Preadipocyte Factor-1 Is Associated with Marrow Adiposity and Bone Mineral Density in Women with Anorexia Nervosa

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Context: Despite having low visceral and sc fat depots, women with anorexia nervosa (AN) have elevated marrow fat mass, which is inversely associated with bone mineral density (BMD). Adipocytes and osteoblasts differentiate from a common progenitor cell, the human mesenchymal stem cell. Therefore, understanding factors that regulate this differentiation process may provide insight into bone loss in AN.

Objective: The objective of the study was to investigate the relationship between preadipocyte factor-1 (Pref-1), a member of the epidermal growth factor-like family of proteins and regulator of adipocyte and osteoblast differentiation, and fat depots and BMD in AN.

Design: This was a cross-sectional study.

Setting: The study was conducted at a clinical research center.

Patients: Patients included 20 women with AN (26.8 ± 1.5 yr) and 10 normal-weight controls (29.2 ± 1.7 yr).

Interventions: There were no interventions.

Main Outcomes Measure: Pref-1, leptin, IGF-I, IGF binding protein (IGF-BP)-2 and estradiol levels were measured. BMD of the spine and hip was measured by dual-energy x-ray absorptiometry. Marrow fat content of the L4 vertebra and femur was measured by ^{1}H-magnetic resonance spectroscopy.

Results: Pref-1 levels were significantly higher in AN compared with controls ($P = 0.01$). There was a positive correlation between Pref-1 and marrow fat of the proximal femoral metaphysis ($R = 0.50$, $P = 0.01$) and an inverse association between leptin and L4 marrow fat ($R = -0.45$, $P < 0.05$). There was an inverse association between Pref-1 and BMD of both the anteroposterior spine and lateral spine ($R = -0.54$, $P = 0.003$; $R = -0.44$, $P = 0.02$, respectively).

Conclusions: Pref-1 is elevated in AN. Pref-1, IGF-I, IGF-BP2 and leptin are associated with marrow adiposity and BMD. (*J Clin Endocrinol Metab* 95: 407–413, 2010)

Abbreviations: AN, Anorexia nervosa; AP, anteroposterior; BMD, bone mineral density; BMI, body mass index; CV, coefficient of variation; DXA, dual-energy x-ray absorptiometry; EGF, epidermal growth factor; hMSC, human mesenchymal stem cell; IGF-BP, IGF binding protein; Pref-1, preadipocyte factor-1; SAT, sc adipose tissue; TAT, total adipose tissue; VAT, visceral adipose tissue.

Anorexia nervosa (AN) is a primary psychiatric disorder, characterized by extreme self-imposed starvation, affecting 0.5–1% of college-aged women in the United States (1, 2). There are many significant medical complications and comorbidities associated with the disease, and bone loss is among the most common. An estimated 50% of women with AN have osteopenia, with an additional 35% having evidence of osteoporosis (3).

Low bone mass in adolescents and adults occurs in the setting of low sc and visceral fat depots (4), and loss of nutritionally dependent factors are important in the pathogenesis of bone loss. Recent advances in mesenchymal stem cell differentiation have demonstrated a possible inverse relationship between osteoblast differentiation and adipocyte differentiation (5, 6). Because osteoblasts and adipocytes originate from a common progenitor, the human mesenchymal stem cell (hMSC), understanding the factors that potentially regulate the differentiation process of hMSCs into bone and fat may be of great importance in understanding clinical states of low bone mass.

We have previously shown that although peripheral and visceral fat stores are low in AN, bone marrow adiposity is increased and inversely associated with bone mineral density (BMD) (7). The clinical significance of bone marrow adiposity has been shown in a number of studies. For example, Schellinger *et al.* (8) demonstrated that individuals with radiographic evidence of vertebral bone weakness, including wedging of vertebrae or vertebral body compression fractures, had higher percentages of vertebral marrow fat content compared with those without such findings.

