Murine $\text{TNF}^{\Delta\text{ARE}}$ Crohn’s Disease Model Displays Diminished Expression of Intestinal $\text{Ca}^{2+}$ Transporters

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Background: Patients suffering from Crohn’s disease (CD) show increased incidence of low bone mineral density. Investigating this complication is difficult because the exact etiology of CD remains elusive. Mice carrying a deletion in the tumor necrosis factor (TNF) AU-rich elements (ARE) are reported as a model for human CD and are characterized by elevated TNF-α levels and inflammations in the terminal ileum. To evaluate whether these mice have a $\text{Ca}^{2+}$ handling problem, this study analyzed the $\text{Ca}^{2+}$ homeostasis in heterozygous $\text{TNF}^{\Delta\text{ARE}}$ mice ($\text{TNF}^{\Delta\text{ARE}+/+}$) in comparison to wildtype littermates.

Methods: Beside serum $\text{Ca}^{2+}$ and vitamin D levels, the expression of $\text{Ca}^{2+}$ transporters was analyzed in intestine, kidney and bone using quantitative real-time PCR, Western blot and immunohistochemistry. Bone scans were performed to measure bone parameters.

Results: $\text{Ca}^{2+}$ transporters in duodenum (TRPV6, calbindin-D$_{9K}$, PMCA1b) and kidney (TRPV5, calbindin-D$_{28K}$, NCX1) showed significantly reduced mRNA expression levels in $\text{TNF}^{\Delta\text{ARE}+/-}$ mice, except for renal TRPV5. In bone, only calbindin-D$_{9K}$ mRNA displayed a significant down-regulation. These findings were supported by declined duodenal calbindin-D$_{9K}$ and renal calbindin-D$_{28K}$ protein values. Likely, this down-regulation of $\text{Ca}^{2+}$ transporters in $\text{TNF}^{\Delta\text{ARE}+/-}$ mice is mediated by the $\text{TNF}$-α homeostasis in heterozygous $\text{TNF}^{\Delta\text{ARE}}$ mice ($\text{TNF}^{\Delta\text{ARE}+/+}$) in comparison to wildtype littermates.

Indeed, microcomputed tomography scanning demonstrated reduced trabecular and cortical bone thickness and volume in $\text{TNF}^{\Delta\text{ARE}+/-}$ mice. This finding is further supported by increased total deoxypyridinoline in serum.

Conclusions: Our results imply that $\text{TNF}^{\Delta\text{ARE}+/-}$ mice have a disturbed $\text{Ca}^{2+}$ homeostasis characterized by reduced duodenal and renal $\text{Ca}^{2+}$ transporters, diminished 1,25(OH)$_2$D$_3$ levels, and increased bone resorption associated with profound bone abnormalities.

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Key Words: IBD, calcium, TRPV5, TRPV6, osteoporosis

Crohn’s disease (CD) patients suffer from severe gastrointestinal inflammations that are mainly confined to the terminal ileum and colon. To date, the exact etiology of CD has not been elucidated; however, a multifactorial cause is implicated. This includes both genetic factors, such as carrying a variant of the nucleotide oligodimerization domain 2 (NOD2) gene$^{1,2}$ and environmental influences like nutrition and bacterial flora of the intestine.$^{3–5}$ Furthermore, immunologic factors are likely to play a primary role. An immune response is mediated by T cells that produce large amounts of proinflammatory cytokines. CD patients are characterized by elevated production of the Th1 cytokines IL-2, interferon (IFN)-γ, and tumor necrosis factor alpha (TNF-α).$^6$ Moreover, anti-TNF-α therapy has shown to be highly effective to inhibit inflammation.$^7$

Additionally, it has been revealed that patients with CD have an increased incidence of low bone mineral density (BMD). Previous studies reported that 36%–55% of CD patients are osteopenic, whereas 6%–58% are osteoporotic.$^8–12$ This low BMD has been associated with increased fracture risk and its cause is, therefore, extensively studied. The main potential risk factors for developing low BMD are the presence of intestinal $\text{Ca}^{2+}$ and vitamin D malabsorption, high circulating levels of inflammatory cytokines which can directly affect osteoclast and osteoblast activity, sex hormone deficiency, corticosteroid therapy and malnourishment.

