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In vivo effects of metreleptin treatment on immune system of females with acquired hypoleptinemia

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Abstract

Leptin, a peptide hormone secreted by adipocytes in proportion of the amount of energy stored in fat, plays a central role in regulating human energy homeostasis, controlling food intake, reproductive and immune functions. As a cytokine, leptin can affect thymic homeostasis and the secretion of acute-phase reactants such as interleukin-1 and tumour-necrosis factor (1). Decreased levels of leptin, also known as hypoleptinemia, signal to the brain a state of energy deprivation. Hypoleptinemia can be a congenital or acquired condition, and is associated with alterations of several neuroendocrine axes, including the hypothalamic-pituitary-gonadal, -thyroid, -growth hormone, and -adrenal axes (2). A model of acquired chronic hypoleptinemia induced by negative energy balance is the hypothalamic amenorrhea (HA), previously it has been demonstrated that hypoleptinemia underlies the dysfunction of neuroendocrine axes and bone metabolism associated with HA (3). In uncontrolled human studies, low leptin levels are associated with impaired immune responses and reduced T cell counts but effects of leptin replacement on the adaptive immune system have not yet been reported in the context of randomized controlled studies and/or in conditions of chronic, acquired leptin deficiency. To address these questions, we performed the first randomized, double-blinded, placebo-controlled trial of recombinant methionyl-human-leptin (metreleptin) administration in replacement doses over 36 weeks in women with HA. Metreleptin restored both $CD4^+$ T cell counts and their *in vitro* proliferative responses, these changes were accompanied by a transcriptional signature where relevant genes of cell survival and hormonal response were up-regulated and apoptosis genes were down-regulated in circulating immune cells. We also observed that signaling pathways of cell growth/survival/proliferation were directly activated by acute *in vivo* metreleptin administration in subjects with both chronic hypoleptinemia and normoleptinemic lean female subjects. These data show that metreleptin administration, in doses that normalize circulating leptin levels, induce transcriptional changes, activate intracellular signaling pathways and restore $CD4^+$ T cell counts. Thus, metreleptin may prove to be a safe and effective therapy for selective $CD4^+$ T cell immune-reconstitution in

hypoleptinemic states associated with cachexia such as tuberculosis and HIV infection in which $CD4^+$ T cells are reduced.

Introduction

Leptin as an immunoendocrine mediator

Leptin circulates as a nonglycosylated protein of relative molecular mass 16 kDa in mouse and human plasma. Leptin is a peptide coded by the *obese* (ob) gene, which is located on human chromosome 7 and on mouse chromosome 6, and secreted with circadian rhythm mainly by adipocytes. The plasma levels of leptin are highly correlated with adipose tissue mass, indeed its levels are increased in obese humans and in several genetic forms of rodent obesity (4). Mutations of the *ob* gene are associated with many of the abnormalities seen in starved animals, such as hyperphagia, reduced energy expenditure, decreased immune function and other reproductive, neuroendocrine, and metabolic dysfunctions (5). Leptin initially described as an anti-obesity hormone, has subsequently been shown also to influence basal metabolism, hematopoiesis, thermogenesis, reproduction, and angiogenesis, regulating the balance between food intake and energy expenditure, signaling to the brain the changes in stored energy. Leptin mainly produced by the adipose tissue in proportion to the body fat mass and, at lower levels by other tissue, such as placenta, stomach and skeletal muscle; its receptor (Ob-R) was first isolated from mouse choroid plexus by expressing cloning. It was identified as a member of the cytokine family of receptors and binds leptin with nanomolar affinity (6). There are five spliced form of Ob-R: Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd and Ob-Re; four of the five have transmembrane domains, but only Ob-Rb, known as Ob-RL, is the isoform with long cytoplasm region containing several motif required for signal transduction, through Jak-Stat pathway (7). Mutations in Ob-R lead to massive obesity in *db* mice with phenotype identical to that of *ob* mice; most of the mutations affect all of the splice forms. Ob-Rb is normally expressed in hypothalamic neurons and in other cell type including T cells and vascular endothelial cells (8-10). Leptin induces activation of the transcription factor Stat3, and increases the expression of *fos* and other genes in the hypothalamus of mice (11). Leptin gene expression is regulated by several factors, including hormones such as insulin, glucocorticoids, and sex hormones. Insulin stimulates leptin secretion during feeding, and a decrease in insulin levels

anticipates a fall in leptin during starvation. Glucocorticoids also operate synergistically with insulin in the secretion of leptin from cultured adipocytes, although an inverse relationship between leptin and glucocorticoids is generally observed (**7)**. Finally, leptin expression is inhibited by testosterone, increased by ovarian sex steroids, and directly influences the hypothalamic-pituitary-adrenal axis, the reproductive system, hematopoiesis, and angiogenesis. A series of studies has linked the immune and neuroendocrine systems, indeed mediators that are common to neuroendocrine and immune systems can modulate inflammation through the hypothalamus-pituitary-adrenal axis (12, 13). In the immune system, leptin, together with C-reactive protein (CRP), IL-1, and IL-6, can act as an early acute-phase reactant, produced at high levels during inflammation, sepsis, and fever, and it can be induced by other inflammatory mediators such as TNF and IL-1 (14, 15) (Figure 1). However, although these findings have been demonstrated in several systems, other studies have not found increased leptin in inflammatory conditions in humans, including acute experimental endotoxaemia, newborn sepsis, and HIV infection and during anti-inflammatory therapy (16-18). The neuroendocrine role of leptin is most evident in conditions such as fasting, during which the production of leptin by adipose tissue is markedly reduced, or in relation to the effects of sex hormones on its production, indeed testosterone reduces the secretion of leptin, whereas estrogens increase its production. In this context the link between leptin and sex hormones is also indicated by the marked gender dimorphism, manifested by a higher serum concentration in females than in males with similar body-fat mass (7). Since there is a functional connection and anatomical contiguity between adipocytes and lymphoid cells, it is not surprising that leptin has effects on both the neuroendocrine and immune systems (Figure 1). Morphologically, aggregations of lymphoid tissue, including the lymph nodes, thymus, and bone marrow, are associated with adipose tissue (19). Fat deposits do not simply have a structural, metabolic, and heat-insulating function but also provide a microenvironment that helps the immune system to sustain immune responses. In particular, lymphoid and adipose tissues interact locally through common mediators known as adipokines, adipocyte-derived molecules that bridge

metabolism, and immune homeostasis, these molecules include leptin, adiponectin, chemokines, and other proinflammatory cytokines.

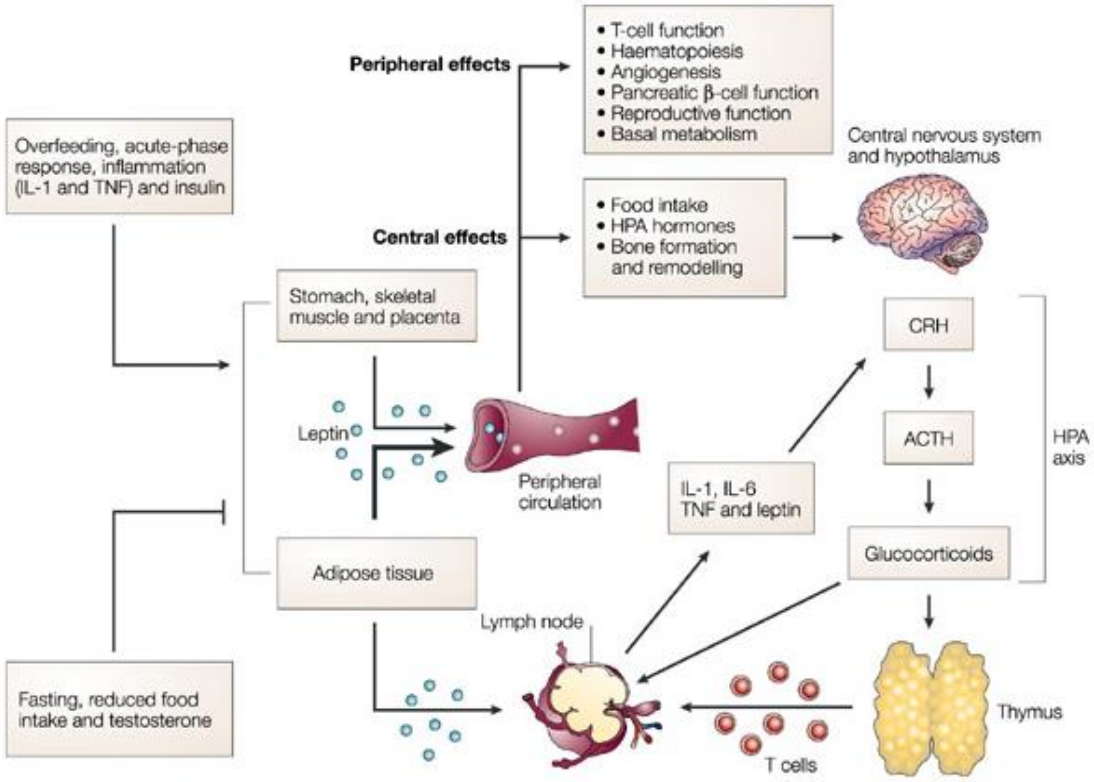

Figure1. The neuroendocrine effects of leptin.

Leptin in innate and adaptive immunity

The functional long form of the leptin receptor (LepRb) is expressed in the hypothalamus where it regulates energy homeostasis and neuroendocrine function; it is also expressed in cells of the innate and adaptive immune system where leptin exerts key regulatory functions (1). On the basis of studies in rodents as well as observational and uncontrolled studies in a limited number of human subjects with congenital leptin deficiency, leptin has also been proposed to act as a signal that conveys information on energy availability to the immune system. The immune system, similar to the neuroendocrine system, requires an adequate supply of energy for optimal functioning (8, 20). Evidence of leptin's importance can be found in animal studies where mice that lack either leptin or LepRb show defects in cell-mediated pro-inflammatory T-helper-1 (Th1)-type immune responses (8, 20). Human with congenital leptin deficiency show an increased risk for infection-related deaths during childhood and have reduced lymphocyte subpopulation numbers. Particularly, the T cell $CD4^+$ fraction and the T-cell-receptor (TCR)-specific proliferative responses are reduced when compared with the general population. Indeed, recent evidence has demonstrated in children with congenital leptin deficiency ,that a direct effect of recombinant methionyl-human leptin (metreleptin) replacement to correct immunophenotypic changes, specifically increasing circulating naive $CD4^+CD45RA^+$ T-cell numbers and reversing impaired T-cell proliferation/cytokine release in response to TCR stimulation (21). Studies in mice have shown that the effect of leptin on the immune system is direct and indirect, via modulation of central or peripheral pathways. Leptin affects both innate and adaptive immunity, indeed in innate immunity it seems to promote activation of and phagocytosis by monocytes/macrophages and their secretion of leukotriene B4 (LTB4), cyclo-oxigenase 2 (COX2), nitric oxide and pro-inflammatory cytokines (22-24) (Figure 2). The products of the inducible form of COX2—PGs and LTs (also known as eicosanoids)—as well as NO are involved in the regulation of inflammation, chemotaxis, and cytokine production and therefore, markedly impact the immune response. Moreover, leptin can induce chemotaxis of neutrophilis and the release of oxygen radicals (25, 26). These mediators can be particularly harmful to cells, as they can

