentrate in the vicinity of active osteoclasts, rather than at sites of bone formation [8]. In a rat study, more than 70% of the osteoclast surface was densely labelled 24 h after administration of a single dose of [^{3}H]- ALN (0.4 mg/kg), in contrast to only 2% of the bone forming surface [9]. When osteoclasts secrete proteolytic enzymes and hydrochloric acid for the purpose of resorption, ALN is released and rapidly engulfed by the osteoclasts [10]. Hence, osteoclasts are obviously the cells exposed to the highest concentration of ALN. The level of ALN that can be achieved in the osteoclast resorption lacuna has been calculated to be as high as 10^{-4} M to 10^{-3} M (100–1000 μM) in newborn rats [9].

The *in vivo* and *in vitro* actions of BPs are well-described for osteoclasts [11,12], whereas the effects on osteoblast function remain to be fully elucidated. As elaborated on above, the osteoclasts are exposed to substantially higher concentrations than osteoblasts *in vivo* [13]. Notably, concentrations of BPs as low as 10^{-11} M have been shown to have an effect on osteoblasts, whereas concentrations above 10^{-5}

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Supplemental data for this article can be accessed here.

M appear to be toxic [14]. In addition, Li et al. found that the minimum inhibitory concentration of the BP zoledronic acid (ZA) on mouse osteoclasts was 10^{-6} M [15].

In order to achieve a more comprehensive understanding of how BPs affect the cells involved in osteogenesis, the concentration of the drug applied *in vitro* should ideally be as close to relevant therapeutic concentrations as possible. Accordingly, the *in vitro* dosage that promotes similar effects in osteoblasts as described *in vivo* would be the preferable. The aim of the present study was to delineate the effects of different concentrations of ALN on human osteoblasts *in vitro*, and relate this to *in vivo* effects of ALN in order to identify a therapeutically relevant concentration or range of concentrations for *in vitro* studies.

Materials and methods

Study design

Primary human osteoblasts at passage 4 from tibia of a one-day old female donor (Cambrex BioScience, Walkersville, MD, USA) were grown in Lonza Osteoblast Growth Media (OGM) (Cambrex BioScience), containing ascorbic acid, foetal calf serum and gentamicin. Cells were subcultured at 37 °C in a humidified atmosphere of 5% CO_2 prior to confluence, according to manufacturers' instructions.

Cells were seeded in 12-well plates and incubated with ALN (Sigma-Aldrich Biotechnology, Saint Louis, MO, USA) dissolved in OGM at concentrations of 5, 20 and 100 μM. For protein quantification, cells and cell culture media were harvested after 1, 3, 7 or 14 days of incubation, with the last change of medium with or without ALN 24 hrs prior to harvest. Unexposed cells at each time point were used as control. Cells and cell culture supernatants were collected and stored at −80 °C until analysis.

Cell viability and proliferation

Cell viability was confirmed by monitoring the activity of lactate dehydrogenase (LDH) in cell culture medium. LDH was measured using the microplate-based Cytotoxicity Detection Kit (LDH; Boehringer, Mannheim, Germany). In accordance with the manufacturers' protocol, 50 μL aliquots of cell culture medium was used, and the absorbance was read using a microplate reader (Elx800, BioTek, Bad Friedrichshall, Germany) at 450 nm.

The proliferation rate of the cells was measured by [^{3}H]-thymidine incorporation and the MTT colorimetric assay. In the [^{3}H]-thymidine incorporation assay, the cells (1.7×10^4 cells/well in 12-well plates) were incubated with cell culture medium containing 5, 20 and 100 μM ALN for 1 and 3 days ($n = 3$). The cells were pulsed with 1 μCi [^{3}H]-thymidine/well 12 h prior to harvest, and upon harvest, the medium was removed, and the cells were washed twice with PBS and twice with 5% trichloroacetic acid (TCA) to remove unincorporated [^{3}H]-thymidine. The cells were solubilized in 500 μl of 1 M sodium hydroxide (NaOH), and 200 μL of the solubilized cell solution was transferred to 4 mL scintillation fluid (Lumagel LSC BV; GE Groningen, Netherlands) and counted for 3 min in a liquid scintillation counter (Packard 1900 TR, Packard Instruments, Meriden, CT, USA).

The MTT colorimetric Cell Growth Assay (CT02 Chemicon, Merck KGaA, Darmstadt, Germany) was performed according to manufactures' instruction. Cells (4×10^3 cells/well in 96-well plates) were harvested after 3 days of incubation with the various concentrations of ALN ($n = 3$). Unexposed cells were used as control. The absorbance was measured using an ELISA plate reader (ELx800, BioTek, Vermont, USA) at a test wavelength of 570 nm with reference wavelength of 630 nm.

Protein quantification in cell culture medium

Multianalyte profiling was performed using the Luminex 200TM system (Luminex Corporation, Austin, TX, USA), and acquired fluorescence data were analysed by the xPONENT 3.1 software (Luminex).

Prior to analysis, aliquots of the cell culture medium were concentrated 5 times using Microsep Centrifugal tubes with 3 kDa cut-off (Pall Life Science, Ann Armour, MI, USA). A simultaneous quantification of 29 cytokine and chemokine biomarkers in 25 μL of cell culture media were ascertained using the 29-Milliplex Human Cytokine Immunoassay kit (Millipore, Billerica, MA, USA). The evaluated biomarkers included epidermal growth factor (EGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon alpha-2 (IFN-a2), IFN-γ, interleukin-1a (IL-1a), IL-1b, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-13, IL-15, IL-17, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1a (MIP-1a), MIP-1b, tumour necrosis factor-a (TNF-a), TNF-b and vascular endothelial growth factor (VEGF). In addition, the levels of osteoprotegerin (OPG), osteocalcin (OC), leptin, TNF-a, sclerostin, and fibroblast growth factor 23 (FGF-23)) were simultaneously determined in 25 μL of cell culture media using Milliplex Human Bone Panel Immunoassay kit, HBNMAG-51K-7plex (Millipore).

Luminex Multiplex Bead Immunoassays are solid phase sandwich immunoassays, which are designed to be analysed with a Luminex instrument. All the reagents and tools needed were provided in the kit and analyses were performed according to the manufacturers' protocols. In brief, in each of the above-described multiplex kits, 25 μl cell culture medium was applied in 96 cell plates, diluted with assay buffer, and incubated over night at 2–8 °C with antibody coated fluorescent magnetic beads. After washing, analyte-specific biotinylated detector antibodies are added and incubated with the beads. Excess biotinylated detector antibodies were removed by washing and streptavidin conjugated to the fluorescent protein, R-Phycoerythrin (Streptavidin-RPE), was added and the mixture incubated. After the final washing step acquired fluorescence data were quantified based on the spectral properties of the beads and the amount of associated R-Phycoerythrin (RPE) fluorescence in a Luminex 200^{TM}. The concentration (pg/mL) of each biomarker in the

Q2 **Table 1.** ■■■

Method	Day	5 μM	20 μM	100 μM
LDH	**1**	100.5 ± 4.5	90.8 ± 44.5	110.4 ± 69.1
	3	103.4 ± 5.1	75.2 ± 17.8	84.6 ± 12.8
3H-Thymidin	**1**	84.1 ± 37.0	65.9 ± 19.8	37.3 ± 20.7
	3	128.9 ± 12.2	124.5 ± 21.1	7.2 ± 2.5††§
MTT	**3**	95.5 ± 7.8	83.2 ± 6.0†	34.9 ± 15.2††§

$^{\dagger}p \leq .05$; †† $p \leq .001$ compared to 5 μM.
$^{\S}p \leq .001$ compared to 20 μM.
LDH, lactate dehydrogenase; [^{3}H]-thymidine incorporation in proliferating cells; MTT, abbreviation for the dye compound 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Data are calculated relative to unexposed control cells at each time point and presented as percentages ($n = 3$).

samples was determined based on standard curves for each of the individual analytes in the kit.

