Dissociation of Serum Leptin Concentration and Body Fat Content During Long Term Dietary Intervention in Obese Individuals

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High serum leptin concentrations are observed in humans with high body fat content, indicating leptin resistance. Reducing leptin levels by lowering body fat could restore leptin sensitivity. Our study was designed to clarify the relationship between changes in body composition and circulating leptin during a long term hypocaloric diet. 12 obese women and 10 obese men were included in a 1000 kcal/day dietary intervention trial for 10 weeks. Body composition was measured by body impedance analysis and leptin by radioimmunoassay every 2 weeks. Body fat was reduced in females from 39.0 ± 1.5 kg to 32.9 ± 1.5 kg ($p < 0.001$) and in males from 30.4 ± 1.4 kg to 26.3 ± 1.3 kg ($p < 0.005$). Plasma leptin decreased in females from 38.07 ± 4.17 ng/ml to 18.90 ± 2.75 ng/ml ($p < 0.001$) and in males from 10.58 ± 2.16 ng/ml to 6.33 ± 1.25 ng/ml ($p < 0.001$). Non-linear regression analysis of leptin kinetics showed a comparable one-phase exponential decline ($y = \text{Span} \cdot e^{-K \cdot x} + \text{Plateau}$) in females ($\bar{x} \pm$ SEM: K = 0.48 ± 0.01) and males (K = 0.60 ± 0.01). Kinetics of body fat differed significantly from leptin data for females (K = 0.10 ± 0.001, $p < 0.001$) but not for males (K = 0.27 ± 0.02, $p > 0.05$). The leptin plateau was reached in both groups after 6–8 weeks and the fat plateau in men after 10 weeks. Compared to healthy controls, some obese individuals had higher absolute values of leptin, but seemed to have a relative leptin deficiency when leptin was adjusted to body mass index according to a non-linear regression model of a large group of healthy women (n = 561) and men (n = 393). We conclude that during a long-term hypocaloric diet leptin uncouples from changes in body fat mass.

Key words: Obese Gene – ob Gene – Food Intake – Body Weight – Weight Reduction

Introduction

Leptin, a hormone which is able to reduce body fat in mice (1, 2,3) and rats (4) is exclusively produced by peripheral and visceral adipose tissue in mice (5), rats (4) and also in humans (6,7). In rodents, it regulates food intake and energy consumption. In *ob/ob* mice, a lack of physiologically active leptin, due to a mutation in the leptin gene (5), leads to obesity and non-insulin dependent diabetes with insulin resistance, hyperphagia, hypothermia, cold intolerance, infertility, decrease in lean body mass and a reduced linear growth. Many of these symptoms can be reversed by leptin treatment in rodents (8).

In obese humans, there is no evidence for a mutation of the leptin gene so far (6,9). Obesity in humans is related to higher serum leptin levels compared to normal weight individuals because of an elevated amount of fat mass and a higher production rate of leptin per unit of body fat with increased weight (10,11). Because the enhanced serum leptin concentrations did not prevent weight gain, leptin resistance is assumed. A leptin receptor mutation, as observed in the leptin resistant *db/db* mice or *fa/fa* rat, was not found in humans (12). In humans, certain metabolites and hormones, including leptin itself, might contribute to the resistant state by leptin receptor down-regulation or inhibition of post-receptor events. With this assumption in mind, we hypothesized that decreasing leptin levels could normalize leptin sensitivity by reducing body fat. We therefore investigated the relationship of serum leptin levels and body fat kinetics during long term dietary intervention in obese humans.

Patients

Weight-stable (at least for 3 month) obese patients (12 females and 10 males) of middle age (females: 45.6 ± 3.1 years, males: 54.6 ± 1.6 years) willing to participate in a dietary intervention trial were included, and their progress was followed up in a prospective manner for at least 10 weeks. Patients with heart failure, renal disorders, diabetes or other endocrine diseases were excluded. The study protocol was approved by the ethics committee of the University of Leipzig.

Study Design

Before and every 2 weeks during the study, participants were instructed according to the following nutritional recommendations. Food intake and behavioral changes were reviewed by the investigator to obtain a maximum of compliance. The individuals were weighed and received body impedance (BIA)-analysis, which was discussed with the participant in the light of food composition and consumption. At each visit, capillary blood was drawn for hormone measurements.

