CHAPTER 7

Long-term Vitamin D Deficiency in Older Adult C57BL/6 Mice Does not Affect Bone Structure, Remodeling and Mineralization

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ABSTRACT

Animal models show that vitamin D deficiency may have severe consequences for skeletal health. However, most studies have been performed in young rodents for a relatively short period, while in older adult rodents the effects of long-term vitamin D deficiency on skeletal health have not been extensively studied. Therefore, the first aim of this study was to determine the effects of long-term vitamin D deficiency on bone structure, remodeling and mineralization in bones from older adult mice. The second aim was to determine the effects of long-term vitamin D deficiency on mRNA levels of genes involved in vitamin D metabolism in bones from older adult mice.

Ten months old male C57BL/6 mice were fed a diet containing 0.5% calcium, 0.2% phosphate and 0 (n=8) or 1 (n=9) IU vitamin D₃/gram for 14 months. At an age of 24 months, mice were sacrificed for histomorphometric and micro-computed tomography (micro-CT) analysis of humeri as well as analysis of CYP27B1, CYP24 and VDR mRNA levels in tibiae and kidneys using RT-qPCR. Plasma samples, obtained at 17 and 24 months of age, were used for measurements of 25-hydroxyvitamin D (25(OH)D) (all samples), phosphate and parathyroid hormone (PTH) (terminal samples) concentrations.

At the age of 17 and 24 months, mean plasma 25(OH)D concentrations were below the detection limit (<4 nmol/l) in mice receiving vitamin D deficient diets. Plasma phosphate and PTH concentrations did not differ between both groups. Micro-CT and histomorphometric analysis of bone mineral density, structure and remodeling did not reveal differences between control and vitamin D deficient mice. Long-term vitamin D deficiency did also not affect CYP27B1 mRNA levels in tibiae, while CYP24 mRNA levels in tibiae were below the detection threshold in both groups. VDR mRNA levels in tibiae from vitamin D deficient mice were 0.7 fold lower than those in control mice.

In conclusion, long-term vitamin D deficiency in older adult C57BL/6 mice, accompanied by normal plasma PTH and phosphate concentrations, does not affect bone structure, remodeling and mineralization. In bone, expression levels of CYP27B1 are also not affected by long-term vitamin D deficiency in older adult C57BL/6 mice. Our results suggest that mice at old age have a low or absent response to vitamin D deficiency probably due to factors such as a decreased bone formation rate or a reduced response of bone cells to 25(OH)D and 1,25(OH)₂D. Older adult mice may therefore be less useful for the study of the effects of vitamin D deficiency on bone health in older people.
**INTRODUCTION**

Vitamin D deficiency is a common condition in older persons [1;2]. Older persons are particularly at risk of developing vitamin D deficiency due to a lack of sun exposure, a decline of the cutaneous production of vitamin D, reduced dietary intake and an impaired 1α-hydroxylase activity in the kidney [3;4]. In Europe, up to 35% of adults has serum 25(OH)D levels lower than 30 nmol/l, but in older persons percentages may increase up to 90% [5;6]. Due to the role of vitamin D in the maintenance of skeletal health, vitamin D deficiency is a major cause of metabolic bone diseases [6]. Vitamin D deficiency results in a decreased intestinal calcium absorption which in turn leads to a reduced bone mineralization [6;7]. In the long-term, vitamin D deficiency may lead to osteomalacia, a metabolic bone disease characterized by bone mineralization defects and an increased osteoid volume [6;8]. Vitamin D deficiency also leads to the release of parathyroid hormone (PTH) from the parathyroid glands due to low serum calcium levels which results in an increased bone turnover, bone loss and osteoporosis [6;7]. However, the response of bone and the parathyroid glands to vitamin D deficiency is highly variable in older persons [6;9].