Statistical analysis

Statistical evaluation was performed using the software SigmaPlot 13.0 and 14.0 (Systat Software, San Jose, CA, USA); statistical significance was assessed by Student's t-test and *p*-value set to .05, given a passed test of normality and equality.

Results

Viability and proliferation

There was no significant difference in the cell viability (LDH) between the cultures after exposure to 5, 20 or 100 μM of ALN at any of the time points tested (Table 1). Evaluation of thymidine incorporation indicated that there was a marked reduction in proliferation of the osteoblasts after three days of exposure to 100 μM ALN compared to 5 and 20 μM ALN ($p \leq .001$). This correlates with the findings in the MTT assay, where incubation with 100 μM ALN for three days resulted in a diminished proliferation compared to incubation with 5 and 20 μM ALN ($p \leq .001$). Moreover, the MTT assay disclosed a reduced proliferation of the cells exposed to 20 μM ALN for three days, compared to 5 μM ALN ($p \leq .05$) (Table 1).

The effect of ALN on secretion of biomarkers relevant to osteogenesis

As a consequence of the abolished proliferation after exposure to 100 μM ALN for 3 days, we chose to focus on 5 and 20 μM for the remainder of the study. Following an initial rise in secretion of sclerostin from cells exposed to 5 μM ALN ($p = .008$), there was no change in the concentration compared to control after 14 days (Figure 1). Sclerostin secretion increased gradually up to 14 days in cells incubated with 20 μM ALN ($p = .044$), however, not significantly compared to 5 μM ALN exposed cells. The release of granulocyte colony-stimulating factor (G-CSF) was transiently enhanced after incubation with 5 μM ALN ($p = .026$ day 3 and $p = .025$ day 7) (Figure 2a). After 14 days of exposure to 20 μM ALN, G-CSF was reduced by 50% compared to the cells incubated with 5 μM ALN ($p = .020$) and unexposed cells ($p = .017$).

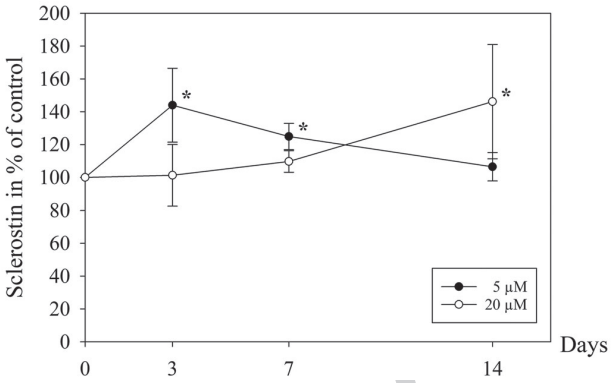

Figure 1. Sclerostin in cell culture media from human osteoblasts. The cells were exposed to 5 μM or 20 μM ALN. Data are presented in % relative to unexposed control at each time point. *$p \leq 0.05$ compared to control, † $p \leq .05$ compared to 5 μM.

The effect of ALN on secretion of biomarkers relevant to angiogenesis

There was a non-significant initial reduction in the release of VEGF from osteoblasts after exposure to both 5 and 20 μM ALN. This was followed by an increase of VEGF to control level after 14 days of incubation with 5 μM ALN, and a reduction of VEGF by 50% in cells exposed to 20 μM ALN compared to 5 μM ALN ($p = .003$) and unexposed cells ($p = .006$) (Figure 2b). The secretion of MCP-1 rose to 155% of control after 3 days of incubation with 5 μM ALN ($p = .019$) (Figure 2c), with a subsequent decline to control level after 14 days. In cells exposed to 20 μM ALN, the level of MCP-1 was reduced by 70% compared to cells incubated with 5 μM after 14 days ($p \leq .001$).

The effect of ALN on secretion of biomarkers relevant to inflammation

Compared to unexposed cells, there was a significant rise in the secretion of IL-8 after 1 day of exposure to 5 μM of ALN ($p = .002$) (Figure 2d). 20 μM ALN promoted a drop in IL-8 from day 3, resulting in 50% and 75% lower levels after 7 days ($p = .007$) and 14 days ($p \leq .001$), respectively, compared to 5 μM. A transient non-significant increase in IFN-a2 was observed in cells incubated with 20 μM ALN (Figure 2e), thereafter IFN-a2 decreased, and was 30% lower after 3 days and 35% lower after 14 days when compared to 5 μM ($p = .022$ and .015, respectively).

Most of factors tested were not found to change significantly at any time point (leptin, OC, OPG, IFN-g, and IL-1ra), or the concentrations were lower than the set levels of detection for the analyses (FGF-23, EGF, eotaxin, IL-10, IL-12p40, IL-13, IL-15, IL-17, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IP-10, MIP-1a, MIP-1b, TNF-a and TNF-b).

Discussion

In this study, exploring the *in vitro* effects of different concentrations of ALN on human osteoblasts, the viability was not affected by any of the dosages. Exposure to 5 μM ALN

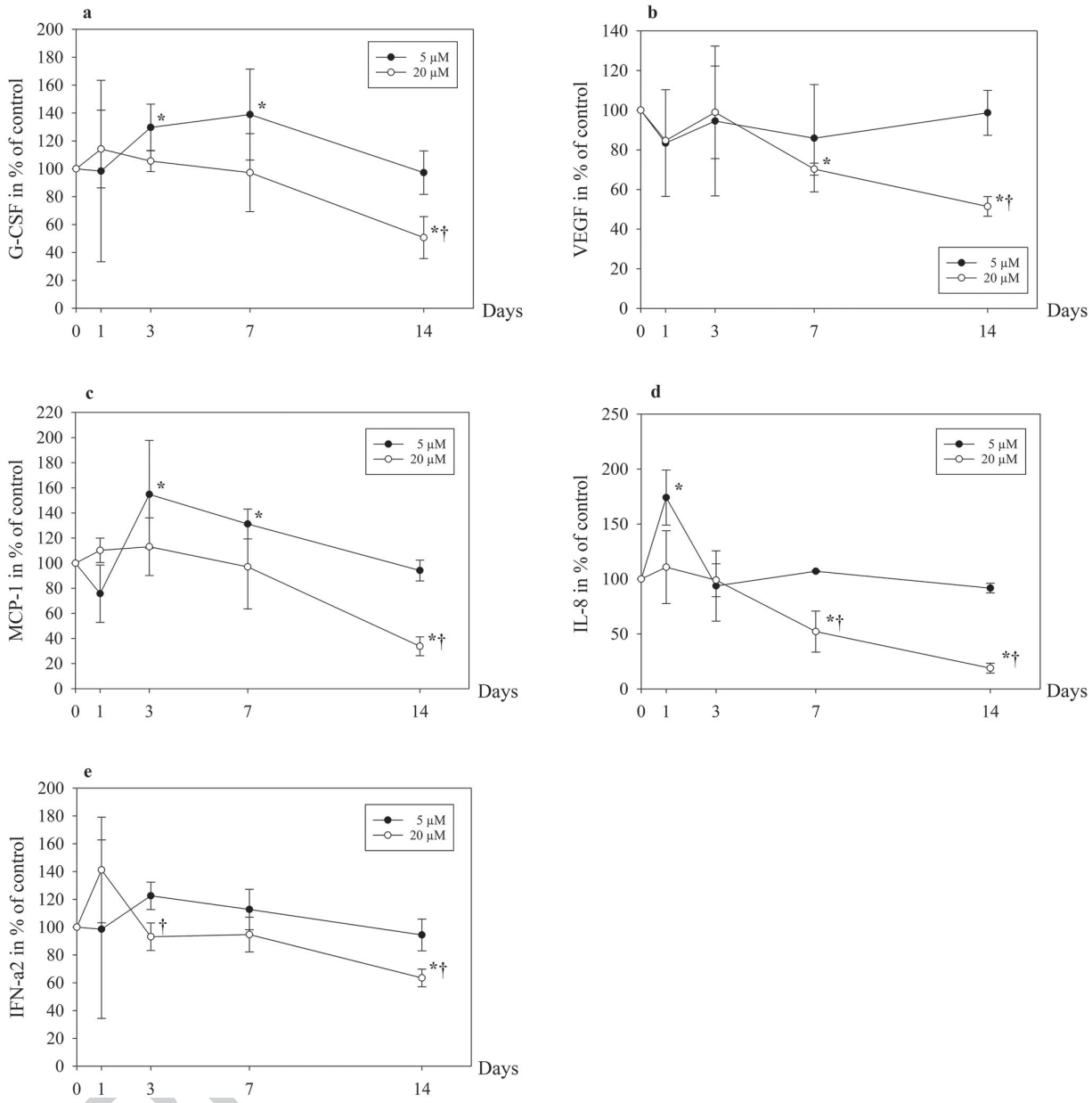

Figure 2. Granulocyte colony-stimulating factor (G-CSF) (a), vascular endothelial growth factor (VEGF) (b), monocyte chemoattractant protein (MCP-1) (c), interleukin-8 (IL-8) (d) and interferon-alpha2 (IFN-a2) (e) in cell culture media from human osteoblasts. The cells were exposed to 5 µM or 20 µM ALN. Data are presented in % relative to unexposed control at each time point. * $p \leq .05$ compared to control, † $p \leq .05$ compared to 5 µM.

