

# 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

## 1 Abstract

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

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

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

11 **Results:** Cross-sectionally, baseline leptin was positively associated with FMI in girls ( $0.211 \text{ kg/m}^2 \text{ pr. } \mu\text{g/ml}$  (0.186; 0.236),  $p < 0.001$ ) and boys ( $0.231 \text{ kg/m}^2 \text{ pr. } \mu\text{g/ml}$  (0.200; 0.261),  $p < 0.001$ ). IGF-1 in both  
12 genders and iPTH in boys were positively associated with FMI. An inverse association between  
13 adiponectin and FFMI in boys and a positive association between IGF-1 and FFMI in girls were found.  
14 In longitudinal models, baseline leptin was inversely associated with subsequent changes in FMI ( $-0.018$   
15  $\text{kg/m}^2 \text{ pr. } \mu\text{g/ml}$  (-0.034; -0.002),  $p = 0.028$ ) and FFMI ( $-0.014 \text{ kg/m}^2 \text{ pr. } \mu\text{g/ml}$  (-0.024; -0.003),  $p = 0.006$ )  
16 in girls.  
17

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

## 21 Introduction

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

34  
35 Several hormones have purported effects on the regulation of appetite and body composition, such as  
36 leptin, ghrelin, adiponectin and insulin. However, evidence regarding the relationship between these  
37 hormones and growth and body composition in children is still limited. The best studied of these  
38 hormones is leptin, which, according to rare monogenic human cases and animal experimental studies,  
39 should act as a satiating factor that restrains weight gain. In contrast, most prospective studies in school-  
40 aged children point towards a positive relationship between circulating leptin levels and subsequent  
41 gains in body fat mass (FM) (8-13). However, most of those studies were in obese populations, and in  
42 contrast to these studies Ahmed *et al.* found that among girls low levels of leptin at the beginning of  
43 puberty predicted larger gains in body fat percentage during puberty (14), and Byrnes *et al.* also showed  
44 that leptin levels were inversely associated with weight gain in prepubertal children (15). These two

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

51  
52 There is also increasing evidence for a bidirectional relationship between bone growth and energy  
53 metabolism (20-22). Hormones coupled to the mineralization or demineralization of bones, like the bone  
54 formation marker osteocalcin, and the calcium-mobilizing parathyroid hormone (PTH), have been  
55 linked with energy metabolism and body fat deposition (23-26), but more knowledge is needed for  
56 children.

57  
58 Thus, the role of hormones produced by FM or involved in energy metabolism or bone growth in  
59 regulation of body composition in childhood is unclear. Large longitudinal studies are needed that can  
60 take into account the possible interrelationship of these hormones.

61  
62 The aim of the present paper is to examine whether baseline fasting blood concentrations of ghrelin,  
63 adiponectin, leptin, insulin, insulin-like growth factor I (IGF-1), osteocalcin, and intact parathyroid  
64 hormone (iPTH) are cross-sectionally and longitudinally associated with body composition over a six  
65 months period in children participating in the OPUS (Optimal well-being, development and health for  
66 Danish children through a healthy New Nordic Diet) School Meal Study , which involved 8-11-year-  
67 olds from third and fourth grades at 9 schools (27). Most emphasis will be put on the longitudinal results  
68 as these are closest to a causal relationship going from hormones to body composition.

## 69 **Materials and Methods**

70 The OPUS School Meal Study was a cluster-randomized, controlled, and unblinded cross-over study  
71 with the primary outcomes to investigate the impact of free school meals based on a so-called New  
72 Nordic Diet on concentration performance and a metabolic syndrome score. In this paper data from the  
73 study were used in an exploratory way not focusing on the effects of the dietary intervention.  
74 The study design has been described in detail previously (27). Briefly, children from third and fourth  
75 grades (8-11-year-olds) at 9 schools in Denmark were invited to participate in the study. Each child  
76 participated in two 3-month periods: an intervention period with provision of meals based on the New  
77 Nordic Diet and a control period. Randomization to order of periods was performed in clusters  
78 corresponding to year group within school. The schools entered the study sequentially, one to three  
79 weeks apart. Measurements were carried out from August 2011 to June 2012. The study was conducted  
80 according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human  
81 subjects were approved by the Regional Committee on Biomedical Research Ethics of the Capital  
82 Region of Denmark (no. H-1-2010-124). Written informed consent was obtained from custody holders  
83 of the child. Exclusion criteria for the children were strong food allergies or food intolerances or  
84 concomitant participation in other scientific studies that involved radiation or blood sampling. The trial  
85 was registered in the Clinical Trials database (clinicaltrials.gov; no. NCT01457794).