Animal models of dietary vitamin D deficiency are often used to study effects on skeletal health in more detail. However, the impact of dietary vitamin D deficiency on skeletal health depends on a variety of factors such as the age of the animal, calcium and phosphate intake and duration of vitamin D deficiency. Most studies use young vitamin D deficient rodents or second generation vitamin D deficient rodents, born to vitamin D deficient mothers [10]. These studies in young growing rodents show that severe vitamin D deficiency accompanied by hypocalcaemia results in the development of rickets including an increased width of the epiphyseal growth plates, abundant osteoid tissue and mineralization defects [11-13]. These signs of rickets in young rats can largely or entirely be prevented through the maintenance of normal calcium and phosphate levels by infusions or high calcium and phosphate intake [11-14]. Only a few studies have described the development of osteomalacia in adult rodents due to vitamin D insufficiency and inadequate dietary calcium and phosphate intake [15;16]. When vitamin D levels are insufficient in adult rodents, but dietary calcium intake is adequate, osteoporosis may occur [15;16]. Regarding the duration of vitamin D deficiency, most models of dietary vitamin D deficiency have been applied for a relatively short period, especially in young rodents. To our knowledge, the effects of long-term dietary vitamin D deficiency on skeletal health in older adult rodents have not been studied yet.

Vitamin D deficiency has not only indirect negative consequences for bone mineralization and turnover, but also affects direct activity of vitamin D in bone cells. Direct effects are exerted by the active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)₂D), through binding to the vitamin D receptor (VDR) which is present in bone cells [17]. The metabolite 1,25(OH)₂D is not only derived from the kidney, but
all major types of bone cells express $1\alpha$-hydroxylase and have the ability to convert 25(OH)D to 1,25(OH)$_2$D [18-23]. Although bone cells have the ability to metabolize 25(OH)D similar to the kidney, expression and activity of $1\alpha$-hydroxylase in bone cells is modulated differently from renal $1\alpha$-hydroxylase [19;24-27]. More specifically, studies in vivo and in vitro show that the expression of CYP27B1 in bone cells, i.e., the gene encoding $1\alpha$-hydroxylase, is not regulated by PTH which is an important regulator of renal $1\alpha$-hydroxylase [19;24;26]. When $1\alpha$-hydroxylase activity in bone is not regulated by renal regulators as PTH, the amount of substrate, i.e., 25(OH)D levels, may become more important. This raises the question what are the consequences of low 25(OH)D levels regarding the activity of $1\alpha$-hydroxylase. One study in vitamin D deficient nine months old rats shows that bone CYP27B1 expression increases in response to high dietary calcium compared to low dietary calcium [26]. Another study in seven months old rats with 25(OH)D levels ranging from 15–120 nmol/l, shows a trend toward increased CYP27B1 expression levels with higher serum 25(OH)D levels [28]. However, the effect of long-term vitamin D deficiency on the expression of CYP27B1 in older adult rodents has not been well described.

The first aim of this study was to determine the effects of long-term vitamin D deficiency on bone structure, remodeling and mineralization in bones from older adult mice. The second aim was to determine the effects of long-term vitamin D deficiency on mRNA levels of genes involved in vitamin D metabolism in bones and kidneys from older adult mice.

**MATERIALS AND METHODS**

**Animals**

The local Committee for Care and Use of Laboratory Animals at Wageningen University approved the experiment, as described previously [29]. All procedures were conducted in conformity with the institutional and national guidelines for the care and use of animals.

Ten months old male C57BL/6 mice (Janvier Laboratories, Le Genest Saint Isle, France) were fed a diet containing 0.5% calcium, 0.2% phosphate and 0 (n=8) or 1 (n=9) IU vitamin D$_3$/gram for 14 months. Food and water were provided ad libitum. Blood samples were collected on two occasions: at the age of 17 months (withdrawal from tail tip cut; sample volume approximately 100 µl) and at sacrifice at an age of 24 months (cardiac puncture; sample volume approximately 400 µl), as previously described [29]. Plasma samples were stored at $-20^\circ$C until measurements of 25(OH)D (all samples), phosphate and PTH concentrations (terminal samples). After sacrifice, humeri, tibiae as well as kidneys were dissected. Humeri were processed for histomorphometric analysis and micro-computed tomography. Tibiae and kidneys were frozen in liquid nitrogen and stored at $-80^\circ$C until the RNA isolation procedure.
**Plasma biochemistry**

Plasma 25-hydroxyvitamin D concentrations were analyzed using liquid chromatography–tandem mass spectrometry (LC–MS/MS). The terminal plasma sample (150 µl) from each individual mouse and a pooled sample which consisted of equal volumes (25 µl) of six randomly selected mice per group were analyzed [29]. Briefly, samples were incubated with deuterated internal vitamin D standards (d6-25(OH)D$_3$ and d6-24R,25(OH)$_2$D$_3$) and protein-precipitated using acetonitrile. Supernatant was, after PTAD derivatization, 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).

Plasma phosphate concentrations were analyzed on a Modular system (Roche Diagnostics, Mannheim, Germany).

