

## METABOLIC EFFECTS

### Bone Mineral Density, Bone Turnover Markers and Cytokines in Alcohol-Induced Cirrhosis

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**Abstract** — **Aims:** Liver cirrhosis is a risk factor for osteoporosis. However, the pathogenesis of the bone mass loss in patients with alcohol-induced cirrhosis (AC) is not well understood. Serum concentrations of soluble tumour necrosis factor receptor (sTNF-R55), neopterin and soluble interleukin 2 receptor (sIL-2R), activation markers of cellular immunity, correlate with clinical activity and severity of the AC. The aim of this study is to evaluate the association of these soluble markers with the development of osteoporosis in patients with AC. **Methods:** We studied 33 consecutive patients with AC and 24 healthy volunteers. Bone mineral density (BMD) was measured by X-ray absorptiometry in the lumbar spine (LS) and femoral neck (FN). Neopterin was measured by radioimmunoassay. Serum concentrations of sTNF-R55 and sIL-2R were measured by enzyme immunoassay. We also determined serum levels of osteocalcin and bone alkaline phosphatase as biochemical markers of bone formation, and deoxypyridinoline urinary excretion (D-Pyr) as marker of bone resorption. **Results:** Patients with AC had reduced BMD (expressed as z-score) in all sites (LS:  $P < 0.001$  and FN:  $P < 0.05$ ). Serum concentrations of sTNF-R55 were significantly higher in patients with both AC and osteoporosis than in those with only AC ( $P < 0.001$ ). Serum levels of sTNF-R55 positively correlated with D-Pyr urinary excretion ( $r = 0.354$ ;  $P = 0.01$ ). Serum levels of sIL-2R were significantly higher in patients with both AC and osteoporosis than in those with only AC ( $P < 0.05$ ). **Conclusions:** There is a relation between activation of the cellular immunity and osteoporosis in AC. Bone mass loss could be related to the increased bone resorption found in these patients.

#### INTRODUCTION

Osteoarticular alterations as extrahepatic manifestations of chronic liver disease (CLD) are well known (Diamond *et al.*, 1990; Gallego-Rojo *et al.*, 1998; Rouillard and Lane, 2001; Pares and Guañabens, 2008). Cirrhosis is considered to be a risk factor for osteoporosis. Nevertheless, the pathogenesis of bone mass loss in AC is still unknown (Giouleme *et al.*, 2006). The prevalence of osteoporosis in CLD can reach up to 50% (Bonkovsky *et al.*, 1990; Gallego-Rojo *et al.*, 1998). Several factors are related to the pathogenesis of osteoporosis in cirrhotic patients, i.e. physical inactivity, smoking, malnutrition, vitamin D deficiency or the use of corticoids could affect mineral metabolism in CLD patients (Schiefke *et al.*, 2005; Collier, 2007).

The pathogenesis of bone mass loss in chronic alcoholic subjects without liver disease seems to be related, at least in part, to the reduced osteoblastic activity associated with chronic alcoholism (García-Sánchez *et al.*, 1995; González-Calvin *et al.*, 1999; Maran *et al.*, 2001).

In AC patients, other factors related to liver disease could participate apart from the toxic effect of alcohol (Kimble 1997; Santolaria *et al.*, 2000). Several evidences show that immune alterations and an increase in production of cytokines are related to the severity of CLD (Neuman, 2003; García-Ruiz *et al.*, 2004; Feng, 2005; Leevy and Elbeshbeshy, 2005; García-Valdecasas *et al.*, 2006). These changes could be involved in other systemic manifestations of liver cirrhosis such as bone mass loss of these patients.

Therefore, we determined serum concentrations of soluble tumour necrosis factor receptor (sTNF-R55), soluble interleukin 2 receptor (sIL-2R) and macrophage product neopterin to study the possible role of immune changes on the pathogenesis of osteoporosis and bone turnover markers in patients with AC.