## 88 **Measurements**

### 89 *Anthropometric measurements*

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

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

96  
97 Total body composition of the children was measured by Dual Energy X-ray Absorptiometry (DXA)  
98 scanning (Lunar Prodigy; GE Medical Systems (Madison, Wisconsin) with Encore software version  
99 13.5). Most of the children had a standardized breakfast prior to the scan. Fat mass index (FMI) and fat-  
100 free mass index (FFMI) were calculated as originally described by Van Itallie *et al.* (30):

$$101 \quad \text{FMI (kg/m}^2\text{)} = (\text{FM (kg)}) / (\text{height (m)})^2$$

$$102 \quad \text{FFMI (kg/m}^2\text{)} = (\text{lean mass (kg)} + \text{bone mineral content (kg)}) / (\text{height (m)})^2$$

103  
104 In a study on the reproducibility of whole body scans of 5-17 year old children using the GE Lunar,  
105 coefficients of variation of 1.94 % (FM) and 0.48 % (FFM) were found for two repeated scans in thin  
106 mode (31).

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

### 111 *Pubertal status*

112  
113 Baseline pubertal status (breast development in girls and emergence of pubertal hair in boys) was  
114 assessed by self-reported questionnaires on Tanner staging (32). Since very few children (6 %)   
115 categorized themselves as being at stage 3-5, the variable was recoded to a binary variable: not entered  
116 puberty (stage 1) or entered puberty (stage 2-5).



117 *Blood analyses*

118 At each examination fasting blood samples were collected and plasma stored at -80°C until analysis.  
119 Families were provided with local anaesthetic patches (EMLA, Astra Zeneca). Leptin, adiponectin and  
120 total ghrelin were analyzed using ELISA (leptin and adiponectin: R&D Systems Europe, Ltd.,  
121 Abingdon, UK and ghrelin: Millipore, Hellerup, Denmark). Inhibitors (Pefabloc, DPP-IV and Trasylol;  
122 Sigma-Aldrich, Gentofte, Denmark) were added to tubes used for the collection of blood for ghrelin  
123 analysis, and tubes were kept on ice throughout the process to avoid degradation of acylated ghrelin.  
124 IGF-1 and osteocalcin were analyzed using a chemiluminescent immunoassay on an Immulite 1000  
125 (Siemens Healthcare Diagnostics, Ballerup, Denmark and Siemens Medical Solutions Diagnostics,  
126 Newark, Delaware). One osteocalcin sample was above the detection limit of 100 ng/ml and was  
127 excluded from the data set. Serum was stored at -80°C for analyses of insulin and iPTH. Serum insulin  
128 was measured by an automated chemiluminescent immunoassay on an ADVIA Centaur XP (Siemens  
129 Healthcare, Ballerup, Denmark) and expressed in pmol/l. Serum iPTH concentrations were determined  
130 using CLIA technique on ADVIA Centaur XP (Siemens Healthcare, Ballerup, Denmark). One iPTH  
131 value was below the detection limit of 0.265 pmol/l and was excluded from the analyses. The inter- and  
132 intra-assay coefficients of variation were: 9.2% and 3.7% (leptin), 9.0% and 3.7% (ghrelin); 11% and  
133 3.8% (adiponectin); 2.5% and 3.1% (insulin); 2.4% and 2.9% (IGF-1), 5.9% and 4.1% (osteocalcin), and  
134 7.4% and 7.9% (iPTH). For each analysis, all samples were run on the same device with the same  
135 reagent lot, all samples from each child were analyzed on the same day, and all samples from each  
136 school were analyzed in one assay.

137

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

141 data on all the seven hormones at baseline (n=656). One child with achondroplasia, 21 children who did  
142 not meet fasting for the examinations, and one child with a doubtful iPTH value (109 pmol/l and  
143 25(OH)D was 89.1 nmol/l) were excluded from the analyses.

