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Laakso, Saila

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## Testicular function and bone in young men with severe childhood-onset obesity

Saila Laakso<sup>1</sup>, Heli Viljakainen<sup>1,2</sup>, Marita Lipsanen-Nyman<sup>1</sup>, Ursula Turpeinen<sup>3</sup>, Kaisa K Ivaska<sup>4</sup>,

Ravinder Anand-Ivell<sup>5</sup>, Richard Ivell<sup>5</sup>, Outi Mäkitie<sup>1,2,6,7</sup>

<sup>1</sup> Children's Hospital, Helsinki University Hospital and University of Helsinki, Helsinki, Finland

<sup>2</sup> Folkhälsan Research Center, Biomedicum, Helsinki, Finland

<sup>3</sup> HUSlab, Helsinki University Hospital, Helsinki, Finland

<sup>4</sup> Department of Cell Biology and Anatomy, University of Turku, Turku, Finland

<sup>5</sup> School of Biosciences, University of Nottingham, Nottingham, United Kingdom

<sup>6</sup> Department of Molecular Medicine and Surgery and Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden

<sup>7</sup> Department of Clinical Genetics, Karolinska University Hospital, Stockholm, Sweden

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**Correspondence:** Saila Laakso, MD, PhD  
Children's Hospital  
Helsinki University Hospital  
PO Box 281  
FI-00029 Helsinki  
Finland  
Fax: +358 9 471 75888  
Phone: +358 50 372 2400  
E-mail: saila.laakso@helsinki.fi

**ESPE members:** Marita Lipsanen-Nyman, Outi Mäkitie

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## ABSTRACT

Background: Previous studies suggest increased risk for hypoandrogenism and fractures in men with obesity. We aimed to describe the effects of severe childhood-onset obesity on the cross talk between metabolic state, testes and skeleton at late puberty.

Methods: A cohort of adolescent and young adult males with severe childhood-onset obesity (n=21, mean age 18.5 yrs) and an age-matched control group were assessed for testicular hormones and DXA-derived bone mass.

Results: Current median body mass indexes for the obese and control subjects were 37.4 kg/m<sup>2</sup> and 22.9 kg/m<sup>2</sup>. Severe early-onset obesity manifested with lower free testosterone [median (inter-quartile range) 244 (194–332) vs. 403 (293–463) pmol/l, P=0.002]. Lower insulin-like 3 [1.02 (0.82–1.23) vs. 1.22 (1.01–1.46) ng/mL, P=0.045] and lower ratio of testosterone to LH [2.81 (1.96 – 3.98) vs. 4.10 (3.03 – 5.83) nM/IU, P=0.008] suggested disrupted Leydig cell function. Degree of current obesity correlated inversely with free testosterone ( $\tau$  =-0.516, P=0.003), which in turn correlated positively with bone area at all measurement sites in males with childhood-onset obesity.

Conclusions: Severe childhood-onset obesity associates with impaired Leydig cell function in young men and lower free testosterone may contribute to impaired skeletal characteristics.

## INTRODUCTION

Childhood-onset obesity is an increasing concern worldwide. Both pubertal development and skeletal growth are sensitive to nutritional status and excessive fat mass [1,2]. Some studies indicate that fat mass may associate negatively with testicular volume in boys with obesity [3] while other studies have found no effect on testicular volume [4]. Further, obesity has been associated with lower testosterone levels in males during [5,6] and after puberty [4,7], but also with lower SHBG levels and similar free testosterone levels during puberty in comparison with lean peers [4,8]. Recent studies have associated obesity in pubertal boys with decreased LH secretion and lower levels of insulin-like 3 (INSL3), another hormone produced by the Leydig cells as puberty proceeds, suggesting impaired Leydig cell stimulation and function, respectively [6,9]. Impaired LH secretion may be due to increased aromatization of androgens to estrogens in fat mass [10]. However, details related to progression of puberty until maturity remain obscure in boys with obesity [11] and the mechanisms leading to decreased testosterone levels in male obesity are far from being captured.

