

Differences in zinc status and the leptin axis in anorexic and recovered adolescents and young adults: a pilot study

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Abstract

Background: Evidence from animal studies suggests that leptin metabolism is associated with zinc (Zn) status. However, research investigating this relationship in adolescents and young adults with anorexia nervosa (AN) is scarce; the present study aims to fill that gap.

Methods: Serum concentrations of leptin, the soluble leptin receptor (sOB-R) and the free leptin index (FLI) were obtained in healthy control subjects ($n = 19$), acutely ill individuals ($n = 14$) and recovered patients with AN ($n = 15$). Serum Zn concentrations noted in previous research data were also incorporated for all groups.

Results: Leptin, FLI and Zn concentrations were higher in recovered subjects with AN when compared with acutely ill AN patients. Remitted patients showed higher sOB-R concentrations but no difference in FLI compared with the control group. Leptin and FLI were lower in the acutely ill patients compared with the control subjects, who showed no differences in Zn concentrations. Zn concentrations were not correlated with leptin, sOB-R or FLI concentrations in any of the three investigated subgroups.

Conclusions: The present investigation does not entirely support an association between Zn, Leptin and FLI concentrations in subjects with AN, possibly due to limited statistical power. Further research and replication of the present findings related to the interaction between leptin and Zn is warranted. However, with respect to serum leptin levels the data of the present investigation indicate that acutely ill and remitted patients with AN differ as regards serum leptin concentrations and FLI, which is in line with previous research.

Keywords: *anorexia nervosa; leptin; leptin receptor (sOB-R); free leptin index (FLI); zinc*

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Past research has indicated that the hormone leptin is a major player when it comes to triggering the adaptation of the human body to severely restricted food intake (1–3). Studies focusing on the regulation of leptin levels during a zinc (Zn) deficiency showed that circulating leptin concentrations are diminished during

Zn deficiency in rats and humans (4, 5). There seem to be two mechanisms responsible for this phenomenon: a decrease in the amount of leptin produced per gram of adipose tissue (6), and an overall reduction in body fat during Zn deficiency (7). With respect to previous findings, marginal Zn deficiency in rats decreased leptin

expression independently of food intake, while decreasing corticotrophin-releasing hormone in relation to food intake (8), suggesting that leptin expression may be directly affected by Zn. Further research suggests that decreased food intake, rather than Zn deficiency itself, is associated with changes in plasma leptin, metabolic rate and activity levels in Zn deficient rats; thus, it seems that low serum leptin concentrations, hypometabolism, and decreased activity are more likely the result of anorexia than of Zn deficiency *per se* (9, 10). However, data investigating this relationship in adolescent humans and young adults with anorexia nervosa (AN) do not exist.

Research has indicated that the free leptin index (FLI) is a valuable tool for investigating the leptin axis during growth and sexual maturation (11). FLI is calculated using the ratio of leptin to the soluble leptin receptor (sOB-R). In human blood, leptin is bound by a high-affinity binding protein; this particular protein appears to be identical to the sOB-R. We previously reported differences in serum Zn concentrations between remitted and acutely ill patients with AN (12), with elevated serum Zn concentrations in remitted patients. This difference was most likely linked to reported dietary preferences, in particular as regards Ca^{2+} and phosphorus containing foods which can influence absorption of Zn.

Ca^{2+} and phosphate can decrease Zn absorption, and previous findings in this sample demonstrated that acutely ill patients had higher serum Ca^{2+} and inorganic phosphate concentrations when compared to controls, and also preferred foods containing Ca^{2+} and phosphorus (12).

In view of the expected correlation between altered serum leptin levels and Zn metabolism, we aimed to investigate potential interactions between Zn status and the leptin axis in acutely ill and remitted AN patients in comparison with healthy controls. The data on Zn concentrations referred to in this paper are published separately (12). In line with previous research, we hypothesised that acutely ill patients would present with lower serum leptin concentrations when compared with remitted patients. We also expected that altered serum leptin levels would be correlated with Zn concentrations (4, 5), in particular in the group of acutely ill patients.

