CHAPTER 6

The Effects of Vitamin D Deficiency on Bone Structure and Remodeling in Adult Rats with a Normal Mineral Homeostasis

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ABSTRACT

Skeletal health problems caused by vitamin D deficiency are mainly attributed to hypocalcemia, hypophosphatemia and hyperparathyroidism, but whether low serum 25(OH)D and 1,25(OH)_2D concentrations directly affect bone cell activity remains subject of debate. Therefore, the aim was to investigate whether vitamin D deficiency accompanied by normal serum calcium and phosphate concentrations in adult rats affects bone remodeling and bone structure as well as genes involved in local vitamin D metabolism in bone. Three months old male Wistar rats were fed either a diet containing 1.5 IU vitamin D/gram, 1% calcium and 0.7% phosphate (n=10) or a vitamin D deficient diet containing 2% calcium, 1.25% phosphate and 20% lactose in combination with six injections of paricalcitol during the first two weeks (n=10) to deplete vitamin D stores. Within three weeks, vitamin D deficiency was induced in the rats receiving the paricalcitol injections in combination with the vitamin D deficient diet, while serum calcium and phosphate concentrations were kept within the normal range. After ten weeks, rats were sacrificed. Histomorphometric analysis revealed that vitamin D deficiency in adult rats accompanied by normal serum calcium and phosphate concentrations did not have negative effects on bone structure and remodeling. Bone cells did also not respond to low serum 25(OH)D and 1,25(OH)_2D levels by altered expression levels of CYP27B1, CYP24 and VDR. These results suggest that vitamin D deficient adult rats receiving a diet high in calcium, phosphate and lactose do not develop a vitamin D deficient bone phenotype.
**INTRODUCTION**

An adequate vitamin D status is essential for the maintenance of the skeleton throughout life [27]. Vitamin D status is assessed by the measurement of serum 25-hydroxyvitamin D (25(OH)D) concentration which is the major circulating metabolite [28]. The metabolite 25(OH)D is hydroxylated to the active metabolite 1,25-dihydroxyvitamin D (1,25(OH)₂D) at the 1α-position by the renal 1α-hydroxylase enzyme derived from the CYP27B1 gene [57]. The positive effects of 1,25(OH)₂D on the skeleton are primarily achieved by increasing the intestinal absorption of calcium, which facilitates mineral deposition in bone matrix [27]. Additional effects of 1,25(OH)₂D on the skeleton can be exerted by binding to the vitamin D receptor (VDR) found in bone cells [55;56]. In vitro, 1,25(OH)₂D inhibits proliferation of osteoblasts [52;56], enhances osteoblast differentiation as well as matrix mineralization [4;52;54;56], and increases RANKL expression which subsequently stimulates osteoclastogenesis [16]. In osteocytes, 1,25(OH)₂D stimulates the transcription and synthesis of fibroblast growth factor 23 (FGF23) [43].

An adequate vitamin D status is also important for the autocrine and paracrine activities of 1,25(OH)₂D in bone tissue, for instance regulation of cell growth and function [3;52]. Bone cells express the enzyme 1α-hydroxylase (gene CYP27B1) and are able to synthesize 1,25(OH)₂D from 25(OH)D [4;52;54]. An excess of local 25(OH)D and 1,25(OH)₂D concentrations is prevented by the activity of the enzyme 24-hydroxylase (gene CYP24) which is responsible for the conversion to 24R,25(OH)₂D and 1,24R,25(OH)₃D, respectively [2-4;52;54]. In contrast to the renal synthesis of 1,25(OH)₂D which is tightly regulated by hormones, the synthesis of 1,25(OH)₂D by 1α-hydroxylase in bone cells may depend on the serum 25(OH)D concentration [52]. Therefore, an adequate serum 25(OH)D concentration may be essential for the local availability of 1,25(OH)₂D in bone tissue [38].

