Preadipocyte factor-1 concentrations in patients with anorexia nervosa: the influence of partial realimentation

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Short title: Pref-1 in anorexia nervosa

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SUMMARY:

Preadipocyte factor-1 (Pref -1) is a member of epidermal growth-factor like family of proteins that regulates adipocyte and osteoblast differentiation. Experimental studies suggest that circulating Pref-1 levels may be also involved in the regulation of lipid and glucose metabolism and energy homeostasis. **We hypothesized that alterations in Pref-1 levels may contribute to the ethiopathogenesis of anorexia nervosa or its underlying metabolic abnormalities**. We measured Pref-1 concentrations and other hormonal, biochemical and anthropometric parameters in eighteen patients with anorexia nervosa and sixteen healthy women and studied the influence of partial realimentation of anorexia nervosa patients on these parameters. **The mean duration of realimentation period was 46 ± 2 days.** At baseline, anorexia nervosa patients had significantly decreased body mass index, body weight, body fat content, fasting glucose, serum insulin, TSH, free T4, leptin and total protein. Partial realimentation improved these parameters. Baseline serum Pref-1 levels did not significantly differ between anorexia nervosa and control group **(0.26 ± 0.02 vs. 0.32 ± 0.05 ng/ml, p = 0.295)** but partial realimentation significantly increased circulating Pref-1 levels **(0.35 ± 0.04 vs. 0.26 ± 0.02 ng/ml, p < 0.05)**. Post-realimentation Pref-1 levels significantly positively correlated with the change of body mass index after realimentation **(r = 0.49, p < 0.05)**. We conclude that alterations in Pref-1 are not involved in the ethiopathogenesis of anorexia nervosa but its changes after partial realimentation could be involved in the regulation of adipose tissue expansion after realimentation.

INTRODUCTION:

Anorexia Nervosa (AN) is a psychiatric disorder characterized by malnutrition due to chronically decreased food intake caused by distorted body image and inappropriate fear of obesity (Hoek and van Hoeken 2003; Sigman 2003). Detailed ethiopathogenesis of AN is still not completely known (Foppiani *et al.* 1998). Numerous alterations in circulating levels of appetite-regulating hormones, including leptin, ghrelin and others together with various other hormonal and metabolic abnormalities have been previously documented in these patients (Baranowska *et al.* 1997; Dostalova and Haluzik 2009; Dostalova *et al.* 2008; Stoving *et al.* 1999; Tanaka *et al.* 2003). However, most if not all of these changes appear rather secondary to compensate for chronically decreased food intake and malnutrition.

One of the typical features of patients with anorexia nervosa is severely decreased body fat content (Frisch and McArthur 1974). Consequently, endocrine function of adipose tissue is markedly altered with significantly decreased serum leptin concentrations and increased serum adiponectin levels (Dolezalova *et al.* 2007; Grinspoon *et al.* 1996; Haluzikova *et al.* 2009; Housova *et al.* 2005; Tagami *et al.* 2004).

Preadipocyte factor-1 (Pref -1) is a member of epidermal growth-factor like family of proteins that plays an important role in the regulation of adipocyte and osteoblast differentiation (Smas and Sul 1993; Wang *et al.* 2006). **Recent studies in mesenchymal stem cells have demonstrated a possible inverse relationship between osteoblast and adipocyte differentiation since both osteoblasts and adypocytes has common progenitor (Beresford et *al.* 1992, Verma *et al.* 2002) suggesting that Pref-1 might be also indirectly involved in the regulation of bone metabolism and bone mineral density.**

Within adipose tissue, Pref-1 is specifically expressed by preadipocytes but not by adipocytes and therefore it can be used as a preadipocyte marker (Lee *et al.* 2003). Other tissues such as the liver can also produce Pref-1 (Wang *et al.* 2006). Pref-1 is released into the circulation suggesting that it may

have also systemic role in other organs and tissues. Experimental studies have shown that overexpression of Pref-1 in mice lead to lower adipose tissue mass, hypertriglyceridemia, decreased glucose tolerance and lower insulin sensitivity implying a possible role for this factor in the regulation of energy homeostasis and glucose metabolism (Villena *et al.* 2008).

