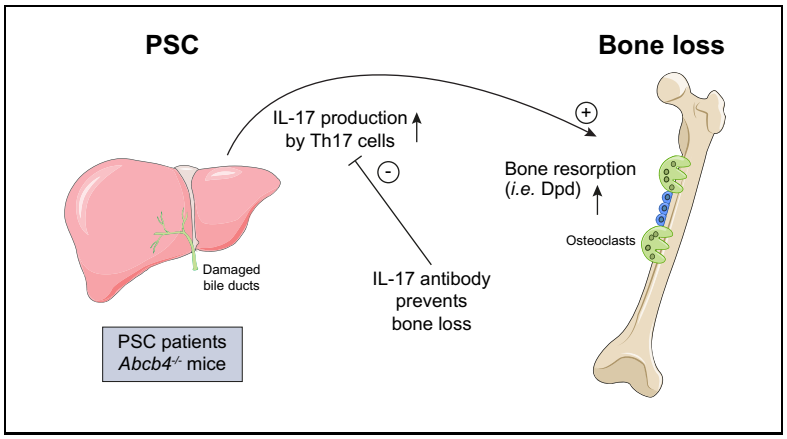
Research Article
Cholestasis and Autoimmune Diseases

JOURNAL
OF HEPATOLOGY

Th17 cell frequency is associated with low bone mass in primary sclerosing cholangitis

Graphical abstract



Highlights

- Decreased bone mass in patients with PSC is associated with increased bone resorption.
- Th17 cell frequency correlates with bone resorption indices in patients with PSC.
- Increased osteoclastogenesis is corrected by IL-17 inactivation in *Abcb4*$^{-/-}$ mice.

Authors

Tobias Schmidt, Dorothee Schwinge, Tim Rolvien, ..., Christoph Schramm, Thorsten Schinke, Michael Amling

Correspondence

amling@uke.de
(M. Amling)

Lay summary

Primary sclerosing cholangitis (PSC) is a cholestatic liver disease characterized by progressive bile duct destruction. One serious complication of PSC is reduced bone mass resulting in increased fracture risk. Herein, we demonstrate that Th17 cells mediate bone loss in PSC by inducing bone resorption, which suggests that antibody-based IL-17 blockade might be beneficial for the treatment of bone loss in affected patients.

http://dx.doi.org/10.1016/j.jhep.2018.12.035
 J. Hepatol. 2019, 70, 941–953

Research Article
Cholestasis and Autoimmune Diseases

JOURNAL OF HEPATOLOGY

Th17 cell frequency is associated with low bone mass in primary sclerosing cholangitis

Tobias Schmidt[1,2], Dorothee Schwinge[3], Tim Rolvien[1,2], Anke Jeschke[1], Constantin Schmidt[1], Mona Neven[1], Sebastian Butscheidt[1], Marvin Kriz[3], Lilly Kunzmann[3], Haider Mussawy[2], Jan Hubert[2], Thelonius Hawellek[2], Wolfgang Rüther[2], Ralf Oheim[1,4], Florian Barvencik[1,4], Ansgar W. Lohse[3,4], Christoph Schramm[3,4], Thorsten Schinke[1], Michael Amling[1,4,*]

[1]*Department of Osteology and Biomechanics, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany;* [2]*Department of Orthopedics, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany;* [3]*First Department of Medicine, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany;* [4]*Martin Zeitz Center for Rare Diseases, University Medical Center Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg, Germany*

Background & Aims: Osteoporotic fractures are a major cause of morbidity and reduced quality of life in patients with primary sclerosing cholangitis (PSC), a progressive bile duct disease of unknown origin. Although it is generally assumed that this pathology is a consequence of impaired calcium homeostasis and malabsorption, the cellular and molecular causes of PSC-associated osteoporosis are unknown.

Methods: We determined bone mineral density by dual-X-ray absorptiometry and assessed bone microstructure by high-resolution peripheral quantitative computed tomography in patients with PSC. Laboratory markers of liver and bone metabolism were measured, and liver stiffness was assessed by FibroScan. We determined the frequency of Th17 cells by the *ex vivo* stimulation of peripheral blood mononuclear cells in a subgroup of 40 patients with PSC. To investigate the potential involvement of IL-17 in PSC-associated bone loss, we analyzed the skeletal phenotype of mice lacking *Abcb4* and/or *Il-17*.

Results: Unlike in patients with primary biliary cholangitis, bone loss in patients with PSC was not associated with disease duration or liver fibrosis. However, we observed a significant negative correlation between the bone resorption biomarker deoxypyridinoline and bone mineral density in the PSC cohort, indicating increased bone resorption. Importantly, the frequency of Th17 cells in peripheral blood was positively correlated with the urinary deoxypyridinoline level and negatively correlated with bone mass. We observed that *Abcb4*-deficient mice displayed a low-bone-mass phenotype, which was corrected by an additional *Il-17* deficiency or anti-IL-17 treatment, whereas the liver pathology was unaffected.

Conclusions: Our findings demonstrate that an increased frequency of Th17 cells is associated with bone resorption in PSC. Whether antibody-based IL-17 blockade is beneficial against bone loss in patients with PSC should be addressed in future studies.

Lay summary: Primary sclerosing cholangitis (PSC) is a cholestatic liver disease characterized by progressive bile duct destruction. One serious complication of PSC is reduced bone mass resulting in increased fracture risk. Herein, we demonstrate that Th17 cells mediate bone loss in PSC by inducing bone resorption, which suggests that antibody-based IL-17 blockade might be beneficial for the treatment of bone loss in affected patients.

Introduction

Primary sclerosing cholangitis (PSC) is a severe idiopathic disease characterized by the progressive fibrosis of intrahepatic and extrahepatic bile ducts with a median age at onset of between 30–40 years.[1–3] In contrast to primary biliary cholangitis (PBC), which predominantly affects middle-aged or elderly women,[2] approximately 60% of PSC patients additionally develop colitis. Genetic variations associated with PSC can involve genes related to the immune system (human leukocyte antigen) and pathways that mediate inflammation. These associations are clearly different from those of ulcerative colitis.[3] In addition to genetic risk factors, environmental influences also play important roles in the development of biliary inflammation and fibrosis.[4] In this context, changes in microbial flora in the gut and subsequent activation of the immune system may be an important driving factor.[5] In fact, recent studies have shown a clear difference in the gut microbiome between patients with PSC and healthy controls.[6] We have previously shown that the bile fluid of patients with PSC is frequently colonized with different pathogens. Moreover, patients with PSC displayed a higher Th17 cell frequency after the stimulation of peripheral blood mononuclear cells with these pathogens, suggesting an important role of Th17 cells in the pathogenesis of PSC.[7] However, the impact of microbial alterations on immune dysregulation and the perpetuation of biliary inflammation remain to be determined.

One serious complication in patients with cholestatic liver diseases is osteoporosis. In contrast to the occurrence of osteoporosis accompanied by low bone formation in PBC, which has

Keywords: Bone remodeling; Osteoporosis; Metabolic bone disease; Inflammatory bone loss.
Received 16 July 2018; received in revised form 19 December 2018; accepted 26 December 2018; available online 11 January 2019
* Corresponding author. Address: Department of Osteology and Biomechanics, University Medical Center Hamburg-Eppendorf, Lottestraße 59, 22529 Hamburg, Germany. Tel: +49-40-7410-56373.
E-mail address: amling@uke.de (M. Amling).

been reported repeatedly,[8–10] this pattern is less established for PSC. However, in a previous study involving 237 patients with PSC, osteoporosis was found in 15% of patients.[11] These authors also reported that osteoporosis was associated with age, body mass index (BMI) and the presence of inflammatory bowel disease (IBD) in their PSC cohort. However, the respective study also had some limitations, as the patients did not undergo examination for the analysis of biomarkers of bone turnover, trabecular and cortical bone architecture, or distinct immune cell populations. It therefore remains to be established which bone remodeling cell type is primarily affected in PSC. Considering that liver transplantation may be required in most patients and that subsequent immunosuppression is an additional risk factor for osteoporosis, determination of the bone status and providing adequate treatment is crucial for preventing fractures in affected patients.

Given the clinical relevance of this research question, our translational study aimed to investigate the mechanism for PSC-associated bone loss. We show that low bone mass in patients with PSC is not associated with disturbed calcium homeostasis but with increased bone resorption and an increased Th17 cell frequency. Moreover, the deletion of IL-17 in a mouse model of PSC resulted in a correction of the observed osteopenia by reducing osteoclastogenesis.

Patients and methods

Human patients

Between 2012 and 2016, a total of 238 adult patients with a diagnosis of PSC and a total of 242 adult patients with a diagnosis of PBC were admitted to the Department of Osteology and Biomechanics at the University Medical Center Hamburg-Eppendorf, Germany (Table 1). The diagnosis of PSC and PBC was established by the First Department of Medicine (University Medical Center Hamburg-Eppendorf, Germany) using clinical, imaging, biochemical, immunological and histological criteria as defined in the European Association for the Study of the Liver clinical practice guidelines.[12,13] Secondary causes of sclerosing cholangitis were excluded in patients with PSC. Liver stiffness measurements were obtained in 211 patients with PSC and 159 with PBC using FibroScan (Echo-Sens, Paris, France) as previously reported.[14] An osteological assessment was performed within the first 3 months after the hepatic analysis. We only present data from the first visit, before vitamin D supplementation and antiresorptive treatment was initiated for some patients according to the guidelines of the German Society for Osteology. Of note, a small number of patients had already received bisphosphonates (n = 8), initiated by other physicians because of prednisolone medication. Additionally, there were 37 other patients receiving only prednisolone treatment, mostly because of associated colitis. Importantly, all patients receiving bisphosphonates and/or prednisolone were excluded from all analyses except the initial description of the study cohort (*i.e.* correlation of dual-energy X-ray absorptiometry [DXA] values with age, disease duration and FibroScan).

This study was performed in accordance with the Declaration of Helsinki. The local ethics committee of the University Medical Center Hamburg-Eppendorf approved this study (PV4081-Z). Written informed consent was obtained from all patients.