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Nutritional recommendation

The participants were allowed one mixed meal of 500 to 800 kcal/day according to their calculated body cell mass (BCM) and basal energy consumption. The participants were advised to choose mixed meals with carbohydrate/protein/fat energy ratios of 50:20:30 mainly for breakfast. For lunch and dinner, a dietary product was allowed with 174 kcal each containing 223.8 g water, 18.6 g protein, 4.4 g fat and 15.0 g carbohydrate as well as vitamins, minerals and trace elements. A daily intake of 2 to 3 liters of water (tea, mineral water) was suggested.

Methods

Capillary blood (ear) was collected from patients at late morning or afternoon postprandially, where leptin levels have their nadir (13), to avoid a significant influence of circadian rhythms on leptin kinetics. EDTA-plasma was stored at −20°C until assay.

Leptin radioimmunoassay

Using recombinant human leptin, provided by Dr. Heiman (Eli Lilly Research Laboratories, Indianapolis, U.S.A.), antiserum was produced in rabbits. Leptin from the same source was used for standardisation and for iodination by the Chloramin T method. Assay buffer contained 0.05 mol/l sodium phosphate, pH 7.04, 0.1 mol/l NaCl, 0.05% (w/v) NaN_3, 0.1% (v/v) gelatine from teleost fish (Sigma Chemicals, Munich, Germany), 0.1% Triton X100 (Serva, Heidelberg, Germany). After overnight incubation at room temperature, a second antibody technique was used for separation of unbound and bound tracer. The assay volume was 0.3 ml. Sensitivity of the assay with undiluted samples was 0.03 ng/ml with intra- and interassay coefficients of variation of 0.8% and 8.5% respectively. For construction of reference ranges of leptin versus BMI, leptin and BMI from 393 normal healthy men and 561 women (BMI-range $\bar{x} \pm SD$): females: 24.4±4.0, males: 24.6±3.3; age range in both groups 20–80 years, no age-dependence of leptin levels), were measured. The best fit regression curves were: $leptin = 0.0237 \cdot e^{(0.1985 \cdot BMI)}$ for men and $leptin = 0.3204 \cdot e^{(0.1448 \cdot BMI)}$ for women. Standard deviation scores (SDS), providing a gender and BMI-adjusted measure of leptin levels, were calculated by the following equations: $SDS = (\ln(leptin) - \ln(0.3204) - (0.1448 \cdot BMI))/0.52483$ for females and $SDS = (\ln(leptin) - \ln(0.0237) - (0.1985 \cdot BMI))/0.63859$ for males. Measuring leptin concentrations in capillary and venous blood (EDTA-plasma) yielded identical values with a correlation coefficient of $r = 0.998$ ($n = 30$). Because leptin binding proteins exist in the circulation (14), which might interfere with leptin measurements, exclusion chromatography was used to study the influence of the leptin-leptin binding protein interaction on our assay system. Due to the very high affinity of our antibody to leptin and the fast-off kinetics of the leptin-leptin binding protein interaction, there was no interference of leptin binding proteins in our assay, which measures total leptin.

Body impedance analysis (BIA)

Body impedance (Z) was measured with the Akern-RJL systems body impedance analyser BIA 101/S (RJL Systems, Detroit, U.S.A.) at 800 µA and 50 kHz under standard conditions (15) according to the guidelines of the NIH technology assessment statement on BIA (16). EUROBODY®-software (Data Input GmbH, Frankfurt, Germany) was used to calculate body fat and BCM. In brief, impedance has two components, resistance (R) and reactance (X) according to $Z = (R^2 + X^2)^{1/2}$. From the R value, total body water (TBW) can be calculated ($TBW = a \cdot Ht^2/R + b \cdot Wt + c$, with Ht = height, R = resistance, a = proportionality constant specific for a given subject population, b · Wt = independent weight term correction, c = constant, constants derived for selected population by Data Input GmbH, Frankfurt). The standard error for TBW estimates by BIA is generally less than 4% (16). Lean body mass (LBM) is calculated from TBW ($LBM = TBW/0.732$). Body fat is calculated from body weight minus LBM. Body cell mass (BCM) is calculated according to $BCM = a \cdot LBM \cdot \log$ phase angle (phase angle = $\arctan(X/R)$, a = constant for selected population). BMI was calculated as body weight/height^2.

Statistics

Statistical analysis and graphics were done with GraphPad Prism™ version 2.01 (GraphPad Software, Inc., San Diego, U.S.A.).

For determining the kinetics of total body fat and serum leptin concentrations, a one-phase exponential non-linear regression analysis was performed for each individual, and the degree of fit was proven by comparing R^2, plot of residuals and runs tests.