Little is known about the hormonal determinants of marrow fat. We therefore investigated preadipocyte factor-1 (Pref-1), an important factor in mesenchymal stem cell differentiation. We also investigated other hormonal mediators of low bone mass in AN including leptin, an adipokine linked to bone mass and decreased in AN, IGF-I, IGF binding protein (IGF-BP)-2 and estradiol. Pref-1 is a member of the epidermal growth factor (EGF)-like-family of proteins and is expressed in several progenitor cell types including hMSCs and preadipocytes (9). Pref-1 is present on the extracellular membrane and is cleaved before it is released into the extracellular space to exert suppressive effects in an autocrine and paracrine manner on adipocyte and osteoblast differentiation (10, 11). Interestingly, Pref-1 circulates in relatively high concentrations, although it is unclear how the circulating levels relate to target tissues. IGF-I is a nutritionally dependent hormone that is known to stimulate bone formation through effects on osteoblastic function (12–14). IGF-BP2 is one of the six IGF-BPs that binds IGF-I in the circulation and has been previously shown to be abnormally elevated

in adult women with AN (15–17) and has been shown to be inversely associated with markers of bone formation in AN (16). Although estrogen therapy has not been shown to increase BMD in women with AN (18, 19), estrogen therapy in postmenopausal women has been shown to decrease marrow adipocyte volume and prevent increases in marrow adipocyte number (20). Leptin has been shown to increase trabecular bone volume and trabecular number in ovariectomized rats when administered peripherally (21). In humans with hypothalamic amenorrhea, a state characterized by low leptin levels, treatment with leptin has been shown to increase markers of bone formation including osteocalcin and bone-specific alkaline phosphatase (22). Given the phenotypic nature of AN with very little peripheral adipose tissue, low leptin, and reduced bone formation, we hypothesized that Pref-1, leptin, IGF-I, IGF-BP2 and estradiol would be associated with marrow adiposity in AN.

Subjects and Methods

Subjects

Thirty women were studied: 20 women with AN (aged 19–41 yr) and 10 normal-weight controls of comparable age (aged 25–42 yr). The 20 women with AN were recruited through referrals from local eating disorder providers and on-line advertisements, and the 10 normal-weight controls were recruited through on-line advertisements. Subjects met *Diagnostic and Statistical Manual of Mental Disorders*, fourth edition, weight and psychiatric criteria for AN. None of the subjects had received estrogen within 3 months of the study. All control subjects had a normal body mass index (BMI), a history of regular menstrual cycles and were receiving no medications known to affect bone mass. Control subjects did not have a past or present history of an eating disorder. Subjects with abnormal TSH, elevated FSH, chronic diseases known to affect BMD (other than AN), or diabetes mellitus were excluded from participation.

All subjects were examined and blood was drawn for laboratory studies at a single study visit at our Clinical Translational Science Center. Height was measured as the average of three readings on a single stadiometer, and subjects were weighed on an electronic scale while wearing a hospital gown. BMI was calculated using the formula [weight (kilograms)/ height (meters)^2].

The study was approved by the Partners Institutional Review Board and complied with the Health Insurance Portability and Accountability Act guidelines. Written informed consent was obtained from all subjects. The clinical characteristics and magnetic resonance imaging and dual-energy x-ray absorptiometry (DXA) data of nine subjects with AN and all of the control subjects have been previously reported (7).

Biochemical assessment

Pref-1 was measured with the Quantikine human Pref-1 immunoassay (ELISA) (R&D Systems, Minneapolis, MN) with a mean minimum detectable level of 0.012 ng/ml and intraassay

coefficient of variation (CV) of 3.1–4.3%. RIA was used to measure serum leptin (Linco Diagnostics, Inc., St. Louis, MO; sensitivity 0.5 ng/ml, CV 3.4–8.3%). Estradiol was measured by automated immunoassay (ARCHITECT; Abbott Diagnostics, Chicago, IL) with a minimum reportable concentration of 10 pg/ml with interassay CV of 2–9.6%. IGF-I and IGF-BP2 were measured by RIA in the laboratory of David Clemmons (Chapel Hill, NC) as previously described (23). The intraassay CV for IGF-I was 3.3% and the interassay CV was 5.4% (23). For IGF-BP2, the intraassay CV was 5.6% and the interassay CV was 5.9% (23).

