Hepatic osteodystrophy in rats results mainly from portasystemic shunting

S W van der Merwe, J B van den Bogaerde, C Goosen, F F Maree, R J Milner, C M Schnitzler, A Biscardi, J M Mesquita, G Engelbrecht, D Kahn, J Fevery

Background and aims: In chronic liver disease, bone disease frequently develops. The contributions of the different features of liver disease such as parenchymal inflammation, portal hypertension, and portasystemic shunting on bone metabolism have not been systematically studied. The aim of this study was to identify the features of liver disease contributing to bone disease using rat models.

Methods: Parenchymal liver disease was induced by carbon tetrachloride administration, portal hypertension by partial portal vein ligation, and portasystemic shunting by end-to-side anastomosis of the portal vein to the inferior vena cava. Normal and sham operated surgical animals served as controls. Serum calcium, 25-hydroxy vitamin D (25-OH vit D), and osteocalcin levels, and urinary deoxypyridinoline excretion were analysed. Testosterone and oestradiol levels were determined in male and female rats, respectively. Interleukin 1, interleukin 6, and tumour necrosis factor α (TNF-α) were determined in serum. Bone density was measured in all groups and in addition, in the surgical groups, histomorphometry was performed on undecalciﬁed specimens of the proximal tibia. The calcium content of the femurs, removed at termination and ashed, was determined.

Results: Early parenchymal disease and portal hypertension did not affect bone metabolism or body mass. Portasystemic shunting increased bone resorption, decreased bone formation, bone density, and trabecular bone volume which were commensurate with a reduction in body mass. TNF-α levels were elevated and testosterone levels were low in male portasystemic shunted rats.

Conclusions: Portasystemic shunting in the rat adversely affects bone metabolism as part of a generalised catabolic state where high TNF-α and low testosterone and 25-OH vit D levels may play a role.

Animal models and experimental design

The experimental design consisted of two non-concurring protocols. In the first protocol, parenchymal liver disease was established and compared with a non-surgical control. In the second protocol, portal hypertension and portasystemic shunting was established and compared with a surgical control group.

Early parenchymal liver disease model: n=10 (males=5; females=5)

Liver cirrhosis in rats can be induced by weekly administration of carbon tetrachloride (CCL4) for 16 weeks. To induce early parenchymal disease, animals were dosed for eight weeks with CCL4 (Merck, Darmstadt, Germany). No phenobarbital was added to the drinking water because of its recognised effects on bone metabolism. Animals received 80 µl of CCL4 at the outset with subsequent dosing based on weight loss. The intended weekly weight loss was 6–9%. An additional five CCL4 exposed rats were sacrificed between five and 10 weeks and the livers removed for histology.

Control rats (n=8) received 0.9% sodium chloride weekly by gavage.

Abbreviations: PSS, portasystemic shunting; PPVL, partial portal vein ligation; SC, sham operated surgical controls; 25-OH vit D, 25-hydroxy vitamin D; CCl4, carbon tetrachloride; u-DPD, urinary deoxypyridinoline; BMD, bone mineral density; BMC, bone mineral content; CRP, C reactive protein; TNF-α, tumour necrosis factor α; ALT, alanine aminotransferase; ALP, alkaline phosphatase; IL, interleukin.

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Presinusoidal portal hypertension model (PPVL): n=9
(males=5; females=4)

Portal hypertension was induced by reduction of the diameter of the portal vein. A midline laparotomy was performed and a blunt 21 gauge needle was placed alongside the portal vein. A 3-0 silk ligature was placed around both needle and portal vein and the needle was carefully removed. Portal hypertension was confirmed at termination by portal vein pressure measurements in all PPVL animals.

Portasystemic shunt model (PSS): n=10 (males=5; females=5)

A laparotomy was performed, the portal vein, inferior vena cava identified, and the pyloric vein ligated. The adventitia of the inferior vena cava was carefully removed. The portal vein was ligated at the hilum of the liver, transacted, and the distal limb anastomosed end to side to the inferior vena cava. The patency of the shunt was confirmed histologically in all animals at termination.

Sham operated surgical controls (SC): n=10 (males=5; females=5)

A laparotomy was performed at baseline. Portal pressure measurements were conducted at termination.

The surgical groups were terminated after 16 weeks. At termination, the liver, femur, and tibia of all animals were carefully removed. The bones were carefully stripped of all soft tissue.

