Supporting Information

Chou et al. 10.1073/pnas.1015674108

SI Materials and Methods

Subjects. Eligible subjects were women between 18 and 35 y old who had had secondary hypothalamic amenorrhea for at least 6 mo coincident with a period of strenuous exercise and/or low body weight. All participants were within 15% of ideal body weight, and their weights had been stable for at least 6 mo at the time of screening. Fasting morning leptin levels were <5 ng/mL before entering the study. Study participants did not have significant coexisting medical conditions, including active eating disorder, depression or other psychiatric disease, alcoholism, drug or tobacco use, malignancies, renal or hepatic disease, diabetes mellitus, myocardial ischemia, gastrointestinal malabsorption, or anemia (hemoglobin ≤10 g/dL). Women with amenorrhea secondary to other causes, specifically hyperprolactinemia, hypothyroidism, hyperthyroidism, Cushing's syndrome, congenital adrenal hyperplasia, polycystic ovarian syndrome, or primary ovarian failure, were excluded from the study. Women who had taken medications that might affect hormone measurements or bone mineral density, including glucocorticoids, antiseizure medications, thyroid hormones, or estrogens, within 3 mo of screening were excluded also. Additionally, women who were breastfeeding, pregnant, or planning pregnancy were excluded from study participation. Pregnancy tests were performed at the screening and all follow-up visits. Participants were required to use a double-barrier methods (diaphragm with intravaginal spermicide, cervical cap, male or female condom with spermicide) and/or abstinence to prevent pregnancy.

Study Design. Twenty participants provided written informed consent to participate in this randomized, double-blinded, placebo-controlled study. The protocol was approved by the institutional review board of Beth Israel Deaconess Medical Center (BIDMC).

The participants were assigned randomly in a 1:1 ratio to receive either metreleptin or placebo. All participants were provided calcium (600 mg twice daily) and vitamin D (400 international units daily) supplements. During the 36-wk study, participants had visits to the General Clinical Research Center (GCRC) of BIDMC every 4 wk for assessments (including blood tests, body composition, and/or resting energy expenditure) as well as to monitor for potential side effects and review the injection technique. Before the inpatient assessment every 12 wk, participants were requested to keep a 3-d food diary (recording all caloric intake for 1 weekend day and 2 weekdays). They were asked to keep exercise records and to document any menstrual bleeding throughout the study. Participants returned at week 52 for a follow-up visit 16 wk after discontinuing the study medication. Subjects who had a menstrual cycle were instructed to return at day 21 for measurement of serum progesterone to assess whether ovulation had occurred.

Metreleptin or matching placebo was self-administered by s.c. injection once daily between 7:00 PM and 11:00 PM for 36 wk. The dose of metreleptin or placebo was calculated based on the subject's weight to achieve physiologic to supraphysiologic leptin levels. The initial dose of metreleptin or placebo for all subjects was 0.08 mg/kg and was continued for 12 wk. At the end of 12 wk, subjects who had begun menstruating continued the same dose through week 36. Subjects who had not started menstruating at week 12 had their dose increased to 0.12 mg/kg. Their weights were checked at each visit, and the treatment doses were adjusted to maintain stable weights. Specifically, doses were reduced by 0.04 mg/kg if a subject lost >5% of her baseline weight. Subjects were withdrawn from the study if body weight decreased to <8% of baseline for more than one visit or to <80% of ideal body weight.

Fasting blood samples were collected at each visit for measurement of serum leptin, free leptin, antileptin antibodies, luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, progesterone, testosterone, inhibin B, thyroid-stimulating hormone (TSH), free thyroxine (fT4), free triiodothyronine (fT3), cortisol, insulin-like growth factor-1 (IGF1), IGF binding protein 3 (IGFBP-3), prolactin, osteocalcin, bone-specific alkaline phosphatase (BSAP), osteocalcin, osteoprotegerin, and C-telopeptides of type 1 collagen (CTX). A urine sample also was collected approximately 2 h after the first morning void for urinary N-telopeptides of type 1 collagen (NTX), creatinine, and free cortisol measurements.

