Glucocorticosteroid-induced osteoporosis in adult primiparous Göttingen miniature pigs: effects on bone mineral and mineral metabolism


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PREVALENCE OF DISEASES of the musculoskeletal system increases with age, and thus will raise the individual and socioeconomic burden in the future in societies with anticipated increasing life expectancy. In Germany, annual costs for diseases of the musculoskeletal system were €25.2 billion in 2002. Most of the total costs per inhabitant were required in the group of subjects more than 65 years old (5). The total medical care expenditures associated with arthritis and other rheumatic conditions in the United States in 1997 amounted to $186.9 billion (68), whereas the actual costs for musculoskeletal disease in the United States were estimated to add up to $215 billion a year, with the prospect of an increase in the future (27).

Because of its anti-inflammatory and immunosuppressive action, glucocorticosteroids (GC) are used for therapy of various symptoms and diseases, including rheumatoid arthritis and related diseases, asthma bronchiale, neurodermitis, and, with high doses, after organ transplantation (24, 29). One of the side effects of GC treatment is an accelerated bone loss and an increased risk for bone fracture, especially after a prolonged exposure (2, 8). GC treatment is the third leading cause of osteoporosis, following the loss of sex steroids and old age (67). GC-treated patients are at a twofold higher risk of suffering from a fracture compared with controls, irrespective of their bone mineral density (BMD; see Ref. 66). Limited information is available on its pathophysiology, since the clinical picture of glucocorticosteroid-induced osteoporosis (GIO) mostly reflects a combined effect of the underlying systemic disease and of the secondary effects induced by GC treatment.

To develop strategies for the treatment of GIO, the mechanisms of how GC act on bone have to be known. In GIO, bone loss predominantly affects the trabecular bone, and thus the risk for bone loss is greater at the spine than the hip (8). From regulatory and metabolic effects, it is assumed that the development of GIO, and with it the degree of bone loss, is biphasic. The key action of GC is thought to be the long-term depression of osteoblast number and activity and thus bone formation (8, 67). The initiation step, however, is suggested to be a stimulation of bone resorption as indicated by enhanced RANK-ligand expression and reduced osteoprotegerin production in the presence of GC (8). Both substances are osteoblast-derived cytokines that modulate osteoclastogenesis.

Observations on effects of different GC made in cell culture should be verified in animal models before, finally, applications are tested in clinical trials in human subjects. Animal models, however, have to be tested for suitability. Beyond the testing of new pharmaceuticals in small animal models like the rat or mouse, the U.S. Food and Drug Administration requires

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that they have to be tested in a large-animal model before a step III clinical study can be performed (38).

In the present experiment, we investigated the effect of GC on calcium metabolism and bone development in adult primiparous Göttingen minipigs. Bone loss, assessed by quantitative computed tomography, changes of markers of bone formation, and resorption and mineral balances were analyzed in a cross-sectional and longitudinal design. Bone morphometry, chemical analyses of bone, and bone mechanical property were analyzed cross-sectionally. The hypothesis was that GC induces the loss of BMD with similar physiological signs as in humans and thus makes the adult minipig a suitable large animal model for research in GC-induced osteoporosis and its treatment.

MATERIALS AND METHODS

The experiment was approved by the German institution for legalization of animal experiments (Ministerium für Umwelt, Natur und Forsten des Landes Schleswig-Holstein). The experiment was part of a larger study in which we tested the physiological response of a large-animal model to various treatments cross-sectionally and longitudinally. The present paper is restricted to describe the effects of short-term and long-term GC treatment.

Animals. We used adult female primiparous Göttingen minipigs of the Institute’s own breeding herd because earlier experiments in domestic pigs have shown that osteoporosis induced by ovarectomy was more pronounced in adult primiparous than in growing nulliparous animals (53). At the age of delivery, the sows were 20 mo old. Because pregnancy and lactation may have effects on bone mineral content (BMC) and turnover (4), the lactation productivity was standardized to a litter size of five to six piglets and to a nursing time of six weeks.

Experimental groups, time schedule. The experiment was initiated with 50 primiparous sows. Before intervention, blood, urine, and fecal samples were collected, and BMD measurements were performed. Next, the sows were assigned to the following three groups: animals on GC treatment for 8 mo (GC-short), n = 30, animals on GC treatment for 15 mo (GC-1, n = 10), and animals without treatment for 15 mo (control, n = 10). To longitudinally describe the short-term effect of GC on bone markers in plasma and urine, repeated samples were taken, and samples of all animals on GC treatment (GC-short and GC-1) were pooled (GC-s-all, n = 40) during the first 8 mo. After 8 mo, 10 animals from the GC-short group were killed to assess the short-term effects on bone samples (GC-s), whereas the remaining 20 animals switched to another experiment with therapeutic treatments. The results of the therapeutic treatment have been published in a separate paper (16). To describe the long-term effect of GC, animals of the control group and of GC-1 continued for another 7 mo before they were killed.

Diets, housing, intervention. Before starting the intervention, the sows switched from the Institute’s standard nutrition regimen for lactating minipigs to the Institute’s semipurified diets for adult minipigs for an adaptation time of 4 wk (preexperimental). This diet has been described previously (52). In brief, it consisted of the following components (g/kg diet): 290 corn starch, 240 sucrose, 150 casein, 80 cellulose, 75 lard, 75 margarine, 60 mineral and vitamin premix, and 30 lactulose. The mineral and vitamin premix provided all nutrients necessary for minipigs in sufficient amounts but were otherwise adapted to an average human diet (rich in fat and protein, poor in fiber). The diet contained 14 MJ metabolizable energy/kg: 6 g/kg calcium and 6.6 g/kg phosphorus from CaCO₃ and Na₂H₄PO₄·12H₂O, respectively; and 6,500 IU of vitamin D₃. The content of vitamin D₃ (Deutsche Vilomix Tierernährung, Neuenkirchen-Vörden, Germany) was higher than in standard pig diets and higher than the recommendations for humans, since this was a long-term experiment on semi-purified diets with no other vitamin D sources. Moreover, the pigs were kept indoors since birth with artificial light, i.e., almost no vitamin D production in the skin from precursors.