Plasma PTH concentrations were analyzed using a mouse PTH 1-84 ELISA kit (Immutopics Inc., San Clemente, CA, USA) according to the manufacturer’s protocol.

**Histomorphometry**

Humeri were fixed in 4% phosphate-buffered formaldehyde for 24 h, dehydrated in graded ethanol and embedded in methylmethacrylate (MMA; BDH Chemicals, Poole, England) supplemented with 20% dibutyl phtalate (Merck, Darmstadt, Germany), 8.0 g/l dibenzoyl peroxyde (AKZO Nobel, Deventer, The Netherlands) and 22 µl/10 ml N,N-dimethyl-p-toluidine (Merck). Undecalcified sections of 5 µm thickness were cut with a Leica Jung K Polycut microtome (Nussloch, Germany) and subsequent 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 [30]. Measurements on trabecular bone were performed on Goldner sections in a total area of 3.5–11.4 mm$^2$ per mouse defined as 2 mm from the cortex of the humeral head [31]. Measurement was started at one cortical thickness medially of the endosteme 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 surface per bone surface (Oc.S/BS). Von Kossa staining was used to assess bone formation by determining osteoid surface per bone surface (OS/BS).
**Micro-computed tomography (micro-CT)**

Humeri were scanned with a high-resolution micro-CT system (µCT 40, Scanco Medical AG, Brüttisellen, Switzerland). All humeri were embedded in methylmethacrylate before scanning. Scan settings for each cross section were as follows: peak voltage 55 kV, electric current 145 µA, spatial resolution 15 µm, scan integration time 0.8 s. Beam hardening effects were reduced using both an aluminum filter in the micro-CT scanner and a correction algorithm in the reconstruction software. To separate cortical and trabecular bone from background thresholds of 543.5 and 526.6 mg hydroxyapatite (HA)/cm³, respectively, were used.

To compensate for small differences in bone length, a relative scale was introduced to define the locations of the regions of interest. The proximal end of the humeral head was set as 0% and the distal part of the deltoid tuberosity was set as 100%. The trabecular volume was defined manually by drawing contours and trabecular bone morphometry was performed in the region between 5% and 35%. The length of this region ranged from 109 to 128 slices (=1635–1920 µm). Cortical bone morphometry of the midshaft was performed in the region between 110% and 115%. This region had a length of 19–22 slices (=285–330 µm). The morphometry was performed with the program uct_evaluation v6.5-3 (Scanco Medical AG, Brüttisellen, Switzerland).

Mineral density maps of the humeral cortical bones were made for comparison of the distribution pattern between both groups, as described previously [32].

**RNA isolation and RT-qPCR**

After pulverizing of whole tibiae in lysis buffer (Qiagen, Hilden, Germany), total RNA was isolated using the Rneasy Mini kit (Qiagen) according to the manufacturer’s protocol. Total RNA isolation of whole kidneys was performed using the Rneasy Midi Kit (Qiagen) according to the manufacturer’s protocol. For removing residual DNA amounts an additional on-column DNase treatment was accomplished during the RNA isolation procedure. Total RNA concentration was measured with 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, Madison, WI, USA), 1 mmol/l dATP, 1 mmol/l dCTP, 1 mmol/l dGTP, 1 mmol/l dTTP (Roche Diagnostics, Mannheim, Germany), 1 mmol/l betaïne, 10 ng/µl random primer, 0.4 U/µl RNAsin (Promega) and 5 U/µl M-MLV RT-enzym (Promega). The PCR reaction of total 25 µl contained 3 µl cDNA, 300 nmol/l reverse and forward primer and SYBR Green Supermix (Bio-Rad Laboratories Inc., Veenendaal, The Netherlands). The following primer sets were used: CYP27B1 forward: 5’-CATCATGGGCGAGACCCGTG-3′ reverse: 5’-TCACCACATCCGCGGTTAGCAA-3’; CYP24 forward: 5’-AACAGCAGCACACTGGCAGA-3′ reverse: 5’-CTCGGCCGAGCCCCAGATGCAG-3′; VDR forward: 5’-TCCTGCTCGATGCCACCACA-3’
reverse: 5′-TGACCAATTGGAGGCCGGA-3′; PBGD forward: 5′-AGTGATGAAA-GATGGCA-3′ reverse: 5′-TCTGGACATCTTCTGTGCTGA-3′; β2-microglobulin forward: 5′-TGCTATCCAGAAACCCCCTCAA-3′ reverse: 5′-GCGGGGGAAGCTGTTACG-3′. The PCR was performed on an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad): 3 mins at 95°C, 40 cycles consisting of 15 s at 95°C and 1 min at 60°C. The relative gene expression was calculated by the 2^{ΔCt} method and PBGD as well as β2-microglobulin were used as housekeeping 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

