BONE LOSS
IN RELATION TO
RENAL TRANSPLANTATION

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A thesis performed at the Department of Pharmacology

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CONTENTS

CONTENTS…….. **4**

SELECTED ABBREVIATIONS…… **8**

ABSTRACT…….. **9**

1. INTRODUCTION…… **10**

1.1 Background……... 10

1.2 Bone metabolism and disorders…….……. 11

1.2.1 Structure and composition……. 11

1.2.2 Extracellular and bone mineral homeostasis……. 11

1.2.3 Bone remodelling…….. 13

1.2.4 Osteoporosis and renal osteodystrophy……..... 16

1.3 Dual energy X-ray absorptiometry……. ……17

1.4 Biochemical markers of bone turnover……...20

1.4.1 Osteocalcin……20

1.4.2 Telopeptide……20

1.5 The kidney……..21

1.5.1 Outline of renal function……21

1.5.2 Glomerular filtration…….. 21

1.5.3 Chronic renal failure…….. 22

1.5.4 Complications following RTx……23

1.6 Immunosuppressive treatment…… 24

1.6.1 The immune system……... 24

1.6.2 Glucocorticoids……. 25

1.6.3 Calcineurin inhibitors……...……..26

1.6.4 Noncalcineurin immune modulators……..28

1.7 Aim……..... 30

1.8 Hypotheses…… 30

2. MATERIAL AND METHOD…….....**31**

2.1 Patient material…… 31

2.1.1 Inclusion criteria…… 31

2.1.2 Exclusion criteria……... 31

2.1.3 Study design……...31

2.1.4 Study population…… 32

2.1.5 Ethical considerations…… 34

2.2 DEXA……. 35

2.3 Blood sampling and analyses……35

2.3.1 Blood sampling…….. 35

2.3.2 Analysis of osteocalcin…….. 36

2.3.3 Analysis of telopeptide……...38

2.3.4 Other biochemical data, drugs and GFR …….……..39

2.4 Calculations……... 40

2.4.1 Cumulative glucocorticoid dose…….. ……..40

2.4.2 Estimated GFR……...41

2.5 Statistics …… 41

3. RESULTS……...**42**

3.1 Posttransplant status……42

3.2 Bone mineral density……44

3.2.1 Baseline data.…..……..…….. 45

3.2.2 Longitudinal data……..……. 47

3.3 Bone turnover…… 50

4. DISCUSSION…… **52**

4.1 Validity……... 52

4.2 Bone mineral density……53

4.2.1 Baseline data……..... 53

4.2.2 Longitudinal data……... 56

4.3 Bone turnover…… 59
4.4 Limitations of the study…… 60
5. CONCLUSION……..**61**
6. PERSPECTIVES……...**62**
REFERENCES…….. **63**

APPENDIX

I Protocol

II Patient information

III Flowcharts; clinical and biochemical data

IV Letters from the Regional Committee for Medical Research Ethics, Sør-Norge (REK Sør)

V Letter from the Directorate for Health and Social Affairs

VI Letter from the Rikshospitalet University Hospitals Personal Information Protection Commission

SELECTED ABBREVIATIONS

BGP	Bone GLA protein
BL	Baseline
BMD	Bone mineral density
BMI	Body mass index
BMU	Basic multicellular unit
CI	Calcineurin inhibitor
CML	Cumulative
CMV	Cytomegalovirus
CRF	Chronic renal failure
CsA	Cyclosporin A
CT	Calcitonin
CTRL	Control
DEXA	Dual energy X-ray absorptiometry
D-Pyr	Deoxypyridinoline
ESRF	End-stage renal failure
ELISA	Enzyme linked immunosorbent assay
TF	Total femur
GC	Glucocorticoid
GFR	Glomerular filtration rate
GLA	γ-carboxyglutamic acid
HDL	High density lipoprotein
IGF	Insulin growth factor
IRMA	Immunoradiometric assay
LDL	Low density lipoprotein
LS	Lumbar spine
MMF	Mycophenolate mofetil
Non-CI	Noncalcineurin inhibitor
PTH	Parathyroid hormone
Pyr	Pyridinoline
PF	Proximal forearm
RTx	Renal transplantation
SD	Standard deviation
TGF-β	Transforming growth factor-β
TB	Total body
UD	Ultra distal
WHO	World Health Organization
$1,25(OH)_2D_3$	1,25-dihydroxycholecalciferol = calcitriol
$25OHD_3$	25-hydroxycholecalciferol = calcifediol

ABSTRACT

***Background*:** Patients with chronic renal failure (CRF) who are going through a renal transplantation (RTx) are at high risk of experiencing bone loss and fractures. Abnormalities of the skeleton in CRF, collectively known as renal osteodystrophy, are an important cause of morbidity and decreased quality of life. In addition, bone loss occurs rapidly following renal transplantation because of aggravating factors that emerge after procedure. Among these factors, the key pathophysiological contributor to bone disease is immunosuppressive agent application, especially glucocorticoids (GC).

***Aim*:** The purpose of the present study was to examine the bone mineral density of CRF patients at the point of transplantation, and thereafter the early bone loss following transplantation by doing a prospective descriptive study. Moreover the purpose was to identify predictors of the bone status prior to and the bone loss following transplantation in these patients.

***Material and method*:** 46 patients were measured between the fifth and tenth day following RTx as baseline, and again 10 to 12 weeks later, in a descriptive longitudinal study. At both visits lumbar spine (LS), total neck (TN), total body (TB), ultra distal and proximal forearm were measured using the dual-energy X-ray absorptiometry machine Lunar Prodigy Advance. In addition, blood samples were collected for analyses of the bone markers osteocalcin and telopeptide. Questionnaires were used to gain information about variables which possibly could be implicated in bone loss. Test results from standard procedure analyses were collected from the patients journals.

***Results*:** According to the Z-scores the patients had significantly lower BMD at baseline compared to normative data. At baseline, significant determinants of low BMD were age, gender, smoking and former transplantations. A highly significant bone loss from baseline to follow-up was observed in LS, TF and TB, within the range 1.2 to 2.5 %. The significant determinants of change in BMD were age (LS), change in osteocalcin levels (TF) and CMV infection (TB), explaining about 9 %, 18 % and 10 % of the bone loss, respectively.

***Conclusion*:** The study gives further evidence to the fact that patients suffering from CRF who goes through RTx is in danger of a continuing bone loss with a major risk of bone fractures.

1. INTRODUCTION

1.1 Background

«A systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture risk» is the definition of osteoporosis (1) The abnormalities of the skeleton in chronic renal failure (CRF), collectively known as renal osteodystrophy, are an important cause of morbidity and decreased quality of life. Renal osteodystrophy is a multifactorial and complex disorder of compromised bone strength (2)

Renal transplantation offers the best prognosis for patients with severe end-stage renal failure: 1-year survival following renal transplantation now exceeds 90%, and new immunosuppressants introduced in the last decade have reduced rejection episodes and prolonged graft survival. Consequently, improving the long term quality of life for these patients is becoming more important. One potential problem relates to the adverse effects of fractures, which may result from the well-recognized disturbances in bone metabolism among patients with end-stage renal disease. When the amount of bone tissue is reduced the skeleton is not able to withstand normal forces and fragility fractures occur. These fractures most commonly occur in the sites of the skeleton rich in trabecular bone as in the spine, distal forearm and proximal femur. Several recent reports have suggested an above-average fracture rate in these patients (3-5)

Bone loss occurs rapidly following renal transplantation because of aggravating factors that emerge after procedure. Among these factors, the key pathophysiological contributor to bone disease is immunosuppressive agent application, especially glucocorticoids (GC). The pathogenesis of glucocorticoid-induced bone loss is multifactorial and has been reviewed extensively (6) The main deleterious effect of glucocorticoids is a direct and profound inhibition of bone formation. GCs inhibit osteoblast differentiation and induce apoptosis in mature osteoblasts as well as osteocytes (7) They also decrease gastrointestinal calcium absorption, resulting in a negative calcium balance and secondary hyperparathyroidism. In addition, glucocorticoids directly suppress gonadotropins and may cause hypogonadism (8)

With most solid organ transplants, bone loss is greatest at sites rich in cancellous bone, and spinal bone mineral density (BMD) losses of 3–10% have been reported in the first 6 months following renal transplantation, with continued slower bone loss thereafter. Long term studies have shown the cumulative incidence of fracture to be three times higher than expected 15 years after the renal transplantation. (3-5)

1.2 Bone metabolism and disorders of bone

1.2.1 Structure and composition

The human skeleton consists of 80 % cortical bone and 20 % trabecular bone. Cortical bone is the dense, compact outer part and trabecular bone the inner meshwork. The former predominates in the shafts of long bones, the later in the vertebrae, the epiphyses of long bones and the iliac crest. Trabecular bone, having a large surface area, is metabolically more active and more affected by factors that lead to bone loss. The proximal femur consists of 60 % cortical and 40 % trabecular bone, while the lumbar spine (LS) is comprised of a 50 to 50 % distribution, respectively. The ultra distal (UD) forearm consists of 25 % trabecular bone, while the proximal forearm (PF) is the compartment with least trabecular bone, with less than 10 % (9).

The main mineral in bone is calcium salts and phosphates. More than 99 % of the calcium in the body is in the skeleton, mostly as crystalline hydroxyapatite but some as non-crystalline phosphates and carbonates; together, these make up half the bone mass.

The organic matrix of bone is osteoid, the principal component of which is collagen, other components are proteoglycans, osteocalcin and various phosphoproteins. The phosphoprotein osteonectin binds to both calcium and collagen and thus links these two major constituents of bone matrix. Calcium phosphate crystals are deposited in the osteoid, converting it into hard bone matrix (10).

1.2.2 Extracellular and bone mineral homeostasis

A highly integrated and complex endocrine system maintains calcium, phosphate, and magnesium homeostasis. It involves the interplay between the actions of two polypeptide hormones, parathyroid hormone (PTH) and calcitonin (CT), and a sterol hormone, 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$) or calcitriol.

Calcitriol is the active metabolite of vitamin D_3, or cholecalciferol. Cholecalciferol is synthesized in the skin by ultraviolet radiation of 7-dehydrocholesterol. Another source of vitamin D is from vitamin D_2, or ergocalciferol, in the diet, which is produced by ultraviolet irradiation of the plant sterol ergosterol. The parent compound, cholecalciferol, essentially lacks biological activity and requires metabolic transformation to attain potency. The first step involves 25-hydroxylation by microsomal enzymes, a process that occurs chiefly in the liver. The product of this reaction is 25-hydroxycholecalciferol ($25OHD_3$), also known as

calcifediol a metabolite with little biological activity. It is in this form vitamin D is stored in the fat tissue of the body. Calcifediol is transported to the kidneys through the blood for further synthesis. There are two different kinds of hydrolyzing enzymes in the kidneys that can further metabolise calcifediol. One of these enzymes leads to a 1α-hydroxylation and the active form of vitamin D, calcitriol. The other enzyme leads to the inactive metabolite 24,25-dihydroxycholecalciferol ($24,25(OH)_2D_3$ (11).

Biosynthesis and secretion of the polypeptide hormones, PTH and CT, are regulated by a negative feedback mechanism that involves the activity of ionic calcium in the extracellular fluid. The biosynthesis of the active form of vitamin D, calcitriol, is regulated by PTH and CT, as well as by the extracellular fluid concentrations of calcium and phosphate. Parathyroid hormone, CT and calcitriol regulate flow of minerals into and out of the extracellular fluid compartment through their actions on intestine, kidney, and bone (Table 1) (11).

Hormone	Bone	Kidney	Intestine
Parathyroid hormone (PTH)	Increases resorption of calcium and phosphate	Increases reabsorption of calcium; decreases reabsorption of phosphate; increases conversion of $25OHD_3$ to $1,25(OH)_2D_3$; decreases reabsorption of bicarbonate	No direct effect
Calcitonin (CT)	Decreases resorption of calcium and phosphate	Decreases reabsorption of calcium and phosphate. Questionable effect on vitamin D metabolism	No direct effect
Vitamin D	Maintains Ca^{2+} transport system	Decreases reabsorption of calcium	Increases absorption of calcium and phosphate

Table 1. Actions of major calcium-regulating hormones

Under normal circumstances, PTH prevents serum calcium from falling below physiological concentrations by stimulating calcium movement from intestinal and renal tubular lumina and from the bone fluid compartment (adjacent to mobilizable bone mineral) into the blood. Whereas its effect on bone and kidney is direct, PTH acts indirectly on the intestine, through the mediation of vitamin D. The hormone stimulates $25OHD_3$ 1α-hydroxylase in the mitochondria of the renal tubule in the kidney which converts calcifediol to calcitriol, as mentioned earlier. Calcitriol stimulates intestinal calcium absorption. PTH also prevents

serum phosphate levels from rising above normal by increasing renal tubular excretion of phosphate. This regulatory action is action is important because phosphate, like calcium, is also released into the blood by PTH-induced bone resorption. This function can be particularly appreciated in patients with end-stage renal failure associated with severe hyperthyroidism. These patients develop hyperphosphatemia, as discussed later, because large quantities of phosphate are released from bone, and the kidney can no longer excrete them (11).

The role of calcitonin is to prevent increases in both serum calcium and serum phosphate. It decreases the translocation of calcium from the renal tubule and bone fluid compartment into the blood and thus can be considered as a counter regulator of PTH. The effects of CT on vitamin D metabolism and on the intestinal absorption of calcium are uncertain (11).

1.2.3 Bone remodelling

In mature and healthy adults, skeletal size is neither increasing nor decreasing. Despite this, bone is continuously being turned over, so that the net activity of bone resorbing cells equals the net activity of bone forming cells. In the adult, this activity is largely accounted for by bone remodelling which is a mechanism that provides self repair and adaptation to stress. The process of bone remodelling involves the activity of two main cell types, osteoblasts and osteoclasts, the action of a variety of cytokines, the turnover of bone minerals and the actions of several hormones, some of which has been described above. Together the osteoblasts and osteoclasts form the basic multicellular unit (BMU) in which they have a close cooperation where osteoblasts secrete bone matrix while osteoclasts break it down. The osteoblasts are the prime mover in the process in that it controls osteoclast differentiation during cell-to-cell contact(10;11).

A cycle of remodelling starts with the recruitment of osteoclast precursors by cytokines (Figure 1). Osteoblast action regulates the differentiation of these to mature osteoclasts, which adhere to an area of trabecular bone and move along it digging a pit by secreting H^+ and proteolytic enzymes. This process gradually liberates cytokines such as insulin growth factor (IGF-1), transforming growth factor-β (TGF-β) and others that have been embedded in the osteoid. The cytokines in turn recruit and activate successive teams of osteoblasts that have been stimulated to develop from precursor cells. The osteoblasts invade the site, synthesising

and secreting the organic matrix of bone, the osteoid, and secreting IGF-1 and TGF-β which become embedded in the osteoid. Some osteoblasts also become embedded in the osteoid, forming terminal osteocytes, others interact with and activate osteoclast precursors, and a new cycle begins (10;12).

The daily turnover of bone minerals during remodelling involves about 700 mg calcium. Intracellular Ca^{2+} constitutes only a small proportion of body calcium, but it has a major role in cellular function, so the concentration of Ca^{2+} in the extracellular fluid and the plasma needs to be controlled with great precision. The concentration is regulated by complex interactions between PTH, the various forms of vitamin D, and calcitonin as described earlier (10;12).

In normal healthy individuals bone formation is couples to bone resorption in a tight equilibrium. When this delicate balance is disturbed, the net result is pathological situations such as osteopetrosis or osteoporosis.