At an average age of 30 mo, the sows were allocated to the experimental groups on the basis of their body weights, as described above. Feed intake was controlled individually and was restricted to 370 g/day to allow slow, steady growth as it is typical for this age, while avoiding fattening. Feed was completely consumed by all animals. Deionized water was available ad libitum. Animals were housed individually for the whole time. The sows were kept on strawless floor cages.

The glucocorticosteroid prednisolone (Lichtenstein Pharmazeutika, Mühleheim-Kärlich, Germany) was given orally at a dose of 1.0 mg·kg body wt⁻¹·day⁻¹ for 8 wk and thereafter at a dose of 0.5 mg·kg body wt⁻¹·day⁻¹ until the end of the experiment. The tolerability was tested previously in a pilot set of three sows. At the end of the experiment, all animals were killed by exsanguination after anesthesia with a combination of dormicium (Midazolam, 1 mg/kg body wt; Synopharm, Barsbüttel, Germany) and ketamine hydrochloride (5 mg/kg body wt; CuraMed Pharma, Karlsruhe, Germany), and representative bone specimens were taken and stored at −18°C until further procedures and analyses.

BMD in vivo. BMD was assessed in vivo by quantitative computed tomography (QCT) before intervention (t₀), after 8 mo (t₈), and after 15 mo (t₁₅) with a Siemens Sensation 16 Scanner. Animals were anesthetized with a combination of dormicium and ketamine hydrochloride (see above), and an accessory medication with atropine. At the beginning, three animals of the GC-treated animals died after the second QCT measurement (t₈) as a consequence of prolonged anesthesia, which was sometimes necessary if the measurements had to be interrupted because of constrains within the clinic routine. For the remaining QCT measurements, the anesthesia was completed by injections with prednisolone (5 ml of a solution of 10 mg/ml prednisolone-21-acetate) for animals on GC treatment. No more animals died. Trabecular BMD of a central 10-mm-thick slice and an elliptical region in the anterior part of lumbar vertebras L₁–L₄ were evaluated. The Siemens OsteoPackage was used to calculate calibrated BMD results (25). Reproducibility of the measurement was assessed by duplicate measurements in four minipigs with interim repositioning. The root-mean-square average of precision errors (15) was 0.83% (3.4 mg/cm²).

Plasma parameters. Blood samples were taken before intervention (t₀) and after 2, 5, 7, 10, 13, and 15 mo (t₂–t₁₅) after an overnight fast, always at the same time between 08:00 and 10:00 A.M., and stored in ice-cooled water until centrifugation and aliquotting. Calcium was analyzed directly by atomic absorption spectroscopy (PerkinElmer 1100) using an air/acetylene-flame at 2,300°C after dilution with a 0.5% lanthanum chloride solution. Five standards were used for calibration. Phosphorus was estimated in EDTA plasma as inorganic phosphorous with an automatic analyzer (Cobas Bio; Hoffmann La Roche, Basel, Switzerland) using a test kit (Roche Diagnostics, Mannheim, Germany). Parathyroid hormone (PTH) was determined in EDTA plasma using an RIA (Immundiagnostik, Bennheim, Germany). We selected the PTH kit containing a goat anti-serum directed against the COOH-terminal end of PTH-(53–84), with a donkey-anti-goat immunoglobulin as precipitation reagent. This kit was the most sensitive to physiological changes like pregnancy and lactation in the minipigs (unpublished observations) compared with the midregional or NH₂-terminal kit, or the kit containing antibodies against intact human PTH. This kit showed significantly higher values for ovarectomized pigs compared with intact controls (53). Values for sensitivity, and intra- and interassay variability were 3.4 pg/ml, 9.2% coefficient of variation (CV) and 12.5% CV, respectively. 25-Hydroxyvitamin D₃ [25(OH)D₃] was measured in EDTA plasma by RIA (Immundiagnostik) after acetonitrile precipitation. Values for sensitivity and intra- and interassay variability were 2.5 nmol/L, 9.8% CV, and 14% CV, respectively. 1,25-Dihydroxyvitamin D₃ [1,25(OH)₂D₃]
was measured in EDTA plasma by a radioreceptor assay (RRA; Immundiagnostik). For this test, the plasma was cleaned, and the biologically effective vitamin D metabolite was extracted by Extrelut columns followed by a separation on silica columns, where the 1,25-(OH)2D3 metabolite was separated from other vitamin D metabolites. For the quantitative determination of 1,25-(OH)2D3 by RRA, [3H]1,25-(OH)2D3 tracer was used, which competed with the sample for a limited number of 1,25-(OH)2D3 receptors. Values for intra- and interassay variability were 12% CV and 17% CV, respectively.

Osteoprotegerin (OPG) was analyzed in serum by sandwich enzyme immunoassay (Immundiagnostik). For technical reasons, only a limited number of samples could be analyzed at baseline and after 8 mo, i.e., in the short-term samples. Total alkaline phosphatase (TAP) and bone alkaline phosphatase (BAP) were analyzed in heparin-plasma directly and as the difference after lectin precipitation, respectively. The kit (Roche Diagnostics) was based on a kinetic color reaction. Values for intra- and interassay variability were 1.6% CV and 2.5% CV, respectively. In our validation studies, we observed that diets with low calcium content raised BAP and those with high calcium content depressed it in ovariectomized minipigs (54).

