Journal of Cystic Fibrosis 9 (2010) 239–245

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Original Article

Osteopenia in Cftr-deltaF508 mice

Josée Paradis, Martina Wilke, Christina K. Haston*

Meakins-Christie Laboratories, Department of Medicine, McGill University, Canada
Department of Cell Biology, Erasmus Medical Centre, Rotterdam, The Netherlands

Received 17 February 2010; received in revised form 23 April 2010; accepted 5 May 2010
Available online 31 May 2010

Abstract

Background: Mice with the cystic fibrosis transmembrane conductance regulator (*Cftr*) gene knocked out develop osteopenia. To determine whether this phenotype is present in cystic fibrosis mouse models with the ΔF508 *Cftr* mutation we assessed the femora of adult FVB/N *Cftr*^{tm1Eur} and C57BL/6 *Cftr*^{tm1Kth} mice.
Methods: Bone disease, relative to littermate controls, was measured using histology, densitometry and quantitative imaging.
Results: C57BL/6 *Cftr*^{tm1Kth} mice had shorter femurs and bones of lower volume due to thinner trabeculae, compared to wild type littermates. FVB/N *Cftr*^{tm1Eur} mice also presented a lower bone volume which was due to significantly fewer trabeculae in this strain. Osteoblast and osteoclast numbers did not differ between CF and controls, for either of FVB/N *Cftr*^{tm1Eur} or C57BL/6 *Cftr*^{tm1Kth} mice. The bone architecture of FVB/N *Cftr*^{tm1Eur} mice did not significantly differ from that of C57BL/6 *Cftr*^{tm1Kth} mice.
Conclusions: An osteopenic bone disease is evident in adult ΔF508-*Cftr* cystic fibrosis mouse models.

Keywords: Bone disease; Genetically modified mouse; Cftr; deltaF508; Femur

1. Introduction

Cystic fibrosis (CF) is caused by defects in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and these lead to well described pathologies affecting the lung, intestine, and pancreas [1]. An additional phenotype developing in CF patients is bone disease, which is manifest by low bone mineral density (BMD) and increased fracture rates [2,3] in this population. Although factors such as malnutrition, steroid therapy and lung disease likely contribute to the bone complications in CF patients [2,4], King et al. [5] have shown that the common ΔF508 mutation in the chloride-ion channel encoded by *CFTR* is an independent risk factor for bone disease in CF.

Mouse models with specific alterations in *Cftr* have been created to investigate the pathophysiology cystic fibrosis [6] and these include mice which produce no Cftr protein, such as the *Cftr*^{tm1Unc} line developed by Snouwaert et al. [7], or mice producing the Cftr protein with the ΔF508 mutation. Mice of the latter type include the *Cftr*^{tm1Eur} line which was created with a hit and run technique [8] and these mice express the mutant allele at the same level at which wild type mice express the wild type allele in assayed tissues including the intestine [9]. A second line, *Cftr*^{tm1Kth} mice [10], express the mutated allele at a level of 25% relative to that of wild type mice expressing the wild type allele and this low level of expression in the intestine [10], but not other tissues including the lung, likely contributes to the lethal intestinal phenotype mice of this strain can develop. Although known to be expressed in bone [11,12] and in chondrocytes [13], the level of expression of *Cftr* in the bones of these ΔF508 *Cftr* mouse models has not been reported.

Previous studies have shown that *Cftr*^{tm1Unc} mice on both a genetically mixed [14] and inbred strain, BALBc/J [15], background present a bone disease characterised by reduced bone volume of the femoral tissue which featured fewer thinner trabeculae. Whether FVB/N *Cftr*^{tm1Eur} or C57BL/6 *Cftr*^{tm1Kth}

* Corresponding author. Meakins-Christie Laboratories, 3626 rue St. Urbain, Montreal Quebec, Canada H2X 2P2. Tel.: +1 514 398 3864x089714; fax: +1 514 398 7483.
E-mail address: christina.haston@mcgill.ca (C.K. Haston).

1569-1993/$ - see front matter © 2010 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jcf.2010.05.001

mice also develop a bone phenotype, which would enable these mice to be used as a model of this pathology of cystic fibrosis, is unknown. The current study was undertaken to determine whether the ΔF508 *Cftr* mutation leads to a bone phenotype in mice.

