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# Mitochondrial Point Mutation m.3243A>G Associates With Lower Bone Mineral Density, Thinner Cortices, and Reduced Bone Strength: A Case-Control Study

Jakob Høgild Langdahl,<sup>1,2,3</sup> Anja Lisbeth Frederiksen,<sup>1,2</sup> Stinus Jørn Hansen,<sup>2,4</sup> Per Heden Andersen,<sup>3</sup> Knud Bonnet Yderstræde,<sup>4</sup> Morten Dunø,<sup>5</sup> John Vissing,<sup>6</sup> and Morten Frost<sup>4</sup>

<sup>1</sup>Department of Clinical Genetics, Odense University Hospital, Odense, Denmark

<sup>2</sup>Institute of Clinical Research, University of Southern Denmark, Odense, Denmark

<sup>3</sup>Department of Endocrinology, Hospital of Southwest Jutland, Esbjerg, Denmark

<sup>4</sup>Department of Endocrinology, Odense University Hospital, Odense, Denmark

<sup>5</sup>Department of Clinical Genetics, Rigshospitalet, Copenhagen, Denmark

<sup>6</sup>Copenhagen Neuromuscular Center, Department of Neurology, Rigshospitalet, Copenhagen, Denmark

## ABSTRACT

Mitochondrial dysfunction is associated with several clinical manifestations including diabetes mellitus (DM), neurological disorders, renal and hepatic diseases, and myopathy. Although mitochondrial dysfunction is associated with increased bone resorption and decreased bone formation in mouse models, effects of alterations in mitochondrial function on bone remodeling and mass have not been investigated in humans. We recruited 45 carriers (29 females, 16 males) with the m.3243A>G mutation and healthy controls matched for gender, age, height, and menopausal status. DXA and HRpQCT scans were performed, and bone turnover markers (BTMs) P1NP and CTX were measured. Cases and controls were well matched except for body weight, which was lower in cases ( $63.6 \pm 18.1$  kg versus  $74.6 \pm 14.8$  kg,  $p < 0.01$ ), and manifest DM was present in 25 of 45 cases (none in controls). Bone scans showed lower BMD at the lumbar spine, total hip, and femoral neck in cases. Mean lumbar spine, total hip, and femoral neck *T*-scores were  $-1.5$ ,  $-1.3$ , and  $-1.6$  in cases, respectively, and  $-0.8$ ,  $-0.3$ , and  $-0.7$  in controls (all  $p < 0.05$ ). The m.3243A>G mutation was associated with lower BMD, cortical but not trabecular density, cortical thickness, and estimated bone strength. Furthermore, BTMs were lower in the m.3243A>G group before but not after adjustment for DM. The mitochondrial point mutation m.3243A>G was associated with decreased bone mass and strength. Although the coexistence of DM may have influenced bone turnover, the bone phenotype observed in m.3243A>G cases appeared to mirror age-related deterioration in bone, suggesting that mitochondrial dysfunction may cause a premature aging of bone. © 2017 The Authors. *Journal of Bone and Mineral Research* Published by Wiley Periodicals Inc.

**KEY WORDS:** M.3243A>G; MITOCHONDRIAL; BONE TURNOVER MARKERS; DIABETES; HRPQCT

## Introduction

The mitochondrion is the principal site of generation of adenosine triphosphate (ATP) and reactive oxygen species (ROS), and it is generally agreed that molecular damage caused by ROS is central in the pathogenesis of human aging and contributes to the accompanying gradual loss of bone mass.<sup>(1,2)</sup> Clinical data corroborating potentially deleterious effects of mitochondrial dysfunction on bone mass and fracture risk as observed in osteoporosis are limited.

Several animal studies support causative roles of mitochondrial dysfunction on bone metabolism. Mice with mitochondrial dysfunction due to accumulation of mutations in mitochondrial

DNA (mtDNA) display premature onset of age-related disorders, including reduced bone mineral density (BMD) and multiple compression fractures of the spine.<sup>(3)</sup> Depletion of intracellular ATP increases osteoclast resorption activity and apoptosis and inhibits bone formation in mice, supporting that intracellular ATP levels regulate bone remodeling.<sup>(4)</sup> Furthermore, ROS increases bone resorption by promoting receptor activator of nuclear factor kappa-B ligand (RANKL)-induced proliferation, differentiation, and lifespan of osteoclasts,<sup>(5)</sup> whereas oxidative stress reduces osteoblast differentiation and subsequently bone formation, by inhibition of the Wnt/ $\beta$ -catenin signaling pathway.<sup>(6)</sup> In addition, increased oxidative stress in osteocytes specifically increases osteoclast resorption and decreases bone

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Address correspondence to: Morten Frost, MD, PhD, Dept. of Endocrinology, Odense University Hospital, Sdr. Boulevard 29, 5000 Odense C, Denmark. E-mail: frostnielsen@yahoo.com

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formation due to increased expression of RANKL and the Wnt-pathway inhibitor sclerostin.<sup>(7)</sup>

The clinical phenotypes of mitochondrial diseases in humans may include several conditions, including but not restricted to impaired growth, myopathy, neurological deficits, and liver and kidney diseases.<sup>(8)</sup> Severe osteoporosis has been reported in a single case of a young man with an mDNA deletion,<sup>(9)</sup> but it remains unknown if mitochondrial dysfunction influences bone remodeling in humans.