### 145 **Statistical analyses**

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

149  
150 To be able to tell which hormones were related to each other and to what extent, Spearman  
151 correlation coefficients and corresponding p-values were calculated for correlation between the different  
152 hormones at baseline.

153  
154 Analyses of the cross-sectional associations between hormones and body composition at baseline were  
155 based on ANCOVA-type multiple linear regression and adjusted for age and pubertal status at baseline,  
156 and in case of FFMI also for FMI at baseline. Analyses of the longitudinal associations between baseline  
157 hormones and body composition at three months/six months were based on a one-level ANCOVA-type  
158 hierarchical linear mixed model with individual as random effect. Results were adjusted for time (three  
159 or six months), age and pubertal status at baseline and baseline value of FMI/FFMI, and analyses on  
160 FFMI were also adjusted for FMI at baseline and at three months or six months. We have not adjusted  
161 for the dietary intervention or order of dietary periods as the intervention did not influence FMI and  
162 FFMI (33). Both cross-sectional and longitudinal analyses tested two different models – firstly including  
163 only one hormone at a time, and secondly with all hormones in the same model. Bonferroni correction  
164 of p-values for multiple comparisons was done based on the gender subgroups (all p-values were

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

170  
171 For significant longitudinal associations between hormones and measures of body composition, we also  
172 tested the opposite theory; that the change in the hormone over three to six months could be predicted  
173 from body composition at baseline. The analysis used was similar to those longitudinal analyses  
174 described previously with the only difference being that hormone was the dependent variable and the  
175 measure of body composition was an independent variable.

176  
177 Analyses were carried out using STATA/IC 13.0 (Texas, USA).  
178

## 179 **Results**

### 180 181 *Baseline characteristics*

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

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

191  
192 *Inter-correlations between hormones at baseline*

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

197  
198 *Relationship of baseline hormones with fat mass index*

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

208  
209 *Relationship of hormones with fat free mass index*

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

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

## 216 **Discussion**

217

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

221  
222 The results on leptin are consistent with the well-known physiological role of leptin as a signal of  
223 energy repletion leading to satiety and decreased energy intake, but they are opposite to the reports of  
224 many similar studies on leptin and changes in adiposity over time in children and adolescents (8;10-  
225 13;34). However, the majority of those other studies on school-aged children that found a positive  
226 association between leptin and either weight or body fat gain over time were based on overweight  
227 populations or populations with a high prevalence of overweight and therefore likely leptin resistance  
228 (8;9;11-13), which was not the case for the two studies finding an inverse association (14;15). Our  
229 results may thus reflect the low prevalence of overweight in this child population and therefore probable  
230 leptin sensitivity. However, in a study on the impact of leptin during early growth Boeke *et al.* found  
231 that maternal leptin and cord blood leptin were negative predictors of 3-year adiposity, while 3-year  
232 leptin was associated with greater weight gain and adiposity through age 7(35). The authors suggested  
233 that the latter results were due to the development of leptin resistance within the first three years of life  
234 across the whole BMI spectrum (no modifying effect of BMI on the positive relation between leptin at  
235 three years and adiposity at 7 years) (35). Like our findings, Ahmed *et al.* also found an inverse  
236 association between leptin and fat-free mass (FFM) when adjusting for body fat mass (FM) in 8-16

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

245

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

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

264  
265 We chose to express FM as FMI although FMI does correlate positively with height. If we were to  
266 minimize the correlation with height in this data material, FM should be divided with height raised to  
267 the fifth (4.47 in girls and 6.24 in boys), which is in line with results by Wells *et. al.* (38). However, we  
268 are not convinced that minimizing the correlation with height is necessarily the most correct approach.  
269 Children with a large FM have faster prepubertal growth, and therefore must be expected to be taller  
270 than children with less body fat within this age range (39). FFMI did not show residual correlation with  
271 height.

272  
273 When studying hormonal regulation of body composition it is difficult to distinguish the effects of  
274 individual hormones from each other or explain the causal direction. We chose a relatively simple  
275 analysis strategy allowing for comparison of cross-sectional and longitudinal results, and comparison of  
276 results for hormones when they are studied one at a time or together with other hormones. The  
277 hormones regulate the secretion and sensitivity of each other and are confounded by the same factors  
278 (eg. level of testosterone or oestrogen). Adjustment for pubertal status is important because of the  
279 simultaneous influence of puberty on the body composition and hormonal profile. For logistical reasons  
280 pubertal status was assessed at baseline only and children may have changed their pubertal status during  
281 this six month period.