Growth velocity is increased in obese children as compared with normal-weight children [3-5,12]. Bone formation and structure are altered in children with obesity [13,14] and their fracture risk during puberty is increased in relation to lean peers [15]. Testosterone and estrogens are crucial for skeletal maturation and bone mass accrual. Recent evidence suggests the presence of endocrine regulation between bone and testes beyond sex steroids - the bone-testis axis. Osteoblast-derived uncarboxylated osteocalcin (OC) stimulates testosterone production in the testes in mice [16]. In humans, serum total OC levels correlate positively with testosterone levels in adult men [17,18] and pubertal boys [19]. Leydig-cell derived INSL3 may be the feedback signal from the testes that modulates the activity of osteoblasts [20,21]. However, the various physiological functions of the bone-testis axis during growth, and the influence of obesity therein, remain inadequately characterized.

We previously observed that in subjects with severe childhood-onset obesity bone strength is more likely to be compromised in men than in women [22]. This prompted us to evaluate testicular function and related skeletal features in males with severe childhood-onset obesity. We evaluated parameters reflecting metabolic state and pituitary and testicular function in relation to DXA-derived bone characteristics in a case-control cohort of young men.

## METHODS

### *Subjects*

The study was designed to assess skeletal and metabolic characteristics of severe childhood-onset obesity at young adulthood and was carried out at Children's Hospital, Helsinki University Central Hospital, Finland as previously reported [23]. In the present study assessing testicular function, inclusion criteria for the patients with early-onset severe obesity were: i) males with age and sex adjusted body mass index (BMI) corresponding to severe obesity (BMI > 35 kg/m<sup>2</sup> in adult males), according to Finnish growth standards [24], ii) referral because of severe obesity to Children's Hospital, Helsinki University Hospital, during childhood, iii) living in the capital region of Helsinki at age 9 years, and iv) aged between 15 and 25 years at the time of the study. A pediatrician had followed all patients, and endocrine and genetic disorders underlying obesity had been excluded (e.g. Prader-Willi syndrome, hypercortisolism, hypothyroidism, pseudohypoparathyroidism). Control subjects were selected from the national population register based on their age and residential area (hospital's catchment area). Exclusion criteria for the controls were obesity (age and sex adjusted BMI > 30 kg/m<sup>2</sup> in adult males) before the age of 10 years. Patients and controls were studied similarly. An ethical approval was obtained from the Research Ethics Committee of the Hospital District of Helsinki and Uusimaa. Informed written consent was obtained from all study participants or their guardians (subjects aged <18 years).

Anthropometry including height and weight was collected during the study visit. Weight was measured in light clothing with Seca digital scale ([www.seca.com](http://www.seca.com)) to the nearest 0.1 kg. Height was measured with a stadiometer connected to the scale to the nearest 0.1 cm. BMI was calculated as weight divided by square height ( $\text{kg}/\text{m}^2$ ). Lifestyle factors including smoking, alcohol use and current total physical activity, and use of medications were evaluated with questionnaires as described previously [22]. Less than 10 min of total physical activity per day was considered a threshold for a sedentary lifestyle.