Methods

Study sample

Forty-eight participants were enrolled in the study (for characteristics see Table 1). The study sample and the respective subgroups are identical to those used in previous research studies (12). The present work focuses on the interaction of serum Zn concentrations with leptin, sOB-R and FLI.

Acutely ill patients ($n = 14$) met weight and diagnostic criteria for AN (ICD-10, BMI <10th percentile or

BMI <17.5, with a mean duration of illness of 6.1 ± 3.7 years). All subjects were administered the Structured Interview for Anorexia and Bulimia Nervosa (SIAB) (13–15) and the Eating Disorders Inventory Symptom Checklist (EDI-SC) (16). Secondary school students, medical students and associated staff of a major mental health service served as a control group ($n = 15$); they did not display eating disorder symptoms and had normal menses (12). Other co-morbid psychiatric disorders were ruled out using clinical interviews. The remitted patients ($n = 19$) were sent letters inviting them to participate in the study. A BMI of at least 18 was required for the group of remitted patients; all remitted subjects were symptom-free based on the SIAB. Moreover, recovered patients needed to have maintained a normal weight for at least 1 year prior (mean period of recovery 6.0 ± 2.7 years) without vomiting, bingeing, or irregular menses (mean duration of regular menstruation after amenorrhea during acute illness: 6.3 ± 3.3 years; mean duration of amenorrhea during acute illness: 11.2 ± 10.5 months (12). Participants in the remitted group with abnormal blood chemistry (e.g. serum thyroid hormones, liver enzymes and electrolytes) were not included in the analysis. Previous and current medical diseases were ruled out using patient history, and acute as well as recent infections met exclusion criteria for participation. Data on the intake of dietary supplements as well as medications are provided in a different publication (12). Subjects who took oral contraceptives did not differ from subjects who did not in regard to serum Zn concentrations (12), which was examined because the intake of oral contraceptives has been associated with lower Zn concentrations (17, 18). The study protocol was assessed and approved by the Ethics Committee of the Faculty of Medicine at J.W. Goethe University, Frankfurt am Main, Germany. The investigation was carried out in accordance with the Declaration of Helsinki.

Assessment

Blood samples from the healthy control group and the remitted group were obtained during a single visit at the above-mentioned mental health service (in addition, assessment of weight, height, BMI, SIAB and EDI-SC took place). Blood samples for the assessment of Zn status in acutely ill patients were obtained following admission. Trace mineral-free plastic tubes containing 2% sodium oxalate were used for blood sampling. The obtained blood samples were then placed on ice. An atomic absorption spectrophotometer (PerkinElmer, Germany; intra-assay imprecision was between 2.7 and 3.2%, inter-assay imprecision was between 4.3 and 5.3%) was used to determine serum Zn concentrations (Johannes Gutenberg-University) (12). Other routine laboratory tests (e.g. electrolytes, liver enzymes) were

Table 1. Characteristics of the control group ($n = 15$), acutely ill patients with anorexia nervosa (AN, $n = 14$) and remitted patients with AN ($n = 19$)

