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Modelling hepatic osteodystrophy in *Abcb4* deficient mice

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Abstract

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Hepatic osteodystrophy (HOD) denotes the alterations in bone morphology and metabolism frequently observed in patients with chronic liver diseases, in particular in case of cholestatic conditions. The molecular mechanisms underlying HOD are only partially understood. In the present study, we characterized the bone phenotypes of the ATP-binding cassette transporter B4 knockout mouse (*Abcb4*^{-/-}), a well-established mouse model of chronic cholestatic liver disease, with the aim of identifying and characterizing a mouse model for HOD. Furthermore, we investigated the influence of vitamin D on bone quality in this model. The bone morphology analyses revealed reduced bone mineral contents as well as changes in trabecular bone architecture and decreased cortical bone densities in *Abcb4*^{-/-} mice with severe liver fibrosis. We observed dysregulation of genes involved in bone remodeling (osteoprotegerin, osteocalcin, osteopontin) and vitamin D metabolism (7-dehydrocholesterol reductase, Gc-globulin, *Cyp2r1*, *Cyp27a1*) as well as alterations in calcium and vitamin D homeostasis. In addition, serum RANKL and TGF- β levels were increased in *Abcb4*^{-/-} mice. Vitamin D dietary intervention was only partially able to restore the bone phenotypes of *Abcb4*^{-/-} animals. We conclude that the *Abcb4*^{-/-} mouse provides an experimental framework and a preclinical model to gain further insights into the molecular pathobiology of HOD and to study the systemic effects of therapeutic interventions.

Keywords

chronic cholangitis; liver fibrosis; osteoporosis; RANK ligand; vitamin D

Introduction

Patients with chronic liver disease frequently demonstrate alterations in bone mineral metabolism, leading to osteopenia and osteoporosis. The metabolic bone disease that causes defective bone remodelling in the setting of chronic liver diseases is termed hepatic osteodystrophy (HOD) [1, 2]. To date, the exact prevalence of HOD is unknown. However previous studies estimated that up to 75% of all patients with chronic liver disease suffer from severe osteoporosis [3, 4].

Depending on the aetiology of the liver disease, different patterns of progressive bone diseases can be detected. Specifically, chronic cholestasis appears to affect bone metabolism and structure. Indeed, the reported prevalence of osteopenia and osteoporosis in chronic viral hepatitis is approximately 20%, whereas up to 60% of patients with chronic cholestatic diseases display decreases in bone mass [5, 2]. Low bone mass and diseases associated structural deterioration in patients with chronic cholestasis results in increased frequency of fractures of spine, hip and femoral neck as well as other peripheral fractures [1]. However, it is well understood that in addition to low bone mass, other factors such as bone geometry and altered states of bone turnover, contribute to the risk of fracture [6]. In patients with chronic liver diseases fractures do severely affect the quality of life and result in increased morbidity, which in turn compromises long-term prognosis.

HOD, once it has developed, is difficult to treat and special care is required to support healing of existing fractures [7]. Better understanding of the pathogenesis of HOD is essential to develop adequate treatment strategies. To date several factors have been

identified to be associated with HOD, but the pathobiological mechanisms have yet to be fully defined. In particular, bilirubin, insulin-like growth factor 1 (IGF-1) deficiency and the receptor activator of nuclear factor κ B ligand (RANKL) - osteoprotegerin (OPG) system have been investigated [6]. Distorted calcium and vitamin D homeostasis seem to play a prominent role in cholestasis-induced bone disease. In short, vitamin D represents a key regulator of calcium homeostasis and is therefore essential for bone formation and metabolism. It can be absorbed from food or synthesized endogenously from cholesterol derivatives by ultraviolet irradiation in the skin. Here, 7-dehydrocholesterol is converted to cholecalciferol, which then undergoes 25-hydroxylation in hepatocytes, a process mediated primarily by cytochrome P450 enzyme CYP2R1 as well as CYP27A1, CYP2J2 and CYP3A4 [8]. The hydroxylation product, 25-hydroxyvitamin D (25(OH)-vitamin D, also termed calcidiol), enters the systemic circulation, where it is transported by vitamin D binding-protein, (also known as Gc, group-specific component or (Gc)-globulin) [9]. Calcidiol undergoes further hydroxylation by CYP27B1 mainly, but not exclusively, in the kidney. The resulting hormonal metabolite of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)₂-vitamin D) is, known to act on genes involved in bone metabolism, such as *BGLAP* (osteocalcin), *RANKL* (a.k.a. tumour necrosis factor ligand superfamily member 11, *TNFSF11*) and *SPPI* (osteopontin) [10].

The ATP-binding cassette transporter B4 knockout mouse (*Abcb4*^{-/-}) is a well-established mouse model of chronic liver diseases with a distinct and well-characterized hepatic phenotype. Due to the lack of the hepatobiliary phosphatidylcholine floppase ABCB4, the mice develop bile acid-induced liver damage, leading to sclerosing cholangitis and biliary fibrosis [11, 12, 13]. Since, to our knowledge there are no data outlining the bone phenotype of these mice, we sought to characterize *Abcb4*^{-/-} mice with regard to bone mass, structure and metabolism, with the goal of ascertaining the suitability of the *Abcb4*^{-/-} mouse as a model for HOD. Second, we investigated the influence of vitamin D treatment on bone quality in this new model.