### *Laboratory measurements*

Blood samples were obtained between 8 and 10 am after an overnight fast of at least 10 hours for serum estradiol, testosterone, LH, FSH, anti-müllerian hormone (AMH), SHBG, leptin, OC, and INSL3. Serum estradiol and testosterone were analysed on an LC-MS/MS system (API4000, AB Sciex, Concord, Canada). Interassay CVs for estradiol were 5.6% at 60 pM concentration and 5.1% at 360 pM. Interassay CVs for testosterone were 7.6% at 3.3 nM and 5.6% at 23 nM. LH and FSH were measured with electrochemiluminescence immunoassays (Elecsys 2010, Roche Diagnostics, Mannheim, Germany). The limit of detection (LOD) was 0.1 mIU/mL for LH and <0.1 mIU/mL for FSH, with inter-assay CVs across the range of measurement of <6%. AMH was quantitated with an ELISA assay (AMH Gen II ELISA, Beckman Coulter, Brea, CA, USA). The LOD was 0.08  $\mu\text{g}/\text{L}$ . Intra-assay and inter-assay CV was <6% in the range 3.8-16.5  $\mu\text{g}/\text{L}$ . Total CV was <8%. SHBG was quantified with an automatic immunoanalyzer (Immulite 2000XPi, Siemens Healthcare Diagnostics, UK). The LOD of the SHBG assay is 0.02 nmol/L and the total CV  $\leq 7\%$  at SHBG concentrations 1.2-80 nmol/L. Serum leptin was determined with Human Leptin R Quantikine ELISA Kit (R&D Systems, Minneapolis, USA) with intra- and interassay CVs of <12%. Serum total OC was determined by two-site immunoassay protocol [25], as described in detail previously [26]. Free testosterone level (pmol/l) was calculated with the formula *serum testosterone* (nmol/l)  $\times 10 \times [2.28 - 1.38 \times \log(\text{SHBG (nmol/l)} \times 0,1)]$ . INSL3 was measured using an established and well

validated time-resolved fluorescent immunoassay [27], modified for higher sensitivity [28]. The LOD was 10 pg/mL, with intra- and inter-plate CVs across the range of measurement of <3% and <10%, respectively.

### *Bone mineral density and body composition*

Bone area (BA) and bone mineral density (BMD) for whole body, lumbar spine and femoral neck, and total fat and lean mass were measured with Lunar Prodigy Advance DXA on subjects with weight < 160 kg. Prodigy enCORE software was used to estimate relative android and gynoid fat. The android region is the area between the ribs and the pelvis. The upper demarcation is 20% of the distance between iliac crest and neck. Lower demarcation is at the top of pelvis. The gynoid region includes hips and upper thighs. The upper demarcation is below the top of the iliac crest at a distance of 1.5 times the android height and lower demarcation is distal two times the height of android region. Calibration of the measurement was performed with a spine phantom; inter-CV% for BA was 0.38%. Reducibility of DXA measurement for total body is: BMD = 0.85% and BA = 0.78% [29].

### *Statistics*

Results are reported as median (interquartile range, IQR). Differences between two groups were tested with Mann-Whitney U-test and Student's t test. Fischer's exact test was applied for the categorical variables. General linear model was used to test for differences in bone parameters between groups as relative fat mass or relative muscle mass as covariate. We used Kendall's rank correlation test for correlation analyses and partial correlation analysis correcting for age and BMI for normally distributed variables. In order to investigate the effects of the hormones and relative fat mass on skeletal characteristics, we included age, relative fat mass, estradiol, free testosterone, INSL3 and osteocalcin in linear regression models explaining whole body BMD or BA, lumbar spine BMD or BA in the whole group. Significance level was determined as  $P < 0.05$ . No statistical adjustment for multiple testing was performed. All the statistical analyses were

performed with SPSS Statistical package (version 24.0.0.1).