2. Materials and methods

2.1. Mice

Cftr^{tm1Eur} heterozygous mice [8], which had been backcrossed for 13 generations to the FVB/N strain, were obtained from Dr. B. Scholte of the University Medical Centre Rotterdam, and the C57BL/6 (B6) *Cftr*^{tm1Kth} strain [10] from Dr. C. Bear of the Hospital for Sick Children (Toronto, Canada). Cystic fibrosis mice of each strain and *Cftr* +/+ littermate controls were derived from heterozygous breeding pairs, genotyped as in [16, www.jax.org] and maintained at the Meakins-Christie Laboratories of McGill University. To circumvent the premature death due to intestinal disease, all mice were fed standard chow and received PEGLYTE® (17.8 mmol/L polyethylene glycol, Pharma Science, Montreal, Canada) in their drinking water as described previously [15,17,18]. At 11–15 weeks of age the mice were weighed, euthanised with a lethal dose of anaesthetic and one femur was removed. Relative to sexual maturity at 6–8 weeks of age, the bone disease assessment is of young adult mice. All animal procedures were performed in accordance with the McGill University guidelines set by the Canadian Council on Animal Care.

2.2. Radiological imaging

Phenotyping procedures were performed as described previously [15]. High resolution X-rays of the femora were obtained with a Faxitron MX20/DC2 (Faxitron X-ray Corporation, Wheeling IL, USA), equipped with an FPX-2 Imaging system (Dalsa Medoptics, Waterloo, Ontario, Canada), and used to measure femur length. A Lunar PixiMUS 1.46 instrument (GE-Lunar, Madison, WI, USA) was then used to measure bone mineral density on bones dissected free of soft tissue. Bones were fixed overnight in 4% paraformaldehyde, rinsed three times with PBS and scanned on a Skyscan 1072 micro CT instrument (Skyscan, Antwerp, Belgium) to assess bone morphometry. Image acquisition was performed at 45 kV, 222 μA for a 2.24 second exposure at 50× with a 0.9° rotation between frames. These two-dimensional images were used to reconstruct three-dimensional images for quantitative analysis using 3D Creator software supplied with the instrument. The area of interest selected for quantification of trabecular bone was located immediately distal to the growth plate and extended for 2.25 mm towards the metaphysis of the femur.

2.3. Histochemistry

The bone tissue was embedded in polymethylmethacrylate at low temperature and 4 μm sections were cut and stained for alkaline phosphatase (ALP) activity in osteoblasts or tartrate resistant acid phosphatase (TRAP) in osteoclasts as described previously [15]. Cell numbers were scored by an investigator, blinded to mouse strain and *Cftr* genotype, using a single section from 4 to 7 mice per group. Osteoclast percentage was determined by measuring the area of TRAP positive cells present per unit area of the metaphysis. Osteoblast cell numbers were counted and normalised to the length of the growth plate in each section.

2.4. Statistical analysis

Results are expressed as the mean±SD and differences between groups of mice were determined using Student's *t* test.

3. Results

3.1. Femur length and bone mineral density in CF mice

To determine whether the bone disease in *Cftr* knockout mice is evident in those with the ΔF508 mutation in *Cftr* length and mineral density measures were made in femora extracted from mice of two strains and from age, sex and strain matched *Cftr* +/+ (control) mice. As shown in Fig. 1 the bones of the B6 *Cftr*^{tm1Kth} mice were shorter than those of control mice but this difference was not evident in the mice of the FVB/N *Cftr*^{tm1Eur} strain. In addition,

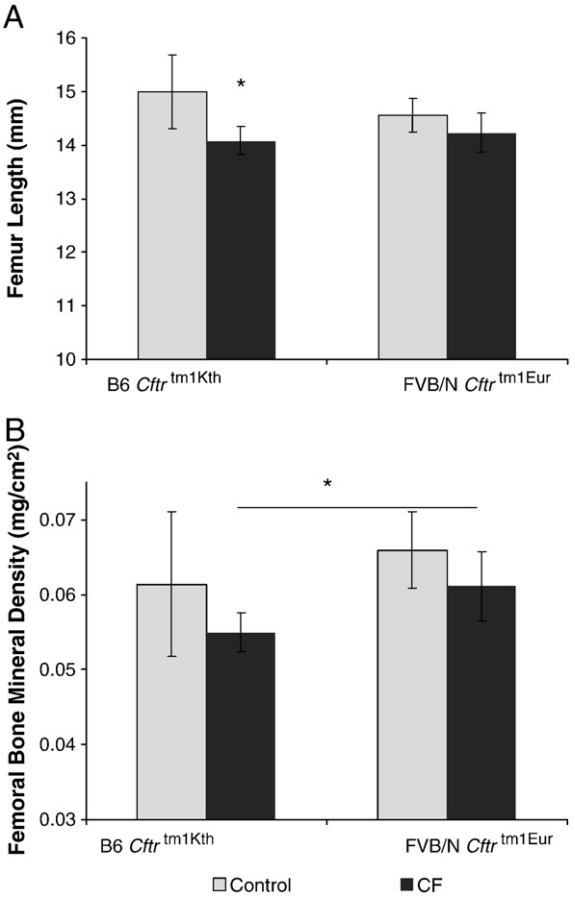

Fig. 1. Femur length and bone mineral density in ΔF508 *Cftr* mouse models. CF and control (*Cftr* +/+) mice were maintained on PEGLYTE® treated drinking water until euthanasia at 11–15 weeks of age. Bone lengths (A) were measured on high resolution Faxitron X-rays and bone mineral density (B) on a PixiMus densitometer. Values represent the mean±SD of $n=4–7$ mice per group. *Significant difference between groups, $p<0.05$.

