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A high fat diet increases bone marrow adipose tissue (MAT) but does not alter trabecular or cortical bone mass in C57BL/6J mice

Casey R. Doucette^{1,2}, Mark C. Horowitz³, Ryan Berry³, Ormond A. MacDougald⁴, Rea Anunciado-Koza¹, Robert A. Koza¹, and Clifford J. Rosen^{1,2,*}

¹Center for Clinical and Translational Research, Maine Medical Center Research Institute, Scarborough, Maine 04074

²University of Maine Graduate School of Biomedical Science and Engineering, Orono, Maine 04469

³Department of Orthopaedics and Rehabilitation, Yale School of Medicine, New Haven, Connecticut 06510

⁴Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, Michigan 48105

Abstract

Obesity has been associated with high bone mineral density (BMD) but a greater propensity to fracture. Some obese individuals have increased marrow adipose tissue (MAT), but the impact of MAT on bone turnover remains controversial, as do changes in BMD associated with a high fat diet (HFD). In this study we hypothesized that MAT volume would increase in response to HFD but would be independent of changes in BMD. Hence, we fed C57BL/6J (B6) male mice at 3 weeks of age either a high fat diet (60 kcal %) or regular diet (10 kcal %) for 12 weeks (n=10/group). We measured MAT volume by osmium staining and micro-CT (μ CT) as well as bone parameters by μ CT, histomorphometry, and dual-energy X-ray absorptiometry. We also performed a short-term pilot study using 13-week-old B6 males and females fed a HFD (58 kcal %) for 2 weeks (n=3/sex). Both long- and short-term HFD feedings were associated with high MAT volume, however, femoral trabecular bone volume fraction (BV/TV), bone formation rate and cortical bone mass were not altered in the long-term study. In the short-term pilot study, areal BMD was unchanged after two weeks of HFD. We conclude that, for B6 mice fed a HFD starting at wean or 13 weeks of age, MAT increases whereas bone mass is not altered. More studies are needed to define the mechanism responsible for the rapid storage of energy in the marrow and its distinction from other adipose depots.

Keywords

high fat diet; obesity; bone mineral density; marrow adipose tissue

*Corresponding Author: Clifford J. Rosen, MD Maine Medical Center Research Institute 81 Research Drive Scarborough, Maine 04074 cjrofen@gmail.com Tel: 207-396-8228 Fax: 207-396-8174.

Introduction

Obesity is a chronic condition associated with significant morbidity and a long-term risk of cardiovascular disease and cancer. The effects of visceral obesity are pronounced on target tissues such as the vasculature and are likely related to the inflammatory nature of the adipose depot and the cytokines released from those cells. Notwithstanding, the relationship of obesity to osteoporosis is controversial. Obese individuals tend to have higher areal bone mineral density (aBMD) based on body size (Rosen & Klibanski 2009, Zaidi et al. 2012). However, a recent cohort study of older men (MrOs) as well as a registry study of postmenopausal women from England suggested that obesity was a major risk factor for fractures (Nielson et al. 2012, Premaor et al. 2010). In addition, several studies in children have shown that obesity increases the risk for radial fractures (Ryan 2010). With respect to skeletal microarchitecture, Bredella and colleagues demonstrated that visceral adiposity was negatively associated with lumbar volumetric BMD measured by QCT, but positively related to marrow adipose volume by MRI (Bredella et al. 2011). In contrast, it was shown that obese young and old individuals have higher trabecular bone volume fractions in the radius and tibia than healthy controls (Dimitri et al. 2014). Therefore, the role of obesity in regulating bone turnover and skeletal fragility remains unclear.

A similar paradox has been noted in mice; C57BL/6J (B6), the most commonly used inbred strain, has a genetic predisposition to obesity and diabetes, particularly when fed a high fat diet (HFD). This weight gain, seen in both young and aging mice, is associated with visceral adiposity, insulin resistance and, in some instances, bone loss (Cao et al. 2010, Patsch et al. 2011). Previous studies have demonstrated that marrow adipose tissue (MAT) is increased in B6 mice fed a HFD (Lecka-Czernik et al. 2015). Strain, age, gender, composition of the diet, duration of feeding, and the microbiome of the laboratory mouse likely play important roles in determining the effects of a HFD on bone marrow composition and ultimately the skeletal response.