	Healthy control subjects ($n = 15$)			Acutely ill patients with AN ($n = 14$)			Remitted patients with AN ($n = 19$)			Controls vs. acutely ill patients	Controls vs. remitted patients	Acutely ill vs. remitted patients		
	$M \pm SD$	Min.	Max.	$M \pm SD$	Min.	Max.	$M \pm SD$	Min.	Max.	p_1	p_2	p_3		
Age (years)	24.80 \pm 2.78	20.42	29.91	20.75 \pm 4.71	14.58	29.50	24.24 \pm 3.31	17.42	29.91	0.008	ns	0.018		
Weight (kg)	59.06 \pm 6.18	50.00	71.30	45.92 \pm 6.14	39.00	58.9	58.22 \pm 6.00	50.10	70.80	0.000	ns	0.000		
Height (cm)	167.43 \pm 5.60	158	176	166.63 \pm 6.95	157	181	165.53 \pm 5.4	158	177	ns	ns	ns		
BMI (kg/m ²)	21.06 \pm 2.02	17.81	24.98	16.45 \pm 1.26	13.68	16.45	21.13 \pm 1.86	18.18	24.50	0.000	ns	0.000		
Zinc (μ g/dl)	59.95 \pm 11.25	33.40	77.80	62.23 \pm 13.78	40.30	89.50	81.75 \pm 16.2	52.67	114	ns	0.000	0.001		
Leptin (ng/ml)	10.10 \pm 6.42	0.03	25.9	4.53 \pm 5.8	0.001	23.4	11.92 \pm 7.37	2.77	27.30	0.021**	ns	0.004*		
sOB-R (ng/ml)	18.94 \pm 5.77	9.0	28.6	21.30 \pm 6.77	13.4	34.4	24.79 \pm 9.81	12.8	47.5	ns	0.051**	ns		
FLI	60.8 $\times 10^3 \pm 48.6 \times 10^3$	0.1 $\times 10^3$	74.70	159.6 $\times 10^3$	24.6 $\times 10^3$	35.0 $\times 10^3$	0.005 $\times 10^3$	141.8 $\times 10^3$	54.3 $\times 10^3 \pm 36.0 \times 10^3$	13.6 $\times 10^3$	128.8 $\times 10^3$	0.031**	ns	0.027**

Data are presented with mean (M), minimum (Min.), maximum (Max.), and standard deviation (SD). Mean values were compared (t -tests, two-tailed) for significant differences between controls and acutely ill patients with AN (p_1), controls and remitted patients with AN (p_2) and acutely ill patients with AN versus remitted patients with AN (p_3).

sOB-R – soluble leptin receptor; FLI – free leptin index.

*Excellent power (0.99); **moderate power (0.8–0.9).

performed at the Department of Laboratory Medicine at J.W. Goethe University in Frankfurt am Main.

Leptin

Leptin was measured by an in-house RIA (11, 19), with intra-assay and inter-assay coefficients of variation lower than 12.5% (range: 1 and 8 ng/ml). Recovery of dilution experiments (undiluted to 1:20) was 88–112% for the concentration range between 4 and 6 ng/ml. Leptin levels of the in-house RIA were comparable with the levels of commercially available leptin RIA (Mediagnost, Tuebingen, Germany) in the sera of normal-weight and obese subjects.

sOB-R and FLI

Concentrations were determined by an in-house ligand-immunofunctional assay (11). Briefly, microtiter plates with anti-sOB-R IgG coated wells were used, with the anti-sOB-R IgG having been raised in rabbits against recombinant sOB-R (R&D, Wiesbaden, Germany). Incubation with PBS, 1% bovine serum albumin for 1 h at room temperature was performed in order to block potentially unspecific binding sites. Either rsOB-R standards (10 μ l), ranging from 6.25 and 100 ng/ml, or the serum samples were added to the wells. The rsOB-R standards were incubated overnight at 4°C with an excess of biotinylated leptin in assay buffer (1% rabbit globulin, 0.1% BSA and 0.05% Tween 20 in PBS). An established recombinant sOB-R preparation was used for calibration purposes (11, 20). A parallel course between rsOB-R and native sOB-R was found. Europium-labeled streptavidin (PerkinElmer, Germany) was used to detect the complex of biotinylated leptin and sOB-R. The plate was washed with PBS (0.05% Tween 20) each time between coating, blocking, incubation and detection. The Victor-system (PerkinElmer, Germany) was used to measure the solid phase-linked fluorescence signal of Europium. The resulting signal was dependent on both immunological recognition of the sOB-R and leptin binding. Intra-assay and inter-assay coefficients of variation for two of the control samples were lower than 11.7%. As was shown previously, the assay used was nearly insensitive to the interference of leptin (11).

The free leptin index (FLI) was determined by calculating the ratio of the levels of leptin to sOB-R, multiplied by 100.