the average body weight of B6 *Cftr*^{tm1Kth} mice was less than that of control mice (20.7±1.6 (std. dev. vs. 26.9±3.0 g, $p=0.03$) while, again, this difference was not evident in the mice of the FVB/N *Cftr*^{tm1Eur} strain (26.1±3.8 vs. 29.8±2.5 g, $p=0.08$). Femoral bone mineral density was not significantly different between CF and control mice of either strain (Fig. 1B), but the density of the FVB/N *Cftr*^{tm1Eur} bones exceeded that of B6 *Cftr*^{tm1Kth} mice ($p=0.027$). Finally, in *Cftr* +/+ control mice, no significant differences between strains were evident in either bone mineral density or in length (each $p>0.19$).

3.2. Bone architecture of CF mice

To investigate whether the osteopenia of BALB *Cftr*^{tm1Unc} CF mice is evident in the ΔF508 *Cftr* mouse models microCT imaging of the femoral tissue was completed. As shown in Figs. 2 and 3 and quantified in Table 1, the bones from the FVB/N *Cftr*^{tm1Eur} mice and of B6 *Cftr*^{tm1Kth} mice were thinner and had fewer, or narrower, trabeculae compared to controls. Among the CF mouse models, no strain dependence in bone architecture was detected ($p>0.11$), while in the *Cftr* +/+ (control) mice, trabecular thickness was dependent on strain ($p=0.029$), and was lower for the FVB/N mice. The other parameters did not significantly differ by strain among the control mice (all $p>0.10$), as shown in Table 1.

3.3. Osteoblast and osteoclast quantification

To determine whether the lower bone volume of B6 *Cftr*^{tm1Kth} and FVB/N *Cftr*^{tm1Eur} mice coincided with reduced levels of osteoblasts, or increased numbers of osteoclasts in the tissue, bone