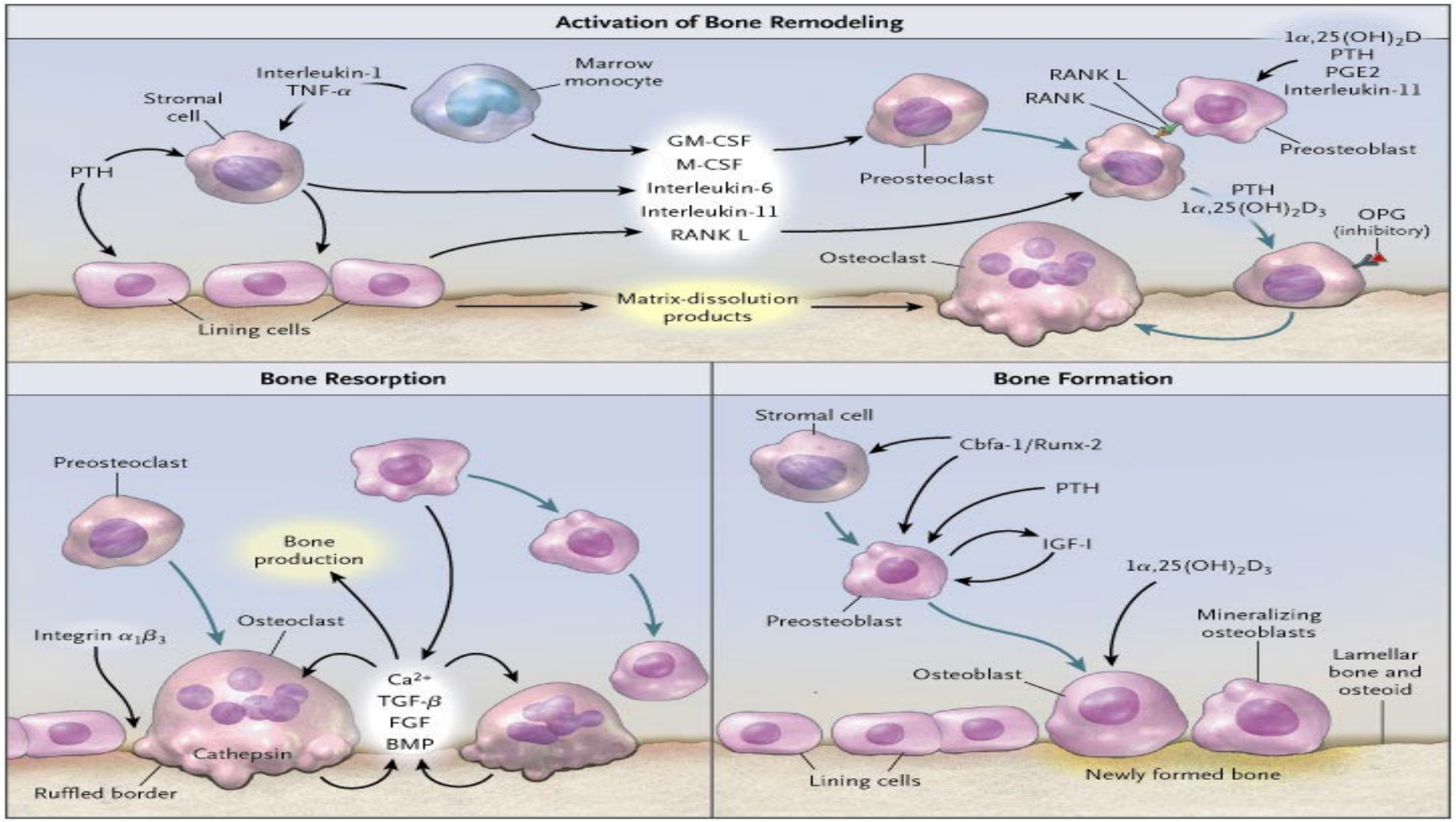

Figure 1. Regulation of bone remodelling.

The schematic illustrates the key factors that are thought to be involved in the activation, resorption, and formation phases of the bone-remodelling cycle. PTH denotes parathyroid hormone, TNF- tumor necrosis factor, GM-CSF granulocyte–macrophage colony-stimulating factor, M-CSF macrophage colony-stimulating factor, RANK receptor activator of nuclear factor B; RANK L RANK ligand, 1,25(OH)2D 1,25-dihydroxyvitamin D, OPG osteoprotegerin, PGE2 prostaglandin E2,TGF- transforming growth factor , FGF fibroblast growth factor, BMP bone morphogenetic protein, Cbfa-1 core binding factor 1, Runx-2 runt-related transcription factor 2, and IGF-I insulin-like growth factor I (13).

1.2.4 Osteoporosis and renal osteodystrophy

Osteoporosis is a progressive disease characterized by low bone mass and microarchitectural deterioration of bone tissue resulting in increased bone fragility and susceptibility to fractures. The increased bone fragility is caused by decreased quantity of bone, the bone quality is not affected (Figure 2) (11;14).

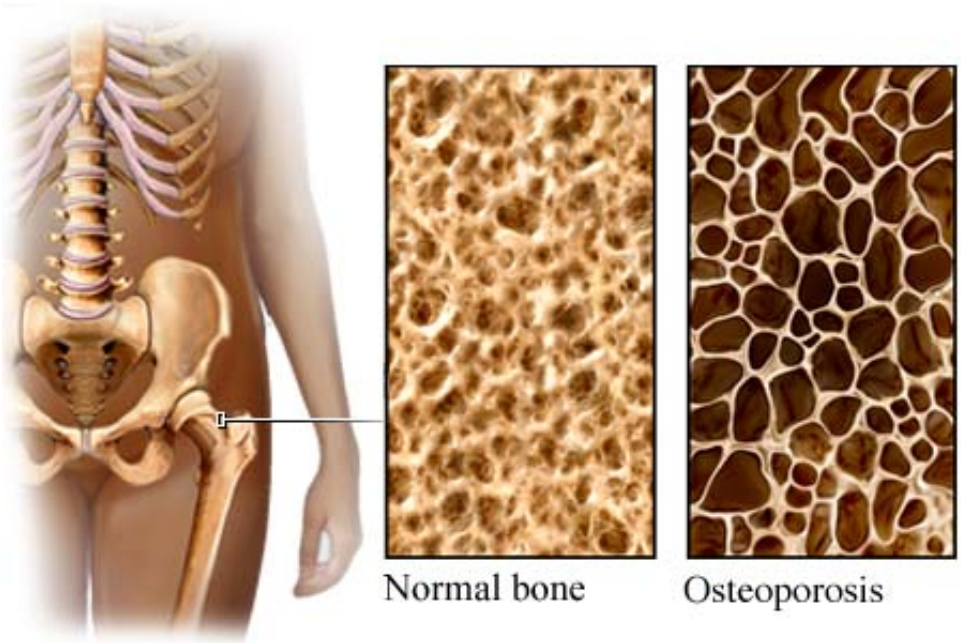

Figure 2. Normal bone vs. osteoporotic bone

As the life expectancy of patients with CRF has become longer with dialysis and transplantation, the problems of chronic management has attracted more and more attention. One of the problems that need to be addressed in these patients is renal osteodystrophy. Renal osteodystrophy describes the four types of bone disease associated with CRF; secondary hyperparathyroidism, osteomalacia, mixed renal osteodystrophy (both hyperparathyroidism and osteomalacia), and adynamic bone disease with reduced bone formation and resorption (15).

An increase in PTH secretion that is adaptive and unrelated to intrinsic disease of the parathyroid glands is called secondary hyperparathyroidism. The disorder is associated with prolonged stimulation of the parathyroid glands by chronic decreases in concentration of ionic calcium in the blood. Serum levels of PTH progressively increase in CRF patients as glomerular filtration rate (GFR) decreases below 40 ml/min. Decreased renal filtration affects

the filtration of phosphate and subsequently leads to a hyperphosphatemia in the genesis of renal hyperparathyroidism. Each decrement in GFR is accompanied by a transient increase in serum phosphorus, which in turn leads to a transient decrease in serum calcium and a compensatory increase in secretion of PTH. Restoration of serum calcium and serum phosphorus toward normal occurs as a result of the effects of increased serum PTH on mobilization of bone mineral and renal tubular reabsorption of phosphate, respectively. As the cycle is repeated, secondary hyperparathyroidism progressively worsens since the failed kidney is unable to respond to PTH by increasing renal calcium reabsorption. This leads to a persistently elevated PTH level and hyperplasia of the thyroid glands develop (11;15).

Osteomalacia is failure of the organic matrix (osteoid) of bone to mineralize normally. Because of this the strength of the skeleton is reduced and there is a higher risk of fractures. Several disorders can lead to osteomalacia; one of them is impaired renal synthesis of calcitriol which occur in CRF patients. In chronic renal disease there is an extensive nephron loss which makes the kidney unable to convert calcifediol to calcitriol, even if the body has enough vitamin D_3 in storage. A decreased level of calcitriol is compounded by hyperphosphatemia caused by reduced phosphate excretion, which in turn reduces the concentration of ionized serum calcium by sequestering calcium phosphate in bone or, eventually, in soft tissue. Hypocalcaemia and a reduction in the direct suppressive action of calcitriol on the parathyroid glands results in an increased secretion of PTH (11;15).

1.3 Dual energy X-ray absorptiometry

Dual energy X-ray absorptiometry (DEXA) is a widely used technique to measure bone mineral density (BMD). It measures the amount of bone in a given area. In this study the DEXA technology were used to take measurements at the spine, hip, wrist, and a whole body scan. As the patient lie on the table or sits on a chair beside the table, the machine moves over the part of the body that is going to be measured while it sends a thin, invisible beam of low-dose x-rays (Figure 3). The scans are shown on the operator's computer screen where the pictures are analyzed before a medical doctor evaluates the results.

Figure 3. An examination of a patients BMD using DEXA

The amount of radiation is very small with an effective patient dose at 0,7-1,3 µSv, which is less than 1/10 the dose of a standard chest x-ray (16). Based on how much the x-rays have changed after passing through the body, a picture of the skeleton will be generated on a computer screen. The X-ray beams are sent through the body from the moving arm over the patients and a detector register the amount of X-ray beams that are let through to the other side of the body.

The DEXA instruments differentiate body mass into the components of lean soft tissue, fat soft tissue and bone, based on the differential attenuation by tissues of the x-rays. This means that lean soft tissue is everything else but fat soft tissue and bone, that means for the most part muscle and water. Bone mineral content (BMC) is expressed in grams (g) and the area scanned is expressed in cm^2, from these values the computer software calculates the BMD which therefore is expressed in g/cm^2. Historically, BMD values themselves have not been used for estimating fracture risk. Instead, BMD values are most often expressed in comparison to an established normative range. All densiometry manufacturers provide normative databases for this purpose. These databases are derived from measurements of large groups of both men and women of different ages and races. Comparisons are expressed both as the percentage of age-matched and young normal values, as well as standard deviation

scores. The most commonly used standard deviation score is the T-score. It compares a patient's bone density to the expected value for young adults. The young adult mean and standard deviation (SD) are usually derived from a group of healthy subjects aged 20 to 35 years and matched for sex and race. The young normal or T-score is defined as the difference between the patient's BMD and the young adult mean BMD called the reference value (YA), divided by the standard deviation of the reference population (SD):

$$\text{T-score} = \frac{\text{Measure BMD - YA}}{\text{Young adult SD}}$$

For the diagnosis of osteoporosis, The World Health Organisation (WHO) has defined the following criteria for the assessment of osteoporosis based on the T-scores:

- Normal: A BMD not more than 1 standard deviation below young adult (T-score ≥ -1)
- Low bone mass (osteopenia): A BMD between 1 and 2,5 standard deviations below young adult (T-score < -1 and > -2,5)
- Osteoporosis: A BMD 2,5 or more standard deviations below young adult (T-score ≤ -2,5)
- Severe osteoporosis: A BMD 2,5 or more standard deviations below young adult (T-score ≤ -2,5) and the presence of one or more fragility fractures

The WHO definition is intended for use in defining populations and not for the diagnosis of osteoporosis in individual subjects. However, in the absence of other criteria, the WHO definition has become the standard for diagnosis in clinical practice. These guidelines cannot be directly transferred to patients with renal osteodystrophy, since they have been drawn up especially with postmenopausal women in mind, but they can give a pointer as to if the patient is in a risk zone. (1)

Another standard deviation score in use is the Z-score. This is a value that shows the amount of bone the patient has in comparison to healthy people of his/her age group, gender, and size (17):

$$\text{Z-score} = \frac{\text{Measure BMD – Age-Matched Mean BMD}}{\text{Population SD}}$$

Z-scores are not used to define osteoporosis, since they would not reflect the increasing prevalence of osteoporosis with age. For example, elderly patients may have a Z-score of zero, based on comparison to their own age group, but a T-score would put them in the osteoporotic category. Z-scores are useful if they show that a patient's BMD is significantly below an age-matched group(17).

1.4 Biochemical markers of bone turnover

1.4.1 Bone formation; Osteocalcin

Osteocalcin is a biochemical marker of bone formation. It contains two or three amino acid residues of γ-carboxyglutamic acid (GLA), hence it is also known as bone GLA protein (BGP). Osteocalcin is the most abundant noncollagenous protein in bone and is predominantly synthesised by the osteoblasts (2;18;19). It is incorporated into the extracellular matrix of bone, but a fraction of newly synthesised osteocalcin is released into the circulation where it can be measured by radioimmunoassay (18;20). Because of this there has been considerable interest in its assay as a possible means for the evaluation of patients with bone disease, particularly in osteoporosis. Histological studies have shown significant correlations between the rates of bone formation and serum values for osteocalcin. Care is required in interpreting values in the presence of renal failure since the kidney is a site of its metabolism (18-20).

1.4.2 Bone resorption; Telopeptide

Telopetptide is a biochemical marker of bone resorption. Type I collagen accounts for more than 90 % of the organic matrix of bone and is synthesised primarily in bone. Pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) are nonreducible cross-links that stabilise the collagen chains within the extracellular matrix (12;20-22). They cross-link at two locations in type-I collagen, N-telopeptide-to-helix and C-telopeptide-to-helix.

Pyridinoline is present in bone and cartilage matrix and in minute amounts in some other connective tissues. Significant amounts of D-Pyr are only found in bone collagen, at a concentration of 0.07 mol/mol collagen. The Pyr/D-Pyr ratio in human bone matrix is 2:3 (12;20-22).

During bone remodelling, type I collagen is degraded as described above, and small peptide fragments are excreted into the bloodstream. These fragments can be analysed in serum by an enzyme linked immunosorbent assay (ELISA), as described earlier: (12;20-22).

1.5 The kidney

1.5.1 Outline of renal function

The main function of the kidney is the excretion of waste products such as urea, uric acid and creatinine. In the course of this activity, it also regulates the NaCl and electrolyte content and the volume of the extracellular fluid, a function which is crucially important in homeostasis. The kidneys receive about a quarter of the cardiac output. From the several hundred litres of plasma which flows through them each day, they filter an amount equivalent to about 11 times the extracellular fluid volume. This filtrate is similar in composition to plasma, the main difference being that it has very little protein or protein-bound substances. As it passes through the renal tubule, about 99 % of it is reabsorbed while some substances are secreted, and eventually about 1.5 litres of filtered fluid are voided as urine. In structure, each kidney consists of an outer cortex, an inner medulla and a hollow pelvis, which empties into the urethra. The functional unit is the nephron, of which there are about 1.3 millions of in each kidney (23).

1.5.2 Glomerular filtration

Fluid is driven from the capillaries into the tubular capsule of the nephron, known as Bowman's capsule, by hydrodynamic force. There it crosses three layers; the capillary endothelium, the basement membrane and the epithelial cell layer of the capsule. Together these form a complex filter that excludes large molecules. Normally, all constituents in the plasma, except the plasma proteins and the blood cells themselves, appear in the filtrate (23).

Creatinine is a by-product of normal muscle metabolism and is formed at a rate proportional to muscle mass. It is freely filtered by the glomerulus, with little secretion or reabsorption by the tubule. When muscle mass is stable, any change in serum creatinine levels reflects a change in its clearance by filtration. Consequently, measurement of creatinine clearance gives an estimate of the glomerular filtration rate (GFR).

One method to estimate GFR involves measurement of the serum creatinine concentration at a given time, compensating for those factors that affect creatinine levels, including age, sex and weight. An estimate can be made from average data using the equation of Cockroft & Gault:

$$Cl_{\mathrm{Cr}} = \frac{F\,(140 - \text{age (years)}) \times \text{weight (kg)}}{\text{Serum creatinine (}\mu\text{mol/l)}}$$

Where F = 1.04 (females) or 1.23 (males) (15).

A patient with a clearance of 100 ml/min is said to have a 100 % kidney function (24).

1.5.3 Chronic renal failure

Chronic renal failure may be defined as a condition characterized by uraemia, anaemia, acidosis, osteodystrophy, neuropathy and general debility frequently accompanied by hypertension, oedema and susceptibility to infection. This is the result of a significant reduction in the excretory, homeostatic, metabolic and endocrine functions of the kidneys that occur over a period of months or years. The disease generally progresses through four stages. The kidneys have a greater capacity to function than what is needed under normal conditions, the renal reserve. In the first stage of the disease this reserve is eradicated by damage to the kidneys. Normal renal function is maintained, but responses to conditions that place additional demands upon the kidney, such as pregnancy or increased dietary protein, cannot be met. During the second stage, toxins such as creatinine and urea that are normally excreted by the kidney begin to accumulate. Electrolyte levels often remain within normal limits as a result of homeostatic adaptations. Compensation will inevitably result in imbalance elsewhere, such as acidaemia, bone disease and changes in hormone levels, for example, PTH. The third stage is called CRF and is a result of progressive decline in renal function that produces a wide range of both biochemical and hormonal abnormalities. Symptoms may still be insignificant despite severe disturbances of homeostasis. End-stage renal failure (ESRF) is the last stage of the disease and it is characterized by uraemia and a wide spectrum of gastrointestinal, dermatological and CNS symptoms (15).

The reduction in renal function in CRF results from damage to the infrastructure of the kidney. It is thought that nephrons are lost as complete units with all functions lost

simultaneously. The remaining nephrons initially manage to cope with the increased demand upon them. But as the damage continues the GFR progressively declines. The patient may remain symptom free until GFR falls as low as 15 – 20 ml/min. Often CRF is discovered during investigation of other medical problems or following routine screening.

Chronic renal failure can arise from a variety of causes; for example chronic glomerulonephritis, diabetes, hypertension, pyelonephritis and obstructive uropathy. The cause is not always identified, but establishing a cause is useful in the identification and elimination of reversible factors, in planning for likely outcomes and treatment needs, and for appropriate counselling when genetic basis is established.

When the GFR has declined to about 20 ml/min, a continuing deterioration in renal function to end-stage renal failure appears inevitable in most patients. Renal transplantation remains the treatment of choice for patients with ESRF, as a relatively normal lifestyle is usually re-established (15).

