The F508del Mutation in Cystic Fibrosis Transmembrane Conductance Regulator Gene Impacts Bone Formation

The F508del mutation in the cystic fibrosis transmembrane conductance regulator (Cftr) gene is believed to be an independent risk factor for cystic fibrosis-related bone disease. In this study, we evaluated the bone mineral density as well as the histomorphometric parameters of bone formation and bone mass in both F508del-Cftr homozygous mice (F508del Cftr\(^{tm1Eur}\)) and Cftr\(^{+/+}\) littermate controls at 6 (prepubertal), 10 (pubertal), and 14 (young adult) weeks of age in both sexes. The bone architecture of F508del Cftr\(^{tm1Eur}\) and wild-type (WT) littermate mice was evaluated by bone densitometry, microcomputed tomography, and analysis of the dynamic parameters of bone formation. Serum levels of both insulin-like growth factor 1 and osteocalcin also were determined. Reduced bone mineral density, lower femoral bone mass, and altered trabecular bone architecture were observed in F508del Cftr\(^{tm1Eur}\) mice compared with controls at 6, 10, and 14 weeks of age. A decrease in the bone formation rate in F508del Cftr\(^{tm1Eur}\) mice was shown compared with control mice, independently of age and sex. In addition, we found lower insulin-like growth factor 1 levels in F508del Cftr\(^{tm1Eur}\) mice compared with age-matched controls, whereas osteocalcin levels were normal. Severe osteopenia and altered bone architecture were found in young and mature adult F508del Cftr\(^{tm1Eur}\) mice. Our findings show that the F508del mutation in CFTR impacts trabecular bone mass by reducing bone formation.

Cystic fibrosis (CF) is an autosomal-recessive disorder caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent anion channel leading to progressive pulmonary damage and, ultimately, to death. Over the past 3 decades, reduced bone mineral density (BMD) and low bone mass has been reported in children, adolescents, and adults with CF, independently of sex, compared with the general population. Brittle bones in CF disease were confirmed by densitometric data, fracture incidence, and impaired quality of life in young patients. The cause of the CF-related low BMD is multifactorial including vitamin K and D insufficiencies, calcium malabsorption, malnutrition, glucocorticoid use, delayed puberty, and pulmonary infection/systemic inflammation. The bone disease occurs around puberty owing to a poor acquisition of peak bone mass and worsens with age. However, low BMD has been observed in CF children younger than the age of 6 years with mild disease and normal nutritional status, suggesting that CF-related low BMD also may be owing to the intrinsic defect (ie, CFTR dysfunction). Whether CFTR activity plays a direct role in the low bone mass in children with CF is unknown, but can be hypothesized on the basis of animal studies in the CFTR-null mouse. Smaller bones with decreased BMD, cortical bone thinning, and altered trabecular architecture were found in young and mature adult F508del Cftr\(^{tm1Eur}\) mice.
Cftr<sup>−/−</sup> mice despite no difference in osteoblast and osteoclast numbers as compared with control mice, suggesting that CFTR protein may influence bone cell activity rather than number. Another study reported sex-related differences in the bone formation rate of gut-corrected Cftr<sup>−/−</sup> mice, suggesting inadequate bone formation in females but increased formation in males. The expression of CFTR protein has been identified by immunohistochemistry in human bone cells. We previously reported the expression of CFTR mRNA and protein in primary human osteoblasts (cells that form bone) and showed that inhibition of CFTR-mediated Cl<sup>−</sup> channel activity affects the release of osteoprotegerin and prostaglandin E2, two key regulators of bone formation. We recently discovered a defective CFTR Cl<sup>−</sup> channel activity and a deficit of osteoprotegerin production by primary osteoblasts from a 25-year-old CF patient with the F508del/G542X mutation in CFTR. One study in patients with CF with at least one F508del allele showed a direct association between the F508del mutation and low BMD in both sexes. However, the impact of the F508del allele mutation in CFTR on bone formation and bone mass remains unknown. In this study, we evaluated the impact of the F508del mutation in CFTR on the BMD status and bone formation in F508del-CFTR homozygous mice in relation to age (prepubertal, pubertal, and young adult) and sex compared with normal Cftr<sup>+/+</sup> littermates.

