JARKKO JOKIHAARA

Chronic Renal Insufficiency and Bone

ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the small auditorium of Building K,
Medical School of the University of Tampere,
Teiskontie 35, Tampere, on August 24th, 2007, at 12 o’clock.

UNIVERSITY OF TAMPERE
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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>aBMD</td>
<td>Areal bone mineral density</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Anteroposterior direction</td>
</tr>
<tr>
<td>APD</td>
<td>Pamidronate</td>
</tr>
<tr>
<td>BMC</td>
<td>Bone mineral content</td>
</tr>
<tr>
<td>cBMD</td>
<td>Cortical volumetric bone mineral density</td>
</tr>
<tr>
<td>cCSA</td>
<td>Cortical bone cross-sectional area</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CKD-MBD</td>
<td>Chronic kidney disease-mineral and bone disorder</td>
</tr>
<tr>
<td>CRI</td>
<td>Chronic renal insufficiency</td>
</tr>
<tr>
<td>CSMI</td>
<td>Cross-sectional moment of inertia</td>
</tr>
<tr>
<td>CV$_{rms}$</td>
<td>Average root-mean-square coefficient of variation</td>
</tr>
<tr>
<td>CWT</td>
<td>Cortical wall thickness</td>
</tr>
<tr>
<td>DXA</td>
<td>Dual energy X-ray absorptiometry</td>
</tr>
<tr>
<td>ML</td>
<td>Mediolateral direction</td>
</tr>
<tr>
<td>NTX</td>
<td>Subtotal surgical 5/6 nephrectomy</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PAR</td>
<td>Paricalcitol</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>pQCT</td>
<td>Peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor activator for nuclear factor (\kappa B)</td>
</tr>
<tr>
<td>RANK-L</td>
<td>Receptor activator for nuclear factor (\kappa B )-ligand</td>
</tr>
<tr>
<td>ROD</td>
<td>Renal osteodystrophy</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SEV</td>
<td>Sevelamer hydrochloride</td>
</tr>
<tr>
<td>Sham</td>
<td>Surgical sham-operation</td>
</tr>
<tr>
<td>TBMC</td>
<td>Total bone mineral content of a whole bone</td>
</tr>
<tr>
<td>tCSA</td>
<td>Total bone cross-sectional area</td>
</tr>
<tr>
<td>vBMD</td>
<td>Total volumetric bone mineral density</td>
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ABSTRACT

The rationale for this thesis was to study the effects of chronic renal insufficiency (CRI) on bone structure. CRI has been associated with skeletal lesions and this related bone disorder has been termed renal osteodystrophy or more extensively as chronic kidney disease-bone and mineral disorder (CKD-BMD). The definition and evaluation of renal osteodystrophy has predominantly focused on changes in the trabecular bone and circulating systemic biochemical markers of bone turnover. Accordingly, one objective of this study was to assess the changes in the cortical bone with peripheral quantitative tomography, and particularly, in the bone structural strength with mechanical testing. In this study, experimental CRI was shown to be associated with decreased volumetric cortical bone mineral density and that along with the progression of CRI bone strength may eventually deteriorate. Other main objective of this study was to assess the efficacy of four different pharmacological treatments (calcium carbonate, sevelamer, paricalcitol, pamidronate) on bone in CRI. These were chosen to represent clinical treatment options for the consequences of impaired renal function. It is hypothesized that the general treatment of renal condition is beneficial also to the bones, but there is also some concern about the possible adverse skeletal side effects. In this study, calcium carbonate, sevelamer, and paricalcitol treatments were observed to alleviate the CRI-induced detrimental changes in bone. Calcium salts and sevelamer are phosphate binding agents are clinically used to alleviate hyperphosphatemia in CRI. Sevelamer treatment was associated with positive effects on bone, and although calcium carbonate treatment simultaneously suppressed parathyroid hormone secretion, no detrimental effects on bone strength were observed. Similarly, the amelioration of secondary hyperparathyroidism with vitamin D analogue paricalcitol was beneficial also on bone. In addition, it was shown that pamidronate treatment was associated with increased bone mineral content in CRI. Altogether these findings suggest that experimental CRI exerts detrimental effects on bone, but however, these above mentioned treatments were shown to be effective in preventing these changes.
INTRODUCTION

The main function of the skeleton is locomotion and appropriate mechanical competence is crucial for bones to function properly. Bones must be strong enough to bear loading without fracturing, but simultaneously bone mass should be reasonable to achieve overall energy efficiency. It seems evident that each bone has adapted a precise three-dimensional structure that is an optimum in the light of these requirements. Sufficiently high mineral content of bone tissue is needed to achieve the appropriate structural properties. Minerals are equally essential in numerous vital biochemical processes in the body and systemic concentrations and metabolism of minerals, notably calcium and phosphorous, are therefore controlled delicately. Kidneys and bones are among the organs that are critical to this intricate regulation system. However, if body is subjected to metabolic distress, such as the general disorder of mineral metabolism associated with chronic kidney disease, the homeostatic control system may be influenced by inappropriate factors. Thus, as a consequence of impaired renal function the properties of bone may be affected so that the bone becomes inadequate for competent mechanical function. Particularly, increased bone fragility is a detrimental consequence of unsatisfactory bone structure as it may predispose to bone fractures.

Chronic impairment of renal function, chronic renal insufficiency (CRI), has been recognized as a growing public health problem and prevalence of CRI is constantly increasing worldwide. This may be explicable in terms of increased prevalence of risk factors that have been associated with CRI, including diabetes mellitus and hypertension. The pathogenesis of CRI originates in gradual decrease in glomerular filtration and loss of metabolically active kidney tissue. In other words, the capacity of kidneys to excrete substances and to produce active vitamin D (calcitriol) becomes deficient and results in disturbances in the homeostasis of phosphorous and calcium, particularly hyperphosphatemia and hypocalcemia, and low levels of calcitriol. Circulating serum calcium and phosphorous concentrations are regulated to a large extent by parathyroid hormone (PTH) and calcitriol, which act on three target organs: kidney, intestine, and bone. Increased PTH levels are needed to maintain physiological calcium and phosphorous concentrations in CRI and this leads to the development of secondary hyperparathyroidism. Mild CRI is generally asymptomatic, but if the condition is left untreated, the disturbances of mineral metabolism may eventually result in cardiovascular calcifications, skeletal lesions, and other detrimental complications. This condition is extensively termed as chronic kidney disease-bone and mineral disorder (CKD-BMD), and the bone lesions
have traditionally been specified as renal osteodystrophy. CKD-BMD is widely considered the most complex and least predictable form of metabolic bone diseases. Advanced CRI has been associated with increased bone fragility, whereby the skeletal changes in CKD-BMD have attracted considerable scientific interest. However, information on changes in the bone structure and mechanical competence of bones is scarce.

The rationale for this series of experiments was to utilize a structurally oriented approach to characterize the effects of CRI on bone. Mechanical competence is a fundamental feature of bone and therefore characterization of the bone structural strength and its determinants is warranted, and indispensable, in the evaluation of bone. One objective of the clinical treatment of patients with CRI is to prevent and overcome the consequences of the underlying hyperphosphatemia, hypocalcemia, low calcitriol levels, and the associated secondary hyperparathyroidism. There are distinct pharmacological treatment options available, and because of the complexity of the consequences of CRI, there are a myriad of mechanisms and possible treatment outcomes to consider. In general, the cornerstones of the pharmacological management of disturbances in mineral metabolism are alleviation of hyperphosphatemia and inhibition of excessive parathyroid function. In this thesis, the treatments were chosen to represent the current clinical pharmacological treatment options for the general management of CRI and the rationale was to evaluate the influence of these widely used interventions on bone structure. Specifically, the objectives were to evaluate the effects of treatment of hyperphosphatemia with calcium salts and sevelamer, and the effects of vitamin D analogue paricalcitol on bone. Additionally, the effects of bisphosphonate treatment on bone in CRI were also explored; bisphosphonates are shown to be potent inhibitors of bone resorption, but however, the net effect may not be desirable in CKD-BMD. Furthermore, outcome of any bone affecting drug treatment is somewhat unpredictable in CKD-BMD.
REVIEW OF THE LITERATURE

1 Bone biology

1.1 Bone anatomy

Bones are the hard and rigid constituents of skeleton. In each bone, the solid bone tissue is enclosed between outer periosteal envelope and inner endosteal envelope (Figure 1). Inside the bone endosteal perimeter demarcates the solid portion and the marrow cavity (medullary canal which encompasses the bone marrow). The texture of the solid portion is either dense cortical bone or honeycomb-like trabecular bone. Periosteal envelope includes mainly cortical bone, and articular cartilage in joints, while endosteal perimeter is considered to include trabecular, endocortical, and intracortical subdivisions which are in continuity. The solid bone tissue consists of bone cells and extracellular matrix, which comprises of mineral compound that resembles hydroxyapatite.

The shape of bones varies greatly according to the specific function of each given bone. The overall shape of each bone may be grossly described as long (femur, tibia, humerus) or flat (skull, ilium, scapula). The proportion of cortical to trabecular bone is distinct in different bones and also in different parts within a given bone: shaft (diaphysis) of a long bone resembles a cylinder made of cortical bone, while in the ends of a long bone (metaphysis and epiphysis), and in flat bones overall, a thinner cortical shell is filled with a spongy mesh of trabecular bone.

Figure 1. Schematic structure of a long bone.
1.2 Function of bone

The purpose of the skeleton is to contribute to the survival of the organism in the wild. Bones have locomotive, protective, and metabolic function, and like all organs, tissue builds up functional structures that are optimum to carry out these functions (Einhorn 1992). Each bone presents a species-specific size, shape and internal structure to outcome the evolutionary driven genetic adaptation in the population and this is the starting point for physiological bone adaptation in the individual during its lifespan (Currey 2003a, Currey 2003b).

1.2.1 Locomotive function

The primary function of bones is locomotion (Einhorn 1992). Bones build up skeleton, which may be considered as a mechanism that consists of supports, levers, joints, and sites for muscle attachment that are needed for movement. Skeleton also bears the body. Bone strength and stiffness are of greatest importance in the locomotive function of bones (Einhorn 1992, Currey 2003b, Currey 2004). Bones must be able to resist bending and compression to perform as levers in musculoskeletal system (Currey 2004). Equally, bones must resist the forces applied to them by muscle contraction and gravity without breaking, and only adequately strong bones make movement possible. The precise three-dimensional structure and composition of each bone is critical to its ability to function effectively in locomotion (Einhorn 1992, Burr 1997, Frost 1997, Parfitt 1998, Currey 2001, van der Meulen et al. 2001, Turner 2002, Currey 2003a, Currey 2003b, Currey 2004). When considering the attributes of the other constituents of skeleton; cartilage, tendons and ligaments are less rigid but appropriate in toughness, tensile strength, and flexibility thus providing for particular features such as bone adhesion and joint movement.

Cortical bone is strong and stiff because of the high mineral content and combination of dense cortical bone and hollow marrow cavity provides for combination of strength and low weight (Einhorn 1992, Turner and Burr 1993, Currey 2001, van der Meulen et al. 2001). Cortical bone is superior to trabecular bone in terms of whole bone strength and load bearing in the skeleton is mostly carried out by cortical bone (Augat et al. 1996, Bell et al. 1999, Haidekker et al. 1999, Crabtree et al. 2001, Muller et al. 2003, Pistoia et al. 2003). For example, the thickness of the cortical shell has been shown to be as small as 0.3–0.4 mm in the vertebral body, but still the biomechanical role of this thin cortical shell can be substantial, being about 45% at the midtransverse section (Eswaran et al. 2006).