Very little is known about regulation of circulating Pref-1 levels in patients with anorexia nervosa and the influence of partial realimentation on its levels. **Based on the experimental data showing decreased adipose tissue mass and other energy homeostasis alterations in Pref-1 overexpressing mice we hypothesized that alterations in Pref-1 levels may contribute to the ethiopathogenesis of anorexia nervosa or to regulation of response to partial realimentation.** To this end, we measured Pref-1 and other hormonal, biochemical and anthropometric parameters in patients with anorexia nervosa and healthy normal-weight women and studied the influence of partial realimentation on these parameters.

METHODS:

Study subjects

Eighteen previously untreated female patients with AN (age: 24.39 ± 1.21 years; body mass index (BMI): 15.58 ± 0.28 kg/m^2; body fat content 10.31 ± 1.8 %) and sixteen age- and sex-matched healthy controls (age: 22.69 ± 0.76 years; BMI: 21.8 ± 0.51 kg/m^2; body fat content 20.07 ± 1.4 %) were included in the study. The diagnosis of eating disorder was based on Diagnostic Statistical Manual IV diagnostic system (DSM-IV). **Patients were referred by local psychiatrist and they were hospitalized in the Department of Psychiatry, General University Hospital throughout the entire study period.**

A clinical evaluation of the patients was performed by an experienced psychiatrist. The Structured Clinical Interview MINI 5.0 was used for diagnostic assessment of the patients. None of the studied subjects suffered from diabetes mellitus, thyroid disorder and/or acute infectious disease. None of the

studied subjects had malignant tumor. All women included in the study had no allergies. The duration of AN disorder was 9 ± 1.8 years. All of the patients were firstly examined before entering specific treatment programs and were drug-free for more than three months prior to first sampling.

During the realimentation period (46 ± 2 days), all AN patients were prescribed 3 main dishes, two snacks and one second dinner. The realimentation diet contained 50% carbohydrates, 25% fats and 25% proteins; the total daily energy intake was 2300 kcal/day. All meals and snacks were eaten under supervision.

Healthy control women had been free of any medication and had no history of obesity or malnutrition, hypertension, gastrointestinal disease, eating disorder or other psychiatric disorder. Blood tests confirmed normal blood count, liver and renal functions. **All healthy women had regular menstrual cycle while all patients with AN had amenorrhea which was not affected by the realimentation. All patients with AN were hospitalized in the Department of Psychiatry, General University Hospital in Prague. All healthy control women were enrolled into study by local advertisement.**

Written informed consent was provided by all participants before being enrolled in the study. The study was approved by the Human Ethical Review Committee, First Faculty of Medicine and General University Hospital, Prague, Czech Republic, and was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

Anthropometric examination and blood sampling

All patients with AN were examined twice; at a basal state before the beginning of any treatment and after two months of partial realimentation while normal-weight healthy women were examined only once. All subjects were measured and weighted and BMI was calculated. Body fat content was estimated by bioimpedance analysis (Bodystat 1500, Bodystat Ltd., UK) and by dual energy X-ray absorptiometry (DEXA). Bone mineral density was also estimated by DEXA on QDR 4500A bone

densitometer (Hologic, Waltham, MA, USA). Resting energy expenditure was determined by indirect calorimetry (Vmax ENCORE Viasys[TH] HEALTHCARE, SensorMedics BV, Netherlands). Blood samples for Pref-1, other hormonal and biochemical parameters measurements were withdrawn between 0700 and 0800 h after 12 h of overnight fasting. **All of the blood drawings in control group were performed at the beginning of follicular phase of menstrual cycle 3-5 days after the end of menstrual period. Both the AN patients and control subjects were instructed to refrain from food intake after 7 PM on the day preceding the blood drawing. Water was the only drink allowed to all subjects after 7 PM.** Serum was separated by centrifugation and stored at - 80°C until being assayed.

Hormonal and biochemical assays

 Serum Pref-1 concentrations were measured by a commercial ELISA kit (R&D Systems, Minneapolis, U.S.A.). The sensitivity was 12 pg/ml, and the intra- and interassay variability was less than 5.0 and 15.0 %, respectively.

