Gender- and region-specific alterations in bone metabolism in *Scarb1*-null female mice

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Abstract

A positive correlation between plasma levels of HDL and bone mass has been reported by epidemiological studies. As scavenger receptor class B, type I (SR-BI), the gene product of *Scarb1*, is known to regulate HDL metabolism, we recently characterized bone metabolism in *Scarb1*-null mice. These mice display high femoral bone mass associated with enhanced bone formation. As gender differences have been reported in HDL metabolism and SR-BI function, we investigated gender-specific bone alterations in *Scarb1*-null mice by microtomography and histology. We found 16% greater relative bone volume and 39% higher bone formation rate in the vertebrae from 2-month-old *Scarb1*-null females. No such alteration was seen in males, indicating gender- and region-specific differences in skeletal phenotype. Total and HDL-associated cholesterol levels, as well as ACTH plasma levels, were increased in both *Scarb1*-null genders, the latter being concurrent to impaired corticosterone response to fasting. Plasma levels of estradiol did not differ between null and WT females, suggesting that the estrogen metabolism alteration is not relevant to the higher vertebral bone mass in female *Scarb1*-null mice. Constitutively, high plasma levels of leptin along with 2.5-fold increase in its expression in white adipose tissue were measured in female *Scarb1*-null mice only. *In vitro* exposure of bone marrow stromal cells to ACTH and leptin promoted osteoblast differentiation as evidenced by increased gene expression of *osterix* and *collagen type I alpha*. Our results suggest that hyperleptinemia may account for the gender-specific high bone mass seen in the vertebrae of female *Scarb1*-null mice.

Key Words

- *Scarb1*
- SR-BI
- high bone mass
- osteoblasts
- leptin
- corticosterone
- adrenocorticotropin

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Introduction

Bone tissue is constantly renewed in a process called remodeling. Remodeling relies on the activities of specialized cells, bone-forming osteoblasts, and bone-resorbing osteoclasts, to maintain bone mass (Clarke 2008). The balance between bone formation and resorption involves the regulation of bone cell activities by several endocrine and paracrine axes including steroid hormones (Frenkel

et al. 2010), adrenocorticotrophic hormone (ACTH; Isaacs *et al.* 2010), and cytokines (Hughes *et al.* 2006). Uncoupling of the formation and resorption processes results in improper bone mass maintenance. Bone mass results from the difference between peak bone mass, defined as the completion of skeletal growth typically occurring in early adulthood, and age-related bone loss (Rizzoli *et al.* 2001,

Stagi *et al.* 2013). It is generally accepted that the key determinant in healthy bone is the achievement of a higher peak bone mass, which delays or even prevents the onset of osteoporosis later on in life (Stagi *et al.* 2013).

Several factors influence peak bone mass, predominantly gender, genetics, and physical activity, as well as vitamin D and calcium intake (Rizzoli *et al.* 2001, Stagi *et al.* 2013). It is a well-known fact that men tend to achieve higher peak bone mass than do women, presumably due to the longer period of bone mass gain in the former (Rizzoli *et al.* 2001). Bone accrual is also site specific within the same skeleton, with preferential bone mineral accumulation in the lumbar vertebrae and femoral neck (Rizzoli *et al.* 2001, Bonjour *et al.* 2009); these also happen to be common sites for osteoporotic fractures and show the greatest gender-related dimorphism (Raisz 2005, Bonjour *et al.* 2009). Several determinants of peak bone mass are common to that of osteoporosis; factors that compromise bone mass are attributed to race, smoking, inactive lifestyle, malnutrition, and underlying metabolic disorders such as diabetes and atherosclerosis are often associated with lower bone mass (Leidig-Bruckner & Ziegler 2001, Sweet *et al.* 2009, Stojanovic *et al.* 2011). Genetic control of bone mass is polygenic and the specific genes involved are beginning to be enumerated, such as genetic variants/mutations of vitamin D and estrogen receptors (Mitchell & Yerges-Armstrong 2011). Recent attention has been given to atherogenic conditions with low HDL plasma levels as factors compromising bone health, and numerous associations, have been revealed between lower bone mass and cardiovascular diseases (Banks *et al.* 1994, Uyama *et al.* 1997, Barengolts *et al.* 1998, von der Recke *et al.* 1999, Hegsted 2001, Yamaguchi *et al.* 2002, Poli *et al.* 2003, Adami *et al.* 2004, Orozco 2004). In accordance, a positive correlation between plasma HDL levels and bone mass has been reported by epidemiological studies (reviewed in Ackert-Bicknell (2012)), suggesting that, in addition to protecting against atherosclerosis, HDL contribute to a healthy environment for bones.

The product of the *Scarb1* gene, the scavenger receptor class B, type I (SR-BI) is recognized as a high-affinity HDL receptor (Acton *et al.* 1996) and is known for its role in HDL metabolism (Cuchel & Rader 2006). *Scarb1*-null mice show high HDL-associated cholesterol (HDL-C) levels due to impaired selective uptake of cholesterol by the liver (Rigotti *et al.* 1997). Also, *Scarb1*-null male mice exhibit lack of SR-BI-mediated cholesterol uptake from HDL by the adrenal glands and concurrent impaired corticosterone response to stress and high plasma levels of ACTH

(Hoekstra *et al.* 2008, 2013, Martineau *et al.* 2014). Given that we reported the expression of SR-BI by osteoblasts (Brodeur *et al.* 2008), we recently characterized bone metabolism in *Scarb1*-null mice (Martineau *et al.* 2014). Higher trabecular bone mass in femora and greater proliferation rate and differentiation of bone marrow mesenchymal stromal cells (MSCs) were found in *Scarb1*-null mice, concurrent to enhanced bone formation. Of interest, differences between male and female *Scarb1*-null mice were observed in cortical bone volume of the femurs from *Scarb1*-null female mice which had increased volume compared with WT mice, whereas no difference was noted in males.