had a neutral effect on osteoblast proliferation, and on secretion of osteogenic and inflammatory markers, while enhancing synthesis of MCP-1, a marker of angiogenesis. 20 µM ALN induced a decline in proliferation and affected angiogenic and osteogenic biomarkers adversely after 14 days of exposure. ALN at a concentration of 100 µM reduced proliferation dramatically, and this dosage was excluded from further experiments.

Higher concentrations of ALN reduce proliferation

As previously mentioned, the positive skeletal effects of BPs are mainly mediated by suppression of osteoclastic activity. This is achieved through inhibition of the mevalonate pathway, ultimately leading to apoptosis of the osteoclasts [16]. *In vivo* studies indicate that BPs also may affect osteoblasts and osteocytes [17,18]. As elaborated on in the introduction, osteoclasts are exposed to higher concentrations of BPs than cells of the osteoblastic lineage [13]. Thus, when exploring the effects of BPs on osteoblasts *in vitro*, it is of importance to identify a dosage that is close to the *in vivo* concentration.

ALN in concentrations of 10^{-5} M or higher have been reported to affect osteoblasts adversely, inducing apoptosis and inhibiting cell differentiation [19]. We observed no effect on viability after exposure to 5, 20 and 100 µM ALN for three

days, as assessed by LDH activity. The effect on proliferation was, however, dose-dependent. In accordance with the findings of Garcia-Moreno et al. [20], incubation with 100 μM ALN resulted in a steep decline in osteoblast proliferation after 3 days. The proliferation assessed with an MTT-assay was also reduced after 3 days of exposure to 20 μM ALN, compared to 5 μM. We did, however, not observe enhancement of osteoblast proliferation by 5 μM ALN. Probably an even lower dosage of ALN would be needed to exert this effect.

Notably, ALN has been shown to abolish the glucocorticoid-induced apoptosis in vertebral cancellous bone osteocytes and osteoblasts in mice [21]. These findings suggest that the effect of BPs in glucocorticoid-induced osteoporosis and osteoporosis in general may be due, in part, to their ability to prevent osteocyte and osteoblast apoptosis [3], this has also been confirmed in other studies [22]. Preservation of the bone-forming function of mature osteoblasts and maintenance of the osteocytic network is decidedly of significance for normal function of bone. Thus, to mimic *in vivo* conditions, a dosage of 5 μM is clearly the better option.

Low dose of ALN has the least impact on biomarkers affecting osteogenesis

The Wnt signalling pathway is essential in bone formation, and bone homeostasis, as well as bone repair and regeneration following injury [23]. This signalling pathway plays an important role in controlling the differentiation of mesenchymal stem cells (MSCs), in favour of the osteoblasts, as well as promoting osteoblast maturation and survival [24]. Bone remodelling is constantly ongoing to replace old bone tissue by new bone tissue. Activation of the Wnt/β-catenin signalling pathway leads to increased proliferation and differentiation of osteoblast precursor cells, reduces apoptosis of mature osteoblasts, and promotes the ability of differentiated osteoblasts to inhibit osteoclast differentiation [25]. Sclerostin, which is a protein predominantly secreted by osteocytes, is a potent antagonist to the Wnt pathway [26], and as such potentially inhibits bone formation and stimulates bone resorption [24]. We found that 5 μM ALN induced a temporary rise in secretion of sclerostin after 3 days of exposure, whereas 20 μM ALN induced a gradual rise in sclerostin throughout the duration of the experiment. In postmenopausal osteoporotic women treated with ZA, an early rise in sclerostin serum levels was observed [27]. On the other hand, no significant alteration in circulating sclerostin levels was seen in a retrospective observational study after long-term treatment of postmenopausal women with the oral BPs ALN and risedronate [28].

Furthermore, we found that ALN at a dosage of 5 μM promoted a transient increase in release of G-CSF from osteoblasts, followed by a decline to control level after 14 days. 20 μM ALN induced a pronounced decrease in G-CSF, with a 50% reduction after 14 days of exposure. A rise in G-CSF has also been reported in mice in response to a BP [29]. G-CSF has been mainly associated with the recruitment of stem cells from bone marrow [30,31]. Effects on osteoclast and osteoblast activity have also been reported, the data are, however, diverging [32–34]. The significance of our findings regarding G-CSF under *in vivo* conditions is uncertain.

Low dose of ALN promotes angiogenesis

The development of a microvasculature and microcirculation is critical for the homeostasis and regeneration of living bone, without which, the tissue would simply degenerate and die [35,36]. Thus, it is essential that drugs used in the treatment of osteoporosis do not inhibit angiogenesis. This complex process is orchestrated by multiple factors and mechanisms, which when in balance will contribute to insure a sufficient supply of nutrients and minerals. Osteoblasts have been shown to secrete VEGF, an essential angiogenic growth factor which is critical in the initial stages of wound healing and bone repair [37]. ALN in the concentration range 10^{-5}–10^{-3} M has been reported to enhance VEGF secretion from osteoblasts [38], whereas 10^{-12}–10^{-6} M of ZA and ALN reduced the VEGF secretion from osteoblastic cell lines in a dose-dependent manner [19]. We observed no significant changes in VEGF secretion from osteoblasts exposed to 5 μM ALN, however a pronounced decline occurred after exposure to 20 μM ALN, which reduced VEGF by 50% compared to 5 μM ALN.

MCP-1 has been recognized as another important angiogenic chemokine, which is involved in induction of VEGF-A gene expression [39]. We noted a substantial rise in MCP-1 after 3 days of incubation with 5 μM ALN, thereafter levelling off to control values after 14 days. In contrast, there was a marked drop in secretion of MCP-1 after 14 days of exposure to 20 μM ALN. Accordingly, our data indicate that ALN in a dosage of 5 μM could favour angiogenesis, whereas 20 μM seemed to affect it negatively.

5 μM Aln with marginal impact on inflammatory biomarkers after 14 days of exposure

The osteoblasts are capable of producing a wide range of cytokines and growth factors that are involved in bone damage and repair [40,41]. These substances include among others IL-1, IL-6, IL-8, TNF-a, IFN-a and IFN-g which interact in balancing bone metabolism [42]. The expression of several proinflammatory cytokines from osteoblasts and osteoblast-like cells are affected by lower concentrations of BPs *in vitro* [43,44]. We have previously shown that 5 μM ALN stimulated release of proinflammatory cytokines [45]. In the present study, an initial spike in the expression of IL-8 was seen after exposure to 5 μM ALN, whereas 20 μM induced a decline after 7 and 14 days. This is at odds with another study, showing that 100 μM ALN promoted an increase in the secretion of IL-8 [46]. IL-8 has for decades been known as an important cytokine regarding development of inflammation, mainly through its activation of neutrophils [47].