Urinary parameters. Urine was collected over 24 h before intervention and after 2, 5, 7, 10, 13, and 15 mo and frozen at −18°C until analysis. For technical reasons, urine samples of only 40 animals were collected at t2 (5–6/group). Calcium in urine was measured directly after dilution with a lanthanum chloride solution by atomic absorption spectrometry (PerkinElmer 1100) using an air-acetylene flame at 2,300°C. For lissylpyridinoline, denoted as deoxypyridinoline (DPD), the urine samples were centrifuged, lyophilized, and hydrolyzed with 6 M HCl at 110°C for 24 h and centrifuged again. Each hydrolysate (1 ml) was added to a mixture of 1 ml glacial acetic acid, 2 ml n-butanol-1-ol, and 5 ml of a 10% CF-1-slurry (fibrous cellulose powder; Whatman, Maidstone, UK). A column was prepared by adding the mixture of hydrolysate and CF-1-slurry to a polypropylene econocolumn (Bio-Rad, München, Germany). The resin was washed, the pyridinium-containing eluate was eluted, and the lyophilized eluate was redisolved and analyzed by HPLC. The intra- and interassay variability was 2% CV and 4.8% CV, respectively. The hydroxylysyl- and lissylpyridinolines were quantified by external standards with established linearity gained from a commercially available adult bovine bone gelatin (Deutsche Gelatine-Fabriken Stoess, Eberbach/Baden, Germany). They were purified by a preparative reverse-phase HPLC column, and the degree of purity was verified by amino acid analysis (>98% dry wt) according to a method previously described (1). In this paper, only lissylpyridinoline results are reported, since DPD is specific for bone. All parameters in plasma and urine were analyzed in a blind fashion and in duplicate.

Metabolic mineral balance. Four metabolic calcium and phosphorus balances were performed: before the intervention with glucocorticosteroids (i.e., the beginning of the experiment, t0) and after 5 (t5) 10 (t10), and 15 mo (t15). For this, the minipigs were transferred to metabolic cages that allowed the separate collection of feces and urine. Thereafter, the dry weight in group GC-l increased to 37.7 kg or 12.6% after 8 mo and 38.9 kg or 12.2% after 15 mo. In both GC-treated groups, body weight increased nonsignificantly during the study period. After 15 mo, the body weight in group GC-l increased to 37.7 ± 1.7 kg or +12.6% from baseline (P = 0.23), which was significantly different from the control group (P < 0.05). When all animals on GC treatment (GC-s-all) were pooled at t8 (Table 2, n = 35–40), their weight was significantly different from baseline (P < 0.0003).

BMD in vivo. During the experiment, BMD was stable in the control group but decreased significantly with GC treatment (Fig. 1, top). In the control group, the change of BMD was −11.75 ± 12.64 mg/cm2 or −2.6% after 8 mo and −2.28 ±
Plasma phosphorous changed only slightly from \( t_0 \) to 11002 GC-s group, BMD was 11002 Ca retention, mg/7 days a PTH, pmol/l Plasma Ca, mg/l Body wt, kg BMD, mg/cm² Body weight, BMD, bone markers, and mineral balances after short-term treatment (8 mo) in minipigs

### Table 1. Baseline values in minipigs before intervention

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>GC-s</th>
<th>GC-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mo</td>
<td>30.0 ± 0.4</td>
<td>30.3 ± 0.8</td>
<td>30.5 ± 0.7</td>
</tr>
<tr>
<td>Body wt, kg</td>
<td>33.1 ± 1.6</td>
<td>32.4 ± 1.5</td>
<td>33.5 ± 1.7</td>
</tr>
<tr>
<td>BMD, mg/cm²</td>
<td>450.2 ± 15.9</td>
<td>446.8 ± 17.1</td>
<td>448.2 ± 14.7</td>
</tr>
<tr>
<td>Plasma calcium, mg/l</td>
<td>110.1 ± 3.3</td>
<td>109.9 ± 3.1</td>
<td>109.9 ± 4.0</td>
</tr>
<tr>
<td>Plasma phosphorous, mg/l</td>
<td>49.7 ± 1.9</td>
<td>52.4 ± 2.6</td>
<td>51.2 ± 3.3</td>
</tr>
<tr>
<td>BAP, U/l</td>
<td>29.6 ± 4.6</td>
<td>34.9 ± 4.5</td>
<td>34.4 ± 5.8</td>
</tr>
<tr>
<td>OPG, pmol/l</td>
<td>0.86 ± 0.13</td>
<td>0.77 ± 0.04</td>
<td>1.21 ± 0.32</td>
</tr>
<tr>
<td>DPD/creatinine, nmol/mmol</td>
<td>10.1 ± 3.2</td>
<td>6.9 ± 1.4</td>
<td>11.2 ± 4.4</td>
</tr>
<tr>
<td>PTH, pmol/l</td>
<td>18.5 ± 2.2</td>
<td>20.2 ± 3.5</td>
<td>32.0 ± 11.3</td>
</tr>
<tr>
<td>25(OH)D, nmol/l</td>
<td>74.8 ± 10.8</td>
<td>70.0 ± 12.4</td>
<td>56.6 ± 9.0</td>
</tr>
<tr>
<td>1,25(OH)₂D₃, pg/ml</td>
<td>42.2 ± 5.8</td>
<td>45.3 ± 4.8</td>
<td>46.5 ± 7.6</td>
</tr>
<tr>
<td>Urinary Ca, mg/7 days</td>
<td>430.7 ± 78.8</td>
<td>385.5 ± 20.0</td>
<td>388.9 ± 37.7</td>
</tr>
<tr>
<td>Ca absorption, mg/7 days</td>
<td>1,841 ± 850</td>
<td>3,349 ± 1,227</td>
<td>2,225 ± 1,245</td>
</tr>
<tr>
<td>Ca retention, mg/7 days</td>
<td>1,410 ± 893</td>
<td>2,963 ± 1,223</td>
<td>1,836 ± 1,248</td>
</tr>
<tr>
<td>Urinary P, mg/7 days</td>
<td>1,103 ± 198</td>
<td>1,252 ± 161</td>
<td>1,554 ± 271</td>
</tr>
<tr>
<td>P absorption, mg/7 days</td>
<td>661 ± 1,669</td>
<td>1,618 ± 1,442</td>
<td>1,674 ± 2,191</td>
</tr>
<tr>
<td>P retention, mg/7 days</td>
<td>-441 ± 1,666</td>
<td>365 ± 332</td>
<td>120 ± 2,231</td>
</tr>
</tbody>
</table>

Values are least square means ± SE, n = 9–10 animals. GC-s and GC-I, short-term and long-term glucocorticoid, respectively; BMD, bone mineral density; BAP, bone alkaline phosphatase; OPG, osteoprotegerin; DPD, deoxypyridinoline; PTH, parathyroid hormone; 25(OH)D, 25-hydroxyvitamin D₃; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; P, phosphorus.

