The role of leptin and other hormones related to bone metabolism and appetite-regulation as determinants of gain in body fat and fat-free mass in 8-11 year old children

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Abbreviations

BMI, body mass index; CI, confidence interval; DXA, dual energy X-ray absorptiometry; FFM, fat-free mass; FFMI, fat-free mass index; FM, fat mass; FMI, fat mass index; GH, growth hormone; IGF-1, insulin-like growth factor 1; iPTH, intact parathyroid hormone; IQR, interquartile range; OPUS, acronym for ´Optimal well-being, development and health for Danish children through a healthy New Nordic Diet´; PTH, parathyroid hormone

Abstract

Background: Regulation of body composition during childhood is complex. Numerous hormones are potentially involved. Leptin has been proposed to restrain weight gain, but results are inconsistent.

Objectives: We examined if baseline fasting levels of ghrelin, adiponectin, leptin, insulin, insulin-like growth factor I (IGF-1), osteocalcin and intact parathyroid hormone (iPTH) were associated with body composition cross-sectionally and longitudinally in 633 8-11-year-olds.

Design: Data on hormones and body composition by Dual-energy X-ray absorptiometry from OPUS School Meal Study were used. We looked at baseline hormones as predictors of baseline fat mass index (FMI) or fat-free mass index (FFMI), and also subsequent changes (three and six months) in FMI or FFMI using models with hormones individually or combined.

Results: Cross-sectionally, baseline leptin was positively associated with FMI in girls (0.211 kg/m^2 pr. µg/ml (0.186; 0.236), p<0.001) and boys (0.231 kg/m^2 pr. µg/ml (0.200; 0.261), p<0.001). IGF-1 in both genders and iPTH in boys were positively associated with FMI. An inverse association between adiponectin and FFMI in boys and a positive association between IGF-1 and FFMI in girls were found. In longitudinal models, baseline leptin was inversely associated with subsequent changes in FMI (-0.018 kg/m^2 pr. µg/ml (-0.034; -0.002), p=0.028) and FFMI (-0.014 kg/m^2 pr. µg/ml (-0.024; -0.003), p=0.006) in girls.

Conclusions: Cross-sectional findings support that leptin is produced in proportion to body fat mass, but the longitudinal observations support that leptin inhibits gains in FMI and FFMI in girls, a finding which may reflect preserved leptin sensitivity in this predominantly normal weight population.

Introduction

Regulation of growth and body composition during childhood is complex and the interrelationship between the numerous hormones involved has to be taken into account when studying the impact of individual hormones. Growth hormone (GH) is the dominant stimulator of linear growth in childhood and it also important for gain in muscle mass (1;2). Its effects are mainly mediated through the insulin-like growth factor (IGF) system. Insulin-like growth factor I (IGF-I) is associated with obesity in early life, but the relation is complex and differs with age (3). Thus, a high level of IGF-I in infancy is associated with lower levels of IGF-I in childhood and adolescence (3). The insulin system and the GH/IGF system share a common evolutionary origin, but diverged in higher animal species so that insulin primarily has metabolic functions while the GH/IGF system plays a critical role in growth and development (4). A longitudinal study on children suggests insulin to be a promoter of weight or body fat gain over time (5;6), a plausible finding considering its peripheral effects on body fat storage and oxidation (7). Insulin may also stimulate growth in fat-free mass (FFM) (6).

Several hormones have purported effects on the regulation of appetite and body composition, such as leptin, ghrelin, adiponectin and insulin. However, evidence regarding the relationship between these hormones and growth and body composition in children is still limited. The best studied of these hormones is leptin, which, according to rare monogenic human cases and animal experimental studies, should act as a satiating factor that restrains weight gain. In contrast, most prospective studies in school-aged children point towards a positive relationship between circulating leptin levels and subsequent gains in body fat mass (FM) (8-13). However, most of those studies were in obese populations, and in contrast to these studies Ahmed *et al.* found that among girls low levels of leptin at the beginning of puberty predicted larger gains in body fat percentage during puberty (14), and Byrnes *et al.* also showed that leptin levels were inversely associated with weight gain in prepubertal children (15). These two

studies finding inverse associations between leptin and gain in fat or weight gain both were based on a relatively low number of children (Ahmed *et al.* n=40 and Byrnes *et al.* n=52). Circulating levels of adiponectin, an anti-inflammatory and insulin-sensitizing adipocytokine, decrease with increasing amount of body fat (16). Whether, in turn, adiponectin influences changes in body composition over time is less clear (10;17-19). One study reported that adiponectin levels were inversely associated with subsequent one-year gains in FFM in boys (17).

There is also increasing evidence for a bidirectional relationship between bone growth and energy metabolism (20-22). Hormones coupled to the mineralization or demineralization of bones, like the bone formation marker osteocalcin, and the calcium-mobilizing parathyroid hormone (PTH), have been linked with energy metabolism and body fat deposition (23-26), but more knowledge is needed for children.