Materials and Methods

Mice

Rotterdam homozygous F508del-CFTR mice (F508del Cftr<sup>tm1Eur</sup>) that express the clinically common F508del mutation in Cftr at wild-type (WT) protein level and their normal Cftr<sup>+/+</sup> homozygous littermates (in the FVB background) were obtained from the Centre de Distribution, Typage et Archivage Animal, Centre National de la Recherche Scientifique (Orléans, France). At 6, 10, and 14 weeks of age, F508del Cftr<sup>tm1Eur</sup> and Cftr<sup>+/+</sup> littermate (WT-CFTR) male and female mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg; Virbac, Carros, France) and xylazine (20 mg/kg Rompun; Bayer, Leverkusen, Germany) as previously described.

Dual-Energy X-Ray Absorptiometry, Scanning Electron Microscopy, and X-Ray Spectra

The BMD (mg/cm<sup>2</sup>) of total body and femoral bones free of soft tissue were measured from groups of 8 to 10 F508del Cftr<sup>tm1Eur</sup> and WT-CFTR mice using a PIXImus mouse densitometer (software version 1.44; Lunar GE Medical Systems, Paris, France). The femoral bones were stored in 70% ethanol and dehydrated in graded ethanol, defatted in xylene, and embedded in methyl methacrylate. Measurements of total calcium (Ca<sup>2+</sup>) and phosphate (P) atomic concentrations in undecalcified femoral sections from X-ray spectra were performed to calculate a mean value of the Ca/P atomic ratio, indicative of calcium phosphate levels in the bone, as reported in our laboratory.

Bone Histomorphometry

Two nonconsecutive sections of femoral bones were stained for tartrate-resistant acid phosphatase detection using naphthol AS-TR phosphate (Sigma, St. Quentin Fallavier, France) as substrate and then counterstained with toluidine blue (pH 4.3). The bone surface, trabecular bone volume, trabecular bone width, and trabecular separation were measured using a software package developed for bone histomorphometry (Microvision, Evry, France). Osteoclast numbers expressed as N.Oc/T.Ar (per mm<sup>2</sup>) were evaluated on tartrate-resistant acid phosphatase–stained sections. Histomorphometric parameters were recorded at this standard sampling site in compliance with the recommendations of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee. Double labeling of tetracycline and calcein in vivo was performed as described. The mineral apposition rate was calculated according to the American Society for Bone and Mineral Research nomenclature. Femoral bones of 6-week-old F508del Cftr<sup>tm1Eur</sup> and WT-CFTR mice were scanned with microcomputed tomography (µCT-40; Scanco Medical) for 8 to 10 animals per group. Statistical differences are indicated by analysis of variance.

Figure 1. F508del Cftr<sup>tm1Eur</sup> mice had lower body weight compared with WT control mice at 6, 10, and 14 weeks of age. A: Six-week-old WT control and F508del Cftr<sup>tm1Eur</sup> male mice. B: Femur length was not significantly different between F508del Cftr<sup>tm1Eur</sup> and WT control mice between both sexes and at all ages. C: A lower weight in F508del Cftr<sup>tm1Eur</sup> was found compared with WT control mice in both sexes and at all ages. Data are mean ± SEM for 8 to 10 animals per group. Statistical differences are indicated by analysis of variance: **P < 0.01, ***P < 0.001.
AG, Brüttiselen, Switzerland) to assess three-dimensional bone morphology.

**Serum Analysis**

Sera were obtained from F508del CFTRtm1Eur and WT-CFTR mice and stored at −85°C until analysis of duplicate samples. In the active insulin-like growth factor 1 (IGF-1) enzyme-linked immunosorbent assay, IGF-1 was separated from its binding proteins in serum by using an ethanol and HCl-based extraction solution provided by the manufacturer (Millipore, Molsheim, France). Analysis of bone turnover biomarkers was undertaken with a commercial assay (enzyme-linked immunosorbent assays for osteocalcin, IL-6, and tumor necrosis factor-α; Millipore).