Experimental Procedures

Generation of BALB-*Abcb4*^{-/-} mice

To generate the fibrosis-susceptible BALB-*Abcb4*^{-/-} mouse line, the FVB-*Abcb4*^{tm1Bor} strain was backcrossed into the BALB/cJ background for more than 10 generations. BALB/cJ inbred mice were obtained from Charles River (Sulzfeld, Germany). Mice were kept in 12-h light-dark cycles and were provided with water and standard diet (Altromin 1314, Lage, Germany) *ad libitum*. Temperature and humidity were regulated to 22 ± 1°C and 55 ± 5%, respectively.

To confirm the *Abcb4*^{-/-} genotype, we used the polymerase chain reaction (PCR) of tail DNA with *neo* (5'-CTT GGG TGG AGA GGC TAT TC-3', 5'-AGG TGA GAT GAC AGG AGA TC-3') and *Abcb4* (5'-CAC TTG GAC CTG AGG CTG TG-3', 5'-TCA GGA CTC CGC TAT AAC GG-3') specific primer pairs. The PCR reaction contained 10× PCR buffer (Applied Biosystems, Darmstadt, Germany), 2 mM MgCl₂, 10 μM dNTPs, 10 μM primer, 1.25 U *Taq* DNA polymerase (Invitrogen, Darmstadt, Germany), and 20 – 100 ng DNA in

25 µl-reactions. PCR cycling conditions were 30 s @ 94°C, 60 s @ 55°C and 30 s @ 72°C for 35 cycles, and a final extension step of 10 min @ 72°C.

The experimental protocols were performed with permission of the federal states of Baden-Württemberg, Bavaria and Saarland according to §8 of the German Law for the Protection of Animals and the Directive 2010/63/EU of the European Parliament.

Phenotypic characterization of hepatic fibrosis

Histopathology and hydroxyproline assay—Liver samples for histopathological evaluation were fixed in 4% neutral buffered formalin at 4°C for 24 h and embedded in paraffin. Sections (2 – 5 µm) were stained with haematoxylin-eosin (H&E), Masson Goldner trichrome, and Sirius red.

Liver injury was scored at 5, 15, 20, 30, and 44 weeks of age in groups of 4 animals per genotype and point in time. In detail, slices of the left lateral, the right, the median and the caudate liver lobe were scored (0 – 20) separately based on the presence of periductal connective tissue, oedema, inflammatory infiltrations, periportal fibrosis, spongy or bridging necrosis, connective tissue septa, proliferation, atrophy and diminution of bile canaliculi, and biliary cirrhosis.

Liver fibrosis was quantified in 15-week-old mice using histomorphometric semi-automatic system of image analysis (Leica microscope, equipped with Leica application suite software; Wetzlar, Germany). The percentage of collagenous area was calculated from 10 microscopic fields (magnification 100×) randomly chosen in each liver section. Hepatic fibrosis was staged according to Batts and Ludwig [14] and the Ishak [15] scoring system. The F-scores were defined as follows: 0, no fibrosis; 1, scatter periportal and perineoductular fibrosis; 2, periportal, perineoductular fibrosis (complete lamellae with beginning septa); 3, periportal, perineoductular fibrosis with portal-portal septa; 4, complete cirrhosis.

In addition, hepatic collagen contents were quantified calorimetrically via the collagen specific amino acid hydroxyproline (Hyp), as described by Jamall et al. [16]; [17].

Clinical chemical and enzyme-linked immunosorbent assays—Blood samples for chemical analyses were obtained from isoflurane-anesthetized mice by puncturing the retro-orbital sinus with capillaries and subsequently collected in heparinized tubes. Plasma alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (AP) activities as well as calcium and inorganic phosphate concentrations were measured with the Olympus AU 400 autoanalyzer (Olympus, Hamburg, Germany) using adapted reagent kit (Olympus, Hamburg, Germany) or Hitado (Möhnesee, Germany) kits, alternatively.

Serum 25(OH)-vitamin D levels were determined using the chemiluminescence immunoassay LIAISON® 25 OH VitaminD TOTAL assay (DiaSorin, Dietzenbach, Germany). Transforming growth factor-β (TGF-β) levels were measured by TGF-β-receptor cells (MFB-F11), provided by Dr. Ina Tesseur [18] with slight modifications as described [19]. Each sample was measured in triplicate, using active recombinant human TGF-β₁ as control. Receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG)

concentrations in serum were measured in duplicates by enzyme-linked immunosorbent assay (ELISA) (Quantikine mouse RANK ligand and OPG immunoassays, R&D Systems, Minneapolis, USA).