From the evolutionary point of view, the skeleton is subjected to an obvious need for a mechanism to attain perfect temporospatial specificity for metabolic needs of systemic hormones without compromising the mechanical competence of locomotive apparatus, the bottom line in terms of viability of vertebrates. While hormones affect the bone mineral mass, the ultimate currency in the
economy of survival is the metabolic energy efficiency (Currey 2003b). Bones being twice as dense as other tissues, bone mass has a profound effect on body mass, which in turn governs metabolic energy requirements. Accordingly, it appears that basic physical principles have simply resulted in evolutionary pressures to specifically reduce and optimize bone mass required to function in a given evolutionary niche.

1.2.2 Protective and supportive function

In addition to locomotive function, strength and stiffness of bone is also utilized to form protective and supportive tissues. Skeleton defines the general shape of the body and acts as a supporting framework for all other systems of the body. In addition, skeleton forms support for body cavities in which internal structures are located, and equally, skeleton provides mechanical protection for certain organs and vital structures. For example, the vertebral column protects the spinal cord, the skull protects the brain, ribs protect the heart and lungs, and long bone diaphysis encompasses hematopoietic red bone marrow (Moore and Agur 2007).

1.2.3 Metabolic function

The overall shape or precise distribution and orientation of bone are of less importance to the successful execution of the metabolic functions, particularly the former, whereas the existence of metabolically active surfaces is obviously more critical. In the whole skeleton, cortical bone represents about 75% of the volume and mass, but only 25% of the surface area (Parfitt 1998). Thus, the importance of trabecular bone becomes readily apparent concerning the mineral transfer function. Because of the honeycomb-like texture, the trabecular bone possesses a large surface area with close proximity to circulation and bone marrow. Bone tissue is a reserve of ions, particularly calcium and phosphate, and 99% of total body calcium is deposited in bone (Moe 2005). Systemic control of calcium and phosphorous metabolism is a complex and closely regulated process involving the kidneys, intestine, parathyroid glands, and the skeleton (Figure 2). In addition to mineral metabolism, skeleton is also a source of hematopoietic cells, cytokines and growth factors.
1.3 Bone turnover

Bone remodeling is a coupled multi-step process of bone resorption and formation at discrete locations in the skeleton. The rate of bone remodeling is referred to as bone turnover. In bone physiology, turnover means replacement referring to proportional volume replacement per time unit, usually expressed as percent/year (Parfitt 2002). The primary objective of bone remodeling is to prevent bone becoming too old, remodeling makes less direct contribution to mineral homeostasis (Parfitt 2003). In human adult skeleton, bone resorption and formation is continuously occurring at about 1–2 million microscopic sites (Rodan and Martin 2000). Bone turnover is mainly affected by mechanical stimuli, hormones, cytokines, and growth factors that influence the recruitment, differentiation and activity of osteoclasts, osteoblasts and osteocytes.

During the formation phase of remodeling, the collagenous structure of the bone becomes calcified in a process called mineralization. The density of unmineralized bone is lower and it increases as all free matrix water is being replaced by mineral. Thus, the amount of bone mineral in a particular volume is decreased if bone turnover is high and the mean age of bone is low, or bone mineralization is impaired (Parfitt 2003). Reciprocally, the degree of mineralization is increased if bone turnover is low and mean bone age is high, however, complete mineralization is normally prevented presumably by some property of living osteocytes (Parfitt 2003). Formation rate is frequently in balance with that of the resorption rate, but not always and the imbalance can affect bone volume (Parfitt 1998, Salusky and Goodman 2001, Parfitt 2003).

The instrument of remodeling is a multicellular unit which consists of osteoclasts, osteoblasts, and precursors of those (Figure 3). Osteoclasts are bone

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**Figure 2.** A simplified diagram of the regulation of calcium and phosphorous balance.
resorbing cells that are derived from the circulating progenitor cells. Activation of osteoclasts is responsible for bone resorption and that takes place for about 3 weeks per microscopic site (Rodan and Martin 2000). During bone resorption osteoclasts endocytose the degradation products of bone matrix, which are then transported through the resorbing osteoclast, and thus osteoclasts can simultaneously remove large amounts of bone matrix and penetrate into bone (Salo et al. 1997). Osteoblasts, the bone forming cells, are derived from the marrow stromal cells and some of them eventually become osteocytes or lining cells. Once the osteoclasts have resorbed bone, the osteoblasts fill the resorption pit with unmineralized matrix, the osteoid. To complete the remodeling cycle, the osteoid is finally mineralized. The entire rebuild process takes about 3–4 months (Rodan and Martin 2000).

Figure 3. Basic multicellular unit of bone remodeling and a simplified diagram of the regulation of osteoclast activation. OB, osteoblast; SC, stromal cell; OC, osteoclast; OPG, osteoprotegerin; RANK, receptor activator for nuclear factor κB; RANK-L, receptor activator for nuclear activator κB -ligand.

Osteoblasts and its precursors (marrow stromal cells) regulate osteoclast activity via osteoprotegerin / receptor activator for nuclear factor κB -ligand (OPG/RANK-L) system (Suda et al. 1999, Boyle et al. 2003, Harada and Rodan 2003, Martin and Sims 2005). Receptor activator for nuclear factor κB (RANK) is located on osteoclasts precursor cells, whereas osteoblasts and stromal cells express RANK-L, which activates the development and controls the activity of mature osteoclasts (Figure 3) (Boyle et al. 2003). The interactions of RANKL and RANK are also controlled by OPG: soluble OPG acts as a decoy receptor and binds also to RANK-L and subsequently blocks the binding of RANK to RANK-L, and thus decreases the activation of the osteoclasts (Suda et al. 1999, Boyle et al. 2003). This OPG/RANK-L system is regulated by a large number of different hormones, growth factors and cytokines that are known to have an effect on bone turnover (Suda et al. 1999, Boyle et al. 2003, Harada and Rodan
Parathyroid hormone (PTH) is among the best known factors that affect bone formation and resorption (Boyle et al. 2003, Harada and Rodan 2003).

1.4 Functional bone adaptation

Differences between corresponding individual bones may be classified as outcome of long-term (evolutionary) or short-term (during a single lifetime) adaptation (Currey 2003a). Different species have distinct bones because of evolutionary adaptation driven by natural selection. During individual’s lifetime, bones are adapted to the prevailing predominant functional requirements (Currey 2003b) and this phenomenon is referred as functional bone adaptation. In theory, optimal locomotive structure is light for speed and energy efficiency but still strong for load bearing. These conflicting properties are balanced in bone; functional adaptation seems to optimize appropriate structures of minimal weight and maximal strength (Einhorn 1992, Turner and Burr 1993, van der Meulen et al. 2001, Currey 2003a, Currey 2003b).

The concept of bone functional adaptation dates back to the writings by Julius Wolff (1892), which introduced the premise that biological processes could be regulated by mechanical loading. Mechanical forces can modulate the incessant regulation of bone turnover at specific desired sites within bone (Frost 2003). Loading-induced strains in bone are associated with corresponding changes in bone structure which seems to gradually optimize the bone structure for the prevailing loading environment (Frost 2003). Bones are also able to some extent counteract the deleterious effect on some material property by an alteration in the architecture of the whole bone (Currey 2004).

Functional bone adaptation may influence bone strength by changing the absolute bone mass and the distribution of the given bone mass (Turner 2002, Currey 2003a, Currey 2003b, Currey 2004, Ruff et al. 2006). Larger bones can carry more loads and optimized distribution minimizes the mass needed to accomplish structures for the predominant mechanical demands. In addition to these structural changes of adding and redistributing of bone mass, bone strength is also influenced by material properties of bone tissue (Turner 2002).

2 Characterization of bone

The characteristics of bone can be described at different levels: tissue level (material) properties are defined as qualities which are independent of bone size or structure, while characteristics of whole bone or an intact anatomical unit may be considered as organ level structural properties (morphology) (Einhorn 1992, Currey 2001, van der Meulen et al. 2001).
2.1 Material

The solid bone tissue is characterized by physiologically mineralized organic matrix and water. In adult human bone, mineral accounts for 60% to 70% and organic matrix for 30% to 40% of the dry weight (Standring 2005). In living bone, the matrix is moderately hydrated and water accounts for 10% to 20% of the mass. The organic matrix consists predominantly of type I collagen and bone mineral resembles geological hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$). The matrix mineral accumulates at the expense of water so that the tissue volume does not change along the mineralization process.

Mineral density of bone is a measure of mass per unit volume. The definition of apparent mineral density must be distinguished from material density (true bone density). Apparent density of bone equals its total mass divided by its total volume (volumetric bone mineral density, $\text{vBMD}$), whereas in determination of true bone material density only a certain volume of solid cortical bone is included in the analysis (cortical volumetric bone mineral density, $\text{cBMD}$). Computed analysis methods have enabled the attainment of apparent volumetric bone density for any given precise volume of interest, defined anatomically or arbitrarily, since the mass encompassed by this given particular volume can be unambiguously determined. Nevertheless, the determination of true material density for the whole bone is impracticable, because the outer edges of a bone encompass cortical and trabecular bone compartments, and bone marrow, and all these constituents have different tissue densities.

Furthermore, true bone density is the density of bone material when it is considered as uniform material over the particular volume, i.e. material density. The volume to which the mass is referred should therefore be defined to include only bone tissue, and marrow cavity as well as the cavities within trabecular bone need to be excluded. For greatest accuracy, the bone volume should even exclude osteocyte lacunae and canaliculae within cortical bone, but in practice this refinement is usually disregarded. Accordingly, the usual interpretation of increased cortical bone porosity is that bone tissue density is decreased (Parfitt 1998).

2.2 Mass and geometry

The bulk of bone is usually referred as bone mass, which is influenced by absolute size of the whole bone and properties of bone tissue and structure within, and it is usually defined as bone mineral content (BMC). Each bone has specific spatial dimensions and mass distribution which define its geometrical attributes. Periosteal bone formation defines the total cross-sectional area ($\text{tCSA}$), which includes cortical bone, trabecular bone and marrow area. Endocortical bone formation and resorption define cortical wall thickness (CWT) and cortical bone cross-sectional area ($\text{cCSA}$). However, the separation between cortical and trabecular bone is not always definite as the shift is a continuum.
The marrow area in diaphysis of a long bone is defined as the area surrounded by endosteal surface. The outside diameter of bone (width, thickness) is defined as the distance between the opposite periosteal surfaces, and accordingly, the inside diameter of bone marrow cavity (inside width, inside thickness) is defined as the diameter between the opposite endosteal surfaces.

2.3 Biomechanics

Whole bone strength equals to the ultimate force the bone can sustain without a fracture. Bone fractures occur when applied load exceeds the bone strength, and accordingly, bone strength may also be defined as the breaking load (unit newton, N) needed to break the bone structure. Whole bone strength is influenced by the tissue level material properties and organ level structural properties of bone. (Einhorn 1992, Turner and Burr 1993, Currey 2001, van der Meulen et al. 2001, Turner 2002)

The relationship between the load applied to structure (unit newton, N) and deformation (unit millimeter, mm) in response to the load is called a load-deformation curve (Figure 4). The load-deformation curve can be divided into the elastic deformation region and plastic deformation region; elastic deformation is reversible, while plastic deformation refers to permanent structural damage (Turner and Burr 1993).

Figure 4. A typical load-deformation curve of bone mechanical testing until failure.
Mechanical competence of whole bone represents the net influence of all factors acting at lower physical scales (i.e. material properties, mineral mass or geometry) when considering bone as a hierarchical structure (Hernandez and Keaveny 2006). Determination of bone strength by structural testing measures both structural and material properties of bone (Currey 2001, van der Meulen et al. 2001, Turner 2002, Hernandez and Keaveny 2006). Furthermore, it has been argued that conclusions regarding bone mechanical function based solely on properties of bone material, mineral content or geometry are inappropriate and likely to be misleading (van der Meulen et al. 2001, Turner 2002).