Bone density (total, spine, hip, and radius) and body composition (total body mass, fat mass, and lean mass) were measured using dual-energy X-ray absorptiometry (DEXA) (Hologic QDR-4500; Hologic) in whole-body array mode at baseline and every 12 wk after an overnight stay at the GCRC. The resting energy expenditure was measured directly using a Sensormedics V_{max} Encore equipment (VIASYS Respiratory Care Inc.) in the morning on awakening and in the fasted state before any other testing. The food diaries were analyzed by Nutritionist Pro, version 4.3.0 (Axxya Systems).

Biochemical Analysis. The following hormone levels were measured using immunoassays: leptin (Millipore); LH, estradiol, progesterone, testosterone, cortisol, IGF1, IGFBP-3, prolactin, and inhibin B (Diagnostic Systems Laboratory); FSH and TSH (ALPCO); BSAP and osteocalcin (Quidel); osteoprotegerin (R&D Systems); fT3 and fT4 (Immulite Siemens Healthcare Diagnostics); CTX (Immunodiagnostic Systems Inc.); and urinary NTX (Wampole Laboratories). Creatinine was assayed using the Creatinine Jaffe Method on the Roche Modular P Analyzer (Roche Diagnostics). All samples from each subject were analyzed in duplicate in the same assay. Assay sensitivities as well as interassay and intraassay coefficients of variation (CV) were similar to those reported by the manufacturer or in previous studies (1–3).

Serum free leptin was measured by RIA (Millipore) with a sensitivity of 0.5 ng/mL, intraassay CV of 3.4–8.9%, and interassay CV of 3.0–6.2%. Serum for all free leptin measurements underwent a preincubation water bath for 2 h at 37 °C, followed by treatment with equal volume of Gamma PEG (Immucor, Inc.) for 10 min and vortexing of the tubes every 2–5 min. After incubation the samples were centrifuged at 585 × *g* for 20 min, and the sample was removed for assaying without disturbing the pellet. Reported free leptin values have been corrected for 1:2 dilution by pretreatment PEG solution.

Serum antileptin antibody levels were determined by a colorimetric sandwich ELISA developed in house. Briefly, 50 μL of recombinant human leptin (R&D Systems) at a final concentration of 10 μg/mL in PBS was plate-bound to a 96-well ELISA plate (PBI International SpA). After 16-h incubation at 4 °C, the plates were washed extensively with PBS-Tween 20 0.05%, blocked with 200 μL of PBS/10% FCS for 2 h, and washed repeatedly. Diluted sera in PBS-0.05% Tween20/10% FCS (1:10–1:1,000) were added at 100 μL per well and incubated for 4 h at room temperature. After five washes, goat anti-human polyvalent immunoglobulins alkaline phosphatase-conjugated Abs (Sigma-Aldrich), diluted 1:30,000 in PBS-Tween20 0.05%/10% FCS, was added at 100 μL per well for 1 h. The reaction was developed with Sigma-Fast PNPP (p-nitrophenyl phosphate, al-

kaline phosphatase substrate) (Sigma) and read after 30 min at 405 in an ELISA plate-reader (Bio-Rad Laboratories). Standard curves of antileptin antibodies were developed in each assay using an antileptin-neutralizing monoclonal antibody generated in our laboratory (971212 mAb). Quantification of optical density values was performed after extrapolation from standard curves of known concentrations of antileptin antibodies.

The functional activity of antileptin antibodies was assessed with the human leptin receptor-transfected BAF3 (hLepR$^+$ BAF3) cell line, whose proliferation is leptin dependent. This cell line was kindly provided by Arieh Gertler (Hebrew University, Rehovot, Israel). Briefly, hLepR$^+$BAF3 cells were cultured in flat-bottomed 96-well microtiter plates (Becton-Dikinson Falcon) at a density of 5×10^3 cells per well in a total volume of 100 μL of RPMI medium 1640 supplemented with 2% FCS (HyClone-Pierce), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). Cells were cultured at 37 °C in 100% humidity and 5% CO_2 in the presence of increasing doses of metreleptin ranging from 0.01 to 10 ng/mL Purified IgG immunoglobulins from sera of metreleptin-treated subjects, placebo-treated subjects, and healthy controls were added to the cells in the different conditions at a final concentration of 50 μg/mL. After 48 h [^{3}H]-thymidine (0.5 μCi per well) (Perkin-Elmer) was added to the cultures, and the cells were harvested after 12 h. Radioactivity was measured with a β-cell plate scintillation counter (Wallac). As a standard of leptin neutralization, we used our anti-human leptin mAb 971212 at increasing concentrations from 0.1 to 25 μg/mL.