1.5.4 Complications following RTx

Cytomegalovirus is a herpes virus that is acquired by approximately 50 % of the general population. Like other herpes viruses, once infection has occurred the virus remains dormant thereafter. In individuals with advanced immunosuppression reactivation may occur and cause disease (25). Human cytomegalovirus (CMV) infection is the single most frequent infectious complication in the early period after kidney transplantation. CMV infection is an independent risk factor for acute kidney graft rejection. There is also evidence that CMV infection is associated with an increased long-term mortality and post-transplant diabetes mellitus. Studies has shown that more than 60 % of the patients have an active CMV infection following RTx, and that more than 20 % of these patients experience clinical symptoms as well (26;27). Infection usually occurs between the first and fourth month after RTx if no prophylactic or pre-emptive treatment is given (28).

The incidence of CMV disease is almost 3 times higher in seronegative recipients of seropositive donors (D_/R-) than in CMV-seropositive recipient (D-/R_ and D_/R_). The seronegative patients develop a primary CMV infection, while the seropositive patients experience a reactivation of the CMV virus. At present at Rikshospitalet University Hospital,

patients are kept under weekly surveillance for CMV, and those who have positive CMV polymerase chain reaction results are treated with oral valganciclovir (26). This is a method called pre-emptive therapy, which is associated with fewer late onsets of CMV disease, less expenses related to pharmaceutics, and probably a lower risk of developing infections with ganciklovir resistant viruses (29). Diagnosing CMV infection or CMV disease depends on the absence of clinical symptoms and signs of disease (30).

1.6 Immunosuppressive treatment

The most important therapeutic aspect of transplantation is immunosuppression to prevent rejection. The major disadvantage of all immunosuppressive agents is their relative non-specificity, in that they cause a general depression of the immune system. This exposes the patient to an increased risk of malignancy and infection, which remains an important cause of morbidity and mortality. In order to minimize side-effects, doses of immunosuppressants are gradually reduced over a 2-6 months period to the lowest that will maintain effective immunosuppression. In addition, transplants appear to become less immunogenic over a period of time so lower prophylactic levels of immunosuppression are required. The treatment is continued for as long as the transplanted kidney remains in situ. The drug regimes vary widely between individual transplant centres (15). At Rikshospitalet University Hospital, the most common regime is a triple therapy involving prednisolone, cyclosporin and mycophenolate mofetil. Tacrolimus and sirolimus sometimes replace cyclosporin in the triple therapy, and in other cases everolimus is given in addition to prednisolone, mycophenolate mofetil and lower doses of cyclosporin. In the present study we included all patients independent of immunosuppressant regimen.

1.6.1 The immune system

A mammalian organism facing an invasion by disease-causing organism, a pathogen, can call on a prodigious array of powerful defensive responses. The deployment of these responses constitutes the acute immune reaction. When the strength of these defences is reduced because of disease or because they are suppressed by drugs, as they are in posttransplant patients, organisms that are not normally pathogens can cause disease (opportunistic infections). An example of this is the CMV virus as mentioned in section 1.5.4; Complications following RTx. Rejection of the allograft is a result of the immune systems reaction to an alien organ in the body. The immune system initiates reactions that results in

rejection of the transplanted organ. By adjusting the immune system downwards the body easier accepts the new organ (31).

The key cells of the adaptive immune system, the lymphocytes, is described below for a better understanding of the action of the immunosuppressive drugs discussed further on. There are three main groups of lymphocytes; B cells, T cells and natural killer cells. B cells are responsible for antibody production. T cells are responsible for cell-mediated immune reactions and are important in the induction phase of the immune response. Natural killer cells are specialized lymphoid cells that are active in the non-immunological innate response, and are therefore not a part of the adaptive system. The involvement of lymphocytes in the specific immune response involves two phases; an induction phase and an effector phase. During the induction phase, antigen is presented to T cells by large dendritic cells, antigen presenting cells (APC). This is followed by complex interactions of the T cells which have interacted with the APCs and with B cells and other T cells. On first contact with an antigen, a foreign protein or polysaccharide, the lymphocytes that have recognised it by binding with surface receptors specific for that antigen, undergo a series of cell divisions. This gives rise to a large clone of cells that have the capacity to recognise and respond to that particular antigen. These latter cells are responsible for the next phase. In the effector phase, these cells differentiate into plasma or memory cells. The B cells that turn into plasma cells produce antibodies, while the T cells are involved in cell-mediated immune response by activating macrophages or by killing virus-infected host cells. The memory cells forms an increased population of antigen-sensitive memory cells. A second exposure to the antigen will therefore result in a multiplied response (31).

1.6.2 Glucocorticoids

Prednisolone and methylprednisolone are synthetic glucocorticoids with anti-inflammatory and immunosuppressive effects. Methylprednisolone has 20 % higher potency than prednisolone. Immunosuppression involves both their effects on the immune response and their anti-inflammatory actions. Glucocorticoids restrain the clonal proliferation of T cells, through decreasing the transcription of the gene coding interleukin-2 (IL-2). They also decrease the transcription of many other cytokine genes through inhibition of the action of transcription factors (32).

At Rikshospitalet University Hospital the patients are given high doses of intravenous methylprednisolone as a booster at the day of transplantation and the day after. Then the oral agent prednisolone is given with a gradual daily reduction until a maintenance dose of 10-20 mg/day is reached. Methylprednisolone is used intravenously to reverse acute rejection. The use of glucocorticoid therapy often leads to complications, particularly if high doses are given for long periods. In addition to a cushingoid state there may be gastrointestinal bleeding, hypertension, dyslipidaemia, diabetes, osteoporosis and mental disturbances (15). Glucocorticoids enhance bone resorption and decrease bone formation, consequently decreasing the bone mass and increasing the risk of fractures. The increased bone resorption is in part due to direct effects of GCs on the skeleton and in part the result of a decrease in intestinal calcium absorption and an increase in the urinary excretion of calcium (33). Parathyroidectomy prevents the excessive bone resorption associated with GCs, suggesting that *in vivo*, a cause of excessive bone resorption is enhanced secretion or activity of PTH. In addition, GCs enhance the responsiveness of osteoblasts to PTH by increasing the expression of PTH receptors in these cells. As the bone-resorbing actions of PTH require the presence of osteoblasts, an increase in PTH receptors in osteoblasts could explain some of the bone loss observed (33). Studies on transgenic mice which had blocked out GC action in osteoblasts and osteocytes showed that excess GCs directly affect bone forming cells *in vivo*. Furthermore, the results suggested that GC-induced loss of bone strength results in part from increased death of osteocytes, independent of bone loss (34). Later on the same group of researchers carried out another similar study, but this time they knocked out the GC action specifically in the osteoclasts. The reason for this study was that while excess GCs reduce osteoblast and osteoclast precursors, cancellous osteoclast numbers surprisingly does not decrease as does osteoblast numbers. Their study showed that GCs decreased cancellous osteoclast numbers in the transgenic but not the wild-type mice, demonstrating that early, rapid loss of bone caused by GC excess results from direct actions on osteoclasts (35).

1.6.3 Calcineurin inhibitors

Cyclosporin

The discovery and development of cyclosporin immunosuppression regimens have greatly increased transplant survival rates. Cyclosporin is a cyclic peptide of 11 amino acid residues with potent immunosuppressive activity but no effect on the acute inflammatory reaction. It inhibits the activation of T cells by forming a complex with an intracellular protein,

cyclophilins, which then binds to one of the activators of the T cells, calcineurin (36-39). The action of cyclosporin is partially selective in that it suppresses cytotoxic T cell production and to some extent spares B lymphocyte activity, permitting a greater response to infection than can normally be mounted by patients using older forms of immunosuppression. Thus, there is a relatively low incidence of severe infection associated with cyclosporin therapy, although the incidence of malignancies appears to be similar to that found with other immunosuppressants. Cyclosporin carries a high risk of side-effects, including nephrotoxicity, hypertension, fine muscle tremor, gingival hyperplasia, nausea and hirsutism (15;32).

Tacrolimus

Tacrolimus (earlier known as FK506) is a macrolide antibiotic with a very similar mechanism of action to cyclosporin by forming a complex with an intracellular protein and binding to and inhibiting calcineurin (36;37;39). The difference is the protein it binds to, called FK-binding proteins, and it has considerably more potency than cyclosporin. The unwanted effects of tacrolimus are similar to those of cyclosporin, but neurotoxicity is more common, which also causes disturbances of glucose metabolism. In contrast, hirsutism is less of a problem. Tacrolimus appears to be particularly useful in attempts to reverse acute rejection episodes (15;32).

Calcineurin inhibitors (CIs), such as cyclosporine and tacrolimus, also have serious effects causing rapid and severe bone loss in animal models and humans (40). Histomorphometric studies in rats have demonstrated that both drugs cause acute, rapid, and severe bone loss (41-44). Administration of immunosuppressive doses to normal, young and old, male or female rats produced significant bone loss of both trabecular and cortical bone within weeks. Bone loss was dose dependent and reversible after discontinuing the drug. Histomorphometry characterized the phenomenon as extremely high-turnover bone loss with increases in resorption and formation markers (41).

The impact of immune modulators on humans is difficult to study. The multidrug regimens employed in immunosuppressive treatment following transplantations tend to compound each other's effect. This makes it difficult to assess the contribution of each individual drug to bone loss. If the glucocorticoid dose is decreased or discontinued, it still would be very unclear which drug is to be blamed for the initial versus ongoing bone loss. The initial rapid bone loss

is mainly the result of a glucocorticoid effect and occurs typically in the first three to six months (45). There have not yet been carried out a large multicenter trials, and because immunosuppressive regimes and dosages vary from one transplant centre to another, there are only small studies available. However, these smaller studies implicate the CIs in bone loss following organ transplantation (40;46). The nonsteroidal immunosuppressants of the CI family have been shown experimentally and clinically to produce severe and rapid high-turnover bone loss. These drugs are often used with glucocorticoids, which are known to compound that effect. However, while GCs lead to low-turnover bone loss, the bone biopsy in patients taking CIs reveals a high turnover state (40). There is a need for larger studies before any final conclusion about the CIs effect on bone is drawn.

1.6.4 Noncalcineurin immune modulators

Sirolimus and everolimus

These two drugs are macrolides and similar in mechanism of action. They inhibit T cell activation specifically by binding to a protein in the cytosol, and this drug-protein complex then inhibits the activation of an essential enzyme in the cell cycle so that cycle is arrested. The cytosolic protein that the drugs bind to are different for the two different drugs, but the result is the same; it inhibits the mammalian target of rapamycin (mTOR) and blocks the cell cycle of various cell types, including T- and B-lymphocytes. Calcineurin inhibitors (CIs), like cyclosporin and tacrolimus, are associated with important side effects, such as nephrotoxicity, and thus there is an interest in developing CI-sparing protocols using agents such as the proliferation signal inhibitors/mammalian target of rapamycin inhibitors; sirolimus and everolimus (47). Sirolimus dos not cause bone loss in rats but may interfere with longitudinal bone growth and, at high doses, may decrease cortical bone in young, rapidly growing animals (40;48). In human subjects, there have not been any reports of studies on the effect of sirolimus on bone without concomitant administration of glucocorticoids or CIs, and they may not be feasible in a population of transplant recipients. However, the strategy of combining sirolimus with low-dose CsA to mitigate the bone loss seen with CIs has been shown to be an effective strategy in rats, and it does not comprise immune suppression (49). A study, which examined the effect of everolimus on mouse and human bone cells *in vitro* and on bone in an ovariectomized rat model, concluded that everolimus directly inhibits bone resorption by osteoclasts and thus could at least be neutral or protective for bone *in vivo* (50).

There has to this point in time not been performed any studies on the effect of everolimus on human bone cells *in vivo* (40).

Mycophenolate mofetil

Mycophenolate mofetil (MMF) is a semisynthetic derivate of a fungal antibiotic. It is converted *in vitro* to mycophenolic acid, a natural product of *penicillium fungi*. The drug retains proliferation of both T and B cells and reduces the production of cytotoxic T cells by inhibiting inosine monophosphate dehydrogenase, an enzyme crucial for *de novo* purine biosynthesis. T and B cells, as well as arterial wall smooth muscle cells, are unique in obtaining the purines needed for DNA synthesis by synthesising them *de novo* whereas other cells can obtain purines by an alternative pathway. In this way MMF has a fairly selective action on T and B cells. Unwanted gastrointestinal effects are common with use of this drug. There is evidence that compared to azathioprine, a drug that is replaced by mycophenolate mofetil in several transplant centres today, MMF reduces the risk of acute rejection episodes and causes less bone marrow suppression. However the risk of opportunistic infections and the occurrence of blood disorders such as leucopenia may be higher (15;32;40). No evidence of alterations in bone metabolism and no loss of bone volume have been noted experimentally *in vivo* (51). However, recent reports suggest that prednisone and MMF, in the absence of CsA, may also be associated with high-turnover bone loss on bone histomorphometry (40).

1.7 Aim

The purpose of the present study was to examine the bone mineral density of CRF patients at the point of transplantation, and thereafter the early bone loss following transplantation by doing a prospective descriptive study. Moreover the purpose was to identify predictors of the bone status prior to and the bone loss following transplantation in these patients.

1.8 Hypotheses

The primary hypothesis of this study was that we would be able to register a significant bone loss in patients as soon as 10-12 weeks after RTx.

A secondary hypothesis was that we would be able to identify predictors of low bone mass prior to the RTx and explain some of the bone loss after the RTx by variables registered in association with the patients and their treatment after the RTx.

2. MATERIAL AND METHOD

2.1 Patient material

2.1.1 Inclusion criteria

- Patients at the Section of Nephrology at Rikshospitalet University Hospital, who had been cleared for a renal transplantation.
- ≥18 years of age

2.1.2 Exclusion criteria

- < 18 years of age
- Pregnant women
- Competing medical disease
- Psychological unstableness
- Psychiatric disease that demands medical treatment

2.1.3. Study design

The project was performed in a prospective study from late January to early July. The subjects were investigated at the Day Clinic of the Section of Endocrinology as well as at the Section of Nephrology at Rikshospitalet University Hospital.

The first examination was performed during the fifth and tenth day after the transplantation. This time limit was set to assure that every patient participating in the study would be examined at an approximately similar time in connection with the procedure, regardless if the kidney had been harvested from a deceased or living donor. The second examination was performed contemporary to a follow-up at the Day Clinic of the Section of Nephrology, ten to twelve weeks after the renal transplantation. During these sessions it is a part of the routine examination to measure the glomerular filtration (GFR), as an indication on how the kidney is functioning. The result of the measurement was recorded for each patient and it was one of the variables that were investigated in the study in relation to BMD.

The following data was registered during the study:

- The patients went through a DEXA examination at both visits, which included measurements of the lumbar spine (LS), total femur (TF) (both sides), the ultra distal forearm (UD), proximal forearm (PF) and total body (TB). Not everybody could complete

all the measurements, either because of pain or the fact that they had gone trough hip surgery.

- Blood samples were collected at the ward and at the Section of Nephrology as a part of the routine control in relations to the renal transplantation. A 6 ml test tube was then delivered to the Section of Endocrinology for analyses of osteocalcin and telopeptide.
- The GFR was only measured at the second examination. This is because the kidneys have very little or no ability to filtrate fluid at the point of transplantation and therefore the GFR is approximately zero.
- Other biochemical data which were available because of the routine blood tests taken following an RTx, and that could be of interest to the study, was collected from the patients' journals.
- From the journals, all the immunosuppressant drugs were noted, in doses and blood concentrations. In addition, use of other drugs which affects the kidney and bone metabolism was registered.

2.1.4 Study population

A total of 70 patients received a kidney allograft at Rikshospitalet University Hospital during the period of including patients in the study, which was from late January to late April 2006. Eleven of these patients were not asked to participate in the study due to age below 18 years (n = 4), their mental health (n = 2), because they could not speak Norwegian or English (n = 2), or because the clinic was closed during Easter (n = 3).

Of the remaining 59 patients that were asked to participate 13 were not included in the final study. Six of these patients were not included in the first round because they did not have the strength. The rest of the patients wanted to participate but were lost during the study because of early graft loss (n = 2), transfer to the local hospital (n = 2), cerebral infarction (n = 1), other complications (n = 1), or death (n = 1). Thus a total of 46 recipients were included in the final prospective descriptive study.

The demographics and baseline characteristics for the population are given in Table 2 and Table 3. The average age of the group was middle-aged, but there was a large span with the youngest being just under 19 and the oldest soon turning 83 years old. The wide range of different ages was the same in both genders, but the mean age of the women were almost ten years higher then for the men. About 65 % of the patients who carried through the study were

men. This corresponds with a larger study performed at Rikshospitalet University Hospital where 69 % out of 172 patients were men (52).

Body Mass Index (BMI) is a simple index of weight-for-height that is commonly used to classify underweight, overweight and obesity in adults. It is defined as the weight in kilograms divided by the square of the height in metres (kg/m^2). The WHO has used this definition as a tool to define what could be looked at as underweight (> 18.5), normal weight (18.5 – 25), overweight (≥ 25) and obese (≥ 30). According to this definition the mean of the population in the study was at the border of being overweight at baseline (Table 2).