Release of IFN-a2 was also affected differently by the low and high dosages, 20 μM evoking an initial rise, followed by a decline, whereas the cells exposed to 5 μM ALN responded in a similar manner as control cells. The cytokine IFN-a2 is

mainly accredited as an important factor in the immune response to a microbial infection [48], but has also been applied in the treatment of several malignant diseases [49]. Moreover, it has been suggested as an inhibitor of osteoclasts differentiation [50].

In this study we have compared the effects of 5, 20 and 100 μM of ALN on osteoblasts *in vitro*, addressing effects on viability, proliferation, and the release of factors affecting inflammation, angiogenesis and osteogenesis. We intended to identify a therapeutically relevant concentration of ALN for *in vitro* studies on osteoblasts. Taken together, we observed that osteoblasts exposed to 5 μM ALN responded in a similar manner as control cells after 14 days, whereas the higher dosages negatively affected proliferation and factors regarding angiogenesis and osteogenesis. Therefore, it is reasonable to propose that $\leq$ 5 μM ALN *in vitro* are concentrations that best mimic observations made *in vivo*.

Osteonecrosis of the jaw (BRONJ) and atypical femur fractures are rare adverse effects of BPs. Under these circumstances, the concentration of ALN is probably higher. These adverse effects tend to occur after long-term treatment and subsequent accumulation of ALN in bone. Schaudinn et al. applied energy-dispersive X-ray spectroscopy (EDS) to estimate the concentrations of ALN in jaw bone by measurement of percent nitrogen incorporation [51]. They observed that ALN concentrations correlated with both duration of therapy and BRONJ stage. Notably, in an inflammatory setting, peripheral blood mononuclear cells seem to increase their release of pro-inflammatory cytokines (TNF-a and IFN-g) as a response to internalizing BPs [52]. Moreover, a local acidic environment, i.e. periodontitis, has been reported to amplify the dose-dependent cytotoxic effects of BPs, potentially triggering further osteolysis and an increased concentration of BPs [53]. It should be recalled that there may be differences between individuals and between skeletal sites that affect the *in vitro* response to ALN. Hence, when conducting *in vitro* studies on primary human osteoblasts, both skeletal site of origin and donor age are variables of significance [54].

Conclusion

With only marginal changes in viability, proliferation and secretion of factors of growth, angiogenesis and inflammation, incubation with 5 μM ALN had the least negative impact on the osteoblasts of the concentrations tested in this study. Altogether, based on the current available literature and our own findings, we suggest that $\leq$ 5 μM is a therapeutically relevant concentration of ALN for *in vitro* studies on primary human osteoblasts.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Alendronate and omeprazole in combination reduce angiogenic and growth signals from osteoblasts

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ABSTRACT

Objective: Due to gastrointestinal side effects of oral bisphosphonates (BPs), proton pump inhibitors (PPIs) are often prescribed. PPIs may enhance the risk of osteonecrosis of the jaw, a rare side effect of BPs. Therefore, the objective of this study was to evaluate the effects of the oral BP alendronate (ALN) and the PPI omeprazole (OME) alone and in combination on primary human osteoblasts and gingival fibroblasts in vitro.
Methods: Human gingival fibroblasts and normal human osteoblasts were incubated with either 5 μM of ALN or 1 μM of OME, or ALN + OME for 1, 3, 7 or 14 days. Effect on viability was evaluated by the lactate dehydrogenase activity in the medium and on proliferation by quantifying 3H-thymidin incorporation. Multianalyte profiling of proteins in cell culture media was performed using the Luminex 200TM system to assess the effect on selected bone markers and cytokines.
Results: The proliferation of osteoblasts and fibroblasts was reduced upon exposure to ALN + OME. ALN induced an early, temporary rise in markers of inflammation, and OME and ALN + OME promoted a transient decline. An initial increase in IL-13 occurred after exposure to all three options, whereas ALN + OME promoted IL-8 release after 7 days. OME and ALN + OME promoted a transient reduction in vascular endothelial growth factor (VEGF) from osteoblasts, whereas ALN and ALN + OME induced a late rise in VEGF from fibroblasts. Osteoprotegerin release was enhanced by ALN and suppressed by OME and ALN + OME.
Conclusions: ALN + OME seemed to exaggerate the negative effects of each drug alone on human osteoblasts and gingival fibroblasts. The anti-proliferative effects, modulation of inflammation and impairment of angiogenesis, may induce unfavorable conditions in periodontal tissue facilitating development of osteonecrosis.

1. Introduction

Adverse effects of bisphosphonates (BPs), like osteonecrosis of the jaw (ONJ), have got increasing attention in the last decade (Yang et al., 2019; Yarom et al., 2019). BP-related osteonecrosis of the jaw (BRONJ) was first described in cancer patients receiving high dose IV BPs, and subsequently also in osteoporosis patients treated with oral or IV BPs, preferentially amino-BPs (Marx, 2003). Alendronate (ALN) given orally is the most widely used BP in treatment of osteoporosis (Pazianas et al., 2007). The incidence of BRONJ among patients with osteoporosis is very low, ranging from 0.02–0.4%, however, an underestimation has been suggested (Galis et al., 2017; Hansen et al., 2013). Medications other than BPs, such as denosumab and antiangiogenic drugs, have also been related to ONJ (Hansen et al., 2013), and the term medication- related ONJ (MRONJ) is applied to cover all drugs (Migliorati et al., 2019).

The pathophysiology of BRONJ is not settled, and it is unknown whether necrosis precedes or follows infection. Several authors have proposed that it may be attributed to over-suppression of osteoclasts by long-term BP therapy, resulting in impairment of osteoblast function and bone renewal (Aghaloo et al., 2015). BPs have been reported to exert an anti-proliferative effect on osteoblasts (Koch et al., 2010), the data are, however, diverging (Krüger et al., 2016). Impairment of angiogenesis is

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another factor suggested in the pathophysiology. Accordingly, treatment with BPs has been reported to reduce vascular endothelial growth factor (VEGF) (Santini et al., 2002). Moreover, the inflammation induced by BPs is postulated to promote BRONJ (Endo et al., 2017). Healing of oral soft tissue has also been shown to be affected in a negative manner after treatment with BPs. The migration and growth capacity of oral fibroblasts were blocked, as well as a downregulation of type-1 collagen, which is necessary for re-epithelization (Ravosa et al., 2011). Under these circumstances, events such as tooth extractions and dental infections might result in tissue death, vascular loss, and eventually osteonecrosis (Wan et al., 2020).

Comorbidity and certain drugs like glucocorticoids seem to enhance the risk of BRONJ (Khan et al., 2015). H2-blocking agents have also been mentioned in this context (Rejnmark, 2008). In a Danish study including more than 60,000 subjects using ALN, use of proton pump inhibitors (PPIs) was independently associated with surgically treated BRONJ (Eiken et al., 2017). ONJ was also observed in a patient using the PPI esomeprazole without simultaneous BP therapy (Marconcini et al., 2019). Concomitant treatment with BPs and PPIs has also been reported to increase the risk of atypical femur fractures, another rare adverse effect of BPs (Giusti et al., 2010). PPIs are often prescribed to patients treated with oral BPs, as gastrointestinal complaints are the most common adverse effects. Use of PPIs is associated with a modest increase in fracture risk, whereas few studies have shown a reduction in bone mineral density (BMD) (Ozdil et al., 2013).

At a cellular level, esomeprazole, lansoprazole and omeprazole (OME) have been observed to exert inhibitory effects on osteoclasts and osteoblasts at concentrations similar to the plasma levels attained with therapeutic dosages (Costa-Rodrigues et al., 2013). These results suggest that PPIs might have a direct deleterious effect on bone cells, with the possibility of decreased bone turnover. OME has been reported to stimulate osteoblast proliferation, but keeping the level of mineralization unchanged (Salai et al., 2013). There are few, if any, in vitro studies addressing the effect of BPs and PPIs on human gingival fibroblasts, or osteoblasts.

Hence, the aim of this study was to investigate the effect of ALN and OME alone and in combination on human osteoblasts and gingival fibroblasts, addressing cell viability, proliferation and secretion of selected bone markers and cytokines.