Skeletal analysis

Bone mineral density (BMD) was measured by DXA (Lunar Prodigy enCore 2007, GE Healthcare; Madison, WI, USA). Two skeletal areas, the left proximal femur and the lumbar spine (L1-L4), were evaluated by DXA. DXA measurements of the lumbar spine had to be excluded due to degenerative osteoproliferative changes in 6 patients with PSC and 15 with PBC, and DXA measurements of the proximal femur were not possible due to bilateral hip replacement in 6 patients with PSC and 8 with PBC. A total of 138 patients with PSC were also assessed by high-resolution peripheral quantitative computed tomography (HR-pQCT, XtremeCT, Scanco Medical, Brüttisellen, Switzerland) using the default *in vivo* settings, namely, 60 kVp, 1,000 μA, integration time of 100 ms and voxel size of 82 μm at the distal left tibia and distal right radius. The manufacturer's standard protocol was used to analyze the bone microstructure, including the total volumetric density (Tt.BMD), trabecular volumetric density (Tb.BMD), cortical volumetric density (Ct.BMD), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and cortical thickness (Ct.Th), at the distal left tibia and distal right radius. For further interpretation, we used recently published age- and sex-specific reference values,[15] which were previously validated in our own cohort.[16]

Biochemical analyses

Biochemical analyses, including determination of the serum levels of 25-hydroxyvitamin D (25-OH-D), calcium, phosphate, parathyroid hormone (PTH), osteocalcin, bone-specific alkaline phosphatase (ALP), bilirubin, ALP, gamma-glutamyl transferase, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and urinary levels of deoxypyridinoline (Dpd) crosslinks, were performed in the Department of Clinical Chemistry, University Medical Center Hamburg-Eppendorf. The reference values used for each parameter were in accordance with the local laboratory. We did not measure the level of estrogen, testosterone or adrenal androgens, since their determination does not impact clinical management in our experience.

FACS analyses

For intracellular cytokine staining, peripheral whole blood was restimulated with PMA (25 ng/ml) and ionomycin (5 μg/ml, both Sigma-Aldrich, Taufkirchen, Germany) in the presence of brefeldin (10 μg/ml, BD Pharmingen, Heidelberg, Germany) for 5 h. Extracellular staining was performed with antibodies to CD3 and CD4 (BioLegend, Fell, Germany) following the lysis of erythrocytes using FACS Lysing Solution (BD Biosciences). Dead cells were stained with Pacific Orange-NHS (Life Technologies, Darmstadt, Germany). Cells were fixed and perforated with saponin buffer (0.3% saponin, 0.1% bovine serum albumin in PBS) (Sigma-Aldrich) and stained for IFN-γ and IL-17. Flow cytometry data were analyzed with FACS Diva software (BD Biosciences).

For the FACS analysis of murine cells, the liver was perfused with PBS via the vena portae and mechanically dissected to generate a single-cell suspension. Hepatocytes and debris were sedimented, and non-parenchymal cells were recovered via centrifugation over a 21% OptiPrep gradient (Sigma-Aldrich). The spleen was mechanically dissected to generate a single-cell suspension. For intracellular cytokine staining, isolated cells were restimulated with PMA (25 ng/ml) and ionomycin (5 μg/ml, both Sigma-Aldrich, Taufkirchen, Germany) in the presence of

Table 1. Demographic, disease-specific, laboratory and osteologic data of patients with PSC and PBC.

	PSC		PBC	
	Mean (SD) or n (%)	n	Mean (SD) or n (%)	n
Demographic characteristics				
Females (%)	102/238 (42.9)	238	210/242 (86.8)	242
Age (yr)	47.1 (13.8)	238	60.7 (11.0)	242
Height (m)	1.75 (0.10)	238	1.65 (0.08)	242
Weight (kg)	75.9 (14.7)	238	73.86 (14.8)	242
BMI (kg/m^2)	24.8 (4.3)	238	27.1 (5.7)	242
Smoking	24/238 (10.1)	238	25/242 (10.3)	242
Alcohol consumption	6/238 (2.5)	238	11//242 (4.5)	242
Disease characteristics				
Small-duct PSC	12/238 (5.0)	238	–	
Associated colitis (%)	115/238 (48.3)	238	–	
Associated autoimmune hepatitis	–		39/242 (16.1)	242
Duration of disease (mo)	79.1 (75.4)	238	71.9 (62.7)	242
Fibroscan (kD)	11.1 (13.4)	211	7.0 (3.4)	159
History of vertebral fracture	10/238 (4.2)	238	16/242 (6.6)	242
History of peripheral fracture	15/238 (6.3)	238	19/242 (7.9)	242
Treatment regime				
Glucocorticoid (within the last 6 mo)	45/238 (18.9)	238	33/242 (13.6)	242
Ursodeoxycholic acid	220/238 (92.4)	238	230/242 (95)	242
Bisphosphonate	8/238 (3.4)	238	9/242 (3.7)	242
Proton pump inhibitor	26/238 (10.9)	238	41/242 (16.9)	242
Azathioprine	40/238 (16.8)	238	43/242 (17.8)	242
Laboratory values				
Bilirubin (mg/dl)	1.1 (1.4)	224	0.65 (1.5)	217
AST (U/L)	43.8 (39.5)	223	31.1 (18.5)	217
ALT (U/L)	62.9 (73.9)	223	35.3 (28.0)	220
AP (U/L)	186.4 (150)	223	128.3 (90.2)	220
GGT (U/L)	178.2 (205.7)	223	84.17 (137.5)	220
25-OH-D3 (μg/L)	24.5 (11.4)	222	24.5 (11.4)	211
Vitamin D deficiency (<20 μg/L)	75/222 (33.8)	222	75/211 (35.5)	211
Calcium (mmol/L)	2.26 (0.12)	192	2.30 (0.11)	131
Phosphate (mmol/L)	0.98 (0.16)	141	1.0 (0.15)	97
Bone AP (μg/L)	28.0 (21.8)	145	20.8 (14.1)	62
Osteocalcin (μg/L)	19.1 (7.52)	130	18.8 (7.1)	59
Parathyroid hormone (ng/L)	71.1 (40.5)	140	74.0 (37.0)	61
Dpd (nmol/mmol)	5.7 (2.2)	122	6.2 (2.7)	36
Leukocytes (Mrd/L)	7.1 (2.8)	215	7.2 (2.3)	207
Lymphocytes %	24.3 (8.7)	196	26.0 (8.1)	157
Neutrophils %	64.2 (10.7)	193	63.2 (9.3)	161
T17 cells % after stimulation	2.24 (0.89)	40	–	–
Dual-energy X-ray absorptiometry				
Z-score lumbar spine	−0.37 (1.3)	232	−0.11 (1.3)	227
Z-score left femur	−0.75 (1.1)	232	−0.1 (1.0)	234
T-score lumbar spine	−0.58 (1.35)	232	−0.76 (1.4)	227
T-score left femur	−0.75 (1.16)	232	−0.74 (1.2)	234

25-OD-D, 25-hydroxyvitamin D; ALT, alanine aminotransferase; aP, available phosphorus; AST, aspartate aminotransferase; BMI, body mass index; Dpd, deoxypyridinoline; GGT, gamma glutamyltransferase; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

GolgiPlug (10 μg/ml, BD Pharmingen, Heidelberg, Germany) for 4 h. Immunofluorescence staining of cells was performed with antibodies to CD3, CD4, and IL-17A (BioLegend, Fell, Germany). Dead cells were stained with Pacific Orange-NHS (Life Technologies, Darmstadt, Germany).

Animals

Abcb4- and *Il17af*-deficient mice (on a C57BL/6J genetic background) were kindly provided by Dr. Frank Lammert[17] and Dr. Immo Prinz,[18] respectively. We first mated *Abcb4*$^{-/-}$ and *Il17af*$^{-/-}$ mice to generate compound heterozygous animals. These were then intercrossed to obtain littermates with the genotypes of interest. At least n = 4 animals per group were used (females and males). The exact group size is indicated in the respective figures. The anti-IL-17A antibody (Bio X Cell, clone 17F3) was injected intraperitoneally 3 times a week at a dose of 5 mg/kg. All mice were kept in a specific pathogen-free environment with a 12 h light/dark cycle, 45–65% relative humidity and 20–24 °C ambient temperature in open or individually ventilated cages with wood shavings bedding and nesting material in groups not surpassing 6 animals. The mice had access to tap water and standard rodent chow (1328P, Altromin Spezialfutter GmbH & Co. KG). All animal experiments were approved by the animal facility of the University Medical Center Hamburg-Eppendorf and by the "Amt für Gesundheit und Verbraucherschutz" (Org529, 46/16).

Analysis of mouse skeletons

Dissected skeletons were fixed in 3.7% PBS-buffered formaldehyde for 18 h. The lumbar vertebral bodies (L1-L4) and one tibia were dehydrated in ascending alcohol concentrations and then embedded in methyl methacrylate and sectioned as previously described.[19] Histomorphometry was performed according to the ASBMR guidelines using the OsteoMeasure system (OsteoMetrics, Inc.). TRAP activity staining was performed on decalcified sections using naphthol AS-MX phosphate (Sigma) and Fast Red Violet LB salt (Sigma) in 40 mM acetate buffer (pH 5). For microcomputed tomography (μCT) scanning, we used a μCT 40 desktop cone-beam system (Scanco Medical) with a voxel size of 10 μm (for femora) or 10 μm (for skull bones).

Analysis of hepatic defects in mice

Sirius red staining was performed on formalin-fixed liver tissue for fibrillary collagen, as previously described.[20] For cytokeratin staining, paraffin-embedded liver sections were deparaffinized, rehydrated; then, endogenous peroxidases were blocked, and antigen retrieval was carried out. Staining was performed using anti-cytokeratin (Wide spectrum, #Z0622 DAKO, Hamburg, Germany) and polyclonal swine anti-rabbit horseradish peroxidase (#P0217 DAKO, Hamburg, Germany). Peroxidase activity was visualized using 3,3′-diaminobenzidine, and sections were counterstained with hematoxylin. Both stains were visualized by confocal microscopy (Biorevo BZ-9000; Keyence, Osaka, Japan). Sirius red-positive or cytokeratin-positive areas were quantified in at least 10 low-power fields using Adobe Photoshop and ImageJ in a blinded fashion, as described previously.[20] Liver damage was assessed by measuring the plasma enzyme activity of AST and ALT using an automated procedure (Cobas Mira; Roche, Basel, Switzerland).

Primary osteoblasts

Primary osteoblasts were obtained from the calvariae of 5-day-old C57BL/6 wild-type mice by sequential digestion with collagenase/dispase. After 3 days, the cells were cultured in medium supplemented with ascorbic acid (50 μg/ml, Sigma-Aldrich) and β-glycerophosphate (10 mM, Sigma-Aldrich) for 10 days to induce osteogenic differentiation and matrix mineralization. To determine the influence of bilirubin, unconjugated bilirubin or conjugated bilirubin was added to a final concentration of 1, 10 or 100 μM to the differentiation medium. To quantify matrix mineralization, we performed alizarin red staining with subsequent photometric quantification. To analyze the immediate effects of bilirubin, cells were serum-starved overnight at day 10 of differentiation and then treated with unconjugated bilirubin or conjugated bilirubin for 6 h.