Table 2. Demographics baseline

Age at Tx	50±17	yr
Male	47±17	yr
Female	56±15	yr
Gender		
Male	65.2	%
Female	34.8	%
Post menopause	75.0	%
Age at menarche	12.8±1.4	yr
Ethnicity		
White	91.3	%
Asian	8.7	%
BMI	25.3±4.1	kg/m²
Smoking status		
Ever smoked	58.7	%
Smoking now	19.6	%

Mean ± SD

Table 3. Baseline characteristics

Previous Tx	8.7	%
Dialysis/Pre-emptive	69.6/30.4	%
Diabetic nephropathy	8.7	%
Rheumatoid arthritis	2.2	%
Cardiovascular disease	2.2	%
Total parathyroidectomy	0.0	%
Pretransplant fractures		
1 fracture	19.6	%
>1 fracture	13.0	%
Z-score Lumbar Spine	-0.7±1.2	
Male	-0.8±1.2	
Female	-0.5±1.3	
Z-score Total femur	-0.9±1.1	
Male	-0.9±1.1	
Female	-0.5±1.3	
Z-score Total Body	-0.7±1.4	
Male	-1.0±1.3	
Female	-0.3±1.5	
PTH	45.1±35.4	pmol/l

Mean ± SD

About 9 % of the population had gone through a transplant procedure in the past (Table 3). About two thirds of the population had gone through dialysis before the RTx, either haemodialysis or peritoneal dialysis, while one third was transplanted pre-emptive. This also reflects the distribution in the larger study done earlier at the hospital, where 72 % had had dialysis before the transplantation (52). Usually, the registration of fractures in studies is limited to fractures that have occurred after the patients have become middle-aged. But since this study also embraced younger patients the age-limit was set to the same age-limit as the inclusion criteria, which means any fracture that had occurred from the age of 18. It was only registered if there had been one or more fractures, not if it was brought on by a lot of force or

if it was a low energy fracture. The Z-scores show that both men and women consistently had a somewhat lower BMD in all three compartments than what healthy adults at the same age and size usually have according to the database. The Z-scores do also indicate that the men had a poorer bone status than the women initially. Table 3 gives a mean PTH value for the population that is high above normal range, but there is a large standard deviation. The normal range for PTH levels is 1.6 – 6.9 pmol/l.

2.1.5 Ethical considerations

This study was necessary to gain increased knowledge about the development of osteoporosis as a secondary disease in relation to chronic kidney disease and renal transplantation. Participation in this study involved very little extra for the patients as it was a descriptive study. It means that there was no interference in the treatment by introducing new drugs or methods. The study collected data from patients who received the treatment available at that time.

DEXA measurements have not been fully integrated as a part of the routine examinations in relation to a renal transplant at the Rikshospitalet University Hospital yet. But it is about to become a part of this routine, as it already has become in several hospitals worldwide. Also today, many of the CRF patients go through DEXA measurements during their treatment to observe the status of the bone mass at Rikshospitalet University Hospital because of its benefits. A reason for the implementation at the point in time was that the methods and machines for DEXA measurements have improved. In that way the advantage of using the measurements has become greater as it contributes with precise information about the patients BMD, from which the medical practitioners can evaluate the risk of developing osteoporosis. The dose of radiation during the measurement is very low, the procedure takes no more than 15 minutes, and it is non-invasive and does not cause any pain or discomfort for the patient. So even if the DEXA measurement is was not fully a part of the standard routine at that point, we had no hesitations about carrying out this procedure during the study. Data acquired from the measurements that was of importance to an individual patient was registered in the patient's journal and the necessary treatment was given.

Blood sampling is a part of the standard routine at the Section of Nephrology in the initial examinations and the follow up of renal transplantation patients. Those who agreed to participate in the study were asked to give an extra test tube of blood at the same time as they

were giving blood to other analyses. In that way there was no extra piercing of the skin for the patient.
A test of the GFR is a part of the standard procedure in the follow up of a patient with a renal transplantation, as explained earlier; therefore it was not done especially for the study.

When evaluating the ethical aspect of the study we did not find anything ethically alarming, since most of the examinations that were done are a part of the routine in the evaluation and follow up of patients with chronic kidney disease who are going through a renal transplantation at the Rikshospitalet University Hospital.

The study was approved by the local ethical committee (Appendix II) and conducted according to the Declaration of Helsinki II. Informed written consents were obtained from the patients.

2.2 DEXA

Bone mineral density of the lumbar spine (LS), in this study defined as the measurement of the region L2-L4, ultra distal (UD) forearm, proximal forearm (PF), total femur (TF) and total body (TB) were measured by dual-energy X-ray absorptiometry with Lunar Prodigy Advance which is based on a narrow fan-shaped X-ray principle (fanbeam) that scan the patient with transverse movement. The software used was enCORE 2006, General Electrics Healthcare, analysis version 10.10. The reference population of young adults was from a group of healthy subjects aged 20 to 40 years from the United States of America, the age-matched group was also from the USA. According to the manufacturer, the DEXA measurements have a precision error of ± 1 %, independent of the operator (16).

2.3 Blood sampling and analyses

2.3.1 Blood sampling

Blood samples were collected from each patient at the same day as the BMD measurements. At the first visit the patients were admitted at the ward, so the samples were collected together with the routine blood samples before breakfast at the ward, between 0800 and 0900 am. At the second visit the patients were called in for various tests at the Section of Nephrology, so this time the samples were collected together with the routine blood samples before breakfast at the day clinic, between 0800 and 0900 am.

Osteocalcin has been shown to exhibit a marked diurnal variation with a maximum level at night and a minimum in late morning or early afternoon. Because of this, time of sampling must be taken into account when interpreting results. All of our samples were collected early in the morning, so they are comparable.

The blood samples were collected in 6 ml Vacutainer™ tubes with clots. Continuously, as the samples were collected, the tubes were centrifuged at room temperature for 10 minutes at 3200 rcf (Megafyge 1.0, Heraeus). The serum was aliquoted and dispersed into three marked 1.8 Nunc tubes, and frozen at - 80 ºC. One of the smaller test tubes from each patient and from both visits was used for the analysis of osteocalcin and another one for the analysis of telopeptide. In that way the serum was only thawed once before each of the analyses, and we had one extra test tube from both visits from each patient in case of mistakes.

2.3.2 Analysis of osteocalcin

The analysis of osteocalcin was done by using an immunoradiometric assay (IRMA) named N-tact® Osteo SP Osteocalcin IRMA kit made by DiaSorin, Stillwater, Minnesota, USA, and delivered by Boule Nordic. The intra-assay (variation within the assay) and inter-assay (variation between assays) coefficients of variation were both < 10 % for the assay, according to the factory.

Chemical principle of the assay

The assay measures intact human osteocalcin 1-49 quantitatively, with no cross-reactivity to the 1-43 fragments. It utilizes human osteocalcin 1-49 for calibrators and controls and two polyclonal antibodies that have been purified using affinity chromatography. The purified antibodies are specific for two different regions of the osteocalcin molecule. The first antibody, specific for the amino terminus of human osteocalcin, is bound to a solid phase (polystyrene beads). The second antibody, specific for the carboxy terminus of human osteocalcin, is labelled with ^{125}I. Samples are incubated on an orbital agitator at room temperature for two hours. Intact human osteocalcin 1-49 is the only form of osteocalcin that will be bound by both the antibody on the bead and the ^{125}I labelled antibody. Following the incubation period, each bead is washed to remove unbound labelled antibody. The radioactivity present in the remaining bound labelled antibody is then measured using a

gamma counter. The concentration of osteocalcin 1-49 present in the sample is directly proportional to the radioactivity measured. (53;54)

Assay procedure

Serum collected at the time of baseline and 10 – 12 weeks after the surgery were used. All samples were assayed in duplicate to ensure confidence in the values obtained.

Borosilicated glasses were filled with 20 µl of standards, controls or unknown samples. There were six standards and two controls, and in addition there were two empty glasses functioning as blanks, or total counts. 300 µl of tracer were added to each glass. The tracer contained affinity purified goat antibody, specific for the carboxy terminus of human osteocalcin, labelled with iodine-125 and diluted in buffered goat serum containing 0.2% sodium azide and 0.2% gentamicin sulfate. All the glasses were vortexed. One bead was added to each tube with a forceps, except the total count tubes. The polystyrene beads were coated with affinity purified goat antibody specific for the amino terminus of human osteocalcin. Then the tubes were covered with parafilm and agitated at 200 rpm (Edmund Bühler, Swip) for 2 hours ± 5 minutes in room temperature (20 – 25 ºC). The reaction mixture was aspirated from each tube, except the total count tubes. Afterwards the beads were washed by vigorously dispensing 1 ml of wash solution, containing phosphate buffer and Tween 20, in the tubes and then aspirating it again. This procedure was repeated two more times. Then the radioactivity present in each tube was measured using a gamma counter (Cobra A™ II, Auto Gamma, Packard). The gamma counter was set to count the radioactivity in each tube for 3 minutes.

Interpretation of results

The computer calculated the average count per minute (CPM) for the standards, controls and unknown samples. Thereafter the average CPM for the zero standards was subtracted from all other average counts to obtain the correct CPM. To determine the concentration of osteocalcin found in controls and unknown samples, a calibrator curve was prepared using the calibrator concentrations stated on the vial labels. The corrected CPM of each calibrator was plotted on the ordinate against the calibrator concentration on the abscissa. This curve could then be used to interpolate the levels of osteocalcin in the controls and unknown samples.

2.3.3 Analysis of telopeptide

The analysis of telopeptide was done by using an enzyme linked immunosorbent assay (ELISA) named Serum CrossLaps® ELISA made by Nordic Bioscience Diagnostics A/S, Herlev, Denmark, and delivered by Nordic Bioscience Diagnostics. The intra- and inter-assay coefficients of variation were both < 10% for the assay, according to the factory.

Chemical principle of the assay

The assay measures the degradation product of C-terminal telopeptides of Type-I collagen in human serum and plasma quantitatively. The test is based on two highly specific monoclonal antibodies against the amino acid sequence of EKAHD-β-GGR, where the aspartic acid residue (D) is β-isomerised. In order to obtain a specific signal in the ELISA, two chains of EKAHD-β-GGR must be cross-linked.

Standards, control, or unknown serum samples are pipetted into the appropriate microtitre wells coated with streptavidin, followed by application of mixture of biotinylated antibody and a peroxidase-conjugated antibody. Then a complex between the CrossLaps® antigens, biotinylated antibody and peroxidase-conjugated antibody is generated, and this complex binds to the streptavidin surface via the biotinylated antibody. Following the one-step incubation at room temperature, the wells are emptied and washed. A chromogenic substrate is added and the colour reaction is stopped with sulfuric acid. Finally, the absorbance is measured. (21;22)

Assay procedure

Serum collected at the time of baseline and 10 – 12 weeks after the surgery was used. All samples were assayed in duplicate to ensure confidence in the values obtained.

Charts were drawn up of the wells so that the content of the different wells could be identified. Then 50 µl of standard, control or unknown sample was drawn up into the appropriate well. There were six standards and two controls, and in addition there was a duplicate of blanks. This was followed by 150 µl antibody solution, a mixture of the two antibodies, biotinylated and peroxidase-conjugated antibody and incubation buffer. The plate of wells were covered with sealing tape and incubated for 120 ± 5 minutes at room temperature (18 – 22 ºC) on a microtitre plate mixing apparatus at 300 rpm (Edmund Bühler, Swip). Afterwards an automated plate washer was used to wash the wells in six washing

cycles with the washing buffer from the kit. Then 100 µl substrate solution, containing tetramethylbenzidine substrate in an acidic buffer, was added in each well. The plates were covered with sealing tape and incubated for 15 ± 2 minutes at room temperature in the dark on the mixing apparatus (300 rpm). The reaction was stopped with 100 µl of 0.18 mol/l sulfuric acid. Then the absorbance was measured at 450 nm with 540 nm as reference with a ELISA reader (Labsystems Multiskan RC Original).

Interpretation of results

The computer constructed a standard curve by plotting the mean absorbance of the six standards against the corresponding concentrations. The concentrations controls and unknown samples were then determined by an interpolation on the standard curve. The mean values of the concentrations from each duplicate were used.

2.3.4 Other biochemical data, drugs and GFR

Just before a renal transplantation, and as a part of the follow-up, there are run a lot of different blood tests. We agreed on several different variables that would be of interest to the study. The test results were registered from the patient's journals as close as possible to the two visits during the study. The variables of interest were; HbA_{1c}, corrected ionized calcium, calcium, phosphor, urea, creatinine, albumin, C-reactive protein (CRP), PTH, cholesterol, triglycerides, high density lipoproteins (HDL), low density lipoproteins (LDL) and lipoprotein a. Also, for the second visit the concentrations of immunosuppressive drugs in the blood could be registered; the concentrations of cyclosporin, tacrolimus, sirolimus, everolimus and mycophenolate mofetil.

In addition to the immunosuppressant drugs, there were other drugs of interest for the study because of their affect on the kidneys and bone metabolism. For these drugs, it was only registered if the patient was on the drug or not. We used the journals to see if the patient had been using any of the drugs up to the day of transplantation and what they were using at the day of follow-up measurements. The drugs of interest and the percentage of the population using the drugs before RTx and at the time of follow-up are listed in Table 4 and Table 7, respectively. Prior to the transplantation, most of the patients who were on dialysis were given calcitriol. Very few in the group were taking calcium as an additive, and only 4.3 % of the population, that is two patients, were using bisphophonates before the RTx.

Table 4. Drugs of interest to the study and use prior to the RTx.

	Pre RTX
Loop diuretics	52.2 %
Tiazides	2.2 %
Statin	37.0 %
Aluminium containing phosphate binders	0.0 %
Calcium containing phosphate binders	0.0 %
Anabolic steroid	0.0 %
Glucocorticoids	10.9 %
Bisphosphonate	4.3 %
Estrogen	2.2 %
Vitamin D3 (calcitriol)	52.2 %
Calcium	6.5 %
Vitamin K	0.0 %
Vitamin K antagonist	17.4 %

Glomerular filtration rate (GFR) was measured at the Section of Nephrology 10 – 12 weeks after the transplantation, as a part of the routine follow-up after RTx, and the results were registered in this study.

2.4 Calculations

2.4.1 Cumulative glucocorticoid dose

The cumulative glucocorticoid dose was defined as the amount of glucocorticoid divided by the patients weight divided by the number of days; mg/kg/days.

The number of days was defined as number of days from the day of transplantation to the day of DEXA measurement, at baseline and 10 -12 weeks after transplantation. When glucocorticoid was given intravenously as methylprednisolone, the dose had to be multiplied by 1.2 before it could be added with glucocorticoid given orally as prednisolone.

2.4.2 Estimated GFR

Estimated GFR is the GFR corrected for size so that the result can be compared to an "average person" with a surface area of 1.73 m^2. The patient's weight and height is used to calculate the surface by means of the following formula (55):

$$\text{Surface area } (m^2) = \frac{\text{Weight}^{0.425} \times \text{Height}^{0.725} \times 71.84}{10000}$$

Then the patient's absolute GFR, the measured value, is corrected according to a surface area of 1.73 m^2:

$$\text{Estimated GFR (ml/min)} = \frac{\text{Absolute GFR} \times 1.73}{\text{Calculated surface area}}$$

For the measurement and calculation of absolute GFR, see section 1.5.2 *Glomerular filtration*.

2.5 Statistics

The statistical analysis was performed using Statistical Package for the Social Sciences, SPSS®. Normality was evaluated by the Kolmogorov-Smirnov test. Differences between and within groups were analyzed by the Unpaired- and Paired-Samples T-test, respectively. To examine the relationship between BMD, at baseline and change in BMD after 10 to 12 weeks, and potential bone loss predictors, we used simple linear (bivariate) regression analysis. This was subsequently followed by multiple linear regression analyses with stepwise addition of variables that had *P* values less than 0.2 in our a priori analysis and upon bivariate regression. As the multiple regression analyses were based on parametric principles, we based the simple regression analysis on parametric principles (Pearson) as well. Statistical significance was accepted as $p < 0.05$. In figures shown in the next section, *Results*, the level of significance is labelled in the following manner; * = $p < 0.050$, ** = $p < 0.010$, *** = $p < 0.001$.

3. RESULTS

3.1 Posttransplant status

Pre- and posttransplant characteristics of the study population are given in tables 2-7.

At baseline, according to the WHO definition, 35 %, 37 % and 28 % of the patients were classified as osteopenic in the LS, TF and TB, respectively, and likewise, 13 %, 15 % and 15 % as osteoporotic. During the time to follow-up the group of patients defined as osteoporotic from LS T-scores had increased to 20 %, and in TF and TB the fraction of osteopenic patients had been amplified to 44 % and 35 %, respectively. Furthermore, patients had very high PTH levels compared to normal range (1.6 – 6.9 pmol/l), which decreased markedly following RTx but were still higher than normal (Table 3, 5). Moreover, almost 48% of the patients experienced rejection of the allograft, while 41% had CMV infection (Table 5). A person is said to have a 100 % renal function if the GFR is 100 ml/min (24). The patients in the study have about half of this capacity 10 – 12 weeks after the RTx (Table 5).

Table 5. Posttransplant characteristics.