Vitamin D deficiency is a highly prevalent condition in humans worldwide [29]. Vitamin D deficiency has been associated with a lower bone mineral density [23], an increased bone turnover [23] and an increased fracture risk [53]. Skeletal health problems caused by vitamin D deficiency are mainly attributed to changes in serum calcium and parathyroid hormone (PTH) concentrations [29]. On the one hand severe vitamin D deficiency results in a reduced intestinal calcium absorption leading to a defective bone mineralization in the long term; on the other hand vitamin D deficiency causes secondary hyperparathyroidism, which results in a high bone turnover and an increased bone resorption [26;27]. In addition to the indirect effects of vitamin D deficiency on bone health, the activity of bone cells may also directly be affected by low 25(OH)D and 1,25(OH)₂D concentrations. To study the effects of both low 25(OH)D and low 1,25(OH)₂D concentrations on bone remodeling and local vitamin D metabolism in bone in more detail, the effects of vitamin D deficiency need to be separated from those of hypocalcaemia and hyperparathyroidism. Several normocalcemic mod-
els such as VDR knock-out mouse have been developed [1;10;11;37;42], but these models have limited value to study the effects of acquired vitamin D deficiency on local vitamin D metabolism in bone. Normocalcemic animal models of dietary vitamin D deficiency have also been developed [24;41;49;59], but the induction of both low serum 25(OH)D and 1,25(OH)₂D levels in adult rats requires a long period of time as it only occurs in case of severe vitamin D deficiency [36;45].

Recently, a novel rat model of acquired vitamin D deficiency was introduced for the investigation of vitamin D deficiency without concurrent changes in PTH, calcium and phosphate serum concentrations [45]. In this model, rats were fed a vitamin D deficient diet during the whole study and received six intraperitoneal injections of paricalcitol in the first two weeks [45]. These paricalcitol injections accelerated the catabolism of vitamin D stores by increasing the expression of CYP24. Within three weeks, serum 25(OH)D and 1,25(OH)₂D concentrations were extremely low [45]. Due to high dietary concentrations of calcium and phosphate in combination with lactose, the serum concentrations of calcium, phosphate and PTH were kept in the normal range [45]. For this reason, this model is very suitable for studying the isolated effects of deficiency of both 25(OH)D and 1,25(OH)₂D serum concentrations on bone structure, bone remodeling, bone mineralization and local vitamin D metabolism in bone tissue.

The first aim of this study was to investigate whether vitamin D deficiency in the absence of abnormal serum calcium and phosphate concentrations affects bone remodeling, bone mineralization and bone structure in adult rats. With the presence of normal calcium and phosphate serum concentrations in the rats, we hypothesize that bone mineralization is not affected by vitamin D deficiency. The second aim of this study was to investigate the isolated impact of vitamin D deficiency on the expression of CYP27B1, CYP24 and the gene encoding VDR in bone metabolism of adult rats.

**MATERIALS AND METHODS**

**Animals**
Ten male Wistar rats (Harlan CPB, Horst, The Netherlands) at the age of 14 weeks were fed a vitamin D deficient diet (TD.87095 Brown C.C. Vit.D Defic; Harlan Laboratories, Madison, WI, USA) containing 2% calcium, 1.25% phosphate and 20% lactose. At day 1, 3, 5, 8, 10 and 12, the rats received intraperitoneal injections of 32 ng 19-nor-1,25-dihydroxyvitamin D₂ (paricalcitol; Zemplar, kindly provided by Abb-Vie) for induction of CYP24 to accelerate the catabolism of endogenous 25(OH)D and 1,25(OH)₂D leading to vitamin D deficiency, as published previously [45]. Ten age- and sex-matched Wistar rats (Harlan CPB, Horst, The Netherlands) were used as external control group. The control rats were fed a diet containing 1.5 IU vitamin D/gram, 1% calcium and 0.7% phosphate (2016 Teklad Global 16% Protein Rodent
Diet; Harlan Laboratories, Madison, WI, USA). Food and water were provided ad libitum and rats were maintained under conventional housing conditions. After 0 and 3 weeks, blood samples were collected from the vitamin D deficient rats by vena puncture to determine whether vitamin D deficiency was induced [45]. After 10 weeks, blood samples were collected at sacrifice by cardiac puncture from both vitamin D deficient and control rats. Serum samples were stored at -20°C until measurements of 25(OH)D, 1,25(OH)₂D, calcium, phosphate, PTH and creatinine concentrations. After sacrifice, tibiae and kidneys were dissected for further analysis. The Animal Care Committee at the VU University Medical Center in Amsterdam approved the experimental protocol, as described previously [45].