Statistical Analysis. We did not have any data from randomized, placebo-controlled clinical trials to perform a priori power analysis. Thus, we performed an interim analysis after seven subjects per arm had completed 24 wk of the study to perform power calculations and determine whether additional subjects were needed and/or multicenter extension was warranted. This interim analysis was done at a fixed $\alpha = 0.001$ to minimize the impact on the α-level at the end of the study.

The results are presented as mean values ± SEM. SAS version 9.1 (SAS Institute) was used for statistical analysis, and $P < 0.05$ (two-tailed) was considered statistically significant for all analyses. To compare changes between the metreleptin-treated group and the placebo-treated group across the entire study duration, we used repeated-measures analysis as major analysis to evaluate changes in body composition parameters, hormone levels, and levels of bone markers by giving overall P values by treatment effect and treatment over time interaction. All P values reported in the text are by treatment over time interaction unless otherwise specified. One-way ANOVA followed by the protected least significant differences technique was used to compare differences between the two treatment groups for baseline characteristics and for follow-up measurements.

Values of leptin, LH, FSH, fT3, and estradiol were normalized logarithmically for analysis. Baseline values were adjusted in the model for analysis of inhibin B and the urinary NTX:creatinine ratio. Statistical analyses were conducted using both on-treatment analysis and intention-to-treat analysis (last observation carried forward, $n = 9$ for placebo-treated group and $n = 10$ for metreleptin-treated group). Similar results were found. One participant who completed only the baseline visit was excluded from all analyses of the study.

1. Welt CK, et al. (2004) Recombinant human leptin in women with hypothalamic amenorrhea. *N Engl J Med* 351:987–997.
2. Taylor AE, Khoury RH, Crowley WF, Jr. (1994) A comparison of 13 different immunometric assay kits for gonadotropins: Implications for clinical investigation. *J Clin Endocrinol Metab* 79:240–247.
3. Welt CK, McNicholl DJ, Taylor AE, Hall JE (1999) Female reproductive aging is marked by decreased secretion of dimeric inhibin. *J Clin Endocrinol Metab* 84:105–111.

Table S1. Baseline characteristics of subjects with hypothalamic amenorrhea

	Subjects receiving metreleptin (n = 10)		Subjects receiving placebo (n = 9)		
Characteristic	Mean ± SE	Range	Mean ± SE	Range	P value
Age (y)	26.6 ± 1.4	20.6–34.4	25.4 ± 1.2	19.9–24.1	0.5341
Weight (kg)	56.9 ± 1.8	49.6–69.7	54.9 ± 2.1	45.7–67.1	0.4899
Height (cm)	164.2 ± 1.8	154.7–175.7	166.4 ± 2.1	159.0–178.8	0.4565
Body mass index (kg/m^2)	20.9 ± 0.6	18.7–24.5	19.8 ± 0.7	17.6–23.9	0.2458
Duration of hypothalamic amenorrhea (y)	5.1 ± 1.3	1.5–15	4.0 ± 1.1	0.75–10	0.5337
Leptin (ng/mL)	4.6 ± 0.6	0.9–8.0	4.1 ± 0.6	1.0–6.9	0.8702
LH (IU/L)	8.8 ± 3.3	0.6–31.7	14.3 ± 5.0	0.4–47.2	0.3648
FSH (IU/L)*	5.3 ± 0.5	3.4–8.1	4.9 ± 0.5	3.0–6.6	0.6241
Estradiol (pg/mL)	23.0 ± 9.0	6.6–108.6	14.0 ± 1.7	8.1–23.0	0.3756

* n = 8 for placebo.