Rejection	47.8	%
CMV status		
Infection	41.3	%
disease	4.3	%
GFR		
Absolute	55±15	ml/min
Estimated	51±14	ml/min/1.73m²
PTH	17.5±13.5	pmol/l

Mean ± SD

Table 6. Immunosuppressant treatment at 10-12 weeks.

Prednisolone dose	15±7	mg/day
Cumulative GC dose	0.6±0.3	mg/kg/day
CsA	56.5	%
Dose	261±81	mg/day
Concentration; C_0	218±76	ug/l
Tacrolimus	19.6	%
Dose	5.8±1.6	mg/day
Concentration	7.5±2.1	ug/l
Sirolimus	17.4	%
Dose	5.1±2.0	mg/day
Concentration	10.9±2.0	ug/l
Everolimus and CsA	6.5	%
Everolimus dose	2.2±0.8	mg/day
Everolimus concentration	3.4±0.3	ug/l
CsA dose	150±50	mg/ day
CsA concentration;C_0	52±16	ug/l
MMF	91.3	%
Dose	1560±458	mg/day
Concentration	1.9±1.9	mg/l

Mean ± SD

Doses and concentrations of the immunosuppressive drugs were registered (Table 6). All patients receive prednisolone after a renal transplantation. The percentage of patients on the different kinds of immunosuppressive drugs is displayed in Table 6. Over half of the population were treated with cyclosporin in a combination with prednisolone and mycophenolate mofetil. Much smaller groups received tacrolimus and sirolimus, while only

three patients were given everolimus in combination with low-dose cyclosporin. In statistical analyses of the different immunosuppressants affect on change in BMD, the group was dichotomized according to whether the patient received a calcineurin inhibitor (cyclosporin, tacrolimus) or if the patients received sirolimus or everolimus with low-dose CsA. There were no significant differences between the groups (LS: p = 0.656, TF: p = 0.790, TB: p = 0.301). Then the group was dichotomized according to whether or not the patient received CIs, independent of dose and combination with non-CIs. The analyses revealed no statistical significant findings (LS: p = 0.221, TF: p = 0.732, TB: p = 0.351).

Other medications besides the immunosuppressants at the time of follow-up are presented in Table 7. Nobody was using calcium as an additive to their meals. In contrast to baseline data, only a few were still receiving calcitriol at the time of follow-up. The journals showed that the administration for most of the patients receiving active vitamin D_3 was stopped just prior to the transplantation. There were still only two patients on bisphosphonates. There were no significant findings between the use of other medications besides immunosuppressants prior to the transplantation (Table 4) and BMD at baseline or the change in BMD. However, statistical analyses showed that those who where still receiving loop diuretics at the time of the second visit had a larger loss of BMD in total body, compared to those who had been taken of the drug and those who were not using it prior to the RTx (p = 0.046). At the time of follow-up there were only three patients on calcitriol. Compared to those who were not receiving calcitriol any longer or who never had been on it, this group had a significantly lower loss of BMD in total femur (p = 0.043).

Table 7. The use of other drugs of interest 10-12 weeks after RTx

	Follow-up
Loop diuretics	30.4 %
Tiazides	0.0 %
Statin	15.2 %
Aluminium containing phosphate binders	0.0 %
Calcium containing phosphate binders	0.0 %
Anabolic steroid	0.0 %
Bisphosphonate	4.3 %
Estrogen	4.3 %
Vitamin D3 (calcitriol)	6.5 %
Calcium	0.0 %
Vitamin K	0.0 %
Vitamin K antagonist	13.0 %

3.2 Bone mineral density

Following RTx, a significant loss of bone mass was observed in LS, TF and TB (Figure 5), in the scale of 2.1 %, 2.5 % and 1.2 %, respectively. In contrast, no significant findings were revealed in UD or PF.

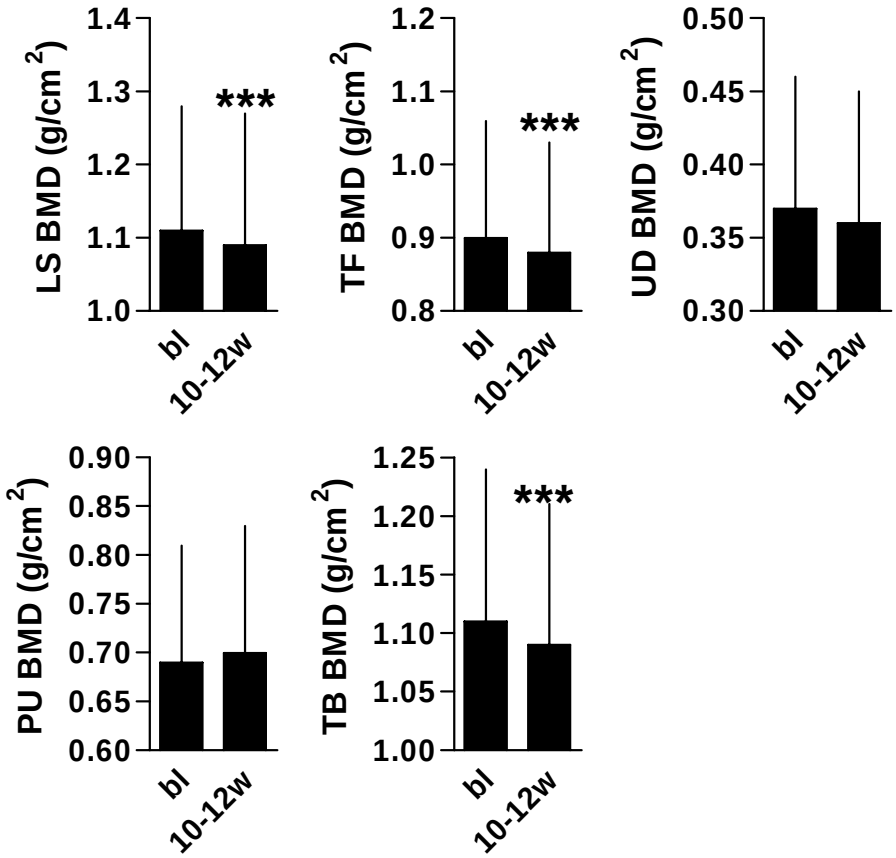

Figure 5. Bone mineral density at baseline and follow-up in RTx patients.
* = p < 0.050, ** = p < 0.010, *** = p < 0.001

To further elucidate the impact of RTx on bone mass we investigated baseline and change in BMD in relation to demographics, disease activity and treatment by simple regression analyses (Table 8, 10, 11) in those sites that changed significantly post RTx (LS, TF, TB). This was subsequently followed by stepwise linear regression analyses of the variables that had *P* values less than 0.2 in the simple regression analyses (Table 9, 12).

3.2.1 Baseline data

The mean amount of days from transplantation to baseline was nine days range. At baseline, we found strong negative correlations between BMD and osteocalcin, and BMD and PTH at all sites in the simple regression analyses (Table 8). Former transplantations had a highly significant negative effect on BMD at LS, TF and TB. Age and smoking correlated negatively with BMD at TF and TB. Women had a significantly lower in BMD in TB, while BMI had a positive correlation at LS BMD.

Table 8. Correlation analysis of possible determinants of bone mass in RTx patients at baseline.

Variable	Lumbar Spine		Total femur		Total Body	
	β	*p*	β	*p*	β	*p*
Age	-0.13	0.408	-0.35	**0.027** [m]	-0.33	**0.025** [m]
Gender	0.20	0.188 [m]	0.28	0.076 [m]	0.31	**0.034** [m]
Former Tx	-0.41	**0.004** [m]	-0.43	**0.005** [m]	-0.42	**0.003** [m]
Dialysis	-0.27	0.076 [m]	-0.21	0.189 [m]	-0.22	0.144 [m]
Smoking	-0.19	0.204	-0.33	**0.034** [m]	-0.34	**0.019** [m]
BMI	0.33	**0.026** [m]	0.14	0.374	0.19	0.199 [m]
PTH	-0.45	**0.002** [m]	-0.38	**0.017** [m]	-0.43	**0.004** [m]
Osteocalcin	-0.44	**0.002** [m]	-0.42	**0.006** [m]	-0.44	**0.002** [m]
Telopeptide	-0.04	0.793	0.01	0.929	0.01	0.972
CML GC BL	-0.04	0.774	0.09	0.585	0.05	0.724

[m] *$p < 0.2$ included in multiple regression*

The following multiple regression analyses showed that osteocalcin level and BMI were the significant predictors for LS BMD, explaining 28 % of the variation in BMD at baseline, with osteocalcin level as the major determinant (19 %). For the TF, the three variables age, former transplantation and osteocalcin level together accounted for 41 % of the variation in BMD, with former transplantation as the major determinant (14 %). In TB, 50 % of the variation was explained by a number of factors; age, gender, former transplantation, smoking and osteocalcin level, and with the latter as the dominant predictor (19 %).

Table 9. Stepwise multiple regression of determinants of bone mass in RTx patients at baseline.

Variable	Lumbar Spine			Total femur			Total Body		
	β	*p*	r^2	β	*p*	R^2	β	*p*	r^2
Age				-0.43	0.002	0.102	-0.34	0.006	0.096
Gender							0.24	0.046	0.043
Former Tx				-0.42	0.004	0.144	-0.33	0.009	0.113
Smoking							0.25	0.033	0.054
BMI	0.33	0.014	0.097						
Osteocalcin	-0.45	0.001	0.187	-0.28	0.045	0.168	-0.28	0.024	0.191

r^2*: Adjusted r square*

3.2.2 Longitudinal data

The mean amount of days from baseline to follow-up was 63 days. By simple regression analyses of the changes from baseline to follow-up we showed a negative correlation between the change in osteocalcin level and BMD in LS and TF (Table 10). Moreover, age correlated positively with the change in LS BMD. Finally, those with CMV infection had a larger decrease in TB BMD than those without.

Table 10. Correlation analysis of possible determinants of change in BMD from BL to 10-12 weeks control.

Variable	Δ Lumbar Spine		Δ Total femur		Δ Total Body	
	β	*p*	β	*p*	β	*p*
Age	0.34	**0.022** [m]	0.17	0.296	0.05	0.732
Gender	-0.09	0.556	-0.15	0.350	-0.13	0.393
Former Tx	-0.13	0.380	-0.19	0.233	-0.10	0.528
Dialysis	-0.04	0.791	-0.01	0.935	-0.25	0.090 [m]
Smoking	-0.19	0.333	-0.18	0.263	0.04	0.770
Δ BMI	-0.05	0.746	0.00	0.982	-0.15	0.309
Δ PTH	0.28	0.097 [m]	0.22	0.228	-0.25	0.148 [m]
Δ osteocalcin	-0.31	**0.034** [m]	-0.34	**0.033** [m]	-0.20	0.182 [m]
Δ telopeptide	-0.16	0.283	-0.00	0.994	-0.12	0.442
GFR; absolute	0.01	0.958	-0.13	0.456	-0.15	0.345
Transplant rejection	-0.28	0.057 [m]	0.15	0.370	0.09	0.535
CMV infection	0.08	0.594	-0.09	0.584	-0.42	**0.004** [m]

[m] *$p < 0.2$ included in multiple regression*

Δ *Change from baseline to follow-up*

To investigate the impact of anti-rejection therapy on BMD a simple regression analysis was also performed on BMD in the three compartments with significant bone loss and immunosuppressive drug treatment data (Table 11). We did not use the change in blood concentration for those who received CsA because their samples were drawn at the time of C_2 at the first visit, and at the time of C_0 at the second visit. It is not a part of the routine to measure the concentration of cortisol in blood in RTx patients; therefore we did not have such results to use in our statistical analyses in the study. There were only three patients receiving everolimus in a combination with CsA, and because of the small number of patients the results of a statistical analysis would not be that reliable. The only variable that came through in the simple correlation analyses as significant was the change in blood concentration of sirolimus in correlation to BMD in TF. The positive association means that those of the patients who had lower concentrations of the drug in their blood had a larger loss of bone in total femur.

Table 11. Correlation analysis of possible determinants of change in BMD from BL to 10-12 weeks control in relation to immunosuppressive treatment.

Variable	Δ Lumbar Spine		Δ Total femur		Δ Total Body	
	β	*p*	β	*p*	β	*p*
Δ Prednisolone dose	0.18	0.225	0.11	0.488	-0.03	0.842
Δ CML GC dose	0.25	0.092 [m]	0.08	0.607	0.04	0.819
Δ CsA dose	-0.01	0.954	0.00	0.996	0.10	0.614
Δ Tacrolimus dose	-0.31	0.613	0.48	0.419	-0.61	0.277
Δ Sirolimus dose	-0.23	0.590	-0.18	0.671	0.10	0.812
Δ MMF dose	0.03	0.877	0.19	0.281	0.11	0.492
Δ [Tacrolimus]	0.87	0.053 [m]	0.84	0.077 [m]	0.39	0.511
Δ [Sirolimus]	0.31	0.543	0.72	**0.042** [m]	0.47	0.237
Δ [MMF]	0.01	0.967	0.06	0.741	0.04	0.818

[m] *$p < 0.2$ included in multiple regression*

Δ *Change from baseline to follow-up*

[ ] *concentration in blood*

Multiple regression analyses were subsequently performed to determine which variables were most closely associated with the change in BMD following RTx. In this stepwise analysis we included all the variables that had a *P* values less than 0.2 from both Table 8 and Table 9.

The analysis revealed that age was the only significant predictor explaining about 9 % of the change in BMD in LS (Table 12). The difference in osteocalcin level explained 18 % of the change in TF BMD. And in the TB, CMV infection accounted for 10 % of the variation in BMD.

Table 12. Stepwise multiple regression of determinants of change in BMD from baseline to 10-12 weeks after RTx.

Variable	Δ Lumbar Spine			Δ Total femur			Δ Total Body		
	β	*p*	r^2	β	*p*	r^2	β	*p*	r^2
Age	0.33	0.050	0.086						
Δ osteocalcin				-0.47	0.008	0.181			
CMV infection							-0.36	0.032	0.095

Δ *Change from baseline to follow-up*

r^2*: Adjusted r square*

3.3 Bone turnover

To investigate bone metabolism in the RTx patients we first determined osteocalcin and telopeptide, as biochemical markers of bone turnover, at baseline and at the second visit. We found that patients had normal osteocalcin levels at baseline, which increased significantly following RTx (Figure 4A). In contrast, telopeptide levels were markedly higher in RTx patients at baseline compared to reference values, in addition it increased significantly following RTx (Figure 4B).

Simple regression analysis revealed significant associations between osteocalcin and telopeptide at baseline and at 10-12 weeks control (figure 4C and 4E). At baseline, a total of 12 % of the variation in telopeptide concentrations is explained by the variation in serum osteocalcin levels (r^2). At the time of second visit, a total of 40 % of the variation in telopeptide concentrations is explained by the variation in serum osteocalcin levels. The change in telopeptide and osteocalcin levels were not significantly correlated (Figure 4D).

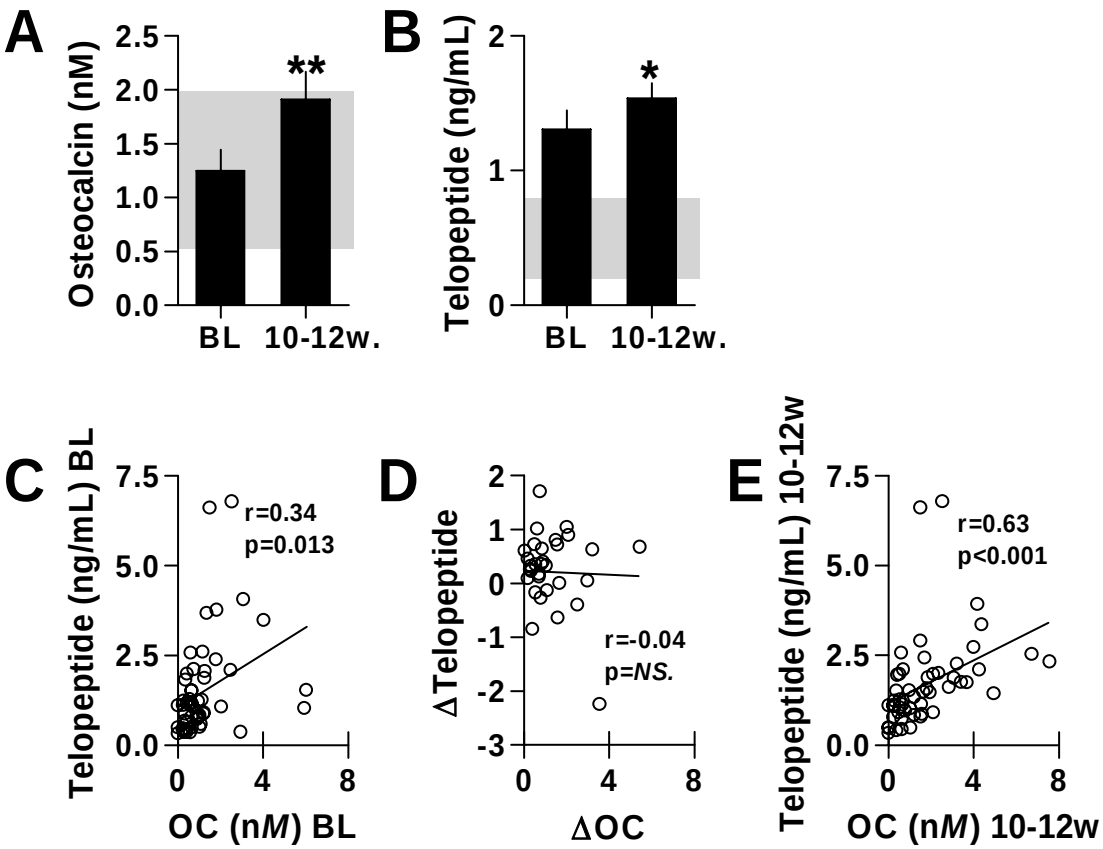

Figure 4. Illustration of bone markers.