3 Chronic kidney disease-bone and mineral disorder

3.1 Physiology of mineral metabolism

Physiological extracellular calcium and phosphate concentrations are essential to life and the deliberate mineral homeostasis is regulated by controlling the serum concentrations of the corresponding ions. Kidneys excrete water and electrolytes, as well as various other substances, in order to control the appropriate concentrations of these in body fluids. Calcitriol and PTH are the principal hormones mediating the regulation of calcium and phosphorous concentration through actions on kidney, bone and intestine (Figure 2).

There is approximately 1 000 g of calcium and 600 g of phosphorous in human body stores. Ninety-nine percent of the calcium is in bone, 0.9% is intracellular and 0.1% in extracellular space. For phosphorous, the distribution is 85% in bone, 14% in intracellular, and 1% in extracellular space. The average daily intake is from 0.05% to 0.10% (from 0.5 g to 1 g) for calcium and from 0.15% to 0.23% (from 0.9 g to 1.4 g) for phosphorous when calculated as percentage of the total reserve in body (absolute amounts in parenthesis). The absorption of calcium and phosphorous in intestine is both active and passive, and one third of the absorbed excess calcium and two thirds of excess phosphorous is excreted in urine by the kidneys and the rest in stool (Moe 2005).

Calcitriol acts on bone to increase mineralization and enhance osteoclast activity, and there are also other minor effects. Calcitriol acts also on parathyroid gland to inhibit PTH secretion, and on intestine to increase calcium and phosphorous absorption. For the present, the action of calcitriol on kidney calcium and/or phosphorous uptake/excretion is controversial. The sources of calcitriol are dietary ergocalciferol and conversion of 7-dehydrocholesterol to cholecalciferol. These precursors are hydroxylated in liver to 25-hydroxycholecalciferol, which is then further hydroxylated by 1-α-hydroxylase to calcitriol (1,25-dihydroxycholecalciferol) in the proximal nephron of kidney (Fraser and Kodicek 1970). The synthesis is regulated by altering 1-α-
hydroxylase activity, which is increased by low phosphorous, low calcium, low calcitriol, and increased by PTH.

PTH increases calcium reabsorption and decreases phosphorous reabsorption from the kidney and increases bone resorption and formation by multiple mechanisms. PTH is released from parathyroid glands in response to lowering of \( \text{Ca}^{2+} \) concentration (hypocalcemia) via \( \text{Ca}^{2+} \) sensing receptor. Hyperphosphatemia stimulates PTH secretion (Fine et al. 1993). Calcitriol inhibits PTH secretion and PTH enhances the synthesis of calcitriol, and thus PTH indirectly increases intestinal absorption of calcium, thereby completing the regulatory feedback loop. Active PTH is cleaved from pre-pro PTH to pro PTH in parathyroid gland, and then secreted and catabolized to active N-terminal 1-84 PTH and less active C-terminal 7-84 fragments. Kidneys are responsible for the plasma clearance and subsequent degradation of 1-84 PTH and a variety of the truncated derivatives of PTH (Freitag et al. 1978).

Phosphatonin is a group of substances (e.g. fibroblast growth factor 23, frizzled related protein 4, and matrix extracellular phosphoglycoprotein) that appear to be involved in regulation of serum phosphorous levels in certain diseases, such as tumor-induced osteomalacia, X-linked hypophosphatemic rickets, and autosomal dominant hypophosphatemic rickets, but for the present, the role of phosphatoinins in normal physiology remains unresolved.

3.2 Definition of chronic kidney disease-bone and mineral disorder

Chronic kidney disease (CKD) is defined as long-term kidney damage and it is commonly associated with deterioration of kidney function. CKD may be staged according to severity of functional impairment, but in literature on experimental studies this staging is often omitted. Accordingly, in experimental studies deteriorated kidney function is widely referred as CRI regardless of the severity of the impairment. The use of the terms renal failure and end-stage renal disease are somewhat problematic in experimental settings due to their clinical definitions. Therefore, throughout the results and discussion sections of this thesis, CRI of the utmost severity is referred as advanced CRI.

The complex and multifactorial skeletal changes associated to CKD have been traditionally described as renal osteodystrophy (Stanbury and Lumb 1962, Stanbury et al. 1969, Parfitt 1972, Parkinson et al. 1979, Hruska and Teitelbaum 1995, Martin et al. 2004), although other terms have also been used, such as renal bone disease and uremic bone disease. In recent years, organizations called Kidney Disease: Improving Outcomes Quality Initiative (K/DOQI) and Kidney Disease Improving Global Outcomes (KDIGO) have defined renal osteodystrophy (ROD) as a constellation of bone disorders present or exacerbated by CKD that lead to bone fragility and fractures, abnormal mineral metabolism, and extraskeletal manifestations (Massry et al. 2003, Moe and Drueke 2004, Moe et al. 2006), or concisely as an alteration of bone morphology in patients with CKD (Moe et al. 2006). In addition, the recent KDIGO position
statement also introduced a broad systemic entity or syndrome called chronic kidney disease-mineral and bone disorder (CKD-MBD) which covers all the clinical, biochemical, and imaging abnormalities due to CKD manifested by the following: abnormalities of calcium, phosphorous, PTH, or vitamin D metabolism; abnormalities in bone turnover, mineralization, volume, linear growth, or strength; and vascular or other soft tissue calcification (Moe et al. 2006). The term osteoporosis is not recommended when describing skeletal complications that are associated with CKD (Cunningham et al. 2004, Moe et al. 2006).

3.3 Epidemiology of chronic kidney disease-bone and mineral disorder

First notes on bone abnormalities associated with renal diseases date back to 19th century and the first epidemiological reports were published during the 1970s and 1980s when fractures in significant number were associated to the use of aluminum-containing dialysis fluids (Parfitt et al. 1972, Ward et al. 1978, Parkinson et al. 1979). The identification of aluminum-related osteomalacia led to changes in composition of dialysis fluids and thereafter the clinical entity of aluminum-related osteodystrophy has practically vanished.

Prevalence of impaired renal function in general adult population is estimated to be approximately 11% (Chadban et al. 2003, Coresh et al. 2003). Rix et al. (1999) have analyzed biochemical markers of bone turnover together with bone mineral density data in mild-to-moderate CRI patients and concluded that skeletal changes seem to initiate early in the course of CKD. In unselected population of predialysis CRI patients, abnormal bone histology was found in 68% of the patients with severe impairment of renal function (Spasovski et al. 2003). Among dialysis patients, 46% have been reported to display gross histological bone abnormalities (Barreto et al. 2006).

Epidemiological studies on U.S. dialysis population report approximately four-fold increases in the incidence of hip fractures in comparison to age-matched population (Alem et al. 2000, Stehman-Breen et al. 2000). In other populations on dialysis, the prevalence of vertebral fracture has been reported to be as high as 21% (Atsumi et al. 1999) and even higher incidence of hip fractures has been reported (Coco and Rush 2000). Further, there is also epidemiological data that older women with less severe (moderate) renal dysfunction are at increased risk of hip fracture (Ensrud et al. 2007). However, in this context, one should recall that that CKD-BMD is usually asymptomatic and the clinical complications appear late in the course of CKD (Llach and Fernandez 2003).
3.4 Pathogenesis of chronic kidney disease-bone and mineral disorder

Disturbances in mineral metabolism and the resulting bone changes are common complications of CKD. The major factors in the pathogenesis of CKD-BMD include renal insufficiency-associated secondary hyperparathyroidism with the associated phosphorous retention (hyperphosphatemia) as renal glomerular filtration rate falls and a decrease in calcitriol levels as metabolically active renal mass is reduced (Figure 5). As a consequence, the serum levels of calcium are affected (Slatopolsky et al. 1971, Goodman 2001, Llach and Velasquez Forero 2001, Martin and Gonzalez 2001a). In incipient CRI, the plasma levels of PTH increase in an obvious attempt to maintain physiological calcium levels and to overcome hyperphosphatemia, but elevated PTH levels may also be observed in absence of hypocalcemia (Lopez-Hilker et al. 1986). Due to this response, the early stage of CKD may be present with elevated PTH levels and normal serum phosphate, calcium and calcitriol levels (Fukagawa et al. 1991, Fajtova et al. 1995, Martinez et al. 1997). But concurrently, particularly the incessant incidence of high phosphorous levels causes activation and progressive growth of parathyroid glands resulting in a process called secondary hyperparathyroidism (Slatopolsky and Delmez 1994, Naveh-Many et al. 1995, Slatopolsky et al. 1996). In addition, abnormal regulation of calcitriol metabolism is also observed with the secondary hyperparathyroidism (Fukuda et al. 1993). Nevertheless, if the renal insufficiency persists and advances, serum phosphorous and calcium levels cannot be maintained within an optimal range despite the development of secondary hyperparathyroidism.

![Figure 5. Some of the crucial factors that contribute to the development of secondary hyperparathyroidism in CRI.](image-url)

In clinical CKD-BMD, persistent high PTH may result in representative histological characteristics of high bone turnover, and in contrast, prevailing low PTH levels are associated with histological features of low bone turnover.
(Salusky and Goodman 2001, Llach and Fernandez 2003, Lehmann et al. 2005). In general, the histological representation of ROD based on determination of bone turnover and mineralization varies greatly from such entities as osteitis fibrosa, osteomalacia, adynamic bone disease, to mixed uremic bone disease. However, it needs to be noticed that in the clinical setting the histological features of ROD are often obscured by the simultaneous existence of diabetes mellitus, immobilization, glucocorticoid-related osteoporosis, and changes induced by prior parathyroid surgery (Salusky and Goodman 2001), which are common in CRI patient population. Further, the histological features may also be influenced by such factors as the race, sex, age, type of kidney disease, nutrition, and menopausal status (Martin et al. 2004).

In addition to the determination of PTH levels, several circulating biochemical markers of bone formation and resorption have also been used in the clinical characterization of bone turnover related to CRI: total or bone-specific alkaline phosphatase, osteocalcin, and carboxy-terminal propeptide of type I collagen as indicators of bone formation, and, tartrate-resistant acid phosphatase, cross-linked C-telopeptide of type I collagen, and N-telopeptide of collagen cross-links as indicators of bone resorption, but their significance and applicability are poor or remain to be established (Urena et al. 1996, Coen et al. 1998, Rix et al. 1999, Urena and De Vernejoul 1999, Salusky and Goodman 2001, Moe et al. 2006). Particularly in CRI, the measures of several of these biomarkers are unreliable, because their elimination depends on kidney function and the markers accumulate in serum if there is dysfunction of glomerular filtration and excretion (Martin et al. 2004, Moe et al. 2006).

3.5 Effects of chronic kidney disease-bone and mineral disorder on bone

3.5.1 Experimental chronic kidney disease-bone and mineral disorder

Subtotally nephrectomized rat has been widely used as an experimental model of CRI and it has been shown to resemble the characteristics of CKD-BMD in biochemistry and bone histology (Kaye 1974, Jablonski et al. 1993, Jablonski et al. 1994, Slatopolsky et al. 1996, Turner et al. 1996, Miller et al. 1998, Sanchez et al. 1998, Geng et al. 2000, Sanchez et al. 2000, Freesmeyer et al. 2001, Sanchez and He 2003, Slatopolsky et al. 2003, Sanchez et al. 2004). More recently, adenine-containing diet has been introduced as a novel means to impair renal function (Katsumata et al. 2003, Nagano et al. 2006), but data on the effects of this model of CKD-BMD is scarce.