**Statistical Analysis**

Data are presented as mean ± SEM for 6 to 10 animals per group. Significance of the in vivo response was determined using one-way analysis of variance with Dunnett post hoc analysis. Significance between groups was determined using two-way analysis of variance with Bonferroni post hoc analysis. A P value <0.05 was considered significant.

**Results**

**F508del CFTR Mutation Results in a Decreased Bone Mass**

In a recent study it was reported that femurs of adult F508del CFTRtm1Eur mice of 11 to 15 weeks of age presented with a significantly lower bone volume compared with CFTR+/+ controls whereas weight, length, and BMD were similar in the two mouse groups.29 In our study, measures of body weight, femur length, and BMD were performed in growing F508del CFTRtm1Eur mice at 6 weeks (prepubertal), 10 weeks (pubertal), and 14 weeks (young adult) of age in both sexes. No significant difference in femur length was observed between F508del CFTRtm1Eur mice and sex-matched WT controls. We further assessed BMD of F508del CFTRtm1Eur mice and sex-matched controls in all ages. As expected, the average body weight of F508del CFTRtm1Eur mice was 24%, 20%, and 14% less than control mice at 6, 10, and 14 weeks of age, respectively (Figure 1, A–C).

We then examined bone microarchitecture in 6-week-old mice of both genotypes by microcomputed tomography. Representative three-dimensional images of the distal femur are shown in Figure 2A. The plate-like structure of the trabecular bone was reduced markedly, and the connecting rods of trabeculae were disrupted in F508del CFTRtm1Eur mice compared with sex-matched WT controls. We further assessed BMD of F508del CFTRtm1Eur mice and their littermate controls by dual-energy X-ray absorptiometry at 6, 10, and 14 weeks of age. At 6 weeks, the whole-body BMD in F508del CFTRtm1Eur mice was reduced significantly (6% and 15% in males and females, respectively) compared with sex-matched controls (Figure 2B). The reduction in BMD was more pronounced in F508del CFTRtm1Eur mice when femurs were analyzed separately in both sexes, namely a 14% and 22% reduction of BMD in femurs of male and female 6-week-old F508del CFTRtm1Eur mice, respectively, compared with sex-matched controls (Figure 2C). The reduction in BMD persisted in both whole body and femurs of 10-week-old F508del CFTRtm1Eur mice in both sexes. However,
there was a trend toward BMD normalization in both whole body and femoral bone in 14-week-old F508del Cftr<sup>tm1Eur</sup> mice, which was more pronounced in females (Figure 2C).

**F508del CFTR Mutation Results in Altered Bone Architecture**

We next assessed architectural parameters in femoral sagittal sections from F508del Cftr<sup>tm1Eur</sup> mice by micro-computed tomography and histologic measurements (Figure 3). In the femur of 6-week-old F508del Cftr<sup>tm1Eur</sup> mice, two-dimensional coronal sections adjacent to the growth plate (Figure 3A) showed reduced trabecular bone compared with sex-matched controls in both sexes. Figure 3B shows a reduction of trabecular bone in distal femoral sections of 6-week-old F508del Cftr<sup>tm1Eur</sup> mice in both sexes. Quantification of structural parameters revealed that bone volume/tissue volume (percentage of trabecular bone volume) was decreased by 40% and 36% in 6-week-old F508del Cftr<sup>tm1Eur</sup> male and female mice, respectively, compared with sex-matched controls. Trabecular thickness was decreased by 17% and 9% in 6-week-old F508del Cftr<sup>tm1Eur</sup> male and female mice, respectively, confirming the microcomputed tomography findings. Trabecular separation was increased by 53% and 69% in 6-week-old F508del Cftr<sup>tm1Eur</sup> mice, respectively, in both sexes (Figure 3, C and D). As shown in Figure 3E, the decrease of trabecular bone volume in femurs of F508del Cftr<sup>tm1Eur</sup> mice persisted at both 6 and 14 weeks whereas trabecular bone volume was normalized in F508del Cftr<sup>tm1Eur</sup> mice at 14 weeks of age, in females but not in males. Data are mean ± standard error of the mean for 8 to 10 animals per group. Statistical differences are indicated by analysis of variance. *P < 0.05 and ***P < 0.001.
Because the F508del Crttm1Eur mice showed decreased bone mass, we examined whether these changes were related to the alterations in tartrate-resistant acid phosphatase–positive osteoclasts, a specific marker indicative of the rate of bone resorption (Figure 4A). Quantitative analysis of osteoclasts (N.Oc/T.Am) lining the femoral trabecular bone at 6, 10, and 14 weeks of age is shown in Figure 4B. No significant difference in osteoclast number was observed in 6- and 14-week-old F508del Crttm1Eur mice compared with sex-matched control mice. In contrast, a significant increase in osteoclast number was found in F508del Crttm1Eur mice at 10 weeks in both sexes (Figure 4B).