### Table 2. Longitudinal effects of GC treatment on body weight, BMD, bone markers, and mineral balances after short-term treatment (8 mo) in minipigs

<table>
<thead>
<tr>
<th>Delta (t8-t0)</th>
<th>Control (n = 8–10)</th>
<th>GC-s-all (n = 35–40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, kg</td>
<td>0.46 ± 1.10</td>
<td>1.7 ± 0.42[^a]</td>
</tr>
<tr>
<td>BMD, mg/cm²</td>
<td>-11.8 ± 12.6</td>
<td>-47.5 ± 5.13[^a]</td>
</tr>
<tr>
<td>Plasma Ca, mg/l</td>
<td>-0.30 ± 3.33</td>
<td>-0.89 ± 1.79</td>
</tr>
<tr>
<td>Plasma P, mg/l</td>
<td>-2.76 ± 3.32</td>
<td>-3.88 ± 1.53[^f]</td>
</tr>
<tr>
<td>BAP, U/l</td>
<td>-1.43 ± 4.80</td>
<td>-1.73 ± 2.29[^a]</td>
</tr>
<tr>
<td>OPG, pmol/l</td>
<td>0.05 ± 0.05</td>
<td>-0.22 ± 0.15[^b]</td>
</tr>
<tr>
<td>DPD/creatinine, nmol/mmol</td>
<td>-5.18 ± 3.71</td>
<td>-7.12 ± 1.34[^c]</td>
</tr>
<tr>
<td>PTH, pmol/l</td>
<td>-4.37 ± 4.81</td>
<td>1.45 ± 3.99</td>
</tr>
<tr>
<td>25(OH)D, nmol/l</td>
<td>143.2 ± 30.5[^g]</td>
<td>160.6 ± 14.1[^g]</td>
</tr>
<tr>
<td>1,25(OH)₂D₃, pg/ml</td>
<td>-8.11 ± 4.58</td>
<td>-3.61 ± 3.23</td>
</tr>
<tr>
<td>Urinary Ca, mg/7 days[^a]</td>
<td>73.0 ± 93.7</td>
<td>34.0 ± 31.1</td>
</tr>
<tr>
<td>Ca absorption, mg/7 days[^a]</td>
<td>-1,380 ± 1,297</td>
<td>-2,488 ± 684[^b]</td>
</tr>
<tr>
<td>Ca retention, mg/7 days[^a]</td>
<td>-1,453 ± 1,286</td>
<td>-2,522 ± 698[^b]</td>
</tr>
<tr>
<td>Urinary P, mg/7 days[^a]</td>
<td>260.3 ± 335</td>
<td>-317 ± 147[^b]</td>
</tr>
<tr>
<td>P absorption, mg/7 days[^a]</td>
<td>-954 ± 2,128</td>
<td>-1,137 ± 842</td>
</tr>
<tr>
<td>P retention, mg/7 days[^a]</td>
<td>-1,212 ± 2,206</td>
<td>-821 ± 821</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of animals; t8 = 18. ^aData after 5 mo. Significantly different from baseline with \( P < 0.05 \), \(^bP < 0.01\), and \(^cP < 0.001\). Significantly different from the control group with \(^dP < 0.05\) and \(^eP < 0.01\). Significantly different from the group with \(^fP < 0.05\) and \(^gP < 0.01\). Significantly different from the group with \(^hP < 0.05\) and \(^iP < 0.01\). Significantly different from the group with \(^jP < 0.05\) and \(^kP < 0.01\). Significantly different from the group with \(^lP < 0.05\) and \(^mP < 0.01\). Significantly different from the group with \(^nP < 0.05\) and \(^oP < 0.01\).

11.77 or −0.5% after 15 mo [both not significant (NS)]. In the GC-s group, BMD was −25.07 ± 3.62 mg/cm² or −5.6% for completers after 8 mo (\( P < 0.001 \) vs. baseline; NS vs. control). In the GC-I group, BMD was −52.54 ± 9.82 mg/cm³ or −11.7% for completers after 8 mo (\( P < 0.005 \) vs. baseline; \( P < 0.05 \) vs. control) and −56.73 ± 8.31 mg/cm² or −12.7% for completers after 15 mo (\( P < 0.001 \) vs. baseline and \( P < 0.01 \) vs. control). When all completers on GC were pooled at \( t_8 \) (GC-s-all), their change of BMD of −47.43 ± 5.13 mg/cm³ from baseline was significantly different from baseline (\( P < 0.000 \); Table 2) and from the change in the control group of −11.76 ± 12.64 (\( P < 0.05 \)).