Radiological imaging

All control subjects and a subset of 10 women with AN underwent ^{1}H-magnetic resonance spectroscopy of bone marrow of the L4 vertebral body, the proximal femoral epiphysis, metaphysis, and diaphysis to determine lipid content using a 3.0T magnetic resonance imaging system (Siemens Trio, Siemens Medical Systems, Erlangen, Germany); fitting of the ^{1}H-magnetic resonance spectroscopy data were performed using LC-Model software (version 6.1–4A; Stephen Provencher, Oakville, Ontario, Canada) as previously described (7). A single axial magnetic resonance imaging slice through the abdomen at the level of L4 and a single slice through the midthigh were obtained (Siemens Trio, 3T; Siemens Medical Systems) to determine abdominal sc adipose tissue (SAT), visceral adipose tissue (VAT), and total adipose tissue (TAT) as well as SAT of the thigh.

All subjects (20 with AN and 10 healthy controls) underwent DXA to measure BMD of the anteroposterior (AP) lumbar spine (L1-L4), lateral spine (L2-L4), distal radius, total hip, femoral neck, and total body and body composition including fat mass (kilograms), lean mass (kilograms), and percent body fat using a Discovery A densitometer (Hologic Inc., Waltham, MA). CVs of DXA have been reported as less than 1% for bone (24), 1.1% for lean body mass, and 2.7% for fat mass (25).

Statistical analysis

Statistical analysis was performed using JMP software (SAS Institute, Carry, NC). The means and SEM measurements were calculated for AN and the control group, and the means were compared using the Student's t test. Correlations are for the group as a whole unless otherwise noted. Where data were not normally distributed, we either performed a transformation to approximate a normal distribution or used nonparametric tests. Log transformations were performed for leptin, abdominal SAT, VAT, and TAT.

Results

Clinical characteristics

Clinical characteristics of the study subjects are presented in Table 1. Subjects with AN had lower weight, BMI, percent ideal body weight and percent body fat compared with controls.

Hormonal parameters

Pref-1, leptin, and estradiol

Pref-1 was significantly higher in AN compared with controls [AN: 0.46 ± 0.03 ng/ml (mean $\pm$ 1 SEM) vs. con-

TABLE 1. Clinical characteristics of subjects with AN and healthy controls

	AN (n = 20)	Healthy controls (n = 10)	P value
Age	26.8 ± 1.5	29.2 ± 1.7	0.13
Weight (kg)	48.2 ± 1.0	61.7 ± 2.2	<0.0001
BMI	17.6 ± 0.2	21.9 ± 0.5	<0.0001
Percent IBW	78.3 ± 1.0	99.5 ± 1.8	<0.0001
AP spine BMD (g/cm²)	0.84 ± 0.02	1.03 ± 0.03	<0.0001
Lateral spine BMD	0.69 ± 0.02	0.80 ± 0.02	0.004
Total hip BMD (g/cm²)	0.80 ± 0.03	0.94 ± 0.03	0.004
Total body BMD (g/cm²)	1.03 ± 0.02	1.14 ± 0.04	0.04[a]
Percent body fat by DXA	18.7 ± 1.1	27.6 ± 1.9	0.0002
VAT of abdomen[b]	23 ± 1.9	47.8 ± 6.2	0.0006[a]
SAT of abdomen[b]	81.6 ± 6.8	170.2 ± 24.8	0.007
TAT of abdomen[b]	104.6 ± 8.1	218 ± 29.8	0.005
SAT of thigh[b]	46.7 ± 5.2	83.6 ± 9.0	0.002

[a] Wilcoxon test.