To analyze bone formation dynamics, the rate of new bone apposition (ie, the trabecular mineral appositional rate) was evaluated in vivo after double labeling with tetracycline and calcine in F508del Crttm1Eur mice (Figure 5A). We found that the mineral apposition rate in F508del Crttm1Eur mice was consistently lower than in sex-matched controls, at all ages (Figure 5B). To investigate whether bone defects observed in F508del Crttm1Eur mice may be related to alteration in changes of circulating levels of bone-growth factors, we measured levels of serum IGF-1 and osteocalcin in F508del Crttm1Eur mice compared with control mice at 6, 10, and 14 weeks of age (Figure 6). We found lower levels of serum IGF-1 in F508del Crttm1Eur mice compared with age-matched control mice (Figure 6A). Control WT mice showed an expected increase in IGF-1 with time, which was less pronounced in F508del Crttm1Eur mice. No significant difference was observed in the serum level of osteocalcin between F508del Crttm1Eur and control WT mice (Figure 6B).

Discussion

Our data clearly indicate that F508del mutation in CFTR results in decreased bone mass and bone formation,
independently of sex. A severe osteopenic phenotype was seen in 6-week-old F508del Cftrm1Eur mice, with reduced BMD, lower bone mass, and reduction in the number and thickness of trabeculae compared with control littermates. This phenotype was associated with a decreased rate of new bone formation at all ages. In addition, at 10 weeks (pubertal age), we found that the number of osteoclasts was increased in F508del Cftrm1Eur female and male mice compared with sex-matched controls, supporting the idea that high bone turnover was increased in F508del Cftrm1Eur mice at this puberal period. We found a persistent deficit in both BMD and bone mass in F508del Cftrm1Eur mice despite significant, quantitative improvements in females at 14 weeks of age (young adult age). Although F508del Cftrm1Eur mice are, on average, smaller than their control littermates, we show that the observed osteopenic phenotype cannot be ascribed only to the reduced size of CF mice. Compared with sex-matched controls, a lower trabecular bone volume consistently was found in F508del Cftrm1Eur mice, which was more pronounced in males. Our data therefore do not support the sex-related differences in bone loss described in the gut-corrected Cftr+/− mouse model, reporting inadequate bone formation in females but increased bone formation in males.18