Gene expression

RNA was isolated from liver tissue or cultured osteoblasts using the RNeasy Mini kit (Qiagen), and DNase digestion was performed according to the manufacturer's instructions. The concentration and quality of RNA were determined using a NanoDrop ND-1000 system (NanoDrop Technology). For RT-PCR analysis, 1 μg of RNA was reverse transcribed using SuperScript III (Invitrogen) according to the manufacturer's instructions. The expression of *Col1a1, Col3a1, Mmp13, Tgfb1* or *Casp4* was determined with predesigned TaqMan assays (Applied Biosystems) and TaqMan gene expression master mix. Reactions were performed on a StepOnePlus system (Applied Biosystems). Target gene expression was normalized to *Gapdh* expression by employing the [ΔΔCT] method.

Statistical analyses

SPSS 22 software (version 22.0, IBM, Armonk, New York, USA) and GraphPad Prism® (GraphPad Software, La Jolla, CA) were used for statistical analyses. Quantitative characteristics are presented as the mean ± SD or the number (proportion) of patients with a condition. The normality of the data distributions was tested with the Kolmogorov–Smirnov test. For correlation analyses, Pearson's correlation test was used for parametric data, and Spearman's rank correlation test was used for nonparametric data. To determine potential predictors of bone loss in patients with PSC, we applied a multivariate regression model including age, BMI, disease duration, the presence of colitis, liver elastography data (FibroScan), smoking status, and 25-OH-D and Dpd levels. To test for differences between the study groups, we used the unpaired 2-sided *t* test for normally distributed data and the Mann–Whitney *U* test for non-normally distributed data. When comparing more than 2 groups, significance was calculated by 1-way analysis of variance (ANOVA) with Tukey's *post hoc* analysis. *p* values of 0.05 were considered statistically significant.

Results

Low bone mass in patients with PSC is not associated with age, disease duration or liver fibrosis

We recruited 238 patients with PSC and 242 patients with PBC between 2012 and 2016 (Table 1). Consistent with the literature, patients in the PSC group were younger on average, and the percentage of female patients was higher in the PBC group. In most patients with PSC, the large ducts were affected, and approximately half of these patients were diagnosed with associated colitis, but none of them had autoimmune hepatitis. The 2 cohorts did not substantially differ with respect to previous medications, and most of the patients had received ursodeoxycholic acid. The fracture rate in the PBC cohort was only slightly greater than that in the PSC cohort, with 6.6% *vs.* 4.2% of patients with a history of a vertebral fracture and 7.9% *vs.* 6.3% of patients with a history of a peripheral fracture.

To characterize the bone pathologies in the patients with PSC, we first determined the BMD and respective T-scores by DXA of the lumbar spine and the femoral neck (Fig. 1A). Among the 238 patients analyzed, 19 individuals (8.2%) were diagnosed with osteoporosis (T-score <−2.5), whereas 80 individuals (34.5%) displayed osteopenia (T-score <−1.5) of the lumbar spine. There was overall heterogeneity in the cohort, and 4 individuals (1.7%) even displayed high bone mass (T-score >2.5). Similar results were observed in the femoral neck, with 15 individuals (6.5%) being in the range of osteoporosis and 88 individuals (37.9%) displaying osteopenia. In total, 27 patients (11.4%) were diagnosed with osteoporosis according to the WHO guidelines.

In a comparative approach, we also determined DXA T-scores in 242 individuals with PBC (Fig. 1b). Although these patients were significantly older (60.7 ± 11.0 *vs.* 47.1 ± 13.8) than those in the PSC group, they showed comparable T-score heterogeneity, with 34 individuals (14.0%) diagnosed with osteoporosis. In the PBC cohort, we found a significant negative correlation of T-score with age, in contrast to the PSC cohort. Since the T-score is related to the BMD of young individuals with peak bone mass,

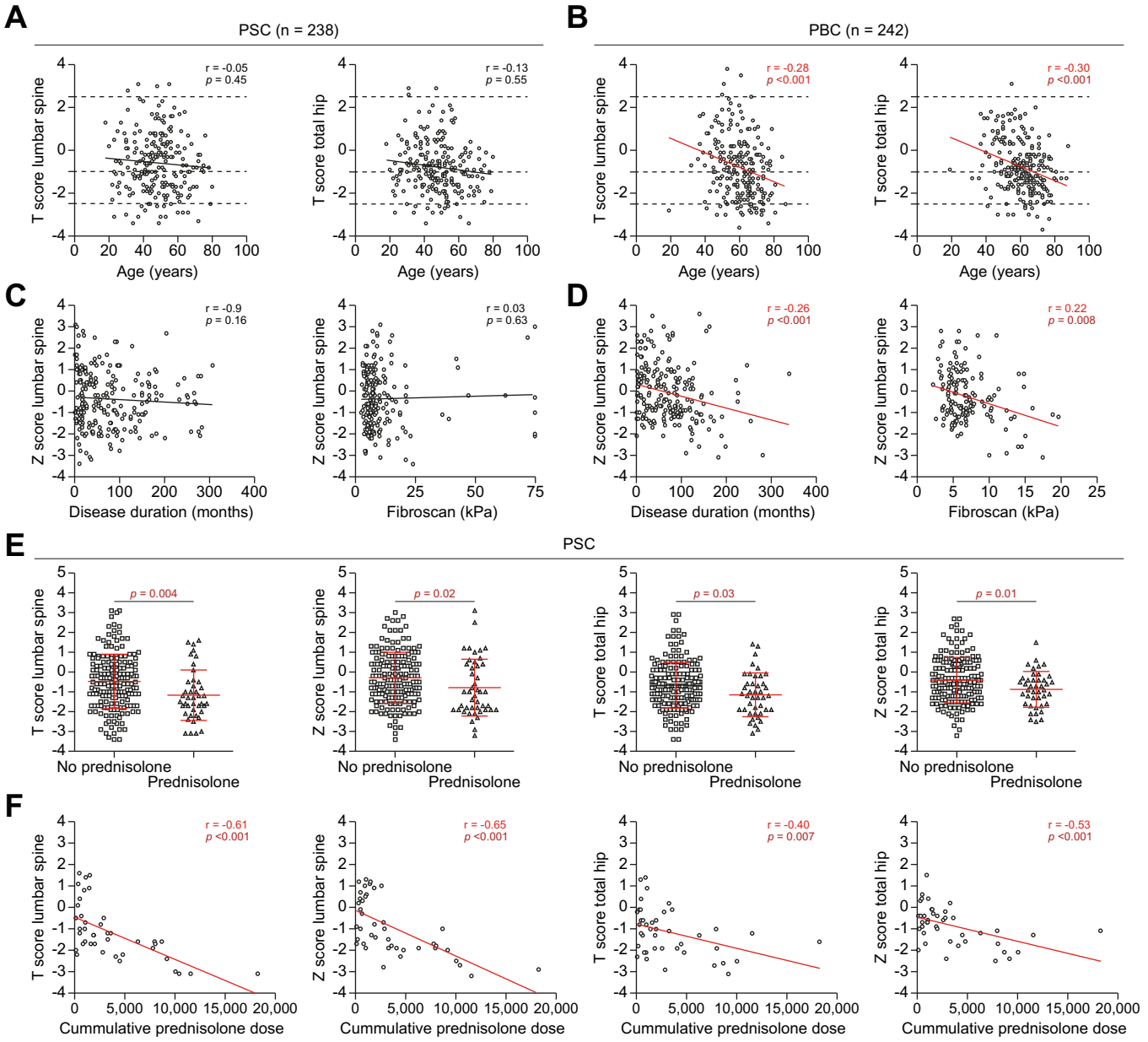


Fig. 1. Low bone mass in patients with PSC is not associated with age, disease duration or liver fibrosis. (A, B) The DXA T-score in 238 patients with PSC and 242 with PBC at the lumbar spine (left) and femoral neck (right) is correlated with age. Dotted lines indicate the range of osteoporosis (<−2.5), osteopenia (<−1.0 to −2.5), reference (−1.0 to 2.5) and high bone mass (>2.5). (C) In patients with PSC, the DXA Z-score at the lumbar spine is correlated with disease duration (left, 238 patients) and transient liver elastography (FibroScan) values (right, 211 patients). (D) In patients with PBC, the DXA Z-score at the lumbar spine is correlated with disease duration (left, 242 patients) and transient liver elastography (FibroScan) values (right, 159 patients). Blue lines indicate non-significant correlations; red lines indicate significant correlations (all linear correlation). (E) DXA T-scores and Z-scores of the lumbar spine and total hip were compared in patients with or without prednisolone treatment within the last 6 months prior to DXA measurement (232 patients) (*t* test). (F) The cumulative prednisolone dose (mg) was correlated with the DXA T-scores and Z-scores of the lumbar spine and total hip. Values p <0.05 were considered statistically significant (linear correlation). DXA, dual-energy X-ray absorptiometry; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis.

we focused on DXA Z-scores for the remainder of the study, since this score is related to age-matched reference BMD values. Comparing the Z-scores of both patient groups revealed another difference between PSC and PBC. In fact, whereas DXA Z-scores displayed a significant negative correlation with disease duration and liver stiffness in the PBC cohort, there was no such correlation in the PSC cohort (Fig. 1C,D). Moreover, the DXA scores in the PSC cohort were not influenced by coexisting colitis (Fig. S1), PSC subtype, or treatment with azathioprine or proton pump inhibitors (Fig. S2). The only significant influence of an existing medication was found in the case of prednisolone treatment (Fig. 1E,F), which confirms general knowledge obtained from several clinical studies.[21]

Low bone mass in patients with PSC is associated with high bone resorption

To further characterize the bone status of patients with PSC, we correlated various serum parameters with the DXA Z-scores. Here, we excluded all patients who had been treated with prednisolone (which includes the 8 patients previously receiving