## RESULTS

### *Characteristics*

The characteristics of the males with childhood-onset obesity and control subjects are shown in Table 1. The current median BMI was 37.4 kg/m<sup>2</sup> in case subjects (n=21) and 22.9 kg/m<sup>2</sup> in control subjects (n=21, P<0.001). Males with childhood-onset obesity had higher fasting serum leptin concentration and relative android, gynoid and total fat mass, whereas their relative muscle mass and OC concentration were lower than in controls. Serum FSH and LH concentrations did not differ between the groups. Males with childhood-onset obesity had lower serum total testosterone and SHBG concentrations. Moreover, the calculated free testosterone concentration and the ratio of free testosterone to LH were significantly lower in males with childhood-onset obesity than in control subjects. Serum INSL3 levels were decreased in comparison to controls, as well as serum AMH concentration. Serum estradiol concentrations did not differ between the groups, but the ratio of serum estradiol to total testosterone concentration was higher in males with childhood-onset obesity. Subjects with childhood-onset obesity had higher BMD at all measurement sites: whole body, femoral neck and lumbar spine; BA was larger in whole body measurement (Table 1). When we adjusted bone parameters for relative fat mass or total muscle mass, the only statistically significant difference was observed for femoral neck BMD between groups after adjusting for total muscle mass (p = 0.043). Males with childhood-onset obesity smoked more often (32 % vs 5 %, p = 0.044) and fourth of them had sedentary lifestyle (25% vs. 5%, p = 0.093), but the difference in total physical activity did not reach statistical significance (Table 1). Alcohol use did not differ between the groups (74 % vs. 65 %, p = 0.7).

Based on the medical records, 14 males with childhood-onset obesity had reached pubertal stage of G4-5



at mean age of 15.6 yrs (range, 12.8-17.8), while age at G4 stage was not recorded on seven males. One male with childhood-onset obesity (19.5 yrs) and one control subject (15.4 yrs) had testosterone below 10 umol/l, and FSH and LH below 2 IU/l suggesting that they were still at late pubertal state. Exclusion of these two subjects did not change the statistical significance of comparative results reported in Table 1. Age correlated positively with estradiol concentration in the control group ( $\tau = 0.415$ ,  $P=0.009$ ), but no correlation between age and free testosterone or INSL3 concentrations was observed in either group (data not shown). However, the youngest tertile of the whole cohort had significantly lower INSL3 concentration than older subjects [ $n=10$  vs. 32; 0.89 (0.77 – 1.05) vs 1.19 (1.00 – 1.40),  $P=0.027$ ].

Among the males with childhood-onset obesity, two had daily medication for type 2 diabetes mellitus, two for hypertension, one for depression, and one had used sildenafil for erection difficulties.

#### *Associations between BMI and hormone concentrations*

To describe correlations between body composition and circulating hormone concentrations, BMI was plotted against free testosterone, estradiol, and INSL3 concentrations in Figure 1. We found a strong inverse correlation between BMI and free testosterone ( $\tau = -0.414$ ,  $P<0.001$ , Figure 1A). We observed no significant correlation between BMI and estradiol concentration ( $\tau = 0.107$ ,  $P=0.3$ , Figure 1B), but an inverse correlation between BMI and INSL3 concentration was evident ( $\tau = -0.281$ ,  $P=0.022$ , Figure 1C). To define the associations between fat distribution and testicular hormones, we calculated correlations between android fat, gynoid fat, free testosterone and INSL3. Android fat correlated inversely with free testosterone concentration in males with childhood-onset obesity ( $\tau = -0.410$ ,  $P=0.03$ ), while no other statistically significant association was found (data not shown).

We found no correlation between OC and free testosterone or estradiol in the group of males with severe

childhood-onset obesity. Total OC concentration correlated negatively with free testosterone ( $\tau = -0.343$ ,  $P=0.030$ ) and estradiol ( $\tau = -0.482$ ,  $P=0.002$ ) concentrations only in the control group. To study if these associations were concealed by BMI we adjusted for BMI: no statistically significant correlation was observed between OC and free testosterone, while correlation between OC and estradiol remained significant in the control group ( $r = -0.500$ ,  $P=0.025$ ).