**Serum biochemistry**
Serum 25(OH)D concentrations were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) [21]. Briefly, samples were incubated with deuterated internal vitamin D standards (d6-25(OH)D and d6-24R,25(OH)₂D) and protein-precipitated using acetonitrile. After PTAD derivatization, supernatant was purified using a Symbiosis online solid phase extraction (SPE) system (Spark Holland, Emmen, The Netherlands), followed by detection with a Quattro Premier XE tandem mass spectrometer (Waters Corp., Milford, MA, USA). Serum 1,25(OH)₂D concentrations were analyzed using a radioimmunoassay (Immunodiagnostic Systems, Boldon, UK). Serum PTH concentrations were analyzed using a rat intact PTH ELISA kit (Scantibodies Laboratory, Santee, CA, USA) according to the manufacturer’s protocol. Serum calcium, phosphate and creatinine concentrations were analyzed on a Modular system (Roche Diagnostics, Mannheim, Germany).

**Histomorphometry**
Right tibiae were fixed in 4% phosphate-buffered formaldehyde for 24 h, dehydrated in graded ethanol and embedded in methylmetacrylate (MMA; BDH Chemicals, Poole, England) supplemented with 20% dibutylphtalate (Merck, Darmstadt, Germany), 8.0 g/L dibenzoylperoxide (AKZO Nobel, Deventer, The Netherlands) and 22 µl/10 ml N,N-dimethyl-p-toluidine (Merck), as described previously [51]. Undecalcified sections of 5 µm thickness were cut with a Leica Jung K Polycut microtome (Nussloch, Germany) and sections were stained with Goldner’s trichrome, tartrate-resistant acid phosphatase (TRAP) and Von Kossa. Histomorphometric analysis was performed using a Nikon microscope equipped with NIS-Elements AR 3.1 (Nikon GmbH, Düsseldorf, Germany). All measurements were performed according to the guidelines of the American Society of Bone and Mineral Research (ASBMR) nomenclature [12]. Measurements on trabecular bone were performed on two Goldner sections in a total area of 20.3 ± 4.7 mm² per rat and started at one trabecular thickness medially of the endostem to be certain that the area contained only trabecular bone. Goldner’s trichrome staining was used to measure bone structural parameters including
trabecular bone volume (BV) and trabecular bone surface (BS), which were subsequently used to calculate trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp). TRAP staining was used to assess bone resorption by determining osteoclast number per bone surface (Oc.N/BS). Von Kossa staining was used to assess bone formation by determining osteoid surface per bone surface (OS/BS).

RNA isolation and RT-qPCR
One week before the RNA isolation procedure, kidneys and left tibiae were placed in the RNAlater-ICE solution (Ambion, Austin, TX, USA). Total RNA isolation of kidneys was performed using the RNeasy Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. For removing residual DNA amounts an additional on-column DNase treatment was performed. The cortices of diaphyseal tibiae were pulverized in the presence of Trizol (Life Technologies, Bleiswijk, The Netherlands) using the Freezer Mill (Spex, Metuchen, NY, USA). After pulverization, a Trizol extraction, a chloroform/isoamylalcohol extraction and a second Trizol extraction was performed. For removing residual DNA amounts, samples were treated with DNase (Promega, Madison, WI, USA). Total RNA concentrations of both kidneys and tibiae were determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

RNA was reverse transcribed from 100 ng total RNA in a 20 µl reaction mixture containing 5 mmol/l MgCl₂ (Eurogentec, Maastricht, The Netherlands), 1x RT buffer (Promega), 1 mmol/l dATP, 1 mmol/l dCTP, 1 mmol/l dGTP, 1 mmol/l dTTP (Roche Diagnostics), 1 mmol/l betaine, 10 ng/µl random primer, 0.4 U/µl RNAsin (Promega) and 5 U/µl M-MLV RT-enzym (Promega) [52]. The PCR reaction of total 25 µl contained 3 µl cDNA, 300 nmol/l reverse and forward primer for several genes of interest (Table 1) and SYBR Green Supermix (Bio-Rad Laboratories, Veenendaal, The Netherlands). The PCR was performed on an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad): 3 min at 95°C, 40 cycles consisting of 15 sec at 95°C and 1 min at 60°C. The relative gene expression was calculated by the 2⁻ΔΔCt method and PBGD as well as HPRT were used as reference genes.