282  
283 Using data from both three months and six months as the dependent variable in the longitudinal analyses  
284 has strengths as well as limitations. There may be differences in the “effects” of the hormones whether

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

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

296  
297 In conclusion, these cross-sectional findings support that leptin is produced in proportion to the size of  
298 body FM, but the longitudinal observations support that leptin appeared to inhibit subsequent gains in  
299 FMI and FFMI over time in girls, a finding which may reflect preserved leptin sensitivity in this  
300 predominantly normal weight childhood population. Our findings demonstrate the importance of  
301 longitudinal study designs with repeated body composition and hormonal data.



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415

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

|  | Girls (n=308)        | Boys (n=325)         | p <sup>†</sup>   |
|--|----------------------|----------------------|------------------|
| Age (yrs)                                    | 9.9 (9.4; 10.4)      | 10.1 (9.5; 10.5)     | <b>0.001</b>     |
| Pubertal status (% entered puberty)          | 45.8                 | 24.9                 | <b>&lt;0.001</b> |
| 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 <sup>2</sup> )                     | 16.8 (15.4; 18.2)    | 16.8 (15.7; 18.4)    | 0.40             |
| Prevalence (%) <sup>‡</sup>                  |                      |                      |                  |
| Overweight (incl. obese)                     | 14.3                 | 12.6                 | 0.54             |
| Underweight                                  | 11.7                 | 8.0                  | 0.12             |
| FMI (kg/m <sup>2</sup> )                     | 4.13 (2.88; 5.73)    | 3.14 (2.20; 4.76)    | <b>&lt;0.001</b> |
| FFMI (kg/m <sup>2</sup> )                    | 12.44 (11.83; 13.02) | 13.49 (12.80; 14.09) | <b>&lt;0.001</b> |
| Plasma leptin (µg/ml)                        | 5.14 (2.84; 9.77)    | 2.80 (1.75; 5.64)    | <b>&lt;0.001</b> |
| Plasma leptin pr. kg body fat (µg/ml pr. kg) | 0.65 (0.48; 0.89)    | 0.47 (0.36; 0.67)    | <b>&lt;0.001</b> |
| 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)    | <b>&lt;0.001</b> |
| Plasma IGF-1 (ng/ml)                         | 211 (177; 268)       | 180 (140; 210)       | <b>&lt;0.001</b> |
| Plasma osteocalcin (ng/ml)                   | 30.4 (24.1; 38.4)    | 24.7 (20.8; 31.5)    | <b>&lt;0.001</b> |
| Serum iPTH (pmol/l)                          | 3.3 (2.4; 4.2)       | 3.0 (2.2; 3.9)       | <b>0.010</b>     |