$\text{Ca}^{2+}$ is an essential bone component and plays a key role in bone mineralization. In the body, serum $\text{Ca}^{2+}$ is maintained at constant levels via the concerted action of intestinal uptake from food, renal reabsorption from pro-
urine, and skeletal storage. Intestinal Ca^{2+} absorption and renal tubular reabsorption of filtered Ca^{2+} are modulated on the basis of the body’s overall need for Ca^{2+} gain or loss. Part of the Ca^{2+} (re)absorption in kidney and intestine occurs through the active transcellular pathway. This is a 3-step process in which Ca^{2+} first enters the epithelial cell via the apical (luminal) Ca^{2+} channels, TRPV5 and TRPV6, respectively. Subsequently, Ca^{2+} is transported across the cell in association with Ca^{2+}-buffering proteins, calbindin-D_{9K} and calbindin-D_{28K}, and finally Ca^{2+} is extruded across the basolateral membrane into the bloodstream via the Na^{+}/Ca^{2+}-exchanger (NCX1) in the kidney and the plasma membrane Ca^{2+}-ATPase (PMCA1b) in duodenum.

This active transport occurs in the distal convoluted tubule (DCT) and connecting tubule (CNT) in kidney and in the enterocytes of the duodenum. In bone, Ca^{2+} transporters are present in osteoblasts and osteoclasts, where they are thought to be involved in bone modeling. Moreover, these Ca^{2+} absorptive cells are the primary targets for regulation by calcitropic hormones, including 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the active metabolite of vitamin D, and parathyroid hormone (PTH). Alterations in expression of the above-mentioned active Ca^{2+} transporters in duodenum, kidney, and bone can disturb the Ca^{2+} homeostasis that might contribute to the establishment of low BMD.

However, to study the involvement of active Ca^{2+} absorption in the occurrence of low BMD in CD, a proper animal model is required. In the literature, several murine models have been developed to mimic this human disease. Besides chemically, immunologically, or genetically induced models have been developed to mimic this human disease. A new murine model of Crohn’s-like ileitis has been reported, the TNF^{ARE/-} model, that closely resembles human characteristics of CD. These mice have a deletion in the TNF AU-rich elements (ARE), which are responsible for TNF-α mRNA destabilization and translational repression. As a consequence, they exhibit markedly increased TNF-α serum levels and develop a specific human CD-like phenotype characterized by inflammations primarily localized to the terminal ileum and occasionally to the proximal colon. Heterozygous TNF^{ARE} mice (TNF^{ARE/-}) at 4 months of age displayed severe inflammation in the ileum illustrated by mucosal and submucosal infiltration of chronic and acute inflammatory cells. No prominent signs of diarrhea were observed. Furthermore, these mice show features of arthritis.

The aim of the present study, therefore, was to evaluate the Ca^{2+} homeostasis in heterozygous TNF^{ARE/-} mice compared to its wildtype littermates. To this end we investigated serum Ca^{2+}, 1,25(OH)₂D₃, and 25-hydroxyvitamin D (25(OH)D) levels and analyzed the abundance of active Ca^{2+} transporters in the small intestine, kidney, and bone. Finally, bone conditions were examined performing microcomputed tomography scanning and total deoxypyridinoline (tDPD) serum measurements. Ultimately, this information could be important to develop new therapies for CD-related bone abnormalities.

**MATERIALS AND METHODS**

**Animal Protocol**

Ca^{2+} homeostasis was analyzed in female heterozygous TNF^{ARE/-} mice which were generated as previously described. Homozygous TNF^{ARE/ARE} mice die between 5 and 12 weeks of age; therefore, experiments were performed with heterozygous TNF^{ARE/-} mice that develop severe intestinal inflammation and arthritis. The mice (n = 8) were 4 months old with established inflammation in the ileum and occasionally in the proximal colon. Wildtype littermates were used as controls (n = 8). All mice were bred and maintained on a mixed C57BL/6J×129S6 genetic background in the animal facilities of the Biomedical Sciences Research Center “Alexander Fleming” under specific pathogen-free conditions. The mice were used in accordance with the guidance of the Institutional Animal Care and Use Committee of B.S.R.C. “Alexander Fleming.” After 4 months, when the inflammations in the ileum were fully developed, blood samples were taken and the mice were sacrificed. Duodenum, kidney, and femurs were sampled and immediately frozen in liquid nitrogen. Samples were stored at −80°C until further processed. The animal ethics board of the Radboud University Nijmegen approved all experimental procedures.