Parameters in plasma and urine. Plasma calcium and plasma phosphorous changed only slightly from \( t_0 \) values in all three groups. They did not differ from the control group, not even when all GC-treated animals were pooled at \( t_8 \) (Table 2). 25(OH)D₃ increased significantly compared with baseline in all three study groups (control, GC-s, and GC-I) until \( t_8 \), with no significant differences between groups but a transient trend for lower values in the long term in GC-treated animals (Fig. 2, top). 1,25(OH)₂D₃ did not change with time until \( t_8 \) (Table 2) and slightly increased thereafter, but with a trend for a lower raise in GC-treated animals with absolute concentrations of 49.7 ± 6.6 pmol/ml at \( t_{10} \) and 54.9 ± 7.5 pmol/ml at \( t_{13} \) compared with 58.1 ± 11.3 pmol/ml at \( t_{10} \) and 62.8 ± 14.2 pmol/ml at \( t_{13} \) in control animals. Longitudinally, we observed a tendency for a decline in PTH in the long term in the control group with changes from baseline of −7.04 ± 3.78, −9.94 ± 3.34, and −4.15 ± 5.29 pmol/l at \( t_{10} \), \( t_{13} \) and \( t_{15} \), respectively, which reached significance at \( t_{13} \) (\( P = 0.05 \)). The decline in GC-treated animals was more pronounced with −14.96 ± 7.29, −20.51 ± 12.24, and −25.32 ± 12.22 pmol/l at \( t_{10} \), \( t_{13} \) and \( t_{15} \), respectively, but failed to reach significance because of higher variation. The difference between groups was not significant. TAP in plasma was not significantly affected by GC treatment at any time (data not shown). BAP in
positive in all groups and not different between groups (Table 1). During the study, calcium retention in the control group decreased slightly until t10 and then almost returned to baseline at t15 (Fig. 1, bottom). In the GC-treated groups, the decrease in calcium retention was more pronounced. In GC-I animals, calcium retention remained low until t15 (Fig. 1, bottom). In spite of the impressive changes from baseline in the GC-s and GC-I groups, this decline became significant only at t5 in GC-s animals. The changes within a group and the differences between groups did not reach significance because of the large individual variation and the relatively low number of animals. However, when the steroid-treated animals were pooled (GC-s-all), the decrease in calcium retention of −2,522 ± 698 mg/7 days was different from baseline after 5 mo (P < 0.001). This was because of significantly higher fecal calcium excretion (and thus lower calcium absorption; Table 2). The decline was not significant for control animals, with a calcium retention of −1,453 ± 1,286. The difference between the groups did not attain significance.

Phosphorus intake (15.54 g/7 days) was the same in each group and animal at any time. Phosphorus absorption (Table 2) and retention decreased nonsignificantly in the short term independent of the treatment. At t15, phosphorus retention was positive in the control group (294 ± 1,158 mg/7 days) but still negative in the GS-I animals (−2,312 ± 2,677 mg/7 days). None of the changes longitudinally or differences cross-sectionally reached significance, except for the change of urinary phosphorus excretion from baseline, which significantly increased in the control group but significantly decreased in animals treated with GC (Table 2).

**BMC.** The wet weight of the fourth lumbar vertebra specimen (mg) did not differ between groups [595 ± 17 (control), 578 ± 17 (GC-s), and 540 ± 26 (GC-I)]. Compared with the control group, the bone dry weight (mg) tended to be lower in the GC-s group and was significantly lower in the GC-I group (Table 3). The same was true for the content of dry matter (%; Fig. 3, top, P < 0.05). The mineral content (mg ash/bone specimen) tended to be lower in sows after the short-term

<table>
<thead>
<tr>
<th>Fourth LV</th>
<th>Control</th>
<th>GC-s</th>
<th>GC-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone dry wt, mg</td>
<td>350.1 ± 12.7</td>
<td>333.5 ± 13.3</td>
<td>302.3 ± 14.2</td>
</tr>
<tr>
<td>Organic matter, mg</td>
<td>142.6 ± 6.9</td>
<td>137.2 ± 6.0</td>
<td>128.6 ± 6.6</td>
</tr>
<tr>
<td>Ash, mg</td>
<td>207.5 ± 11.2</td>
<td>196.3 ± 10.9</td>
<td>173.6 ± 11.0</td>
</tr>
<tr>
<td>Calcium, mg</td>
<td>128.7 ± 4.1</td>
<td>126.2 ± 4.5</td>
<td>122.2 ± 4.2</td>
</tr>
<tr>
<td>Phosphorus, mg</td>
<td>59.6 ± 2.1</td>
<td>58.1 ± 2.0</td>
<td>55.2 ± 1.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2nd LV</th>
<th>Control</th>
<th>GC-s</th>
<th>GC-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>BVTV, %</td>
<td>34.0 ± 3.1</td>
<td>33.3 ± 1.6</td>
<td>29.0 ± 2.0</td>
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<tr>
<td>OVTV, %</td>
<td>0.54 ± 0.1</td>
<td>1.33 ± 0.4</td>
<td>0.76 ± 0.2</td>
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<tr>
<td>BS/BV, %</td>
<td>14.7 ± 3.4</td>
<td>13.2 ± 2.3</td>
<td>19.1 ± 4.5</td>
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<tr>
<td>ES/BV, %</td>
<td>28.1 ± 5.3</td>
<td>24.3 ± 5.2</td>
<td>22.3 ± 3.2</td>
</tr>
<tr>
<td>Tb.Sp, μm</td>
<td>245 ± 26</td>
<td>211 ± 15</td>
<td>251 ± 11</td>
</tr>
</tbody>
</table>

Values are means ± SE, n = 8–10 animals. All values of chemical analysis are gained from 5 × 50-mm cylindrical specimens from the 4th lumbar vertebra by a diamond-covered hole saw. Significantly different from the control group with *P < 0.1 (borderline) and †P < 0.05. Significantly different from the GC-s group with ‡P < 0.1 (borderline) and §P < 0.05.

**Table 3. Cross-sectional effects on bone weight, BMC, and histomorphometric parameters in vertebrae of minipigs after short-term (8 mo, GC-s) and long-term (15 mo, GC-I) treatment with GC**

**Metabolic mineral balance.** Calcium intake was 15.54 g/7 days and was the same for each animal in each group at any time. Basal values of calcium absorption and retention were
term GC treatment. The alteration after long-term intervention was more pronounced ($P < 0.09$) in the GC group compared with the control group.

