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Author: Krol, Charlotte Georgette

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Pitfalls in the diagnosis and management of skeletal complications of liver transplantation

Charlotte Krol

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Pitfalls in the diagnosis and management of skeletal complications of liver transplantation

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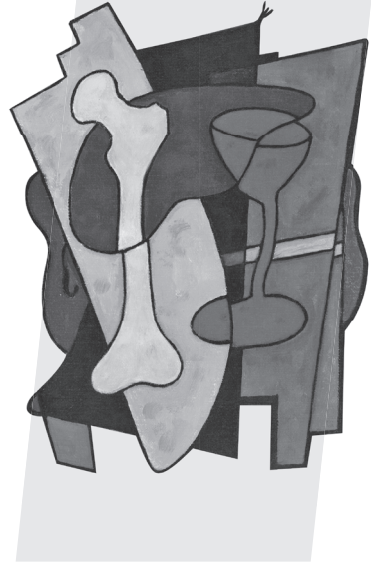
time is short and work is long
[...] you are forced to omit so many needful studies,
and pass by so many needy souls, for want of time

yet if you look not well about you,
and will not bear some censure and offence
you shall lose even the rest of the time
which now you do improve

Richard Baxter (1615-1691)

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1

General Introduction

Skeletal complications before and after liver transplantation

INTRODUCTION

Skeletal pathology is common in patients with end-stage liver disease before and after orthotopic liver transplantation (OLT), and this is associated with significant morbidity and decreased quality of life. Significant bone loss occurs and the incidence of new fractures is high within the first year after OLT, despite recovery of liver function. (1-4) However, the pathophysiology of increased bone fragility in end-stage liver disease and after liver transplantation remains incompletely understood. A number of risk factors, such as vitamin D deficiency, hypogonadism, malnutrition, alcohol abuse and glucocorticoid use where applicable contribute to bone loss and increased fracture risk, but the persistence of increased fracture risk after correction of metabolic abnormalities after liver transplantation suggests that other factors, as yet to be identified, are likely to be involved in the pathophysiology of skeletal complications after OLT.

The aim of this Chapter is to provide an overview on the role of the liver in bone metabolism and on the pathophysiology of bone loss and increased fracture risk observed in liver diseases before and after liver transplantation.

NORMAL BONE REMODELING

The skeleton is constantly renewed by the process of bone remodeling which plays a major role in the preservation of calcium and phosphate homeostasis, and in the maintenance of the structural integrity of bone by preserving bone mass. Bone remodeling takes place in the so called bone multicellular units (BMU's) through the coordinated activity of the 3 types of bone cells: osteoblasts, osteoclasts and osteocytes. The remodeling cycle starts with the resorption of bone by osteoclasts, followed by the formation of new bone by osteoblasts, which fills the resorption cavities created by the osteoclasts. Mineralization of the newly formed osteoid completes the bone remodeling cycle. (Figure 1)

Bone remodeling is regulated by several factors including hormones, signaling pathways and mechanical factors. Two signaling pathways are crucial for the differentiation, maturation, activation and survival of bone cells, the RANK/RANKL/OPG pathway for osteoclasts and the Wnt signaling pathway for osteoblasts. Differentiation of osteoclasts from hematopoietic stem cells is mediated through the RANK/RANKL/OPG pathway (5;6) in the presence of the macrophage-colony stimulating factor (M-CSF), which induces RANK expression in osteoclast progenitor cells. Binding of the ligand of RANK (RANKL), produced by the osteoblast, to its receptor RANK on the surface of pre-osteoblasts induces a cascade of signaling pathways, which affects all steps of osteoclastogenesis. All these steps are in turn inhibited by osteoprotegerin (OPG), a natural decoy receptor also produced by the osteoblast, which binds to RANKL, and

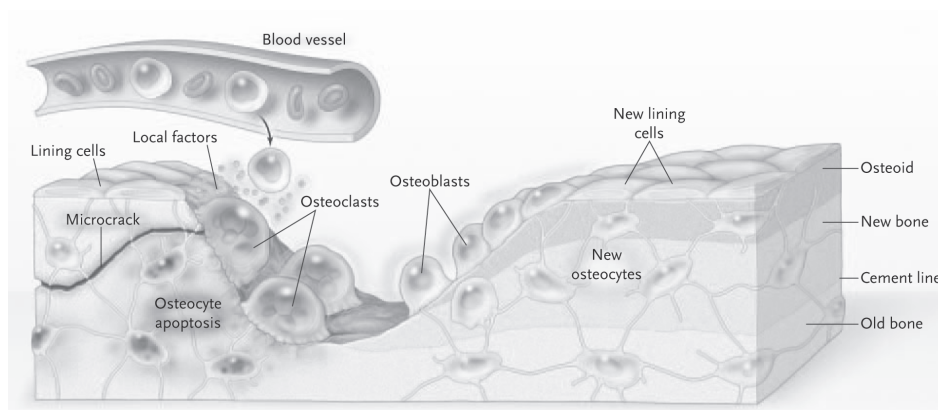


Figure 1: The bone remodeling cycle, which starts with the resorption of bone by osteoclasts, followed by the formation of new bone by osteoblasts, which fills the resorption cavities created by the osteoclasts. Mineralization of the newly formed osteoid completes the bone remodeling cycle. Osteoclasts that are trapped in the matrix become osteocytes; others die or form new, flattened osteoblast lining cells. (Reproduced with permission from Seeman E, Delmas PD N Engl J Med 2006; 354(21):2250-2261, Copyright Massachusetts Medical Society).

preventing its binding to RANK, and thus inhibiting all steps of osteoclastogenesis when this is no longer required.(Figure 2)

The Wnt signaling pathway is a critical regulator of bone formation (6;7), which when activated, results in binding between Wnt proteins (Figure 3), Frizzled and low density lipoprotein receptor-related protein 5 (LPR-5) or LPR-6 receptors. This results in inactivation of GSK-3 β , inhibition of beta-catenin degradation and accumulation of beta-catenin in the nucleus.(8) Beta-catenin binds to the TCF/LEF family of transcription factors and induces target gene expression, which is essential for the differentiation of mesenchymal stem cells to osteoblasts through effects on proliferation and maturation of osteoblast progenitor cells. The transcriptional factor RUNX2 is a target of beta-catenin for the stimulation of bone formation through the initiation of differentiation of multipotent mesenchymal stem cells into osteoblasts.(9) Osterix, another transcription factor, acts downstream of RUNX2 and is responsible for the transition from osteoprogenitor cells to pre-osteoblasts. Later in the process of osteoblast differentiation, beta-catenin influences bone resorption by decreasing osteoclastogenesis through stimulation of the expression of OPG.(10) The Wnt signalling pathway is antagonized by sclerostin, a protein produced by osteocytes, which is a product of the *Sost*-gene and binds to LPR-5 and LPR-6, thereby preventing the formation of the complex between Wnt proteins and Frizzled.(11) In addition to sclerostin, members of the Dickkopf (DKK) family such as DKK-1 also inhibit the Wnt signalling pathway by binding to the LPR-5 and LPR-6 receptors.(12) Secretion of sclerostin by the osteocyte is suppressed by mechanical stress, leading to increased bone formation.(6)

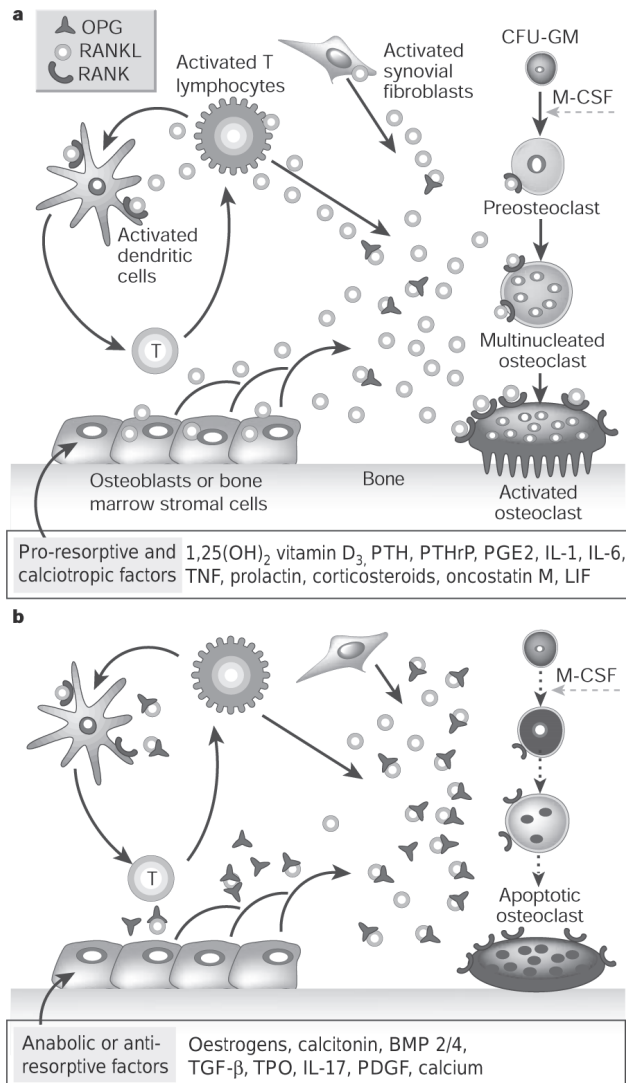


Figure 2: The RANK/RANKL/OPG pathway. Binding of the ligand of RANK (RANKL), produced by the osteoblast, to its receptor RANK on the surface of pre-osteoblasts induces a cascade of signaling pathways, which affects all steps of osteoclastogenesis (upper panel, a). All these steps are in turn inhibited by OPG, a natural decoy receptor also produced by the osteoblast, which binds to RANKL, and preventing its binding to RANK, and thus inhibiting all steps of osteoclastogenesis when this is no longer required (lower panel, b). OPG = osteoprogenin; RANK = receptor activator of nuclear factor kappa b; RANKL = receptor activator of nuclear factor kappa B- ligand; PTH = parathyroid hormone; PTHrP = parathyroid hormone related peptide; PGE-2 = prostaglandin E2; IL = interleukin; TNF = tumor necrosis factor; BMP = bone morphogenic protein; TGF-beta = transforming growth factor beta; TPO= thrombopoietin; PDGF = platelet derived growth factor (Adapted from Boyle WJ, Simonet WS, Lacey DL. Nature 2003; 423(6937):337-342, reproduced with permission)

The tightly regulated process of bone remodeling is thus controlled by a number of regulatory signals, which have been largely although as yet somewhat incompletely unraveled.(6) Metabolic bone activity can be evaluated in the clinic by measuring circulating products of bone remodeling activity. Biochemical markers of bone

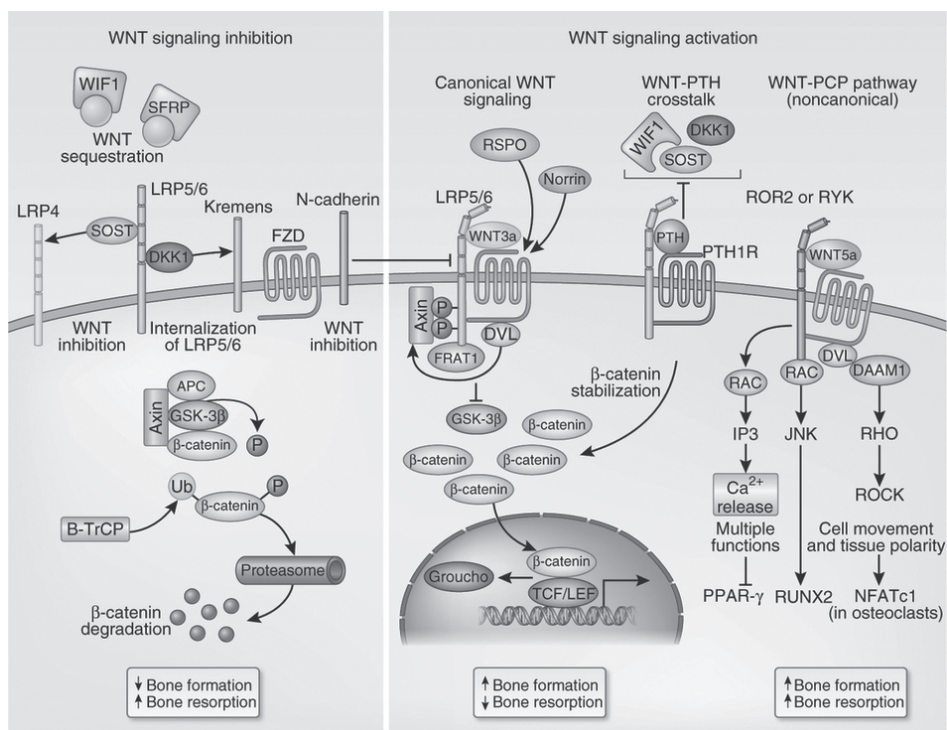


Figure 3: The Wnt signaling pathway. Activation of Wnt signaling results in binding between Wnt proteins, FZD and LPR5 or LPR6 receptors (right panel). This results in inactivation of GSK-3 β , inhibition of beta-catenin degradation and accumulation of beta-catenin in the nucleus. Beta-catenin binds to the TCF/LEF family of transcription factors and induces target gene expression, which is essential for the differentiation of mesenchymal stem cells to osteoblasts through effects on proliferation and maturation of osteoblast progenitor cells. The Wnt signaling pathway is antagonized by sclerostin, a protein produced by osteocytes, which is a product of the *Sost*-gene and binds to LPR-5 and LPR-6, thereby preventing the formation of the complex between Wnt proteins and FZD (left panel). In addition to sclerostin, members of the DKK family also inhibit the Wnt signalling pathway by binding to the LPR-5 and LPR-6 receptors. SFRP= secreted frizzled-related proteins; LRP= low density lipoprotein receptor-related protein; FZD=Frizzled; APC= adenomatous polyposis; P= phosphorylation; Ub = polyubiquitination; DVL = dishevelled; TCF/LEF = T cell factor/lymphoid enhancer factor; PTH= parathyroid hormone; DKK1 =Dickkopf-1; WIF1= Wnt inhibitory factor 1; DAAM1= dishevelled associated activator of morphogenesis 1; IP3 = inositol 1,4,5-trisphosphate, type 3; JNK = Jun kinase; ROCK = RHO-associated kinase; PPAR- γ = peroxisome proliferator activated receptor- γ . NFATc1 = nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 1; ROR = receptor-tyrosine-kinase-like orphan receptor; PLC = phospholipase C; DAG = dystroglycan; RUNX2 = Runt-related transcription factor 2. (Adapted from Baron R, Kneissel M. Nat Med 2013; 19(2):179-192, reproduced with permission)

remodeling activity, the bone turnover markers, include circulating crosslink fragments of degraded collagen: serum beta-crosslaps (CTX), as well as the urinary excreted crosslink fragments: hydroxyproline, pyridinoline, deoxypyridinoline and type 1 collagen cross-links (NTX and CTX). The products of various stages of bone formation, procollagen type 1 N-terminal propeptide (P1NP), osteocalcin and bone alkaline phosphatase (BALP), each representing a different stage of maturation of the osteoblast, can also be measured in serum. The gold standard of evaluation of metabolic bone disease remains the double tetracycline labeled bone biopsy, which provides information on bone cell activity as well as mineralization.

ROLE OF THE LIVER IN NORMAL BONE REMODELING

The two main physiological functions of the liver are metabolic and excretory, and various aspects of both functions play an important role in normal bone remodeling and thus in the maintenance of skeletal health.

Metabolic functions of the liver of relevance for skeletal health

Vitamin D metabolism

The liver plays an important role in skeletal and calcium homeostasis through its role in vitamin D metabolism. Vitamin D, a fat soluble vitamin, is synthesized in the skin by the effect of UV radiation from sun light on 7-dehydrocholesterol. Intestinal absorption of dietary vitamin D is a much less important source of vitamin D as this is mainly found in fatty fish and essentially scarce in a normal diet. Dietary vitamin D is absorbed by enterocytes, incorporated into micelles, packaged into chylomicrons and transported to the liver via the portal circulation. Vitamin D is metabolically converted in the liver to the pro-vitamin D hormone, 25-hydroxy vitamin (25(OH)D), by cytochrome p450 enzymes, which exhibit a high capacity for the substrate vitamin D₃. 25(OH)D is converted to the biologically active ligand for the widely distributed vitamin D receptor (VDR) through activity of the renal one-alpha hydroxylase (CYP27B1) enzyme. The proximal renal tubular epithelial cell is the largest source of the 1-alpha hydroxylase enzyme but the enzyme has also been found to be present in a large number of cells resulting in the extra-renal intracellular production of biologically active 1,25(OH)₂D (calcitriol), which is primarily governed by the availability of extracellular 25(OH)D as substrate. The VDR is expressed in virtually all nucleated cells and influences a wide variety of processes such as proliferation, differentiation, apoptosis, mineral homeostasis, immune response and metabolism.(6;13) The vitamin D-binding protein (DBP), a member of the albumin family of proteins, is mainly produced by the liver and

is essential for binding of vitamin D and its metabolites in serum and for delivery of its biologically active form $1,25(\text{OH})_2\text{D}$ to target tissues expressing the VDR. The most important biological action of vitamin D in the control of calcium homeostasis is the promotion of enterocyte differentiation, the intestinal absorption of calcium and the suppression of parathyroid hormone (PTH) synthesis and release by the parathyroid gland. In bone, the VDR regulates osteoblast and osteoclast function and is also important for bone mineralization.(6;14;15) VDR controls bone remodeling by regulating the production of RANKL and OPG by osteoblasts and osteocytes, thus playing a major role in the coupling of bone formation and resorption and in the maintenance of bone mass and integrity. Bone resorption is decreased by VDR activity by suppression of the RUNX2 gene transcription in the Wnt signaling pathway . Activation of the VDR also leads to production of osteopontin (OPN) by the osteoblasts, a peptide believed to be involved in bone mineralization and remodeling by anchoring osteoblasts to the mineral matrix.(6;15) VDR also affects the osteocalcin gene, the function of which in bone is incompletely understood, (6;15) although it has been suggested this osteoblast-specific protein may contribute to the density and structural integrity of bone.

A feedback loop is formed by the formation of fibroblast growth factor (FGF-23) by VDR signaling in the osteocyte.(6;15) FGF-23 inhibits the renal 1-alpha-hydroxylase enzyme and thereby the conversion of calcidiol to the biologically active calcitriol ($1,25(\text{OH})_2\text{D}$), simultaneously inducing the degradation of $1,25(\text{OH})_2\text{D}$ to the biologically inactive metabolite $24,25(\text{OH})_2\text{D}$ by increasing the expression of the 24-alpha-hydroxylase enzyme.

Metabolism of gonadal steroids

Gonadal steroids play an important regulatory role on bone and mineral metabolism. Sex steroid deficiency results in high bone turnover in favor of increased bone resorption, leading to loss of bone mass and bone strength and to an increase in fracture risk. The liver is the main site for peripheral inactivation and clearance of estrogens. Furthermore, sex-hormone binding globulin (SHBG) and albumin, the most important serum binding proteins for testosterone and estradiol, are synthesized in the liver.

Insulin-like growth factor 1

Bone remodeling is influenced by insulin-like growth factor-1 (IGF-1), a growth factor synthesized in the liver under the influence of growth hormone (GH).(16-18) To a lesser extent, IGF-1 is also produced in bone under the influence of GH as well as of local factors, with bone-derived IGF-1 also playing an important role in bone remodeling.(17) IGF-1 circulates mostly as a complex of IGF-1 bound to IGF-binding-protein-3 (IGFBP-3), one of the IGF-binding-proteins (IGFBP's).(19) IGFBP-concentrations and IGF-1 expression are regulated by various hormones, including GH, testosterone, estrogen and thyroid

hormones.(18) IGF-1 stimulates osteoblast function and bone formation by mitogenic activity and by stabilization of beta-catenin, thereby enhancing Wnt-dependent activity. (20-22) On the other hand, IGF-1 also induces RANK-L synthesis thereby promoting osteoclastogenesis and osteoclast function.(20;23) Furthermore, IGF-1 maintains the bone matrix by influencing collagen transcription and collagenase production.(20) IGF-1 is also believed to be necessary for the action of PTH on bone; PTH increases IGF-1 expression in osteoblasts and IGF-1 in turn mediates the anabolic action of PTH on the skeleton.(18;20;24;25) Systemic IGF-1 appears to contribute more to cortical bone integrity, whereas locally bone-derived IGF-1 is thought to play a more important role in the maintenance of trabecular bone integrity (20)

Excretory function of the liver

One of the main excretory functions of the liver is the synthesis and excretion of bile salts, which contribute significantly to normal calcium and bone homeostasis as they are essential for the absorption of the lipid soluble vitamin D.(26) Bile acid synthesis starts with the synthesis of the two primary bile acids, cholic acid and chenodeoxycholic acid, by the process of oxidation of cholesterol in liver cells. These bile acids are secreted into the lumen of the intestine, where intestinal bacteria partly dehydroxylate them to the secondary bile acids, deoxycholic acid and lithocholic acid. The secondary bile acids are in turn reabsorbed in the ileum and transported to the liver via the portal circulation, and are re-secreted, creating an enterohepatic circulation. Bile acids are secreted after conjugation with glycine or taurine, forming a total of eight possible conjugated bile acids. These are referred to as bile salts. Bile salts are more water-soluble than bile acids. They are able to emulsify lipids and fat-soluble vitamins (such as vitamin D) in the intestine, to form micelles that can be transported to the liver.(26)

Vitamin K metabolism and bone

Bile salts are necessary for the absorption of vitamin K, a fat-soluble vitamin necessary for the production of osteocalcin in bone. Vitamin K1 is mainly found in leafy vegetables and absorbed in the small intestine, and vitamin K2 is synthesized by bacterial flora and found in animal liver and fermented foods.(27) Absorption of vitamin K is dependent on pancreatic function and on the presence of bile salts and it is excreted as its catabolic product in bile and urine. A proportion of vitamin K is stored in the liver.(27)

Osteocalcin is the most abundant non-collagenous protein in bone, which is produced by the osteoblasts and incorporated into the bone matrix, where it is involved in bone calcification. Vitamin K is essential for the post-translational carboxylation of glutamic acid, which is necessary for binding of calcium to the proteins found in bone such as osteocalcin. Vitamin K has also been shown to inhibit osteoclast formation by affecting

RANK-L production, and to stimulate bone formation by enhancing osteoblastogenesis and promoting osteoblast to osteocyte formation.(27;28)

FACTORS AFFECTING BONE REMODELING IN LIVER DISEASE

In liver disease, the metabolic and excretory functions of the liver are compromised to a lesser or greater extent by the underlying pathology, leading to disturbances in calcium and bone metabolism. Viral pathogens, toxins such as alcohol or accumulated bile salts, and medication potentially used to treat the liver disease such as corticosteroids in auto-immune hepatitis may also exert direct deleterious effects on bone independently of the disturbed hepatobiliary function.

Vitamin D deficiency

Vitamin D deficiency is common in chronic liver disease, with a prevalence ranging from 64-92%, which increases with the severity the disease.(29;30) In liver disease, the pathogenesis of vitamin D deficiency is multifactorial, including poor nutritional status, decreased sun light exposure due to chronic illness and decreased vitamin D absorption in cholestatic liver diseases. In the late stages of liver disease, the production of the 25 hydroxylase enzyme is also impaired and increased breakdown of 25(OH)D to biologically inactive metabolites decreases substrate availability for the renal production of the biologically active 1,25(OH)₂D. This leads to decreased intestinal absorption of calcium, hypocalcemia, and secondary hyperparathyroidism.(31) Decreased VDR signaling results in further stimulation of PTH synthesis and increased bone turnover in favour of bone resorption. Bone mineralization is also significantly compromised by vitamin D deficiency, resulting first in a decrease in bone mass and eventually in case of longstanding severe vitamin D deficiency to osteomalacia.

In patients with chronic liver disease, vitamin D deficiency is mainly due to decreased sunlight exposure and malnutrition.(32) 25-hydroxylation of vitamin D remaining generally intact until the late stages of liver failure when more than 90% of liver function is lost.(30;33) Cholestasis such as occurs in primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and overlap syndromes with auto-immune hepatitis (AIH), negatively influences vitamin D metabolism.(32;34;35) In these diseases, in which transportation of bile salts to the intestine is compromised, the resulting decrease in bile salts leads to defective absorption of fat-soluble vitamins including dietary vitamin D.(32;34;35) In patients with PSC the often concomitant inflammatory bowel disease, Crohn's disease, may in its own right contribute to decreased intestinal re-absorption of bile salts and of vitamin D through mucosal inflammation or surgical resection of significant length of the small intestine to control disease activity.(35) In addition to

the effect of cholestasis on the intestinal absorption of vitamin D, cholestatic toxins may also directly influence bone remodeling through a direct toxic effect on various steps of the RANK/RANKL signaling pathway and on mediators of osteoblast function, thereby inhibiting bone formation and increasing bone resorption.(30). A large fraction of vitamin D is bound to DBP and albumin so that reduced production of these proteins in the liver also leads to a decrease in circulating vitamin D and in its availability for metabolic functions, particularly in the late stages of liver failure.(30)

Hypogonadism

Gonadal hormone deficiency is common in liver disease. Estrogen and testosterone attenuate the maturation of osteoclasts and inhibit bone resorption by the stimulation of osteoclast apoptosis while preventing osteoblast and osteocyte apoptosis thus maintaining a healthy balance between bone resorption and formation leading to preservation of bone mass and integrity. A decrease or loss of sex steroids therefore leads to increased osteoclast activity and accelerated bone resorption.

However, the aetiology of hypogonadism in liver failure is not completely understood. It may develop as a result of primary gonadal failure as well as a result of failure of the pituitary-hypothalamic gonadal axis. Alcohol abuse is associated with central as well as primary gonadal hypogonadism, independently of cirrhosis or nutritional status. Alcohol also accelerates the metabolic clearance of testosterone, leading to decreased circulating testosterone levels.(36) Some studies also suggest that alcohol increases hepatic aromatization of androgens to estrogen in men, although this would not be deleterious to the skeleton.(37) In hereditary and secondary hemochromatosis, pituitary iron deposition leads to destruction of gonadotroph cells with consequent secondary hypogonadism.(38-40) In patients with end-stage liver disease, testosterone levels decline while SHBG increases, leading to decreased free testosterone levels.(4)

Renal impairment

Renal disease is common in patients with liver disease with a reported prevalence of 20-25% of patients.(41) The later stages of liver failure may be associated with renal failure: the hepatorenal syndrome. Renal IgA deposition is often seen in liver disease and may result in IgA nephropathy, especially in the case of alcoholic liver disease. Viral hepatitis is associated with membranous and membranoproliferative glomerulonephritis and focal segmental glomerulosclerosis. In patients with diabetes and steatohepatitis, co-existing diabetic nephropathy may also lead to renal impairment.

Renal impairment has complex deleterious effects on bone, on one hand through phosphate retention and consequent progressive stimulation of FGF-23 production, and on the other hand through chronic stimulation of the parathyroids resulting in hyperplasia and proliferation of these cells.(42;43) Secondary hyperparathyroidism

results from phosphate retention and from decreased calcium- and calcitriol concentrations.(43) Calcitriol concentrations are reduced due to suppressed renal 1-alpha hydroxylase activity, which result from phosphate retention and increased FGF-23 concentrations.(43;44) In severely impaired renal function, loss of functional renal mass leads to decreased 1-alpha hydroxylase production, also contributing to the decline in calcitriol concentration.(44) Nutritional vitamin D deficiency and loss of 25(OH)D are also very common in renal failure, also contributing to secondary hyperparathyroidism. High or low bone turnover may both be associated with osteoporosis and increased fracture risk in patients with renal failure.(45-48)

IGF-1

Liver diseases are associated with low IGF-1 levels, and these have been shown to be associated with low bone mass and increased fracture risk in growth hormone deficiency states.(49) The contribution of low IGF-1 levels to decreased bone mass in patients with liver disease has not however been adequately addressed so far.(50)

A decrease in IGF-1 levels is observed in the early stages of liver disease, such as fibrosis and steatosis, and in alcoholics (51-53) and IGF-1 levels progressively decline with further deterioration in liver function.(52-57) In liver diseases, the aetiology of decreased IGF-1 levels is multifactorial. A decrease in hepatocyte function leads to a decrease in hepatic IGF-1 and IGFBP-3 secretion. (50-56;58-63) Interestingly, GH levels have been shown to be increased in these patients (50-56;58-63), suggesting hepatic GH-resistance, which may be related to malnutrition.(59). Low IGF-1 levels are also common in inflammatory bowel disease, such as Crohn's disease which is associated with PSC. Moreover, IGF-1 production may be inhibited by inflammatory cytokines such as IL-6, which are released during infections such as spontaneous bacterial peritonitis.(64) Although hormone deficiencies such as testosterone, insulin and thyroxine deficiencies which are prevalent in liver diseases may lead to an increase in IGF-1 and therefore to an increase in unbound IGF-1, a decrease of IGFBP-3 is actually observed in most patients with compromised liver function.

Vitamin K

In liver disease, vitamin K deficiency may occur as a result of malnutrition and of decreased intestinal absorption of vitamin K in cholestatic disease due to a decrease in bile salt excretion in the intestine. Small-intestine pathology such as Crohn's disease may also lead to decreased vitamin K absorption. In case of vitamin K insufficiency, osteocalcin fails to become carboxylated in bone, and it is released from the osteoblasts into the circulation.(65) Low circulation levels of vitamin K have been associated with low bone mineral density (BMD) and osteoporotic fractures.(27;65;66)

Other factors associated with skeletal complications in liver disease

Pro-inflammatory cytokines, such as IL-1 and TNF-alpha, are increased in patients with liver cirrhosis and stimulate osteoclast activity and thereby bone resorption. Hyperhomocysteinemia may occur in patients with liver disease as a result of stimulation of the hepatic production of homocysteine by a number of factors such as nutritional vitamin deficiencies and cigarette smoking or because of accumulation of the protein due to reduced excretion in case of renal impairment. Hyperhomocysteinemia has been associated with increased risk for fractures independently of BMD, possibly by altering bone quality through abnormal collagen crosslinking.

Effect of underlying liver disease on the skeleton

Alcoholic liver disease

Alcohol abuse is associated with decreased bone mass and increased fracture risk. (67-70) Ethanol exerts direct toxic effects on osteoblasts, resulting in decreased bone formation.(71) In addition, ethanol also stimulates osteoclast activity through stimulating the RANK/RANK-L system.(72;73) Ethanol also alters the metabolism of several hormones, including vitamin D, IGF-1 and cortisol. In alcoholics, malnutrition may also contribute to bone loss when alcohol represents the main caloric intake.(74) Heavy alcohol intake is also associated with increased risk of falls and thereby increased risk of fracture.(75)

Cholestatic liver disease

Cholestasis, as seen in cholestatic liver diseases such as primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and overlap syndromes with auto-immune hepatitis (AIH), negatively influences vitamin D metabolism.(32;34;35) A decrease in bile salts availability is associated with defective absorption of dietary vitamin D when transportation of bile salts to the intestine is compromised.(32;34;35) In patients with PSC a concomitant inflammatory bowel disease, such as Crohn's disease, may in its own right contribute to decreased intestinal re-absorption of bile salts and vitamin D through mucosal inflammation or surgical resection of significant length of the small intestine to control disease activity.(35) In addition to the effect of cholestasis on absorption of vitamin D, cholestatic toxins may also directly influence bone remodeling through an effect on the RANK/RANKL pathway and on mediators of osteoblast function, thus contributing to decreased bone formation and increased bone resorption and thereby to bone loss.(30;76;77)

Viral hepatitis

Chronic hepatitis B and C infections (HBV and HCV) have been reported to be associated with bone loss, regardless of their effect on liver function (78-81). High bone turnover and increased bone resorption have been observed in viral liver diseases possibly related to the inflammatory process associated with increased levels of cytokines, leading to stimulation of the RANK/RANKL pathway and of all aspects of osteoclastogenesis. Higher levels of soluble tumor necrosis factor (TNF) receptor-55 have also been shown to be associated with increased bone resorption and lower BMD in patients with HCV.(81) Fracture rate has also been shown to decline after virus clearance.(82) Antiviral therapy used to treat viral hepatitis may also affect bone turnover. Ribavirin has been shown to impair osteoblast proliferation, with one study showing lower BMD in patients treated with this agent (83), although this deleterious effect of this antiviral agent on bone is controversial, as other studies have showed stable or increasing BMD during ribavirin therapy.(84-86)

Iron storage disease

In haemochromatosis, an autosomal recessive disease with a *HFE*-gene mutation leading to increased iron absorption due to inappropriate hepcidin secretion (87), iron excess is associated with low bone mass and increased risk of fractures.(38;40;88;89) The main mechanism of action of iron overload is to impair osteoblast activity.(38-40) Iron overload may also contribute to the development of central hypogonadism due to pituitary iron deposition, with associated increased bone resorption and decreased bone formation.(38-40)

SKELETAL COMPLICATIONS AFTER LIVER TRANSPLANTATION

Bone loss and fractures are common after solid organ transplantation, including liver transplantation, and these skeletal complications are associated with significant morbidity and mortality. The pathogenesis of these skeletal complications remains incompletely understood. Corticosteroids and other drugs used for immunosuppression play an important role. Main additional contributing factors include the underlying primary liver pathology and skeletal status at the time of transplantation. Potential other contributing factors include persistent vitamin D deficiency and hypogonadism or high bone turnover.

Significant bone loss at the lumbar spine (LS) as well as the femoral neck (FN) occurs during the first six months after liver transplantation with a more variable course thereafter. (90-98) Fracture incidence is very high despite recovery of liver function, with new fractures occurring in 15-34% of patients within the first year after OLT, mainly

vertebral and rib fractures.(1-4) LS BMD has been shown to spontaneously recover thereafter, reaching pre-transplant values within two years after OLT.(4;99-103) FN BMD has been reported to remain low in most studies, with BMD stabilizing or even decreasing further beyond the first year after OLT.(4;102;104;105)

Underlying liver disease

Significant differences in degree of bone loss and in fracture incidence have been reported after OLT according to type of liver disease (2;102;103;105;106;106-108), with patients with cholestatic liver disease being at higher risk for fractures than patients with other liver diseases.

Underlying skeletal pathology

Low bone mass and fractures are already highly prevalent before liver transplantation. Osteoporosis is thus reported in 15-40% of patients with end-stage liver disease (94;95;107;109;110) and fractures are reported to be prevalent in 6-30% of patients(107;111-113). Whereas pre-existing low bone mass is a risk factor for fractures after transplantation (1;2;4;114-116), fractures have also been shown to occur in patients with osteopenia or normal bone mineral density (BMD).

Corticosteroids

Corticosteroids are used postoperatively in almost all immunosuppressive protocols after solid organ transplantation. Corticosteroids exert their deleterious effects on bone through direct and indirect effects on bone cells, mainly leading to suppression of bone formation, but also to a certain extent by increasing bone resorption, with the resulting bone loss being more prominent at trabecular sites. The mechanism bone of loss due to corticosteroids is complex. Glucocorticoids lead to osteoblast apoptosis and to suppression of osteoblast differentiation by suppression of markers genes such as Runx2 and Wnt/beta catenin signaling, leading to decreased bone formation.(117) On the other hand, glucocorticoids also induce bone resorption by reducing osteoclast apoptosis, resulting in net bone loss. Glucocorticoids also indirectly contribute to bone loss by reducing intestinal calcium absorption and increasing urinary calcium excretion. Vitamin D metabolism does not appear to be influenced by glucocorticoids.(118) Corticosteroids also decrease the stimulatory effect of IGF-1 on osteoblasts. The effect of glucocorticoids on bone have been shown to be dose dependent (4;105;119), which may explain the bone loss and increased fractures risk observed in liver transplant recipients particularly within the first few months after transplantation.