Recent work from our laboratory and elsewhere has shown that some murine strains lose bone with a HFD and have higher MAT, while others do not (Rosen et al. 2004, Beamer et al. 2011). These findings are reminiscent of earlier studies with inbred strains treated with rosiglitazone (Ackert-Bicknell et al. 2009). For example, Le and colleagues showed that 13 weeks of HFD feeding beginning at weaning caused bone loss in mice with a deletion of Alox5, a lipoxygenase important in the synthesis of inflammatory mediators, but not in B6 littermates (Le et al. 2012). In the 6T congenic mouse strain, which is 99.6% genetically identical to B6 but carrying a small segment of the C3H/HeJ (C3H) genome, a modified HFD resulted in greatly enhanced MAT volume and profound bone loss due to increased bone resorption (Bonnet et al. 2014). On the other hand, in the same experiment, B6 littermates showed little change in bone mass and only slight increases in MAT. To better understand the interrelationship of diet, MAT, and BMD, we studied the effects of an obesogenic diet on bone mass and MAT in B6 mice following long- (84 days, starting at 3 weeks of age in males) or short-term (14 days, starting at 13 weeks of age in males and females) feeding. We found that consumption of a HFD rapidly increases the volume of MAT, but has little or no effect on trabecular and cortical compartments as measured by dual-energy X-ray absorptiometry, histomorphometry, and micro-computed tomography.

Materials and Methods

Animals

C57BL/6J were derived from a stock strain (stock# 000664, The Jackson Laboratory, Bar Harbor, Maine) and were maintained at the Maine Medical Center Research Institute animal facility on a 12/12 hour light/dark cycle with *ad libitum* access to food and water. Mice were euthanized by CO₂ inhalation and decapitation. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at the Maine Medical Center Research Institute.

Diet manipulation

Long-term study: At 3 weeks of age, C57BL/6J male mice (n=10 per group) were randomly assigned to either control (10% kcal from fat, cat# D12450B, Research Diets, Inc., New Brunswick, NJ, USA) or high fat diet (HFD, 60% kcal from fat, cat# D12492, Research Diets, Inc.). Mice were group-housed and fed *ad libitum* for a period of 12 weeks, at which time they were sacrificed and tissues were collected for analysis.

Short-term pilot study: 13-week-old male (n=3) and female (n=3) C57BL/6J mice were maintained on a Surwit diet (58% kcal from fat, cat# D12331, Research Diets, Inc.) for 14 days, at which time they were sacrificed and tissues were collected for analysis.

Serum analysis

Serum concentrations of dickkopf-related protein-1 (DKK1), fibroblast growth factor 23 (FGF23), interleukin 6 (IL-6), insulin, leptin, osteoprotegerin (OPG), osteocalcin (OC), sclerostin (SOST), and tumor necrosis factor alpha (TNF α) were measured using the Milliplex mouse bone metabolism multiplex assay (cat# MBNMAG-41K, EMD Millipore, Billerica, MA, USA) and a Luminex 200 xMAP platform for detection (cat# 40-012, EMD Millipore).

Body composition

Dual-energy X-ray absorptiometry (DXA): areal bone mineral density (BMD) and body composition measurements (excluding the head) were taken using a PIXImus Densitometer (GE-Lunar Corp., Madison, WI, USA). The instrument was calibrated daily using a phantom standard provided by the manufacturer.

Nuclear magnetic resonance spectroscopy (NMR): body composition (body fat, lean mass and free fluid) was analyzed by Minispec NMR (Bruker). A quality control check of NMR parameters using a standard provided by the manufacturer was performed prior to testing.

Bone length

Bone length was measured using Traceable® digital calipers (mod# 62379-531, Control Company, Friendswood, TX, USA). Measurements were taken from the greater trochanter to the lateral and medial condyles.

