Reduced Bone Density and Cortical Bone Indices in Female Adiponectin-Knockout Mice

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A positive association between fat and bone mass is maintained through a network of signaling molecules. Clinical studies found that the circulating levels of adiponectin, a peptide secreted from adipocytes, are inversely related to visceral fat mass and bone mineral density, and it has been suggested that adiponectin contributes to the coupling between fat mass and bone. Our study tested the hypothesis that adiponectin affects bone tissue by comparing the bone phenotype of wild-type and adiponectin-knockout (APN-KO) female mice between the ages of 8–37 weeks. Using a longitudinal study design, we determined body composition and bone density using dual energy x-ray absorptiometry. In parallel, groups of animals were killed at different ages and bone properties were analyzed by microcomputed tomography, dynamic histomorphometry, 3-point bending test, nanoindentation, and computational modelling. APN-KO mice had reduced body fat and decreased whole-skeleton bone mineral density. Microcomputed tomography analysis identified reduced cortical area fraction and average cortical thickness in APN-KO mice in all the age groups and reduced trabecular bone volume fraction only in young APN-KO mice. There were no major differences in bone strength and material properties between the 2 groups. Taken together, our results demonstrate a positive effect of adiponectin on bone geometry and density in our mouse model. Assuming adiponectin has similar effects in humans, the low circulating levels of adiponectin associated with increased fat mass are unlikely to contribute to the parallel increase in bone mass. Therefore, adiponectin does not appear to play a role in the coupling between fat and bone tissue. (Endocrinology 157: 3550–3561, 2016)

Low body weight is a major risk factor for osteoporosis and fracture, as body weight affects bone turnover and mineral density (1). In recent years, it became apparent that adipose tissue has endocrine functions and adipocytes secrete soluble factors, or “adipokines,” that affect multiple target tissues and play important physiological roles. Adiponectin is an adipokine that affects a large number of target cells and tissues; it promotes insulin sensitivity, induces fat oxidation, and acts as an anti-inflammatory agent (2). Although adiponectin is predominantly secreted from adipocytes, its circulating concentrations are inversely related to visceral fat mass and body mass index (BMI) (3). A large number of clinical studies found low levels of circulating adiponectin in obese, insulin-resistant individuals and increased serum adiponectin in people who are lean and have normal insulin sensitivity (2, 4). Because adiponectin circulating levels relate to fat mass, the possibility

Abbreviations: APN-KO, adiponectin-knockout; BMC, bone mineral content; BMD, bone mineral density; BMI, body mass index; DXA, dual energy x-ray absorptiometry; FDR, false discovery rate; microCT, microcomputed tomography; 3D, three dimensional; WT, wild type.
that bone is a target of adiponectin and that adiponectin has a role in the bone-fat relationship has been investigated. Inverse correlation between circulating adiponectin concentrations and bone mineral density (BMD) were reported in numerous epidemiological studies, a relationship that persists after adjustment for potential confounding factors, including BMI and central fat mass (5–8).

A number of genetically modified animal models have been used to study the physiological role of adiponectin (9–12). Early studies focused mainly on energy homeostasis and insulin sensitivity in adiponectin-knockout (APN-KO) mice, but the findings in the different animal models were inconsistent, showing no difference in insulin sensitivity between APN-KO and wild-type (WT) animals, moderate insulin-resistance in APN-KO mice or development of insulin resistance in APN-KO mice on a high-fat diet (9–11). The bone phenotype of APN-KO mice has also been investigated, but again, results from different experimental models varied. A number of studies reported no differences between the bone phenotype of APN-KO and WT mice (13, 14). A recent study found that adiponectin regulates bone metabolism in 2 opposite manners; young APN-KO had higher bone mass in comparison with WT animals, whereas older APN-KO mice had reduced bone mass (15). In another study adiponectin deficiency was found to protect against ovariectomy-induced osteoporosis, suggesting a role for adiponectin in mediating the negative effect of estrogen deficiency on bone (14). Studies have shown higher BMD in APN-KO mice (16, 17) but overexpression of adiponectin was also found to increase bone mass (18). Therefore, the activity of adiponectin in bone and the role it might play in mediating the fat-bone relationship are still unclear.