Reverse transcription and quantitative real-time PCR—Total mRNA from grinded snap frozen liver tissue specimens was isolated using peqGOLD TriFast™ (Peqlab, Erlangen, Germany) or RNeasy Mini kits (Qiagen, Hilden, Germany). cDNA was synthesized from 1 – 2 µg RNA using cDNA reverse transcription (RT) kits from Applied Biosystems (Carlsbad, USA) or Fermentas (St. Leon-Rot, Germany). Table 1 summarizes primer sequences (5′ – 3′) and RT-PCR conditions. Products, resolved by gel electrophoresis in a 2 % (w/v) agarose gel, were visualized with ethidium bromide. Densitometric analysis of signals was performed using Image J software (NIH, Bethesda, USA).

mRNA transcript levels were determined by reverse transcriptase quantitative real-time PCR (qPCR; *TaqMan*, Applied Biosystems, Carlsbad, USA), with one cycle for 10 min @ 95°C, followed by 45 cycles with 30 s @ 95°C and 60 s @ 60°C. The relative expression level of each gene was calculated by the $\Delta\Delta C_t$ -method [20], utilizing 18S RNA as endogenous control, and in relation to wild-type controls similar in age and gender. The relative quotient (RQ, $2^{-\Delta\Delta C_t}$) was normalized to the values of BALB/cJ controls.

Phenotypic characterization of osteodystrophy

Dual-energy x-ray absorptiometry (DXA)—Phenotypic DXA analyses have been performed at the German Mouse Clinic (GMC, [21], [22]) in *Abcb4*^{-/-} mice and wild-type control animals at the age of 15 to 17 weeks (10 animals per genotype and sex). After isoflurane anaesthesia, weight and length of each mouse were recorded. Bone mineral content (BMC) and areal bone mineral density (aBMD) of the whole body, excluding the skull was ascertained using a Sabre X-ray bone densitometer (Norland Medical Systems, Hampshire, UK), using a scan speed 20 mm/s and a resolution of 0.5 mm × 1.0 mm and a histogram averaging width (HAW) setting of 0.02. The calibration of the system was performed using the QC and QA phantoms supplied by the manufacturer.

Micro-computed tomography (µCT) and histology—Bones of knockout and wild-type mice at the ages of 5, 15, 20, 30 and 44 weeks were collected (4 per genotype and point in time). The bone structure and the mineralization of mouse femora was determined by µCT scans on a Viva µCT40 (Scanco Medical, Wangen-Brüttisellen, Switzerland). Femora were placed into phosphate buffered saline-filled reaction tubes and fixed by Pasteur pipettes. To assess the trabecular bones, the distal and mid metaphyses were scanned with standard parameters (70 kV, 114 µA, 10.5 µm voxel size). The volume of interest (VOI) included 135 slices at the distal area and 50 scans in the middle of the femur. Two different global thresholds were used to separate mineralized tissue from bone marrow and soft tissues: These were set to 429 and 682 hydroxyapatite (HA) mg/cm³ for trabecular bone.

From these measurements the following parameters were determined: bone volume (BV), total volume (TV), bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp), trabecular thickness (Tb.Th), connective density (Conn.D), and the

structure-model index (SMI), which defines the trabecular structure (disc-shape: 0 to rod-shape: 3) [23].

Femura of these animals were used to visualize mineralized tissue. Therefore, femoral bones of knockout and wild-type mice (4 animals per point in time) were embedded after formalin fixation and dehydration into polymethylmethacrylate (Technovit 9100; Heraeus Kulzer, Wehrheim, Germany). Longitudinal sections (6 μ m) were cut and stained by use of von Kossa staining (3% silver nitrate and 5% sodium thiosulfate).

Peripheral quantitative computed tomography (pQCT)—The hind axial skeleton with the attached musculature was fixed in 10% neutral buffered formalin overnight and placed subsequently in 95% ethanol until analysis (6 – 14 animals per genotype and sex, at 15 – 17 weeks of age). The total femur bone density was quantified using the SA Plus densitometer (Orthometrics, Stratec SA Plus Research Unit, White Plains, USA). The SA Plus instrument was calibrated using HA standards (50 – 1000 mg/mm³) with cylindrical diameters of 2.4 mm and lengths of 24 mm to approximate mouse femurs. Quality control was performed with a phantom supplied by the manufacturer. The precision of the SA Plus for repeated measurements of a single femur was found to be 1.2 – 1.4%. As thresholds to separate bone from soft tissue, we used 710 and 570 HA mg/cm³ for cortical bone areas and surfaces, respectively; to determine mineral contents, a second analysis was carried out with thresholds of 220 and 400 HA mg/cm³ to include mineral from most partial voxels (0.07 mm) in the analysis. Isolated femurs were scanned at seven locations at 2 mm intervals, beginning 0.8 mm from the distal ends of the epiphyseal condyles. Density values were calculated from the summed areas and associated mineral contents.

Vitamin D intervention study—In parallel experiments, we performed a vitamin D dietary intervention study in *Abcb4*^{-/-} mice on the FVB/N background. At 4 weeks of age, FVB-*Abcb4*^{-/-} and wild-type mice were assigned to three intervention groups. These groups encompassed a total of 6 to 14 mice per genotype and sex. Mice were fed one of: a control vitamin D diet (600 IE vitamin D₃/kg food), a vitamin D-sufficient diet (2,400 IE vitamin D₃/kg food), or a vitamin D-insufficient diet (100 IE vitamin D₃/kg food). Apart from vitamin D concentrations, the diets (Altromin, Lage, Germany) were otherwise equal in nutrient and energy contents. The dietary interventions were continued until the age of 15 to 17 weeks.