2. Materials and methods

2.1. Study design

Commercially available cells were used in all aspects of this study. Primary human osteoblasts at passage 4 from tibia of a one-day old female donor (Cambrex BioScience, Walkersville, MD, USA) were grown in Lonza Osteoblast Growth Media (OGM) (Cambrex BioScience), containing ascorbic acid, fetal calf serum and gentamicin.

Human gingival fibroblasts, passage 4 (LGC Standards GmbH, Mercatorstr. 51, 46485 Wesel, Germany) isolated from a 28-year-old male donor were cultivated with Lonza Fibroblast Growth Media (FGM) (Cambrex BioScience) containing fetal calf serum and gentamicin. Cells were subcultured at 37 °C in a humidified atmosphere of 5% CO_2 prior to confluence according to manufacturers' instructions.

Cells were seeded in 12-well plates and incubated with ALN and/or OME (Sigma-Aldrich Biotechnology, Saint Louis, MO, USA) dissolved in OGM/FGM at concentrations of 5 and 1 μM, respectively ($n = 3$). Cells and cell culture media were harvested after 1, 3, 7 or 14 days of incubation, with the last medium change, with or without factors, 24 h prior to harvest. Unexposed cells were used as control at each time point.

2.2. Cell viability and proliferation

Cell viability was evaluated by monitoring the activity of lactate dehydrogenase (LDH) in the cell culture medium. The LDH was measured using the microplate-based Cytotoxicity Detection Kit (LDH; Boehringer, Mannheim, Germany). In accordance with the manufacturers' protocol, 50 μl aliquots of cell culture medium were used and the absorbance was read using a microplate reader (Elx800, BioTek, Bad Friedrichshall, Germany) at 450 nm.

The proliferation rate of the cells (approx. 5×10^3 cells/cm^2) was measured by [3H]-thymidine incorporation into the new strands of DNA during replication. Sub confluent cells were incubated with cell culture medium containing either 5 μM of ALN, 1 μM of OME, or a combination of these drugs. Unexposed cells were used as control at each time point. The cells were pulsed with 1 μCi ^{3}H-thymidine/well 12 h prior to harvest. The medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS) and twice with 5% trichloroacetic acid (TCA) to remove unincorporated [3H]-thymidine. The cells were solubilized in 500 μl of 1 M sodium hydroxide (NaOH), and 200 μl of the solubilized cell solution was transferred to 4 ml scintillation fluid (Lumagel; Lumac LSC BV; Packard, Groningen, Netherlands) and counted for 3 min in a liquid scintillation counter (Packard 1900 TR, Packard Instruments, Meriden, CT, USA).

2.3. Quantification of specific proteins in cell culture medium

Prior to analysis, the cell culture medium was concentrated five times using Microsep Centrifugal tubes with 3-kDa cut-off (Pall Life Science, Ann Armor, MI, USA).

Multianalyte profiling was performed using the Luminex 200TM system (Luminex Corporation, Austin, TX, USA) and the XY-platform, and acquired fluorescence data were analyzed by the 3.1 x PONENT software (Luminex).

The concentrations of cytokines in cell culture media were determined using the 29-Milliplex Human Cytokine Immunoassay kit (Millipore, Billerica, MA, USA). The cytokines include epidermal growth factor (EGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon alpha-2 (IFN-a2), IFN-g, interleukin-10 (IL-10), IL-12p40, IL-13, IL-15, IL-17, IL-1ra, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1a (MIP-1a), MIP-1b, tumor necrosis factor-a (TNF-a), TNF-b and vascular endothelial growth factor (VEGF). Further, the level of bone markers IL-6, OPG, osteocalcin (OC), leptin, TNF-a, sclerostin, fibroblast growth factor 23 (FGF-23) were determined using Milliplex Human Bone Panel Immunoassay kit, HBNMAG-51K-7plex (Millipore). All analyses were performed according to the manufacturers' protocols.

2.4. Statistical analysis

Statistical evaluation was performed using the software SigmaPlot version 13.0 and 14.0 (Systat Software, San Jose, CA, USA). Statistical significance was assessed by Student's *t*-test and *P*-value set to 0.05, all data groups passed tests for normality and equality.

3. Results

3.1. Viability and proliferation

ALN, OME and ALN + OME induced no changes in cell viability of either cell type tested after three days of incubation compared to controls (Table 1). Administration of ALN and OME reduced the proliferation of osteoblasts at day 1, followed by an enhancement to control levels at day 3 and a reduction at day 7. ALN + OME, however, caused a time dependent reduction in osteoblast proliferation to 34% ($p = 0.001$) of control at day 7. The proliferation of fibroblasts was reduced to 81% ($p = 0.041$) of control when exposed to ALN + OME (Table 2).

Table 1
Lactate dehydrogenase (LDH) activity in cell culture medium presented in % of control after exposure to ALN, OME and ALN + OME.

Osteoblasts						
Day	Alendronate 5 μM	P-value	Omeprazole 1 μM	*P*-value	Alendronate + omeprazole	P-value
1	100.5 ± 2.6	0.872	95.8 ± 2.1	0.140	99.1 ± 0.9	0.492
3	103.4 ± 2.9	0.378	93.3 ± 3.4	0.150	98.1 ± 3.6	0.658
Fibroblasts						
Day	Alendronate 5 μM	P-value	Omeprazole 1 μM	P-value	Alendronate + omeprazole	P-value
1	107.9 ± 2.6	0.070	102.4 ± 2.2	0.442	98.8 ± 1.2	0.609
3	99.4 ± 3.0	0.866	98.9 ± 3.5	0.770	101.1 ± 1.7	0.580

The effect was measured after 1 and 3 days of incubation with 5 μM ALN, 1 μM OME and ALN + OME.

Table 2
Effects of ALN, OME and ALN + OME on cell proliferation, measured by [3H]-thymidine incorporation.

Osteoblasts						
Day	Alendronate 5 μM	*P*-value	Omeprazole 1 μM	P-value	Alendronate + omeprazole	P-value
1	55.9 ± 3.1	0.007	51.4 ± 2.3	0.004	65.6 ± 14.6	0.108
3	103.6 ± 22.2	0.881	140.9 ± 30.6	0.259	58.1 ± 5.6	0.006
7	41.2 ± 2.6	0.001	79.3 ± 6.6	0.08	33.8 ± 3.1	0.001
Fibroblasts						
Day	Alendronate 5 μM	P-value	Omeprazole 1 μM	*P*-value	Alendronate + omeprazole	P-value
1	91.2 ± 2.5	0.037	87.3 ± 3.7	0.031	84.6 ± 6.8	0.090
3	92.7 ± 2.9	0.214	96.7 ± 21.9	0.891	84.2 ± 3.1	0.036
7	98.9 ± 6.6	0.896	92.7 ± 4.1	0.301	81.4 ± 4.3	0.041

Subconfluent cells incubated for 1, 3 or 7 days with 5 μM ALN, 1 μM OME and the drugs combined. Data are presented in % relative to control at each time point.

3.2. Factors affecting angiogenesis

An immediate decline in secretion of VEGF from osteoblasts to 20% of control was observed when incubated with OME ($p = 0.011$), and with ALN + OME to 30% of control ($p = 0.030$), whereas there was no significant effect of ALN (Fig. 1A). Secretion of VEGF from the fibroblasts was more than doubled after 14 days of administration of ALN + OME ($p \leq 0.001$), while at the same time point ALN caused increase in secretion to 170% of control ($p = 0.003$). In contrast, OME alone did not result in any significant changes in VEGF secretion (Fig. 1B). ALN + OME induced a transient reduction in secretion of IFN-g from fibroblasts after one day of incubation ($p = 0.013$). A non-significant rise occurred after exposure to ALN for three days and OME for 14 days (Fig. 1C).