**Serum Biochemistry**

Serum Ca^{2+} concentrations were determined using a colorimetric assay as described previously. Serum 25(OH)D and 1,25(OH)₂D₃ plasma levels were measured using immunoextraction followed by 125I-RIA quantification (Diasorin, Stillwater, MN, and IDS, Boldon, UK, respectively). tDPD, a marker for the amount of bone resorption, was analyzed in serum utilizing the Metra total DPD EIA kit (Quidel, San Diego, CA).

**Quantitative Real-time PCR Analysis**

Total RNA was extracted from duodenum, kidney, and bone using Trizol Total RNA Isolation Reagent (Life Technologies, Breda, the Netherlands). For isolation of bone mRNA, femurs were pulverized using a Dismembrator (Satorius, Goettingen, Germany) at 2800 rpm for 1 minute. The obtained total RNA was subjected to DNase treatment to prevent genomic DNA contamination. Thereafter, 1.5 μg of total RNA was reverse transcribed by Moloney-murine leukemia virus-reverse transcriptase (Life Technologies), as described previously. The obtained cDNA was used to determine TRPV5, calbindin-D_{28K}, and NCX1 mRNA levels in kidney cortex as well as TRPV6, calbindin-D_{9K}, and
PMCA1b mRNA expression in duodenum. Moreover, all 6 above-mentioned Ca\(^{2+}\) transporters were analyzed in bone. Additionally, the enzyme 25-hydroxyvitamin D\(_3\)-1α-hydroxylase (1α-OHase) was measured in renal tissue. The housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) was used as an endogenous control. Expression levels were quantified by real-time quantitative polymerase chain reaction (PCR) on an ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland). The primers and fluorescent probes used are as previously described (Biolegio, Malden, the Netherlands).\(^{21,22}\)

**Immunoblotting**

For protein analysis, frozen duodenal and kidney tissues were homogenized in ice-cold solubilization buffer as previously described.\(^{23}\) Protein concentration of the homogenates was determined (Bio-Rad Protein Assay; Bio-Rad, Munich, Germany) and samples were normalized accordingly. Subsequently, total protein fractions (10 μg) were separated on 12% or 16.5% (wt/vol) SDS-PAGE gels for renal calbindin-D\(_{28K}\) and duodenal calbindin-D\(_{9K}\), respectively, and blotted to polyvinylidine difluoride (PVDF)-nitrocellulose membranes (Immobilon-P, Millipore, Bedford, MA). Blots were incubated overnight at 4°C with calbindin-D\(_{28K}\) antibody (Swant, Bellizona, Switzerland) (1:5000), calbindin-D\(_{9K}\) antibody (Swant, Bellizona, Switzerland) (1:5000), or β-actin antibody (1:25,000) as an internal control. Thereafter, blots were incubated with a goat antirabbit (calbindin-D\(_{28K}\) and calbindin-D\(_{9K}\)) or goat antimouse (β-actin) peroxidase coupled secondary antibody (Sigma Chemical, St. Louis, MO) (1:10,000). Immunoreactive protein was detected using the chemiluminescence (ECL) method as described by the manufacturer (Amersham, Buckinghamshire, UK). Immunopositive bands were scanned using an imaging densitometer (Bio-Rad Gs-690) to determine pixel density (Molecular Analyst Software; Bio-Rad Laboratories, Hercules, CA).

**Immunohistochemistry**

Staining of TRPV5 protein in kidney samples was performed on periodate-lysine-paraformaldehyde-fixed cryosections as described previously.\(^{24}\) For semiquantitative determination of protein abundance, images were made using a Zeiss fluorescence microscope equipped with a digital camera (Nikon DXM1200), and subsequently analyzed with the Image Pro Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD), resulting in quantification of the protein levels as the mean of integrated optical density (IOD).