denature proteins and damage membrane lipids (by peroxidation of unsaturated fatty acids), carbohydrates, and nucleic acids. At least in human neutrophils, leptin seems to mediate its effects through an indirect mechanism, probably involving the release of TNF from monocytes (25, 26). Leptin also affects natural killer (NK)-cell development and activation both *in vitro* and *in vivo* (27, 28) (Figure 2). As NK cells express OBRb, and *db/db* mice have a deficit of NK cells resulting from abnormal NK cell development, it is possible that leptin might influence the development/maintenance of a normal, peripheral NK cell pool. Indeed, an important role of OBRb in NK cell physiology is indicated by the ability of OBRb to influence NK cell cytotoxicity through direct activation of STAT3 and the transcription of genes encoding IL-2 and perforin. In addition to above mentioned activity, leptin can stimulate the production of growth hormone by peripheral-blood mononuclear cells (PBMCs) through protein kinase C (PKC) and nitric oxide-dependent pathways. This last effect might be important in immune homeostasis, given the fact that this cytokine-like hormone has marked influences on immune responses by controlling the survival and proliferation of immune cells (24). Instead regard the effects of leptin on adaptive immune responses, have been investigated extensively on human $CD4^+$ T cells (Figure 2). Addition of physiological concentrations of leptin to a MLR induces a dose-dependent increase in $CD4^+$ T cell proliferation (8). However, leptin has different effects on proliferation and cytokine production by human naive ($CD45RA^+$) and memory ($CD45RO^+$) CD4+ T cells (both of which express OBRb). Leptin promotes proliferation and IL-2 secretion by naive T cells, whereas it minimally affects the proliferation of memory cells (on which it promotes a bias toward Th1 cell responses). Another important role of leptin in adaptive immunity is highlighted by the observation that leptin deficiency in *ob/ob* mice is associated with immunosuppression and thymic atrophy—a finding similar to that observed in acute starvation (8). Acute caloric deprivation causes a rapid decrease of serum leptin concentration accompanied by reduced delayed-type hypersensitivity (DTH) responses and thymic atrophy, which are reversible with administration of leptin (8, 29, 30). The thymic atrophy in *ob/ob* mice (or wild-type, starved animals) affects the cortex of the thymus, in

which most $CD4^+CD8^+$ T cells are found, and leptin replacement reduces the rate of apoptosis of such cells (29). Despite the evidence of direct effects of leptin on immune responses in vitro, a major problem remains whether leptin can influence immune responses in vivo. This task is particularly difficult, because of the complexity of the network of interactions that link leptin to several endocrine pathways. It is notable that T cells are sensitive to the supply of cellular nutrients, such as glucose (31), as they do not have glycogen stores and therefore, depend on the import of extracellular glucose to meet their metabolic needs (32). By stimulating glucose uptake through ERK1/ERK2- and PI-3K-dependent pathways, leptin might help to restore the impaired T cell function caused by starvation (29). In this context, it is worth mentioning that other long-chain helical cytokines similar to leptin (such as IL-3, IL-7, and IL-15) are important in promoting the uptake and metabolism of glucose (32).

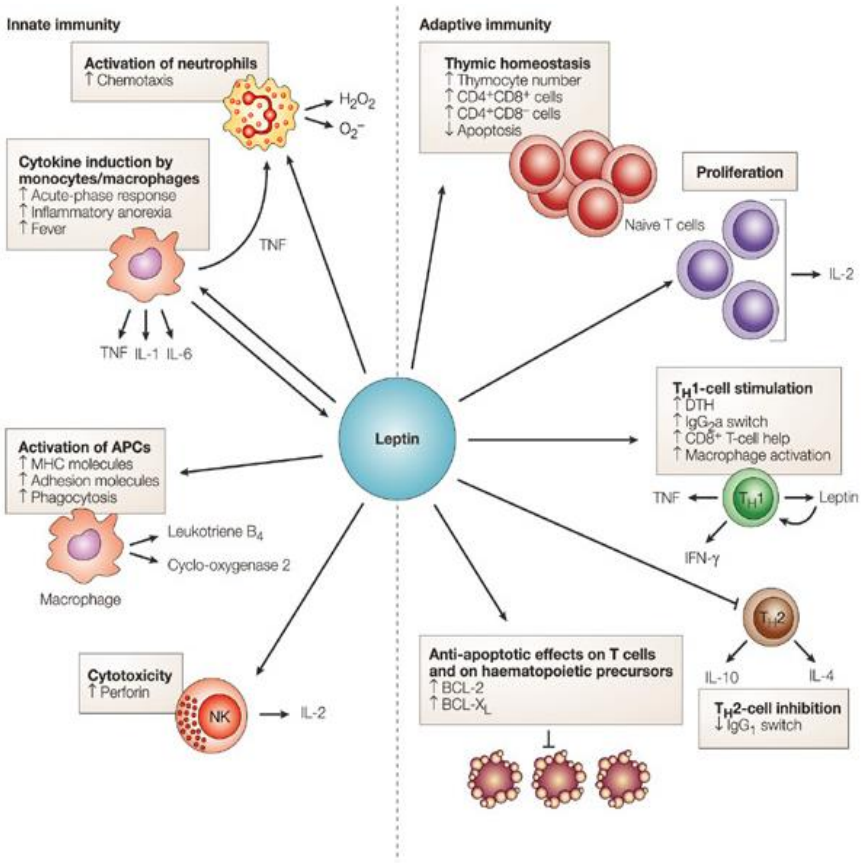

Figure 2. Schematic representation of the effects of leptin on both innate and adaptive immunity.

Leptin in hypothalamic amenorrhea

In 1994, the discovery of leptin has changed the perspective of adipose tissue from that of an inert energy store to a true endocrine organ that secretes metabolically active hormones (33). Several studies in mice (34) and human (35) have shown that leptin has a role in the neuroendocrine adaption to starvation, which includes changes in hormone concentrations that probably have a protective effect. These findings are clinically relevant for common disease states associated with low leptin concentrations and neuroendocrine abnormalities (energy-deficient states such as exercise-induced amenorrhoea, non-athletic forms of hypothalamic amenorrhea and anorexia nervosa). Hypothalamic amenorrhea (HA), either organic or functional, (36) is characterized by the absence of menstrual cycle, low or normal levels of gonadotropins. Functional hypothalamic amenorrhea occurs when a relative energy deficit (owing to weight loss, excessive exercise, or eating disorders) disrupts the secretion of hypothalamic gonadotropin-releasing hormone (GnRH) and other neuroendocrine axes (37, 38). Leptin, a hormone that regulates the energy homeostasis, is a prime candidate. There are leptin receptors in the hypothalamus and these found in both the area associated with eating behaviour and energy balance and the area concerned with reproduction regulation (arcuate, ventro-medialis and paraventricular nucleus) (39). The long isoform, expressed abundantly in hypothalamus, activates mainly the Janus kinase signal transducer and activator of transcription system to change the expression of hypothalamic neuropeptides and thus regulate energy homeostasis (40). In the hypothalamus the target of leptin is neuropeptide-Y (NPY) and pro-opiomelanocortin (POMC), both involved in regulation of body weight and reproduction (41). An inadequate leptin production as occurs in women with nutritional amenorrhea, could induce hypercortisolism through an increase in corticotrophin-releasing hormone (CRH) secretion and adrenergic activation; leptin acts directly or indirectly on hypothalamic receptors, by affecting NPY or POMCS (41). Evidence from *in vitro* studies and models of leptin deficiency in animals and man, suggest that leptin has an important role in reproduction and regulation of the hypothalamic-pituitary-gonadal axis,

indeed *in vitro*, leptin stimulates gonadotropin-releasing hormone pulsatility and release (42). Leptin-deficient *ob/ob* mice are morbidly obese but also sterile, and leptin treatment is able to correct the sterility of these mice (43, 44). The hypothalamic menorrhea condition arises when impaired secretion of gonadotropin-releasing hormone leads to low or normal gonadotropin concentrations, low oestrogen concentrations and subsequently the absence of menstrual cycle (37). This condition can be deemed to represent a regression to a prepubertal or peripubertal pattern of gonadotropin secretion. Several evidence has shown that amenorrheic female athletes have lower serum leptin concentrations than weigh-matched controls, associated with neuroendocrine abnormalities, supporting the idea that leptin plays an important role in reproductive and neuroendocrine function associated with this disorder. Studies conducted by Mantzoros have shown that the administration of recombinant methionyl human leptin (r-metHuLeptin) at replacement doses to woman with hypothalamic amenorrhea, improved ovarian parameters, increased levels of LH, estradiol, IGF-1 and thyroid hormone (45), suggesting that leptin is a peripheral signal of adequate energy stores necessary for normal reproductive and neuroendocrine functions and leptin replacement could became a potential new therapeutic option (33).

Aim of study

During the last decade, there has been a growing understanding of how host nutritional status and metabolism can affect the immune response. In this context, studies *in vitro* and in animal models suggest that leptin, the prototype adipose tissue derived cytokine, is able to participate in a wide range of biological functions that include—in addition to its function as an adipostat—glucose metabolism and $CD4^+$ T lymphocyte proliferation, cytokine secretion and apoptosis, underlying the link between immune function/homeostasis, metabolism and nutritional state. The aim of the present study was to characterize the role of leptin on $CD4^+$ T cell homeostasis in a state of acquired leptin deficiency, such as HA, a condition in which adipose tissue is particularly reduced and consequently serum leptin concentrations are also chronically lower. This is the first randomized, placebo-controlled trial on the effects on immune function of rrecombinant methionyl-human-leptin (metreleptin) administration in humans over a period of 36 weeks. We report for the first time that metreleptin in replacement doses restored the number and function of $CD4^+$ T cells and their proliferative potential, which was impaired in hypoleptinemic women. We also assessed the modulation of gene expression in T cells from these subjects at various time points during metreleptin treatment and we observed that metreleptin induced the up-regulation of genes involved in cell survival and in hormonal response while induced down-regulation of cell death/apoptosis genes. Our data have relevance in hypoleptinemic states associated with negative energy balance, such as recurrent infections associated with reduced T cell counts, immunodeficiencies such as HIV and Tuberculosis all of which are very frequently associated with low body weight and decreased adipocyte mass. Our findings might pave the way for clinical researchers to translate our findings to new therapeutic options that could selectively affect the crosstalk between immune system and metabolic status through leptin administration.

Materials and Methods

Placebo-controlled trial of metreleptin replacement in women with HA

Eligible subjects were women between 18 and 35 years old with secondary HA, defined by an absence of menstrual cycles for 6 months or more, in the setting of strenuous exercise or negative relative energy balance. All had normal weight (within 15% of ideal body weight for six months or more) that had not fluctuated more than +/- 5 lb over the last 6 months. All subjects had baseline serum leptin levels of less than 5 ng/ml, but were otherwise healthy, with an absence of significant coexisting medical conditions and psychiatric diseases, including depression or past/active eating disorders. This was determined using a screening questionnaire, physical examination, and routine blood tests. Other causes of absent menstrual cycles were excluded, including hypothyroidism or hyperthyroidism, polycystic ovarian syndrome, hyperprolactinemia, Cushing's syndrome, congenital adrenal hyperplasia, or primary ovarian failure. None of the subjects had been taking medications known to affect neuroendocrine, immunological or bone density measurements, including glucocorticoids, anti-seizure medications, thyroid hormones, and estrogens. Also excluded were women who were breastfeeding, pregnant, or planning pregnancy within one year, and those with a known history of hypersensitivity to E. coli derived proteins. Because the effects of metreleptin on the developing fetus are not known, all subjects were required to use double barrier methods of contraception (diaphragm with intravaginal spermicide, cervical cap, male or female condom with spermicide). All participants were tested for pregnancy at screening and at all subsequent visits.

