CHAPTER 7

Summary and General discussion
This thesis focuses on the effect of inflammation on bone tissue. In Chapter 1 the characteristics and pathogenesis of inflammatory bowel disease (IBD) and osteoporosis are introduced. IBD comprises primarily two disorders: Crohn’s disease (CD) and ulcerative colitis (UC). Although IBD primarily involves a chronic inflammation of the bowel, multiple other organ systems may be affected, including the bones and joints. Osteopenia and osteoporosis are diseases characterized by bone loss, and are relatively common in IBD patients. Factors contributing to bone loss in IBD patients include malabsorption and vitamin D deficiency, disturbances of calcium homeostasis, sex hormone deficiency, low body mass index, and drug use, e.g. glucocorticosteroid use. The importance of the inflammatory process itself in IBD-associated bone loss has gradually become evident since the emergence of the scientific field of osteoimmunology. Currently more and more complex networks of cytokine interactions are thought to be involved in the pathogenesis of bone loss in IBD. This concept of bone loss in IBD has mainly been achieved through the use of animal models, or relies on extrapolation from data on the pathogenesis of other inflammation-induced bone diseases.

This thesis addresses the etiopathogenesis of bone loss in patients with IBD, in particular with CD, from an interdisciplinary perspective. The objectives of this thesis are (1) to explore bone metabolism in patients with IBD at the tissue level, and (2) to explore bone metabolism in patients with IBD at the cellular level. In this chapter the main findings of this thesis are summarized and discussed. Subsequently, findings at the tissue level and cellular level are integrated. In the light of future directions, at the end of this chapter recommendations for further research are presented, including speculations on new treatment modalities for IBD-associated bone loss.

Bone metabolism in IBD patients at the tissue level

Summary | Chapter 2 describes a histomorphometric study in which bone mass and structure, bone formation, bone resorption and osteocyte apoptosis were assessed in transiliac bone biopsies obtained from patients with CD in a quiescent state of disease activity. In comparison to data from age and sex-matched healthy controls, quiescent CD patients showed a reduction in trabecular bone mass that was characterized by thinning of trabeculae. The extent of bone loss was more severe in male CD patients than in female patients, and additionally characterized by loss of trabeculae. The CD-associated bone loss was shown to be caused by a reduced bone formation. Osteoclast number and surface seemed to be low in comparison to data obtained from literature, and as such suggested a reduced resorption as well. Osteocyte density and apoptosis were normal, but the percentage of empty lacunae was increased in CD patients. As empty lacunae mark sites where osteocytes have died previously, a possible causative factor for the observed reduction in bone remodeling was suggested to be a decreased osteocyte viability in the patients’ past.

In Chapter 6 a randomized, placebo-controlled trial is described. Bone micro-architecture and bone remodeling were assessed in a population of quiescent CD patients with osteopenia after treatment with risedronate, a potent inhibitor of bone resorption, or placebo. Both groups received in addition calcium and vitamin D supplementation. Bone volume remained unchanged over
the 2-year follow-up period in bone biopsies of both the risedronate and placebo group. Bone structure improved in risedronate-treated CD patients, as reflected by an increase in trabecular thickness. Bone remodeling showed a marked reduction by risedronate treatment and was moderately reduced by calcium and vitamin D supplementation alone. In view of the observed effects of risedronate on bone structure and bone turnover, risedronate is being sufficiently absorbed by the mucosa of patients with CD. Taken together these findings indicate that risedronate is effective in treating bone loss in quiescent CD patients with a mild bone mass deficit.

