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Long-term metreleptin treatment increases bone mineral density and content at the lumbar spine of lean hypoleptinemic women

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ABSTRACT

Strenuously exercising young women with hypothalamic amenorrhea are hypoleptinemic and have low bone mineral density (BMD) and content (BMC), which predispose them to increased fracture risk. Short-term leptin replacement in these women corrects many neuroendocrine abnormalities and increases circulating levels of bone formation markers. Whether treatment with recombinant methionyl human leptin (metreleptin) for a long period improves BMD and BMC remains unknown. We studied 20 strenuously exercising young women with hypoleptinemia (leptin concentration <5 ng/mL) and hypothalamic amenorrhea of at least 6 months' duration. Eleven were randomized to metreleptin (initial dose, 0.08 mg/[kg·d] for 3 months; altered thereafter to 0.12 mg/kg for lack of efficacy or 0.04 mg/[kg d] for more than 5% weight loss) and 9 were randomized to placebo for 9 months. After a 3-month washout period, subjects were reexamined at the 1-year time point. Six subjects elected to continue on open-label metreleptin treatment for another 12 months. Two subjects dropped out after 18 months, and 4 completed the entire 2-year study. The BMD and BMC of the total body, lumbar spine (L1-L4), hip, and radius were assessed by using dual-energy x-ray absorptiometry at baseline and at 3, 6, 9, 12, 18, and 24 months of treatment. Metabolic and hormonal parameters and bone markers were measured in blood and urine. Metreleptin significantly increased BMC ($P = .034$) and tended to increase BMD ($P = .069$) at the lumbar spine at 9 months in the entire study group (intention-to-treat analysis). In subjects who completed the entire 2-year study (n = 4), metreleptin significantly increased BMD ($P = .024$) and BMC ($P = .049$) at the lumbar spine by 4% to 6%. Changes were not significant at the whole body, hip, and radius. Changes in hormonal and metabolic

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parameters and bone markers were moderate during the first year of treatment, but metreleptin further increased insulin-like growth factor 1 and decreased cortisol and cross-linked C-terminal telopeptide of type 1 collagen concentrations in serum during the second year of treatment ($P < .05$). The incremental area under the estradiol concentration curve over the 2-year course of the study correlated positively with the corresponding increase in lumbar spine BMD ($\rho = 0.42$, $P = .039$). Long-term metreleptin administration in strenuously exercising young women with hypothalamic amenorrhea and hypoleptinemia increases lumbar spine BMD and BMC and alters bone remodeling milieu to favor bone accretion. Results from this pilot study should be confirmed by future, larger clinical trials and need to be extended by studying bone microarchitecture and fracture risk.

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1. Introduction

Chronic energy deficiency and the resulting hypoleptinemia are closely linked to hypothalamic amenorrhea, a condition encompassing a clinical spectrum ranging from hormonal abnormalities in strenuously exercising women athletes to anorexia nervosa [1-5]. Hypothalamic amenorrhea is caused by suppressed gonadotropin-releasing hormone pulsatility, and endocrine evaluation of these patients often reveals not only suppression of the midcycle gonadotropin surge with decreased downstream gonadal sex steroid production but also decreased serum leptin because of low adipose tissue mass [1-4,6]. An imbalance between energy intake and exercise-induced energy expenditure results in the female athletic triad of decreased energy availability, amenorrhea with anovulatory infertility, and decreased bone mineral density (BMD) that increases risk for osteoporosis and stress fractures.

Women with hypothalamic amenorrhea have low BMD and bone mineral content (BMC) and may not attain optimal peak bone mass [6-10]. Many of the therapies designed to improve the hormonal abnormalities associated with this condition (estrogen, insulin-like growth factor 1 [IGF-1], and dehydroepiandrosterone), isolated or in combination [11], have no consistent or only mild favorable effects on BMD [12,13]. Thus, the search remains for a treatment that resolves both the reproductive dysfunction and the increased risk of osteoporosis in these women, possibly involving a molecule upstream of all neuroendocrine abnormalities.

Low body weight and adipose tissue mass in strenuously exercising women with hypothalamic amenorrhea raise the possibility that the relative lack of molecules normally secreted from adipose tissue could be responsible, at least in part, for the endocrine- and bone-related abnormalities associated with this condition. Leptin, a pleiotropic, adipocyte-secreted hormone, the circulating levels of which reflect the amount of energy stored in adipose tissue, has recently emerged as a key player in the complex system that regulates bone metabolism [14-17]. Low leptin concentrations (hypoleptinemia) are frequently observed in young women with hypothalamic amenorrhea, the female athlete triad, and/or anorexia nervosa [1-4]. We have previously shown that increasing leptin concentrations in women with hypothalamic amenorrhea by administering exogenous recombinant methionyl human leptin (metreleptin) leads to resumption of ovulatory menses and improves many of the underlying metabolic and neuroendocrine abnormalities [18,19]. In addition, we found that metreleptin treatment significantly increases circulating levels of bone formation markers and/or decreases circulating levels of bone resorption markers [18,19]. The duration of metreleptin treatment in our previous studies was probably too short to allow for changes in BMD to manifest [18, 19]. In this proof-of-concept, pilot study, we examined the effects of metreleptin treatment for 2 years on BMD, BMC, hormonal profile, and circulating markers of bone metabolism in lean, hypoleptinemic women with hypothalamic amenorrhea.

2. Methods

2.1. Subjects

Eligible subjects were strenuously exercising lean women between the ages of 18 and 35 years, with hypothalamic amenorrhea for at least 6 months and hypoleptinemia (fasting morning leptin concentrations <5 ng/mL at screening), recruited through advertisements in the community. At the time of screening, all subjects' body weights had been stable for at least 6 months before and were within 15% of their ideal body weight. Participants were free of any significant coexisting medical conditions, including active eating disorders (screened for on the basis of questionnaires), and were not taking any medications known to affect bone metabolism [18]. Women with amenorrhea secondary to other causes, particularly hyperprolactinemia, hypothyroidism, Cushing's syndrome, congenital adrenal hyperplasia, polycystic ovarian syndrome, or primary ovarian failure, were excluded from the study [18]. Pregnancy test results were negative at baseline, and tests were repeated at each follow-up visit.