The m.3234A>G is the most common mitochondrial point mutation (3.5 per 100,000).<sup>(8)</sup> Carriers of the m.3234A>G may be asymptomatic or develop specific clinical phenotypes including mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) and maternally inherited diabetes mellitus (DM) and deafness (MIDD).<sup>(10)</sup> Although infrequent, hypoparathyroidism has been reported in m.3234A>G carriers.<sup>(11–13)</sup> Hypoparathyroidism may increase bone mass due to lower bone turnover,<sup>(14)</sup> but accounts of bone mass in carriers of m.3234A>G mutations are absent. Exercise, disuse, and age-related changes in muscle and bone mass are correlated in humans, and low BMD has been reported in patients with myopathy.<sup>(15,16)</sup> Although BMD is lower in type 1 DM (T1D) and normal or increased in type 2 DM (T2D), bone turnover is lower in both T1D and T2D.<sup>(17,18)</sup> Mitochondrial DM is generally explained by decreased insulin secretory capacity,<sup>(19)</sup> suggesting that the bone phenotype of individuals with m.3243A>G could resemble that observed in T1D.

Although mitochondrial dysfunction appears to have significant impact on bone remodeling in mice, effects on human bone turnover, mass, and structure are unknown. The aim of this case-control study was to characterize bone turnover, mass, structure, and estimated strength in subjects carrying the m.3243A>G mitochondrial mutation.

## Subjects and Methods

### Design, setting, and participants

This cross-sectional study was conducted between June 2013 and June 2015. The study population consisted of 45 adult subjects carrying the mitochondrial point mutation m.3243A>G recruited from a Danish cohort of m.3243A>G-positive subjects from 25 families. These families included 79 individuals, three of whom were excluded due to pregnancy ( $n = 1$ ), cancer ( $n = 1$ ), or coexistence of a *PHEX* mutation ( $n = 1$ ). Twelve individuals were below the age of 18 years, and 19 declined participation.

Forty-five control subjects were included from a cohort of 499 healthy individuals participating in a separate study aimed at establishing HRpQCT reference data in the adult Danish population. The participants in the HRpQCT study were recruited in 2010 to 2011 using population listings in the municipality of Odense.<sup>(20)</sup> Each m.3243A>G positive subject was matched with a control subject from this cohort with regard to gender, age, height, and menopausal status (no menstruation for at least 6 months).

Examinations and scans were performed at Odense University Hospital. Information on lifestyle, comorbidities, and treatment was retrieved by interview and a structured questionnaire. Body weight was measured with participants wearing casual indoor clothing and barefoot to the nearest 0.1 kg on a Seca model 708 scale (Seca, Hamburg, Germany). Body height was measured to the nearest 0.1 cm on a wall-mounted Harpenden stadiometer (Holtain Ltd., Crymich, UK).

All participants provided informed consent, and the study was performed according to the guidelines from the Declaration of Helsinki. The Regional Scientific Ethical Committees for Southern Denmark approved both investigations (ID S-20100112 and ID S-20090069).

### Genetic investigations

DNA extractions were carried out using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany), using the manufacturer's protocol. An allele-specific polymerase chain reaction (PCR) assay was used for the detection of the m.3243A>G mutation. Two sets of primers were used in one reaction tube: 3014L (forward), GTGCAGCCGCTA TTAAGGT, and 3262HM17 (reverse), TTTTATGCG ATTACCGCGCC harboring two mismatches, which allow for a selective amplification of a 249-bp product if the m.3243A>G mutation is present. A control 614-bp product was co-amplified using the primers 5289 to 5309 (forward) and 5903 to 5584 (reverse). PCR reactions were carried out using GoTaq (Promega, WI, USA) on an ABI 2720 (Thermo Fisher, MA, USA).