Statistics

Phenotypic data of mice are given as means $\pm$ standard errors of the mean (SEM) and assessed by one-way analysis of variance (ANOVA) with post-hoc multiple comparison tests (Bonferroni) or Student's t-tests. For bone data acquired by pQCT, analysis of covariance (ANCOVA) was used.

To determine whether *Abcb4*^{-/-} mice display a bone phenotype, a subtractive model fitting was performed. All covariates (body weight, femoral length, genotype) were initially considered and then removed in a step-wise fashion until only significant factors remained.

For all tests, p-values < 0.05 were regarded as significant, unless otherwise stated. Statistical analyses were performed using SPSS 20 (IBM, Ehningen, Germany) and GraphPad Prism (GraphPad Software, El Camino Real, USA).

Results

***Abcb4*^{-/-} mice with severe liver injury show alteration of the bone structure and density**

The phenotypic characterization of hepatic fibrosis in *Abcb4*^{-/-} mice showed that liver damage increased most rapidly in the first 15 weeks of life (average liver damage score 15.5 ± 0.8 out of 20). Later on the rate of liver injury progressed slowly (average liver damage score 18.3 ± 0.7) until the age of 44 weeks (Fig. 1). Liver pathology was reflected by significantly elevated serum activities of liver enzymes, including alanine aminotransferase and alkaline phosphatase (Fig. 2).

Whole-body densitometry (DXA) was employed to determine whether bone content and density were affected in *Abcb4*^{-/-} mice at 15 weeks of age. This analysis revealed a significant reduction of whole body bone mineral contents (BMC) in *Abcb4*^{-/-} males (Fig. 3A) and of the bone fraction of the total tissue weight in knockout females (Fig. 3B) in comparison to dedicated wild-type controls. However, whole body bone mineral density (aBMD) did not differ (Fig. 3C).

Serial micro-computed tomography (μCT) scanning of *Abcb4*^{-/-} mice revealed alternations in the trabecular bone compartment during progression of liver disease. The alterations in bone structure were reflected by significantly decreased trabecular number (Fig. 4A) and increased trabecular separation (Fig. 4B) in knockout mice at the age of 20 weeks. A similar trend was observed at later time points. In addition, connective density and trabecular tissue mineral density were significantly lower in *Abcb4*^{-/-} mice as compared to controls at 20 and 30 weeks, respectively (data not shown). The μCT analysis revealed significant differences in trabecular bone volume fraction in young knockout mice (i.e. 5 weeks of age), but not in older animals. However, trabecular thickness did not differ between *Abcb4*^{-/-} and wild-type mice (data not shown). Supplementary table 1 summarizes all outcomes for trabecular bones as measured by μCT.

Femur sections after von Kossa staining (Fig. 4C) complement the μCT results. In both groups, the trabeculae of young knockout (Fig. 4a) and wild-type (Fig. 4d) mice were found close to the cartilage growth plate arranged mainly in parallel order. At increasing age a change in trabecular order and connectivity was observed with reduced mineralization of the trabecular bone meshwork in *Abcb4*^{-/-} mice (Fig. 4C, a – c) as compared to wild-type animals (Fig. 4C, d – f).

***Abcb4*^{-/-} mice display decreased femoral mineral contents and volumes as well as lower cortical bone densities**

Detailed analyses of femurs by pQCT demonstrated a significant reduction in femoral mineral content (Fig. 5A) and commensurately in total femoral volume (Fig. 5B) in both female and male *Abcb4*^{-/-} mice in comparison to wild-type controls at 15 weeks of age. As

a result, total femoral vBMD, which is the amount of mineral per unit volume of the entire femur, was not affected by genotype (Fig. 5C).

Interestingly, cortical density, which reflects the material density of the bone, was significantly decreased in female knockout mice as compared to wild-type mice (1.166 ± 0.004 vs. 1.177 ± 0.004 mg/mm³; $p < 0.05$) (Fig. 6A). Additionally, as illustrated in Fig. 6B, the circumference of the bone at the exact mid-diaphysis (periosteal circumference) of female *Abcb4*^{-/-} animals was significantly lower in comparison to controls. Accordingly, we detected a trend for a decreased in the mid-diaphysal endosteal circumference in these mice (3.00 ± 0.04 vs. 3.11 ± 0.04 mm; $p = 0.06$) resulting in no significant differences in the thickness of the cortical bone between knockout and wild-type controls (data not shown).

***Abcb4*^{-/-} mice present with distorted calcium homeostasis and vitamin D metabolism**

In comparison to controls, *Abcb4*^{-/-} mice show lower plasma calcium concentrations (male: 2.24 ± 0.01 vs. 2.15 ± 0.03 mmol/l, $p < 0.05$; female: 2.22 ± 0.02 vs. 2.18 ± 0.02 mmol/l, $p > 0.05$), whereas phosphate levels were markedly increased in both sex ($p < 0.01$; Fig. 7A). Consistent with the alteration in calcium-phosphate homeostasis, 25(OH)-vitamin D serum concentrations (Fig. 7B) are almost 50% lower in *Abcb4*^{-/-} than in wild-type controls.