Zn

As reported previously, trace mineral-free plastic tubes containing 2% sodium oxalate were used to collect blood samples and then placed on ice (12). An atomic absorption spectrophotometer (PerkinElmer, Germany) was used to assess serum Zn concentrations.

Data analysis

The PASW Statistics 18 software package (SPSS, Chicago, USA) was used for data analysis. Serum leptin concentrations, sOB-R and FLI were normally distributed (Kolmogorov-Smirnov goodness of fit test). Statistical significance was assumed for $p < 0.05$. *t*-tests (two-tailed) were implemented for between-group comparisons. Pearson correlations (two-tailed) were used for additional dimensional analyses in regard to correlations between Zn concentrations and leptin, sOB-R and FLI in the different subgroups. Because of the explorative nature of this investigation, significant *p*-values were not subjected to alpha-adjustment to avoid overlooking potential effects and interactions. The data on Zn concentrations had been previously subjected to alpha-adjustment (12) in accordance with the Bonferroni–Holm procedure (21). Adjusted *p*-values in regard to Zn concentrations are indicated by p_{adj} .

Results

Comparison of subgroups

Acutely ill versus remitted

Serum leptin concentrations ($t = -3.108$, $F = 4.338$, $p = 0.004$, $df = 1,31$) and FLI ($t = -2.329$, $F = 1.578$, $p = 0.027$, $df = 1,30$) were higher in remitted subjects compared with acutely ill patients, but there were no differences in sOB-R concentrations between those two subgroups (see Table 1, Fig. 1a–c). As previously mentioned, remitted AN patients with had significantly higher serum Zn levels compared with the acutely ill AN patients ($t = -3.642$, $F = 0.522$, $p_{\text{adj}} = 0.002$, $df = 1,31$; see Fig. 1d and (12)).

Remitted versus controls

There was nearly a statistically significant difference in sOB-R concentrations ($t = -2.031$, $F = 4.565$, $p = 0.051$, $df = 1,31$), with remitted patients showing higher sOB-R concentrations (see Fig. 1b). There were no differences in FLI concentrations between controls and remitted patients ($t = 0.440$, $F = 0.906$, $p > 0.05$, $df = 1,31$; see also Fig. 1c). Remitted AN patients had significantly higher Zn levels than the control subjects ($t = -4.435$, $F = 2.427$, $p_{\text{adj}} = 0.000$, $df = 1,32$; see Fig. 1d and (12)).

Acutely ill versus controls

As shown in Fig. 1a and c, serum leptin concentrations ($t = 2.447$, $F = 1.310$, $p = 0.021$, $df = 1,27$) and FLI were higher in control subjects compared with acutely ill AN patients ($t = 2.276$, $F = 2.905$, $p = 0.031$, $df = 1,27$). There was no significant difference in Zn serum levels between acutely ill patients and controls ($t = -0.490$, $F = 0.644$, $p > 0.05$, $df = 1,27$; see Fig. 1d and (12)).