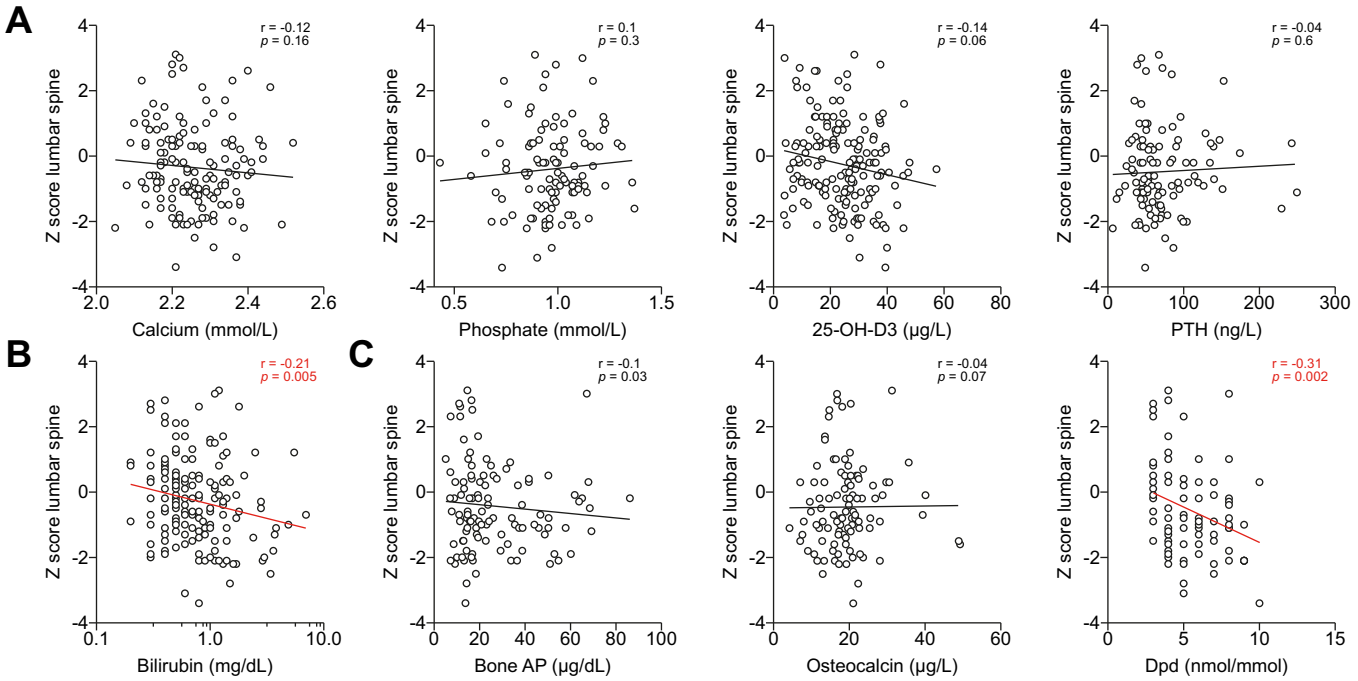


Fig. 2. Low bone mass in patients with PSC is associated with increased bone resorption. (A) The DXA Z-score at the lumbar spine is correlated with serum parameters of calcium homeostasis (calcium, 149 patients; phosphate, 110 patients; 25-OH-D, 176 patients; PTH, 115 patients). (B) The DXA Z-score at the lumbar spine is correlated with serum bilirubin levels (177 patients). (C) The DXA Z-score at the lumbar spine is correlated with markers of bone formation (bone-specific ALP, 114 patients; osteocalcin, 108 patients) and bone resorption (urinary Dpd, 95 patients). Blue lines indicate nonsignificant correlations; red lines indicate significant correlations (linear correlation for all panels). 25-OD-D, 25-hydroxyvitamin D; ALP, alkaline phosphatase; Dpd, deoxypyridinoline; DXA, dual-energy X-ray absorptiometry; PSC, primary sclerosing cholangitis; PTH, parathyroid hormone.

bisphosphonates). While there were no significant correlations with parameters of mineral homeostasis (Fig. 2a), we found that the bilirubin levels were associated with BMD in patients with PSC (Fig. 2b). Moreover, while the serum concentrations of bone-specific ALP and osteocalcin (biomarkers of bone formation) were not associated with BMD in patients with PSC, we observed a significant negative correlation between the urinary Dpd level (a biomarker of bone resorption) and the DXA Z-score (Fig. 2c).

Data on systemic bone loss in patients with PSC have only been assessed by evaluating the BMD by DXA so far, whereas detailed analyses of bone structure in PSC have never been performed. Hence, we also evaluated bone quality by HR-pQCT, which allows a separate analysis of trabecular and cortical bone (Fig. 3A). HR-pQCT data were obtained for 138 patients with PSC and are expressed as a percentage of recently published age- and sex-specific reference values.[15] Using this approach, we found an overall reduction in the Ct.Th (Fig. 3B) at the distal tibia in patients with PSC compared to the healthy reference population. While the Tb.Th was slightly decreased, the Tb.N was within the reference range, suggesting that trabecular bone is only moderately affected in patients with PSC. However, consistent with the DXA BMD assessment, there was overall heterogeneity in the cohort, in both the cortical and trabecular bone compartments. The same analysis was performed for the distal radius, and similar results were obtained (Fig. S3A,B).

We next correlated various serum parameters with the HR-pQCT data and found no associations between serum parameters for mineral homeostasis and bone formation and the Tb.N or Ct.Th (Fig. 3C,D). The same was the case for bilirubin. Importantly, however, we found a significant negative correlation between the urinary Dpd level with both the Tb.N (Fig. 3C) and Ct.Th (Fig. 3D). We additionally used a multivariate regression model including all patients to search for independent predictors of T-score at the lumber spine and both the Ct.Th and Tb.Th at the distal tibia. Age, BMI, disease duration, the presence of colitis, liver stiffness (FibroScan), smoking status (current or longer than 6 months within the last 10 years), and the 25-OH-D and Dpd levels were included as variables (Table S1). In this regression model, the Dpd level was identified as an independent predictor of all 3 parameters. Additionally, BMI was an independent predictor of Tb.Th. In contrast, no independent associations were found for age, BMI, disease duration, the presence of colitis, liver stiffness, smoking or 25-OH-D level. Taken together, these data demonstrate that the bone mass reduction in a subset of patients with PSC is likely explained by increased bone resorption.

Bone loss in patients with PSC is associated with an increased Th17 cell frequency

Because inflammation can affect bone homeostasis, we investigated whether bone mass in patients with PSC is associated with inflammatory cell types. We did not observe a significant correlation between the DXA Z-score and the number of leukocytes or the percentage of neutrophils and lymphocytes in our PSC cohort (Fig. 4a). Since Th17 cells have been reported to mediate bone loss under different pathological conditions,[22,23] we hypothesized that an increased frequency of Th17 cells, as observed in patients with PSC,[7] is involved in PSC-associated bone pathologies. To investigate this question, we quantified the percentage of Th17 cells by the *ex vivo* stimulation of peripheral blood mononuclear cells with PMA/ionomycin in a subgroup of 40 patients with PSC not treated with prednisolone (Table S2). We found that the frequency of Th17 cells correlated

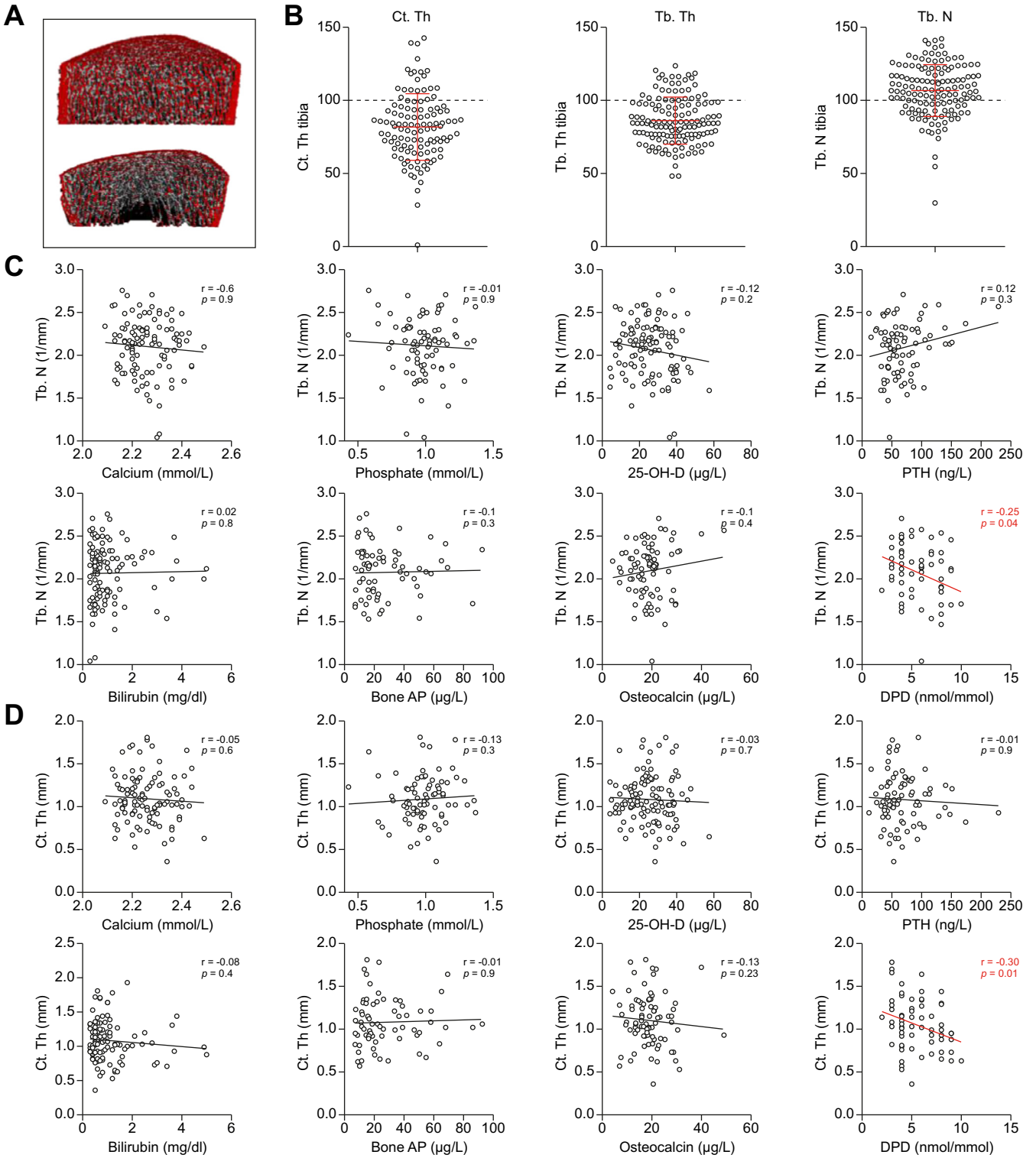


Fig. 3. Cortical and trabecular bone loss in patients with PSC is associated with increased bone resorption. (A) Representative three-dimensional reconstructions of HR-pQCT scans of the distal tibia of a patient with a well-preserved bone microstructure (top) and a patient with a severely deteriorated bone microstructure (bottom). (B) HR-pQCT microstructure parameters, including the cortical thickness (Ct.Th), trabecular thickness (Tb.Th) and trabecular number (Tb.N), of 124 patients with PSC were normalized to age- and sex-specific reference values and are expressed as percentages of reference values. (C) The Tb.N is correlated with serum parameters of calcium homeostasis (calcium, 108 patients; phosphate, 80 patients; 25-OH-D, 115 patients; PTH, 85 patients), bilirubin (115 patients), and bone remodeling biomarkers (bone-specific ALP, 86 patients; osteocalcin, 85 patients; urinary Dpd, 66 patients). (D) The Ct.Th is correlated with serum parameters of calcium homeostasis (calcium, 108 patients; phosphate, 80 patients; 25-OH-D, 115 patients; PTH, 85 patients), bilirubin (116 patients), and bone remodeling biomarkers (bone-specific ALP, 86 patients; osteocalcin, 85 patients; urinary Dpd, 66 patients). Blue lines indicate non-significant correlations; red lines indicate significant correlations (linear correlation for all panels). 25-OD-D, 25-hydroxyvitamin D; ALP, alkaline phosphatase; Dpd, deoxypyridinoline; HR-pQCT; high-resolution peripheral quantitative computed tomography; PSC, primary sclerosing cholangitis; PTH, parathyroid hormone.

