SERUM OSTEOPROTEGERIN AND RANKL LEVELS IN CHRONIC ALCOHOLIC LIVER DISEASE

ELENA GARCÍA-VALDECASAS-CAMPELO1, EMILIO GONZÁLEZ-REIMERS1,8, FRANCISCO SANTOLARIA-FERNÁNDEZ1, MARÍA JOSÉ DE LA VEGA-PRIETO2, ANTONIO MILENA-ABRIL3, MARÍA JOSÉ SÁNCHEZ-PÉREZ1, ANTONIO MARTÍNEZ-RIERA1 and MARÍA DE LOS ÁNGELES GÓMEZ-RODRÍGUEZ3

1Servicios de Medicina Interna, 2Servicio de Laboratorio and 3Servicio de Medicina Nuclear, Hospital Universitario, Universidad de La Laguna, Tenerife, Canary Islands, Spain

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Abstract — Objectives: Osteoprotegerin (OPG) is a decoy receptor that binds RANK-ligand (RANKL) and prevents osteoclast activation. Oestrogens, androgens, corticosteroids, parathyroid hormone (PTH), vitamin D, and several cytokines exert their effects on bone modulating the OPG/RANKL system. Since these substances become altered in chronic alcoholic liver disease, we investigated the OPG/RANKL system in alcoholic liver disease, its relation with bone mineral density (BMD) and with several hormones and cytokines. Methods: Serum OPG, RANKL, C-terminal cross-linking telopeptide of type 1 collagen, osteocalcin, insulin-like growth factor 1 (IGF-1), 1,25 dihydroxyvitamin D, IL-6, tumour necrosis factor (TNF)-α, PTH, estradiol, free testosterone and corticosterone were measured in 77 male alcoholic patients, 25 of them cirrhotics. All these patients underwent assessment of BMD at lumbar spine and left hip by a Hologic QDR-2000 (Waltham, MA) bone densitometer. Nineteen non-drinkers male sanitary workers of similar age served as controls. Results: Serum OPG levels were higher in patients (12.66 ± 6.44 pmol/l) than in controls (6.59 ± 1.58 pmol/l, P < 0.005), especially in cirrhics (15.97 ± 7.03 pmol/l) vs non-cirrhics (10.96 ± 5.45 pmol/l, P < 0.001). Patients also showed higher telopeptide levels (0.60 ± 0.36 vs 0.20 ± 0.10 nmol/100 ml, P < 0.001), less IGF-1 (median = 192, interquartile range (IQR) = 46.7–175.99 ng/ml vs 150, IQR = 118.8–239.4 ng/ml, P < 0.001), vitamin D (25.5, IQR = 18.25–35 pg/ml vs 77.89, IQR = 57.48–98.53 pg/ml, P < 0.001) and osteocalcin (1.8, IQR = 1–3.6 ng/ml vs 6.04, IQR = 4.63–8.20 ng/ml, P < 0.001) than controls, but no differences in PTH and RANKL. Patients also showed lower Z-scores than controls at trochanter (~0.36 ± 0.10 vs 0.26 ± 0.87 in controls, P = 0.026), intertrochanteral area (~0.56 ± 1.16 vs 0.46 ± 1.01, P = 0.001), and total hip (~0.44 ± 1.12 vs 0.42 ± 1, P = 0.003). TNF-α levels were higher in patients (7.40, IQR = 4.30–17.80 pg/ml) than in controls (5.10, IQR = 4.40–8 pg/ml, P = 0.009), especially in cirrhics (median = 13.90, IQR = 6.10–21.10 pg/ml). OPG levels showed strong correlations with TNF-α (r = 0.57, P < 0.001) and IL-6 (r = 0.62, P < 0.001), but not with BMD. Estradiol levels (31.83 ± 13.11 pg/ml) were higher and free testosterone lower (13.62 ± 11.96 pg/ml) in patients than in controls (20.36 ± 3.08 and 18.19 ± 4.68 pg/ml, respectively, P < 0.001 in both cases). Conclusion: OPG is raised in alcohols, especially in cirrhics, showing no relationship with decreased BMD. Also, raised TNF and IL-6 were observed, and were strongly, directly related with OPG levels. Since TNF and IL-6 enhance bone resorption, their relation with OPG suggests a protective effect of raised OPG on bone loss.