Osteocalcin (A) and telopeptide (B) at the time of baseline and control. Grey squares indicate interval of reference values for normal individuals. Correlation between osteocalcin and telopeptide at baseline (C), change from baseline to second visit (D) and at second visit (E).

* = $p < 0.050$, ** = $p < 0.010$, *** = $p < 0.001$

4. DISCUSSION

The present study was a pilot-study prior to a larger study which will include medical intervention in connection to osteoporosis in renal transplantation patients at Rikshospitalet University Hospital. Our study observed the development in BMD in a longitudinal, descriptive study with 46 patients from baseline to 10 to 12 weeks after transplantation. The baseline status in BMD and the change in BMD from baseline to follow-up were investigated in correlation to variables registered throughout the study in an effort to identify predictors of bone loss. Our main findings were that the CRF patients had reduced BMD at baseline compared to normative data, and that there was statistically significant loss of BMD from baseline to follow-up in the range of 1.2 to 2.5 % in three compartments; lumbar spine, total femur and total body. This was observed with a mean of only 63 days between the two visits. In addition, predictors significant for the BMD prior to and for change in BMD following the transplantation were identified. At baseline, the known risk factors age, gender, smoking and former transplantations were the significant determinants. For change in BMD, the predictors were age (LS), change in osteocalcin levels (TF) and CMV infection (TB).

4.1 Validity

The Rikshospitalet University Hospital is the only transplant centre in Norway, therefore the selection of patients in the study originated from different parts of the country. Because of this the study is based on an unbiased, nationwide population. The conformity in the population characteristics between this study and the population in a larger study from the same hospital, with about four times the number of patients, is very convincing (52). Only 10 % of the patients who were asked were unwilling to participate in the study. In addition, there was a 13 % dropout rate from baseline to second visit. These are low numbers in comparison to the normal drop-out rate for the control after 10-12 weeks at the nephrologic day clinic, which normally is about 20 %. In total, all of the above makes us confident in that the patient material in the present study is representative for patients going through RTx in Norway.

The number of patients we were able to include during a three month period was large enough to disclose reduction in BMD from baseline to follow-up in three compartments with highly statistical significance. The risk for our observation to be caused by random variation, a type 1 error, is less than 0.1 %. From other studies we know that glucocorticoids contribute to loss of bone following transplantation (33;56). This did not come through in any of our results. An

explanation may be that the differences in cumulative glucocorticoid dose between the patients were too small to identify a greater loss of bone in relation to this variable. Differences between patients receiving GCs might be difficult to identify, since most transplant patients receive standard doses. Another study with approximately the same number of patients could not find any dose-dependent effects of glucocorticoids either (57). An additional explanation might be that the study was not powered enough to show significant differences, possibly a result of a type 2 error at this point. In the intervention study which will follow this pilot-study, there will be a higher number of patients and because of this we expect to find significant differences between subgroups, such as for example smoker vs. non-smoker, dialysis vs. pre-emptive, high or low cumulative glucocorticoid doses and others.

The analytical validity in the study is high. We used an osteodensitometer with high precision and low analytical variance. Moreover, all analyses of the measurements were supervised by an experienced operator. The analyses of the bone markers, osteocalcin and telopeptide, have a greater variation than the DEXA measurements, but is in the normal area of similar tests. According to the factory, the intra-assay and inter-assay coefficients of variation were < 10 % for both assays. Both analyses were supervised by an experienced laboratory engineer.

4.2 Bone mineral density

4.2.1 Baseline data

As the Z-scores for LS, TF and TB indicated (Table 3), the mean BMDs in the population were lower than average for gender-, size-, and age-matched normative data. This signifies that the group already had experienced a loss of bone prior to the transplantation. The men had a consistently lower BMD in all three compartments compared to the women, implying that they had a poorer bone status initially. However, a Norwegian group of researchers performed a study which compared healthy middle-aged and older Norwegian men and women to the database of healthy middle-aged and older Americans provided by the manufacturer of the densitometer (Lunar) (58). They found that the BMD in total femur in healthy Norwegian middle-aged and elderly men and elderly women seemed to be slightly lower than the reference population. In addition, middle-aged women had significantly higher total body BMD. Because of this, the Z-scores might be giving a somewhat incorrect picture of the starting point of the population. But this is small differences, so never the less the

population had lower BMD than the average population. Another reason that could explain why the men had lower BMD than the women in the study is the fact that there were more men than women in the study. In that way we may have captured a more authentic picture of the bone status in CRF patients in the group of men because of higher numbers of observations, since a lower BMD prior to transplant correlates to other previous studies (56).

The multiple regression analyses of baseline data gave us several predictors for low BMD in all three compartments. Osteocalcin level was the major determinant in all three compartments, and the higher the level of osteocalcin, the lower the BMDs. This relationship has also been seen in a similar study with 125 renal patients in Denmark (59). Another study found that serum osteocalcin is increased in patients with osteomalacia (53). As described in *1.2.3 Bone remodelling*, the resorption and formation of bone is coupled to each other, and increased levels of serum osteocalcin indicate an escalated turnover in total (56). Therefore, those who had higher turnover were those who had lower BMD.

In total femur and total body, age predicted a lower baseline BMD. Age is a well known factor which predisposes for bone loss and osteoporosis (60;61). As an individual is aging, distinct changes take place with trabecular bone and cortical bone. Reductions in trabecular bone have been well documented, and the alterations are due to a variety of factors, including changes in gonadal status, nutrition, and physical activity. Furthermore, with the menopause comes a reduction in the estrogen levels, thereby favouring osteoclast formation and inhibition of osteoclast apoptosis (61). Changes are also seen in cortical bone, were the reduction primarily is attributed to bone resorption on the endosteal surface that occurs at a greater rate than periosteal appositional growth. This results in a reduction in cortical bone width as aging progresses, and the bone at the interface adjacent to trabecular bone becomes porous. The architecture no longer resembles cortical bon, but trabecular bone. In addition, osteoblasts have a shorter life span and more prone to undergo apoptosis with aging (61). As a result of these factors, age predisposes for a low BMD.

Former transplantation was another parameter that was highly significant in correlation to low BMD in total femur and total body in the multiple analyses. This group of patients was quite small, but the significance can be explained by long term regimens with glucocorticoids and immunosuppressants in the past. The patient may have been inactive for longer periods of time because of their severe illness. If this has been the case, the skeleton has had less

mechanical stimulation, which causes less regeneration and synthesis of new bone matrix (62).

The female patients in the group had lower BMD in total body compared to the males. Literature states that male gender has a positive effect on bone mass (60;63;64). These results have mainly been explained based on postmenopausal status of the female patients studied. However, the same significant difference between the genders has been seen in study populations where all female patients were premenopausal and had regular menstrual periods (60). Therefore, more investigation is needed regarding the effects of sex hormones on bone mass in transplantation patients. In our study, 75 % of the females were postmenopausal and this probably accounted for some of the difference between the genders. The mean Z-scores indicate that this might have been the case since the women had values closer to zero (normal) than the men. The reduced levels of estrogen signalling after menopause favours a milieu that is detrimental to bone maintenance by increasing local factors (interleukins, tumour necrosis factors, and colony-stimulating factor), thereby favouring osteoclast formation and inhibition of osteoclast apoptosis (61;65).

Patients who were smokers at the time of the study had a lower BMD in TB than non-smokers. This is one of the recognized risk factors and in accordance with other studies (66-68). The mechanism by which smoking affects bone metabolism and bone mass remains inadequately elucidated. Nicotine, the principle pharmacologically active component of cigarette smoke, has been investigated in relation to bone cell function. It has been shown to have direct effects on osteoblast cell proliferation, mediated by specific receptors, and to be able to induce expression of the bone matrix protein osteopontin, suggesting direct toxic effects of nicotine on bone cells (66;69).

In lumbar spine, a higher BMI had a beneficial effect on bone mass, findings in line with reports in literature (60;62;64). The higher load associated with increased weight may protect against bone loss by stimulating osteoblast production, thereby increasing bone formation (62). Alternatively, a protective effect may be attributed to a higher level of estrogens, particularly estrone, resulting from the conversion of androgens to estrogen in adipose tissue (70). The positive correlation between BMI and BMD will not be an indefinite fact, since extreme values of BMI will be associated with inactivity and diverse health problems, and

subsequently bone loss. But none of the patients in this study group were obese, so their weight was not restraining in relation to everyday life and exercise.

4.2.2 Longitudinal data

The DEXA measurements revealed a significant loss of BMD in three compartments 10 to 12 weeks after transplantation; LS, TF and TB. In the two other areas measured, UD and PU, no significant loss of BMD was observed. The reason for this is uncertain, but a theory may be the distribution of cortical and trabecular bone in these two areas. The remodelling activity in cortical bone is only about 10 % of that in trabecular bone (71;72), and therefore the negative effects are more readily apparent within trabecular bone, even though both types of bone are affected (13). But the total body and the ultra distal forearm have approximately the same distribution of cortical and trabecular bone. So a part of the explanation may be regional differences in turnover in the body, or the fact that sources of error are induced because the arm has been used in dialysis which has caused impaired regional blood flow. Seeman et al (71) found that endocrine dysfunction had differential effects on the axial and the appendicular skeleton. They concluded that induced alterations in BMD are both site and disease specific, and that the changes reflects regional differences in the profile of skeletal response rather than merely differences in level of bone turnover. This supports the theory of regional differences, and since the total body is a sum of the entire axial and appendicular skeleton, the result is a loss of bone in TB even though it is not registered in all compartments. The degree of bone loss in the three compartments reflect the distribution of trabecular versus cortical bone, with the largest loss in the compartment most rich in trabecular bone, LS, and the lowest loss in the compartment with the least amount of trabecular bone, TB. This is in according to other findings in other studies (73).

In the multiple analyses of determinants of change in BMD none of the variables were significant for all three compartments. In lumbar spine higher age was associated with a larger loss of BMD. This may be a result of the convention of standard administration of GCs in renal transplant patients. A study compared the cortisol pharmacodynamics after methylprednisolone administration in young and elderly males and found that the elderly had a greater exposure to and a more significant response of cortisol to the exogenous glucocorticoid. In addition, a slower clearance of methylprednisolone was noted in the elderly group (74). Therefore, those of age may possibly have a higher degree of accumulation of

glucocorticoids in their body and because of this have a larger loss of bone. Another reason for a greater loss of bone in relation to increasing age is that age is a predisposing factor for low BMD, as shown in the baseline data in this study and others. Studies show that those who have lower BMD before transplantation are those who experience a greater fall in BMD (56;60;61).

In total femur, the more the level of osteocalcin increased, the more the BMD was preserved. Both osteocalcin and telopeptide levels increased from baseline to control, but osteocalcin increase the most. When a massive process of bone resorption is initiated, the formation process is not mobilized straight away. First the osteoclasts break down the osteoid in a limited vascular area, and then the osteoblasts follow and construct new osteoid afterwards (75). In this way, the formation process is delayed. When the resorption is activated in an uncoupled manner, there is an uneven relationship between formation and resorption. The balance between the two processes is gone, and the total result will in this case be a loss of bone. The patients who have more of their BMD preserved at follow-up are those who have the highest values of osteocalcin. That is, a greater ability to initiate compensatory formation processes results in a lower total loss of bone.

CMV infection had a significant association with loss of bone in total body. To our knowledge, this is the first study suggesting a correlation between CMV infection and bone loss. Our first hypothesis was that since CMV is known to predispose for transplant rejection, the correlation could be the result of an indirect effect through the need for increased GCs doses in treatment of rejections. But there was no significant correlation between CMV and transplant rejection in our study group, and we did not observe any direct association between a decrease in BMD and transplant rejection either. Another theory is that there may be a direct correlation between bone loss and the immune reactions initiated by the CMV infection. There has been found evidence for such a relationship between CMV infection and new-onset posttransplantation diabetes mellitus (76). The theory in this study was that the CMV-induced proinflammatory cytokines lead to apoptosis or functional disturbances of the β-cells. Some of the same mechanisms as seen in bone loss in relation to rheumatoid arthritis might be involved, although this is a disease with chronic inflammation over long periods of time (77). In rheumatoid arthritis, cytokines, among other inflammatory species, play a part in complex mechanisms leading to increased osteoclast survival and decreased osteoblast differentiation and survival, with a net loss of bone as the consequence. There has been performed a study on

IL-6-transgenic mice to evaluate the effect of chronic interleukin-6 (IL-6) overexpression on the skeleton of growing prepubertal mice (78). The transgenic mice developed a skeletal phenotype closely resembling growth and skeletal abnormalities observed in children with chronic inflammatory disease. Although these studies do not address the association between CMV infection and bone loss, they show a trend for negative impact of cytokines on bone. We cannot conclude with any certainty that the bone loss seen in correlation to CMV infection is owing to a direct effect of the exposure to active infection; this is a topic that needs further investigation before any conclusions can be drawn.

None of the immunosuppressive drugs were shown to be significant determinants of change in BMD in the multiple analyses. This may be because of the short time of observation, and that we did not have any matched group of patients receiving another type of treatment that we could compare with. In contrast, the simple regression analysis of the change of concentration in blood in correlation to BMD in total femur resulted in a positive correlation, meaning that the larger the decrease in blood concentration the lower BMD in TF. This could be an indication of the bone sparing effect that the drug has been postulated to have (79). But the correlation was not strong enough to be significant in the multiple regression analysis. Even though the statistical analyses could not reveal any relationship with the immunosuppressants in this study, we know from several other reports that most of the immune modulators are contributory to loss of bone like we have observed (40). The escalated bone turnover may in part be explained by adverse drug actions. The effect of glucocorticoids on bone loss is associated with high resorption and low formation, a dissociation of the two processes in bone turnover (33). The analyses of the bone markers reflect this. The study revealed a high turnover bone loss, which is also associated with the use of calcineurin inhibitors in various studies (40;56). Therefore, we will argue that the bone loss observed is a consequence of the high dose immunosuppressant treatment that the patients are exposed to, especially during the first weeks after transplantation.

Of the other medications used besides immunosuppressants, loop diuretics had a significant association with lower BMD in total body. This negative effect on the skeleton is explained in literature by the urinary calcium loss that the drug causes (13;80). The few patients who were still using calcitriol at the time of control had a significantly lower bone loss in total femur than those who had quit prior to the transplantation or who had never used it. Several studies have confirmed this positive effect of calcitriol on bone (81-84). These results have been

found to be in agreement with the simultaneous observation of a disturbed vitamin D metabolism (83).

4.3 Bone turnover

At baseline, dissociation between bone resorption and formation was found, as illustrated in Fig. 4C, with a relatively poor r-value (0.34). In normal healthy individuals bone formation is coupled to bone resorption in a tight equilibrium. As this result show, this delicate balance had been disturbed in the study population, which resulted in a shift in turnover causing more resorption than formation of bone. Studies have raised the possibility that there might not only be the resorptive activity of osteoclasts that controls the coupling between resorption and formation of bone, but more likely the osteoclasts themselves that secrete or produce anabolic factor(s) contributing to induction of bone formation by osteoblasts (85). In addition, remnants from osteoclasts, and the preparation of the formation area seem to play important roles in the bone formation process. Osteopetrotic mutations and pharmacological intervention in animal models have given evidence to this theory by demonstrating dissociation when these mechanisms are disrupted. The same dissociation is seen in postmenopausal osteoporosis (85), and in patients using GCs (33), indicating that there might be some of these mechanism that had been affected in the study population at the time of baseline as well.

The fact that the levels of telopeptide already were above normal range at baseline might have been because blood samples were collected at the day of DEXA measurement, which took place on the fifth to tenth day after the RTx. At this point the patients had already been exposed to high doses of glucocorticoids and other immunosuppressants, and this could already have affected bone turnover negatively, escalating it to a higher level than normal at a very early stage following transplantation.

Following RTx, a much stronger correlation was observed (r = 0.63), as shown in Fig. 4E, indicating a tighter coupling between the two processes of bone turnover. Even so, 40 % association between the two bone markers show that the patients were still a long way from achieving equilibrium in turnover, and they were still in a situation where bone resorption was much higher than bone formation. As illustrated in Fig. 4D, no significant correlation between the changes in markers of bone turnover was found following RTx, reflecting the

relatively greater increase in osteocalcin compared to telopeptide. High levels of osteocalcin, which the study group had at follow-up (Fig. 4E), indicate escalated bone turnover (56). Within the group, those who had the largest increase in osteocalcin levels, as discussed in the previous section; *4.2.2 longitudinal data,* were those who experienced slighter change in BMD, because they were the patients with the best recovery in total and therefore managed to compensate more of the bone resorption (59).