Subsequently, we examined gene expression of key enzymes in vitamin D metabolism. *Abcb4*^{-/-} animals show significantly ($p < 0.001$) higher hepatic expression of *Dhcr7*, which converts the vitamin D precursor 7-dehydrocholesterol to cholesterol, at the age of 5 weeks and a trend to higher steady-state mRNA levels at every consecutive time-point in comparison to controls (Fig. 8A). As illustrated in panels B and C of Fig. 8, *Abcb4*^{-/-} animals are also characterized by markedly reduced hepatic expression of *Cyp2r1* and *Cyp27a1*. In line with these findings, the expression of the vitamin D binding protein *Gc* is constantly low in *Abcb4*^{-/-} as compared to wild-type controls, with significant differences in expression levels at early age (from 5 to 15 weeks of age; Fig. 8D). This expression pattern acts in concert to decrease vitamin D levels in *Abcb4*^{-/-} mice.

***Abcb4*^{-/-} mice have altered gene expression of bone modeling markers**

Next we examined expression levels of genes associated with bone turnover. Specifically, levels of *tumor necrosis factor receptor superfamily, member 11b* (*Tnfrsf11b*, also known as OPG, an inhibitor of osteoclastogenesis), *bone γ -carboxyglutamate protein* (*Bglap*, alias ‘osteocalcin’, a marker of osteoblastogenesis) and *secreted phosphoprotein* (*Spp1*, also named ‘osteopontin’, a component of the bone matrix) in livers and bones of *Abcb4*^{-/-} and wild-type animals. Interestingly, the hepatic expression of *Tnfrsf11b*, the natural antagonist of RANKL, increased markedly in *Abcb4*^{-/-} animals until 20 weeks of age and was significantly ($p < 0.05$) lower than in wild-type controls afterwards (Fig. 9A). In contrast, the expression levels of osteocalcin in liver increased in a time-shifted fashion, starting with significantly ($p < 0.001$) higher and later on lower expression in knockout mice as compared to controls at the age of 20 and 44 weeks, respectively (Fig. 9B). Gene expression levels of *Spp1* in liver did not significantly vary in *Abcb4*^{-/-} mice and controls (data not shown).

In contrast to these data, bone expression levels of *Spp1* decreased significantly ($p < 0.05$) with age, starting at 30 weeks, in *Abcb4*^{-/-} mice, whereas expression levels of wild-type controls remained constant (Fig. 9C). Bone expression of osteocalcin was markedly lower in *Abcb4*^{-/-} mice as compared to controls at every age, with the largest difference in relative expression detected at the age of 5 weeks ($p < 0.001$; Fig. 9D).

***Abcb4*^{-/-} mice have increased systemic TGF- β and RANKL levels**

As shown in Fig. 10A, *Abcb4*^{-/-} animals displayed elevated serum levels of activated TGF- β as compared to wild-type mice. Whereas TGF- β concentrations increased in *Abcb4*^{-/-} animals until the age of 30 weeks (from 3.4 ± 0.3 at week 5 to 11.6 ± 2.0 ng/ml at week 30) and subsequently declined to basal levels (4.3 ± 0.1 ng/ml at week 45), the levels continuously decreased throughout the life span of controls (from 2.4 ± 0.2 ng/ml to 0.1 ± 0.1 ng/ml). Of note, *Abcb4*^{-/-} mice exhibited strikingly higher concentrations of the osteoclastogenesis inducing factor RANKL in serum as compared to controls (258 ± 39 vs. 120 ± 10 pg/ml at week 15, $p < 0.01$; Fig. 10B), whereas serum levels of OPG did not differ (2894 ± 308 vs. 3140 ± 131 pg/ml; $p > 0.05$).

Vitamin D supplementation does not restore bone phenotypes in *Abcb4*^{-/-} mice

The influence of the vitamin D dietary interventions on bone phenotypes was determined in *Abcb4*^{-/-} female mice. The femoral bone mineral densities were significantly ($p < 0.05$) affected by vitamin D treatment. As illustrated in Fig. 11A, *Abcb4*^{-/-} mice that were fed the vitamin D insufficient diet showed a significantly ($p < 0.001$) decreased mineral density as compared to knockout females receiving the control diet (0.66 ± 0.01 vs. 0.70 ± 0.11 mg/mm³). Fig. 11B illustrates that cortical bone mineral densities measured by pQCT were also significantly ($p < 0.001$) influenced by vitamin D intervention. Interestingly, both *Abcb4*^{-/-} mice that received vitamin D insufficient (100 IE/kg) and mice that received vitamin D supplemented diets (2400 IE/kg) displayed reduced cortical bone mineral density in comparison to corresponding controls (1.11 ± 0.01 and 1.12 ± 0.01 vs. 1.14 ± 0.01 ; $p < 0.001$), and a similar pattern was observed for femoral bone mineral densities (Fig. 11). However, cortical thickness and periosteal circumference were not affected by the vitamin D diets in *Abcb4*^{-/-} mice (data not shown). Moreover, no changes of bone phenotypes were observed in wild-type mice fed any of the three vitamin D diets (data not shown).