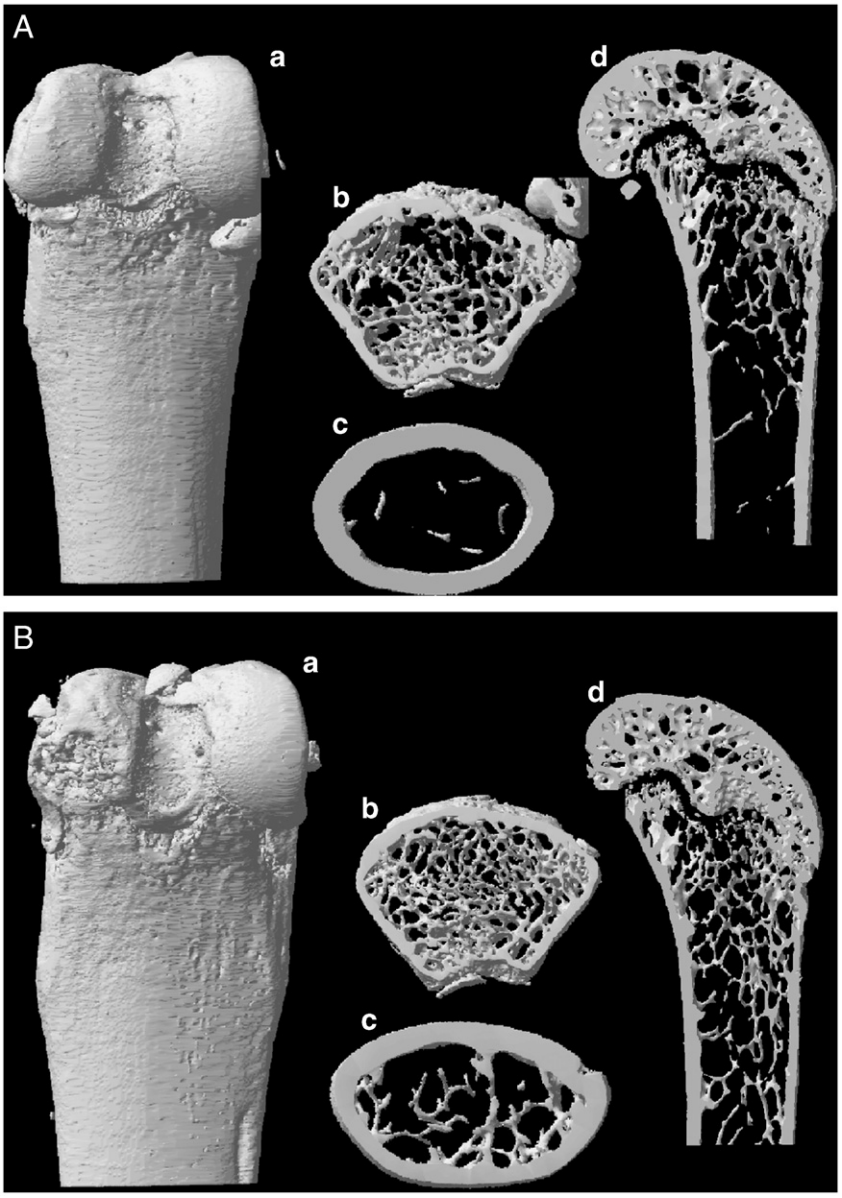

Fig. 2. Architecture of the distal femur of C57BL/6 *Cftr*^{tm1Kth} CF and control mice. Bones from CF (upper) and *Cftr* +/+ control (lower) mice were dissected free of soft tissue, fixed and scanned on a Skyscan 1072 micro CT equipped with 3D Creator analytical software. Three-dimensional re-construction (a) and two-dimensional sectional scans (b–d) demonstrated thinner trabeculae and cortical thinning of CF bones.

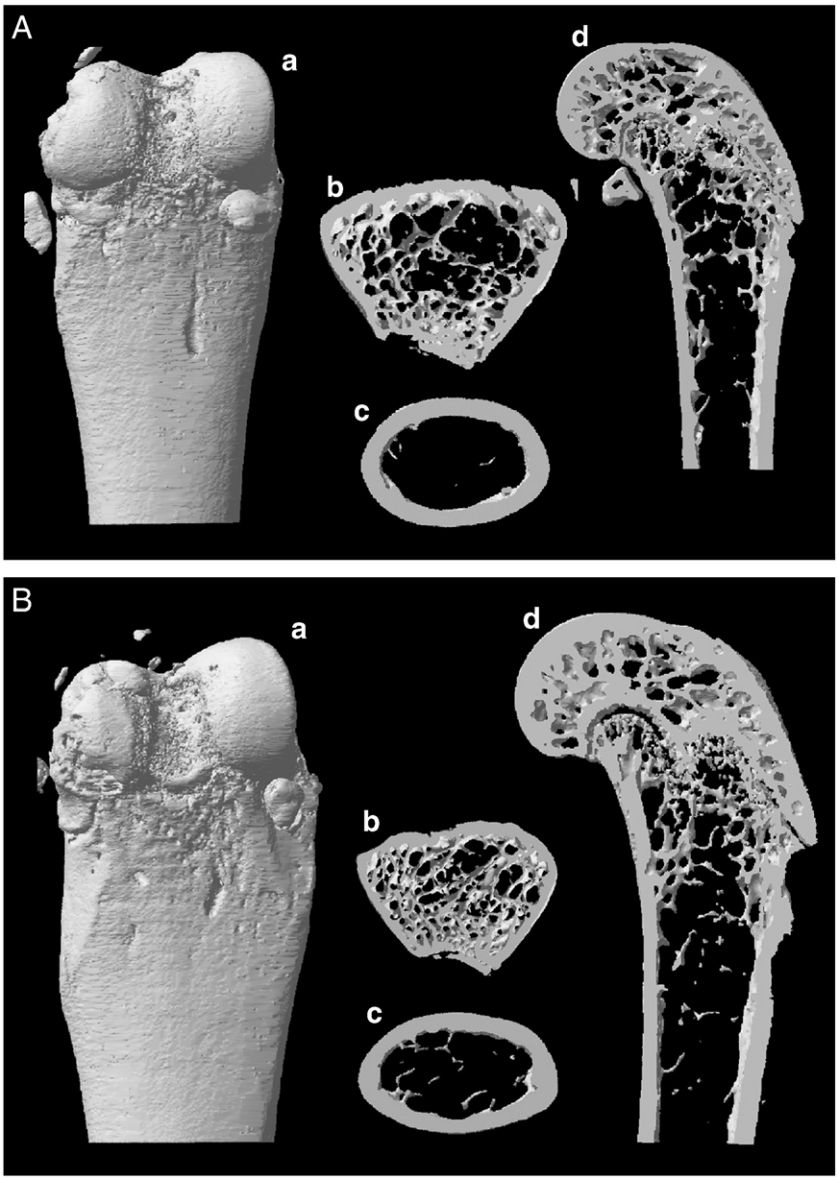

Fig. 3. Architecture of the distal femur of FVB/N *Cftr*^{tm1Eur} and control mice. Bones from CF (upper) and *Cftr* +/+ control (lower) mice were dissected free of soft tissue, fixed and scanned on a Skyscan 1072 micro CT equipped with 3D Creator analytical software. Three-dimensional re-construction (a) and two-dimensional sectional scans (b–d) demonstrated fewer trabeculae and cortical thinning of FVB/N *Cftr*^{tm1Eur} bones.