INTRODUCTION

Osteoprotegerin (OPG) is a soluble tumour necrosis factor (TNF) receptor produced mainly by osteoblasts, that binds RANK-ligand (RANKL) (a TNF family protein)—also synthesized by osteoblasts—which activates RANK (receptor activator of nuclear factor kappa B). Activated RANK mediates osteoclast differentiation. Thus, binding of OPG to RANKL prevents osteoclast activation and preserves bone (Boyle et al., 2003). In addition to RANKL, OPG binds to TNF related apoptosis induced ligand. It, therefore, seems that, in addition to blocking the effect of TNF on osteoclast activation, osteoprotegerin also protects osteoblast from the apoptotic signals induced by TNF (Bu et al., 2003).

Osteoporosis is frequently observed in the alcoholic patient (Leslie et al., 2003). Ethanol decreases bone formation (Diamond et al., 1989) in a dose-dependent fashion (Turner, 2000), mainly through a direct toxic effect on osteoblast function. It also alters, both directly and indirectly, bone mineral metabolism, including parathyroid hormone (PTH), vitamin D, testosterone, insulin-like growth factor 1 (IGF-1), and cortisol levels. Recent research has shown that the majority of these hormones influence on bone metabolism by modulating the OPG/RANKL system. In this sense it is known that oestrogens lead to increased OPG levels, but cause no change in RANKL levels (Hofbauer et al., 2004); in vitro, OPG production is upregulated by oestrogens (Bord et al., 2003). An opposite effect has been shown for androgens (Hofbauer et al., 2002). Vitamin D in its active form promotes osteoclastogenesis by reciprocally upregulating the expression of RANKL and downregulating that of OPG (Kondo et al., 2004). Some controversy exists, however, regarding the effect of corticosteroids on OPG. Whereas some data suggest an inhibitory effect (Vidal et al., 1998), others reflect raised OPG levels in patients affected by Cushing’s syndrome (Ueland et al., 2001). Thus, it is possible that alterations in the aforementioned hormones lead to changes in OPG, and alter bone homeostasis. In addition several cytokines, as TNF-α and IL-6, may regulate the OPG/RANKL system (Lorenzo, 2000; Yamada et al., 2002; Nagata et al., 2003), and become also altered by chronic alcohol consumption. Indeed, ethanol seems to stimulate IL-6 production, causing via induction of RANKL, activation of osteoclastogenesis (Dai et al., 2000). This is another way by which ethanol and/or alcoholic liver disease may be associated to osteoporosis, and may lead to alterations in the OPG/RANKL system.

Two recent studies on patients affected by chronic liver disease—including alcoholics—have shown an increase in OPG levels, perhaps in response to enhanced bone loss (Gaudio et al., 2005; Moschen et al., 2005).
In the present study we investigate the OPG/RANKL system in alcoholic liver disease, its relation with bone mineral density (BMD) and with several hormones and cytokines which exert active effects on bone and may become altered in chronic alcoholic liver disease, such as estradiol, testosterone, vitamin D and corticosteroids, TNF-α and IL-6.

PATIENTS AND METHODS

Patients and controls

We included 77 male alcoholic patients consecutively admitted to our hospitalization unit. All of them were heavy drinkers of >150 g ethanol/day for prolonged (>5 years) time periods. Twenty-five of these patients were cirrhotics and 52, non-cirrhotics. The diagnosis of liver cirrhosis was established on clinical grounds, including liver ultrasound, scintigraphy, and biopsy when necessary. The mean age of the cirrhotics was 51.6 ± 10.3 years, and that of the non-cirrhotics, 47.2 ± 11.3 years.

The control group was composed of 19 healthy sanitary workers, drinkers of <10 g ethanol day, aged 43.89 ± 11.55 years (Table 1). Age differences among the three groups were not statistically significant (F = 2.70, P = 0.072), nor between patients (as a whole) and controls (t = 1.64, P = 0.10). There were also no differences in body mass index (BMI) (24.95 ± 3.63 kg/m² for patients and 25.60 ± 2.84 kg/m² for controls, P = 0.45).

After giving informed consent, all the patients and controls (Table 1) underwent an assessment of BMD by double energy X-ray absorptiometry at the lumbar spine and hip. BMD, Z-scores, and T-scores were recorded for the femoral neck, trochanter, Ward’s triangle, intertrochantereal area, total hip, and lumbar spine with a HOLOGIC QDR-2000 (Waltham, MA) using the array spine medium and array left hip medium programmes.