Thus, the role of hormones produced by FM or involved in energy metabolism or bone growth in regulation of body composition in childhood is unclear. Large longitudinal studies are needed that can take into account the possible interrelationship of these hormones.

The aim of the present paper is to examine whether baseline fasting blood concentrations of ghrelin, adiponectin, leptin, insulin, insulin-like growth factor I (IGF-1), osteocalcin, and intact parathyroid hormone (iPTH) are cross-sectionally and longitudinally associated with body composition over a six months period in children participating in the OPUS (Optimal well-being, development and health for Danish children through a healthy New Nordic Diet) School Meal Study , which involved 8-11-year-olds from third and fourth grades at 9 schools (27). Most emphasis will be put on the longitudinal results as these are closest to a causal relationship going from hormones to body composition.

Materials and Methods

The OPUS School Meal Study was a cluster-randomized, controlled, and unblinded cross-over study with the primary outcomes to investigate the impact of free school meals based on a so-called New Nordic Diet on concentration performance and a metabolic syndrome score. In this paper data from the study were used in an exploratory way not focusing on the effects of the dietary intervention.

The study design has been described in detail previously (27). Briefly, children from third and fourth grades (8-11-year-olds) at 9 schools in Denmark were invited to participate in the study. Each child participated in two 3-month periods: an intervention period with provision of meals based on the New Nordic Diet and a control period. Randomization to order of periods was performed in clusters corresponding to year group within school. The schools entered the study sequentially, one to three weeks apart. Measurements were carried out from August 2011 to June 2012. The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Regional Committee on Biomedical Research Ethics of the Capital Region of Denmark (no. H-1-2010-124). Written informed consent was obtained from custody holders of the child. Exclusion criteria for the children were strong food allergies or food intolerances or concomitant participation in other scientific studies that involved radiation or blood sampling. The trial was registered in the Clinical Trials database (clinicaltrials.gov; no. NCT01457794).

Measurements

Anthropometric measurements

Clinical examinations were performed at baseline, three months and six months. Height was measured to the nearest 0.1 cm using a mobile height measure (Tanita Leicester Portable Height Measure) and body weight measured to the nearest 0.1 kg using a digital weight (Tanita BWB 800 S). Measurements

were carried out after an overnight fast. Prevalence of underweight and of overweight including obesity were based on age- and sex-specific cut-offs defined to pass through body mass index (BMI) of 18.5 and 25 kg/m^2 at age 18 years according to Cole *et al.* (28;29).

Total body composition of the children was measured by Dual Energy X-ray Absorptiometry (DXA) scanning (Lunar Prodigy; GE Medical Systems (Madison, Wisconsin) with Encore software version 13.5). Most of the children had a standardized breakfast prior to the scan. Fat mass index (FMI) and fat-free mass index (FFMI) were calculated as originally described by Van Itallie *et al.* (30):

$$\text{FMI (kg/m}^2\text{)} = (\text{FM (kg)}) / (\text{height (m)})^2$$

$$\text{FFMI (kg/m}^2\text{)} = (\text{lean mass (kg)} + \text{bone mineral content (kg)}) / (\text{height (m)})^2$$

In a study on the reproducibility of whole body scans of 5-17 year old children using the GE Lunar, coefficients of variation of 1.94 % (FM) and 0.48 % (FFM) were found for two repeated scans in thin mode (31).

Anthropometric measures and scans were carried out by a team of investigators throughout the project period, but investigators were carefully trained using standard operating procedures. All scans were evaluated by two investigators who assessed if scans were usable, and also checked if the divisions of the body into different compartments automatically carried out by the device were correct.

Pubertal status

Baseline pubertal status (breast development in girls and emergence of pubertal hair in boys) was assessed by self-reported questionnaires on Tanner staging (32). Since very few children (6 %) categorized themselves as being at stage 3-5, the variable was recoded to a binary variable: not entered puberty (stage 1) or entered puberty (stage 2-5).