### DXA, HRpQCT, and biochemical analysis

Areal BMD (aBMD) at the lumbar spine (L<sub>1</sub>–L<sub>4</sub>), total hip, femoral neck (all participants), and whole-body composition (cases only) were measured using dual-energy X-ray absorptiometry (DXA) (Hologic Discovery, Waltham, MA, USA). Bone geometry, volumetric BMD (vBMD), and microarchitecture were assessed using a HRpQCT system (Xtreme CT; Scanco Medical AG, Brüttisellen, Switzerland) as described.<sup>(21)</sup> The quality of the HRpQCT scans of the forearm was unacceptable due to motion artifacts (grade <3) in five of the participants, and these images and those from matching controls were excluded from the analyses.

Blood samples were drawn between 7:30 a.m. and 10:00 a.m. in the fasting state and stored at  $-80^{\circ}\text{C}$  until measurement of serum procollagen type I amino-terminal propeptide (P1NP) and C-telopeptide of type I collagen (CTX) by chemiluminescence method. The samples were analyzed in a single step with the same batch of the reagents and assay.<sup>(22)</sup>

### Statistical analyses

Data were expressed as mean  $\pm$  standard deviation, median (interquartile range [IQR]), or numbers as appropriate. Normality was evaluated using normal probability plots. m.3243A>G-positive subjects and controls were compared using chi square test for categorical variables and unpaired Student's *t* test or Mann-Whitney *U* test for normally distributed or nonparametric data, respectively. Differences in bone mass, structure, and strength in m.3243A>G-positive subjects and controls were presented graphically as mean percentage differences. These relationships were further investigated in three regression models. The first model included mutation status as the only independent variable; the second model included mutation status, body weight, age, and gender as independent variables. In addition, the third model also included DM status. Logarithmic transformation was applied if variables were not normally distributed. Statistical analyses were performed using STATA statistical package version 14 (StataCorp LP, College Station, TX, USA).

## Results

### General characteristics of study participants

Characteristics of m.3243A>G subjects and controls are presented in Table 1. Gender, age, body height, fracture history, ethnicity, smoking status (present smoker or nonsmoker), alcohol consumption (dichotomized as intake of more than 14 units per week or less), menopausal status, age at menopause, and length of radius and tibia did not differ between the groups. However, mean body weight and BMI were 11.0 kg and 3.8 kg/m<sup>2</sup> lower in the m.3243A>G group. The mean BMI of the m.3243A>G group was within the normal range (BMI 23.0 ± 5.3 kg/m<sup>2</sup>), but included eight individuals with a BMD <18.5 kg/m<sup>2</sup>, whereas the control subjects were slightly overweight (BMI 26.8 ± 4.1 kg/m<sup>2</sup>). Furthermore, calcium intake (dietary and supplement combined) was higher and use of vitamin D supplements were prevalent in the m.3243A>G subjects.

### Case-specific characteristics

Twenty-five of the m.3243A>G subjects had manifest DM and 31 reported hearing impairment (HI). None of the cases were known to have hepatic disease. Two cases had impaired renal function (creatinine >200 μmol/L). Those with DM were treated

**Table 1.** General Characteristics of the Study Population

	m.3243A>G (n = 45)	Control (n = 45)
Gender (male/female), n	16/29	16/29
Age (years)	47.6 ± 15.2	47.8 ± 14.4
Body weight (kg)	63.6 ± 18.1**	74.6 ± 14.8
Body height (cm)	165.4 ± 9.3	166.4 ± 8.4
Body mass index (kg/m <sup>2</sup> )	21.4 (20.1–25.0)**	26.3 (23.9–28.7)
Any previous fracture (yes/no), n	15/30	13/32
Diabetes status (DM/non-DM), n	25/20	0/45
Hearing impairment (HI/non-HI), n	31/14	0/45
Ethnicity (white/Middle Eastern/ Hispanic), n	43/1/1	45/0/0
Current smoking (yes/no), n	11/34	10/35
Alcohol consumption (yes/no), n <sup>a</sup>	1/44	5/39
Menopause (post/pre/male), n	16/13/16	16/13/16
Age at menopause (years)	48.2 ± 6.8	48.4 ± 3.6
Daily calcium intake (mg)	1300 (775–1700)**	600 (400–850)
Vitamin D supplement (yes/no/ unknown), n	29/16/0**	4/40/1
Radius length (cm)	24.7 ± 2.0	25.2 ± 2.2
Tibia length (cm)	36 (34–38)	36 (34–38)
Total spine aBMD (g/cm <sup>2</sup> )	0.89 ± 0.14**	0.97 ± 0.13
Total hip aBMD (g/cm <sup>2</sup> )	0.81 ± 0.16**	0.94 ± 0.13
Femoral neck aBMD (g/cm <sup>2</sup> )	0.68 ± 0.16**	0.80 ± 0.13
N-terminal propeptide of type 1 procollagen (μg/L)	45.3 ± 23.4*	57.8 ± 25.1
C-terminal telopeptide of type 1 collagen (μg/L)	0.41 ± 0.31	0.55 ± 0.30

Values are mean ± SD, median (interquartile range), or n.