#### METHODS

##### Subjects

Thirty-three consecutive males outpatients were studied, with a mean age of 53.1 years (SD: 9.59 years); 26 of them were diagnosed by liver biopsy. Patients were not active drinkers for the last 6 months at the moment of the study. Previous alcohol consumption was in a range from 60 to 200 g/day for at least 10 years. The diagnosis of liver cirrhosis was based on liver biopsy in 28 patients; in the remaining four patients, diagnosis was based on the association of clinical, analytical and image data. Patients with malignancy, endocrine, cardiac, immune system or bone disease were excluded. Also, those patients under treatment with corticosteroids, and other medications affecting the immune system or mineral metabolism, were excluded.

Patients were classified into groups depending on the severity of the cirrhosis (Child–Pugh criteria) and the presence of osteoporosis (T-score  $< 2.5$ ) (Pugh *et al.*, 1973). Accordingly, 10 patients were included in Child–Pugh Group A, 13 in Group B and 10 in Group C, with no significant differences in ages among different groups. The control group was formed by 24 healthy subjects (age:  $52.1 \pm 11.7$  years) with an alcohol intake  $< 30$  g/day. All of them were studied to prove the absence of liver, bone metabolism and immune system diseases. The health status was checked by clinical history, physical examination and analytical study.

All subjects gave their informed consent to participate in the study. The protocol was approved by our hospital's ethics committee in February 2005, and the study was performed between April 2005 and October 2008.

##### Bone mineral density measurements

Bone mineral density (BMD) was measured in all patients in lumbar spine (LS) and femoral neck (FN) by X-ray absorptio-

metry (Hologic QDR-1500, S/N 1535; Hologic Corp., Waltham, MA, USA). Osteopaenia and osteoporosis have been defined according to the World Health Organization criteria depending on the BMD values (Kanis, 2000).

#### Laboratory analyses

Serum concentrations of sTNF-R55 and sIL-2R were measured by enzyme immune analysis (T Cell Sciences, Cambridge, MA, USA). Neopterin concentrations were measured by the neopterin RIAcid kit (BRAHMS, Hennigsdorf, Germany). In addition, serum concentrations of insulin-like growth factor I (IGF-I; Nichols Institute Diagnostics, San Juan Capistrano, CA, USA), BGP-osteocalcin and intact PTH (Incstar Corporation, Cat 22800, Stillwater, MN, USA), sexual steroids binding globulin (SHBG; Farnos SF 90460, Oulunsalo, Finland) and 25-hydroxyvitamin D (25OHD; RIA, Incstar) were measured by radioimmunoassay.

Urinary free deoxypyridinoline (D-Pyr) was measured by enzyme immune analysis (Pyrilinks; Metra Biosystems Inc., Santa Clara, CA, USA) according to a previously described method (Delmas *et al.*, 1993). Urinary excretion was expressed as the ratio of D-Pyr to creatinine.

#### Statistical analysis

Statistical analyses were performed using the programme SPSS 15.1 for Windows. All results were expressed as the mean  $\pm$  SEM. Normal distribution of values in controls and cirrhotic patients were verified with the Kolmogorov–Smirnov test. The mean values in different groups were compared by one-way ANOVA and unpaired two-tailed *t*-tests or non-parametric Mann–Whitney *U*-test as appropriate. A one-sample *t*-test was used to assess the differences between the mean BMD z-score at each site and zero. Correlation studies were performed with Pearson standard linear regression analysis (normal distribution) or the Spearman test (non-normal distribution). Risk factors for the development of osteoporosis were analysed by backward stepwise regression analysis. Variables entered into the model included the patient's age, Child score, free testosterone index and serum values of 25OHD, IGF-I, PTH, sTNF-R55, sIL-2R and neopterin.

*P*-values <0.05 were regarded to indicate significant differences between groups or correlations between variables tested.

## RESULTS

Table 1 shows biochemical data. Data of the main variables of this study are summarized in Table 2.