We recorded the presence or absence of ascites, encephalopathy, and BMI. We determined serum bilirubin, prothrombin activity and serum albumin, mean corpuscular volume (MCV), and serum gamma-glutamyl transpeptidase (GGT) (Table 1). We also collected blood samples after overnight fast. Samples were stored at –80° until the following hormones and cytokines were determined.

We determined TNF-α by immunometric chemiluminescent assay [intra-assay variation coefficient ranging 4–6.5%, inter-assay variation coefficient ranging 2.6–3.6%, recovery 92–112%, Diagnostic Products Corporation (DPC), Los Angeles, CA]; IL-6, by chemiluminescent assay (inter-assay variation coefficient ranging 5.3–7.5%, recovery = 85–104%, DPC, Los Angeles, CA); OPG, by sandwich enzyme-linked immunosorbent assay (ELISA), with a sensitivity of 0.14 U/l, and intra- and inter-assay variation coefficients <10% (Biovendor, Brno, Czech Republic) and RANKL by ELISA, with a sensitivity of 0.08 pmol/l and a variation coefficient of 5% or less (intra-assay) and 9% or less (Immundiagnostik, Bensheim, Germany); serum osteocalcin, by immunometric chemiluminescent assay (recovery = 97–121%; variation coefficients of assays ranging from 3.5 to 7.1%; DPC, Los Angeles, CA), as a marker of bone synthesis, and C-terminal telopeptide of type I collagen (CrossLaps), by one step ELISA, with a recovery ranging from 94 to 107% and an intra- and inter-assay variation coefficient ranging 4.7–4.9% and 5.4–8.1%, respectively (Osteometer Bio Tech A/S, Herlev, Denmark), as a marker of bone breakdown.

We also determined serum IGF-1 (Chemiluminescent assay, DPC, Los Angeles, CA), estradiol (competitive immunoassay, DPC, Los Angeles, CA), 1,25 dihydroxyvitamin D3 [radioimmunoassay (RIA), Nichols, San Juan Capistrano, CA), serum free testosterone (RIA, DPC, Los Angeles, CA), free thyroxine, cortisol, PTH, and routine laboratory evaluation. IGF-1 was determined only in 56 patients (22 cirrhotics), RANKL, in 59 patients (20 cirrhotics), and telopeptide, in 62 patients (24 cirrhotics).

The study protocol was approved by the local ethical committee of our Hospital and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Statistics

The Kolmogorov–Smirnov test was used to test for normal distribution, a condition not fulfilled by TNF-α, IGF-1, vitamin D, and osteocalcin. Therefore, non-parametric tests,
RESULTS

Bone mineral density

T- and Z-score values for lumbar spine and hip of cirrhotics, non-cirrhotics and controls are shown in Table 2. Decreased BMD was observed in our patients (both cirrhotics and non-cirrhotics), who showed lower Z-scores than controls at trochanter (−0.36 ± 1.10 vs 0.26 ± 0.87 in controls, P = 0.026), intertrochanteral area (−0.56 ± 1.16 vs 0.46 ± 1.01, P = 0.001), and total hip (−0.44 ± 1.12 vs 0.42 ± 1, P = 0.003, Table 2). Although differences in Z-scores were not statistically significant between cirrhotics and non-cirrhotics, the former showed a higher prevalence of osteoporosis at the

