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Circulating levels of the components of the GH/IGF-1/IGFBPs axis total and intact IGF-binding proteins (IGFBP) 3 and IGFBP 4 and total IGFBP 5, as well as PAPPA, PAPPA2 and Stanniocalcin-2 levels are not altered in response to energy deprivation and/or metreleptin administration in humans

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ABSTRACT

Objective: It remains unclear whether food deprivation induces changes in components of the GH/IGF-1/IGFBPs axis and if yes, which ones are mediated by leptin, an adipocyte secreted hormone regulating neuroendocrine response to energy deprivation in animals and humans. We aimed to investigate components of the axis that have not been studied to date, i.e. IGF-binding proteins (IGFBPs) and related proteases (total and intact IGFBP 3 and IGFBP 4, total IGFBP 5, PAPPA, PAPPA2 and Stanniocalcin-2), during acute (short-term fasting in healthy subjects) and chronic (women with hypothalamic amenorrhea [HA] due to excessive exercise) energy deprivation and whether metreleptin administration, in replacement, supraphysiologic or pharmacologic levels, may mediate any changes of circulating levels of the above molecules in healthy individuals and in women with hypothalamic amenorrhea.
Methods: We studied: 1) 11 healthy men and women during three four day admissions i.e. a baseline admission in the fed isocaloric state and two admissions in the complete food deprivation state for 72-h with either placebo (resulting in a hypoleptinemic state) or metreleptin administration in doses designed to normalize circulating leptin levels for the duration of the study, 2) 15 healthy men and women during three 72-hour long admissions in a complete food deprivation state receiving three escalating doses of metreleptin designed to bring circulating leptin levels to physiologic, supraphysiologic, or pharmacologic levels, and 3) 18 women with HA randomized to either metreleptin treatment in replacement doses or placebo for nine months.
Results: There were no significant changes in the circulating profiles of the above molecules in the fasting vs. fed state and/or with metreleptin administration during acute and chronic energy deprivation.
Conclusions: The studied components of the GH/IGF-1/IGFBPs axis are not affected by energy deprivation, leptin deficiency associated with energy deprivation, or by metreleptin administration in physiologic, supraphysiologic or pharmacologic doses.

1. Introduction

Acute energy deprivation, i.e. fasting or weight loss, and chronic energy deprivation (e.g. anorexia nervosa and exercise-induced hypothalamic amenorrhea) alter pulsatile secretion of growth hormone (GH) by increasing the frequency of GH pulses as well as GH levels in humans [1–6], while decreasing circulating and total insulin-like growth factor-1 (IGF-1) [1–5,7–12]. States of acute and chronic energy deficiency, such as fasting and anorexia nervosa, are also characterized by low levels of leptin, an adipose tissue-secreted hormone, which reflects the amount of energy stored in adipose tissue and mediates adaptation to starvation [13]. On the basis of animal experiments, it has been hypothesized that falling leptin levels may mediate the alterations in the GH/IGF-1 axis in states of energy deprivation [5]. Our group has previously published that leptin replacement during acute energy deprivation fails to alter the IGF-1 decline observed during fasting [5]. During chronic energy deprivation, metreleptin administration increased total IGF-1 levels and tended to increase free IGF-1 in an uncontrolled study, but the magnitude of its effect was only marginal and nonsignificant in a randomized controlled trial [5,14]. In addition, distinct changes in total IGF-binding proteins (IGFBPs), have been described in response to energy deprivation [5,14,15], but no studies thus far have fully investigated all members of

Abbreviations: BMI, body mass index; GH, growth hormone; HA, hypothalamic amenorrhea; IGF-1, insulin-like growth factor-1; IGFBPs, IGF-binding proteins; PAPPA, pregnancy-associated plasma protein A; STC-2, stanniocalcin-2.

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the GH/IGF-1/IGFBPs axis in response to acute or chronic energy deprivation and no prior studies have studied intact circulating IGFs and/or the related proteases/cleavage proteins. More specifically, no prior study has explored whether intact IGFBPs and the related proteases [pregnancy-associated plasma protein A (PAPPA), PAPPA2, Stanniocalcin-2] change in response to metreleptin administration. It is important to fully elucidate human physiology in this regard, by not only studying not yet studied IGFBPs in response to changes in energy intake and/or metreleptin administration, but also by studying intact circulating IGFBPs and/or the related proteases/cleavage proteins given that intact IGFBPs are binding with higher affinity to IGF-1 compared to the fragmented ones, thus reducing IGF-1's bioavailability.

Therefore, we aimed to study total and intact levels of novel IGFBPs along with related proteases in acute (short-term food deprivation in healthy subjects) and chronic energy deprivation states [women with hypothalamic amenorrhea (HA) due to excessive exercise] as well as in response to metreleptin administration in replacement physiologic, supraphysiologic or high pharmacologic doses. Total and intact IGFBP 3 and 4, total IGFBP 5, PAPPA, PAPPA2 and Stanniocalcin-2 were measured herein.