Plasma biochemistry
At the age of 24 months, mean 25(OH)D plasma concentrations were lower in the vitamin D deficient mice compared to the concentrations in the control mice (<4.0 and 57.4 ± 5.3 nmol/l, respectively; p<0.001; Table 1), as published previously [29]. The 25(OH)D concentration of the pooled sample collected at the age of 17 months was also below detection level (<4.0 nmol/l) which confirms the presence of a vitamin D deficient status of a duration of at least 7 months [29]. Plasma phosphate concentrations did not differ between the vitamin D deficient and control mice (1.6 ± 0.1 and 1.5 ± 0.1 mmol/l, respectively), nor did plasma PTH concentrations (308.1 ± 28.1 and 323.8 ± 55.8 pg/ml, respectively).

Table 1. Plasma biochemistry

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Control</th>
<th>Deficient</th>
</tr>
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<tr>
<td>25(OH)D</td>
<td>nmol/l</td>
<td>57.4 ± 5.3</td>
<td>&lt;4.0 *</td>
</tr>
<tr>
<td>Phosphate</td>
<td>mmol/l</td>
<td>1.5 ± 0.1</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>PTH</td>
<td>pg/ml</td>
<td>323.8 ± 55.8</td>
<td>308.1 ± 28.1</td>
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</table>

NOTE. Results are expressed as mean ± SEM from 8-9 mice and were statistically analysed using the Mann–Whitney U test.
a control vs. deficient p<0.001
**CYP27B1, CYP24 and VDR mRNA levels in kidneys and tibiae**

In kidneys, CYP27B1, VDR and CYP24 mRNA levels from vitamin D deficient mice did not significantly differ from control mice (Fig. 1A–C). In tibiae, CYP27B1 mRNA levels from vitamin D deficient mice were also not different from those in control mice (Fig. 1D). In tibiae, however, VDR mRNA levels from vitamin D deficient mice were 0.7 fold lower than those in control mice (p<0.05; Fig. 1E). In tibiae, CYP24 mRNA levels were below the detection threshold in both groups.

![Figure 1. Levels of CYP27B1, VDR and CYP24 mRNA in tibiae and kidneys from vitamin D deficient and control mice.](image)