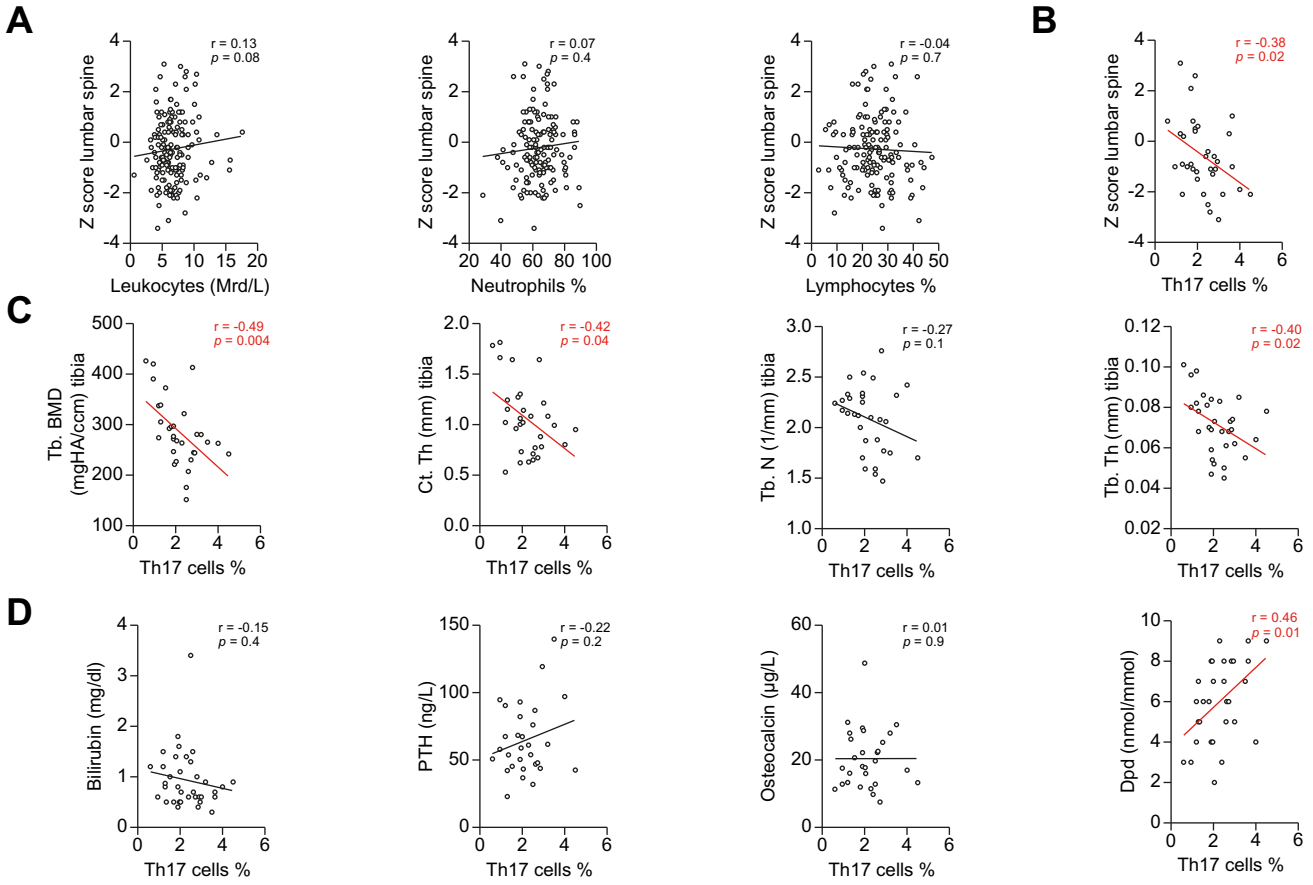


Fig. 4. Th17 cell frequency in patients with PSC is associated with bone mass and bone resorption. (A) The DXA Z-score at the lumbar spine is correlated with the number of leukocytes (215 patients) and the percentage of neutrophils (193 patients) and lymphocytes (196 patients). (B) Peripheral blood mononuclear cells from 40 patients with PSC were stimulated with PMA and analyzed for cytokine production using FACS. The number of Il-17-producing $CD4^+$ cells (Th17 cells) was then correlated with the DXA Z-score at the lumbar spine. (C) HR-pQCT microstructure parameters (Tb.BMD, volumetric trabecular bone mineral density; Ct.Th, cortical thickness; Tb.N, trabecular number; Tb.Th, trabecular thickness) are correlated with the Th17 cell frequency. (D) The serum bilirubin, PTH, and osteocalcin levels and urinary Dpd level are correlated with the Th17 cell frequency. Blue lines indicate non-significant correlations; red lines indicate significant correlations (linear correlation for all panels). Dpd, deoxypyridinoline; DXA, dual-energy X-ray absorptiometry; HR-pQCT; high-resolution peripheral quantitative computed tomography; PSC, primary sclerosing cholangitis; PTH, parathyroid hormone.

significantly with the DXA Z-score in these patients (Fig. 4B). Likewise, the Th17 cell frequency was also associated with HR-pQCT parameters, *i.e.*, the trabecular BMD and the Ct.Th and Tb.Th in both the distal tibia (Fig. 4C) and distal radius (Fig. S3C). Importantly, while there was no association between the Th17 cell frequency and the osteocalcin, PTH or bilirubin level, the urinary Dpd level was positively correlated with the Th17 cell frequency (Fig. 4D). These data suggest that increased IL-17 production by Th17 cells is one mediator of PSC-associated bone loss in that it potentially causes increased osteoclastogenesis, similar to what has been reported for other conditions.[23]

IL-17 deficiency corrects osteopenia in *Abcb4*-deficient mice

Abcb4-deficient mice are considered a mouse model of PSC, since they develop hepatic inflammation, fibrosis and features of sclerosing cholangitis caused by an accumulation of toxic bile acids.[17] Moreover, *Abcb4*$^{-/-}$ mice have been reported to display osteopenia, yet their bone remodeling phenotype has never been analyzed on the basis of undecalcified histology or bone-specific histomorphometry.[24] We first performed FACS analysis and found that the *Abcb4*$^{-/-}$ mice displayed an increased Th17 cell frequency in the liver compared to their wild-type littermates (Fig. 5a). As a control, we generated *Il17af*-deficient *Abcb4*$^{-/-}$ mice (*Abcb4*$^{-/-}$/*Il17af*$^{-/-}$), in which no IL-17-positive cells were detected. We next analyzed the skeletal phenotype of 20-week-old female wild-type, *Abcb4*$^{-/-}$ and *Abcb4*$^{-/-}$/*Il17af*$^{-/-}$ mice by µCT of the femora. Here, we observed that the *Abcb4*$^{-/-}$ animals displayed osteopenia and that their trabecular bone volume was significantly increased by the additional *Il17af* deficiency (Fig. 5B).

The skeletal phenotype in spine sections was further analyzed by non-decalcified histology and histomorphometry (Fig. 6B). We thereby confirmed that the *Abcb4*$^{-/-}$ animals displayed reduced trabecular bone mass and that this phenotype was prevented by the additional *Il17af* deficiency (Fig. 6B). We also analyzed *Il17af*$^{-/-}$ littermates, but no skeletal phenotype was observed here (Fig. S4). To identify the underlying cause of osteopenia in *Abcb4*$^{-/-}$ mice, we next applied cellular and dynamic histomorphometry. Here, we did not observe a significant difference in the osteoblast number or bone formation rate, and neither parameter was influenced by *Il17af* deficiency (Fig. 6C,D). Importantly, however, as confirmed by TRAP activity staining (Fig. 6E), we detected increased osteoclastogenesis parameters in *Abcb4*$^{-/-}$ mice. Moreover, both the osteoclast number and surface area were normalized to the wild-type