Other immunosuppressive agents

A number of immunosuppressive agents may also have adverse effects on bone with a tendency for calcineurin inhibitors such as cyclosporine or tacrolimus to cause rapid and profound bone loss.(120;121) High bone turnover and increase in bone turnover markers of bone formation and resorption have been demonstrated in experimental animal studies of calcineurin use (122) and also confirmed by histomorphometric studies of bone biopsies, with greater bone loss shown with tacrolimus than with cyclosporine A.(123) However, data on the effects of calcineurin inhibitors in humans are scarce and results are conflicting.(124-126) Several studies have shown a less deleterious effect of tacrolimus on bone compared to cyclosporine A. A histomorphometric study showed earlier recovery of bone turnover after OLT in patients treated with tacrolimus compared to those treated with cyclosporine A (127), which was confirmed by two other studies, showing less bone loss in patients treated with tacrolimus and an earlier recovery of bone mass to pre-transplant values.(128;129) Small subgroup analysis in one study (114) and an observational study (130) indeed show that incident fractures occur less in OLT recipients treated with tacrolimus compared to cyclosporine A. In contrast, another study showed no difference in bone mass between cyclosporine A and tacrolimus treated patients. (131) Taken together, these conflicting data suggest that the exact effect of tacrolimus on the skeleton remains to be established in patients with a liver transplantation. Mycophenolate mofetil (MMF) has not been shown thus far to have a deleterious effect on bone.(125;129) Data on the effect of sirolimus on bone turnover are conflicting, with studies showing increased as well as decreased osteoclast activity. (132;133)

Renal failure

A decline in renal function is common after OLT and this may contribute to further bone loss.(94;96;100;134) During the early post-operative period, acute kidney injury occurs in up to 64% of patients.(135) Pre-existing renal impairment and comorbidity lead to increased susceptibility to kidney injury. Several factors may lead to acute kidney injury after transplantation, including acute tubular necrosis, calcineurin inhibitor toxicity and sepsis. In the long-term, some degree of chronic renal impairment is highly prevalent in OLT recipients. One study showed a prevalence of moderate renal impairment in over 50% of OLT recipients at twenty years after OLT (136), and the cumulative incidence of renal failure (glomerular filtration rate of 29 ml/ minute/ 1.73m² body surface or less) is reported to be 18% at five years after OLT.(137) As previously mentioned, renal impairment contributes to bone loss and to mineralization disorders due to abnormalities such as phosphate retention and calcitriol deficiency leading to secondary hyperparathyroidism.(42;43)

Other factors

Recovery of liver function leads to reversal of cholestasis and reversal of metabolic disturbances early after transplantation. IGF-1 and IGFBP-3 levels increase rapidly after OLT, normalizing within the first week after successful transplantation.(60;62;138) Gonadal hormones have been shown to increase (4;100;139) and 25(OH)D levels normalize in the majority of patients(100;104;134;139;140). The introduction of new immunosuppressive regimens has allowed early tapering of corticosteroids (141-145), substantially contributing to recovery of bone mass.

Vitamin D deficiency

Even though vitamin D status improves after OLT, as shown by post-transplantation increases in 25(OH)D (100;100;104;134;139;140), vitamin D levels do not normalize in all patients, and a degree of vitamin D deficiency persist in a number of patients. Furthermore, even though 25(OH)D levels increase after transplantation, this does not always translate in increases in the metabolically active calcitriol.(4;100)

Persistent hypogonadism

Gonadal function improves after transplantation, with marked increases in free testosterone levels observed within the first year after OLT.(4) However, recovery to normal levels is not achieved in all patients, with hypogonadism persisting in a substantial proportion of OLT recipients.(4;100;139)

THERAPEUTIC CONSIDERATIONS

In solid organ transplantation, prevention of post-transplantation bone disease involves optimization of pre-transplant skeletal health and prophylaxis of post-transplantation bone loss. Patients with end-stage liver disease should be screened for skeletal pathology and treated accordingly. Life-style measures, including cessation of smoking and of alcohol use, good nutrition and weight bearing exercise, are to be advised to all patients. Calcium and vitamin D should be adequately supplemented where required. Vitamin D deficiency should be corrected to levels above 50 nmol/L, using follow-up measurements of 25(OH)D. This is the level suggested by the Institute of Medicine (146) although the International Osteoporosis Foundation (IOF) suggests a higher threshold of 75 nmol/L for men and women above the age of 60 and those with osteoporosis (147). It is to be noted that in clinical practice, it is sometimes difficult to reach the threshold of 75 nmol/L with the recommended daily use of 800 units of colecalciferol. At the moment, there is still no consensus on the desirable cut-off point of 25(OH)D to reach in liver transplant recipients but in view of the important role of the liver in vitamin

D metabolism, it remains highly recommended to measure these 25(OH)D levels in patients with chronic liver disease before and after liver transplantation.

Bisphosphonates

Most studies showed a short-term beneficial effect of bisphosphonate treatment on BMD when treatment is initiated at or shortly after OLT.(148-155) Bisphosphonates used in these studies were largely nitrogen-containing bisphosphonates including intravenously administered zoledronate, ibandronate and pamidronate (148-150;154-163) and orally administered alendronate and risedronate (149;151-153). Time of initiation of treatment was at or shortly after OLT in most studies (148-152;154-157;162), and 1-2 years after OLT in 3 studies(158;161;163) and patients were usually treated for one year (148-150;152;155-157;161) or 2-3 years (151;153;158;163). Most studies showed a decrease of biochemical markers of both bone formation, including procollagen type 1 N-terminal propeptide (P1NP), bone alkaline phosphatase and osteocalcin, and of those of bone resorption, including beta crosslaps (CTX) and urinary deoxyhydroxyproline, after initiation of treatment with bisphosphonates in some (148;151;152;155;158;163) but not all studies.(149;150;157;160) A decrease in bone turnover was also demonstrated in two bone histomorphometry studies.(156;162) The authors of a meta-analysis published in 2011 concluded that bisphosphonate treatment administered during the first year after OLT appeared to reduce accelerated bone loss and to improve LS BMD.(164) Data on long-term effect of treatment with bisphosphonates administered shortly after OLT on BMD are scarce and conflicting, with two studies showing a persistent beneficial effect on BMD (150;153), whereas this was not confirmed in a second study.(157) Three studies in which treatment was initiated after the first year after OLT show a beneficial effect on BMD.(158;161;163) Treatment with bisphosphonates in the form of pamidronate administered as a single infusion before OLT did not prevent post-transplantation bone loss.(160) Data on both short-term and long-term effect of bisphosphonate treatment on fracture risk are conflicting, with studies showing a decreased fracture incidence in treated patients with oral as well as intravenous bisphosphonates, initiated before, at or shortly after and 1-2 years after OLT and for a duration of 9-24 months (149;150;152;153;158;159), whereas other studies did not show any effect on fracture risk. (151;154;157;160;163) In the only published meta-analysis, the effect of bisphosphonate treatment on fracture risk could not be analysed because of heterogeneity of the available data.(164)

Whereas bisphosphonates have thus been shown to reduce post-transplantation bone loss and to some degree fracture risk in most but not all studies, there is still no consensus on which patients should be treated with these agents. Antiresorptive therapy should be considered in patients with low bone mass particularly in the presence of fractures. However, since pre-transplant BMD poorly identifies patients at

risk for fractures, and these have been shown to occur in patients with normal BMD (2;114;165), the question raised is whether all liver transplant recipients should be treated with antiresorptive agents at time of transplantation.

CONCLUSION

The metabolic and excretory functions of the liver play an important role in normal bone remodeling. In liver diseases, disturbances in both physiological functions of the liver are associated with loss of bone mass as well as bone quality, leading to increased fracture risk. Significant bone loss occurs after liver transplantation despite recovery of the metabolic and excretory functions of the liver, mainly due to immunosuppressive regimens including glucocorticoids. The incidence of new fractures is also very high during the first year after liver transplantation, with fractures occurring in up to a third of transplant recipients, leading to significant morbidity and to a decrease of quality of life. The aetiology of bone loss and increased fracture risk observed in OLT recipients is multifactorial and still incompletely understood. It has thus been proven difficult to identify patients at risk. Moreover, data on efficacy of bisphosphonate treatment after liver transplantation are scarce and heterogeneous.

Awareness of the magnitude of skeletal pathology in patients with liver disease remains a clinically unmet need before and after liver transplantation. Candidates for liver transplantation and patients with liver diseases need to be thoroughly screened for low bone mass and prevalent fractures and disturbances in calcium and mineral homeostasis need to be adequately corrected in order to prevent further bone loss and decrease post-transplantation fracture risk.

QUESTIONS TO BE ADDRESSED

Mechanisms leading to bone loss in patients with end-stage liver disease need to be further elucidated. Since immunosuppressive regimen have changed during the last decade with the introduction of new immunosuppressive agents and early tapering of corticosteroids, the magnitude of bone loss and fracture risk in OLT recipients under current transplantation protocols needs to be re-examined. Risk factors for post-transplantation bone loss and fractures need to be elucidated in order to identify patients at risk. The value of bone turnover markers, BMD measurements and prevalent fractures in the identification of patients at risk need to be examined. Furthermore, the optimal time of initiation of treatment, the modality of treatment and its duration

remain to be established in OLT recipients. Finally, it needs to be clarified whether the persistent high fracture rate a year after OLT may be decreased or prevented.

REFERENCE LIST

1. Guichelaar MM, Schmoll J, Malinchoc M, Hay JE. Fractures and avascular necrosis before and after orthotopic liver transplantation: long-term follow-up and predictive factors. *Hepatology* 2007; 46(4):1198-1207.
2. Hardinger KL, Ho B, Schnitzler MA, Desai N, Lowell J, Shenoy S, Chapman W, Crippin JS. Serial measurements of bone density at the lumbar spine do not predict fracture risk after liver transplantation. *Liver Transpl* 2003; 9(8):857-862.
3. Leidig-Bruckner G, Eberwein S, Czechatka D, Dodidou P, Pritsch M, Klose C, Otto G, Theilmann L, Zimmermann R, Lange R, Ziegler R. Incidence and determinants of osteoporotic fractures after liver or heart transplantation. *Experimental and Clinical Endocrinology and Diabetes* 1997; 105(4):A61-A62.
4. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, Ordi J, Rimola A, Rodes J, Munoz-Gomez J. Bone disease after liver transplantation: a long-term prospective study of bone mass changes, hormonal status and histomorphometric characteristics. *Osteoporos Int* 2001; 12(6):484-492.
5. Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. Modulation of osteoclast differentiation and function by the new members of the tumor necrosis factor receptor and ligand families. *Endocr Rev* 1999; 20(3):345-357.
6. Imai Y, Youn MY, Inoue K, Takada I, Kouzmenko A, Kato S. Nuclear receptors in bone physiology and diseases. *Physiol Rev* 2013; 93(2):481-523.
7. Karsenty G, Kronenberg HM, Settembre C. Genetic control of bone formation. *Annu Rev Cell Dev Biol* 2009; 25:629-648.
8. Tamai K, Semenov M, Kato Y, Spokony R, Liu C, Katsuyama Y, Hess F, Saint-Jeannet JP, He X. LDL-receptor-related proteins in Wnt signal transduction. *Nature* 2000; 407(6803):530-535.
9. Gaur T, Lengner CJ, Hovhannisyan H, Bhat RA, Bodine PV, Komm BS, Javed A, van Wijnen AJ, Stein JL, Stein GS, Lian JB. Canonical WNT signaling promotes osteogenesis by directly stimulating RUNX2 gene expression. *J Biol Chem* 2005; 280(39):33132-33140.
10. Glass DA, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA, Karsenty G. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell* 2005; 8(5):751-764.
11. van Bezooijen RL, Svensson JP, Eefting D, Visser A, van der Horst G, Karperien M, Quax PH, Vrieling H, Papapoulos SE, ten DP, Lowik CW. Wnt but not BMP signaling is involved in the inhibitory action of sclerostin on BMP-stimulated bone formation. *J Bone Miner Res* 2007; 22(1):19-28.
12. Diarra D, Stolina M, Polzer K, Zwerina J, Ominsky MS, Dwyer D, Korb A, Smolen J, Hoffmann M, Scheinecker C, van der Heide D, Landewe R, Lacey D, Richards WG, Schett G. Dickkopf-1 is a master regulator of joint remodeling. *Nat Med* 2007; 13(2):156-163.
13. Kato S. The function of vitamin D receptor in vitamin D action. *J Biochem* 2000; 127(5):717-722.
14. Baldock PA, Thomas GP, Hodge JM, Baker SU, Dressel U, O'Loughlin PD, Nicholson GC, Briffa KH, Eisman JA, Gardiner EM. Vitamin D action and regulation of bone remodeling: suppression of osteoclastogenesis by the mature osteoblast. *J Bone Miner Res* 2006; 21(10):1618-1626.
15. Haussler MR, Haussler CA, Whitfield GK, Hsieh JC, Thompson PD, Barthel TK, Bartik L, Egan JB, Wu Y, Kubicek JL, Lowmiller CL, Moffet EW, Forster RE, Jurutka PW. The nuclear vitamin D receptor controls the expression of genes encoding factors which feed the "Fountain of Youth" to mediate

- healthful aging. *J Steroid Biochem Mol Biol* 2010; 121(1-2):88-97.
16. Takahashi Y. Essential roles of growth hormone (GH) and insulin-like growth factor-I (IGF-I) in the liver. *Endocr J* 2012; 59(11):955-962.
17. Ohlsson C, Mohan S, Sjogren K, Tivesten A, Isgaard J, Isaksson O, Jansson JO, Svensson J. The role of liver-derived insulin-like growth factor-I. *Endocr Rev* 2009; 30(5):494-535.
18. Mohan S, Kesavan C. Role of insulin-like growth factor-1 in the regulation of skeletal growth. *Curr Osteoporos Rep* 2012; 10(2):178-186.
19. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev* 2002; 23(6):824-854.
20. Canalis E. Growth factor control of bone mass. *J Cell Biochem* 2009; 108(4):769-777.
21. Canalis E. Effect of insulinlike growth factor I on DNA and protein synthesis in cultured rat calvaria. *J Clin Invest* 1980; 66(4):709-719.
22. Playford MP, Bicknell D, Bodmer WF, Macaulay VM. Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of beta-catenin. *Proc Natl Acad Sci U S A* 2000; 97(22):12103-12108.
23. Mochizuki H, Hakeda Y, Wakatsuki N, Usui N, Akashi S, Sato T, Tanaka K, Kumegawa M. Insulin-like growth factor-I supports formation and activation of osteoclasts. *Endocrinology* 1992; 131(3):1075-1080.
24. Canalis E, Centrella M, Burch W, McCarthy TL. Insulin-like growth factor I mediates selective anabolic effects of parathyroid hormone in bone cultures. *J Clin Invest* 1989; 83(1):60-65.
25. McCarthy TL, Centrella M, Canalis E. Parathyroid hormone enhances the transcript and polypeptide levels of insulin-like growth factor I in osteoblast-enriched cultures from fetal rat bone. *Endocrinology* 1989; 124(3):1247-1253.
26. Hofmann AF. Bile Acids: The Good, the Bad, and the Ugly. *News Physiol Sci* 1999; 14:24-29.
27. Shearer MJ, Newman P. Metabolism and cell biology of vitamin K. *Thromb Haemost* 2008; 100(4):530-547.
28. Atkins GJ, Welldon KJ, Wijenayaka AR, Bonewald LF, Findlay DM. Vitamin K promotes mineralization, osteoblast-to-osteocyte transition, and an anticatabolic phenotype by {gamma}-carboxylation-dependent and -independent mechanisms. *Am J Physiol Cell Physiol* 2009; 297(6):C1358-C1367.
29. Fisher L, Fisher A. Vitamin D and parathyroid hormone in outpatients with noncholestatic chronic liver disease. *Clinical Gastroenterology and Hepatology* 2007; 5(4):513-520.
30. Stokes CS, Volmer DA, Grunhage F, Lammert F. Vitamin D in chronic liver disease. *Liver Int* 2013; 33(3):338-352.
31. Fonseca V, Epstein O, Gill DS. Hyperparathyroidism and low serum osteocalcin despite vitamin D replacement in primary biliary cirrhosis. *Journal of Clinical Endocrinology and Metabolism* 1987; 64(5):873-877.
32. Dibble JB, Sheridan P, Hampshire R, Hardy GJ, Losowsky MS. Osteomalacia, vitamin D deficiency and cholestasis in chronic liver disease. *Q J Med* 1982; 51(201):89-103.
33. Skinner RK, Sherlock S, Long RG, Wilis MR. 25-Hydroxylation of vitamin D in primary biliary cirrhosis. *Lancet* 1977; 1(8014):720-721.
34. Danielsson A, Lorentzon R, Larsson S-E. Intestinal absorption and 25-hydroxylation of vitamin D in patients with primary biliary cirrhosis. *Scandinavian Journal of Gastroenterology* 1982; 17(3):349-355.
35. Dibble JB, Sheridan P, Losowsky MS. A survey of vitamin D deficiency in gastrointestinal and liver disorders. *Q J Med* 1984; 53(209):119-134.
36. Gordon GG, Altman K, Southren AL, Rubin E, Lieber CS. Effect of alcohol (ethanol) administration on sex-hormone metabolism in normal men. *N Engl J Med* 1976; 295(15):793-797.

37. Purohit V. Can alcohol promote aromatization of androgens to estrogens? A review. *Alcohol* 2000; 22(3):123-127.
38. Guggenbuhl P, Deugnier Y, Boisdet JF, Rolland Y, Perdriger A, Pawlotsky Y, Chales G. Bone mineral density in men with genetic hemochromatosis and HFE gene mutation. *Osteoporos Int* 2005; 16(12):1809-1814.
39. Diamond T, Pojer R, Stiel D, Alfrey A, Posen S. Does Iron Affect Osteoblast Function - Studies Invitro and in Patients with Chronic Liver-Disease. *Calcified Tissue International* 1991; 48(6):373-379.
40. Conte D, Caraceni MP, Duriez J, Mandelli C, Corghi E, Cesana M, Ortolani S, Bianchi PA. Bone involvement in primary hemochromatosis and alcoholic cirrhosis. *Am J Gastroenterol* 1989; 84(10):1231-1234.
41. Gonwa TA, Wadei HM. Kidney disease in the setting of liver failure: core curriculum 2013. *Am J Kidney Dis* 2013; 62(6):1198-1212.
42. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl* 2009;(113):S1-130.
43. Cunningham J, Locatelli F, Rodriguez M. Secondary hyperparathyroidism: pathogenesis, disease progression, and therapeutic options. *Clin J Am Soc Nephrol* 2011; 6(4):913-921.
44. Gutierrez O, Isakova T, Rhee E, Shah A, Holmes J, Collerone G, Juppner H, Wolf M. Fibroblast growth factor-23 mitigates hyperphosphatemia but accentuates calcitriol deficiency in chronic kidney disease. *J Am Soc Nephrol* 2005; 16(7):2205-2215.
45. Cannata-Andia JB, Rodriguez GM, Gomez AC. Osteoporosis and adynamic bone in chronic kidney disease. *J Nephrol* 2013; 26(1):73-80.
46. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). *Kidney Int Suppl* 2009;(113):S1-130.
47. Klawansky S, Komaroff E, Cavanaugh PF, Jr., Mitchell DY, Gordon MJ, Connelly JE, Ross SD. Relationship between age, renal function and bone mineral density in the US population. *Osteoporos Int* 2003; 14(7):570-576.
48. Jassal SK, von MD, Barrett-Connor E. Measures of renal function, BMD, bone loss, and osteoporotic fracture in older adults: the Rancho Bernardo study. *J Bone Miner Res* 2007; 22(2):203-210.
49. Thomas JD, Monson JP. Adult GH deficiency throughout lifetime. *Eur J Endocrinol* 2009; 161 Suppl 1:S97-S106.
50. George J, Ganesh HK, Acharya S, Bandgar TR, Shivane V, Karvat A, Bhatia SJ, Shah S, Menon PS, Shah N. Bone mineral density and disorders of mineral metabolism in chronic liver disease. *World J Gastroenterol* 2009; 15(28):3516-3522.
51. Fusco A, Miele L, D'Uonno A, Forgione A, Riccardi L, Cefalo C, Barini A, Bianchi A, Giampietro A, Cimino V, Landolfi R, Grieco A, De ML. Nonalcoholic fatty liver disease is associated with increased GHBP and reduced GH/IGF-I levels. *Clin Endocrinol (Oxf)* 2012; 77(4):531-536.
52. Ichikawa T, Nakao K, Hamasaki K, Furukawa R, Tsuruta S, Ueda Y, Taura N, Shibata H, Fujimoto M, Toriyama K, Eguchi K. Role of growth hormone, insulin-like growth factor 1 and insulin-like growth factor-binding protein 3 in development of non-alcoholic fatty liver disease. *Hepatol Int* 2007; 1(2):287-294.
53. Santolaria F, Gonzalez-Gonzalez G, Gonzalez-Reimers E, Martinez-Riera A, Milena A, Rodriguez-Moreno F, Gonzalez-Garcia C. Effects of alcohol and liver cirrhosis on the GH-IGF-I axis. *Alcohol Alcohol* 1995; 30(6):703-708.
54. Helaly GF, Hussein NG, Refai W, Ibrahim M. Relation of serum insulin-like growth factor-1 (IGF-1) levels with hepatitis C virus infection and insulin resistance. *Transl Res* 2011; 158(3):155-162.

55. Nielsen MF, Aagaard NK, Grofte T, Frystyk J, Greisen J, Christiansen JS, Holland-Fischer P, Vilstrup H. Normalisation of insulin-like growth factor-I does not improve insulin action in cirrhosis. *Liver Int* 2011; 31(10):1511-1518.
56. Scharf JG, Schmitz F, Frystyk J, Skjaerbaek C, Moesius H, Blum WF, Ramadori G, Hartmann H. Insulin-like growth factor-I serum concentrations and patterns of insulin-like growth factor binding proteins in patients with chronic liver disease. *J Hepatol* 1996; 25(5):689-699.
57. Gonzalez-Reimers E, Alvisa-Negrin J, Santolaria-Fernandez F, Ros-Vilamajo R, Martin-Gonzalez MC, Hernandez-Betancor I, Garcia-Valdecasas-Campelo E, Gonzalez-Diaz A. Prognosis of osteopenia in chronic alcoholics. *Alcohol* 2011; 45(3):227-238.
58. Arturi F, Succurro E, Procopio C, Pedace E, Mannino GC, Lugara M, Procopio T, Andreozzi F, Sciacqua A, Hribal ML, Perticone F, Sesti G. Nonalcoholic fatty liver disease is associated with low circulating levels of insulin-like growth factor-I. *J Clin Endocrinol Metab* 2011; 96(10):E1640-E1644.
59. Assy N, Hochberg Z, Amit T, Shen-Orr Z, Enat R, Baruch Y. Growth hormone-stimulated insulin-like growth factor (IGF) I and IGF-binding protein-3 in liver cirrhosis. *J Hepatol* 1997; 27(5):796-802.
60. Bassanello M, De Palo EF, Lancerin F, Vitale A, Gatti R, Montin U, Ciarleglio FA, Senzolo M, Burra P, Brolese A, Zanus G, D'Amico DF, Cillo U. Growth hormone/insulin-like growth factor 1 axis recovery after liver transplantation: a preliminary prospective study. *Liver Transpl* 2004; 10(5):692-698.
61. Khoshnood A, Nasiri TM, Faravash MJ, Esteghamati A, Froutan H, Ghofrani H, Kalani M, Miroliaee A, Abdollahi A, Yasir A. A survey of correlation between insulin-like growth factor-I (igf-I) levels and severity of liver cirrhosis. *Hepat Mon* 2013; 13(2):e6181.
62. Weber MM, Auernhammer CJ, Lee PD, Engelhardt D, Zachoval R. Insulin-like growth factors and insulin-like growth factor binding proteins in adult patients with severe liver disease before and after orthotopic liver transplantation. *Horm Res* 2002; 57(3-4):105-112.
63. Gaudio A, Lasco A, Morabito N, Atteritano M, Vergara C, Catalano A, Fries W, Trifiletti A, Frisina N. Hepatic osteodystrophy: does the osteoprotegerin/receptor activator of nuclear factor- κ B ligand system play a role? *J Endocrinol Invest* 2005; 28(8):677-682.
64. Bonefeld K, Hobolth L, Juul A, Moller S. The insulin like growth factor system in cirrhosis. Relation to changes in body composition following adrenoreceptor blockade. *Growth Horm IGF Res* 2012; 22(6):212-218.
65. Yamauchi M, Yamaguchi T, Nawata K, Takaoka S, Sugimoto T. Relationships between undercarboxylated osteocalcin and vitamin K intakes, bone turnover, and bone mineral density in healthy women. *Clin Nutr* 2010; 29(6):761-765.
66. Gundberg CM, Lian JB, Booth SL. Vitamin K-dependent carboxylation of osteocalcin: friend or foe? *Adv Nutr* 2012; 3(2):149-157.
67. Klein RF. Alcohol-induced bone disease: impact of ethanol on osteoblast proliferation. *Alcohol Clin Exp Res* 1997; 21(3):392-399.
68. Tsai CF, Liu CJ, Chen TJ, Chu CJ, Lin HC, Lee FY, Su TP, Lu CL. Increased incidence of orthopedic fractures in cirrhotic patients: a nationwide population-based study. *J Hepatol* 2013; 58(4):706-714.
69. Drake MT, Murad MH, Mauck KF, Lane MA, Undavalli C, Elraiyah T, Stuart LM, Prasad C, Shahrour A, Mullan RJ, Hazem A, Erwin PJ, Montori VM. Clinical review. Risk factors for low bone mass-related fractures in men: a systematic review and meta-analysis. *J Clin Endocrinol Metab* 2012; 97(6):1861-1870.
70. Mukamal KJ, Robbins JA, Cauley JA, Kern LM, Siscovick DS. Alcohol consumption, bone density, and hip fracture among older adults: the cardiovascular health study. *Osteoporos Int* 2007; 18(5):593-602.

71. Gonzalez-Reimers E, Perez-Ramirez A, Santolaria-Fernandez F, Rodriguez-Rodriguez E, Martinez-Riera A, Duran-Castellon MC, Aleman-Valls MR, Gaspar MR. Association of Harris lines and shorter stature with ethanol consumption during growth. *Alcohol* 2007; 41(7):511-515.
72. Garcia-Valdecasas-Campelo E, Gonzalez-Reimers E, Santolaria-Fernandez F, De la Vega-Prieto MJ, Milena-Abril A, Sanchez-Perez MJ, Martinez-Riera A, Gomez-Rodriguez ML. Serum osteoprotegerin and RANKL levels in chronic alcoholic liver disease. *Alcohol* 2006; 41(3):261-266.
73. Dai J, Lin D, Zhang J, Habib P, Smith P, Murtha J, Fu Z, Yao Z, Qi Y, Keller ET. Chronic alcohol ingestion induces osteoclastogenesis and bone loss through IL-6 in mice. *J Clin Invest* 2000; 106(7):887-895.
74. Santolaria F, Gonzalez-Reimers E, Perez-Manzano JL, Milena A, Gomez-Rodriguez MA, Gonzalez-Diaz A, de la Vega MJ, Martinez-Riera A. Osteopenia assessed by body composition analysis is related to malnutrition in alcoholic patients. *Alcohol* 2000; 22(3):147-157.
75. Cawthon PM, Harrison SL, Barrett-Connor E, Fink HA, Cauley JA, Lewis CE, Orwoll ES, Cummings SR. Alcohol intake and its relationship with bone mineral density, falls, and fracture risk in older men. *J Am Geriatr Soc* 2006; 54(11):1649-1657.
76. Guichelaar MM, Malinchoc M, Sibonga J, Clarke BL, Hay JE. Bone metabolism in advanced cholestatic liver disease: analysis by bone histomorphometry. *Hepatology* 2002; 36(4 Pt 1):895-903.
77. Vedi S, Greer S, Skingle SJ, Garrahan NJ, Ninkovic M, Alexander GA, Compston JE. Mechanism of bone loss after liver transplantation: A histomorphometric analysis. *J Bone Miner Res* 1999; 14(2):281-287.
78. Schiefke I, Fach A, Wiedmann M, Aretin AV, Schenker E, Borte G, Wiese M, Moessner J. Reduced bone mineral density and altered bone turnover markers in patients with non-cirrhotic chronic hepatitis B or C infection. *World J Gastroenterol* 2005; 11(12):1843-1847.
79. Orsini LG, Pinheiro MM, Castro CH, Silva AE, Szejnfeld VL. Bone mineral density measurements, bone markers and serum vitamin D concentrations in men with chronic non-cirrhotic untreated hepatitis C. *PLoS One* 2013; 8(11):e81652.
80. Duarte MP, Farias ML, Coelho HS, Mendonca LM, Stabnov LM, do C, Lamy RA, Oliveira DS. Calcium-parathyroid hormone-vitamin D axis and metabolic bone disease in chronic viral liver disease. *J Gastroenterol Hepatol* 2001; 16(9):1022-1027.
81. Gonzalez-Calvin JL, Gallego-Rojo F, Fernandez-Perez R, Casado-Caballero F, Ruiz-Escolano E, Olivares EG. Osteoporosis, mineral metabolism, and serum soluble tumor necrosis factor receptor p55 in viral cirrhosis. *J Clin Endocrinol Metab* 2004; 89(9):4325-4330.
82. Arase Y, Suzuki F, Suzuki Y, Akuta N, Kobayashi M, Sezaki H, Hosaka T, Kawamura Y, Yatsuji H, Hirakawa M, Ikeda K, Hsieh SD, Oomoto Y, Amakawa K, Kato H, Kazawa T, Tsuchi H, Kobayashi T, Kumada H. Virus clearance reduces bone fracture in postmenopausal women with osteoporosis and chronic liver disease caused by hepatitis C virus. *J Med Virol* 2010; 82(3):390-395.
83. Solis-Herruzo JA, Castellano G, Fernandez I, Munoz R, Hawkins F. Decreased bone mineral density after therapy with alpha interferon in combination with ribavirin for chronic hepatitis C. *J Hepatol* 2000; 33(5):812-817.
84. Hofmann WP, Kronenberger B, Bojunga J, Stamm B, Herrmann E, Bucker A, Mihm U, von WM, Zeuzem S, Sarrazin C. Prospective study of bone mineral density and metabolism in patients with chronic hepatitis C during pegylated interferon alpha and ribavirin therapy. *J Viral Hepat* 2008; 15(11):790-796.
85. Trombetti A, Giostra E, Mentha G, Negro F, Rizzoli R. Lack of evidence for ribavirin-

- induced bone loss. *Hepatology* 2002; 36(1):255-257.
86. Urganci N, Gulec SG, Arapoglu M, Vural S, Nuhog A. The effect of ribavirin on bone density in patients with chronic hepatitis C treated with interferon-ribavirin therapy. *J Pediatr Gastroenterol Nutr* 2005; 41(5):650-652.
 87. Bridle KR, Frazer DM, Wilkins SJ, Dixon JL, Purdie DM, Crawford DH, Subramaniam VN, Powell LW, Anderson GJ, Ramm GA. Disrupted hepcidin regulation in HFE-associated haemochromatosis and the liver as a regulator of body iron homeostasis. *Lancet* 2003; 361(9358):669-673.
 88. Sinigaglia L, Fargion S, Fracanzani AL, Binelli L, Battafarano N, Varenna M, Piperno A, Fiorelli G. Bone and joint involvement in genetic hemochromatosis: role of cirrhosis and iron overload. *J Rheumatol* 1997; 24(9):1809-1813.
 89. Richette P, Ottaviani S, Vicaut E, Bardin T. Musculoskeletal complications of hereditary hemochromatosis: a case-control study. *J Rheumatol* 2010; 37(10):2145-2150.
 90. Abdelhadi M, Eriksson SA, Ljusk ES, Ericzon BG, Nordenstrom J. Bone mineral status in end-stage liver disease and the effect of liver transplantation. *Scand J Gastroenterol* 1995; 30(12):1210-1215.
 91. Crosbie OM, Freaney R, McKenna MJ, Curry MP, Hegarty JE. Predicting bone loss following orthotopic liver transplantation. *Gut* 1999; 44(3):430-434.
 92. Eastell R, Dickson ER, Hodgson SF, Wiesner RH, Porayko MK, Wahner HW, Cedel SL, Riggs BL, Krom RA. Rates of vertebral bone loss before and after liver transplantation in women with primary biliary cirrhosis. *Hepatology* 1991; 14(2):296-300.
 93. Floreani A, Fries W, Luisetto G, Burra P, Fagioli S, Boccagni P, Della Rovere GR, Plebani M, Piccoli A, Naccarato R. Bone metabolism in orthotopic liver transplantation: a prospective study. *Liver Transpl Surg* 1998; 4(4):311-319.
 94. Floreani A, Mega A, Tizian L, Burra P, Boccagni P, Baldo V, Fagioli S, Naccarato R, Luisetto G. Bone metabolism and gonad function in male patients undergoing liver transplantation: a two-year longitudinal study. *Osteoporos Int* 2001; 12(9):749-754.
 95. Hardinger KL, Ho B, Schnitzler MA, Desai N, Lowell J, Shenoy S, Chapman W, Crippin JS. Serial measurements of bone density at the lumbar spine do not predict fracture risk after liver transplantation. *Liver Transpl* 2003; 9(8):857-862.
 96. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, Ordi J, Rimola A, Rodes J, Munoz-Gomez J. Bone disease after liver transplantation: a long-term prospective study of bone mass changes, hormonal status and histomorphometric characteristics. *Osteoporos Int* 2001; 12(6):484-492.
 97. Ninkovic M, Skingle SJ, Bearcroft PW, Bishop N, Alexander GJ, Compston JE. Incidence of vertebral fractures in the first three months after orthotopic liver transplantation. *Eur J Gastroenterol Hepatol* 2000; 12(8):931-935.
 98. Scolapio JS, DeArment J, Hurley DL, Romano M, Harnois D, Weigand SD. Influence of tacrolimus and short-duration prednisone on bone mineral density following liver transplantation. *JPEN J Parenter Enteral Nutr* 2003; 27(6):427-432.
 99. Eastell R, Dickson ER, Hodgson SF, Wiesner RH, Porayko MK, Wahner HW, Cedel SL, Riggs BL, Krom RA. Rates of vertebral bone loss before and after liver transplantation in women with primary biliary cirrhosis. *Hepatology* 1991; 14(2):296-300.
 100. Feller RB, McDonald JA, Sherbon KJ, McCaughan GW. Evidence of continuing bone recovery at a mean of 7 years after liver transplantation. *Liver Transpl Surg* 1999; 5(5):407-413.
 101. Floreani A, Fries W, Luisetto G, Burra P, Fagioli S, Boccagni P, Della Rovere GR, Plebani M, Piccoli A, Naccarato R. Bone metabolism in orthotopic liver

- transplantation: a prospective study. *Liver Transpl Surg* 1998; 4(4):311-319.
102. Hamburg SM, Piers DA, van den Berg AP, Slooff MJ, Haagsma EB. Bone mineral density in the long term after liver transplantation. *Osteoporos Int* 2000; 11(7):600-606.
 103. Mart i, Gomez R, Jodar E, Loinaz C, Moreno E, Hawkins E. Long-term follow-up of bone mass after orthotopic liver transplantation: effect of steroid withdrawal from the immunosuppressive regimen. *Osteoporos Int* 2002; 13(2):147-150.
 104. Crosbie OM, Freaney R, McKenna MJ, Curry MP, Hegarty JE. Predicting bone loss following orthotopic liver transplantation. *Gut* 1999; 44(3):430-434.
 105. Giannini S, Nobile M, Ciuffreda M, Iemmolo RM, Dalle CL, Minicuci N, Casagrande F, Destro C, Gerunda GE, Sartori L, Crepaldi G. Long-term persistence of low bone density in orthotopic liver transplantation. *Osteoporos Int* 2000; 11(5):417-424.
 106. Navasa M, Monegal A, Guanabens N, Peris P, Rimola A, Munoz-Gomez J, Visa J, Rodes J. Bone fractures in liver transplant patients. *Br J Rheumatol* 1994; 33(1):52-55.
 107. Carey EJ, Balan V, Kremers WK, Hay JE. Osteopenia and osteoporosis in patients with end-stage liver disease caused by hepatitis C and alcoholic liver disease: not just a cholestatic problem. *Liver Transpl* 2003; 9(11):1166-1173.
 108. Trautwein C, Possienke M, Schlitt HJ, Boker KH, Horn R, Raab R, Manns MP, Brabant G. Bone density and metabolism in patients with viral hepatitis and cholestatic liver diseases before and after liver transplantation. *Am J Gastroenterol* 2000; 95(9):2343-2351.
 109. Guichelaar MM, Kendall R, Malinchoc M, Hay JE. Bone mineral density before and after OLT: long-term follow-up and predictive factors. *Liver Transpl* 2006; 12(9):1390-1402.
 110. Premaor MO, Das TK, Debiram I, Parker RA, Ninkovic M, Alexander GT, Compston JE. Fracture incidence after liver transplantation: results of a 10-year audit. *QJM* 2011; 104(7):599-606.
 111. Leidig-Bruckner G, Hosch S, Dodidou P, Ritschel D, Conrad C, Klose C, Otto G, Lange R, Theilmann L, Zimmerman R, Pritsch M, Ziegler R. Frequency and predictors of osteoporotic fractures after cardiac or liver transplantation: a follow-up study. *Lancet* 2001; 357(9253):342-347.
 112. Monegal A, Guanabens N, Suarez MJ, Suarez F, Clemente G, Garcia-Gonzalez M, De la Mata M, Serrano T, Casafont F, Tome S, Barrios C, Navasa M. Pamidronate in the prevention of bone loss after liver transplantation: a randomized controlled trial. *Transpl Int* 2009; 22(2):198-206.
 113. Ninkovic M, Love S, Tom BD, Bearcroft PW, Alexander GJ, Compston JE. Lack of effect of intravenous pamidronate on fracture incidence and bone mineral density after orthotopic liver transplantation. *J Hepatol* 2002; 37(1):93-100.
 114. Leidig-Bruckner G, Hosch S, Dodidou P, Ritschel D, Conrad C, Klose C, Otto G, Lange R, Theilmann L, Zimmerman R, Pritsch M, Ziegler R. Frequency and predictors of osteoporotic fractures after cardiac or liver transplantation: a follow-up study. *Lancet* 2001; 357(9253):342-347.
 115. McDonald JA, Dunstan CR, Dilworth P, Sherbon K, Sheil AGR, Evans RA, McCaughan GW. Bone loss after liver transplantation. *Hepatology* 1991; 14(4 1):613-619.
 116. Meys E, Fontanges E, Fourcade N, Thomasson A, Pouyet M, Delmas PD. Bone loss after orthotopic liver transplantation. *Am J Med* 1994; 97(5):445-450.
 117. Canalis E, Mazziotti G, Giustina A, Bilezikian JP. Glucocorticoid-induced osteoporosis: pathophysiology and therapy. *Osteoporos Int* 2007; 18(10):1319-1328.
 118. Davidson ZE, Walker KZ, Truby H. Clinical review: Do glucocorticosteroids alter vitamin D status? A systematic review with meta-analyses of observational studies. *J Clin Endocrinol Metab* 2012; 97(3):738-744.