3.3. Factors affecting osteoclastogenesis

After 24 h, the secretion of OPG from osteoblasts incubated with OME was reduced to 55% ($p = 0.005$) and to 45% after incubation with ALN + OME ($p \leq 0.001$) (Fig. 2A). ALN induced a rise in OPG after three days ($p \leq 0.001$). Incubation with OME and ALN + OME for 24 h reduced the secretion of G-CSF from osteoblasts to 50% ($p = 0.033$) and 30% ($p = 0.007$), respectively (Fig. 2B). ALN induced an increase in G-CSF after three days ($p = 0.026$). Both exposure to OME and ALN + OME reduced the secretion of MCP-1 from osteoblasts to 40% of control after one day of incubation ($p \leq 0.001$). ALN induced a rise in MCP-1 after three days ($p = 0.019$) persisting to day 7 (Fig. 2C).

3.4. Factors affecting inflammation

ALN induced a rise in secretion of IL-6 from osteoblasts after three days ($p = 0.008$), whereas a decline to 70% and 50% of control was seen at day three after incubation with OME ($p = 0.010$) and ALN + OME ($p \leq 0.001$), respectively (Fig. 3A). The secretion of IL-8 from osteoblasts was increased after 24 h of exposure to ALN ($p = 0.002$), whereas OME evoked a decrease after three days of incubation. Exposure to ALN + OME promoted a decline in IL-8 after three days ($p = 0.006$), followed by a rise to 160% of control at day seven ($p = 0.021$) (Fig. 3B). OME reduced the release of IFN-a2 from the osteoblasts to 40% ($p = 0.020$), and ALN + OME to 45% ($p = 0.017$) of control after 24 h of incubation. ALN induced a non-significant rise in IFN-a2 after three days (Fig. 3C). ALN, OME and ALN + OME resulted in a near 2-fold increase in the release of IL-13 from fibroblasts after 24 h (Fig. 3D).

Several of the factors tested were not found to change significantly at any time point (leptin, OC, sclerostin, IL-1ra), or the concentrations were lower than the set levels of detection for the analyses (FGF-23, EGF, eotaxin, IL-10, IL-12p40, IL-15, IL-17, IL-1a, IL-1b, IL-2, IL-3, IL-4, IL-5, IL-7, IP-10, MIP-1a, MIP-1b, TNF-a and TNF-b).

4. Discussion

This is the first study to address the effects of concomitant exposure with ALN and OME on cellular functions of human osteoblasts and gingival fibroblasts. We show that the drugs in combination exerted effects that may favor development of ONJ. The combination of ALN and OME induced a marked decline in proliferation of both cell types. The viability of osteoblasts and fibroblasts was unchanged after exposure to ALN, OME or the combination. ALN promoted an initial, transient increase in pro-inflammatory cytokines, whereas OME seemed to act anti-inflammatory. After an initial suppression of IL-8, ALN + OME evoked a rise after seven days. All three drug options stimulated IL-13 release from fibroblasts, followed by a decline. OME both alone and combined with ALN caused an immediate reduction in the secretion of VEGF from the osteoblasts. Moreover, the combination of drugs resulted in a reduced secretion of OPG from osteoblasts and INF-g from fibroblasts.

The majority of cases with MRONJ, including BRONJ, exhibit exposed bone with a duration of more than 8 weeks (Nicolatou-Galitis et al., 2019). Hence, factors that disrupt the process of bone repair may promote ONJ. As a response to physical damage, a cascade of processes

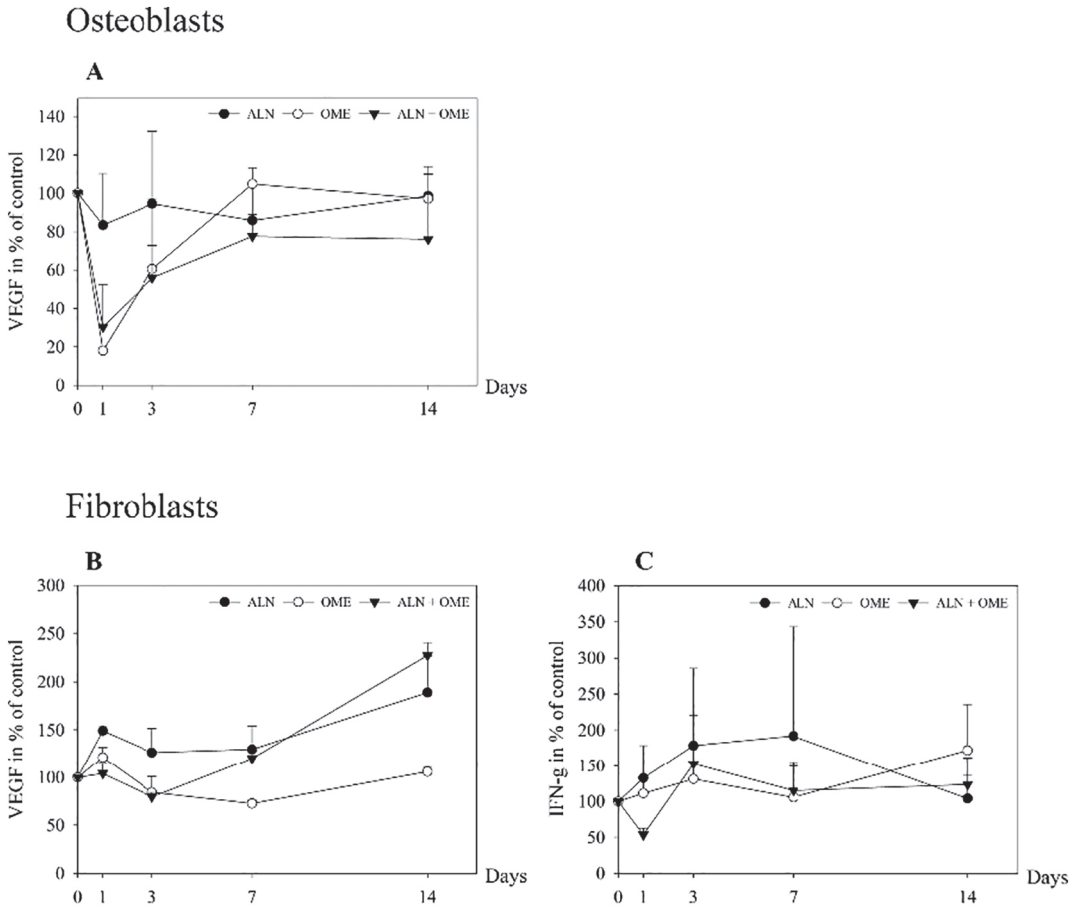

Fig. 1. Measured levels of vascular endothelial growth factor (VEGF) (A) in cell culture media from human osteoblasts and VEGF (B) and interferon gamma (INF-g) (C) levels in cell culture media from human gingival fibroblasts. The cells were exposed to 5 μM ALN, 1 μM OME and ALN + OME. Data are presented in % relative to untreated control at each time point.

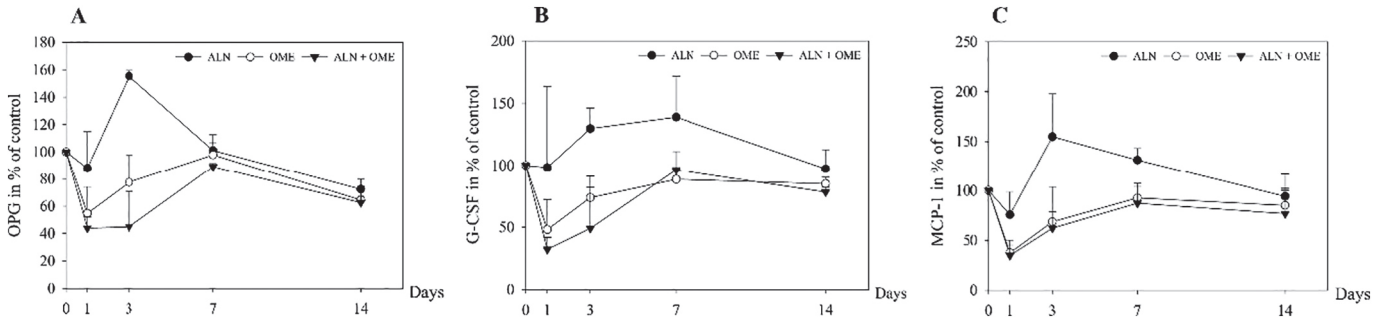

Fig. 2. Measured levels of osteoprotegerin (OPG) (A), granulocyte colony-stimulating factor (G-CSF) (B) and monocyte chemoattractant protein (MCP-1) (C) in cell culture media from human osteoblasts. The cells were exposed to 5 μM ALN, 1 μM OME and ALN + OME. Data are presented in % relative to unexposed control at each time point.

is induced to promote bone healing. This is accomplished through complex interactions between inflammatory cells, fibroblasts, osteoblasts, and cells of the monocyte-macrophage-osteoclast lineage. Acute inflammation is the first stage of bone repair, as signified by release of proinflammatory cytokines, including IL-6 (Loi et al., 2016). Thereafter angiogenic and osteogenic pathways are activated. The multifunctional cytokine IL-6 is also involved in promotion of angiogenesis and it seems to be crucial for all stages of fracture healing (Imaculada de Queiroz Rodrigues et al., 2020).