2. Material and methods

2.1. Study population

2.1.1. Study 1: Inpatient study investigating the regulation of total and intact levels of novel IGFBPs along with related proteases by short term fasting (acute energy deprivation state) and/or metreleptin administration in healthy individuals

Eight men and seven women were assessed during 4-day admissions in the fed and fasting states as previously described (ClinicalTrials.gov no. NCT00140231) [4,16]. The fed admission involved an isocaloric weight-maintaining diet, while the 72-hour fasting studies involved administration of either placebo or replacement dose of recombinant human metreleptin for the whole duration of the studies to normalize the fasting-induced hypoleptinemia (Fig. 1). A washout period of at least 8 weeks was allowed between each of the studies for hematocrit, leptin levels and weight to recover to baseline. The order of the studies in the fasting state was random in women, while in men the placebo study preceded the leptin one, except for one subject (where the metreleptin study preceded the placebo one). The total dose of metreleptin was 0.04 (men) or 0.08 (women) mg/kg per day the first day and 0.1 (men) or 0.2 (women) mg/kg per day the second and third days injected subcutaneously starting at 8 am and every 6 h thereafter. Placebo administration followed the same dosing schedule. Subjects were admitted the night before the first study day and consumed a snack. During the fed admission they received four meals at predetermined times every day to maintain an isocaloric state and thus their baseline body weight. During the fasting studies, subjects had free access only to water and calorie-free liquids as well as NaCl (500 mg), KCl (40 mEq), and a standard multivitamin with minerals daily for the first 3 days and until 10 am of day 4. Blood was collected at 8 am (men and women) on day 1 and 8 am on day 3 (men) or day 4 (women) during all three studies. Two men and two women did not complete one of the three studies and thus, were excluded from the analysis. In addition, one more subject was removed from the intact IGFBP 4 analysis as an outlier, as per Section 2.3.

2.1.2. Study 2: Inpatient double-blinded study investigating the regulation of total and intact levels of novel IGFBPs along with related proteases by short term fasting (acute energy deprivation state) and metreleptin administration in physiologic replacement, supraphysiologic and high pharmacologic doses in healthy subjects

As previously described [17,18], fifteen healthy men and women were studied during three 72 hour admissions following the above described paradigm (ClinicalTrials.gov no. NCT00140205). Three different

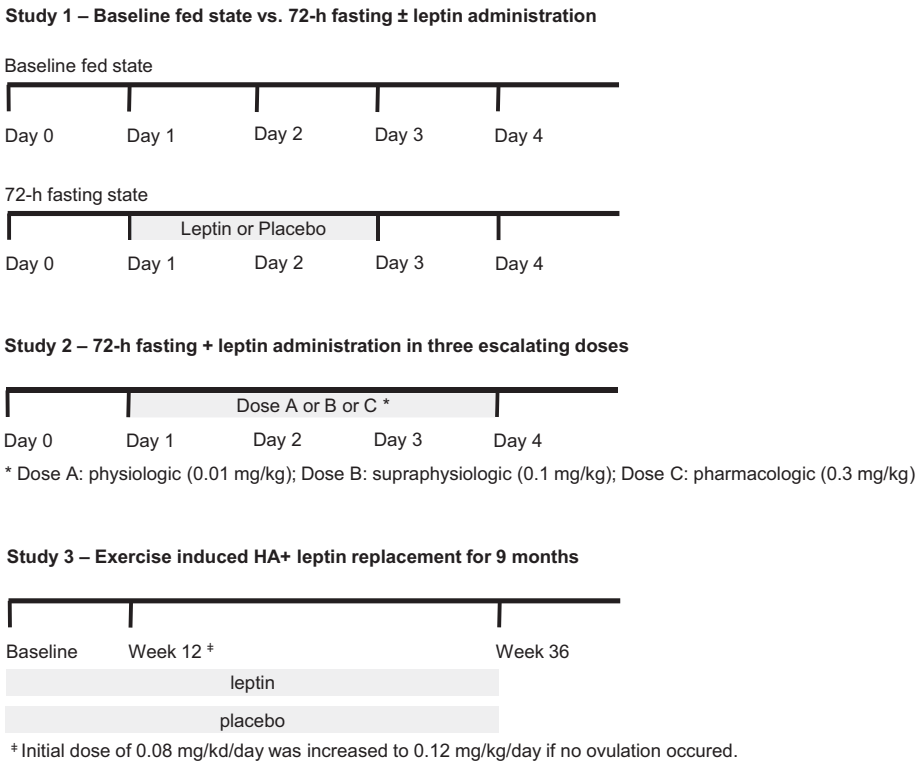

Fig. 1. Schematic of the protocols of the three clinical trials included in our study. Study 1 involved three four-day admissions: a baseline in the fed isocaloric state, and two admissions in the complete food deprivation state for 72-h with either placebo or metreleptin administration in healthy subjects. Study 2 consisted of three 72-hour long admissions in a complete food deprivation state with administration of three escalating doses of metreleptin in healthy subjects as outlined above. Study 3 was a randomized controlled trial of metreleptin vs. placebo administration over 9 months in women with hypothalamic amenorrhea (HA).

metreleptin doses [physiologic (0.01 mg/kg per day), supraphysiologic (0.1 mg/kg per day), and pharmacologic (0.3 mg/kg per day)] were administered under fasting conditions during three consecutive admissions (Fig. 1). Participants were admitted the night before the first study day and consumed a snack (748 kcal) at 10 pm, followed by fasting (except consumption of calorie-free liquids, NaCl [500 mg], KCl [40 meq], and one minerals-containing multivitamin every day) until study day 3 at 10 pm when they consumed another snack (225 kcal). For each fasting study, metreleptin was administered subcutaneously once daily at 8 am for 3 consecutive days. We measured the molecules of interest in blood collected at time 0 before each dose and +2 h and +8 h after the dose. The time point 2 h after metreleptin administration corresponds to the peak of leptin levels, while the one 8 h afterwards to the beginning of the decline in leptin levels [17,19]. The first two visits were 2–4 weeks apart, while the third followed 3–10 weeks after the second.

