Chapter 7.

General discussion
Bone resorption by osteoclasts is often seen as the initial step of bone remodeling. Together with the other bone cells, i.e. osteoblasts, osteocytes, and bone-lining cells, osteoclasts are essential to maintain bone and mineral homeostasis. The last decades, it has become clear that osteoclasts are bone-site specific, a phenomenon termed osteoclast heterogeneity [1,2]. When osteoclastic bone resorption outbalances bone formation, bone is lost and the skeleton becomes more fragile. In degenerative bone diseases, such as osteoporosis and bone cancer, bisphosphonates (BPs) are widely used as a treatment to counteract excessive bone resorption by causing osteoclast apoptosis and inhibiting resorptive activity.

BP use is associated with a rare, though severe side effect in the jaw bone: osteonecrosis of the jaw (ONJ), which is defined as exposed necrotic bone that does not heal for at least 8 weeks [3,4]. Depending on the dose, prevalence of BP-related ONJ is estimated between 0.1 and 10% for people that receive high doses for cancer treatment [5,6]. Risk factors for this condition are dental extractions and other oral trauma, however it is not clear why specifically the jaw is negatively affected by BPs. With the increasing life expectancy these drugs will be subscribed more often, and the side effects will become an emerging problem. Gaining more insight into the pathogenesis of ONJ is therefore highly relevant for researchers in the fields of bone and dentistry, the clinicians subscribing BPs, the patients using BPs, and the dentists and maxillofacial surgeons treating those patients.

In this thesis, we investigated the relation between two bone-site-specific phenomena: osteonecrosis of the jaw and osteoclast heterogeneity. We hypothesized that bisphosphonates may have a different effect on osteoclasts and their precursors in the long bones and in the jaw. We used in vitro and in vivo approaches to investigate this hypothesis and revealed novel findings that will be discussed in this chapter. The major findings are summarized in Table 1 (the effect of BPs) and Table 2 (gene expression in long bone and jaw osteoclast precursors).

First, we used a fluorescently labeled BP and investigated its internalization by long bone and jaw osteoclast precursors in vitro (chapter 2). Jaw osteoclast precursors internalized more BPs than long bone osteoclast precursors, both after 4 and 24 hours. After 24 hours, this was accompanied by the accumulation of more unprenylated Rap1a in jaw cells. Surprisingly, this did not result in a different effect on osteoclastogenesis. These findings indicate that jaw osteoclast precursors might be less sensitive to BPs than long bone osteoclast precursors after BP uptake. A possible explanation for this might be the lower activity of the apoptosis marker caspase 3/7 in jaw osteoclast precursors than in long bone osteoclast precursors after BPs. Therefore, we propose that jaw osteoclast precursors are more resistant to BP-induced apoptosis than those from long bone.
Table 1. The effect of bisphosphonates (BPs) on long bone and jaw osteoclasts. Overview of the main findings in this thesis. + indicate a relative stimulation (or levels of internalization/accumulation), - indicate an inhibition, and ++ means that there was no effect.