Blood analyses

At each examination fasting blood samples were collected and plasma stored at -80°C until analysis. Families were provided with local anaesthetic patches (EMLA, Astra Zeneca). Leptin, adiponectin and total ghrelin were analyzed using ELISA (leptin and adiponectin: R&D Systems Europe, Ltd., Abingdon, UK and ghrelin: Millipore, Hellerup, Denmark). Inhibitors (Pefabloc, DPP-IV and Trasylol; Sigma-Aldrich, Gentofte, Denmark) were added to tubes used for the collection of blood for ghrelin analysis, and tubes were kept on ice throughout the process to avoid degradation of acylated ghrelin. IGF-1 and osteocalcin were analyzed using a chemiluminescent immunoassay on an Immulite 1000 (Siemens Healthcare Diagnostics, Ballerup, Denmark and Siemens Medical Solutions Diagnostics, Newark, Delaware). One osteocalcin sample was above the detection limit of 100 ng/ml and was excluded from the data set. Serum was stored at -80°C for analyses of insulin and iPTH. Serum insulin was measured by an automated chemiluminescent immunoassay on an ADVIA Centaur XP (Siemens Healthcare, Ballerup, Denmark) and expressed in pmol/l. Serum iPTH concentrations were determined using CLIA technique on ADVIA Centaur XP (Siemens Healthcare, Ballerup, Denmark). One iPTH value was below the detection limit of 0.265 pmol/l and was excluded from the analyses. The inter- and intra-assay coefficients of variation were: 9.2% and 3.7% (leptin), 9.0% and 3.7% (ghrelin); 11% and 3.8% (adiponectin); 2.5% and 3.1% (insulin); 2.4% and 2.9% (IGF-1), 5.9% and 4.1% (osteocalcin), and 7.4% and 7.9% (iPTH). For each analysis, all samples were run on the same device with the same reagent lot, all samples from each child were analyzed on the same day, and all samples from each school were analyzed in one assay.

A total of 834 children had been enrolled in OPUS School Meal Study. Children were included in the present analyses if they had data on age and pubertal status at baseline, data on body weight, height and body composition at baseline plus minimum one post-baseline occasion (month 3 and/or month 6) and

data on all the seven hormones at baseline (n=656). One child with achondroplasia, 21 children who did not meet fasting for the examinations, and one child with a doubtful iPTH value (109 pmol/l and 25(OH)D was 89.1 nmol/l) were excluded from the analyses.

Statistical analyses

Baseline characteristics for boys and girls were compared by Wilcoxon rank-sum test or Pearson´s chi-squared test. All further analyses were carried out for boys and girls separately due to their different body composition and different hormone levels.

To be able to tell which hormones were related to each other and to what extent, Spearman correlation coefficients and corresponding p-values were calculated for correlation between the different hormones at baseline.

Analyses of the cross-sectional associations between hormones and body composition at baseline were based on ANCOVA-type multiple linear regression and adjusted for age and pubertal status at baseline, and in case of FFMI also for FMI at baseline. Analyses of the longitudinal associations between baseline hormones and body composition at three months/six months were based on a one-level ANCOVA-type hierarchical linear mixed model with individual as random effect. Results were adjusted for time (three or six months), age and pubertal status at baseline and baseline value of FMI/FFMI, and analyses on FFMI were also adjusted for FMI at baseline and at three months or six months. We have not adjusted for the dietary intervention or order of dietary periods as the intervention did not influence FMI and FFMI (33). Both cross-sectional and longitudinal analyses tested two different models – firstly including only one hormone at a time, and secondly with all hormones in the same model. Bonferroni correction of p-values for multiple comparisons was done based on the gender subgroups (all p-values were

multiplied by two) and 97.5% confidence intervals (CIs) were presented to fit the corrected p-values. A Bonferroni corrected p-value of < 0.05 was used to denote statistical significance. To allow comparison of estimated effect sizes across different hormones measured in different units, we also expressed a multiplication of the regression coefficients and CIs with the size of the IQRs for the relevant hormones at baseline.

For significant longitudinal associations between hormones and measures of body composition, we also tested the opposite theory; that the change in the hormone over three to six months could be predicted from body composition at baseline. The analysis used was similar to those longitudinal analyses described previously with the only difference being that hormone was the dependent variable and the measure of body composition was an independent variable.

Analyses were carried out using STATA/IC 13.0 (Texas, USA).

Results

Baseline characteristics

Of the 834 children enrolled in the OPUS School Meal Study 633 children (308 girls and 325 boys) were included in the present analyses. Of these 633 children 585 (~ 92 %) had data from both three months and six months, 35 (~ 6 %) had data from three months only and 13 (~ 2 %) had data from six months only. At baseline boys were older and had higher FFMI than girls (**Table 1**). More girls than boys had entered puberty and girls had higher FMI, leptin, leptin pr. kg body fat, insulin, IGF-1, osteocalcin and iPTH than the boys (Table 1). Height, ghrelin and adiponectin were not different between the genders (Table 1). Most of the children were normal weight, 14.3 % of girls and 12.6 % of

boys were overweight or obese, and 11.7 % of girls and 8.0 % of boys were underweight with no significant differences between the genders (Table 1).

Inter-correlations between hormones at baseline

Leptin, insulin and IGF-I values were all positively inter-related in both genders (**Table 2**). The strongest association was between insulin and leptin with correlation coefficients of 0.60 and 0.54, for girls and boys, respectively. In contrast, ghrelin was inversely associated with all these three hormones with correlation coefficients between -0.22 and -0.31.