Micro-computed tomography (μ CT)

Microarchitecture of femoral trabecular and cortical bone was assessed postmortem by high-resolution micro-computed tomography (MicroCT40, Scanco Medical AG, Switzerland). Approximately 100 CT slices with an isotropic voxel size of 12 μ m were taken just proximal to the distal growth plate of the femur for trabecular bone measurements. For cortical bone measurements, 18 CT slices were obtained from the mid-femoral diaphysis.

Marrow adipose tissue quantification (Osmium- μ CT)

Quantification and visualization of marrow adipose tissue was performed as described in (Scheller et al. 2014). Briefly, long bones were dissected free of soft tissues and fixed in 10% neutral buffered formalin (cat# SF100-4, Fisher Scientific, Pittsburgh, PA, USA) overnight at 4°C with gentle agitation. The following day, bones were washed in cool running tap water. The bones were then decalcified in 4% EDTA for 14 days at 4°C, with EDTA changes every 3-4 days. The bones were then stained for lipid using a 1:1 mixture of 2% aqueous osmium tetroxide (cat# 23310-10, Polysciences Inc., Warrington, PA) and 5% potassium dichromate for 48 hours. The bones were then washed in cool running tap water for 2 hours. Whole bones were imaged using micro-computed tomography (μ CT) performed in water with energy of 55kVp, an integration time of 500 ms, and a maximum isometric voxel size of 10 μ m using a μ CT-35 (Scanco Medical, Bruttisellen, Switzerland).

Histomorphometry

Mice received intraperitoneal injections of 20 mg/kg calcein and 50 mg/kg demeclocycline 10 and 3 days prior to sacrifice, respectively, for the determination of bone formation rates. At the time of sacrifice, limbs were placed in 70% ethanol and maintained in the dark at room temperature. Quantitative static and dynamic histomorphometry measurements were taken at the secondary spongiosa of the proximal tibial metaphysis with the OsteoMeasure morphometry system (Osteometrics, Atlanta, GA, USA).

Statistical analysis

All data are expressed as the mean $\pm$ the standard error of the mean (SEM) unless otherwise noted. Statistically significant differences for the long-term study were determined using an unpaired Student's *t*-test. A paired Student's *t*-test was used for the short-term pilot study. Differences were considered statistically significant when $p < 0.05$.

Results

In the long-term study, HFD-fed mice had significantly higher body weight than control-fed animals after 2 weeks of feeding (HFD), and this parameter remained higher throughout the study (Fig. 1A). Longitudinal measurements of whole-body composition by dual-energy x-ray absorptiometry (DXA) showed a significant increase in body fat percentage following 12 weeks of HFD feeding as compared to controls (Fig. 1B). In addition, MAT volume, quantified by osmium staining and μ CT, was more than five-fold higher in mice fed the HFD compared to the control diet (Fig. 1C). However, areal bone mineral density (aBMD) was not significantly different between control and HFD-fed groups (Fig. 1D). Bone length was also unchanged as compared to controls after 12 weeks of HFD in B6 mice (data not

shown). Micro-computed tomography (μ CT) analysis at the end of the 12-week diet study showed that B6 mice on a HFD had no changes in femoral trabecular bone volume fraction (BV/TV), yet displayed a slight but significant decrease in trabecular number (Tb.N) and small increases in spacing (Tb.Sp) and connectivity (Conn.D; Table 1). Analysis of cortical bone at the mid-diaphysis indicated no significant differences between control- and HFD-fed B6 mice. Histomorphometric measurements revealed no changes in static parameters including trabecular number, thickness, and spacing, nor in dynamic parameters such as bone formation rate (BFR) and mineral apposition rate (MAR) in response to a HFD (Table 2). Similarly, osteoblast and osteoclast number did not differ between HFD-fed mice and controls.