Originally, 20 subjects (19 Caucasian and 1 Asian) were enrolled in a randomized, double-blinded, placebo-controlled study of metreleptin treatment for 9 months followed by a 3-month washout period. Seven subjects dropped out (Fig. 1) [18]. Subjects who completed the initial 9 months of double-blinded treatment and the 3-month washout period (phase A) had the opportunity to enroll in an open-label extension of metreleptin treatment for another 12 months (phase B). A subset of 6 subjects (all Caucasian) proceeded beyond the washout period and participated in the long-term open-label extension study for up to a total of 2 years of treatment; 2 of them dropped out after 18 months (Fig. 1). The baseline characteristics of the subjects who participated in the initial 9-month trial (phase A) and those who were enrolled in the 2-year study (phase B) are presented in Table 1. The study protocol was approved by the Institutional Review Board at Beth Israel Deaconess Medical

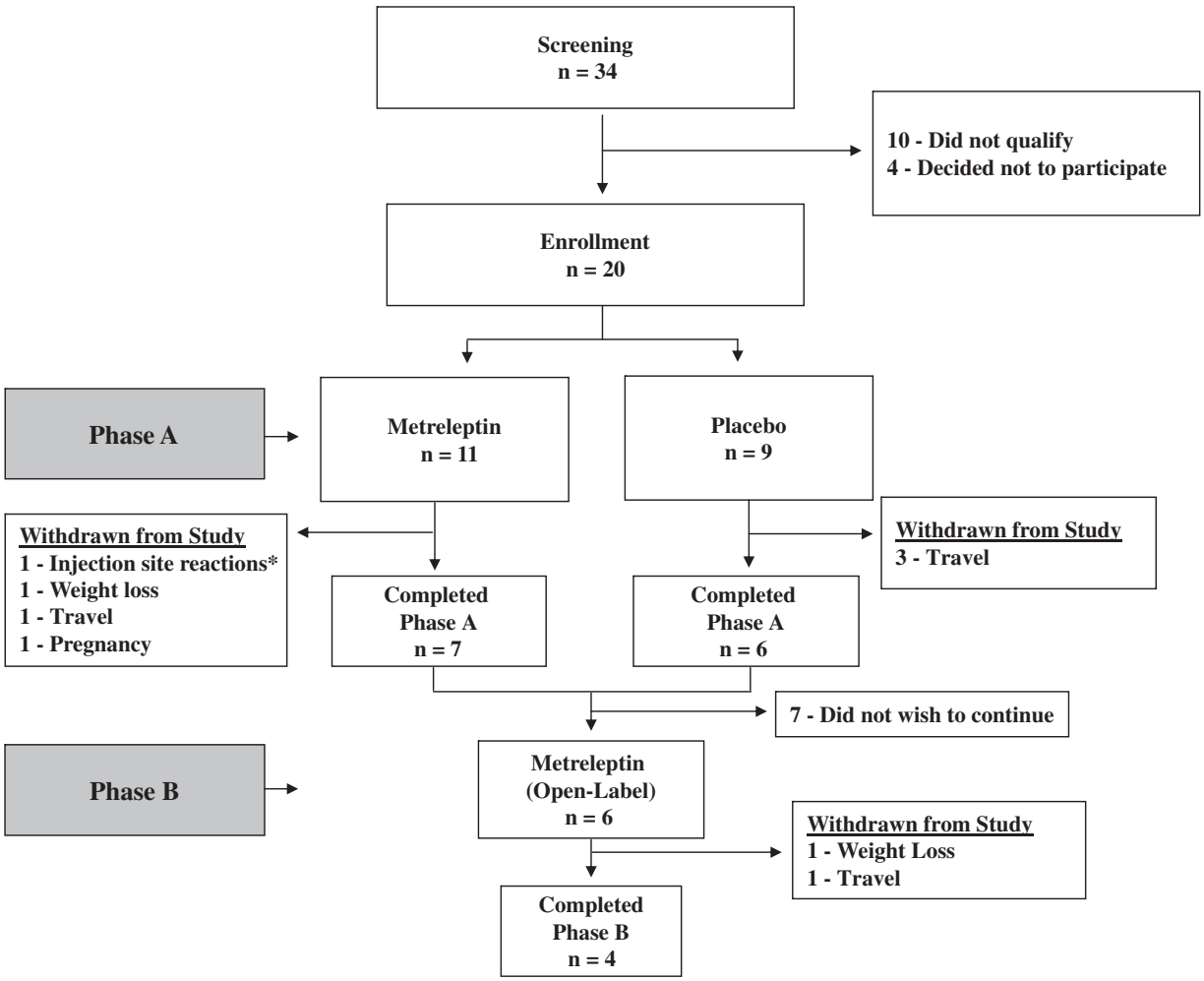

Fig. 1 – Study enrollment schema.

Center (BIDMC), and all subjects provided their written informed consent.

2.2. *Experimental design*

During phase A, participants were randomized in a 1:1 ratio to receive either metreleptin (n = 11) or placebo (n = 9), self-administered subcutaneously every day between 7:00 PM and 11:00 PM, for 9 months. Placebo and metreleptin (A-100, formerly known as *met-Hu-leptin*; phase II clinical trial) were provided by Amylin Pharmaceuticals, San Diego, CA. One subject randomized to receive metreleptin was withdrawn from the study after the baseline visit (shown with asterisk in Fig. 1) and was thus excluded from all analyses [18]. All subjects began metreleptin administration at a dose of 0.08 mg/kg for 12 weeks. If menstruation occurred during this time, subjects continued on the 0.08-mg/kg dose. Otherwise, the dose was increased to 0.12 mg/kg (for 3 out of 6 subjects during the first 9 months and for 2 out of 6 during the second year; Supplementary Table 1). If weight dropped below 5% of baseline weight, the dose was decreased by 0.04 mg/kg (in 4 out of 11 metreleptin-treated subjects during phase A and 2 out of 6 subjects during phase B; Supplementary Table 1). Dose adjustments for subjects who participated in the randomized phase A have been reported previously [18].