The most common forms of ROD are likely to be largely attributable to variations in the plasma levels of PTH. Continuous PTH excess seems to induce an overall catabolic effect on the cortical bone in ROD, the suggested mechanism being an increase in the porosity due to an increase in bone turnover
(Parfitt 1996, Miller et al. 1998, Schober et al. 1998, Slatopolsky et al. 2003, Jamal et al. 2006a). Quite intriguingly, the effect of PTH excess on trabecular bone is generally anabolic in ROD, but there seems to be a myriad of possible mechanisms (Miller et al. 1998, Parfitt 1998, Schober et al. 1998, Slatopolsky et al. 2003). Furthermore, Miller et al. (1998) have also suggested that slightly elevated PTH levels may result in decreased amount of trabecular bone.

Sanchez et al. have studied the effects of CRI on bone growth in weanling rats (Sanchez et al. 1998, Sanchez et al. 2000, Sanchez and He 2003, Sanchez et al. 2004). The findings are somewhat controversial, as the authors have shown data that CRI restricts longitudinal bone growth in one experiment (Sanchez et al. 2004), whereas another experiment failed to show this effect (Sanchez et al. 2000). Others have shown no change in bone length in the nephrectomized (NTX) rats (Kaye 1974). All in all, these studies suggest that when evaluating the effect of CRI on bone longitudinal growth, the changes in body weight need to be included in the analysis. Reduced bone longitudinal growth has been associated with reduced weight gain (Sanchez et al. 2004), whereas in most of the studies NTX has not been associated with either reduced gain in bone length or body weight (Kaye 1974, Sanchez et al. 2000). However, in general NTX does not seem to influence body weight in pair fed (Russell and Avioli 1972, Kaye 1974) or in ad libitum fed rats (Kaye 1974, Slatopolsky et al. 1996, Miller et al. 1998, Sanchez et al. 1998, Sanchez et al. 2000, Freesmeyer et al. 2001, Sanchez and He 2003).

Abnormal mineralization is observed in subtotally nephrectomized rats after 2 weeks (Russell and Avioli 1972). Turner et al. (1996) have estimated bone mineral density 6 months after a NTX operation from a section of rat femoral midshaft by dividing wet weight by volume and concluded that advanced CRI is associated with a decrease in bone density. In a 12-week experiment, Miller et al. (1998) determined BMC and bone width by single-photon absorptiometry at 10 equidistant sites in femur and calculated bone mineral density as the BMC divided by bone width (i.e. areal bone mineral density, aBMD). They reported that aBMD is uniformly decreased in mild ROD and unchanged in moderate disease, while in severe ROD, aBMD is decreased only in proximal femur and increased in distal femur (Miller et al. 1998). However, they did not provide data on actual changes in BMC or bone width at these sites. In the histomorphometrical determination of rat tibial metaphysis the trabecular bone volume per total volume is increased and in tibial shaft cortical bone porosity ratio is increased (Miller et al. 1998, Slatopolsky et al. 2003). In rat lumbar vertebrae CRI is associated with increased bone volume per tissue volume (BV/TV) and osteoid thickness (Turner et al. 1996).

The changes in the gross bone geometry of NTX rats include a decrease in outer diameters of femur (Jablonski et al. 1993); or no change in the outer diameter, an increase in inner diameter, and a decrease in CWT (Jablonski et al. 1994). Miller et al. (1998) observed no change in tCSA, cCSA, or marrow area. More recently, Slatopolsky et al. (2003) have reported a slight increase in tCSA
with no change in cCSA, marrow area or CWT after 8 weeks of CRI, and an additional decrease in marrow area and an increase in CWT after 16 weeks of CRI.

There is sparse data on the effects of CRI on whole bone strength. The group of Jablonski has reported that bone strength is maintained during 28 or 33 weeks of CRI (Jablonski et al. 1993, Jablonski et al. 1995), but decreased bone strength was reported after 38 weeks of CRI (Jablonski et al. 1993). After 6 months of advanced CRI, Turner et al. (1996) have determined breaking load by three-point bending in femoral midshaft and by compression in lumbar vertebrae, and observed a decrease in breaking strength in both locations.

3.5.2 Clinical kidney disease-bone and mineral disorder

Bone measurements with dual energy X-ray absorptiometry (DXA) in patients with CKD have provided data on changes in bone mineral density along the course of the disease and most of the studies conclude that reduced renal function is associated with decreased bone mineral density (Bianchi et al. 1992, Gabay et al. 1993, Rix et al. 1999, Hsu et al. 2002, Klawansky et al. 2003, Lobao et al. 2004). Furthermore, when patients are classified according to the impairment of renal function, there seems to be a close relationship between renal function and bone mineral density (Bianchi et al. 1992, Rix et al. 1999, Hsu et al. 2002). These studies also suggest that deleterious processes affecting bone mineral properties commence early in the course of CRI. However, although a large general population sample (National Health and Nutrition Examination Survey of USA, NHANES III) did indeed confirm that renal function correlates with bone mineral density, it was also shown that when the data was adjusted for age and weight, the negative association between renal function and bone mineral density extinguishes (Hsu et al. 2002). On the contrary, Klawasky et al. (2003) have analyzed the same data and concluded that decreased renal function was associated with reduced bone mineral density, but the adjustments for age and weight were absent.

The most consistent finding regarding end-stage renal disease patients in dialysis is a decrease in the DXA-derived bone mineral density, but similarly to predialysis patients, the relationship was affected by age, gender or body mass index (BMI) (Bianchi et al. 1992, Gabay et al. 1993, Russo et al. 1998, Atsumi et al. 1999, Taal et al. 1999, Jamal et al. 2006a). In a detailed evaluation of bone loss in patients on dialysis by single-photon absorptiometry, Schober et al. (1998) found that particularly cortical bone volume is reduced and this is due to an increase in cortical porosity and a decrease in CWT. Russo et al. (1998) used computed tomography to assess the cortical compartment showed that cBMD and cCSA were reduced in long bone diaphysis. Recent data from Jamal et al. (2006a) corroborated these changes observed in cortical bone, and furthermore, they suggest that after adjusting for age, weight and sex, a decrease in cBMD, cCSA and CWT are associated with an increase in fracture risk in dialysis
patients. The changes associated with ROD in bone sites which are rich in trabecular bone seem to be more complex (Schober et al. 1998); Russo et al. (1998) reported no differences in trabecular vBMD while Jamal et al. (2006a) observed a decrease in vBMD. However, the separation between cortical and trabecular bone was defined as a fixed percentage of total area in both studies and this may generate inaccuracy particularly in patients with ROD, as the disease is associated with changes in the mineralization of the endosteal surface.

3.6 Pharmacological treatments for chronic kidney disease-bone and mineral disorder

3.6.1 Calcium-based phosphate binders

Data from experimental studies show that excess intake of dietary phosphorous is associated with an increase in plasma levels of phosphorous and growth of the parathyroid glands in CRI rats (Kaye 1974). In vivo and in vitro data has also shown that phosphorous has a direct stimulatory effect on PTH secretion and parathyroid cell proliferation, and that restriction of dietary phosphate prevents the development of these changes independently of changes in calcium and calcitriol levels (Kilav et al. 1995, Naveh-Many et al. 1995, Almaden et al. 1996, Denda et al. 1996, Slatopolsky et al. 1996). Reduced phosphate intake has been associated with an increase in calcitriol levels in healthy adults (Portale et al. 1986) and amelioration of secondary hyperparathyroidism in CRI patients (Martinez et al. 1997).

The mechanism of action of the phosphate-binding agents is to form non-absorbable compounds with phosphorous in the intestine lumen. Calcium salts (carbonate or acetate) are the most widely used phosphate binders and have been shown to be effective in lowering serum phosphorous and PTH levels in experimental and clinical studies with different degrees of CRI (Kaye 1974, Slatopolsky et al. 1986, Johnson et al. 2002, Coladonato 2005, Salusky 2006). The use of phosphate-binders is well established in the clinical treatment of CKD-BMD.

However, calcium salts contain a high proportion of elemental calcium and from 20% to 30% of that amount is absorbed from the intestine. The resulting increase in the calcium intake may affect bone metabolism in CKD (Salusky and Goodman 2001). Particularly, there is a great concern that the use of calcium salts as phosphate binders together with long-term imbalance between calcium and phosphorous may contribute to the development and progression of cardiovascular calcification in CKD patients (Goodman et al. 2000, Llach and Fernandez 2003, Goldsmith et al. 2004, London et al. 2004).
3.6.2 Sevelamer

Sevelamer hydrochloride is a specifically designed hydrogel of cross-linked poly-allylamine which is not absorbed and does not contain calcium or aluminum (Burke et al. 1997, Plone et al. 2002). Sevelamer acts as a phosphate binder in the intestine and the treatment has been associated with reduced phosphorous and PTH levels in experimental CRI (Rosenbaum et al. 1997, Nagano et al. 2001, Cozzolino et al. 2002, Cozzolino et al. 2003, Katsumata et al. 2003, Nagano et al. 2003a, Nagano et al. 2003b). The efficacy of the compound in phosphate binding seems fairly similar to calcium salts, but the use of sevelamer is not associated with the above noted potentially harmful elevation in calcium levels (Cozzolino et al. 2002, Cozzolino et al. 2003). These experimental findings are in agreement with the data from clinical studies and sevelamer has been considered a potentially valuable treatment option for patients with CRI. (Chertow et al. 1997, Bleyer et al. 1999, Chertow et al. 1999a, Chertow et al. 1999b, Slatopolsky et al. 1999, Chertow et al. 2002, Qunibi et al. 2004, Asmus et al. 2005, Raggi et al. 2005, Salusky 2006).

3.6.3 Calcitriol and paricalcitol

Calcitriol controls the growth of the parathyroid gland and inhibits the synthesis and secretion of PTH in parathyroid cells (Llach and Velasquez Forero 2001, Martin and Gonzalez 2001a) and therefore calcitriol has been used in the treatment of secondary hyperparathyroidism. However, calcitriol also increases the intestinal absorption of calcium and phosphorous and therefore inappropriate treatment may contribute to the development of toxic hypercalcemia and hyperphosphatemia (Llach and Velasquez Forero 2001, Llach and Fernandez 2003). Long-term calcitriol therapy may also contribute to the suppression of bone turnover in CRI (Goodman et al. 1994, Salusky and Goodman 2001, Llach and Fernandez 2003).

Certain synthetic analogs of calcitriol have been developed with an expectation of exhibiting a similar suppressive activity on the parathyroid but lesser potential to induce hypercalcemia and hyperphosphatemia than native calcitriol. Currently the most salient such compounds are paricalcitol (19-nor-1,25-(OH)2D2), 22-OCT (22-oxa-1,25-(OH)2D3), alfacalcidol (1-α-OH-D3), and doxercalciferol (1-α-OH-D2). Paricalcitol has been shown to effectively suppress CKD related increase in plasma PTH with less pronounced effects on serum calcium and phosphorous levels than calcitriol in experimental (Brown et al. 1990, Slatopolsky et al. 1995, Takahashi et al. 1997, Brown et al. 2002, Slatopolsky et al. 2002, Slatopolsky et al. 2003) and clinical studies (Llach et al. 1998, Martin et al. 1998a, Martin et al. 1998b, Martin and Gonzalez 2001b, Sprague et al. 2001, Sprague et al. 2003). It has also been suggested that there may be a general survival advantage when paricalcitol is compared to calcitriol treatment (Drueke and McCarron 2003, Teng et al. 2003).
3.6.4 Bisphosphonates

Bisphosphonates are inhibitors of bone resorption. The compound concentrates in bone wherein they are taken up by osteoclasts, and subsequently, acts by inhibiting osteoclast activity. Bisphosphonate treatment has been shown to result in reduced bone resorption and positive net bone balance (Rodan and Fleisch 1996, Fleisch 1998). Alendronate and risendronate are well proven agents in the prevention and treatment of postmenopausal osteoporosis (Chavassieux et al. 1997, Cranney et al. 2002), but data on the fracture-prevention efficacy of pamidronate is scarce.