The lack of reproducibility of preclinical studies has raised major concerns in the field of biomedical research and was recently addressed specifically in the bone field (19, 20). Studies of the bone phenotype of APN-KO mice suggested a physiological role of adiponectin in bone metabolism, but due to the lack of consistency in the different models the bone effects of adiponectin are still unclear. The aim of the current study was to compare the bone phenotype of APN-KO and WT female mice between the ages of 8–37 weeks. Paying careful attention to details that were pointed out as crucial for reproducibility in preclinical studies, we studied the effect of adiponectin-deficiency on total-skeleton BMD and on local bone indices in the femur.

Materials and Methods

The overall experimental design and the methods used are summarized in Table 1. In all experiments, samples were analyzed in a random order by assessors who were blinded.

Animals

Animals were maintained in a virus- and parasite-free barrier facility in a 12-hour light, 12-hour dark cycle. Mice were given a standard chow diet (Harlan 2018; Teklad Global 18% protein rodent diet), with free access to food and water. Animals were killed by lethal inhalation of CO2. All protocols were approved by the Animal Ethics Committee at the University of Auckland (under the Animal Welfare Act 1999), project license 001240. The animals’ health status was monitored throughout the study according to Institutional Guidelines for Welfare Monitoring in Mice. APN-KO mice, kindly provided by Baylor College of Medicine (Houston, TX), were backcrossed to C57BI/6J for at least 12 generations and were genotyped using genomic DNA isolated from tail clips, with specific primers described previously (10). The animals used in the study were produced by breeding pairs of APN-KO animals and pairs of matching WT animals. All mice used in the experiments were either 5 or 6 generation away from the last backcross. There was no difference in serum glucose levels between WT and APN-KO mice (data not shown). The study was conducted using groups of 10 WT C57BI/6J and 10 APN-KO female mice at 8, 14, 21, and 28 weeks of age, and groups of 12 animals at 37 weeks of age. The general appearance of the WT and APN-KO mice was similar. There were no significant differences in the average weights of the APN-KO and WT groups up to 28 weeks of age, but at 37 weeks, the overall appearance of the WT and APN-KO mice was different (Supplemental Figure 1A). The lengths of the femurs were similar in the 2 groups at all the ages (Supplemental Figure 1B).

Body composition and bone density, dual energy x-ray absorptiometry (DXA)

Total bone mineral content (BMC), total areal BMD, and body composition were measured in 12 WT/12 APN-KO mice at 10, 14, 21, 28, and 37 weeks by DXA, using a Lunar PIXI-
and area were determined using Osteomeasure, in an area
drated and stained with toluidine blue. Adipocyte number
femurs were deplasticized in 2-ethoxyethyl acetate, rehy-
bone marrow adipocytes, longitudinal sections of the distal
interlabel distance were measured using Osteomeasure system
the growth plate and extending for 5 mm. Bone perimeter,
Mathematical modeling

Histomorphometry
Ten days before killing, animals were injected sc with cal-
cein (30 mg/kg) and 7 days later with alizarin (20 mg/kg).
The distal femurs were fixed and then dehydrated in a graded
series of ethanol and embedded, nondecalcified, in methyl-methac-
lar bone and extending for 0.5 mm in the proximal direction.

Microcomputed tomography (microCT)
Soft tissue was removed and the bones fixed in 70% eth-
anol at 4°C. The distal end of femurs were scanned using a
SkyScan 1172 microCT scanner (x-ray voltage 50 kV, 0.25 or
0.5 mm aluminum filter; isotropic voxel size 5 μm), as pre-
viously described (17). After standardized reconstruction us-
ing NRecon software, the datasets were analyzed using CTan
software (Bruker microCT). The trabecular region of interest
had to be adjusted according to age, the distance from the
growth plate varied from 0.75 mm in the youngest age group
to 0.4 mm in the oldest, extending to 1.75 mm in the proximal
direction in all groups apart from the oldest animals, where it
extended to 1.5 mm. The cortical region of bone was 3.5 mm
proximal to the growth plate and extended 0.5 mm in the proximal direction.