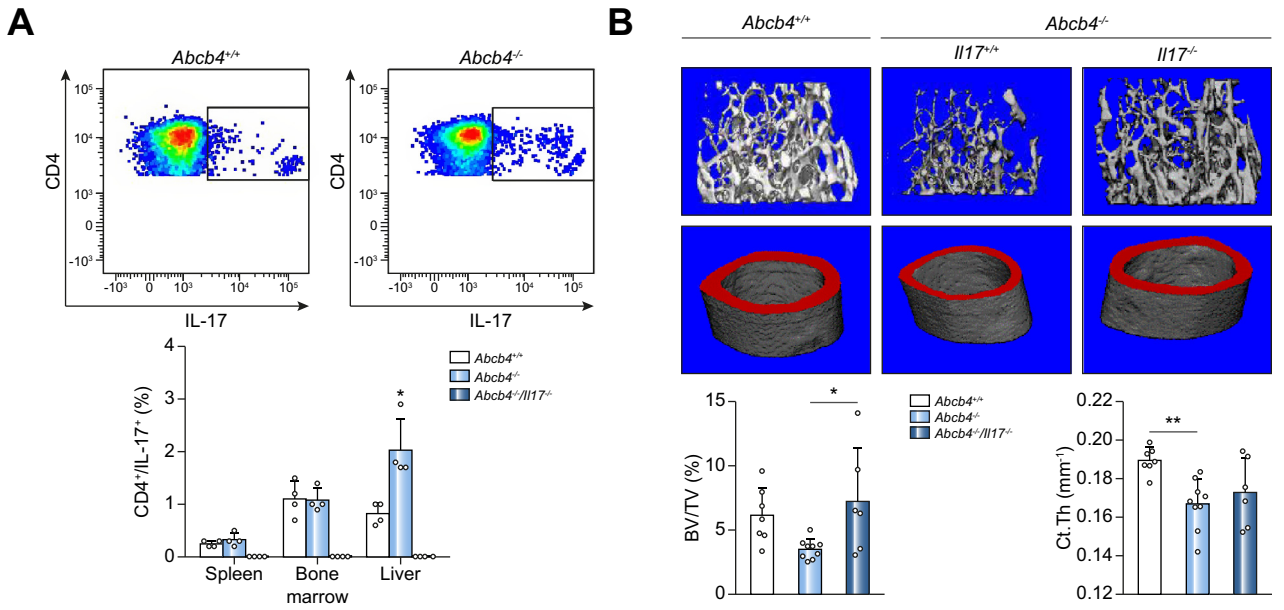


Fig. 5. *Il17af* deficiency corrects osteopenia in *Abcb4*-deficient mice. (A) Representative FACS analysis (left) showing an increased frequency of Th17 cells in the liver of *Abcb4*$^{-/-}$ mice. Quantification (right) of the Th17 population in the spleen, bone marrow and liver of 20-week-old female wild-type, *Abcb4*$^{-/-}$ and *Abcb4*$^{-/-}$/*Il17af*$^{-/-}$ littermate mice (n = 4). (B) Representative μCT scans (left) of the femora of 20-week-old female wild-type, *Abcb4*$^{-/-}$ and *Abcb4*$^{-/-}$/*Il17af*$^{-/-}$ littermate mice. Quantification of trabecular (BV/TV, bone volume per tissue volume) and cortical bone mass (Ct.Th, cortical thickness) is given on the right (n ≥6). **p* <0.05; ***p* <0.005 (ANOVA for all panels).

levels in the *Abcb4*$^{-/-}$/*Il17af*$^{-/-}$ mice (Fig. 6F). To investigate whether the neutralization of IL-17 could prevent osteopenia in *Abcb4*$^{-/-}$ mice, we additionally treated them with a specific IL-17A blocking antibody for 4 weeks, starting at 16 weeks of age. The treatment significantly increased the trabecular bone volume in *Abcb4*$^{-/-}$ mice (Fig. 7A,B), which was related to a reduction in osteoclastogenesis (Fig. 7C).

Since a recently published study demonstrated that intrahepatic IL-17 production is involved in promoting liver fibrosis in *Abcb4*$^{-/-}$ mice,[25] we finally assessed the hepatic pathology in the different groups of mice (Fig. 8A). We did not observe a significant influence of *Il17af* deficiency or the anti-IL-17 treatment on liver fibrosis in *Abcb4*$^{-/-}$ mice (Fig. 8b). Likewise, the serum ALT and AST levels were similarly increased in all groups of *Abcb4*$^{-/-}$ mice. Since these data were also confirmed by cytokeratin staining and gene expression analyses (Fig. S5), our combined analysis demonstrates that the *Il17af* deficiency-mediated correction of the *Abcb4*$^{-/-}$ bone phenotype is not explained by reduced liver pathologies.

Discussion

In this study, we report that bone loss in a large cohort of patients with PSC occurs independently of age, disease duration and severity, unlike in patients with PBC. We found a strong positive association between BMD and both bone resorption and the Th17 cell frequency, and we demonstrate that *Il17af* deletion prevents increased osteoclastogenesis and osteopenia in an established mouse model of PSC.

The pathogenesis of liver disease-associated bone loss is not well understood. It has been speculated that increased levels of bilirubin may inhibit bone formation in affected patients.[26] However, molecular studies regarding the effects of bilirubin on cultured osteoblasts have generally been performed using the unconjugated form, whereas patients with PSC usually show increased levels of conjugated bilirubin in cases of biliary obstruction or advanced disease. In this regard, it is important to state that we observed no negative influence of conjugated bilirubin on primary murine osteoblasts, whereas unconjugated bilirubin inhibited the formation of mineralized matrix and increased *Casp4* expression (Fig. S6). Moreover, reduced bone formation in patients with PSC, together with increased bone resorption, has only been described in one study, where the authors applied histomorphometry to examine iliac crest biopsies obtained from patients with PSC at the time of liver transplantation.[27] Importantly, however, the mean value of bilirubin was 8.39 mg/dl in this analyzed cohort, whereas most patients analyzed in our study were at an early stage of disease, with a mean serum bilirubin level of 1.1 mg/dl. This difference indicates that cholestatic disorders may differentially affect bone remodeling at an early or advanced stage of the disease. Therefore, the large heterogeneity in bone mass parameters in our cohort enabled us to identify specific associations potentially explaining the bone remodeling disturbances in patients with early stage PSC.

Interestingly, we found that unlike in patients with PBC, bone loss in PSC patients was not associated with liver fibrosis as measured by FibroScan. This finding is in line with that of a study demonstrating that the severity of liver disease does not predict bone loss in patients with PSC.[28] However, in contrast to the largest study of bone mineral status in patients with PSC to date,[11] age, BMI and the presence of IBD were not associated with bone loss in our patient cohort. Although it is difficult to provide a full explanation for this inconsistency, it is important to state that the respective manuscript represents a longitudinal study in which patients with PSC were followed for 10 years, whereas we performed a cross-sectional study. Moreover, since previous studies have not included determinations of bone turnover markers or structural parameters, we cannot fully relate our own findings to those published data.

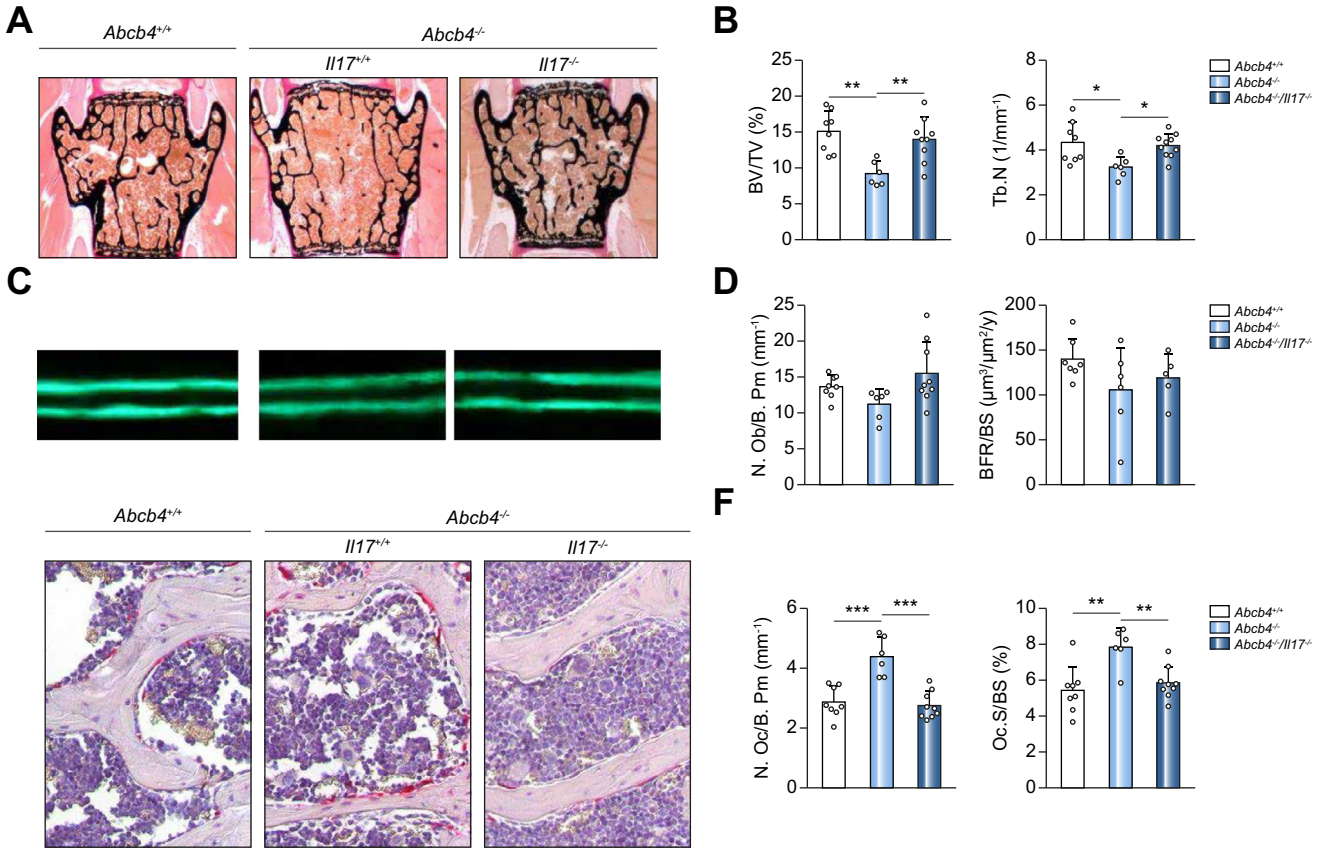


Fig. 6. Increased osteoclastogenesis in *Abcb4*$^{-/-}$ mice is corrected by additional *Il17af* deficiency. (A) Representative von Kossa/van Gieson-stained vertebral body sections (left) obtained from 20-week-old female wild-type, *Abcb4*$^{-/-}$ and *Abcb4*$^{-/-}$/*Il17af*$^{-/-}$ littermate mice. (B) Histomorphometric quantification of the trabecular bone volume (BV/TV) and the trabecular number (Tb.N.) is given on the right (n ≥6). (C) Representative fluorescence micrographs (left) showing calcein-labeled bone surfaces of 20-week-old female wild-type, *Abcb4*$^{-/-}$ and *Abcb4*$^{-/-}$/*Il17af*$^{-/-}$ littermate mice. (D) Histomorphometric quantification of the osteoblast number (N.Ob/B.Pm, number of osteoblasts per bone perimeter) and the bone formation rate (BFR/BS, bone formation rate per bone surface) is given on the right (n ≥5). (E) Representative images of TRAP activity staining (left) showing increased osteoclastogenesis in *Abcb4*$^{-/-}$ mice. (F) Histomorphometric quantification of the osteoclast number (N.Oc/B.Pm, number of osteoclasts per bone perimeter) and surface (OcS/BS, osteoclast surface per bone surface) in the 3 groups of mice is given on the right (n ≥6). Bars represent the mean ± SD. Asterisks indicate statistically significant differences between the groups. **p* <0.05; ***p* <0.005; ****p* <0.0005 (ANOVA for all panels).