Study design: placebo-controlled, randomized trial of metreleptin replacement in women with HA

This protocol was approved by the Institutional Review Board of Beth Israel Deaconess Medical Center, and clinical quality human recombinant leptin (metreleptin, A-100, formerly known as met-Hu-leptin; phase II clinical trial) was provided by Amylin Pharmaceuticals. Metreleptin was administered in the context of an Investigator Initiated Study protocol and under an Investigational

New Drug application by CSM to the Food and Drug Administration. Twenty women were enrolled and provided written informed consent to participate in a randomized, double-blind, placebo-controlled trial. Fourteen of these subjects were eventually studied for immune phenotype and function outcomes. Data from all twenty subjects was utilized in the microarray analysis. Subjects were randomized in a 1:1 fashion to receive either metreleptin or placebo, for a period of 36 weeks in the form of a self-administered daily subcutaneous injection (given between 7pm and 11pm). Eight subjects were randomized to receive metreleptin and six placebo. The initial dose of metreleptin was 0.08mg/kg, aiming at achieving a concentration of endogenous leptin within the normal range. If by week 12 participants had no clinical response i.e. had not begun menstruating, their doses were increased to 0.12 mg/kg and continued on this dose until week 36. Weight was monitored at monthly visits, and metreleptin doses were adjusted accordingly. Metreleptin doses were reduced by 0.04 mg/kg if the participant lost >5% of baseline weight. Participants with weight loss of > 8% of their baseline weight or found to be < 80% of ideal body weight were withdrawn from the study. All subjects also received the current standard of care for HA, including calcium (600 mg twice a day) and vitamin D (400 international units daily) supplementation. Fasting blood samples were obtained at visits to our General Clinical Research Center (GCRC) at baseline and every four weeks. In all phases of the study, heparinized blood samples were collected at the baseline visit and every 12 weeks thereafter and were shipped to Naples, Italy, by express courier to be processed within 48h for immune assays. Processing of PBMCs within this time frame assures their viability, according to standard immunology procedure and verified by our extensive studies in healthy controls and leptin-deficient subjects using a similar protocol (46). PBMCs were also collected at these time points at C.S.M's BIDMC laboratory using the same standard immunology procedure as that used in Italy. RNA for microarray analysis was isolated from PBMCs at the BIDMC Genomics and Proteomics Center using the manufacturer's protocol for Trizol Reagent (Invitrogen, Grand Island, NY).

Study design: healthy controls

In this study we additionally included 13 healthy volunteer donors. The healthy controls were matched for age, BMI and leptin levels with HA patients at baseline (Table 1). None of these had a history of endocrine disease, autoimmune disorders or infection. The study was approved by the institutional ethics committee and all individuals gave written informed consent. The experimental procedures for the immunephenotype analysis of peripheral blood and lymphocyte cultures and stimulation after isolation of human PBMCs were the same of those described for the hypothalamic amenorrhea patients.

Acute Metreleptin Signaling study of PBMCs in vivo and $CD4^+$ cells in vitro

Normal, healthy lean volunteers were recruited from the community and screened at the Clinical Research Center (CRC) at BIDMC. Subjects were excluded if they had a history of any illness, other than obesity, that may affect insulin sensitivity, use of medications that are known to influence glucose metabolism, history of anaphylaxis or anaphylactoid-like reactions or a known hypersensitivity to E. coli-derived proteins or anesthetic agents such as Lidocaine or Novocaine. All subjects provided written informed consent to participate and the study was approved by the institutional review board at BIDMC. Subjects were provided with take-home meals and consumed an isocaloric diet, specifically designed for each subject, for 48 hours prior to their main study visit to ensure stable dietary intake. On the morning of the main study visit, subjects attended the CRC after a 12 hour fast. For an acute *in vivo* metreleptin signaling study**,** we performed experiments with PBMCs before and after a bolus of metreleptin (0.01 mg/kg body weight for 20 min) or placebo (10 cc of normal saline for 20 min) injection. Either metreleptin or placebo was administered to 3 lean females (BMI 22.21±0.32 kg/m^2), by slow intravenous injection over one minute. In another group of lean subjects, human $CD4^+$ T cells were purified from PBMCs by magnetic cell separation with Dynabeads $CD4^+$ T cell Kit (Invitrogen). Soon after isolation, CD4 cells

were 95-98% pure as verified by FACS analysis. Isolated CD4 cells were used for acute in vitro molecular signaling studies.

Immunephenotype

Immunophenotypic analysis of peripheral blood was performed with an EPICS XL flow cytometer (Beckman Coulter). Triple combinations of different anti-human mAbs, e.g., FITC- and phycoerythrin (PE)-anti-CD3, PE- and PC-5-anti-CD4, PC5-anti-CD8, PE-anti-CD16, PC5-anti-CD19, PE-anti-CD25, FITC-anti-CD45, and PE-anti-CD56 (Coulter Immunotech), were used for immunofluorescence staining.

Lymphocyte cultures and stimulation

Human PBMCs were isolated by stratifying 15 mL of whole blood on 5 mL of Ficoll-Paque PREMIUM (GE Healthcare) and centrifuging the solution at 1.2 x *g* for 20 minutes. Next, the lymphocyte layer was removed and washed twice by resuspending the pellet with 40 ml of serum free RMPI medium and a centrifugation of 1700 rpm for 5 minutes, at RT. Viability of cells was verified at 85-95% by using tryphan blue staining and annexin-5 binding during flow cytometry, and serum glucose levels were measured as a marker of metabolic activity, and T cells were cultured in medium supplemented with 5% (vol/vol) autologous subject serum. T cell cultures were performed in triplicate in 96-well round-bottom plates. PBMCs (2×10^5 per well) were stimulated in parallel with OKT3, 0.1 μg/ml (Ortho); phytohemagglutinin, 2 μg/ml (Sigma); phorbol 12-myristate 13-acetate, 0.01 μM and ionomicin, 0.5 μM (Sigma); and purified protein derivative (PPD) 10 μg/ml (Northern Serum Institut) stimuli. Stimulated PBMCs were maintained for 72h in culture, and H_3 thymidine was added (0.5μCi/well) over the last 12h. Cells were harvested on glass-fiber filters using a 96-well cell harvester (Tomtec Inc.), and counted in a 1205 Betaplate liquid scintillation counter (Wallac). Results are expressed as mean (SD) counts per minute from triplicate cultures.

Cytokines and hormonal measurements

Human obesity kit (Bender Medsystems GmbH) was used for quantitative detection of soluble MPO, ICAM-1, CD40L, TNF-R, MCP1 and OPG by flow cytometry (FACSCanto-BD) according to manufacturer's instructions. Data were analyzed using FlowCytomix Pro 2.2 Software (Bender Medsystems GmbH). For leptin, IL-2, IL-7, IL-15, and leptin receptor measurements commercially available ELISA kits from R&D Systems, were utilized as previously described. The following hormone levels were measured using immunoassays: cortisol, IGF1, insulin and ACTH (Immulite; Siemens Healthcare Diagnostics). Serum leptin and free leptin were measured by RIA (Millipore) with a sensitivity of 0.5 ng/mL, intraassay coefficient of variability (CV) of 3.4–8.9%, and interassay CV of 3.0–6.2%.

Levels of serum anti-metreleptin antibodies and their functional activity

Levels of serum anti-metreleptin antibodies were determined with an in-house ELISA. In brief, 50 μL metreleptin at a final concentration of 10 μg/mL in PBS, pH 7.4, was plate-bound to a 96-well ELISA plate (PBI International SpA). After 16 h at 4°C, the plates were extensively washed with PBS-0.05% Tween 20, blocked with 200 μL PBS/10% FCS for 2 h, and repeatedly washed. Diluted sera in PBS-0.05% Tween 20/10% FCS (from 1/10 to 1/1,000) were added at 100 μL/well and incubated for 4 h at room temperature. After five washes, goat anti-human polyvalent immunoglobulins, such as alkaline phosphatase–conjugated antibodies (Sigma-Aldrich), were added at a concentration of 100 μL/well for 1 h. The reaction was developed with Sigma-Fast p-nitrophenyl phosphate and alkaline phosphatise substrate (PNPP) (Sigma-Aldrich) and read after 30 min at 405 nm in an ELISA plate-reader (Bio-Rad Laboratories). Standard curves of antileptin antibodies were developed in each assay using an antileptin monoclonal antibody (mAb) generated in our laboratory (mAb 971212). Quantification of optical density values was performed after extrapolation from standard curves of known concentration of antileptin antibodies. The functional activity of antileptin

antibodies was assessed with the human leptin-receptor (hLepR)-transfected BAF3 cell line, provided by Dr. Arieh Gertler (The Hebrew University). In brief, because hLepR+BAF3 cell proliferation is leptin dependent, hLepR+BAF3 cells were cultured in flat-bottom 96-well microtiter plates (Becton-Dickinson Falcon) at a density of $5x10^3$ cells/well in a total volume of 100 μL RPMI-1640 medium supplemented with 2% FCS (Hyclone-Pierce; Thermo Fisher Scientific), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies). Cells were cultured at 37°C in 100% humidity and 5% CO2 in the presence of increasing doses of metreleptin ranging from 0.01 to 10 ng/mL. Purified IgGs from sera of metreleptin-treated subjects, placebo–treated subjects, and healthy controls were added to cells in all the different conditions at a final concentration of 50 μg/mL. After 48 h, [^{3}H] thymidine (0.5 μCi/well) (Perkin-Elmer) was added to the cultures, and cells were harvested after 12h. Radioactivity was measured with a β-cell plate scintillation counter (Wallac). As standard of leptin neutralization, anti-human leptin mAb 971212 was used at increasing concentrations usually from 0.1 to 25 μg/mL. We also assessed at the biochemical level the capacity of antileptin IgGs, isolated from leptin or placebo-treated subjects and healthy controls, to affect LepR signaling in BAF3 cells. In brief, hLepR+BAF3 cells were incubated for 1 h at 37°C with recombinant leptin at 2 ng/mL in the presence or absence of IgGs (50 μg/mL) purified from leptin- or placebo-treated subjects and healthy controls. After a 1-h incubation, cell lysates were generated to perform Western blotting analyses for STAT3 phosphorylation as a readout of LepR signaling.

Protein extraction and western blotting

For total cell extracts, collected samples were suspended in a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.05% aprotinin, and 0.1% Igepal and then incubated for 30 min at 4°C. The suspension was centrifuged for 25 min at 12,000 rpm and the supernatant was

saved as the total extract. For Western Blotting, proteins were loaded in each lane. After SDS-polyacrylamide gel electrophoresis, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked for 1hr in TBS containing 5% nonfat dry milk and 0.1% Tween 20. Incubation with primary antibodies was performed in TBS containing 5% nonfat dry milk for overnight and then incubated with horseradish peroxidase secondary antibodies for two hour. After incubation with antibodies, membranes were washed with TBS containing 0.1% Tween-20. Enhanced chemiluminescence was used for detection. The antibodies used were the following: anti-STAT3, anti-phospho-STAT3 (Y705), anti-mTOR, anti-phospo-mTOR, anti-SHP2, anti-phospho-SHP2 (Cell Signaling Technology), anti-ERK, anti-phospho-ERK, anti AMPK, anti-phospho-AMPK, anti-AKT, anti-phospho-AKT (Santa Cruz). Measurement of signal intensity on nitrocellulose membranes after Western Blotting with various antibodies was performed using Image J processing and analysis software (http://rsbweb.nih.gov/ij/).

Microarray analyses

Total RNA was isolated from PBMCs using the manufacturer's protocol for Trizol Reagent (Invitrogen) and further purified using RNAeasy spin columns (Qiagen). RNA concentration and purity were determined from 260nm/280 nm absorbances. RNA integrity was determined using the Agilent 2100 BioChip (Agilent Technologies). The RNA was then subjected to a single amplification run, labeled with biotin nucleotides, digested into proper size fragments, and hybridized to the HT-HG-U133A and HT-HG-U133B gene microarrays (Affymetrix) following a standard protocol established by the manufacturer. Hybridized chips were reacted with FITC-avidin and raw fluorescence intensities were read with a laser scanner. Affymetrix GeneChip raw data (CEL files) were imported into R v. 2.13 and analyzed with the bioinformatics facilities present in the BioConductor packages (47). Data was quality checked with the BioConductor packages affyPLM and affy (48). Subsequently, the data was re-annotated according to the updated information present in the

NCBI Entrez Gene database (brainarray website http://brainarray.mbni.med.umich.edu/Brainarray/Database/CustomCDF/14.1.0/entrezg.asp) and preprocessed by the RMA algorithm (49). The quality checked, re-annotated and preprocessed gene expression data were screened for differentially expressed genes by a linear model including the interaction of the treatment-by-time and the subject (pairing) terms (Y ~ treatment * time + subject + error) followed by moderated t-test for the pairwise comparisons of interest. Genes with nominal p-value < 0.01 were considered to be significantly differentially expressed. The lists of differentially expressed genes were then searched for over-represented biological themes by the DAVID annotation software with the default parameters (50). The microarray data used in this study is available at NCBI Gene Expression Omnibus (GEO) database under the accession number: GSE36990 (see site:

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rtmtdyekqooksxa&acc=GSE36990).