**DISCUSSION**

For evaluation of the minipig as an animal model for GIO, we assessed the short-term and long-term effects of GC treatment on BMD, metabolic mineral balances, bone markers, BMC, bone structure, and biomechanical properties. This array of parameters was selected because it is known from fluoride and bisphosphonate therapy that BMD does not always correlate well with bone stability or fracture risk (37, 60). Parameters other than density, particularly trabecular structure, may reflect bone quality or stability more accurately (26).

**Animal model.** The rat seems to have limitations as model for GIO, since there was no bone loss after intravenous GC administration (28). There are few studies on GC-induced bone loss in large animals, including dogs (35, 40, 49) and sheep (9, 32, 55). The data published on sheep showed detrimental effects of GC only in animals that had additional causes for bone loss, like ovariecctomy or calcium/vitamin D deficiency. Moreover, 0.25 mg prednisolone/kg body wt daily was not sufficient on its own to induce loss of BMD or bone strength (11). In fact, these parameters were slightly higher (NS) compared with the untreated group. A significant reduction of histomorphometric variables of bone formation was observed after 3 mo of treatment but without significant changes in markers of bone formation (9).

We used the adult primiparous Göttingen minipig. This large animal model is an omnivore and thus has more similarities with humans than dogs or sheep. Earlier experiments in domestic pigs have shown that effects of ovariecctomy-induced estrogen deficiency were more pronounced in adult primiparous than in young nulliparous sows (53), and ovariecctomy-induced loss of BMC was seen in adult primiparous minipigs aged 2.5–3 yr (54). Our animals were 30 mo old at the beginning of the experiment. It is difficult to extrapolate this age to humans. With respect to fertility, which starts between 6 and 7 mo, the pigs have multiplied their age about four to five times. In humans, this would come to about 50 yr. If the age is related to epiphyseal closure, which occurs in minipigs at 2–3 yr (unpublished observation), this is comparable to 20-yr-old human subjects (34). Thus we used the model of after epiphyseal closure, which is closer to the target group of adult humans that is mainly affected by osteopathies than studies using animals in the rapid-growing stage (23).

The time of intervention of 15 mo corresponds to 6–7% of the life expectancy of a minipig (up to 20 yr; see Ref. 20). In humans, this would correspond to an intervention time of ~5 yr, which is rather long and allows us the ability to draw conclusions on long-term effects.

Information on GC doses applied to pigs or minipigs are rarely reported. Tolerable doses of 1 mg·kg$^{-1}$·day$^{-1}$ orally for 3 mo after three bolus doses of 250 mg on the day of operation were observed in minipigs after organ transplantation (3). In piglets, 0.5 mg/day dexamethasone, equivalent to 3–4 mg prednisolone, was used (65). In humans, maximum doses of 1–3 g/day are given after organ transplantation over a few days (24). Typical initial doses were reported of 20–80 mg/day, chronic doses of 1–10 mg/day for rheumatoid arthritis, and...
10–50 mg/day for others (64). The effect of GC on bone is dose dependent; 1–7.5 mg/day, corresponding to up to 0.1 mg/kg body wt prednisolone equivalent, is expected to result in no or small side effects (24), whereas higher doses (60 mg/day) induced osteoporosis (58).

We used prednisolone given orally at a dose of 1.0 mg·kg body wt $^{-1}·$day$^{-1}$ for 8 wk, which corresponds to 70–90 mg/day in humans, and 0.5 mg·kg body wt $^{-1}·$day$^{-1}$ thereafter. Because the set of pilot sows did not show any signs of intolerability or complications, the dose was not changed during the study.

**Body weight.** The increase in body weight observed in the short-term and in the long-term interval is also observed in humans, especially after long-term therapy. Weight gain is correlated with the cumulative dose (61). In young minipigs, no effect on body weight was reported, probably because they were still in the rapid growing stage that superimposes on the effect of GC (23).

**BMD in vivo.** For practical reasons, the minipig is easy to handle for BMD measurements. The size allowed us to use a standard QCT in the university hospital. The mean BMD was $\sim$450 mg/cm$^3$ and thus much higher than that of adult premenopausal women (133 mg/cm$^3$; see Ref. 46). BMD was depressed $\sim$45–50 mg/cm$^3$, or $\sim$10% from baseline, in the short term. In the long term, only a slight further decline of 1% from t8 values was observed. Bone loss following GC treatment is thus biphasic like in humans (8, 24, 64) with values of $\sim$12% during the first few months and 2–3% annually thereafter (36). Bone loss occurs in cortical and cancellous bone, but predominantly in the axial skeleton (36, 45). It is assumed that $\sim$5 mg prednisolone/day induces bone loss and in 30–50% of patients fractures will occur (63) with strong correlations between cumulative dose and loss of BMD and between daily dose and fracture risk (63). In minipigs after 0.5 mg/kg body wt of 5 days/wk prednisolone subcutaneously for 26 wk, BMD of the femur, but not the lumbar vertebra, was lower compared with control animals (23). In contrast to the sows we used, these pigs were much younger, still growing, and nulliparous. Moreover, the duration was shorter than the short-term observation in our experiment, which may explain their lack of effect in the lumbar spine.

**Parameters in plasma and urine.** GIO is the consequence of several systemic and local effects on different organs, namely gut, kidney, gonads, parathyroid, and bone (14). It is assumed that GC reduce intestinal calcium absorption, increase urinary calcium excretion, induce hyperparathyroidism, depress gonadal hormone secretion and suppress osteoblast number and activity directly, and cause secondary hypogonadism (14), but the results were not consistent.