 Serum insulin concentrations were measured by a commercial RIA kit (Cis Bio International, Gif-sur-Yvette, France). Sensitivity was 2.0 μIU/ml, and the intra- and interassay variability was 4.2 and 8.8 %, respectively.

Serum leptin concentrations were measured by commercial ELISA kit (BioVendor, Brno, Czech Republic). Sensitivity was 0.12 ng/ml and the intra- and interassay variability was 1.7 and 8.0 %, respectively.

Serum levels of TSH and free T4 were measured by chemiluminiscence immunoassay (CLIA) on ADVIA Centauer analyzer (Siemens, Healthcare Diagnostics Inc, Tarrytown, NY, USA). Sensitivity were 0,004 mIU/L for TSH and 0,1 ng/dl for free T4, and the intra- and interassay variability was less than 5% for both measurement. Serum biochemical parameters (glucose, total and HDL-

cholesterol, triglycerides and albumin) were measured by standard laboratory methods on Modular SWA analyzer (Roche Diagnostics, GmbH, Mannheim, Germany), the value of LDL-cholesterol was calculated by Friedewald formula.

Statistical analysis

The statistical analysis was performed on SigmaStat software (Jandel Scientific, San Rafael, CA). Results are expressed as mean ± S.E.M (the standard error of the mean). Unpaired t-test or Mann-Whitney test were used for comparisons of serum parameters between control group and group of patients with AN as appropriate. Paired t-test or Wilcoxon signed rank test were used for the comparisons of serum Pref-1 levels and of other studied parameters in patients with AN before and after partial realimentation as appropriate. The correlations between the values were estimated by Spearman correlation test. A p value < 0.05 denoted statistical significance.

RESULTS

Anthropometric, hormonal and biochemical characteristics of control group of normal-weight healthy women and patients with anorexia nervosa before and after partial realimentation are shown in Table 1. At baseline, body weight, body mass index (BMI), body fat content, fasting glucose, serum insulin, TSH, free T4, leptin, total serum protein and resting energy expenditure, femoral and lumbar bone mineral density in patients with anorexia nervosa were significantly lower relative to control subjects. Age and serum albumin concentrations did not significantly differ beween the groups.

Partial realimentation of patients with anorexia nervosa significantly increased body weight, body mass index, body fat content, fasting glucose, serum insulin, serum leptin, total serum protein, serum albumin and resting energy expenditure.

Serum Pref-1 concentrations are shown in Figure 1. **Pre-treatment serum Pref-1 levels in patients with anorexia nervosa did not significantly differ from those in control subjects (0.26 ± 0.02 vs. 0.32 ± 0.05 ng/ml, p= 0.295), also post-treatment serum Pref-1 levels in patients with AN did not significantly differ from those in control subjects (0.35 ± 0.04 vs. 0.32 ± 0.05 ng/ml, p= 0.578)**. Partial realimentation significantly increased serum Pref-1 levels (p= 0.015) (Fig. 3).

Relationships between Pref-1 concentrations and other anthropometric, biochemical and hormonal parameters were calculated using Spearman correlation test in our whole group of study subjects which included both the pre-alimentation patients with AN and the control subjects.

At baseline, no significant relationships between circulating Pref-1 and other studied parameters were found. **The same was true for relationship between changes in Pref-1 levels and changes in BMI after realimentation (p= 0.161).** The only significant relationship detected was a positive correlation between post-treatment Pref-1 levels and the change of BMI after realimentation of anorexia nervosa patients (Fig. 2) while no significant

DISCUSSION

The most important finding of this study is that partial realimentation of patients with anorexia nervosa significantly increased circulating concentrations of Pref-1. **On the contrary, nor the baseline or postreatment Pref-1 levels in anorexia nervosa group significantly differed from those of healthy normal-weight women suggesting that dynamic change in circulating Pref-1 levels might be more important that its absolute concentrations under the circumstances of realimentation.**