**Discussion**

Extensive research on bone loss in IBD patients during the last decades showed that patients with a more severe disease course, glucocorticoid treatment, and/or a postmenopausal status are at increased risk of developing osteoporosis. The histomorphometric study described in Chapter 2 revealed low bone mass in CD patients in a quiescent state of disease. Interestingly, a high variability between patients was observed. CD patients by definition are part of a heterogeneous population differing with respect to location of the disease in the inflammatory tract, the number and severity of relapses in the past, time since the last exacerbation, and medication use, amongst many other variables. All these factors together define the net effect of the disease on bone health. Unfortunately, in this study we were not able to define the specific clinical factor(s) that accounted for the observed interpatient variability. However, in the population described in Chapter 6, patients with baseline vitamin D insufficiency (25(OH)D levels of 25-50 nmol/L) seemed to have a decreased lumbar spine BMD after 2 years of follow-up, whereas patients with sufficient vitamin D levels at baseline showed an increase in lumbar spine BMD. As such, our data suggest that specific subsets of patients exist that are more prone to bone disease than others. It is conceivable that specific combinations of CD-related factors, possibly other factors than currently known, predispose to bone loss. Therefore, additional research is warranted to identify such factors.

Besides ‘between-patient’ variation, a ‘between-gender’ variation was observed. Bone of male CD patients was more severely affected than that of female patients (Chapter 2). The tendency towards a longer disease duration in men might reflect a higher number of relapses in the past. Consequently, medication to treat the disease, e.g. corticosteroids, could have negatively influenced bone metabolism in men for a longer period of time compared to the women in this cohort. Another explanation for the observed difference might be a poor compliance in male patients. IBD patients in remission have been reported to show a high non-adherence to therapy, with a possibly higher non-adherence in men. This would imply that bone of male CD patients has been exposed more to the negative influences of inflammation than female patients.

The observed bone loss in quiescent CD patients was shown to be associated with a state of low bone turnover, as indicated by a reduced bone formation (Chapter 2). Bone loss in for example post-menopausal osteoporosis and hyperparathyroidism is known to be due to an increased bone turnover. Therefore, our data implicate a difference in bone turnover between inflammation-induced bone loss and the more traditional forms of osteoporosis. From this perspective, one could wonder whether traditional osteoporosis therapies should be the choice of treatment in
inflammation-induced bone loss. In Chapter 6, risedronate, an effective treatment modality in amongst others post-menopausal osteoporosis, was shown to prevent bone loss but not to increase bone mass in the hip of quiescent CD patients with osteopenia. Possibly, other or concomitant therapies are needed when improvement of bone mass in inflammation-induced bone loss is intended. In relation to the observed reduced bone formation, a self-evident possibility is the use of anabolic therapies which directly stimulate bone formation. In Chapter 2 osteocyte apoptosis was observed to positively correlate with IBD disease activity and in Chapter 6 disease duration was shown to inversely correlate with hip BMD. Moreover, from literature a relationship between inflammation and bone loss is becoming more evident. Therefore, modulation of inflammatory cytokines involved in IBD pathogenesis is another promising treatment option to control IBD-associated bone loss.

**Bone metabolism in IBD patients at the cellular level**

**Osteoblasts**

| Summary | Inflammatory factors are known to affect numerous characteristics of osteoblasts. Thus, bone loss in IBD patients might be the result of a disease-related change in the osteoblast phenotype and/or be caused by the numerous inflammatory mediators present in the circulation. In Chapter 3 functional characteristics of bone cells from quiescent CD patients were studied *in vitro*. Bone cells from CD patients showed a reduced growth potential and an impeded maturation in comparison to bone cells from healthy controls. The overall responsiveness of bone cells from CD patients to inflammatory cytokines, known to be involved in active disease, was largely unchanged. Therefore, disease-specific, phenotypic alterations in CD patient-derived bone cells seem to provide a new insight in the understanding of CD-associated bone loss. In addition, the unaffected sensitivity to inflammatory cytokines suggests that bone cells from CD patients remain susceptible to elevated levels of circulating inflammatory cytokines during periods of active CD. Therefore, the effect of serum from IBD patients on bone cells was studied in Chapter 4. Serum from approximately one-third of CD and UC patients, both in quiescent and active state of disease, decreased bone cell proliferation by up to 30% in comparison to bone cell proliferation in the presence of a healthy control (HC) serum pool. Serum from male IBD patients more often reduced bone cell proliferation than serum from female IBD patients. In order to elucidate the inflammatory factors involved in the reduction of bone cell proliferation, the cytokine/chemokine profile of sera was determined. From the 174 inflammatory factors tested, sera from IBD patients showed altered levels of IL-8, ICAM-1, Axl and IL-13 in comparison to the HC serum pool. However, these proteins could not be related to the observed inhibitory effect of IBD patients’ serum on bone cell proliferation. Specifically studying the differential protein expression in relation to the reduction in proliferation revealed a potential role for CC-chemokines and hormones, in particular for MCP-1 (CCL2) and possibly sex hormones. |