Bisphosphonates seem to reduce bone turnover regardless of its cause, and accordingly, bisphosphonates may be considered as potential treatment options for CKD-BMD. Accordingly, it has been speculated that bisphosphonates could be particularly favorable in the case of high bone turnover ROD and the associated increased cortical porosity. However, although bisphosphonates are widely used in the treatment of postmenopausal osteoporosis, it has been speculated whether bisphosphonate treatment is associated with impaired microdamage repair (Burr et al. 1997, Mashiba et al. 2000, Fan and Cunningham 2001) or with inappropriate increase in mineralization causing increased brittleness (Meunier and Boivin 1997, Currey 2004). Reduction of bone turnover may be a therapeutic objective in ROD, but currently the use of bisphosphonates in ROD is not recommended (Rodan and Martin 2000, Fan and Cunningham 2001, Turner 2002, Klawansky et al. 2003, Massry et al. 2003, Langman et al. 2005).

Pamidronate (3-amino-1-hydroxypropylidene-1,1-biphosphonate) contains nitrogen in the side chain and it is called an amino-substituted second-generation bisphosphonate. The alteration in the side chain substitution influences the affectivity of the bisphosphonate. The structure of bisphosphonates resembles inorganic pyrophosphate but instead of the oxygen bridge (P-O-P) there are two C-P bonds on the same carbon and two additional lateral side chains (P-C-P). This structure is resistant to enzymatic hydrolysis and therefore bisphosphonates are excreted unaltered by an active process in the kidneys (Rodan and Fleisch 1996).

3.7 Evidence for the efficacy of the prevention and treatment of chronic kidney disease-bone and mineral disorder

An effective treatment for bone fragility should improve the biomechanical properties of bone as organ, i.e. a combination of positive effects on the various particular of bone structural strength (material properties, mineral mass, geometry, etc.) that result in an increase in bone mechanical competence (Turner 2002).

In adenine-induced model of CRI in rats, Katsumata et al. (2003) evaluated the effects of 4-week sevelamer treatment on bone histology and found that
cortical bone porosity was decreased. Fifty-two week treatment with calcium salts has been associated with reduction in thoracic trabecular and cortical bone attenuation evaluated by computed tomography as an index of bone mineral density in dialysis patients (Raggi et al. 2005). Raggi et al. (2005) also compared the effects of the treatment with calcium salts to a treatment with sevelamer, and they concluded that the sevelamer treatment is associated with more beneficial effect on both cortical and trabecular bone mineral amount than the treatment with calcium salts. Asmus et al. (2005) reported that calcium salt administration was associated with decreased trabecular vBMD while sevelamer treatment prevented the change in a two-year follow up study comparing the effects of sevelamer and calcium administration in dialysis patients. However, in this study, no difference was observed in cBMD between the groups (Asmus et al. 2005). On the other hand, Salusky et al. (2005) analyzed bone histology in dialysis patients treated with either sevelamer or calcium salts and concluded that both treatments are equally efficient in controlling ROD. However, there is no data on the effects of sevelamer treatment on any indices of bone strength in CRI.

Paricalcitol has been shown to be approximately ten times less effective in mobilizing calcium and phosphorous from bone than calcitriol (Finch et al. 1999). Finch et al. (2001) also suggested that it is unlikely that paricalcitol treatment would have a deleterious effect on bone. Subsequently, it was also shown that paricalcitol treatment efficiently reduced cortical porosity and ameliorated trabecular bone pathology that is associated with ROD (Slatopolsky et al. 2003). Calcitriol treatment has been shown to enhance bone mechanical properties in CRI (Jablonski et al. 1995), but there is no data on the effects of paricalcitol treatment on bone strength.

In moderate experimental CRI, ibandronate (bisphosphonate) treatment was associated with decreased bone turnover and erosion with increased bone volume (Geng et al. 2000). Data on the efficacy of bisphosphonates in patients with predialysis CKD or in dialysis patients is scarce, presumably because bisphosphonate treatment is not used in CKD patients. However, Torregrosa et al. (2003) have shown that pamidronate treatment is associated with an increase in aBMD in lumbar spine and proximal femur in dialysis patients, although the study was devoid of a control group. There is no data on the effects of bisphosphonate treatment on any indices of bone strength in CRI. As a remark, bisphosphonate treatment has been shown to result in a positive effect on bone mineral density when used in renal transplant patients (Fan et al. 2000, Grotz et al. 2001, Coco et al. 2003, Fan et al. 2003, Haas et al. 2003). It has, however, been suggested that in this context the positive effect of bisphosphonate treatment is not long-lasting (Schwarz et al. 2004).
AIMS OF THE STUDY

Since the principal task of bones is to allow locomotion and bear incident loads without breaking (Einhorn 1992, Parfitt 1998, van der Meulen et al. 2001), an organ level approach was established to assess the skeletal changes associated with CRI. Equally, the objective was to evaluate the effects of certain pharmacological treatments on bone structure in CKD-BMD. Accordingly, the specific objectives of the individual studies were the following:

1. To characterize the changes in bone structure and mechanical strength attributable to experimental CRI.

2. To determine the effects of calcium carbonate and sevelamer treatments on bone in the management of CRI associated hyperphosphatemia.

3. To evaluate the efficacy of vitamin D analogue paricalcitol treatment on bone in CRI.

4. To explore the effects of bisphosphonate pamidronate administration on bone in CRI.
MATERIALS AND METHODS

1 Animals

Male rats of Sprague-Dawley strain were used in the experiments of this thesis. The specific objective of this series of experiments was to assess the effects of the disease per se (CRI) and the potential agents used to counteract these deleterious effects on bone structure and strength. Being obvious that human experimentation cannot generate a suitable sample for such analysis, it is readily apparent that an appropriately designed animal experiment is reasoned and justified approach (Marcus et al. 1996). All the experimental study designs were approved by the Animal Experimentation Committee of the University of Tampere, Finland, and the Provincial Government of Western Finland Department of Social Affairs and Health, Finland. The studies conformed to the NIH Guide for the Care and Use of Laboratory Animals.

Animals were housed two per cage in an animal laboratory at 22°C temperature with light cycle of 12 hours and fed standard laboratory food pellets containing 0.9% calcium, 0.8% phosphate, 0.27% sodium, 0.2% magnesium, 0.6% potassium, 1 500 IU/kg 1,25(OH)2D3, and 12 550 kJ/kg energy (Lactamin; AnalyCen, Lindköping, Sweden), except in the treatment groups of the study II (see below), and in studies III and IV in which the calcium content of the diet was 0.3% for all groups during the treatment period.

2 Nephrectomy

Animals were operated at the age of 8 weeks in all these experimental studies. Operations were performed under 75 mg/kg ketamine (Parker-Davis Scandinavia, Solna, Sweden) and 2.5 mg/kg diatepam (Orion Pharma, Espoo, Finland) anesthesia that was given intraperitoneally. Rats were randomly subjected to either surgical bilateral kidney decapsulation (Sham) or subtotal 5/6 nephrectomy surgery, except for study IV, in which the rats were subjected to unilateral nephrectomy instead of kidney decapsulation. In the 5/6 nephrectomy operation, the entire right kidney was removed, along with the upper and lower poles of the left kidney (Jolma et al. 2003, Pörsti et al. 2004). In the decapsulation, the kidney capsule was surgically removed but kidneys were left otherwise intact. The unilateral nephrectomy was performed as a Sham-operation.
in study IV to create equal inflammatory conditions in all groups and accordingly, to be able to eliminate the potential confounding influence of surgical trauma on the renal function and drug pharmacodynamics. In unilateral nephrectomy right kidney and small sections of the upper and lower poles of the left kidney were removed. It was confirmed that unilateral nephrectomy did not result in CRI. The surgical operations were followed by 4 week (II), 14 week (additional separate assessment of effects of sevelamer treatment), 15 week (III), and 14 week (IV) periods of disease progression.

3 Pharmacological treatments

3.1 Phosphate binding therapies

3.1.1 Calcium salts

In the study II, after the disease progression the animals were allocated to 3.0% calcium diet (Lactamin; AnalyCen, Lindköping, Sweden) or continued on the previous 0.3% calcium diet for the subsequent 8 weeks. The extra calcium was supplied as calcium carbonate salt and otherwise the chows were identical to the control groups (Figure 6).

3.1.2 Sevelamer

The effects of sevelamer treatment were assessed in an additional experiment (Figure 6). Accordingly, after the disease progression the animals were allocated to untreated and sevelamer treated, and the latter groups received diet containing 3% sevelamer (SEV; sevelamer hydrochloride; Genzyme, Cambridge, MA, USA) for 9 weeks.

3.2 Paricalcitol

In the study III, animals in the treatment group were given intraperitoneal injections of paricalcitol (PAR; 19-nor-1,25-(OH)2D2; Abbott Laboratories, IL, USA) 100 ng per rat three times a week for 12 weeks (Figure 6).
Figure 6. Flowchart of the individual studies. AS, additional study assessing the effects of sevelamer and CRI.
3.3 **Pamidronate**

In the study IV, pamidronate (APD; 3-amino-1-hydroxypropyridene-1,1-biphosphonate; Novartis, Switzerland) was administered 3 mg/kg subcutaneously once a week for 8 weeks (Figure 6). The drug dose was chosen on the basis of previous publications on APD treatment in rat, in which the weekly dose has ranged between 0.5-10.5 mg/kg (Bourrin et al. 2002, Mayahara and Sasaki 2003, Mekraldi et al. 2005).

4 **Samples**

4.1 **Blood**

At sacrifice, the carotid artery was cannulated and blood samples for creatinine, urea, phosphate, and PTH were drawn into chilled tubes (II-IV). The tubes contained ethylenediaminetetraacetic acid (EDTA) or heparin as anticoagulants, as appropriate, after which the samples were centrifuged, and the plasma stored at -70ºC until analysis. Plasma creatinine was measured by the colorimetric assay according to Jaffe, urea by colorimetric enzymatic dry chemistry, and phosphate by colorimetric dry chemistry (Vitros 950 analyzer, Johnson & Johnson Clinical Diagnostics, NY, USA). PTH was measured using immunometric assay (Immutopics, San Clemente, California, USA).

4.2 **Kidney**

Five-micrometer thick sections of aortic artery and kidney were stained with the von Kossa method and processed for light microscope evaluation (II-IV). An expert who was blinded to the experiments qualified all histology at x200 magnification. The calcification was counted from 10 random sections. Each field was divided into 100 grids, and each grid containing foci of calcification denoted one score. Total scores of each field were counted. The index of calcification was determined for each rat by the mean score of the calculated 10 fields (Pörsti et al. 2004).

4.3 **Bone**

At sacrifice, both hindlimbs were excised and all the surrounding skin, muscle and soft tissue were carefully excised to collect both femora. The femora were then wrapped in saline soaked gauze bandage and stored at -20ºC in small, sealed freezer bags. At the day of measurement, the bones were slowly thawed at room
temperature at least 15 h before actual mechanical testing and kept wrapped in the saline-soaked gauzes except during measurements. For each rat, all measurements were performed successively in the same order. For histological determination, certain femora were placed in 70% ethanol solution after excision.