Statistical analysis
Data are presented as mean ± standard error of the mean (SEM). Differences between two groups were assessed using the Mann-Whitney U test. A p-value <0.05 was considered statistically significant. Data were analyzed using SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA).
RESULTS

Serum biochemistry

Vitamin D deficiency was successfully induced within three weeks in rats receiving paricalcitol injections in combination with a vitamin D deficient diet, as published previously [45]. After three weeks, mean serum 25(OH)D concentrations were 13.7 ± 0.5 nmol/l and mean serum 1,25(OH)\(_2\)D concentrations were undetectable (<30.0 pmol/l) in the vitamin D deficient rats (Table 2) [45]. Mean serum PTH concentrations in the vitamin D deficient rats at baseline were 47.0 ± 7.3 pg/ml and after three weeks 30.3 ± 5.9 pg/ml [45]. After 10 weeks, both mean serum 25(OH)D and 1,25(OH)\(_2\)D concentrations were still significantly lower in the vitamin D deficient rats compared to concentrations in the control rats (11.7 ± 0.3 and 72.2 ± 1.7 nmol/l, respectively;
45.6 ± 2.2 and 162.2 ± 12.9 pmol/l, respectively; p<0.001; Table 2). Serum calcium concentrations were slightly higher in vitamin D deficient rats than in control rats (3.4 ± 0.0 and 3.1 ± 0.1 mmol/l, respectively; p<0.01), while serum phosphate concentrations did not differ between vitamin D deficient and control rats (3.9 ± 0.1 and 3.8 ± 0.1 mmol/l, respectively). Serum creatinine concentrations were increased in the vitamin D deficient rats compared to those in the control rats (29.4 ± 1.0 and 26.0 ± 0.6 µmol/l, respectively; p<0.01).

Table 2. Serum biochemistry

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<td>t=10</td>
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<td>25(OH)D (nmol/l)</td>
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<td>13.7 ± 0.5</td>
<td>11.7 ± 0.3 a</td>
<td>72.2 ± 1.7</td>
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<td>1,25(OH)_{2}D (pmol/l)</td>
<td>475.5 ± 19.3</td>
<td>&lt;30.0</td>
<td>45.6 ± 2.2 a</td>
<td>162.2 ± 12.9</td>
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<tr>
<td>PTH (pg/ml)</td>
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<td>30.3 ± 5.9</td>
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<td>Calcium (mmol/l)</td>
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<td>Phosphate (mmol/l)</td>
<td>2.3 ± 0.1</td>
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<tr>
<td>Creatinine (µmol/l)</td>
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<td>29.4± 1.0 c</td>
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NOTE. Results are expressed as mean ± SEM from 10 rats and were statistically analysed using the Mann–Whitney U test.

* p<0.001 control (t=10) vs. deficient (t=10)

Bone histomorphometry

Von Kossa-stained sections of bones from vitamin D deficient rats did not reveal increases of osteoid tissue as a sign of osteomalacia (Fig. 1A). Histomorphometric analysis of bone structure parameters, i.e. bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp), did not reveal differences between vitamin D deficient and control rats (Fig. 1B-E). Regarding remodeling parameters, trabecular osteoid surface (Tb.OS/BS) was 41% lower in the vitamin D deficient rats compared to control rats (p<0.05; Fig. 1F). The number of osteoclasts (N.Oc/BS) in the vitamin D deficient rats did not differ from control rats (Fig. 1G).
mRNA expression of genes involved in bone formation and resorption
Expression levels of collagen type 1α2 (COL1α2; 1.5 fold, p<0.05, Fig. 2A), alkaline phosphatase (ALP; 1.5 fold, p<0.05, Fig. 2B), osteocalcin (2.0 fold, p<0.01, Fig. 2C) and osteopontin (2.3 fold, p<0.01, Fig. 2D) were all higher in the vitamin D deficient rats than those in the control rats. MEPE, RANKL and OPG mRNA levels did not differ between the vitamin D deficient and control rats (Fig. 2E-G), nor did the RANKL/OPG ratio (Fig. 2H).