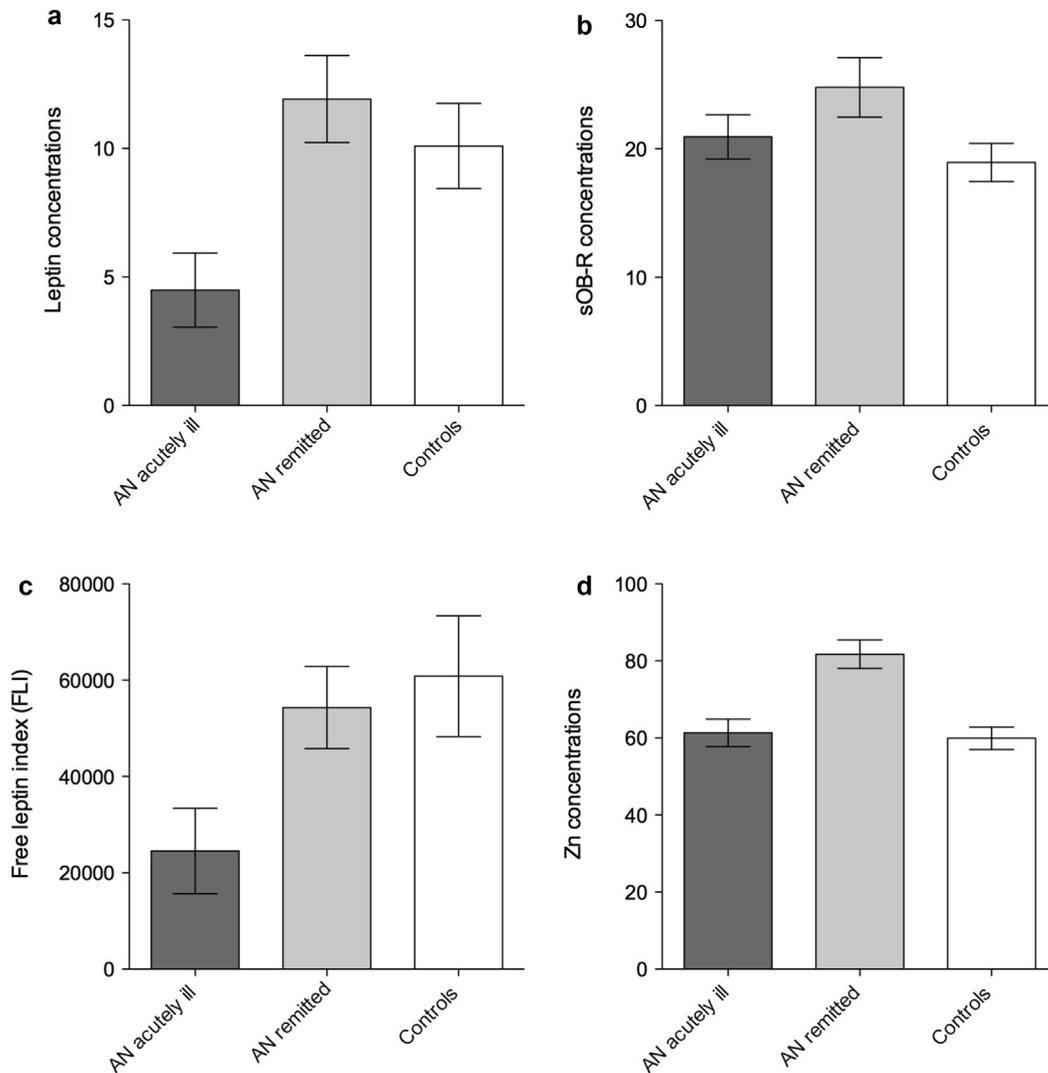


Fig. 1. Serum concentrations obtained in adolescents and young adults of (a) leptin (ng/ml), (b) the soluble leptin receptor (sOB-R, ng/ml), (c) free leptin index (FLI) and (d) zinc (Zn, µg/dl) with mean and SD for acutely ill ($n = 14$) and recovered patients ($n = 15$) with anorexia nervosa (AN) and for healthy controls ($n = 19$).

Dimensional analysis

Controls

Serum Zn concentrations were not correlated with serum leptin ($r_{xy} = -0.114$; $p > 0.05$), sOB-R ($r_{xy} = 0.091$; $p > 0.05$) or FLI ($r_{xy} = -0.173$; $p > 0.05$).

AN acutely ill

Zn concentrations were not correlated with serum leptin ($r_{xy} = 0.253$; $p > 0.05$), sOB-R ($r_{xy} = -0.006$; $p > 0.05$) or FLI ($r_{xy} = 0.229$; $p > 0.05$).

AN remitted

There were no correlations between serum Zn concentrations and serum leptin ($r_{xy} = 0.200$; $p > 0.05$), sOB-R ($r_{xy} = -0.158$; $p > 0.05$) or FLI ($r_{xy} = 0.342$; $p > 0.05$).