4.3.1 Peripheral quantitative computed tomography

The cross-sections of the femoral necks, femoral midshaft and distal femur were scanned with a commercial peripheral quantitative computed tomography (pQCT) system (Stratec XCT Research M with Software version 5.40B; Stratec Medizintechnik, Birkenfield, Germany) (Figure 7).

Figure 7. A longitudinal slice of rat proximal femur (left) and corresponding representative (magnified) cross-sectional slices of femoral midshaft (bottom right) and femoral neck (top right).
For femoral neck analysis, the femur was inserted into a special plastic tube with femoral neck in axial direction. The scan line was adjusted to midneck using scout view option of the software and one cross-sectional image was scanned with a voxel size of 0.070 x 0.070 x 0.5 mm\(^3\) and scan speed 3.0 mm/s. Total cross-sectional area (tCSA), total bone mineral content (BMC), and total volumetric bone mineral density (vBMD) were recorded as given by the pQCT software. Each bone was measured twice with repositioning of the sample between the measurements, and the average of the measurements was used as the variable. In our laboratory, the average (root-mean-square) coefficient of variation (CV\(_{\text{rms}}\)) were 3.9\% for tCSA and 2.1\% for vBMD, respectively (Pajamäki et al. 2003).

For femoral midshaft and distal femur analysis, the femur was inserted into a specially constructed plastic tube. One cross-sectional slice from each bone was scanned at midshaft (at 50\% of the measured femur length) and distal femur (10\% of the femur length, measured from the distal end) with a voxel size, 0.070 x 0.070 x 0.5 mm\(^3\) and scan speed 3.0 mm/s. The tCSA, cortical cross-sectional area (cCSA), cortical volumetric bone mineral density (cBMD), and BMC of the femoral midshaft, and tCSA and BMC of the distal femur were recorded as given by the pQCT software. In our laboratory, the CV\(_{\text{rms}}\) in the femoral midshaft were 0.9\% for the tCSA, 1.5\% for the cCSA, and 0.6\% for the cBMD (Pajamäki et al. 2003).

4.3.2 Geometrical measurements

A digimatic caliper (Mitutoyo 500, Andover, United Kingdom) and pQCT image analysis, both providing a resolution of 0.01 mm, were used to determine the bone dimensions and geometry. The length of the femur was measured from the tip of the greater trochanter to the intercondylar notch. The outside diameters of the femoral shaft were measured in the mediolateral (ML\(_{\text{OUTSIDE}}\)) and anteroposterior (AP\(_{\text{OUTSIDE}}\)) directions. Likewise, the orthogonal inside diameters (ML\(_{\text{INSIDE}}\), AP\(_{\text{INSIDE}}\)) of the medullary canal of the femoral shaft were determined at the break line after three-point bending of the shaft (described in the subsequent text). Additionally, the femoral shaft was considered a hollow elliptic shaped structure, and following geometric indices were determined: the average cortical wall thickness as given by the pQCT software; cortical wall thickness (CWT) in ML (CWT\(_{\text{ML}}\)) and AP (CWT\(_{\text{AP}}\)) directions as CWT\(_{\text{ML}} = (\text{ML}_{\text{OUTSIDE}} - \text{ML}_{\text{INSIDE}})/2\) and CWT\(_{\text{AP}} = (\text{AP}_{\text{OUTSIDE}} - \text{AP}_{\text{INSIDE}})/2\); principal cross-sectional moments of inertia (CSMI\(_{\text{MAX}}\) and CSMI\(_{\text{MIN}}\)) and the polar section modulus (SSI\(_{\text{POLAR}}\)) according to common engineering principles. In our laboratory, CV\(_{\text{rms}}\) for repeated measurements of femur dimensions range from 0.2\% (femur length) to 4.0\% (midshaft inside width) (Järvinen et al. 1998a, Järvinen et al. 1998b).
4.3.3 Dual-energy X-ray absorptiometry

The total bone mineral content of the whole femur (TBMC) was measured with dual-energy X-ray absorptiometry (DXA) (II, IV). All measurements were done using a standard DXA scanner (Norland XR-26; Norland Corporation, Fort Atkinson, Wisconsin, USA) with additional scanner modification for small animal measurements (Sievänen et al. 1994a). The standard general scan option (version 2.2.2) was applied for scanning and the scan data was analyzed with the research scan option (version 2.5.2) Pixel spacing was 0.5 x 0.5 mm and scan speed 10 mm/s. For scanning, the femur was placed at a constant location of the scan table on its posterior surface. The baseline point was located on the soft tissue equivalent. The scanner was calibrated daily according to manufacturer’s recommendations, and the scanner performance was controlled by the quality assurance protocol of our laboratory (Sievänen et al. 1994b). In our laboratory, the precision (CV<sub>rms</sub>) for repeated measurements of TBMC of rat femur is 1.6% (Järvinen et al. 1998a, Järvinen et al. 1998b).

4.3.4 Bone histomorphometry

The histomorphometric measurements were performed using the OsteoMeasure Analysis System (OsteoMetrics, Atlanta GA, USA) (IV). The distal femoral metaphysis was dehydrated and embedded without demineralization. The tissue was sectioned at 5 microns and the sections stained with toluidine blue. An area of 7.5 mm<sup>2</sup>, immediately distal to the primary spongiosa and excluding the cortical bone margins, was analyzed to obtain the following static bone measurements and calculated values as described (Turner et al. 1998): bone volume normalized to tissue volume (BV/TV), trabecular number, trabecular thickness, and trabecular separation (Turner et al. 1998).

4.3.5 Biomechanical testing

Femora were mechanically tested using a Lloyd material testing device (LR5K, J.J. Lloyd Instruments, Southampton, United Kingdom). Anteroposterior three-point bending, mediolateral three-point bending, and compression of femoral neck were performed (Sogaard et al. 1994, Järvinen et al. 1998a, Järvinen et al. 1998b, Leppänen et al. 2006).

For the anteroposterior three-point bending, the femur was placed on its posterior surface on the supports of the bending apparatus (Figure 8A). For each bone, these supports were placed individually just distal to the trochanter minor and the other just proximal to the condyles of the femur. Before the actual testing, a small stabilizing preload was applied on the superior (anterior) surface of the femur at a rate of 0.1 mm/s using a crossbar fixture (a plate with rounded edges of 10 mm diameter). The load was then applied at a rate of 1.0 mm/s until
the failure of the specimen, and the breaking load and energy absorption were determined.

For the mediolateral three-point bending, the femur was placed on its lateral surface on the lower supports of the bending apparatus (Figure 8B). For each bone, these supports were placed individually so that one was under the trochanter major and the other under the distal femur. To prevent the otherwise unavoidable twisting of the bone to the anteroposterior position during loading, the intercondylar fossa of femur was gently pressed between the blades of blunt pliers tightly attached to the bending apparatus. The preloading and actual loading were carried out as described above (Leppänen et al. 2006).

![Figure 8](image)

**Figure 8.** (A) Three-point bending of the rat femoral midshaft in anteroposterior direction; (B) three-point bending of the femoral midshaft in mediolateral direction; and (C) compression test of the femoral neck.

After the three-point bending of the femoral shaft, the proximal part of each specimen was collected and femoral neck was subjected to compression test using the materials testing machine. In the femoral neck compression test, the proximal half of each femur was mounted in a specially constructed fixation device (Sogaard et al. 1994). The specimen was then placed under the materials testing device, and the vertical load was applied to the top of the femoral head using a brass crossbar until failure of the femoral neck (Figure 8C). The
preloading and actual loading were carried out as described above. The breaking load was determined from a load-deformation curve. In our laboratory the $CV_{rms}$ of the breaking load for anteroposterior three-point bending, mediolateral three-point bending and femoral neck compression are 5.0%, 3.8%, and 7.6%, respectively (Järvinen et al. 1998a, Järvinen et al. 1998b, Leppänen et al. 2006).

5 Statistical analysis

All measurements and analysis were blinded to the group assignments. The descriptive variables were reported as mean and standard deviations, and measurement results were reported as mean and standard deviations, standard error of mean or 95% confidence intervals. Two-way analysis of variance (ANOVA) was used to determine the effects of CRI and the effects of calcium salt, sevelamer, and pamidronate treatment. One-way ANOVA was used to determine the effect of paricalcitol treatment in CRI. Differences were considered significant when $P < 0.05$. The predominant stresses in the long bones of the lower extremities stem from weight bearing, concomitant bending, and torsional loading produced by the muscles attached to long bones (Burr 1997, Frost 1997). Accordingly, to eliminate the inherent bias arising from comparisons between study groups that may differ in body weight and size (e.g. uremic versus control), data pertaining to bone mechanical competence was equalized to in terms of the animal’s apparent loading environment by using the body weight and femoral length of each rat as covariates in the analysis (Järvinen et al. 2003a, Järvinen et al. 2003b, Pajamäki et al. 2003).
RESULTS

The surgical subtotal nephrectomy was found effective in inducing a disease state mimicking that of typical CRI in humans, characterized by elevated plasma creatinine, urea, phosphorous and PTH levels. These values are generally used as indices of impairment of renal function and to approximate the prevailing metabolic disorder, and accordingly, these values may be considered to reflect the severity of CRI. In the histological analyses of kidney tissue, the CRI rats showed moderate increases in glomerulosclerosis, interstitial damage and kidney calcifications when compared with the Sham rats. Thus, the morphology of the kidneys confirmed the CRI which was indicated by changes in serum chemistry. The determination of whole bone strength effectively covers virtually all of the individual variability that may be instantaneously, sporadically or permanently present in structural particulars (size and shape of the bone, cortical thickness and specific cortical geometry, trabecular architecture, etc.), providing the ultimate assay on bone functional capacity (I). Accordingly, an organ-level assessment was reasoned approach to explore the effects of experimental CRI on bone (I).

The thesis includes data that has not yet been published, since a discrete additional experimental study was carried out to assess the effects of hyperphosphatemia treatment with sevelamer administration. To make the results from the individual studies commensurate with each other, the severity of the renal condition must be addressed, and accordingly, indices of renal function were determined by serum biochemistry in each study (Figure 9). When these characteristics are combined with the mortality data (Figure 6) over the course of the disease, it is possible to approximate the severity of CRI in each study. Accordingly, studies II and IV may be considered to represent mild-to-moderate CRI, whereas study III and the additional study on effects of sevelamer treatment are models of severely advanced CRI.
Figure 9. Characteristics of serum biochemistry in each study (roman numerals, II-IV; AS, additional study assessing the effects of sevelamer and CRI). Plasma levels of (A) creatinine; (B) urea; (C) phosphate; and (D) PTH. Bars represent the mean in untreated CRI group of each study. The dashed line represents the mean value in Sham and solid lines limit the range of standard deviation.

1 The effects of experimental chronic renal insufficiency on bone structure and strength

In this structurally oriented (I) series of experiments the CRI-induced changes on bone were assessed and CRI was associated with deterioration of bone in all experiments. Already mild-to-moderate CRI induced a significant decrease in the BMC of the whole femur, a deficit that ranged from -4.8% (data from the additional study on effects of sevelamer treatment) to -6.2% (II). In the evaluation of the trabecular structure in the distal femoral metaphysis, the present model of CRI resulted in the characteristic histomorphometric features of ROD, including increased bone turnover and peritrabecular fibrosis (IV). In the site-specific analysis of the femur, it was possible to depict the extent to which the vBMD or cBMD, cross-sectional geometry, BMC, and breaking load of the different regions of rat femur were affected by the disease in all experiments.