2.1.3. Study 3: Outpatient randomized placebo controlled clinical trial of metreleptin administration in women with hypothalamic amenorrhea (chronic energy deprivation state) over 9 months

As reported previously [20], this was a double-blinded trial which randomized 20 women with HA to either metreleptin ($n = 11$) or placebo ($n = 9$) for 9 months (ClinicalTrials.gov no. NCT00130117). Participants self-injected metreleptin or placebo subcutaneously twice daily between 7 and 11 pm for 36 weeks (Fig. 1). The dose of metreleptin was 0.08 mg/kg per day for the first 12 weeks; subjects who had begun menstruating received this dose until the end of the study, while those who had not increased the dose to 0.12 mg/kg per day. Upon weight loss of >5% of the baseline weight, dose was decreased by 0.04 mg/kg. There was a follow up period of 16 weeks after the 36th week of metreleptin injection. Clinical and hormonal evaluations were performed every four weeks and blood was withdrawn after a ten-hour overnight fasting. One of the subjects randomized to metreleptin discontinued the study after the first visit due to injection-site reaction and one of those randomized to placebo did not continue the study after week 4 due to travelling; both were excluded from the analysis. Three subjects from the metreleptin group [one due to travelling (week 24), one due to pregnancy (week 24) and one due to persistent weight loss (week 28)] and two from the placebo group [both due to travelling (week 16, week 24)] also discontinued, but were not excluded from the analysis.

Inclusion-exclusion criteria, participants' characteristics, primary outcomes and secondary outcomes for the three studies have been previously published [5,17,20]. Herein, we performed a secondary analysis of the three studies. All three study protocols were approved by the Institutional Review Board of the Beth Israel Deaconess Medical Center (BIDMC) and follow the guidelines of the Declaration of Helsinki and the International Conference on Harmonization for Good Clinical Practice. All participants signed written informed consent. Recombinant human metreleptin was provided by Amgen, Inc. (Thousand Oaks, CA).

2.2. Hormone measurements

Total IGFBP 3 (Intra-assay Variability: 2.96%, Inter-assay Variability: 2.95%, sensitivity: 0.3 ng/ml), Intact IGFBP 3 (intra-assay CV < 4.94%, inter-assay CV < 5.05%, sensitivity: 1.37 ng/ml), Total IGFBP 4 (intra-assay CV < 3.47%, inter-assay CV < 4.43%, sensitivity: 4.735 ng/ml), Intact IGFBP 4 (intra-assay CV < 4.05%, inter-assay CV < 4.31%, sensitivity 0.669 ng/ml), Total IGFBP 5 (intra-assay CV < 5.47%, inter-assay CV < 4.06%, sensitivity: 4.4 ng/ml), PAPPA (intra-assay CV < 2.69%, inter-assay CV < 2.87%, sensitivity: 10.1 ng/ml), PAPPA2 (intra-assay CV < 4.26%, inter-assay CV < 2.54%, sensitivity: 0.071 ng/ml), Stanniocalcin-2 (intra-assay CV < 10.44%, inter-assay CV < 6.39%, sensitivity: 0.033 ng/ml), were measured using commercially available immunoassays, all from Ansh Laboratories (Webster, TX, USA). We performed an additional validation of the above assays in terms of sample's freeze thaw cycles and we report it herein in addition to information provided by the manufacturer in the insert package (Supplementary Appendix). Measurements were performed in duplicate and were repeated if any sample's coefficient of variation was >20%. The value deriving from the repetition was considered the valid one if the coefficient of variation was <20%.

2.3. Statistical analysis

Statistical analysis was performed with SPSS v25.0 (SPSS, Inc., Chicago, IL) for Windows and with GraphPad prism 7 (GraphPad Software Inc., La Jolla, CA). Values measured below assay sensitivity were replaced with half the value of the lowest standard of the assay and baseline missing values were replaced with the mean of the baseline values of the rest of the admissions as needed. Values above 3-fold of interquartile range were identified as outliers. Shapiro-Wilk test was performed to evaluate the normality of distribution of the variables and those not normally distributed were either logarithmically transformed or analyzed with nonparametric test if applicable. Data are reported as mean ± SD for normally distributed or median [25th, 75th percentile] for non-normally distributed variables. In Study 1, the percentages of change between the first and last days were calculated for each state and analyzed with one way repeated measures ANOVA or Friedman test. The difference in the levels of the molecules between the first and last day among the three conditions were explored with two-way repeated measures ANOVA adjusted for the corresponding baseline values. In Study 2, primary outcomes were changes in the molecules of interest during the 72-h fasting among the three escalating metreleptin doses. An intention-to-treat analysis with last observation carried forward was performed. Areas under the curve from fold changes of the continuous variables were calculated and analyzed with one-way repeated measures ANOVA or Friedman test. In study 3, differences in the aforementioned molecules between baseline and post-intervention (up to 36 weeks) for the metreleptin versus placebo group were estimated using one-way repeated measures ANOVA adjusted for the corresponding baseline values. An intention-to-treat analysis with last observation carried forward was performed.