**Bone Structural Analysis**

After fixation in 10% formalin and measuring their lengths, femurs from both wildtype (\(n = 7\)) and TNF\(^{ΔARE/+}\) (\(n = 8\)) mice were studied in detail by scanning them in a microcomputed tomography (μCT) scanner (Skyscan 1076, Skyscan, Aartselaar, Belgium). Scans were processed and a 3D morphometric analysis of the bone was performed using free software of the 3D-calculator project (http://www2.eur.nl/fgg/orthopaedics/variious.php?sm=6&item=downloads) as described earlier.\(^{25,26}\) All parameters were expressed according to the bone histomorphometry nomenclature.\(^{27}\) The region of interest was confined to a region from halfway the femoral head until the metaphysis. This region contained both cortical and trabecular bone structures, enabling accurate analysis of a number of parameters in both compartments.

**Statistical Analyses**

Data are expressed as means ± SE. Statistical comparisons were performed by Student’s \(t\)-test. \(P < 0.05\) was considered statistically significant. All analyses were performed using the StatView Statistical Package software (Power PC version 4.51, Berkeley, CA) on an Apple iMac computer.

**RESULTS**

**Serum Biochemistry**

Blood was collected from TNF\(^{ΔARE/+}\) mice and wildtype littermates after which serum and plasma analyses were performed to determine Ca\(^{2+}\), 25(OH)\(_2\)D, and 1,25(OH)\(_2\)D\(_3\) concentrations as well as the amount of tDPD. The results are shown in Table 1. Serum Ca\(^{2+}\) concentrations were comparable between both groups, while both 25(OH)\(_2\)D and 1,25(OH)\(_2\)D\(_3\) values were reduced in TNF\(^{ΔARE/+}\) mice, of which serum 1,25(OH)\(_2\)D\(_3\) illustrated a 2-fold decline. Furthermore, tDPD was significantly elevated in TNF\(^{ΔARE/+}\) mice compared to their wildtype littermates.

**Duodenal Expression of Active Ca\(^{2+}\) Transporters**

By quantitative real-time PCR analysis, mRNA expression levels of the Ca\(^{2+}\) transporters TRPV6, calbindin-D\(_{9K}\), and PMCA1b in the intestine were quantified in wildtype and TNF\(^{ΔARE/+}\) mice. The results demonstrated a reduction in TRPV6, calbindin-D\(_{9K}\), and PMCA1b mRNA levels to 30 ± 9%, 38 ± 8%, and 71 ± 8%, respectively, in TNF\(^{ΔARE/+}\) mice.

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**Table 1. Serum Parameters of Wildtype and TNF\(^{ΔARE/+}\) Mice**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wildtype</th>
<th>TNF(^{ΔARE/+})</th>
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<tbody>
<tr>
<td>Ca(^{2+}) (mmol/L)</td>
<td>2.36 ± 0.03</td>
<td>2.32 ± 0.04</td>
</tr>
<tr>
<td>1,25(OH)(_2)D(_3) (pmol/L)</td>
<td>178 ± 8</td>
<td>79 ± 16*</td>
</tr>
<tr>
<td>25(OH)D (mmol/L)</td>
<td>18 ± 2</td>
<td>13 ± 1*</td>
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<tr>
<td>tDPD (mmol/L)</td>
<td>5.8 ± 0.3</td>
<td>6.8 ± 0.3*</td>
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tDPD, total deoxypyridinoline.

Data are presented as mean ± SE (\(n = 8\)).
*\(P < 0.05\) vs. wildtype.
mice compared to wildtype littermates (Fig. 1A). In line with the mRNA data, calbindin-D_{28K} protein abundance was declined in the TNF^{ΔARE/Δ} group, as confirmed by immunoblotting (Fig. 1B). Furthermore, the pixel density of the obtained immunopositive bands was semiquantified, disclosing a 2-fold downregulation of duodenal calbindin-D_{28K} protein in TNF^{ΔARE/Δ} mice.