Post-hoc power analysis (for a significance level [α] of 0.05, two-tailed)

In order to estimate sample sizes for future large-scale studies, we calculated the power of the present study after completion.

Leptin

The experiment had a power of 0.60 to detect the smallest average difference in serum leptin concentrations (5.21 ng/ml) between pairs of controls ($n = 15$) and acutely ill patients ($n = 14$); the difference between controls and acutely ill patients was found to be 5.56 ng/ml. We also estimated the statistical power with respect to the limitations of the intra- and interassay variance. To detect a difference between means of 3.40 ng/ml [a difference calculated for a mean in the control group of

8.84 ng/ml (actual mean value of 10.10 ng/ml – 12.5%) and a mean of 5.80 ng/ml for acutely ill patients (actual mean value of 4.53 ng/ml + 12.5%)] an adjusted power of 0.30 was calculated *post-hoc*. Future studies should take such limitations into account with respect to the used assay. When comparing remitted patients and controls, a power of 0.85 was calculated, detecting a difference between means of 7.46 ng/ml (the found difference was 7.39 ng/ml). For the comparison of remitted and acutely ill patients, a power of 0.10 was calculated to detect a difference between means of 1.67 ng/ml (the found difference was 1.83 ng/ml). Consequently, the present study had good power to detect differences in the means between controls and remitted patients, and only moderate power to detect differences between controls and acutely ill patients. However, the present study was underpowered in regard to the comparison of remitted patients and acutely ill patients.

FLI

In regard to FLI, the study had a power of 0.60 to detect a smallest average difference between pairs of 36.4×10^3 between controls and acutely ill patients (the difference found was 36.3×10^3). A power of 0.6 was also calculated to detect a smallest difference between pairs of 28.7×10^3 (the difference was 30.0×10^3) between remitted and acutely ill patients. For the comparison between remitted patients and controls, a power of 0.10 was calculated to detect a difference between means of 10.2×10^3 (but the difference was discovered to be 6.5×10^3). With this in mind, with respect to FLI, the present study had moderate power to detect significant differences in means between controls and acutely ill patients, as well as between acutely ill and remitted patients. However, in regard to the FLI comparison between remitted patients and controls, the present study was underpowered.

sOB-R

The study had a power of 0.50 to detect a smallest difference between means of control and remitted subjects of 5.80 ng/ml (the difference found was 5.85 ng/ml). When comparing acutely ill and remitted patients, a power of 0.2 was calculated to detect a difference between pairs of 3.54 ng/ml (the difference found was 3.49 ng/ml). For the comparison of acutely ill patients and controls, a power of 0.20 was calculated to detect a difference between these groups of 2.71 ng/ml (the difference found was 2.36 ng/ml). As a consequence, the present study only had low moderate power to detect differences between controls and remitted patients, but was underpowered in finding differences between acutely ill and remitted patients, as well as acutely ill patients and controls.

Discussion

Along with data from previous research, the main results of the present work are:

1. Serum leptin levels and FLI, as well as serum Zn concentrations – mainly due to dietary preferences as regards Ca^{2+} and phosphorus containing foods (12) – were higher in remitted AN subjects compared with acutely ill patients.
2. Remitted patients showed significantly higher concentrations of the sOB-R but no difference in FLI when compared with healthy controls (while remitted patients were shown to have higher serum Zn concentrations).
3. Serum leptin levels and FLI were lower in acutely ill patients compared with controls, who displayed no differences in serum Zn concentrations. Additionally, serum Zn concentrations were not correlated with serum leptin, sOB-R or FLI concentrations in any of the three investigated subgroups (acutely ill patients, remitted patients and controls).

