Interaction between bone, the neuroendocrine system and metabolism
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General discussion
In this chapter, the results of the studies presented in this thesis are put into perspective. A short summary of the main results is given, along with an overview of where we currently stand, the clinical implications of the present findings and directions for future research.

**Part I: Sympathetic control of bone remodeling**

In this part we performed a series of experiments to investigate the role of the sympathetic nervous system (SNS) in human bone remodeling. In rodents, the SNS signals through the beta-2 adrenergic receptor located on the osteoblast to enhance bone resorption (1, 2). Likewise, pharmacological stimulation and inhibition of the beta-2 adrenergic receptor decreases and increases bone mass, respectively (3, 4). It is uncertain whether a similar role for the beta-2 adrenergic receptor in bone metabolism exists in humans. Studies on the association between beta-blocker and beta-agonist use and fracture risk have shown conflicting results (5-8). In combination with the observational nature of these studies, it remains difficult to draw any definite conclusions. We therefore performed a prospective intervention trial, in which we did not find an effect of treatment with a selective beta-2 adrenergic agonist and a non-selective beta-adrenergic antagonist on bone turnover in healthy postmenopausal women (9) (chapter 2). These results are supported by a previously published randomized controlled trial, which showed that non-selective beta-adrenergic blockade does not affect bone turnover (10). In addition, polymorphisms in the beta-2 adrenergic receptor known to influence receptor function, were not associated with bone mineral density or fracture risk in two independent cohorts (11). Taken together, these data do not support a critical role for the beta-2 adrenergic receptor in human bone metabolism.

Since there is convincing evidence that the SNS regulates bone metabolism in humans (12, 13), the mechanism of signal transduction may involve other adrenergic receptors. Both mice with a global genetic inactivation of the beta-1 adrenergic receptor and double beta-1/beta-2 adrenergic receptor knockout mice have decreased bone mass (14). On the other hand, genetic inactivation of all three beta-adrenergic receptors results in a high bone mass phenotype (15). In addition, in an animal model with chronic increased sympathetic activity due to an alpha-2A and alpha-2C adrenergic receptor knockout, female mice showed an increased bone mass (16). These observations suggest a complex interplay of regulation by different adrenergic receptors. To further delineate the role of the SNS in human bone metabolism we determined the acute effect of a single oral dose of the selective alpha-2 adrenergic receptor agonist clonidine on bone turnover. In healthy male and female volunteers, clonidine enhanced bone resorption marker levels, despite the reduced sympathetic tone (17) (chapter 3). The results of this study oppose the general view that increased SNS activity has a catabolic effect on bone or it may indicate that the observed effects of clonidine are mediated via direct stimulation of alpha-2 adrenergic receptors on bone cells. Although human osteoclast-like cells did express alpha-2 adrenergic receptor mRNA, in vitro osteoclast formation and activity was not affected by clonidine (17) (chapter 3), suggesting that direct osteoclast stimulation is not the mechanism of action.
The clinical relevance of the increase in bone resorption observed following pharmacological alpha-2 adrenergic stimulation needs to be explored. Alpha-2 adrenergic agonists are licensed to treat postmenopausal vasomotor symptoms. Studies on the long-term effects of alpha-2 adrenergic stimulation on bone turnover are needed. Based on our results, clonidine could potentially aggravate the risk of developing osteoporosis in a group already at risk. The aforementioned in vitro data do not support a role for the alpha-2 adrenergic receptor in the differentiation and activity of human osteoclasts. Since it is not certain that osteoclast-like cells generated from monocytes behave similar to in vivo grown osteoclasts, it would be useful to perform additional experiments with primary osteoclasts harvested, e.g., during orthopaedic surgeries (18). It is also possible that alpha-2 adrenergic stimulation increases osteoclast function indirectly via other bone cells. In vitro experiments examining osteocyte and/or osteoblast activity following alpha-2 adrenergic administration would therefore be informative.