Table 1
Bone architecture measures of the femora from ΔF508 *Cftr* and control mice.

	FVB/N		C57BL/6	
	Cftr^{tm1Eur} (n=6)	Control (n=7)	*Cftr*^{tm1Kth} (n=4)	Control (n=6)
BV/TV (%)	6.57*±1.05	7.86±1.13	6.81*±1.93	11.22±4.14
SMI	2.15*±0.08	2.06±0.04	2.27±0.17	2.02±0.35
TrTh (μm)	50.6±3.1	50.5±5.0	51.8*±2.8	56.7**±3.9
Tr Sp (μm)	291±11	254±44	251±36	216±44
TrNo	1.29*±0.16	1.56±0.22	1.32±0.40	1.95±0.68

CF and control (*Cftr* +/+) mice were maintained on PEGLYTE® treated drinking water and euthanised at 11–15 weeks of age. Bone volume (BV/TV), the ratio of plate/rod-like structures (SMI), and trabecular thickness (TrTh) separation (TrSp) and number (TrNo) were quantified using micro computed tomography. Values represent the mean±SD. * Significant difference between CF and control mice; $p<0.05$. ** Significant difference between strains; $p<0.05$.

sections from CF and control mice were stained for these cell types and positive cells enumerated. As shown in Fig. 4A tissue from CF and control mice, for both the B6 *Cftr*^{tm1Kth} and FVB/N *Cftr*^{tm1Eur} mice contained numerous ALP positive (osteoblast) cells adjacent to the growth plate. The numbers of osteoblasts per unit length of growth plate did not differ between CF and control mice in either strain. Similarly, TRAP positive cells (osteoclasts) were evident in the region of the growth plate and the distal metaphysis in both CF and control mice and as shown in Fig. 4C the percentage of bone tissue with TRAP positive cells did not differ between CF and controls, in either of FVB/N or B6 mice.

4. Discussion

This study was undertaken to determine whether the bone phenotype evident in cystic fibrosis mouse models created with