VEGF is released from both osteoblasts and fibroblasts to support the upregulation of angiogenesis (Hu and Olsen, 2016). Furthermore, fibroblasts also increase the secretion of TGF-b, underlining the cells' key role in the formation of new blood vessels (Kellouche et al., 2007). A satisfactory vascularization is a prerequisite for osteogenesis, thus failing to promote this escalation in angiogenesis could result in incomplete healing (Dickson et al., 1994). Notably, VEGF also plays a role in coupling of angiogenesis and osteogenesis, and stimulates bone formation and remodeling (Grosso et al., 2017).

We observed that exposure to ALN induced a rise in several proinflammatory cytokines, including IL-6 and IL-8 from osteoblasts and IL-13 from fibroblasts. This concords with in vivo studies, showing elevation of pro-inflammatory cytokines after treatment with ALN (Morita et al., 2017). IL-13 has been shown to stimulate the formation of the pro-inflammatory cytokine IL-6 (Frost et al., 2001). Markedly increased

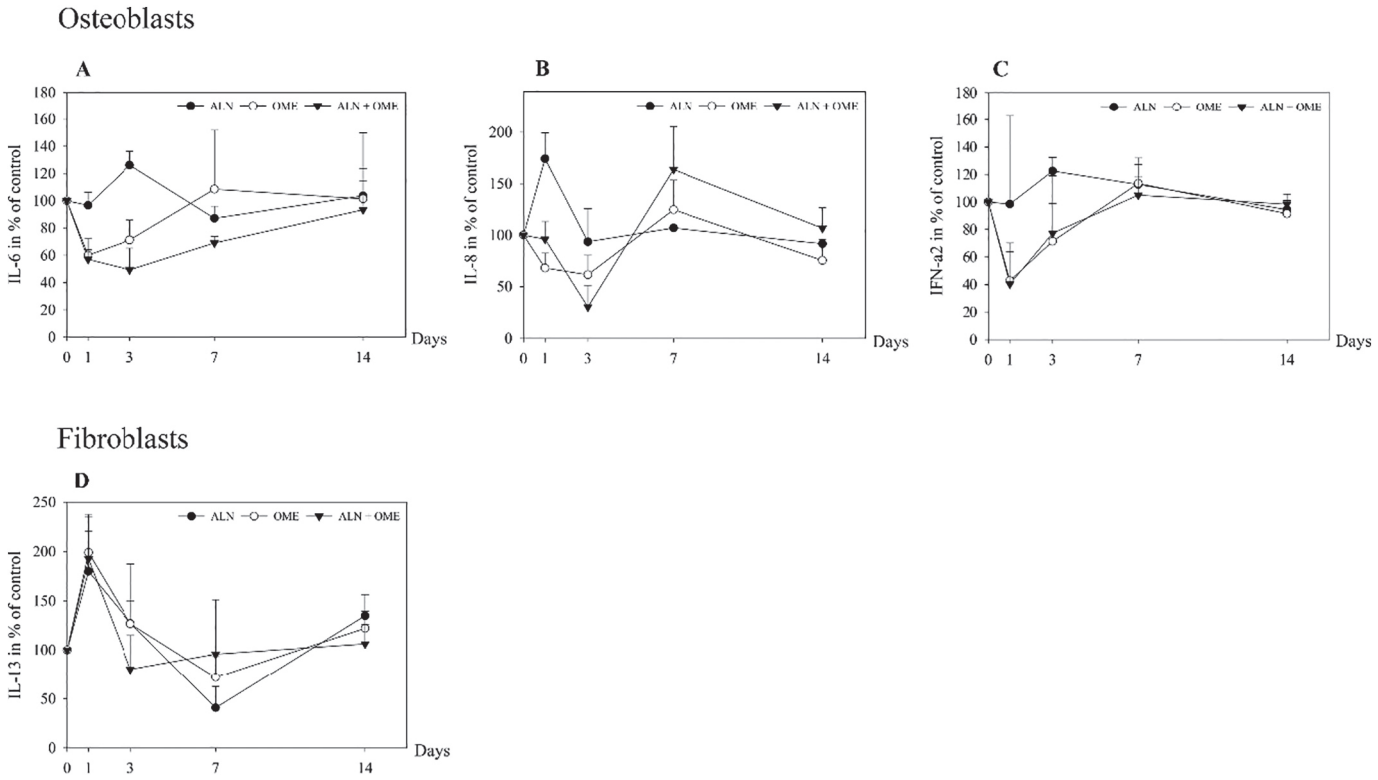

Fig. 3. Measured levels of interleukin-6 (IL-6) (A), interleukin-8 (IL-8) (B), interferon-alpha2 (IFN-a2) (C) in media from human osteoblasts and interleukin-13 (IL-13) (D) in cell media from human gingival fibroblasts. The cells were exposed to 5 μM ALN, 1 μM OME and ALN + OME. Data are presented in % relative to untreated control at each time point.

plasma and salivary levels of IL-6 have been reported in advanced stages of BRONJ (Bagan et al., 2014).

As elaborated on, an initial inflammation also occurs in the bone healing process. Thus, we speculate that the transient rise promoted by ALN may not interfere with bone repair. However, in the case of the systemic and sustained inflammation induced by diseases such as diabetes and rheumatoid arthritis, bone healing may be impaired or delayed, and risk of non-unions is increased (Claes et al., 2012). This concords with the increased risk of BRONJ observed in diabetes patients treated with BPs (Peer and Khamaisi, 2015). Moreover, it is reasonable that BPs may provoke a more pronounced and persistent inflammation in some individuals, hence impeding bone healing. Indeed, inflammation is a hallmark of BRONJ and of ONJ in general (Chang et al., 2018).

Several studies report enhancement of VEGF secretion from osteoblasts and gingival fibroblasts after exposure to BPs (Yuan et al., 2019; Manzano-Moreno et al., 2018). We observed no initial effect on angiogenic factors after exposure of osteoblasts to ALN alone. A rise in VEGF from fibroblasts was seen after 14 days of exposure to ALN and the combined drugs. We interpret this as a secondary response evoked by IL-13, as this cytokine has been shown to stimulate VEGF (Lee et al., 2004). Hence, this may be a mechanism to counteract impairment of angiogenesis, and to favor osteogenesis. Likewise, the increment in IL-6 and IL-8 release from osteoblasts induced by ALN could play a role in maintenance of angiogenesis (Martin et al., 2009; Kayakabe et al., 2012).

Given that these findings translate to in vivo conditions, exposure to ALN alone would affect bone healing modestly. In support of this, a meta-analysis including eight eligible randomized controlled trials with 2508 patients, showed no clinically detectable delay to fracture healing in users of BPs (Xue et al., 2014). It has been postulated that some are predisposed to develop BRONJ in general. Accordingly, a recent study by Lee et al. suggested that patients with impaired function of angiogenesis, osteoclast activity and tissue repair treated with high dose BPs were more prone to BRONJ (Lee et al., 2019). In support of this, some of the candidate genes identified by genome-wide association studies were *TGF-b*, *MMP2*, *PPARG*, *CYP2C8*, *VEGF*, *COL1A1*, *RANK* and *OPG* (Lee et al., 2019).