119. Guthery SL, Pohl JF, Bucuvalas JC, Alonso MH, Ryckman FC, Balistreri WF, Heubi JE. Bone mineral density in long-term survivors following pediatric liver transplantation. *Liver Transpl* 2003; 9(4):365-370.
120. Movsowitz C, Epstein S, Ismail F, Fallon M, Thomas S. Cyclosporin A in the oophorectomized rat: unexpected severe bone resorption. *J Bone Miner Res* 1989; 4(3):393-398.
121. Schlosberg M, Movsowitz C, Epstein S, Ismail F, Fallon MD, Thomas S. The effect of cyclosporin A administration and its withdrawal on bone mineral metabolism in the rat. *Endocrinology* 1989; 124(5):2179-2184.
122. Epstein S, Dissanayake IR, Goodman GR, Bowman AR, Zhou H, Ma Y, Jee WS. Effect of the interaction of parathyroid hormone and cyclosporine a on bone mineral metabolism in the rat. *Calcif Tissue Int* 2001; 68(4):240-247.
123. Abdelhadi M, Ericzon BG, Hultenby K, Sjoden G, Reinholt FP, Nordenstrom J. Structural skeletal impairment induced by immunosuppressive therapy in rats: cyclosporine A vs tacrolimus. *Transpl Int* 2002; 15(4):180-187.
124. Hay JE. Osteoporosis in liver diseases and after liver transplantation. *J Hepatol* 2003; 38(6):856-865.
125. Compston JE. Osteoporosis after liver transplantation. *Liver Transpl* 2003; 9(4):321-330.
126. Thiebaud D, Krieg MA, Gillard-Berguer D, Jacquet AF, Goy JJ, Burckhardt P. Cyclosporine induces high bone turnover and may contribute to bone loss after heart transplantation. *Eur J Clin Invest* 1996; 26(7):549-555.
127. Guichelaar MM, Malinchoc M, Sibonga J, Clarke BL, Hay JE. Immunosuppressive and postoperative effects of orthotopic liver transplantation on bone metabolism. *Liver Transpl* 2004; 10(5):638-647.
128. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, Rimola A, Rodes J, Munoz-Gomez J. Bone mass and mineral metabolism in liver transplant patients treated with FK506 or cyclosporine A. *Calcif Tissue Int* 2001; 68(2):83-86.
129. Smallwood GA, Burns D, Fasola CG, Steiber AC, Heffron TG. Relationship between immunosuppression and osteoporosis in an outpatient liver transplant clinic. *Transplant Proc* 2005; 37(4):1910-1911.
130. Segal E, Baruch Y, Kramsky R, Raz B, Tamir A, Ish-Shalom S. Predominant factors associated with bone loss in liver transplant patients - after prolonged post-transplantation period. *Clin Transplant* 2003; 17(1):13-19.
131. Bjoro K, Brandsaeter B, Wiencke K, Bjoro T, Godang K, Bollerslev J, Schrupf E. Secondary osteoporosis in liver transplant recipients: a longitudinal study in patients with and without cholestatic liver disease. *Scand J Gastroenterol* 2003; 38(3):320-327.
132. Westenfeld R, Schlieper G, Woltje M, Gawlik A, Brandenburg V, Rutkowski P, Floege J, Jahnhen-Dechent W, Ketteler M. Impact of sirolimus, tacrolimus and mycophenolate mofetil on osteoclastogenesis--implications for post-transplantation bone disease. *Nephrol Dial Transplant* 2011; 26(12):4115-4123.
133. Luo LM, Wang LL. Sirolimus protective effects on bone: the need to be demonstrated. *Nephrol Dial Transplant* 2012; 27(6):2602-2603.
134. Guichelaar MM, Kendall R, Malinchoc M, Hay JE. Bone mineral density before and after OLT: long-term follow-up and predictive factors. *Liver Transpl* 2006; 12(9):1390-1402.
135. O'Riordan A, Wong V, McQuillan R, McCormick PA, Hegarty JE, Watson AJ. Acute renal disease, as defined by the RIFLE criteria, post-liver transplantation. *Am J Transplant* 2007; 7(1):168-176.
136. Schoening WN, Buescher N, Rademacher S, Andreou A, Kuehn S, Neuhaus R, Guckelberger O, Puhl G, Seehofer D, Neuhaus P. Twenty-year longitudinal follow-up after

- orthotopic liver transplantation: a single-center experience of 313 consecutive cases. *Am J Transplant* 2013; 13(9):2384-2394.
137. Ojo AO, Held PJ, Port FK, Wolfe RA, Leichtman AB, Young EW, Arndorfer J, Christensen L, Merion RM. Chronic renal failure after transplantation of a nonrenal organ. *N Engl J Med* 2003; 349(10):931-940.
 138. Castro GR, Coelho JC, Parolin MB, Matias JE, de Freitas AC. Insulin-like growth factor I correlates with MELD and returns to normal level after liver transplantation. *Ann Transplant* 2013; 18:57-62.
 139. Floreani A, Mega A, Tizian L, Burra P, Boccagni P, Baldo V, Fagioli S, Naccarato R, Luisetto G. Bone metabolism and gonad function in male patients undergoing liver transplantation: a two-year longitudinal study. *Osteoporos Int* 2001; 12(9):749-754.
 140. Haagsma EB, Thijn CJ, Post JG, Slooff MJ, Gips CH. Bone disease after orthotopic liver transplantation. *J Hepatol* 1988; 6(1):94-100.
 141. Meadows HB, Taber DJ, Pilch NA, Tischer SM, Baliga PK, Chavin KD. The impact of early corticosteroid withdrawal on graft survival in liver transplant recipients. *Transplant Proc* 2012; 44(5):1323-1328.
 142. Hu AB, Wu LW, Tai Q, Zhu XF, He XS. Safety and efficacy of four steroid-minimization protocols in liver transplant recipients: 3-year follow-up in a single center. *J Dig Dis* 2013; 14(1):38-44.
 143. Bonaccorsi-Riani E, Sempoux C, Piette N, Julliard O, Kabamba B, Ciccarelli O, Roggen F, De RC, Hassoun Z, Lerut J. Impact of steroid-avoidance immunosuppression on long-term outcome after liver transplantation for HCV cirrhosis: the need for well documented long-term follow-up. *Acta Gastroenterol Belg* 2012; 75(4):411-418.
 144. Ramirez CB, Doria C, Frank AM, Armenti ST, Marino IR. Completely steroid-free immunosuppression in liver transplantation: a randomized study. *Clin Transplant* 2013; 27(3):463-471.
 145. Takada Y, Kaido T, Asonuma K, Sakurai H, Kubo S, Kiuchi T, Inomata Y, Isaji S, Tsumura H, Teramukai S, Matsubara Y, Sakabayashi S, Uemoto S. Randomized, multicenter trial comparing tacrolimus plus mycophenolate mofetil to tacrolimus plus steroids in hepatitis C virus-positive recipients of living donor liver transplantation. *Liver Transpl* 2013; 19(8):896-906.
 146. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs CS, Mayne ST, Rosen CJ, Shapses SA. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *J Clin Endocrinol Metab* 2011; 96(1):53-58.
 147. Dawson-Hughes B, Mithal A, Bonjour JP, Boonen S, Burckhardt P, Fuleihan GE, Josse RG, Lips P, Morales-Torres J, Yoshimura N. IOF position statement: vitamin D recommendations for older adults. *Osteoporos Int* 2010; 21(7):1151-1154.
 148. Crawford BA, Kam C, Pavlovic J, Byth K, Handelsman DJ, Angus PW, McCaughan GW. Zoledronic acid prevents bone loss after liver transplantation: a randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 2006; 144(4):239-248.
 149. Shane E, Cohen A, Stein EM, McMahon DJ, Zhang C, Young P, Pandit K, Staron RB, Verna EC, Brown R, Restaino S, Mancini D. Zoledronic acid versus alendronate for the prevention of bone loss after heart or liver transplantation. *J Clin Endocrinol Metab* 2012; 97(12):4481-4490.
 150. Kaemmerer D, Lehmann G, Wolf G, Settmacher U, Hommann M. Treatment of osteoporosis after liver transplantation with ibandronate. *Transpl Int* 2010; 23(7):753-759.
 151. Guadalix S, Martinez-Diaz-Guerra G, Lora D, Vargas C, Gomez-Juaristi M, Cobaleda B, Gonzalez EM, Hawkins F. Effect of early risedronate treatment on bone mineral density and bone turnover markers after

- liver transplantation: a prospective single-center study. *Transpl Int* 2011; 24(7):657-665.
152. Atamaz F, Hepguler S, Karasu Z, Kilic M, Tokat Y. The prevention of bone fractures after liver transplantation: experience with alendronate treatment. *Transplant Proc* 2006; 38(5):1448-1452.
 153. Millonig G, Graziadei IW, Eichler D, Pfeiffer KP, Finkenstedt G, Muehllechner P, Koenigsrainer A, Margreiter R, Vogel W. Alendronate in combination with calcium and vitamin D prevents bone loss after orthotopic liver transplantation: a prospective single-center study. *Liver Transpl* 2005; 11(8):960-966.
 154. Monegal A, Guanabens N, Suarez MJ, Suarez F, Clemente G, Garcia-Gonzalez M, De la Mata M, Serrano T, Casafont F, Tome S, Barrios C, Navasa M. Pamidronate in the prevention of bone loss after liver transplantation: a randomized controlled trial. *Transpl Int* 2009; 22(2):198-206.
 155. Pennisi P, Trombetti A, Giostra E, Mentha G, Rizzoli R, Fiore CE. Pamidronate and osteoporosis prevention in liver transplant recipients. *Rheumatol Int* 2007; 27(3):251-256.
 156. Bodingbauer M, Wekerle T, Pakrah B, Roschger P, Peck-Radosavljevic M, Silberhumer G, Grampp S, Rockenschaub S, Berlakovich G, Steininger R, Klaushofer K, Oberbauer R, Muhlbacher F. Prophylactic bisphosphonate treatment prevents bone fractures after liver transplantation. *Am J Transplant* 2007; 7(7):1763-1769.
 157. Bodingbauer M, Pakrah B, Kristo I, Marschalek J, Burghuber C, Gyori G, Kainz A, Rasoul-Rockenschaub S, Klaushofer K, Muehlbacher F, Oberbauer R. Non-persistent effect of short-term bisphosphonate treatment in preventing fractures after liver transplantation. *Transpl Int* 2010; 23(1):118-120.
 158. Dodidou P, Bruckner T, Hosch S, Haass M, Klar E, Sauer P, Ziegler R, Leidig-Bruckner G. Better late than never? Experience with intravenous pamidronate treatment in patients with low bone mass or fractures following cardiac or liver transplantation. *Osteoporos Int* 2003; 14(1):82-89.
 159. Reeves HL, Francis RM, Manas DM, Hudson M, Day CP. Intravenous bisphosphonate prevents symptomatic osteoporotic vertebral collapse in patients after liver transplantation. *Liver Transpl Surg* 1998; 4(5):404-409.
 160. Riemens SC, Oostdijk A, van Doormaal JJ, Thijn CJ, Drent G, Piers DA, Groen EW, Meerman L, Slooff MJ, Haagsma EB. Bone loss after liver transplantation is not prevented by cyclical etidronate, calcium and alphacalcidol. The Liver Transplant Group, Groningen. *Osteoporos Int* 1996; 6(3):213-218.
 161. Valero MA, Loinaz C, Larrodera L, Leon M, Moreno E, Hawkins F. Calcitonin and bisphosphonates treatment in bone loss after liver transplantation. *Calcif Tissue Int* 1995; 57(1):15-19.
 162. Vedi S, Ninkovic M, Garrahan NJ, Alexander GJ, Compston JE. Effects of a single infusion of pamidronate prior to liver transplantation: a bone histomorphometric study. *Transpl Int* 2002; 15(6):290-295.
 163. Wagner D, Amrein K, Dimai HP, Kniepeiss D, Tscheliessnigg KH, Kornprat P, Dobnig H, Pieber T, Fahrleitner-Pammer A. Ibandronate and calcitriol reduces fracture risk, reverses bone loss, and normalizes bone turnover after LTX. *Transplantation* 2012; 93(3):331-336.
 164. Kasturi KS, Chennareddygar S, Mummadi RR. Effect of bisphosphonates on bone mineral density in liver transplant patients: a meta-analysis and systematic review of randomized controlled trials. *Transpl Int* 2010; 23(2):200-207.
 165. Krol CG, Dekkers OM, Kroon HM, Rabelink TJ, van HB, Hamdy NA. Longitudinal Changes in BMD and Fracture Risk in Orthotopic Liver Transplant Recipients Not Using Bone Modifying Treatment. *J Bone Miner Res* 2014.



2

No association between BMD and prevalent vertebral fractures in liver transplant recipients at time of screening before transplantation

Charlotte G. Krol, Olaf M. Dekkers, Herman M. Kroon, Ton J. Rabelink, Bart van Hoek, Neveen A.T. Hamdy

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ABSTRACT

Context: Osteoporosis and fractures are prevalent after orthotopic liver transplantation (OLT), but data on these skeletal complications are scarce in patients with end-stage liver disease awaiting liver transplantation.

Objective: To evaluate the prevalence of vertebral fractures (VF) in OLT recipients at time of screening for transplantation and establish the association between bone mineral density (BMD) and these fractures before transplantation

Design and setting: Retrospective study of consecutive OLT recipients at the Leiden University Medical Centre between 2000 and 2011 at time of screening for transplantation. Clinical, laboratory and BMD data were extracted from electronic hospital records. Conventional spinal radiographs were assessed for VF by two independent observers using the Genant's semi-quantitative method.

Patients: In total, 162 of 223 OLT recipients (median age 51 yrs, 75% men) who had available BMD and spinal radiographs but who were not receiving bone-modifying treatment at screening for OLT were included in the study.

Results: Osteoporosis and osteopenia were prevalent in respectively 19 and 38% at the lumbar spine and in 10 and 42 % at the femoral neck. VF mostly grade 1 were prevalent in 56% . There was no association between BMD and prevalent VF before transplantation.

Conclusions: Vertebral fractures are prevalent in liver transplant recipients at time of screening for transplantation, but there was no association between BMD and prevalent fractures. Spinal radiographs should be routinely performed as part of screening protocols before liver transplantation to enable identification of VF and allow timely intervention to potentially decrease or prevent skeletal morbidity after transplantation

INTRODUCTION

Osteoporosis and fractures are common after liver transplantation (1-3), but data on these skeletal complications are scarce in patients with end-stage liver disease, awaiting orthotopic liver transplantation (OLT). (2;4-6) Published data suggest low bone mass in over 50% of patients, and prevalent vertebral fractures varying from 3.5% to 36% of patients before transplantation, depending on the method used for identification of these fractures. (1;4;5;7;8) The liver plays an important role in bone and mineral metabolism. The aetiology of bone loss and increased fracture risk associated with disturbed liver function is multifactorial, including vitamin D deficiency, hypogonadism, decreased mobility, corticosteroid use (in autoimmune hepatitis) and alcohol toxicity where relevant. Candidates for liver transplantation with underlying skeletal pathology are potentially at higher risk for skeletal morbidity, particularly increased fracture risk, after transplantation. (1;2;9) Cholestatic liver disease has been reported to be associated with osteoporosis and vertebral fractures (10-12), and alcohol abuse is a common cause of osteoporosis and increased fracture risk, particularly in men (5;13) but less is known about these skeletal complications in other liver pathologies.

Prevalent vertebral fractures represent an important predictive factor for future fractures. (1;2;14) Data indeed show that risk of future fracture significantly increases with increasing number and/or grade of severity of vertebral fractures, also shown to be true for mild grade 1 vertebral fractures. (15) Identification of these fractures before transplantation would thus be of significant clinical relevance, not only to predict future fracture risk, but also to allow timely initiation of therapeutic interventions potentially able to decrease or prevent skeletal morbidity after transplantation.

The main objectives of our study were to evaluate the prevalence of vertebral fractures in liver transplant recipients at time of screening for transplantation, and to establish the association between bone mineral density (BMD) and these fractures before transplantation.

PATIENTS AND METHODS

Patients

Consecutive patients who underwent a first OLT at the Leiden University Medical Centre between January 2000 and January 2011, and who had available BMD measurements and conventional radiographs of the thoracic and lumbar spine, performed at the time of their extensive in-hospital screening program before liver transplantation, were eligible for inclusion in the study. Patients treated with corticosteroids or bisphosphonates before or at time of screening for transplantation were excluded from

the present analysis because of the bone-modifying nature of these therapeutic agents. The Leiden University Medical Centre is a tertiary referral centre for liver diseases, and our first encounter with the majority of patients is often only at time of screening for liver transplantation. Correction of vitamin D deficiency and treatment of osteoporosis with bone-modifying agents such as bisphosphonates has thus been very variable despite clear guidelines, and highly dependent on the clinical judgement of the referring physician, rather than dictated by a specific protocol.

Methods

Demographic, clinical, laboratory and BMD data at time of screening before liver transplantation were obtained from individual patients electronic hospital records. Availability of conventional spinal radiographs was ascertained from the records. Non-vertebral fractures were poorly documented so that data on these fractures could not be analyzed.

1. Demographic data

Data on age, gender, smoking, height, weight and primary liver disease were documented. Data on medication including use of corticosteroids, calcium- and vitamin D supplements and bone modifying agents such as bisphosphonates were recorded.

2. Biochemical investigations

Biochemical data extracted from the patients' records included serum calcium, corrected for an albumin of 40 g/L, phosphate, creatinine, total bilirubin, anticoagulant international normalized ratio (INR), parathyroid hormone (PTH) and 25-hydroxy-vitamin D (25-OHD) concentrations. Severity of liver disease was determined using the laboratory-based Model for End-stage Liver Disease (MELD) score, shown to be correlated with 3-month survival in patients with end-stage liver disease. Scores were calculated on the basis of serum measurements of bilirubin, creatinine and INR in SI units, using the following accepted formula (16):

$$\text{MELD} = 10 * (0.957 * \ln(\text{Creatinine}/88.4)) + (0.378 * \ln(\text{Bilirubin}/17.1)) + (1.12 * \ln(\text{INR})) + 6.43$$

3. Bone mineral density measurements

Bone mineral density (BMD) was measured at the lumbar spine (LS) and at the femoral neck (FN) using dual energy X-ray absorptiometry (Hologic QDR 4500, Hologic inc. Waltham, MA, USA, equipped with reference values based on the National Health and Nutrition Examination Survey (NHANES III)). Data collected included absolute measurements of BMD in g/cm², T-scores (standard deviation above or below that of a young adult reference population at peak bone mass) and Z-scores (standard deviation

above or below that of an age- and sex matched reference population). World Health Organization (WHO) criteria were used to define osteoporosis: T-score of -2.5 or less and osteopenia: a T-score between -1 SD and -2.5 SD.

Due consideration was given to the need to adjust mean BMD scores of the LS in the presence of one or more lumbar vertebral fractures at the site of the LS BMD measurement (L1-L4).

4. Vertebral fracture assessment

Conventional anterior-posterior and lateral radiographs of the thoracic spine and postero-anterior and lateral radiographs of the lumbar spine were performed by an experienced radiology technician using a standardized protocol, at a distance of 115 cm, with the film centralized on Th7 for the thoracic spine and on L3 for the lumbar spine.

All radiographs were blindly assessed for the presence and severity (grade) of vertebral fractures by two independent observers, an experienced musculoskeletal radiologist (H.K.) and an experienced bone and mineral disorders specialist (N.H.). Fractures were assessed using the Genant's semi-quantitative method of vertebral fracture assessment.⁽¹⁷⁾ Using this method, a decrease in height of 20-25% is considered to be a "mild" grade 1 fracture, a decrease of 25-40% a "moderate" grade 2 fracture and a decrease of > 40% a "severe" grade 3 fracture. Uniform loss of vertebral height compared to adjacent vertebrae were additionally documented using the same grading scores. Radiographs were assessed in a random order, using random numbers generated by SPSS software (version 20). A unique number was assigned to each series of radiographs performed at screening for OLT. Radiographs were individually scored by the two independent observers and in case of discrepancy in scores, mostly about fracture grading, consensus was achieved by both observers reviewing the radiographs together. Careful attention was given to the interpretation of vertebral deformities, excluding vertebral height reductions or deformities due to development abnormalities, degenerative changes, osteochondritis, (Scheuermann's disease) or localised metabolic bone disorders such as Paget's disease of bone.

The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre.

Statistical analysis

For descriptive statistics, categorical variables were expressed as numbers and as percentages. Data containing continuous variables were summarized using mean and standard deviation in case of an estimated normal distribution. Median and 5th and 95th percentiles were used otherwise.

A regression model was used to evaluate the influence of potential risk factors for the development of osteoporosis and for the prevalence of vertebral fractures. A univariate

regression model was first used, followed by a multivariate regression model with corrections for multiple variables including age and sex. A linear regression model was used in case of continuous variables and a logistic regression model in case of dichotomous variables.

We chose to calculate odds ratios rather than risk ratios because of the regression models used. A p-value < 0.05 was considered significant.

We evaluated the direct effect of various risk factors, such as cholestatic or alcoholic liver disease, on the outcome parameter, such as prevalence of fractures, as well as the indirect effect of the risk factor on the outcome of through various mediators, such as vitamin D deficiency, using a logistic regression model. We calculated confidence intervals of the effect of mediators using the bootstrap method.

Calculations were performed using STATA/SE 12.0 software (StataCorp LP, TX, USA).

RESULTS

Patients characteristics

In total, 162 of the 201 patients, who received an elective OLT in the 10-year period between 2000 and 2011, who had available BMD measurements and conventional spinal radiographs performed at the time of screening before liver transplantation but who were not treated with bisphosphonates, corticosteroids or other bone-modifying agents were included in the study. Of the 39 excluded patients, 21 had for logistics reasons no

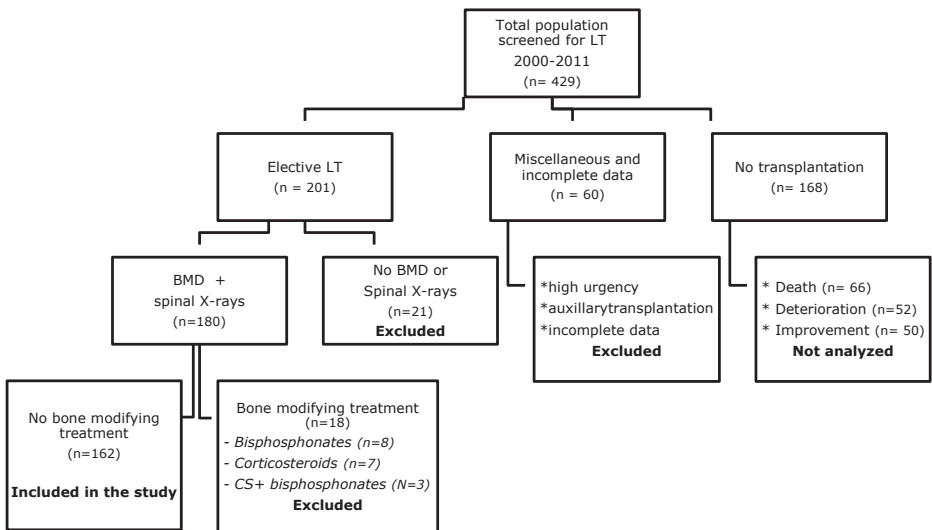


Figure 1: Study Flow Chart; LT = liver transplantation; BMD = bone mineral density; CS = corticosteroids

BMD measurements and/or conventional spine radiographs at time of screening for OLT, and 18 patients were using bone modifying treatments. Details of patients included and excluded are shown in a Study Flow Chart (Figure 1). Median age for the total population studied was 51 years (range 17-69 years), and 122 of the 162 patients were men (75%). Median age for women was also 51 years (range 16 - 66), and 23 women were younger than 50 years. Menopausal status was not documented. Most common liver pathology was viral or alcoholic liver disease (31 and 29% respectively), followed by cholestatic liver disease (14%). 8% of patients were using calcium and vitamin D supplements, usually as a combination preparation at a fixed dose of 500mg calcium and 400 IU cholecalciferol, which had been initiated at some stage before screening for transplantation. Details of patients' characteristics are shown in Table 1.

Table 1 Characteristics of patients at time of screening for liver transplantation

Demographic data	
No of patients	162
Male – no of patients (%)	122 (75%)
Age at the time of screening- Median (5 th - 95 th percentile), years	51 (30-65)
BMI – mean (SD), kg/m ²	26 ± 5
Smoking – no of patients (%)	54 (35%)
Primary liver disease – no of patients (%)	
Viral	50 (31%)
Alcoholic	47 (29%)
Combined alcoholic and viral	4 (2%)
Cholestatic (PSC / PBC/ overlap syndrome)	22 (14%)
Malignancy	8 (5%)
Auto-immune hepatitis	4 (2%)
Metabolic	5 (3%)
Other ^a	22 (14%)
Severity of liver disease- mean lab MELD-score ± SD	14 ± 6
Bone-specific medication – no of patients (%)	
Calcium- and vitamin D supplements	12 (8%)
Vitamin D supplements	2 (1%)

^aOther primary liver disease including cryptogenic cirrhosis, malignant tumor, vascular, rare metabolic disorders; BMI = body mass index; SD = standard deviation; OLT = orthotopic liver transplantation; PSC = primary sclerosing cholangitis; PBC = primary biliary cirrhosis; LS = lumbar spine; FN = femoral neck. MELD = Model for End-stage Liver Disease.

Biochemical data

Mean serum creatinine concentration was 89 ± 47 µmol/L (normal < 104 µmol/L).

Median bilirubin level was 32 µmol/L (range 4-1172, normal < 17 µmol/L). Mean INR was

1.3 ± 0.33 . Mean 25-OHD level was 32 ± 23 nmol/L (normal 50-250 nmol/L). 25-OHD levels were also low, with a mean of 31 ± 24 nmol/L in patients using vitamin D supplements. Of the 162 patients studied 110 patients had 25-OHD levels < 50 nmol/L, of whom 54 patients had levels of < 25 nmol/L. Mean laboratory MELD-score, reflecting severity of liver disease, was 14 ± 6 SD (range 6-35).

BMD measurements

Osteoporosis and osteopenia were prevalent in respectively 19 and 38 % of patients at the LS, and in 11 and 42% of patients at the FN. Mean T-score was -1.15 ± 1.44 at the LS and -1.06 ± 1.23 at the FN. Mean Z-score was -0.63 ± 1.49 at the LS and -0.15 ± 1.21 at the FN. Distribution of BMD at the LS and FN are shown for men and women in Figure 2.

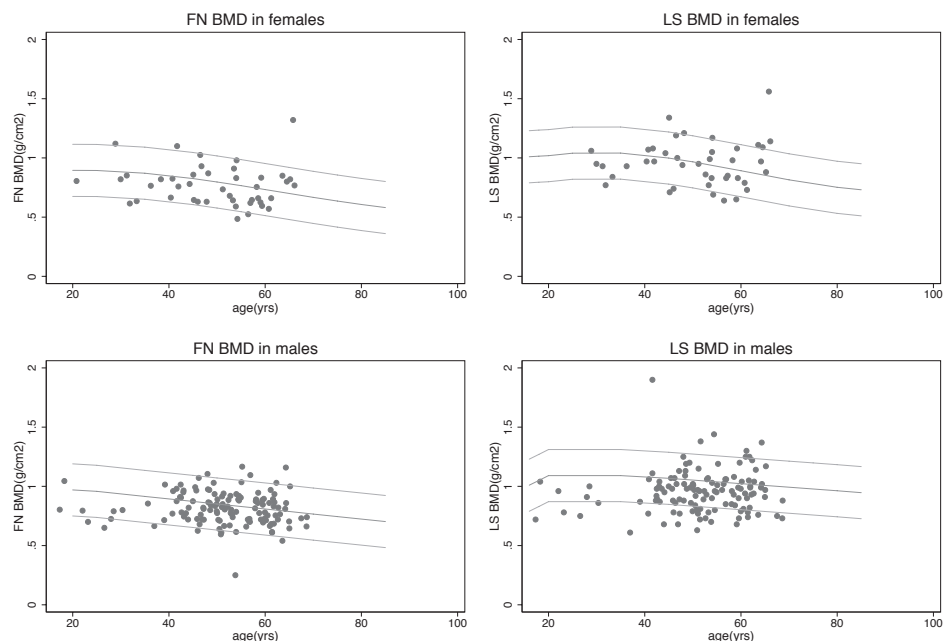


Figure 2 Distribution of bone mineral density in 40 women (upper panel) and 122 men (lower panel) with end-stage liver disease awaiting liver transplantation.

Middle, upper and lower lines represent respectively +1 and -1 standard deviation of an age and sex matched reference population; FN = femoral neck; LS = lumbar spine; BMD = bone mineral density.

Factors associated with a low BMD

We evaluated a number of potential risk factors for low BMD at time of screening for transplantation (Table 2). Age and gender were not associated with increased risk for osteoporosis. Cholestatic liver disease was associated with significant increased risk for osteoporosis at the FN in the univariate model (OR 1.23, 95% CI 1.07-1.41, $p = 0.004$), but

not in the multivariate model. There was no indirect effect of cholestatic liver disease through vitamin D deficiency on low bone mass. (OR 1.00, 95%CI 0.96-1.05). Compared to other liver pathologies, alcoholic liver disease was not associated with an increased risk for low bone mass at the LS in the multivariate model (OR 0.27, 95%CI 0.08-0.87, $p=0.028$), an effect independent of smoking (OR 1.02, 95%CI 0.98-1.06). Severity of liver disease as reflected by the laboratory MELD-score, was associated with an increased risk of low bone mass at the FN in both the univariate and multivariate model (multivariate model: OR 1.15, 95%CI 1.02-1.29, $p = 0.021$), but not at the LS. Vitamin D deficiency (25-OHD levels < 25 nmol/L) was associated with an increased risk for osteoporosis at the LS in the univariate model (OR 1.16, 95%CI 1.01-1.32, $p = 0.029$), but not in the multivariate model or at the FN.

Table 2 Risk factors for osteoporosis

Risk factor	Univariate				Multivariate			
	LS Osteoporosis		FN Osteoporosis		LS Osteoporosis		FN Osteoporosis	
	OR (95%CI)	P-value	OR (95%CI)	P-value	OR (95%CI)	P-value	OR (95%CI)	P-value
Male gender	1.03 (0.89-1.20)	0.660	0.88 (0.79-0.98)	0.025*	1.90 (0.62-5.82)	0.262	0.45 (0.13-1.55)	0.208
Age (years)	1.00 (0.97-1.05)	0.675	1.00 (0.95-1.06)	0.885	1.03 (0.98-1.08)	0.216	0.99 (0.93-1.05)	0.726
Smoking	1.05 (0.92-1.20)	0.446	0.93 (0.85-1.03)	0.153	1.78 (0.69-4.60)	0.234	0.65 (0.15-2.77)	0.562
Cholestatic liver diseases	1.17 (0.97-1.42)	0.106	1.23 (1.07-1.41)	0.004*	1.38 (0.37-5.26)	0.631	2.00 (0.45-8.85)	0.363
Alcoholic liver disease	0.89 (0.77-1.02)	0.086	0.91 (0.82-1.01)	0.070	0.27 (0.08-0.87)	0.028*	0.38 (0.07-2.16)	0.276
Vitamin D deficiency ^a	1.16 (1.01-1.32)	0.029*	1.01 (0.91-1.12)	0.827	2.10 (0.83-5.28)	0.116	0.73 (0.19-2.77)	0.645
Bilirubin level (mmol/L)	1.00 (1.00-1.00)	0.676	1.00 (1.00-1.00)	0.487				
Serum creatinine (μmol/L)	1.00 (0.99-1.01)	0.930	1.01 (1.00-1.01)	0.151	1.00 (0.99-1.01)	0.505	1.00 (0.99-1.01)	0.896
Lab MELD-score	1.032 (0.976-1.0910)	0.531	1.10 (1.02-1.19)	0.012*	1.07 (0.97-1.19)	0.186	1.15 (1.02-1.29)	0.021*

* significant effect, p -value < 0.05; ^avitamin D deficiency 25-OHD level < 25 nmol/L; MELD = Model for End-stage Liver Disease

Prevalence of radiographic vertebral fractures

We documented 186 vertebral fractures in 90 patients, representing a prevalence of 56%. 43 patients (44%) had one prevalent fracture, 25 had two fractures (15%), and 22 had three or more fractures (14%). Over 50% of patients had one or more mild grade 1 fracture (52%), of whom eleven patients (10%) also had moderate grade 2 fractures. None of the patients studied had severe grade 3 vertebral fractures. The anatomical distribution of the fractures is shown in Figure 3; 47 patients had lumbar vertebral

fractures, of whom 32 had one fracture, 24 of which of the first lumbar vertebra. Five patients had 2 lumbar fractures, 2 patients 3, and 2 patients had fractures of all 4 lumbar vertebrae. The majority of these fractures were single grade 1 fractures, with no significant impact on total LS BMD, so that they were not excluded from the analysis of total BMD.

Mid-thoracic compression fractures, usually involving 2-3 vertebrae, most commonly thoracic vertebrae 7 to 9, were prevalent in 7 patients (6%).

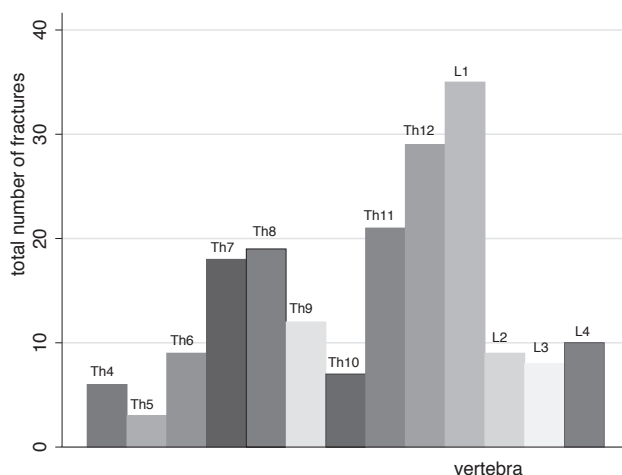


Figure 3 Distribution of 186 vertebral fractures in 90 patients with end-stage liver failure awaiting liver transplantation; Th = thoracic vertebra, L= lumbar vertebra.

Factors associated with radiographic vertebral fractures

We found no significant relationship between BMD and prevalent vertebral fractures using either univariate or multivariate analysis. In patients with prevalent fractures, 38% had a normal BMD, 40 % had osteopenia and only 23% had osteoporosis. There was no significant association between BMD as a continuous variable, the presence or absence of low BMD, and the presence or absence of vertebral fractures, number of fractures per individual patient or grade of severity of the fractures.

Male gender was associated with a higher prevalence of vertebral fractures than female gender, with an odds ratio of 4.10 (95% CI 1.73-9.67, $p = 0.001$), independently of age, underlying disease pathology or severity of liver disease as expressed by the laboratory MELD score (Table 3). Age as a continuous parameter, vitamin D status, underlying liver disease and severity of liver disease were not associated with an increased prevalence of vertebral fractures.