[b] Data reported are for subset of 10 ANs.

trol: 0.37 ± 0.02 ng/ml, $P = 0.01$] (Fig. 1A). Leptin was significantly lower in AN compared with controls (AN: 3.6 ± 0.5 ng/ml vs. control: 6.4 ± 1.3 ng/ml, $P = 0.01$) (Fig. 1B). Estradiol levels were significantly lower in AN than healthy controls (median estradiol level in AN $\pm$ interquartile range: 34.5 ± 58.5 pg/ml vs. controls: 152 ± 245 pg/ml, $P = 0.02$).

IGF-I and IGF-BP2

IGF-I was significantly lower in AN compared with controls (AN: 146.4 ± 11.1 ng/ml vs. control: 203.5 ± 17 ng/ml, $P = 0.01$). IGF-BP2 levels were significantly higher in AN compared with controls (AN: 188.3 ± 19.1 ng/ml vs. control: 131.4 ± 14.2 ng/ml, $P = 0.03$).

Hormonal parameters associated with body composition

Percent body fat as measured by DXA, VAT and SAT were significantly lower in AN compared with the controls (Table 1). TAT as well as SAT of the thigh were also sig-
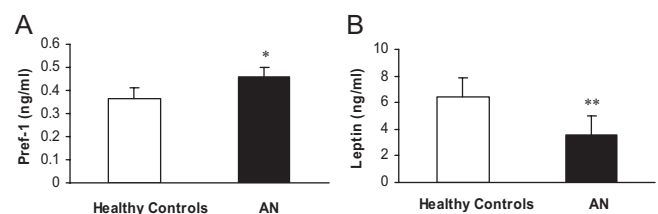

FIG. 1. Pref-1 was significantly higher in women with AN compared with healthy controls (*, $P = 0.01$) (A). Leptin was significantly lower in women with AN compared with healthy controls (**, $P = 0.01$ with logarithmic transformation) (B).
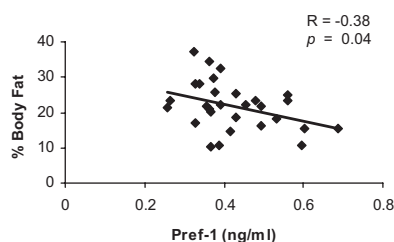
FIG. 2. Pref-1 was inversely associated with percent body fat as measured by DXA.

nificantly lower in AN compared with controls (Table 1). There was an inverse correlation between Pref-1 and percent body fat (Fig. 2) and a positive association between leptin and percent body fat (R = 0.84, $P < 0.0001$), SAT of the abdomen (R = 0.63, $P < 0.005$), VAT of the abdomen (R = 0.52, $P < 0.03$), TAT of the abdomen (R = 0.63, $P = 0.005$), and SAT of the thigh (R = 0.76, $P = 0.0002$). There was a significant positive association between estradiol and percent body fat (Spearman's rho: 0.49, $P = 0.007$). There was also a significant positive correlation between IGF-I and percent body fat (R = 0.51, $P = 0.02$). There were no correlations between IGF-I and VAT, SAT, or TAT of the abdomen or SAT of the thigh, and there were no significant correlations between IGF-BP2 and percent body fat or VAT, SAT, or TAT of the abdomen or SAT of the thigh.

Hormonal parameters associated with bone marrow fat content

There was a positive correlation between Pref-1 and marrow fat of the proximal femoral metaphysis (R = 0.50, $P = 0.01$) (Fig. 3A). There was a negative correlation between leptin and marrow fat of L4 (Spearman's rho = -0.45, $P < 0.05$) (Fig. 3B). There was a significant inverse association between IGF-I and marrow fat of L4 in AN (R = -0.70, $P = 0.02$) and a positive correlation between IGF-I and L4 marrow adiposity in the controls (R = 0.76, $P = 0.01$). There was a significant positive association between IGF-BP2 levels and marrow adiposity of L4 in the group as a whole (R = 0.46, $P = 0.04$). In healthy controls there was a significant inverse association between IGF-

BP2 levels and marrow adiposity of L4 (R = -0.74, $P = 0.01$). There were no associations between estradiol levels and bone marrow fat content.

