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 Fever

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.

Materials and methods

Ten week old male and female Sprague-Dawley rats were used in all experiments. Rats were housed in individual stainless steel cages in a controlled environment at a constant room temperature (22°C), humidity, and 12 hour light-darkness cycles. Rats were fed standard chow (Epol, Johannesburg, South Africa) and water was given ad libitum. The approval of the ethics committee of the University of Pretoria was obtained and animals were treated according to ethical guidelines established by the University of Pretoria.

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

Portasystemic shunt model (PSS): n=10 (males=5; females=4)

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

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

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>CCI exposed animals</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>CCI exposed animals</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>300.3 (215.3-392.5</td>
<td>292.8 (182.2-432.8</td>
<td></td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>60.22 (5 [2.5]</td>
<td>62.93 (4.35</td>
<td></td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>53.67 (2.83)</td>
<td>57.15 (4.83)</td>
<td></td>
</tr>
<tr>
<td>5-Carboxylate (mmol/l)</td>
<td>2.63 (0.05)</td>
<td>2.62 (0.13)</td>
<td></td>
</tr>
<tr>
<td>u-DPD (nmol/mmol creatinine)</td>
<td>127.1 (18.58)</td>
<td>132.1 (13.48)</td>
<td></td>
</tr>
<tr>
<td>Osteocalcin (pg/ml)</td>
<td>128.0 (11.86)</td>
<td>127.8 (19.31)</td>
<td></td>
</tr>
<tr>
<td>25-OH vit D (ng/ml)</td>
<td>25.70 (2.69)</td>
<td>26.12 (1.81)</td>
<td></td>
</tr>
<tr>
<td>Ca2+ (mmol/l)</td>
<td>2.63 (0.05)</td>
<td>2.62 (0.13)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Week 8</th>
<th>CCI exposed animals</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>CCI exposed animals</td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>377.7 (277.1-495.5</td>
<td>346.4 (219.5-457.5</td>
<td>NS</td>
</tr>
<tr>
<td>AST (IU/l)</td>
<td>59.22 (4.80)</td>
<td>88.75 (6.74)</td>
<td>0.02</td>
</tr>
<tr>
<td>ALT (IU/l)</td>
<td>58.67 (6.10)</td>
<td>101.1 (24.01)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>5-Carboxylate (mmol/l)</td>
<td>2.58 (0.08)</td>
<td>2.65 (0.12)</td>
<td>NS</td>
</tr>
<tr>
<td>u-DPD (nmol/mmol creatinine)</td>
<td>48.91 (13.37)</td>
<td>54.99 (11.24)</td>
<td>NS</td>
</tr>
<tr>
<td>Osteocalcin (pg/ml)</td>
<td>60.53 (3.30)</td>
<td>71.78 (11.16)</td>
<td>NS</td>
</tr>
<tr>
<td>25-OH vit D (ng/ml)</td>
<td>29.33 (2.89)</td>
<td>20.52 (1.72)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ca2+ (mmol/l)</td>
<td>2.63 (0.05)</td>
<td>2.62 (0.13)</td>
<td></td>
</tr>
</tbody>
</table>

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

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 isofluorane 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 then dissolved in 2 ml of 6 N HCl and diluted 400× 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.
Hepatic osteodystrophy in rats results mainly from portasystemic shunting

Figure 3 Tibial bone mineral density (BMD) in sham operated surgical control (SC), partial portal vein ligation (PPVL), and portasystemic shunt (PSS) rats at termination at 16 weeks. Boxes are 25th and 75th percentiles; horizontal lines within boxes are 50th percentiles; vertical lines below and above the boxes are 5th and 95th percentiles; symbols indicate values outside the 5th and 95th percentiles.

Figure 4 Tibial bone mineral content (BMC) in sham operated surgical control (SC), partial portal vein ligation (PPVL), and portasystemic shunt (PSS) rats at termination at 16 weeks. Boxes are 25th and 75th percentiles; horizontal lines within boxes are 50th percentiles; vertical lines below and above the boxes are 5th and 95th percentiles; symbols indicate values outside the 5th and 95th percentiles.

Surgical series

Similar to previously reports, liver histology showed varying degrees of atrophy in acinar zone 3 in both PPVL and PSS groups, although atrophy was more pronounced in PSS animals. Mild fatty change was only observed in the PSS group. Portal pressures were significantly elevated in PPVL animals compared with SC (19.8 (6.25) v 6.8 (2.49) mmHg; p=0.002).

The results of biochemical and other parameters studied are presented in tables 1 and 2. Body mass was significantly lower in PSS animals at 16 weeks, as has been reported previously. Serum calcium concentrations, corrected for serum albumin, did not differ between the groups at termination. ALT levels were elevated in both PPVL and PSS groups at termination. As shown in table 2, albumin, testosterone, and 25-OH vit D levels were significantly decreased, and tumour necrosis factor α (TNF-α) and CRP levels significantly increased only in the PSS group at 16 weeks. Alkaline phosphatase (ALP) levels were significantly higher in PSS rats at 16 weeks, indicating greater bone resorption. Bone formation, as assessed by serum osteocalcin levels, is shown in fig 2. Osteocalcin had decreased in all groups by weeks 8 and 16, again indicating an age related change. Osteocalcin levels were significantly lower in PSS animals at eight and 16 weeks compared with SC, indicating a decrease in bone formation. Similar to early parenchymal disease, 25-OH vit D was significantly lower in shunted rats compared with controls at 16 weeks (table 2).

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 tibial 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. IL-1, IL-6, and TNF-α are known to be elevated in liver disease. In our study, TNF-α levels were increased in PSS animals at termination and may have contributed to the development of osteopenia.

### Table 3

<table>
<thead>
<tr>
<th></th>
<th>SC</th>
<th>PPVL</th>
<th>PSS</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>&lt;0.001</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>0.051 0.001</td>
<td>0.001</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>&lt;0.001</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>&lt;0.001</td>
</tr>
</tbody>
</table>

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

### Table 4

<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>&lt;0.001</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>0.001</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 0.037</td>
<td>&lt;0.001</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>&lt;0.001</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>0.01</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>0.002</td>
</tr>
<tr>
<td>mg Ca²⁺/mg bone</td>
<td>0.164 (0.03)</td>
<td>0.172 (0.03)</td>
<td>0.172 (0.03)</td>
<td>0.21 0.034</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tibial BMC (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>&lt;0.001</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>0.004</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.
estrogen levels. Interestingly, portosystemic shunting, but not portal hypertension, has been reported to result in gonadal injury in a rat model. 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 and the development of osteopenia are mainly due to the loss of osteoblasts. Earlier studies suggested that a decrease in 25-hydroxyvitamin D, calcium, and calcitomin for severe osteodystrophy in primary biliary cirrhosis. J Clin Gastroenterol 1997;24:239–44.

In summary, this study demonstrated that of the three features found in chronic liver disease, namely parenchymal inflammation, portal hypertension, and portosystemic 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.

ACKNOWLEDGEMENTS
The authors acknowledge the contributions of Professor Ian Simpson, Department of Pathology, University of Pretoria, for liver histology, and Professor Roger Boullion, 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.

REFERENCES
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 and J Fevery

Gut 2003 52: 580-585
doi: 10.1136/gut.52.4.580

Updated information and services can be found at:
http://gut.bmj.com/content/52/4/580

These include:

References
This article cites 31 articles, 5 of which you can access for free at:
http://gut.bmj.com/content/52/4/580#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/