**PTH.** We observed no significant effect of chronic treatment with GC on PTH. This is in contrast to an anticipated hyperparathyroidism after GC treatment in patients (58) but is in agreement with results in healthy men (17) and in men who were treated with 50 mg prednisolone daily for 3.7 mo because of problems with fertility not related to systemic disease (45), and with reports by others (39, 56, 59). Pearce et al. (45) concluded that, rather than causing bone loss, PTH may be increased as a consequence of lost bone because of illness and immobility. Recently, it was shown that GC reduced the PTH tonic secretory rate and increased the fractional pulsatile PTH secretion, although the overall PTH concentration was similar among normal and GC-treated subjects (6). This indicated that a secondary hyperparathyroidism was not involved. 25(OH)D$_3$. In our experiment, we observed a significant increase in plasma 25(OH)D$_3$ concentration in all groups within the short term. This was because of the vitamin D content of our semipurified diet (6,500 IU/kg), which was higher compared with our standard pig chow (2,000 IU/kg) the animals consumed before the study. Basal values between 57 and 75 nmol/l in the minipigs are higher than concentrations in young men with very low vitamin D intakes of $\sim$100 IU/day, who had mean values of 32 nmol/l before intervention, but were comparable with values of these men after they had been supplemented with 1,800 IU/day. This amount refers to a total of $\sim$25 IU vitamin D·kg body wt $^{-1}·$day$^{-1}$ compared with the 60–70 IU vitamin D·kg body wt $^{-1}·$day$^{-1}$ in the minipigs. In young growing minipigs aged 5 mo, we observed plasma 25(OH)D$_3$ concentrations of 340 nmol/l when animals were on semisynthetic diets containing 8 g calcium/kg diet and 6,500 IU/kg diet vitamin D, whereas their levels were only 40 nmol/l when they consumed the same diet but without vitamin D (51).

In intact and ovariectomized adult domestic pigs on conventional feed for breeding sows, we observed 25(OH)D$_3$ concentrations between 180 and 250 nmol/l in multiparous sows and between 180 and 210 nmol/l in nulliparous sows (53).

In the long term, GC treatment tended to transiently slower the increase in plasma 25(OH)D$_3$ compared with the control animals. We speculate that this indicates a lower absorption rate of vitamin D from the gut after GC treatment (12) and/or its reduced hydroxylation in the liver. This observation is in accordance with reports by others (30, 56) showing that GC excess resulted in small but significant decreases of plasma 25(OH)D$_3$ concentrations. A third possibility to explain a lowered bone mass after GC treatment was recently discussed by Pascucci et al. (43) and Zhou et al. (69). They suggest a mechanism via reduced plasma levels of 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$ as a consequence of increased catabolism. This is mediated by GC-induced activation of pregnane X receptor (PXR), which is involved in the catabolism and detoxification of xenobiotics and drugs and which is able to cross-talk with the vitamin D-responsive gene that activates the respective hydroxylase that catalyses the 24-hydroxylation of 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ down to water-soluble and biologically inactive calcitriol (42). As a consequence, vitamin D deficiency, lower calcium absorption, and finally bone disease will occur (22). At present, there is still debate whether the hydroxylase responsible for 25(OH)D$_3$ or 1,25(OH)$_2$D$_3$ catabolism activated by PXR is CYP24 (43, 44) or CYP3A4 (69).

To overcome the impeding effects of increased PXR production on vitamin D metabolism following the treatment with GC or other drugs, it is recommended to maintain 25(OH)D$_3$ at levels of 30–50 ng/ml (22). Whether the 25–30% lower levels of 25(OH)D$_3$ in GC-treated animals compared with controls had a marked impact on the loss of BMD is difficult to rate, because the absolute values were still relatively high with 187 $\pm$ 43 nmol/l compared with 255 $\pm$ 40 nmol/l in controls. These values were lower than those we observed earlier in growing minipigs on a semisynthetic diet with an adequate supply of calcium and vitamin D with 262–342 nmol/l but were much higher than those of vitamin D-depleted growing minipigs with 31–40 nmol/l (51).
The effects of GC on plasma 1,25(OH)₂D₃ are contradictory. Increases (21, 39, 62), decreases (62), and no changes (47, 56) have been reported. From our results, we conclude that plasma 1,25(OH)₂D₃ seems to play a minor role in the development of GIO, since its plasma level after 8 mo in the GC-treated group was not different from baseline and there were no significant differences between the groups. This effect is in accord with observations in healthy men (17). In the long term, we observed a trend for lower values in the GC-treated animals compared with control animals (e.g., 54.9 ± 7.5 vs. 62.8 ± 14.2 pg/ml after 13 mo). Both values were within the range that we have found earlier in intact domestic pigs of 40 and 100 pg/ml (53) but were significantly higher than values that we have seen before in vitamin D-depleted growing minipigs of 32 ± 6.2 pg/ml (51). The trend for lower 1,25(OH)₂D₃ concentrations in GC-treated minipigs vs. control animals may be explained by a possibility recently published by Zhou et al. (69). They proposed that activation of a steroid and xenobiotic receptor (PXR or SXR) by GC stimulates the PXR-mediated expression of CYP3A4, the enzyme that degrades 1,25(OH)₂D₃ by 24-hydroxylation. This mechanism could also contribute to the reduction of the intestinal calcium absorption that occurred in GC-treated animals (Table 2).

**BAP.** The most striking physiological effect of GC treatment seen in this study is in lowering BAP activity, which is in agreement with studies in men who were treated with 50 mg prednisolone daily for ~4 mo. Their BAP decreased significantly by 24.2 ± 8.6%, and their serum osteocalcin decreased strongly by 28.5 ± 15.5% (P = 0.08; see Ref. 57). The men were treated with GC because of fertility problems and not because of a systemic disease. Similar indications of a reduced bone formation were reported for healthy adult volunteers on a short-term treatment with GC (21), for asthmatics with long-term treatment with doses of ≥1.5 mg/day (13), for postmenopausal women on acute high-dose prednisone (60 mg/day), for growing minipigs (23), and for adult minipigs on a diet with a high compared with a low calcium content as an expression of reduced bone turnover (54).