<table>
<thead>
<tr>
<th>Lumbar spine</th>
<th>Cirrhotics (n = 25)</th>
<th>Non-cirrhotics (n = 52)</th>
<th>Controls (n = 19)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>T-score</td>
<td>−1.44 ± 1.13</td>
<td>−0.81 ± 1.14</td>
<td>P &lt; 0.05</td>
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<tr>
<td>Z-score</td>
<td>−1.03 ± 1.08</td>
<td>−0.51 ± 1.20</td>
<td>1.83, NS (0.07)</td>
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<td>Femoral neck</td>
<td>−1.56 ± 1.31</td>
<td>−1.04 ± 1.29</td>
<td>1.64, NS (0.11)</td>
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<tr>
<td>T-score</td>
<td>−0.29 ± 1.33</td>
<td>0.00 ± 1.33</td>
<td>0.91, NS (0.37)</td>
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<tr>
<td>Z-score</td>
<td>−1.04 ± 1.06</td>
<td>−0.73 ± 1.01</td>
<td>1.17, NS (0.25)</td>
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<td>Trochanter</td>
<td>−0.51 ± 1.27</td>
<td>−0.28 ± 1.01</td>
<td>0.88, NS (0.38)</td>
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<tr>
<td>T-score</td>
<td>−1.28 ± 1.13</td>
<td>−1.11 ± 1.16</td>
<td>0.60, NS (0.55)</td>
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<td>Z-score</td>
<td>−0.62 ± 1.15</td>
<td>−0.53 ± 1.17</td>
<td>0.33, NS (0.74)</td>
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<td>Ward’s triangle</td>
<td>−2.32 ± 1.61</td>
<td>−1.45 ± 1.45</td>
<td>P &lt; 0.05</td>
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<td>T-score</td>
<td>−0.59 ± 1.62</td>
<td>−0.02 ± 1.41</td>
<td>1.58, NS (0.12)</td>
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<tr>
<td>Z-score</td>
<td>−1.25 ± 1.13</td>
<td>−0.99 ± 1.12</td>
<td>0.94, NS (0.35)</td>
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<tr>
<td>Total hip</td>
<td>−0.54 ± 1.15</td>
<td>−0.40 ± 1.12</td>
<td>0.54, NS (0.59)</td>
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</table>

Results are given as mean ± standard deviation. In addition, median and IQR are given for IGF-1, osteocalcin, vitamin D, and TNF-α. * = Some cases were missing, so n values are cirrhotics = 22; non-cirrhotics = 34; *** = comparisons were made using non-parametric tests (Mann–Whitney and Kruskall–Wallis); **** = some cases were missing, so n values are cirrhotics = 20; non-cirrhotics = 39; ** = some cases were missing, so n values are cirrhotics = 22; non-cirrhotics = 34; NS, non-significant; figures in brackets after NS correspond to P-values. Results are given as mean ± standard deviation.
Ward’s triangle (14/25) than the latter (12/52, $\chi^2 = 8.1$, $P = 0.004$). Inverse correlations were observed between T-score at Ward’s triangle and serum TNF-α (rho = −0.26, $P = 0.025$) and serum PTH ($r = −0.26$, $P = 0.028$), and also between IL-6 and T-score at the total hip (rho = −0.24, $P = 0.041$), but no correlations were observed between Z-scores and any of the parameters analysed.

**Osteoprotegerin and other cytokines**

In Table 3 we compare the values of hormones and cytokines in cirrhotics and non-cirrhotics (Student’s t-test/Mann–Whitney’s Z) and in cirrhotics, non-cirrhotics and controls (ANOVA/Kruskall–Wallis). Serum OPG levels were significantly higher in patients (both cirrhotics and non-cirrhotics, 12.66 ± 6.44 pmol/l) than in controls (6.59 ± 1.58, $P < 0.005$ pmol/l), and among the former, higher in cirrhotics than in non-cirrhotics (Table 3), a result which is independent of age or creatinine by covariance analysis. Indeed, although both creatinine ($F = 6.53$, $P = 0.013$) and cirrhosis ($F = 10.50$, $P = 0.002$) were independently related with serum OPG, differences among cirrhotics (14.43 ± 4.91 pmol/l) and non-cirrhotics (10.81± 5.45 pmol/l) were still observed when six patients with serum creatinine >1.4 mg/dl were excluded ($P = 0.011$). Patients also showed higher telopeptide levels (0.60 ± 0.38 nmol/100 cm$^3$) than controls (0.20 ± 0.10 nmol/100 cc, $P < 0.001$). Patients showed significantly less IGF-1 [median = 102, interquartile range (IQR) = 46.7–175.99 ng/ml] vitamin D (median = 25.5, IQR = 18.25–35 pg/ml) and osteocalcin (median = 1.8, IQR = 1–3.6 ng/ml) than controls (Table 2, $P < 0.001$ in all the cases), but no differences in PTH. RANKL levels were also similar in patients (0.15 ± 0.22) to controls (0.09 ± 0.07, $P = 0.33$). TNF-α levels were significantly higher in patients (median = 7.4, IQR = 4.05–16.58 pg/ml) when compared with controls (Table 3, $P < 0.02$). TNF-α levels were also higher in cirrhotics than in non-cirrhotics ($Z = 2.75$, $P < 0.01$). Estradiol levels (31.83 ± 13.11 pg/ml) were higher and free testosterone lower (13.62 ± 11.96 pg/ml, respectively, $P < 0.001$ in both cases, Table 3).