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Three-point bending and nanoindentation

Bones used for biomechanical testing were wrapped in ab-
sorbent sheets soaked in PBS and stored in the freezer. Bones
were thawed completely and submersed in PBS before testing.
Femurs were mechanically tested using an Instron device (In-
tron, Inc), as previously described (23, 24). Briefly, each bone
sample was placed in a rig designed to hold it at 2 points and to
prevent it from rolling. A load was applied at a constant rate of
1 mm/min at the midpoint, perpendicular to the long axis of the
bone. The load and deformation of the bones were recorded.
Nanoindentation experiments were conducted using a TI-950
Hysitron TribolIndenter (Hysitron, Inc) equipped with a Berk-
ovich diamond tip, as previously described (23, 24). Briefly, bone
samples were mounted and set in low temperature curing epoxy
resin (EpoFix cold setting resin, Struers) and then ground
through a series of silicon carbide grinding papers followed by diamond
polishing. Indents were generated on a cross-section of the
trabecular and cortical bone surfaces by linear loading for 5
seconds up to a peak force of 2000 mN. Peak load was held
for 3 seconds to monitor creep and a linear unload of 5 seconds
followed. Each bone was subjected to 20 cortical and 20 tra-
becular indents.

Statistical analysis

The statistical tests used are indicated in the figure legends.
The unit of analysis in all the experiments was the group of
mice (WT or APN-KO), all tests were 2-tailed and a 5% sig-
nificance level was maintained throughout. Data of the lon-
gitudinal study were analyzed using a mixed models approach
repeated measures (Proc Mixed SAS v 9.4; SAS Institute,
Inc) using maximum likelihood estimation and an unstruc-
tured covariance design with false discovery rate (FDR)-pro-
tected pairwise comparisons. Other data were analyzed by
way-two ANOVA with Bonferroni’s correction for multiple
comparisons, using GraphPad Prism 6.0 (GraphPad, Inc). Ad-
ditional post hoc analysis of the data using FDR had no major
effects on the results and the conclusions of the study.

Results

APN-KO mice have lower BMD and percentage fat
than WT mice

Groups of 12 APN-KO and 12 WT female mice were
anesthetized at 10, 14, 21, and 28 weeks of age, and bone
and soft tissue composition were measured using Lunar
PIXImus densitometer. At 37 weeks, the animals were
culled and the final measurements taken. As shown in
Figure 1A, whole-skeleton BMD was significantly lower
in APN-KO mice (P<0.04). The difference in
BMD was quite modest, and was not age dependent
(P<0.16). BMC was similar in the 2 groups,
but bone area was greater in the APN-KO group compared
with the control from week 21 onwards (Figure 1, B and
C). Body composition also varied significantly between the 2 groups (Figure 1, D and E): total body tissue mass was reduced in APN-KO in comparison with WT, with a marked reduction of about 30% in the percentage fat mass.

Further analysis of BMD was carried out in specific regions of interest in 5 skeletal sites: femoral midshaft, distal femur, proximal tibia, tibial midshaft, and lumbar vertebrae L1–L4. As shown in Figure 2, the differences between the BMD of APN-KO and WT mice were mainly restricted to the femur, with lower BMD in APN-KO mice in the femoral midshaft ($P_{\text{genotype}} < 0.0001$) and in the distal femur ($P_{\text{genotype}} = 0.0098$). A modest reduction in BMD was also found in the proximal tibia, whereas in the tibial midshaft and in the lumbar vertebrae (L1–L4) there were no significant differences between the groups ($P_{\text{genotype}} = 0.56$ and $0.38$, respectively).

**Figure 1.** Body composition and whole-skeleton BMD of WT and APN-KO mice at different ages. In this longitudinal experiment animals were briefly anesthetized and measured by DXA on a Lunar Piximus densitometer, until week 37, when the animals were culled and the last measurements taken. A–C, Whole-skeleton BMD, BMC, and bone area. D and E, Soft tissue composition. The graphs present the mean ± SEM, $n = 12$ in each group. Data were analyzed using a mixed models approach to repeated measures using maximum likelihood estimation and an unstructured covariance design, with FDR-protected pairwise comparisons; *, $P < 0.05$; **, $P < 0.01$ vs WT.