The level of statistical significance was set at 0.017 after Bonferroni correction was applied to account for multiple comparisons. Prior data on IGFBPs were not available for power calculations and thus by extension we relied on previously performed power calculations for detecting differences in IGF-1 using a smaller number of participants and we also performed generic power calculations to detect specific differences per unit of standard deviation [5]. Power calculations using our data indicate that we had 80% power at the $a = 0.05$ level to demonstrate differences of 0.94 per unit of standard deviation in Study 1, 0.78 per unit of standard deviation in Study 2 and 1.42 per unit of standard deviation in Study 3. In all three studies, analyses with and without outliers yielded similar results. Notably, on treatment analysis using Mixed Models (data not shown) and intention-to-treat analysis (shown herein as described above), yielded similar results.

3. Results

3.1. Short-term energy deprivation with and without metreleptin replacement does not alter circulating levels of IGFBPs and related proteases in healthy subjects (Study 1)

Baseline characteristics of the subjects participating in the study have been previously published [4,16,17]. As previously reported [5], weight and consequently BMI decreased in response to fasting and fasting plus metreleptin administration (s. Table 1). Leptin levels declined with fasting and significantly increased after leptin replacement as compared to the baseline-fed state (s. Table 1) [5]. We investigated whether IGFBPs and the related proteases are affected by fasting and whether normalizing fasting induced hypoleptinemia could reverse any potential associated changes. Fasting plus placebo did not affect

Table 1
Circulating levels of the IGFBPs and related proteases at the beginning and end of a fed isocaloric admission and two 72-hour fasting admissions with either placebo or metreleptin administration.

	Fed		% change	Fasting + placebo		% change	Fasting + metreleptin		% change	p-Value % change	p-Value[†] p-Value[‡] p-Value[¥]
	B/L	LD		B/L	LD		B/L	LD			
Weight (kg)	66.2 ± 12.4	66.8 ± 12.1	0.8	66.4 ± 12.6	64.5 ± 12.1	−2.8[a]	67.1 ± 13.5	64.8 ± 13.1	−3.4[a]	<0.001*	0.27 <0.001 <0.001
BMI (kg/m^2)	22.6 ± 1.9	22.8 ± 1.8	0.9	22.7 ± 2.2	22.1 ± 2.1	−2.6[a]	22.9 ± 2.5	22.1 ± 2.4	−3.5[a]	<0.001*	0.50 <0.001 <0.001
Leptin (ng/ml)	5.7 ± 7.4	8.6 ± 7.9	51	7.8 ± 1.2	1.3 ± 6.6	−83	7.2 ± 14.2	19.2 ± 13.5	165[b]	<0.001≠	0.03 <0.001 <0.001
Intact IGFBP3 (ng/ml)	1643 ± 499	1928 ± 665	17	1559 ± 698	1559 ± 719	−13	1493 ± 478	1247 ± 700	−16	0.23≠	0.20 0.05 0.05
Intact IGFBP4 (ng/ml)	5.9 [4.2, 8.7]	5.6 [4.3, 9.2]	4	6.3 [4.3, 3.8]	7.9 [4.0, 3.4]	16	4.0 [3.0, 1.4]	7.0 [4.0, 16.0]	19	0.81≠	0.001 0.75 0.75
Total IGFBP5 (ng/ml)	229 [210, 263]	213 [177, 233]	−11	260 [189, 269]	187 [176, 232]	−21	240 [185, 296]	173 [167, 259]	−17	0.69*	0.43 0.89 0.89
PAPPA (ng/ml)	282 [263, 391]	338 [152, 383]	1	349 [216, 463]	301 [162, 414]	−12	325 [216, 404]	238 [180, 332]	−17	0.22≠	0.13 0.40 0.40
PAPPA2 (ng/ml) [§]	0.08 ± 0.06	0.10 ± 0.06	16	0.08 ± 0.04	0.10 ± 0.06	29	0.11 ± 0.06	0.10 ± 0.06	−10	0.60≠	0.01 0.73 0.73
STC-2 (ng/ml)	24.9 ± 5.2	22.5 ± 7.4	−9	24.7 ± 6.1	21.9 ± 5.7	−11	25.7 ± 5.1	20.4 ± 4.1	−21	0.73≠	0.17 0.41 0.41

B/L: Baseline; LD: Last Day (3rd day for men, 4th day for women).
Values represent mean ± SD, median [25th, 75th percentile] and percentage of change between first and last study day. Total IGFBP 5 and PAPPA were logarithmically transformed the two way repeated measures ANOVA.
The level of significance is set to p-value < 0.017. Bonferroni correction was applied ($\alpha_2 = \alpha_1/m$) to account for multiple comparisons performed at a 3-level analysis (3 conditions).
BMI, body mass index; IGFBP, insulin-like growth factor 1 binding protein; PAPPA, pregnancy-associated protein A; STC-2, stanniocalcin-2.
[†] p-Value calculated with two way repeated measures ANOVA for effect of time adjusted for baseline.
[‡] p-Value calculated with two way repeated measures ANOVA for effect of condition (fed vs. fasting + placebo vs. fasting + metreleptin) adjusted for baseline.
[¥] p-Values calculated with two way repeated measures ANOVA for interaction of time by condition adjusted for baseline.
* p-Values calculated with one way repeated measures ANOVA.
≠ p-Values calculated with Friedman test.
[a] Vs. fed p < 0.017.
[b] Vs. placebo p < 0.017.
[§] Total number of subject is 10.