Real-Time PCR Validation

Differentially expressed genes in microarray analyses were verified by real-time PCR using ABI7500FAST (Applied Biosystems) with a 20-μL reaction volume consisting of cDNA transcripts, primer pairs, and SYBR Green PCR Master Mix (Applied Biosystems). Quantifications were normalized to 18S in each reaction. The sequences for the primers are listed in Table 5.

Statistics

Differences in baseline characteristics between HA and control subjects were calculated using unpaired t tests. Where data was not normally distributed, we used Nonparametric Mann-Whitney U tests. The statistical software used was GraphPad InStat3 version 3.0. For the acute *in vivo* metreleptin signaling study, data were analyzed student t-test and/or one-way ANOVA followed by post-hoc test for multiple comparisons. Analyses were carried out using SPSS (version 11.5, SPSS).

Results

Baseline characteristics of the leptin and placebo treated subjects and healthy controls are presented in Table 1. There was no significant difference between the HA groups and normal females as controls with regards to age, weight, BMI, and leptin levels.

Characteristics	**Baseline in Subjects Receiving Placebo (n=6)**		**Baseline in Subjects Receiving metreLeptin (n=8)**		**Healthy controls (n=13)**	
	mean±SD	*range*	*mean±SD*	*range*	*mean±SD*	*range*
Age (yr)	25 ± 4.1	18-31	26 ± 4.7	19-34	26 ± 1.4	24-28
Body-mass index (kg/m^2)	20.1 ± 2.1	18.5-23.8	21.1 ± 1.8	18.5-23.8	19.5 ± 1.8	17-22
Leptin (ng/ml)	2.70 ± 1.8	1.05-6.7	3.64 ± 1.6	1.69-7.1	4.73 ± 2.27	2.3-7.8

Table 1. Baseline characteristics of all enrolled subjects. No significant differences were found among the three groups with regards to age, body-mass index and leptin levels.

Efficacy of metreleptin to increase $CD4^+$ T cell number in HA subjects

We first studied whether leptin treatment could affect the immune phenotype of HA subjects. We initially compared the total number of lymphocytes and several sub-populations between the HA subjects (n=14) and normal matched control subjects (n=13) at baseline. The HA subjects had a significantly lower number of total lymphocytes, B, and NK cells. The difference in the populations of CD4 and CD8 T cells followed the same trend but the difference was not statistically significant (Figure 1). After 36 weeks of metreleptin administration in replacement doses the lymphocyte sub-populations of $CD3^+$ and $CD4^+$ cells increased in terms of Δ fold change (calculated as the difference between week of the treatment and baseline) ($p < 0.05$) and in terms of absolute cell number compared with controls (Figures 2 and 1, respectively). B and NK cell populations did not change significantly. Within the $CD3^+$ T cell population, we observed an increase in both the naïve and memory $CD4^+$ cells,

expressing the CD45RA$^+$ and CD45RO$^+$ markers, respectively (Figures 1 and 2).

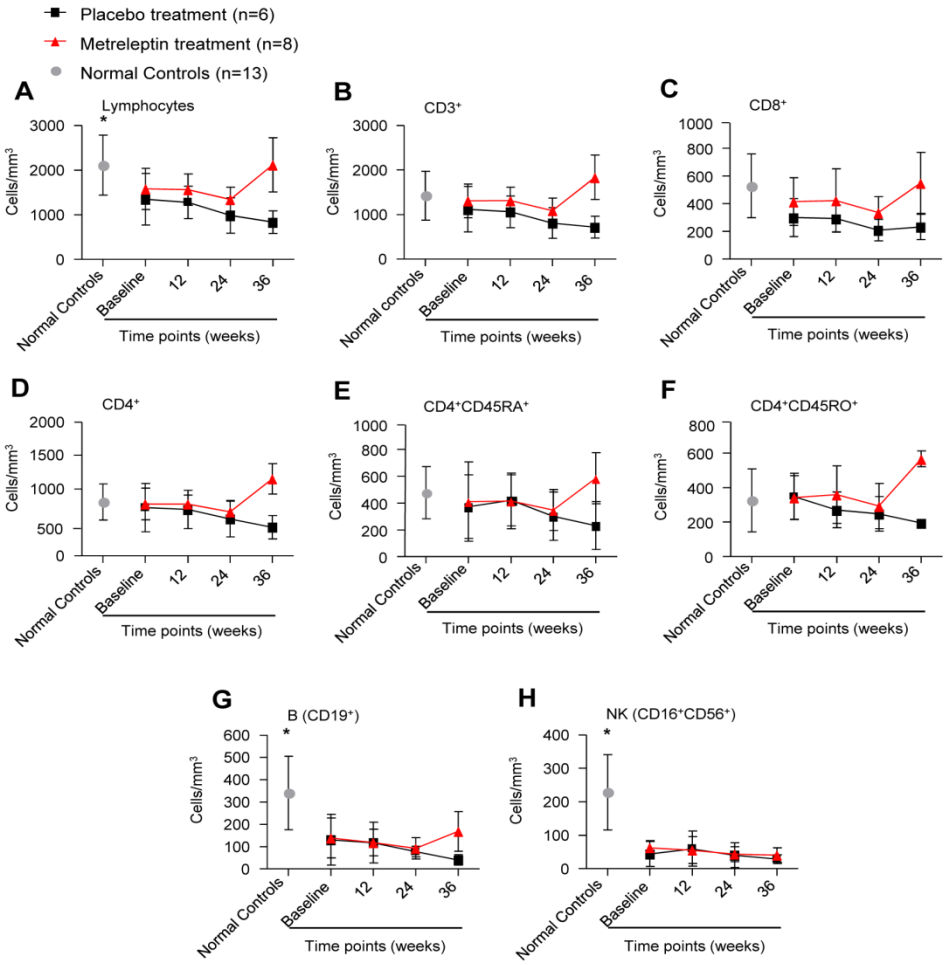

Figure 1 Effects of metreleptin on immune phenotype in terms of absolute number. Absolute number of lymphocytes, T, B and natural killer cells in HA subjects over time compared with healthy controls. (A, G and H). At baseline, the total populations of lymphocytes, B cells and NK cells were significantly lower in HA subjects than in controls (*P < 0.05). (B-F) CD3$^+$, CD4$^+$, CD8$^+$ T, CD4$^+$CD45RA$^+$ and CD4$^+$CD45RO$^+$ cell population were not different in HA subjects and normal controls at baseline.

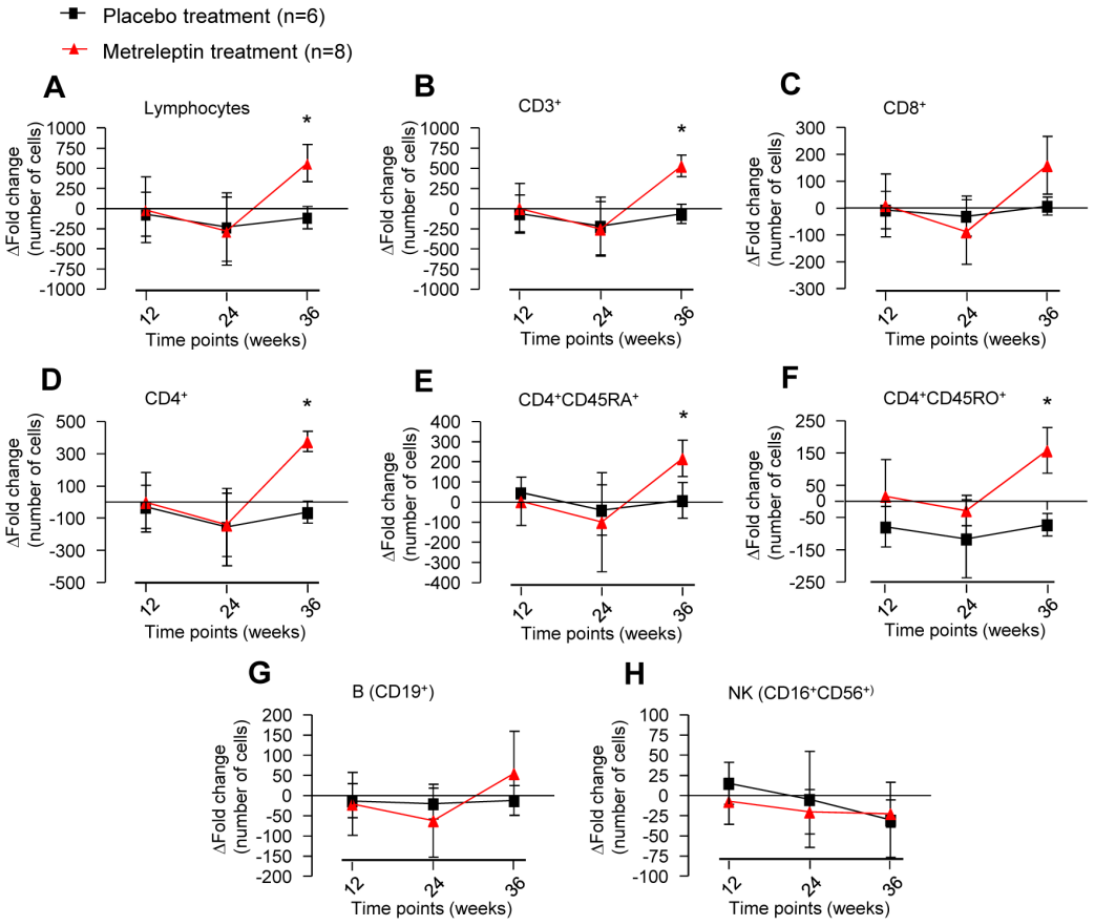

Figure 2. Effects of metreleptin on immune phenotype in terms of Δ fold change. 36 weeks of metreleptin administration in replacement doses induced a statistically significantly increase in terms of Δ fold change (week of treatment minus baseline) in total lymphocytes, $CD3^+$, $CD4^+$, $CD4^+CD45RA^+$ naïve and $CD4^+CD45RO^+$ memory cells (* $p < 0.05$) (A, B and D-F). No significant effect was observed on $CD8^+$, B and NK cells (C, G, H).

Partial efficacy of metreleptin in restoring T cell proliferation in HA subjects

We next evaluated the effect of metreleptin treatment on the subjects' peripheral blood mononuclear cell (PBMC) proliferative profile. We stimulated PBMCs isolated from subjects' blood, using either physiologic T-cell specific stimuli (OKT3 mAb or recall antigen PPD) or using polyclonal unspecific stimuli (phytohemagglutinin or phorbol 12-myristate 13-acetate plus ionomycin). We first evaluated the impact of HA on in vitro T-cell proliferative responses and found a significant reduction in proliferation in HA subjects as compared with normal subjects (Fig. 3), particularly in TCR-specific stimulations such as OKT3 (polyclonal) and PPD (antigen specific) ($P < 0.05$). Furthermore, using in vitro T-cell assays, we evaluated the effect of metreleptin treatment in the HA subjects over time and observed a significant increase in proliferation at week 36 by OKT3 and PPD stimulation ($P < 0.05$) in metreleptin-treated patients as compared with placebo-treated patients (Fig. 4) .