Serum analyses showed increased insulin and leptin levels in HFD-fed mice compared to controls (Fig. 2A, B), whereas osteoprotegerin (OPG) levels were significantly reduced in HFD-fed mice (Fig. 2C). We observed no significant differences in levels of IL-6, DKK1, sclerostin (SOST), osteocalcin, or FGF23 (data not shown). Additionally, there was a decrease in serum levels of TNF α in B6 HFD-fed mice as compared to controls (Fig. 2D).

We also initiated a short-term longitudinal study of male and female B6 mice to assess the time course for generation of MAT and its effects on bone mass. After two weeks of a HFD, there were significant sex differences as the total body weight of male B6 mice increased by 10.1% following 2 weeks of HFD whereas the change in total body weight of female mice was less than 1% (total body weight, male baseline: 27.53 ± 0.99 g, male post-2wk HFD: 30.30 ± 0.93 g, $p=0.055$ by paired t -test; female baseline: 20.03 ± 0.22 g, female post-2wk HFD: 20.20 ± 0.45 g, $p=0.45$ by paired t -test). NMR measurements across the same interval showed nearly identical results (data not shown). By contrast, both males and females exhibited very high MAT volumes following 2 weeks of HFD feeding that were markedly different from MAT measurements in an independent cohort of B6 males fed a control diet (Age-matched B6 VIO2: $1.8 \pm 1.5\%$ versus 2wk HFD male VOI2: $58.7 \pm 8.4\%$ and 2wk HFD female VOI2: $58.9 \pm 10.6\%$) (Fig. 3). There was no difference in MAT volume by sex at the two week time point; $p=0.98$.

Discussion

In this study, we performed high-fat diet feeding in B6 mice to test the hypothesis that increased MAT would negatively affect the skeleton. We varied diet, sex, content of the diet, and timing of HFD feeding, but maintained the same inbred strain in order to minimize confounders. We found that bone microarchitecture and cortical bone mass measured by μ CT showed little change after 13 weeks of a 60% HFD in young B6 male mice. Moreover, dynamic histomorphometry revealed no statistically significant changes in bone formation, osteoblast or osteoclast number, osteoclast surface/bone surface, or mineral apposition rate. Yet MAT volume increased dramatically during this dietary intervention compared to control B6 mice fed a regular diet. To clarify this response, we also undertook a pilot study comparing aBMD and MAT measurements before and after a two-week 58% HFD (i.e. Surwit diet) in male and female B6 mice. Total and femoral aBMD were unchanged compared to baseline, while MAT was high in comparison to an independent cohort of 15-week-old male B6 mice. The skeletal changes we found are in contrast to earlier studies

demonstrating that significant bone loss occurs in B6 mice fed a HFD (Patsch et al, 2011, Cao et al, 2010). However, in contrast to our work (i.e. 3-week-old mice, n=10 per diet), in the Patsch study, high fat feeding was started at an older age (i.e. 7 weeks of age) and with smaller numbers of mice (n=6).

HFD feeding increases adipocyte size and may also enhance adipogenic proliferation which is associated with activation of several adipogenic transcription factors including *PPAR* γ , *C/EBP* α and *C/EBP* β (Harms & Seale 2013, Skurk et al. 2007). These in turn lead to downstream regulation of many adipocyte proteins such as CD36, FABP4, FASN, SREBP, and an array of fatty acid transporters (Kajimura et al. 2008). As the adipocyte enlarges, it can also secrete adipokines and cytokines such as TNF α , IL-6, Pref-1, resistin, leptin, and adiponectin that could influence surrounding bone remodeling units by increasing resorption and suppressing formation (Skurk et al. 2007). However, we found no evidence by histomorphometry of changes in the remodeling sequence in B6 male mice following 12 weeks of high-fat feeding despite a marked increase in MAT. Moreover, circulating IL-6 did not change during a HFD and TNF α was lower in the HFD group as compared to controls.