<sup>a</sup>Yes: ≥14 units of alcohol per week.

\*p < 0.05.

\*\*p < 0.01.

using several types of antidiabetic drugs or dietary restrictions, but none of the diabetics were treated with peroxisome proliferator-activated receptor gamma agonists or metformin. Table 2 presents the m.3243A>G group stratified according to DM status. Cases with DM were more likely to report HI. Furthermore, body weight, but not body fat percentage, was lower in mutation carriers with DM.

### aBMD

aBMD of the lumbar spine, total hip, and femoral neck were lower in cases than controls (Table 1, Fig. 1), and femoral neck BMD was lower in cases with DM compared to those without DM (Table 2). T-scores of the lumbar spine, total hip, and femoral neck in cases were -1.54 ± 1.27, -1.27 ± 1.20, and -1.63 ± 1.17, respectively, and -0.80 ± 1.22, -0.30 ± 0.99, and -0.72 ± 0.97, respectively, in controls (all p < 0.05). The m.3243A>G mutation

**Table 2.** Characteristics of Cases Stratified According to Coexistence of DM

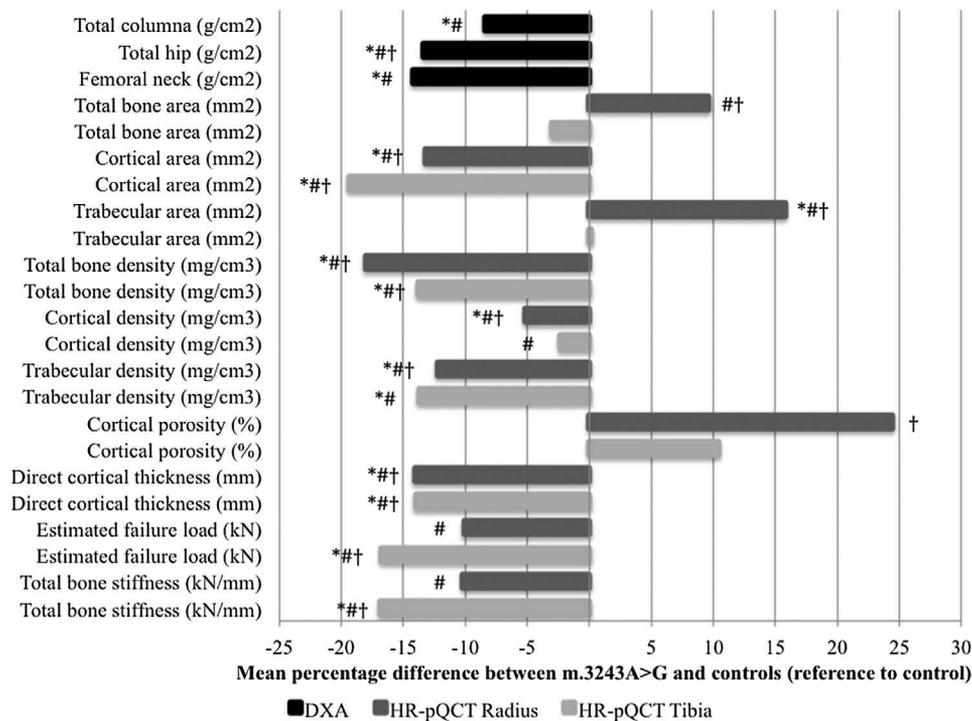
	DM (n = 25)	non-DM (n = 20)
Gender (male/female), n	8/17	8/12
Age (years)	49.7 ± 14.0	44.9 ± 16.5
Body weight (kg)	55.0 (51.9–64.1)*	70.1 (57.6–78.2)
Body height (cm)	163.5 ± 8.8	167.8 ± 9.7
Body mass index (BMI) (kg/m <sup>2</sup> )	21.1 (19.2–23.3)	24.8 (20.8–27.3)
Any previous fracture (yes/no), n	11/14	4/16
Hearing impairment (HI/non-HI), n	22/3**	9/11
Current smoking (yes/no), n	5/20	6/14
Alcohol consumption (yes/no), n <sup>a</sup>	1/24	0/20
Menopause (post/pre/male), n	8/9/8	8/4/9
Age at menopause (years)	48.2 ± 6.8	48.4 ± 3.6
Daily calcium intake (mg)	1350 (900–1925)	1225 (762.5–1600)
Vitamin D supplement (yes/no/ unknown), n	18/7/0	11/9/0
Radius length (cm)	24.0 (23.0–26.0)	24.5 (23.3–26.5)
Tibia length (cm)	35.4 ± 2.7	37.1 ± 3.1
Whole body fat (kg)	18.5 (13.9–22.0)	22.28 (14.9–29.3)
Whole body lean and BMC (kg)	36.7 (32.5–46.2)	43.76 (41.8–52.2)
Whole body mass (kg)	55.8 (49.9–67.0)*	71.02 (58.7–79.4)
Whole body fat percentage (%)	31.8 ± 7.9	32.2 ± 10.0
Vitamin D (nmol/L)	93.2 ± 7.6	73.6 ± 6.8
Total spine aBMD (g/cm <sup>2</sup> )	0.86 ± 0.13	0.90 ± 0.15
Total hip aBMD (g/cm <sup>2</sup> )	0.78 ± 0.16	0.85 ± 0.16
Femoral neck aBMD (g/cm <sup>2</sup> )	0.64 ± 0.13*	0.73 ± 0.17
N-terminal propeptide of type 1 procollagen (μg/L)	34.12 ± 1.08**	48.91 ± 1.11
C-terminal telopeptide of type 1 collagen (μg/L)	0.23 ± 1.16**	0.46 ± 1.16