Table 1. Biochemical data in control subjects and alcoholic cirrhotic patients

Biochemistry	Controls ( <i>n</i> =24)	Viral cirrhosis ( <i>n</i> =33)
Serum albumin (g/dl)	4.50 $\pm$ 0.95	3.73 $\pm$ 0.12*
Serum bilirubin (mg/dl)	0.86 $\pm$ 0.08	2.14 $\pm$ 0.23*
Prothrombin activity (%)	99.83 $\pm$ 0.14	66.97 $\pm$ 2.51*
Serum alkaline phosphatase (U/l)	150.67 $\pm$ 5.03	282.91 $\pm$ 17.22*
Serum aspartate transaminase (U/l)	22.96 $\pm$ 0.94	56.91 $\pm$ 4.97*
Serum alanine transaminase (U/l)	26.08 $\pm$ 2.05	40.00 $\pm$ 4.09**
Serum glutamyltransferase (U/l)	31.83 $\pm$ 2.58	130.88 $\pm$ 18.05*

Values are the mean $\pm$ SEM.

\**P*<0.001 vs controls.

\*\**P*<0.01 vs controls.

#### Bone mineral density

Osteoporosis was found in 39.4% of the patients with AC. When patients were classified according to the severity of liver disease, the percentages of osteoporosis were: Child A 30.0%, Child B 23.1% and Child C 70.0%. BMD in the LS and FN, expressed as the z-score, were significantly lower in patients with liver cirrhosis than in the reference population at both LS (*P*<0.001) and FN (*P*<0.05; Table 2).

#### Serum concentrations of soluble factors and other biochemical markers

Table 2 shows that serum values of sTNF-R55 (*P*<0.001), sIL2-R (*P*<0.001) and neopterin (*P*<0.001) were significantly higher in patients than in healthy controls. Child B (*P*<0.01) and C (*P*<0.001) patients also had higher serum sTNF-R55 than Child A patients.

Patients with osteoporosis had serum concentrations of sTNF-R55 (8.63 $\pm$ 2.42 pg/ml) significantly higher than patients without osteoporosis (5.08 $\pm$ 2.33 pg/ml, *P*<0.001; Figure 1). Serum concentrations of sIL-2R were significantly elevated in AC patients with osteoporosis in the LS and FN (1290 $\pm$ 474 pg/ml) compared with those without osteoporosis (845 $\pm$ 450 pg/ml, *P*<0.05; Figure 2). IGF-I serum concentrations and D-Pyr urinary excretion in AC were significantly higher than in the control group (*P*<0.001; Table 2).

Table 3 shows significant differences between parameters in the Child groups.

#### Relationship between variables

Serum concentrations of sTNF-R55 were inversely correlated with the BMD in LS (*r*=0.364; *P*<0.05) and positively with the urinary concentrations of D-Pyr/creatinine (*r*=0.354; *P*=0.01).

Serum concentrations of sIL-2R were also inversely correlated with the BMD in LS (*r*=0.383; *P*<0.05) and in FN (*r*=−0.309; *P*<0.05).

We found a negative correlation between neopterin and BGP (*r*=−0.33; *P*<0.05). No correlation was found between neopterin concentrations and BMD. We also found a highly significant positive correlation between IGF-I and serum parathormone concentrations (*r*=0.500; *P*<0.01).

#### Regression analysis

The results of the backward stepwise regression analysis determining the main predictor of LS and FN osteoporosis are shown in Table 4. Concentrations of sTNFR-55 and sIL-2R and Child score were the most important independent variables of both LS and FN (*P*<0.05).

## DISCUSSION

Osteoporosis is a complication of advanced liver disease. We found decreased BMD in patients with AC compared with the controls, and this difference is even higher in advanced liver disease. Bone mass loss in viral cirrhosis has been related to an increased bone resorption (Corazza *et al.*, 2000; Crawford *et al.*, 2003; Gonzalez-Calvin *et al.*, 2004; Schiefke *et al.*, 2005). However, the pathogenesis of bone mass loss in alco-

Table 2. Serum concentrations of soluble factors and biochemical parameters of bone mineral metabolism in control subjects, patients with cirrhosis and patients classified according to Child–Pugh score