In an attempt to look further into the role of the alpha-2 adrenergic receptor, we investigated associations between several alpha-2 adrenergic gene single nucleotide polymorphism (SNPs) and bone mineral density and fracture risk, but were unable to demonstrate an effect (chapter 4). Functionality of these alpha-2 adrenergic SNPs has never been examined, but in the literature these SNPs have previously been associated with various disease phenotypes. The results from SNP association studies give some indication regarding possible mechanisms involved. However, a major disadvantage of these studies is that genomic variations say little about a specific target tissue. Therefore, subsequent experiments with cell specific knockouts are needed to establish the exact role of alpha-adrenergic receptors in bone metabolism.

Part II: Humoral control of bone remodeling

In the second part of this thesis we focussed on the humoral control of bone. Over the last several years, an increasing interest in bone marrow fat has emerged, partly facilitated by the development of noninvasive marrow fat imaging techniques. Bone marrow fat increases with aging and is inversely related to bone mass (19-22). This balance between bone marrow adipose tissue and bone is thought to emerge from the common mesenchymal progenitor cell shared by osteoblasts and adipocytes (23). Sex steroids may be involved in the regulation of bone marrow fat, because men have higher bone marrow fat than women and bone marrow fat increases with menopause (19). To date, only long-term changes in bone marrow fat have been investigated (24-26), which does not allow the study of bone marrow fat independent of (detectable) changes in bone mass. We demonstrated that short-term changes in bone marrow fat do occur. In healthy postmenopausal women, treatment for two weeks with oral 17-β estradiol administration rapidly decreases bone marrow fat content and increases bone formation marker levels (27) (chapter 5). These results indicate that 17-β estradiol rapidly reduces bone marrow fat independent of bone mass, which supports the existence of a reciprocal relationship between adipocytes and osteoblast formation and/or activity. Considering this link between osteoblasts and adipocytes, factors modifying lineage commitment of the mesenchymal stem cell could lead to novel bone loss treatments. In line with our results, in vitro studies have shown that mesenchymal stem cell differentiation is affected by estrogen (28-31), but future studies are needed to understand the exact pathways involved.
It has been recognized that the number of mature osteoblasts is not only determined by progenitor cell faith. As adipokines and fatty acids, secreted by marrow adipocytes in the local microenvironment, are known to influence osteoblast differentiation and function in a negative way (32, 33). Lipotoxicity can be one of the reasons why marrow fat accumulation is negatively associated with measures of bone integrity and fracture risk (34, 35). This link between marrow fat and measures of bone integrity and fracture risk could potentially serve as an additional predictor for fracture risk in the future. In fact, one study performed in elderly female subjects showed that marrow fat content has a moderate to high sensitivity in discriminating between fast and slow bone losers (36). Additional prospective studies are needed to provide more evidence that marrow fat can predict bone loss. In addition, treatments targeted at marrow adipocyte toxic release could potentially serve as new bone loss prevention modalities.

Another potentially interesting aspect of bone marrow fat is its endocrine role by the secretion of adiponectin, a hormone with beneficial systemic metabolic effects (37-40). In food restricted mice and in patients with anorexia nervosa, an increase in bone marrow fat is observed, despite marked reductions in peripheral body fat (41, 42). A recent study demonstrated that secretion of adiponectin from bone marrow adipose tissue is greater than from other fat sources. Moreover, the expansion of bone marrow adipose tissue is necessary for the increased adiponectin levels observed during caloric restriction (40). This suggests that in lean states expansion of bone marrow adipose tissue and the resulting increase in adiponectin might serve to promote metabolic health. The longitudinal effect of weight loss on marrow fat in humans is unknown. A recent pilot study in small number of subjects showed that bone marrow fat did not change in nondiabetic subjects six months after gastric bypass surgery while it decreased in patients with diabetes (26). Larger studies with a longer follow-up period are needed to understand the unique metabolic behavior and regulation of marrow fat.