Pref-1 is a 385 amino acid protein belonging to the family of epidermal growth factor-like repeat containing proteins (Sul 2009). In humans, it is highly expressed by preadipocytes but not by adipocytes. Other Pref-1 expressing cells include pancreatic islet cells, thymic stromal cells and adrenal gland cells (Sul 2009). Experimental studies have demonstrated that circulating Pref-1 could directly contribute to the regulation of adipose tissue stores and lipid and glucose metabolism (Lee et al. 2003; Moon *et al.* 2002; Villena et al. 2008). Mice with Pref-1 knockout show growth retardation and skeletal abnormalities as well as increased adiposity when fed a high fat diet (Moon et al. 2002) supporting the role of Pref-1 in the regulation of adipocyte differentiation. On the contrary, young adult mice overexpressing soluble Pref-1 exhibit a marked reduction in white adipose tissue mass as a result of impaired adipocyte differentiation (Lee et al. 2003). These mice are resistant to high fat diet-induced obesity but they develop hypetriglyceridemia, impaired glucose tolerance and insulin resistance mostly due to inability to expand adipose tissue mass (Villena et al. 2008).

Only one human study to date was focused on the changes circulating Pref-1 levels in humans **(Fazeli *et al.* 2010)**. In this study, Fazeli and colleagues describe significantly higher circulating Pref-1 levels in untreated patients with anorexia nervosa compared to healthy normal weight women. Furthermore, Pref-1 concentrations in this study positively correlated with marrow fat of the proximal femoral metaphysis. In contrast to Fazeli´s results, we did not find significant differences in circulating Pref-1 levels between anorexia nervosa patients and healthy normal-weight women. The reason for the differences in Pref-1 concentrations in Fazeli´s and ours study is unclear. It may be caused by the fact that our group of anorexia nervosa patients was leaner compared to patients in Fazeli´s study and possibly also by different disease duration or other unknown factors. Importantly, partial realimentation in our study significantly increased circulating Pref-l levels suggesting that the change in nutritional status can significantly influence Pref-1 production and/or its release from adipose tissue. This finding is interesting given the fact that we were not able to detect any significant relationship of circulating Pref-1 levels with anthropometric or biochemical

nutritional parameters in our group of study subjects. Overall, these findings suggest that dynamic changes of nutritional state but not its chronic status influence Pref-1 concentrations. **Nevertheless, it should be mentioned that our study only tested dynamic changes of Pref-1 in anorexia nervosa group after realimentation while no overalimentation was performed in control group. Therefore the changes observed in anorexia nervosa group cannot be generally extrapolated to other realimentation or overalimentation states such as obesity.**

The role of circulating Pref-1 mostly remains to be elucidated (Lee et al. 2003). Both *in vitro* and *in vivo* experimental studies have shown that Pref-1 is a strong inhibitor of adipocyte differentiation suggesting its major paracrine role in adipose tissue (Smas and Sul 1993). Increased Pref-1 levels in Pref-1 overexpressing transgenic mice were also associated with increased triglyceride concentrations, impaired glucose tolerance and decreased insulin sensitivity (Villena et al. 2008). Experimental data suggest that metabolic abnormalities in Pref-1 overexpressing mice were probably secondary due to partial lipodystrophy caused by impaired adipocyte differentiation rather than due to direct negative metabolic effects of Pref-1 in other tissues or organs (Lee et al. 2003).

In addition to increased Pref-1 levels in patients with anorexia nervosa after partial realimentation we also found that post-realimentation Pref-1 levels significantly correlated with change of BMI after partial realimentation. This relationship may suggest that the production of Pref-1 by adipose tissue after realimentation reflects its differentiation capacity and/or number of preadipocytes. The patients with higher Pref-1 levels and higher preadipocytes number in adipose tissue thus could gain more weight compared to patients with lower number of preadipocytes and Pref-1 levels. However, at this point this hypothesis remains only speculative and has to be directly tested in other studies.

In conclusion, we have demonstrated that circulating levels of Pref-1 in untreated patients with anorexia nervosa do not differ from those of healthy normal-weight women but they are significantly increased by partial realimentation. The relationship between post-realimentation Pref-1 levels and

increase in body mass index of anorexia nervosa patients suggest that Pref-1 concentration after

realimentation may reflect the differentiation capacity of adipose tissue in anorexia nervosa patients.