Values are mean ± SD, median (interquartile range), or n.

<sup>a</sup>Yes: ≥14 units of alcohol per week.

\*p < 0.05.

\*\*p < 0.01.



\*  $p < 0.05$  in model 1. #  $p < 0.05$  in model 2. †  $p < 0.05$  in model 3

**Fig. 1.** Comparison of DXA and HRpQCT results in cases and controls, expressed as mean percentage difference.

was associated with lower aBMD in the unadjusted and the gender, age, and weight-adjusted models (Table 3), but only significantly associated with lower total hip BMD after adjustment for DM. DM was not associated with aBMD in regression models.

### Bone geometry

Total and trabecular bone areas in radius, but not tibia, were larger in the m.3243A>G group in all models with the exception

**Table 3.** Association Between aBMD and Mutation Status ( $n = 90$ )

	Model 1	Model 2	Model 3
Total spine aBMD (g/cm <sup>2</sup> )			
m.3243A>G (+/-)	-0.081**	-0.060*	-0.062
DM status (+/-) <sup>a</sup>			0.005
Total hip aBMD (g/cm <sup>2</sup> )			
m.3243A>G (+/-)	-0.125**	-0.068**	-0.065*
DM status (+/-) <sup>a</sup>			-0.007
Femoral neck aBMD (g/cm <sup>2</sup> )			
m.3243A>G (+/-)	-0.113**	-0.079**	-0.058
DM status (+/-) <sup>a</sup>			-0.042

Model 1: m.3243A>>G status as independent variable. Model 2: m.3243A>G status, weight, age and gender as independent variables. Model 3: m.3243A>G status, weight, age, gender, and DM status as independent variables.

<sup>a</sup>Independent effect of diabetes.

\* $p < 0.05$ .

\*\* $p < 0.01$ .

of total bone area in the unadjusted model (Table 4, Fig. 1). The cortical areas of both the radius and tibia were smaller in the m.3243A>G group in all regression models.

### vBMD

The presence of m.3243A>G was associated with lower total vBMD, cortical vBMD, and trabecular vBMD in the radius in all models (Table 4). Both total and trabecular tibial vBMD were lower in the m.3243A>G group before and after adjustment for weight, gender, and age, and total vBMD remained lower after adjustment for DM status. Tibial cortical vBMD was only associated with mutation status after adjustment for weight, gender, and age (Table 4).

### Bone microarchitecture

The m.3243A>G was associated with a lower ratio between trabecular bone volume and tissue volume in both radius and tibia, but this was not significant in tibia after adjustment for DM status (Table 4, Fig. 1). The m.3243A>G mutation was associated with lower trabecular number in the unadjusted model and the fully adjusted model, but trabecular thickness was not associated with mutation status in radius and tibia. Cortical thickness was lower in m.3243A>G carriers in all models in both radius and tibia, and radial cortical porosity was higher in the m.3243A>G group in the fully adjusted model (Table 4).