Table 3 Risk factors for vertebral fractures

Risk factor	Univariate		Multivariate	
	OR (95%CI)	P-value	OR (95%CI)	P-value
Male gender	1.36 (1.14-1.62)	0.001*	4.10 (1.73-9.67)	0.001*
Age (years)	1.02 (0.99-1.06)	0.136	1.03 (0.99-1.07)	0.093
Smoking	0.91 (0.77-1.08)	0.266	0.64 (0.30-1.38)	0.256
Cholestatic liver diseases	0.76 (0.61-0.95)	0.016*	0.33 (0.10-1.12)	0.076
Alcoholic liver disease	1.07 (0.86-1.42)	0.443	0.80 (0.35-1.86)	0.607
Lab MELD-score	1.01 (0.96-1.07)	0.712	1.03 (0.96-1.11)	0.361
LS BMD (g/cm ²)	1.34 (0.21-8.21)	0.755	0.78 (0.10-5.96)	0.808

* significant effect, p –value <0.05; MELD = Model for End-stage Liver Disease; BMD = bone mineral density; LS = lumbar spine

DISCUSSION

Our data from this retrospective cohort study suggest a high prevalence of vertebral fractures, mostly grade 1 fractures, in 56% of liver transplant recipients at time of screening for transplantation, independently of BMD measurements, although low bone mass, predominantly osteopenia, was documented in about two thirds of these patients. At time of screening for liver transplantation, there was, thus, no association between BMD as a continuous variable or the presence or absence of low BMD and the presence or absence of vertebral fractures, higher number of fractures per individual patient or higher grade of fractures. Of all factors other than BMD potentially associated with increased fracture risk, only male gender was associated with increased fracture prevalence, independently of age, underlying liver disease or severity of liver failure. The absence of a significant association between BMD and fracture prevalence suggests that factors affecting bone quality, rather than just bone quantity, may significantly contribute to fracture risk in patients with end-stage liver failure.

Our findings are in keeping with previously published studies, which report low bone mass in over 50% of patients awaiting liver transplantation, with the majority having osteopenia and 20-40% osteoporosis.(2;5;7;8;10;11;18-21) A high prevalence of osteoporosis and fractures reported in patients with cholestatic liver disease (10-12) is believed to be due to vitamin D deficiency and/or to the toxic effects of accumulated metabolites of cholestasis on bone remodelling. In our cohort, cholestatic liver disease also represented an increased risk for osteoporosis at the FN and for prevalent vertebral fractures in univariate but not multivariate analysis. We did not observe an increased prevalence of vertebral fractures in patients with alcoholic liver disease, possibly because of the removal of the inhibitory effect of alcohol on osteoblast function by the prerequisite alcohol abstinence for at least six months prior to screening for

transplantation, which may have also contributed to the improvement in BMD at time of screening.(22)

The high prevalence of vertebral fractures of 56% observed in our cohort at time of screening before transplantation is much higher than the overall prevalence of fractures of 7% observed in a recent Dutch population-based cohort of men and women aged > 55 years, in which all subjects were screened by conventional spinal X-rays.(23) The observed prevalence of vertebral fractures in our study was also higher than that of previously published studies, which showed a widely variable and overall lower prevalence of these fractures (3 to 36%), in patients awaiting liver transplantation (1-3;5;7;8;19;24). The main reason for this discrepancy in prevalence of vertebral fractures between our study and that of others probably lies in the method used for the identification of these fractures. Since vertebral fractures are often silent, actual fracture prevalence may have been underestimated in studies in which spinal radiographs were only performed in case of clinical suspicion of a vertebral fracture. (5;8;25;26) In contrast, conventional radiographs of the spine formed an integral part of the pre-transplantation work-up of our candidates for liver transplantation, and were consequently available in all but a few patients. In our study, we also judged it of clinical relevance to report grade 1 fractures, whereas mild grade fractures might have been underreported in previous studies. This is partly because radiologists have been generally reluctant worldwide to report mild vertebral fractures, so that relying on radiology reports for documentation of these fractures may have resulted in an underestimate of their true prevalence.(27;28) Mild grade fractures may have also been consciously underreported because of doubt about their clinical relevance, although these mild fractures have also now been shown to be associated with increased fracture risk.(15)

The relationship between BMD and prevalent fractures has been poorly studied in patients awaiting liver transplantation, and available data are not concordant. Some studies thus show an association between low BMD and prevalence of vertebral fractures (1;29), but other studies found no such association.(3;30) In our study, there was intriguingly no association between BMD as a continuous variable or as low vs normal BMD and fracture prevalence. 33% of patients with fractures had thus normal BMD and 35% had osteopenia, suggesting that factors affecting bone quality independently of BMD may significantly contribute to increased skeletal fragility in patients with advanced liver disease. This may have significant implications in the management of skeletal complications of advanced liver disease before and after liver transplantation, as tools to measure bone quality in vivo such as micro-indentation are just emerging and remain to be validated, and data on means to specifically improve bone quality with a view to decrease fracture risk are as yet to be established.

Notwithstanding, the accurate identification of prevalent vertebral fractures is essential in fracture risk assessment, as these fractures represent one of the strongest predictor of subsequent vertebral and non-vertebral osteoporotic fractures.(31;32) There is also evidence that mild-grade 1 vertebral fractures are also predictive for future fracture risk.(15) Of further clinical relevance is that up to 50% of vertebral fractures, even high grade ones, may be asymptomatic (33), and not necessarily associated with a decrease in BMD, so that they may not come to the attention of treating physicians. The small number of patients treated with bisphosphonates despite the high prevalence of osteoporosis and vertebral fractures and the overall poor vitamin D status of patients awaiting liver transplantation underscores the clinically unmet need for treating physicians to address the issue of skeletal health in patients with end-stage liver disease.

Our study has strengths as well as limitations. Its major strength lies in the evaluation of consecutive transplant recipients at time of screening for transplantation, in one center, over a 10-year period, providing a representative pre-transplantation cohort, with available BMD measurements and conventional radiographs of the spine in all but a few patients. Another strength of our study is the blinded assessment of all spinal radiographs for vertebral fractures by two experienced independent observers using the Genant's standardized semi-quantitative method advocated by the International Osteoporosis Foundation (IOF) for evaluation of vertebral fractures in the clinic (34), rather than relying on routinely obtained radiology reports shown to often underestimate the true prevalence of fractures. (25) In most other studies in liver transplantation so far reported, (semi-)quantitative assessments were performed by one observer (1;7;29;30) or the method of evaluation of vertebral fractures is not mentioned (5;6;19;35). A further strength of our study is the inclusion of the mild grade 1 vertebral fractures in the evaluation of prevalent fractures.

A limitation of our study is its retrospective study design. However, the laboratory, radiological and densitometric investigations were not randomly undertaken, but formed an integral part of a pre-planned pre-transplantation screening protocol for skeletal complications, performed in patients with end-stage liver failure, who in this case went on to receive a liver transplantation. Although most candidates for liver transplantation were screened for skeletal complications, we only studied those who subsequently received a liver transplant, which may have led to an underestimate of the true prevalence of skeletal pathology, since the excluded patients would have been more likely to have had the more severe liver disease and thus potentially the more severe skeletal complications. We also chose to exclude patients using corticosteroids and /or bisphosphonates, which could have also led to the exclusion of patients with the worst skeletal pathology. However, since this was the case in a relatively small number of patients, this is unlikely to have affected the outcome of our study. The

absence of prior spinal radiographs precluded the identification of the precise time of occurrence of a documented fracture, resulting in the association between BMD and prevalent fractures being potentially limited by the time lag between the development of a fracture and BMD measurement. We could collect no reliable data on non-vertebral fractures, as these were poorly documented. However, previous studies report a generally low incidence of these fractures in end-stage liver disease.(2;5;6;8;19;29)

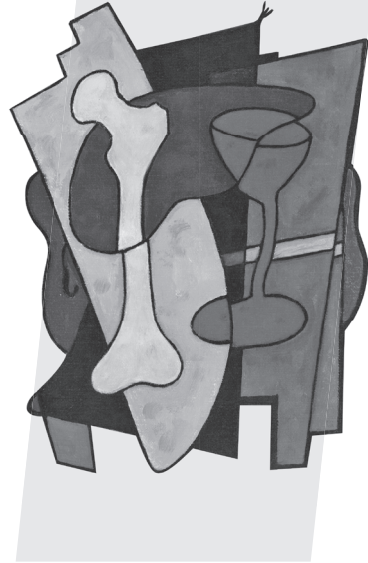
In conclusion, our finding of increased fracture risk independently of BMD in liver transplant recipients at time of screening for transplantation, adds to the accumulating body of evidence of the contributory role of bone quality rather than just bone quantity to fracture risk in patients with liver disease. This finding has significant clinical implications in the evaluation and management of prospective liver transplant recipients, as it suggests that spinal radiographs should be routinely performed in these patients to enable identification of vertebral fractures and thus of skeletal fragility. Whether this pre-transplantation skeletal fragility persists, worsens or improves after liver transplantation, and whether bone fragility could be modulated by available bone-modifying agents to decrease or prevent skeletal morbidity after transplantation remains to be established by long-term studies specifically addressing the issue of skeletal health in liver transplant recipients.

REFERENCE LIST

1. Guichelaar MM, Schmoll J, Malinchoc M, Hay JE. Fractures and avascular necrosis before and after orthotopic liver transplantation: long-term follow-up and predictive factors. *Hepatology* 2007; 46(4):1198-1207.
2. Leidig-Bruckner G, Hosch S, Dodidou P, Ritschel D, Conradt C, Klose C, Otto G, Lange R, Theilmann L, Zimmerman R, Pritsch M, Ziegler R. Frequency and predictors of osteoporotic fractures after cardiac or liver transplantation: a follow-up study. *Lancet* 2001; 357(9253):342-347.
3. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, Ordi J, Rimola A, Rodes J, Munoz-Gomez J. Bone disease after liver transplantation: a long-term prospective study of bone mass changes, hormonal status and histomorphometric characteristics. *Osteoporos Int* 2001; 12(6):484-492.
4. Alcalde VA, Pascasio Acevedo JM, Gutierrez D, I, Garcia JR, Sousa Martin JM, Ferrer Rios MT, Sayago MM, Giraldez GA, Gomez Bravo MA. Prevalence and characteristics of bone disease in cirrhotic patients under evaluation for liver transplantation. *Transplant Proc* 2012; 44(6):1496-1498.
5. Carey EJ, Balan V, Kremers WK, Hay JE. Osteopenia and osteoporosis in patients with end-stage liver disease caused by hepatitis C and alcoholic liver disease: not just a cholestatic problem. *Liver Transpl* 2003; 9(11):1166-1173.
6. Guichelaar MM, Malinchoc M, Sibonga JD, Clarke BL, Hay JE. Bone histomorphometric changes after liver transplantation for chronic cholestatic liver disease. *J Bone Miner Res* 2003; 18(12):2190-2199.
7. Monegal A, Guanabens N, Suarez MJ, Suarez F, Clemente G, Garcia-Gonzalez M, De la Mata M, Serrano T, Casafont F, Tome S, Barrios C, Navasa M. Pamidronate in the prevention of bone loss after liver transplantation: a randomized controlled trial. *Transpl Int* 2009; 22(2):198-206.
8. Premaor MO, Das TK, Debiram I, Parker RA, Ninkovic M, Alexander GT, Compston JE. Fracture incidence after liver transplantation: results of a 10-year audit. *QJM* 2011; 104(7):599-606.
9. Hardinger KL, Ho B, Schnitzler MA, Desai N, Lowell J, Shenoy S, Chapman W, Crippin JS. Serial measurements of bone density at the lumbar spine do not predict fracture risk after liver transplantation. *Liver Transpl* 2003; 9(8):857-862.
10. Bai XL, Liang TB, Wu LH, Li DL, Geng L, Wang WL, Shen Y, Zhang M, Zheng SS. Elevation of intact parathyroid hormone level is a risk factor for low bone mineral density in pretransplant patients with liver diseases. *Transplant Proc* 2007; 39(10):3182-3185.
11. Guichelaar MM, Kendall R, Malinchoc M, Hay JE. Bone mineral density before and after OLT: long-term follow-up and predictive factors. *Liver Transpl* 2006; 12(9):1390-1402.
12. Trautwein C, Possienke M, Schlitt HJ, Boker KH, Horn R, Raab R, Manns MP, Brabant G. Bone density and metabolism in patients with viral hepatitis and cholestatic liver diseases before and after liver transplantation. *Am J Gastroenterol* 2000; 95(9):2343-2351.
13. Gonzalez-Reimers E, Alvisa-Negrin J, Santolaria-Fernandez F, Ros-Vilamajo R, Martin-Gonzalez MC, Hernandez-Betancor I, Garcia-Valdecasas-Campelo E, Gonzalez-Diaz A. Prognosis of osteopenia in chronic alcoholics. *Alcohol* 2011; 45(3):227-238.
14. Klotzbuecher CM, Ross PD, Landsman PB, Abbott TA, III, Berger M. Patients with prior fractures have an increased risk of future fractures: a summary of the literature and statistical synthesis. *J Bone Miner Res* 2000; 15(4):721-739.
15. Siris ES, Genant HK, Laster AJ, Chen P, Misurski DA, Kregge JH. Enhanced prediction

- of fracture risk combining vertebral fracture status and BMD. *Osteoporos Int* 2007; 18(6):761-770.
16. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; 33(2):464-470.
 17. Genant HK, Wu CY, van KC, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. *J Bone Miner Res* 1993; 8(9):1137-1148.
 18. Dolgos S, Hartmann A, Isaksen GA, Simonsen S, Bjortuft O, Boberg KM, Bollerslev J. Osteoporosis is a prevalent finding in patients with solid organ failure awaiting transplantation - a population based study. *Clin Transplant* 2010; 24(5):E145-E152.
 19. Millonig G, Graziadei IW, Eichler D, Pfeiffer KP, Finkenstedt G, Muehllechner P, Koenigsrainer A, Margreiter R, Vogel W. Alendronate in combination with calcium and vitamin D prevents bone loss after orthotopic liver transplantation: a prospective single-center study. *Liver Transpl* 2005; 11(8):960-966.
 20. Ninkovic M, Love SA, Tom B, Alexander GJ, Compston JE. High prevalence of osteoporosis in patients with chronic liver disease prior to liver transplantation. *Calcif Tissue Int* 2001; 69(6):321-326.
 21. Sokhi RP, Anantharaju A, Kondaveeti R, Creech SD, Islam KK, Van Thiel DH. Bone mineral density among cirrhotic patients awaiting liver transplantation. *Liver Transpl* 2004; 10(5):648-653.
 22. Peris P, Pares A, Guanabens N, Del RL, Pons F, Martinez de Osaba MJ, Monegal A, Caballeria J, Rodes J, Munoz-Gomez J. Bone mass improves in alcoholics after 2 years of abstinence. *J Bone Miner Res* 1994; 9(10):1607-1612.
 23. Van der Klift M, De Laet CE, McCloskey EV, Hofman A, Pols HA. The incidence of vertebral fractures in men and women: the Rotterdam Study. *J Bone Miner Res* 2002; 17(6):1051-1056.
 24. Ninkovic M, Love S, Tom BD, Bearcroft PW, Alexander GJ, Compston JE. Lack of effect of intravenous pamidronate on fracture incidence and bone mineral density after orthotopic liver transplantation. *J Hepatol* 2002; 37(1):93-100.
 25. Floreani A, Fries W, Luisetto G, Burra P, Fagioli S, Boccagni P, Della Rovere GR, Plebani M, Piccoli A, Naccarato R. Bone metabolism in orthotopic liver transplantation: a prospective study. *Liver Transpl Surg* 1998; 4(4):311-319.
 26. Pennisi P, Trombetti A, Giostra E, Mentha G, Rizzoli R, Fiore CE. Pamidronate and osteoporosis prevention in liver transplant recipients. *Rheumatol Int* 2007; 27(3):251-256.
 27. Delmas PD, van de Langerijt L, Watts NB, Eastell R, Genant H, Grauer A, Cahall DL. Underdiagnosis of vertebral fractures is a worldwide problem: the IMPACT study. *J Bone Miner Res* 2005; 20(4):557-563.
 28. Kim N, Rowe BH, Raymond G, Jen H, Colman I, Jackson SA, Siminoski KG, Chahal AM, Folk D, Majumdar SR. Underreporting of vertebral fractures on routine chest radiography. *AJR Am J Roentgenol* 2004; 182(2):297-300.
 29. Giannini S, Nobile M, Dalle CL, Ciuffreda M, Germoni V, Iemmolo RM, Gerunda GE, Sartori L, Crepaldi G. Vertebral morphometry by X-ray absorptiometry before and after liver transplant: a cross-sectional study. *Eur J Gastroenterol Hepatol* 2001; 13(10):1201-1207.
 30. Ninkovic M, Skingle SJ, Bearcroft PW, Bishop N, Alexander GJ, Compston JE. Incidence of vertebral fractures in the first three months after orthotopic liver transplantation. *Eur J Gastroenterol Hepatol* 2000; 12(8):931-935.
 31. Black DM, Arden NK, Palermo L, Pearson J, Cummings SR. Prevalent vertebral deformities predict hip fractures and new vertebral deformities but not wrist fractures. Study of Osteoporotic Fractures Research Group. *J Bone Miner Res* 1999; 14(5):821-828.
 32. Melton LJ, III, Atkinson EJ, Cooper C, O'Fallon WM, Riggs BL. Vertebral fractures predict

- subsequent fractures. *Osteoporos Int* 1999; 10(3):214-221.
33. Melton LJ, III, Lane AW, Cooper C, Eastell R, O'Fallon WM, Riggs BL. Prevalence and incidence of vertebral deformities. *Osteoporos Int* 1993; 3(3):113-119.
 34. Hernlund E, Svedbom A, Ivergard M, Compston J, Cooper C, Stenmark J, McCloskey EV, Jonsson B, Kanis JA. Osteoporosis in the European Union: medical management, epidemiology and economic burden : A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch Osteoporos* 2013; 8(1-2):136.
 35. Meys E, Fontanges E, Fourcade N, Thomasson A, Pouyet M, Delmas PD. Bone loss after orthotopic liver transplantation. *Am J Med* 1994; 97(5):445-450.



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Longitudinal changes in BMD and fracture risk in orthotopic liver transplant recipients not using bone modifying treatment

Charlotte G. Krol, Olaf M. Dekkers, Herman M. Kroon, Ton J. Rabelink, Bart van Hoek, Neveen A.T. Hamdy

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ABSTRACT

Introduction Osteoporosis is prevalent in end-stage liver disease, but data on long-term changes in bone mineral density (BMD) and related fracture incidence are scarce after orthotopic liver transplantation (OLT).

Method We evaluated BMD changes up to 5 years in consecutive recipients of a successful OLT at the Leiden University Medical Centre between 2000 and 2011, in whom sequential BMD data were available. Spinal radiographs, available at time of screening and at six and twelve months post-OLT, were assessed for vertebral fractures by two independent observers using Genant's semi-quantitative method. Patients were excluded when started on bisphosphonates.

Results 201 patients (71% men), median age 53 years (range 18-70 years) were included in the study. Most common liver pathology was viral (27%) or alcoholic liver disease (25%). All patients received prednisone for at least 6 months after transplantation and the majority either tacrolimus or cyclosporine for immunosuppression. At time of screening for OLT, osteoporosis and osteopenia were found in respectively 18 and 36 % of patients at the lumbar spine (LS) and in respectively 9 and 42 % at the femoral neck (FN). T-scores declined significantly at both sites 6 months after OLT, but increased thereafter at the LS, reaching pre-transplant values at 2 years, and remaining stable thereafter. FN T-scores remained consistently lower than pre-transplant values. The prevalence of vertebral fractures increased from 56% at screening to 71% at one year after OLT, with a fracture incidence of 34%. BMD changes did not predict fracture risk.

Conclusion Osteoporosis, osteopenia and vertebral fractures are prevalent in patients with end-stage liver disease. An overall decline in BMD is observed within the first 6 months after OLT, with subsequent recovery to pre-transplant values at the LS, but not at the FN. Vertebral fracture risk is high after OLT regardless of changes in BMD.

INTRODUCTION

Osteoporosis is reported to be prevalent in end-stage liver disease, and accelerated bone loss associated with increased fracture risk has been observed in 25-35% of patients in the first twelve months after orthotopic liver transplantation (OLT).⁽¹⁻¹¹⁾ The initial decline in bone mineral density (BMD), believed to be predominantly due to corticosteroid use for immunosuppression, is generally followed by a stabilization in bone mass between 6 and 12 months after OLT, with no further decrease in BMD thereafter, independently of the use of bone-modifying agents such as bisphosphonates.^(1;3-6;8;9;12) Data on natural changes in BMD beyond the first year after OLT are scarce, however, and mainly based on small studies, or on studies in patients with exclusive cholestatic liver disease as underlying liver pathology.^(1-9;12-16) Studies had also been performed over a decade ago ^(1-5;13-15), before the introduction of currently used immunosuppressive regimens, which enable the use of significantly lower corticosteroid doses post-OLT, thus precluding exposure to a high cumulative dose of these agents. Whether the spontaneous recovery of BMD within the first year after OLT is also associated with a decreased fracture risk has also not been adequately addressed.

The aims of this study were to examine changes in BMD after OLT in a cohort of consecutive liver transplant recipients not receiving treatment with anti-resorptive agents, to identify risk factors for bone loss after successful OLT and to examine the influence of changes in BMD on fracture risk within the first year after OLT.

PATIENTS AND METHODS

Patients

Consecutive patients who underwent a first OLT at the Leiden University Medical Centre between January 1st 2000 and January 1st 2011, and who had sequential bone mineral density (BMD) measurements after OLT, were eligible for the study, including 20 patients who had BMD measurements from 6 months onwards after OLT. Immunosuppressive regimens for OLT consisted of corticosteroids for at least 6 months in all patients, with an additional calcineurin inhibitor: cyclosporine or tacrolimus in the majority, with mofetil mycophenolate (MMF) or sirolimus as additional second or third immunosuppressive agent in a few. International guidelines concerning immunosuppression were complied with in the case of new transplanted patients, but established immunosuppressive regimen were not routinely altered in individual transplant patients. Corticosteroid schedules included methylprednisolone at a dose of 500 mg given during the OLT procedure, followed by oral prednisolone at a dose

of 20 mg daily for one week, 10 mg daily for 3 months, slowly tapering to complete discontinuation of corticosteroids between 3 and 6 months after OLT in all but a few patients who required maintenance doses of prednisolone of 2.5 – 10 mg/day. Treatment with calcium- and vitamin D supplements in a combined fixed dose of 500 mg elemental calcium and 400 IU colecalciferol, and treatment with bisphosphonates were initiated at the discretion of the treating physician. Patients treated with bisphosphonates before or at time of screening for OLT were excluded from the study. Patients receiving bisphosphonates after OLT were censored and thus excluded from further analysis from time of starting treatment.

Methods

Demographic and clinical data

Patients' demographic and clinical data were obtained from electronic hospital records. Data on age, gender, smoking, height, weight and primary liver disease were collected. Concomitant or later renal transplantation was documented. Data were also collected on date and cause of death. Recorded medication included immunosuppressives, calcium and vitamin D supplements and bone-modifying medication such as bisphosphonates.

Laboratory investigations

Laboratory data were also obtained from electronic hospital records. Biochemical data collected included serum calcium, corrected for an albumin of 40 g/L, phosphate, creatinine, parathyroid hormone (PTH) and 25-hydroxy-vitamin D (25-OHD) concentrations, which were measured at screening for OLT and at various time points thereafter. Severity of liver disease was determined using the laboratory- Model for End-stage Liver Disease (MELD) scores(17) calculated on the basis of serum creatinine, bilirubin and INR measurements obtained at time of screening for OLT. The following accepted formula was used to calculate the scores:

$$MELD = 10 * (0.957 * \ln(Creatinine/88.4)) + (0.378 * \ln(Bilirubin/17.1)) + (1.12 * \ln(INR))) + 6.43$$

Bone mineral density measurements

Bone mineral density (BMD) was measured at the lumbar spine and at the femoral neck using dual energy X-ray absorptiometry (DXA- Hologic QDR 4500, Hologic inc. Waltham, MA, USA, equipped with reference values based on the National Health and Nutrition Examination Survey (NHANES III)). Absolute measurements of BMD in g/cm² as well as T-scores (matched to young adult reference populations at peak bone mass) and Z-scores (age- and sex-matched reference populations) were recorded. World

Health Organization (WHO) criteria were used to define osteoporosis (T-score of -2.5 SD or less) and osteopenia (T-score between -1 SD and -2.5 SD). BMD measurements were performed at time of screening for OLT (baseline), at 6 and 12 months post-OLT, and yearly thereafter for up to 60 months after OLT. BMD measurements at 36 and 48 months post-OLT were not analyzed because too few measurements were available at these time points.

Vertebral fracture assessment

Spinal radiographs were routinely performed at time of screening and at six and twelve months after OLT. Conventional antero-posterior and lateral radiographs of the thoracic spine and postero-anterior and lateral radiographs of the lumbar spine were performed by an experienced radiology technician following a standardized protocol, at a distance of 115 cm, with the film centralized on Th7 for the thoracic spine and on L3 for the lumbar spine.

All conventional radiographs of the thoracic and lumbar spine were blindly assessed for the prevalence and individually scored for the severity (grade) of vertebral fractures by two independent observers, an experienced musculoskeletal radiologist (H.K.) and an experienced bone and mineral disorders specialist (N.H.). Vertebral fractures were assessed using the Genant's semi-quantitative method.⁽¹⁸⁾ Using this method, a decrease in height of 20-25% is considered to be a "mild" grade 1 fracture, of 25-40% a "moderate" grade 2 fracture and of $> 40\%$ a "severe" grade 3 fracture. Uniform loss of vertebral height compared to adjacent vertebrae was additionally documented using the same grading scores. Radiographs were assessed in a random order, using random numbers generated by SPSS software (version 20, SPSS inc., Chicago, IL, USA). A unique number was assigned to each series of radiographs. In case of discrepancy in scores, consensus was achieved by both observers reviewing the radiographs together.

Laboratory, BMD and fracture data collected at time of screening for OLT were considered to be baseline data.

The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre.

Statistical analysis

For descriptive statistics, categorical variables were expressed as numbers and as percentages. Continuous variables were summarized using mean and standard deviation in case of normal distribution of data; median and 5th and 95th percentiles were used otherwise. An extension of a Generalized Linear Model, Generalized Estimation Equations (GEE), was used for calculation of differences in laboratory measurements and BMD, to account for repeated measurements. Patients treated with bisphosphonates

were excluded from time of initiation of treatment for the duration of follow-up. Incidence rates for osteoporosis after OLT (number of new cases/person years at risk) were calculated with follow-up time starting after transplantation. Patients already diagnosed as having osteoporosis at time of screening for OLT were not included in the calculation of osteoporosis incidence. An incident fracture was defined as the occurrence of a new vertebral fracture or an increase in grade of a prevalent fracture between serial radiographs before and during the first year after OLT. Follow up duration until incident osteoporosis, or last measurement of BMD, was used to calculate person-years at risk. GEE model was used to evaluate the influence of various factors on changes in BMD, corrected for age and gender and accounting for repeated measurements.

Prevalence of fractures was calculated by dividing the number of patients with at least one vertebral fracture by the total number of patients with available spinal radiographs. A GEE model was used to evaluate the influence of change of BMD on fracture risk, corrected for age and gender and accounting for repeated measurements. A univariate and multivariate logistic regression model was used to evaluate risk factors for incident fractures.

Calculations were performed using STATA/SE 12.0 software (Stata Corp LP, College Station, TX, USA).

RESULTS

A. Baseline data

Demographic characteristics

Nine of the 223 eligible transplant recipients were not included in the study because bone densitometry data were not available after OLT and thirteen patients were also not included because of treatment with bisphosphonates before or at time of screening for OLT.

Of the 201 patients included in the study, 142 were men (71%). Median age at time of OLT was 53 years (range 18-70 years, with only 4 patients being younger than 20 years). The most common primary liver diseases were viral hepatitis (27%) and alcoholic liver disease (25%), followed by cholestatic liver disease (14%) (Table 1). At time of screening for OLT, 9 patients (5%) were using corticosteroids, and calcium- and vitamin D supplements were prescribed as combination treatment to 21 patients (10%).

Laboratory data

Mean creatinine level was $92 \pm 67 \mu\text{mol/L}$ (normal $< 104 \mu\text{mol/L}$). Mean vitamin D level was $32 \pm 23 \text{ nmol/L}$ (normal 50-250 nmol/L), 125 patients (83%) had 25-OHD levels < 50

Table Demographic data at time of screening and after liver transplantation

Demographic data	
Number of patients	201
Gender – number of male patients (%)	142 (71%)
Age at the time of OLT- Median (range), years	53 (18-70)
Death during follow-up – no of patients (%)	34 (17%)
Time of death after OLT – Median (5 th - 95 th percentile), month	10 (1-57)
BMI – mean (SD), kg/m ²	26 (5)
Smoking – number of patients (%)	64 (34%)
Primary liver disease – number of patients (%)	
Viral	54 (27%)
Alcoholic	51 (25%)
Combined alcoholic and viral	4 (2%)
Cholestatic (PSC / PBC/ overlap syndrome)	29 (14%)
Malignancy	12 (6%)
Auto-immune hepatitis	10 (5%)
Metabolic	7 (3%)
Other*	34 (17%)
Previous or concomitant renal transplantation - number of patients (%)	6 (3%)
Time between screening and OLT - median (5 th – 95 th percentile), months	9 (1-27)
Second OLT - number of patients (%)	28 (14%)
- time to second OLT – median (5 th – 95 th percentile), days	165 (2-1064)
Rejection episodes – number of episodes (cumulative incidence, %)	
6 months after OLT	27 (14%)
12 months after OLT	30 (15%)
24 months after OLT	42 (22%)
60 months after OLT	42 (22%)
Calcium – and vitamin D supplements at time of screening– - number of patients (%)	21 (10%)
Bisphosphonates after OLT - number of patients (%)	
12 months	58 (32%)
24 months	66 (43%)
60 months	42 (48%)
Corticosteroids at time of screening - number of patients (%)	9 (5%)
Immunosuppressive medication initiated at OLT - number of patients (%)	
Prednisone, tacrolimus	88 (44%)
Prednisone, cyclosporine	44 (22%)
Prednisone, cyclosporine and MMF	21 (10%)
Prednisone, tacrolimus and MMF	18 (9%)
Other	30 (15%)

OLT = orthotopic liver transplantation; BMI = body mass index; PSC = primary sclerosing cholangitis; PBC = primary biliary cirrhosis; LS = lumbar spine; FN = femoral neck; MMF = mycophenolate mofetil. * including cryptogenic cirrhosis, Budd-Chiari syndrome, portal vein thrombosis, hereditary polycystic liver-disease, acute liver failure of pregnancy, medically induced cirrhosis, Caroli disease, cystic fibrosis, Rendu-Osler-Weber's disease.

nmol/L, 87 of whom had levels of <30 nmol/L. Mean bilirubin level was 79 ± 140 mmol/L (normal < 17 mmol/L).

Severity of liver disease

Severity of liver disease at screening for OLT, as expressed by laboratory-MELD score, could be calculated in 196 of the 201 patients (98%). Mean score was 14 ± 6 (range 6-35).

BMD data

BMD measurements were available in 181 patients at time of screening for OLT, but technically non- evaluable in 11 patients at the lumbar spine. Evaluation was precluded in these 11 patients by the presence of residual oral contrast material from abdominal CT-scans, required as part of the screening protocol for OLT, but chronologically performed by error before DXA. Osteoporosis and osteopenia were respectively found in 18% and 36% of patients at the lumbar spine (LS) and in 9 and 42 % of patients at the femoral neck (FN).

Fracture data

176 patients had available conventional spinal radiographs at time of screening for OLT. 99 patients (56 %) had one or more vertebral fractures, mostly grade 1 fractures (87%). Of the patients with documented prevalent vertebral fractures, 20 had osteoporosis (22%), 38 osteopenia (42%) and 41 had a normal BMD (36%).

B. Data after OLT

Clinical data

Median time between screening and OLT was 9 months (range 0-66 months, 5th – 95th percentile 1-27 months). All patients received prednisone during the first 6 months after OLT. Prednisone was combined with tacrolimus in 44% of patients or with tacrolimus and MMF in 9% of patients. Cyclosporine was given to 22% of patients and combined with MMF in 10% of patients. Rejection episodes occurred mainly in the first six months after OLT (27 patients) with a cumulative incidence of 22% at five years after OLT. A second OLT was performed in 28 patients (14%) after a median time of 7 months (range 0-37 months). Calcium and vitamin D supplements were increasingly prescribed after OLT with 26 patients (14%) receiving these agents at screening, 107 (58%) at one year after OLT and 68 patients (77%) at 5 years after OLT. Within the first year after OLT, bisphosphonate treatment was initiated in 58 patients (32%), and 42 patients (48%) were using these agents at 5 years after OLT. Median time of initiation of bisphosphonates was 12 months after OLT (range 12-48 months).

Six patients (3%) received a previous or concomitant renal transplantation. Thirty four patients (17%) died during the follow-up period, at a median time of 10 months after OLT (range 1 – 58 months). The most common cause of death was infection (n=7) and recurrence of hepatocellular carcinoma (n=5). Other causes included myocardial infarction, hypoglycemia and suicide.

Laboratory data

There was a significant improvement in liver function tests with time after OLT, with mean bilirubin level decreasing from 78 ± 141 mmol/L at screening to 20 ± 35 mmol/L ($p < 0.005$) at twelve months and to 20 ± 60 mmol/L at two years after OLT ($p < 0.005$), stabilizing thereafter with bilirubin levels of 15 ± 13 mmol/L at 5 years after OLT. There was a mild but significant decrease in renal function after OLT, with mean creatinine level increasing from 92 ± 67 μ mol/L at time of screening for OLT to 113 ± 50 μ mol/L at 12 months after OLT ($p < 0.005$), stabilizing thereafter with mean creatinine level of 111 ± 66 μ mol/L at five years after OLT. Mean vitamin D levels increased from 32 ± 23 nmol/L at time of screening to 51 ± 28 nmol/L at twelve months after OLT ($p < 0.005$) and to 60 ± 29 nmol/L at five years after OLT ($p < 0.005$).

Changes in BMD after OLT

Sequential changes in BMD were analyzed in the 201 patients included in the study. BMD measurements were not available at screening in 20 of these patients but sequential data were available from six months onward after OLT. BMD declined significantly at both LS and FN sites between screening and six months after OLT: LS -0.02 g/cm² (-2.5%), $p = 0.002$; FN -0.05 g/cm² (-6.5%), $p < 0.005$), stabilizing thereafter until 12 months after OLT (Figure 1). Between one and two years after OLT, LS T-scores increased by 1.2 % (0.02 g/cm², $p = 0.012$), reaching pre-transplant values at two years after OLT and subsequently remaining stable. In contrast, FN BMD remained consistently below pre-transplantation values, but after the initial significant loss demonstrated no further decline up to 5 years of follow-up (Figure 1). A second OLT did not influence the course of changes in BMD. Incidence rates of osteoporosis at the LS and FN were respectively 4.2 and 4.7 per 100 person-years after OLT. The cumulative incidence of osteoporosis at the lumbar spine was 7% at six months, 12.1 % at twelve months and 14.7% at five years (Figure 2). The cumulative incidence of osteoporosis at the femoral neck was 8.5 % at six months, 10.5% at twelve months and 19.5% at five years after OLT.

Incidence of fractures after OLT

Spinal radiographs were available and evaluable in 115 patients at six months and in 106 patients at twelve months after OLT. The prevalence of fractures increased from 56% at time of screening to 78 out of 115 patients (68%) at six months after OLT, and to 75 out

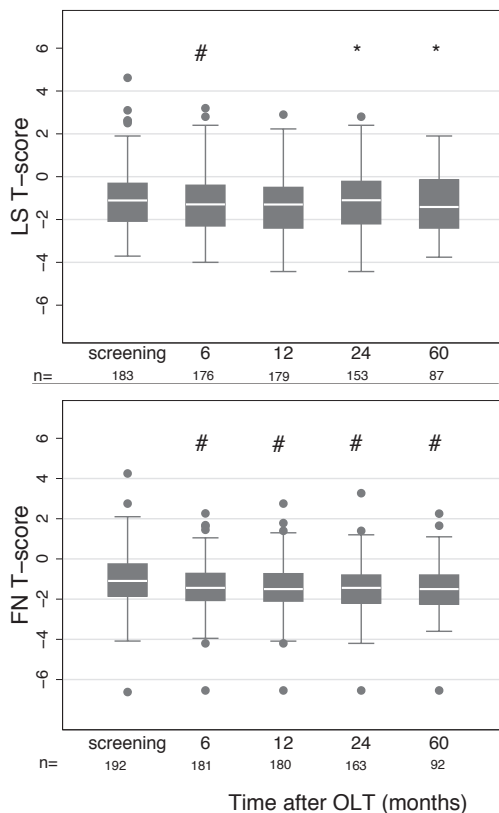


Figure 1: Changes in LS and FN T-scores in patients not treated with bone-modifying agents after OLT. # = Significant decrease $p < 0.05$ compared to values at the time of screening; * = Significant increase $p < 0.05$ when compared to values at 12 months after OLT; LS = lumbar spine; FN = femoral neck; OLT = orthotopic liver transplantation.

of 106 patients (71%) at twelve months after OLT. Incident fractures occurred in 45 out of 133 patients (34%) in whom a second spinal radiograph was available during the first year after transplantation.