Skeleton sexual dimorphism is generally attributed to sex steroids such as estradiol (E₂), but also to growth hormone, insulin-like growth factor, and mechanical sensitivity to loading (Callewaert *et al.* 2010). Gender differences in lipoprotein metabolism have also been evidenced (for a review, Knopp *et al.* (2005)), but it is unknown if and how this affects bone. Of interest, lower levels of SR-BI are found in the liver of female mice (Brodeur *et al.* 2005) and in accordance, estrogen was shown to reduce hepatic SR-BI expression. In contrast, SR-BI expression in adrenal glands is increased by estrogen (Landschulz *et al.* 1996) and its expression is enhanced by ACTH (Sun *et al.* 1999). In addition, the adipokine leptin was shown to stimulate SR-BI expression in hepatocytes (Lundasen *et al.* 2003). Sex differences in adipose tissue, the main source of leptin, were documented and recently, leptin has been identified as an important direct bone growth modulator (Turner *et al.* 2013); gender-related differences in regard to effects of leptin on bone were also noted (Thomas *et al.* 2001). Thus, the observed gender differences in the bone structure of *Scarb1*-null mice may be related to the estrogen, ACTH, and/or leptin hormonal axes. This study therefore aimed at assessing how *Scarb1* deficiency could produce gender-specific skeletal alterations in mice through analysis of vertebral architecture, serology, and marrow-derived stromal cells' response to relevant endocrine factors.

Materials and methods

Animals

Scarb1-null mice on a C57BL/6J129 background were obtained from Jackson Laboratories (stock no. 003379; Bar Harbor, ME, USA) and crossbred with WT C57BL/6 mice. Owing to their low fertility, *Scarb1*-null females were fed a 0.5% probucol diet (Research Diet, New-Brunswick,

NJ, USA) for 10 days before mating (Miettinen *et al.* 2001). The heterozygous (HZ) progeny was mated to obtain first generation (F1) WT and null couples; HZ, WT, and null couples were bred in parallel throughout each generation to allow littermate and inter-litter comparisons, as well as to insure that probucol did not affect the observed phenotype. All animals were kept under a 12 h light:12 h darkness cycle at 25 °C, with free access to food and water, unless specified otherwise. Two- and four-month-old mice from F7–F10 generations were used throughout the study. The mice used for the ACTH/glucocorticoid (GC) study were the same as the ones used by Martineau *et al.* (2014); animals were added to complete the leptin study and lumbar vertebrae analyses. All experiments were performed according to UQÀM Institutional Animal Care Committee guidelines (#611). Following euthanasia, whole body, abdominal fat, and uterine weights were determined.

Serology

Whole blood sample was harvested through cardiac punctures and collected in 3 ml heparinized tubes (68 USP, BD Bioscience, Mississauga, ON, Canada). Plasma was obtained by centrifugation at 2000 *g* for 25 min at 4 °C and stored at –80 °C until analyses. Calcium, phosphate, and glucose were evaluated by the QuantiChrom assays (BioAssay Systems, Hayward, CA, USA), and alkaline phosphatase (ALP) and total/lipoprotein-associated cholesterol were quantified using the EnzyChrom assays

(BioAssay Systems), all according to manufacturer's recommendations. 17β -E₂ levels were measured with the commercially available ELISA Kits (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. For determination of plasma levels of corticosterone (Enzo Life Sciences, Farmingdale, NY, USA), ACTH (MyBioSource, San Diego, CA, USA), leptin (Millipore, Billerica, MA, USA), and adiponectin (Millipore), mice were fasted overnight (16 h); half of them were refed 2 h before being anaesthetized with Isoflurane (PPC, Richmond Hill, ON, Canada) and whole blood collection. All mice were killed by CO₂ asphyxiation following anesthesia and whole blood collection.

Microcomputed X-ray tomography

After the animals were killed, lumbar vertebrae were harvested and fixed in 4% paraformaldehyde (PF) in PBS for 16–18 h at 4 °C. The vertebrae were scanned using a Skyscan 1172c tabletop MicroCT system (Soquelec, Montreal, QC, Canada) with the following parameters: 5 µm pixel size, 70 kV/100 µA tube voltage/current, 1200 ms exposure time, and a 0.5 mm aluminium filter. Raw images were optimized and reconstructed using NRecon Software (Skyscan, Aartselar, Belgium); the volumes of interest were defined as 230 slices, 100 slices below the intervertebral space (Fig. 1A). The threshold was set at 80 to discriminate bone from soft tissue and binary images were subsequently analyzed with CTAn (Skyscan) to obtain the following microCT parameters: relative bone

Figure 1

MicroCT and histological analysis of *Scarb1*-null mouse vertebrae. (A) Representative 3D renderings of 2- and 4-month-old mouse fourth lumbar vertebrae bone volumes of interest (VOI) and representative 3D renderings of VOI. VOI was defined as a 230 slice thick section of the vertebral body (upper and lower dashed lines) that were selected 100 slices from the intervertebral space (IV, full line), with caudal growth plate

facing up. (B) Representative Von Kossa (VK, scale bar=500 µm), alkaline phosphatase (ALP, scale bar=200 µm), tartrate-resistant acid phosphatase (TRAP, scale bar=200 µm) stainings, and calcein fronts (top, 10× digital zoom of typical double-label front and bottom, general labeling, scale bar=200 µm) from lumbar vertebrae of 2-month-old mice.

volume (BV/TV), trabecular thickness (Tb.Th), number of trabeculae (Tb.N), and trabecular spacing (Tb.Sp). Bone mineral density (BMD) was evaluated using a standard curve obtained from hydroxyapatite rods of 2 mm diameter (Skyscan) scanned with the same parameters.