FGF23 mRNA expression in bone and Klotho mRNA expression in the kidney
FGF23 mRNA levels in tibiae were 2.0 fold higher in the vitamin D deficient rats compared to those in the control rats (Fig. 3A). In the kidneys, Klotho mRNA levels were 39% lower in the vitamin D deficient compared to those in the control rats (Fig. 3B).
mRNA expression of genes involved in vitamin D metabolism in bone and kidney

CYP27B1, CYP24 and VDR mRNA levels in tibiae did not differ between vitamin D deficient and control rats (Fig. 4A-C). In kidneys, CYP27B1 mRNA levels were 77% lower and CYP24 mRNA levels were 3.9 fold higher in vitamin D deficient rats compared to those in control rats (Fig. 4D and E). VDR mRNA levels in kidneys from vitamin D deficient rats were not different from control rats (Fig. 4F).

Figure 2. mRNA expression of genes involved in bone formation and resorption in tibiae from vitamin D deficient and control rats. (A) Collagen type 1a2 (COL1a2). (B) Alkaline phosphate (ALP). (C) Osteocalcin. (D) Osteopontin. (E) Matrix extracellular phosphoglycoprotein (MEPE). (F) Receptor activator of nuclear factor kappa-B ligand (RANKL). (G) Osteoprotegerin (OPG). (H) RANKL/OPG ratio. Results are expressed as mean from 8-10 rats and were statistically analyzed using the Mann–Whitney U test (*p<0.05, **p<0.01).
Figure 3. FGF23 mRNA expression in tibiae and Klotho mRNA expression in kidneys from vitamin D deficient and control rats. (A) Fibroblast growth factor 23 (FGF23). (B) Klotho. Results are expressed as mean from 8-10 rats and were statistically analyzed using the Mann–Whitney U test (*p<0.05, **p<0.001).

Figure 4. mRNA expression of genes involved in vitamin D metabolism in tibiae and kidneys from vitamin D deficient and control rats. (A) CYP27B1 in the tibia. (B) CYP24 in the tibia. (C) Vitamin D receptor (VDR) in the tibia. (D) CYP27B1 in the kidney. (E) CYP24 in the kidney. (F) Vitamin D receptor (VDR) in the kidney. Results are expressed as mean from 8-10 rats and were statistically analyzed using the Mann–Whitney U test (**p<0.01, ***p<0.001).
DISCUSSION

Skeletal health problems caused by vitamin D deficiency are assumed to be due mainly to hypocalcemia, hypophosphatemia and hyperparathyroidism. However, direct effects of low serum 25(OH)D and 1,25(OH)\textsubscript{2}D concentrations on bone cell activity may occur as well. Therefore, a recently published rat model of vitamin D deficiency, but with unchanged serum levels of minerals and PTH, was used to study the effects of vitamin D deficiency on skeletal health [45]. The current study presents the effects of both low 25(OH)D and 1,25(OH)\textsubscript{2}D concentrations on bone structure, remodeling, mineralization and expression of genes involved in vitamin D and bone metabolism.

As we hypothesized, the Von Kossa-stained bone sections of the vitamin D deficient rats did not show mineralization defects. The absence of mineralization defects is most likely due to the administration of a diet containing high calcium, high phosphate and lactose to the vitamin D deficient rats leading to normal calcium and phosphate serum concentrations. Because bone mineralization is mainly a passive process [9], the presence of normal calcium and phosphate concentrations may be sufficient to maintain an adequate mineralization of bone as shown by previously published vitamin D deficient rat studies [44;49].