Subjects were assessed at baseline and every 3 months thereafter. After 9 months of double-blinded treatment, subjects discontinued treatment for 3 months and returned to BIDMC at month 12 for a follow-up visit. Metreleptin treatment was resumed as described above for all subjects who decided to continue (open label), and follow-up visits were conducted at months 18 and 24. All subjects were provided with calcium (600 mg twice daily) and vitamin D (400 IU daily) and were asked to keep diary cards documenting exercise habits, time and dose of study drug administration, and any menstrual bleeding. Of the 6 subjects who participated in phase B, 5 received metreleptin and 1 received placebo for the first 9 months of the study. Four of the 6 subjects completed the entire 2-year study, whereas 2 metreleptin-treated subjects dropped out of the study following 18 months, one because she was concerned about treatment-induced weight loss and one because of relocation (Fig. 1).

2.3. *Main outcome measures*

For each visit, subjects arrived at BIDMC after an overnight fast. A fasting blood sample was obtained and BMC and BMD

Table 1 – Baseline characteristics of study subjects

	Subjects participating in phase A			Subjects who continued on to phase B
	Metreleptin n = 10	Placebo n = 9		Metreleptin n = 6
			P value	
Age (y)	26.3 ± 1.5	25.3 ± 1.2	.589	25.5 ± 1.4
Body mass index (kg/m^2)	21.1 ± 0.6	19.9 ± 0.7	.233	20.9 ± 0.7
Body fat (%)	23.9 ± 1.4	20.8 ± 1.3	.116	23.3 ± 2.1
Leptin (ng/mL)	3.1 ± 0.6	2.8 ± 0.5	.783	1.9 ± 0.7
LH (IU/L)	4.5 ± 1.5	4.6 ± 0.9	.992	3.9 ± 1.9
FSH (IU/L)	5.4 ± 0.5	4.9 ± 0.3	.320	5.4 ± 0.7
Prolactin (ng/mL)	6.6 ± 0.7	6.9 ± 0.8	.763	7.9 ± 0.8
Estradiol (pg/mL)	11.5 ± 1.4	14.5 ± 2.0	.227	11.5 ± 2.3
Testosterone (ng/dL)	33.4 ± 4.3	41.9 ± 4.3	.181	37.2 ± 5.7

Data are presented as means ± SEM. Baseline characteristics (at screening) between metreleptin and placebo treatment groups were evaluated with Student unpaired *t* test. LH indicates luteinizing hormone; FSH = follicle-stimulating hormone.

of the total body, lumbar spine (L1-L4), hip, and radius were determined by using dual-energy x-ray absorptiometry (DXA; Discovery 4500A Densitometer, Hologic, Waltham, MA). Scans were performed in ARRAY mode (total body: 2 minutes; hip: 1 minute; lumbar spine: 1 minute; forearm/radius: 45 seconds), and data were analyzed with software version 12. Z-scores were calculated on the basis of the National Health and Nutrition Examination Survey reference data. Serum or plasma was isolated from whole blood and stored at –70°C until analysis of samples for plasma total and free leptin and serum leptin binding protein (LBP), estradiol, testosterone, cortisol, insulin, IGF-1, insulin-like growth factor-binding protein 3 (IGFBP-3), free thyroxine (fT4), total T4, thyroid-stimulating hormone (TSH), thyroxine-binding globulin (TBG), osteocalcin, osteoprotegerin, sclerostin, bone-specific alkaline phosphatase (BSAP), cross-linked C-terminal telopeptide of type 1 collagen (CTX), procollagen type 1 amino-terminal propeptide (P1NP), 25-hydroxy (OH) vitamin D, calcium, and intact parathyroid hormone (PTH). A urine sample (second void of the day) was also collected at each study visit for measurement of urinary creatinine, CTX, and cross-linked N-telopeptide of type I collagen (NTX) levels.

2.4. *Biochemical analysis*

The following hormone levels were measured using immunoassays: leptin (intra- and interassay coefficients of variation = 3.2% and 4.5%, respectively), free leptin (3.2% and 4.5%, respectively), and LBP (8.4% and 6.7%, respectively) (R&D Systems, Minneapolis, MN); estradiol (6.4% and 7.6%, respectively), testosterone (8.1% and 7.3%, respectively), TSH (4.0% and 7.3%, respectively), TBG (3.1% and 3.0%), and free and total T4 (3.6% and 6.5%, and 3.8% and 3.6%, respectively) (ALPCO, Salem, NH); insulin (5.7% and 6.7%, respectively), IGF-1 (3.6% and <9.0%, respectively), IGFBP-3 (4.2% and 10.0%, respectively), cortisol (7.1% and <10%, respectively), and intact PTH (6.0% and 8.3%, respectively) (Immulite; Siemens Healthcare Diagnostics, Deerfield, IL); serum and urinary CTX (2.4% and 6.7%, and 3.4% and 5.3%, respectively) (Immuno-Diagnostic Systems, Fountain Hills, AZ); osteocalcin (7.4% and 7.3%, respectively) and BSAP (4.9% and 6.3%, respectively) (Quidel, San Diego, CA); osteoprotegerin (4.0% and <10.0%, respectively) and sclerostin (4.0% and <10.0%, respectively) (Meso Scale Diagnostics, Gaithersburg, MD); urinary NTX (7.6% and 4.0%, respectively) (Inverness Medical, Princeton, NJ); 25-OH vitamin D (~10.0% and ~10.0%, respectively) (Diasorin, Stillwater, MN); P1NP (8.3% and 7.8%, respectively) (Orion Diagnostica, Espoo, Finland); calcium (<1.0% and <1.2%, respectively) and urinary creatinine (<2.2% and <2.5%, respectively) (Jaffe method) were measured on the Roche Cobas c311 (Roche Diagnostics, Indianapolis, IN).