C. Risk factors for bone loss and for vertebral fractures after OLT

We studied the effect of age, gender, primary liver disease, severity of liver disease, BMD at screening for OLT and immunosuppressive regimens, including corticosteroids, on bone loss and fracture risk within the first year after OLT. Of these potential risk factors which may influence BMD, the only ones with a significant relationship with bone loss were age 52 years or older in women (pragmatic cut-off age for menopause) (OR 0.89, 95%CI 0.81-0.99, $p=0.0024$), and the use of the calcineurin inhibitor tacrolimus for immunosuppression (OR 0.95, 95% CI 0.91-0.98, $p=0.003$). Vitamin D deficiency was not

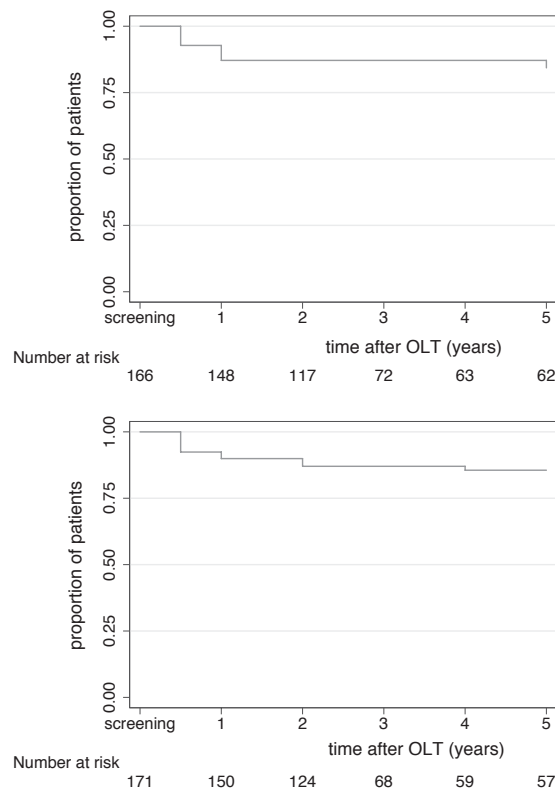


Figure 2: Incidence of osteoporosis. Kaplan Meier survival curve showing the proportion of patients without osteoporosis at the LS (upper panel) and FN (lower panel) after OLT. LS = lumbar spine, FN = femoral neck, OLT = orthotopic liver transplantation.

predictive for bone loss either at the LS (OR 0.95, 95%CI 0.90-1.01, $p=0.080$) or at the FN (OR 0.99, 95%CI 0.95-1.04, $p=0.674$). Patients with alcoholic liver disease demonstrated an increase, rather than a decrease, in LS but not FN BMD after OLT (at LS OR 1.08, 95% CI 1.02-1.14, $p = 0.005$; at FN OR 1.03, 95% CI 0.99-1.08, $p = 0.187$).

Of the potential risk factors studied, only male gender (OR 5.15, 95%CI 1.41-18.8, $p = 0.013$), and higher age (OR 1.06, 95%CI 1.00-1.11, $p = 0.045$) were associated with an increased risk for incident fractures. Other factors, including cholestatic liver disease, severity of liver disease, lumbar spine BMD and prevalent fractures at time of screening for OLT, were not predictive for increased fracture risk. There was no relationship between immunosuppressive medication and fracture risk. There was no difference in pattern of change in BMD at the LS or FN in patients with incident vertebral fractures within the first year after OLT compared to those who did not sustain a fracture (LS BMD: OR 0.97, 95%CI 0.91-1.03, $p = 0.293$; FN BMD: OR 0.97, 95%CI 0.93-1.02, $p = 0.272$).

DISCUSSION

Our findings from this study of longitudinal changes in BMD and vertebral fracture incidence in a cohort of recipients of an orthotopic liver transplantation show a high prevalence of osteopenia/ osteoporosis and vertebral fractures at time of screening for OLT. 54% of patients with diverse primary liver pathology had thus some degree of bone loss and 56% had prevalent vertebral fractures. Despite the high prevalence of skeletal pathology in these patients with end-stage liver disease awaiting liver transplantation, only a few received treatment with a bisphosphonate, which provided us the unique opportunity of studying sequential changes in BMD and fracture risk in a relatively large number of OLT recipients not receiving bone-modifying treatment. In keeping with previously published studies, with up to 2 years follow-up after OLT (1-3;7;9;10;12;13;16), we observed significant bone loss at both the LS and FN sites between screening and 6 months after OLT. Spontaneous recovery of BMD to pre-transplant values was observed at the LS within two years after OLT, with our longer term data showing stabilization of LS BMD thereafter for up to five years after OLT. In contrast to the increase in BMD observed at the lumbar spine after the initial rapid post-OLT decline in BMD, there was no similar recovery of BMD at the femoral neck. Data on long-term changes in FN BMD after OLT are scarce and not always concordant.(1;5;9;13) Whereas some studies show an increase in FN BMD during the second year after OLT (1;5), other studies show a persistently low FN BMD compared to pre-transplant values, or a further decline in bone mass at cortical sites.(6;9;13;15;16) The discrepancy in behavior of BMD between LS and FN sites is likely to be due to the difference in proportion of trabecular and cortical bone between these two sites. Trabecular bone comprises 66 % of total bone at the vertebrae, whereas cortical bone comprises >75% of the total bone composition of the femoral neck. Trabecular bone is much more susceptible to changes in the bone microenvironment resulting from disease processes than cortical bone, just as it is more readily susceptible to pharmacologic interventions. BMD at the LS thus shows the greatest magnitude and speed of change in response to reversal of diseases processes as well as to pharmacological interventions such as has been repeatedly demonstrated with the use of anti-resorptives or anabolic agents in the management of osteoporosis. After successful OLT, reversal of cholestasis, of vitamin D deficiency and of hypogonadism, which follow the restoration of liver function, is likely to significantly contribute to the spontaneous improvement in LS BMD, with recovery of FN BMD lagging behind that of LS BMD up to 5 years after OLT.

Corticosteroids form the mainstay of the induction and maintenance of immunosuppression after solid organ transplantation, and these agents are used in nearly all immunosuppressive protocols. An association between corticosteroid use and early post-transplantation bone loss has been observed in the early stages of solid organ

transplantation.(8) However, the introduction of new immunosuppressive regimens has allowed the early tapering of corticosteroids, thereby substantially contributing to recovery of bone mass in the long-term after transplantation. Nonetheless, acute rejection episodes occur more commonly in the first 3 months after transplantation, so that corticosteroids withdrawal is only practical thereafter.

In our study, only few of the risk factors evaluated at screening for OLT, which may potentially influence BMD, were able to predict bone loss or its severity post-OLT. Alcoholic liver disease was interestingly, but perhaps not surprisingly, associated with no decrease in BMD after OLT. Alcohol is toxic to the osteoblast and alcohol abuse is associated with a low turnover osteoporosis. Alcohol withdrawal is a prerequisite for liver transplantation, and candidates for OLT have to be completely dry for at least six months before being eligible for transplantation. In transplant recipients with alcoholic liver disease, removal of the toxic effect of alcohol on the osteoblast before transplantation, in addition to the improvement in nutritional status, when alcohol does no longer represent the major source of caloric intake, could well explain the lack of deterioration of bone mass after OLT in patients with this liver pathology.

We found no relationship between graft function, corticosteroid use, number of graft rejection episodes or persistent vitamin D deficiency on the degree of bone loss after OLT. This is also in keeping with data from a number of studies addressing the role of these factors on post-OLT bone loss.(1;3;4;8;9;12-14), although some other studies did find an association between cholestatic liver disease, cumulative prednisone dose and bone loss after OLT.(5;6;8)

Previously published data on fracture incidence after OLT show wide variability, ranging from 10-33% within the first year after OLT.(5;7;9;11;14;15;19) Our data on one-year fracture incidence are in keeping with only one previously published study, in which a semi-quantitative method of evaluation of vertebral fractures on routinely performed conventional spinal radiographs was also used, and the evaluation was performed by two independent observers on sequential radiographs.(9) Another reason for the discrepancy in fracture incidence between our study and that of others may be that previous studies were performed in smaller number of patients (5;9;14;15;20), or in patients solely with cholestatic liver disease as primary liver pathology.(19) Furthermore, although conventional spine radiographs were performed in some studies, these were not evaluated by semi-quantitative methods, which may have also led to underestimation of fracture incidence since routine radiology reports tend to underreport specially mild grade fractures.

Data on risk factors for incident fractures after OLT are conflicting. BMD-measurements have been found to be predictive in some studies (9;14;15;19), but not in others (7;11). In our study, both BMD at time of screening as well as changes of BMD within the first year after OLT were not predictive for fracture risk. Whereas

some studies showed prevalent fractures before OLT to be associated with increased fracture risk after transplantation (11;19;20), this was not the case in our study. These data suggest that the absence of low bone mass and of prevalent fractures before OLT does not preclude an increased risk for fracture after transplantation so that it is of significant clinical relevance that liver transplant recipients should be regularly screened for the presence of these fractures and treated accordingly.

Our study has a number of strengths as well as limitations. Its main strength lies in the relatively large representative cohort of patients studied, the availability of sequential BMD measurements as only inclusion criterion, providing a minimal selection bias, and the opportunity to study changes in BMD for a relatively long period after OLT without the confounding influence of bone-modifying treatment on BMD. Another strength of our study is the availability of serial spinal radiographs within the first year after transplantation in a large number of patients, and the blinded semi-quantitative assessment of all spinal radiographs for vertebral fractures by two experienced independent observers.

Our study has also a number of limitations. There were no pre-defined treatment protocols for immunosuppressive regimens, or for indication for bisphosphonate therapy. These patient management decisions were left to the clinical judgment of the treating physician and may have provided some degree of bias. Another limitation may be our choice to exclude patients from the time of initiation of bisphosphonate treatment, using 'last observation carried forward' to minimize the effect of this censoring on our data. Since patients excluded from further analysis because of starting therapy were most likely to have continuing bone loss if left untreated, the increase in BMD reported in the remaining untreated patients may have been overestimated, although numbers of patients treated are smaller than those of untreated patients. Since treatment with different immunosuppressive regimens was not randomly assigned, effect of these regimens on patterns of bone loss after OLT could also not be compared. A further limitation of our study is the variable time lag between screening for OLT and date of transplantation. Since the median time between screening and OLT was 9 months, it is conceivable that further bone loss and new fractures may have occurred between screening and transplantation, with possible further decrease in liver function and worsening cholestasis potentially contributing to worsening of skeletal pathology. However, this limitation is common to most published studies with the majority failing to report time lag between pre- and post-transplantation measurements.(1-5;7;12-14) Although the heterogeneity of the population studied may be perceived as a limitation, we did not consider this to be the case, as the distribution of primary liver pathology was fairly representative of OLT populations, patients receiving bisphosphonates were excluded from the analysis and patients receiving a second liver transplant did not have a different course of changes of BMD.

Only 12 patients (6%) were using corticosteroids at screening and use of these agents post-transplantation would override the effect of the pre-transplantation use of these agents.

In conclusion, osteopenia, osteoporosis and vertebral fractures are prevalent in prospective liver transplant recipients. An overall decline in BMD is observed within the first 6 months after OLT, with subsequent spontaneous recovery to pre-transplant values at the LS, but not at the FN up to five years after OLT. The incidence of vertebral fractures is high within the first year after OLT with poor association between pre-transplantation factors, changes of BMD and fracture risk. Whereas our findings do highlight the spontaneous recovery of BMD associated with restoration of liver function, and its maintenance in the long-term after liver transplantation, our data also demonstrate that the spontaneous recovery in BMD was not associated with a corresponding decrease in fracture risk, at least within the first year after orthotopic liver transplantation, suggesting a potentially persistent or irreversible effect of liver disease on bone quality and fracture risk. Whether treatment with bisphosphonates or other bone-modifying agents may be able to decrease or prevent fracture risk within the first year after liver transplantation and in the longer term thereafter remains to be established.

REFERENCE LIST

1. Crosbie OM, Freaney R, McKenna MJ, Curry MP, Hegarty JE. Predicting bone loss following orthotopic liver transplantation. *Gut* 1999; 44(3):430-434.
2. Eastell R, Dickson ER, Hodgson SF, Wiesner RH, Porayko MK, Wahner HW, Cedel SL, Riggs BL, Krom RA. Rates of vertebral bone loss before and after liver transplantation in women with primary biliary cirrhosis. *Hepatology* 1991; 14(2):296-300.
3. Floreani A, Fries W, Luisetto G, Burra P, Fagioli S, Boccagni P, Della Rovere GR, Plebani M, Piccoli A, Naccarato R. Bone metabolism in orthotopic liver transplantation: a prospective study. *Liver Transpl Surg* 1998; 4(4):311-319.
4. Feller RB, McDonald JA, Sherbon KJ, McCaughan GW. Evidence of continuing bone recovery at a mean of 7 years after liver transplantation. *Liver Transpl Surg* 1999; 5(5):407-413.
5. Giannini S, Nobile M, Ciuffreda M, Iemmolo RM, Dalle CL, Minicuci N, Casagrande F, Destro C, Gerunda GE, Sartori L, Crepaldi G. Long-term persistence of low bone density in orthotopic liver transplantation. *Osteoporos Int* 2000; 11(5):417-424.
6. Hamburg SM, Piers DA, van den Berg AP, Slooff MJ, Haagsma EB. Bone mineral density in the long term after liver transplantation. *Osteoporos Int* 2000; 11(7):600-606.
7. Hardinger KL, Ho B, Schnitzler MA, Desai N, Lowell J, Shenoy S, Chapman W, Crippin JS. Serial measurements of bone density at the lumbar spine do not predict fracture risk after liver transplantation. *Liver Transpl* 2003; 9(8):857-862.
8. Mart G, Gomez R, Jodar E, Loinaz C, Moreno E, Hawkins E. Long-term follow-up of bone mass after orthotopic liver transplantation: effect of steroid withdrawal from the immunosuppressive regimen. *Osteoporos Int* 2002; 13(2):147-150.
9. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, Ordi J, Rimola A, Rodes J, Munoz-Gomez J. Bone disease after liver transplantation: a long-term prospective study of bone mass changes, hormonal status and histomorphometric characteristics. *Osteoporos Int* 2001; 12(6):484-492.
10. Scolapio JS, DeArment J, Hurley DL, Romano M, Harnois D, Weigand SD. Influence of tacrolimus and short-duration prednisone on bone mineral density following liver transplantation. *JPEN J Parenter Enteral Nutr* 2003; 27(6):427-432.
11. Leidig-Bruckner G, Hosch S, Dodidou P, Ritschel D, Conrad C, Klose C, Otto G, Lange R, Theilmann L, Zimmerman R, Pritsch M, Ziegler R. Frequency and predictors of osteoporotic fractures after cardiac or liver transplantation: a follow-up study. *Lancet* 2001; 357(9253):342-347.
12. Floreani A, Mega A, Tizian L, Burra P, Boccagni P, Baldo V, Fagioli S, Naccarato R, Luisetto G. Bone metabolism and gonad function in male patients undergoing liver transplantation: a two-year longitudinal study. *Osteoporos Int* 2001; 12(9):749-754.
13. Abdelhadi M, Eriksson SA, Ljusk ES, Ericzon BG, Nordenstrom J. Bone mineral status in end-stage liver disease and the effect of liver transplantation. *Scand J Gastroenterol* 1995; 30(12):1210-1215.
14. McDonald JA, Dunstan CR, Dilworth P, Sherbon K, Sheil AG, Evans RA, McCaughan GW. Bone loss after liver transplantation. *Hepatology* 1991; 14(4 Pt 1):613-619.
15. Meys E, Fontanges E, Fourcade N, Thomasson A, Pouyet M, Delmas PD. Bone loss after orthotopic liver transplantation. *Am J Med* 1994; 97(5):445-450.
16. Ninkovic M, Skingle SJ, Bearcroft PW, Bishop N, Alexander GJ, Compston JE. Incidence of vertebral fractures in the first three months

- after orthotopic liver transplantation. *Eur J Gastroenterol Hepatol* 2000; 12(8):931-935.
17. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; 33(2):464-470.
 18. Genant HK, Wu CY, van KC, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. *J Bone Miner Res* 1993; 8(9):1137-1148.
 19. Guichelaar MM, Schmoll J, Malinchoc M, Hay JE. Fractures and avascular necrosis before and after orthotopic liver transplantation: long-term follow-up and predictive factors. *Hepatology* 2007; 46(4):1198-1207.
 20. Navasa M, Monegal A, Guanabens N, Peris P, Rimola A, Munoz-Gomez J, Visa J, Rodes J. Bone fractures in liver transplant patients. *Br J Rheumatol* 1994; 33(1):52-55.



4 | Pitfalls in the interpretation of bone turnover markers in liver transplantation

Charlotte G. Krol, Desi C.M. Meiland, Olaf M. Dekkers,
Eveline van der Veer, Herman M. Kroon, Ton J. Rabelink,
Bart van Hoek, Neveen A.T. Hamdy

ABSTRACT

Introduction Osteoporosis and fractures are common in chronic liver disease and fracture incidence increases after orthotopic liver transplantation (OLT). The value of bone turnover markers (BTMs) in the prediction of bone loss and fracture risk pre- and post-OLT remains unclear.

Study design The BTMs P1NP, osteocalcin, BALP and CTX were measured de novo or in Biobank stored serum at screening and at 3, 6 and 12 months post-OLT in consecutive OLT recipients between 2008 and 2011. A prerequisite for inclusion was the availability of BMD data and of spinal radiographs at screening and 6 and 12 months post-OLT.

Results 51 patients (80% male, median age 59 yrs) were studied. Most common liver pathology was alcoholic (41%) and viral liver disease (26%). At screening, osteoporosis and osteopenia were prevalent in respectively 16 and 33 % at the lumbar spine (LS) and in 4 and 44% at the femoral neck (FN). Prevalence of vertebral fractures was 67%. Post-OLT, LS BMD remained stable but FN BMD decreased and 43% of patients developed new fractures. At screening, serum P1NP and CTX levels were high and osteocalcin levels were low. Only CTX levels were predictive for bone loss and fracture risk. An increase in BALP post-OLT was predictive for fracture risk a year post-OLT.

Conclusion Despite the many pitfalls in the interpretation of particularly collagen-derived BTMs in liver disease, persistently high CTX levels pre- and post-OLT and an increase in BALP post-OLT were predictive for bone loss and/or fracture risk during the first post-OLT year.

INTRODUCTION

Osteoporosis is prevalent in 20-50% of patients with chronic liver disease awaiting orthotopic liver transplantation (OLT) (1-9), and prevalent vertebral fractures have been reported in 3.5-36% of these patients. (1;2;8-11) After OLT, accelerated bone loss of multifactorial aetiology further increases fracture risk, leading to new vertebral fractures in 14-30% of patients within the first year after OLT.(2;5;6;8;11-13) It has been proven difficult, however, to identify factors predictive for increased fracture risk in liver transplant recipients. The association between clinical parameters, such as primary liver pathology or severity of liver disease, or of bone mineral density (BMD), and fracture risk has thus been shown to be inconsistent and generally poor.(5;9;12-17)

Over the past two decades, there has been increasing interest in the use of bone turnover markers (BTMs) in the prediction of bone loss and of fracture risk, and in the monitoring of the efficacy of treatment of osteoporosis with bone modifying agents particularly in postmenopausal women.(18-21) BTMs are biochemical products of bone remodelling, which reflect the metabolic activity of bone. Alterations in bone remodelling are in turn associated with changes in bone microarchitecture and increased fracture risk. BTMs are released in the circulation where they can be measured in serum, and excreted by the kidneys, when they can be measured in urine.

In patients with chronic liver disease, there has been considerable heterogeneity of data on indices of bone remodelling particularly when set against data from the gold standard histomorphometric analysis of bone biopsy specimens obtained mostly before and within the first 6 months after OLT. Reported BTM data suggest no increase in bone turnover (15;22-24), or increased bone turnover particularly increased bone resorption before transplantation (4;25-28). Findings after transplantation suggest increased bone turnover, particularly bone resorption in the early post-transplant period.(15;17;24;29) In 2011, the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) have issued a Position Paper recommending the use of serum procollagen type I N-terminal propeptide (P1NP) as the preferred bone formation marker, and of the cross-linked C-terminal telopeptide of type I collagen (CTX) as the preferred bone resorption marker in clinical studies, in patients with osteoporosis, also supporting a role for BTMs in the prediction of fracture risk independent of BMD in postmenopausal women and in the monitoring of treatment. (30) However, the position paper also underlined that the clinical value of BTMs remains somewhat limited by amongst other causes the inadequate appreciation of the sources of variability of these markers. Interpreting data on bone turnover markers represents an even greater challenge in patients with liver disease as collagen type I is produced and degraded at increased rates in patients with liver fibrosis as previously reported by Guanabens et al in 22 patients with primary biliary cirrhosis.(31) Data on BTMs before

and in the short-term after OLT are conflicting (3;32-37), and there are no long-term data on sequential changes in BTMs after OLT.

The aim of our study was to assess the value of BTMs in the prediction of bone loss and fracture risk within the first year after OLT in a well characterized population of liver transplant recipients in whom sequential BTMs, BMD data and conventional spinal radiographs were available before and during the first year after liver transplantation.

PATIENTS AND METHODS

Patients

Consecutive patients who underwent a first OLT at the Leiden University Medical Centre between January 1st 2008 and January 1st 2011 and who had available BTM measurements (either directly measured or measured from stored sera) at time of screening for OLT were eligible for inclusion the study. Excluded were patients who were treated with bisphosphonates, and those who received a second OLT or a renal transplant during the study period. The date of January 1st 2008 for start of the study was selected because of the availability of P1NP measurements in our Clinic from this time onwards. A period of alcohol abstinence of at least six months was a prerequisite for screening for OLT in patients with alcoholic liver disease. Post-OLT immunosuppressive regimens consisted of corticosteroids for at least 6 months in all patients, combined with a calcineurin inhibitor: cyclosporine or tacrolimus in the majority, with mofetil mycophenolate (MMF) or sirolimus as additional second or third immunosuppressive agent in a few. Basiliximab was administered in the anhepatic phase and on day four after transplantation. Corticosteroid schedules included methylprednisolone at a dose of 500 mg given twice during the OLT procedure, followed by oral prednisolone at a dose of 20 mg/day for one week, 10 mg/day for 3 months, slowly tapering to complete discontinuation of corticosteroids between 3 and 6 months after OLT in all but a few patients who required maintenance doses of prednisolone of 20-40mg/day in divided doses. Treatment with calcium and vitamin D supplements at a combined fixed dose of 500 mg elemental calcium and 400 IU cholecalciferol was initiated in the majority of patients at some stage after OLT.

The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre.

Methods

Demographic and clinical data

Demographic and clinical data were obtained from the patients' electronic hospital records. Data on age, gender, smoking, height, weight and primary liver disease were collected. Medication including immunosuppressive agents, calcium and vitamin D supplements and bone-modifying medication such as bisphosphonates was recorded. Data were also collected on date and cause of death.

Biochemical investigations

Laboratory data were obtained from the patients' electronic hospital records. Biochemical data collected included serum calcium, corrected for an albumin of 40 g/L, phosphate, creatinine, parathyroid hormone (PTH) and 25-hydroxy-vitamin D (25(OH) D) and P1NP concentrations, which were measured at screening for OLT and at 3, 6 and 12 months after OLT. Normal values were < 104 µmol/L for creatinine levels, 50-250 nmol/L for 25-vitamin D levels, < 17 mmol/L for bilirubin levels. Data on serum creatinine, bilirubin and INR obtained at screening for OLT were used to calculate the MELD score reflecting severity of liver disease before transplantation, using the following formula:

$$MELD = 10 * (0.957 * \ln(Creatinine/88.4)) + (0.378 * \ln(Bilirubin/17.1)) + (1.12 * \ln(INR)) + 6.43$$

In patients in whom P1NP measurements were not available, this was retrospectively measured from stored sera using the same Electro-Chemo-Luminescence Immunoassay (ECLIA) on a Modular E170 immunoanalyzer (Roche Diagnostics, Mannheim, Germany) with an analytical variation of 6%. Serum stored at -80° was made available from the Liver Transplantation Biobank for missing measurements of P1NP, and for the measurement of the bone turnover markers: osteocalcin, bone-alkaline phosphatase (BALP) and CTX at screening before OLT and when available at 3, 6 and 12 months after OLT. Stored serum for the measurement of CTX was obtained in the non-fasting state. BALP was measured by enzyme-linked immunosorbent assay (ELISA; Metra Biosystems, Mountain View, CA, USA; inter-assay coefficient of variation (CV_{inter}) 5.5%), osteocalcin by immunoradiometric assay (IRMA; BioSource Europe S.A; CV_{inter} 9.4%), and CTX by electrochemiluminescence immunoassay (ECLIA; Elecsys 2010 Roche Mannheim, Germany; CV_{inter} 10.8%). Serum samples were stored within one hour of collection at -20°C until analysis was performed. BTM Z-scores, the number of standard deviations (SD) from the normal mean corrected for age and gender, were calculated using a Dutch control reference group (200 men and 350 women aged ≥ 50 years) checked with laboratory confirmed serum 25-hydroxyvitamin D levels ≥ 50 nmol/L and no evidence

for osteoporosis (BMD T-score >-2.5) on DXA evaluation. BTM Z-scores were calculated as follows:

$$\text{Z-score} = (\text{BTM value of individual patient} - \text{mean BTM value of reference group}) / \text{SD of matched reference cohort}$$

Bone mineral density measurements

Bone Mineral Density (BMD) measurements were performed time of screening for OLT (baseline) and at 6 and 12 months post-OLT. BMD was measured at the lumbar spine and femoral neck using dual energy X-ray absorptiometry (DXA- Hologic QDR 4500, Hologic inc. Waltham, MA, USA, equipped with reference values based on the National Health and Nutrition Examination Survey (NHANES III)). Absolute measurements of BMD in g/cm^2 as well as T-scores (matched to young adult reference populations at peak bone mass) and Z-scores (age- and sex-matched reference populations) were recorded. World Health Organization (WHO) criteria were used to define osteoporosis (T-score of -2.5 SD or less) and osteopenia (T-score between -1 SD and -2.5 SD).

Vertebral fracture assessment

Spinal radiographs were routinely performed at time of screening for OLT and at six and twelve months after OLT. Conventional antero-posterior and lateral radiographs of the thoracic spine and postero-anterior and lateral radiographs of the lumbar spine were performed by an experienced radiology technician following a standardized protocol, at a distance of 115 cm, with the film centralized on Th7 for the thoracic spine and on L3 for the lumbar spine. All plain radiographs of the thoracic and lumbar spine were blindly assessed for the prevalence and severity (grade) of vertebral fractures by two independent observers: an experienced musculoskeletal radiologist (H.M.K.) and an experienced bone and mineral disorders specialist (N.A.H.). Vertebral fractures were assessed using the Genant's semi-quantitative method.(38) Using this method, a decrease in height of 20-25% is considered to be a "mild" grade 1 fracture, of 25-40% a "moderate" grade 2 fracture and of $> 40\%$ a "severe" grade 3 fracture. Uniform loss of vertebral height compared to adjacent vertebrae were additionally documented using the same grading scores. Radiographs were assessed in a random order, using random numbers generated by SPSS software (version 20). A unique number was assigned to each series of radiographs, which included anterior-posterior and lateral radiographs of the thoracic and lumbar spine. Radiographs were individually scored by the two independent observers and in case of discrepancy in scores, consensus was achieved by both observers reviewing the radiographs together.

Statistical analysis

For descriptive statistics, categorical variables were expressed as numbers and as percentages. Continuous variables were summarized using mean and standard deviation in case of an estimated normal distribution. Median and 5th and 95th percentiles were used otherwise. An incident fracture was defined as the occurrence of a new vertebral fracture or an increase in grade of a prevalent fracture between serial radiographs before and during the first year after OLT. An extension of a Generalized Linear Model, Generalized Estimation Equations (GEE), was used to account for repeated measurements.

At time of screening before OLT, the relationship between biochemical parameters, prevalent BMD, prevalent fractures and BTMs was calculated using a multivariate regression model, correcting for age and gender. The relationship between CTX and the BTMs of bone formation: P1NP, osteocalcin and BALP was analyzed using a linear regression model. Predictive value of changes in BTM's for bone loss and for fracture incidence after OLT was analyzed also using a regression model. For the analysis of the influence of changes in BMT's on fracture incidence, difference of BTMs between evaluation points was calculated and a regression model was subsequently used to analyze the predictive value of the change of BTM on fracture incidence. Calculations were performed using STATA/SE 12.0 software (Stata Corp LP, TX, USA).

RESULTS

A. Baseline data at time of screening for OLT

Demographic and clinical characteristics

P1NP measurements and stored sera were available at screening in 66 of the 73 patients who received an orthotopic liver transplantation in the study period 2008-2011. Of these 66 patients, 6 were excluded because they were receiving a second or third liver transplant, and 3 because they also received a renal transplant. A further 6 patients were excluded because of start of treatment with bisphosphonates before transplantation ($n = 2$) or during follow-up ($n = 4$). A total number of 51 patients were thus included in the study (Figure 1).

41 patients were male (80%) and median age was 59 years (range 23-66 years) (Table 1). Most common primary liver disease was alcoholic liver disease (41%) followed by viral liver disease (26%) and cholestatic liver disease (4%). Only one patient died of an unknown cause 211 days after OLT. At time of screening, 4 patients (8%) were using calcium and vitamin D supplements and 1 patient was receiving treatment with corticosteroids.

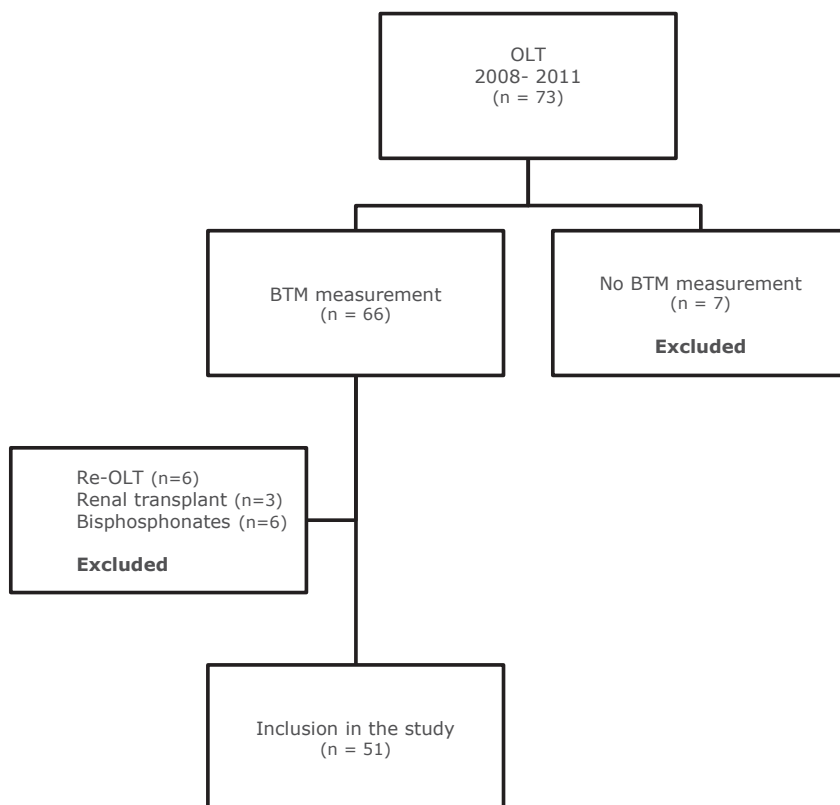


Figure 1: Study flow-chart. OLT = orthotopic liver transplantation; BTM = bone turnover marker.

Biochemical parameters

At time of screening, mean creatinine level was $88 \pm 37 \mu\text{mol/L}$. Mean 25(OH)D level was $36 \pm 27 \text{ nmol/L}$, with 82% of patients having levels of $< 50 \text{ nmol/L}$ and 43% of patients of $< 25 \text{ nmol/L}$. Mean bilirubin level was $96 \pm 212 \text{ mmol/L}$ and mean MELD score was 13 ± 6 .

Prevalent BMD

BMD was available at screening in 50 of the 51 patients. Lumbar spine (LS) data were not interpretable in two patients because of residual oral contrast material from abdominal CT-scans, required as part of the screening protocol for OLT, but chronologically performed by error before DXA. Mean LS BMD was $1.00 \pm 0.16 \text{ g/cm}^2$ and mean femoral neck (FN) was $0.80 \pm 0.14 \text{ g/cm}^2$ at the FN. The prevalence of osteoporosis and osteopenia was respectively 16 and 33 % at the LS and 4 and 44% at the FN.

Table 1 Demographic data at time of screening for liver transplantation.

Demographic data	
Number of patients	51
Gender – number of male patients (%)	41 (80%)
Age at the time of OLT- Median (range), years	59 (23-66)
Death during follow-up – no of patients (%)	1 (2%)
Time of death after OLT – days	211
BMI – mean (SD), kg/m ²	27 ± 6
Smoking – number of patients (%)	18 (38%)
Primary liver disease – number of patients (%)	
Alcoholic	21 (41%)
Viral	13 (26%)
Combined alcoholic and viral	2 (4%)
Cholestatic (PSC / PBC/ overlap syndrome)	2 (4%)
Malignancy	2 (4%)
Metabolic	2 (4%)
Other*	9 (18%)
Time between screening and OLT - median (5 th – 95 th percentile), months	9 (1-33)
Rejection episodes – number of episodes (cumulative incidence, %)	
6 months after OLT	2 (4%)
12 months after OLT	0 (4%)
Calcium – and vitamin D supplements – number of patients (%)	4 (8%)
Corticosteroids at the time of screening - number of patients (%)	1 (2%)
Immunosuppressive medication initiated at OLT - number of patients (%)	
Prednisone, tacrolimus	40 (78%)
Prednisone, tacrolimus and MMF	6 (12%)
Prednisone, cyclosporine and MMF	1 (2%)
Other	4 (8%)

OLT = orthotopic liver transplantation; BMI = body mass index; PSC = primary sclerosing cholangitis; PBC = primary biliary cirrhosis; MMF = mycophenolate mofetil. * including polycystic liver disease, cryptogenic cirrhosis, non-alcoholic steatosis hepatitis, Caroli syndrome

Prevalent vertebral fractures

Conventional spinal radiographs were available in all patients. Vertebral fractures were prevalent at time of screening in 34 patients (67%), mostly grade 1 fractures.

Bone turnover markers

Mean P1NP level was 82 ± 47 ng/mL (normal < 59 ng/mL). Mean osteocalcin levels was 9 ± 4 ug/L (mean Z-score -0.9 ± 1.6), mean BALP was 22 ± 9 U/L (mean Z-score 0.82

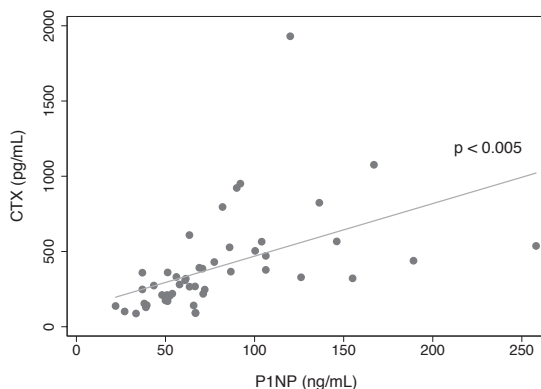


Figure 2: Association between the bone resorption marker serum CTX and the bone formation marker serum P1NP. CTX= C-terminal telopeptide of type I collagen; P1NP = serum procollagen type 1 N-terminal propeptide.

± 1.7) and mean CTX level was 397 ± 326 pg/mL (mean Z-score 2.16 ± 3.5). There was a significant relationship between P1NP and CTX levels ($p < 0.005$), which was no longer significant after correcting for creatinine levels ($p = 0.060$). (Figure 2) There was no relationship between CTX and osteocalcin or BALP

Higher creatinine levels were associated with higher levels of P1NP ($p < 0.005$), osteocalcin ($p < 0.005$) and CTX ($p < 0.005$). There was no association between creatinine levels and BALP ($p = 0.984$).

At time of screening, higher MELD scores were associated with higher P1NP levels ($p < 0.005$) and higher CTX levels ($p < 0.005$). There was no association between MELD scores and BALP or osteocalcin levels ($p = 0.070$ and $p = 0.260$ respectively). There was no significant association between bilirubin levels or INR and any of the BTM's before transplantation. Higher albumin levels were associated with lower CTX levels ($p = 0.015$).

Despite the low mean vitamin D levels at screening for OLT, there was no association between any BTM measured and 25(OH)D levels at this time point.