**Relationships between cytokines and BMD, liver function parameters and ethanol consumption**

OPG levels showed no correlation with BMD. A strong, positive correlation was observed between OPG and TNF-α (rho = 0.57, Fig. 1) and IL-6 ($r = 0.62$, $P < 0.001$ in both cases, Fig. 2). TNF-α values were below the detection limit in 20 cases. Excluding these cases, correlation between OPG and TNF-α is still statistically significant (rho = 0.48, $P < 0.001$). Also, OPG levels kept a significant, inverse relationship with IGF-1 (rho = −0.54, $P < 0.001$), serum albumin ($r = −0.37$, $P = 0.001$), and prothrombin activity ($r = −0.33$, $P = 0.005$), and also a significant relationship with age ($r = 0.33$, $P = 0.004$) and years of consumption ($r = 0.38$, $P = 0.001$). There were also significant correlations between OPG and creatinine ($r = 0.31$, $P = 0.008$). No differences were observed in serum OPG between patients with and without ascites or encephalopathy. Stepwise multiple regression analysis between serum OPG and liver function parameters, such as prothrombin, albumin, and bilirubin; hormones, such as estradiol, testosterone, IGF-1, osteocalcin, vitamin D, PTH, and cortisol; cytokines, such as TNF-α and IL-6; RANKL; age, creatinine, and telopeptide, disclosed that only TNF-α (beta = 0.47, $P < 0.001$), IL-6 (beta = 0.33, $P = 0.004$) and IGF-1 (beta=−0.23, $P = 0.017$) were significantly, independently related with serum OPG. When cytokines and hormones were excluded from the analysis, serum albumin (beta = −0.31, $P = 0.004$), creatinine (beta = 0.29, $P = 0.006$) and age (beta = 0.25, $P = 0.016$) were significantly, independently related with serum OPG.
Serum TNF-α levels also showed an inverse correlation with T-score at Ward’s triangle (\( r = -0.26, P = 0.025 \)); however, correlation was no longer statistically significant if the cases with TNF-α below the detection limit were excluded (\( r = -0.19, P = 0.13 \)). On the other hand, IL-6 levels were related with TNF-α levels (\( r = 0.50, P < 0.001 \)) and with serum PTH (\( r = 0.32, P = 0.005 \)).

**DISCUSSION**

As expected, in accordance with other authors (Oppenheim, 1977; Lindsell et al., 1982; Schnitzler and Solomon, 1984; Wilkinson et al., 1985; Lalor et al., 1986; Lindholm et al., 1991), we found osteopenia in alcoholics, especially in the cirrhotic subgroup. Indeed, since the first observations performed by Saville (1965), several authors have described osteoporosis in alcoholics, which is more intense when liver cirrhosis is also present (Farley et al., 1985; Feitelberg et al., 1987; Jorge-Hernández et al., 1988; Diamond et al., 1989; Santolaria et al., 2000).

In this study we showed elevated serum OPG in patients with chronic alcoholic liver disease, in accordance with other authors (Szalay et al., 2003; Fabrega et al., 2005; Gaudio et al., 2005; Moschen et al., 2005), especially in the cirrhotic subgroup. In a recent report Fabrega et al. (2005) also showed raised OPG levels in 30 cirrhotics compared with 20 controls, although non-cirrhotic alcoholics were not included. However, in Fabrega’s work, BMD was not recorded, so no data relative to the relationships of OPG with BMD are available. We found statistically significant correlations between OPG and serum albumin and prothrombin activity, suggesting a relationship between elevated OPG and deranged liver function. This result is in accordance with Fabrega’s finding: OPG levels were higher in Child–Pugh C patients than in Child–Pugh A patients (Fabrega et al., 2005), and also with Moschen’s study on 193 patients with chronic liver disease, 66 of them alcoholics (Moschen et al., 2005). Thus, although it is assumed that liver synthesizes OPG (Simonet et al., 1997), and, indeed, expression of OPG mRNA has been detected in several liver cells, including hepatocytes, bile duct epithelium, endothelial cells, activated Kupffer cells, lymphocytes and plasma cells (Moschen et al., 2005) among other organs, our results show an increase of OPG levels in parallel with liver function derangement. There are other sources of OPG, as osteoblasts, which are quantitatively more important than hepatic ones (Knaus et al., 1998). It is possible that these cells and/or the inflammatory cells which infiltrate the liver in chronic alcoholic liver disease play a predominant role in serum elevation of OPG in these patients, although this possibility was not tested in this study.