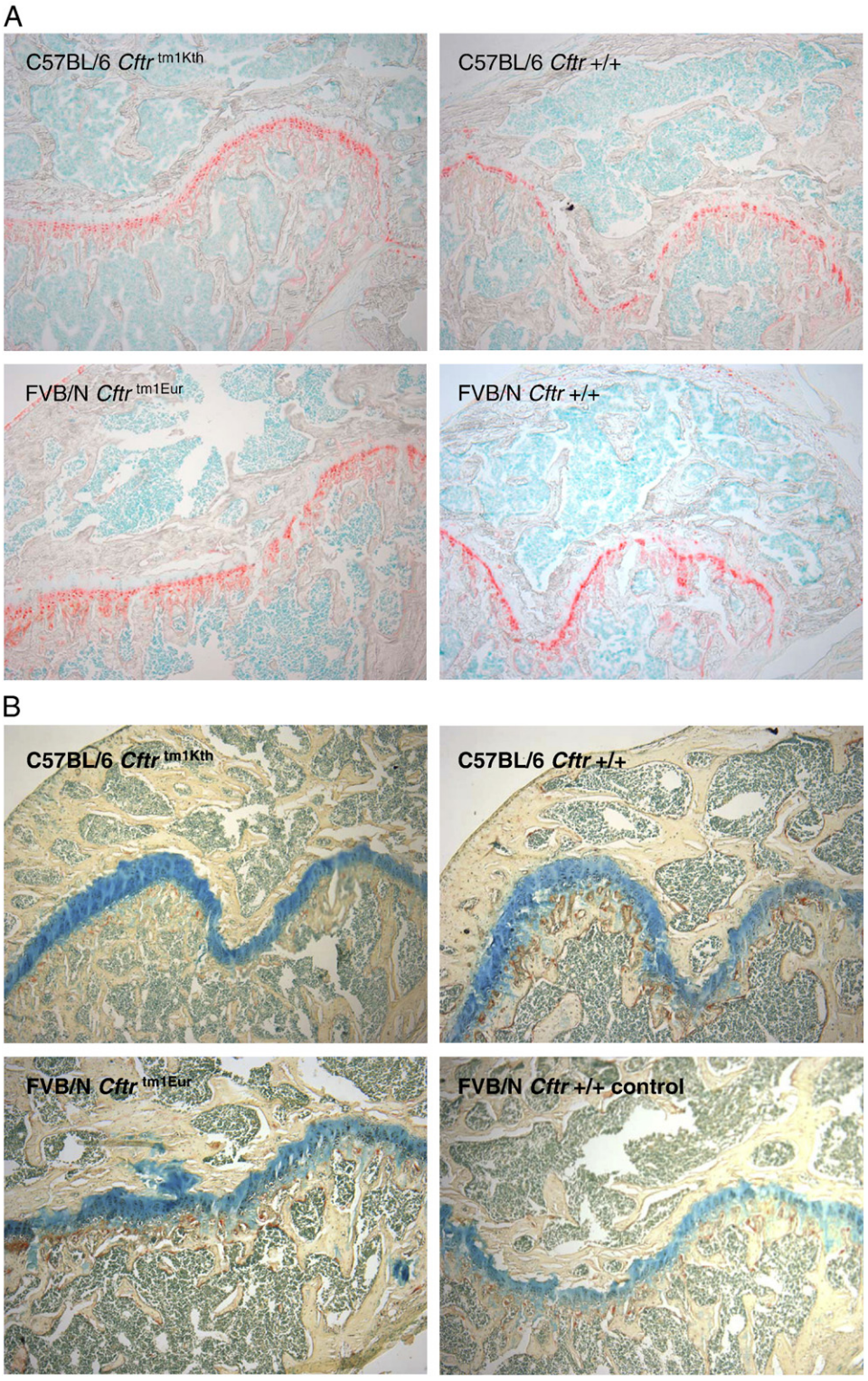

Fig. 4. Osteoblast and osteoclast numbers in ΔF508 *Cftr* mouse models. A. Osteoblasts were counted on representative alkaline phosphatase stained sections (red cells) and B. Osteoclasts were counted on representative tartrate resistant acid phosphatase stained sections (brownish red cells) of plastic embedded femoral bone from 4 to 7 mice per group. Representative images from C57BL/6 *Cftr*^{tm1Kth} (upper left); C57BL/6 *Cftr* +/+ control (upper right); FVB/N *Cftr*^{tm1Eur} (lower left); and FVB/N *Cftr* +/+ control (lower right); magnification = 100×. C. Positive cells (osteoblasts) per mm of growth plate (left) and % osteoclast staining in tissue. Values are expressed as mean ± SD. Significantly different from control, *$p < 0.05$.

the UNC, or knockout, mutation in *Cftr* [14,15,19] is present in CF mouse models carrying a ΔF508 mutation in *Cftr*. We have shown that in adult B6 *Cftr*^{tm1Kth} mice the femora are significantly shorter and of compromised trabecular architecture compared with wild type control mice. Secondly, FVB/N *Cftr*^{tm1Eur} mice had similar bone mineral density as their littermates but still

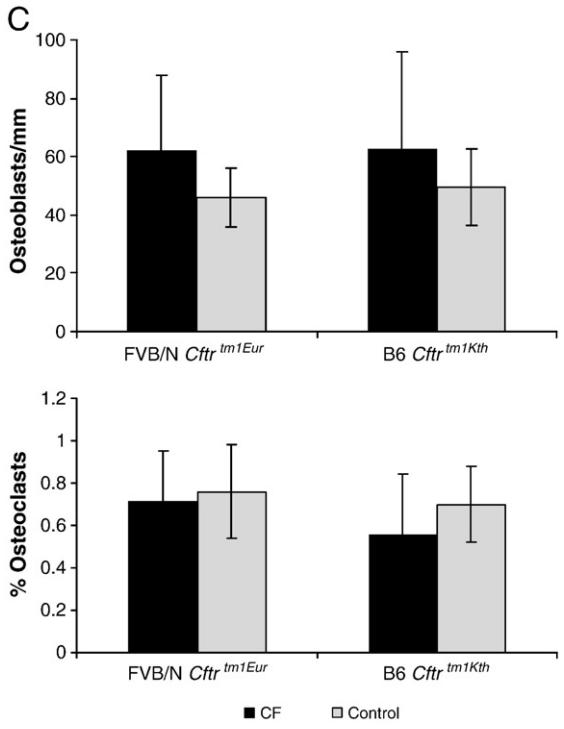

Fig. 4 (*continued*).

exhibited lower bone volume. Given the prior data of B6 [19] and BALB [15] *Cftr*^{tm1Unc} mice, an osteopenic phenotype has now been documented in four adult cystic fibrosis mouse models, including FVB/N *Cftr*^{tm1Eur} mice which express the clinically common ΔF508 mutation in *Cftr* at wild type levels.