Static and dynamic histology

Two-month-old mice were injected twice with 20 mg of calcein (Sigma–Aldrich)/kg body weight 10 and 2 days before being killed. Lumbar vertebrae were dissected and fixed for 16–18 h in PBS with 4% PF at 4 °C. Bones were processed and embedded in low-temperature polymerizing polymethylmethacrylate (Erben 1997), sectioned transaxially starting 20 slices below the caudal growth plate at a 6 µm thickness with a rotary HM 360 microtome (Thermo Fisher, Ottawa, ON Canada). Calcein fronts were observed with a fluorescent microscope (Nikon FN1 Eclipse, Mississauga, ON, Canada) and averaged from the top (sections 20–25), middle (sections 35–40), and bottom (sections 45–50) for each sample. In parallel, bone sections were stained with the Von Kossa technique, and for ALP (Millipore) and tartrate-resistant acid phosphatase (TRAP; Dako, Burlington, ON, Canada) activities. Von Kossa, ALP, and TRAP-stained bone sections were visualized under an inverted phase-contrast microscope (TE-300, Nikon) and analyzed with ImageJ (NIH, Bethesda, MD, USA) to evaluate the following static and dynamic parameters: relative osteoblast surface per bone surface (Ob.S/BS on ALP-stained sections), number of osteoclasts (N.Oc/mm on TRAP-stained sections), percent mineralizing surface (MS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS). MS/BS was calculated as $dL + (sL/2)/BS$, where dL and sL are double- and single-labeled surfaces respectively.

Marrow-derived stromal cultures

Femora and tibiae from 2-month-old female WT and null mice were denuded of any soft tissue, sterilized twice for 30 min in PBS supplemented with 200 U/ml penicillin–streptomycin (Invitrogen) and 0.2% FungiZone (Invitrogen) (PBS–PSF), and then transferred into fresh PBS–PSF under a sterile hood. The epiphyses were cut off and marrow was flushed out into an Eppendorf tube, centrifuged at 5900 g for 1 min in an Eppendorf tabletop centrifuge (Eppendorf, Mississauga, ON, Canada), suspended in PBS, and cell clumps were broken up by repetitive extrusions. The suspension was diluted in αMEM (Invitrogen), containing 25 µg/ml L-ascorbic acid (Sigma–Aldrich).

The marrow cells were plated in 100 mm dish (Sarstedt, Montréal, QC, Canada; bones from one mouse per dish) and left to adhere for 7 days in αMEM with 10% FBS (Cansera, Etobicoke, ON, Canada) and 25 µg/ml of L-ascorbic acid (Sigma–Aldrich). Nonadherent cells were then vigorously washed away with PBS–PSF. The cells were left to reach confluence, washed twice with PBS, and then harvested for experimentation.

Osteoblastic differentiation

Adherent MSCs were harvested and seeded at 50 000 cells/cm² in 24-well plates (Sarstedt), left to reach confluence, and treated for 21 days in osteogenic medium consisting of MEM (Invitrogen) with 10% FBS, 50 µg/ml L-ascorbic acid, and 3 mM glycerol-2-phosphate (Sigma–Aldrich). Osteoblastic differentiation was assessed through ALP (Millipore) and Alizarin S (Sigma–Aldrich) staining. Alizarin S was solubilized in 10% cetylpyridinium chloride and its optical absorbance was read at 575 nm.

Real-time PCR

The cells were kept for 24 h in αMEM with 10% FBS or in MEM containing 2% FBS with or without 100 ng/ml leptin (Sigma–Aldrich) or 10 nM ACTH (Tocris Bioscience, Bristol, UK), lysed with RiboZol (Amresco, Solon, OH, USA), and total RNA was extracted as recommended by the manufacturer. Total RNA was reversed transcribed using AMV RT (Roche) accordingly to the manufacturer's instructions. cDNA was used to carry out PCR with the following primer pairs: reference gene β-microglobulin (*B2m*; F, 5'-TACTCACGCCACCCACCGGAG-3' and R, 5'-GCTCGGCCATACTGGCATGCT-3'), osteoblastic markers osterix (*Osx/Sp7*; F, 5'-TTCGCATCTGAAAGCCCACT-3' and R, 5'-TGCGCTGATGTTTGCTCAAG-3'), collagen type I (*Col1a1*; F, 5'-ACTTCAGCTTCCTGCCTCAG-3' and R, 5'-GCTTCTTTTCCTTGGGGTTC-3'), and sclerostin (*Sost*; F, 5'-CAGGAATGATGCCACAGAGGT-3' and R, 5'-GTCTGTCAGGAAGCGGGTG-3'). In parallel, RNA was extracted from white adipose tissue of 2-month-old mice, reverse transcribed, and used to observe the expression of leptin (*Lep*; F, 5'-TGCGGCCAGGAGAGGTGAG-3' and R, 5'-AAGCAGCACCCAGGAGCCT-3'). PCRs were carried out in a MyiQ Thermocycler (Bio-Rad) for 40 cycles, with a denaturation temperature at 95 °C (10 s), an annealing temperature at 58 °C (30 s), and a 5 min terminal elongation at 72 °C. Relative fluorescence was analyzed with the iQ5 Software (Bio-Rad).