Relationship of baseline hormones with fat mass index

In cross-sectional analyses, leptin and IGF-1 in both genders and iPTH in boys showed independent positive associations with FMI (**Table 3a**) whereas cross-sectional associations between ghrelin and insulin and FMI disappeared after adjustment for other hormones (Table 3a). In longitudinal analyses, the only hormone independently associated with FMI was leptin; only among girls baseline leptin was inversely associated with subsequent change in FMI (**Table 3b**), which was directionally discordant with the cross-sectional association. Additional adjustment for FFMI at baseline and at three/six months did not change the results (results not shown). In support of a possible bi-directional relationship between leptin and FMI in an additional longitudinal model, baseline FMI was positively associated with subsequent change in leptin (β: 2.28 ug/ml (97.5 % CI: 1.87 to 2.70), $p<0.001$).

Relationship of hormones with fat free mass index

In the cross-sectional analyses, adiponectin was inversely associated with FFMI in boys, while IGF-1 was positively associated with FFMI in girls; both associations remained significant after adjustment for other hormones (**Table 4a**). In longitudinal analyses, leptin was inversely associated with subsequent

change in FFMI in girls (**Table 4b**). None of the other hormones were associated with FFMI in longitudinal analyses (Table 4b). In an additional longitudinal model, baseline FFMI was not associated with subsequent change in leptin (-0.23 ug/ml (-0.94 to 0.48), p=0.92).

Discussion

Our main findings were that baseline leptin is a negative predictor of subsequent gain in FMI and FFMI in girls and that ghrelin, adiponectin, insulin, IGF-1, osteocalcin and iPTH do not seem to be involved in regulation of body composition in 8-11 year old children.

The results on leptin are consistent with the well-known physiological role of leptin as a signal of energy repletion leading to satiety and decreased energy intake, but they are opposite to the reports of many similar studies on leptin and changes in adiposity over time in children and adolescents (8;10-13;34). However, the majority of those other studies on school-aged children that found a positive association between leptin and either weight or body fat gain over time were based on overweight populations or populations with a high prevalence of overweight and therefore likely leptin resistance (8;9;11-13), which was not the case for the two studies finding an inverse association (14;15). Our results may thus reflect the low prevalence of overweight in this child population and therefore probable leptin sensitivity. However, in a study on the impact of leptin during early growth Boeke *et al.* found that maternal leptin and cord blood leptin were negative predictors of 3-year adiposity, while 3-year leptin was associated with greater weight gain and adiposity through age 7(35). The authors suggested that the latter results were due to the development of leptin resistance within the first three years of life across the whole BMI spectrum (no modifying effect of BMI on the positive relation between leptin at three years and adiposity at 7 years) (35). Like our findings, Ahmed *et al.* also found an inverse association between leptin and fat-free mass (FFM) when adjusting for body fat mass (FM) in 8-16

year-old girls (14). If leptin does indeed lower appetite in the present population, this would naturally also limit the increase in FFM. The inverse associations between baseline leptin and subsequent gains in both FMI and FFMI were only significant in girls. We wonder if this is due to the higher levels of leptin in girls due to larger FM, the role of leptin in female pubertal development (36) or has something to do with gender differences in leptin sensitivity. Leptin sensitivity is often judged from the concentration of leptin for a given size of FM, and based on this approach females are considered less leptin sensitive than males (37). Also our girls exhibit higher concentrations of leptin pr. kilo body fat at baseline, but still the longitudinal inverse association between leptin and FMI is only significant in girls.

Our results cannot be used to establish a causal relationship between the hormones examined and changes in body composition. However, longitudinal results on ghrelin, adiponectin, insulin and PTH could indicate that these hormones do not play an important role in regulation of body composition, at least not in this age group and/or in a population with relatively low prevalence of overweight and obesity. The cross-sectional associations between ghrelin, insulin and FMI disappeared after adjusting for other hormones, and thus their initial associations with FMI may reflect their correlations with IGF-1 and leptin as demonstrated in table 2. The positive association between PTH and FMI in boys may very well be due to body fat influencing on PTH rather than the opposite. PTH has been claimed to be an independent predictor of obesity (23). However, based on a weight loss trial Reinehr *et al*. concluded that the higher PTH levels observed in the obese children was a consequence rather than a cause of overweight (25). With regards to insulin and ghrelin, it might be more relevant to study postprandial levels, but it was not possible in this study. No associations between osteocalcin and FMI and FFMI were found. In cross-sectional studies in obese children Lenders *et al.* found inverse associations between osteocalcin and both visceral adipose tissue and BMI, but not with FM (26); and Wang *et al.* found negative associations between osteocalcin and both fat percentage and visceral fat area and

positive associations of osteocalcin with FFMI (24). It may be that any possible association between osteocalcin and body composition is more pronounced in more obese child populations. We have no measures of visceral fat in the present study.