2.5. *Statistical analysis*

Analysis was carried out with SPSS version 18 (SPSS, Chicago, IL) and Stata version 11.1 (Stata, College Station, TX). All data sets were tested for normality according to the Shapiro-Wilk procedure, and not-normally distributed data were appropriately transformed or ranked for analysis. Differences between metreleptin- and placebo-treated groups at baseline were evaluated with Student unpaired *t* test. The effect of metreleptin treatment over time was evaluated by using repeated-measures analysis of variance (ANOVA). When no statistical transformation could satisfy normality, data were analyzed with the nonparametric Friedman test (repeated-measures ANOVA on ranks). Results are presented as medians and quartiles, except for percentage changes from baseline that are shown as means ± SEM. The proportion of subjects who menstruated at each time point was evaluated with Cochran Q test, and individual pairwise comparisons with baseline were performed with the McNemar test. The primary analysis was carried out on intention-to-treat data (missing data for noncompleters were imputed by carrying forward the last observation); secondary analysis was performed on the basis of on-treatment data. To compare changes between placebo and metreleptin treatment groups, we used hierarchical linear models. Each dependent variable was modeled as a linear function of time, and we examined the fixed effect of metreleptin treatment on the intercept and slope of each dependent variable's trajectory. To adjust the effect of metreleptin treatment for changes in body weight, we introduced body weight as a time-varying variable in the level-1 model specification in a sequential model. Incremental areas under the curve (iAUCs) were estimated for select

hormones and lumbar BMD and BMC across the study period using the trapezoid rule. These iAUCs serve as unbiased estimates of the average change of the hormone levels and lumbar BMD and BMC throughout the study. Spearman linear correlation coefficients (ρ) were calculated between iAUCs for the variables of interest. Statistical significance was accepted at a 2-tailed P value $\leq .05$.

3. Results

3.1. Body weight and composition

During the first 9 months of the study, metreleptin treatment tended to reduce body weight and significantly decreased fat mass (Fig. 2, left panels). For the subjects who participated in both phases of the study (intention-to-treat: n = 6, Fig. 2, middle panels; on-treatment: n = 4, Fig. 2, right panels), body weight and fat mass appeared to decrease during the first 6 months of metreleptin treatment, stabilize between months 6 and 9 (possibly because of adjustment of metreleptin dosing), return toward baseline during washout (months 9-12), and decrease again during the open-label metreleptin treatment (months 12-24) (range: –9.6% to 1.2% and –35.6% to –9.6% from baseline, respectively). These changes did not always reach significance possibly because of the small number of subjects and/or metreleptin dose adjustments (Fig. 2).

3.2. BMD and BMC

During phase A, treatment with metreleptin significantly increased BMC and tended to increase BMD at the lumbar spine (time × treatment interaction: $P = .034$ and $P = .069$, respectively; Fig. 3, left panels), but did not affect total body, hip, and radial BMD and BMC (Supplementary Table 2). Likewise, for those subjects who continued on to phase B and completed the 2-year study (on-treatment: n = 4), treatment with metreleptin significantly increased BMD ($P = .024$) and BMC ($P = .049$) at the lumbar spine (range: 2.2% to 10.8% and 1.4% to 6.5% from baseline, respectively; Fig. 3, right panels). The increase in lumbar BMC persisted in the intention-to-treat analysis (n = 6), but the increase in lumbar BMD did not reach significance (Fig. 3, middle panels). Generally, the increase in lumbar BMD and BMC was predominantly evident after 12 to 18 months of treatment (Fig. 3). Accordingly, lumbar BMD Z-score increased from the osteopenic range to the reference range whether in the intention-to-treat ($P = .068$; Supplementary Table 3) or the on-treatment ($P = .050$; Supplementary Table 4) analyses. Metreleptin treatment did not affect whole-body, hip, and radial BMD and BMC (Supplementary Table 3 and 4).