Table 1 Plasmatic parameters in fasted 2-month-old female mice. Values are means $\pm$ S.E.M.; number of mice per group is indicated in parentheses

Genotype	WT (5)	Null (5)
ALP ((μ mol p-NP/(l $\times$ min)))	597.6 $\pm$ 47.9	521.3 $\pm$ 30.3
Ca (mg/dl)	9.0 $\pm$ 0.5	9.0 $\pm$ 0.8
Pi (mg/dl)	10.0 $\pm$ 0.7	10.2 $\pm$ 0.6
Ca $\times$ Pi ³ (mg ² /dl ²)	90.2 $\pm$ 11.5	91.7 $\pm$ 13.6
Glucose (mg/dl)	227.9 $\pm$ 27.5	219.4 $\pm$ 20.7

^aThe Ca $\times$ Pi indices were obtained by multiplying the Ca and Pi means within each group, and their relative errors were added to obtain the absolute error of the product.

Statistical analyses

Data were analysed with the Prism5 Software (GraphPad, La Jolla, CA, USA) for statistical differences. Two-way ANOVAs and multiple comparisons by Bonferroni, as indicated in the figure legend, were applied to determine statistical significance; a *P* value of 0.05 was considered as the significance threshold. All data are presented as mean $\pm$ S.E.M.

Results

Higher vertebral bone mass in *Scarb1*-null female mice

Previously, we observed a high femoral bone mass phenotype associated with enhanced bone formation in both male and female *Scarb1*-null mice (Martineau *et al.* 2014). However, the cortical bone volume of *Scarb1*-null female mice was increased compared with WT mice, whereas no difference was noted for males (Martineau *et al.* 2014). Therefore, we further investigated gender-specific bone phenotype and endocrine dysfunction in *Scarb1*-null mice. Total body weight was not significantly

different between WT and *Scarb1*-null female mice (18.2 $\pm$ 1.1 vs 21.5 $\pm$ 3.8 g), and no significant difference was observed in abdominal fat (0.14 $\pm$ 0.03 vs 0.19 $\pm$ 0.09 g) and uterus (0.12 $\pm$ 0.04 vs 0.14 $\pm$ 0.06 g) weights between 2-month-old WT and *Scarb1*-null female mice. Similarly, total body weight was not different between male *Scarb1*-null and WT mice (Martineau *et al.* 2014). To assess whether SR-BI deficiency caused any metabolic, renal, or mineral disorder, plasmatic levels of calcium, phosphate, ALP, and glucose were measured in 2-month-old WT and *Scarb1*-null females; no significant alterations were detected between WT and *Scarb1*-null female mice (Table 1), being similar to male mice (Martineau *et al.* 2014).

Representative microtomographic renderings from lumbar vertebrae qualitatively showed alterations in bone architecture (Fig. 1A). Two-month-old *Scarb1*-null female mice showed higher relative bone volume, increased Tb.N, and BMD, as well as lower Tb.Sp compared with female WT mice (Table 2). The relative vertebral bone mass of *Scarb1*-null female mice thereafter was similar to control values at 4 months of age. Tb.Th values remained unaffected at 2 months, yet they were significantly lower at 4 months. In contrast, microtomographic analysis revealed unchanged vertebral structure in 2-month-old *Scarb1*-null male mice (Table 2), but Tb.Th was also reduced in 4-month-old *Scarb1*-null male mice.

Enhanced bone formation in the vertebrae from *Scarb1*-null female mice

To assess whether this higher lumbar bone mass was due to either altered osteoblastic or osteoclastic functions, histochemistry was performed on samples from 2-month-old mice (Fig. 1B for representative bone sections).

Table 2 MicroCT analysis parameter values in 2- and 4-month-old WT and null mice lumbar vertebrae. Values are mean $\pm$ S.E.M.; number of mice per group is indicated in parentheses

	Female				Male			
	2 months		4 months		2 months		4 months	
	WT (13)	Null (13)	WT (12)	Null (12)	WT (12)	Null (10)	WT (12)	Null (12)
BV/TV (%)	32.7 $\pm$ 0.4	37.9 $\pm$ 0.7 [‡]	37.8 $\pm$ 1.6	37.3 $\pm$ 1.0	35.9 $\pm$ 1.2	34.5 $\pm$ 1.0	35.0 $\pm$ 0.6	32.3 $\pm$ 0.8
Tb.N (/mm)	6.2 $\pm$ 0.1	7.0 $\pm$ 0.1 [†]	6.3 $\pm$ 0.2	6.5 $\pm$ 0.1	6.6 $\pm$ 0.1	6.9 $\pm$ 0.3	6.0 $\pm$ 0.1	6.4 $\pm$ 0.1
Tb.Sp (μ m)	150.5 $\pm$ 2.3	136.9 $\pm$ 2.2*	157.0 $\pm$ 5.4	164.7 $\pm$ 4.1	130.7 $\pm$ 3.1	136.2 $\pm$ 5.9	146.4 $\pm$ 2.8	136.7 $\pm$ 3.0
Tb.Th (μ m)	55.2 $\pm$ 1.9	52.3 $\pm$ 1.9	65.1 $\pm$ 1.7	56.4 $\pm$ 1.1 [†]	54.5 $\pm$ 2.3	50.6 $\pm$ 1.9	57.0 $\pm$ 1.4	49.7 $\pm$ 0.8*
BMD (g/cm ³)	0.21 $\pm$ 0.01	0.28 $\pm$ 0.02*	0.32 $\pm$ 0.02	0.23 $\pm$ 0.01	0.25 $\pm$ 0.02	0.26 $\pm$ 0.02	0.23 $\pm$ 0.06	0.21 $\pm$ 0.01

Significant differences vs WT of corresponding age group: **P* < 0.05, [†]*P* < 0.01 and [‡]*P* < 0.001, Bonferroni's *post hoc* test. BV/TV, relative bone volume; Tb.N, number of trabeculae; Tb.Sp, trabecular spacing; Tb.Th, trabecular thickness; BMD, bone mineral density.