Hormonal parameters associated with BMD

Subjects with AN had lower BMD of the total hip, AP spine, lateral spine, and total body compared with the controls (Table 1). There was an inverse correlation between Pref-1 and BMD of the AP spine (R = -0.54, $P = 0.003$) (Fig. 4A) and lateral spine (R = -0.44, $P = 0.02$) (Fig. 4B). Significant correlations were not found between Pref-1 and BMD of the hip, distal radius, or total body. Leptin was positively correlated with BMD of the AP spine (Spearman's rho = 0.38, $P = 0.04$) (Fig. 4C) and hip (Spearman's rho = 0.42, $P = 0.03$) (Fig. 4D). Significant correlations were not found between leptin and BMD of the lateral spine, distal radius, or total body. In AN, Pref-1 was inversely associated with BMD of the AP spine (R = -0.51, $P = 0.02$), and leptin was positively correlated with hip BMD (R = 0.50, $P = 0.02$). Estradiol levels were positively associated with BMD of the lumbar spine (Spearman's rho: 0.39, $P = 0.04$). IGF-I was positively correlated with BMD of the AP spine (R = 0.46, $P = 0.048$) and BMD of the hip (R = 0.49, $P = 0.03$) in the group as a whole and with hip BMD in AN (R = 0.65, $P = 0.04$). IGF-BP2 was inversely associated with BMD of the lateral spine (R = -0.48, $P = 0.04$) and hip (R = -0.59, $P = 0.008$) in the group as a whole and with BMD of the hip in AN (R = -0.64, $P = 0.048$).

Discussion

We have shown that women with AN have elevated levels of Pref-1. In addition, our data support the role of Pref-1 as a regulator of adipocyte and osteoblast differentiation. We have also shown that IGF-I is negatively associated with L4 marrow adiposity in AN in contrast to IGF-BP2, which is positively associated with L4 marrow fat. Our data, demonstrating an inverse association between leptin and L4 marrow adiposity, support the hypothesis that the role of marrow fat is distinct from that of sc and visceral fat depots.
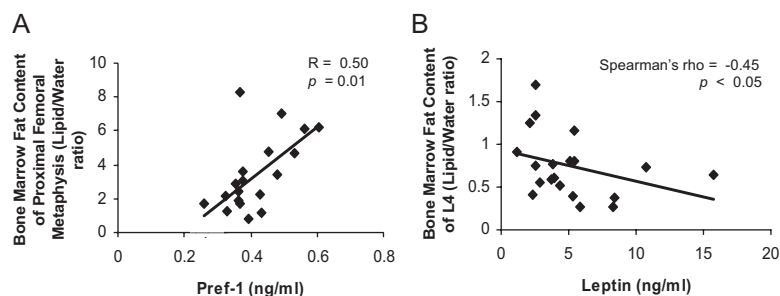
AN is a psychiatric disorder characterized by extreme low body weight and is associated with multiple medical comorbidities including significant bone loss. Fracture risk is also significant in this population. A prospective study of 27 women with AN demonstrated a 7-fold increased risk of nonvertebral fracture during a mean of 2 yr of follow-up (26). A retrospective population-based study demonstrated a 3-fold increased risk of fracture many years after the
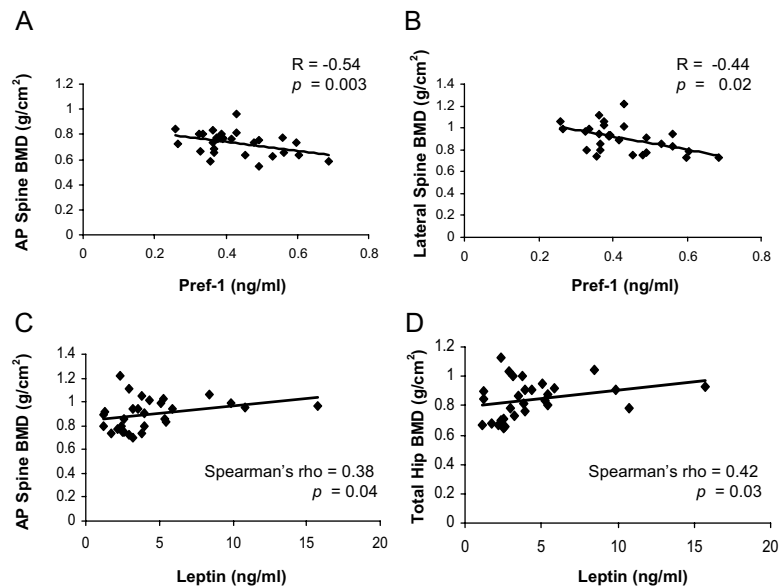