Some long-term studies of different groups of transplant patients have shown an improvement in BMD after the initial rapid loss of bone during the first six months, especially (5;86). The fact that the bone loss may be reversible to some extent indicates that the use of bisphosphonates as prophylaxis or as a mean of intervention to prevent bone loss might be the right way to go (87-89).

4.4 Limitations of the study

The study is based on a relatively small patient material, and a larger study will be able to reveal more significant predictors of low BMD and change in BMD, especially with the effect of the immune modulators in mind. There were no matched control group that we could compare our patient data with, which would have been desirable for the evaluation of the immune modulators effects. Nevertheless, at baseline, we confirmed a significantly low bone mineral density in comparison to a database of normative data, which is harder than finding the same in comparison to a matched group, and this makes our findings more valid. A problem is that there is no Norwegian database of normative data available, but the correlation between the normative data from Norwegians and Americans is satisfactory according to a Norwegian study (58).

5. CONCLUSION

At baseline, patients with end staged renal failure had significantly reduced bone mass as given by osteodensitometry and compared to normative data. The predictors for low mineral density were the same as those that apply the population in general; age, gender and smoking. In addition, patients who had gone through former transplantations were at higher risk of having a lower BMD. Following RTx we found a significant loss of bone in lumbar spine, total femur and total body. The data showed a connection between the early posttransplant bone loss and age, and surprisingly there was a significant correlation with CMV infection.

The study gives further evidence to the fact that patients suffering from CRF who goes through RTx is in danger of a continuing bone loss with a major risk of bone fractures. In addition, the study has shown that bone loss can be seen as soon as 10 to 12 weeks after a renal transplantation, which further raises the question of prophylaxis and intervention.

6. PERSPECTIVES

The group of chronic renal patients who are going through a renal transplantation is in high risk of developing osteoporosis which in turn leads to a high risk of low energy fractures. There are several areas in relation to this problem that needs further investigation. One such area is the role of immunosuppressant medications in bone loss, other than the glucocorticoids. The present study has revealed a negative association between bone and CMV infection, a connection that has not been investigated earlier. The use of prophylaxis and intervention with calcium, calcitriol and bisphosphonates to prevent or decelerate the bone loss in this group of patients is another field with several questions still unanswered. To gain more knowledge randomized controlled trials are needed. Bone loss is a topic that must to be taken seriously in transplantation medicine, so that the recipients of donor organs can look forward to a good quality of life following a life changing procedure.

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PROTOCOL

INTRODUCTION

Background

«A systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture risk» is the definition of osteoporosis [1]. The abnormalities of the skeleton in chronic kidney disease (CKD), collectively known as renal osteodystrophy, are an important cause of morbidity and decreased quality of life. Renal osteodystrophy is a multifactorial and complex disorder of compromised bone strength [2].

Renal transplantation offers the best prognosis for patients with severe end-stage renal failure: 1-year survival following renal transplantation now exceeds 90%, and new immunosuppressants introduced in the last decade have reduced rejection episodes and prolonged graft survival. Consequently, improving the long term quality of life for these patients is becoming more important. One potential problem relates to the adverse effects of fractures, which may result from the well-recognised disturbances in bone metabolism among patients with end-stage renal disease. When the amount of bone tissue is reduced the skeleton is not able to withstand normal forces and fragility fractures occur. These fractures most commonly occur in the sites of the skeleton rich in trabecular bone as in the spine, distal forearm and proximal femur. Several recent reports have suggested an above-average fracture rate in these patients [3;4;5].

Bone loss occurs rapidly following renal transplantation because of aggravating factors that emerge after procedure. Among these factors, the key pathophysiological contributor to bone disease is immunosuppressive agent application, especially glucocorticoids (GC). The pathogenesis of corticosteroid-induced bone loss is multifactorial and has been reviewed extensively [6]. The main deleterious effect of corticosteroids is a direct and profound inhibition of bone formation. GCs inhibit osteoblast differentiation and induce apoptosis in mature osteoblasts as well as osteocytes [7]. They also decrease gastrointestinal calcium absorption, resulting in a negative calcium balance and secondary hyperparathyroidism. In addition, corticosteroids directly suppress gonadotropins and may cause hypogonadism [8]. With most solid organ transplants, bone loss is greatest at sites rich in cancellous bone, and spinal bone mineral density (BMD) losses of 3–10% have been reported in the first 6 months

following renal transplantation, with continued slower bone loss thereafter. Long term studies have shown the cumulative incidence of fracture to be three times higher than expected 15 years after the renal transplantation. [3;4;5]

Aim

The purpose of the present study is to examine bone loss following renal transplantation in CKD patients by doing a prospective descriptive study. Moreover the purpose is to identify predictors of bone loss in these patients.

Dual energy X-ray absorptiometry (DEXA)

Dual energy X-ray absorptiometry (DEXA) measures the bone mineral density (BMD), which is a measure for the amount of bone in a given area. In this study the DEXA technology will be used to take measurements at the spine, hip, wrist, and a whole body scan. As the patient lie on the table or sits on a chair beside the table, the machine moves over the part of the body that is going to be measured while it sends a thin, invisible beam of low-dose x-rays. The amount of radiation is very small with an effective patient dose at 0,7-1,3 μSv, which is less than 1/10 the dose of a standard chest x-ray [9]. Based on how much the x-rays have changed after passing through the body, a picture of the skeleton will be generated on a computer screen. The X-ray beams are sent through the body from one side of the table and a detector register the amount of X-ray beams that are let through to the other side. The DEXA instruments differentiate body mass into the components of lean soft tissue, fat soft tissue and bone, based on the differential attenuation by tissues of the x-rays.

Bone mineral content (BMC) is expressed in grams (g) and the area scanned is expressed in cm^2, from these values the computer software calculates the BMD which therefore is expressed in g/cm^2. Historically, BMD values themselves have not been used for estimating fracture risk. Instead, BMD values are most often expressed in comparison to an established normative range. All densiometry manufacturers provide normative databases for this purpose. These databases are derived from measurements of large groups of both men and women of different ages and races. Comparisons are expressed both as the percentage of age-matched and young normal values, as well as standard deviation scores. The most commonly used standard deviation score is the T-score. It compares a patient's bone density to the expected value for young adults. The young normal or T-score is defined as the difference between the patient's BMD and the young adult reference value (YA) divided by the standard deviation of the reference population (SD):

$$\text{T-score} = \frac{\text{BMD - YA}}{\text{SD}}$$

For the diagnosis of osteoporosis, The World Health Organisation (WHO) has defined the following criteria for the assessment of osteoporosis based on the T-scores:

- Normal: A BMD not more than 1 standard deviation below young adult (T-score = -1)
- Low bone mass (osteopenia): A BMD between 1 and 2,5 standard deviations below young adult (T-score < -1 and > -2,5)
- Osteoporosis: A BMD 2,5 or more standard deviations below young adult (T-score = -2,5)
- Severe osteoporosis: A BMD 2,5 or more standard deviations below young adult (T-score = -2,5) and the presence of one or more fragility fractures

The WHO definition is intended for use in defining populations and not for the diagnosis of osteoporosis in individual subjects. However, in the absence of other criteria, the WHO definition has become the standard for diagnosis in clinical practice. These guidelines cannot be directly transferred to patients with renal osteodystrophy, since they have been drawn up especially with postmenopausal women in mind, but they can give a pointer as to if the patient is in a risk zone. [10]

Another standard deviation score in use is the Z-score. This is a value that shows the amount of bone the patient has in comparison to other people of his/her age group, gender, and size [11].

Biochemical markers of bone turnover

Bone formation

Osteocalcin is a biochemical marker of bone formation. It contains two or three amino acid residues of γ-carboxyglutamic acid (GLA), hence it is also known as bone GLA protein (BGP). Osteocalcin is the most abundant noncollagenous protein in bone and is predominantly synthesised by the osteoblasts [12;13]. It is incorporated into the extracellular matrix of bone, but a fraction of newly synthesised osteocalcin is released into the circulation where it can be measured by radioimmunoassay [12;14]. Because of this there has been considerable interest in its assay as a possible means for the evaluation of patients with bone disease, particularly in osteoporosis. Histological studies have shown significant correlations

between the rates of bone formation and serum values for osteocalcin. Care is required in interpreting values in the presence of renal failure since the kidney is a site of its metabolism [12;13].

Bone resorption

Telopetptide is a biochemical marker of bone resorption. Type I collagen accounts for more than 90 % of the organic matrix of bone and is synthesised primarily in bone. Pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) are nonreducible cross-links that stabilise the collagen chains within the extracellular matrix [14-17]. They cross-link at two locations in type-I collagen, N-telopeptide-to-helix and C-telopeptide-to-helix.

Pyridinoline is present in bone and cartilage matrix and in minute amounts in some other connective tissues. Significant amounts of D-Pyr are only found in bone collagen, at a concentration of 0,07 mol/mol collagen. The Pyr/D-Pyr ratio in human bone matrix is 2:3 [14-17].

During bone remodelling, type I collagen is degraded as described above, and small peptide fragments are excreted into the bloodstream. These fragments can be analysed in serum by an enzyme linked immunosorbent assay (ELISA), as described earlier: [14-17].

Renal function

Creatinine clearance gives an estimate on how the kidneys function when it comes to filtrating fluid. Creatinine clearance can be calculated by doing a clinical measurement of the glomerular filtration. This is done by collecting the patient's urine over a definite period of time. The volume is measured and the concentration of creatinine in the urine and blood serum is determined [18].

The calculations are done after the following formula:

$$\text{Creatinine clearance} = \frac{\text{(volume of urine/minute)* [urine creatinine]}}{\text{[serum creatinine]}}$$

During 24 hours the kidneys normally filtrate 150 litres of fluid. A patient with a clearance of 100 ml/min is said to have a 100 % kidney function [18].

Parameters and variables

The risk for low BMD and fractures in the CKD patients will be evaluated in relations to several risk factors. These are:

Demographic parameters:

- Age
- Gender
- Ethnicity
- Body mass index (BMI)
- Smoking habits
- Activity level
- Post menopause
- Age at menarche

Kidney and bone related variables:

- Diabetic nephropathy
- Dialysis time and mode
- Previous transplantations
- Pre- and posttransplant fractures
- Pre- and posttransplant use of calcium
- Pre- and posttransplant use of vitamin D
- Pre- and posttransplant use of vitamin K
- Pre- and posttransplant use of vitamin K antagonist
- Pre- and posttransplant use of drugs used in osteoporosis
- Posttransplant use of immunosuppressants
- Rejection episodes

MATERIAL AND METHOD

Inclusion criteria

- Patient at the Section of Nephrology at the Rikshospitalet University Hospital who has been cleared for a renal transplantation.
- = 18 years of age

Exclusion criteria

- < 18 years of age
- Pregnant women
- Competing medical disease
- Psychological unstableness
- Psychiatric disease that demands medical treatment

Study design

The project will be performed in a three month prospective study. The subjects will be investigated at the Day Clinic of the Section of Endocrinology as well as at the Section of Nephrology at the National University Hospital.

The first examination will be performed sometime during the fifth and tenth day after the transplantation. This time limit has been set to assure that every patient participating in the study will be examined at an approximately similar time in connection with the procedure, regardless if the kidney has been harvested from a deceased or living donor. The second examination will be performed contemporary to a follow-up at the Section of Nephrology, ten to twelve weeks after the renal transplantation. It is a part of the routine to measure the glomerular filtration during this session. The outcome of this test will be used to calculate the renal clearance and, the result can give us an indication on how the kidneys are functioning.

The following data will be registered during the study:

- The patients will go through a DEXA examination at both visits, which will include measurements of the lumbar spine (LS), both of the proximal femoral necks (FN), the distal forearm (DF), and total body (TB).
- Blood samples will be collected at both visits at the Section of Nephrology as a part of the routine control in relations to the renal transplantation. A 6 ml testtube will be delivered to the Section of Endocrinology for analysis to the study.

- The renal clearance will only be calculated at the second examination. Since the kidneys have very little or no ability to filtrate fluid at the point of transplantation the clearance is approximately zero.

Analysing methods

Osteocalcin will be analysed from serum by an IRMA kit (Boule Nordic).
The assay measures intact human osteocalcin 1-49 quantitatively, with no cross-reactivity to the 1-43 fragments. It utilises human osteocalcin 1-49 for calibrators and controls and two polyclonal antibodies that have been purified using affinity chromatography. The purified antibodies are specific for two different regions of the osteocalcin molecule. The first antibody, specific for the amino terminus of human osteocalcin, is bound to a solid phase (polystyrene beads). The second antibody, specific for the carboxy terminus of human osteocalcin, is labelled with 125I. Samples are incubated on an orbital agitator at room temperature for two hours. Intact human osteocalcin 1-49 is the only form of osteocalcin that will be bound by both the antibody on the bead and the 125I labelled antibody. Following the incubation period, each bead is washed to remove unbound labelled antibody. The radioactivity present in the remaining bound labelled antibody is then measured using a gamma counter. The concentration of osteocalcin 1-49 present in the sample is directly proportional to the radioactivity measured. [19;20]
The kit states the analytical specificity to be between 4,5 - 6,3 % for the intra-assay precision, that is variation within the assay, and between 7,1 – 9,5 % for the inter-assay precision, which means the variation between assays.

Telopeptides will be analysed form serum by a Serum CrossLaps®ELISA analysis (Nordic Bioscience Diagnostics A/S).
The assay measures the degradation product of C-terminal telopeptides of Type-I collagen in human serum and plasma quantitatively. The test is based on two highly specific monoclonal antibodies against the amino acid sequence of EKAHD-β-GGR, where the aspartic acid residue (D) is β-isomerized. In order to obtain a specific signal in the ELISA, two chains of EKAHD-β-GGR must be cross-linked.
Standards, control, or unknown serum samples are pipetted into the appropriate microtitre wells coated with streptavidin, followed by application of mixture of biotinylated antibody and a peroxidase-conjugated antibody. Then a complex between the CrossLaps® antigens,

biotinylated antibody and peroxidase-conjugated antibody is generated, and this complex binds to the streptavidin surface via the biotinylated antibody. Following the one-step incubation at room temperature, the wells are emptied and washed. A chromogenic substrate is added and the colour reaction is stopped with sulphuric acid. Finally, the absorbance is measured [15;16].
The kit states the analytical specificity to be between 5,0 - 5,4 % for the intra-assay precision, that is variation within the assay, and between 5,4 – 8,1 % for the inter-assay precision, which means the variation between assays

Creatinine will be measured at the Section of Nephrology as described above, and the test results will be delivered to the Section of Endocrinology for calculation of the patients' renal clearance.

There will be performed statistical analyses using the statistical software SPSS and the analytical answers will be discussed.

ETHICAL CONSIDERATIONS

This study is necessary to gain increased knowledge about the development of osteoporosis as a secondary disease in relations to chronic kidney disease and renal transplantation. Participation in this study involves very little extra for the patients as it is a descriptive study. It means that there will be no interfering in the treatment by introducing new drugs or methods. The study will collect data from patients who receive the treatment available today. DEXA measurements have not been fully integrated as a part of the routine examinations in relation to a renal transplant at the Rikshospitalet University Hospital yet. But it is about to become a part of this routine, as it already has become in several hospitals worldwide. Also today, many of the CKD patients go through DEXA measurements during their treatment to observe the status of the bone mass at the Rikshospitalet University Hospital because of its benefits. A reason for the implementation at this point in time is that the methods and machines for DEXA measurements have improved. In that way the advantage of using the measurements has become greater as it now contributes with precise information about the patients BMD, from which the medical practitioners can evaluate the risk for osteoporosis. The dose of radiation during the measurement is very low, the procedure takes no more than 15 minutes, it is non-invasive and does not cause any pain or discomfort for the patient. So

even if the DEXA measurement is not fully a part of the standard routine at this point, we have no hesitations about carrying out this procedure during the study. Data acquired from the measurements that is of importance to an individual patient will be registered in the patient's journal and the necessary treatment will be given.
Blood sampling is a part of the standard routine at the Section of Nephrology in the initial examinations and the follow up of renal transplantation patients. Those who agree to participate in the study will be asked to give an extra testtube of blood while they give blood to other analyses. In this way there will be no extra piercing of the skin for the patient.
A test of the renal clearance is a part of the standard procedure in the follow up of a patient with a renal transplantation; therefore it will not be done especially for the study.

When evaluating the ethical aspect of the study we do not find anything ethically alarming, since most of the examinations are a part of the routine in the evaluation and follow up of patients with chronic kidney disease who are going through a renal transplantation.

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FORESPØRSEL OM DELTAGELSE I FORSKNINGSPROSJEKT

Osteoporose i forbindelse med nyretransplantasjon

Osteoporose, beinskjørhet i dagligtale, har en klar sammenheng med nedsatt nyrefunksjon og nyretransplantasjon. Studier har vist at hver femte pasient har sammenfall i ryggsøylen ved tidspunktet for transplantasjon. Risikoen for et lårhalsbrudd er 14 ganger høyere enn hos resten av befolkningen.
Etter en nyretransplantasjon må pasienten bruke legemidler som hemmer immunforsvaret for å hindre at nyren avvises av kroppen. Disse livsviktige legemidlene har dessverre en bivirkning som fører til ytterligere tap av beinmasse, og man regner med et nytt beintap på 8-10 % i løpet av det første året etter transplantasjonen. En konsekvens av dette er at opptil halvparten av pasientene vil kunne oppleve nye brudd.