Table 3 Static and dynamic analyses parameter values in 2-month-old WT and null female and male mice vertebrae. Values are mean $\pm$ S.E.M.; number of mice per group is indicated in parentheses

	Female		Male	
	WT (8)	Null (7)	WT (6)	Null (7)
Single-labeled surface (%)	27.0 $\pm$ 2.3	36.8 $\pm$ 3.2*	30.1 $\pm$ 3.4	35.9 $\pm$ 2.4
Double-labeled surface (%)	15.0 $\pm$ 1.9	21.4 $\pm$ 1.0	11.5 $\pm$ 0.9	10.9 $\pm$ 1.1
Trabecular MAR (μ m/day)	2.79 $\pm$ 0.21	3.62 $\pm$ 0.21*	2.71 $\pm$ 0.27	3.02 $\pm$ 0.19
MS/BS (%)	27.5 $\pm$ 2.8	33.1 $\pm$ 2.2 [†]	23.9 $\pm$ 1.7	25.1 $\pm$ 1.4
Ob.S/BS (%)	22.3 $\pm$ 1.8	33.4 $\pm$ 3.1 [†]	30.5 $\pm$ 2.2	27.5 $\pm$ 1.6
N.Oc/mm	3.5 $\pm$ 0.2	3.9 $\pm$ 0.8	4.2 $\pm$ 0.3	4.6 $\pm$ 0.7
BFR/BS (μ m ² / μ m per day)	0.83 $\pm$ 0.12	1.15 $\pm$ 0.11*	0.63 $\pm$ 0.06	0.75 $\pm$ 0.07

Significant differences vs WT: * P < 0.05 and [†] P < 0.01, Bonferroni's *post hoc* test. MAR, mineral apposition rate; MS/BS, mineralizing surface; Ob.S/BS, relative osteoblast surface per bone surface; N.Oc/mm, number of osteoclasts; BFR/BS, bone formation rate.

Von Kossa staining showed more mineralized tissue in the vertebrae from *Scarb1*-null female mice. Concomitantly, the ALP staining was visibly enhanced in the vertebrae from *Scarb1*-null female mice, whereas ALP staining of bone sections from male WT and *Scarb1*-null mice was similar. The TRAP staining remained similar between WT mice and *Scarb1*-null mice of both genders, indicating a predominantly osteoblastic alteration. In accordance, the relative Ob.S/BS was significantly higher in the vertebra from *Scarb1*-null female mice, as the N.Oc/mm remained unaffected (Table 3). Dynamic histological evaluation yielded similar results, as lumbar MARs and BFRs were enhanced in the *Scarb1*-null female mice (Fig. 1B for typical calcein fronts).

Disrupted GC/ACTH and leptin/adiponectin axes in null females

As SR-BI is involved in lipoprotein metabolism (van der Velde & Groen 2005), we measured the cholesterol plasma profile in *Scarb1*-null mice. The fractions of total plasma cholesterol and HDL-C were increased at similar levels in *Scarb1*-null males and females (Fig. 2), which suggests that gender-specific differences in vertebral bone mass in *Scarb1*-null mice is not related to differences in HDL level.

As we and others have previously reported an impaired corticosterone response to fasting and constitutive high plasma levels of ACTH in male *Scarb1*-null mice (Hoekstra *et al.* 2008, Martineau *et al.* 2014), we asked whether differences in the GC/ACTH axis may also be exhibited by female *Scarb1*-null mice. The *Scarb1*-null females showed impaired corticosteroid response following fasting (Fig. 3A); this condition was accompanied by higher levels of ACTH under refed condition in

Scarb1-null females (Fig. 3B). This relationship is better illustrated in Fig. 3C, as the GC/ACTH ratio is reduced in the refed condition for *Scarb1*-null females. Though being significantly lower in all null mice vs WT mice, the GC and ACTH variation between fasting and refed states tended to be greater in female *Scarb1*-null mice when compared to the null males, however, without reaching statistical significance (Fig. 3D). Our results suggest that gender-specific differences in vertebral bone mass in *Scarb1*-null mice are not related to GC/ACTH axis. As vertebral high bone mass was specific to female *Scarb1*-null mice, we measured plasma estrogen levels. No difference for total E₂ plasma levels between WT and *Scarb1*-null female mice was detected (Fig. 4).

As E₂ status was unaffected in our model and given that several studies have observed region- and

Figure 2

Plasma cholesterol levels in fasted 2-month-old male and female WT and *Scarb1*-null mice. Total, HDL- and LDL-associated cholesterol fractions from plasma were quantified the BioAssay systems Enzychrom Kit. Values are mean $\pm$ S.E.M. from four mice in each group. Significant differences (* P < 0.05, ** P < 0.01, and *** P < 0.001) for WT vs null mice, a two-way ANOVA followed by Bonferroni's *post hoc* test.