### Estimated bone strength

The m.3243A>G mutation was associated with lower estimated bone stiffness and failure loads in tibia in all models (Table 4, Fig. 1). Conversely, m.3243A>G was associated with estimated

**Table 4.** Association Between HRpQCT-Derived Measures of Bone Mass, Structure, and Strength, and Mutation Status

	Radius ( <i>n</i> = 80)			Tibia ( <i>n</i> = 90)		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
<b>Bone geometry</b>						
Total bone area (mm <sup>2</sup> )						
m.3243A>G (+/-)	26.545	33.289**	39.393**	-21.656	14.876	22.216
DM status (+/-) <sup>a</sup>			-12.383			-14.546
Cortical area (mm <sup>2</sup> )						
m.3243A>G (+/-)	-8.658*	-7.941*	-8.641*	-25.413**	-20.669**	-18.580**
DM status (+/-) <sup>a</sup>			1.421			-4.139
Trabecular area (mm <sup>2</sup> )						
m.3243A>G (+/-)	32.678*	38.430**	44.759**	0.696	32.211	38.481
DM status (+/-) <sup>a</sup>			-12.839			-12.425
Cortical perimeter (mm)						
m.3243A>G (+/-)	3.825	4.888**	5.288**	-2.080	0.705	1.217
DM status (+/-) <sup>a</sup>			-0.811			-1.016
<b>Volumetric bone mineral density</b>						
Total bone density (mg/cm <sup>3</sup> )						
m.3243A>G (+/-)	-65.035**	-64.043**	-73.005**	-42.440**	-37.281**	-37.291*
DM status (+/-) <sup>a</sup>			18.182			0.021
Cortical density (mg/cm <sup>3</sup> )						
m.3243A>G (+/-)	-46.438**	-50.672**	-68.185**	-20.427	-26.588*	-26.038
DM status (+/-) <sup>a</sup>			35.528			-1.091
Trabecular density (mg/cm <sup>3</sup> )						
m.3243A>G (+/-)	-20.448*	-18.607*	-22.299*	-23.569**	-15.647*	-15.579
DM status (+/-) <sup>a</sup>			7.489			-0.135
<b>Bone microarchitecture</b>						
Trabecular bone volume/tissue volume						
m.3243A>G (+/-)	-0.018*	-0.017**	-0.018*	-0.020**	-0.013*	-0.013
DM status (+/-) <sup>a</sup>			0.004			-0.000
Trabecular number (1/mm)						
m.3243A>G (+/-)	-0.193*	-0.159	-0.205*	-0.192*	-0.078	-0.185*
DM status (+/-) <sup>a</sup>			0.093			0.211*
Trabecular thickness (mm)						
m.3243A>G (+/-)	-0.002	-0.003	-0.002	-0.003	-0.003	0.002
DM status (+/-) <sup>a</sup>			-0.000			-0.010**
Direct cortical thickness (mm)						
m.3243A>G (+/-)	-0.143**	-0.143**	-0.145*	-0.180**	-0.166**	-0.147*
DM status (+/-) <sup>a</sup>			0.004			-0.037
Cortical porosity (%)						
m.3243A>G (+/-)	1.121	1.167	1.314**	1.073	1.114	1.133
DM status (+/-) <sup>a</sup>			0.705*			0.951
<b>Estimated bone strength</b>						
Total bone stiffness (kN/mm)						
m.3243A>G (+/-)	-9,032.7	-7,293.4*	-6,839.1	-36,735.9**	-25,016.3**	-16,954.7*
DM status (+/-) <sup>a</sup>			-891.9			-16,236.8
Estimated failure load (kN)						
m.3243A>G (+/-)	-451.1	-356.7*	-351.3	-1,836.6**	-1,234.9**	-903.2*
DM status (+/-) <sup>a</sup>			-10.6			-668.0

Model 1: regression with m.3243A>G status as independent variables. Model 2: m.3243A>G status, weight, age, and gender as independent variables. Model 3: m.3243A>G status, weight, age, gender, and DM status as independent variables.

<sup>a</sup>Independent effect of diabetes.

\**p* < 0.05.

\*\**p* < 0.01.

radial bone stiffness and failure loads after adjustment for gender, age, and weight, but not after further adjustment for DM.

#### Biochemical markers of bone turnover

Bone turnover markers (BTMs) (CTX and P1NP) were lower in the m.3243A>G group than in controls (Table 2). However, only

P1NP was lower in cases after adjustment for gender, age, and weight in the regression analyses, and mutation status was not associated with the BTMs after adjustments for DM (Table 5). Both BTMs were lower in individuals with manifest DM in the latter model. Among m.3243A>G carriers, BTMs were lower in those with DM (Table 2).

**Table 5.** Association Between Bone Turnover Markers and Mutation Status

	Model 1	Model 2	Model 3
N-terminal propeptide of type 1 procollagen ( $\mu\text{g/L}$ ) ( $n = 78$ )			
m.3243A>G (+/-)	-0.774*	-0.760*	-0.895
Diabetes (+/-) <sup>a</sup>			-0.691*
C-terminal telopeptide of type 1 collagen ( $\mu\text{g/L}$ ) ( $n = 72$ )			
m.3243A>G (+/-)	-0.703*	-0.741	-0.976
Diabetes (+/-) <sup>a</sup>			-0.552*

Model 1: m.3243A>G status as independent variables. Model 2: m.3243A>G status, weight, age, and gender as independent variables. Model 3: m.3243A>G status, weight, age, gender, and DM status as independent variables.