Trabecular bone fraction is reduced in young APN-KO animals

Analysis of trabecular indices of the distal femur showed that as expected, in both groups trabecular bone volume fraction declined with age. The phenotype of the APN-KO mice changed over time: at 8 and 14
weeks of age, the bone volume fraction of APN-KO mice was lower than that of the WT, but at older ages, the 2 groups were similar (Figure 3A). Trabecular number was lower and trabecular separation was higher in APN-KO in comparison with WT only at 8 weeks. Trabecular thickness varied between older APN-KO and WT mice, with APN-KO mice having 13% (\( P < .0001 \)) and 16% (\( P = .0001 \)) reduced thickness in weeks 28 and 37, respectively. Three-dimensional reconstructions of representative bones are shown in Figure 3B. The dense appearance of the trabeculae at 8 weeks, when the bone volume fraction in both groups is below 10%, is a result of the presentation of the 3D reconstructions in a 2D image. The decrease of trabecular bone with age is apparent in both groups, and the decrease in trabecular thickness in the APN-KO mice at 37 weeks can also be seen.

Cortical bone area is reduced in APN-KO mice

A number of cortical indices measured in the distal femur differed significantly between APN-KO and WT mice. At all the time points, cortical bone area fraction was significantly lower in the APN-KO mice, with an overall average reduction of 11% in comparison with the WT (\( P < .01 \)) (Figure 4A). The total cross-sectional area was similar between the groups throughout the study period,
but the cortical bone area was smaller in the APN-KO mice and the average cortical thickness was reduced from week 14 onwards. Representative images (Figure 4B) demonstrate the increase in medullary area and the reduced average cortical thickness of the APN-KO bone compared with the WT.

In order to determine whether the lower cortical bone volume in APN-KO mice results from attenuated bone formation, dynamic parameters of bone formation were measured in the femur using double-fluorochrome labels. The mineralizing surface and bone formation rates were similar between WT and APN-KO mice at all the time points (Figure 5), whereas mineral apposition rate was reduced in 37-week-old APN-KO mice.

The effect of adiponectin deficiency on bone marrow adipocytes

Bone marrow adipocytes were studied in sections of the distal femur from APN-KO and WT mice at the ages of 8, 21, and 37 weeks. Analysis of adipocyte number and area and representative images of stained bone marrow sections are presented in Figure 6. At 37 weeks of
age, the average number of bone marrow adipocytes in the investigated region was significantly higher in APN-KO mice in comparison with WT mice, but the total adipocyte area was similar in the 2 groups at all ages.

**Computational modeling of bone strength**

Computational analysis of normalized bone strength due to bone architecture alone was performed on the microCT-derived trabecular and cortical bone geometries of mice at 8 and 37 weeks of age. No significant
differences were found in normalized failure force between the WT and APN-KO groups (Figure 7). Large variability was identified within the groups in the trabecular bone samples for 37-week-old mice, reflecting great variation in geometric strength and the mechanical path of failure.

Femoral whole-bone strength and cortical/trabecular bone material properties

Analysis of bone strength by 3-point bending tests showed that the measures of peak load, Young’s modulus and toughness were similar between WT and APN-KO mice at 8, 21, and 37 weeks of age (Figure 8, A–C). Nanoindentation was used to determine bone hardness and reduced modulus, a measure of material stiffness (Figure 8, D–G). Using this technique the measurements made are independent of bone thickness and relate only to the local properties of the bone. Cortical bone hardness was 15% higher in the APN-KO mice at 8 weeks (*P < .001) (Figure 8F). All other comparisons showed no significant differences between the WT and APN-KO groups.