### *Bone parameters in comparison to serum free T, estradiol, and INSL3*

To describe the testis-bone associations, the correlations between bone parameters and free testosterone, estradiol, and INSL3 concentrations were calculated in patient and control groups and controlled for age and BMI (Table 2). Higher free testosterone concentration correlated with higher BA at all measurement sites, and with higher muscle mass in patient group. When analyses were corrected for both age and BMI, no correlation between serum estradiol concentration and bone parameters or muscle mass was found. Serum INSL3 concentration correlated positively with lumbar spine BMD and negatively with BA at femoral neck in males with childhood-onset obesity. In order to investigate the effects of the hormones and relative fat mass, we included them all (age, relative fat mass, estradiol, free testosterone, INSL3 and osteocalcin) in linear regression models explaining whole body BMD or BA, lumbar spine BMD or BA in the whole group. Age [yr, coefficient (95% CI), 55 (2.7 – 108),  $P = 0.040$ ] and relative fat mass [% , 11 (1.5 – 20),  $P = 0.026$ ] had significant associations with whole body BA, while all the other factors in linear regression models lost their power to explain bone parameters (Supplemental Table 1).

## DISCUSSION

Adiposity and altered energy metabolism may have profound effects on pubertal development in boys with childhood-onset obesity. Our key finding demonstrates that severe obesity before the onset of puberty manifests with lower circulating free testosterone levels in late puberty or at early adulthood. Lower

circulating INSL3 concentration and lower ratio of testosterone to LH may indicate disrupted Leydig cell function in the males with childhood-onset obesity as compared with age-matched control subjects. In males with childhood-onset obesity, free testosterone concentration correlated positively with BA at all measurement sites, and INSL3 concentration positively with BMD at lumbar spine.

Our case-control cohort represented a unique group of young adult men who had developed severe childhood-onset obesity before the onset of puberty, and the control group was selected from the same population, excluding subjects who had been obese before puberty. In line with previous studies [6,30], we saw lower total testosterone and free testosterone concentrations in males with childhood-onset obesity. Free testosterone concentration correlated inversely with current BMI. We observed a higher ratio of estradiol to total testosterone in males with childhood-onset obesity, suggesting increased aromatization to estradiol. However, serum estradiol concentrations were similar between males with childhood-onset obesity and control subjects. This may suggest that reduced testosterone concentration may provide less substrate for aromatization to estradiol in the adipose tissue. In line with our results, a previous study on obese males with 14-20 yrs of age demonstrated lower total and free testosterone concentrations, while no difference was observed in LH and FSH levels in comparison to healthy control subjects. The study showed lower total and free estradiol concentrations in males with subnormal testosterone concentration, which indicates that lower testosterone concentration is not secondary to an increase in estradiol concentration [7].

We observed lower free testosterone and INSL3 concentrations in addition to lower T/LH ratio suggesting Leydig cell impairment in males with childhood-onset obesity when compared with controls. Lower INSL3 concentration in males with childhood-onset obesity and the inverse correlation between BMI and INSL3 are in line with previous studies showing significantly lower INSL3 concentration in addition to an inverse correlation between INSL3 and BMI Z-score and leptin concentrations already during puberty [6] and in a cross-sectional study on 31 healthy obese men aged 22-49 years [30]. The differences in INSL3 may also reflect functional changes in pulsatile secretion of LH as suggested in males with Prader-Willi syndrome.

Adolescents with Prader-Willi syndrome and severe obesity may have low LH and INSL3 concentrations, but adult men with Prader-Willi syndrome have normal INSL3 concentrations despite low testosterone [31]. In addition, AMH was lower in cases when compared to controls. Similar inverse correlation between BMI and AMH has been shown previously in a cohort of 166 men aged 22-61 years that included 38 severely obese men with BMI  $\geq$  35 [32].

Leptin, inflammatory cytokines or osteocalcin may be factors connecting severe obesity with decreased testosterone production. A study on mouse testicular cell line has shown that high leptin level decreases cAMP-dependent activation of steroidogenic enzymes Star and Cyp11a1 [33]. Severe obesity results in low-grade inflammation. Interleukin 6 has been shown to inhibit the differentiation of rat stem Leydig cells leading to down-regulation of steroidogenic enzymes and lower serum testosterone levels [34]. Previous studies have suggested that osteoblast-derived OC may stimulate testosterone production [16]. Serum OC concentrations were lower in males with childhood-onset obesity than in controls, but did not correlate with serum testosterone concentrations in the males with childhood-onset obesity. In previous studies, OC concentration has been shown to correlate with testosterone concentration [17-19], but the results are inconsistent [35]. Lower OC concentrations in cases may merely reflect suppressed bone turnover in the obesity [23].