We chose to express FM as FMI although FMI does correlate positively with height. If we were to minimize the correlation with height in this data material, FM should be divided with height raised to the fifth (4.47 in girls and 6.24 in boys), which is in line with results by Wells *et. al.* (38). However, we are not convinced that minimizing the correlation with height is necessarily the most correct approach. Children with a large FM have faster prepubertal growth, and therefore must be expected to be taller than children with less body fat within this age range (39). FFMI did not show residual correlation with height.

When studying hormonal regulation of body composition it is difficult to distinguish the effects of individual hormones from each other or explain the causal direction. We chose a relatively simple analysis strategy allowing for comparison of cross-sectional and longitudinal results, and comparison of results for hormones when they are studied one at a time or together with other hormones. The hormones regulate the secretion and sensitivity of each other and are confounded by the same factors (eg. level of testosterone or oestrogen). Adjustment for pubertal status is important because of the simultaneous influence of puberty on the body composition and hormonal profile. For logistical reasons pubertal status was assessed at baseline only and children may have changed their pubertal status during this six month period.

Using data from both three months and six months as the dependent variable in the longitudinal analyses has strengths as well as limitations. There may be differences in the "effects" of the hormones whether

or not the length of the follow-up is three months or six months. With our models we do not capture such differences, and the resulting regression coefficients were not expressed relative to time. On the other hand our models allow for adjustment for individual as random effect with three data time points available for most of the individuals. Among the major strengths of the present study are the longitudinal design, the large number of children, the repeated measurements of both FM and FFM by DXA scanning, and not at least the large number of hormones measured whereby their interrelationship could be taken into account.

The children in the present study consisted of a representative sample of Danish school children of similar age range, which can be considered both a strength and a limitation. Thus, we did not exclude children based on dieting behavior, level of physical activity (high/low) or due to use of medication that may have influenced body composition e.g. Ritalin.

In conclusion, these cross-sectional findings support that leptin is produced in proportion to the size of body FM, but the longitudinal observations support that leptin appeared to inhibit subsequent gains in FMI and FFMI over time in girls, a finding which may reflect preserved leptin sensitivity in this predominantly normal weight childhood population. Our findings demonstrate the importance of longitudinal study designs with repeated body composition and hormonal data.

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Table 1. Baseline characteristics[*] of the study population (n=633).

	Girls (n=308)	Boys (n=325)	p[†]
Age (yrs)	9.9 (9.4; 10.4)	10.1 (9.5; 10.5)	**0.001**
Pubertal status (% entered puberty)	45.8	24.9	**<0.001**
Height (cm)	142.4 (137.7; 146.6)	142.9 (138.1; 147.6)	0.27
Weight (kg)	33.9 (29.7; 38.3)	34.3 (30.2; 39.7)	0.31
BMI (kg/m^2)	16.8 (15.4; 18.2)	16.8 (15.7; 18.4)	0.40
Prevalence (%)[‡]			
Overweight (incl. obese)	14.3	12.6	0.54
Underweight	11.7	8.0	0.12
FMI (kg/m^2)	4.13 (2.88; 5.73)	3.14 (2.20; 4.76)	**<0.001**
FFMI (kg/m^2)	12.44 (11.83; 13.02)	13.49 (12.80; 14.09)	**<0.001**
Plasma leptin (µg/ml)	5.14 (2.84; 9.77)	2.80 (1.75; 5.64)	**<0.001**
Plasma leptin pr. kg body fat (µg/ml pr. kg)	0.65 (0.48; 0.89)	0.47 (0.36; 0.67)	**<0.001**
Plasma ghrelin (pg/ml)	954 (738; 1208)	977 (792; 1276)	0.16
Plasma adiponectin (µg/ml)	11.28 (8.29; 14.77)	10.47 (7.69; 14.12)	0.09
Serum insulin (pmol/l)	46.3 (34.9; 63.1)	38.9 (30.3; 53.9)	**<0.001**
Plasma IGF-1 (ng/ml)	211 (177; 268)	180 (140; 210)	**<0.001**
Plasma osteocalcin (ng/ml)	30.4 (24.1; 38.4)	24.7 (20.8; 31.5)	**<0.001**
Serum iPTH (pmol/l)	3.3 (2.4; 4.2)	3.0 (2.2; 3.9)	**0.010**

Abbreviations: BMI, body mass index; FFMI, fat free mass index; FMI, fat mass index; IGF-1, insulin-like growth factor I; iPTH, intact parathyroid hormone.

[*]Median (interquartile range) or percentages are presented.

[†]Differences between sexes were determined by Wilcoxon rank-sum test or Pearson's chi-squared test.

[‡]Based on age- and sex-specific cut-offs defined to pass through BMI of 18.5 and 25 kg/m^2 at age 18 years, as according to Cole *et al.* (28;29).