One-phase decline non-linear regression analysis ($y = SPAN \cdot e^{-K \cdot X} + PLATEAU$) has 3 parameters describing the curve. PLATEAU is the value when the regression curve reaches a calculated stable minimum. SPAN is the difference between the initial (high) value and the PLATEAU. K is a rate constant, determining the half life of the decline (half life = 0.6932/K). It is expressed as the inverse of the units used on the X axis. For overall group calculations of regression curves, SPAN, PLATEAU and K of each individual were summarized and an overall one-phase non-linear regression curve calculated from these data. To get an overall impression of BMI-adjusted leptin kinetics, a two-phase non-linear regression model was applied, but because of the relatively small numbers of data points, the regression data were not used for statistical comparisons. Data of males and females at 0, 2 and 10 weeks were given as $\bar{x} \pm SEM$, if not otherwise stated, and compared by paired and unpaired nonparametric two-tailed t-tests.

Results

BMI decreased in the 10 week-study interval comparably in females ($\bar{x} \pm SEM$: 33.9±0.72 kg/m^2 to 30.9±0.72 kg/m^2, $p < 0.001$) and males (33.1±0.72 kg/m^2 to 31.0±0.84 kg/m^2, $p < 0.001$), representing a significant change in body weight of females (92.7±1.9 kg to 84.6±1.8 kg, $p < 0.001$) and males (104.7±2.8 kg to 97.9±2.7 kg, $p < 0.001$) with a small, but significant change in waist-hip-ratio (females: 0.88±0.02 to 0.85 ±0.02, $p < 0.05$, males: 1.05±0.02 to 0.99±0.02, $p < 0.001$). A greater decrease of body fat was found in females (39.0± 1.5 kg to 32.9±1.5 kg, $p < 0.001$) compared to males (30.4± 1.4 kg to 26.3±1.3 kg, $p < 0.005$). Body cell mass (BCM) did not significantly change in females (27.2±0.6 kg to 26.7±0.7 kg, $p = 0.1$) but was reduced in males (39.6±0.9 kg to 37.5±1.1 kg, $p < 0.002$), contributing to the change in BMI and body weight in both groups. Leptin decreased in females (38.07±4.17 ng/ml