<table>
<thead>
<tr>
<th>Effect BPs</th>
<th>Long bone</th>
<th>Jaw</th>
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<tbody>
<tr>
<td></td>
<td>In vitro</td>
<td></td>
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<tr>
<td>BF internalization</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>uRap1a accumulation</td>
<td>+</td>
<td>++</td>
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<tr>
<td>Apoptosis</td>
<td>++</td>
<td>+-</td>
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<tr>
<td>Bone resorption</td>
<td>--</td>
<td>-</td>
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<tr>
<td>Osteoclast numbers</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>In vivo</td>
<td></td>
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<tr>
<td>Bone marrow cells</td>
<td>+ -</td>
<td>-</td>
</tr>
<tr>
<td>Osteoclast numbers</td>
<td>+ -</td>
<td>+ -</td>
</tr>
<tr>
<td>Bone formation</td>
<td>--</td>
<td>-</td>
</tr>
<tr>
<td>BV/TV</td>
<td>++</td>
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<td>TMD</td>
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Several mechanisms may explain lower apoptotic activity in jaw than in long bone osteoclast precursors. Jaw osteoclast precursors expressed a higher level of anti-apoptotic genes B cell lymphoma 2 (Bcl-2) and Bcl-2-like protein 1 (Bcl-xL) than long osteoclast precursors. Yet, the pro-apoptotic gene Bcl-2-associated X protein (Bax) was similarly expressed in both cell types, leading to a higher anti-apoptotic to pro-apoptotic gene expression ratio in jaw cells (chapter 2, Table 2). This ratio was previously shown to be increased by CXCL12 [7], a chemoattractant for osteoclast precursors and other cells, highly expressed in the bone marrow [8]. Since we showed in chapter 6 that jaw osteoclast precursors expressed more CXCL12, as well as its receptor CXCR4, a similar mechanism may apply to our findings. The exact mechanism behind higher anti-apoptotic gene expression and lower caspase 3/7 activity in jaw bone marrow cells, and a possible resistance to BPs, requires further research. Besides exploring the response of jaw and long-bone marrow cells to CXCL12 in more detail, the effect of two key regulators of osteoclast formation could be investigated. Since macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor κB ligand (RANKL) were shown to induce Bcl-2 and Bcl-xL expression [9], they are interesting anti-apoptotic candidates in this regard.
Another question remaining after our internalization studies is which cell type within the bone marrow internalized more BPs. Long bone and jaw bone marrow cells were shown to have a different composition and freshly isolated long-bone marrow contained more osteoclast precursors than jaw bone marrow [10]. Also, the differentiation stage of the long bone and jaw osteoclast precursors at day 3 of culture is different [10], which likely results in a different cellular composition of the osteoclast cultures. Uptake by cells other than osteoclast precursors may be another explanation why despite a different uptake, osteoclastogenesis from jaw and long-bone marrow was similarly affected by BPs. In chapter 2, we did not detect different levels of the osteoblast marker ALP, and the macrophage markers F4/80, MafB, and Irf8 in long bone and jaw bone marrow cultures at the end of the culture period of 6 days with M-CSF and RANKL. Yet, in an earlier stage of differentiation, after 3 days of culture with M-CSF and RANKL and when BP uptake was studied, jaw bone marrow cultures expressed more MafB and F4/80 than long-bone marrow (chapter 6), which could imply that jaw cultures have a more macrophage-like phenotype than long bone osteoclast cultures. Since macrophages are highly endocytic cells, BP uptake by macrophages might explain why jaw bone marrow cultures internalize more BP than those from long bone, and also explains why the actual precursors were similarly affected by BPs. Yet, in chapter 2, we distinguished between small and large cells, and both populations internalized more BPs in jaw cultures. Since macrophages likely belong to the small cell population, strong BP uptake by macrophages cannot fully explain higher BP uptake by jaw osteoclast cultures. Moreover, uptake of a fluid-phase endocytosis marker, dextran [11], was similar in long bone and jaw cells indicating that they have similar rates of

### Table 2. Gene expression in long bone and jaw osteoclast precursors (unless mentioned otherwise).

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>Long bone</th>
<th>Jaw</th>
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<tbody>
<tr>
<td>Bcl-xL&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Bcl-2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>CXCL12&lt;sup&gt;*&lt;/sup&gt;</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Bax</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MafB</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>MafB (mature)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F4/80</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>F4/80 (mature)</td>
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fluid phase endocytosis. Therefore, our findings suggest that long bone and jaw osteoclast precursors have distinct mechanisms of BP uptake specifically and therefore, these data provide more evidence that osteoclast precursors from long bone and jaw are different.

A third explanation why jaw osteoclasts might be less sensitive to BPs, may be a compensation mechanism during the process of osteoclastogenesis. Osteoclast formation consists of several steps, starting with the attraction and migration of osteoclast precursors towards the target site. There the precursors interact with bone-lining cells and other bone-associated cells, and they fuse to become multinucleated osteoclasts. In chapter 5, we used time-lapse microscopy and proved it to be a useful tool to study osteoclastogenesis. We showed that next to fusion, osteoclasts can undergo fission and may be able to get rid of apoptotic nuclei. Possibly, using this mechanism, osteoclasts can also get rid of their cellular compartments that became apoptotic due to BP treatment and therefore compensate for the induced apoptotic pathways.