Renal Expression of Active Ca^{2+} Transporters

To evaluate the expression of renal Ca^{2+} transporters in TNF^{ΔARE/Δ} versus wildtype mice, TRPV5, calbindin-D_{28K}, and NCX1 mRNA levels were determined by quantitative real-time PCR analysis, and protein abundance was analyzed by immunohistochemistry and immunoblotting. Calbindin-D_{28K} and NCX1 mRNA levels were significantly downregulated to 59 ± 6% and 43 ± 10%, respectively, compared to wildtype littermates (Fig. 2A). In addition, calbindin-D_{28K} protein measurements illustrate that the observed decline in calbindin-D_{28K} mRNA is accompanied by a similar reduction on protein level (Fig. 2B). In contrast, renal TRPV5 mRNA levels were unaffected in TNF^{ΔARE/Δ} mice (Fig. 2A). This observation was confirmed by additional immunohistochemical staining of kidney sections with a TRPV5 antibody showing no difference in TRPV5 protein abundance between TNF^{ΔARE/Δ} and wildtype mice (Fig. 3). Finally, the mRNA level of 1α-OHase, the enzyme responsible for 1,25(OH)_{2}D_{3} biosynthesis in kidney, was quantified. However, no significant difference (P = 0.14) in mRNA expression level was observed in the TNF^{ΔARE/Δ} group (68 ± 13%) compared to control littermates (100 ± 17%).

FIGURE 1. Duodenal expression of Ca^{2+} transporters in TNF^{ΔARE/Δ} mice. Using quantitative real-time PCR analysis, duodenal mRNA expression of the Ca^{2+} transporters was measured, corrected for HPRT, and presented as relative percentage of expression in wildtype mice (A). Immunoblot from mouse duodenal homogenates labeled with a calbindin-D_{9K} antibody and quantified by measuring pixel density as a relative percentage of expression in wildtype mice (B). Wildtype (closed bars), TNF^{ΔARE/Δ} (open bars). Data are presented as mean ± SE (n = 8), *P < 0.05 versus wildtype.

FIGURE 2. Renal expression of Ca^{2+} transporters in TNF^{ΔARE/Δ} mice. Using quantitative real-time PCR, renal mRNA expression of the Ca^{2+} transporters was measured, corrected for HPRT, and presented as relative percentage of expression in the wildtype group (A). Immunoblot from mouse kidney homogenates labeled with a calbindin-D_{28K} antibody and quantified by measuring pixel density as a relative percentage of expression in wildtype mice (B). Wildtype (closed bars), TNF^{ΔARE/Δ} (open bars). Data are presented as mean ± SE (n = 8), *P < 0.05 versus wildtype.
Bone mRNA Expression of Active Ca\(^{2+}\) Transporters

Total RNA was isolated from wildtype and \(\text{TNF}\text{-ARE/}\text{ARE}\) mouse femurs and subsequently analyzed by quantitative real-time PCR for the presence of the active Ca\(^{2+}\) transporters TRPV5, TRPV6, calbindin-D\(_{9K}\), calbindin-D\(_{28K}\), NCX1, and PMCA1b. The results are illustrated in Figure 4. The mRNA values indicate that the amount of active Ca\(^{2+}\) transporters was comparable between both groups except for calbindin-D\(_{9K}\), which was significantly downregulated in \(\text{TNF}\text{-ARE/}\text{ARE}\) mice. Calbindin-D\(_{28K}\) could not be detected in mouse femur.

Bone Structural Analysis

Detailed structural analysis of the femurs demonstrated dramatic changes in \(\text{TNF}\text{-ARE/}\text{ARE}\) mice compared to wildtype littermates (Fig. 5A). The femurs were smaller in size, as shown by reduced femur length, circumference (perimeter), and femoral head volume (Fh.V) (Fig. 5B). In the trabecular compartment of \(\text{TNF}\text{-ARE/}\text{ARE}\) mice femurs, the trabeculae were thinner (trabecular thickness, Tb.Th) and less numerous (trabecular number, Tb.N) compared to wildtype mice, leading to a smaller trabecular bone fraction (BV/TV) with less connections between trabeculae (connectivity density, CD/Ec.V) (Fig. 5B). At the cortical bone level, cortical thickness (Ct.Th) and volume (Ct.V) were dramatically reduced, which can be partially explained by the reduced perimeter and femoral head volume, but the moment of inertia (MOI), a proxy for bone strength, was also strongly decreased (Fig. 5B).