**Micro-CT analysis of the humerus**

Views of the humeral cortices showed similar patterns of bone mineral density in both vitamin D deficient and control mice (Fig. 2). The highest mineral densities were found in the distal part of the deltoid tuberosity at the cranial as well as caudal site and proximal of the crista supracondylaris medialis at the medial side of the shaft, extending to the teres major tuberosity in a few humeri. Lowest mineral densities were found on the crest of the greater tubercle, in the region surrounding the radial groove, on the proximal part of the deltoid tuberosity at the cranial as well as the caudal site and at the caudal side of the shaft at the height of the teres major tuberosity.
Micro-CT analysis showed that overall bone mineral density of humeri from vitamin D deficient mice was not different from control mice (Table 2). Evaluation of trabecular microarchitecture revealed no differences between vitamin D deficient and control mice regarding trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), connectivity density (Conn.D), structure model index (SMI), degree of anisotropy (DA) and trabecular bone mineral density. Midshaft analysis did also not show differences between vitamin D deficient and control mice regarding total area (Tt.Ar), cortical bone area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar), cortical thickness (Ct.Th), maximum as well as minimum moment of inertia ($I_{\text{max}}$ and $I_{\text{min}}$) and midshaft bone mineral density.
<table>
<thead>
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<th>Parameter</th>
<th>Abbreviation (unit)</th>
<th>Control</th>
<th>Deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Overall bone morphometry</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Humerus length</td>
<td>(mm)</td>
<td>12.4 ± 0.1</td>
<td>11.9 ± 0.2</td>
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<tr>
<td>Overall bone mineral density</td>
<td>BMD (mg/cm$^3$)</td>
<td>989.1 ± 13.6</td>
<td>996.4 ± 13.6</td>
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<td><strong>Trabecular bone morphometry</strong></td>
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<tr>
<td>Total volume</td>
<td>TV (mm$^3$)</td>
<td>3.3 ± 0.1</td>
<td>3.3 ± 0.1</td>
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<tr>
<td>Bone volume</td>
<td>BV (mm$^3$)</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.0</td>
</tr>
<tr>
<td>Bone volume fraction</td>
<td>BV/TV (%)</td>
<td>12.0 ± 1.1</td>
<td>13.0 ± 1.0</td>
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<tr>
<td>Trabecular number</td>
<td>Tb.N (1/mm)</td>
<td>2.6 ± 0.3</td>
<td>2.6 ± 0.1</td>
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<tr>
<td>Trabecular thickness</td>
<td>Tb.Th (µm)</td>
<td>61.3 ± 2.1</td>
<td>64.9 ± 2.6</td>
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<tr>
<td>Trabecular separation</td>
<td>Tb.Sp (µm)</td>
<td>420.8 ± 51.1</td>
<td>406.6 ± 12.2</td>
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<tr>
<td>Connectivity density</td>
<td>Conn.D (1/mm$^3$)</td>
<td>59.9 ± 9.2</td>
<td>57.7 ± 6.5</td>
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<tr>
<td>Structure model index</td>
<td>SMI</td>
<td>1.8 ± 0.2</td>
<td>1.5 ± 0.2</td>
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<tr>
<td>Degree of anisotropy</td>
<td>DA</td>
<td>1.3 ± 0.0</td>
<td>1.3 ± 0.0</td>
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<tr>
<td>Trabecular bone mineral density</td>
<td>BMD (mg/cm$^3$)</td>
<td>827.8 ± 7.5</td>
<td>851.6 ± 13.4</td>
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<td><strong>Cortical bone morphometry of the midshaft</strong></td>
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<tr>
<td>Total area</td>
<td>Tt.Ar (mm$^2$)</td>
<td>1.1 ± 0.0</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>Cortical bone area</td>
<td>Ct.Ar (mm$^2$)</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.0</td>
</tr>
<tr>
<td>Cortical area fraction</td>
<td>Ct.Ar/Tt.Ar (%)</td>
<td>46.5 ± 1.9</td>
<td>47.3 ± 1.2</td>
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<tr>
<td>Cortical thickness</td>
<td>Ct.Th (µm)</td>
<td>157.9 ± 6.9</td>
<td>158.0 ± 4.5</td>
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<tr>
<td>Maximum moment of inertia</td>
<td>$I_{max}$ (µm$^4$)</td>
<td>877.3 ± 32.0</td>
<td>932.8 ± 127.3</td>
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<tr>
<td>Minimum moment of inertia</td>
<td>$I_{min}$ (µm$^4$)</td>
<td>505.0 ± 32.9</td>
<td>515.2 ± 33.4</td>
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<tr>
<td>Midshaft bone mineral density</td>
<td>BMD (mg/cm$^3$)</td>
<td>965.8 ± 32.5</td>
<td>944.0 ± 25.2</td>
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</table>

**Histomorphometric analysis of the humerus**

Regarding bone structure, no differences were observed in trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N) and trabecular separation (Tb.Sp) between control and vitamin D deficient mice (Fig. 3A–D), which confirms the results from micro-CT analysis.

With the Goldner and Von Kossa stainings, increases of osteoid tissue as a sign of osteomalacia were not observed in the vitamin D deficient mice (Fig. 4A and B). Histomorphometric analysis of bone remodeling parameters, which are endocortical and trabecular osteoid surface (resp. Ec.OS/BS and Tb.OS/BS) as well as endocortical and trabecular osteoclast surface (resp. Ec.Oc.S/BS and Tb.Oc.S/BS), did also not reveal differences between control and vitamin D deficient mice (Fig. 4C–F).
Long-term vitamin D deficiency in older adult mice

Figure 3. Bone structure parameters in humeri from vitamin D deficient and control mice. (A) Bone volume/tissue volume (BV/TV). (B) Trabecular thickness (Tb.Th). (C) Trabecular number (Tb.N). (D) Trabecular separation (Tb.Sp). Results are expressed as mean from 7-8 mice and were statistically analysed using the Mann–Whitney U test.