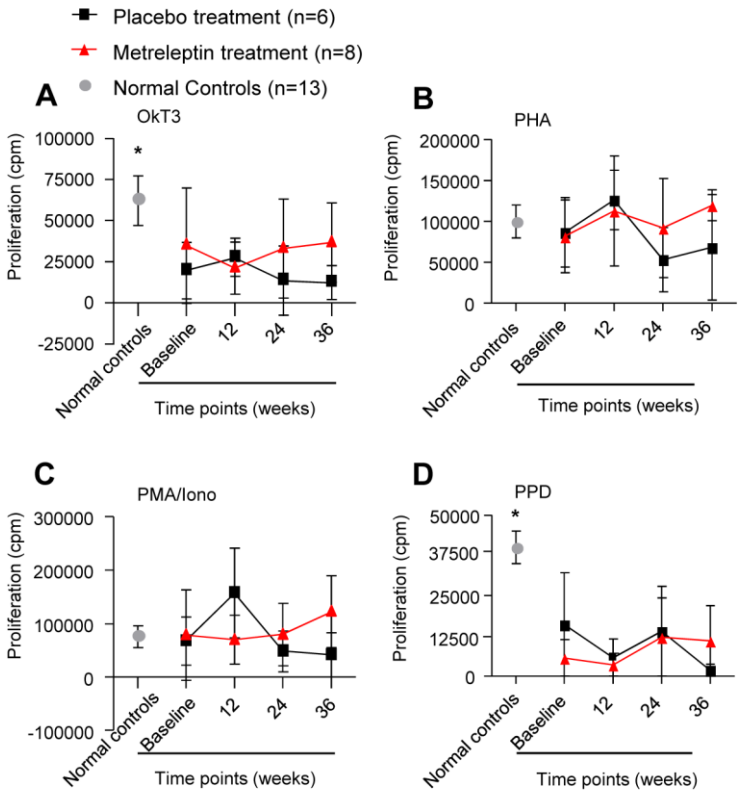

Figure 3. Effects of metreleptin on PBMCs proliferation in terms of cpm. Proliferative capacity of PBMCs increased when stimulated with OKT3, phytohemagglutinin (PHA), phorbol 12-myristate 13-acetate with ionomicin (PMA/Iono), and purified protein derivative (PPD). At baseline the proliferative response to OKT3 and PPD (* $p < 0.05$) stimulation in HA subjects significantly less than in healthy controls (A, D).

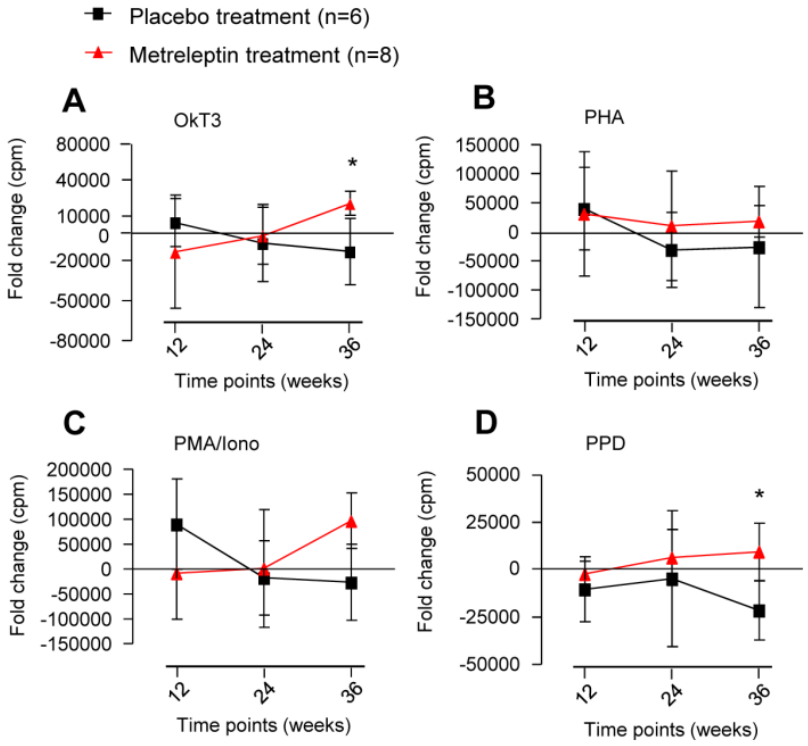

Figure 4. Effects of metreleptin on PBMCs proliferation in terms of fold change. After 36 weeks of metreleptin administration in replacement doses, the proliferative response to T-cell-specific OkT3 (A) and antigen-specific, PPD (D) (* $p < 0.05$) stimulation was increased in metreleptin-treated patients vs. placebo-treated patients in terms of d fold change in proliferation over time (calculated as the cpm at week of treatment minus the number cpm at baseline T0). This effect was not observed during unspecific stimulations such as PHA (B) and PMA/Iono (C).

Immune changes induced by metreleptin are not associated with significant changes in serum hormonal patterns, metabolic/inflammatory parameters and survival cytokines or metabolic/inflammatory parameters

We next assessed the impact of metreleptin treatment on hormonal and cytokine levels which could be responsible for altered immune cell survival. We observed no difference in leptin binding protein (LBP), cortisol, adrenocorticotropic hormone (ACTH), and insulin (Figure 5). We also investigated the impact of metreleptin treatment on circulating survival cytokines such as interleukin (IL)-2, IL-7, IL-15. Again no significant difference was detected in the three groups (Figure 5). Finally, no difference was observed in the levels of serum metabolic/inflammatory parameters such

as CD40L, soluble Tumor Necrosis Factor Receptor (sTNFR1 and sTNFR2), osteoprotegerin (OPG), monocyte chemoattractant protein-1 (MCP-1), myeloperoxidase (MPO), soluble intercellular adhesion molecule-1 (sICAM-1), resistin, or C-reactive protein (Figure 6).

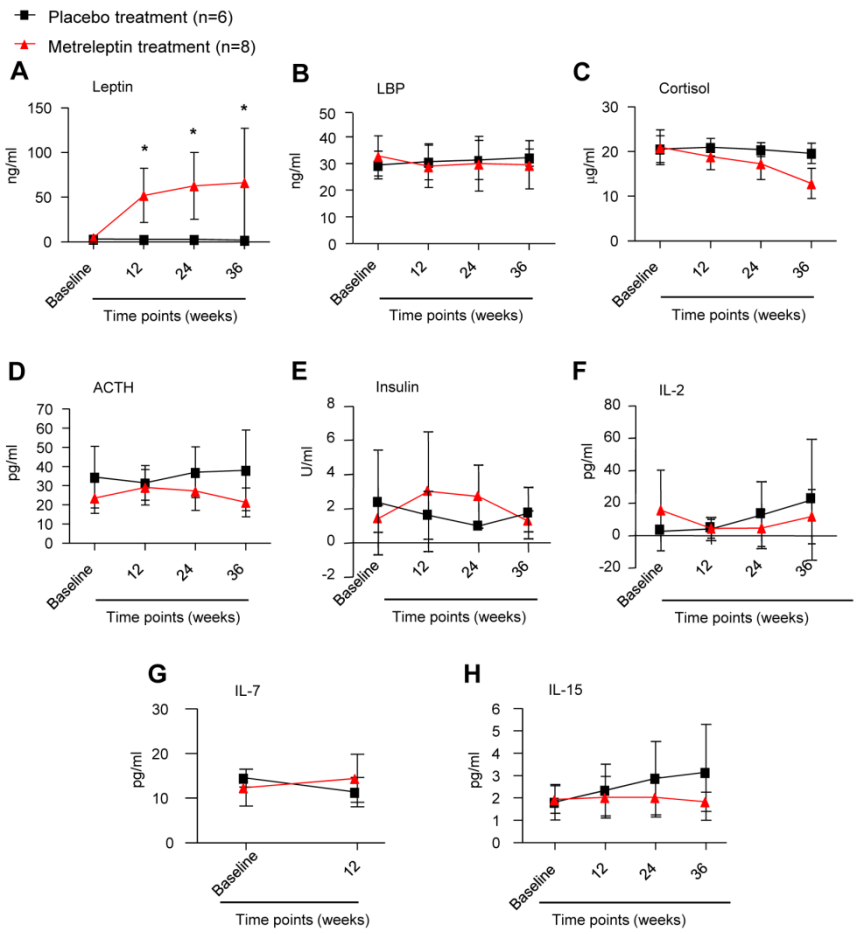

Figure 5. Levels of hormones and cytokines. Only leptin levels were significantly higher in time curse (A). There were no significant differences in these parameters analysed (B-H).

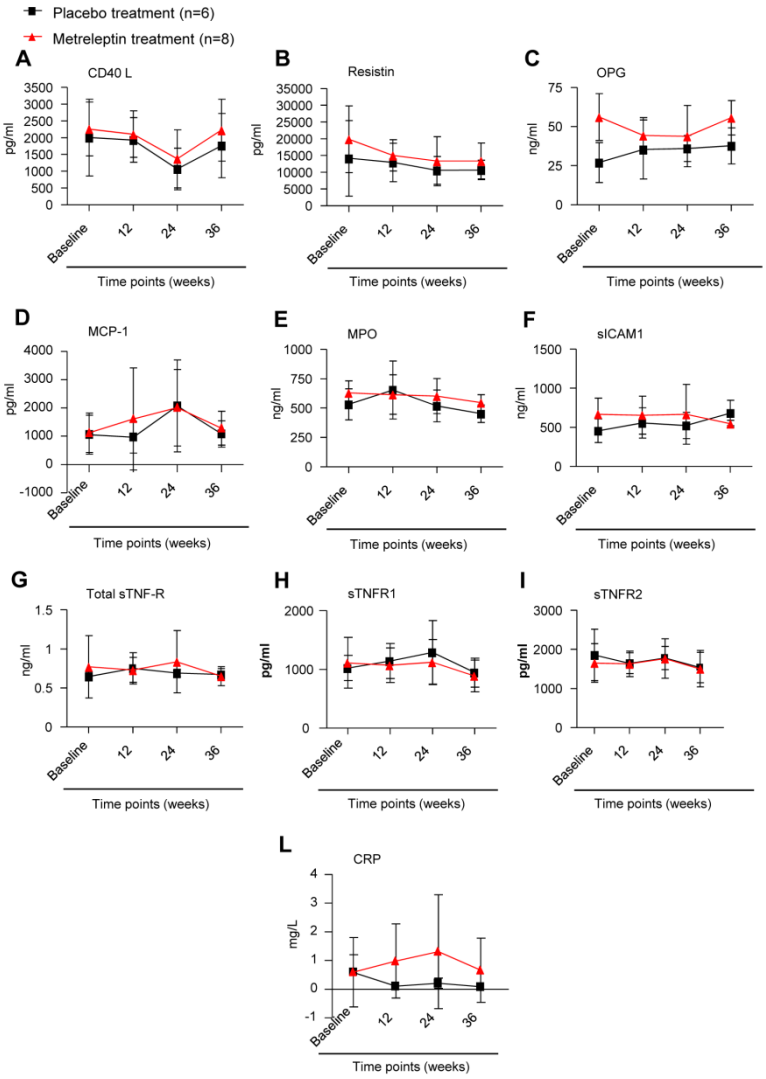

Figure 6. Levels of inflammatory and metabolic parameters. There were no significant differences in metabolic/inflammatory parameters analysed (A-L).

