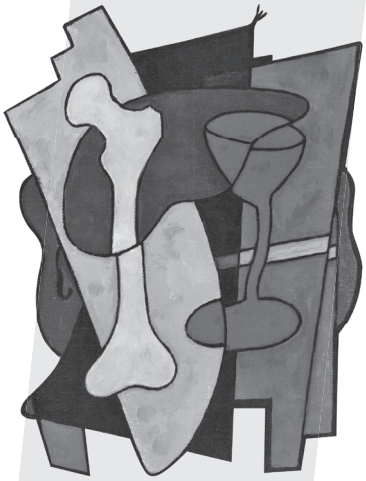

4 Pitfalls in the interpretation of bone turnover markers in liver transplantation

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 phosphoatase (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:

Z-score = (BTM value of individual patient – mean BTM value of reference group) / 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 5^{th} and 95^{th} 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.

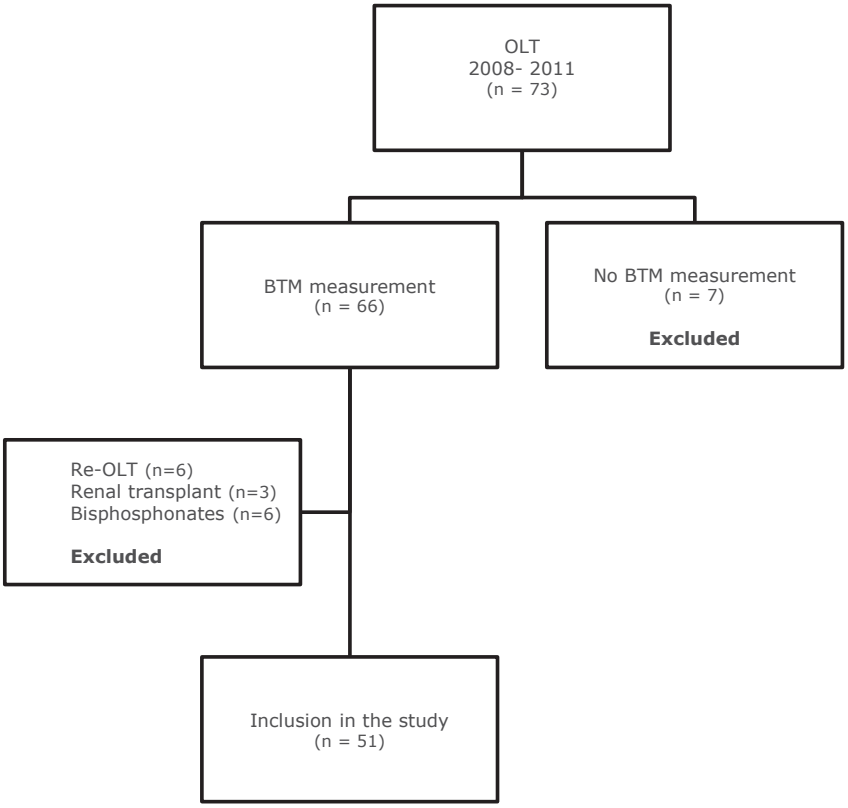

Figure 1: Study flow-chart. OLT = orthotopic liver transplantation; BTM = bone turnover marker.

Biochemical parameters

At time of screening, mean creatinine level was 88 ± 37 µmol/L. Mean 25(OH)D level was 36 ± 27 nmol/L, with 82% of patients having levels of < 50 nmol/L and 43% of patients of < 25 nmol/L. Mean bilirubin level was 96 ± 212 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 ± 0.16 g/cm^2 and mean femoral neck (FN) was 0.80 ± 0.14 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 (5th – 95th 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 = mycofenolate mofetil. * including polycystic liver disease, cryptogenic cirrhosis, non-alcoholic steatosis hepatis, 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

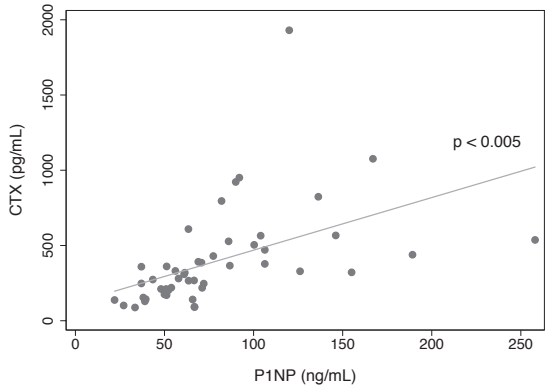

Figure 2: Association between the bone resorption marker serum CTX and the bone formation marker serum P1NP. CTX= C-terminal telopeptide of type I collagen; P1NP = serum procollagen type 1 N-terminal propeptide.