Using time-lapse microscopy, we also studied other steps during osteoclastogenesis and investigated whether long bone and jaw osteoclast precursors differ in their rates of proliferation, migration, and fusion; three important steps during osteoclastogenesis (chapter 6). All rates were similar for long bone and jaw osteoclast precursors and surprisingly, in neither of the populations, the migration velocity was affected by the BP pamidronate. The used nitrogen-containing BP inhibits the prenylation, and thereby the cellular location and function of small GTPases. Those proteins are important for cytoskeletal rearrangements, and therefore play a role in cell adhesion and migration. In our system using time-lapse microscopy and a non-toxic BP concentration (10 µM), we did not find an effect on migration velocity. A drawback of this system is the substrate that was used. Carbon-coated chamber slides that were used for time-lapse microscopy were shown to improve fibroblast cell adhesion [12]. Adhesion to this substrate by osteoclast precursors and culture conditions for those cells, may not have been optimal. Although osteoclastogenesis did take place, the number of fusion events varied between experiments. Therefore, it would be worthwhile to optimize these osteoclastogenesis cultures on carbon-coated chamber slides. Another interesting improvement, resembling the in vivo situation more closely, would be to coat the slides with hydroxyapatite and image the cells on this substrate. To circumvent culturing on chamber slides, the effect of BPs on directional migration towards CXCL12 could also be investigated in the transwell culture system that we used. Yet, as far as we know, we are the first to study the effect of nitrogen-containing BPs on migration velocity of primary bone marrow cells. We did not find a BP concentration that inhibited migration without being toxic to the cells. This may indicate that BPs do not affect osteoclast precursors migration, and do therefore not interfere with osteoclastogenesis at this differentiation stage.
Altogether, our results showed that jaw osteoclasts or precursors may be less sensitive to BPs than long bone osteoclasts or their precursors. Most likely, this was caused by higher anti-apoptotic gene expression and lower caspase 3 activity in jaw than in long bone osteoclast precursors (chapter 2), which might have been the result of higher CXCL12 expression in the jaw than in long bone osteoclast cultures (chapter 6).

In chapter 3, we investigated whether BPs differently affected osteoclasts in the long bones and jaw in vivo. Zoledronic acid (ZA) was administered to 3-month-old female C57BL/6J mice for up to 6 months. At baseline, and after 1, 3, and 6 months of treatment, mice were sacrificed and bone marrow cells were isolated from the mandible, femur, and tibia. Interestingly, after 6 months, significantly fewer cells were isolated from the jaw from BP-treated mice than from controls, whereas the number of long-bone marrow cells was unaffected by BP treatment. This might have been the result of an increased jaw bone volume to over 90% after BPs, which was highly likely accompanied by a decreased bone marrow space. Another explanation could be that BPs are more toxic to the bone marrow cells in the jaw than in the long bones. Although we showed in chapter 2 that jaw bone marrow cells might be less sensitive to BPs than long-bone marrow cells, they may be exposed to higher BP concentrations in vivo. Jaw and long bones from rats were shown to contain a similar amount of BPs per unit of dry weight or calcium [13,14]. Since the jaw contains more mineral per bone volume ([14], chapter 3), it likely adsorbed more BPs than long bones. Together with the finding that jaw osteoclasts, in vitro, resorb at least as much bone or dentin as long bone osteoclasts ([10], chapter 2), more BP is likely released from the jaw than from long bone, making it available for the surrounding cells in the bone marrow [15,16]. The resulting high local BP concentration might cause apoptosis of bone marrow cells, leading to a reduced number of jaw bone marrow cells.