Table 2. Spearman's rank correlations between hormones at baseline

	Plasma leptin	**Plasma ghrelin**	**Plasma adiponectin**	**Serum insulin**	**Plasma IGF-1**	**Plasma osteocalcin**	**Serum iPTH**
Plasma leptin	1.00						
Plasma ghrelin	Girls -0.30 (**p<0.001**) Boys -0.30 (**p<0.001**)	1.00					
Plasma adiponectin	Girls -0.08 (p=0.18) Boys 0.04 (p=0.48)	Girls 0.15 (**p=0.010**) Boys 0.04 (p=0.50)	1.00				
Serum insulin	Girls 0.60 (**p<0.001**) Boys 0.54 (**p<0.001**)	Girls -0.29 (**p<0.001**) Boys -0.31 (**p<0.001**)	Girls -0.19 (**p=0.001**) Boys 0.05 (p=0.36)	1.00			
Plasma IGF-1	Girls 0.33 (**p<0.001**) Boys 0.37 (**p<0.001**)	Girls -0.27 (**p<0.001**) Boys -0.22 (**p<0.001**)	Girls -0.17 (**p=0.003**) Boys -0.06 (p=0.28)	Girls 0.48 (**p<0.001**) Boys 0.42 (**p<0.001**)	1.00		
Plasma osteocalcin	Girls -0.05 (p=0.38) Boys 0.05 (p=0.36)	Girls -0.14 (**p=0.014**) Boys 0.04 (p=0.46)	Girls -0.08 (p=0.15) Boys -0.08 (p=0.15)	Girls 0.12 (**p=0.038**) Boys 0.004 (p=0.94)	Girls 0.31 (**p<0.001**) Boys 0.04 (p=0.46)	1.00	
Serum iPTH	Girls -0.11 (p=0.06) Boys 0.01 (p=0.87)	Girls 0.02 (p=0.78) Boys -0.02 (p=0.77)	Girls -0.13 (**p=0.025**) Boys -0.08 (p=0.148)	Girls -0.08 (p=0.169) Boys -0.17 (**p=0.002**)	Girls 0.12 (**p=0.037**) Boys 0.003 (p=0.96)	Girls 0.19 (**p=0.001**) Boys 0.20 (**p<0.001**)	1.00

IGF-1, insulin-like growth factor 1; iPTH, intact parathyroid hormone.

Table 3. Baseline hormone levels associated with a) cross-sectional fat mass index (FMI); b) longitudinal change in FMI