Discussion

Metabolic bone diseases are common complications of chronic cholestatic liver diseases. Nevertheless, the underlying mechanisms are still to be fully characterized. This is, at least in part, due to the lack of a representative preclinical model that allows the in-depth analysis of the association between liver disease and abnormal bone metabolism. Hence, the present study was designed to establish the *Abcb4*^{-/-} mouse as a novel model for hepatic osteodystrophy and to characterize the systemic consequences of chronic cholangiopathy on bone morphology and metabolism. To date, the phenotypic analyses of *Abcb4*^{-/-} mice have focused exclusively on the hepatic alterations caused by deficiency of the hepatobiliary phospholipid transporter. The present study is the first demonstrating that *Abcb4*^{-/-} animals develop osteodystrophy accompanied by alteration of bone structure and density.

Abcb4^{-/-} mice are characterized by chronic hepatobiliary injury as a result of low phospholipid concentrations in bile [11, 12]. Consequently, *Abcb4*^{-/-} mice develop age-dependent chronic cholangitis and biliary fibrosis in response to persistent inflammation. Consistent with previously published reports [24, 25, 26], we detected marked liver damage that rapidly increased up to the age of 15 weeks with slower progression afterwards. In parallel, we observed initial changes of the bone in *Abcb4*^{-/-} mice up to the age of 15 to 20 weeks. Furthermore, reduced number and increased separation of trabeculae, in line with diminished connective density, were observed at 20 weeks of age. In humans, trabecular bone architecture is key maintaining for bone strength, especially in locations, which experience high strain, such as in the proximal femur [27, 28]. Our results suggest that chronic cholestasis induced loss of trabecular bone may lead to decreased bone strength and stability.

The whole body (DXA) femoral bone analyses (pQCT) demonstrated significantly lower mineral contents in the knockout mice at 15 to 17 weeks of age. It is well established that even minor changes in mineralization can lead to several-fold changes of static strength and elasticity [29]. Detailed analyses revealed also differences between cortical bone in *Abcb4*^{-/-} female and matched control mice. Cortical bone is usually very compact and constitutes about 80% of bone material. It has been previously demonstrated that cortical thinning and increased cortical porosity are important factors of bone strength [30]. Other studies suggested that weakened cortical bone is primarily responsible for intracapsular hip fracture [31]. Overall, the phenotypic characterization identified reduced bone mineralization and trabecular bone alterations in our mouse model, which could compromise bone strength.

In line with the histomorphological observations, we detected aberrations in calcium-phosphate homeostasis and low vitamin D levels, which are also frequently observed in patients with chronic cholestatic liver diseases [2, 32]. Vitamin D deficiency together with altered gene expression of key enzymes in vitamin D metabolism, especially the low expression of *Cyp2r1* and *Cyp27a1*, implies impairment of vitamin D synthesis or at least a systemic effect compromising vitamin D metabolism under cholestatic conditions. However, the role of calcium and vitamin D in cholestasis-associated bone diseases is not clear.

Although the supplementation of vitamin D is generally recommended, there is no unequivocal data confirming the efficacy of vitamin D for preventing bone loss in patients with chronic liver diseases [33, 34]. These clinical observations indicate that other mediators of bone remodeling might be critical in this specific model.

In general, a bone disorder is an acquired dissociation between bone formation and resorption caused by an imbalance of osteoblast and osteoclast activity. RANKL and OPG represent two key regulatory molecules that are produced by osteoblasts. While RANKL enhances osteoclast activity and therefore bone resorption, OPG as its soluble decoy receptor inhibits osteoclast differentiation. The assessment of these mediators in chronic liver diseases produced previously contradictory results [35]. In our model we observed increased RANKL serum levels in *Abcb4*^{-/-} mice with advanced liver injury, whereas OPG serum levels did not differ from controls. Furthermore, *Abcb4*^{-/-} animals displayed elevated serum levels of TGF- β , which promotes RANKL-induced osteoclastogenesis [36, 37]. In

line with the *Abcb4*^{-/-} model, our recently published clinical study has demonstrated that chronically increased serum levels of TGF- β might be a potential inducer of bone density loss in humans [38].

Osteocalcin is produced by osteoblasts and is a surrogate marker of bone osteoblastic activity and bone remodeling. Low mRNA expression of osteocalcin is consistent with reduced osteoblastogenesis, albeit it might also represent a compensatory response. As highlighted recently [39, 40], osteocalcin - apart from its role in bone remodeling - acts as a hormone that influences energy expenditure. It improves glucose tolerance by increasing β -cell proliferation as well as insulin secretion and sensitivity in mice [41]. Of note, we observed that *Abcb4*^{-/-} animals display improved glucose tolerance [42]. In fact, low mRNA levels of osteocalcin might result from a compensatory effect but could also be due to the low levels of vitamin D in *Abcb4*^{-/-} animals, which regulates osteocalcin expression at the post-translational level [43].