Analytical methods in sera and urine

Blood samples were obtained at baseline and at week 8 by puncture of the right jugular vein with a 22 gauge needle, as well as at termination in the surgical groups. Urine was collected from rats individually housed in metabolic cages following an adaptation period of 24 hours. Serum and urine samples were frozen at −70°C. Calcium was measured and routine liver tests and C reactive protein (CRP) were performed using a Perkin-Elmer 3030 atomic adsorption spectrophotometer and a Beckman CX-7 autoanaylser, respectively. Calcium levels were corrected for serum albumin in all samples. Testosterone and oestradiol levels were analysed using a radioimmunoassay kit (IAASorf, Stillwater, USA). Osteocalcin levels were determined according to an inhouse method developed in Leuven. Cytokine levels were analysed using an ELISA kit (Biorad; Amersham Pharmacia Biotech, Buckinghamshire, UK). Urinary deoxypyridinoline crosslinks were assessed using a competitive ELISA kit (Meta Biosystems, Mountain View, USA).

Table 1

| Table 1 Comparison of control versus carbon tetrachloride (CCl4) treated animals at baseline and at eight weeks, detailing liver inflammation, parameters of bone metabolism, and bone densitometry |
|---|---|---|---|---|---|
| | Baseline | CCl4 exposed animals | p Value |
| Weight (g) | 300.3 (213.3–392.5) | 292.8 (182.2–432.8) | 377.7 (277.1–495.5) | 264.4 (219.5–457.5) | NS |
| AST (IU/l) | 60.22 (5.12) | 62.93 (4.35) | 59.22 (4.80) | 88.75 (6.74) | 0.02 |
| ALT (IU/l) | 53.67 (2.83) | 55.75 (4.83) | 58.67 (6.10) | 80.24 (4.20) | 0.01 |
| S-Ca2+ (mmol/l) | 2.63 (0.05) | 2.62 (0.13) | 2.58 (0.08) | 2.65 (0.12) | NS |
| u-DPD (µM/mM creatinine) | 127.11 (18.58) | 132.12 (13.48) | 48.91 (13.37) | 54.99 (11.24) | NS |
| Osteocalcin (pg/ml) | 128.0 (11.86) | 127.8 (19.31) | 60.53 (3.30) | 71.78 (11.16) | NS |
| 25-OH vit D (ng/ml) | 24.10 (2.69) | 26.26 (1.81) | 29.33 (2.89) | 20.52 (1.72) | 0.01 |
| High resolution BMD (g/cm2) | 0.3065 (0.014) | 0.3044 (0.031) | 0.3793 (0.044) | 0.3786 (0.018) | NS |
| Proximal femur | 0.1726 (0.011) | 0.1721 (0.016) | 0.1982 (0.021) | 0.2005 (0.013) | NS |
| Proximal tibia | 0.2674 (0.025) | 0.2654 (0.037) | 0.3311 (0.019) | 0.3430 (0.021) | NS |
| Whole body | 0.2674 (0.025) | 0.2654 (0.037) | 0.3311 (0.019) | 0.3430 (0.021) | NS |

There were no significant differences between control and CCl4 treated animals at baseline.

Bone densitometry

Rats were anaesthetised and bone densitometry performed by dual energy x ray absorptiometry using a DXA Hologic QDR 4500 (Hologic, Inc, Waltham, Massachusetts, USA). The stability of the measurement was controlled daily by scanning a phantom. Whole body and high resolution scans of the femur were carried out in CCl4 exposed animals and their controls at baseline and at week 8. Bone densitometry was performed in the surgical groups on whole body and high resolution scans of the femur at baseline, and on individual tibiae and fibulae removed at termination. The tibiae and fibulae were placed on a non-opaque polyester resin block and high resolution bone densitometry was performed using appropriate software for small animals (Hologic, Inc). Tibial measurements were used for analysis.

Histomorphometry

Individual tibiae removed at termination in the surgical groups were processed undecalcified, embedded in methylmethacrylate, and cut at 7 µm thickness on a Jung K heavy duty microtome. Sections were stained with Masson-Goldner trichrome. Trabecular bone in the proximal 4 mm of the tibial metaphysis was examined for bone volume and static bone turnover variables by routine histomorphometry, using the point and intersect count method with the aid of a 100 point Zeiss eyepiece (Integrations Platte II, Zeiss, Germany) at a magnification of x100. The variables and units used are those approved by the American Society for Bone and Mineral Research.