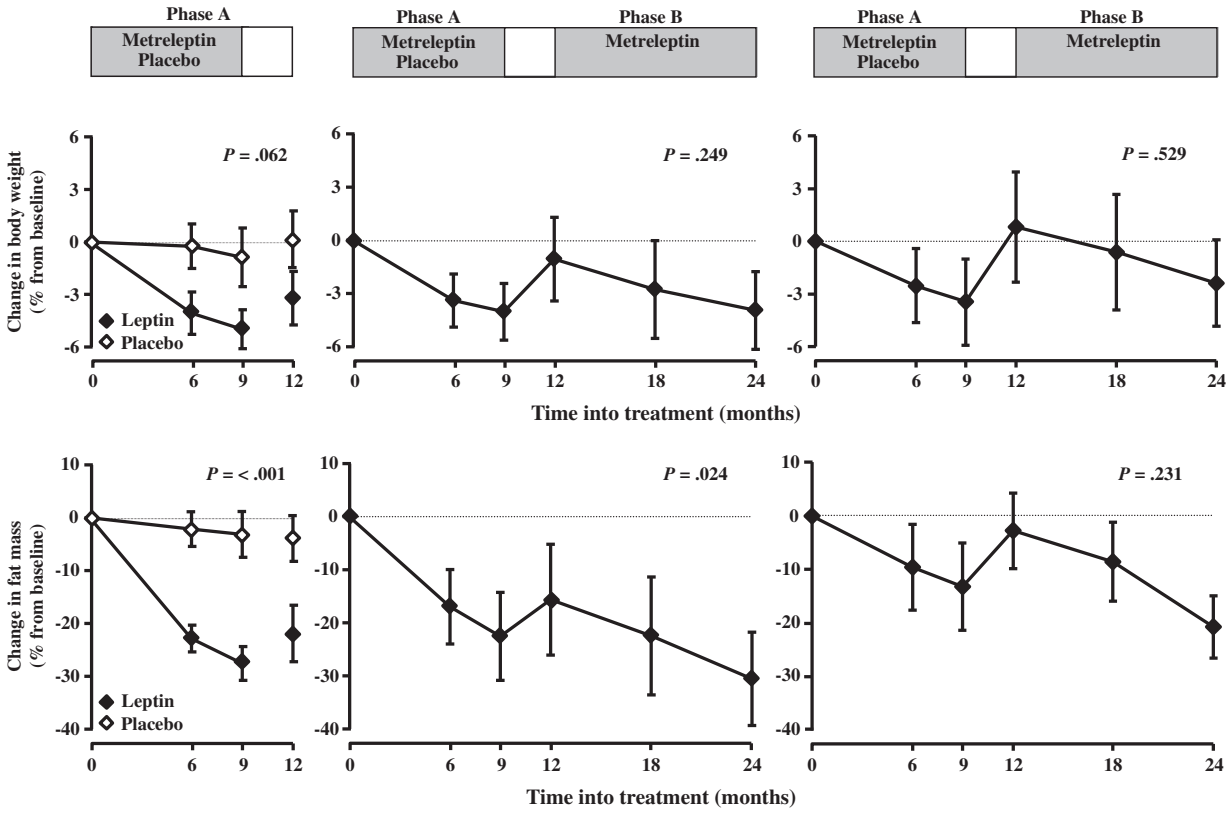

Fig. 2 – Percentage change from baseline in body weight (top) and fat mass (bottom) in lean hypoleptinemic women who participated in the initial 9-month randomized trial of metreleptin treatment (phase A, n = 20, intention-to-treat) (left) and the women who continued on to the 12-month open-label extension study (phase B, n = 6, intention-to-treat) (middle) as well as those who completed the entire 2-year study (n = 4, on-treatment). Values are means ± SEM. The *P* values represent the time × treatment interaction from repeated-measures analyses.

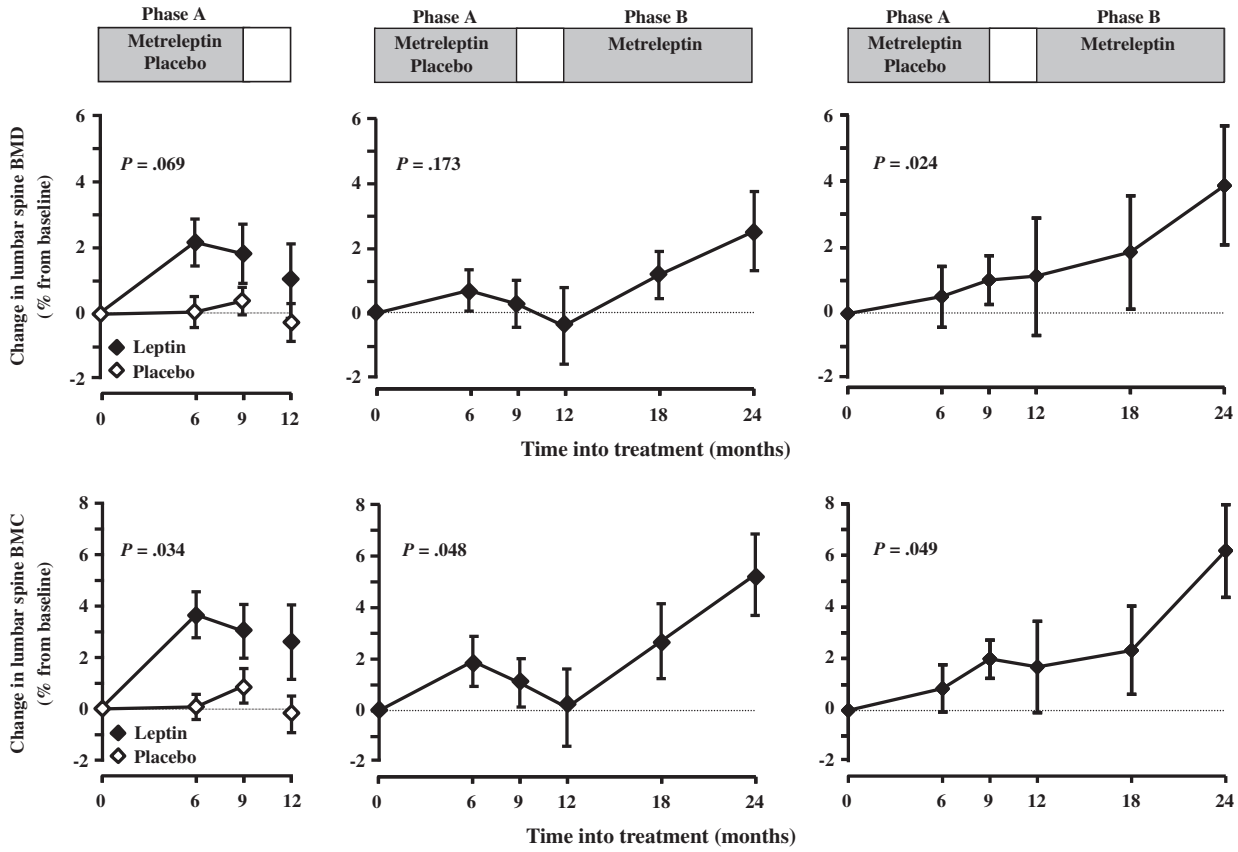

Fig. 3 – Percentage change from baseline in lumbar spine (L1-L4) BMD (top) and BMC (bottom) in lean hypoleptinemic women who participated in the initial 9-month randomized trial of metreleptin treatment (phase A, n = 20, intention-to-treat) (left) and the women who continued on to the 12-month open-label extension study (phase B, n = 6, intention-to-treat) (middle) as well as those who completed the entire 2-year study (n = 4, on-treatment). Values are means ± SEM. The P values represent the time × treatment interaction from repeated-measures analyses.

Adjusting for changes in body weight during the study did not affect these results but intensified the significance of metreleptin's effects. Metreleptin treatment significantly increased both lumbar BMC ($P = .04$) and BMD ($P = .03$) during phase A, and the same held true for phase B ($P \leq .001$ and $P = .002$, respectively).

3.3. *Hormonal and metabolic parameters*

The effects of 9 months of metreleptin treatment on hormonal and metabolic parameters, neuroendocrine function, and menstruation during phase A have been previously published [18].

For the subjects who continued on to phase B, total and free leptin concentrations and LBP increased significantly, as expected, during metreleptin administration (Table 2). Overall, metreleptin treatment decreased cortisol concentration and tended to increase 25-OH-vitamin D, TSH, and total T4 concentrations (Table 2). During the second year of treatment (12-24 months), metreleptin treatment significantly increased IGF-1 concentration ($P = .01$).