The bone phenotype presented for B6 *Cftr*^{tm1Kth} mice, on PEGLYTE® treatment to reduce the incidence of lethal intestinal obstructions, agrees well with that reported by Pashuck et al. [19] for gut-corrected B6 *Cftr*^{tm1Unc} mice in that both investigations describe the adult CF mice to have reductions in femur length and thinner trabeculae, compared to littermates. As gut-corrected CF mice do not suffer the lethal intestinal complications which necessitated dietary intervention to overcome limited survival in our study, this finding indicates that the bone phenotype presented is not that of a selected population.

Also in agreement with previously reported data [10] the B6 *Cftr*^{tm1Kth} mice studied here were significantly smaller than the wild type littermates of the same age, and the reported bone phenotype in part reflects this size difference. Indeed, in a prior study [15] we showed control mice to have increased weight and bone mineral density at 12 weeks of age compared to at 8 weeks, while in BALB *Cftr*^{tm1Unc} CF mice these changes were evident at a later age, in 28 week old mice compared to those at 12 weeks. The CF bone disease features of low bone volume due to fewer, thinner, trabeculae, however, were still evident in the 28 week old mice. The growth delay, therefore, appears to affect the density of bones in mice, more than the architecture. This conclusion is reflected in the phenotype of the FVB/N *Cftr*^{tm1Eur} mice which displayed significantly reduced bone volume while weight, length and mineral density measures were similar to those of wild type mice.

The mouse strains evaluated here have not been reported to develop overt lung disease [8,10], but studies of the bronchoalveolar lavage of *Cftr*^{tm1Eur} mice have revealed it to differ from that of wild type mice. In detail, increased numbers of lavage neutrophils and macrophages have been reported [16,20,21] for *Cftr*^{tm1Eur} mice of a mixed 129/FVB genetic background while Meyer et al. [21] also demonstrated the peritoneal lavage of these animals to have greater inflammatory cell counts. Finally, in their work the lavage was shown to have an increased amount of chemokine C–C motif ligand-2 (CCL2) relative to that in control littermates. As altered levels of cytokines and chemokines, including CCL2 [22], can influence osteoclastogenesis, the reduced bone volume in these mice may be related to an inherent inflammatory state in CF mice. Supporting this conjecture is the work of Haworth et al. [23], who showed bone mineral content measures to be negatively correlated with serum interleukin-6 and C reactive protein levels in CF patients. Whether the FVB *Cftr*-^{tm1Eur} evaluated here present the same inflammation as that of mixed background *Cftr*^{tm1Eur} mice is not known.

Cell-type specific staining was used to show the CF and control mice of the strains evaluated here to not differ in numbers of osteoblasts or osteoclasts, in agreement with the work of Pashuck et al. [19]. Given the lower bone volume in the FVB/N *Cftr*^{tm1Eur} and B6 *Cftr*^{tm1Kth} CF mouse models and previous reports of altered bone formation rates in CF mice [14,19] this finding may indicate that a deficiency in Cftr does not influence bone cell numbers but may alter the cell function. More study is needed to elucidate how a deficiency in Cftr could influence cell function but a recent report [24] showed inhibition of CFTR in primary human osteoblast cultures to significantly decrease osteoprotegerin secretion, a condition which, as this protein negatively regulates osteoclast recruitment and activation [25], if presented by CF patients or mice, would be expected to result in increased bone resorption. Secondly, as Cftr functions in both chloride and bicarbonate transport [26] defects in the latter function could contribute to bone disease, given that bicarbonate levels influence osteoclast function [27].

In summary, through microCT imaging we have shown that cystic fibrosis mice created with reduced expression levels of *ΔF508-Cftr*, or wildtype expression levels of *ΔF508-Cftr* to present an osteopenic bone disease which supports the conclusion that a primary defect in *Cftr* contributes to the bone disease evident in cystic fibrosis.

Acknowledgements

The work was supported by the Canadian Cystic Fibrosis Foundation and the Canadian Institutes of Health Research, the Basic Research and Therapy (BREATHE) Program, and Fonds de la Recherche en Santé Québec. The FVB/N *Cftr*^{Tm1Eur} mice were developed with the support of EUROCARECF.

References

[1] Davis PB, Drumm M, Konstan MW. Cystic fibrosis. Am J Respir Crit Care Med 1996;154:1229–56.