Table 2
Areas under the curve of fold changes of circulating IGFBPs and related proteases during short-term energy deprivation with administration of escalating doses of metreleptin.

	Dose A = 0.01 mg/kg	Dose B = 0.1 mg/kg	Dose C = 0.3 mg/kg	p-Value
Total IGFBP 3 (ng/ml)	64.5 ± 17.1	54.3 ± 12.2	60.0 ± 22.3	0.28[a]
Intact IGFBP 3 (ng/ml)	58.2 ± 8.2	70.1 ± 15.3	63.3 ± 14.1	0.08[a]
Total IGFBP 3/Intact IGFBP 3	70.8 ± 24.6	51.2 ± 14.7	66.1 ± 38.7	0.14[a]
Total IGFBP 4 (ng/ml)	47.7 ± 10.0	52.5 ± 9.8	52.3 ± 6.4	0.42[a]
Intact IGFBP 4 (ng/ml)	80 [44, 170]	58 [39, 134]	56 [42, 137]	0.45[b]
Total IGFBP 4/Intact IGFBP 4	36 [19, 90]	53 [32, 92]	87 [40, 124]	0.19[b]
Total IGFBP 5 (ng/ml)	52 ± 16	50 ± 14	51 ± 14	0.91[a]
PAPPA (ng/ml)	60 [47, 68]	60 [52, 69]	60 [48, 84]	0.82[b]
PAPPA2 (ng/ml)	51 [40, 86]	44 [35, 51]	47 [43, 66]	0.84[b]
STC-2 (ng/ml)	56.9 ± 8.6	53.6 ± 7.2	53.1 ± 9.3	0.84[a]

Values are reported as mean ± SD or median [25th, 75th percentile]. Last observation carried forward was performed for intention to treat analysis.
IGFBP, insulin-like growth factor 1 binding protein; PAPPA, pregnancy-associated protein A; STC-2, stanniocalcin-2.
[a] p-Values calculated with one-way repeated measures ANOVA.
[b] p-Values calculated with Friedman test.

intact IGFBP 4, total IGFBP 5, PAPPA, PAPPA2 and Stanniocalcin-2 as compared to the baseline-fed state. Similarly, the normalization of leptin levels with administration of metreleptin during fasting did not significantly change any of the above observations.

3.2. Metreleptin administration in escalating doses during short-term energy deprivation does not change circulating profile of IGFBPs and related proteases in healthy individuals (Study 2)

In study 2, subjects were matched properly in terms of age [17]. Leptin levels were higher in obese men and lean women versus lean men as expected [17]. We examined whether three escalating doses of metreleptin during 72-h fasting admissions affect circulating levels of IGFBPs and related proteases in healthy individuals with normal weight or obesity. In intention-to-treat analysis AUCs of fold changes did not differ among the three doses of metreleptin, indicating that leptin does not exhibit a dose response pattern on the GH/IGF-1/IGFBPs axis in fasting (s. Table 2). On treatment analysis yielded similar results (data not shown).

3.3. Metreleptin administration in women with chronic energy deprivation does not alter circulating profile of IGFBPs and related proteases (Study 3)

In study 3, age, weight, BMI and leptin levels were similar between the treatment groups, indicating proper randomization of the participants [14]. We tested whether the IGFBPs and related proteases change in response to increasing leptin levels in a study with women with HA (chronic energy deprivation state due to strenuous exercise). Our results from intention-to-treat analysis suggest that metreleptin administration for 36 weeks does not cause any distinct patterns of changes in the molecules of interest as compared to placebo (s. Table 3).

4. Discussion

Our study examined the effect of energy deprivation on the IGFBPs and related proteases and the potential role of leptin replacement in the regulation of these molecules under acute and chronic energy deprivation. We measured total and intact IGFBP 3 and 4, which bind most of the circulating IGF-1 along with total IGFBP 5, and the proteases PAPPA, PAPPA2 and Stanniocalcin-2, which also control IGF-1 bioavailability by cleaving IGF binding proteins and altering their intact levels; these molecules have not been studied previously. We are the first to show that short- or long term energy deprivation does not alter the levels of intact IGFBP 3 and 4, total IGFBP 5, PAPPA, PAPPA2 and Stanniocalcin-2. In addition, metreleptin administration in replacement physiologic, supraphysiologic or pharmacologic doses does not alter the levels of intact IGFBP 3, intact IGFBP 4, total IGFBP 5, PAPPA, PAPPA2 and Stanniocalcin-2 during short- and long-term energy deprivation.