Discussion

Our study found that adiponectin-deficiency has negative effects on bone microarchitecture and mineral density, but
the modified bone properties do not have a significant impact on bone strength, as judged by 3-point bending. Comparison of the microarchitecture of the distal femur in female APN-KO mice with WT controls showed a reduction in 3 of the 4 main indices used to describe cortical bone: bone area, bone area fraction and cortical thickness, whereas the total cross-sectional area was similar in the 2 groups. Cortical bone formation rate, determined in the same bone region by dynamic histomorphometry, was not significantly attenuated in APN-KO mice, and is thus unlikely to be causing the differences in cortical bone microarchitecture. Indices of trabecular bone were also reduced, but bone volume fraction and trabecular number were only lower in young APN-KO animals. Thus, adiponectin appears to increase trabecular volume fraction in the young, actively growing bone, whereas in adult mice, with the decrease of the trabecular component of long bones, adiponectin’s effect diminishes. Taken together, the microCT analysis demonstrated that adiponectin deficiency has a negative effect on bone geometry, principally affecting cortical bone. DXA analysis determined a decrease in the femoral midshaft and distal femur BMD in APN-KO mice in comparison with WT controls, and a modest decrease was measured in the total skeleton BMD. Although indices of both microarchitecture and density of cortical bone were reduced in APN-KO animals, 3-point bending tests found no differences in bone strength. Thus, it appears that the magnitude of the changes in cortical BMD and bone microarchitecture were not sufficient to affect bone strength.

Previous studies of the bone phenotype of APN-KO mice produced conflicting results, perhaps reflecting the variability among the knockout animals generated by different groups, as well as the age and sex of the animals, the methods used to analyze bone properties and the specific bones studied. In one of the early studies, Shinoda et al examined the skeleton of 8-week-old APN-KO mice by DXA and histomorphometry (13). Similar to our results, in this age group no differences were found between the APN-KO and WT mice. Tu et al investigated the effect of short-term exposure of bone to an adiponectin-deficient environment, by studying the properties of explants from 3-day-old mice that were transplanted into WT or APN-KO mice for 4 weeks (25). The growth of the bone explants was significantly retarded in the APN-KO mice as a result of enhanced osteoclastogenesis, implying that adiponectin plays an important role in the inhibition of osteoclastogenesis and therefore has an anticatabolic activity in the growing bone (25). Although this experimental system is very different from the one we used here, similar to our system, it suggests a positive effect of adiponectin in bone. In contrast to these results, a number of studies determined increases in indices of trabecular bone in APN-KO mice that suggest a negative effect of adiponectin on bone (16, 17). A recent study found that adiponectin plays a dual role in the regulation of bone mass (15). At 6 and 12 weeks of age, APN-KO mice had increased vertebral bone mass, a result of the direct action of adiponectin to inhibit osteoclast proliferation. At 36 weeks, APN-KO mice showed a decrease in vertebral bone mass, as in these older animals an indirect effect of adiponectin on bone, through the decrease in sympathetic tone, becomes predominant. Thus, adiponectin regulates bone mass in 2 opposing manners. Our study focused on the properties of long bones and the skeleton as a whole, and in all the age groups we found evidence for a positive effect of adiponectin in bone, as described for the older animals in the study by Kajimura et al (15).

A number of studies examined the bone phenotype of transgenic mice overexpressing adiponectin, but similar to the APN-KO mice studies, results varied. Overexpression of adiponectin in the liver had no effect on the bone phenotype measured by DXA and histomorphometry in a study by Shinoda et al (13), whereas Ealey et al (26) found that overexpression of adiponectin inhibited the acquisition of bone mass in growing mice and decreased bone strength. Positive effects were also produced by overex-
pression of adiponectin in mice; an increase in trabecular bone volume associated with a decrease in osteoclast number (18), and an increase in BMD and BMC with an increase in osteoblast number and bone formation rate (27).

The effect of adiponectin on bone cells has also been studied in vitro. Most studies found stimulation of osteoblast proliferation and differentiation by adiponectin in primary cells and in osteoblast-like cell lines (17, 28–30), but results of studies of the effect of adiponectin on osteoclast formation and differentiation were somewhat less consistent (18, 25, 31). Results from in vitro studies of adiponectin activity should be interpreted cautiously as there is evidence that commercially available preparations of adiponectin are often contaminated with lipopolysaccharide (32, 33). Generally, the in vitro studies of adiponectin activity in bone cells suggest it has an anabolic bone effect through enhancement of osteoblast formation and activity and inhibition of osteoclast formation. The lower BMD and bone volume of APN-KO mice determined in the current study are congruent with the in vitro results in suggesting a positive effect of adiponectin in bone, although bone formation rate was not significantly different between APN-KO and WT mice in our dynamic histomorphometry analysis.