Portal vein pressure measurements

Portal pressure measurements were performed at termination in the SC and PPVL animals under isoflurane 2% anaesthesia. In control rats, portal pressures were measured by insertion of a fluid filled 20 gauge needle directly into the portal vein and in PPVL animals by inserting the needle into the distal superior mesenteric vein. The needle was connected to a transducer (Medex Medical Instrumentation, UK) calibrated for venous pressure. The external zero reference point was placed at the level of the atria. The pressure was recorded as soon as a stable reading with respiratory variations was observed.

Liver histology

Liver sections were stained with haematoxylin and eosin and Masson’s trichrome.

Analytical methods of bone calcium content

Rat femurs were ashed for eight hours in a muffle furnace at 600°C, then weighed, and measured using a flexible image processing system (FIPS, CSIR, South Africa). The femurs were carefully removed. The bones were carefully stripped of all soft tissue.

Table 1 Comparison of control versus carbon tetrachloride (CCl4) treated animals at baseline and at eight weeks, detailing liver inflammation, parameters of bone metabolism, and bone densitometry
were then dissolved in 2 ml of 6 N HCl and diluted 400-fold with demineralised/deionised water. Calcium content was determined using a Perkin-Elmer 3030 atomic absorption spectrophotometer.

Statistical analysis
Data were analysed using SigmaStat for Windows version 2.0 and SigmaPlot for Windows version 4.0. Data were checked for normality and equal variance; if passed, one way analysis of variance (ANOVA) was performed and where it failed, analysis of variance on ranks (Kruskal-Wallis) was conducted. Results are presented as means (SD), medians, or range where appropriate. Results were considered significant at p<0.05.

RESULTS
Early parenchymal liver disease model
CCl4 exposure led to a significant increase in aspartate aminotransaminase and alanine aminotransferase (ALT) levels (table 1). At histology, characteristic changes associated with CCl4 exposure, consisting of inflammatory cell infiltrates, necrosis, and fatty changes, were observed, as has been reported previously by others.11 Except for 25-OH vit D levels, no statistically significant differences between control and CCl4 exposed rats were observed at eight weeks in any of the variables studied relating to bone metabolism, including bone density. 25-OH vit D levels at eight weeks were significantly lower (table 1) in the treated animals compared with controls.

Figure 1 Urinary deoxypyridinoline levels in the rat surgical models (surgical controls, portasystemic shunt (PSS) model, and partial portal vein ligation (PPVL) model) at baseline and at week 8 and week 16. Values are given as means (SD).

Figure 2 Serum osteocalcin levels in the rat surgical models (surgical controls, portasystemic shunt (PSS) model, and partial portal vein ligation (PPVL) model) at baseline, and at week 8 and week 16. Values are given as means (SD).

Table 2 Comparison of body mass, liver enzymes, C reactive protein, testosterone, and cytokine levels in the rat models at baseline and at 16 weeks

<table>
<thead>
<tr>
<th>Variable</th>
<th>SC</th>
<th>PPVL</th>
<th>PSS</th>
<th>p Value</th>
<th>SC vs PPVL</th>
<th>SC vs PSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body mass (g)</td>
<td>320.15</td>
<td>301.56</td>
<td>320.15</td>
<td>0.567</td>
<td>1.00</td>
<td>0.63 &lt;0.001</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>17.78</td>
<td>17.57</td>
<td>18.22</td>
<td>0.68</td>
<td>0.42</td>
<td>0.03 &lt;0.001</td>
</tr>
<tr>
<td>ALP (IU/l)</td>
<td>307.15</td>
<td>252.35</td>
<td>230.50</td>
<td>0.59</td>
<td>0.58</td>
<td>0.89 &lt;0.001</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>0.23</td>
<td>0.23</td>
<td>0.23</td>
<td>0.99</td>
<td>0.58</td>
<td>0.89 &lt;0.001</td>
</tr>
<tr>
<td>25-OH vit D (ng/ml)</td>
<td>29.23</td>
<td>27.16</td>
<td>25.97</td>
<td>0.23</td>
<td>0.092</td>
<td>0.89 &lt;0.001</td>
</tr>
<tr>
<td>IL-1 (pg/ml)</td>
<td>27.84</td>
<td>39.57</td>
<td>38.92</td>
<td>0.14</td>
<td>0.30</td>
<td>0.76 0.56</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>9.95</td>
<td>12.3</td>
<td>5.71</td>
<td>0.55</td>
<td>0.16</td>
<td>0.89 0.28</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>58.99</td>
<td>67.7</td>
<td>64.71</td>
<td>0.13</td>
<td>0.27</td>
<td>0.93 &lt;0.001</td>
</tr>
</tbody>
</table>
| SC, sham operated surgical controls; PPVL, partial portal vein ligation; PSS, portasystemic shunting; ALP, alkaline phosphatase; ALT, alanine aminotransferase; 25-OH vit D, 25-hydroxy vitamin D; CRP, C reactive protein; TNF-α, tumour necrosis factor-α; IL, interleukin.
DISCUSSION

Osteopenia has been previously reported in rats made cirrhotic by CCl₄ administration. However, as in humans, it remains unclear which aspect of cirrhosis is responsible for bone loss. The present study demonstrated that experimental portasystemic shunting had the most deleterious effect on bone.