At screening, higher CTX levels but none of the other BTMs were predictive for lower BMD at the LS ($p = 0.009$) but not at the FN. None of the markers were predictive for prevalent vertebral fractures.

B. Follow-up data in the first year after OLT

Clinical parameters

Median time between screening and OLT was 9 months. After OLT, all patients were treated with prednisone, which was combined with tacrolimus in the majority of patients (78%) or tacrolimus and MMF (12%). Cyclosporine combined with MMF was added to

prednisone in 2% of patients. 2 patients had rejection episodes during the first year after OLT. The cumulative incidence of rejection episodes, requiring high dose of corticosteroids, was 4% at 12 months after OLT. At twelve months after OLT, calcium and or vitamin D supplements were prescribed to 40% of patients compared to 8 % at time of screening.

Biochemical parameters

After OLT, bilirubin levels significantly decreased from 96 ± 212 mmol/L at time of screening to 18 ± 34 mmol/L at three months after OLT ($p = 0.002$) with complete normalization to < 17 mmol/L in 38 patients (76%) at this time point, remaining stable thereafter with a mean value of 23 ± 66 mmol/L at six months and 16 ± 19 mmol/L at twelve months after OLT. Creatinine levels remained mildly elevated after OLT with a value of 107 ± 35 μ mol/L at twelve months after OLT. 25(OH)D levels increased within the first year after OLT, only reaching significance at twelve months after OLT with a mean value of 50 ± 27 nmol/L ($p < 0.005$).

Changes in BMD

There was no significant change in LS BMD at 6 or 12 months after OLT compared to baseline. Mean BMD at the LS was 1.01 ± 0.15 g/cm² at twelve months after OLT compared to 1.00 ± 0.16 g/cm² at baseline ($p = 0.236$). There was however, a significant decrease in FN BMD after OLT from 0.79 ± 0.13 g/cm² at time of screening to 0.75 ± 0.12 g/cm² at six months after OLT ($p < 0.005$) remaining stable thereafter with a mean BMD of 0.77 ± 0.14 g/cm² at twelve months after OLT ($p = 0.438$ compared to values six months after OLT).

Incident fractures during the first year after OLT

Conventional spinal radiographs were available and evaluable in 49 of the 51 patients included in the study: in 45 at 6 months after OLT and in 37 patients at 12 months after OLT. Within the first year after OLT, new fractures were documented in 21 of the 49 patients (43%).

Bone turnover markers

Changes in BTMs were analysed in all patients included in the study. A separate analysis was also conducted in patients without incident vertebral fractures ($n = 28$) to avoid the possible effect of a recent fracture on the interpretation of BTMs. In the whole group, P1NP decreased after OLT from 82 ± 47 ng/mL at screening to 68 ± 38 ng/mL at three months and 77 ± 52 ng/mL at six months, both non-significantly, only reaching significance at twelve months after OLT with a level of 65 ± 50 ng/mL ($p = 0.005$) (Figure 3). In patients without incident vertebral fractures during the first year after OLT, P1NP also decreased significantly ($p = 0.003$ at 12 months after OLT) whereas it did not in the 21 patients with incident vertebral fractures ($p = 0.207$). Changes in P1NP levels

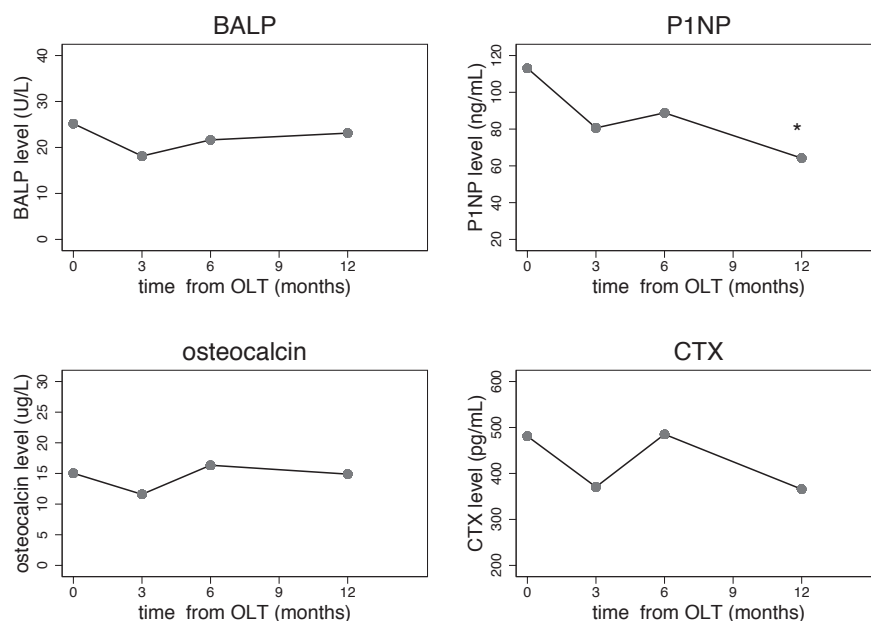


Figure 3: Changes in bone turnover markers after OLT. BALP = bone specific alkaline phosphatase; P1NP = procollagen type 1 N-terminal propeptide; CTX = C-terminal telopeptide of type I collagen; * = significant change compared to screening, p -value < 0.05 .

in relation to changes in bilirubin, changes in creatinine and the presence or absence of a fracture within the first year after transplantation, highlighting the difficulty in interpreting this marker in liver transplant recipients, are shown in Figure 4.

Osteocalcin increased albeit not significantly in the whole group from 9 ug/L at screening to 12 ug/L at 3 months, 16 ug/L at 6 months and 15 ug/L at 12 months after OLT. However, in patients without new vertebral fractures, osteocalcin increased significantly from 9 ug/L at screening to 13.8 ug/L at six months ($p = 0.010$), remaining stable thereafter with a mean value of 14.5 ug/L at twelve months ($p = 0.001$ compared to screening).

There were no significant changes in mean BALP levels after OLT, although 17 out of 37 patients with measurements at both evaluation points (46%) demonstrated an increase in BALP at 6 months post-OLT. However, BALP decreased significantly from 23 U/L to 11 U/L at three months ($p = 0.004$) in patients without incident vertebral fractures, remaining stable thereafter with a mean value of 14 U/L at twelve months after OLT ($p = 0.011$ compared to screening).

The high CTX levels observed at screening remained unchanged during the first year after OLT. (Figure 3) In patients without vertebral fractures, CTX only showed an increased significantly at six months after OLT ($p = 0.042$) but not at 12 months ($p = 0.628$).

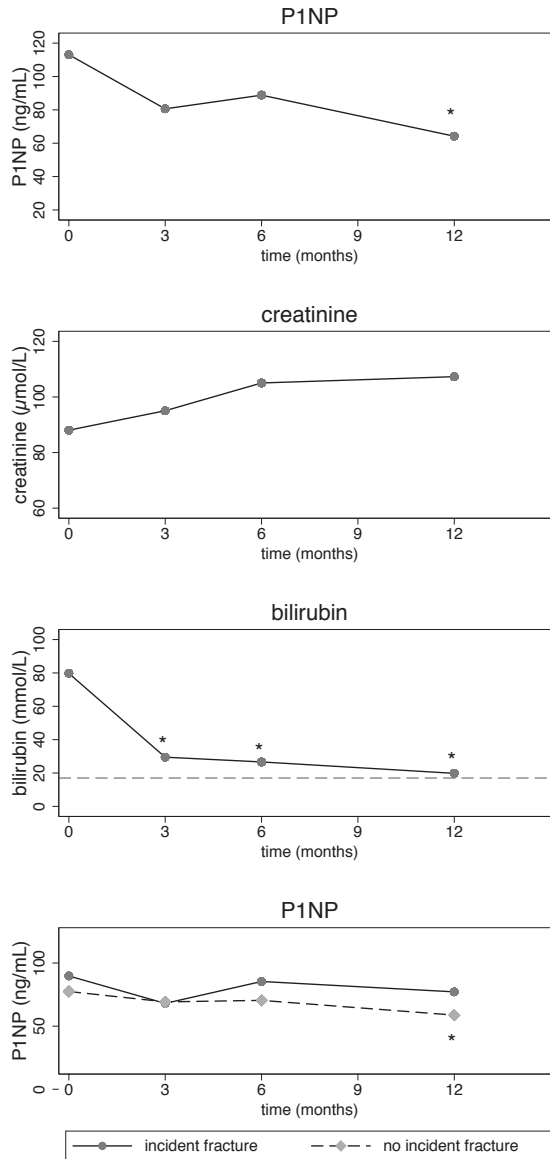


Figure 4: Highlight of factors affecting interpretation after transplantation. P1NP = procollagen type 1 N-terminal propeptide. * = significant change compared to screening, p-value < 0.05.

C. Predictive value of BTMs at screening for OLT for bone loss and fracture incidence up to a year after transplantation

Higher CTX levels were associated with a decrease in LS ($p = 0.008$) as well as FN BMD ($p=0.039$). P1NP, BALP and osteocalcin levels measured at screening were not predictive

for bone loss in the first year after OLT (P1NP LS $p = 0.472$ and FN $p = 0.064$, BALP LS $p = 0.100$ and FN $p = 0.321$, osteocalcin LS $p = 0.278$ and FN $p = 0.308$).

Higher CTX values were also predictive for increased fracture risk ($p = 0.038$ for absolute CTX values, $p = 0.014$ for Z-scores after correction for age and gender). None of the other BTMs predicted incident fractures after OLT either considering absolute values (P1NP $p = 0.373$, BALP $p = 0.429$ and osteocalcin $p = 0.993$) or Z-scores (BALP $p = 0.215$, osteocalcin $p = 0.857$). Absolute values of BTMs measured at three months after OLT were also not predictive for incident fractures (P1NP $p = 0.927$, osteocalcin $p = 0.414$, BALP $p = 0.458$, CTX $p = 0.559$) and neither were BTM Z-scores (CTX $p = 0.823$, BALP $p = 0.937$, osteocalcin $p = 0.161$).

D. Predictive value of changes in BTMs for fracture risk within the first year post-OLT

An increase of BALP levels between screening and 6 months after OLT was associated with an increased risk of fractures ($p = 0.001$). Changes in any of the other BTMs between screening and six months after OLT were not predictive for incident fractures a year after OLT (P1NP $p = 0.798$, osteocalcin $p = 0.066$, CTX $p = 0.205$).

DISCUSSION

Our data from this study demonstrate the limited value of the widely used bone turnover markers in the prediction of bone loss and of fracture risk before and after orthotopic liver transplantation. Although P1NP levels were increased and osteocalcin levels were decreased at screening before OLT, these markers as well as baseline bone alkaline phosphatase were neither predictive for prevalent BMD or prevalent vertebral fractures before transplantation, nor were they predictive for bone loss or increased fracture risk after transplantation. Only increased CTX levels at time of screening for OLT were predictive for bone loss and increased fracture risk after OLT. An increase in BALP at 6 months after OLT was also predictive for increased fracture risk a year after OLT.

Bone turnover markers are biochemical products of bone remodelling, which reflect the metabolic activity of bone, and can be easily measured in serum or urine using semi-automated techniques. The measurement of BTMs is non-invasive, free of radiation and easily incorporated into routine follow-up protocols. Serum and urine examinations are routinely used in the monitoring of patients with liver transplantation, so that measurements of bone turnover markers may be easily scheduled in these patients. The question which arises is whether measuring bone turnover markers would be of value in the management of the commonly encountered skeletal complications of end-stage liver disease, before and after liver transplantation. BTMs have been shown to predict

bone loss and fracture risk in postmenopausal women.(18;39) However, there are scarce data on the value of BTMs in patients with end-stage liver disease before and after liver transplantation and available data are conflicting, particularly after transplantation. There are a number of potential reasons for the difficult interpretation of BTMs after all solid organ transplantation including the effect of glucocorticoids on bone remodelling and the effect of calcineurin inhibitors possibly because of their potential nephrotoxic effect often associated with increases in PTH concentrations.

The Position Paper on bone markers issued by the IOF in 2011 recommends the use of P1NP as the preferred bone formation marker, as it reflects the synthesis of the most abundant protein of the organic bone matrix. P1NP is a peptide derived from the posttranslational cleavage of the type I pro-collagen molecule by specific protease enzymes during bone formation, which is released into the circulation where it can be easily and reliably measured. However, although most circulating P1NP is mainly derived from pro-collagen secreted by osteoblasts during the process of bone formation (39) and collagen type I constitutes more than 90% of the organic bone matrix. P1NP is not strictly speaking a bone-specific marker as it is also released in the circulation by fibroblasts in soft connective tissue, including hepatic connective tissue. P1NP is thus as much a serum marker of connective tissue metabolism as it is of bone metabolism. P1NP is cleared from the circulation by both renal and liver extraction so that impairment of either or both clearing mechanisms by liver and/or renal impairment may influence its serum levels. On the other hand, P1NP measurements are stable with minimal diurnal variation and no effect of food on circulating levels.

In our study, we could find no association between the high baseline P1NP values and either prevalent BMD or prevalent fractures before transplantation, or bone loss or increased fracture risk post-transplantation. In chronic liver disease, the marked increase in connective tissue synthesis and degradation of hepatic collagen type I (40;41), which does not reflect bone remodelling but rather liver fibrogenicity, has been previously demonstrated in animal models of liver fibrosis (42), in patients with progressive fibrosis of the liver due to primary biliary cirrhosis and in patients with alcoholic liver cirrhosis (31;43). In our study, we observed a significant decrease in P1NP levels after OLT, parallel with increases in osteocalcin levels, both closely related to improvement in hepatobiliary function as shown by significant post-OLT decreases in bilirubin levels(Figure 4). Intriguingly, we could not demonstrate a significant relationship between bilirubin levels and P1NP either at baseline or within the first year after transplantation, although both markers showed parallel decreases with time. A reason for this is likely to be that although bilirubin is a useful marker of liver function, it may not be a sufficient surrogate marker for all aspects of liver function.

Osteocalcin is a non-collagenous protein of the bone matrix, exclusively synthesized by the osteoblast during the process of bone formation, which should have potentially

been a more useful marker of bone turnover in patients with liver disease as it is a non-collagen-derived marker and it is mainly cleared by the kidney so that it would not be affected by abnormal hepatic collagen production. (44;45) Our data confirm this premise as we could show no association between osteocalcin and MELD scores, bilirubin, INR or albumin. However, osteocalcin was disappointingly also not predictive for either bone loss or increased fracture risk after OLT. A possible explanation for this may be that osteocalcin synthesis is vitamin K dependent (45), and that levels may decrease due to decreased synthesis of vitamin K in patients with end-stage liver failure. Osteocalcin is also rapidly degraded in serum to heterogeneous fragments and its circulating levels are influenced by circadian rhythms and by renal function, which is often disturbed in patients with end-stage liver disease before and after OLT.

Alkaline phosphatase (ALP) is secreted by osteoblasts in the extracellular fluid when newly formed osteoid undergoes maturation and mineralization. However, in healthy adults, only half of circulating ALP is derived from bone, the other half being predominantly of hepatic origin, with an even higher contribution of liver-derived ALP in patients with end-stage liver disease. The bone specific isoform BALP can be separately measured and has been shown to predict osteoporotic fractures in postmenopausal women.(46) In our study, we did observe that an increase in BALP 6 months after OLT was predictive of fracture risk within the first year after OLT. There was no association between BALP and creatinine levels or MELD scores suggesting that BALP may be less influenced by changes in liver or renal function after OLT.

The IOF recommends CTX as the reference marker for bone resorption.(39) CTX is a degradation product of type 1 collagen and consists of the C-terminal crosslinks of the collagen molecule, which are released into the circulation during bone resorption. CTX has been shown to be one of the most sensitive markers of bone resorption with a more consistent association with fracture risk than bone formation markers (46). However, CTX is a marker of bone resorption based on collagen degradation, which has also been found to be increased in patients with primary biliary cirrhosis just as P1NP is.(31) It is thus likely that in liver failure, measured levels of CTX would not exclusively reflect degradation of bone collagen, but also that of increased hepatic collagen synthesis and degradation. Higher MELD scores, reflecting severity of liver disease, were indeed associated with higher CTX levels which supports a relationship between hepatobiliary function and CTX.

Interestingly, however, high pre-transplantation levels of CTX were predictive not only of post-transplantation bone loss, but also of increased fracture risk. The better predictive value of a bone resorption marker over that of a bone formation marker for fracture risk is that bone formation lags behind bone resorption, needing some 3 months or more to fill the newly formed resorption cavities, in the process of which, the unfilled resorbed areas would significantly weaken the microarchitecture of bone

resulting in a decrease in bone quality and a consequent increase in bone fragility, independently of the actual amount of bone lost. Our data are in keeping with this premise, showing an increased fracture risk associated with persistently increased levels of the bone resorption marker CTX and with increasing bone turnover as suggested by increasing bone alkaline phosphatase levels, despite the absence of significant bone loss at the lumbar spine as suggested by stable BMD values at this site in the first year after OLT.

Our study has strengths as well as limitations. A main strength of the study is that the cohort studied is representative of a liver transplantation cohort. A predefined protocol was applied with BMD measurements and conventional radiographs of the spine being routinely undertaken and thus available at screening and at regular intervals thereafter in the majority of patients. A further strength of our study is the long established practice of storage of sera in a Biobank in the Liver Transplantation Program, as a result of which stored serum samples could be analysed in case of missing data and further used for the measurement of P1NP, osteocalcin, BALP and CTX.

Our study has also a number of limitations. One of the limitations is the lack of a well-defined marker of liver function except for the validated laboratory MELD score. This score includes in addition to bilirubin and INR, creatinine and albumin as surrogate markers for the poorly prognostic hepatorenal syndrome. Whereas MELD scores are commonly used to evaluate severity of liver disease and thus of prognosis at the time of screening before OLT, these scores are not actually validated for the evaluation of liver function after OLT. A further limitation of our study is the relatively small number of patients included in the study, which may have precluded the detection of significant differences due to lack of power.

The fact that serum measured for CTX was not collected as recommended in the fasting state due to the retrospective nature of our study may also be perceived as a limitation in the interpretation of our results due to the known circadian rhythm of the marker and the influence of food intake on its circulating levels. However, it has been shown that non-fasting levels of CTX are about 20% lower than fasting ones, so that measured non-fasting levels would represent if anything an underestimate of the actual levels. It has also been previously shown that the afternoon level of CTX was more closely related to fracture risk than the morning sample.⁽⁴⁷⁾

Although biochemical markers of bone turnover are widely available for use in the Clinic, their role in the prediction of bone loss and of fracture risk remains inconclusive and their value in the management of post-transplantation bone disease remains highly debatable. This is indeed the case in chronic liver disease, before and after liver transplantation, because of the many pitfalls encountered in the interpretation of these markers in the presence of disturbed connective tissue metabolism as

well as the confounding effect of corticosteroids and calcineurin inhibitors used for immunosuppression.

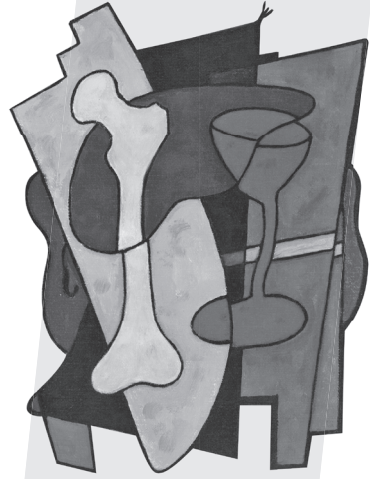
In conclusion, our data highlight the pitfalls in the interpretation of BTMs in chronic liver disease before and after liver transplantation. Based on our findings, we would recommend that caution should be advocated with the use of BTMs in the clinic in patients with chronic liver disease before and after liver transplantation until further evidence is obtained for the value of available or newer BTMs for therapeutic decision making in the management of skeletal complications in these patients.

REFERENCE LIST

1. Alcalde VA, Pascasio Acevedo JM, Gutierrez D, I, Garcia JR, Sousa Martin JM, Ferrer Rios MT, Sayago MM, Giraldez GA, Gomez Bravo MA. Prevalence and characteristics of bone disease in cirrhotic patients under evaluation for liver transplantation. *Transplant Proc* 2012; 44(6):1496-1498.
2. Carey EJ, Balan V, Kremers WK, Hay JE. Osteopenia and osteoporosis in patients with end-stage liver disease caused by hepatitis C and alcoholic liver disease: not just a cholestatic problem. *Liver Transpl* 2003; 9(11):1166-1173.
3. Floreani A, Mega A, Tizian L, Burra P, Boccagni P, Baldo V, Faggioli S, Naccarato R, Luisetto G. Bone metabolism and gonad function in male patients undergoing liver transplantation: a two-year longitudinal study. *Osteoporos Int* 2001; 12(9):749-754.
4. Guichelaar MM, Malinchoc M, Sibonga JD, Clarke BL, Hay JE. Bone histomorphometric changes after liver transplantation for chronic cholestatic liver disease. *J Bone Miner Res* 2003; 18(12):2190-2199.
5. Leidig-Bruckner G, Hosch S, Dodidou P, Ritschel D, Conradt C, Klose C, Otto G, Lange R, Theilmann L, Zimmerman R, Pritsch M, Ziegler R. Frequency and predictors of osteoporotic fractures after cardiac or liver transplantation: a follow-up study. *Lancet* 2001; 357(9253):342-347.
6. Millonig G, Graziadei IW, Eichler D, Pfeiffer KP, Finkenstedt G, Muehllechner P, Koenigsrainer A, Margreiter R, Vogel W. Alendronate in combination with calcium and vitamin D prevents bone loss after orthotopic liver transplantation: a prospective single-center study. *Liver Transpl* 2005; 11(8):960-966.
7. Ninkovic M, Love SA, Tom B, Alexander GJ, Compston JE. High prevalence of osteoporosis in patients with chronic liver disease prior to liver transplantation. *Calcif Tissue Int* 2001; 69(6):321-326.
8. Premaor MO, Das TK, Debram I, Parker RA, Ninkovic M, Alexander GT, Compston JE. Fracture incidence after liver transplantation: results of a 10-year audit. *QJM* 2011; 104(7):599-606.
9. Krol CG, Dekkers OM, Kroon HM, Rabelink TJ, van HB, Hamdy NA. No association between BMD and prevalent vertebral fractures in liver transplant recipients at time of screening before transplantation. *J Clin Endocrinol Metab* 2014; jc20141469.
10. Guichelaar MM, Kendall R, Malinchoc M, Hay JE. Bone mineral density before and after OLT: long-term follow-up and predictive factors. *Liver Transpl* 2006; 12(9):1390-1402.
11. Monegal A, Guanabens N, Suarez MJ, Suarez F, Clemente G, Garcia-Gonzalez M, De la Mata M, Serrano T, Casafont F, Tome S, Barrios C, Navasa M. Pamidronate in the prevention of bone loss after liver transplantation: a randomized controlled trial. *Transpl Int* 2009; 22(2):198-206.
12. Hardinger KL, Ho B, Schnitzler MA, Desai N, Lowell J, Shenoy S, Chapman W, Crippin JS. Serial measurements of bone density at the lumbar spine do not predict fracture risk after liver transplantation. *Liver Transpl* 2003; 9(8):857-862.
13. Krol CG, Dekkers OM, Kroon HM, Rabelink TJ, van HB, Hamdy NA. Longitudinal Changes in BMD and Fracture Risk in Orthotopic Liver Transplant Recipients Not Using Bone-Modifying Treatment. *J Bone Miner Res* 2014; 29(8):1763-1769.
14. Guichelaar MM, Schmol J, Malinchoc M, Hay JE. Fractures and avascular necrosis before and after orthotopic liver transplantation: long-term follow-up and predictive factors. *Hepatology* 2007; 46(4):1198-1207.
15. McDonald JA, Dunstan CR, Dilworth P, Sherbon K, Sheil AG, Evans RA, McCaughan GW. Bone loss after liver transplantation. *Hepatology* 1991; 14(4 Pt 1):613-619.

16. Meys E, Fontanges E, Fourcade N, Thomasson A, Pouyet M, Delmas PD. Bone loss after orthotopic liver transplantation. *Am J Med* 1994; 97(5):445-450.
17. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, Ordi J, Rimola A, Rodes J, Munoz-Gomez J. Bone disease after liver transplantation: a long-term prospective study of bone mass changes, hormonal status and histomorphometric characteristics. *Osteoporos Int* 2001; 12(6):484-492.
18. Chopin F, Biver E, Funck-Brentano T, Bouvard B, Coiffier G, Garnero P, Thomas T. Prognostic interest of bone turnover markers in the management of postmenopausal osteoporosis. *Joint Bone Spine* 2012; 79(1):26-31.
19. Garnero P. New developments in biological markers of bone metabolism in osteoporosis. *Bone* 2014; 66C:46-55.
20. Vasikaran S, Eastell R, Bruyere O, Foldes AJ, Garnero P, Griesmacher A, McClung M, Morris HA, Silverman S, Trenti T, Wahl DA, Cooper C, Kanis JA. Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. *Osteoporos Int* 2011; 22(2):391-420.
21. Civitelli R, Armamento-Villareal R, Napoli N. Bone turnover markers: understanding their value in clinical trials and clinical practice. *Osteoporos Int* 2009; 20(6):843-851.
22. Stellon AJ, Webb A, Compston J, Williams R. Low bone turnover state in primary biliary cirrhosis. *Hepatology* 1987; 7(1):137-142.
23. Stellon AJ, Webb A, Compston JE. Bone histomorphometry and structure in corticosteroid treated chronic active hepatitis. *Gut* 1988; 29(3):378-384.
24. Vedi S, Greer S, Skingle SJ, Garrahan NJ, Ninkovic M, Alexander GA, Compston JE. Mechanism of bone loss after liver transplantation: A histomorphometric analysis. *J Bone Miner Res* 1999; 14(2):281-287.
25. Cuthbert JA, Pak CY, Zerwekh JE, Glass KD, Combes B. Bone disease in primary biliary cirrhosis: increased bone resorption and turnover in the absence of osteoporosis or osteomalacia. *Hepatology* 1984; 4(1):1-8.
26. Lalor BC, France MW, Powell D, Adams PH, Counihan TB. Bone and mineral metabolism and chronic alcohol abuse. *Q J Med* 1986; 59(229):497-511.
27. Hay JE, Lindor KD, Wiesner RH, Dickson ER, Krom RA, LaRusso NF. The metabolic bone disease of primary sclerosing cholangitis. *Hepatology* 1991; 14(2):257-261.
28. Hodgson SF, Dickson ER, Eastell R, Eriksen EF, Bryant SC, Riggs BL. Rates of cancellous bone remodeling and turnover in osteopenia associated with primary biliary cirrhosis. *Bone* 1993; 14(6):819-827.
29. Guichelaar MMJ, Malinchoc M, Sibonga JD, Clarke BL, Hay JE. Bone histomorphometric changes after liver transplantation for chronic cholestatic liver disease. *Journal of Bone and Mineral Research* 2003; 18(12):2190-2199.
30. Vasikaran S, Cooper C, Eastell R, Griesmacher A, Morris HA, Trenti T, Kanis JA. International Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine position on bone marker standards in osteoporosis. *Clin Chem Lab Med* 2011; 49(8):1271-1274.
31. Guanabens N, Pares A, Alvarez L, Martinez de Osaba MJ, Monegal A, Peris P, Ballesta AM, Rodes J. Collagen-related markers of bone turnover reflect the severity of liver fibrosis in patients with primary biliary cirrhosis. *J Bone Miner Res* 1998; 13(4):731-738.
32. McDonald JA, Dunstan CR, Dilworth P, Sherbon K, Sheil AGR, Evans RA, McCaughan GW. Bone loss after liver transplantation. *Hepatology* 1991; 14(4 1):613-619.
33. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, Ordi J, Rimola A, Rodes J, Munoz-Gomez J. Bone disease after liver transplantation: a long-term prospective study of bone mass changes,

- hormonal status and histomorphometric characteristics. *Osteoporos Int* 2001; 12(6):484-492.
34. Rabinovitz M, Shapiro J, Lian J, Block GD, Merkel IS, Van Thiel DH. Vitamin D and osteocalcin levels in liver transplant recipients. Is osteocalcin a reliable marker of bone turnover in such cases? *J Hepatol* 1992; 16(1-2):50-55.
 35. Fahrleitner A, Prenner G, Kniepeiss D, Iberer F, Tscheliessnigg KH, Piswanger-Solkner C, Obermayer-Pietsch B, Leb G, Dobnig H. Serum osteoprotegerin levels in patients after liver transplantation and correlation to bone turnover, bone mineral density and fracture status. *Wien Klin Wochenschr* 2002; 114(15-16):717-724.
 36. Crosbie OM, Freaney R, McKenna MJ, Curry MP, Hegarty JE. Predicting bone loss following orthotopic liver transplantation. *Gut* 1999; 44(3):430-434.
 37. Giannini S, Nobile M, Ciuffreda M, Iemmolo RM, Dalle CL, Minicuci N, Casagrande F, Destro C, Gerunda GE, Sartori L, Crepaldi G. Long-term persistence of low bone density in orthotopic liver transplantation. *Osteoporos Int* 2000; 11(5):417-424.
 38. Genant HK, Wu CY, van KC, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. *J Bone Miner Res* 1993; 8(9):1137-1148.
 39. Vasikaran S, Eastell R, Bruyere O, Foldes AJ, Garnero P, Griesmacher A, McClung M, Morris HA, Silverman S, Trenti T, Wahl DA, Cooper C, Kanis JA. Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. *Osteoporos Int* 2011; 22(2):391-420.
 40. Melkko J, Hellevik T, Risteli L, Risteli J, Smedsrod B. Clearance of NH₂-terminal propeptides of types I and III procollagen is a physiological function of the scavenger receptor in liver endothelial cells. *J Exp Med* 1994; 179(2):405-412.
 41. Qvist P, Petersen KR, Jespersen J, Vinberg N, Christiansen C. Lack of hepatic metabolism of C-telopeptides of type I collagen. *Clin Chim Acta* 2006; 366(1-2):344-347.
 42. Veidal SS, Vassiliadis E, Bay-Jensen AC, Tougas G, Vainer B, Karsdal MA. Procollagen type I N-terminal propeptide (PINP) is a marker for fibrogenesis in bile duct ligation-induced fibrosis in rats. *Fibrogenesis Tissue Repair* 2010; 3(1):5.
 43. Schytte S, Hansen M, Moller S, Junker P, Henriksen JH, Hillingsø J, Teisner B. Hepatic and renal extraction of circulating type I procollagen aminopropeptide in patients with normal liver function and in patients with alcoholic cirrhosis. *Scand J Clin Lab Invest* 1999; 59(8):627-633.
 44. Farrugia W, Yates NA, Fortune CL, McDougall JG, Scoggins BA, Wark JD. The effect of uninephrectomy on osteocalcin metabolism in sheep: a direct evaluation of renal osteocalcin clearance. *J Endocrinol* 1991; 130(2):213-221.
 45. Hauschka PV. Osteocalcin: the vitamin K-dependent Ca²⁺-binding protein of bone matrix. *Haemostasis* 1986; 16(3-4):258-272.
 46. Chopin F, Biver E, Funck-Brentano T, Bouvard B, Coiffier G, Garnero P, Thomas T. Prognostic interest of bone turnover markers in the management of postmenopausal osteoporosis. *Joint Bone Spine* 2012; 79(1):26-31.
 47. Chapurlat RD, Garnero P, Breart G, Meunier PJ, Delmas PD. Serum type I collagen breakdown product (serum CTX) predicts hip fracture risk in elderly women: the EPIDOS study. *Bone* 2000; 27(2):283-286.



5

Bisphosphonates in the prevention of bone loss after liver transplantation

a comprehensive review of the literature and a case series from the Leiden Liver Transplantation Cohort Study

Charlotte G. Krol, Olaf M. Dekkers, Herman M. Kroon, Ton J. Rabelink, Bart van Hoek, Neveen A.T. Hamdy

ABSTRACT

Introduction Bone loss and fractures are common after orthotopic liver transplantation (OLT). Data on the efficacy of bisphosphonates to decrease skeletal complications are not concordant and therapeutic strategies to decrease skeletal morbidity after OLT remain to be established.

Materials and methods A review was undertaken of published studies on the effect of bisphosphonates on bone loss and fracture risk in liver transplant recipients. Changes in bone mineral density (BMD) measurements routinely obtained before and 6 months and one and two years after OLT were analyzed in consecutive recipients of a successful OLT at the Leiden University Medical Centre between 2000 and 2011, who were treated with the bisphosphonate alendronate within the first year after OLT. Non bisphosphonate-treated transplant recipients were used as controls. Conventional spinal radiographs available at time of screening for OLT and at six and twelve months post-OLT were assessed for vertebral fractures using the Genant's semi-quantitative method.

Results Review of the published literature generated 16 studies, in all but 3 of which bisphosphonates were started around the time of OLT at various doses, frequency of administration and duration. Decreased fracture risk was the primary end point in only one study. A short-term beneficial effect of bisphosphonate treatment on BMD was observed in most studies. Data on long-term effect of treatment are conflicting.

Leiden case series: Data of 39 OLT recipients (mean age 49 yrs, 72% male) treated with oral alendronate within the first year after OLT (median 185 days, range 22-365) were compared to those of 104 controls (mean age 53 yrs, 68% male) transplanted within the same period and not receiving bisphosphonates. At baseline, BMD was lower in the bisphosphonate-treated group ($p < 0.005$) at one or both sites measured with osteoporosis observed in 41% and osteopenia in 56% of patients compared to 39 and 11% respectively in the control group. Radiological vertebral fractures were equally prevalent in both groups (61% of patients in the treatment group and 55% in the control group). LS BMD increased equally in treated and non-treated patients. FN BMD remained stable in the treatment group, but decreased in the control group. There was no significant difference in changes in BMD at either site between treated and untreated patients or between patients in whom treatment was initiated between 3 and 6 months or more than 6 months after OLT.

Conclusion Our data suggest that although preventing further bone loss, bisphosphonate treatment not initiated shortly after OLT does not significantly

influence post-transplantation changes in BMD compared to controls. Whether bisphosphonate use is associated with a significant decrease in fracture risk remains to be established by long-term randomized controlled studies using these agents.

INTRODUCTION

Vertebral fractures are prevalent in patients with end-stage liver disease awaiting orthotopic liver transplantation (OLT).⁽¹⁾ Accelerated bone loss is observed in the first six months after transplantation despite correction of the metabolic abnormalities associated with disturbed liver function, and fracture risk remains high despite post-OLT recovery of bone mineral density (BMD).⁽¹⁻¹²⁾ The initial rapid decline in BMD after OLT has been consistently shown to be followed by a recovery of bone mass to pre-transplant values one year after OLT.^(1;4-7;9;10;13) However, we and others have demonstrated that this recovery in BMD is not associated with a corresponding decrease in the incidence of new fractures which have been documented in 25-34% of transplant recipients at one year after OLT.^(1;8;10;12;14-17) A meta-analysis conducted in 2011 provides evidence that treatment with bisphosphonates generally prevents or decreases bone loss and decrease fracture risk in patients with solid organ transplantation.⁽¹⁸⁾ The rapid decline in bone mass observed within the first 6 months after OLT combined with the increased bone turnover demonstrated by the histomorphometric analysis of paired bone biopsies obtained at transplantation and 3-6 months after OLT as well as the prevention of this increased bone turnover by very early use of bisphosphonates provide the rationale for the use of bisphosphonates in OLT recipients to prevent or decrease the associated high fracture risk observed within the first year after OLT.^(19;20) However, data on the potential beneficial effect of bisphosphonates to decrease or prevent bone loss and fracture risk after OLT remain scarce with available data obtained from 16 studies with short follow-up duration or including a small number of patients, with a new fracture being the primary end-point in only one randomized controlled trial (RCT).^(19-34;34) A meta-analysis conducted in liver transplant recipients did demonstrate that bisphosphonates had a beneficial effect on bone loss but fracture risk could not be evaluated because of heterogeneity of available data.⁽³⁵⁾ Three studies have previously addressed the efficacy of the orally administered nitrogen-containing bisphosphonate alendronate to decrease or prevent bone loss and fracture risk within the first year after OLT.^(31;34;36)

The aims of our study were to review published data on the effect of bisphosphonate treatment on bone loss and fracture risk after OLT, and to evaluate the effect of bisphosphonate treatment (oral alendronate administered weekly) initiated within the first year after OLT on BMD in liver transplant recipients.

MATERIALS AND METHODS

A. Comprehensive review of the literature

Objective

To review available data on the effect of bisphosphonate treatment to decrease or prevent bone loss and fracture risk when administered to liver transplant recipients before, at or after OLT.

Design

Review of all RCT's and controlled or open observational studies published until May 2014 on the effect of a bisphosphonate of any type administered at any time around OLT, at any dose or schedule, and for any duration after OLT, on BMD and on fracture risk after OLT.

Data source

Pubmed, Embase, reference lists of published papers.