Figure 3

Serological analysis of the glucocorticoid (GC)/ACTH axis in 2- and 4-month-old WT and *Scarb1*-null female mice. (A) Corticosterone and (B) ACTH levels were measured by ELISA in fasting (16 h) and refed (2 h) female mice. Values are mean $\pm$ S.E.M. and number of mice is indicated in parentheses. Significant difference ($^*P < 0.05$) for fasted vs refed state and $^*P < 0.05$ for WT vs null mice in refed condition, two-way ANOVA followed by Bonferroni's *post hoc* test. (C) GC/ACTH ratio obtained from the averages of

datasets A and B. Significant differences ($^*P < 0.05$) for WT vs null mice in refed condition, two-way ANOVA followed by Bonferroni's *post hoc* test. (D) Differences between female and male mice for plasma levels of corticosterone (Δ GC) and ACTH (Δ ACTH) obtained from the averages of datasets A and B for females, and data from the Martineau *et al.* (2014) study for males. Significant differences ($^*P < 0.05$ and $^{EEE}P < 0.001$) for WT vs null mice, two-way ANOVA followed by Bonferroni's *post hoc* test.

gender-specific effect of leptin (Thomas *et al.* 2001, Hamrick *et al.* 2004, Wang *et al.* 2007), we questioned whether leptinemia was affected in null mice. As expected, plasma levels of leptin were increased by feeding in both WT genders as well as null males (Fig. 5A and B). However, plasma levels of leptin were constantly elevated in fasted *Scarb1*-null female mice compared with the corresponding WT mice, which translates in reduced leptin induction in refed mice (Fig. 5C). This was not observed in males. No difference between WT and *Scarb1*-null mice was noticed for adiponectin (fasting values in 2-month-old females: 1.9 ± 0.1 ng/ml in WT vs 2.0 ± 0.2 ng/ml in null mice, four per group). As the white adipose tissue is the main source of leptin production (Guerre-Millo 2004), we verified its expression in WT and null mice. We measured a 2.5-fold increase in *Lep* gene expression in white adipose tissue from the female null mice compared with their WT counterparts (Fig. 5D). Such difference was not observed in male mice.

In vitro osteoblasts' response to leptin and ACTH

We recently reported that MSCs from *Scarb1*-null male mice have enhanced proliferation rate and differentiation potential (Martineau *et al.* 2014). Similarly, enhanced rates of proliferation (1.5-fold) and higher ALP activity with increased matrix mineralization (1.8-fold) were observed in null MSCs from female mice (data not shown). In addition, gene expression of the osteoblastic transcription factor *Osx/Sp7* was also upregulated in null MSCs from females (Fig. 6A), whereas *Col1a1* was not different (Fig. 6B) and *Sost* was downregulated (Fig. 6C); similar observations were made in cells isolated from male mice (*Osx/Sp7*, 2.3-fold; *Sost*, 0.4-fold vs WT, data not shown).

Following treatment with leptin or ACTH, gene expression of *Col1a1* was increased in MSCs from WT and *Scarb1*-null female mice (Fig. 6B). In contrast, gene expression of *Osx/Sp7* and *Sost* was not further regulated by ACTH and leptin in MSCs from *Scarb1*-null female mice (Fig. 6A and C).

Discussion

Recently, we have reported enhanced trabecular bone formation in the femora from *Scarb1*-null mice (Martineau *et al.* 2014). Interestingly, differences between males and females were observed in cortical bone volume of *Scarb1*-null mice, which was increased compared with WT mice in females only (Martineau *et al.* 2014). This current study further assessed gender-specific bone alterations in *Scarb1*-null mice. Our data indicate that 2-month-old *Scarb1*-null

Figure 4

Estradiol (E₂) plasma levels in WT and *Scarb1*-null mice. Total E₂ levels were measured by ELISA in fasted 2- and 4-month-old female mice. Values are mean $\pm$ S.E.M. from five mice per group.

Figure 5

Plasma levels of leptin and gene expression of *Lep* in adipose tissue from WT and *Scarb1*-null mice. (A and B) Plasma levels of leptin were measured by ELISA in fasting and refed WT and *Scarb1*-null mice. Values are mean $\pm$ s.e.m. and number of mice is indicated in parentheses. Significant differences when compared to fasted state (* P < 0.05) or compared with WT mice ($^{**}P$ < 0.05), two-way ANOVA followed by Bonferroni's *post hoc* test. (C) Leptin induction in refed mice from the

averages of datasets A and B. Significant differences (* P < 0.05) for WT vs null mice, two-way ANOVA followed by Bonferroni's *post hoc* test. (D) Gene expression of *Lep* in adipose tissue from 2-month-old WT and *Scarb1*-null mice. Values are mean $\pm$ s.e.m. and number of mice is indicated in parentheses. Significant difference ($^{**}P$ < 0.01) vs WT mice, two-way ANOVA followed by Bonferroni's *post hoc* test.

females have higher vertebral bone mass concurrent with greater BFR, whereas no difference was seen in male *Scarb1*-null mice compared with WT mice. Therefore, we explored the potential conditions responsible for this gender difference. Higher levels of total cholesterol and HDL-C were measured in both male and female *Scarb1*-null mice, in accordance with the role of SR-BI in HDL metabolism. Impaired corticosterone response to fasting and constitutive high plasma ACTH levels were measured in female null mice, as in males (Martineau *et al.* 2014). ACTH promoted *in vitro* osteoblast differentiation of WT MSCs as evidenced by increased gene expression of *Osx/Sp7* and *Col1a1* and reduced *Sost* gene expression, indicating that ACTH may contribute to the high bone mass phenotype of null mice. In contrast, high plasma leptin levels and enhanced *Lep* gene expression in adipose tissue were seen in null females only. Moreover, leptin had anabolic effects *in vitro* by increasing differentiation of WT MSCs through upregulation of *Osx/Sp7* and *Col1a1* expression and reduced *Sost* expression, suggesting that increased plasma leptin may account for the gender-specific high bone mass seen in the femoral cortical region and vertebrae of null females. In addition to these anabolic systemic conditions, MSCs from *Scarb1*-null females displayed enhanced proliferation (data not shown) and differentiation potentials, as observed in null MSCs from male mice (Martineau *et al.* 2014).