Complete portasystemic shunting led to significantly lower bone formation, greater bone resorption, lower trabecular bone volume, and smaller bone size compared with controls. The lower trabecular bone volume was reflected in lower bone density, and both lower trabecular bone volume and smaller bone size in lower BMC in PSS rats. The smaller bone size is interpreted as an expression of an overall failure to thrive, and of stunted growth. Indeed, the reduction in bone mass together with body mass suggests that bone loss occurs as part of a generalised catabolic state. The presumed partial shunting induced by collaterals in the portal vein ligation model did not have the same adverse effect on body mass, growth, or bone, nor did early parenchymal disease.

Suppression of bone formation in the PSS group was expressed by lower values for osteocalcin, osteoid surface, and osteoid volume, and greater bone resorption by higher values for u-DPD and increased resorption surfaces observed in PSS animals but not in controls. This picture suggests uncoupling of bone formation from resorption in favour of resorption. Contributions to these bone abnormalities by failure to thrive, stunted growth, and malnutrition cannot be ruled out.
The discrepancy between the rise in femur mass per 100 g of body weight and the decline in trabecular bone volume in the PSS group may be due to the fact that the femur consists predominantly of cortical bone whereas histomorphometry is routinely carried out on trabecular bone. Trabecular bone loss is known to occur earlier and more rapidly than cortical bone loss because of the larger surface area available for resorption. The duration of the experiment may have been insufficient for cortical bone loss to become apparent. The rise in femur mass per 100 g of body mass in PSS rats at 16 weeks may be explained on similar grounds: body fat and lean body mass are presumably lost earlier and more rapidly than cortical bone mass in the femur so that the lag in cortical bone loss gives rise to an apparent increase in bone mass relative to body mass. Whether the findings in the rat can be applied to adult humans remains unclear: rat bone continues to grow throughout life and does not exhibit remodelling. A similar study in a larger animal that has both closed physes in adulthood and bone remodelling may be more applicable to human bone pathology.

The cytokines IL-1, IL-6, and TNF-α have been shown to influence different aspects of bone metabolism.24–28 IL-1, IL-6, and TNF-α are known to be elevated in liver disease.24 In our study, TNF-α levels were increased in PSS animals at termination and may have contributed to the development of osteopenia. Chronic liver disease, especially in males, can result in gonadal dysfunction with low testicular mass and decreased

### Table 3
Comparison of histomorphometric parameters: trabecular bone volume, osteoid volume, and osteoid surface, and resorption surface in the proximal tibia of rats at 16 weeks

<table>
<thead>
<tr>
<th></th>
<th>SC (n=10)</th>
<th>PPVL (n=9)</th>
<th>PSS (n=10)</th>
<th>p Value SC v PPVL</th>
<th>p Value SC v PSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trabecular bone volume (%)</td>
<td>13.2 (1.35)</td>
<td>14.17 (2.23)</td>
<td>7.92 (0.99)</td>
<td>0.36 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Osteoid volume (%)</td>
<td>7.16 (1.23)</td>
<td>5.17 (1.47)</td>
<td>4.74 (0.74)</td>
<td>0.021 0.001</td>
<td></td>
</tr>
<tr>
<td>Osteoid surface (%)</td>
<td>9.71 (1.04)</td>
<td>11.33 (2.34)</td>
<td>6.06 (1.33)</td>
<td>0.234 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Resorption surface (%)</td>
<td>0.94 (0.07)</td>
<td>1.73 (0.59)</td>
<td>3.38 (0.96)</td>
<td>0.061 &lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

SC, sham operated surgical controls; PPVL, partial portal vein ligation; PSS, portasystemic shunting.