These findings are in contrast to inbred strains of mice, including B6, fed rosiglitazone, which directly activates *PPAR* γ , and MAT increases significantly in parallel with bone loss (Ackert-Bicknell & Rosen 2006, Lazarenko et al. 2007, Ackert-Bicknell et al. 2009). Rosiglitazone has been shown to have a dual effect on skeletal remodeling; i.e. suppressing bone formation through enhancement of *PPAR* γ activity and inducing osteoclastogenesis by stimulation of *Pgc1* β expression (Wei et al. 2010). It seems likely that ligand activation of *PPAR* γ differs between rosiglitazone and HFD because the latter does not stimulate *Pgc1* α or β , nor is there evidence from histomorphometry that eroded surface, osteoclast number, or bone resorption is increased. This is reinforced by studying HFD-induced skeletal changes between B6 and the congenic 6T mouse. The latter strain is associated with a gain-of-function polymorphism in *Ppar* γ which leads to activation of this nuclear receptor in a manner that is analogous to rosiglitazone-induced activation. Thus it is conceivable that with a HFD in B6 mice, specific fatty acid ligands may not activate *Ppar* γ to the extent that this occurs with rosiglitazone or in the congenic 6T background.

We found that circulating leptin levels increased after 12 weeks of HFD feeding in parallel with substantial weight gain and greater fat mass, but these changes were not correlated with changes in bone mass by μ CT or bone remodeling measured by histomorphometry. Osteocalcin levels also increased on the HFD as was serum OPG. On the other hand, markers of inflammation such as TNF α and IL-6 were not increased systemically by a HFD. Notwithstanding, we cannot exclude the possibility that HFD-induced MAT releases factors that counterbalance any negative effects from a HFD resulting in little change in the remodeling sequence and therefore no changes in bone mass.

The rapid increase in MAT volume observed in our pilot study is of particular interest due to our recent efforts to characterize marrow adipogenesis and identify the MAT progenitor. It has been hypothesized that marrow adipocytes may be unique in their mesenchymal lineage and arise from a distinct progenitor that differs from osteoblasts, white adipocytes, and brown adipocytes (Horowitz et al, 2014). For example, calorie restriction in mice and

anorexia nervosa in humans were associated with a marked increase in MAT as well as a progressive decline in bone mass despite the relative paucity of adipose stores in peripheral tissues (Devlin et al. 2010, Bredella et al. 2009). Similarly in aging mammals, MAT is markedly increased while bone volume fraction is reduced and peripheral adipose depots are low (Duque 2008). On the other hand, during the early phases of lactation we reported that MAT almost completely disappears, despite the very pronounced loss of bone mass and the massive lipolysis in peripheral adipocytes (Bornstein et al. 2014). We report here that HFD feeding results in both increased peripheral and marrow adipose tissue. Importantly, in the inbred strain C3H/HeJ, MAT volume is much greater than in B6, yet both cortical and trabecular bone mass are also high (Ackert-Bicknell & Rosen 2006). These data suggest that the inducer of MAT (e.g. HFD, aging, calorie restriction, genetic background) may signal not only the adipocyte progenitor but other cells in the bone marrow resulting in changes in bone remodeling. Thus, the purported reciprocal relationship between MAT and trabecular bone mass is not a constant, nor is there a definitive relationship between changes in peripheral white and marrow adipose tissues. Therefore, the relationship among MAT, white adipose tissue, and trabecular bone mass is influenced by numerous factors that could mediate a shift in MSC lineage.

In the longitudinal study, we sought to limit the confounding variables that could affect the marrow and skeletal response to a HFD. These included duration of high fat feeding, sex, inbred strain, and diet content. Interestingly, mice fed a Surwit HFD (58 kcal %) for just two weeks had extremely high MAT volumes, but no change in areal BMD as compared to baseline. Importantly, female mice showed the same high MAT volume as the male mice even though only the latter gained weight. Diet composition did not appear to influence the MAT volume or bone response; the Surwit diet contains coconut oil as the source of fat as opposed to the 60% HFD, which contains lard, yet both diets appeared to have similar effects. Thus, it is clear that MAT is exquisitely sensitive to nutritional influences and is independent of body weight changes. This would raise the possibility that MAT possesses unique fatty acid transporters that can recycle these compounds more rapidly and efficiently than other depots, either paradoxically in response to low substrate availability or during dietary excesses. Whether this reflects the unique location of this depot (i.e. the marrow) or extrinsic factors needs to be elucidated by further studies including lineage tracing experiments.