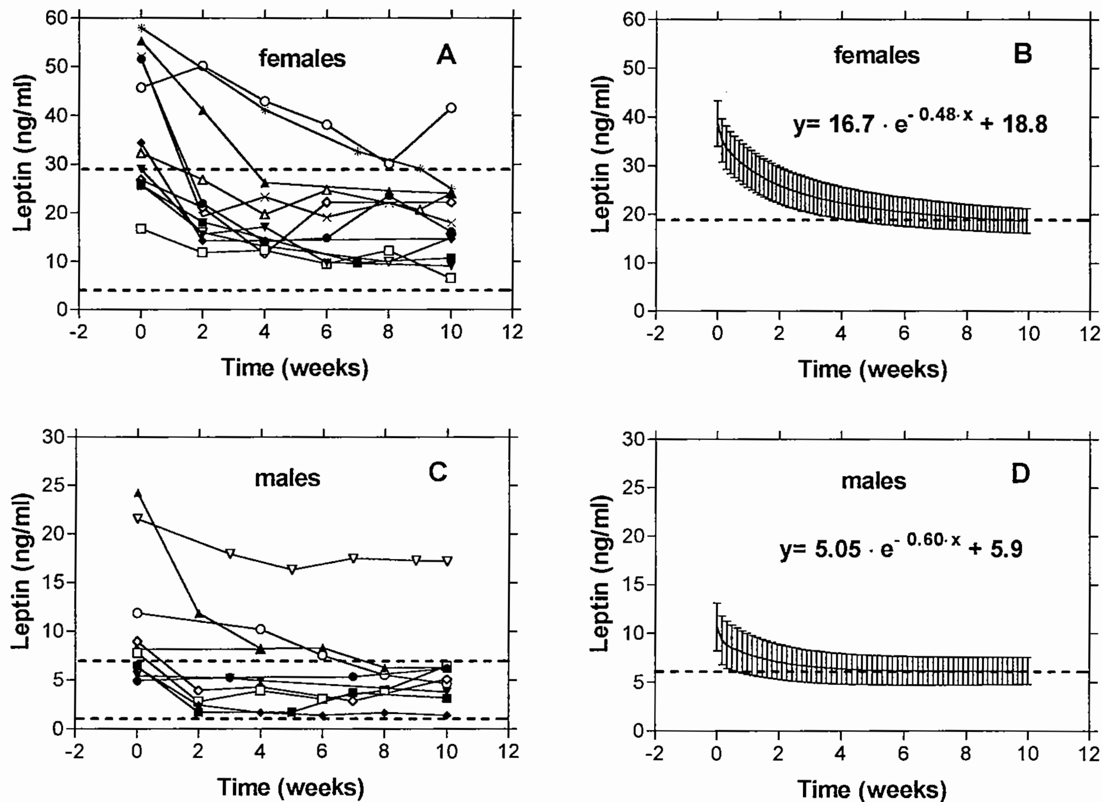

Fig. 1 Single leptin kinetics of 12 females (**A**) and 10 males (**C**) during long term hypocaloric diet (1000 kcal/d). The dotted lines indicate the upper (95th percentile) and lower (5th percentile) normal values at a BMI of 25 kg/m^2, derived from a non linear regression model of healthy females (n = 561) and males (n = 393) over a broad range of BMI values. A one-phase decline non-linear regression model was fitted to the individual kinetic data and a summary curve derived (**B** and **D**). The resulting equations are given, describing the amplitude of the leptin change (SPAN), the half life of the decline (0.6932/K) and the plateau value (dotted).

to 18.90±2.75 ng/ml, $p < 0.001$) and males (10.58±2.16 ng/ml to 6.33±1.25 ng/ml, $p < 0.001$).

Serial measurements in single females (Fig. **1A**) and males (Fig. **1C**) showed a clear decrease in leptin during the beginning of the study interval. However, leptin kinetics were different between the individuals. Most participants showed a sharp decrease in the first 2 weeks, whereas others had a slower decline of leptin. In some patients, leptin did not change after the second week. Comparing the changes in leptin during the first two weeks and during ten weeks, more than $^3/_4$ of the changes resulted from the decrease in leptin during the first two weeks in females (77.5±9.1%) and males (95.3±34.49%) compared to much lower changes in fat (females: 28.8±4.9%, males: 41.3±11.1%; Fig. **2A** and **C**).

In accordance with single kinetic measurements for leptin, non-linear regression analysis (Fig. **1B** and **D**) revealed a similar, not significantly different ($p > 0.05$), exponential decline for leptin in females (K = 0.458±0.01) and males (K = 0.60±0.01) leading to a plateau phase after about 6–8 weeks. Single kinetic data for body fat demonstrated no plateau in most females during the study period (Fig. **2A**), whereas in most males a plateau phase is evident (Fig. **2C**). In females, kinetics derived from non-linear regression of body fat (K = 0.10±0.001; Fig. **2B**) differed significantly ($p < 0.001$) from the leptin data (Fig. **1B**). A nearly continuous decline in body fat was seen, but there was no evidence for a plateau phase of body fat kinetics during the study interval of 10 weeks, although leptin levels reached a plateau at 8 weeks. In men, no significant difference between K values of body fat (K = 0.27±0.02, Fig. **2D**) and leptin kinetics (Fig. **1D**) was found, although the plateau of the regression curve of body fat was reached later (6 weeks versus 10 weeks).

At the beginning of the study, leptin levels were clearly elevated in most individuals when compared to those of normal weight males or females in most individuals (Fig. **1A** and **C**), but mean standard deviation scores of the study were low ($\bar{x}$ ±SEM): −0.48±0.24 for females and −0.98±0.25 for males when adjusted for BMI (for description of the statistical analysis see methods section). The BMI-adjusted SDS values decreased on average further mainly during the first 2 weeks and showed a trend to increase thereafter (Fig. **3A** and **C**). Two-phase non-linear regression curves and the corresponding equations are given in Fig. **3B** and **D**).

Discussion and Conclusions

Few data are available about changes of leptin during hypocaloric diet in humans. Maffei et al. (17) found decreased serum leptin concentrations after weight loss by dieting in 14 obese females. These data were supported by Considine et al. (18), who found a reduction of serum leptin concentration by about