<sup>a</sup>Independent effect of diabetes.

\* $p < 0.05$ .

## Discussion

We have demonstrated that bone mass, structure, and estimated strength are compromised in carriers of m.3243A>G mitochondrial mutations. Additionally, BTMs were lower in carriers with DM. To our knowledge, this is the first comprehensive investigation of bone composition and turnover in individuals with a mitochondrial mutation.

The m.3243A>G mutation causes mitochondrial dysfunction and enhances oxidative stress, which is considered part of the pathogenesis of DM and other manifestations of mitochondrial disease.<sup>(23)</sup> Previous investigations of bone status in monogenic mitochondrial disorder are lacking. Negative correlations between BMD and oxidative stress and oxidative damage have been observed in postmenopausal women,<sup>(24,25)</sup> supporting that the bone phenotype observed in our investigation is explained by mitochondrial dysfunction. With age, vBMD, cortical thickness, and trabecular number decline, cortical porosity increases, and estimated bone strength regresses,<sup>(21)</sup> which is largely compatible with the pattern of differences among the carriers of m.3243A>G mutation and healthy individuals observed in our study. Although speculative, this could indicate that the m.3243A>G bone phenotype exemplify premature bone aging.

Nearly all carriers of the m.3243A>G develop impaired glucose tolerance or DM before the age of 70 years,<sup>(26)</sup> and it is generally agreed that mitochondrial DM primarily is caused by impaired glucose-stimulated insulin secretion.<sup>(26)</sup> Increased heteroplasmy is associated with earlier debut of DM in carriers of m.3243A>G.<sup>(27)</sup> Therefore, the bone phenotype observed in mutation carriers with DM may illustrate the effects of severe forms of mitochondrial dysfunction and DM. This notion is supported by a higher prevalence of HI and lower body weight in m.3243A>G with DM, indicating that these individuals had a higher level of mitochondrial dysfunction.

The present study shows that the bone phenotype observed in m.3243A>G carriers, in most aspects differ from that seen in healthy controls, and appear to share similarities with the bone phenotype reported in T1D, which includes decreased cortical bone thickness and density as well as lower trabecular vBMD.<sup>(22,28)</sup> With respect to bone turnover, the regression analyses showed that bone turnover appeared to be preserved in nondiabetic but lower in diabetic mutation carriers. However, the relatively small size of the study population refrained us from assessing BTMs at different age ranges in mutation carriers and

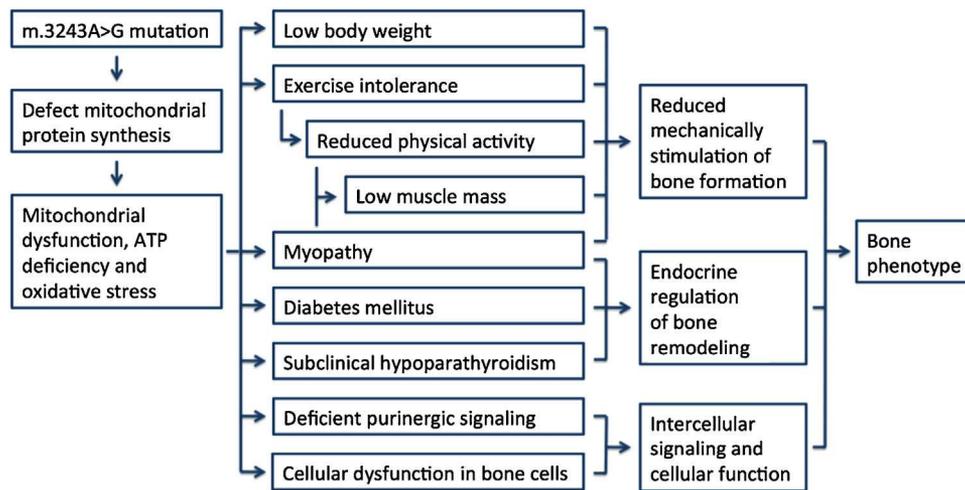
controls. Therefore, we cannot exclude the possibility that bone turnover is lower in elderly mutation carriers. However, because heteroplasmy levels were not assessed in our study, we were unable to investigate whether the degree of mitochondrial dysfunction, assessed using heteroplasmy levels as a surrogate marker, was associated with any of the available measures of bone mass, structure, or turnover.