High bone mass phenotype in *Scarb1*-null mice seemingly contradicts a positive correlation between efficient HDL metabolism and bone mass, suggested by several studies (Ackert-Bicknell 2012). In accordance with its role in reverse cholesterol transport (RCT), *Scarb1*-null mice were shown to be prone to atherosclerosis (Huby *et al.* 2006) due to impaired selective uptake of cholesteryl esters

from HDL by the liver and associated with abnormally cholesterol-enriched HDL particles (Rigotti *et al.* 1997). Therefore, the contribution of SR-BI to efficient RCT appears to be unrelated to the bone alterations in *Scarb1*-null mice. Moreover, a positive correlation between levels of HDL-C and bone mass has also been reported in experimental mouse strains (Mouse Phenome Database, Jackson Laboratories), underscoring its significance for bone metabolism. In accordance, both male and female *Scarb1*-null mice show similar high levels of total cholesterol and HDL-C that could account for the higher bone mass phenotype of these mice, but not for gender differences in bone status. Our results show that 2-month-old *Scarb1*-null female mice have higher bone mass and enhanced formation rate in lumbar vertebrae, whereas no significant alteration was observed in males. Such gender-associated bone features were also noted in femoral cancellous and cortical bone from *Scarb1*-null female mice (Martineau *et al.* 2014). These data indicate that SR-BI deficiency is associated with gender-specific differences in skeletal phenotype. Overall, the absence of SR-BI translates in a more generalized high bone mass phenotype in female mice, whereas this was limited to trabecular bone in *Scarb1*-null males (Martineau *et al.* 2014).

Considering the high expression of SR-BI in ovaries and adrenal glands (Rigotti *et al.* 1997), relevant hormonal axes were explored. Impaired corticosterone response to fasting accompanied by constant high ACTH levels was observed in *Scarb1*-null females, similarly to *Scarb1*-null males (Hoekstra *et al.* 2008, Martineau *et al.* 2014). As ACTH is recognized to have anabolic effects on bone metabolism (Isales *et al.* 2010), its higher plasma levels correlate with the high bone mass of both male and female *Scarb1*-null mice. Therefore, such systemic alterations in

Figure 6

Response of MSCs from WT and *Scarb1*-null mice to ACTH and leptin. Real-time gene expression analysis of osteoblastic markers *Osx/Sp7* (A), *Col1a1* (B), and *Sost* (C) without (CTL) or with addition of 10 nM ACTH or 100 ng/ml leptin to the culture medium for 24 h. Values are mean $\pm$ s.e.m. and number of cell preparations is indicated in parentheses. Significant differences when compared with CTL condition (* P <0.05 and ** P <0.01) or compared with WT mice (ε P <0.05), two-way ANOVA followed by Bonferroni's post hoc test.

both genders of *Scarb1*-null mice constitute anabolic conditions for bone formation, agreeing with the observed high bone mass phenotype. Nevertheless, gender differences in bone phenotypes of *Scarb1*-null mice may not rely on the GC/ACTH axis alterations. We next investigated whether E_2 , a powerful bone growth modulator (Frenkel *et al.* 2010), was affected in *Scarb1*-null females. Total E_2 levels were normal in *Scarb1*-null females,

therefore discarding the disruption of this hormonal axis as a major factor for the gender-specific bone alterations. Though female *Scarb1*-null females show reduced fertility (Miettinen *et al.* 2001), their ovarian functions are normal in terms of progesterone and E_2 production (Rigotti *et al.* 2003), indicating existing compensatory mechanisms in some aspects. Moreover, HDL carries a large proportion of esterified estrogens in the bloodstream (Hockerstedt *et al.* 2002); it is unknown whether *Scarb1* deficiency impacts the distribution of E_2 esters vs free E_2 .

As SR-BI was shown to be abundantly expressed in fat tissue (Acton *et al.* 1994), plasma levels of leptin and adiponectine were measured in null mice. Leptin is recognized as an important bone growth modulator (Fleet 2000, Turner *et al.* 2013). Null females showed high plasma levels of leptin under fasting conditions, as leptinemia was normal in *Scarb1*-null males, suggesting that leptin may explain the gender differences observed in bone status. Leptin is primarily produced by white adipose tissue and its plasma levels generally correlate with body fat. Though no difference in abdominal fat weight was noticed between WT and *Scarb1*-null females, greater *Lep* expression was observed in white adipose tissue from null females, implying alterations of adipocyte function. To note, the majority of adipocyte cholesterol originates from circulating lipoproteins (Yu *et al.* 2010). As SR-BI is abundantly expressed in fat tissue and promotes cholesterol uptake from HDL (Tondou *et al.* 2005), cholesterol balance in *Scarb1*-null adipocytes might be deregulated. Such cholesterol imbalance is the characteristic of hypertrophied adipocytes that translates to alterations in their metabolism and gene expression (Le Lay *et al.* 2001, Jernas *et al.* 2006, Yu *et al.* 2010).