Because adipocytes and osteoblasts originate from the same precursor cell, a correlation between obesity and bone metabolism has been assumed. While traditionally obesity was thought to be beneficial to bone, recently detrimental effects of excessive body fat on bone have been described (43-45). As a result of the worldwide obesity rise, diets have become increasingly popular. While successful diets are linked to improved health outcome in obese subjects, numerous studies in rodents and children with epilepsy have shown that low-carbohydrate/high-fat diets can act negatively on bone (46-70). To distinguish between the effect of differences in energy intake and the effect of variation in dietary composition, we determined the effect of short-term eucaloric diets, with different fat and carbohydrate amount, on bone turnover. An eucaloric high-fat, low-carbohydrate diet decreases bone resorption, while an eucaloric low-fat, high-carbohydrate diet increases bone resorption (chapter 6). These results indicate that short-term alterations in dietary content may impact on bone metabolism. Since this is the first study investigating the effects of eucaloric variation in dietary carbohydrate and fat content on bone metabolism, additional studies are required to confirm our results and to investigate the possible mechanisms involved. These studies should also determine the long-term effect of dietary carbohydrate and fat content on bone and look into other outcome measures including bone mineral density and fracture risk. In the future,
carbohydrate and fat could possibly serve as additional modifiable dietary factors to maintain bone health, next to calcium and vitamin D.

**Part III: Bone as an endocrine organ**

In this part we aimed to demonstrate that bone is not only a recipient of hormonal input, but acts as an endocrine organ itself, by the secretion of osteocalcin by osteoblasts. The study of knockout animals and in vitro experiments have identified osteocalcin, in particular its undercarboxylated form, to have important hormonal functions, with a role in energy metabolism and reproduction (71-75). Osteocalcin knockout mice present with a metabolic phenotype of glucose intolerance, insulin resistance, increased fat mass and decreased energy expenditure (73). In addition, male osteocalcin knockout mice breed poorly and are characterized by decreased testis and epididymal weights, a decreased sperm count and low circulating testosterone levels (71, 72). In humans, in two systematic surveys of the literature, the majority of the studies observed a negative correlation of serum undercarboxylated or total osteocalcin levels with blood glucose, insulin resistance, diabetes, obesity and markers metabolic syndrome (76, 77). Less information is available on the role of osteocalcin in human gonadal function. The first human genetic evidence that osteocalcin fulfils a hormonal function in humans, came from a loss-of-function mutation in the osteocalcin receptor found in two males with a subfertile phenotype (71). However, in a unselected cohort of older Dutch men, serum osteocalcin did not show to be an important positive modifier of testosterone levels (78) (chapter 7). Because of the correlative nature of the currently available studies, as well as the use of different assays to measure osteocalcin and the presentation of different forms of osteocalcin, at this point it is impossible to draw definitive conclusions. There is an urgent need for prospective intervention studies investigating the hormonal actions of osteocalcin in humans. Osteocalcin is not available for administration in humans, but endogenous osteocalcin increases 2-3 fold during daily PTH administration (79). Therefore we studied the effect of 12 weeks of PTH treatment on testosterone levels and metabolic indices in male subjects with primary osteoporosis. The preliminary results from this study do not support a role for osteocalcin in the regulation of glucose homeostasis and testosterone levels in humans (chapter 8). However, the small sample size of this study does not allow for a firm conclusion at this stage. Additional large-scale prospective studies are needed to establish once and for all whether osteocalcin has similar hormonal actions in humans as demonstrated earlier in animal experiments. In addition, such studies will provide information whether dysregulation of glucose metabolism and testosterone can be expected in patients treated with antiresorptive and anabolic therapy for osteoporosis.

**Concluding remark**

In conclusion the beta-2 adrenergic receptor does not seem to be crucial in the regulation of human bone metabolism by the sympathetic nervous system. At this point, in vivo and in vitro studies on a possible role for the alpha-2 adrenergic receptor are inconclusive. Still, there is convincing evidence that the SNS influences bone turnover is humans. For the future, the challenge remains to identify the pathways and mechanisms involved.
The close relationship between bone marrow fat and bone has been recognized for years. Only recently, progress has been made in understanding the unique metabolic behaviour and regulation of bone marrow fat. In the long run, bone marrow fat may play an important role in the diagnostics and therapeutics of osteoporosis. Another potentially interesting modifiable factor which can contribute to bone health is dietary fat and carbohydrate content. Although many of the novel roles of bone have been well established in animal models, the majority of longitudinal studies do not support a similar endocrine or metabolic role for bone in humans.

In this thesis we show several aspects of bone metabolism that could not direct be extrapolated from mouse models to humans. Therefore when interpreting the currently available literature, caution needs to be taken, while future human studies will establish whether or not this field holds diagnostic and/or therapeutic promise.
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