[2] Sparks AA, McGee SJ, Boone CE, Neuringer IP, Jones SK, Aris RM. 'Old' bones in young bodies: the tale of cystic fibrosis. Curr Opin Endocrinol Diabetes Obes 2009;16:407–14.
[3] Paccou J, Zeboulon N, Combescure C, Gossec L, Cortet B. The prevalence of osteoporosis, osteopenia, and fractures among adults with cystic fibrosis: a systematic literature review with meta-analysis. Calcif Tissue Int 2010;86:1–7.
[4] Stalvey MS, Flotte TR. Endocrine parameters of cystic fibrosis: back to basics. J Cell Biochem 2009;108:353–61.
[5] King SJ, Topliss DJ, Kotsimbos T, et al. Reduced bone density in cystic fibrosis: DeltaF508 mutation is an independent risk factor. Eur Respir J 2005;25:54–61.
[6] Scholte BJ, Davidson DJ, Wilke M, De Jonge HR. Animal models of cystic fibrosis. J Cyst Fibros 2004;3:183–90.
[7] Snouwaert J, Brigman KK, Latour AM, et al. An animal model for cystic fibrosis made by gene targeting. Science 1992;257:1083–8.
[8] van Doorninck JH, French PJ, Verbeek E, et al. A mouse model for the cystic fibrosis delta F508 mutation. EMBO J 1995;14:4403–11.
[9] French PJ, van Doorninck JH, Peters RH, et al. A delta F508 mutation in mouse cystic fibrosis transmembrane conductance regulator results in a temperature-sensitive processing defect in vivo. J Clin Invest 1996;98:1304–12.
[10] Zeiher BG, Eichwald E, Zabner J, et al. A mouse model for the delta F508 allele of cystic fibrosis. J Clin Investig 1995;96:2051–64.
[11] Shead EF, Haworth CS, Condliffe AM, McKeon DJ, Scott MA, Compston JE. Cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in human bone. Thorax 2007;62:650–1.
[12] Bronckers A, Kalogeraki L, Jorna HJ, et al. The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in maturation stage ameloblasts, odontoblasts and bone cells. Bone 2009 Dec 29.
[13] Liang H, Yang L, Ma T, Zhao Y. Functional expression of cystic fibrosis transmembrane conductance regulator in mouse chondrocytes. Clin Exp Pharmacol Physiol 2009 Oct 16.
[14] Dif F, Marty C, Baudoin C, de Vernejoul MC, Levi G. Severe osteopenia in CFTR-null mice. Bone 2004;35:595–603.
[15] Haston CK, Li W, Li A, Lafleur M, Henderson JE. Persistent osteopenia in adult cystic fibrosis transmembrane conductance regulator-deficient mice. Am J Respir Crit Care Med 2008;177:309–15.
[16] Legssyer R, Huaux F, Lebacq J, et al. Azithromycin reduces spontaneous and induced inflammation in DeltaF508 cystic fibrosis mice. Respir Res 2006;7:134.
[17] Gawenis LR, Spencer P, Hillman LS, Harline MC, Morris JS, Clarke LL. Mineral content of calcified tissues in cystic fibrosis mice. Biol Trace Elem Res 2001;83:69–81.
[18] Clarke LL, Gawenis LR, Franklin CL, Harline MC. Increased survival of CFTR knockout mice with an oral osmotic laxative. Lab Anim Sci 1996;46:612–8.
[19] Pashuck TD, Franz SE, Altman MK, et al. Murine model for cystic fibrosis bone disease demonstrates osteopenia and sex-related differences in bone formation. Pediatr Res 2009;65:311–6.
[20] Bergamini G, Cigana C, Sorio C, et al. Effects of azithromycin on glutathione S-transferases in cystic fibrosis airway cells. Am J Respir Cell Mol Biol 2009;41:199–206.
[21] Meyer M, Huaux F, Gavilanes X, et al. Azithromycin reduces exaggerated cytokine production by M1 alveolar macrophages in cystic fibrosis. Am J Respir Cell Mol Biol 2009;41:590–602.
[22] Miyamoto K, Ninomiya K, Sonoda KH, et al. MCP-1 expressed by osteoclasts stimulates osteoclastogenesis in an autocrine/paracrine manner. Biochem Biophys Res Commun 2009;383:373–7.
[23] Haworth CS, Selby PL, Webb AK, et al. Inflammatory related changes in bone mineral content in adults with cystic fibrosis. Thorax 2004;59:613–7.
[24] Le Heron L, Guillaume C, Velard F, et al. Cystic fibrosis transmembrane conductance regulator (CFTR) regulates the production of osteoprotegerin (OPG) and prostaglandin (PG) E(2) in human bone. J Cyst Fibros 2010;9:69–72.
[25] Clarke B. Normal bone anatomy and physiology. Clin J Am Soc Nephrol 2008;3(Suppl 3):S131–9.
[26] Riordan JR. CFTR function and prospects for therapy. Annu Rev Biochem 2008;77:701–26.
[27] Geng W, Hill K, Zerwekh JE, Kohler T, Müller R, Moe OW. Inhibition of osteoclast formation and function by bicarbonate: role of soluble adenylyl cyclase. J Cell Physiol 2009;220:332–40.