Remarkably, trabecular osteoid surface was lower in tibiae of vitamin D deficient rats than in those of control rats, while mRNA levels of osteoblast markers including ALP, COL\textsubscript{1a2}, osteocalcin and osteopontin were higher. The lower trabecular osteoid surface can be explained by an increased speed of mineralization or a reduced bone formation. A reduced bone formation is less likely due to the increased mRNA levels of osteoblast markers in tibiae of vitamin D deficient rats. It is possible that the slightly higher serum calcium levels induced by the high calcium intake may have resulted in a higher speed of mineralization and thus a lower trabecular osteoid surface compared to the control rats. The increased mRNA levels of osteoblast markers would be expected to result in an increased trabecular bone volume as well. Probably, the increase in mRNA levels of osteoblast markers did not result in an increased trabecular bone volume yet. Alternatively, it is possible that post-translational processes differ substantially for different vitamin D levels, since we did not measure protein abundance.

Contrary to expectations, this study showed that mRNA levels of osteoblast markers increased in vitamin D deficient normocalcemic rats. According to several other animal and in vitro studies a decrease in osteoblast activity was expected after a period of low 1,25(OH)\textsubscript{2}D levels [17;37;52;54]. For instance, in VDR knockout, 1\alpha-hydroxylase knockout and double knockout mice which showed normal PTH, calcium and phosphate levels due to a rescue diet, osteoblast numbers and bone volume were below levels in wild-type mice [37]. This suggests that the absence of a functional 1,25(OH)\textsubscript{2}D/VDR system has direct negative consequences for bone forma-
Vitamin D deficiency in rats with a normal mineral homeostasis

The stimulating role of 1,25(OH)$_2$D on bone formation has also been demonstrated by in vitro studies in which 1,25(OH)$_2$D enhanced rat osteoblast differentiation by increasing expression levels of alkaline phosphatase, osteocalcin, and osteopontin [8;18;25;30;35;40]. Moreover, VDR overexpression in mature osteoblasts leads to an increase in trabecular and cortical bone due to an increase in formation and a decrease in resorption [5;17]. The studies mentioned above are in contrast to our study in which vitamin D deficiency resulted in an increase, and not in a reduction, of mRNA levels of osteoblast markers. Nevertheless, it has been reported that the absence of the VDR in osteoblasts can result in an increase in bone mass in mice with normal mineral homeostasis, but these effects were caused by a reduced bone resorption and not by an increased bone formation rate [60]. Thus, the increase in mRNA levels of osteoblast markers in vitamin D deficient rats is difficult to explain by low serum 1,25(OH)$_2$D levels, but other factors such as the high calcium diet may play a role as well. In an adult mouse model, a high calcium diet resulted in an increase of trabecular bone volume and bone mineral density [14]. This increase of trabecular bone volume was associated with an increase in osteoblast numbers and an increased gene expression of the calcium sensing receptor (CaSR), ALP, COL1 and osteonectin, but also with a decrease in osteoclast numbers and a reduced RANKL/OPG ratio [14]. Results from the latter study suggest that a high calcium diet can stimulate bone formation. In vitro, it has also been shown that activation of the CaSR by high extracellular calcium can stimulate osteoblast differentiation and mineralization [13;61]. Thus, it is possible that the higher calcium content in the diet of vitamin D deficient animals may have stimulated mRNA levels of osteoblast markers.

The diet used in this study contained, in addition to the high calcium content, also high phosphate which may have resulted in higher FGF23 mRNA levels in tibiae of vitamin D deficient rats than those of control rats. After all, FGF23 responds to phosphate loading as such promoting phosphaturia [32]. This feature is well-established in human subjects as well where phosphate loading increases serum FGF23 concentrations to maintain phosphate balance [58]. An important function of FGF23 is the regulation of 1α-hydroxylase and 24-hydroxylase activity in the kidney. Since it could have been expected that vitamin D deficiency would have induced a compensatory response in expression of genes involved in its regulation, this was not observed. It can be speculated that the reduced CYP27B1 mRNA levels and increased CYP24 mRNA levels in kidneys of vitamin D deficient rats with normal calcium and phosphate concentrations may be due to high FGF23 concentrations in serum. In this way, FGF23 suppresses serum 1,25(OH)$_2$D levels to reduce phosphate absorption from the intestine.