Estradiol concentration approximately doubled in response to metreleptin treatment, but the overall change was not significant ($P = .24$; Table 2). Likewise, metreleptin resulted in resumption of menses in 40% (2 out of 5) of the subjects during months 3 to 12 (the sixth subject received placebo during the first 9 months), in 50% (3 out of 6) of them at 18 months, and in 75% (3 out of 4) of them at 24 months (Supplementary Table 1); however, the change did not reach significance (Cochran Q test: $P = .161$; intention to treat: $P = .134$), probably because of the small number of subjects.

The increase in estradiol concentration over the 2-year course of the study (iAUC) correlated positively with the corresponding increase in lumbar spine BMD ($r = 0.42$, $P = .039$). Correlations of changes in lumbar BMD with changes in other hormonal parameters such as IGF-1 and cortisol were not significant; however, our small sample size does not allow for reliably evaluating such associations.

3.4. *Bone markers*

For subjects participating in phase B, treatment with metreleptin significantly reduced serum concentration of CTX but did not significantly affect other markers of bone remodeling, whether in serum or in urine (Table 3). Nevertheless, during the second year of treatment (12-24 months), metreleptin tended to also decrease urinary NTX concentration and NTX adjusted for creatinine (both $P = .09$).

Table 2 – Changes in metabolic and hormonal parameters over time in response to metreleptin treatment over 2 years in subjects who participated in both phase A and phase B

	Baseline	6 mo	9 mo	12 mo	18 mo	24 mo	OTx	ITT
							P value	*P* value
Leptin (ng/mL) [a]	4.1 (2.4, 5.0)	54.2 (37.1, 67.7)	53.4 (25.4, 80.8)	7.8 (3.8, 26.2)	68.5 (67.2, 69.6)	65.3 (58.0, 72.9)	.02	<.01
Free leptin (ng/mL) [a]	1.4 (0.90, 1.5)	36.8 (17.9, 51.8)	49.8 (5.0, 80.4)	1.3 (0.76, 3.6)	61.8 (43.8, 79.5)	55.9 (44.7, 70.0)	.01	<.01
LBP (ng/mL) [a]	36.1 (22.6, 47.0)	36.5 (28.1, 52.7)	35.7 (26.8, 47.7)	34.7 (22.6, 48.7)	41.8 (27.0, 48.7)	36.1 (26.6, 40.6)	.02	.35
Insulin (μIU/mL) [a]	1.8 (1.0, 2.8)	2.9 (1.0, 4.2)	1.0 (1.0, 2.4)	2.6 (2.3, 3.2)	3.0 (1.0, 3.7)	1.0 (1.0, 2.3)	.16	.38
PTH (pg/mL)	27.0 (22.3, 44.7)	28.2 (19.1, 40.9)	25.3 (12.8, 31.7)	24.8 (18.3, 32.6)	34.5 (15.7, 40.0)	29.8 (12.5, 56.0)	.59	.69
25-OH vitamin D (ng/mL)	27.1 (21.4, 30.8)	32.7 (22.6, 42.8)	18.6 (17.2, 26.2)	31.2 (17.3, 45.7)	48.5 (28.3, 64.0)	37.5 (23.5, 53.8)	.09	.05
Calcium (mg/dL) [a]	9.52 (8.90, 9.60)	9.37 (9.25, 9.50)	9.45 (9.30, 9.65)	9.67 (9.10, 9.95)	8.97 (8.95, 9.45)	9.45 (9.30, 9.7)	.90	.99
Estradiol (pg/mL) [b]	8.7 (5.0, 10.7)	9.3 (9.0, 12.9)	12.1 (8.3, 17.8)	10.3 (3.9, 19.9)	18.4 (6.0, 24.6)	22.0 (14.0, 35.5)	.24	.49
Testosterone (ng/dL) [a]	39.8 (31.0, 82.2)	49.2 (26.0, 184.5)	33.6 (32.2, 71.7)	35.4 (27.9, 71.7)	29.3 (23.4, 60.7)	53.0 (38.1, 135.2)	.90	.41
IGF-1 (ng/mL)	190 (147, 239)	225 (176, 251)	215 (177, 249)	175 (136, 283)	205 (140, 241)	216 (170, 294) [*]	.61	.79
IGFBP-3 (μg/mL) [b]	4.30 (3.58, 5.13)	4.45 (3.70, 5.46)	4.29 (3.48, 4.98)	4.23 (3.59, 5.51)	5.32 (3.73, 7.07)	4.80 (4.37, 7.73)	.60	.68
IGF-1:IGFBP-3 [b]	42.7 (33.6, 56.4)	46.9 (43.7, 51.6)	48.8 (40.7, 58.0)	41.2 (35.2, 54.6)	31.6 (26.6, 51.8)	39.1 (30.9, 57.0)	.66	.68
TSH (μIU/mL) [b]	2.17 (1.36, 3.30)	2.89 (2.36, 4.52)	1.73 (1.35, 2.44)	2.32 (1.50, 2.81)	2.78 (1.64, 3.78)	2.86 (2.15, 3.41)	.10	.03
TBG (mg/L) [a]	15.0 (13.5, 17.3)	16.6 (13.2, 17.7)	15.1 (12.4, 17.1)	14.5 (13.3, 17.1)	12.6 (11.6, 15.0)	16.7 (15.4, 17.7)	.14	.02
Total T4 (μg/dL) [a]	5.57 (4.78, 5.94)	4.93 (4.49, 5.48)	5.14 (4.54, 5.61)	5.08 (4.54, 5.33)	5.64 (4.40, 5.94)	5.98 (5.54, 7.48)	.08	.43
Free T4 (ng/dL) [a]	1.11 (0.88, 1.12)	1.02 (0.98, 1.09)	1.16 (0.89, 1.63)	1.03 (0.96, 1.08)	1.13 (1.06, 1.23)	1.08 (1.01, 1.18)	.96	.60
Cortisol (μg/dL)	19.4 (16.8, 20.5)	18.2 (15.8, 20.6)	12.4 (9.0, 15.3)	14.0 (12.4, 17.9)	13.0 (10.8, 14.3)	16.1 (14.5, 20.4)	.01	<.01

Data are presented as medians and quartiles (on-treatment data, n = 4). Changes over time were analyzed by using repeated-measures ANOVA or Friedman test (nonparametric ANOVA). The *P* value in the last column represents the overall effect of metreleptin from both on-treatment (n = 4) and intention-to-treat (n = 6) analyses. OTx indicates on-treatment; ITT, intention-to-treat.