Målet med denne studien er å undersøke betydningen av kjente og andre mulige risikofaktorer for osteoporose ved transplantasjonstidspunktet og å se på hvilke faktorer som påvirker et ytterligere beintap de første månedene etter transplantasjonen. Studien skal være med på å kartlegge hvor store endringer man kan se i beinmassen så tidlig som 3 måneder etter transplantasjon. Resultatene fra studien skal være en del av grunnlaget for en senere større studie rundt problematikken med osteoporose hos nyrepasienter.

Studien innebærer to undersøkelser, den første i løpet av perioden 5. til 10. dag etter transplantasjonen, og den andre i løpet av 10. til 12. uke etter transplantasjonen. Dette er på et tidspunkt hvor du vil befinne seg på sykehuset, innlagt etter operasjonen og senere til oppfølging. Deltagelse i studien fører dermed ikke med seg ekstra sykehusbesøk. Undersøkelsene som legges til grunn for studien er en del av den rutinemessige oppfølgingen ved en nyretransplantasjon.
Undersøkelsene som vil være en del av studien er beinmineralmålinger og blodprøver, og resultatene av disse vil sammenlignes med andre variabler.

- En beinmineralmåling innebærer at man ligger på og sitter ved siden av en benk mens en detektor beveger seg over den delen av kroppen som skal undersøkes. I studien skal man måle håndledd, rygg, hofter og total kropp, og en slik undersøkelse tar ca 15 minutter. Metoden benytter svært svake røntgenstråler som kun tilsvarer 1/10 av stråledosen ved en vanlig røntgenundersøkelse av brystkassen. Dette er en undersøkelse som ofte er en del av utredning og oppfølging i forbindelse med en nyretransplantasjon, og den innebærer verken stikk eller annet ubehag.
- I studien skal man også se på oppbygning og nedbrytning av beinsubstans, og til det trenger man en blodprøve. Deltagerne vil derfor avgi et ekstra tappeglass på 6 ml under blodprøvetagningen som er en del av rutinen ved nefrologisk avdelingen. Det blir altså ingen ekstra stikk.
- For hver deltager fylles det ut et skjema med informasjon om tidligere type behandling og annen informasjon fra pasientjournalen som er relevant for studien.

Deltagelse i prosjektet vil altså innebære svært lite ekstra for deg som pasient. Analysesvar av betydning vil bli lagret i pasientjournalen. Du vil også få den nødvendige behandling dersom man skulle finne ut at nettopp du har en behandlingstrengende beinskjørhet.

Undersøkelsene vil bli utført ved Endokrinologisk og Nefrologisk Seksjon, Medisinsk avd., Rikshospitalet.

Det er ikke problematisk om du deltar i andre studier samtidig som denne.

Deltagelse i studien er frivillig. Ditt forhold til de som behandler deg vil ikke påvirkes av om du gir ditt samtykke til dette prosjektet eller ikke.

Om du gir ditt samtykke kan dette senere trekkes tilbake uten å oppgi noen begrunnelse. Ved en tilbakekalling av samtykke kan du kreve at biologisk materiale destrueres samt at helse- og personopplysninger om deg utleveres eller slettes. Adgang til å tilbakekalle samtykket eller kreve destruksjon, sletting eller utlevering gjelder ikke dersom opplysningene allerede har inngått i vitenskapelige arbeider. Dette er fordi man i ettertid skal kunne kontrollere resultatene og kvaliteten av studier.

Prosjektmedarbeiderne har taushetsplikt. Alle innsamlede data om deg som pasient oppbevares avidentifisert ved at pasientene tildeles et prosjektnummer. Nummeret brukes til å merke prøver, analysesvar og spørreskjemaer slik at data fra samme pasient kan behandles samlet. Koden som sier hvem dataene gjelder oppbevares nedlåst og vil kun være tilgjengelig for prosjektmedarbeiderne. Materialet vil bli anonymisert ved prosjektavslutning slik at opplysningene som er innhentet ikke kan spores tilbake til pasientene. Dette vil senest skje i desember 2010.

Regional komité for medisinsk forskningsetikk og personvernombudet ved Rikshospitalet har gitt sine tilrådninger til studien.

Dersom det er noe du lurer på i forbindelse med prosjektet må du gjerne ta kontakt med oss.

Vennlig hilsen

Jens Bollerslev	Anders Hartmann	Stine Bønsnes
Overlege	Overlege	Stud.pharm.
Endokrinologisk seksjon	Nefrologisk seksjon	Farmasøytisk Inst., UiO

Ansvarlig for forskningsprosjektet og biobanken:
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Tlf: 23 07 19 02

SAMTYKKEERKLÆRING

For deltagelse i studien
Osteoporose i forbindelse med nyretransplantasjon

Med dette gir jeg mitt informerte samtykke i at materiale fra meg, inkludert beinmineralmålinger, blodprøver og relevante opplysninger fra journalen, blir brukt i forskning på osteoporose i forbindelse med nyretransplantasjon. Jeg er kjent med at data blir anonymisert slik at de ikke kan spores tilbake til min person.

Kryss av her ved samtykke: _____

_____

Sted/dato Underskrift

Flowchart clinical data	Baseline	Follow-up
General information		
Age	x	
Gender	x	
Ethnicity	x	
Height	x	x
Weight	x	x
Body mass index (BMI)	x	x
Anamnesis		
Postmenopause	x	x
Age of menarche	x	
Previous transplantation	x	
Diabetic nephropathy	x	
Rheumatoid arthritis	x	
Total parathyroidectomy	x	x
Pretransplant cardiovascular disease:		
Angina pectoris	x	
Heart attack	x	
Heart failure	x	
Stroke	x	
Pretransplant fractures (one/multiple)	x	
Time on hemodialysis	x	
Time on peritoneal dialysis	x	
Treatment with bisphosphonates during the last year	x	
Smoking habits:		
Daily	x	x
Sporadically	x	x
Never	x	x
Activity level:		
Inactive	x	x
Exercise for 1/2 hour once a week	x	x
Exercise for 1/2 hour 2-3 times pr week	x	x
Exercise for 1/2 hour more than 3 times pr week	x	x
Level of education:		
Primary school	x	x
High school	x	x
College/University	x	x
Current medication		
Loop diuretics	x	x
Tiazides	x	x
Statines	x	x
Aluminum containing phosphate binders	x	x
Calcium containing phosphate binders	x	x
Anabolic steroids	x	x
Glucocorticoids	x	x
Current treatment with bisphosphonates	x	x
Estrogens	x	x
Vitamin D	x	x
Calcium	x	x
Vitamin K	x	x

Vitamin K antagonist	x	x
Immunosuppressive treatment		
Current dose of prednisolone	x	x
Cumulative dose of prednisolone	x	x
Current dose of cyclosporin A (CsA)	x	x
Current does of tacrolimus	x	x
Current dose of sirolimus	x	x
Current dose of everolimus	x	x
Current dose of mycophenolate mofetil	x	x
Complications after transplantation		
Acute rejection episodes (yes/no)		x
Posttransplant fractures		x
Cytomegalovirus (CMV) infection		x
CMV disease		x
Cardiovascular disease:		
Angina pectoris		x
Heart attack		x
Heart failure		x
Stroke		x
Sudden death		x

Flowchart biochemical data	**Baseline**	**Follow-up**
Marker of bone formation	x	x
S-osteocalcin		
Marker of bone resorption		
S-CTX (telopeptide cross laps)	x	x
General biochemical assays		
S-PTH	x	x
S-total calcium	x	x
S-ionized calcium	x	x
S-phosphate	x	x
S-albumin	x	x
HbA1C	x	x
Evaluation of renal function		
GFR		x
S-urea	x	x
S-creatinine	x	x
S-tacrolimus	x	x
S-CsA C2	x	x
S-CsA C0	x	x
Cardiovascular risk factors		
C-reactive protein (CRP)	x	x
Lipid status fasting:		
Total cholesterol	x	x
Triglycerides	x	x
HDL cholesterol	x	x
LDL cholesterol	x	x
Lp(a)	x	x

UNIVERSITETET I OSLO

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Dato: 03.02.06
Deres ref.:
Vår ref.: S-06024

S-06024 **Osteoporose i forbindelse med nyretransplantasjon**

Komiteen behandlet søknaden i sitt møte onsdag 25.01.06.

Komiteen har følgende merknader til prosjektsøknaden.

1. Komiteen gjør oppmerksom på at den kun har vurdert pilotprosjektet. En evt. hovedstudie må senere forelegges komiteen.
2. Under pkt 18 er det er ikke krysset av for melding til Datatilsynet/personvernombud. Det er meldeplikt til Datatilsynet for forskningsprosjekter med personsensitive pasientopplysninger, også når disse er avidentifiserte. Eventuelt kan det meldes til personvernombudet, som ved Rikshospitalet - Radiumhospitalet HF er representert ved Aksel Sogstad. Det er ikke nødvendig å melde til Datatilsynet når melding gis personverneombudet. Mer opplysninger om dette finnes på Datatilsynets hjemmeside: www.datatilsynet.no
3. Eksklusjonskriteriene må spesifiseres nærmere.

Komiteen har ingen merknader til søknad om opprettelse av forskningsbiobank.

Komiteen har følgende merknader til pasientinformasjon:

1. Informasjonsskrivet bør ha heading med logo(er), evt. bør det på annen måte fremgå tidlig hvor informasjonsskrivet kommer fra.
2. Pasientinformasjonen må tilpasses biobankloven jfr. §11-14 fordi forskningsbiobank opprettes. Ansvarshavende for forskningsbiobanken må oppgis samt at den som ønsker å tilbakekalle samtykket, kan kreve det biologiske materialet destruert og innsamlede helse- og personopplysninger slettet eller utlevert. Adgangen til å tilbakekalle samtykket eller kreve destruksjon, sletting eller utlevering gjelder ikke dersom opplysningene allerede har inngått i vitenskapelige arbeider, jf biobankloven § 14 tredje ledd. (Dersom prøver skal sendes til utlandet, må pasientens tillatelse til dette innhentes, og sosial- og helsedirektoratet må søkes om tillatelse til utføring av prøver). Se også Mal for hva som bør inngå i et informasjonsskriv under Forskerportalen på http://www.etikkom.no/REK/forskerportal/infoskriv
3. Regional komité for medisinsk forskningsetikk, Sør-Norge godkjenner ikke men tilrår prosjekter.

Vedtak:
"Komiteen forutsetter at merknadene tas til etterretning, og tilrår at prosjektet gjennomføres og at forskningsbiobank opprettes. Komiteen videresender skjema for opprettelse av forskningsbiobank og informasjonsskriv samt komiteens vedtak til Sosial- og helsedirektoratet for endelig behandling av opprettelse av forskningsbiobanken. Revidert pasientinformasjon sendes komiteen til orientering"

UNIVERSITETET I OSLO
Det medisinske fakultet

Med vennlig hilsen

Kristian Hagestad
Fylkeslege cand.med., spes. i samf.med
Fungerende leder

Tone Haug
Rådgiver
Sekretær

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Dato: 09.03.06
Deres ref.:
Vår ref.: S-06024

S-06024 **Osteoporose i forbindelse med nyretransplantasjon**

Vi viser til e-post av 23.02.06 med vedlegg: revidert pasientinformasjon og samtykkeerklæring datert 20.02.06 final version.

Komiteen kan ikke se å ha fått svar på følgende merknader til prosjektsøknaden:

1. Komiteen gjør oppmerksom på at den kun har vurdert pilotprosjektet. En evt. hovedstudie må senere forelegges komiteen.
2. Eksklusjonskriteriene må spesifiseres nærmere.

Komiteen har følgende merknad til revidert pasientinformasjon og samtykkeerklæring:

1. Regional komité for medisinsk forskningsetikk, Sør-Norge godkjenner ikke men tilrår studier.

Komiteen ber om svar på merknader og revidert pasientinformasjon til orientering.

Med vennlig hilsen

Kristian Hagestad
Fylkeslege cand.med., spes. i samf.med
Fungerende leder

Tone Haug
Rådgiver
Sekretær

UNIVERSITETET I OSLO

DET MEDISINSKE FAKULTET

Seksjonsoverlege Professor dr. med Jens Bollerslev
Endokrinologisk seksjon
Medisinsk avdeling
Rikshospitalet
Rikshospitalet-Radiumhospitalet HF

Regional komité for medisinsk forskningsetikk
Sør- Norge (REK Sør)
Postboks 1130 Blindern
NO-0318 Oslo

Telefon: 228 44 666
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Nettadresse: www.etikkom.no

Dato: 07.04.06
Deres ref.:
Vår ref.: S-06024

S-06024 **Osteoporose i forbindelse med nyretransplantasjon**

Vi viser til e-post av 28.03.06 med vedlegg: revidert pasientinformasjon og samtykkeerklæring datert 21.03.06 final version.

Komiteen tar svar på merknader til etterretning.

Komiteen har ingen merknader til revidert pasientinformasjon og samtykkeerklæring.

Komiteen tilrår at prosjektet gjennomføres.

Vi ønsker lykke til med prosjektet!

Med vennlig hilsen

Kristian Hagestad
Fylkeslege cand.med., spes. i samf.med
Fungerende leder

Jørgen Hardang
Rådgiver
Sekretær

Sosial- og helsedirektoratet

Seksjonsoverlege Professor dr. med Jens Bollerslev
Endokrinologisk seksjon
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Rikshospitalet
Rikshospitalet-Radiumhospitalet HF
0027 OSLO

Deres ref:
Saksbehandler: jte
Vår ref: 06/930
Arkivkode:
Dato: 03.03.2006

Melding om opprettelse av forskningsbiobank: Osteoporose i forbindelse med nyretransplantasjon

Vi viser til brev vedrørende ovennevnte. Sosial- og helsedirektoratet er delegert å vurdere meldinger om opprettelse av forskningsbiobanker i henhold til biobankloven § 4.

Direktoratet har ingen innsigelser til at forskningsbiobanken opprettes i henhold til biobankloven.

Direktoratet forutsetter at opprettelsen av den planlagte forskningsbiobanken oppfyller nødvendige krav til godkjenning, konsesjon m.v. i henhold til annet relevant regelverk, herunder bioteknologiloven, helseregisterloven og legemiddelloven.

Etter direktoratets vurdering bør det gå frem av pasientinformasjonen at de som har avgitt samtykke, til enhver tid kan tilbakekalle slikt samtykke og kreve det biologiske materialet destruert og innsamlede helse- og personopplysninger slettet eller utlevert. Adgangen til å tilbakekalle samtykket eller kreve destruksjon, sletting eller utlevering gjelder ikke dersom opplysningene allerede har inngått i vitenskapelige arbeider, jf. biobankloven § 14. Direktoratet forutsetter at pasientinformasjonen/samtykket blir endret i tråd med dette.

Meldingen om forskningsbiobanken vil bli sendt til Nasjonalt folkehelseinstitutt som har fått ansvaret for å føre et offentlig tilgjengelig register over landets biobanker, jf. biobankloven § 6.

Med vennlig hilsen

Ragnhild Castberg e.f.
seniorrådgiver

Jill Terserus
rådgiver

Kopi:
REK Sør S-06024
Biobankregisteret

Sosial- og helsedirektoratet
Avdeling for spesialisthelsetjenester

Postadr: Pb 7000 St Olavs plass, 0130 Oslo • Besøksadr: Universitetsgaten 2, Oslo
Tel: 24 16 30 00 • Faks: 24 16 30 08 • Org.nr.: 983 544 622 • postmottak@shdir.no • www.shdir.no/ts

Notat

Til:	Stine Bønsnes	Interne tjenester
Kopi:		Postadresse: 0027 OSLO Besøksadresse: Sognsvannsvn. 20
Fra:	Aksel Sogstad	
Saksbehandler:		Sentralbord: 23 07 00 00 Dir. linje: 23 07 50 34 Telefaks: 23 07 50 30
Dato:	07.03.2006	
Offentlighet:	Ikke unntatt offentlighet	Rikshospitalet
Sak:	Tilrådning av forskningsstudie unntatt konsesjon	

Tilrådning til innsamling og databehandling av personopplysninger i forskningsstudiet
"Osteoporose i forbindelse med nyretransplantasjon".

Personvernombudet har vurdert det til at den planlagte databehandlingen av personopplysninger tilfredsstiller forutsetningene for melding gitt i personopplysningsforskriften § 7-27 og derfor er unntatt konsesjon. Personvernombudet har myndighet til å foreta denne avgjørelsen på vegne av Datatilsynet.

Det tilrås at prosjektet igangsettes med følgende betingelser:

- Data lagres avidentifisert på RR HFs forskningsserver, og kodeliste oppbevares avskilt fra prøvematerialet.
- Data anonymiseres (kodelisten slettes) før publisering av resultater eller senest ved prosjektavslutning 31.12.2010.
- Registeret må vurderes og tilrås av den regionale etiske komité for medisinsk forskning (REK), og eventuelle merknader må følges.

Kontaktperson for prosjektet skal hvert tredje år sende personvernombudet bekreftelse på at behandlingen skjer i overensstemmelse med meldingen og helseregisterlovens regler. Hvis formålet eller databehandlingen endres må personvernombudet informeres om dette.

Vennlig hilsen

Aksel Sogstad
Personvernombud RR HF