Leptin has been demonstrated to promote HDL clearance (Lundasen *et al.* 2003) and defective HDL metabolism was reported in leptin-deficient (*ob/ob*) and leptin receptor-deficient (*db/db*) mice (Silver *et al.* 1999, 2000), evoking that a potential cross talk between these two axes may occur. In accordance, leptin induces SR-BI expression in the liver of *ob/ob* mice (Lundasen *et al.* 2003). Of interest, estrogen increases SR-BI expression in adrenals and ovaries (Lopez & McLean 2006). Furthermore, estrogen influences adipogenesis and adipose metabolism (reviewed in Mattsson & Olsson (2007)). Though no difference was observed in E_2 levels between WT and null females, the higher levels naturally occurring in females could induce disorders absent in males. Also, the transient nature of the higher bone mass observed in *Scarb1*-null females despite sustained high leptinemia may underlie an acquired leptin resistance phenomenon,

perhaps through the modulation of leptin receptors as observed in models of age-related or diet-induced obesity (Martin *et al.* 2008). Furthermore, this phenotype may be stage specific, as males tend to achieve peak bone mass later than females (Rizzoli *et al.* 2001, Bonjour *et al.* 2009, Stagi *et al.* 2013); we cannot discard this augmented bone mass as it might occur at another time point. Further studies are warranted to determine how the absence of SR-BI alters the functions of adipocytes in female mice only and whether injection of leptin to male *Scarb1*-null mice mimics the bone phenotype of female *Scarb1*-null mice.

Although the altered endocrine condition in the female *Scarb1*-null mice likely exerts a significant influence on bone metabolism, we explored potential cellular defects in MSCs. Our results indicate that *Scarb1*-null MSCs from females displayed a greater proliferation rate, similar to null MSCs from males (Martineau *et al.* 2014); enhanced proliferation was also reported in SR-BI-deficient lymphocytes (Feng *et al.* 2011). Our data also indicate enhanced ALP activity and matrix mineralization, as well as enhanced gene expression of *Osx/Sp7* in MSCs from *Scarb1*-null females, similarly to male mice (Martineau *et al.* 2014). Therefore, intrinsic alterations of MSCs correlate with the high bone mass phenotype of *Scarb1*-null mice. Moreover, we measured reduced gene expression of *Sost* in null MSCs from females, as observed in males. The *Sost* gene product, sclerostin, is a factor known to inhibit osteoblastic differentiation (Komori 2013), and its down regulation in the absence of SR-BI also correlates with the bone phenotype of *Scarb1*-null mice.

Several studies argue that leptin decreases bone accrual through a hypothalamic relay (Fleet 2000). In contrast, other studies rather suggest that leptin acts directly on osteoblasts and increases cell proliferation and differentiation; it has been proposed that peripheral leptin has anabolic bone effects, whereas its hypothalamic actions are catabolic (Motyl & Rosen 2012). To determine whether *Scarb1* deficiency affected MSC response to this hormone, we verified the expression of *Col1a1* and *Osx/Sp7*, two factors known to be induced by leptin exposure in osteoblasts (Gordeladze *et al.* 2002, Zhou *et al.* 2012). Both WT and null MSCs showed increased *Col1a1* gene expression in response to leptin, indicating that it promotes their osteoblastic differentiation. However, the induction of *Osx/Sp7* expression by leptin treatment was significant in the WT cells only due to its basal overexpression in null cells. Also, leptin treatment shows no effect on *Sost* gene expression in null MSCs due to its reduced basal expression. Thus, *Scarb1* deficiency does not overtly alter the response of MSCs to ACTH or

leptin, but rather influences basal genetic expression in these cells, seemingly rendering them less sensitive to these hormones effects.

There is evidence in humans for gender-specific association of leptin with bone metabolism. Plasma leptin levels correlate positively with bone mass in women, as the opposite is noted in men (Weiss *et al.* 2006). Epidemiological studies also state a positive correlation between blood leptin levels and bone quality in nonobese premenopausal women (Cirmanova *et al.* 2008). Furthermore, gender differences in leptin function in bone development have also been suggested with higher leptin receptor expression in female vs male rats (Li *et al.* 2011). Moreover, subcutaneous leptin replacement in *ob/ob* mice resulted in increased trabecular bone area mainly in lumbar vertebra and to a lower extent in femur (Turner *et al.* 2013), which agrees with the bone phenotype of female *Scarb1*-null mice in which hyperleptinemia is associated with enhanced bone formation in vertebrae and cortical bone; however, work remains to be done to understand why leptinemia is affected in females only.

Conclusions

We have associated SR-BI deficiency to higher vertebral and femoral bone mass in female mice, seemingly related to both systemic and cell-autonomous mechanisms. First, constitutively high plasma ACTH and leptin levels arguably account for an important part of the augmented bone mass. Second, adipose tissue metabolism seems to be altered in females only, leading to gender-specific leptin increases. Finally, MSCs from *Scarb1*-null female mice show enhanced osteoblast functions, which may also contribute to augmented bone mass.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

The authors have made the following declarations about their contributions: study design: L B and R M. Study conduct: C M and L M-F. Data collection: C M and L M-F. Data analysis: C M and R M. Data interpretation: C M and R M. Drafting manuscript: C M and R M. Revising manuscript

content: C M, L B and R M. Approving final version of manuscript: C M, L M-F, L B, and R M. R M takes responsibility for the integrity of the data analysis.

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