[*] Significantly different than 12-month value; *P* = .01.

[a] Data could not be normalized by appropriate transformations; analysis was performed with Friedman test.

[b] Data were transformed as necessary to satisfy normality; analysis was performed with repeated-measures ANOVA.

3.5. *Safety of metreleptin therapy*

During phase A, one subject developed local injection site reactions with erythematous rashes within a few weeks of starting metreleptin treatment. She withdrew from the study, and the symptoms resolved within a week. Another subject treated with metreleptin had persistent weight loss (>8% from her baseline weight) despite dose adjustment and was

Table 3 – Changes in bone markers over time in response to metreleptin treatment over 2 years in subjects who participated in both phase A and phase B

	Baseline	6 mo	9 mo	12 mo	18 mo	24 mo	OTx	ITT
							P value	*P* value
Serum								
Osteoprotegerin (ng/mL) [a]	0.14 (0.12, 0.18)	0.13 (0.11, 0.15)	0.11 (0.09, 0.17)	0.14 (0.13, 0.20)	0.16 (0.15, 0.18)	0.15 (0.15, 0.16)	0.12	0.18
Osteocalcin (ng/mL)	9.3 (5.8, 14.1)	8.3 (6.0, 13.5)	11.3 (7.7, 13.9)	8.9 (4.5, 13.1)	7.8 (3.8, 9.5)	8.8 (4.6, 13.7)	0.27	0.36
BSAP (U/L) [b]	18.6 (17.5, 23.1)	20.6 (14.1, 27.5)	20.7 (15.7, 25.8)	18.8 (13.5, 24.9)	18.4 (15.1, 25.1)	15.5 (13.9, 20.5)	0.90	0.79
Sclerostin (ng/mL) [b]	0.08 (0.07, 0.09)	0.06 (0.06, 0.07)	0.06 (0.04, 0.07)	0.07 (0.05, 0.16)	0.06 (0.04, 0.100)	0.08 (0.04, 0.10)	0.47	0.60
P1NP (μg/L)	44.8 (34.4, 81.4)	53.8 (42.5, 73.2)	46.8 (38.4, 108)	49.7 (36.5, 75.1)	53.3 (37.1, 65.9)	38.5 (33.8, 55.9)	0.56	0.56
CTX (ng/mL) [b]	0.60 (0.39, 0.83)	0.49 (0.22, 1.17)	0.45 (0.38, 0.65)	0.43 (0.35, 0.83)	0.31 (0.29, 0.38)	0.33 (0.30, 0.37)	0.02	0.12
Urine								
CTX (ng/mL) [b]	12.0 (10.0, 26.3)	14.1 (10.0, 22.2)	14.1 (6.2, 34.9)	11.8 (10.6, 12.6)	12.0 (10.0, 16.3)	10.6 (8.0, 11.3)		0.83
NTX (nmol/L) [b]	209 (104, 445)	269 (104, 424)	185 (68, 750)	172 (104, 266)	125 (103, 292)	120 (72, 116)	0.81	0.87
CTX:creatinine (ng CTX/mg Cr) [b]	2.9 (0.90, 4.0)	3.8 (1.4, 5.1)	4.8 (2.5, 6.1)	2.9 (2.1, 9.1)	4.3 (2.6, 6.4)	2.6 (1.5, 3.5)	0.14	0.06
NTX:creatinine ([nmol/L NTX]/[mg Cr]) [b]	0.37 (0.10, 0.95)	0.45 (0.23, 0.92)	0.63 (0.54, 0.89)	0.62 (0.37, 0.76)	0.64 (0.27, 0.87)	0.31 (0.13, 0.43)	0.38	0.62

Data are presented as medians and quartiles (on-treatment data, n = 4). Changes over time were analyzed by using repeated-measures ANOVA or Friedman test (nonparametric ANOVA). The *P* value in the last column represents the overall effect of metreleptin from both on-treatment (n = 4) and intention-to-treat (n = 6) analyses. OTx indicates on-treatment; ITT, intention-to-treat.

[a] Data were transformed as necessary to satisfy normality; analysis was performed with repeated-measures ANOVA.

[b] Data could not be normalized by appropriate transformations; analysis was performed with Friedman test.

withdrawn from the study. During phase B, 2 subjects at 2 time points had mild injection site reactions that did not persist. There were no other clinically significant adverse events related to the study medication.

4. Discussion

We found that increasing leptin concentrations in strenuously exercising, hypoleptinemic lean young women with hypothalamic amenorrhea through metreleptin administration for a prolonged period (2 years) increases lumbar spine BMC and BMD by 4% to 6% but does not significantly affect total body BMC and BMD, or BMC and BMD of the hip and forearm. The increase in lumbar BMD and BMC manifested slowly over the first year of treatment and became more evident after 18 to 24 months. Accordingly, we observed a decrease in serum CTX, which is a marker of bone resorption.

Bone is a highly metabolically active tissue; and therefore, bone metabolism (remodeling) is closely linked to energy metabolism [14]. Leptin has many actions pertaining to the regulation of energy homeostasis, and several lines of evidence indicate that it may be involved in the regulation of bone mass either through changes in the neuroendocrine profile or through a central relay [14-16,20]. The role of leptin in bone remodeling in animals in vivo is not well understood, and available data are inconsistent. It was recently suggested that leptin's role in bone metabolism may be permissive and therefore different in leptin-sufficient as opposed to leptin-deficient models [21]. Animal studies report predominantly antiosteogenic central hypothalamic pathways through which leptin decreases bone density [22-25]. However, this mechanism may not be directly applicable to humans because leptin does not activate this system in human subjects [26,27]. Several other studies have shown that leptin directly stimulates bone growth in vitro and increases bone density in leptin-deficient animals [28-33] by stimulating osteoblast and/or suppressing osteoclast activity [28,31,34-36]. In aggregate, these observations are consistent with a bone trophic effect of leptin, in accordance with our findings of increased lumbar BMC and BMD.