Together, our data suggest an increase in osteoclastogenesis in bone in the absence of *Abcb4*, co-incident with an impact on osteoblastogenesis. This would result in a net decrease in bone remodeling, which might explain both the decrease in bone volume in these young and growing mice, as well as the reduction of bone in the trabecular bone compartment. Osteopontin modifies the migration and attachment of osteoclasts and their resorptive activity [44] and Osteopontin-deficient mice show delayed bone resorption in metaphyseal trabeculae and increased bone rigidity [45]. Osteopontin is expressed by both osteoblasts and osteoclasts, and the lower *Spp1* expression in bones from our mice further supports the hypothesis of an imbalance during bone remodeling in the absence of ABCB4.

Since *Rankl*, *Bglap* and *Spp1* are target genes of 1,25(OH)₂-vitamin D and their expression is modulated by 25(OH)₂-vitamin D [10], a systemic effect of vitamin D on bone in the setting of cholestasis is likely. Further evidence for differential effects of vitamin D on the bone phenotypes in chronic cholestasis is further supported by our finding experiments in female *Abcb4*^{-/-} mice, which develop more severe fibrosis as compared to males [46]. Interestingly, we found that cortical BMD was reduced in both mice receiving vitamin D-insufficient and vitamin D-sufficient diet. This observation could be explained, at least in part, by interactions between vitamin D and bile acid metabolism. Circulating bile acids levels are about ten-fold higher in *Abcb4*^{-/-} as compared to wild-type mice (not shown) and in fact, the secondary bile acid lithocholic acid (LCA) is known to have deleterious effects on osteoblasts viability and affect the expression of *Bglap* and *Rankl* [47, 48]. Moreover, the treatment with LCA in combination with vitamin D decreases the expression of *Cyp24a1*, encoding the hydroxylase involved in catabolism of vitamin D. Additionally to bile acids, elevated serum bilirubin in the ABCB4 deficient mouse model [24] could also affect bone formation since unconjugated bilirubin is known to impair osteoblast proliferation in a dose-dependent fashion [34].

Although HOD is likely to be caused by multiple environmental and genetic factors, it was beyond the attempt of this study to analyze all of these factors. Our experiments demonstrate that mice lacking the hepatic phospholipid transporter ABCB4, primarily known as model for chronic cholestatic liver disease, develop an osteopenic phenotype. We postulate that the

Abcb4^{-/-} mouse might be considered as a genetically defined preclinical HOD model to gain further insights into the molecular pathobiology of this disorder and to analyze the systemic effects of therapeutic interventions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *Abcb4*^{-/-} mice develop progressive liver injury and changes of bone morphology in parallel
- Bones from *Abcb4*^{-/-} mice show decreased mineral contents, trabecular numbers and cortical densities
- Bone and liver expression of genes involved in bone turnover is altered in *Abcb4*^{-/-} mice
- *Abcb4* deficient mice display elevated RANKL and increased TGF- β serum levels
- Vitamin D does not fully restore bone phenotypes in this novel preclinical mouse model

Figure 1. Liver damage score

Liver damage scores in BALB-*Abcb4*^{-/-} and BALB/cJ wild-type mice. Hepatic injury was scored in liver slices stained with Masson Goldner Trichrome based on the presence of periductal connective tissue, oedema, inflammatory infiltrations, periportal fibrosis, spongy or bridging necrosis, connective tissue septa, proliferation, atrophy and diminution of bile canaliculi, and biliary cirrhosis as described in *Methods*. N = 4 per genotype and time point.

Figure 2. Liver enzyme activities

Liver enzymes activities in plasma samples from female and male BALB/cJ wild-type and BALB-*Abcb4*^{-/-} mice at 14 weeks of age. N = 10 per genotype and sex; *** p < 0.001.

Abbreviations: ALT, alanine aminotransferase, AP, alkaline phosphatase; AST, aspartate aminotransferase.

Figure 3. DXA results

Total bone mineral content (A), bone fraction of the total tissue weight (B) and areal bone mineral density (C) in BALB/cJ wild-type and BALB-*Abcb4*^{-/-} mice at the age of 15–17 weeks. N = 10 per genotype and gender; * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 4. Trabecular bone morphometry and meshwork

Trabecular number (**A**) and trabecular separation (**B**) of BALB/cJ wild-type and BALB-*Abcb4*^{-/-} mice at the age of 5 – 44 weeks, determined by μ CT. N = 4 per genotype and time point; * $p < 0.05$, ** $p < 0.01$.

Histological femoral slices (von Kossa) (**C**) in BALB-*Abcb4*^{-/-} (a – c) and BALB/cJ (d – f) mice at the age of 5, 20 and 44 weeks. Mineralized cartilage and bone stain presented in black, non-mineralized cartilage and connective tissue brown.

Figure 5. Bone density parameters of the femur

Femoral mineral content (A), femoral volume (B) and femoral bone mineral density (C) of BALB/cJ wild-type and BALB-Abcb4^{-/-} mice at the age of 15 – 17 weeks, determined by pQCT. N = 6 – 14 per sex and genotype; * p < 0.05, ** p < 0.01.