| Discussion | Cellular dysfunction of osteoblasts, e.g. changes in the level of proliferation and... |
differentiation, alterations in their response to vitamin D and several cytokines, as well as changes in the production of cytokines in vitro, has been described primarily in osteoblasts obtained from patients with osteoporosis. In the study described in Chapter 3, alterations in osteoblast characteristics were shown in CD patient-derived bone cells as well. This finding suggests that aberrations in bone cell phenotype related to CD pathogenesis might be involved in the bone loss frequently observed in CD patients.

Not only an unfavorable bone cell phenotype, but also a negative influence of IBD patients’ serum on bone metabolism seemed to be a causative factor in IBD-associated bone loss (Chapter 4). Prolonged exposure to IBD patients’ serum had a more detrimental effect on bone cells than short term exposure. The effect of serum seemed to be independent of disease type (CD vs. UC) and disease activity (active or quiescent). Bone cells from IBD patients are therefore likely to be exposed to the observed negative influences of serum during a vast part of their lifespan.

Similar to the studies at the bone tissue level, both a high ‘between-patient’ and ‘between-gender’ variability were observed with respect to the deleterious effect of patients’ serum on bone cell metabolism. Seeking for factors responsible for this variability is like looking for a needle in a haystack. The use of a semi-quantitative screening of serum content showed that the variation could be attributed to differences in serum levels of CC-chemokines, hormones, and possibly cell adhesion molecules. Recently, several CC-chemokines have been shown to modulate bone cell metabolism. For example, CCR1−/− mice show osteopenia due to impaired osteoblast and osteoclast function, and CCR2−/− mice have high bone mass owing to a decrease in number, size and function of osteoclasts. Additionally, CCL20 has been shown to play an important role in the bone tissue of RA patients by influencing both osteoblasts and osteoclasts, whereas CCL3 has been shown to be involved in myeloma-induced bone loss through inhibition of osteoclast function. It is therefore conceivable that CC-chemokines also play a role in IBD-associated bone loss. In addition, bone metabolism is under constant regulation of many hormonal factors of which the most widely known are steroid hormones, e.g. estrogen and progesterone, and calcitropic hormones, e.g. vitamin D and calcitonin. As many hormones are also involved in modulation of the immune response, their role in the pathogenesis of IBD and its associated bone loss is likely to be an ongoing and important research topic. Lastly, bone metabolism is known to be regulated by cell-cell and cell-matrix interactions among bone cells, as such implicating a role for integrins and other adhesion molecules. The best characterized adhesion molecule in bone cell metabolism is intercellular adhesion molecule-1 (ICAM-1). Defective bone formation in myeloma patients has amongst others been ascribed to involvement of ICAM-1, and polymorphisms of ICAM-1 have been found to be associated with RA as well as with CD. Therefore, ICAM-1 may be of importance in inflammation-induced bone loss.

The effect of IBD patients’ serum on bone cell differentiation could not be studied in our experiments as a consequence of limitations in the study design. Other studies showed an inhibitory effect of CD patients’ serum on the differentiation of primary rat osteoblasts. However, differences in species hamper a direct extrapolation to the human situation and therefore necessitates additional research using human bone cells.
Osteoclasts