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Table 1 Anthropometric, hormonal and biochemical characteristics of the control group of normal-weight healthy women and patients with anorexia nervosa before and after partial realimentation.

Values are means ± SEM. Statistical significance is from unpaired and paired t-test or Mann-Whitney test.

[a] P < 0.05 vs. controls
[b] P < 0.05 vs. anorexia nervosa before realimentation

|	Controls (n= 16)	AN before realimentation (n=18)	AN after realimentation (n=18)
Age (years)	22.69 ± 0.76	24.39 ± 1.21	24.39 ± 1.21
Weight (kg)	64.23 ± 1.91	42.59 ± 0.98 [a]	47.63 ± 0.91 [a, b]
Body mass index (kg/m^2)	21.80 ± 0.51	15.58 ± 0.28 [a]	17.44 ± 0.24 [a, b]
Body fat content (bodystat) (%)	20.07 ± 1.4	10.31 ± 1.8 [a]	13.3 ± 1.32 [a, b]
Body fat content (DEXA)%	26.19 ± 1.52	14.86 ± 1.58 [a]	18.71 ± 1.41 [a, b]
Resting energy expenditure (kcal/day)	1405.75 ± 30.63	1055.44 ± 35.81 [a]	1166.00 ± 24.02 [a, b]
Total cholesterol (mmol/l)	4.32 ± 0.19	7.64 ± 3.10	5.18 ± 0.24 [a]
HDL-cholesterol (mmol/l)	1.76 ± 0.09	1.57 ± 0.10	1.63 ± 0.1
LDL-cholesterol (mmol/l)	2.15 ± 0.13	2.37 ± 0.17	2.9 ± 0.20 [a]
Triacylglycerides (mmol/l)	0.91 ± 0.12	1.52 ± 0.16 [a]	1.46 ± 0.17 [a]
Fasting glucose (mmol/l)	4.38 ± 0.08	3.97 ± 0.07 [a]	4.26 ± 0.08 [b]
Serum insulin (µUI/ml)	19.29 ± 1.12	15.79 ± 0.75 [a]	16.1 ± 0.95 [a,b]
TSH (mIU)	2.25 ± 0.27	1.45 ± 0.21 [a]	-
Free T4 (mIU)	14.19 ± 0.50	12.35 ± 0.51 [a]	-
Leptin (ng/ml)	11.8 ± 1.78	0.97 ± 0.23 [a]	2.62 ± 0.66 [a, b]
Total serum protein (g/l)	78.4 ± 1.28	69.66 ± 1.84 [a]	75.26 ± 0.97 [b]
Albumin (g/l)	46.36 ± 0.99	44.23 ± 1.03	47.32 ± 0.66 [b]
Total bone mineral density (g/cm2)	1.08 ± 0.02	1.05 ± 0.02	1.04 ± 0.02 [a]
BMD femoral (g/cm2)	0.96 ± 0.02	0.78 ± 0.03 [a]	-
BMD lumbal (g/cm2)	0.99 ± 0.03	0.86 ± 0.03 [a]	-
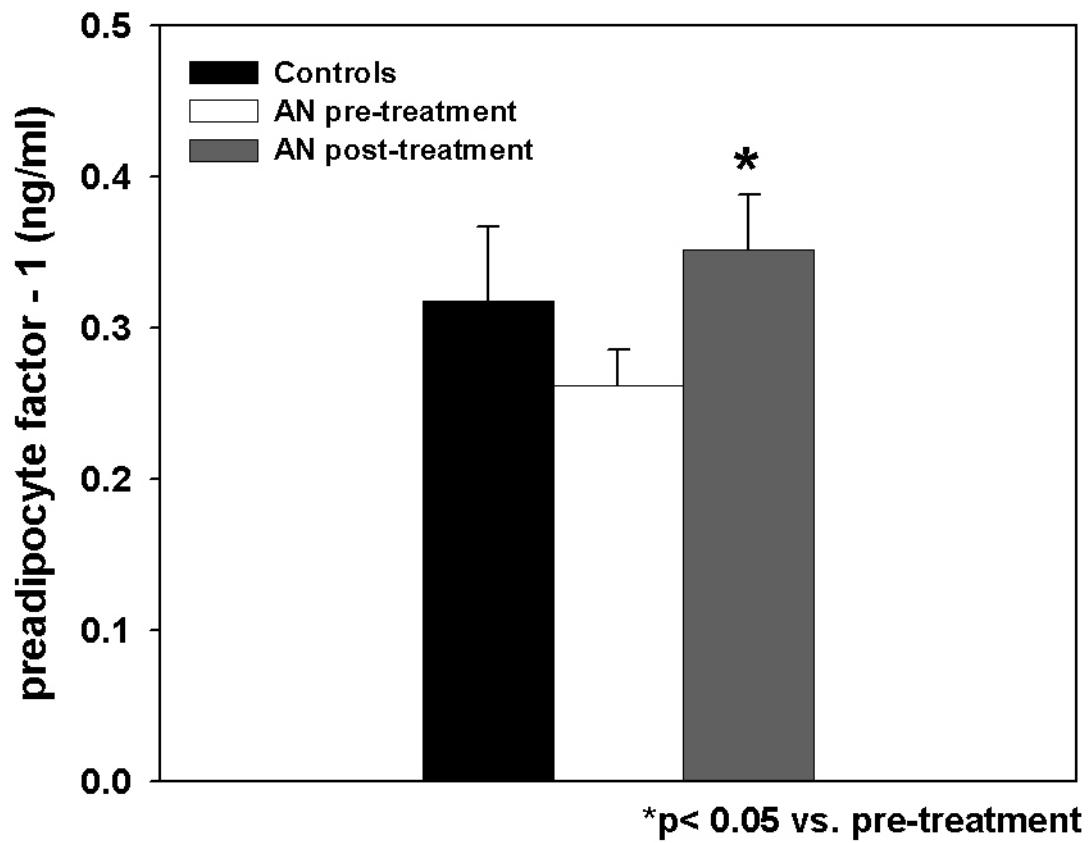
Figure 1 Serum concentrations of preadipocyte factor -1 in control group (black bar) and patients with anorexia nervosa before (open bar) and after realimentation (gray bar).