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<sup>2</sup> 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 |
|---------------------------|---|---|--------------------------------------|--|--|---------------------------------------|------------|
| <b>Plasma leptin</b>      | 1.00                                    |   |                                      |  |  |                                       |            |
| <b>Plasma ghrelin</b>     | Girls<br>-0.30<br>( <b>p&lt;0.001</b> ) |   |                                      |  |  |                                       |            |
|                           | Boys<br>-0.30<br>( <b>p&lt;0.001</b> )  | 1.00                                    |                                      |  |  |                                       |            |
| <b>Plasma adiponectin</b> | Girls<br>-0.08<br>(p=0.18)              | Girls<br>0.15<br>( <b>p=0.010</b> )     |                                      |  |  |                                       |            |
|                           | Boys<br>0.04<br>(p=0.48)                | Boys<br>0.04<br>(p=0.50)                | 1.00                                 |  |  |                                       |            |
| <b>Serum insulin</b>      | Girls<br>0.60<br>( <b>p&lt;0.001</b> )  | Girls<br>-0.29<br>( <b>p&lt;0.001</b> ) | Girls<br>-0.19<br>( <b>p=0.001</b> ) |  |  |                                       |            |
|                           | Boys<br>0.54<br>( <b>p&lt;0.001</b> )   | Boys<br>-0.31<br>( <b>p&lt;0.001</b> )  | Boys<br>0.05<br>(p=0.36)             | 1.00                                   |  |                                       |            |
| <b>Plasma IGF-1</b>       | Girls<br>0.33<br>( <b>p&lt;0.001</b> )  | Girls<br>-0.27<br>( <b>p&lt;0.001</b> ) | Girls<br>-0.17<br>( <b>p=0.003</b> ) | Girls<br>0.48<br>( <b>p&lt;0.001</b> ) |  |                                       |            |
|                           | Boys<br>0.37<br>( <b>p&lt;0.001</b> )   | Boys<br>-0.22<br>( <b>p&lt;0.001</b> )  | Boys<br>-0.06<br>(p=0.28)            | Boys<br>0.42<br>( <b>p&lt;0.001</b> )  | 1.00                                   |                                       |            |
| <b>Plasma osteocalcin</b> | Girls<br>-0.05<br>(p=0.38)              | Girls<br>-0.14<br>( <b>p=0.014</b> )    | Girls<br>-0.08<br>(p=0.15)           | Girls<br>0.12<br>( <b>p=0.038</b> )    | Girls<br>0.31<br>( <b>p&lt;0.001</b> ) |                                       |            |
|                           | Boys<br>0.05<br>(p=0.36)                | Boys<br>0.04<br>(p=0.46)                | Boys<br>-0.08<br>(p=0.15)            | Boys<br>0.004<br>(p=0.94)              | Boys<br>0.04<br>(p=0.46)               | 1.00                                  |            |
| <b>Serum iPTH</b>         | Girls<br>-0.11<br>(p=0.06)              | Girls<br>0.02<br>(p=0.78)               | Girls<br>-0.13<br>( <b>p=0.025</b> ) | Girls<br>-0.08<br>(p=0.169)            | Girls<br>0.12<br>( <b>p=0.037</b> )    | Girls<br>0.19<br>( <b>p=0.001</b> )   |            |
|                           | Boys<br>0.01<br>(p=0.87)                | Boys<br>-0.02<br>(p=0.77)               | Boys<br>-0.08<br>(p=0.148)           | Boys<br>-0.17<br>( <b>p=0.002</b> )    | Boys<br>0.003<br>(p=0.96)              | Boys<br>0.20<br>( <b>p&lt;0.001</b> ) | 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 <sup>2</sup> )   |   |                  |  |   |                  |
|---|-------|--|---|------------------|--|---|------------------|
|   |       | <i>One hormone at a time</i>                                     |   |                  | <i>All hormones in one model</i>                                 |   |                  |
| <b>a) Cross-sectional*</b>              |       | $\beta$ (97.5 % CI) <sup>†</sup>                                 | IQR <sup>‡</sup> ( $\beta$ (97.5 % CI)) | p                | $\beta$ (97.5 % CI) <sup>†</sup>                                 | IQR <sup>‡</sup> ( $\beta$ (97.5 % CI)) | p                |