CRI had the most pronounced effect on vBMD. In the femoral neck the changes ranged from -6.5% to -11% (Figures 10A, 11C, and 12A) and in mild-to-moderate CRI a simultaneous increase in tCSA was observed (Figure 10B). To further examine the relationship between the renal function and bone geometry of the femoral neck, additional 25 Sham and 7 subtotally nephrectomized animals (NTX) were pooled with the data from study II. The added Sham and NTX animals showed similar changes in plasma creatinine and urea to the previous results. In the pooled analyses, the NTX rats showed corresponding increases of plasma creatinine and urea as above, and reduced
bone mineral density and increased cross-sectional area in the femoral neck (Figures 13A and 13B). However, no differences were observed in the breaking load, while vBMD of the femoral neck showed a significant inverse correlation with tCSA in both Sham and NTX rats (Figures 13C and 13D). The mechanical strength of femoral neck was maintained in mild-to-moderate CRI (Figure 10D) (II). However, in groups with more advanced CRI a decrease in breaking load up to -16% was observed (III, and data from the additional study on effects of sevelamer treatment) (Figures 11F and 12D).

The results from the analysis of femoral midshaft showed a decrease in cBMD ranging from -1.2% to -6.6% (Figures 14A and 15A). No CRI-induced differences were observed in tCSA or cCSA between the study groups (Figures 14B, 14C, 15B, 15C, and 16), and CRI was associated with a decrease in BMC (Figures 14D and 15E). However, a localized thinning of the cortex in the medial and lateral aspects of the femoral midshaft was evidenced in the determination of CWT in association with severe CRI (data from the additional study on effects of sevelamer treatment) (Figure 14F). Bone strength was evidently maintained in mild-to-moderate CRI (Figure 15F), whereas the more advanced CRI was associated with a reduction in the breaking load of the femoral midshaft (Figures

Figure 10. The effects of CRI and calcium treatment (Ca) in femoral neck: (A) vBMD; (B) tCSA; (C) BMC; and (D) breaking load. Bars represent the mean + SD. Significant differences are indicated: a P < 0.01 CRI-groups vs. Sham-groups; b P < 0.05 CRI vs. Sham; c P < 0.001 CRI+Ca vs. Sham+Ca; d P < 0.05 CRI groups vs. Sham-groups.
14G and 14H). In distal femur, mild-to-moderate CRI induced histological features of ROD, but no changes were detected in amount of bone, or in the trabecular thickness or number.

Figure 11. The effects of CRI and sevelamer treatment (Sev) in femoral neck: (A) width in mediolateral direction; (B) thickness in cephalocaudal direction; (C) vBMD; (D) tCSA; (E) BMC; and (F) breaking load. Bars represent the mean ± SD. Significant differences are indicated: a P < 0.05 vs. Sham, b P < 0.05 vs. CRI, c P < 0.05 CRI-groups vs. Sham-groups.

2 The effects of the treatment of hyperphosphatemia on bone

CRI results in a progressive hyperphosphatemia (Figure 9C), a condition that is associated with the above described detrimental effects on bone. Accordingly, the effects of two different dietary phosphorous binding agents, calcium carbonate and sevelamer hydrochloride, on bone structure were investigated in
Two separate studies (II, and data from the additional study on the effects of sevelamer treatment). In study II, the administration of calcium salts in experimental CRI was efficient in reducing phosphorous levels and resulted in anticipated suppression of PTH levels. In terms of bone mineral content, calcium administration revoked the CRI-induced decrease in BMC of the whole femur. In this study calcium administration was not associated with any changes in the site-specific analysis of vBMD, tCSA or BMC in the femoral neck (Figures 10A-C) or femoral midshaft, and consistently, no differences were observed in the evaluation of morphological indices (CSMIMAX, CSMIMIN and SSIpolar) of the femoral midshaft. Moreover, the effects of calcium administration did not induce any changes in breaking load of the femoral neck (Figure 10D) or femoral midshaft.

To eliminate the possible effects of increased dietary calcium, the effects of sevelamer treatment in advanced CRI were evaluated in an additional experiment. In this study the effects of sevelamer treatment on bone were clearly evident, although elevated phosphorous (hyperphosphatemia) and PTH levels were observed in the end of the study. Sevelamer administration attenuated the CRI-induced decrease in vBMD of the femoral neck (Figure 11C) and in cBMD of the femoral midshaft (Figure 14C). In addition, sevelamer administration was also associated with an increased amount of cortical bone in femoral midshaft (Figures 14C-F). As a result, we observed that sevelamer treatment ameliorated the deterioration of bone strength in CRI (Figures 11F, 14G and 14H).

Figure 12. The effects of CRI and pamidronate (PAR) treatment in femoral neck: (A) vBMD; (B) tCSA; (C) BMC; and (D) breaking load. Bars represent the mean + SD. Significant differences are indicated: a P < 0.05 vs. Sham, b P < 0.05 vs. CRI.
Figure 13. Scatter plots of (A) vBMD and (B) tCSA in relation to plasma urea (dashed line at 7.5 mmol/l denotes highest urea concentration of normal (Sham) rats), and scatter plots and correlations of vBMD and tCSA in (C) 34 Sham rats and (D) 17 5/6 nephrectomized (NTX) rats.

3 The effects of paricalcitol treatment on bone

In study III, paricalcitol treatment significantly restrained the CRI-induced increase in PTH levels without any effect on calcium or phosphorous levels. CRI is associated with decreased calcitriol levels, and as anticipated, paricalcitol administration suppressed calcitriol levels further. In the bone analysis, paricalcitol treatment was associated with a beneficial effect on CRI-induced decreases in vBMD of the femoral neck (Figure 12A) and in cBMD of the femoral midshaft. Most importantly, paricalcitol treatment completely prevented the CRI associated decrease in breaking load in the femoral neck (Figure 12D).
Figure 14. The effects of CRI and sevelamer treatment (Sev) in femoral midshaft: (A) cBMD; (B) tCSA; (C) cCSA; (D) BMC; (E) cortical thickness in anteroposterior direction; (F) cortical thickness in mediolateral direction; (G) breaking load in anteroposterior and (H) mediolateral direction. Bars represent the mean ± SD. Significant differences are indicated: a P < 0.05 vs. Sham, b P < 0.05 vs. CRI, c P < 0.05 sevelamer treatment groups vs. untreated groups.
4 The effects of pamidronate administration on bone in experimental chronic renal insufficiency

According to the general mechanisms of the action of bisphosphonates, the effects of pamidronate treatment on bone were evident although the general characteristics of CRI in serum biochemistry (creatinine, urea, phosphate and PTH) were not affected (IV). Pamidronate treatment resulted in an increase in total BMC of whole femur, an effect that was observed in both Sham and CRI groups, but intriguingly, the treatment effect was substantially greater in the CRI group. In histological analysis of distal femoral metaphysis, pamidronate treatment was shown to result in an increase in relative bone volume (BV/TV) in both the CRI and Sham groups. This increase in BV/TV is most likely attributable to increases in trabecular thickness and number, and consequently, a decrease in trabecular separation. However, also relative volume of osteoid was increased in association with pamidronate treatment (histomorphometrical data not shown).

In the structural analysis of the femoral midshaft, the most pronounced effect of pamidronate treatment was an increase in the amount of cortical bone particularly in the CRI group (Figures 13C and 13D). Increased cCSA together with decreased tCSA and reduced inside diameters of the marrow cavity (marrow area) indicate that the net bone gain was located on the endosteal surface (Figure 16). As a result, an increase in BMC was observed but no differences were detected in the cBMD between the treated and untreated groups (Figures 15A and 15E). In mild-to-moderate CRI, pamidronate treatment was not associated with changes in bone strength (Figure 15F).
Figure 15. The effects of CRI and pamidronate administration (APD) in femoral midshaft: (A) cBMD; (B) tCSA; (C) cCSA; (D) average cortical thickness; (E) tBMC; (F) breaking load in anteroposterior direction. Bars represent the mean ± SD. Significant differences are indicated: a P > 0.01 CRI-groups vs. Sham-groups; b P < 0.05 CRI vs. Sham; c P < 0.05 pamidronate treatment groups vs. untreated groups; d P < 0.05 CRI+APD vs. CRI; e P < 0.05 for interaction between CRI and pamidronate treatment.
Figure 16. The effects of CRI and pamidronate administration (APD) on marrow cavity (medullary canal) dimensions in femoral midshaft. Cross-section of bone is depicted white (in Sham) or grey (in CRI), and effect of APD treatment is black (in both). APD treatment effect $P < 0.05$ in both mediolateral (vertically in picture) and anteroposterior directions (horizontally in picture); NS, statistically non-significant difference.
The rationale for this thesis was prompted by the fact that traditionally the research on ROD has been mostly focused on non-mechanical functions of bones and on material properties of trabecular bone, although the increased susceptibility to fractures is the most important clinical manifestation of all metabolic bone disorders (Parfitt 1998). Accordingly, albeit the increased risk of fractures is recognized as a skeletal complication of CRI, the assessment of the mechanical properties of bone structure has not been properly included in the definitions of ROD and CKD-MBD (Massry et al. 2003, Moe et al. 2006, Sprague 2007). Furthermore, analysis of cortical bone has been almost completely ignored from the context of ROD despite the fact that cortical bone is the main determinant of bone strength (Ferretti et al. 1995, Augat et al. 1996, Parfitt 1998, Massry et al. 2003, Moe et al. 2006). Inspired by this apparent discrepancy, an organ level structural approach was established to evaluate the consequences of experimental CRI on bone (I). Accordingly, the prime focus of the analysis in this thesis was on the evaluation of mechanical competence of bone (I).

The first main objective of the thesis was to assess the effects of CRI on bone structure, and broaden the knowledge of changes in bone strength and its main determinants in CRI (I). This present study confirms that CRI is associated with detrimental effects on bone and suggest that the mechanical competence of bone is maintained despite evident ROD associated with mild-to-moderate CRI. The present model of experimental mild-to-moderate CRI induced a loss of cortical bone, but there were no differences in mechanical testing of bone strength in femoral midshaft or femoral neck (II, IV). However, in severely advanced CRI (data from the additional study on effects of sevelamer treatment), the mechanical competence of bone was eventually deteriorated. These findings are in agreement with current understanding of the CKD-BMD, and additionally, the results suggest that bones possess quite a substantial capacity to resist the detrimental effects associated with CRI before actual structural decay occurs.

The second main objective of the study was to evaluate the skeletal effects of three pharmacological treatments that are currently used in the clinical management of CRI. The treatments were chosen to represent the cornerstones of the general pharmacological treatment of CRI (Elder 2002, Emmett 2004, Martin et al. 2004, Friedman 2005): alleviation of hyperphosphatemia with two distinct phosphate binding agents, calcium carbonate (II) and sevelamer (data from the additional study), and amelioration of secondary hyperparathyroidism with vitamin D analogue paricalcitol (III). Concerning the efficacy of the
treatment with calcium salts in prevention of the deleterious effects of CRI, the high calcium diet was found to increase the total BMC of the whole femur, although the only statistically significant effect of treatment was observed in the vBMD of the femoral neck (II). Nevertheless, there is a concern of so called adynamic bone in association with administration of therapeutic doses of calcium salts, because high calcium intake may oversuppress PTH levels, and that may have adverse consequences on bone (Salusky and Goodman 2001). Although a significant suppression of PTH levels below the physiological range was observed, calcium salt administration treatment was not associated with any differences in bone strength in Sham or CRI groups (II). Similarly to the calcium salts, sevelamer also binds phosphate in the intestine, but unlike the calcium salts, sevelamer is not absorbed and therefore it is not supposed to have any direct effect on PTH levels (Plone et al. 2002). In the present study, the treatment with sevelamer was shown to prevent CRI-induced decreases in cBMD, BMC, and bone strength in the femoral midshaft, and the beneficial effect of sevelamer treatment was also evident in the femoral neck (data from additional study on the effects of sevelamer treatment). The known mechanism of sevelamer is to act as a phosphate binder, and as no differences were detected in the serum phosphorous levels between the treated and untreated groups at the end of the study, the results of the present study suggest that the beneficial effect of sevelamer was attributable to its action along the course of CRI. In previous experimental studies, sevelamer has been shown to decrease serum phosphorous (Rosenbaum et al. 1997, Nagano et al. 2001, Cozzolino et al. 2002, Cozzolino et al. 2003, Katsumata et al. 2003, Nagano et al. 2003a, Nagano et al. 2003b), and the lack of effect in the end of the study may be explained by the very severely advanced CRI, in which the hyperphosphatemia had become too grave to overcome. Combining the similar findings of calcium carbonate and sevelamer treatments, it may be concluded that the control of hyperphosphatemia is relevant also in terms of prevention of the bone lesions and the use of dietary calcium carbonate or sevelamer as a phosphate binding agents seem viable treatment options. However, the comparison between the efficacy of calcium carbonate and sevelamer treatment was not possible, because the severity of the CRI and ROD was different in study II and in the additional study on the effects of sevelamer treatment.