Figure 4. Bone remodeling parameters in humeri from vitamin D deficient and control mice. (A,B) Representative micrographs of the Goldner and Von Kossa stained sections, respectively. Magnification: 20x. (C) Trabecular osteoid surface/bone surface (Tb.OS/BS). (D) Endocortical osteoid surface/bone surface (Ec.OS/BS). (E) Trabecular osteoclast surface/bone surface (Tb.Oc.S/BS). (F) Endocortical osteoclast surface/bone surface (Ec.Oc.S/BS). Results are expressed as mean from 7-8 mice and were statistically analysed using the Mann–Whitney U test.
DISCUSSION

Animal models show that vitamin D deficiency may have severe consequences for skeletal health. However, most studies have been performed in young rodents for a relatively short period, while the effects of long-term vitamin D deficiency on skeletal health in aging rodents have not been extensively studied. The present study shows that long-term (at least 7 months) vitamin D deficiency, accompanied by normal PTH and phosphate levels, did not affect indices for bone structure, remodeling and mineralization in older adult C57BL/6 mice. Visually, the bone had a normal aspect without mineralization defects after long-term vitamin D deficiency in older adult C57BL/6 mice (see Fig. 4A and B).

We showed that long-term vitamin D deficiency in older adult mice did not result in mineralization defects or a reduced mineral content. This suggests that older adult mice respond less to vitamin D deficiency than young mice in which vitamin D deficiency results in the development of rickets [11-13]. One reason for the absence of mineralization defects in older adult mice may be the decrease of bone formation to low rates during aging [33]. When bone remodeling in older adult mice is very low, osteoid tissue may not be formed and osteomalacia may not develop. This is in contrast to severe vitamin D deficiency in mice at a younger age in which bone remodeling results in an increase of osteoid tissue as an early stage of osteomalacia [34-37]. Studies in older men and women show that the response to vitamin D deficiency is highly variable [6;9]. In patients with hip fracture and severe vitamin D deficiency (serum 25(OH)D <25 nmol/l) only a minority of 12% or 19% respectively, show an increase of osteoid volume or osteoid surface [9]. Similarly, older vitamin D deficient men and women may not always develop increased serum PTH levels [6;38]. Reasons for this variable response to vitamin D deficiency in older persons are not yet completely understood.

Another explanation for the absence of mineralization defects or a reduced mineral content is that the diets in our study may contain sufficient calcium and phosphate. Previous animal studies show that low serum calcium and phosphate levels play an important role in the development of bone mineralization defects [12]. Specifically, in young vitamin D deficient rats, development of rickets can be prevented by calcium and phosphate infusions or a diet high in calcium and phosphate [11-13]. The epiphyseal plate width, trabecular osteoid volume as well as osteoid seam width are not increased and osteoid mineralization is not reduced compared to those in control rats [11;13]. Mouse models with targeted deletion of CYP27B1 (1α-hydroxylase knock-out) or VDR (VDR−/−) also show that bone mineralization can be rescued by a high calcium, high phosphate and lactose diet rather than direct activity of 1,25(OH)2D through the VDR on bone cells [34;35;39]. In our study, vitamin D deficiency did not result in mineralization defects or a reduced mineral content, suggesting that the diets used in our study may contain sufficient calcium and phosphate. Under normal circumstances,
calcium absorption from the diet is regulated by both an active transcellular vitamin D-dependent pathway and a passive paracellular pathway, which depends on the calcium gradient [40]. Vitamin D deficiency leads to a diminished active transcellular pathway, but intestinal calcium absorption through the passive paracellular pathway is still possible. Several in vivo studies in rodents show that the effects of dietary vitamin D deficiency can be restored by either a diet with high calcium, high phosphate and lactose (rescue diet) or with calcium and phosphate infusions, indicating that sufficient calcium is absorbed through the passive paracellular pathway. Concentrations of calcium and phosphate in most of these rescue diets are very high and ranged from 1.1 to 4.0% and 0.8 to 1.4%, respectively [12;14;34;39;41], while the diet used in our study contained 0.5% calcium and 0.2% phosphate. However, rescue diets are used in young growing rodents, which have a high demand for minerals during a period of rapid growth. The mice in our study were much older and did not have a period of rapid bone growth. Moreover, C57BL/6 mice do not show any bone growth after 6 months of age, while in most mice strains longitudinal bone growth slowly continues after puberty [42;43]. In C57BL/6 mice, the femur length does not increase between 6 and 12 months of age [43] and body length, an index of axial bone growth, does not increase between 12 and 24 months of age [42;44]. Therefore, the mice used in our study may have had a lower demand for minerals than rodents used in other studies and passive calcium absorption may have met the demand for calcium. Nevertheless, it has been shown that rats fed on a diet containing 0.4% calcium were able to maintain normal calcium levels [45]. Thus, the concentrations of calcium and phosphate in our diet may have been prevented the development of mineralization defects in older adult C57BL/6 mice.