Which subset of bone marrow cells internalized BPs and which subset was affected by 6 months of ZA requires further investigation. This could be done by staining of specific markers for different bone marrow cells in decalcified sections. Monocytes are promising candidates in this regard, as they were shown to internalize BPs in vivo [17]. Furthermore, a role for diminished macrophage activity after BP treatment was previously proposed to play a role in ONJ [18]. This hypothesis, together with our findings, may provide more insight into the pathogenesis of ONJ, and suggest a combination of hypotheses to play a role in ONJ (chapter 1), as illustrated in Figure 1. We suggest that BP release and the resulting high local BP concentration due to low sensitivity of jaw osteoclasts to BPs, play a more central role in the pathogenesis of ONJ. This hypothesis provides a strong connection between the previously suggested hypotheses of microbial infection and the toxicity of BPs to cells other than osteoclasts (Figure 1).
Another hypothesis regarding the etiology of ONJ is the oversuppression of bone turnover. A reduced bone remodeling capacity and reduced ability to repair bone damage can lead to the accumulation of microdamage [19,20]. Our results however, do not support a long-term, reducing effect on bone turnover markers in the jaw, whereas bone turnover was inhibited in the long bones (chapter 3). Also, the bone surface covered with osteoclasts and osteoclast numbers increased with age in mice only in long bone, to levels that were 2 to almost 4 times higher in the long bones than in the jaw. Therefore, our results suggest that suppression of bone turnover and the resulting microdamage would more likely cause side effects in the long bones than in the jaw. Indeed, BPs were recently associated with atypical femur fractures that are probably caused by the inability to heal microcracks [21].

**Figure 1.** New hypothesis for BP-related ONJ (squares), where the BP release (purple square) due to the mild effect of BPs on jaw osteoclasts specifically plays a central role. A possible toxicity to immune cells and a reduced ability to clear microbial infections provides a link between the 2 previously proposed hypotheses (black boxes). ONJ (in the blue square) consists of 3 events shown in the squares within the blue box. Green arrows represent positive effects, red blunted arrows show inhibitions. The blue arrows point out a result of the inhibitions indicated in red. The dashed arrows with question marks are speculative, since positive effects of BPs on osteoblasts and osteocytes have also been shown.
Dental trauma such as a tooth extraction was reported to be a risk factor for ONJ, however, a causal relationship has never been shown [22]. In our model, we did not perform a tooth extraction and the mice did not develop any signs of ONJ. However, we did find BP-induced osteoclast activity at the molar roots, despite the presence of bisphosphonates. An explanation for this may be that, in contrast to BP adsorbed to the alveolar bone, little BP seems to accumulate at the root cementum and therefore osteoclasts formed at this site were hardly or not exposed to BPs [23]. The tooth loss that might result from osteoclast activity at the root, may be related to the reported relation between tooth extraction and ONJ. It would be of interest to investigate a possible causative role for root resorption and tooth loss in the pathogenesis of ONJ. Therefore, it requires further investigation whether BP use, for instance by applying a longer administration or a higher dose, can result in tooth loss caused by root resorption, and whether this could eventually lead to osteonecrosis of the jaw. Altogether, we reported a stimulating, rather than an inhibiting effect of BPs on osteoclasts at the molar root, thereby revealing another bone-site-specific effect of BPs.

The osteoclasts that were formed at the molar roots of BP-treated mice were located in the periodontal ligament (PDL). Previously, PDL fibroblasts were shown to induce osteoclast formation in a co-culture with osteoclast precursors from peripheral blood mononuclear cells (PBMCs) [24,25]. Also, PDL fibroblast viability was reduced by BPs in vitro [26,27] and in our in vivo study, the periodontal area was only mildly affected by BPs (chapter 3). Therefore, we investigated whether PDL fibroblasts may have played a role in the observed osteoclast formation at the molar root after BP treatment (chapter 4). Molar-root-associated human PDL fibroblasts were allowed to attach to 48-well plates and subsequently pre-treated with pamidronate (1-100 µM) or vehicle for 24 hours. After this pre-incubation, the number of PDL fibroblasts and their viability seemed to be unaffected. BPs were washed away with PBS, and freshly isolated PBMCs were added. After 21 days, the number of osteoclasts was counted. Although directly after a 24-hour treatment the PDL fibroblasts were not affected, after long-term culturing, pre-treatment with 100 µM pamidronate induced PDL fibroblast death. This resulted in a completely abolished osteoclastogenesis in co-culture with PBMCs. Lower concentrations did not affect PDL fibroblasts or their effect on osteoclastogenesis. These data may indicate that non-toxic BP concentrations do not affect osteoclastogenesis through PDL fibroblasts in vitro.