Summary | Inflammatory factors are known to affect the formation and activity of osteoclasts. Therefore, increased osteoclast formation could, at least in part, be responsible for the bone loss associated with IBD. In Chapter 5 osteoclastogenesis from peripheral blood of quiescent CD patients was evaluated. Moreover, the role of lymphocytes and inflammatory cytokines in this process was determined. Peripheral blood precursor cells, when cultured in the absence of the osteoclastogenic cytokines M-CSF and RANKL, showed increased osteoclast formation in patients with quiescent CD when compared to healthy controls. T cells were observed to be critical for the spontaneous osteoclastogenesis, as osteoclast formation was completely abolished in T cell depleted peripheral blood cultures. T cells from CD patients showed higher basal RNA expression levels of the inflammatory cytokines IL-6 and IL-17. Moreover, T cells of CD patients produced higher levels of IL-13 and IL-17. Taken together these findings suggest a higher activation level of T cells from CD patients in comparison to T cells from healthy controls. A novel pathogenic finding was that osteoclast formation was preceded by the formation of cell clusters in cultures where osteoclast precursor cells were present. Cluster formation was induced by the presence of T cells, but exclusively in CD cultures. Cluster formation showed a strong positive relation with osteoclast formation, and as such suggests heterotypic interactions between osteoclast precursor cells and T cells to precede osteoclast formation. Both cluster formation and osteoclast formation were related to IL-17 levels in vitro, proposing a potentially important role for IL-17 in osteoclastogenesis in CD patients, and as such in CD-associated bone loss.

Discussion | Osteoclastogenesis has been studied in many bone diseases with evoked osteoclast activity. Formation of osteoclasts from PBMCs of patients with rheumatic diseases, osteoporosis, periodontitis, chronic liver disease with osteopenia, and osteolytic cancers has been shown to be increased when cultured in the absence of the osteoclastogenic factors M-CSF and RANKL (reviewed by de Vries et al.18). Based on the study presented in Chapter 5, increased osteoclastogenesis might play a role in CD-associated bone loss as well, even in patients with quiescent state of disease.

An intriguing finding was that clustering of osteoclast precursors with T cells seemed to be a potentially decisive step in the formation of osteoclasts. Cell clustering was shown to be mediated by LFA-1, an integrin that coordinates interactions between monocytes and lymphocytes via its adhesion partner intercellular adhesion molecule-1 (ICAM-1). From both in vitro studies and an animal study, LFA-1 and its adhesion molecules have been implicated to play a role in osteoclastogenesis in a very early stage of development.19-21 Therefore, interference with LFA-1 or its adhesion molecules might be an important target to prevent osteoclast formation.

An increased activity of T cells, rather than a change in number, was observed to mediate both cluster formation and osteoclast formation. Besides the traditional pro-inflammatory cytokines IL-6 and TNF-α, IL-17 was shown to be involved in these processes. IL-17 is a cytokine produced by a recently recognized new T helper (Th) cell subset, Th17 cells. IL-17 levels have been reported to be increased in both the intestinal mucosa and serum of IBD patients.22 Therefore, IL-17 is
recognized to be implicated in the pathogenesis of IBD. Our findings add to this with a potential role for IL-17 in the bone loss associated with IBD as well.

Although differences in the number of peripheral blood cells were not observed between CD patients and healthy controls, this could be due to the use of the relatively general classification to analyze blood cell composition. In view of the findings concerning IL-17, the use of a more discriminating classification, i.e. determining the number of Th17 cells, could have resulted in a different outcome. Elaborating on this, elevated numbers of circulating Th17 cells in IBD patients, as already reported in literature, could be an additional explanation for the observed increase in osteoclast formation in these patients. Therefore, further research using distinct subsets of T cells in the experimental set up might be of help to fine-tune which specific T cells are involved in osteoclastogenesis.

Fitting the pieces together

Integrating the findings at the tissue level and those at the cellular level reveals a rather lucid view on the mechanism through which bone metabolism may be affected in IBD patients (Fig. 1). The observed reduced bone mass in CD patients is caused, at least in part, by a lowered bone formation as shown at the tissue level. At the cellular level, CD patient-derived osteoblasts showed a reduced ability to proliferate, which may indicate a decreased number of these cells in vivo. In addition, osteoblasts obtained from bone biopsies of CD patients showed an impeded maturation, which may reflect a disturbed functioning of these cells in vivo. Therefore, both aberrations in number and function of osteoblasts appear to account for the reduced bone formation seen at the tissue level.