Lower testosterone concentration may have effects on bone structure and strength. Higher BMD in our males with childhood-onset obesity may reflect increased mechanical loading of bones due to overweight, as the differences in bone parameters lost their significance when controlled for fat or muscle mass. To exclude such effects of increased mass, we adjusted analyses between hormones and bone parameters for both age and BMI. In males with childhood-onset obesity, lower free testosterone concentration correlated with smaller BA at all measurements sites. A 3-year longitudinal study on boys with overweight and obesity showed that boys who gained BMI extensively during pubertal development increased their bone mineral characteristics less than those with lower BMI increase [14]. Considering the confounding results of

previous studies on obesity and bone, fat mass may confer advantages to the developing bone, but when fat mass accumulation reaches excessive levels, unfavorable metabolic changes may impede skeletal development [13].

One of the indirect mediators between obesity and bone may be INSL3. Bone is a target site for INSL3. We found a positive correlation between INSL3 and lumbar spine BMD in the subjects with childhood-onset obesity. Another study showed that young men with inactivating mutations of the INSL3 receptor have reduced bone mass and are at risk for osteoporosis despite normal testosterone concentrations [20]. A study on human osteoblasts has demonstrated that INSL3 signaling is involved in bone metabolism by stimulating transcription of genes related to osteoblast maturation and signaling between osteoblasts and osteoclasts [21]. We did not find any statistically significant association between estradiol concentration and bone parameters in either group when we corrected the analyses for both age and BMI. Previous studies have shown positive associations between estradiol and bone mineral acquisition during puberty [36,37], but our relatively small group consisted of males at late puberty or early adulthood. When we analyzed all hormonal parameters together in linear regression models explaining bone characteristics, only age and relative fat mass associated with whole body BA. This may reflect the small sample size of the cohort, undefined confounders or unsuitability of linear regression model in this cohort with extreme phenotypes.

A very small sample size may explain some of the contradictory results in relation to previous studies. The small sample size did not allow us to make subgroup analyses concerning smoking and sedentary lifestyle, although it is well known that many physical factors including poor balance and muscle tone may impact fracture risk. The issue of timing of puberty was not under the scope of this study and we did not have data concerning testicular size, which limits the interpretation of the results. On the other hand, the results emphasize the importance of follow-up of boys with obesity through puberty long enough and underline the importance of evaluating gonadal function in males with severe obesity. Prospective studies on pubertal development in boys with severe childhood-onset obesity are urgently needed.

In conclusion, our results suggest that there may be alterations in the coordinated regulation of testicular function and bone mass in males with childhood-onset obesity. Severe obesity before the onset of puberty manifested with lower circulating free testosterone concentrations at age of early adulthood. Lower circulating INSL3 concentration and lower ratio of testosterone to LH suggested disrupted Leydig cell function. The severity of current obesity correlated inversely with free testosterone concentration, which in turn correlated positively with BA in males with childhood-onset obesity.

## CONFLICTS OF INTEREST

Dr. Laakso has nothing to disclose. Dr. Viljakainen reports grants from The Academy of Finland, during the conduct of the study. Dr. Lipsanen-Nyman reports grants from The Finnish foundation for Pediatric research, grants from The Sigrid Juselius Foundation, grants from Helsinki University Hospital Research Funds, during the conduct of the study. Dr. Turpeinen has nothing to disclose. Dr. Ivaska has nothing to disclose. Dr. Anand-Ivell has nothing to disclose. Dr. Ivell has nothing to disclose. Dr. Mäkitie reports grants from Grants as indicated in the manuscript, during the conduct of the study; personal fees from Alexion, personal fees from Kyowa Kirin, outside the submitted work.