A small percentage of IGF-1 circulates free and this is the biologically active IGF-1 which promotes cell migration, proliferation and differentiation and exerts anti-inflammatory, anti-apoptotic and insulin-like effects through the IGF type 1 and 2 receptors and secondarily the insulin receptor [21]. Almost 99% is bound to one of the six IGFBPs: IGFBP 1–6 and acid labile subunit [22]. These molecules are secreted by the liver, muscle, adipose tissue and female reproductive organs and bind IGF-1 with high affinity, mainly preventing it from binding to the IGF-1 receptor, thus prolonging its half-life [23]. IGFBP 3 is the most abundant IGFBP in the circulation, followed by 2 and 4, and creates a complex with acid labile unit, binding 75–80% of free IGF-1 [24]. IGFBPs are subject to post-translational modification, such as proteolysis, which creates two fragments with decreased affinity to IGF-1 compared to the intact (uncleaved) respective molecules and leads to higher levels of free IGF-1 [25]. More specifically, IGF-1 can be liberated from IGFBP 4 through proteolytic cleavage by a metalloproteinase primarily expressed in placenta, PAPPA, and from IGFBP 3 and 5 by PAPP A2 (17). Stanniocalcin-2, a glycoprotein mainly expressed in breast, placenta and pancreas, but also in most tissues, inhibits the action of PAPPA and PAPPA2 irreversibly, increasing the bioavailability of intact IGFBPs and thus, decreasing the ability of IGF-1 to bind to its receptors [26,27]. Additionally, IGFBPs are involved in IGF-1 independent cellular pathways, such as cell proliferation (IGFBP 1: smooth muscle cells, IGFBP 3: cancer cells, IGFBP 5: osteoblasts), survival (IGFBP 3) and migration (IGFBP 1), findings which triggered further investigation of their role as potential biomarkers in various conditions [23,25].

It has been suggested that energy deprivation changes levels of IGFBPs (increasing total IGFBP 1 and 2 and decreasing total IGFBP 3 and 6), but studies have been inconclusive so far [5,7–10,28]. Leptin is considered a key regulator of hormonal responses to energy deprivation. The role of leptin in regulating the GH/IGF-1 axis has been described partially in the past due to the lack of commercial assays to measure intact IGFBPs, the determinants of free IGF-1, and the related cleavage proteins until very recently. Our results confirm previous studies which showed no effect of leptin on total levels of other IGFBPs during acute energy deprivation. Chan et al., had previously demonstrated that fasting for 72 h resulted in a decrease in total and free IGF—1, IGFBP 3 and 4 as compared to the baseline-fed state [5]. Normalization of leptin levels with metreleptin failed to change any of the above observations except for the decline in total IGF—1, which appeared attenuated, albeit not significantly [4,5,16]. This trend was not observed in Schurgin's et al. study who examined healthy women of reproductive age during a 4-day fasting admission plus daily metreleptin or placebo, but the rest of the observations were in agreement with our observations [15]. Total IGFBP 1, 2 and 6 did not change after 72 h of fasting as compared to the baseline fed state in our previous study [5]. The same was observed with intact IGFBP 4, total IGFBP 5, PAPPA, PAPPA2 and stanniocalcin-2 in our current study.

Table 3
Changes in circulating IGFBPs and related proteases under chronic energy deprivation based on a randomized placebo controlled trial with administration of metreleptin.