In conclusion, we found that HFD feeding increases MAT volume but does not adversely affect skeletal remodeling in young and adolescent B6 mice. Understanding the origin of the marrow adipocyte within this niche and its relationship to osteogenesis will provide greater insights into the role of obesity, if any, in modulating skeletal mass.

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Figure 1. 12-week high fat diet leads to increased weight, total body fat, and MAT, but areal BMD is unchanged

A) male B6 mice on a HFD had significantly higher body weight than controls after 12 weeks of feeding ($p < 0.03$) **B)** total percent body fat by DXA showed increased fat in HFD-fed mice ($p < 0.001$) **C)** marrow adipose tissue volume was significantly increased in HFD-fed mice ($p < 0.04$) **D)** aBMD was unchanged with HFD as compared to controls.

Figure 2. High fat diet-fed mice have increased circulating insulin, leptin, and osteocalcin levels
A) Serum insulin was increased in HFD-fed mice after 12 weeks ($p < 0.001$), as was **B)** leptin ($p < 0.04$), while levels of **C)** osteoprotegerin and **D)** TNF α were significantly reduced as compared to control mice ($p < 0.04$).

Figure 3.

Mice fed a Surwit diet for two weeks have high volumes of marrow adipose tissue in both males and females.

Table 1

Micro-computed tomography of the trabecular and cortical regions of femora from control and high fat diet-fed mice.

	C57BL/6J	
	Control	HFD
Femur length (mm)	15.50 ± 0.06	15.61 ± 0.13
<i>Trabecular</i>		
BV/TV (%)	0.156 ± 0.006	0.137 ± 0.019
Tb.N (1/mm)	4.88 ± 0.07	4.39 ± 0.19*
Tb.Th (mm)	0.051 ± 0.001	0.052 ± 0.003
Tb.Sp (mm)	0.194 ± 0.003	0.223 ± 0.012*
Conn.D (1/mm ³)	114.33 ± 7.90	98.61 ± 14.14*
<i>Cortical</i>		
Tt.Ar (mm ²)	2.09 ± 0.04	2.23 ± 0.07
Ma.Ar (mm ²)	1.07 ± 0.03	1.14 ± 0.04
Ct.Ar (mm ²)	1.03 ± 0.01	1.09 ± 0.04
Ct.Ar/Tt.Ar (%)	0.491 ± 0.007	0.489 ± 0.009
Ct.Th (mm)	0.227 ± 0.003	0.227 ± 0.005
Cort. Porosity (%)	7.49 ± 0.13	7.89 ± 0.27

Table 2

Static and dynamic histomorphometry of tibiae from control and high fat diet-fed mice.

	C57BL/6J	
	Control	HFD
BV/TV (%)	19.33 ± 1.91	17.80 ± 1.09
OS/BS (%)	17.52 ± 2.99	24.18 ± 4.38
Ob.S/BS (%)	4.58 ± 1.22	8.29 ± 1.73
Oc.S/BS (%)	5.28 ± 0.85	5.69 ± 0.95
Tb.Th. (μm)	41.68 ± 2.88	43.94 ± 1.87
O.Th. (μm)	3.11 ± 0.12	2.87 ± 0.14
N.Ob/B.Pm (/mm)	4.10 ± 1.08	7.47 ± 1.54
N.Oc/B.Pm (/mm)	1.43 ± 0.24	1.62 ± 0.27
Tb.Sp. (μm)	178 ± 12	206 ± 12
Tb.N. (/mm)	4.61 ± 0.21	4.06 ± 0.21
MS/BS (%)	36.28 ± 2.95	28.84 ± 2.70
MAR (μm/day)	1.36 ± 0.06	1.47 ± 0.07
BFR/BS (μm ³ /μm ² /day)	179.65 ± 15.40	154.53 ± 16.56
BFR/BV (%/year)	1015 ± 113	787 ± 67