BMD and body weight are closely associated, and the difference in weight between cases and controls probably influenced the results of this study.<sup>(29,30)</sup> Obese adults have higher BMD, thicker and denser cortices, and higher trabecular number than non-obese adults,<sup>(31,32)</sup> and these differences are mainly present in tibia and not radius, suggesting that the effects of body weight are explained by positive effects of mechanical loading. When comparing our study with studies of the effect of obesity on BMD it seems that weight has a disproportionately larger impact on BMD in our study.<sup>(31,33)</sup> Furthermore, with trabecular number and CTX as exceptions, we observed that associations between m.3243A>G and bone parameters remained significant after adjustment for weight, gender, and age. Taken together, this suggests that the weight difference cannot explain the differences in the bone phenotype between cases and controls in our study.

Mitochondrial myopathy may cause varying degrees of exercise intolerance, fatigue, myalgia, and a general sense of weakness,<sup>(34)</sup> and lower levels of habitual physical activity have been reported in mitochondrial patients.<sup>(35)</sup> Physical activity is directly correlated with bone mass; therefore, mitochondria-related myopathy could affect bone mass and structure.<sup>(36,37)</sup> Unfortunately, data on physical activity were not recorded in the present investigation, which excluded the possibility of evaluating whether differences in bone status and BTMs between m.3243A>G carriers and controls were related to physical activity.

Use of calcium and vitamin D supplements were higher in the m.3243A>G carriers, which may be explained by higher contact with healthcare services in cases versus controls. These supplements could be beneficial to the bone status of the cases, and any negative effect of m.3243A>G might thus be underestimated. Although unsubstantiated, subclinical disturbances in the calcium metabolism such as hypoparathyroidism could interfere with bone metabolism in cases with m.3243A>G. The prevalence of hypoparathyroidism was 6% among 226 individuals with Kearns-Sayre syndrome (KSS) (mitochondrial encephalomyopathies with early onset, caused by deletions of mtDNA), but similar data is not available for m.3243A>G.<sup>(38)</sup>

Although the present investigation shows that mitochondrial dysfunction due to m.3243A>G mutation may have deleterious effects on bone, the mechanisms linking the mutation and mitochondrial dysfunction to the bone phenotype remains to be determined. Figure 2 presents some of the mechanisms, which may link mitochondrial dysfunction to the bone phenotype. Mitochondrial dysfunction including ATP deficiency and increased oxidative stress may have direct effects on the differentiation and activity of bone cells. Decreased ATP synthesis may disturb purinergic signaling, which has been implicated in several signaling pathways involved in the regulation of bone metabolism,<sup>(39)</sup> such as the response to mechanical strain<sup>(40)</sup> and potentiation of PTH signaling.<sup>(41)</sup> DM, myopathy, low body weight, and exercise intolerance may have secondary effects on the bone phenotype, either by endocrine regulation such as decreased secretion of myokines or lower mechanical loading of the bone. Therefore, it would appear that



**Fig. 2.** Relations between mitochondrial dysfunction and bone metabolism.

the pathogenesis of the bone phenotype observed in m.3243A>G is caused by a complex interplay of factors as illustrated in Fig. 2.

The foremost limitation to this study is the inadequate matching of cases and controls with regard to body weight. This was particularly challenging because the variation in body weight was substantial in cases, which comprised several individuals with a low BMI. However, recruitment of healthy individuals with a low BMI as controls was considered inappropriate because underweight is infrequent in the normal population and often associated with chronic conditions that could have direct effects on bone. Furthermore, we abstained from matching diabetic cases with diabetic controls because DM observed in m.3243A>G is different from T1D and T2D. The coexistence of DM-influenced bone turnover, however, omitting m.3243A>G carriers with manifest DM would have excluded cases with the highest degree of mitochondrial dysfunction, which were most likely to have developed a distinct bone phenotype. Furthermore, we cannot exclude the possibility that selection bias was introduced because controls were recruited from urban population listings and controls from both urban and rural parts of the country.

Acknowledging the shortcoming with regard to matching on body weight, the present study has a number of strengths. Most notably, we included a sizable number of individuals with a rare mutation as well as age- and gender-matched controls. Although validated methods were used to study the bone phenotype in these rare cases, assessment of dynamic bone histomorphometry could have provided knowledge on the effects of mitochondrial dysfunction on bone remodeling, including osteoblast and osteoclast activity in humans.

In conclusion, this study shows that the m.3243A>G mutation is associated with a bone phenotype characterized by impaired bone mass, structure, and strength. Although the mechanisms by which mitochondrial dysfunction affects bone remodeling in humans cannot be determined by this study, we speculate that enhanced oxidative stress and decreased ATP production could lead to a premature ageing of the skeleton.

## Disclosures

All authors state that they have no conflicts of interest.

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