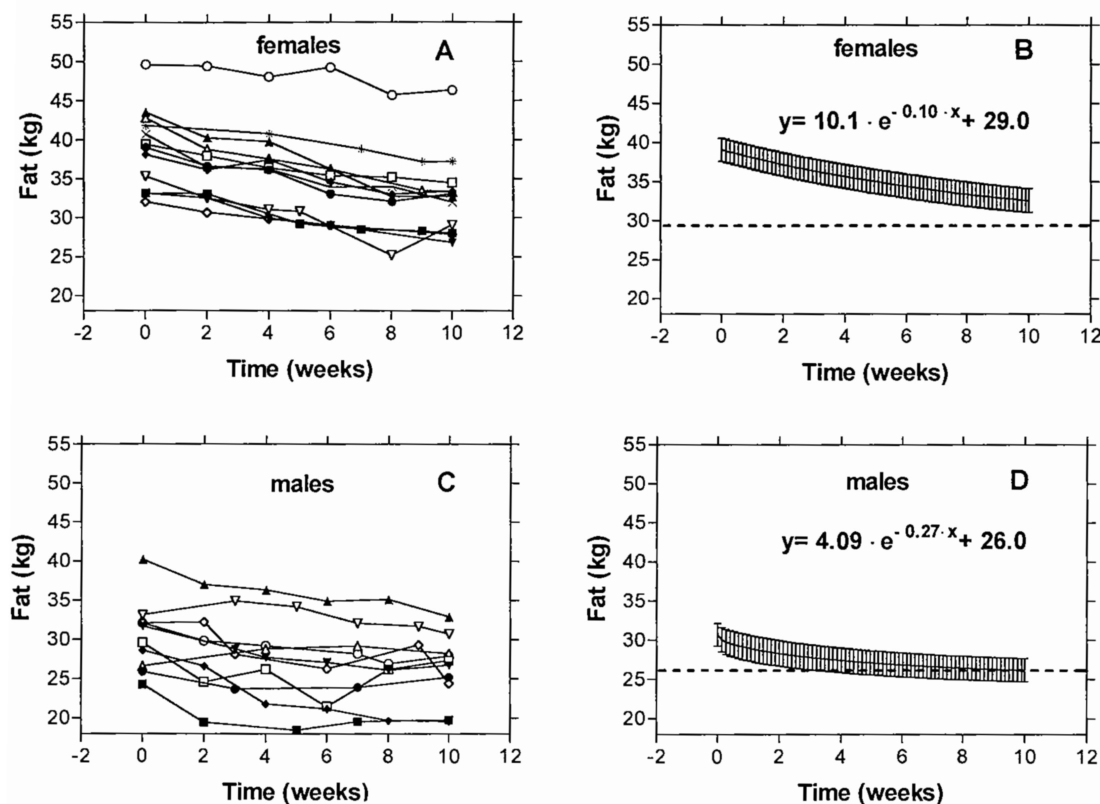

Fig. 2 Single body fat kinetics, measured by BIA for females (**A**) and males (**C**). A one-phase decline non-linear regression model was fitted to the individual kinetic data and a summary curve derived (**B** and **D**). The non-linear regression equations are given.

53 % in 6 females and 1 male after a loss of 10 % of body weight. Our study extends these data by analysing a greater number of obese humans, differentiating between men and women matched for BMI, and investigating in more detail the dynamics of body fat reduction and leptin levels by multiple point measurements during the weight loss period in both groups. In accordance with previous reports (17, 18), leptin levels in the obese patients were clearly higher than in normal weight or lean subjects. This fact lends support to the concept that leptin sensitivity is diminished in obese individuals (17, 18), and that the set point of leptin sensitivity increases with increasing fat mass, although the mechanisms are still unknown.

From a large cohort of healthy male and female adults including a substantial number of obese individuals, we had constructed a gender-specific leptin reference range with BMI as the independent variable. The best-fit regression line was an exponential curve of the form: $\text{leptin} = a \cdot e^{b \cdot \text{BMI}}$. This relationship was used to construct a reference range which would comprise 90 % of all leptin values and which can be used to express leptin levels as standard deviation scores (SDS, for equations see methods section). That is, leptin levels will be adjusted for gender and BMI referring to a healthy normal control group. When leptin levels of the obese patients were transformed to SDS to adjust for BMI, values were on average about 0.5 to 1 standard deviation lower than one would have expected. With the assumption that this approach is applicable to the obese patients, it must be concluded that a significant proportion of those were relatively leptin-deficient in comparison to the average population and to the putative set point of leptin sensitivity in relation to BMI.

Analysis of kinetic data yielded a one-phase non-linear exponential decline of both body fat and leptin levels independent of sex. Surprisingly, the time required for reduction of leptin levels by half did not differ between females and males despite significantly different absolute leptin values and different body fat content and body cell mass. The decline of leptin levels reached a plateau phase after approximately 6–8 weeks while the time required for 50 % reduction of body fat was significantly longer. This dissociation or uncoupling of body fat and leptin kinetics has not been described so far in humans.