Apart from the prevention of phosphate absorption, the results of study III show that amelioration of secondary hyperparathyroidism with paricalcitol decreased PTH levels and prevented the CRI-induced decrease in cBMD of the femoral midshaft and vBMD of the femoral neck. These findings complement the previously published results on the effect of paricalcitol on bone histology (Slatopolsky et al. 2003). The results of the present study further show that the treatment prevented the CRI-induced loss on bone strength in the femoral neck. When considering together the effects of controlling hyperphosphatemia by hindering phosphate absorption and the effects of inhibition of parathyroid function by vitamin D analogue, the results not only highlight the significance of high phosphorous levels in the development of bone abnormalities, but also
pinpoint that PTH is the essential mediator of these effects in ROD. In essence, it may be concluded that the general management of the hyperphosphatemia and secondary hyperparathyroidism with these interventions was proven to be beneficial to bone in present experimental model of CRI.

When considering the effects of pamidronate treatment on bone in CRI, it needs be pointed out that bisphosphonates are not widely used, or even currently recommended, treatment options of ROD and thus the treatment does not represent clinical management of CRI. However, the use of bisphosphonates in CRI population has been considered (Rodan and Martin 2000, Fan and Cunningham 2001, Turner 2002, Klawansky et al. 2003, Massry et al. 2003, Langman et al. 2005). In study IV, pamidronate treatment was associated with an increase in BMC and cCSA, and the effects were particularly evident in CRI group. This interaction suggests that CRI may augment the effect of bisphosphonate treatment on bone. Detailed analysis of the cross-sectional geometry of femoral midshaft showed that the amount of endosteal bone was increased after pamidronate treatment (IV). When combined with the findings of increased BMC and BV/TV in the distal femur, it may be suggested that the effect of pamidronate was pronounced in bone surfaces that are metabolically most active. However, there were no differences between study groups in breaking load of the femoral midshaft and the plausible explanation for this is that the apposition of bone on endosteal surface has only a minor influence on the mechanical strength of the structure (I, IV) (Turner and Burr 1993, Turner 2002).

In addition to increased cortical porosity, another important factor for impaired cortical bone structure in ROD is generalized cortical thinning. An apparent mechanism for thinner cortices is that the increase in net endocortical resorption exceeds the slow rate of net periosteal apposition (Parfitt 2003). It has been suggested that ROD represents a spectrum of disorders of bone turnover that range from high- to low-turnover skeletal lesions (Hruska and Teitelbaum 1995, Salusky and Goodman 1995) and the complex outcome is reflected in the wide scatter of bone abnormalities which have been associated with ROD according to the current literature. However, it has also been discussed that the decrease in cortical thickness is the major determinant of bone strength regardless of subsequent development and regardless of the histological classification or the disorder of bone remodeling that may be present at the time the fracture occurs (Parfitt 1998, Schober et al. 1998).

In general, there are numerous studies which have reported associations between bone mineral density values and bone fragility or even with incidence bone fractures. This has been adapted also to the current literature on ROD, but the use of the term “bone density” has been somewhat vague and confusing (Sievänen 2000). Firstly, the distinction between the different definitions of bone density has not been explicit. DXA is a widely used technique to assess BMC and bone density, and thus derived measures of density may be broadly termed as areal density values. In this thesis, the bone density results refer uniformly to actual volumetric cBMD or vBMD values which are determined by pQCT and
accordingly defined as mass per unit volume (mg/cm$^3$), not per unit area (mg/cm$^2$). Secondly, especially in ROD, the interpretation of numerical changes in areal density values is uncertain, because the calculations are based on assumptions that changes in bone three-dimensional structure and material density are known or predictable. In ROD, these underlying assumptions may no longer hold since the known effects are divergent on cortical and trabecular bone, and the overall pattern of changes on bone structure is complex and unpredictable for the present (Parfitt 1998). Change in such derived areal density value may thus arise from any undefined change(s) in cortical or trabecular bone tissue properties, or from geometrical alteration, and the eventual mechanical significance of the occurrence might not correlate at all with the numerical value (Parfitt 1998, van der Meulen et al. 2001, Martin et al. 2004). On the other hand, the ostensibly unchanged areal density value may conceal some distinctive changes resulting from possible redistribution of bone mineral between cortical and trabecular compartments. Therefore, particularly in ROD, some changes in bone may have influence on bone strength (I) although those changes are not reflected by DXA assessment of bone (van der Meulen et al. 2001, Elder 2002, Turner 2002). Nevertheless, the K/DOQI guidelines recommend the use of DXA in CKD patients for the evaluation of the risk factors for fractures (Massry et al. 2003), although the role of DXA measurements in assessment of ROD is not well established (Martin et al. 2004).

In previous experimental studies the group of Jablonski et al. have shown that the material properties of bones were quite well preserved even 240 days after 5/6 nephrectomy (Jablonski et al. 1993, Jablonski et al. 1994, Jablonski et al. 1995). Further, Turner et al. (1996) have shown that in rats with advanced CRI the bone strength is deteriorated. Thus the finding of reduced bone mineral density with decreased bone strength in response to advanced CRI in the present study is in accordance with previous experimental findings. These are also in agreement with the clinical data on incidence of fractures in patients with pre-dialysis CRI (Ensrud et al. 2007) and in end-stage renal disease patients (Atsumi et al. 1999, Alem et al. 2000, Coco and Rush 2000, Stehman-Breen et al. 2000, Jamal et al. 2006a).

However, although the finding of decreased bone strength may be associated with increased incidence of fractures, the relationship may not be as straightforward as it appears. It should be recalled that that bone mineral density as itself is only a modest risk factor of fractures - some 85% of the contribution to the rise in fracture risk with age is unrelated to bone mineral density (Wilkin and Devendra 2001). According to several well-designed clinical studies on risk factors of fractures among elderly people (Hayes et al. 1993, Nevitt and Cummings 1993, Schwartz et al. 1998, Parkkari et al. 1999, Carter et al. 2000, Palvanen et al. 2000, Wei et al. 2001, Lee et al. 2002, Robinovitch et al. 2003), falling with its determinants – not the decrease in bone mineral density – has been shown to be the strongest single risk factor for a fracture. When a person falls, the type and severity of falling are crucial in determining whether or not a

Therefore, although reduced bone mineral density and elevated levels of PTH, and both bone formation and resorption, have been detected already at an early stage of CRI, the most likely explanation for increased fracture rates in association with CRI is the more frequent occurrence of falls among CRI patients (Roberts et al. 2003, Cook and Jassal 2005, Desmet et al. 2005). The etiology of falls is multifactorial (Kannus et al. 2002, Tinetti 2003, Kannus et al. 2005) and it is plausible that prevalence of some general risk factors of falling is higher among the CRI population. These risk factors may include general frailty with other chronic diseases, gait disabilities, peripheral neuropathy, impaired muscle strength, peripheral vascular disease, and vision impairment (Stehman-Breen 2004, Jamal et al. 2006b). According to Desmet et al. (2005), particularly diabetes, polypharmacy and failed walking test are independent risk factors for falling in dialysis population. And further, epidemiological studies showing increased fracture risk in dialysis population show also that body mass index (BMI), age, gender, race and presence of peripheral vascular disease are independently associated with fracture risk, while PTH, phosphate and calcium levels do not predict fracture risk (Alem et al. 2000, Stehman-Breen et al. 2000, Jamal et al. 2006b).

Assessment of bone biopsy is a widely used method to characterize ROD in clinical and experimental studies. The histomorphometrical data from study IV showed gross changes in association with pamidronate treatment. According to the basic principles of bone biology, the outcome of these histological changes, regardless of the cause, should ultimately translate into changes in bone structure, and possibly, but not necessarily, altered mechanical competence of bone (I). In the data from study IV, the histomorphometrical findings were translated into changes in BMC, but however, no differences were observed between the study groups in strength of femoral midshaft. Thus, this data supports the claims (van der Meulen et al. 2001) that although the characterization of the changes in bone histomorphometry provides information on bone turnover, but the interpretation of these findings in terms of bone strength is inappropriate if the outcome, the strength of whole bone as a structure, remains unknown (I). Geometrical variables and mineral properties may be considered as determinants or surrogate measures of bone strength (I), but the relationship between whole bone strength and these underlying determinants is complex (Ruff et al. 2006). Furthermore, particularly in ROD, the relationship between bone strength and changes in bone geometry and mineral properties is unclear, because the changes in bone tissue properties are diverse and unpredictable (Parfitt 1998). Determination of bone material texture properties (e.g. histomorphometrical variables) and structural properties (e.g., geometry or cortical wall thickness) are essential elements of the characterization of ROD, but when assessing the efficiency of certain intervention on bone structure in ROD, the chances should be evaluated with data on to the total outcome, which is the strength of the bone structure (I) (Turner 2002).
Kidney Disease: Improving Global Outcomes (KDIGO) has stated that in CKD the contribution of bone mass to bone strength is of uncertain value and emphasized the assessment of bone strength (Moe et al. 2006). It is also possible that bone mineral density values altogether may be quite misleading in ROD, in addition to the above mentioned matter, if bone resorption is being replaced with newly formed bone which has defective mechanical properties because of inappropriate microstructure (Parfitt 2003). These statements further emphasize the importance of mechanical testing of bone structure in ROD. Along these lines, Hernandes and Keaveny (2006) have recently stated that fracture is a mechanical event and necessitates that any relevant change in bone structure must change bone biomechanical performance. In this study, mechanical testing of a whole bone was used to determine directly and unambiguously the structural strength of bone as an organ, and as van der Meulen et al. (2001) have discussed, there is no alternative to mechanical testing of whole bones in terms of bone strength (I).
SUMMARY AND CONCLUSIONS

1. Experimental CRI was associated with gradual deterioration of bone structure and the most evident detrimental changes were observed in the measures of cBMD in the femoral midshaft and vBMD in the femoral neck. The mechanical strength of bone structure was maintained in mild-to-moderate CRI (II, IV), but advanced CRI was associated with detrimental changes in bone strength (data from the additional study on effects of sevelamer treatment).

2. The alleviation of CRI-induced hyperphosphatemia through the use of calcium carbonate (II) and sevelamer (data from the additional study on effects of sevelamer treatment) were found to be effective in the prevention of deleterious changes associated with CRI in femoral midshaft and in femoral neck.

3. Administration of vitamin D analogue paricalcitol was observed to prevent the loss of bone mineral and bone strength in femoral neck in CRI (III).

4. Bisphosphonate pamidronate was found to increase BMC in endosteal surface of femoral midshaft and in distal femur, particularly in association with CRI, while no differences were observed in bone strength (IV).
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