FIG. 3. Pref-1 was positively associated with marrow fat content of the proximal femoral metaphysis (A). Leptin was inversely associated with marrow fat content of L4 (B).

FIG. 4. Pref-1 was inversely associated with BMD of the AP spine (A) and lateral spine (B). Leptin was positively associated with BMD of the AP spine (C) and total hip (D).

initial diagnosis of AN, with the long-term cumulative incidence of any fracture being 57% (27). Thus, understanding the mechanisms of bone loss and the factors that regulate low bone mass in this population is of particular importance.

We have recently shown that women with AN have increased marrow fat content in the lumbar spine, femoral metaphysis, and diaphysis and that there is an inverse relationship between marrow adiposity and BMD at multiple skeletal sites (7). The clinical importance of bone marrow fat content has been demonstrated in a number of studies. Schellinger *et al.* (8) demonstrated that subjects with morphological evidence of bone weakness, such as Schmorl's nodes, end plate depression, wedging of vertebrae, and/or compression fractures had elevated levels of vertebral marrow fat content. A recent study also demonstrated that marrow adiposity, unlike visceral and sc fat, is not associated with increased risk of cardiovascular disease, suggesting that the genesis of marrow fat is clinically distinct from that of visceral and sc fat (28).

Several studies have demonstrated an inverse relationship between marrow adiposity and BMD (6, 29, 30). Yet it is provocative that in AN, in which marrow adipose depots are elevated, visceral and sc fat depots are very low (4). This suggests that the role of marrow fat in the human is distinct from that of sc and visceral depots and may include the local regulation of bone formation.

Osteoblasts and adipocytes are derived from a common progenitor, hMSCs. Many factors have been shown to affect differentiation of the hMSC into either osteoblasts or adipocytes. Peroxisome proliferator-activated receptor-γ agonists and glucocorticoids have been shown to induce adi-

pocyte differentiation (31, 32), whereas *in vitro* studies have demonstrated that estrogen administration leads to osteoblastogenesis with concomitant inhibition of adipogenesis (33). Yet it is still unclear whether this process of differentiation is a switch process or independently regulated because there are states, such as puberty, during which both marrow adipocyte differentiation and osteoblast differentiation are increased, arguing against a mutually exclusive switch process. New evidence is also emerging that independent preosteoblast and preadipocyte populations of mesenchymal stem cells may exist, providing a possible explanation for states of concomitant osteogenesis and adipogenesis (34).