At first sight, this may simply mirror the exponential relationship between leptin levels and fat mass. However, when leptin levels where transformed to SDS, the BMI-adjusted values decreased, mainly within the first 2 weeks, and showed an average tendency to rise again after 4 weeks. That is, in the early phase of weight reduction leptin levels decrease faster than would be expected for a corresponding decrease in BMI, suggesting a relative leptin deficiency according to the fat mass and to the putative set point of leptin sensitivity. According to our serial measurements, more than 75 % of the leptin changes are the result of the decrease in leptin during the first two weeks. This is in accordance with the observation of Shina et al. (14), that after short term fasting (24 hours), a 50 % drop in free plasma leptin levels could be observed in obese humans. In our study, the participants did not fast, but ate a low caloric diet, which seems to be of more importance for the decline in leptin in the first two weeks than the modest reduction in body fat of about 28 % in females and 41 % in males over the same time period. Body fat calculated from the difference of body

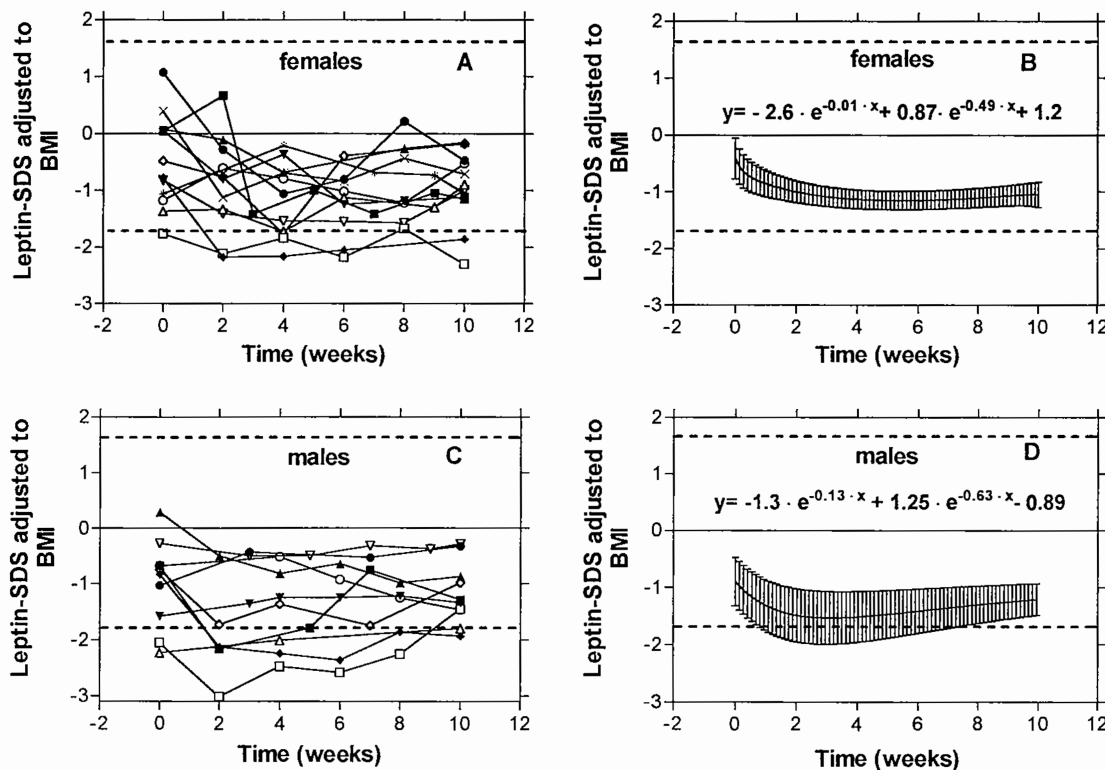

Fig. 3 BMI-adjusted leptin values (SDS) of females (**A**) and males (**C**). Single two-phase non-linear regression models were summarized (see equations above the solid 0-SDS-line) to give an overall impression of the kinetics of scored leptin data (**B** and **D**). The 95th and 5th percentiles are given as dotted lines.

weight and lean body mass, measured by BIA might be overestimated in the first days of a low caloric diet because of a certain degree of dehydration. After 14 days, corresponding to our second sample time point, this effect should be negligible.

The relative deficiency in leptin we have observed in females and males in our study may cause an increase in appetite and a decrease in energy expenditure, in accordance with the observed effects of leptin deficiency in animals (1,2,3). This hypothesis could explain some of the problems encountered during dietary programs for weight reduction.

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