Regulation of phosphate homeostasis by FGF23 in the kidney is mediated through binding to the FGF receptor (FGFR) and co-receptor α-Klotho, a protein predominantly expressed in the distal tubule at the cell surface [39]. The membrane form of Klotho increases the binding affinity of FGF23 to the FGFR whereby the FGF23-
FGFR-Klotho complex can exert its effects including stimulation of phosphaturia and calcium reabsorption [32]. The secreted form of Klotho acts as an anti-aging and organ protection factor and regulates ion channels and transporters in target tissues independent of the FGFR [22]. We found a lower mRNA expression of Klotho in the vitamin D deficient rats than in the control rats which may be caused by the low 1,25(OH)₂D serum levels. Supportive is a study in mice in which 1,25(OH)₂D administration increased Klotho expression in the kidney, while mice fed a vitamin D deficient diet showed decreased Klotho expression levels [47]. In addition, in human and mouse renal cell lines 1,25(OH)₂D is able to induce mRNA levels of Klotho [15;19;20]. Other factors may have influenced the expression of Klotho in the vitamin D deficient rats as well. In mice, renal Klotho expression is reduced by high dietary phosphate (1.0-1.5% phosphate) [34] and in other animal models, high FGF23 levels result in decreased Klotho mRNA levels [31;33]. Moreover, the vitamin D deficient rats had a higher serum creatinine level (for unknown reasons), which is also a well-established factor in the downregulation of Klotho [46]. However, whether these factors act directly on Klotho mRNA levels is unknown.

CYP27B1 mRNA levels in bone appeared not to be affected by low 25(OH)D and 1,25(OH)₂D serum concentrations which is in line with a previous study in long-term vitamin D deficient older adult mice [51]. Because the locally synthesized 1,25(OH)₂D in bone acts via autocrine and paracrine mechanisms, it is most likely that CYP27B1 mRNA is regulated at a local level and not systemically. This is supported by studies in which bone CYP27B1 mRNA is regulated by local factors such as mechanical loading [50], interleukin-1β [54] and TGF-β [48]. CYP24 and VDR mRNA levels were also not affected by low serum 25(OH)D and 1,25(OH)₂D concentrations, suggesting that these mRNA levels in bone are not regulated by circulating 25(OH)D and 1,25(OH)₂D.

In our rat model, paricalcitol injections were administered in the first two weeks to induce CYP24 expression leading to a rapid reduction of the 25(OH)D and 1,25(OH)₂D serum concentrations [45]. In rats, the clearance rate of paricalcitol is similar to that of 1,25(OH)₂D [6]. Although there are no data available about the half-life of paricalcitol in rats, in healthy humans the half-life of paricalcitol is 5-7 hours [7]. Therefore, a carry-over effect of paricalcitol on our results is highly unlikely.

This study has several limitations. The use of an external control group may have led to small differences between the groups, but differences between groups were minimized by the use of age-, sex- and strain-matched rats and the maintenance of the same housing conditions. Furthermore, eight weeks of vitamin D deficiency in 14 weeks old rats may be too short to alter bone structure or remodeling parameters. A vitamin D deficient bone phenotype could not be established for this animal model due to the absence of a vitamin D deficient control group receiving a normal diet in combination with paricalcitol injections. Another point that can be made is that we did not have the possibility to confirm the presence of high concentrations of FGF23
in serum of vitamin D deficient rats due to instability of FGF23 in serum samples. Lastly, we did not measure protein content in bone tissue.

We showed that vitamin D deficiency in young adult rats did not result in the development of bone mineralization defects and an increase in bone resorption which is most likely due to the administration of a diet high in calcium, phosphate and lactose. The high calcium and high phosphate content in the diet may even have resulted in the development of additional effects such as the high FGF23 mRNA levels in the vitamin D deficient rats. In other studies, diets containing 2% calcium and 1.25% phosphate in combination with 20% lactose are not unusual but these rescue diets are mostly administered to growing animals [1;10;37]. We used young adult rats, which did not have a period of rapid growth during the study and probably had a lower need for calcium and phosphate.

We can conclude that vitamin D deficient adult rats receiving a diet high in calcium, phosphate and lactose do not develop a vitamin D deficient bone phenotype. CYP27B1, CYP24 and VDR mRNA levels in bone do also not change after a period of low serum 25(OH)D and 1,25(OH)₂D levels in adult rats accompanied by normal serum calcium and phosphate concentrations.

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