	Controls (n=24)	Cirrhotic patients (n=33)	Child A (n=10)	Child B (n=13)	Child C (n=10)
Age (years)	52.08±2.38	53.09±1.67	57.00±2.44	52.85±2.75	49.50±3.23
sTNF-R55 (pg/ml)	1.28±0.12	5.94±0.48*	3.50±0.65*****	6.23±0.60*,*****	8.00±0.75*,**
sIL-2R (pg/ml)	545.33±37.30	953.06±85.03*	706.70±127.24	998.08±134.89*	1140.90±161.68*,*****
Neopterin (nmol/l)	4.91±0.30	7.69±0.74*	9.67±1.59*	6.85±0.97***	6.81±1.30***
z-score LS	0.05±0.09	-1.33±0.19*	-0.74±0.32*****	-1.42±0.28*,*****	-1.80±0.39*,*****
z-score FN	0.02±0.12	-0.58±0.17***	-0.57±0.30***	-0.27±0.30	-1.01±0.26*****
25OHD (pg/ml)	45.51±5.32	26.42±4.68***	22.43±5.38***	36.13±9.97	17.79±5.68***
FTI	9.64±0.72	7.71±1.23	9.14±2.46	8.79±3.29	7.56±2.42
IGF-I (ng/ml)	161±69.3	82.6±48.2*	110.56±18.87*****	74.11±1.43*	63.68±10.21*,*****
BGP (ng/l)	2.70±0.48	3.36±1.91	2.68±1.31	4.21±2.22	2.95±1.74
SHBG (nmol/l)	31.32±1.83	70.92±7.44*	56.48±12.12***	94.85±13.84*,*****	54.26±5.85***
PTH (pg/ml)	28.51±2.64	23.44±2.45	24.71±3.01	26.75±4.90	17.89±3.88
Urinary D-Pyr (nmol/mmol)	4.22±0.49	7.47±0.97*****	5.13±0.92	7.66±1.08*****	9.57±2.67*****
Urinary calcium/creatinine (mg/mg)	0.08±0.01	0.14±0.28	0.08±0.15	0.19±0.05	0.14±0.05

Data are the mean±SEM. FTI, free testosterone index.

\*P<0.001 vs controls.

\*\*P<0.001 vs Group A.

\*\*\*P<0.05 vs controls.

\*\*\*\*P<0.05 vs Group A.

\*\*\*\*\*P<0.01 vs controls.

\*\*\*\*\*P<0.01 vs Group A.

holic liver diseases is not fully understood, and it has been published that a decreased osteoblastic activity could be involved (Turner, 2000).

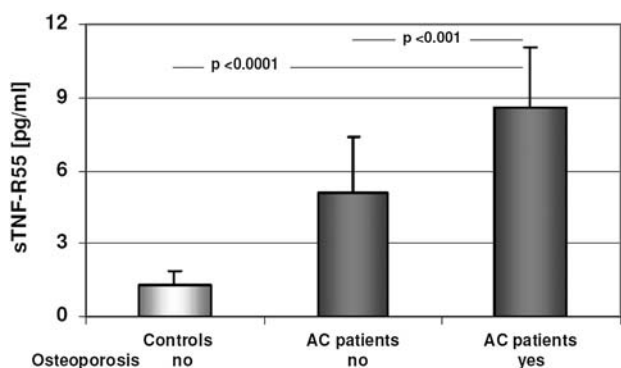


Fig. 1. Serum concentrations of soluble 55-kDa tumour necrosis factor receptor (sTNF-R55) in control subjects and patients with alcoholic cirrhosis (AC) with and without osteoporosis.

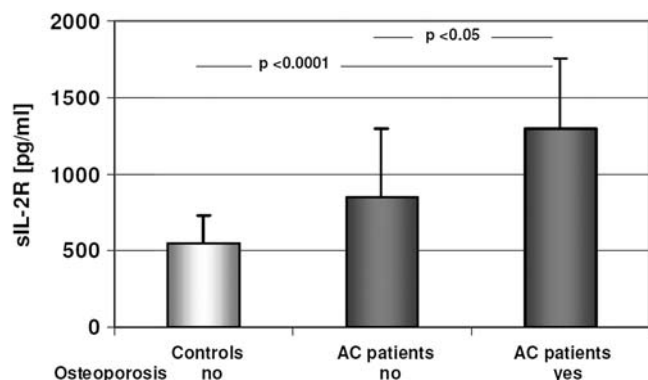


Fig. 2. Serum concentrations of soluble interleukin-2 receptor (sIL-2R) in control subjects and patients with alcoholic cirrhosis (AC) with and without osteoporosis.