DISCUSSION

This study demonstrated that \(\text{TNF}\text{-ARE/}\text{ARE}\) mice, as a model for human CD, have a disturbed Ca\(^{2+}\) homeostasis. This conclusion is supported by a downregulation of the relevant Ca\(^{2+}\) transporters in organs involved in Ca\(^{2+}\) handling, e.g., kidney, duodenum, and bone, accompanied by decreased serum 25(OH)D and 1,25(OH)\(_{2}\)D\(_{3}\) levels. Furthermore, elevated tDPD values and structural bone analysis revealed increased bone resorption in \(\text{TNF}\text{-ARE/}\text{ARE}\) mice, presumably to replenish serum Ca\(^{2+}\), which remained unchanged. Altogether, this indicates that the \(\text{TNF}\text{-ARE/}\text{ARE}\) mouse is a suitable model to study low BMD in CD.

Previous studies have elucidated that the TRPV5 and TRPV6 channels are the rate-limiting step in active Ca\(^{2+}\) (re)absorption in kidney and duodenum, respectively. A causal relationship was observed between diminished renal TRPV5 expression and increased urinary Ca\(^{2+}\) wasting, whereas reduced duodenal TRPV6 was associated with a decline in Ca\(^{2+}\) uptake from food, as analyzed by in vivo \(^{45}\text{Ca}\) absorption assays. This connection was further supported in a TRPV5 knockout (TRPV5\(-/-\) ) mouse model. TRPV5\(-/-\) mice showed markedly increased urinary Ca\(^{2+}\) excretion and a compensatory intestinal Ca\(^{2+}\) absorption accompanied by enhanced duodenal TRPV6 levels. Furthermore, \(\alpha\)-OHase\(-/-\) mice, deficient in renal 1,25(OH)\(_{2}\)D\(_{3}\) biosynthesis, displayed a strong reduction in renal and duodenal active Ca\(^{2+}\) transporters. Consequently, these mice were unable to sufficiently (re)absorb Ca\(^{2+}\) in both kidney and duodenum resulting in severe hypocalcemia. 1,25(OH)\(_{2}\)D\(_{3}\) supplementation completely restored the phenotype of the \(\alpha\)-OHase\(-/-\) mice by normalizing the Ca\(^{2+}\) transporter expression levels. This mouse model illustrates the stimulatory effect of 1,25(OH)\(_{2}\)D\(_{3}\) on the abundance of the Ca\(^{2+}\) transporters.

Hypothetically, reduced vitamin D levels in CD patients could negatively affect the Ca\(^{2+}\) balance via diminished active Ca\(^{2+}\) (re)absorption that eventually could result...
FIGURE 5.