The APN-KO mice were lighter than WT mice at 37 weeks, and analysis of body composition by DXA demonstrated a substantially lower percentage of fat mass in comparison with the WT controls. These findings suggest that adipose tissue is not only a major source of circulating adiponectin but also one of its targets, as adiponectin,

Figure 8. Bone strength and material properties of WT and APN-KO mice. A–C, Three-point bending parameters in WT and APN-KO mice. D–G, Reduced modulus and hardness as determined by nanoindentation of femurs from WT and APN-KO mice. Individual values and means are presented; **, P < .05 vs WT. Data were analyzed by two-way ANOVA with post hoc Bonferroni’s. Cortical area hardness (F): (age) < .0001, (genotype) = .09, (age*genotype) = .0062, for all other comparisons (A–E and G): (age) < .0001, (genotype) = ns, (age*genotype) = ns.
directly or indirectly, promotes the accumulation of fat. A previous study showing significantly lower body weight and fat-pad weight in 9-months old APN-KO mice determined that these were due to a marked increase in energy expenditure (15). An additional mechanism for the effect of adiponectin on adipose tissue was suggested in an in vitro study, showing that the expression of the adiponectin gene in 3T3-L1 fibroblasts induced their differentiation into adipocytes (34). In contrast to these finding, other studies found that mice overexpressing adiponectin in white fat cells had reduced adiposity due to increased energy expenditure and impaired adipocyte differentiation (35). In addition to white adipose tissue, bone marrow adipocytes have recently been recognized as an important source of circulating adiponectin (4). The increase in circulating adiponectin levels in lean states, in which white adipose tissue mass is reduced, is often associated with an increase in bone marrow adiposity. Investigating the effect of adiponectin-deficiency on bone marrow adipocytes in our study, we found that the number of adipocytes in sections of the distal femur were significantly higher in APN-KO mice at 37 weeks in comparison with WT mice. This observation is consistent with an inverse relationship between total body and bone marrow adipose tissue, and identifies bone marrow adipocyte as a potential target tissue of adiponectin in our animal model.

Epidemiological studies have consistently found inverse relationship between circulating adiponectin levels and BMD (36). This relationship was first described in a group of 80 subjects (6), and was subsequently confirmed in many additional studies. For example, in a study of 1735 women, each doubling of serum adiponectin was associated with 2.7% decrease in BMD (8). In this study, the inverse relationship between adiponectin and BMD remained significant after adjustment for potential confounding factors including age, BMI, serum leptin, central fat mass, and exercise (8). Negative relationships between circulating adiponectin and fracture risk have also been determined. A study of 3075 women and men that tested the association of leptin and adiponectin levels with fracture risk found association of adiponectin, but not leptin, and suggested that elevated adiponectin may be a novel risk factor for increased fracture risk independent of body composition and BMD (5).

Given that in most epidemiological studies adiponectin levels are negatively related to BMD, our working hypothesis was that adiponectin deficiency is associated with increased BMD. However, we found the opposite effect, adiponectin-deficient mice had lower BMD, with reduced cortical thickness and cortical bone area fraction in comparison with WT animals. Therefore, our results do not support the suggestion that a reduction in circulating adiponectin improves bone properties. Moreover, the observation that the APN-KO mice had lower BMD and reduced body fat, demonstrates a preservation of the fat-bone correlation in the absence of adiponectin. The apparent discrepancy between our results and those from epidemiological studies has several possible explanations. The mechanisms that operate in rodents might be different from those found in humans, and it is possible that compensatory mechanisms in the knockout mice could be masking the “true” effect of adiponectin. Another potential limitation of our study is the use of age-matched WT and APN-KO mice and not littermate controls. However, it is important to note that all the clinical studies of the relationship between adiponectin levels and bone indices are observational, and therefore can only describe association and not causal relationship. In addition, the multiple physiological activities of adiponectin and the complex relationship between fat and bone tissue suggest the existence of many potential confounding factors in these studies.

In conclusion, our study found reduced body fat and a decrease in whole body BMD and cortical bone indices in APN-KO mice in comparison with WT mice. These bone properties suggest that adiponectin has a small positive effect in bone. Given the inverse relationship between fat mass and circulating adiponectin levels, our results do not support a role for adiponectin in the association between fat mass and bone mass.

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