The observed changes in osteoblast characteristics may be the result of (sub)clinical inflammation. At the tissue level, osteocyte apoptosis was shown to correlate positively with IBD disease activity. Moreover, a possible inverse relation was observed between disease duration and the bone formation marker ALP. Lastly, it was speculated that the number and/or severity of relapses in the patients’ past might correlate inversely with bone formation. At the cellular level, cytokines known to be involved in CD pathogenesis caused amongst others an inhibition of osteoblast maturation. In addition, IBD patients’ serum containing a cocktail of inflammatory mediators inhibited osteoblast proliferation. As one may assume that disease activity and disease duration are related to the presence of inflammatory mediators, these findings together suggest that the reduced bone formation observed at the tissue level is caused by deleterious effects of inflammation on osteoblast characteristics.

Besides a reduced bone formation, a normal or slightly reduced bone resorption was observed at the tissue level. At the cellular level, osteoclast formation from peripheral blood precursor cells was increased in CD patients. This suggests that osteoclast precursor cells are primed, i.e. triggered to partially differentiate, in the circulation already. However, these findings did not correlate with the in vivo levels of the bone resorption marker CTx. In addition, these findings did not fit in with the findings at the tissue level. However, the patients included in the osteoclast study (Chapter 5)
Figure 1. Mechanism through which bone metabolism may be affected by IBD. In IBD, both environmental factors and host factors activate the intestinal immune system resulting in increased levels of inflammatory mediators in both the intestine and the circulation. These inflammatory mediators generally inhibit the number and function of osteoblasts and stimulate the number, and possibly function, of osteoclasts. The result is a decreased bone formation and possibly increased bone resorption (at least during active state of disease). Consequently, the net effect of IBD on the skeletal system is a reduced bone mass.

Abbreviations used: Obl, osteoblast; Ocl, osteoclast; Ocy, osteocyte; TC, T cell; BC, B cell; DC, dendritic cell; MΦ, macrophage.
differed largely from the patients included in the histomorphometry study (Chapter 2). In addition, the apparent discrepancy between the findings at the cellular and tissue level might be due to the fact that osteoclast precursor cells require further local differentiation (priming) in order to actually resorb bone. Possibly, the required microenvironment to activate osteoclasts is present only during active disease, whereby only then the partially triggered osteoclast precursors are able to become an active osteoclast. In that case, osteoclast resorption is high during active disease and normalizes in quiescent state of disease (or even decreases when taking into account coupling with bone formation).

Elevated concentrations of several osteoclastogenic cytokines were produced by both osteoclast precursor cells and T cells of patients with CD. Interestingly, levels of these cytokines correlated with osteoclast formation, pinpointing towards an important role for (T cell-mediated) inflammation in osteoclastogenesis. The studies at the tissue level described in this thesis were not designed to answer the role of inflammation in bone resorption and as such in bone metabolism. However, animal studies previously highlighted the crucial role of TNF-α and IL-1 in bone resorption in vivo.\textsuperscript{24,25}

At the cellular level, osteoclast formation was preceded by formation of cell clusters. The cell clustering process was found to be mediated by heterotypic cell-cell interactions, which suggests that a direct crosstalk between osteoclast precursors and T cells may play a pivotal role in the early phase of osteoclast formation. In vivo, the critical role of T cells in bone loss has been proven by the fact that T cell deficient mice show a diminished infection-induced bone loss and are protected from estrogen-related bone loss.\textsuperscript{26,27} Although these findings were implicated to be the result of a disturbed osteoclastic bone resorption, no information is available on the role of a direct crosstalk between osteoclasts and T cells in this process in vivo.