In fact, by applying HR-pQCT, we show for the first time that bone loss in patients with PSC occurs predominantly at the cortical site. Similarly, we have recently demonstrated that patients with PBC display a significant decrease in the Ct.Th compared to age- and sex-matched healthy controls.[16] Compared to trabecular bone, cortical bone is characterized by a smaller surface/matrix volume ratio and a lower bone remodeling rate. Therefore, cortical bone loss is thought to occur more slowly, yet it significantly diminishes bone strength. In fact, we have previously reported an association between the Ct.Th and prevalent fractures, as have others.[29,30] Consistent with the patient data, the *Abcb4*$^{-/-}$ mice also displayed a decreased Ct.Th, yet this was not significantly increased by the additional *Il17af* deficiency.

Importantly, however, our study clearly identifies increased osteoclastogenesis as a cause of osteopenia in *Abcb4*$^{-/-}$ mice, with full correction of the relevant parameters in *Abcb4*$^{-/-}$/*Il17af*$^{-/-}$ mice. Notably, although a moderate skeletal phenotype in *Abcb4*$^{-/-}$ mice has previously been reported, there was no previous knowledge on the specific disturbances in bone remodeling cell types, since cellular and dynamic histomorphometry analyses had not yet been performed. In full agreement with the data observed in mice, we found that the Th17 cell frequency in our PSC cohort was strongly associated with the BMD and urinary Dpd levels. There is growing evidence suggesting that special subsets of T cells can directly induce osteoclastogenesis by producing certain cytokines. In this context, IL-17A may play a crucial role in promoting osteoclast differentiation by inducing the pro-osteoclastogenic cytokine RANKL[31] and by recruiting and activating other immune cells.[32,33] We have previously found that the administration of IL-17 to murine osteoblasts immediately induces the expression of Rankl in its transmembrane form,[34] which is in full agreement with the results of published studies demonstrating that the pro-osteoclastogenic influence of IL-17 requires a coculture system.[31,35] In line with these studies, Th17 cells have been implicated in the bone pathologies found in several autoimmune diseases, such as lupus erythematosus, multiple sclerosis, psoriasis and rheumatoid arthritis.[36] IL-17- and TNFα-producing cells have also been found to be associated with the induction of osteoclast differentiation in patients with IBD.[23] Hence, the concept of the Th17 cell-mediated induction of bone resorption is broadly accepted.

A recently published study has demonstrated that intrahepatic IL-17 production is involved in promoting liver fibrosis in *Abcb4*$^{-/-}$ mice.[25] It was found that the treatment of

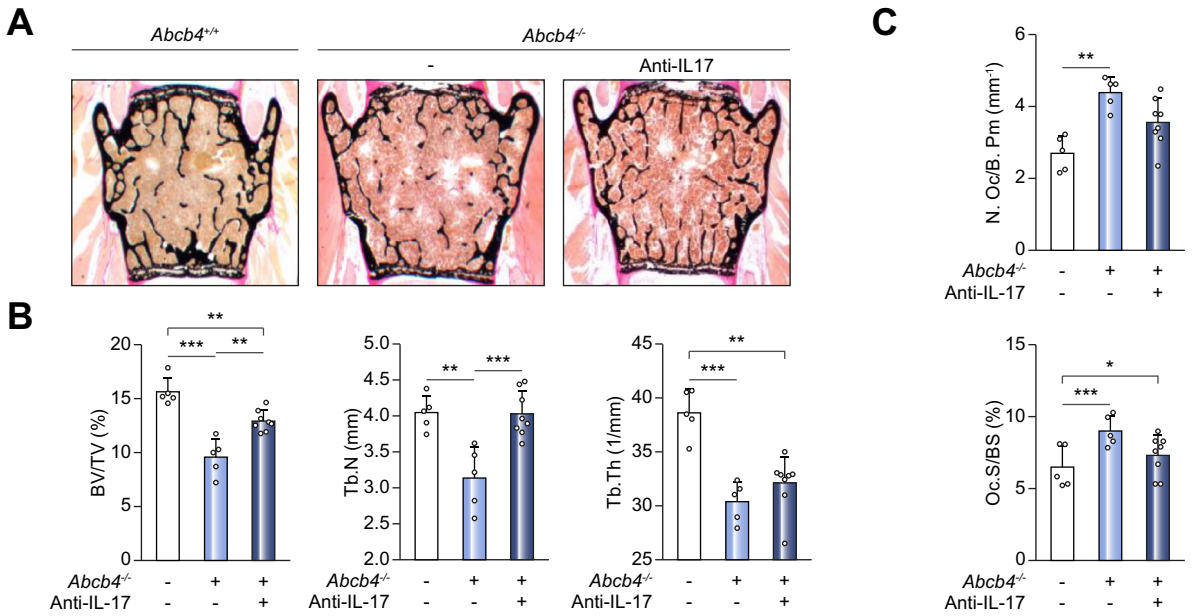


Fig. 7. IL-17A neutralization in *Abcb4*-deficient mice increases trabecular bone mass and decreases osteoclast number. (A) Representative von Kossa/van Gieson-stained vertebral body sections obtained from 20-week-old female wild-type, *Abcb4*$^{-/-}$ mice and *Abcb4*$^{-/-}$ mice treated with a regular (3 times a week) injection of IL-17A-neutralizing antibody for 4 weeks. (B) Histomorphometric quantification of the trabecular bone volume (BV/TV) and trabecular number (Tb. N). (C) Histomorphometric quantification of the osteoclast number (N.Oc/B.Pm, number of osteoclasts per bone perimeter) and surface (OcS/BS, osteoclast surface per bone surface) in the same groups of mice (n = 5–8). Bars represent the mean ± SD. Asterisks indicate statistically significant differences between the groups. *p <0.05; **p <0.005; ***p <0.0005 (ANOVA for all panels).

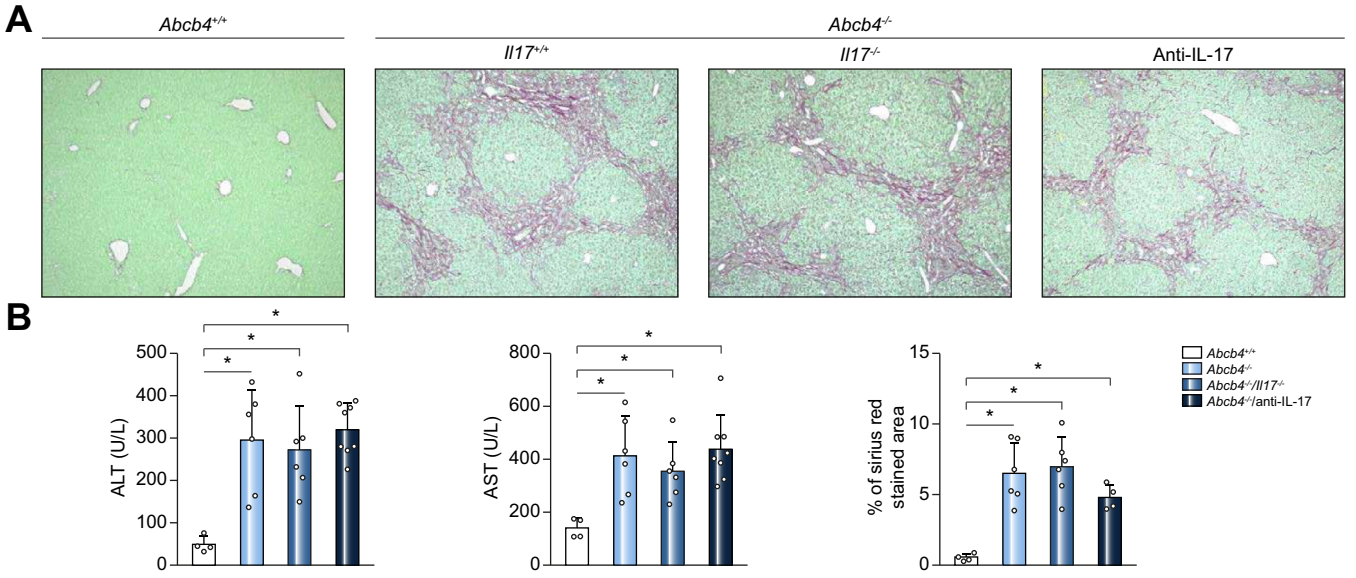


Fig. 8. The influence of *Il17af* deficiency or IL-17 blockade on the *Abcb4*-deficient bone phenotype is independent of liver fibrosis. (A) Representative sirius red-stained liver sections obtained from 20-week-old female wild-type, *Abcb4*$^{-/-}$, *Abcb4*$^{-/-}$/*Il17af*$^{-/-}$ and anti-IL-17-treated *Abcb4*$^{-/-}$ mice. (B) Quantification of hepatic fibrosis (left) and serum ALT/AST levels (right) in the same groups of mice (n = 5–8). Bars represent the mean ± SD. Asterisks indicate statistically significant differences between the groups. *p <0.05 (ANOVA for all panels). ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Abcb4$^{-/-}$ mice with anti-IL-17 caused a moderate yet significant reduction in the hepatic fibrosis score. In contrast to our study, these authors used a longer treatment period (8 to 25 weeks of age) and a different genetic background (FVB/NJ). Since there is experimental evidence for the impact of genetic background on liver fibrosis,[37] this might explain why even *Il17af* deficiency did not impact the liver phenotype of *Abcb4*$^{-/-}$ mice in our experimental setting. On that point, it is also important to state that we did not observe bile duct stones in our group of 20-week-old female mice, as has previously been described in *Abcb4*$^{-/-}$ mice with the FVB/NJ background.[17] Since the same study reported a later phenotype onset in male *Abcb4*$^{-/-}$ mice, we also analyzed 20-week-old male *Abcb4*$^{-/-}$ mice and observed increased osteoclastogenesis, which was corrected by the additional *Il17af* deficiency without affecting the liver pathology (Fig. S7). In any case, although our findings certainly do not rule

out that intrahepatic IL-17 production is involved in the pathogenesis of cholestatic disorders, they clearly show that the correction of the *Abcb4*$^{-/-}$ bone phenotype by IL-17 deficiency or blockade is not explained by beneficial effects on the liver in our experimental setting.