Although in several animal models the development of rickets can be prevented by a high dietary intake or infusions of calcium and phosphate, other studies show that direct activity of 1,25(OH)$_2$D through the VDR is also required for normal bone homeostasis. For instance, in four months old 1α-hydroxylase knock-out, VDR knock-out and double knock-out mice which received a rescue diet resulting in normal serum PTH and calcium levels, osteoblast numbers and trabecular bone volume were lower than those in wild-type mice [34]. Treatment with 1,25(OH)$_2$D, normalized osteoblast numbers and trabecular bone volume in the 1α-hydroxylase knock-out mice, but not in the VDR knock-out and the double knock-out mice [34], suggesting that the 1,25(OH)$_2$D/VDR system is involved in bone formation. This anabolic effect of the 1,25(OH)$_2$D/VDR system on bone is supported by other mouse mutant models [46;47] in which 1,25(OH)$_2$D/VDR system acts on bone by modulating the activity of both osteoblasts and osteoclasts to increase bone matrix formation, which in turn can be mineralized [48]. Thus, in addition to calcium, the 1,25(OH)$_2$D/VDR system is also necessary for normal skeletal homeostasis. In our study, 25(OH)D levels and most likely 1,25(OH)$_2$D levels were undetectable for a long period without any effect on bone turnover and structure. One reason for the absence of any effect of vitamin
D deficiency on bone turnover and structure may be the older age of the mice as most studies which do show effects use young rodents. Aging has pronounced effects on the skeleton in mice including the loss of bone mass caused by a decrease in osteoblast number and bone formation rate [49]. In male C57BL/6 mice between 12 and 24 months of age, trabecular bone volume decreases by 32% at the proximal tibial metaphysis and cortical thickness decreases by 12% at the midtibial diaphysis [50]. The number of osteoclasts in trabecular bone is also diminished in aged mice [51]. These negative effects that occur in the skeleton with advancing age may be a result of decreased cell function due to mechanisms such as increased oxidative stress, deregulated autophagy, abnormal protein folding, loss of sex steroids and higher lipid oxidation [52]. Not only long-lived cells such as osteocytes and mesenchymal stem cells are affected, but also osteoblasts and osteoclasts [52]. The decline in cell function may lead to a reduced response of bone tissue to factors such as mechanical stimuli and 1,25(OH)$_2$D [53-56]. Regarding 1,25(OH)$_2$D, human mesenchymal stem cells show an age-related decline in the stimulation of osteoblastogenesis by 1,25(OH)$_2$D [53;54]. Thus, when aging leads to a reduced bone formation rate and a reduced response of bone cells to 1,25(OH)$_2$D, differences in bone turnover or structure between control and deficient mice may be less pronounced or even almost absent.