All the above mentioned data strongly confirm the role of the inflammatory process and its sequelae in bone (cell) metabolism. Combining the findings from the Chapters 3, 4 and 5, reveals which specific inflammatory factors are of potential importance in the regulation of bone metabolism in vitro. In summary, IL-1 and IL-10 affect osteoblast proliferation and function in both the physiological (healthy controls) and pathophysiological (CD patients) situation. IL-6 and TNF-α seem to interfere with osteoblast characteristics only in CD patients, although this finding was less consistent. The cytokines IL-1, IL-6, IL-13, IL-17, INF-γ and TNF-α as well as the adhesion molecules LFA-1 and ICAM-1 play a role in osteoclast formation. The inhibitory effect of IBD patient serum on osteoblast proliferation is related to levels of MCP-1, and possibly to hormones and cell adhesion molecules. Serum from IBD patients contains altered levels of IL-8, IL-13, ICAM-1 and Axl. Although these cytokines did not correlate to the effect of serum on bone cell proliferation, these factors still may turn out to be of relevance in the regulation of bone (cell) metabolism.
Future directions

In the paragraph ‘Fitting the pieces together’ several assumptions have been made. Additional research is required to confirm and further elaborate the suggested pathophysiological mechanism of IBD-associated bone loss.

The studies described in this thesis aimed to use a clinically well-defined study population. However, already in this experimental setting a high variability between patients was observed. Therefore, it would be interesting to repeat and extend the performed studies using an even more delineated population in which the patient heterogeneity is taken into account. Including patients with accurately recorded differences in for example disease activity, disease duration, gender, vitamin D status, blood cell composition, and severity of bone loss might help to identify subsets of patients at risk for osteoporosis.

An intriguing and enigmatic finding in this thesis was that male IBD patients seem to be at greater risk for bone loss than female patients. As a gender difference may point to the potential importance of sex hormones, the role of estrogen and testosterone in IBD-associated bone loss should be investigated.

Besides cytokines, our findings suggest chemokines as well to be of importance in the regulation of bone metabolism in IBD patients. Recent research has demonstrated a role for several chemokines in bone cell metabolism (in vitro studies), as well as in the development of osteoporosis and bone lesions in multiple myeloma (studies in animals and humans). Therefore, elucidating the role of chemokines, possibly in particular CC-chemokines, in IBD-associated bone loss is an interesting topic for further investigation.

Vitamin D status showed an inverse relation to CTx levels and seemed to relate positively to lumbar spine BMD. In addition, vitamin D status was observed to relate inversely to CRP levels and as such to disease activity, a finding also reported in literature very recently. These findings, in combination with the high prevalence of vitamin D deficiency in IBD patients, highlight the importance of vitamin D, and based on literature possibly its receptor as well, in both bone cell metabolism and IBD pathogenesis. Therefore, the role of vitamin D and its receptor (polymorphisms) in IBD-associated bone loss might currently be underestimated, and as such warrants further research.

The role of the osteocyte in bone metabolism in IBD patients has gained only limited attention in this thesis. At the tissue level, osteocyte apoptosis was observed to relate positively with disease activity and bone loss in quiescent CD patients seemed to be caused by a decreased osteocyte viability in the patients’ past. Therefore, more detailed research on osteocyte characteristics in relation to IBD-associated bone loss would be of value.

New therapeutic insights

Possibilities for new therapeutic strategies have been touched on already in the previous paragraphs. An overview of the most promising ones is given below.
In relation to the observed reduced bone formation in CD patients in this thesis, a self-evident treatment option for IBD-associated bone loss is the use of anabolic therapies, such as recombinant human parathyroid hormone and growth factors. The last years, the role of the Wnt-signalling pathway in bone mass regulation has become more and more evident. Therefore, a novel therapeutic anabolic approach might be, amongst others, targeting sclerostin.

This thesis highlights the role of cytokines in bone metabolism in IBD patients. Besides the widely known factors like IL-1, IL-6 and TNF-\(\alpha\), a particular role for IL-17 is implicated. Anti-IL-17 has been shown to be effective in inhibiting osteoclast formation in various diseases associated with bone loss. Therefore, therapies aimed at blocking the IL-17 pathway may be beneficial to prevent bone loss in IBD patients as well.

The observed role for chemokines in the regulation of bone mass suggests that therapies targeted at this group of proteins may be of potential use in the fight against IBD-associated bone loss.
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