In contrast to ALN, OME and the combination of drugs promoted transient, inhibitory effects on proinflammatory cytokines (IL-6, IL-8, IFN-a2, MCP-1 and G-CSF) secreted from osteoblasts, whereas an early rise occurred in IL-13 release from fibroblasts. As elaborated on above, IL-13 has been shown to stimulate both IL-6 and VEGF (Frost et al., 2001). Finally, a substantially increased secretion of IL-8 occurred after 7 days of exposure to OME and ALN + OME. IL-8 is one of the major mediators of inflammation (Kany et al., 2019). An initial decline in the angiogenic factors VEGF, IFN-g and IL-6 was induced by OME and the combination with ALN, accompanied by an early rise in IFN-g and a late rise in IL8 and VEGF.

The effects exerted by OME and the combination of drugs could potentially induce impairment of all stages of bone healing. This is supported by a study in mice showing that the PPI pantoprazole affected fracture repair adversely (Histing et al., 2012). Moreover, Subaie et al. demonstrated that omeprazole had a negative effect on bone healing and osseointegration of titanium implants in a rat model (Al Subaie et al., 2016). In both studies, these effects seemed to be mediated through inhibition of bone formation and bone remodeling. It is reasonable that similar mechanisms apply in bone repair and regeneration of the jaw. The interference with factors involved in inflammation, angiogenesis and osteogenesis could under certain circumstances like dental extraction promote ONJ. It should be recalled that ONJ also has been reported in a patient using only PPI (Marconcini et al., 2019), and that the use of PPIs in patients on ALN therapy was linked with surgically treated BRONJ (Eiken et al., 2017).

Factors that affect osteoblast proliferation and differentiation could also interfere with bone healing. Data on the effect of BPs on proliferation of osteoblasts and osteoblast-like cells are diverging. In the present

study, ALN induced an initial decline in the proliferation of osteoblasts, followed by an increase after 3 days, and a decrease after 7 days. Koch et al. reported that exposure to zoledronate decreased the proliferation of human osteoblasts (Koch et al., 2010). In contrast, several studies have shown that ALN and other BPs enhance proliferation of osteoblasts and osteoblast-like cells (Krüger et al., 2016; Xiong et al., 2009). This discrepancy may be attributed to differences in the dosage applied and the time points studied. Based on previous in vitro studies, we used a concentration of 5 μM ALN, considered to be in the therapeutic range (Scheper et al., 2009), whereas Koch et al. used 50 μM.

OME evoked a similar pattern as ALN with a transient reduction in proliferation, a rise after 3 days, and a decline after 7 days. Costa-Rodrigues et al. reported that exposure to OME induced an inhibition of both osteoblast proliferation and differentiation (Costa-Rodrigues et al., 2013). Notably, they found a significantly reduced rate of proliferation after exposure to concentrations of 10 μM and higher. In comparison, we used a concentration of 1 μM considered to be at a clinically relevant level (Costa-Rodrigues et al., 2013). ALN and OME in combination induced a decline in osteoblast proliferation at all time points. ALN at a dosage of 2.5 μM has previously been shown to increase proliferation in gingival fibroblasts after one week of exposure (Acil et al., 2018). In the present study, neither ALN, nor OME seemed to affect proliferation of these cells. No previous reports on the effect of OME or other PPIs on fibroblasts were found. The suppression of proliferation of gingival fibroblasts after exposure to both drugs, could result in prolongation of the healing of mucosal and gingival lesions, theoretically stressing the underlying bone (Ravosa et al., 2011).

Osteoclasts are essential for bone homeostasis and fracture healing, and inhibition of osteoclast differentiation has been reported to delay bone healing (Bahney et al., 2019). The OPG/RANKL system plays a major role in osteoclastogenesis. OPG is a decoy receptor that binds RANKL, thereby preventing the binding of RANKL to its receptor RANK and thus reducing the development of osteoclasts (Boyce and Xing, 2007). Several studies, both in vitro and in vivo have reported that BPs promote OPG release from osteoblasts, whereas some observed no effects on the RANKL/OPG system (Kim et al., 2002). However, ibandronate and zoledronate were shown to induce a pronounced enhancement of the RANKL gene expression in human osteoblasts, whereas OPG gene expression was moderately increased (Koch et al., 2012). In concordance with this, we observed that ALN stimulated OPG secretion. On the other hand, OME and the drugs in combination caused an immediate reduction of OPG to less than 50% of control. A similar prompt reduction also occurred in secretion of IFN-g from osteoblasts after exposure to OME and ALN + OME, whereas ALN promoted a rise. The attenuation of both OPG and IFN-g release induced by the combined drugs implies favoring of osteoclastogenesis (Kak et al., 2018). ALN promoted release of factors with opposite effects on osteoclastogenesis, OPG and IFN-g acting inhibitory, and G-CSF and MCP-1 exerting stimulatory effects (Christopher and Link, 2008; Mulholland et al., 2019). The significance of these observations under in vivo conditions is difficult to rule out.

Taken together, the effects evoked by the combined exposure of ALN and OME seemed to be more pronounced than of each drug alone, and may translate to impairment of immune responses, angiogenesis, and osteogenesis in vivo. Under given circumstances, like dental extraction, these changes may affect tissue remodeling and repair adversely, and in some cases lead to BRONJ. The mechanisms revealed may explain the increased risk of ONJ in patients receiving concomitant therapy with BPs and PPIs.

Moreover, recent cohort studies on dental implant outcomes have suggested that PPI intake is associated with an increased risk of implant failure (Altay et al., 2019). Notably, PPI use has also been associated with atypical femoral fracture, another rare adverse effect of BPs (Larsen and Schmal, 2018; Yang et al., 2015). It is reasonable that some of the same mechanisms apply in the pathogenesis of the two conditions. Our findings support the notion that PPIs may further exaggerate the molecular mechanisms that favor development of BRONJ or actually MRONJ. It should be recalled that PPIs also seem to blunt the fracture-reducing effect of ALN (Yang et al., 2015; Abrahamsen et al., 2011). Hence, in patients experiencing gastro-intestinal side effects of oral ALN, treatment with an IV BP should be considered instead of adding a PPI.

We acknowledge that our findings not necessarily translate to an in vivo setting. Most of the effects evoked by the different drug options were short-term, and it may be questioned whether they have pathophysiological consequences in vivo. It is, however, reasonable that the duration of these effects is different and may be more sustained under in vivo conditions. Moreover, the alterations promoted by the drugs are in correspondence with several in vivo studies addressing ONJ (Morita et al., 2017; Xue et al., 2014). Ideally, osteoblasts derived from the mandible should have been applied, as osteoblasts from tibia and the mandible seem to behave a little differently with respect to bone turnover and functionality (Huja and Beck, 2008; Marolt et al., 2012). Furthermore, it would have been beneficial with more than one donor of osteoblasts. We used human primary cells, thus ensuring a cell behavior that reflects the in vivo niche, and that has more preclinical and clinical applicability. The concentration of the drugs was based on what is considered to be the therapeutic level. However, with the relatively high turnover of bone in the jaws and BPs strong affinity for bone matrix, an exact measure of local exposure to the drug is uncertain at best (Khan et al., 1997).

5. Conclusion

The combination of ALN and OME seemed to exaggerate the negative effects of each drug alone on human osteoblasts and gingival fibroblasts. The modulation of pro-inflammatory cytokines, impairment of angiogenesis, and anti-proliferative effects may induce conditions in periodontal tissue favoring development of osteonecrosis. Additional studies are needed to evaluate the clinical relevance.

CRediT authorship contribution statement

Krüger TB: main author; done the experiments, evaluated the results and drafted the manuscript, **Herlofson BB**: manuscript revisions and evaluation of results, **Lian AM**: Luminex analysis, **Syversen U**: manuscript revisions and evaluation of results, **Reseland JE**: designed the study, evaluation of results, supervised the experiments, manuscript revisions.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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