Table S2. Additional changes in neuroendocrine axes and markers of bone turnover over time

Analyte	Group	Week 0 (baseline)	Week 12	Week 24	Week 36	Week 52 (follow-up)	*P* (trt)*	*P* (trt* time)†	*P* (follow-up)‡
Hormones									
Leptin BP(ng/mL)	Metreleptin	34.7 ± 2.6	31.3 ± 2.8	32.9 ± 3.5	31.6 ± 3.2	39.9 ± 3.1	0.93	0.32	0.10
	Placebo	30.8 ± 2.3	31.6 ± 2.4	32.2 ± 2.9	31.5 ± 2.5	31.3 ± 3.6			
LH (IU/L)	Metreleptin	8.8 ± 3.3	11.8 ± 4.4	9.7 ± 3.5	13.4 ± 5.3	8.2 ± 3.2	0.86	0.4	0.40
	Placebo	14.3 ± 5.0	8.7 ± 2.2	12.9 ± 3.9	10.0 ± 3.6	5.4 ± 3.4			
FSH (IU/L)	Metreleptin	5.3 ± 0.5	5.0 ± 0.4	4.9 ± 0.4	5.0 ± 0.6	5.0 ± 0.6	0.69	0.7	0.99
	Placebo	4.9 ± 0.5	4.9 ± 0.4	4.7 ± 0.5	4.8 ± 0.5	5.0 ± 0.7			
Testosterone(ng/mL)	Metreleptin	42.1 ± 4.3	36.0 ± 4.7	31.5 ± 4.0	31.5 ± 4.2	41.3 ± 5.3	0.57	0.88	0.08
	Placebo	41.3 ± 6.9	41.6 ± 7.9	36.5 ± 7.1	37.4 ± 7.2	26.0 ± 6.0			
Inhibin B (pg/mL)	Metreleptin	43.0 ± 10.5	71.4 ± 13.9	65.5 ± 14.9	72.5 ± 17.18	76.5 ± 7.5	0.07	0.27	0.005
	Placebo	64.0 ± 13.0	63.0 ± 10.7	60.9 ± 10.3	54.4 ± 8.4	40.5 ± 4.7			
TSH(μIU/mL)	Metreleptin	1.8 ± 0.2	2.1 ± 0.3	2.2 ± 0.4	1.8 ± 0.3	2.0 ± 0.3	0.78	0.70	0.97
	Placebo	1.9 ± 0.2	2.1 ± 0.2	1.9 ± 0.2	1.9 ± 0.2	2.1 ± 0.3			
fT3 (pg/mL)	Metreleptin	1.90 ± 0.17	2.28 ± 0.19	2.22 ± 0.24	2.05 ± 0.21	1.83 ± 0.22	0.49	0.02	1
	Placebo	2.09 ± 0.13	1.87 ± 0.07	2.04 ± 0.12	1.94 ± 0.12	1.83 ± 0.06			
fT4 (ng/dL)	Metreleptin	1.00 ± 0.08	1.03 ± 0.08	1.07 ± 0.08	1.05 ± 0.08	1.08 ± 0.09	0.43	0.95	0.47
	Placebo	0.91 ± 0.08	0.98 ± 0.09	0.99 ± 0.10	1.00 ± 0.10	0.97 ± 0.11			
Urinary free cortisol:creatinine	Metreleptin	2.2 ± 0.2	2.3 ± 0.3	3.4 ± 0.6	2.3 ± 0.6		0.83	0.95	
	Placebo	2.0 ± 0.2	2.8 ± 0.6	3.5 ± 1.0	3.5 ± 1.0				
IGFBP-3 (μg/mL)	Metreleptin	90.4 ± 4.0	88.0 ± 4.4	88.2 ± 3.6	87.3 ± 3.6	83.9 ± 8.3	0.23	0.81	0.93
	Placebo	83.2 ± 2.7	84.1 ± 2.5	83.5 ± 1.7	83.3 ± 2.5	84.7 ± 3.6			
Prolactin(ng/mL)	Metreleptin	10.0 ± 1.3	10.3 ± 1.6	11.2 ± 1.5	8.5 ± 1.3	7.8 ± 1.5	0.78	0.56	0.38
	Placebo	11.3 ± 1.7	9.6 ± 1.1	10.8 ± 1.7	8.9 ± 1.0	6.3 ± 0.9			
Bone markers									
CTX (ng/mL)	Metreleptin	1.09 ± 0.11	1.16 ± 0.14	1.02 ± 0.15	1.12 ± 0.14	0.96 ± 0.24	0.13	0.39	0.24
	Placebo	0.97 ± 0.14	0.84 ± 0.15	0.79 ± 0.11	0.82 ± 0.10	0.64 ± 0.06			
Osteoprotegerin (pg/mL)	Metreleptin	1023 ± 73	1064 ± 80	1131 ± 77	1142 ± 62	1168 ± 134	0.2371	0.168	0.98
	Placebo	947 ± 103	978 ± 144	936 ± 119	1026 ± 88	1176 ± 208			