### Table 4
Comparison of femur dimensions, composition, and tibial bone mineral density in rats at termination at 16 weeks

<table>
<thead>
<tr>
<th></th>
<th>SC (n=10)</th>
<th>PPVL (n=9)</th>
<th>PSS (n=10)</th>
<th>p Value SC v PPVL</th>
<th>p Value SC v PSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femur length (mm)</td>
<td>30.47 (2.36)</td>
<td>30.78 (1.94)</td>
<td>27.83 (1.25)</td>
<td>0.86 0.008</td>
<td></td>
</tr>
<tr>
<td>Mid shaft thickness (mm)</td>
<td>4.13 (0.46)</td>
<td>3.98 (0.31)</td>
<td>3.63 (0.30)</td>
<td>0.44 0.017</td>
<td></td>
</tr>
<tr>
<td>Femur mass (g)</td>
<td>0.416 (0.09)</td>
<td>0.385 (0.06)</td>
<td>0.295 (0.03)</td>
<td>0.37 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>mg Ca²⁺/femur</td>
<td>70.92 (14.52)</td>
<td>66.29 (14.62)</td>
<td>50.69 (5.40)</td>
<td>0.50 0.003</td>
<td></td>
</tr>
<tr>
<td>Femur mass/100 g body mass</td>
<td>0.108 (0.009)</td>
<td>0.100 (0.02)</td>
<td>0.137 (0.03)</td>
<td>0.08 0.01</td>
<td></td>
</tr>
<tr>
<td>mg Ca²⁺/mm bone</td>
<td>2.31 (0.30)</td>
<td>2.18 (0.36)</td>
<td>1.82 (0.13)</td>
<td>0.40 0.002</td>
<td></td>
</tr>
<tr>
<td>mg Ca²⁺/mg bone</td>
<td>0.164 (0.03)</td>
<td>0.172 (0.02)</td>
<td>0.172 (0.03)</td>
<td>0.21 0.34</td>
<td></td>
</tr>
<tr>
<td>Tibial BMD (g/cm²)</td>
<td>0.223 (0.005)</td>
<td>0.218 (0.009)</td>
<td>0.199 (0.01)</td>
<td>0.12 &lt;0.001</td>
<td></td>
</tr>
<tr>
<td>Tibial BMC (g)</td>
<td>0.244 (0.05)</td>
<td>0.253 (0.05)</td>
<td>0.184 (0.02)</td>
<td>1.00 0.004</td>
<td></td>
</tr>
</tbody>
</table>

SC, sham operated surgical controls; PPVL, partial portal vein ligation; PSS, portasystemic shunting; BMD, bone mineral density; BMC, bone mineral content.

Figure 5 Photomicrograph of undecalcified proximal tibia from (A) sham operated surgical control (SC), (B) partial portal vein ligation (PPVL) rat, and (C) portasystemic shunt (PSS) rat. The PSS rat had the lowest trabecular bone volume. Masson-Goldner trichrome stain; original magnification ×8.
estosterone levels.13 Interestingly, portasystemic shunting, but not portal hypertension, has been reported to result in gonadal injury in a rat model.14 Low testosterone levels were confirmed in the male PSS rats in our study and may have contributed to the development of osteopenia. Despite similar changes in bone parameters, oestradiol levels did not seem to have the same influence in female rats.

The role of vitamin D in hepatic osteodystrophy remains unclear. Vitamin D levels were decreased in the PSS and early parenchymal liver disease groups but despite this, bone mineralisation remained unaffected. Impaired bone formation in chronic liver disease may be responsible for failure of osteomalacia to become apparent as mineralisation defects develop only in newly laid down osteoid. Earlier studies suggested that a decrease in 25-hydroxyvitamin D activity was responsible for the osteodystrophy found in bile duct ligated rats.15 This could not be confirmed in CCl4 treated rats.16 Vitamin D metabolism has not been studied in portacaval shunting. 25-OH vitamin D levels are normal in primary biliary cirrhosis except in the terminal phases when osteoporosis is established.17 In addition, no association was found between vitamin D receptor gene polymorphisms and bone mass in primary biliary cirrhosis.18

In summary, this study demonstrated that of the three features found in chronic liver disease, namely parenchymal inflammation, portal hypertension, and portasystemic shunting, the most pronounced effect on bone as part of a generalised catabolic state. Although the precise mechanism whereby bone disease is induced remains unclear, cytokine activation, low sex hormone and vitamin D levels, or a combination of these factors may play a role.

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The authors acknowledge the contributions of Professor Ian Simpson, Department of Pathology, University of Pretoria, for liver histology, and Professor Roger Bouillon, Department of Endocrinology, Leuven, Belgium, for determination of osteocalcin levels, as well as the excellent secretarial assistance of Ms Gezina Kies. This study was supported by the Hepatology Research fund, ASN 5991, University of Pretoria.

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