Method

The comprehensive search strategy included the following terms: liver transplantation, ibandronate, bisphosphonates, alendronate, etidronic acid, zoledronic acid, risedronic acid, alendronate, bone mineral density, fractures.

The search was conducted in May 2014 through a search of two databases (Pubmed and Embase) and screening reference lists of published papers. All recovered abstracts were screened. All search generated published papers were carefully read to extract details of treatment and its efficacy.

B. Case series from the Leiden Liver transplantation cohort study

Patients and methods

Patients

The electronic hospital records of all consecutive patients who underwent a first OLT at the Leiden University Medical Centre between January 1st 2000 and January 1st 2011 were screened for treatment with bisphosphonates initiated at any time within the first year after transplantation. Only patients with available BMD measurements before start of treatment and at one and two years after OLT were eligible for entry in the study. In our unit, treatment with bisphosphonates uniformly consisted of oral alendronate at a dose of 70 mg per week in addition to calcium- and vitamin D supplementation at a

combined fixed dose of 500 mg elemental calcium and 400 IU colecalciferol. There was no preplanned protocol for, or prerequisites or time for initiation of treatment with these agents after OLT, which was left at the discretion of the treating physician. The remaining consecutive patients who were transplanted within the same 10-year period and who also had BMD data available at one and two years after OLT, but who were never treated with bisphosphonates before or after OLT were used as controls. Patients in whom treatment with bisphosphonates was initiated before OLT were excluded from the study because of the lack of baseline data before or at start of treatment

Immunosuppressive regimens consisted of corticosteroids for at least 6 months in all patients, with a calcineurin inhibitor: tacrolimus or cyclosporine in the majority, and with mofetil mycophenolate (MMF) or sirolimus or everolimus as additional immunosuppressive agent in a few. International guidelines for immunosuppression were complied with in the case of newly transplanted patients, but established successful immunosuppressive regimen were seldom altered in individual transplant recipients. Basiliximab 20 mg was given in the anhepatic phase and on day 4. Corticosteroid schedules included methylprednisolone at a dose of 500 mg given during the OLT procedure, followed by oral prednisolone at a dose of 20 mg daily for one week, 10 mg daily for 3 months, slowly tapering to complete discontinuation of corticosteroids between 3 and 6 months after OLT in all but a few patients who required maintenance doses of prednisolone of 2.5 – 10 mg/day. Treatment of rejection episodes consisted of methylprednisolone at a single dose of 1000 mg or – in severe cases- of three doses of 1000 mg, followed by oral prednisolone at a dose of 140 mg tapered by 20 mg a day until a dose of 20 mg daily was reached. The number of rejection episodes were recorded in individual patients.

Methods

Demographic and clinical data

Demographic and clinical data were obtained from individual patients' electronic hospital records. Data on age, gender, smoking, height, weight, primary liver disease and date and cause of death were extracted from the records. Concomitant or later renal transplantation was documented. Data on immunosuppressive regimen, calcium and vitamin D supplements and date of initiation and discontinuation of alendronate were also recorded.

Laboratory investigations

Laboratory data were also obtained from electronic hospital records. Biochemical data collected included serum calcium, corrected for an albumin of 40 g/L, phosphate, creatinine, parathyroid hormone (PTH) and 25-hydroxy-vitamin D (25-OHD) concentrations, which were measured at screening for OLT and at various time points

thereafter in the two years after OLT. Bone turnover markers were not routinely measured in all patients so that because of the scarcity of available data these were not used in the analysis. Severity of liver disease was determined using the laboratory-Model for End-stage Liver Disease (MELD) scores (37) calculated on the basis of serum creatinine, bilirubin and INR measurements obtained at time of screening for OLT. The following accepted formula was used to calculate the scores:

$$MELD = 10 * (0.957 * \ln(\text{Creatinine}/88.4)) + (0.378 * \ln(\text{Bilirubin}/17.1)) + (1.12 * \ln(\text{INR})) + 6.43$$

Bone mineral density measurements

BMD was measured at the lumbar spine and at the femoral neck using dual energy X-ray absorptiometry (DXA- Hologic QDR 4500, Hologic inc. Waltham, MA, USA, equipped with reference values based on the National Health and Nutrition Examination Survey (NHANES III)). Absolute measurements of BMD in g/cm² as well as T-scores (matched to a young adult reference population at peak bone mass) and Z-scores (age- and sex-matched reference population) were recorded. World Health Organization (WHO) criteria were used to define osteoporosis (T-score of -2.5 SD or less) and osteopenia (T-score between -1 SD and -2.5 SD). BMD measurements were performed at time of screening for OLT (baseline) and at 6, 12 and 24 months after OLT.

Vertebral fracture assessment

Conventional spinal radiographs were performed as part of the standard protocol for evaluating skeletal complications at screening before OLT and at six and twelve months after OLT.

All radiographs of the thoracic and lumbar spine were blindly assessed for the prevalence of vertebral fractures by two independent observers, using the Genant's semi-quantitative method.(38)

Laboratory, BMD and fracture data collected at time of screening for OLT were considered to be baseline data.

The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre.

Statistical analysis

For descriptive statistics, categorical variables were expressed as numbers and as percentages. Continuous variables were summarized using mean and standard deviation in case of normal distribution of data. Median and 5th and 95th percentiles were used otherwise.

Patients in whom treatment was initiated in the first year after OLT were analyzed on an intention to treat basis. BMD changes were compared with those of patients not

receiving treatment with bisphosphonates, using an extension of a Generalized Linear Model, Generalized Estimation Equations (GEE) with correction for BMD, change in BMD compared to screening, fractures prevalent before OLT and incident fractures after OLT, age, gender and bilirubin levels (reflecting hepatobiliary function).

Prevalence of fractures was calculated by dividing the number of patients with at least one vertebral fracture by the total number of patients with available spinal radiographs. The incidence of fractures was defined as the occurrence of a new vertebral fracture or an increase in grade of a prevalent fracture between serial radiographs before and in the first year after OLT. A GEE model was used to evaluate the influence of change of BMD on fracture risk, corrected for age and sex and accounting for repeated measurements. P-values < 0.05 were considered significant.

Calculations were performed using STATA/SE 12.0 software (Stata Corp LP, TX, USA).

RESULTS

A. Comprehensive review of the literature

Review of the literature generated sixteen studies, including nine RCT's and seven observational studies, and a total of 729 patients treated with bisphosphonates orally or intravenously after OLT. Details of the studies elicited by the literature search are shown in Table 1.

1. Details of bisphosphonate treatment

Bisphosphonates used were largely nitrogen-containing bisphosphonates including oral alendronate and risedronate and intravenous zoledronate, ibandronate and pamidronate. Two early studies reported results with the use of the first generation bisphosphonate etidronate.(29;30) Mode of administration was intravenous in most studies (total number of patients treated 499)(19-23;25-30;32;33) and oral in 4 studies (total number of patients treated 235)(24;26;31;34). Time of initiation of treatment was before OLT in 3 studies (total number of patients treated 66 patients with exact number of patients treated not mentioned in one study)(28;29;31), at or shortly after OLT in 10 studies (total number of patients treated 488)(19-22;24-26;32-34;39), and 1-2 years after OLT in 3 studies (total number of patients treated 82)(23;27;30). Duration of treatment was one year in 7 studies (total number of patients treated 303 patients) (19;21;22;25;26;30;32;34) or 2-3 years in 5 studies (total number of patients treated 251) (23;24;27;31;34). Details of duration of other treatments are shown in Table 1. Follow-up duration was 9 months in one study (total number of patients treated 13)(28), one year in 9 studies (total number of patients treated 413)(19-22;24;26;29;30;32-34) and 2-3 years in 6 studies (total number of patients treated 280)(21;23;25;27;31;34).

Table 1: Available data on the effect of various bisphosphonates to prevent bone loss and fracture risk after liver transplantation

Source	Study type	No. of patients (treatment/controls)	Bisphosphonate used	Time start treatment	Duration of treatment (months)	Follow-up after OLT (months)	Effect on BMD	Effect on fracture risk	Effect on bone turnover	Bone markers Histo-morphometry
Valero et al. 1995 (30)	RCT	40 (17/23)	Calcitonin or Etidronate	17 mo (range 1-74)	12	12	+	NA	NS	NA
Riemsens et al. 1996 (29)	Open label	53 (53/0)	Etidronate	Pre-OLT (median 4 mo, r 20-0)	Until OLT	12	NS	NS	NS	NA
Reeves et al. 1998 (28)	Open label	29 (13/16) ^a	Pamidronate	2-10 weeks before OLT	9 post-OLT	9	NA	+ ^{a,b}	NA	NA
Vedi et al. 2002 (20)	RCT	99 (45/54)	Pamidronate	At OLT	Single dose	12	NS	NA	NA	↓
Dodidou et al. 2003 (23)	Obs	128 (29/99) ^a	Pamidronate	Mean 2 yrs after OLT	24	24	+	+ [*]	↓	NA
Millonig et al. 2005 (31)	Open label	136 (98/38)	Alendronate	Pre-OLT, at OLT, 4 mo after OLT	27	27	+	+	NA	NA
Atamaz et al. 2006(34;39)	Open label	98 (49/49) ^a	Alendronate	< 7 days after OLT	24	24	+	+	↓	NA
Crawford et al. 2006 (22)	RCT	62 (32/30)	Zoledronate	< 7 days after OLT	12	12	+	NA	↓	NA
Boddingbauer et al. 2007 (19)	RCT	96 (47/49)	Zoledronate	At OLT	12	12	+	+	↓	↓
Pennisi et al. 2007 (32)	Open label	85 (43/42)	Pamidronate	At OLT	12	12	+	NA	↓	NA
Monegal et al 2009 (33)	RCT	79 (41/38)	Pamidronate	< 14 days after OLT	3	12	+	NS	↓	NA
Boddingbauer et al. 2010 (21)	RCT	57 (28/29)	Zoledronate	At OLT	12	36	NS	NS	NS	NA
Kaemmerer et al. 2010 (25)	RCT	114 (74/40)	Ibandronate	At OLT	12	24	+	+ ^b	NS	NA
Guadalix et al. 2011 (24)	RCT	89 (45/44)	Risedronate	< 14 days after OLT	24	12	NS	NS	↓	NA
Wagner et al. 2012 (27)	Open label	54 (30/24) ^a	Ibandronate	1-2 yrs after OLT	36	36	+	NS	↓	NA
Shane et al. 2012 (26)	RCT	111 (84/27)	Alendronate Zoledronate	<30 days after OLT	12	12	+	+ [*]	NS	NA

RCT = randomized controlled trial; obs = observational study; OLT = orthotopic liver transplantation; yrs= years; + = beneficial effect of bisphosphonate treatment on BMD or fracture incidence; NS = non-significant effect of bisphosphonate treatment; NA = not available; mo = months; ↓ decrease of bone turnover ↑ increase of bone turnover * p-value not mentioned; ^a historical controls used; ^b only clinical fractures documented.

BMD was sequentially evaluated in 15 studies (total number of patients treated 693) (19-27;29-34;39), and incidence of new fractures was reported in 13 studies (total number of patients treated 608)(19-21;23-29;31;33;34;39), in 2 studies on the basis of clinical fractures (25;28), and in 10 studies the basis of sequential X-rays.(19;21;23-27;29;31;33;34;39) Only one study had incidence of new fractures as a primary end-point(19;21).

2. *Effect of bisphosphonate treatment on bone turnover*

Most studies have evaluated the effect of bisphosphonates on bone turnover using changes in bone turnover markers. The bone turnover marker used as bone formation marker was osteocalcin in most studies, bone alkaline phosphatase in some, and in later years procollagen type 1 N-terminal propeptide (P1NP). Bone resorption markers used were mostly urinary deoxypyridinoline and in later years serum beta crosslaps (CTX). A decrease in bone turnover markers is reported after the initiation of treatment with bisphosphonates in the majority of studies (21-27;29;32;34;39). This decrease in bone turnover has also been histologically confirmed by histomorphometric analysis of paired bone biopsies obtained at start of bisphosphonate treatment shortly after transplantation , and 3-6 months after transplantation.(19;20)

3. *Effect of bisphosphonate treatment on bone loss*

Most studies show a short-term beneficial effect of treatment initiated at or shortly after OLT on BMD.(22;24-26;31-34;39) The conclusion reached by authors of a meta-analysis published in 2010 was that bisphosphonate treatment administered during the critical first months after OLT appeared to reduce accelerated bone loss and to improve lumbar spine (LS) BMD.(35) Data on long-term effect of treatment with bisphosphonates administered at or shortly after OLT on BMD are scarce and conflicting. Treatment with bisphosphonates in the form of pamidronate administered as a single infusion before OLT did not prevent post-transplantation bone loss in the long-term.(29) Three studies showed a persistent beneficial effect on BMD up to 27 months after OLT (25;31;34) but this was not confirmed in a fourth study.(21) Three studies in which treatment was initiated longer than a year after OLT show no bone loss after start of treatment. (23;27;30)

4. *Effect of bisphosphonate treatment on fracture risk*

Data on both short-term and long-term effect of bisphosphonate treatment on fracture risk are conflicting, with studies showing a decreased fracture incidence in treated patients with oral as well as intravenous bisphosphonates, when treatment is initiated before, at or shortly after transplantation, or 1-2 years after OLT with a range of treatment duration of 9-24 months (23;25;26;28;31;34;39), whereas other studies do

not show any beneficial effect on fracture risk.(21;24;27;29;33) Of note is that 2 studies report only clinical fractures (25;28) whereas 10 studies radiographic fractures (19;21;23-27;29;31;33;34;39) and 4 studies report only on BMD changes (20;22;30;32). In the only published meta-analysis, the effect of bisphosphonate treatment on fracture risk could not be analysed because of the heterogeneity of the available data.(35)

Conclusion

A comprehensive review of published literature up to May 2014 suggests that bisphosphonate treatment when administered at or shortly after OLT is able to decrease the rapid early post-transplantation bone loss and to improve bone mass in the short term, irrespective of type of bisphosphonate used or duration of treatment. Data on long-term effects of these agents particularly when started 6 months or longer after OLT on BMD and fracture risk are conflicting.

B. Leiden Case Series

Baseline data

Demographic characteristics

223 patients with end-stage liver disease received an orthotopic liver transplant at the Leiden University Medical Centre between 2000 and 2011 and were eligible for the study (Figure 1). Twenty-one patients were excluded from analysis because of use of bisphosphonates at time of screening for OLT. Bisphosphonate treatment was initiated within the first year after OLT (median 185 days after OLT, range 22-365 days) in 44 patients, of whom 39 patients had available BMD measurements before start of treatment and at one and 2 years after OLT. Bisphosphonate treatment was initiated within the first 6 months after OLT (median 100 days after OLT, range 22-179) in 15 out of these 39 patients. The control group consisted of 104 patients not treated with bisphosphonates in whom BMD measurements were also available at the same time points. Demographic details of bisphosphonate treated and untreated OLT recipients are shown in Table 2. 72% and 68% of patients were men in the treatment and control group respectively, with a respective median age of 49 and 53 years. Most common primary liver disease was viral disease in both groups (33 and 29% in the treatment group and control group respectively), followed by alcohol liver disease (23% and 25% respectively) and cholestatic liver disease (15 and 14% respectively). Median time between screening and transplantation was 9 and 10 months in the treatment and control group respectively.

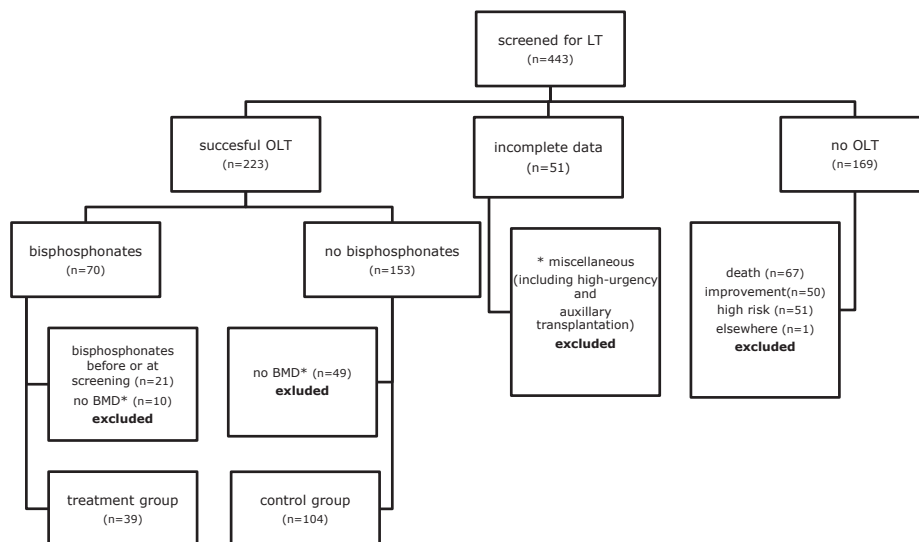


Figure 1: Flow-chart of the study. OLT = orthotopic liver transplantation; BMD = bone mineral density measurement; * none availability of BMD measurement before start of treatment and at one and two years after OLT.

Laboratory data

Vitamin D levels were significantly lower in bisphosphonate treated patients before OLT, with a mean vitamin D level of 24 ± 17 nmol/L compared to a mean level of 35 ± 24 nmol/L in the control group (normal 50-250 nmol/L, $p = 0.043$). In the treatment group, 88% of patients had vitamin D levels < 50 nmol/L, and 53% < 25 nmol/L, compared to the control group, in which 80% of patients had vitamin D levels below 50 nmol/L, and 38% below 25 nmol/L. There was no significant difference in any other laboratory parameter measured at time of screening for OLT between bisphosphonate treated and non-treated OLT recipients, including mean serum creatinine, bilirubin and MELD scores, reflecting severity of liver disease.

BMD data

BMD data were available as per inclusion criterion in all patients studied but LS data were not interpretable in a number of patients ($n = 10$) because of residual oral contrast material from abdominal CT-scans, required as part of the screening protocol for OLT, but chronologically performed by error before DXA in these patients. BMD measurements were significantly lower in the bisphosphonate treated group, with a mean LS BMD of 0.85 ± 0.15 g/cm² compared to 1.00 ± 0.17 g/cm² in the non-treated control group ($p < 0.005$). Femoral neck (FN) BMD was 0.71 ± 0.12 g/cm² in the bisphosphonate treated group compared to 0.85 ± 0.13 g/cm² in the non-treated group

Table 2: Demographic data at time of screening and after liver transplantation

Demographic data	Treatment group	Control group	P-value
Number of patients	39	104	
Gender – number of male patients (%)	28 (72%)	71 (68%)	0.684
Age at the time of OLT- Median (range), years	49 (18-68)	53 (19-67)	0.488
Death during follow-up – no of patients (%)	0	0	
BMI – mean (SD), kg/m ²	25 ± 4	26 ± 5	0.105
Smoking – number of patients (%)	6 (17%)	38 (38%)	0.026 [†]
Primary liver disease – number of patients (%)			
Viral	13 (33%)	30 (29%)	0.133
Alcoholic	9 (23%)	26 (25%)	0.002 [†]
Cholestatic (PSC / PBC/ overlap syndrome)	6 (15%)	15 (14%)	0.058
Auto-immune hepatitis	3 (8%)	4 (4%)	0.706
Malignancy	2 (5%)	5 (5%)	0.273
Metabolic	0 (0%)	3 (3%)	-
Other*	6 (15%)	21 (20%)	0.007 [†]
Previous or concomitant renal transplantation			
- number of patients (%)	2 (5%)	3 (3%)	0.502
Time between screening and OLT			
- median (5 th – 95 th percentile), months	9 (1-19)	10 (1-25)	0.371
Second OLT			
- number of patients (%)	4 (10%)	16 (15%)	0.445
- time to second OLT – median (5 th – 95 th percentile), months	6 (1-11)	4 (0-23)	
Rejection episodes – number of episodes (cumulative incidence, %)			
6 months after OLT	14 (37%)	10 (10%)	< 0.005 [†]
12 months after OLT	0 (0%)	1 (11%)	0.498 [#]
24 months after OLT	2 (45%)	5 (17%)	0.773 [#]
Calcium – and/or vitamin D supplements at time of screening – number of patients (%)	5 (13%)	11 (11%)	0.918
Corticosteroids at time of screening - number of patients (%)	2 (5%)	4 (4%)	0.760
Immunosuppressive medication initiated at OLT			
- number of patients (%)			
Prednisolone, tacrolimus	15 (39%)	42 (40%)	0.834
Prednisolone, cyclosporine	10 (26%)	26 (25%)	0.937
Prednisolone, cyclosporine and MMF	5 (13%)	12 (12%)	0.833
Prednisolone, tacrolimus and MMF	3 (8%)	11 (11%)	0.607
Other	6 (15%)	13 (13%)	-

OLT = orthotopic liver transplantation; BMI = body mass index; PSC = primary sclerosing cholangitis; PBC = primary biliary cirrhosis; LS = lumbar spine; FN = femoral neck; MMF = mycophenolate mofetil.

* including polycystic liver disease, Budd Chiari, portal vein thrombosis, acute fatty liver of pregnancy, cryptogenic cirrhosis, cystic fibrosis, Rendu Osler Weber syndrome; † = p-value < 0.05; # = p-value concerning incident rejection episodes

($p < 0.005$). Mean LS and FN T-scores were respectively -1.98 ± 1.28 and -1.78 ± 1.12 in the bisphosphonate treated group and -0.70 ± 1.23 ($p < 0.005$) and -0.63 ± 1.08 ($p < 0.005$) in the control group. The prevalence of LS osteoporosis was higher in the treatment group than in the control group (35 vs 8%, OR 5.6, 95%CI 1.6-15.9, $p = 0.001$) whereas the prevalence of osteoporosis at the FN did not differ between groups (19 and 8%, OR 2.9, 95%CI 0.89-9.40, $p = 0.078$). Osteopenia was also more prevalent in treated patients, with a prevalence of 52% at the LS compared to 31% in untreated patients (OR 2.3, 95%CI 1.0-5.4, $p = 0.050$), and a prevalence of 59% at the FN compared to 26% in untreated patients (OR 4.3, 95% CI 1.8-10.0, $p = 0.001$).

Prevalent vertebral fractures at screening for OLT

Conventional spinal radiographs were available and evaluable in 33 of 39 patients in the bisphosphonate treated group and in 92 of 104 patients in the non-treated control group at time of screening for OLT. Vertebral fractures were prevalent in 20 patients in the bisphosphonate treated group (61%) and in 51 patients in the control group (55%) with no significant difference between groups ($p = 0.607$). Vertebral fractures were thus equally prevalent in treated and untreated patients at screening for OLT despite significantly lower BMD measurements both at LS and FN in the bisphosphonate treated group.

Follow-up data after transplantation

Clinical course

Immunosuppression included prednisolone in all patients, with the majority also receiving tacrolimus (39% in the treatment group and 40% control group) or tacrolimus and MMF (8% and 11%). Cyclosporine or cyclosporine and MMF were given to 26% and 15% of patients in the treatment group and to 25% and 12% of patients in the control group. Rejection episodes occurred mainly in the first year after OLT, with a significantly higher cumulative incidence of rejection episodes in bisphosphonate treated patients (37% versus 10% in the first six months after OLT), consequently reflecting significantly higher cumulative doses of corticosteroids in bisphosphonate treated than in non-treated patients ($p = 0.002$). All patients in the treatment group were receiving bisphosphonate treatment at 12 months after OLT. At the end of follow-up at 24 months after OLT, 34 out of the 39 patients (87%) in the treatment group were still using alendronate. Reasons for discontinuation of treatment were not clearly documented.

Laboratory data

There was a comparable significant improvement in bilirubin levels during the first year after OLT reflecting improvement in hepatobiliary function with mean bilirubin levels

significantly decreasing from 70 ± 60 mmol/L to 30 ± 66 mmol/L at six months after OLT ($p = 0.038$) in the bisphosphonate treated group and from 88 ± 174 mmol/L to 15 ± 11 mmol/L ($p < 0.005$) in the control group, remaining stable thereafter in both groups. There was no difference in mean bilirubin levels between groups at screening for OLT, or thereafter at any time point measured. There was no significant difference in changes in bilirubin levels between groups ($p = 0.928$).

Changes in renal function were also comparable between groups ($p = 0.337$). Serum creatinine levels increased significantly in the control group with a mean level of 107 ± 44 μ mol/L at 3 months after OLT ($p = 0.003$) but remained stable thereafter. There was also an increase in creatinine levels albeit not significant in the bisphosphonate treated group, reaching levels of 97 ± 31 μ mol/L at 3 months, 102 ± 32 μ mol/L at 6 months and 107 ± 32 μ mol/L at 12 months after OLT ($p = 0.179$). There was no significant difference in serum creatinine concentrations between groups at any time point measured.

Vitamin D levels increased significantly after OLT in both groups, reaching significance at three months after OLT in the bisphosphonate treated group ($p = 0.020$), with a mean level of 36 ± 24 nmol/L increasing to a mean level of 58 ± 25 nmol/L at 12 months after OLT ($p < 0.005$ compared to 3 months after OLT) and remaining stable thereafter with levels of 60 ± 29 nmol/L at 24 months after OLT ($p = 0.573$ compared to 12 months after OLT). In the control group, vitamin D levels increased significantly at 12 months after OLT ($p < 0.005$) with a mean value of 43 ± 24 nmol/L, and increasing further until 24 months after OLT with a mean value of 63 ± 36 nmol/L ($p = 0.001$ compared to 12 months after OLT). There was no significant difference in changes in vitamin D levels between groups ($p = 0.870$).

Sequential changes in BMD

Changes in BMD after OLT are shown in Figure 2 and 3. LS BMD decreased in all patients in the first six months after OLT, albeit significant only in the patients who subsequently did not receive treatment with bisphosphonates ($p = 0.009$), with no further decrease between 6 and 12 months after OLT. LS BMD increased significantly thereafter in both the bisphosphonate-treated and control groups, with values reaching pre-transplantation values in the control group ($p = 0.382$) and exceeding pre-transplant levels after OLT in the bisphosphonate treated group ($p = 0.016$) at 24 months after OLT. Changes in BMD at the LS between 12 and 24 months after OLT did not differ significantly between the treatment and control groups ($p = 0.574$). There was no difference in changes in BMD over the first 2 years of treatment between patients in whom treatment was initiated within the first six months after OLT compared to patients in whom treatment was initiated between six and twelve months after OLT ($p = 0.282$). (Figure 3) There was also no difference in changes in BMD between the group

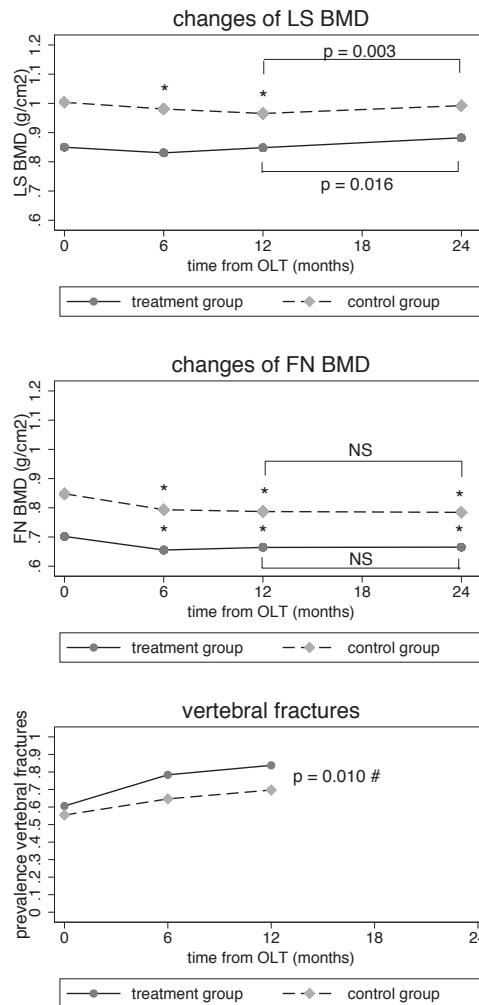


Figure 2: Changes of BMD at the LS (upper panel) and FN (middle panel) and incidence of vertebral fractures (lower panel). P-values concerning increase of BMD between 12 and 24 months after OLT in bisphosphonate treated patients and untreated controls. BMD = bone mineral density; LS = lumbar spine; FN = femoral neck; * = significantly lower when compared to screening ($p < 0.05$); NS = not significant. # = p-value concerning difference in incidence of new vertebral fractures between groups.

of patients in whom treatment with bisphosphonates was initiated within the first 6 months after OLT and the untreated control group ($p = 0.613$).

FN BMD decreased significantly between screening and six months after OLT in both bisphosphonate-treated and untreated patients ($p < 0.005$)(Figure 2). In the bisphosphonate treated group, FN BMD stabilized after start of treatment (mean 167 days after OLT, range 22-364), whereas FN BMD declined further in the control group,

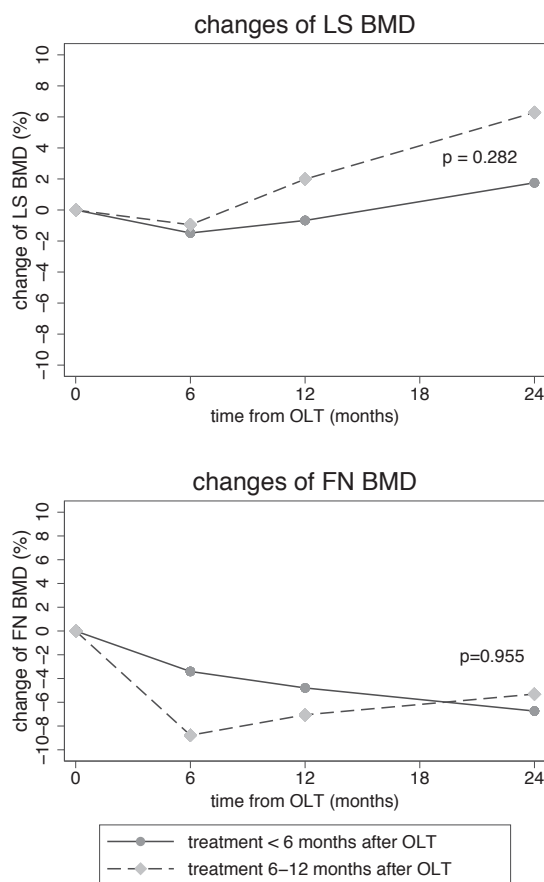


Figure 3: Percentage changes in BMD at the LS (upper panel) and FN (lower panel) in bisphosphonate treated patients in whom treatment was initiated within the first six months after OLT ($n=15$) or between six and twelve months after OLT ($n = 24$). OLT = orthotopic liver transplantation; BMD= bone mineral density; LS lumbar spine; FN = femoral neck; p - value reflecting difference in changes of BMD between patients in whom treatment was initiated before or after 6 months after OLT.

with levels being significantly lower at 24 months than at 6 months after OLT (12 months: $p = 0.091$; 24 months: $p=0.024$). In the treatment group, changes in FN BMD did not differ whether treatment was initiated within the first 6 months after OLT or between 6 and 12 months after transplantation (at 12 months $p = 0.955$)(Figure 3). There was no significant difference in changes in BMD between the treated and control group between 12 and 24 months after OLT ($p = 0.545$), also when comparing the control group with that of patients in whom bisphosphonate treatment was initiated within the first 6 months after OLT ($p = 0.361$).

Incident vertebral fractures during the first year after transplantation

Within the first year after transplantation, the incidence of fractures was significantly higher at 54% in the bisphosphonate treated group compared to 32% in the non-treated control group ($p = 0.010$). At 12 months after OLT, the prevalence of fractures was 84% in the treatment group after an overall median time of initiation of treatment of 180 days (range 343-0), and 70% in the control group ($p = 0.070$) (Figure 2). Data on vertebral fractures were not available beyond the first year after OLT.

DISCUSSION

Our findings from this retrospective analysis of a single centre case series of OLT recipients treated with bisphosphonates indicate that treatment with bisphosphonates initiated within the first year after liver transplantation with a median time of 185 days after OLT and given for a median time of 17 months (range 4-23) does prevent bone loss after start of treatment although there was no significant difference in BMD at one and two years after OLT between treated and untreated patients.

Data on the protective effect of bisphosphonates on post-transplantation bone loss are scarce in liver transplant recipients, with studies conducted in small numbers of patients, treatment initiated either before, at or shortly after OLT or more than a year or later after transplantation.^(22;23;25-29;34;39) Our study addresses the effect of bisphosphonate treatment initiated within the first year after OLT, not directly after transplantation, but after a median time of 185 days after OLT. Whereas treatment initiated within the first six months after OLT did decrease to some extent the accelerated bone loss characteristic of that period, it appears that it was not started soon enough after liver transplantation to prevent it all together. Notwithstanding, our findings do show an increase in LS BMD and a stabilization of FN BMD after initiation of bisphosphonate treatment, regardless of when treatment was initiated after OLT. Intriguingly, BMD changes were not significantly different between bisphosphonate treated and control patients at one and two years after transplantation. We and others have previously shown the spontaneous recovery of BMD to pre-transplant levels in liver transplant recipients not receiving bone-modifying treatment.^(1;2;4;6;8;10;13;15;16;40) This recovery in BMD is believed to be at least partly due to the improvement of gonadal function and of vitamin D status as a result of restoration of hepatobiliary function as well as a result of the tapering and rapid discontinuation of corticosteroids within the first few months after transplantation. We have also shown, however, that this spontaneous recovery in BMD is not associated with a parallel decrease in fracture risk⁽¹⁾, suggesting that bone loss may not be the sole determinant of increased fracture risk, and that altered bone quality may persist and be a significant contributor to fracture

risk after liver transplantation. In our study, the incidence of new vertebral fractures was higher at one year after transplantation in the bisphosphonate treated group, which had also a higher prevalence of osteoporosis and fracture before OLT, with the caveat that at the end of the first year after OLT, only few patients had been treated long enough with bisphosphonates, and more rejection episodes had occurred within the first six months after OLT in treated patients than in non-treated controls, resulting in higher cumulative doses of corticosteroids thereby predisposing to more severe bone loss.

Most studies show a beneficial effect of bisphosphonate treatment on BMD when treatment is initiated at or very shortly after transplantation.(19;22;25;26;34;39) This is in keeping with the beneficial effect of bisphosphonates on the prevention of glucocorticoid-induced bone loss, which is associated with a decrease in fracture risk when these agents are initiated shortly after start of glucocorticoids.(41;42) However, a single peri-operative pamidronate infusion (within 3 months of OLT), or treatment administered only before OLT failed to prevent post-transplantation bone loss (20;29), which suggests that longer treatment may be necessary to achieve bone protection, particularly if high dose prednisolone is used as part of the immunosuppressive regimen in the first months after transplantation when rejection episodes are the most frequent. Only three studies, including one RCT, have previously evaluated the effect of the oral bisphosphonate alendronate in liver transplant recipients, showing an increase in BMD during the first year after OLT.(26;31;39) Data on the effect of treatment with other bisphosphonates are heterogeneous and results are conflicting.(19-30;32-34) Our data are in keeping with a study using oral risedronate treatment, which showed a temporary beneficial effect of treatment at 6 months on LS BMD but not on FN BMD and no effect at either site at 12 months after transplantation.(24) A number of studies have reported beneficial effects of intravenous zoledronate or ibandronate on BMD.(19;22;23;25-27) Only one study compared an oral and intravenous bisphosphonate (alendronate and zoledronate respectively) (26), showing a similar increase in LS BMD and stabilization of FN BMD with the use of either agent. In our study, BMD changes were not different in patients in whom treatment with alendronate was initiated during the first 6 months after OLT compared to those in whom treatment was started 6-12 months after OLT. However, in the majority of the patients in whom treatment was initiated within the first 6 months after OLT, it was started at least 3 months post-transplantation and therefore after the early and most vulnerable postoperative period when the highest corticosteroid dosages are likely to have been administered.

Our study has a number of strengths and limitations. Its main strength lies in the availability of sequential BMD measurements and serial conventional spinal radiographs in the most vulnerable first year after transplantation, and in the blinded semi-quantitative assessment of all spinal radiographs for vertebral fractures by two experienced independent observers. A further strength of the study is the availability of

data in a relatively large and representative group of patients not receiving treatment with bisphosphonates, which was used as control group, and which was largely comparable to the treatment group in every respect except for an expected lower BMD in the bisphosphonate-treated group, which was corrected for in the final analysis of data.

Our study has also a number of limitations. There was no pre-defined protocol for bisphosphonate therapy. The decision to treat and when to start treatment was left to the clinical judgment of the treating physician which may have provided some degree of bias. Even though treatment was not randomly allocated and patients with the lowest BMD and the more prevalent fractures received treatment, we were able to compare treated and untreated patients by correction for BMD as well as for other parameters. Another limitation of our study is the relatively small sample size of bisphosphonate treated patients, although comparable to that of previously published studies. Because of the small number of patients in whom treatment with a bisphosphonate was initiated in referring hospitals before OLT, the lack of data at start of treatment in these patients, and the variable duration of treatment before OLT, we decided to exclude these patients from analysis and were thus unable to assess the effect of starting treatment before OLT on BMD and fracture risk after transplantation. Another limitation of our study is the lack of data on fracture incidence beyond the first year after transplantation, because of which we were unable to evaluate the long-term effect of bisphosphonate treatment on fracture risk. Non-vertebral fractures were poorly documented so that we could also not examine any potential beneficial effect of bisphosphonates on their occurrence.