The present data are in line with previous research on the leptin state in AN showing lowered leptin concentrations in acutely ill patients with AN, and that weight gain is associated with increased leptin levels or even hyperleptinaemia (22). However, the association between the stage and state of AN (i.e. acutely ill vs. remitted) in humans and changed serum leptin levels, FLI and Zn concentrations is not clear, particularly with respect to animal data obtained in rats suggesting that leptin expression may be directly affected by Zn (8). The data of the present investigation do not entirely support an association between serum Zn concentrations and different indices of the leptin axis, in particular because there were no significant correlations between serum Zn and leptin, sOB-R and FLI in any subgroup. In our study, remitted patients showed elevated serum leptin levels; because of this, FLI and Zn concentrations might be considered indicative of such a relationship, with respect to the stage of illness in younger patient populations.

However, the data provided by the present study must be considered preliminary, in particular because differences in serum Zn concentrations are likely to depend on reported food preferences (12). In addition, research proposing decreased food intake, rather than Zn deficiency itself, being associated with changes in plasma leptin availability in Zn-deficient rats must be mentioned here (9, 10). Accordingly, these findings assume that low serum leptin concentrations, hypometabolism and diminished activity levels are more likely the result of anorexia *per se* than of Zn deficiency alone (9, 10). Notably, in our study, remitted patients showed higher sOB-R and Zn concentrations when compared with healthy controls,

but there was no difference in FLI. Moreover, there was no difference in sOB-R concentrations observed between acutely ill and remitted patients with AN. Following this, more studies with larger samples are warranted in order to further investigate if changes in leptin expression are associated with differences in serum Zn status in patients with AN.

Unambiguous clinical conclusions cannot be drawn from the present study. However, if the finding that remitted patients with AN showed increased serum Zn, FLI and leptin concentrations could be replicated in future large-scale studies, one could speculate that leptin expression is associated with altered serum Zn status in remitted patients; this would somewhat align with the aforementioned animal data (8). One could speculate that changed leptin expression in response to normalised or even elevated Zn concentrations in remitted patients, which is likely to be influenced by dietary factors (12), might reflect an adaptive mechanism with respect to the state of the illness, possibly in order to prevent relapse. However, such assumptions remain speculative at this stage. Randomised double-blind placebo-controlled trials with Zn supplementation and determination of serum leptin, sOB-R concentrations and FLI, also involving longitudinal follow-up assessments of the outlined parameters and clinical symptoms, would be necessary in order to address this question.

The present study is subject to limitations. In regard to the noted significant differences between groups, only the reported difference in leptin concentrations between remitted and acutely ill patients had insufficient statistical power; on the other hand, moderate power was calculated for the group differences between control subjects and acutely ill patients (leptin, FLI), as well as low to moderate power for remitted patients versus controls (sOB-R) and remitted versus acutely ill patients (FLI). Apart from the limited sample size (12), it must also be noted that within the group of acutely ill patients, blood samples were obtained at different BMI stages. To overcome this limitation, one would need a sample of patients with nearly equal BMIs upon admission, which is difficult to coordinate as patients generally present in different stages of the illness (and due to this, they display different BMI values upon admission) upon entry to clinical departments and subsequent inpatient treatment. In addition, no data on body fat and energy intake, in particular as regards the total quantity of food intake, were obtained. These aspects should be assessed in future studies, and the present data need to be replicated in larger samples.

In summary, the present study investigated the relationship between serum Zn concentrations and parameters of the leptin axis (sOB-R, FLI) in adolescent and young adult humans with AN. However, the relationship between the leptin axis and Zn concentrations remains unclear, in particular due to limited statistical power;

some of the comparisons investigated only higher leptin concentrations in remitted patients compared with acutely ill patients, which parallel the higher serum Zn concentrations in remitted versus acutely ill patients. Because recent research has indicated that elevated serum Zn concentrations in remitted patients were likely due to dietary preferences (12), the relationship between changes in the leptin axis, as well as in serum Zn levels, should be further elucidated, in particular with respect to the stage of the illness. Altered concentrations of Zn, leptin and FLI in remitted AN patients compared with acutely ill patients, with respect to healthy controls, need to be replicated using larger sample sizes. The present study provides an estimate for recommended sample sizes in regard to this type of future large-scale research.

Conflict of interest and funding

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