Pref-1, a member of the EGF-like family of proteins, has also been shown to be an important regulator of adipocyte and osteoblast differentiation. Pref-1 is highly expressed in osteoblastic cell lines, preadipocytes, and hMSCs (9) and is a negative regulator of adipocyte and osteoblast differentiation. Osteoblast-specific Pref-1 overexpression in a mouse model results in significantly low-body-weight mice and significantly reduced BMD (11). Moreover, Pref-1's role as a regulator of energy stores has been further elucidated with the finding that overexpression of Pref-1 in a mouse model leads to lower adipose tissue mass than wild-type mice but increased insulin resistance (35). Therefore, it appears that Pref-1, an *in vitro* inhibitor of both osteoblast and adipocyte differentiation, may also be an important *in vivo* regulator of several metabolic processes.

Leptin, which is a major regulator of appetite, has been shown to be decreased in AN, most likely as a result of reduced total body fat (36). With respect to the skeleton, leptin acts centrally to enhance sympathetic tone and reduce bone formation, although leptin may also have direct effects on distinct skeletal sites (21, 37, 38). Our findings of a positive association between leptin and BMD in the low leptin state of AN are consistent with findings that leptin, when provided sc to women with low leptin levels, stimulates markers of bone formation (22) and with observational studies that demonstrate a positive association between leptin levels and bone mass in postmenopausal women (39). Our finding that leptin is inversely associated with marrow fat is also consistent with *in vitro* studies in which leptin blocks hMSC differentiation into adipocytes and stimulates osteogenesis (40). Whereas we found an inverse association between leptin and marrow fat at an axial site, we did not find an association between leptin and a peripheral site of marrow fat. One explanation for

this may be that the sample size of our study population was simply too small to detect this difference. Another possible explanation is that leptin acts differentially at different marrow fat sites. Hamrick *et al.* (38) demonstrated that when compared with wild-type mice, leptin-deficient *ob/ob* mice had increased marrow adipocyte number in the femur, whereas in the vertebra there was decreased marrow adipocyte number. Therefore, it is possible that leptin is differentially associated with the various marrow depots in the human as well.

IGF-I is known to be a stimulator of osteoblastogenesis (12–14) and is known to be low in states of nutritional deprivation, such as AN (15, 16), whereas IGF-BP2, a binding protein that binds to IGF-I in the circulation, has been shown to be elevated in adults with AN (15–17). We have shown that IGF-I is positively associated with BMD of the spine and hip and is inversely associated with L4 marrow adiposity in AN and that IGF-BP2 is positively associated with marrow adiposity. Interestingly, IGF-I was positively associated with L4 marrow adiposity in healthy controls, suggesting that IGF-I may have differential actions, depending on an individual's nutritional state and/or the hormonal milieu.

There are several limitations to our study. First, this was a cross-sectional study, and therefore, we cannot determine causation based on these data; therefore, all of the relationships demonstrated in this study are purely associational and cannot imply causation. Second, the source of Pref-1 is not clear from our studies. It is possible that Pref-1, which is found in high levels in preadipocytes, is cleaved during the maturation process and released into the circulation, explaining the elevated levels of circulating Pref-1 observed in anorexia nervosa. Yet Pref-1 is synthesized in several tissues, including the liver, and therefore, we cannot exclude the possibility that this EGF-like protein from other tissue sources may contribute to the suppressed bone formation reported in AN. Third, any conclusions about leptin's effects on bone have to be tempered by the complex nature of its relationship to hypothalamic processing and sympathetic signaling. Fourth, because only a single estradiol level was measured, definitive conclusions regarding the relationship between estradiol and marrow adiposity cannot be made. Given the exploratory nature of our study, further studies, involving larger study populations, will be needed in further understanding the association between Pref-1, leptin, and marrow fat.

In conclusion, our data demonstrate that women with AN have significantly higher levels of Pref-1, an important negative regulator of adipocyte and osteoblast differentiation. We have shown that Pref-1 is associated with marrow adiposity and low bone mass. Further understanding

of the role of Pref-1 as a possible mediator of adipocyte and osteoblast differentiation may be of significant clinical importance.

Acknowledgments

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