Metreleptin induces production of non-neutralizing anti-leptin antibodies with agonistic activity

Since subcutaneous injections of metreleptin have previously been described to induce antibodies we measured anti-metreleptin antibodies over time and functionally tested them to assess possible interference with leptin signaling. Firstly, we asked whether the antibodies produced after treatment with metreleptin in the clinical study were able to affect (either stimulate or neutralize) leptin-related proliferation and intracellular signalling. Thus, we used the BAF3 cell line after transfection with the long form of the human leptin receptor ($hLepR^{+}BAF3$). First, we developed standard curves of anti-leptin antibodies using an anti-leptin mAb generated in the laboratory (see Methods). Antibodies isolated from metreleptin-treated HA subjects were able to stimulate proliferation more than leptin alone but only at lower leptin concentrations (Figure 7). On the contrary, no additional stimulatory effect was observed in the same subjects before treatment, in the placebo-treated subjects, or when using IgGs from normal subjects (Figure 7). These functional effects were also analyzed at the signalling level by measuring STAT3 phosphorylation. IgGs from metreleptin-treated HA subjects significantly increased p-STAT3 levels (Figure 7), whereas no effect was observed after addition of either placebo-treated IgGs or IgGs isolated from normal controls (Figure 7). These results suggest that metreleptin treatment results in the production of anti-leptin antibodies that are not neutralizing but may demonstrate minor agonistic activities on hLepR measured both as increased proliferation of a leptin-dependent cell line and as increased hLepR-mediated STAT3 signalling. The stimulatory effect of these antibodies is small, however, and easily overridden by relatively higher leptin doses. Finally, we also evaluated the free leptin levels to assess possible binding of the antibodies and found that the free leptin levels increased over time even during production of antibodies (Figure 7).

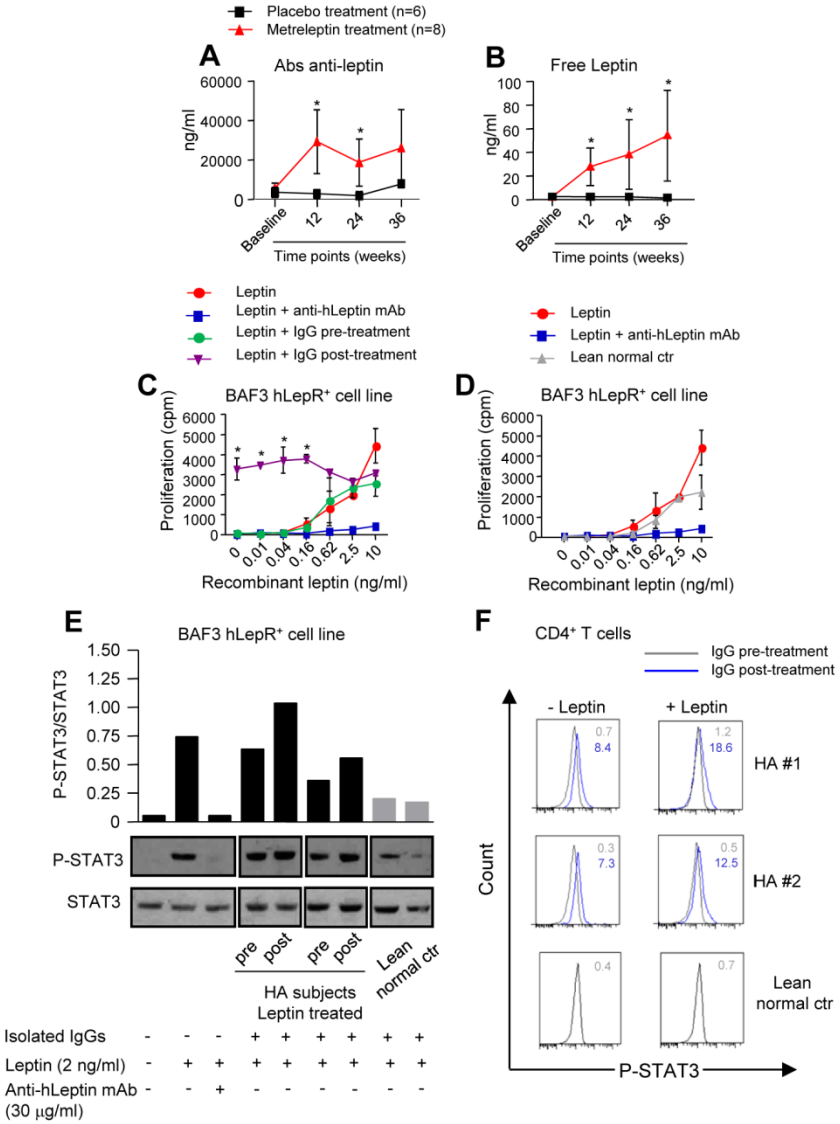

Figure 7. Metreleptin treatment induces anti-leptin antibodies with agonistic activity. In Panel A (*p < 0.05) is shown the anti-leptin levels in HA subjects treated with leptin and placebo, (red and black respectively). Panel B (at week 12, 24, 36 *p < 0.05) shows the free leptin in subjects treated with leptin and placebo. In Panels C and D are shown the proliferation of the leptin-dependent hLepR+BAF3 cell line in the presence of isolated IgGs from metreleptin-treated HA subjects and controls. This assay was performed three times. In Panel E is shown STAT3 phosphorylation induced by IgGs from metreleptin-treated HA subjects. One representative experiment out of two is shown.

Transcriptional signature induced by metreleptin in PBMCs from HA subjects

Given the profound effects of metreleptin on immune cell reconstitution and proliferation, we next evaluated the modulation of gene expression in PBMCs from patients at the different time points during metreleptin treatment (weeks 12, 24, and 36). When comparing the transcriptional profiles at week 12 of treatment vs. baseline (n = 14), we observed 314 differentially expressed genes. Of those, 177 were up-regulated, and 137 were down-regulated compared with baseline. Likewise, at weeks 24 (n = 16) and 36 (n = 11), only 26 and 79 genes changed, respectively, compared with baseline (Fig. 8). Of these, 13 genes were up-regulated and 13 were down-regulated at week 24; 50 genes were up-regulated and 29 were down-regulated at week 36 (Fig. 8). Changes in gene expression at week 12 are summarized in Tables 2a and 2b; changes at weeks 24 and 36 are shown in Tables 3 and 4, respectively. Using the Database for Annotation, Visualization and Integrated Discovery (DAVID; National Institute of Allergy and Infectious Diseases, National Institutes of Health) functional pathway annotation tools, we analyzed the full pathways at weeks 12, 24, and 36 (dataset table 3,4 and 5 respectively). The genes affected at week 12 of treatment included those encoding for proteins involved in the regulation (33 genes) and induction (21 genes) of transcription, cell/biological adhesion (17 genes), biological proteolytic pathways (ubiquitin conjugation) (13 genes), negative regulation of macromolecule metabolic process (17 genes), vacuole formation (eight genes), and protein (17 genes) and macromolecule (20 genes) catabolic process. Among the genes that were affected significantly, metreleptin treatment induced the transcripts involved in cell survival and development such as the cytokines IL-7 and neurotrophin-3 (NT-3) factor in PBMCs at week 12. Both IL-7 and NT-3 are involved in lymphocyte survival, their effector functions, and neural development (51, 52). Conversely, genes involved in cell death/apoptosis, such as the B-cell chronic lymphocytic leukemia/lymphoma 10 (BCL-10) and TP53-regulator of apoptosis-1 (TRIAP-1) genes, were down-modulated significantly, thus confirming an antiapoptotic/prosurvival effect of leptin (53-55). The transcription factor 7–like-1 (TCF7L1), known to be a transcriptional activator

involved in T-lymphocyte differentiation and necessary for survival of $CD4^+$ and $CD8^+$ immature thymocytes (56), was up-regulated. In parallel with these changes, genes involved in cell adhesion and cell–cell/cell–matrix interactions, such as ADAM-metallopeptidase-23 (ADAM-23), actinin-3, vascular adhesion molecule-1 (VCAM-1), laminin-α2, and selectin-E (relevant in fertilization, muscle development, and neurogenesis), also were up-regulated (57), confirming the induction of adhesiveness and activation of immune cells by leptin previously observed in vitro (8). Additionally, some hormones important in maintaining reproductive functions, such as FSH and progestogen-associated endometrial protein (Paep), were up-regulated by metreleptin treatment (3). Notably, in addition to its important role in reproductive function, FSH has relevant immune-modulating effects (58). These effects suggest that leptin, by inducing FSH up-regulation, also could be involved in immunomodulatory processes. Moreover, at week 12 we observed up-regulation of genes involved in the control of immune tolerance, such as the forkhead box-D3 (FOXD3) gene (59), the CD1e molecule [which is necessary for the presentation of lipid antigens to T cells (60), and the triggering receptor expressed on myeloid cells-2 (TREM-2), which may have a role in chronic inflammation and may stimulate production inflammatory chemokines and cytokines (61). It is interesting that at week 24 (Table 3) the transcription of the aryl-hydrocarbon receptor nuclear translocator (ARNT) was up-regulated. ARNT is a key component in the elimination of dioxins, well known to be involved in autoimmune disease susceptibility, generation of Th17 and regulatory T (Treg) cells, and protection from infections (62, 63)The BCL-2–like-11 protein, which is an apoptosis facilitator, and the cannabinoid receptor 2 (Cnr2), which is known to inhibit macrophage-induced inflammation, were both down-regulated (64). Finally, we also observed the down-modulation of the TGF-β receptor-associated protein-1 (TGFBRAP-1), which plays a role in the TGF-β/activin signaling pathways known to be involved in the control of immune responses and down-modulation of inflammation. At week 36 (Table 4), we observed up-regulation of the receptor for the cytotoxic ligand TRAIL, TNF-R-10c. This receptor lacks a cytoplasmic death domain and hence is not capable of inducing apoptosis; rather, it protects against TRAIL-mediated apoptosis by competing

with TRAIL-R1 and -R2 for binding to the ligand. The hormone gastrin also was up-regulated, whereas both interleukin enhancer-binding factor-2 (ILF-2) and the growth factor-independent 1 (GFI-1) transcription repressor were down-regulated (65, 66). The following factors are involved directly and indirectly in lymphocyte survival and function: (*i*) gastrin, in addition to its effect on acid secretion by gastric cells, has hematopoietic and proinflammatory activities (67); (*ii*) ILF-2 encodes for a nuclear factor of activated T cells (NFAT) required for T-cell expression of the IL-2 gene (68); and (*iii*) GFI-1 encodes a nuclear zinc finger protein that functions as a transcriptional repressor (69) GFI-1 plays a role in diverse developmental contexts, including hematopoiesis and oncogenesis (69, 70). Finally, the significant changes in gene expression seen at week 12 vs. baseline in the metreleptin-treated group were validated by real-time PCR (Fig. 9).

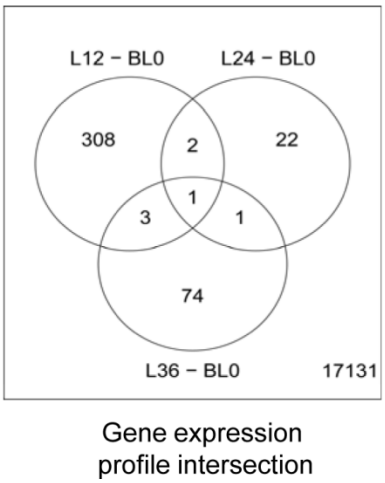

Figure 8. Transcriptional signature induced by metreleptin in PBMCs from HA subjects. Diagram of gene expression profile at week 12, 24 and 36 of treatment with metreleptin versus baseline. 17131 were total number of genes that were analyzed; at week 12 there were more differentially expressed genes, 308 versus 22 and 74 after 24 and 36 week of treatment, respectively.