On the other hand, some interleukins, such as TNF-α and IL-6 exert their actions, enhancing bone resorption, via the RANKL system. The strong, direct relation between osteoprotegerin—the decoy receptor which impedes activation of the osteoclast—and TNF-α and IL-6, which in fact activate osteoclasts, is noteworthy, as is the finding of the strong relationship between raised TNF-α and IL-6 and serum OPG by multivariate analysis. TNF-α and IL-1, and not IL-6, increase OPG expression by osteoblastic cells (Hofbauer et al., 1999a), a fact which could explain, at least in part, our results. These results are in accordance with the hypothesis that OPG increases as a compensatory mechanism in situations of decreased BMD, and also with the observation that OPG ameliorates ethanol-induced bone loss in mice (Zhang et al., 2002). Moreover, raised serum OPG levels have been described in a variety of conditions in which osteopenia ensue, such as hyperthyroidism (Amato et al., 2004), ageing (Szulc et al., 2001), rheumatoid arthritis (Feuer hern et al., 2001), primary biliary cirrhosis (Szalay et al., 2003) or corticosteroid excess (Ueland et al., 2001). Its elevation has been interpreted as a ‘reactive’ or ‘protective’ effect against accelerated bone loss. The strong correlation between activators of bone loss—as TNF and IL-6—and OPG in this study also suggests a compensatory role of OPG, in accordance with the observations mentioned before.

However, some uncertainties are still evident. Osteoporosis induced by alcohol intake is a low turn-over osteoporosis, in which decreased synthesis is superseded by increased resorption, both at a low level of activity (Turner et al., 2001). Osteocalcin is a bone protein which reflects bone synthesis, and in our study, in accordance with others, it is decreased in the alcoholic population when compared with the controls (Rico et al., 1987; Diamond et al., 1989; Friday and Howard, 1991; Wezeman et al., 2000). On the other hand, serum telopeptide is increased, thus reflecting an increased bone breakdown—although liver collagenolysis may also contribute (Guanabens et al., 1998). Increased serum telopeptide has also been found by most other investigators (Schnitzler and Solomon, 1984; Farley et al., 1985). Since OPG is mainly of osteoblastic origin and osteoblast activity seems to be depressed, the finding of increased OPG levels is difficult to explain. Raised serum OPG levels do not seem to depend on deranged renal function, as shown by covariance analysis. Moreover, differences still persist when the few patients with raised serum creatinine levels (from 1.40 to 2.40 mg/dl) were excluded from the analysis. The diseased liver might be a source of increased OPG. It has been recently shown that liver inflammatory cells produce OPG in chronic liver disease (Moschen et al., 2005). Moreover, in our study, OPG is related with prothrombin activity and serum albumin, and when cytokines are not introduced in the stepwise multivariate analysis, serum albumin is the main factor independently related with OPG.

Interestingly, OPG levels show a close correlation with two cytokines, such as TNF-α and IL-6, which can be produced by activated lymphocytes, something in accordance with these findings. In addition, in vitro studies have shown that the production of OPG by osteoblasts is upregulated by oestrogens (Hofbauer et al., 1999b). In our study estradiol levels are raised in cirrhotics, who also showed the highest OPG values. Although no relation was observed between estradiol and OPG, the possible role of raised oestrogen levels on raised OPG in alcoholic cirrhosis remains speculative.

Thus we conclude that OPG is raised in alcoholic subjects, especially in cirrhotics, showing a poor relation with the decrease in BMD. OPG was strongly related to TNF-α and IL-6, which in turn were also weakly related with decreased bone mass. Our results are in accordance with the observations performed by other authors in chronic liver diseases, in which serum OPG levels increase in order to compensate for an excess bone loss.
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