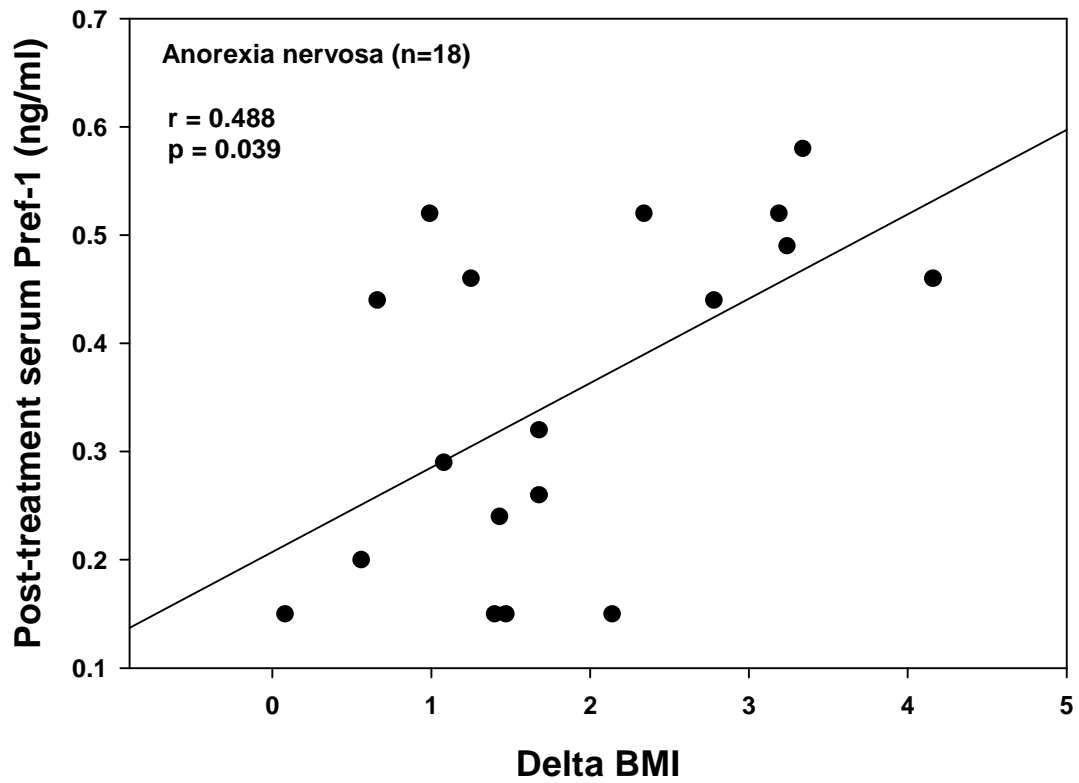## Relationship of post-treatment serum Pref-1 concentrations with delta BMI during nutritional treatment in AN