±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 µmol/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/cm^2 at twelve months after OLT compared to 1.00 ± 0.16 g/cm^2 at baseline ($p = 0.236$). There was however, a significant decrease in FN BMD after OLT from 0.79 ± 0.13 g/cm^2 at time of screening to 0.75 ± 0.12 g/cm^2 at six months after OLT ($p < 0.005$) remaining stable thereafter with a mean BMD of 0.77±0.14 g/cm^2 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

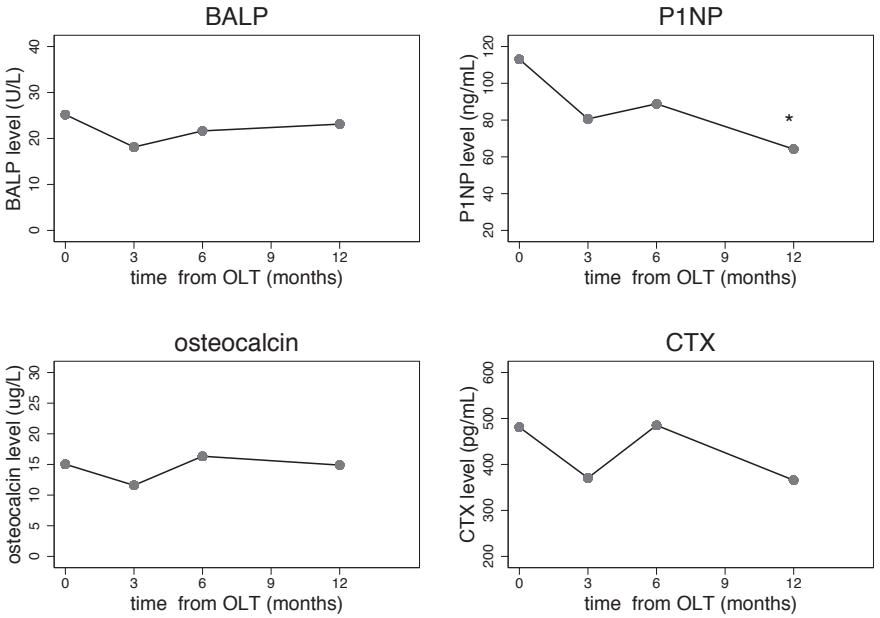

Figure 3: Changes in bone turnover markers after OLT. BALP = bone specific alkaline phosphatase; P1NP = procollagen type 1 N-terminal propeptide; CTX = C-terminal telopeptide of type I collagen; * = significant change compared to screening, p-value < 0.05.

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).

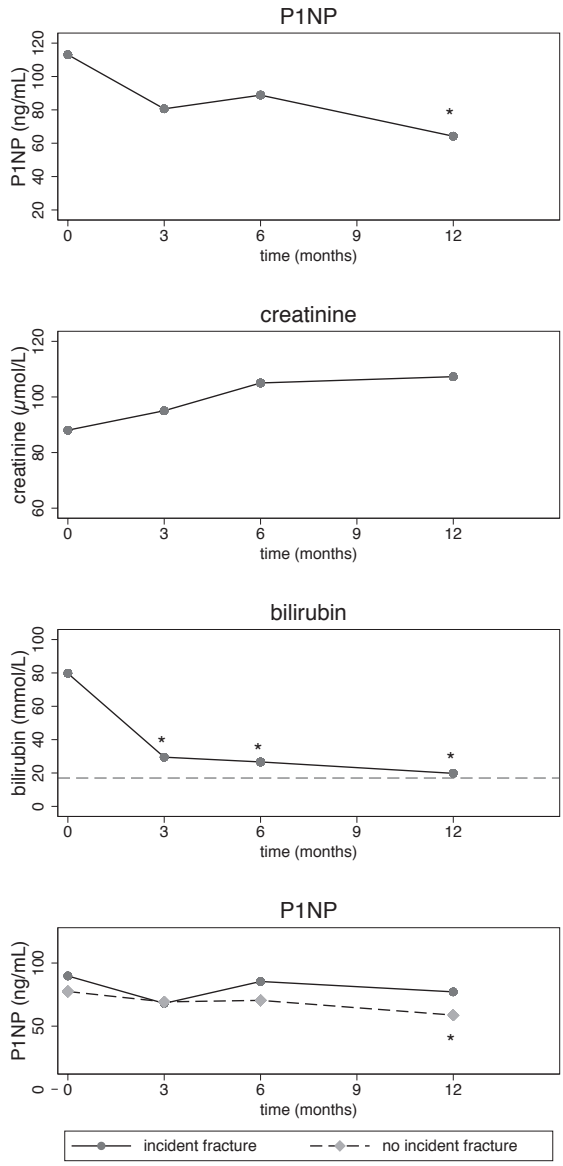

Figure 4: Highlight of factors affecting interpretation after transplantation. P1NP = procollagen type 1 N-terminal propeptide. * = significant change compared to screening, p-value < 0.05.

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 disappointly 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, Fagiuoli 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, 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. 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, 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. 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 I):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, Hillingso 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.