A  X-ray images

1

2

B  Bone parameters

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<tr>
<th></th>
<th>Wild-type</th>
<th>TNF^\text{ΔARE}\text{I}^+</th>
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<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
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<tr>
<td>Femur length (mm)</td>
<td>16.4 ± 0.2</td>
<td>15.1 ± 0.1*</td>
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<tr>
<td>Perimeter (mm)</td>
<td>8.4 ± 0.2</td>
<td>7.8 ± 0.1*</td>
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<td>Fh.V (mm(^3))</td>
<td>3.89 ± 0.11</td>
<td>3.41 ± 0.03*</td>
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<tr>
<td><strong>Trabecular</strong></td>
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<tr>
<td>Tb.Th (µm)</td>
<td>78.7 ± 2.9</td>
<td>67.1 ± 3.1*</td>
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<tr>
<td>Tb.V (mm(^3))</td>
<td>0.39 ± 0.02</td>
<td>0.25 ± 0.02*</td>
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<tr>
<td>Ec.V (mm(^3))</td>
<td>2.02 ± 0.06</td>
<td>2.15 ± 0.05</td>
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<tr>
<td>BV/TV (%)</td>
<td>0.22 ± 0.01</td>
<td>0.21 ± 0.01*</td>
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<tr>
<td>Tb.N</td>
<td>0.58 ± 0.03</td>
<td>0.44 ± 0.03*</td>
</tr>
<tr>
<td>CD/EcV (mm(^3))</td>
<td>112.0 ± 20.4</td>
<td>16.2 ± 12.5*</td>
</tr>
<tr>
<td><strong>Cortical</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct.Th (µm)</td>
<td>196.3 ± 2.7</td>
<td>156.7 ± 6.9*</td>
</tr>
<tr>
<td>Ct.V (mm(^3))</td>
<td>1.86 ± 0.07</td>
<td>1.26 ± 0.04*</td>
</tr>
<tr>
<td>MOI (mm(^4))</td>
<td>1.38 ± 0.08</td>
<td>1.08 ± 0.04*</td>
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in the observed high incidence of low BMD. Data about serum vitamin D levels in CD patients, however, are not unequivocal. Most studies report subnormal serum 25(OH)D levels, a vitamin D precursor that is converted to the active 1,25(OH)2D3 by the enzyme 1α-OHase.32–34 Hypovitaminosis D might contribute to an increased risk in the development of low BMD. Conversely, a study by Abreu et al35 measured increased serum 1,25(OH)2D3 levels and enhanced intestinal 1α-OHase activity. Although the majority of studies reported hypovitaminosis D in CD, controversy exists over whether there is a correlation between serum vitamin D metabolites and low BMD, because both positive36,37 and negative correlations38,39 have been reported. It is postulated that malabsorption of vitamin D from the diet plays an important role in the etiology of hypovitaminosis D. In the situation of inflammation or absence of the ileum, which is one of the main inflammatory sites in CD, interruption of the enterohepatic circulation may reduce bile salt secretion, which affects the absorption of vitamin D.38,39 This malabsorption is in line with the fact that low BMD is more profound in CD when the inflammatory activity in ileum is increased. Our study, investigating TNFΔARE/Δ mice as a model for CD, displayed both decreased 25(OH)D and 1,25(OH)2D3 values, likely caused by malabsorption of vitamin D precursors from the diet. A possible effect on renal 1α-OHase was unlikely since no significant differences in mRNA levels were observed between both groups. The diminished 1,25(OH)2D3 values explain the reduction in duodenal and renal Ca2+ transporters as reported in this study. Surprisingly, although serum 1,25(OH)2D3 levels declined and the renal Ca2+ transporters, calbindin-D28k and NCX1 decreased, renal TRPV5 was unaffected both on the mRNA and protein levels. Hypothetically, the kidney can compensate the negative Ca2+ balance in the body by restoring renal TRPV5 expression to normal levels, resulting in reduced urinary Ca2+ loss. Further research is necessary to unravel the underlying mechanism. Altogether, the inflammation in the ileum of TNFΔARE/Δ mice resulting in reduced serum 25(OH)D and 1,25(OH)2D3 levels is an important similarity with the human CD situation and supports the notion that hypovitaminosis D and disease activity are linked to low BMD.

The majority of the body’s Ca2+ content is stored in bone, where the balanced processes of bone formation in osteoblasts and resorption in osteoclasts maintain bone homeostasis. Previous studies have revealed that the Ca2+ transporters are present in bone. Recently, van der Eerden et al16 localized TRPV5 exclusively to the ruffled border of osteoclasts. They identified TRPV5 as an epithelial Ca2+ channel that is essential for osteoclastic bone resorption and demonstrated the significance of transcellular Ca2+ transport in osteoclastic function. We did not detect changes in bone TRPV5 mRNA in the TNFΔARE model. Remarkably, a significant decrease in calbindin-D28k mRNA expression was observed in TNFΔARE/Δ mouse femurs. However, to date the role of calbindin-D28k in bone homeostasis is still unclear. Besides increased tDPD levels in serum, the bone structural analysis performed using μCT indicated that bone metabolism is severely affected in TNFΔARE/Δ mice. The reduced bone size suggests a developmental delay in bone formation in TNFΔARE/Δ mice. The reduced bone size parameters (maximum 10% less compared to wildtype) can only partly explain the severe decline in cortical and trabecular bone volume and thickness. Together with the fact that despite a smaller bone size, the endocortical volume (Ec.V) is similar to that in wildtype mice, we suggest that increased endocortical bone resorption occurs, thereby confirming the elevated tDPD levels in serum of TNFΔARE/Δ mice. The reduced trabecular thickness may also be explained by enhanced bone resorption. However, dynamic bone formation and mineralization data in these mice is required to address this issue in more detail. Taken together, we suggest that TNFΔARE/Δ mice display a severe phenotype, including enhanced bone resorption and developmentally reduced bone formation.