		Baseline	4wk	8wk	12wk	16wk	20wk	24wk	28wk	32wk	36wk	p-Value[a] p-Value[b] p-Value[c]
Total IGFBP 3 (ng/ml)	L	4568 ± 1076	4823 ± 1405	4845 ± 1376	4383 ± 862	4256 ± 585	4797 ± 1191	5080 ± 1272	4929 ± 1309	5531 ± 1232	5064 ± 1198	0.21
	P	3800 ± 861	4010 ± 1003	4150 ± 766	3716 ± 1031	3828 ± 1196	4536 ± 1328	4134 ± 1171	3925 ± 657	4003 ± 687	4322 ± 1443	0.34
	Δ_{L-P}	769	813	695	667	427	261	946	1004	1528	743	0.74
Intact IGFBP 3 (ng/ml)	L	1524 ± 359	1551 ± 459	1490 ± 401	1400 ± 336	1419 ± 468	1414 ± 543	1316 ± 427	1339 ± 393	1330 ± 442	1282 ± 337	0.12
	P	1473 ± 294	1572 ± 378	1612 ± 375	1785 ± 390	1610 ± 293	1588 ± 334	1771 ± 272	1693 ± 172	1516 ± 287	1526 ± 201	0.17
	Δ_{L-P}	51	−21	−122	−385	−191	−174	−455	−354	−186	−244	0.16
Total IGFBP 3/Intact IGFBP 3	L	2.9 [2.2, 3.9]	3.0 [2.0, 5.1]	3.1 [1.9, 4.1]	3.2 [2.3, 4.0]	3.6 [2.5, 4.0]	3.5 [2.5, 5.2]	4.0 [2.4, 6.0]	3.6 [2.5, 5.5]	3.8 [3.1, 5.7]	3.5 [2.6, 5.5]	0.54
	P	2.4 [1.9, 5.0]	2.2 [1.7, 3.9]	2.5 [2.2, 3.4]	2.1[1.5, 3.8]	2.2 [1.7, 3.2]	2.3 [2.1, 4.6]	2.5 [1.8, 3.1]	2.2 [2.0, 2.5]	2.7 [2.2, 3.7]	2.3 [1.9, 3.2]	0.12
	Δ_{L-P}	0.5	0.8	0.6	1.1	1.4	1.2	1.5	1.4	1.1	1.1	0.11
Total IGFBP 4 (ng/ml)	L	111 ± 17	121 ± 24	117 ± 16	121 ± 20	114 ± 12	119 ± 20	129 ± 21	128 ± 20	123 ± 14	122 ± 15	0.26
	P	120 ± 22	128 ± 18	137 ± 28	128 ± 22	121 ± 22	128 ± 31	130 ± 28	125 ± 29	125 ± 23	128 ± 28	0.86
	Δ_{L-P}	−10	−7	−20	−7	−8	−9	−1	3	−2	−6	0.69
Intact IGFBP 4 (ng/ml)	L	28.4 ± 27.7	38.7 ± 18.4	45.5 ± 23.6	36.9 ± 26.2	58.3 ± 32.2	34.0 ± 27.1	35.1 ± 24.3	42.1 ± 26.8	38.6 ± 25.2	36.3 ± 28.6	0.01
	P	34.4 ± 22.5	37.6 ± 23.0	38.9 ± 27.0	32.0 ± 18.5	45.2 ± 20.2	36.2 ± 28.4	24.3 ± 19.0	34.3 ± 17.5	33.6 ± 24.2	35.8 ± 29.6	0.39
	Δ_{L-P}	−6.0	1.0	6.6	4.8	13.1	−2.2	10.7	7.8	5.1	0.4	0.97
Total IGFBP 4/Intact IGFBP 4	L	4.3 [1.5, 9.8]	2.8 [1.7, 4.2]	2.0 [1.6, 4.3]	4.0 [1.6, 10.9]	1.7 [1.3, 3.9]	4.3 [3.2, 6.6]	3.7 [2.5, 9.7]	4.0 [1.8, 10.1]	4.1 [3.1, 5.9]	4.3 [1.4, 9.0]	0.03
	P	5.0 [2.3, 8.7]	3.4 [1.5, 6.9]	6.3 [2.8, 8.1]	4.2 [3.6, 6.3]	5.0 [2.3, 8.7]	2.8 [1.6, 4.1]	5.0 [2.3, 12.9]	7.1 [4.3, 15.9]	3.4 [2.5, 5.7]	4.7 [1.7, 6.3]	0.40
	Δ_{L-P}	−0.6	−0.6	−4.3	−0.2	−1.1	−0.7	−3.4	0.6	−0.6	−1.8	0.69
Total IGFBP 5 (ng/ml)	L	305 [239, 320]	275 [229, 352]	318 [285, 380]	273 [250, 482]	287 [228, 347]	273 [247, 314]	316 [225, 432]	317 [245, 415]	319 [244, 415]	270 [230, 368]	0.25
	P	283 [253,321]	276 [239, 298]	282 [234, 378]	273 [196, 331]	258 [200, 297]	300 [241, 350]	287 [227, 331]	300 [265, 333]	303 [216, 328]	254 [218, 310]	0.17
	Δ_{L-P}	22	−1	37	−1	29	−27	29	17	15	16	0.44
PAPPA (ng/ml)	L	198 ± 66	204 ± 83	254 ± 106	239 ± 85	230 ± 89	240 ± 101	225 ± 123	243 ± 126	240 ± 123	241 ± 126	0.80
	P	186 ± 56	196 ± 64	200 ± 69	192 ± 67	192 ± 57	206 ± 51	191 ± 68	198 ± 44	183 ± 51	196 ± 49	0.29
	Δ_{L-P}	15	12	55	50	44	47	53	45	61	54	0.63
PAPPA2 (ng/ml)	L	0.16 ± 0.08	0.19 ± 0.10	0.19 ± 0.08	0.17 ± 0.08	0.21 ± 0.08	0.23 ± 0.06	0.20 ± 0.08	0.20 ± 0.07	0.20 ± 0.08	0.18 ± 0.06	0.03
	P	0.11 ± 0.03	0.14 ± 0.06	0.16 ± 0.07	0.13 ± 0.07	0.12 ± 0.05	0.18 ± 0.06	0.12 ± 0.04	0.15 ± 0.06	0.13 ± 0.06	0.11 ± 0.06	0.60
	Δ_{L-P}	0.07	0.05	0.03	0.04	0.09	0.05	0.08	0.05	0.07	0.07	0.30
STC-2 (ng/ml)	L	18.6 ± 4.8	21.2 ± 6.1	21.8 ± 7.1	21.4 ± 6.3	19.9 ± 6.6	21 ± 5.6	20.1 ± 6.1	20.9 ± 5.9	21.1 ± 5.8	20.2 ± 5	0.51
	P	21 ± 5.7	23.7 ± 5.8	24.6 ± 4.3	20.9 ± 6.2	22.6 ± 4.4	25.4 ± 5.3	20.2 ± 2.8	24 ± 4.6	23.3 ± 4.1	22.6 ± 5.7	0.31
	Δ_{L-P}	0.91	0.80	−1.00	0.97	0.12	−3.21	0.79	−0.74	−0.77	−1.33	0.56

Values are reported as mean ± SD or median [25th, 75th percentile]. Last observation carried forward was performed for intention to treat analysis.
IGFBP, insulin-like growth factor 1 binding protein; L: Leptin (metreleptin) treatment; PAPPA, pregnancy-associated protein A; P: Placebo treatment; STC-2, stanniocalcin-2; wk, weeks; Δ_{L-P}: Difference between metreleptin and placebo.
[a] p-Value calculated with repeated measures ANOVA for effect of time.
[b] p-Value calculated with repeated measures ANOVA for effect of treatment.
[c] p-Values calculated with repeated measures ANOVA for interaction of time by treatment.