Figure 6. Bone material density

Cortical bone mineral density (**A**) and periosteal and endosteal circumferences of the femur at the mid-diaphysis (**B**) of BALB/cJ wild-type and BALB-*Abcb4*^{-/-} mice at the age of 15 – 17 weeks, determined by pQCT. N = 6 – 14 per sex and genotype; * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 7. Calcium, phosphate and vitamin D blood concentrations

Calcium and phosphate plasma concentrations (A) and 25(OH)-vitamin D serum level (B) in BALB/cJ wild-type and BALB-Abcb4^{-/-} mice at the age of 15 – 17 weeks. N = 9 – 14 per sex and time point; * p < 0.05, *** p < 0.001.

Figure 8. Hepatic expression of key genes involved in vitamin D metabolism
mRNA steady-state levels of genes encoding 7-dehydrocholesterol reductase (*Dhrc7*) (A), vitamin D 25-hydroxylase (*Cyp2r1*) (B), sterol 27-hydroxylase (*Cyp27a1*) (C), and vitamin D binding protein (*Gc*) (D) in BALB/cJ wild-type and BALB-Abcb4^{-/-} mice at the age of 5 – 44 weeks. All expression levels are presented relative to β -actin expression (RT-PCR). N = 4 per genotype and time point; * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 9. Expression of genes involved in bone metabolism

Hepatic steady-state mRNA levels of *Tnfrsf11b* coding osteoprotegerin (OPG) (A) and bone γ -carboxyglutamate protein (*Bglap*, osteocalcin) (B) and bone steady-state mRNA levels of secreted phosphoprotein 1 (*Spp1*, osteopontin) (C) and *Bglap* (D) in BALB/cJ wild-type and BALB-Abcb4^{-/-} mice at the age of 5 – 44 weeks. All expression levels are presented relative to β -actin expression (RT-PCR). N = 4 per genotype and time point; * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 10. TGF-β and RANKL serum concentrations

Active TGF-β serum levels measured by cell-based bioassay in BALB-*Abcb4*^{-/-} and BALB/cJ wild-type mice. N = 4 per genotype and time point (A). RANKL serum concentration measured by ELISA in BALB/cJ wild-type and BALB-*Abcb4* mice at the age of 15 – 17 weeks. N = 8 – 9 per genotype; ** p < 0.01 (B).

Figure 11. Bone phenotype in *Abcb4*^{-/-} female animals after vitamin D dietary intervention
 Femoral bone mineral density (A) and cortical bone mineral density (B) measured by pQCT in FVB-*Abcb4*^{-/-} female mice at 16 – 18 weeks of age after vitamin D dietary intervention (vitamin D deficient versus control versus vitamin D supplemented diet). The pattern of the bars correspond to the three dietary regimens (100 IE vitamin D/kg food, vertical lines; 600 IE vitamin D/kg food, black bars; 2,400 IE vitamin D/kg food, vertical lines). The dashed lines above the bars illustrate the vitamins D diet intervention effect over all groups ([#] *p* < 0.05, ^{###} *p* < 0.001); the solid lines indicate significant differences between dietary groups (* *p* < 0.05, *** *p* < 0.001). *N* = 8 – 11 per diet group.

Table 1

Primer sequences and PCR conditions for RT-PCR

Gene	GeneBank accession [NM_]	Forward primer	Reverse primer	T _m [°C]	Product length [bp]
<i>Actb</i>	001101.3	CGACAAACGGCTCCGGCATGT	GCACAGTGTGGGTGACCCCG	64	461
<i>Bglap</i>	001037939.1	ACCTTGGCTGCGCTCTGTCT	CCAGGGTCTTGGACATGGGGA	58	241
<i>Cyp24a1</i>	000782.4	TGGCCACTGCTGGGCAGC	TTTGAAAAATGGTGTCCCAAGGCCA	58	651
<i>Cyp27a1</i>	000784.3	GTGGACACGACATCCAAACAC	ATGATCCGGGAGTTTGTGG	60	212
<i>Cyp27b1</i>	000785.3	CGCCTCTGCCGAGACTGGGA	CTCCCCAGCCAGCGAGCTG	58	555
<i>Cyp2r1</i>	177382.3	GGGAGGCTTACTCAATTCCA	GCAATGATGAGTTTCAACCCACT	60	567
<i>Dhcr7</i>	007856.2	ATGGGGCTGCCTCATCTGG	GATTCCAGGCAGCAGGCGGT	60	329
<i>GC</i>	000583.2	AGAGGAGGTGCTGCAAGACT	GCAGCATATTGTGAGCAGACTC	60	707
<i>Spp1</i>	009263.2	GCAGTCTTCTGCGGCAGGCA	CGGCCGTTGGGGACATCGAC	58	487
<i>Tnfrsf11b</i>	008764.3	TGTGCTGCGCACTCCTGGTG	GGTGGCGTTGCACTCCTGCT	60	287
<i>Vdr</i>	000376.2	GCCTGCCGGCTCAACGCTG	CAGCCAGGTGGGGCAGCATG	58	463

Abbreviations: *Actb*, β actin; *Bglap*, bone γ -carboxyglutamate protein (osteocalcin); *Cyp*, cytochrome P450; *Dhcr7*, 7-dehydrocholesterol reductase; *GC*, group specific component; *Spp1*, secreted phosphoprotein 1 (osteopontin); *Tnfrsf11b*, tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin); *Vdr*, vitamin D receptor.