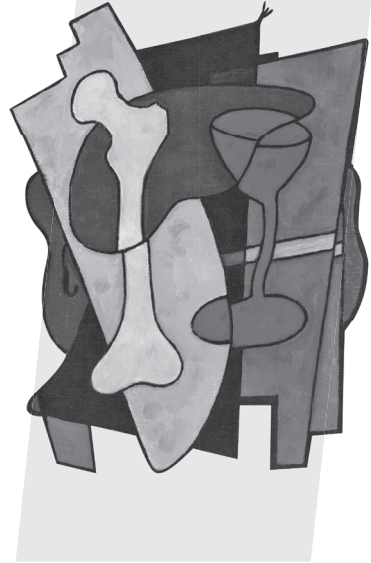
In conclusion, our data suggest that the incidence of vertebral fractures increases in the first year after transplantation and that treatment with bisphosphonates initiated 3 months or later but not immediately after transplantation may be too late to prevent or decrease the crucial and rapid bone loss characteristic of the early post-OLT period and its associated increased long-term fracture risk despite recovery of BMD. Whether this increased fracture risk may be modulated by the earlier and longer use of bisphosphonates after OLT to decrease or prevent skeletal morbidity after OLT remains to be established using RCT's addressing the incidence of new fractures in the long-term as primary end-point.

REFERENCE LIST

1. Krol CG, Dekkers OM, Kroon HM, Rabelink TJ, van HB, Hamdy NA. Longitudinal Changes in BMD and Fracture Risk in Orthotopic Liver Transplant Recipients Not Using Bone Modifying Treatment. *J Bone Miner Res* 2014.
2. Crosbie OM, Freaney R, McKenna MJ, Curry MP, Hegarty JE. Predicting bone loss following orthotopic liver transplantation. *Gut* 1999; 44(3):430-434.
3. Eastell R, Dickson ER, Hodgson SF, Wiesner RH, Porayko MK, Wahner HW, Cedel SL, Riggs BL, Krom RA. Rates of vertebral bone loss before and after liver transplantation in women with primary biliary cirrhosis. *Hepatology* 1991; 14(2):296-300.
4. Floreani A, Fries W, Luisetto G, Burra P, Fagioli S, Boccagni P, Della Rovere GR, Plebani M, Piccoli A, Naccarato R. Bone metabolism in orthotopic liver transplantation: a prospective study. *Liver Transpl Surg* 1998; 4(4):311-319.
5. Feller RB, McDonald JA, Sherbon KJ, McCaughan GW. Evidence of continuing bone recovery at a mean of 7 years after liver transplantation. *Liver Transpl Surg* 1999; 5(5):407-413.
6. Giannini S, Nobile M, Ciuffreda M, Iemmolo RM, Dalle CL, Minicuci N, Casagrande F, Destro C, Gerunda GE, Sartori L, Crepaldi G. Long-term persistence of low bone density in orthotopic liver transplantation. *Osteoporos Int* 2000; 11(5):417-424.
7. Hamburg SM, Piers DA, van den Berg AP, Slooff MJ, Haagsma EB. Bone mineral density in the long term after liver transplantation. *Osteoporos Int* 2000; 11(7):600-606.
8. Hardinger KL, Ho B, Schnitzler MA, Desai N, Lowell J, Shenoy S, Chapman W, Crippin JS. Serial measurements of bone density at the lumbar spine do not predict fracture risk after liver transplantation. *Liver Transpl* 2003; 9(8):857-862.
9. Mart G, Gomez R, Jodar E, Loinaz C, Moreno E, Hawkins E. Long-term follow-up of bone mass after orthotopic liver transplantation: effect of steroid withdrawal from the immunosuppressive regimen. *Osteoporos Int* 2002; 13(2):147-150.
10. Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, Ordi J, Rimola A, Rodes J, Munoz-Gomez J. Bone disease after liver transplantation: a long-term prospective study of bone mass changes, hormonal status and histomorphometric characteristics. *Osteoporos Int* 2001; 12(6):484-492.
11. Scolapio JS, DeArment J, Hurley DL, Romano M, Harnois D, Weigand SD. Influence of tacrolimus and short-duration prednisone on bone mineral density following liver transplantation. *JPEN J Parenter Enteral Nutr* 2003; 27(6):427-432.
12. Leidig-Bruckner G, Hosch S, Dodidou P, Ritschel D, Conrad C, Klose C, Otto G, Lange R, Theilmann L, Zimmerman R, Pritsch M, Ziegler R. Frequency and predictors of osteoporotic fractures after cardiac or liver transplantation: a follow-up study. *Lancet* 2001; 357(9253):342-347.
13. Floreani A, Mega A, Tizian L, Burra P, Boccagni P, Baldo V, Fagioli S, Naccarato R, Luisetto G. Bone metabolism and gonad function in male patients undergoing liver transplantation: a two-year longitudinal study. *Osteoporos Int* 2001; 12(9):749-754.
14. Guichelaar MM, Schmoll J, Malinchoc M, Hay JE. Fractures and avascular necrosis before and after orthotopic liver transplantation: long-term follow-up and predictive factors. *Hepatology* 2007; 46(4):1198-1207.
15. McDonald JA, Dunstan CR, Dilworth P, Sherbon K, Sheil AG, Evans RA, McCaughan GW. Bone loss after liver transplantation. *Hepatology* 1991; 14(4 Pt 1):613-619.
16. Meys E, Fontanges E, Fourcade N, Thomasson A, Pouyet M, Delmas PD. Bone loss after orthotopic liver transplantation. *Am J Med* 1994; 97(5):445-450.

17. Navasa M, Monegal A, Guanabens N, Peris P, Rimola A, Munoz-Gomez J, Visa J, Rodes J. Bone fractures in liver transplant patients. *Br J Rheumatol* 1994; 33(1):52-55.
18. Stein EM, Ortiz D, Jin Z, McMahon DJ, Shane E. Prevention of fractures after solid organ transplantation: a meta-analysis. *J Clin Endocrinol Metab* 2011; 96(11):3457-3465.
19. Bodingbauer M, Wekerle T, Pakrah B, Roschger P, Peck-Radosavljevic M, Silberhumer G, Grampp S, Rockenschaub S, Berlakovich G, Steininger R, Klaushofer K, Oberbauer R, Muhlbacher F. Prophylactic bisphosphonate treatment prevents bone fractures after liver transplantation. *Am J Transplant* 2007; 7(7):1763-1769.
20. Vedi S, Ninkovic M, Garrahan NJ, Alexander GJ, Compston JE. Effects of a single infusion of pamidronate prior to liver transplantation: a bone histomorphometric study. *Transpl Int* 2002; 15(6):290-295.
21. Bodingbauer M, Pakrah B, Kristo I, Marschalek J, Burghuber C, Gyori G, Kainz A, Rasoul-Rockenschaub S, Klaushofer K, Muehlbacher F, Oberbauer R. Non-persistent effect of short-term bisphosphonate treatment in preventing fractures after liver transplantation. *Transpl Int* 2010; 23(1):118-120.
22. Crawford BA, Kam C, Pavlovic J, Byth K, Handelsman DJ, Angus PW, McCaughan GW. Zoledronic acid prevents bone loss after liver transplantation: a randomized, double-blind, placebo-controlled trial. *Ann Intern Med* 2006; 144(4):239-248.
23. Dodidou P, Bruckner T, Hosch S, Haass M, Klar E, Sauer P, Ziegler R, Leidig-Bruckner G. Better late than never? Experience with intravenous pamidronate treatment in patients with low bone mass or fractures following cardiac or liver transplantation. *Osteoporos Int* 2003; 14(1):82-89.
24. Guadalix S, Martinez-Diaz-Guerra G, Lora D, Vargas C, Gomez-Juaristi M, Cobaleda B, Gonzalez EM, Hawkins F. Effect of early risedronate treatment on bone mineral density and bone turnover markers after liver transplantation: a prospective single-center study. *Transpl Int* 2011; 24(7):657-665.
25. Kaemmerer D, Lehmann G, Wolf G, Settmacher U, Hommann M. Treatment of osteoporosis after liver transplantation with ibandronate. *Transpl Int* 2010; 23(7):753-759.
26. Shane E, Cohen A, Stein EM, McMahon DJ, Zhang C, Young P, Pandit K, Staron RB, Verna EC, Brown R, Restaino S, Mancini D. Zoledronic acid versus alendronate for the prevention of bone loss after heart or liver transplantation. *J Clin Endocrinol Metab* 2012; 97(12):4481-4490.
27. Wagner D, Amrein K, Dimai HP, Kniepeiss D, Tscheliessnigg KH, Kornprat P, Dobnig H, Pieber T, Fahrleitner-Pammer A. Ibandronate and calcitriol reduces fracture risk, reverses bone loss, and normalizes bone turnover after LTX. *Transplantation* 2012; 93(3):331-336.
28. Reeves HL, Francis RM, Manas DM, Hudson M, Day CP. Intravenous bisphosphonate prevents symptomatic osteoporotic vertebral collapse in patients after liver transplantation. *Liver Transpl Surg* 1998; 4(5):404-409.
29. Riemens SC, Oostdijk A, van Doormaal JJ, Thijn CJ, Drent G, Piers DA, Groen EW, Meerman L, Slooff MJ, Haagsma EB. Bone loss after liver transplantation is not prevented by cyclical etidronate, calcium and alphacalcidol. The Liver Transplant Group, Groningen. *Osteoporos Int* 1996; 6(3):213-218.
30. Valero MA, Loinaz C, Larrodera L, Leon M, Moreno E, Hawkins F. Calcitonin and bisphosphonates treatment in bone loss after liver transplantation. *Calcif Tissue Int* 1995; 57(1):15-19.
31. Millonig G, Graziadei IW, Eichler D, Pfeiffer KP, Finkenstedt G, Muehllehner P, Koenigsrainer A, Margreiter R, Vogel W. Alendronate in combination with calcium and vitamin D prevents bone loss after orthotopic liver transplantation: a prospective single-center study. *Liver Transpl* 2005; 11(8):960-966.

32. Pennisi P, Trombetti A, Giostra E, Mentha G, Rizzoli R, Fiore CE. Pamidronate and osteoporosis prevention in liver transplant recipients. *Rheumatol Int* 2007; 27(3):251-256.
33. Monegal A, Guanabens N, Suarez MJ, Suarez F, Clemente G, Garcia-Gonzalez M, De la Mata M, Serrano T, Casafont F, Tome S, Barrios C, Navasa M. Pamidronate in the prevention of bone loss after liver transplantation: a randomized controlled trial. *Transpl Int* 2009; 22(2):198-206.
34. Atamaz F, Hepguler S, Akyildiz M, Karasu Z, Kilic M. Effects of alendronate on bone mineral density and bone metabolic markers in patients with liver transplantation. *Osteoporos Int* 2006; 17(6):942-949.
35. Kasturi KS, Chennareddygar S, Mummadi RR. Effect of bisphosphonates on bone mineral density in liver transplant patients: a meta-analysis and systematic review of randomized controlled trials. *Transpl Int* 2010; 23(2):200-207.
36. Shane E, Adesso V, Namerow PB, McMahon DJ, Lo SH, Staron RB, Zucker M, Pardi S, Maybaum S, Mancini D. Alendronate versus calcitriol for the prevention of bone loss after cardiac transplantation. *N Engl J Med* 2004; 350(8):767-776.
37. Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; 33(2):464-470.
38. Genant HK, Wu CY, van KC, Nevitt MC. Vertebral fracture assessment using a semiquantitative technique. *J Bone Miner Res* 1993; 8(9):1137-1148.
39. Atamaz F, Hepguler S, Karasu Z, Kilic M, Tokat Y. The prevention of bone fractures after liver transplantation: experience with alendronate treatment. *Transplant Proc* 2006; 38(5):1448-1452.
40. Abdelhadi M, Eriksson SA, Ljusk ES, Ericzon BG, Nordenstrom J. Bone mineral status in end-stage liver disease and the effect of liver transplantation. *Scand J Gastroenterol* 1995; 30(12):1210-1215.
41. Compston J. Management of glucocorticoid-induced osteoporosis. *Nat Rev Rheumatol* 2010; 6(2):82-88.
42. Homik J, Cranney A, Shea B, Tugwell P, Wells G, Adachi R, Suarez-Almazor M. Bisphosphonates for steroid induced osteoporosis. *Cochrane Database Syst Rev* 2000;(2):CD001347.



6 | Summary and Conclusions

In this Thesis, we establish that skeletal complications are common after liver transplantation. Although this is predominantly in the form of osteopenia and mild grade vertebral fractures, it has nonetheless been clearly shown that both osteopenia and grade 1 fractures are also associated with increased fracture risk. The morbidity and the mortality associated with fractures dictates that all liver transplant recipients should be evaluated for these complications in addition to evaluating and correcting any vitamin D deficiency.

In **Chapter 1**, we review the important role of the liver in the tightly regulated, well-balanced process of bone remodelling. Through its metabolic and excretory function, the liver plays an important role in the absorption of the lipid-soluble vitamins A, E, D and K, the last two important in bone metabolism, and for the metabolism of vitamin D, IGF-1 and gonadal hormones also important for bone remodelling. In liver diseases, factors other than decreased liver function may influence bone turnover such as the underlying primary liver disease if due to inflammation (as seen in viral hepatitis and sclerosing cholangitis associated with inflammatory bowel diseases) or direct toxicity to bone cells (alcohol abuse in alcoholic liver disease or iron overload in hereditary haemochromatosis or bile salts in cholestatic liver disease). In patients awaiting liver transplantation, end-stage liver disease is often associated with a decrease in bone mass and an increase in fracture risk likely to be due to a decrease in bone quality, as not always associated with actual bone loss. After liver transplantation, significant bone loss still occurs despite recovery of liver function due to several factors predominantly including the high dose of corticosteroids used for immunosuppression and in the treatment of rejection episodes and the use of calcineurin inhibitors for immunosuppression with associated risk of renal impairment. Within the first year after OLT, fracture incidence is very high, with fractures occurring in 25-35% of transplanted patients within the first year after OLT.

In **Chapter 2**, we evaluated the prevalence of low bone mass and of vertebral fractures. We found a high prevalence of skeletal pathology in the form of mostly osteopenia and vertebral fractures, predominantly grade 1 fractures. We did take these fractures in consideration because these mild vertebral fractures have recently been shown to be clinically relevant as also predictive of increased future fracture risk. No association could be found between bone mineral density (BMD) and prevalent fractures, with a large number of patients having prevalent fractures despite normal or mildly decreased BMD. The independence of fracture risk from bone mineral density measurements suggest that decreased bone quality rather than bone quantity may play an important role in the increased fracture risk observed in these patients.

In **Chapter 3**, we studied the changes in bone mass after liver transplantation. We demonstrated that after the rapid bone loss observed within the first six months after transplantation, bone mass stabilizes at the femoral neck and improves at the

lumbar spine with spontaneous recovery to pre-transplant values at two years after transplantation. Nevertheless, new vertebral fractures occur in a third of transplanted patients within the first year after transplantation. There was no association between BMD and prevalent fractures at time of screening and fracture risk after liver transplantation. Of all potential risk factors, only male gender and higher age were associated with increased risk of fractures after transplantation but other factors including vitamin D deficiency and severity of liver disease were not.

It has been proven difficult to identify factors associated with increased fracture risk in liver transplant recipients. The association between clinical parameters such as primary liver pathology or severity of liver disease, and of bone mineral density (BMD) and fracture risk has thus been shown to be inconsistent and generally poor. Biochemical markers of bone turnover are widely available for use in the Clinic, although their role in the prediction of bone loss and of fracture risk remains inconclusive and their value in the management of post-transplantation bone disease remains highly debatable. In the case of chronic liver disease, before and after liver transplantation, this is largely due to the many pitfalls in the interpretation of the measurements in the presence of abnormal liver connective tissue metabolism resulting in increased extra-skeletal collagen type I synthesis and degradation as well as because of post - solid organ transplantation confounding factors such as use of corticosteroids and calcineurin inhibitors. In **Chapter 4** we report that although procollagen type 1 N-terminal propeptide (P1NP) levels were increased and osteocalcin levels were decreased at screening before OLT, these markers as well as baseline bone alkaline phosphatase were neither predictive for prevalent BMD or prevalent vertebral fractures before transplantation, nor were they predictive for bone loss or increased fracture risk after transplantation. High C-terminal telopeptide (CTX) levels before liver transplantation and an increase in bone-alkaline phosphatase (BALP) after liver transplantation probably reflect best the risk of bone loss and fracture in patients with end-stage liver disease in the first year after OLT. However, because of the many pitfalls in the interpretation of the most commonly used collagen-derived bone turnover markers in patients with liver disease, caution is strongly advocated with their use in the clinic in therapeutic decision making in the management of skeletal complications before and after liver transplantation.

In the previous Chapters we have shown that skeletal pathology is highly prevalent in liver transplant recipients. However, it is unclear how these patients should be treated in order to prevent further bone loss and decrease or prevent further fractures after transplantation. In **Chapter 5**, we conducted a comprehensive review of the literature on the efficacy of bisphosphonate treatment in liver transplant recipients, which showed that treatment initiated shortly after OLT generally decreases bone loss and improves bone mass. However, data on the long-term effect of bisphosphonate

treatment on BMD are conflicting, and whereas some studies show a beneficial effect of bisphosphonates on fracture risk, others did not. In this Chapter, we also report on a case series of 39 patients treated with bisphosphonates within the first year after transplantation a median of 185 days after OLT, using as controls patients transplanted in the same time period but not receiving bisphosphonates. We observed that BMD increased in both treated and untreated patients with no significant difference between groups. These results suggest that bisphosphonate treatment not immediately initiated after transplantation may be too late to prevent or decrease bone loss characteristic of the early post-transplantation period and its associated increased fracture risk.

CONCLUDING REMARKS AND CLINICAL IMPLICATIONS

Low bone mass and vertebral fractures are highly prevalent in patients with end-stage liver failure awaiting liver transplantation, there is a rapid bone loss early after liver transplantation and the incidence of new fractures significantly increases within the first year after transplantation despite significant recovery of bone mass. Fractures represent a significant cause of morbidity and mortality so that it is of high clinical relevance to identify factors associated with this skeletal complication of liver transplantation. In this Thesis we show that neither clinical parameters such as primary liver pathology or severity of liver disease nor BMD measurements or bone turnover markers can reliably predict fracture risk before or after liver transplantation. It is clear that factors other than bone quantity play a significant role in the increased fracture risk and altered bone quality is a prime contender for this role. There are to date no reliable means of assessing bone quality, and it is not clear whether currently available bone-modifying treatments may positively influence bone quality to decrease or prevent future fractures. Taken together, findings from this Thesis are clinically very relevant, as they suggest that awareness should be raised amongst physicians caring for patients with liver disease about the inevitable skeletal complications associated with failure of the metabolic and excretory function of the liver, and all attempts made at early correction of all reversible aspects of this failure to try and avoid the increased bone fragility associated with altered bone quality.

Nederlandstalige samenvatting

Levertransplantatie wordt vaak gecompliceerd door aandoeningen van het skelet zoals botontkalking (osteoporose) en botbreuken. Zoals wordt beschreven in **Hoofdstuk 1** speelt de lever een cruciale rol in het nauwkeurig afgestemde proces van de botopbouw. De lever is onder andere belangrijk voor de opname van vet-oplosbare vitamines zoals A, E, D en K, waarvan de laatste twee belangrijk zijn voor het botmetabolisme, en voor het omzetten van hormonen zoals IGF-1 en de geslachtshormonen, die ook belangrijk zijn voor normale botopbouw. Bij leverziekten beïnvloeden naast de verminderde leverfunctie ook andere factoren het botmetabolisme. Zo kan een chronische ontsteking door de onderliggende ziekte, zoals virale hepatitis of de inflammatoire darmziekte (Morbus Crohn) bij primaire scleroserende cholangitis, het botmetabolisme negatief beïnvloeden, en hebben langdurig overmatig alcoholgebruik en stapeling van giftige stoffen zoals ijzer bij hereditaire hemochromatose of galzouten bij cholestatische leverziekten een direct nadelig effect op de botcellen. Er wordt dan ook bij patiënten die in afwachting zijn van een levertransplantatie een verminderde botmassa en een sterk verhoogd risico op botbreuken beschreven.

Ondanks het herstel van de leverfunctie treedt na levertransplantatie aanvankelijk verder botverlies op en maakt ongeveer een derde van de getransplanteerde patiënten een nieuwe botbreuk door. Een belangrijke oorzaak van dit botverlies is de afweerremmende medicatie die nodig is om afstotingsreacties na transplantatie te voorkomen en te behandelen. Patiënten worden na levertransplantatie behandeld met onder andere hoge doseringen corticosteroiden, die de botaafbraak versnellen en de botaanmaak onderdrukken, en met calcineurineremmers, die naast hun directe negatieve effecten op de botopbouw ook geassocieerd zijn met een verminderde nierfunctie wat weer een ongunstige invloed heeft op het bot door een verstoorde vitamine D omzetting.

In **Hoofdstuk 2** hebben we de botmassa en het aantal doorgemaakte botbreuken onderzocht bij patiënten die in afwachting waren van een levertransplantatie. Bij het grootste deel van de patiënten was de botmassa al laag ten tijde van de screening voor transplantatie, waarbij bij de meesten een voorloper van botontkalking (osteopenie) werd gezien. Bovendien had ruim de helft van de patiënten op dat moment al één of meerdere milde wervelbreuken doorgemaakt. Er was geen relatie tussen de botminerale dichtheid (BMD) en de botbreuken; een groot deel van de patiënten met een doorgemaakte wervelbreuk had een normale of slechts minimaal verlaagde BMD,

hetgeen suggereert dat bij patiënten met een eindstadium leverziekte een verminderde kwaliteit van het bot een belangrijkere rol speelt dan de botmassa bij het ontstaan van botbreuken.

In **Hoofdstuk 3** hebben we bestudeerd wat er gebeurde met de botmassa en het aantal botbreuken na levertransplantatie. Tijdens de eerste zes maanden na levertransplantatie trad een forse daling van de botmassa op en binnen het eerste jaar na transplantatie maakte twee derde van de patiënten een nieuwe wervelbreuk door. Na de eerste zes maanden na transplantatie stabiliseerde de botmassa van de heupen en trad herstel op van de botmassa van de wervelkolom, waarbij de BMD twee jaar na transplantatie terug was op het niveau ten tijde van de screening voor transplantatie. Het bleek moeilijk om te voorspellen welke patiënten na transplantatie een botbreuk zouden doormaken; er was geen relatie tussen de BMD op het tijdstip van de screening voor transplantatie en het ontstaan van wervelbreuken na levertransplantatie en van alle potentiële risicofactoren waren alleen mannelijk geslacht en hogere leeftijd geassocieerd met verhoogd risico op botbreuken. Alle andere factoren, zoals vitamine D deficiëntie en de mate van leverfalen, waren niet voorspellend.

Het is dus moeilijk gebleken om factoren aan te wijzen bij levertransplantatiepatiënten die geassocieerd zijn met een verhoogd risico op botbreuken. Er was nauwelijks samenhang tussen klinische parameters zoals de aard van de leverziekte, BMD en risico op botbreuken. Er zijn biochemische markers van botaanmaak en botafbraak beschikbaar die men kan meten in bloed en urine en waarvan bekend is dat ze kunnen worden gebruikt om botverlies en botbreuken te voorspellen bij postmenopauzale vrouwen. Een aantal van deze markers zijn afkomstig van de aanmaak en afbraak van het type bindweefselmoleculen dat veel in bot voorkomt, namelijk type 1 collageen. Echter, de voorspellende waarde van deze markers voor botverlies en botbreuken bij transplantatiepatiënten is nog onduidelijk en er is veel discussie over de waarde van deze markers bij het behandelen van skeletcomplicaties na orgaantransplantaties. Bij chronische leverziekten, zowel voor als na transplantatie, zijn er namelijk veel potentiële valkuilen bij het meten en interpreteren van deze markers. Zo is er aan de ene kant een verhoogde aanmaak van bindweefsel in de lever, waaronder van het type 1 collageen, maar is ook er verhoogde afbraak van dit bindweefsel door factoren gerelateerd aan orgaantransplantaties, zoals het gebruik van de afweerremmende medicatie, met name corticosteroiden en calcineurineremmers. In **Hoofdstuk 4** wordt beschreven dat ten tijde van screening voor levertransplantatie de botmarkers N-terminaal pro-peptide en C-terminaal telopeptide van type 1 collageen (P1NP en CTX) bij veel patiënten verhoogd waren en osteocalcine verlaagd, maar dat de hoogte van deze markers alsook het bot-specifiek alkalisch fosfatase (BALP) niet gerelateerd waren aan de verlaagde botmassa of doorgemaakte wervelbreuk. Het bleek ook moeilijk om met de botmarkers botverlies en botbreuken ná transplantatie te

voorspellen. Alleen hoge CTX spiegels ten tijde van screening voor transplantatie en een stijging van BALP na transplantatie waren geassocieerd met botverlies en botbreuken in het eerste jaar na levertransplantatie. Vanwege de vele valkuilen bij de interpretatie van deze van collageen afkomstige markers van botopbouw moet men dus ook voorzichtig zijn bij het gebruik van deze markers bij de besluitvorming rondom de behandeling van skeletcomplicaties bij patiënten met leverziekten en na levertransplantatie.

In de voorgaande hoofdstukken lieten we zien dat skeletcomplicaties veel voorkomen bij levertransplantatiepatiënten. Het is echter nog niet duidelijk hoe deze patiënten zouden moeten worden behandeld om botverlies en botbreuken te voorkomen. In **Hoofdstuk 5** wordt een overzicht gegeven van de resultaten van alle beschikbare wetenschappelijke onderzoeken naar de behandeling van levertransplantatiepatiënten met bisfosfonaten (geneesmiddelen die de botafbraak remmen en het bot versterken). De resultaten laten zien dat behandeling met deze middelen, als die kort na levertransplantatie is gestart, over het algemeen botverlies voorkomt en de botmassa doet verbeteren. Gegevens over effecten van bisfosfonaatbehandeling op botbreuken zijn inconsistent; sommige studies laten een gunstig effect zien van bisfosfonaten op het risico op botbreuken, en andere weer niet. Bovendien is nog onduidelijk of deze behandeling ook op de lange termijn na levertransplantatie de botmassa verbetert en de kans op botbreuken verlaagt.

Verder beschrijven we in dit Hoofdstuk een serie van 39 levertransplantatiepatiënten die werden behandeld met bisfosfonaten en waarbij deze behandeling werd gestart tijdens het eerste jaar na levertransplantatie (meestal een half jaar of later na transplantatie). De behandelde patiënten werden vergeleken met patiënten die in dezelfde periode zijn getransplanteerd, maar die niet werden behandeld met bisfosfonaten. We zagen dat de botmassa verbeterde bij zowel de behandelde als de onbehandelde patiënten en dat er geen verschil was tussen de groepen. Deze resultaten suggereren dat als de behandeling met bisfosfonaten niet direct na transplantatie wordt gestart, deze te laat is om het botverlies, wat vooral optreedt tijdens de eerste periode na transplantatie, te kunnen voorkomen en om het daarbij horende verhoogde risico op botbreuken te verlagen.

Concluderende opmerkingen en implicaties voor de kliniek

Het merendeel van de patiënten met eindstadium leverziekte in afwachting van een levertransplantatie heeft een lage botmassa en heeft al één of meerdere botbreuken doorgemaakt. In de eerste periode na transplantatie treedt fors botverlies op, maar ondanks het uiteindelijk herstel van de botmassa daarna maakt een groot deel van de patiënten in het eerste jaar na levertransplantatie een botbreuk door. Deze botbreuken zorgen voor een grote ziektelast en verhogen de kans op overlijden. Het is dus ook van groot belang om factoren te identificeren die geassocieerd zijn met de

skeletcomplicaties van een levertransplantatie. In dit proefschrift laten wij zien dat noch klinische parameters, zoals de onderliggende leverziekte en de ernst van leverfalen, noch de BMD en biochemische markers van botombouw het risico op botbreuken voor en na levertransplantatie betrouwbaar kunnen voorspellen. Het is duidelijk dat andere factoren dan de botmassa een belangrijke rol spelen bij het verhoogde risico op breuken, waaronder een veranderde kwaliteit van het bot. Er zijn tot op heden geen betrouwbare methoden om de kwaliteit van het bot te meten en het is niet duidelijk of de beschikbare bot-modulerende medicamenten de botkwaliteit dusdanig kunnen beïnvloeden dat botbreuken worden voorkomen en het risico op breuken wordt verkleind.

Concluderend, de bevindingen van dit proefschrift zijn uitermate relevant omdat zij laten zien dat er meer aandacht zou moeten zijn van de behandelend artsen van patiënten met leverziekten voor de onvermijdelijke skeletcomplicaties van het langdurig falen van de lever. Een correctie van alle omkeerbare aspecten van dit falen zou in een zo vroeg mogelijk stadium al moeten worden nagestreefd, zodat een afname van de kwaliteit van het bot wordt voorkomen en zodat het daarmee geassocieerde sterk verhoogde risico op botbreuken wordt verlaagd.

List of abbreviations

25(OH)D	25-hydroxy vitamin D
ALP	alkaline phosphatase
APC	adenomatous polyposis
AIH	auto-immune hepatitis
BALP	bone-alkaline phosphatase
BMD	bone mineral density
BMI	body mass index
BMP	bone morphogenic protein
BMU's	bone remodeling units
BTMs	bone turnover markers
CI	confidence interval
CV	coefficient of variation
CTX	C-terminal telopeptide, beta crosslaps
DAAM1	dishevelled associated activator of morphogenesis 1
DAG	dystroglycan
DBP	vitamin D binding protein
DKK1	Dickkopf-1
DVL	dishevelled
DXA	dual-energy X-ray absorptiometry
ECLIA	Electro-Chemo-Luminescence Immunoassay
ELISA	enzyme-linked immunosorbent assay
FGF-23	fibroblast growth factor-23
FN	femoral neck
FZD	Frizzled
GEE	generalized estimation equations
HBV	hepatitis B virus
HCV	hepatitis C virus
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IGF-1	insulin-like growth factor-1
IGFBP	insulin-like growth factor binding protein
IL	interleukin
IOF	International Osteoporosis Foundation
IP ₃	inositol 1,4,5-trisphosphate, type 3
IRMA	immunoradiometric assay
JNK	Jun kinase
LPR-5	lipoprotein receptor-related protein-5

LS	lumbar spine
LT	liver transplantation
LUMC	Leids Universitair Medisch Centrum / Leiden University Medical Centre
M-CSF	macrophage colony-stimulating factor
MELD	Model for End-stage Liver Disease
MMF	mycophenolate mofetil
NFATc1	nuclear factor of activated T-cells, cytoplasmic, calcineurin dependent 1
NHANES	National Health and Nutrition Examination Survey
NTX	N-terminal telopeptide
OLT	orthotopic liver transplantation
OPG	osteoprogenin
OPN	osteopontin
OR	odds ratio
P1NP	procollagen type 1 N-terminal propeptide
PBC	primary biliary cirrhosis
PDGF	platelet derived growth factor
PGE-2	prostaglandin E-2
PLC	phospholipase C
PPAR- γ	peroxisome proliferator activated receptor- γ
PSC	primary sclerosing cholangitis
PTH	parathyroid hormone
PTHrP	parathyroid hormone related peptide
RANK	receptor activator of nuclear factor kappa B
RANKL	receptor activator of nuclear factor kappa B- ligand
RCT	randomized controlled trial
ROCK	RHO-associated kinase
ROR	receptor-tyrosine-kinase-like orphan receptor
RUNX2	Runt-related transcription factor 2
SD	standard deviation
SFRP	secreted frizzled-related proteins
SHBG	sex hormone binding globulin
TCF/LEF	T cell factor/lymphoid enhancer factor
TGF-beta	transforming growth factor beta
TNF	tumor necrosis factor
TPO	thrombopoietin
Ub	polyubiquitination
VDR	vitamin D receptor
VF	vertebral fractures
WHO	World Health Organisation
WIF1	WNT inhibitory factor 1

Dankwoord

Dit proefschrift vindt haar oorsprong in een uitstapje naar de faculteit Geesteswetenschappen, waar ik colleges van hoogleraren Piet Emmer en Leonard Blussé heb bijgewoond over de geschiedenis van de Nederlandse en Europese expansie overzee. De vrijstelling die mijn opgedane kennis over uitgewisselde ziekten tussen Europa en de Nieuwe Wereld opleverde, gaf mij de ruimte voor een extra wetenschapsstage in mijn derde studiejaar bij **Neveen Hamdy** op de afdeling Endocrinologie en Stofwisselingsziekten van het Leids Universitair Medisch Centrum (LUMC). Vanaf die tijd heeft Neveen mij als co-promotor begeleid en mij veel geleerd over alle aspecten van het opzetten en uitvoeren van wetenschappelijk onderzoek. Daarnaast is zij als interniste met een schat aan kennis en wijze woorden voor mij een mentor geweest tijdens de roerige jaren van mijn co-schappen en opleiding tot internist.

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List of publications

1. Krol CG, Dekkers OM, Kroon HM, Rabelink AJ, van Hoek B, Hamdy NA. Longitudinal changes in BMD and fracture risk in orthotopic liver transplant recipients not using bone-modifying treatment. *Journal of Bone and Mineral Research* 2014; 29(8): 1763-1769
2. Krol CG, Dekkers OM, Kroon HM, Rabelink AJ, van Hoek B, Hamdy NA *No association between BMD and prevalent vertebral fractures in liver transplant recipients at time of screening before transplantation* *Journal of Clinical Endocrinology and Metabolism* 2014; 99(10): 3677-3685
3. Krol CG, Meiland DC, Dekkers OM, Kroon HM, van der Veer E, Rabelink AJ, van Hoek B, Hamdy NA *Pitfalls in the interpretation of bone turnover markers in orthotopic liver transplantation* – in progress
4. Krol CG, Dekkers OM, Kroon HM, Rabelink AJ, van Hoek B, Hamdy NA *Bisphosphonates in the prevention of bone loss after orthotopic liver transplantation: a review of the literature and case series from the Leiden Liver Transplant Cohort Study* - in progress
5. Krol CG, van Hoek B, Hamdy NA *Skeletal complications before and after liver transplantation: a review of the literature* – in progress

Curriculum Vitae

Charlotte Georgette Krol werd op 22 april 1983 in Rijswijk geboren. Na *cum laude* te zijn geslaagd voor haar gymnasium aan het Were Di college in Valkenswaard ging zij in 2001 geneeskunde studeren aan de Rijksuniversiteit Leiden. Vanaf haar derde studiejaar deed zij onderzoek naar botmetabolisme bij niertransplantatiepatiënten onder begeleiding van mw. dr. N.A.T. Hamdy op de afdeling Endocrinologie en Metabole ziekten van het Leids Universitair Medisch Centrum (LUMC). In 2005 behaalde zij haar doctoraal examen met haar scriptie "*Bone and mineral metabolism in kidney transplant patients*".

Na het voltooien van haar co- assistentschappen behaalde zij eind 2007 ook haar artsexamen. Aansluitend begon zij in 2008 aan haar opleiding tot internist in het Rijnland Ziekenhuis in Leiderdorp (opleider dr. M.J.F.M. Janssen) om in 2010 haar opleiding tot internist voort te zetten in het LUMC (opleiders prof. dr. J.A. Romijn, prof. dr. J.H. Bolk, prof. dr. J.W.A. Smit, prof. dr. J.T van Dissel en prof. dr. J.W. de Fijter). In 2012 begon zij aan haar aandachtsgebied op de afdeling Endocrinologie en Metabole ziekten van het LUMC (opleider prof. dr. A.M. Pereira Arias).

Naast de opleiding tot internist zette zij haar onderzoek voort, waarbij zij zich vanaf 2012 richtte op botmetabolisme bij levertransplantatiepatiënten. Dit promotieonderzoek heeft zij uitgevoerd onder begeleiding van mw. dr. N.A.T. Hamdy en prof. dr. B. van Hoek en in samenwerking met de afdelingen Maag-Darm-Leverziekten, Klinische Epidemiologie en Radiologie. Eind 2013 heeft zij haar opleiding tot internist-endocrinoloog afgerond waarna zij werd aangesteld als chef de clinique in het Catharina ziekenhuis in Eindhoven. In die periode heeft zij ook haar proefschrift voltooid. Sinds 1 januari 2015 is zij werkzaam als internist-endocrinoloog in het Amphia ziekenhuis in Breda.