One major limitation of our study is that Th17 cells were only quantified in a subset of patients (n = 40). Nevertheless, the association between Th17 cells and bone mass in these patients with PSC raises the question of whether anti-IL-17A treatment could be beneficial for preventing osteoporosis in affected individuals. In a proof-of-principle experiment, we found that the treatment of *Abcb4*$^{-/-}$ mice with an IL-17A antibody for 4 weeks significantly increased the trabecular bone volume while reducing osteoclastogenesis, which is in line with studies reporting the positive effects of IL-17A neutralization on bone mass in different mouse models of inflammatory disorders.[22,38] Since human monoclonal IL-17A antibodies, *i.e.*, brodalumab or secukinumab, have been found to be effective in patients with psoriasis and ankylosing spondylitis,[39,40] such a treatment is possible in the case of PSC. In our opinion, this is indeed an important issue, since most patients with PSC will require liver transplantation, and their subsequent treatment is a strong risk factor for osteoporosis. In other words, should the respective individuals already display low bone mass at the time of transplantation, they will have a higher risk of skeletal fractures in the future. This latter argument highlights the clinical relevance of our study, as it suggests that patients with PSC should be monitored for bone mass and resorption at an early stage of the disease and that a subset of the patients will require antiresorptive treatment. The same applies for PBC, autoimmune hepatitis and overlapping syndromes, although their molecular impact on bone remodeling still needs to be established.[41]

Financial support

This study was supported by the Deutsche Forschungsgemeinschaft in the context of the clinical research group KFO 306.

Conflict of interest

The authors state that they have no conflicts of interest. CS is supported by the Helmut and Hannelore Greve Foundation and the YAEL Foundation.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design: Wolfgang Rüther, Ralf Oheim, Ansgar W. Lohse, Christoph Schramm, Thorsten Schinke, and Michael Amling. Acquisition of data: Tobias Schmidt, Anke Jeschke, Dorothee Schwinge, Constantin Schmidt, Tim Rolvien, Sebastian Butscheidt, Stephanie Stein, Marvin Kriz, Lilly Kunzmann, Haider Mussawy, Jan Hubert, and Thelonius Hawellek. Analysis and interpretation of data: Tobias Schmidt, Tim Rolvien, Thorsten Schinke, and Michael Amling. Drafting of the manuscript: Tobias Schmidt and Thorsten Schinke. Critical revision of the manuscript for important intellectual content: Ansgar W. Lohse, and Christoph Schramm. Statistical analysis: Tobias Schmidt. Administrative, technical, or material support: Mona Neven. Study supervision: Wolfgang Rüther, Ralf Oheim, Florian Barvencik, Ansgar W. Lohse, Christoph Schramm, Thorsten Schinke, and Michael Amling.

Acknowledgments

We are grateful for the excellent technical assistance of Olga Winter and Angelika Schmidt.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2018.12.035.

References

[1] Lazaridis KN, LaRusso NF. Primary sclerosing cholangitis. N Engl J Med 2016;375:1161–1170.
[2] Boonstra K, Beuers U, Ponsioen CY. Epidemiology of primary sclerosing cholangitis and primary biliary cirrhosis: a systematic review. J Hepatol 2012;56:1181–1188.
[3] Ji SG, Juran BD, Mucha S, et al. Genome-wide association study of primary sclerosing cholangitis identifies new risk loci and quantifies the genetic relationship with inflammatory bowel disease. Nat Genet 2017;49:269–273.
[4] Eaton JE, Talwalkar JA, Lazaridis KN, et al. Pathogenesis of primary sclerosing cholangitis and advances in diagnosis and management. Gastroenterology 2013;145:521–536.
[5] Tilg H, Cani PD, Mayer EA. Gut microbiome and liver diseases. Gut 2016;65:2035–2044.
[6] Kummen M, Holm K, Anmarkrud JA, et al. The gut microbial profile in patients with primary sclerosing cholangitis is distinct from patients with ulcerative colitis without biliary disease and healthy controls. Gut 2017;66:611–619.
[7] Katt J, Schwinge D, Schoknecht T, et al. Increased T helper type 17 response to pathogen stimulation in patients with primary sclerosing cholangitis. Hepatology 2013;58:1084–1093.
[8] Seki A, Ikeda F, Miyatake H, et al. Risk of secondary osteoporosis due to lobular cholestasis in non-cirrhotic primary biliary cholangitis. J Gastroenterol Hepatol 2017;32:1611–1616.
[9] Wariaghli G, Allali F, El Maghraoui A, et al. Osteoporosis in patients with primary biliary cirrhosis. Eur J Gastroenterol Hepatol 2010;22:1397–1401.
[10] Guanabens N, Pares A, Ros I, et al. Severity of cholestasis and advanced histological stage but not menopausal status are the major risk factors for osteoporosis in primary biliary cirrhosis. J Hepatol 2005;42:573–577.
[11] Angulo P, Grandison GA, Fong DG, et al. Bone disease in patients with primary sclerosing cholangitis. Gastroenterology 2011;140:180–188.
[12] EASL Clinical Practice Guidelines. The diagnosis and management of patients with primary biliary cholangitis. J Hepatol 2017;67:145–172.
[13] European Association for the Study of the L. EASL Clinical Practice Guidelines: Management of cholestatic liver diseases. J Hepatol;51:237-267.
[14] Hartl J, Denzer U, Ehlken H, et al. Transient elastography in autoimmune hepatitis: timing determines the impact of inflammation and fibrosis. J Hepatol 2016;65:769–775.
[15] Burt LA, Liang Z, Sajobi TT, et al. Sex- and site-specific normative data curves for HR-pQCT. J Bone Miner Res 2016;31:2041–2047.
[16] Schmidt T, Schmidt C, Schmidt FN, et al. Disease duration and stage influence bone microstructure in patients with primary biliary cholangitis. J Bone Miner Res 2018;33:1011–1019.
[17] Lammert F, Wang DQ, Hillebrandt S, et al. Spontaneous cholecysto- and hepatolithiasis in Mdr2−/− mice: a model for low phospholipid-associated cholelithiasis. Hepatology 2004;39:117–128.
[18] Haas JD, Ravens S, Duber S, et al. Development of interleukin-17-producing gammadelta T cells is restricted to a functional embryonic wave. Immunity 2012;37:48–59.
[19] Schinke T, Schilling AF, Baranowsky A, et al. Impaired gastric acidification negatively affects calcium homeostasis and bone mass. Nat Med 2009;15:674–681.
[20] Thiele ND, Wirth JW, Steins D, et al. TIMP-1 is upregulated, but not essential in hepatic fibrogenesis and carcinogenesis in mice. Sci Rep 2017;7:714.

[21] Briot K, Roux C. Glucocorticoid-induced osteoporosis. RMD Open 2015;1 e000014.
[22] Uluckan O, Jimenez M, Karbach S, et al. Chronic skin inflammation leads to bone loss by IL-17-mediated inhibition of Wnt signaling in osteoblasts. Sci Transl Med 2016;8:330ra37.
[23] Ciucci T, Ibanez L, Boucoiran A, et al. Bone marrow Th17 TNFalpha cells induce osteoclast differentiation, and link bone destruction to IBD. Gut 2015;64:1072–1081.
[24] Hochrath K, Ehnert S, Ackert-Bicknell CL, et al. Modeling hepatic osteodystrophy in Abcb4 deficient mice. Bone 2013;55:501–511.
[25] Tedesco D, Thapa M, Chin CY, et al. Alterations in intestinal microbiota lead to production of interleukin 17 by intrahepatic gammadelta T-cell receptor-positive cells and pathogenesis of cholestatic liver disease. Gastroenterology 2018;154:2178–2193.
[26] Ruiz-Gaspa S, Martinez-Ferrer A, Guanabens N, et al. Effects of bilirubin and sera from jaundiced patients on osteoblasts: contribution to the development of osteoporosis in liver diseases. Hepatology 2011;54:2104–2113.
[27] Guichelaar MM, Malinchoc M, Sibonga J, et al. Bone metabolism in advanced cholestatic liver disease: analysis by bone histomorphometry. Hepatology 2002;36:895–903.
[28] Campbell MS, Lichtenstein GR, Rhim AD, et al. Severity of liver disease does not predict osteopenia or low bone mineral density in primary sclerosing cholangitis. Liver Int 2005;25:311–316.
[29] Sornay-Rendu E, Boutroy S, Munoz F, et al. Alterations of cortical and trabecular architecture are associated with fractures in postmenopausal women, partially independent of decreased BMD measured by DXA: the OFELY study. J Bone Miner Res 2007;22:425–433.
[30] Mussawy H, Ferrari G, Schmidt FN, et al. Changes in cortical microarchitecture are independent of areal bone mineral density in patients with fragility fractures. Injury 2017.
[31] Kotake S, Udagawa N, Takahashi N, et al. IL-17 in synovial fluids from patients with rheumatoid arthritis is a potent stimulator of osteoclastogenesis. J Clin Invest 1999;103:1345–1352.
[32] Dong C. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. Nat Rev Immunol 2006;6:329–333.
[33] Kikuta J, Wada Y, Kowada T, et al. Dynamic visualization of RANKL and Th17-mediated osteoclast function. J Clin Invest 2013;123:866–873.
[34] Heckt T, Keller J, Peters S, et al. Parathyroid hormone induces expression and proteolytic processing of Rankl in primary murine osteoblasts. Bone 2016;92:85–93.
[35] Sato K, Suematsu A, Okamoto K, et al. Th17 functions as an osteoclastogenic helper T cell subset that links T cell activation and bone destruction. J Exp Med 2006;203:2673–2682.
[36] Singh RP, Hasan S, Sharma S, et al. Th17 cells in inflammation and autoimmunity. Autoimmun Rev 2014;13:1174–1181.
[37] Ikenaga N, Liu SB, Sverdlov DY, et al. A new Mdr2(−/−) mouse model of sclerosing cholangitis with rapid fibrosis progression, early-onset portal hypertension, and liver cancer. Am J Pathol 2015;185:325–334.
[38] Li J-Y, D'Amelio P, Robinson J, et al. IL-17A is increased in humans with primary hyperparathyroidism and mediates PTH-induced bone loss in mice. Cell Metab 2015;22:799–810.
[39] Baeten D, Sieper J, Braun J, et al. Secukinumab, an interleukin-17A inhibitor, in ankylosing spondylitis. N Engl J Med 2015;373:2534–2548.
[40] Mease PJ, McInnes IB, Kirkham B, et al. Secukinumab inhibition of interleukin-17A in patients with psoriatic arthritis. N Engl J Med 2015;373:1329–1339.
[41] Kerkar N, Chan A. Autoimmune hepatitis, sclerosing cholangitis, and autoimmune sclerosing cholangitis or overlap syndrome. Clin Liver Dis 2018;22:689–702.