Post-treatment serum Pref-1 (ng/ml)

Anorexia nervosa (n=18)

r = 0.488
p = 0.039

Delta BMI

Figure 2 Relationship of post-treatment preadipocyte factor – 1 concentrations and the change of body mass index after realimentation.
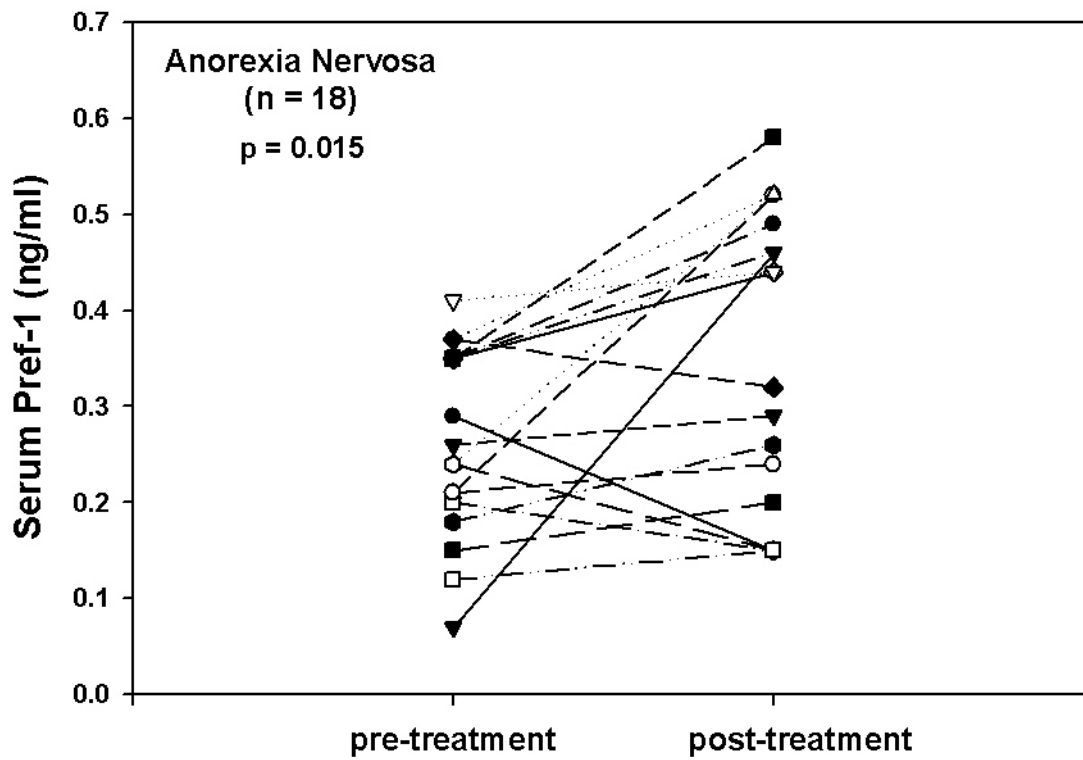
Figure 3 The influence of partial refeeding on serum concentrations of preadipocyte factor – 1 (ng/ml) shown for each patient with anorexia nervosa individually (AN; n = 18).