| Plasma leptin ( $\mu\text{g/ml}$ )      | Girls | <b>0.220 (0.198; 0.241)</b>                                      | <b>1.524 (1.375; 1.673)</b>             | <b>&lt;0.001</b> | <b>0.211 (0.186; 0.236)</b>                                      | <b>1.463 (1.288; 1.638)</b>             | <b>&lt;0.001</b> |
|   | Boys  | <b>0.250 (0.223; 0.278)</b>                                      | <b>0.975 (0.868; 1.083)</b>             | <b>&lt;0.001</b> | <b>0.231 (0.200; 0.261)</b>                                      | <b>0.900 (0.781; 1.018)</b>             | <b>&lt;0.001</b> |
| Plasma ghrelin (pg/ml)                  | Girls | <b>-0.001 (-0.002; -0.001)</b>                                   | <b>-0.602 (-0.891; -0.314)</b>          | <b>&lt;0.001</b> | $-3 \times 10^{-4}$ ( $-7 \times 10^{-4}$ ; $1 \times 10^{-4}$ ) |   | 0.18             |
|   | Boys  | <b>-0.001 (-0.002; -0.001)</b>                                   | <b>-0.569 (-0.831; -0.306)</b>          | <b>&lt;0.001</b> | $-4 \times 10^{-4}$ ( $-8 \times 10^{-4}$ ; $1 \times 10^{-5}$ ) |   | 0.06             |
| Plasma adiponectin ( $\mu\text{g/ml}$ ) | 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 | <b>0.031 (0.023; 0.039)</b>                                      | <b>0.867 (0.640; 1.094)</b>             | <b>&lt;0.001</b> | $-5 \times 10^{-7}$ (-0.007; 0.007)                              |   | 1.00             |
|   | Boys  | <b>0.036 (0.026; 0.046)</b>                                      | <b>0.855 (0.622; 1.088)</b>             | <b>&lt;0.001</b> | 0.002 (-0.007; 0.011)  |   | 1.00             |
| Plasma IGF-1 (ng/ml)                    | Girls | <b>0.006 (0.003; 0.010)</b>                                      | <b>0.587 (0.285; 0.889)</b>             | <b>&lt;0.001</b> | <b>0.003 (0.001; 0.005)</b>                                      | <b>0.263 (0.055; 0.472)</b>             | <b>0.005</b>     |
|   | Boys  | <b>0.011 (0.008; 0.015)</b>                                      | <b>0.805 (0.527; 1.083)</b>             | <b>&lt;0.001</b> | <b>0.005 (0.002; 0.008)</b>                                      | <b>0.355 (0.172; 0.538)</b>             | <b>&lt;0.001</b> |
| 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  | <b>0.160 (0.004; 0.316)</b>                                      | <b>0.272 (0.007; 0.538)</b>             | <b>0.043</b>     | <b>0.185 (0.077; 0.293)</b>                                      | <b>0.314 (0.131; 0.498)</b>             | <b>&lt;0.001</b> |
| <b>b) Longitudinal<sup>§</sup></b>      |       |  |   |                  |  |   |                  |
| Plasma leptin ( $\mu\text{g/ml}$ )      | Girls | <b>-0.019 (-0.034; -0.003)</b>                                   | <b>-0.129 (-0.235; -0.023)</b>          | <b>0.012</b>     | <b>-0.018 (-0.034; -0.002)</b>                                   | <b>-0.122 (-0.233; -0.011)</b>          | <b>0.028</b>     |
|   | Boys  | -0.013 (-0.030; 0.005)   |   | 0.20             | -0.013 (-0.031; 0.006)   |   | 0.24             |
| Plasma ghrelin (pg/ml)                  | Girls | $-1 \times 10^{-4}$ ( $-3 \times 10^{-4}$ ; $2 \times 10^{-5}$ ) |   | 0.11             | $-1 \times 10^{-4}$ ( $-3 \times 10^{-4}$ ; $1 \times 10^{-5}$ ) |   | 0.08             |
|   | Boys  | $-3 \times 10^{-5}$ ( $-2 \times 10^{-4}$ ; $1 \times 10^{-4}$ ) |   | 1.00             | $-2 \times 10^{-5}$ ( $-2 \times 10^{-4}$ ; $1 \times 10^{-4}$ ) |   | 1.00             |
| Plasma adiponectin ( $\mu\text{g/ml}$ ) | 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  | $1 \times 10^{-4}$ (-0.003; 0.003)                               |   | 1.00             | 0.001 (-0.003; 0.004)  |   | 1.00             |
| Plasma IGF-1 (ng/ml)                    | Girls | $4 \times 10^{-4}$ (-0.001; 0.001)                               |   | 0.71             | $4 \times 10^{-4}$ ( $-5 \times 10^{-4}$ ; 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.