IGF-I is a polypeptide that enhances bone growth, and it is essential for normal development of different tissues, including bone, and serum IGF-I levels is known to be a major determinant of BMD in healthy men (Johannsson *et al.*, 1994). The decrease in serum concentrations of IGF-I found in our patients could be involved in bone mass loss. We found increased D-Pyr urinary excretion in patients comparing to control, which probably reflects an increased bone resorption in our patients as D-Pyr is a selective marker of bone resorption that is only found in bone and dentine (Tsuneoka *et al.*, 1996; Gonzalez-Calvin *et al.*, 2004). This result suggests that patients with alcoholic cirrhosis have high bone turnover osteoporosis, as it has also been described in patients with viral cirrhosis (Gallego-Rojo *et al.*, 1998; González-Calvín *et al.*, 1999). Serum concentrations of sTNF-R55 and sIL-2R were

Table 3. Multiple means comparison of variables between Child groups (ANOVA)

Variable	Significance
Serum albumin	P<0.001
Prothrombin activity	P<0.01
Serum bilirubin	P<0.001
Serum glutamyltransferase	P<0.05
Serum alkaline phosphatase	P<0.05
SHBG	P<0.05
PTH	P<0.05
sTNF-R55	P<0.0001

Only variables with P<0.05 are listed.

Table 4. Backward stepwise regression analysis defining the main predictor of BMD in alcohol cirrhotic patients

Independent variables	β coefficient	SE	P-value
sTNF-R55	-0.37	0.01	0.037
sIL-2R	-0.38	0.04	0.028
Child score	-0.35	0.10	0.043

Only variables with P<0.05 are listed.

significantly increased in patients with osteoporosis, comparing to patients without osteoporosis, and serum levels of both cytokines correlated inversely with BMD and inversely with D-Pyr urinary excretion. TNF is an important bone resorbing cytokine, and its high serum values and its inverse correlation with BMD suggest that continuous stimulation of the immune system can play an important role in the evolution of cirrhosis and other associated processes like osteoporosis.

During the inflammatory response associated with the activation of T lymphocytes, IL-2 receptors are expressed in cellular membranes of mononuclear cells, increasing the shedding of the soluble receptor (sIL-2R). Increased concentrations of sIL-2R have been reported in many liver diseases (Kitaoka *et al.*, 2003). In our patients, increased concentrations of sIL-2R, in addition to sTNF-R55, their relationship with the severity of liver disease and their association with bone mass loss suggest a role of altered immune response in the demineralization process of these patients. Nevertheless, it could be also a parallel process without causative relation.

Neopterin is considered to be a marker of cellular immune activation as well as a marker of the activity of macrophages in different processes. It reflects one part of cellular immune activation, proliferation and differentiation (Murr *et al.*, 2002). Increased neopterin concentrations have been reported in cirrhotic patients of different aetiology including alcoholic cirrhosis (Díez-Ruiz *et al.*, 1995; Wilmer *et al.*, 1995). Our findings suggest that the activated macrophages can play a role in the pathogenesis of liver cirrhosis, but from our data we could not confirm previous reports about the value of neopterin as a prognostic marker of cirrhosis (Gonzalez Reimers *et al.*, 1993) nor as a marker of the alterations in BMD associated with cirrhosis.

Some studies have described an association between immune system alterations and osteoporosis (Oelzner *et al.*, 1999; Sugai *et al.*, 2002). Our findings suggest that the decrease in bone mass in AC patients could be related to a persisting activation of the cellular immunity found in these patients. Bone mass loss in AC in our study may be due not only to a decreased osteoblastic activity but also to an increased osteoclastic activity, as shown by the high bone turnover found in our patients.

*Conflict of interest statement.* None declared.

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