Gene symbol	Gene assignment	Gene ID	p-value	Fold change
ADAM23	ADAM metallopeptidase domain 23	8745	0.00657402	0.64445533
Paep	progestagen-associated endometrial protein	5047	0.00268326	0.62717751
SELE	selectin E	6401	0.0076583	0.56464262
Zfp42	zinc finger protein 42 homolog (mouse)	132625	0.00391605	0.54934091
Vcam1	vascular cell adhesion molecule 1	7412	0.00908406	0.53175772
ASB12	ankyrin repeat and SOCS box-containing 12	142689	0.00853215	0.52964533
Tgm5	transglutaminase 5	9333	0.0037842	0.50489131
MMP10	matrix metallopeptidase 10 (stromelysin 2)	4319	0.00792751	0.49938345
FSHB	follicle stimulating hormone, beta polypeptide	2488	0.00645861	0.46332061
NTF3	neurotrophin 3	4908	0.00791533	0.45733451
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	1543	0.00851326	0.4530755
actn3	actn3	89	0.00447152	0.42328933
TUBGCP4	tubulin, gamma complex associated protein 4	27229	0.00366037	0.42254891
LRRK1	leucine-rich repeat kinase 1	79705	0.00171516	0.38451621
TREM2	triggering receptor expressed on myeloid cells 2	54209	0.00797656	0.37922294
Il7	interleukin 7	3574	0.00912747	0.37264077
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	2202	0.00731755	0.36643077
ATP2C2	ATPase, Ca++ transporting, type 2C, member 2	9914	0.00339537	0.36473719
TM4SF4	transmembrane 4 L six family member 4	7104	0.00051904	0.36139
Lama2	laminin, alpha 2	3908	0.00654983	0.35533335
TRPM3	transient receptor potential cation channel, subfamily M, member 3	80036	0.00950655	0.33836606
FUT3	fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, Lewis blood group)	2525	0.00877468	0.32655352
Apobec1	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1	339	0.00504257	0.32323031
tcf7l1	transcription factor 7-like 1 (T-cell specific, HMG-box)	83439	0.00841957	0.31810237
FOXD3	forkhead box D3	27022	0.00455988	0.31602
Gpr20	G protein-coupled receptor 20	2843	0.00566617	0.30375888
Hdac10	histone deacetylase 10	83933	0.00432499	0.28392
Krt32	keratin 32	3882	0.00276204	0.26272288
CD1E	CD1e molecule	913	0.00678964	0.24852336

Table 2a. Upregulated genes in response to metreleptin treatment at week 12 *vs* baseline.

Gene symbol	Gene assignment	Gene ID	p-value	Fold change
CCNI	cyclin I	10983	0.00817352	-0.2289351
TGOLN2	trans-golgi network protein 2	10618	0.00415695	-0.2934237
Psmd11	proteasome (prosome, macropain) 26S subunit, non-ATPase, 11	5717	0.00992041	-0.3663301
Polr2c	polymerase (RNA) II (DNA directed) polypeptide C, 33kDa	5432	0.00923576	-0.3997785
Terf2ip	telomeric repeat binding factor 2, interacting protein	54386	0.00972071	-0.4122286
Ergic3	ERGIC and golgi 3	51614	0.00748465	-0.4385879
NDUFS4	NADH dehydrogenase (ubiquinone) Fe-S protein 4, 18kDa (NADH-coenzyme Q reductase)	4724	0.00853818	-0.5093644
TUBA1C	tubulin, alpha 1c	84790	0.00458926	-0.5157148
TRIAP1	TP53 regulated inhibitor of apoptosis 1	51499	0.00476182	-0.5386698
ARID1B	AT rich interactive domain 1B (SWI1-like)	57492	0.00238552	-0.5438516
Mad2l1bp	MAD2L1 binding protein	9587	0.00620568	-0.544755
LOC100133398	selenoprotein T; similar to Selenoprotein T	51714	0.00627308	-0.5606207
SNRNP27	small nuclear ribonucleoprotein 27kDa (U4/U6.U5)	11017	0.0028898	-0.6324866
BCL10	B-cell CLL/lymphoma 10; hypothetical LOC646626	8915	0.0045983	-0.6407714
arfgap3	ADP-ribosylation factor GTPase activating protein 3	26286	0.00881985	-0.6912068
Abt1	activator of basal transcription 1	29777	0.00359616	-0.7326123
rbm38	RNA binding motif protein 38	55544	0.0085592	-0.816973
TBPL1	TBP-like 1	9519	0.00175630	-0.9431134

Table 2b. Downregulated genes in response to metreleptin treatment at week 12 *vs* baseline.

Gene symbol	Gene assignment	Gene ID	p-value	Fold change
Cacng8	calcium channel, voltage-dependent, gamma subunit 8	59283	0.00197222	0.74792967
ZNF490	zinc finger protein 490	57474	0.00107306	0.59417048
CENPI	centromere protein I	2491	0.00724578	0.45477823
LELP1	late cornified envelope-like proline-rich 1	149018	0.00013992	0.42264562
DPPA5	developmental pluripotency associated 5	340168	0.00743253	0.33456177
ARNT	aryl hydrocarbon receptor nuclear translocator	405	0.00860958	0.31569146
WDR53	WD repeat domain 53	348793	0.00494466	0.29463523
SPHKAP	SPHK1 interactor, AKAP domain containing	80309	0.00351039	0.2461562
DBT	dihydrolipoamide branched chain transacylase E2	1629	0.0099502	0.23485338
BCL2L11	BCL2-like 11 (apoptosis facilitator)	10018	0.00543426	-0.3127599
PRDM2	PR domain containing 2, with ZNF domain	7799	0.00790638	-0.322973
Cnr2	cannabinoid receptor 2 (macrophage)	1269	0.00416617	-0.3281563
TGFBRAP1	transforming growth factor, beta receptor associated protein 1	9392	0.00359646	-0.3439002
ZNF592	zinc finger protein 592	9640	0.00782736	-0.3622582
MED10	mediator complex subunit 10	84246	0.00406945	-0.547997
STX10	syntaxin 10	8677	0.00218949	-0.751147

Table 3. Differences in gene expression profile in response to metreleptin treatment at week 24 *vs* baseline.

Gene symbol	Gene assignment	Gene ID	p-value	Fold change
TNFRSF10C	tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain	8794	0.006717	0.90305714
ZNF200	zinc finger protein 200	7752	0.00610212	0.54233656
f2rl1	coagulation factor II (thrombin) receptor-like 1	2150	0.00829106	0.51888316
GAST	gastrin	2520	0.00128913	0.46499107
GLI1	GLI family zinc finger 1	2735	0.00939256	0.45136181
CYTH4	cytohesin 4	27128	0.00437337	0.36625752
bbs7	Bardet-Biedl syndrome 7	55212	0.00857128	0.27351589
ilf2	interleukin enhancer binding factor 2, 45kDa	3608	0.00451506	-0.692222
TXNIP	thioredoxin interacting protein	10628	0.00791743	-0.4120624
Cdk5r1	cyclin-dependent kinase 5, regulatory subunit 1 (p35)	8851	0.00957235	-0.4554088
GFI1	growth factor independent 1 transcription repressor	2672	0.0062539	-0.5224277
ZBTB16	zinc finger and BTB domain containing 16	7704	0.00228704	-0.7820146

Table 4. Differences in gene expression profile in response to metreleptin treatment at week 36 *vs* baseline.

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Gene name	forward	reverse
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
ADAM23	GCTACAATGGCGAGTGCAAG	CTCCATCCTTCCCGCAGTTT
SELE	GTGAAGCTCCCACTGAGTCC	AGCCAGAGGAGAAATGGTGC
IL7	TTTATTCCGTGCTGCTCGC	CCAGGGCAGCTGGTTTTCTT
Ergic3	GAAGTCAATAAGGTGGCCGGA	AGCTCTGCAAGTCATGGATCT
TRIAP1	CTGTCGCCATGAACAGTGTG	AAATTTCTCGGCGAACCAGC
BCL10	TTCCAGATGGAGCCACGAAC	CGTCGTGCTGGATTCTCCTT
Gene name	**forward**	**reverse**
18s	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
ADAM23	GCTACAATGGCGAGTGCAAG	CTCCATCCTTCCCGCAGTTT
SELE	GTGAAGCTCCCACTGAGTCC	AGCCAGAGGAGAAATGGTGC
IL7	TTTATTCCGTGCTGCTCGC	CCAGGGCAGCTGGTTTTCTT
Ergic3	GAAGTCAATAAGGTGGCCGGA	AGCTCTGCAAGTCATGGATCT
TRIAP1	CTGTCGCCATGAACAGTGTG	AAATTTCTCGGCGAACCAGC
BCL10	TTCCAGATGGAGCCACGAAC	CGTCGTGCTGGATTCTCCTT

Table 5. Sequences of primers for RT-PCR microarray validation.

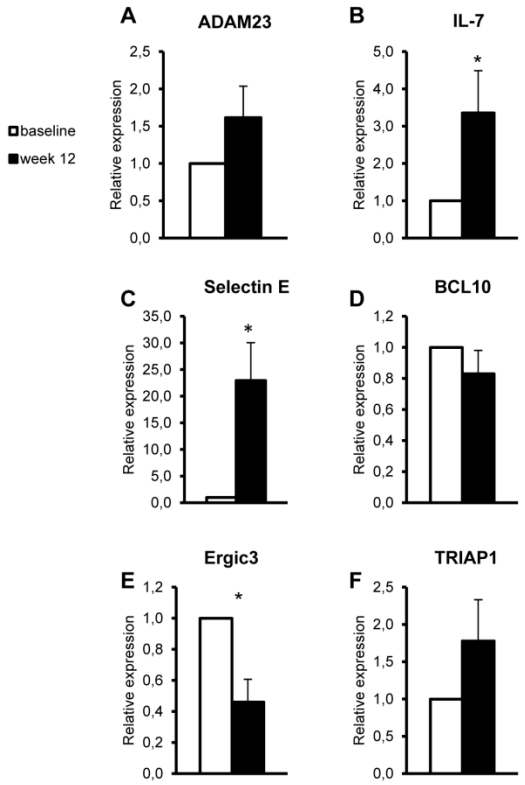

Figure 9. Gene-expression levels were verified by real-time PCR. Metreleptin-treated groups at week 12 were compared with the baseline levels. Gene-expression levels of IL-7 (B), selectin E (C), and Ergic3 (E) were changed significantly (*$P \leq 0.05$) compared with their baseline levels. Gene-expression levels of ADAM-23 (A), BCL-10 (D), and TRIAP-1 (F) did not change significantly from baseline

Acute *in vivo* metreleptin signaling in PBMCs and in vitro signaling on $CD4^+$ T cells from lean, normoleptinemic subjects

We next investigated the effects of acute in vivo metreleptin administration on signaling in both PBMCs and $CD4^+$ T cells from lean subjects. Similar to the transcriptional signature induced by metreleptin in PBMCs taken from subjects with HA, the signaling pathways involved in cell growth/survival/proliferation, such as the STAT3, AMPK, mTOR, ERK1/2, and Akt pathways, were activated directly by acute in vivo metreleptin administration in PBMCs (Fig. 10 A–F) and by acute in vitro stimulation with metreleptin in isolated $CD4^+$ T cells from lean subjects (Fig. 10 G–L). In contrast, acute in vivo metreleptin administration did not regulate SHP2 activation either in PBMCs or in isolated $CD4^+$ T cells, probably because induction of LepR signaling without simultaneous TCR engagement cannot induce high counterregulatory loops mediated by SHP2.