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

|                                    |       | FFMI (kg/m <sup>2</sup> )  |   |                  |  |   |                  |
|------------------------------------|-------|--|---|------------------|--|---|------------------|
|                                    |       | <i>One hormone at a time</i>                                     |   |                  | <i>All hormones in one model</i>                                 |   |                  |
| <b>a) Cross-sectional*</b>         |       | $\beta$ (97.5 % CI) <sup>†</sup>                                 | IQR <sup>‡</sup> ( $\beta$ (97.5 % CI)) | p                | $\beta$ (97.5 % CI) <sup>†</sup>                                 | IQR <sup>‡</sup> ( $\beta$ (97.5 % CI)) | p                |
| Plasma leptin ( $\mu$ g/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 | $2 \times 10^{-5}$ ( $-3 \times 10^{-4}$ ; $3 \times 10^{-4}$ )  |   | 1.00             | $1 \times 10^{-4}$ ( $-2 \times 10^{-4}$ ; $4 \times 10^{-4}$ )  |   | 0.65             |
|                                    | Boys  | $3 \times 10^{-5}$ ( $-3 \times 10^{-4}$ ; $3 \times 10^{-4}$ )  |   | 1.00             | $5 \times 10^{-5}$ ( $-3 \times 10^{-4}$ ; $4 \times 10^{-4}$ )  |   | 1.00             |
| Plasma adiponectin ( $\mu$ g/ml)   | Girls | -0.019 (-0.039; 0.001)   |   | 0.06             | -0.017 (-0.036; 0.003)   |   | 0.11             |
|                                    | Boys  | <b>-0.029 (-0.049; -0.008)</b>                                   | <b>-0.186 (-0.318; -0.053)</b>          | <b>0.003</b>     | <b>-0.027 (-0.048; -0.007)</b>                                   | <b>-0.175 (-0.307; -0.043)</b>          | <b>0.006</b>     |
| Serum insulin (mIU/l)              | Girls | $4 \times 10^{-4}$ (-0.004; 0.005)                               |   | 1.00             | -0.003 (-0.008; 0.002)   |   | 0.26             |
|                                    | Boys  | $4 \times 10^{-4}$ (-0.006; 0.007)                               |   | 1.00             | 0.003 (-0.004; 0.010)  |   | 0.76             |
| Plasma IGF-1 (ng/ml)               | Girls | <b>0.003 (0.001; 0.004)</b>                                      | <b>0.261 (0.122; 0.400)</b>             | <b>&lt;0.001</b> | <b>0.003 (0.001; 0.005)</b>                                      | <b>0.272 (0.120; 0.424)</b>             | <b>&lt;0.001</b> |
|                                    | Boys  | $1 \times 10^{-4}$ (-0.002; 0.003)                               |   | 1.00             | $-5 \times 10^{-5}$ (-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>b) Longitudinal<sup>§</sup></b> |       |  |   |                  |  |   |                  |
| Plasma leptin ( $\mu$ g/ml)        | Girls | <b>-0.012 (-0.022; -0.002)</b>                                   | <b>-0.083 (-0.151; -0.015)</b>          | <b>0.013</b>     | <b>-0.014 (-0.024; -0.003)</b>                                   | <b>-0.095 (-0.167; -0.023)</b>          | <b>0.006</b>     |
|                                    | Boys  | -0.008 (-0.018; 0.003)   |   | 0.21             | -0.006 (-0.017; 0.005)   |   | 0.40             |
| Plasma ghrelin (pg/ml)             | Girls | $-2 \times 10^{-5}$ ( $-1 \times 10^{-4}$ ; $9 \times 10^{-5}$ ) |   | 1.00             | $3 \times 10^{-6}$ ( $-1 \times 10^{-4}$ ; $1 \times 10^{-4}$ )  |   | 1.00             |
|                                    | Boys  | $-3 \times 10^{-5}$ ( $-1 \times 10^{-4}$ ; $6 \times 10^{-5}$ ) |   | 0.99             | $-2 \times 10^{-5}$ ( $-1 \times 10^{-4}$ ; $7 \times 10^{-5}$ ) |   | 1.00             |
| Plasma adiponectin ( $\mu$ g/ml)   | Girls | -0.001 (-0.008; 0.006)   |   | 1.00             | $-2 \times 10^{-4}$ (-0.007; 0.007)                              |   | 1.00             |
|                                    | Boys  | -0.006 (-0.012; $4 \times 10^{-4}$ )                             |   | 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 ( $-4 \times 10^{-4}$ ; 0.003)                             |   | 0.18             |
|                                    | Boys  | $1 \times 10^{-5}$ (-0.002; 0.002)                               |   | 1.00             | $1 \times 10^{-5}$ (-0.002; 0.002)                               |   | 1.00             |
| Plasma IGF-1 (ng/ml)               | Girls | $5 \times 10^{-4}$ ( $-8 \times 10^{-5}$ ; 0.001)                |   | 0.11             | $3 \times 10^{-4}$ ( $-4 \times 10^{-4}$ ; $9 \times 10^{-4}$ )  |   | 0.68             |
|                                    | Boys  | $4 \times 10^{-4}$ ( $-3 \times 10^{-4}$ ; 0.001)                |   | 0.36             | $4 \times 10^{-4}$ ( $-4 \times 10^{-4}$ ; 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.