Moreover, cytokines like IL-1, IL-6, and TNF-α are postulated to stimulate bone resorption through stimulating osteoclast maturation and activity. Because the murine model used has highly increased TNF-α serum levels, the effect of TNF-α on bone resorption could significantly contribute to low BMD, and therefore cannot be neglected. Furthermore, previous studies reported that 1,25(OH)2D3 supplementation can reduce inflammation through inhibition of TNF-α production.40,41 However, the impact of TNF-α on 1,25(OH)2D3 levels, the Ca2+ transporters, and bone has not been extensively studied to date.

Evaluating the Ca2+ homeostasis in TNFΔARE/Δ mice revealed decreased expression of duodenal Ca2+ transporters, suggesting diminished Ca2+ absorption from the diet. Re-

**FIGURE 5.** Bone structural analysis of femurs from wildtype and TNFΔARE/Δ mice. Femurs from wildtype (n = 7) and TNFΔARE/Δ (n = 8) mice were analyzed in detail by μCT (A). The scanned region was from halfway the femoral head (1) until the metaphysis (2). Cross-sections of a wildtype femur (left panels) contained more bone compared to TNFΔARE/Δ femurs (right panels). Arrows indicate more intense trabecular network in femoral head (top panels) and increased cortical thickness in the metaphysis (lower panels) of wildtype mice (B). Femoral length was determined. Parameters measured by μCT included perimeter, femoral head volume (Fh.V), trabecular volume (Tb.V), total bone marrow volume including trabeculæ (Ec.V), trabecular bone volume fraction (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), connectivity density (CD/ Ec.V) (measure of the interconnectivity of the trabecular network), cortical thickness (Ct.Th), cortical volume (Ct.V), and moment of inertia (MOI) (measure of the geometrical distribution indicative of the mechanical strength per transversal cross-section). Data are presented as means ± SE. *P < 0.05 versus wildtype mice.
duced Ca\(^{2+}\) absorption eventually causes an overall Ca\(^{2+}\) shortage in the body. Together with unchanged serum Ca\(^{2+}\) levels, this implies that the Ca\(^{2+}\) resorption in bone is increased to compensate for this deficiency. Indeed, enhanced bone resorption was reported in the TNF\(^{\Delta A R E}^{+/-}\) mice, using the specific bone matrix degradation product tDPD in serum as a bone resorption marker and detailed structural analysis of the femurs. Inevitably, increased bone resorption will result in low BMD. These findings are in line with a study in interleukin-10 knockout (IL-10\(^{-/-}\)) mice. This colitis model also displayed osteoporotic hallmarks including reduced bone mass, increased mechanical fragility, and suppressed bone formation. Those authors concluded that the presence of colitis is an important contributor to osteoporosis in IL-10\(^{-/-}\) mice. However, in contrast with our study, the IL-10\(^{-/-}\) mice did not show decreased vitamin D serum levels compared to wildtype littermates. Most likely the difference in disease location could explain the discrepancy between the mouse models. It is known that disturbance of the integrity of the ileum reduces vitamin D absorption, but the colon is the main site of inflammation in the IL-10\(^{-/-}\) mice and, therefore, malabsorption of dietary vitamin D may be less profound. However, the involvement of Ca\(^{2+}\) transporters was not evaluated in the IL-10\(^{-/-}\) mouse model.

In conclusion, we showed that the TNF\(^{\Delta A R E}^{+/-}\) mouse model maintains normal serum Ca\(^{2+}\) levels despite decreased expression of Ca\(^{2+}\) transporters in duodenum and kidney, presumably as a result of reduced serum 1,25(OH)\(_2\)D\(_3\) levels. This initiated an increased Ca\(^{2+}\) resorption from bones, causing low BMD. Because of the high similarity of this mouse strain with the human situation, we suggest that the TNF\(^{\Delta A R E}^{+/-}\) mouse is an interesting model to further study low BMD in human CD patients.

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