By using quantitative microcomputed tomography imaging of femurs, Paradis et al29 reported reduced bone volume in adult F508del Cftrm1Eur mice although measures of weight, length, BMD, and osteoclast numbers were similar to those of control littermates. Our data support some of these observations by showing a strong reduction (49%) in bone volume and no significant change in BMD of femoral bone in adult F508del Cftrm1Eur mice in both sexes. Also in agreement with the latter, we noted that osteoclast number in femurs of 14-week-old F508del Cftrm1Eur mice did not differ from those in control mice. We also found a slower rate in bone formation in F508del Cftrm1Eur male and female mice compared with sex-matched controls. Given the lower bone volume now reported in two CF animal models (B6 Cftrtm1Kth and Cftrm1Eur mice) with the F508del mutation (this study and the study by Paradis et al29), all data suggest that the F508del mutation in CFTR does not increase the osteoclast number, but rather may modify the activity of bone cells. It is therefore reasonable to hypothesize that the effects of F508del CFTR on bone cells may depend on the stage of development (ie, growing versus adult F508del Cftrm1Eur mice). This may very well reflect a different regulation of the osteoblast/osteoclast balance in growing individuals. Indeed, in growing individuals, bone formation must exceed bone resorption to gain bone mass, allowing skeletal growth. On the other hand, during the growth phase, balanced bone resorption is essential for mineralized cartilage and woven bone erosion, two processes that contribute physiologically to longitudinal development. In our growing 10-week-old F508del Cftrm1Eur mice, we show a higher osteoclast number compared with 6- and 14-week-old F508del Cftrm1Eur mice. Therefore, the regulation of osteoblast and osteoclast activities must be different in growing mice compared with young adult mice. Whatever they might be, our results show that F508del mutation in CFTR in growing mice can physiologically dissociate osteoclast function from osteoblast function, with a higher bone resorption activity in the stage of bone trabecular development, contributing to an osteopenic phenotype. These findings are consistent with clinical studies reporting that levels of bone turnover markers were higher in F508del

### Table 1. Ca/P Atomic Ratio of Femoral Bones in 14-Week-Old F508del Cftrm1Eur and Sex-Matched WT Mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>N</th>
<th>Ca/P atomic ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Male</td>
<td>3</td>
<td>1.62 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>F508del</td>
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<td>3</td>
<td>1.63 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
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<td>1.57 ± 0.02</td>
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</tr>
<tr>
<td>F508del</td>
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<td>3</td>
<td>1.58 ± 0.02</td>
<td>NS</td>
</tr>
</tbody>
</table>

Undecalcified femoral sections coated with a conductive layer (carbon) of 14-week-old WT control and F508del Cftrm1Eur mice were observed using a LaB6 electron microscope (JEOL JSM-5400LV) operating at 15 kV, which is equipped with an ultra-thin window Si (Li) detector for X-ray measurements. The Ca/P values represent the means ± standard error of the mean of three distinct areas analyzed in three femoral sections for three animals per group.

Ca/P, the atomic ratio of total calcium/phosphates in trabecular bone measured on the basis of high resolution X-ray spectra; NS, not significant.

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![Figure 6](image). Serum IGF-1 and osteocalcin levels in F508del Cftrm1Eur mice. A: Serum IGF-1 levels were lower in F508del Cftrm1Eur compared with WT control mice, in all ages. B: No significant difference in serum levels of osteocalcin was observed between F508del Cftrm1Eur and WT control mice. Blood collected by cardiac puncture in anesthetized animals was analyzed at 6 weeks (WT, 5 females and 5 males; F508del, 5 females and 5 males), at 10 weeks (WT, 2 females and 2 males; F508del, 5 females and 5 males), and at 14 weeks of age (WT, 5 females and 6 males; F508del, 5 females and 6 males). Statistical difference is indicated by analysis of variance. **P < 0.01.
homozygous CF patients compared with healthy controls and were more evident in male compared with female patients with CF. Our finding that F508del Cftrtm1Eur mice are characterized by decreased new bone formation, independently of age and sex, may be relevant to patients with CF with the F508del mutation. Consistently, three clinical reports through histomorphometric data in young adult CF patients revealed both a reduced BMD and lower trabecular bone volume compared with age- and sex-matched healthy control subjects, which was ascribed to low bone formation.

A link between reduced BMD, bone resorption, and alteration in bone turnover markers has been described accompanying exacerbations of lung infection with systemic inflammation in CF patients. A recent study showed a positive correlation between osteoclast number and serum levels of tumor necrosis factor-α and osteocalcin in CF patients. The F508del Cftrtm1Eur mouse strain studied here has not been reported previously to develop lung inflammatory disease, although recent data based on quantitative lung histology showed mild inflammation in mature adult (16 to 19 weeks) F508del Cftrtm1Eur mice compared with normal mice, under pathogen-free conditions. In the present study, we found no significant difference in the serum osteocalcin level between F508del Cftrtm1Eur and control mice, as well as in the serum levels of tumor necrosis factor-α and IL-6 cytokines (data not shown).