All data are presented as mean ± SE. For analysis, the last observation was carried forward to yield $n = 10$ for the metreleptin group and $n = 9$ for the placebo group. On-treatment analysis yielded similar results (data not shown). Log transformed data were used for LH and fT3. Baseline level was adjusted for in the model for inhibin B. Overall *P* values were based on repeated measure ANOVA from baseline and every 4 wk through week 36 for all variables except IGFBP-3, which was analyzed using repeated measure ANOVA from baseline, week 12, week 24, and week 36.

*Effect of metreleptin and placebo treatment.

†Metreleptin and placebo treatment over time interaction.

‡One-way ANOVA was used to compare the difference between metreleptin and placebo treatment groups at week 52 follow-up.

Table S3. Individual data on presence of menstruation, metreleptin dose, and weight: Placebo group

		Baseline	Week 4	Week 8	Week 12	Week 16	Week 20	Week 24	Week 28	Week 32	Week 36	Week 52
L	Menstruation	–	–	–	–	–	–	–	–	–	–	–
	Dose (mg/kg)	0.08	0.08	0.08	0.12	0.12	0.12	0.12	0.12	0.12		
	Weight (kg)	52.15	52.20	52.20	53.00	52.20	52.10	52.20	52.25	52.45	52.45	51.70
M	Menstruation	–	–	–	–	–	–	–	–	–	–	–
	Dose (mg/kg)	0.08	0.08	0.08	0.12	0.12	0.12	0.12	0.12	0.08		
	Weight (kg)	67.05	65.00	63.70	65.85	63.65	64.65	63.80	62.95	61.70	60.65	62.35
N	Menstruation	–	–	–	–	–	–	–		Withdrew from the study.		
	Dose (mg/kg)	0.08	0.08	0.08	0.12	0.12	0.12					
	Weight (kg)	49.00	49.95	49.90	50.65	49.20	50.20	50.75				
O	Menstruation	–	+	–	–	–			Withdrew from the study.			
	Dose (mg/kg)	0.08	0.08	0.08	0.08							
	Weight (kg)	56.25	56.15	56.25	55.50	55.95						
P	Menstruation	–	–	–	–	–	–	–	–	–	–	–
	Dose (mg/kg)	0.08	0.08	0.08	0.12	0.12	0.12	0.12	0.12	0.12		
	Weight (kg)	58.70	60.15	61.40	60.15	60.00	60.50	60.75	60.80	61.75	61.40	63.50
Q	Menstruation	–	–	–	–	–	–	–	–	–	–	–
	Dose (mg/kg)	0.08	0.08	0.08	0.12	0.12	0.12	0.12	0.12	0.12		
	Weight (kg)	45.65	46.90	46.95	46.90	47.60	47.45	47.20	48.05	47.10	46.80	46.90
R	Menstruation	–	–				Withdrew from the study.					
	Dose (mg/kg)	0.08	0.08									
	Weight (kg)	51.10	51.85									
S	Menstruation	–	–	–	–	–	–	–	–	–	–	+
	Dose (mg/kg)	0.08	0.08	0.08	0.12	0.12	0.12	0.12	0.12	0.12		
	Weight (kg)	55.55	56.35	55.90	56.75	56.20	56.90	55.50	55.80	55.00	55.05	56.50
T	Menstruation	–	–	–	–	–	–	–	–	+	+	+
	Dose (mg/kg)	0.08	0.08	0.08	0.12	0.12	0.12	0.08	0.08	0.08		
	Weight (kg)	58.70	59.15	58.15	55.90	55.85	56.40	54.25	55.85	56.00	53.55	54.60