		FMI (kg/m^2)					
		One hormone at a time			*All hormones in one model*		
a) Cross-sectional[*]		β (97.5 % CI) [†]	IQR[‡] (β (97.5 % CI))	p	β (97.5 % CI) [†]	IQR[‡] (β (97.5 % CI))	p
Plasma leptin (μg/ml)	Girls	**0.220 (0.198; 0.241)**	**1.524 (1.375; 1.673)**	**<0.001**	**0.211 (0.186; 0.236)**	**1.463 (1.288; 1.638)**	**<0.001**
	Boys	**0.250 (0.223; 0.278)**	**0.975 (0.868; 1.083)**	**<0.001**	**0.231 (0.200; 0.261)**	**0.900 (0.781; 1.018)**	**<0.001**
Plasma ghrelin (pg/ml)	Girls	**-0.001 (-0.002; -0.001)**	**-0.602 (-0.891; -0.314)**	**<0.001**	$-3x10^{-4}$ ($-7x10^{-4}$; $1x10^{-4}$)		0.18
	Boys	**-0.001 (-0.002; -0.001)**	**-0.569 (-0.831; -0.306)**	**<0.001**	$-4x10^{-4}$ ($-8x10^{-4}$; $1x10^{-5}$)		0.06
Plasma adiponectin (μg/ml)	Girls	-0.029 (-0.073; 0.015)		0.27	0.006 (-0.021; 0.034)		1.00
	Boys	0.002 (-0.036; 0.040)		1.00	-0.003 (-0.028; 0.022)		1.00
Serum insulin (mIU/l)	Girls	**0.031 (0.023; 0.039)**	**0.867 (0.640; 1.094)**	**<0.001**	-5x10-7 (-0.007; 0.007)		1.00
	Boys	**0.036 (0.026; 0.046)**	**0.855 (0.622; 1.088)**	**<0.001**	0.002 (-0.007; 0.011)		1.00
Plasma IGF-1 (ng/ml)	Girls	**0.006 (0.003; 0.010)**	**0.587 (0.285; 0.889)**	**<0.001**	**0.003 (0.001; 0.005)**	**0.263 (0.055; 0.472)**	**0.005**
	Boys	**0.011 (0.008; 0.015)**	**0.805 (0.527; 1.083)**	**<0.001**	**0.005 (0.002; 0.008)**	**0.355 (0.172; 0.538)**	**<0.001**
Plasma osteocalcin (ng/ml)	Girls	-0.014 (-0.036; 0.007)		0.27	-0.004 (-0.018; 0.010)		1.00
	Boys	0.013 (-0.014; 0.041)		0.53	0.009 (-0.009; 0.028)		0.52
Serum iPTH (pmol/l)	Girls	-0.079 (-0.216; 0.058)		0.39	0.008 (-0.075; 0.092)		1.00
	Boys	**0.160 (0.004; 0.316)**	**0.272 (0.007; 0.538)**	**0.043**	**0.185 (0.077; 0.293)**	**0.314 (0.131; 0.498)**	**<0.001**
b) Longitudinal[§]							
Plasma leptin (μg/ml)	Girls	**-0.019 (-0.034; -0.003)**	**-0.129 (-0.235; -0.023)**	**0.012**	**-0.018 (-0.034; -0.002)**	**-0.122 (-0.233; -0.011)**	**0.028**
	Boys	-0.013 (-0.030; 0.005)		0.20	-0.013 (-0.031; 0.006)		0.24
Plasma ghrelin (pg/ml)	Girls	$-1x10^{-4}$ ($-3x10^{-4}$; $2x10^{-5}$)		0.11	$-1x10^{-4}$ ($-3x10^{-4}$;$1x10^{-5}$)		0.08
	Boys	$-3x10^{-5}$ ($-2x10^{-4}$;$1x10^{-4}$)		1.00	$-2x10^{-5}$ ($-2x10^{-4}$;$1x10^{-4}$)		1.00
Plasma adiponectin (μg/ml)	Girls	0.002 (-0.009; 0.012)		1.00	0.001 (-0.009; 0.012)		1.00
	Boys	0.006 (-0.004; 0.016)		0.40	0.007 (-0.004; 0.017)		0.29
Serum insulin (mIU/l)	Girls	-0.001 (-0.004; 0.001)		0.50	-0.001 (-0.004; 0.002)		0.81
	Boys	$1x10^{-4}$ (-0.003; 0.003)		1.00	0.001 (-0.003; 0.004)		1.00
Plasma IGF-1 (ng/ml)	Girls	$4x10^{-4}$ (-0.001; 0.001)		0.71	$4x10^{-4}$($-5x10^{-4}$; 0.001)		0.70
	Boys	0.001 (-0.001; 0.002)		0.43	0.001 (-0.001; 0.002)		0.67
Plasma osteocalcin (ng/ml)	Girls	-0.001 (-0.006; 0.004)		1.00	-0.002 (-0.008; 0.003)		0.72
	Boys	0.005 (-0.002; 0.012)		0.25	0.004 (-0.003; 0.012)		0.40
Serum iPTH (pmol/l)	Girls	-0.001 (-0.034; 0.032)		1.00	-0.002 (-0.035; 0.031)		1.00
	Boys	0.013 (-0.031; 0.056)		1.00	0.006 (-0.039; 0.051)		1.00

CI, confidence interval; FMI, fat mass index; IGF-1, insulin-like growth factor 1; iPTH; intact parathyroid hormone; IQR, interquartile range.

* Analyses of the cross-sectional associations between the hormones and FMI at baseline were based on ANCOVA-type multiple linear regression including adjustment for age and pubertal status at baseline.

† P-values were Bonferroni corrected due to the gender sub-groups (=multiplied with 2) and 97.5 % CIs were used to match these corrected p-values.

‡ For significant associations the regression coefficients and CIs were multiplied with the size of the IQR for the hormone at baseline, to better be able to compare the strengths of the associations across hormones.

§ Analyses of the longitudinal associations between the hormones and FMI were based on a one-level ANCOVA-type linear mixed model with individual as random effect. Analyses were adjusted for age, pubertal status and FMI at baseline.

416

417 **Table 4. Baseline hormone levels associated with a) cross-sectional fat-free mass index (FFMI); b) longitudinal change in FFMI**