Previously, we and others showed that the synthesis of 1,25(OH)$_2$D from 25(OH)D in osteoblast cultures has an inhibitory effect on cell growth and a stimulatory effect on the differentiation of osteoblasts and the mineralization of the matrix [18-20]. Due to long-standing low 25(OH)D levels, the synthesis of 1,25(OH)$_2$D from 25(OH)D by bone cells may be impaired which may have negative implications for cell growth and differentiation in bone tissue. The involvement of an impaired local 25(OH)D metabolism has been suggested in animal models in which marked effects on bone structure were found when 25(OH)D levels were reduced [15;28;57]. Seven months old rats with insufficient 25(OH)D levels, but normal PTH, 1,25(OH)$_2$D and calcium levels, do show a loss of trabecular bone due to increased bone resorption [28]. In these rats, only serum 25(OH)D levels are associated with trabecular bone volume and osteoclast surface, but neither 1,25(OH)$_2$D nor PTH levels show a relationship with these parameters [28]. Another study in nine months old rats shows a positive association between serum 25(OH)D levels and cortical bone volume [15], which probably suggests a role for the local synthesis of 1,25(OH)$_2$D in bone. In our study, the local synthesis of 1,25(OH)$_2$D may be impaired for a long period but effects on bone structure or remodeling were not observed. One factor that may explain our findings is the marked decrease of CYP27B1 expression levels in bone during aging [27]. The consequence of lower CYP27B1 expression levels may be a reduced response of bone cells to 25(OH)D due to a decreased synthesis of 1,25(OH)$_2$D. When in aged mice the response to 25(OH)D is already reduced, differences in bone turnover or structure between both groups may not occur.
Previously, seven months old rats with 25(OH)D levels ranging from 20 to 115 nmol/l showed a trend toward increased CYP27B1 expression levels in bone with higher serum 25(OH)D levels [28]. In the present study, long-term vitamin D deficiency did not affect mRNA levels of CYP27B1 in bone cells, but the differences in age of the rats and duration of vitamin D deficiency may explain the different findings. In rats receiving adequate dietary amounts of vitamin D, there is no association between CYP27B1 mRNA levels in bone and serum 25(OH)D or 1,25(OH)_{2}D levels [27]. Moreover, CYP27B1 mRNA levels in cultured primary human bone cells do also not respond to supplementation of medium with 25(OH)D or 1,25(OH)_{2}D [20]. Regarding CYP24, we showed that mRNA levels were below the detection threshold in bones from older adult mice. It has been shown that rats at 26 weeks of age or older have much lower CYP24 mRNA levels than rats aged between 3 and 15 weeks [27]. Thus, CYP24 mRNA levels in bone decrease during aging [27], which may explain the undetectable CYP24 mRNA levels in the older adult mice in our study. In contrast to the CYP24 mRNA levels in bone that decrease during aging, levels of CYP24 mRNA in the kidney increase during aging [27], explaining the detectable expression levels of CYP24 in the kidney. Regarding the VDR in bone, long-term vitamin D deficiency resulted in lower mRNA levels than those in control mice. Because 1,25(OH)_{2}D is able to affect expression levels of the VDR in bone cells [20;58;59], the lower VDR mRNA levels in bones from vitamin D deficient mice may be due to the possible reduced 1,25(OH)_{2}D concentrations in plasma or locally.

Although mice are frequently used as a model, this study raises the question whether older adult C57BL/6 mice are appropriate for the investigation of skeletal consequences of vitamin D deficiency. In older men and women, the response to vitamin D deficiency is highly variable, but skeletal consequences of vitamin D deficiency have commonly been described [6;7;9;38]. However, effects of long-term vitamin D deficiency in older adult C57BL/6 mice on bone structure, remodeling and mineralization in our study were not observed. Due to several factors such as a low bone remodeling [33] or a reduced response of bone cells to 25(OH)D and 1,25(OH)_{2}D [27;53;54], older adult C57BL/6 mice may be less useful for the study of the effects of vitamin D deficiency on bone health in older people. Note, however, that the response of bone to long-term vitamin D deficiency in other mouse strains may be different.

A limitation of the current study is that plasma calcium concentrations could not be determined. However, plasma PTH concentrations were determined which did not differ between the vitamin D deficient and control mice. Because PTH is an important regulator of serum calcium concentrations, calcium concentrations in the vitamin D deficient mice did probably not differ from control mice. Another limitation is that the resolution used during micro-CT scanning may not be high enough to find small differences in trabecular bone microarchitecture between vitamin D deficient and control mice. However, absence of mineralization defects and normal bone structure was
confirmed by histomorphometric analysis. Another point that warrants discussion is the use of the humeral head for analysis of bone structure, remodeling and mineralization in our study, while other skeletal sites, such as the distal femoral metaphysis, proximal tibial metaphysis and L1–L4 vertebral bodies, are most frequently used in adult mice. It is possible that the impact of vitamin D deficiency is somewhat different for various anatomical sites of the skeleton, but sensitivity for vitamin D deficiency seems to be widespread in the skeleton.

In conclusion, long-term vitamin D deficiency in older adult C57BL/6 mice, accompanied by normal plasma PTH and phosphate concentrations, does not affect bone structure, remodeling and mineralization. In bone, expression levels of CYP27B1 were also not affected by long-term vitamin D deficiency in older adult C57BL/6 mice. Our results suggest that mice at old age have a low or absent response to vitamin D deficiency probably due to factors such as a decreased bone formation rate or a reduced response of bone cells to 25(OH)D and 1,25(OH)\(_2\)D. Older adult mice may therefore be less useful for the study of the effects of vitamin D deficiency on bone health in older people.

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