Although much attention has been focused on growth deficits in children, adolescents, and adults, evidence suggests that individuals with CF show abnormalities at birth. Circulating levels of IGF-1 have been shown to directly regulate bone growth and density. Studies have indicated that CF newborns are shorter and have a lower body weight that non-CF newborns. Additional studies have suggested that the reduced levels of serum IGF-1 might be responsible, at least in part, for the growth defect reported in patients with CF, which also was observed in both Cfr−/− mouse and newborn Cfr−/F508 pig models. IGF-1 has been identified as an independent predictor of low BMD in CF patients and in Cfr−/− mice. In the present study, serum IGF-1 level was lower in F508del Cftrtm1Eur mice compared with age-matched controls, as previously reported in the Cfr−/− mice. However, in the study by Haston et al, the median value of serum IGF-1 in 12-week-old Cfr−/− mice was lower (210 ng/mL) compared with the median value of serum IGF-1 of 500 ng/mL that we observed in our 10- and 14-week-old F508del Cfrtm1Eur mice. An alternate explanation for this difference is that no CFTR protein is expressed in the Cfr−/− mouse model, whereas in the F508del Cfrtm1Eur mouse model the F508del mutation in Cfr expresses the F508del CFTR protein similar to the WT protein level. Rogan et al also reported a significant reduction in serum IGF-1 levels in both newborn Cfr−/F508 pigs and humans with CF and suggested that the decrease is not only a consequence of malnutrition or pulmonary inflammation, but that loss of CFTR function may have an additional, more direct effect in reducing IGF-1 levels. These data and the present study make it likely that F508del CFTR expression associated with low circulating IGF-1 level might be a causal factor in reduced bone formation. How the F508del mutation in CFTR decreases the serum IGF-1 level remains, to date, to be investigated.

In humans, vitamin D deficiency also results in impaired bone density owing to decreased calcium absorption. Although lower serum 25-hydroxyvitamin D levels commonly are seen in patients with CF compared with the non-CF population, cross-sectional studies have shown no clear association between the vitamin D status and BMD of CF patients. In addition, vitamin D supplementation shows no evidence of benefits on BMD, fractures, and bone turnover markers in CF individuals. Adequate calcium intake is essential to achieve an optimal peak bone mass. In the present study, we reported a comparable total calcium/phosphate atomic ratio in femoral bone between adult F508del Cfrtm1Eur and control mice, suggesting that bone calcium and phosphate content was not affected in F508del Cfrtm1Eur mice. In support of this contention, Hillman et al showed that in patients with CF the percentage of true calcium absorption was in the normal range based on age and pubertal state compared with the control population.

Our data generate the hypothesis that the diminished bone mass observed in F508del-CFTR mice may imply an inherent defect of the F508del mutation, leading to a reduction of new bone formation in vivo that is not sex- and age-specific. These data are the first to show an important role for CFTR in maintaining bone mass, as well as new bone formation. Most experts in the European CF bone mineralization working group found that bone loss is observed most often in the peripubertal age range (8 to 10 years) in CF patients. In accordance with these clinical data, we show a marked bone mass loss and altered trabecular bone architecture in F508del Cfrtm1Eur mice at the prepubertal state. Our data support the conclusion that the F508del-CFTR mutation may contribute to bone disease by slowing the new bone formation in infants and young children with cystic fibrosis. Additional molecular and cellular studies are required to elucidate how the F508del mutation in CFTR could disrupt the process of osteogenic differentiation, which could take place in bone marrow stromal cells, to affect both the osteoblastogenesis and osteoclastogenesis processes. The murine F508del Cfrtm1Eur model detailed earlier may become a valuable tool to identify new anabolic targets for the treatment of CF-related bone disease.

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