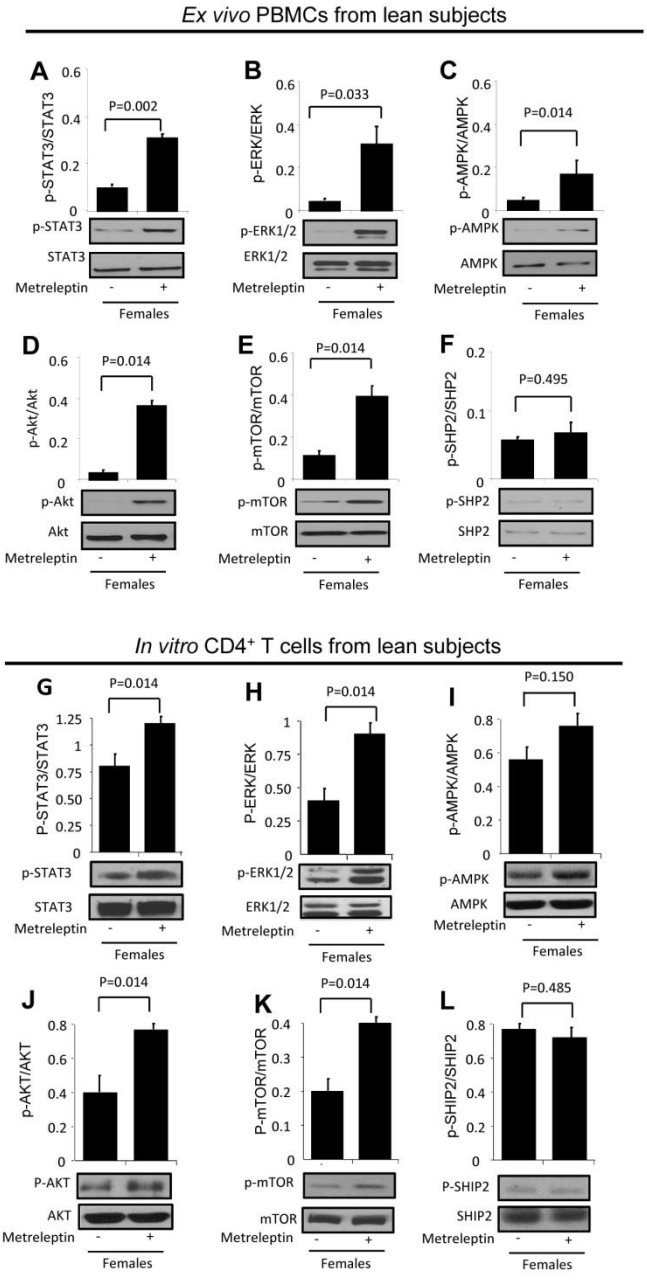

Figure 10. Acute *in vivo* metreleptin-induced signaling in PBMCs and *in vitro* in CD4$^+$ T cells from lean normoleptinemic subjects. In panels A-F, are shown western blotting analyses on PBMCs from lean females upon *in vivo* metreleptin stimulation. In panels G-L, are shown the same analyses performed on isolated highly purified CD4$^+$ T cells stimulated *in vitro* with metreleptin at 1h. Both PBMCs *in vivo* and isolated CD4$^+$ T cells *in vitro* provided similar results. All data were analyzed using student t-test. Values are means (n=3) ± SD.

Discussion

Prior evidence suggests that a link exixts between energy status and immunity as well as metabolic and neuroendocrine functions has been proposed (71). The adipocyte-derived hormone leptin has been suggested as one of the key players in this process. Indeed, leptin represents for the brain a signal of the amount of energy stored in fat and/or acute changes in energy availability and, similarly, it has been proposed that leptin is the signal conveying to the immune cells information on energy availability (7). While extensive experimental evidence has suggested a link between circulating leptin level and immune function/homeostasis in leptin deficient mice and humans, still little is known about the capacity of leptin to affect immune homeostasis in normal individuals and in conditions of relative leptin deficiency in humans. With this study we provide evidence that in an experimental setting where HA women served as subjects with chronic acquired relative leptin deficiency there is a reduction in T cells particularly in the $CD4^+$ T cell subset and in their proliferative capacity. Administration of metreleptin in replacement doses restored the number of $CD4^+$ T cells and partially their proliferative potential. This study demonstrates for the first time, in a placebo controlled, randomized manner, that relative leptin deficiency is associated with reduced number and function of T cells in humans and that metreleptin treatment restores immune cell population (specifically $CD4^+$ T cell) size and function. An important difference, also reaching statistical significance, was observed particularly in the capacity of metreleptin to increase $CD4^+$ T cell numbers thus helping the immune-reconstitution process in HA subjects. This novel aspect of metreleptin as an immune-reconstituting agent could have great impact in settings in which $CD4^+$ T cells are reduced, such as in states of chronic energy deficiency including cachexia associated with tuberculosis (TB), HIV infection or exposure to radiation etc (72, 73). In the context of immune reconstitution of the CD4+ T-cell compartment, the results obtained are highly encouraging. Indeed, the normal range of CD4+ T cells in adult humans generally varies from 750-1,200 cells/mm^3 (74). In placebo-treated HA subjects we observed a trend from low degree lymphopenia to frank lymphopenia over time: basal average $CD4^+$ T-cell count of these subjects was 721 ± 363 cells/mm^3; this cell

count became 428 ± 176 cells/mm^3 at week 36. This progressive decline in the $CD4^+$ T-cell count was significant and resembled the decline in $CD4^+$ T counts seen in the progression of other immunocompromised conditions, such as HIV infection. Although the two study groups (placebo and metreleptin) had similar basal $CD4^+$ T-cell counts (721 ± 363 and 775 ± 234 $CD4^+$ T cells/mm^3, respectively), metreleptin treatment was able to increase subjects' $CD4^+$ cell count from 775 ± 234 at baseline to 1,152 ± 227 cells/mm^3 at week 36; the effect of metreleptin treatment on $CD4^+$ T-cells was even more apparent when we compared the $CD4^+$ T-cell counts of placebo-treated vs. metreleptin-treated subjects at week 36 (428 ± 176 cells for placebo-treated subjects vs. 1,152 ± 227 cells/mm^3 for metreleptin-treated subjects). At the transcriptional level, a specific gene signature was observed for the first time in PBMCs from metreleptin treated subjects where genes involved in cell survival, hormonal response and adhesion were upregulated and those involved in cell death/apoptosis were down-regulated. These observations confirm that leptin administration promotes the expression of genes involved in pathways critical to cell survival, proliferation, and migration through transcriptional regulation (8, 75). These observations also help explain leptin's marked ability to improve $CD4^+$ T-cell survival and to promote immune reconstitution, perhaps suggesting an important role for leptin in the context of susceptibility to autoimmune disease (1). We and others have recently shown that leptin administration is effective in improving the metabolic profile in subjects with HAART associated lipodystrophy (76-79). Also, metreleptin therapy could be considered in other disease states where CD4 T cells play a role such as in the treatment of the most common form of intestinal infection leading to diarrhea in less developed countries: *amebiasis* (80). Indeed, it has been recently demonstrated in observational studies that a specific LepR polymorphism appears to be able to impair leptin signaling and induce higher susceptibility to this parasite (21). We also report herein that we detected anti-metreleptin antibodies in the serum of metreleptin treated HA subjects (3). This finding is not surprising since subcutaneous injections of native proteins can activate immune responses. Also considering the double nature of leptin, as a probable antigenic protein and an immunostimulating agent, it cannot be excluded that

leptin could simultaneously act both subcutaneously as an antibody generating protein and peripherally as a pro-inflammatory agent, allowing thus easier production of antibodies. In spite of this, we found that the antibodies produced may bind endogenous leptin but without having any effects in neutralizing its signaling capacity. Since leptin acts in a pleiotropic manner at multiple levels, it cannot be excluded that immune reconstituting activities observed in HA subjects could be a result of leptin's action on stress hormones or circulating cytokines which control survival of T cells and not necessarily to the direct action of leptin on CD4 T cells. To rule out these actions we evaluated in the serum pro-survival cytokines such as IL-2, IL-7 and IL-15 all involved in T cell survival and thymic selection, and observed no significant change in levels during metreleptin treatment (51). In contrast, IL-7 gene transcript was found to be up-regulated in PBMCs from treated HA patients, thus suggesting that metreleptin treatment was able to induce selectively, not systemically, the pro-survival cytokine IL-7 in immune cells. In addition, the transcript of the FSH hormone was increased at the immune cell level; since FSH is important to promote immune cell homeostasis and inflammatory responses we cannot exclude that some of the peripheral immune-cell improvements could be also ascribed to increased FSH (81). Also stress hormones such as cortisol, ACTH, and other hormones like insulin did not correlate significantly with changes in immune function reported herein. Therefore, we hypothesize that leptin's immune reconstituting actions are partly to be ascribed to an indirect pro-survival action on T cells (*via* induction of IL-7 and FSH) but also to a direct action on the LepR known to be expressed on T cells. Nonetheless, we recently demonstrated that HA subjects treated with metreleptin recovered menstruation and regained normal levels of hormones in the gonadal, thyroid, growth hormone, and adrenal axes (82). We also demonstrated changes in markers of bone metabolism suggestive of bone formation (3, 82) and, in the open label follow up period, we did detect a change in lumbar spine bone mineral density after treatment of leptin for two years (82). Therefore, the improvement in immune cell number and function could be related, in part, to the normalization of the gonadal axis (3, 82), evidence which is also corroborated by the increased FSH transcript also in immune cells. Finally, studies from the Tschöp

group have shown that leptin-initiated neuroendocrine pathways are able to functionally coordinate the systemic immune response (83). More specifically they observed that leptin deficiency was associated with an impaired immune response and lowered survival in a murine model of sepsis, mainly due to impaired neutrophil function. On the contrary, genetic rescue of leptin signalling exclusively and specifically within the central nervous system (CNS) was sufficient to improve mortality and cytokine profiles in sepsis, suggesting that leptin-dependent neurocircuitry in the CNS is required for efficient coordination of the immune response in sepsis to limit organ damage and prevent mortality. All the observations presented herein reveal the existence of a specific CNS leptin-signalling system that controls systemic immune defense in a functionally relevant manner. It is possible to speculate that leptin might act in the brain to directly regulate peripheral immune function and thereby contribute to better outcomes in different infectious diseases compared with states of relative or total leptin deficiency. Therefore, it is possible that the effects observed in HA women could also be ascribed to a specific CNS activity whereby metreleptin partly restores immune functions in the periphery (83). Finally, we extended these studies by performing acute *in vivo* leptin signaling experiments to determine whether pathways induced by metreleptin in PBMCs from lean subjects are similar or different from those induced by metreleptin in PBMCs from HA subjects. Consistent with data obtained in PBMCs from HA subjects, in which acute in vivo metreleptin administration activates signaling pathways involved in cell growth/survival/proliferation (i.e., STAT3, AMPK, ERK1/2, mTOR, and Akt), we replicated these results in highly purified $CD4^+$ T cells from lean subjects using an in vitro method. Although we have not examined all signaling pathways potentially mediating leptin's actions on PBMCs in vivo, we have studied signaling pathways that we believe are of critical importance. The results potentially are biologically important. The mechanisms reported herein lend themselves to future studies with an ultimate goal of identifying in vivo leptin signaling in immune cells and states in which leptin replacement could help the immune-reconstitution process. In conclusion, this study adds novel data on the capacity of

metreleptin treatment to facilitate immune reconstitution in non-obese subjects with acquired hypoleptinemia such as HA.

Conclusions

This is the first randomized, placebo-controlled trial of metreleptin administration in humans over a period of 36 weeks. Our data can have relevance in hypoleptinemic states of negative energy balance, such as recurrent infections associated with reduced T cell counts, immunodeficiencies such as HIV and Tuberculosis very frequently associated with low body weight and adipocyte mass. This study adds significant new information to the understanding of the biology of $CD4^+$ T cells in the context of hypoleptinaemia and how their function can be regulated by the metabolic state through leptin. Moreover, considering that nutritional deprivation or chronic restriction reduce autoimmune diseases onset, manipulation of the leptin axis, and in general of nutritional status, might represent a new means to modulate T cell tolerance in infections and autoimmunity. Thus, the effects we observed herein need to be replicated in larger studies and in different disease states associated with chronic hypoleptinemia, and it is necessary to study whether metreleptin administration could therefore contribute to improved morbidity and mortality in these conditions remains to be shown in future studies.

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