On the contrary, prior data point towards a possible effect of leptin on the GH/IGF-1 axis during chronic energy deprivation. In an open-label uncontrolled study we recruited eight women with HA due to strenuous exercise or low weight to receive metreleptin in replacement doses and followed up them for up to three months [20]. Women with HA had lower total and free IGF-I and higher IGFBP 4 levels at baseline and metreleptin administration increased their IGF-1 levels, while tended to increase free IGF-1 and IGFBP 3 levels after 3 months [5,20]. Another study in women with anorexia nervosa showed that weight regain of ≥10% to a mean BMI of 19.2 mg/kg^2 over a period 1 year restores GH levels, but fails to correct abnormalities in IGF-1 and some IGFBPs observed at baseline versus controls [10], while a previous one had reported normalization of the relevant abnormalities with refeeding and recovery of BMI to a mean of 19.2 kg/m^2 after 2 months [9]. However, based on the primary analysis of our double blinded randomized placebo controlled trial with women with HA (Study 3) with a stable BMI (20.8 kg/m^2 in the metreleptin-treated group and 19.9 kg/m^2 in the placebo group) over the 9-month period of the study there is only a marginal effect of leptin on total IGF-1 and IGFBP 3 [14]. Our current analysis demonstrates that leptin replacement does not affect the additional IGFBPs and related proteases studied herein, expanding and supporting the previous findings [14].

Similarly, a 6-month long placebo controlled trial of overweight and obese participants randomly assigned to metreleptin or placebo in addition to a mild hypocaloric diet over a period of 6 months as part of a weight loss program revealed that even though free leptin levels increased by 6–8-fold in the metreleptin treated group, serum IGF-1 and IGFBP 1 and 3 levels did not change, suggesting no direct role of leptin on these components of the GH/IGF-1/IGFBP axis in leptin resistant overweight and obese women [29].

Congenital leptin deficiency is characterized by hypoleptinemia, normal levels of IGF-1, IGF-2, IGFBP 2, IGFBP 3, and IGFBP 6 and very low postprandial levels of IGFBP 1 [30]. Leptin replacement increased fasting and postprandial IGFBP 1 and IGFBP 2 after 2 months of therapy and resulted in levels at or above normal limit after 18 months of therapy (IGFBP 1: 7-fold and IGFBP 2: 2-fold), without affecting levels of IGF-1, IGFBP 3, and IGFBP 6 but these observations derive from an uncontrolled study [30]. In men with hypoleptinemia and lipoatrophy caused by highly active antiretroviral therapy metreleptin treatment in the context of a randomized, placebo controlled clinical trial for 8 weeks significantly increased leptin levels and improved insulin resistance, but these were not accompanied by any alterations in IGF-1, free IGF-1, or IGFBP 1, IGFBP 2, IGFBP 3 and IGFBP 4 [31].

Serum levels of the molecules measured herein fluctuate throughout the day, but only intact IGFBP 4 exhibits biologically significant fluctuations of >25–35%, peaking at 9 am and falling the most at 9 pm, similar to cortisol's circadian rhythm [32,33]. In our first short-term energy deficit study measurements were performed at 8 am, in the second one at 8 am, 10 am and 4 pm, while in the long-term energy deficit study at 8 am, to minimize any variability stemming from the circadian rhythm of the measured molecules.

Strengths of our study are the double-blinded design of the trials, and the use of accurate, specific and reliable novel ELISA kits validated by both the manufacturer and our laboratory. A limitation of the study is the lack of any preliminary data on the effect of energy deprivation and/or leptin administration on IGFBPs and related proteases that prevented power calculations. For this reason, we enrolled a sufficient number of subjects. This number provides a power of 80% to demonstrate differences of 0.94 per unit of standard deviation in Study 1 and 0.78 per unit of standard deviation in Study 2 at the $a = 0.05$ level. Similarly, for our chronic energy deprivation study, we enrolled a higher number of participants and achieved 80% power to demonstrate differences of 1.42 per unit of standard deviation at the pre-specified $a = 0.05$ level. Subjects were studied during acute energy deprivation in the context of a cross over study design to minimize biological variability while confining them to a Clinical Research Center for the duration of studies 1 and 2 and standardizing diet and exercise to minimize variability due to exogenous environmental factors.

5. Conclusion

We report for the first time that neither acute or chronic energy deprivation nor metreleptin administration, in physiologic replacement, supraphysiologic or pharmacologic doses, significantly alter circulating levels of the intact IGFBPs 3 and IGFBP 4, total IGFBP 5, and/or the proteases PAPPA, PAPPA2 and Stanniocalcin-2.

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Author contributions

CSM designed the study and contributed to the interpretation of data and manuscript writing. EP performed the measurements, conducted the statistical analysis, interpreted the data and wrote the manuscript. NaP performed the measurements and contributed to the interpretation of data. NiP contributed to the interpretation of data and manuscript writing. All authors have read and approved the final version of the manuscript.

Declaration of Competing Interest

EP, NP, NiP have nothing to declare. CSM is advisor of Ansh Labs LLC.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.05.004.

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