Our results indicate that the skeletal effects of metreleptin may be primarily on the trabecular (cancellous) bone in the lumbar spine. Although we found a significant increase in lumbar BMC and BMD over the course of the study, we observed no significant changes in hip or radial BMC and BMD. The lumbar spine has been identified as a key region of low bone density in female athletes [8,9]. Vertebral bone consists of a higher proportion of trabecular relative to cortical bone and has a faster turnover rate [37,38]. Therefore, changes in BMC and BMD of the lumbar spine are detected more quickly and are of greater magnitude than at other sites (hip and radius), as also evidenced in response to other treatments, for example, PTH [39,40], IGF-1, and contraceptive steroids [11,41,42]. Hence, we cannot exclude the possibility that a longer treatment period, beyond 2 years, may be required to detect favorable effects of metreleptin on hip and radial BMC and BMD.

It is well established that neuroendocrine changes characteristic of energy deficiency states, such as low thyroid and sex hormone and IGF-1 concentrations and high levels of cortisol, are thought to contribute to bone loss [17]. It is therefore possible that, besides putative direct effects of leptin, multiple neuroendocrine changes in response to metreleptin treatment, that is, increased IGF-1 concentration and restoration of euthyroid status, might be involved because the lumbar spine is more sensitive to the bone trophic effect of these mediators than the hip and the forearm [11,43,44]. The reduction in cortisol levels could also play a role because cortisol reduces bone formation [45] and it is inversely associated with circulating osteocalcin and lumbar spine BMD, but not with markers of bone resorption or BMD at the hip in humans [46]. Furthermore, treatment of hypercortisolism improves lumbar spine BMD to a greater extent and more quickly than femoral neck or radial BMD [47]. Finally, estradiol levels approximately doubled and menses were resumed in the majority of subjects, consistent with our previous observations [5,18,19]. Estradiol is a major bone trophic hormone, and its lower levels in oligo- and amenorrheic women have been implicated in bone loss [12,13]. We found a positive correlation between the leptin-induced increase in the iAUC of estradiol concentrations and the corresponding increase in lumbar BMD over 2 years of metreleptin treatment. Thus, the effect of leptin on bone is likely multifactorial and may involve several hormonal mediators besides possible direct actions on bone. Larger studies are needed to more comprehensively evaluate the direct and indirect effects of leptin on bone metabolism in humans.

This is the first study to investigate the long-term effect of metreleptin treatment on BMD and BMC and markers of bone turnover in hypoleptinemic women. The increase in lumbar spine BMC and BMD over the course of our 2-year study, albeit mild (4%-6% compared with baseline), may be especially important because it occurred in the face of nonsignificant decreases in body weight and fat mass. Although DXA measurements at the lumbar spine are less accurate compared with those at other skeletal sites because of differences in the amount of fat and soft tissue thickness, it was recently shown that fat thickness overlying bone and adjacent soft tissue shows only minor changes with weight loss [48]. Hence, we do not expect this to have confounded our finding of increased lumbar spine BMD. Consistent with this notion, we assessed changes in BMC and BMD, after adjusting for changes in body weight, and found that the observed increases in bone density remained significant. This is in line with the recent observation that leptin treatment in growing leptin-replete rats prevents normal weight gain but does not adversely affect normal bone accretion and skeletal growth [21]. However, this study has several limitations as well. First, it was a pilot study; and thus, its sample size was small. Therefore, the results from the linear statistical models should be interpreted with caution and be replicated by future larger studies. Nonetheless, we detected a significant increase in lumbar BMD and BMC and provide a basis for future, larger-scale studies designed to evaluate the effect of metreleptin treatment on bone density and metabolism in hypoleptinemic women. Second, we relied on DXA to evaluate macroarchitecture of bone and areal BMD and did

not use more elaborate methods to assess bone microarchitecture, morphometry, and/or volumetric BMD based on computed tomography or magnetic resonance [49]. Such an evaluation could have allowed for a more direct investigation of the effects of metreleptin treatment on cortical vs trabecular bone. Third, by design, phase B of our study did not include a placebo group but instead consisted of an open-label 12-month extension. We cannot take into account possible age-related changes or uncontrolled confounding factors in general. However, other studies have shown that BMD at various skeletal sites, including the ones we measured, does not change significantly over 9 to 30 months in untreated young women with exercise-induced amenorrhea; changes from baseline typically range from an increase of approximately 0.5% to a decline of approximately 2.5% [11,41,42]. This is not surprising, considering that maximal bone accrual in females occurs in mid adolescence and that more than 90% of peak bone mass is achieved by the end of the second decade [50,51]. Hence, we do not believe that lack of a placebo group in phase B could be confounding our observations. In addition, the inclusion in the open-label extension of one subject who started in the placebo arm for phase A of the study would, if anything, dilute the observed effects of treatment (Fig. 2; left vs middle panels). Finally, we cannot exclude the possibility that greater treatment duration might have revealed favorable changes at other skeletal sites as well. These hypotheses remain to be tested by future studies.

In summary, our results suggest that metreleptin treatment may improve BMD and BMC at the lumbar spine and thus counteract the bone loss and increased risk for stress factures and osteoporosis in strenuously exercising lean women with hypothalamic amenorrhea and hypoleptinemia. Aside from minor injection site reactions, metreleptin treatment was well tolerated, in accordance with other reports [52,53]. One would envision that metreleptin treatment could be used, alone or in combination with other treatments proposed for this condition and/or along with efforts to improve dietary intake and/or reduction in exercise intensity aiming at achieving a healthy weight gain. The latter has been shown to be crucial in improving bone density in these women [6,12,54]. Because achieving a normal weight and adipose tissue mass in these women usually requires a prolonged period, metreleptin would be expected to provide protection in terms of bone health for the period before weight gain is achieved, and possibly, even after weight gain is achieved and maintained. Longer, randomized studies are required to confirm our observations, to extend them by studying related clinical outcomes, and to elucidate the mechanisms underlying the effects of leptin on bone remodeling in humans.

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Conflict of Interest

There are no conflicts of interest associated with this manuscript.

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