In vivo, BP administration increases bone volume and therefore likely increases compressive loading on the PDL (chapter 3). Previously, PDL fibroblasts under compressive loading were shown to induce osteoclastogenesis [28] and a similar mechanism might play a role in BP-induced root resorption. Studying the effect of BPs on mechanically
loaded PDL fibroblasts, might therefore give us more insight into the mechanism behind BP-induced root resorption. It also requires further investigation whether in vivo, PDL fibroblasts are exposed to BP concentrations that may affect PDL-mediated osteoclastogenesis.

**Conclusion**

Taken together, the data presented in this thesis suggest that bisphosphonates similarly affect long bone and jaw osteoclasts in vitro and in vivo. Interestingly, this similarity was likely the result of different mechanisms of action (summarized in Table 1). More BPs accumulated in jaw osteoclasts and their precursors than in those from long bone. Yet, jaw osteoclast precursors likely compensate for the higher internal BP concentration by using anti-apoptotic pathways. We provided mechanistic insight into this hypothesis by showing higher expression of CXCL12 in jaw osteoclast precursors, which was previously shown to inhibit apoptosis in osteoclasts. These data provide further evidence that the osteoclasts and their precursors are bone-site specific. How these differences might provide more insight into the pathogenesis of osteonecrosis of the jaw is explained below.

We hypothesize that the more apoptosis-resistant jaw osteoclasts can survive longer than long bone osteoclasts in the presence of BPs. Therefore, jaw osteoclasts might absorb more BPs from the bone, thereby making them available for uptake by other surrounding cells. BP toxicity to monocytes and macrophages, epithelial cells, endothelial cells, osteocytes, and osteoblasts may all contribute to the pathogenesis of ONJ (Figure 1). Thus, the previously postulated hypothesis that BPs may be toxic to other cells, may be explained by the resistance of jaw osteoclasts to BP-induced apoptosis that we reported here. This resistance also explains the paradoxical finding of bone loss in osteonecrosis of the jaw, despite the presence of BPs and subsequent inhibition of osteoclasts. Furthermore, the toxicity to immune cells may reduce the ability to clear infections, thereby supporting the other hypothesis that an infection may cause ONJ. Therefore, this multifactorial disease consisting of soft tissue damage, bone necrosis, and bone loss, probably originates from multifactorial causes (Figure 1). However, our findings contradict the hypothesis that oversuppression of bone turnover may explain its etiology, since bone turnover was inhibited in the long bones, rather than in the jaw.

Our data imply that further research is required to fully elucidate the effect of BPs on osteoclasts and surrounding cells at different bone sites. Uptake by those cells in vivo should be assessed to gain more insight into our hypothesis that a high local BP concentration in the jaw microenvironment plays a more central role in the pathogenesis of ONJ. Also, the effect of RANKL inhibitors on bone-site-specific cells should be investigated, as denosumab was also shown to increase the risk for ONJ. Moreover, the finding that jaw
bone marrow cells may contain more CXCL12 is intriguing, and suggests that more research could focus on the role of bone-site-specific bone marrow cells and osteoclasts in terms of the hematopoietic stem and progenitor cell pool.

This thesis provides additional evidence for bone-site specificity of osteoclasts and their precursors in the bone marrow. Therefore, these studies may contribute to the development of more specific anti-resorptive drugs. To achieve this, more research is necessary to gain more insight into the differences between bone-site-specific osteoclasts and other bone cells.
References