		FFMI (kg/m^2)					
		One hormone at a time			*All hormones in one model*		
a) Cross-sectional[*]		β (97.5 % CI) [†]	IQR[‡] (β (97.5 % CI))	p	β (97.5 % CI) [†]	IQR[‡] (β (97.5 % CI))	p
Plasma leptin (µg/ml)	Girls	-0.001 (-0.029; 0.028)		1.00	0.009 (-0.020; 0.038)		0.93
	Boys	-0.033 (-0.067; 0.002)		0.07	-0.032 (-0.068; 0.005)		0.10
Plasma ghrelin (pg/ml)	Girls	$2x10^{-5}$ ($-3x10^{-4}$;$3x10^{-4}$)		1.00	$1x10^{-4}$ ($-2x10^{-4}$;$4x10^{-4}$)		0.65
	Boys	$3x10^{-5}$ ($-3x10^{-4}$; $3x10^{-4}$)		1.00	$5x10^{-5}$ ($-3x10^{-4}$;$4x10^{-4}$)		1.00
Plasma adiponectin (µg/ml)	Girls	-0.019 (-0.039; 0.001)		0.06	-0.017 (-0.036; 0.003)		0.11
	Boys	**-0.029 (-0.049; -0.008)**	**-0.186 (-0.318; -0.053)**	**0.003**	**-0.027 (-0.048; -0.007)**	**-0.175 (-0.307; -0.043)**	**0.006**
Serum insulin (mIU/l)	Girls	$4x10^{-4}$ (-0.004; 0.005)		1.00	-0.003 (-0.008; 0.002)		0.26
	Boys	$4x10^{-4}$ (-0.006; 0.007)		1.00	0.003 (-0.004; 0.010)		0.76
Plasma IGF-1 (ng/ml)	Girls	**0.003 (0.001; 0.004)**	**0.261 (0.122; 0.400)**	**<0.001**	**0.003 (0.001; 0.005)**	**0.272 (0.120; 0.424)**	**<0.001**
	Boys	$1x10^{-4}$(-0.002; 0.003)		1.00	$-5x10^{-5}$ (-0.003; 0.002)		1.00
Plasma osteocalcin (ng/ml)	Girls	0.008 (-0.001; 0.018)		0.10	0.003 (-0.007; 0.013)		0.90
	Boys	0.001 (-0.014; 0.016)		1.00	-0.002 (-0.017; 0.013)		1.00
Serum iPTH (pmol/l)	Girls	0.061 (-0.001; 0.122)		0.05	0.044 (-0.016; 0.105)		0.20
	Boys	0.057 (-0.031; 0.144)		0.29	0.049 (-0.040; 0.139)		0.44
b) Longitudinal[§]							
Plasma leptin (µg/ml)	Girls	**-0.012 (-0.022; -0.002)**	**-0.083 (-0.151; -0.015)**	**0.013**	**-0.014(-0.024; -0.003)**	**-0.095 (-0.167; -0.023)**	**0.006**
	Boys	-0.008 (-0.018; 0.003)		0.21	-0.006 (-0.017; 0.005)		0.40
Plasma ghrelin (pg/ml)	Girls	$-2x10^{-5}$($-1x10^{-4}$;$9x10^{-5}$)		1.00	$3x10^{-6}$ ($-1x10^{-4}$; $1x10^{-4}$)		1.00
	Boys	$-3x10^{-5}$ ($-1x10^{-4}$; $6x10^{-5}$)		0.99	$-2x10^{-5}$ ($-1x10^{-4}$;$7x10^{-5}$)		1.00
Plasma adiponectin (µg/ml)	Girls	-0.001 (-0.008; 0.006)		1.00	$-2x10^{-4}$ (-0.007; 0.007)		1.00
	Boys	-0.006 (-0.012; $4x10^{-4}$)		0.08	-0.005 (-0.012; 0.001)		0.09
Serum insulin (mIU/l)	Girls	0.001 (-0.001; 0.003)		0.32	0.001 ($-4x10^{-4}$; 0.003)		0.18
	Boys	$1x10^{-5}$ (-0.002; 0.002)		1.00	$1x10^{-5}$ (-0.002; 0.002)		1.00
Plasma IGF-1 (ng/ml)	Girls	$5x10^{-4}$ ($-8x10^{-5}$; 0.001)		0.11	$3x10^{-4}$ ($-4x10^{-4}$;$9x10^{-4}$)		0.68
	Boys	$4x10^{-4}$ ($-3x10^{-4}$; 0.001)		0.36	$4x10^{-4}$($-4x10^{-4}$; 0.001)		0.51
Plasma osteocalcin (ng/ml)	Girls	0.002 (-0.001; 0.005)		0.38	0.001 (-0.003; 0.004)		1.00
	Boys	0.003 (-0.001; 0.008)		0.21	0.003 (-0.002; 0.007)		0.41
Serum iPTH (pmol/l)	Girls	0.011 (-0.010; 0.033)		0.46	0.007 (-0.014; 0.029)		0.89
	Boys	0.009 (-0.017; 0.035)		0.89	0.004 (-0.023; 0.031)		1.00

CI, confidence interval; FFMI, fat-free mass index; IGF-1, insulin-like growth factor 1; iPTH; intact parathyroid hormone; IQR, interquartile range.

[*] Analyses of the cross-sectional associations between the hormones and FFMI at baseline were based on ANCOVA-type multiple linear regression including adjustment for age, pubertal status and fat mass index at baseline.

[†] P-values were Bonferroni corrected due to the gender sub-groups (=multiplied with 2) and 97.5 % CIs were used to match these corrected p-values.

[‡] For significant associations the regression coefficients and CIs were multiplied with the size of the IQR for the hormone at baseline, to better be able to compare the strengths of the associations across hormones.

[§] Analyses of the longitudinal associations between the hormones and FFMI were based on a one-level ANCOVA-type linear mixed model with individual as random effect. Analyses were adjusted for age, pubertal status and FMI and FFMI at baseline and also FMI at three months/six months.