**DPD.** Ikeda et al. (23) reported significantly reduced urinary type 1 collagen N-telopeptides after 13 wk. We did not see such an indication of depressed bone resorption after chronic administration of GC to healthy animals. This is in agreement with reports in chronically GC-treated young men for reasons of infertility, without a chronic disease (45), in euthyroid patients with Graves’ ophthalmopathy treated with GC for one year (47) and in asthmatics with long-term treatment (13). GC treatment may evoke different responses in the growing compared with the mature skeleton. However, in contrast to postmenopausal osteoporosis where bone resorption prevails over bone formation and bone formation is almost unaltered, stimulated bone resorption contributes only slightly to the bone mineral loss in GC-induced osteoporosis, but bone formation is dramatically reduced (8).

**Metabolic mineral balance.** It is assumed that the bone loss observed after GC treatment has its origin in part in a lower calcium retention and that the reason is a decreased renal hydroxylation of 25(OH)D₃, a reduced expression of the duodenal but not the renal calcium-binding protein CaBP-9k (31), an increased catabolism of 25(OH)D₃ and 1,25(OH)₂D₃ (43, 69), and/or a vitamin D-independent defect in transmucosal calcium transport (7, 30). Harrison and Harrison (19) reported on an inhibitory effect on intestinal calcium absorption in rats where dietary cortisol impaired the active transport and passive diffusion.

In some cases, GC treatment increased the urinary excretion of calcium because of a reduced renal tubular calcium reabsorption that is directly mediated by the drug (7, 8).

After acute administration of high-dose GC (40 mg of prednisone) to postmenopausal women, serum calcium and serum phosphate were unchanged (59) like in our minipigs. In contrast to others (39, 58, 59), we did not observe significant changes in urinary calcium. This divergent finding may be explained by the fact that we measured 7-day urinary calcium excretion in healthy adult animals in a long-term intervention with sufficient calcium in the diet. The impact of GC on renal calcium excretion may be different in postmenopausal women (59), in patients that are treated with GC because of a variety of different diseases (58), or in spot urine samples of fasted subjects (39). Moreover, there was no untreated control group and the observation was limited to 5 days (59), or both groups consisted of patients of several different diseases (58). Our results are in accordance with reports by Hahn et al. (18) in which renal calcium excretion of GC-treated patients was not different from normal subjects.

The persistent decline in calcium retention after GC treatment was a result of persistently higher fecal excretion, i.e., lower absorption. Thus the metabolic calcium balance reflected very well the decrease of BMD seen in vivo (Fig. 1), the tendency for a lower BMC (Table 3), and the borderline lower fracture resistance in vitro (Fig. 3).

Our observation is in accordance with a 12% reduction of calcium absorption in normal volunteers within 2 wk of GC treatment (57), and with patients receiving pharmacological doses of prednisone (15–100 mg/day), when calcium absorption correlated inversely with the dose of GC (30). The authors suggested that acute calcium malabsorption in GC-treated patients was the result of an abnormality of vitamin D metabolism because the administration of a physiological dose of synthetic 1,25(OH)₂D₃ stimulated calcium absorption in all patients (30). Since we did not see large effects on plasma vitamin D metabolites, nor on PTH, the chronic application of GC may deteriorate the vitamin D-independent transmucosal absorption of calcium, whereas the change in vitamin D metabolites that was associated with the reduced calcium absorption in patients may be the result of the underlying disease.

There were no strong effects of GC on phosphorus retention. The trend for a lower phosphorus absorption in minipigs receiving GC was compensated by the significant reduction of urinary phosphorus.

**BMC, histomorphometry, bone stability.** The positive association between cumulative dose of GC and loss of BMC was indicated by a trend for lower contents of ash, calcium, and phosphorus. The significantly lower dry matter in the lumbar vertebra that paralleled the development of BMD demonstrates a GC-induced change of bone composition with consequences on mechanical properties. Our results are in agreement with lower ultimate load and maximum absorption energy in the lumbar vertebra of growing minipigs with GC treatment (23). The reason for the bone loss might be a reduced GC-associated bone formation in the presence of an unaltered ongoing bone resorption. It was proposed that the inhibition of osteoblasto-
genesis and a higher rate of apoptosis of osteoblasts and osteocyttes are of primary importance (8, 41, 67).

Consequently, a depressed OPG production could be expected. We observed only slightly more depressed plasma OPG concentrations in GC-treated animals. The biphasic course of GC-induced bone loss may be caused by an initial borderline increase in osteoid formation ($P = 0.059$), a short-term but maintained trend for lower trabecular width, and a significant rise of trabecular separation in the second phase of the study. The resulting trend for ongoing lower trabecular bone volume was in agreement with the direction of histomorphometric parameters of aged women undergoing GC treatment, who had slightly lower bone volume/tissue volume but significantly lower trabecular thickness (50).

Moreover, we could show in this set of minipigs that vascular endothelial growth factor (VEGF) was significantly decreased in the lumbar vertebrae of GC-treated minipigs (48). This observation supports the hypothesis that an impairment of the VEGF-modulated vascularization of the bone remodeling unit by GC contributes to the short-term decrease in osteoblast activity, and thus to the significant decline in plasma BAP (Fig. 2, bottom) and to the trend for a lower number of alkaline phosphatase-positive chondrocytes in articular knee joint cartilage (10).

The controversy over the role of urinary calcium loss, bone resorption, vitamin D metabolites, and hyperparathyroidism to GIO in human studies, and the absence of their contribution to GIO in our study, may be explained by the underlying disease that is treated in patients rather than GC themselves (8, 47), whereas our study was performed in healthy animals.

In summary, the aim was to investigate if the minipig is a suitable animal model for GIO. The main parameter was BMD in vivo as measured by QCT. We have shown a significant and persistent loss of BMD, which was biphasic like in humans. Thus the adult Go¨ttingen miniature pig is a suitable animal model for GC-induced osteoporosis.

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