## AUTHOR CONTRIBUTIONS

All authors are responsible for reported research. Saila Laakso wrote the first draft of the manuscript. Ursula Turpeinen performed the steroid analyses. Kaisa K Ivaska performed the osteocalcin analyses. Ravinder Anand-Ivell and Richard Ivell were responsible for the INSL3 measurements. All authors have participated in the concept and design; analysis and interpretation of data; drafting and revising of the manuscript, and they have approved the manuscript as submitted.

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Table 1 Characteristics, metabolic state and pituitary-testes axis in the case-control cohort of males with severe childhood-onset obesity and healthy age-matched controls.

Characteristic	Cases N=21	Controls N=21	P-value
Age (yrs)	18.5 (16.8 – 19.6)	18.4 (16.8 – 19.4)	0.8
Height (cm)	183 (174 – 186)	183 (179 – 186)	0.4
BMI (kg/m <sup>2</sup> )	37.4 (33.6 – 42.9)	22.9 (20.5 – 24.8)	<b>&lt;0.001</b>
Leptin (ng/mL)	27.7 (20.8 – 45.3)	2.8 (1.2 – 3.9)	<b>&lt;0.001</b>
Osteocalcin (ng/mL)	15.7 (11.5 – 19.7)	23.4 (16.7 – 26.5)	<b>0.001</b>
FSH (IU/L)	3.1 (1.9 – 3.4)	3.6 (2.1 – 5.3)	0.4
LH (IU/L)	4.9 (4.0 – 5.9)	4.2 (3.6 – 6.0)	0.4
Testosterone (nM)	12.3 (10.1 – 16.5)	19.4 (13.8 – 22.1)	<b>0.002</b>
SHBG (nM)	20.0 (12.8 – 25.0)	30.0 (21.0 – 41.0)	<b>0.010</b>
Free testosterone (pM)	244 (194 – 332)	403 (293 – 463)	<b>0.002</b>
Ratio testosterone/LH (nM/IU)	2.81 (1.96 – 3.98)	4.10 (3.03 – 5.83)	<b>0.008</b>
INSL3 (ng/mL)	1.02 (0.82 – 1.23)	1.22 (1.01 – 1.46)	<b>0.045</b>
AMH (µg/L)	6.5 (5.0 – 11.7)	10.1 (7.9 – 14.0)	<b>0.047</b>
Estradiol (pM)	107 (96 – 124)	118 (85 – 148)	0.4
Ratio estradiol/testosterone	8.6 (6.0 – 14.6)	6.7 (4.8 – 7.3)	<b>0.008</b>
Whole body BA (cm <sup>2</sup> )*	2750 (2610 – 2890)	2590 (2490 – 2680)	<b>0.047</b>
Whole body BMD (g/cm <sup>2</sup> )*	1.27 (1.22 – 1.31)	1.19 (1.13 – 1.24)	<b>0.026</b>
Lumbar spine BA (cm <sup>2</sup> )*	64.8 (61.3 – 68.4)	61.6 (58.5 – 64.7)	0.2
Lumbar spine BMD (g/cm <sup>2</sup> )*	1.26 (1.18 – 1.34)	1.13 (1.05 – 1.21)	<b>0.029</b>
Femoral neck BA (cm <sup>2</sup> )*	5.48 (5.29 – 5.67)	5.58 (5.41 – 5.75)	0.4
Femoral neck BMD (g/cm <sup>2</sup> )*	1.23 (1.15 – 1.31)	1.06 (0.98 – 1.14)	<b>0.005</b>
Muscle mass (%)*	53.0 (49.2 – 56.8)	76.8 (72.7 – 80.8)	<b>&lt;0.001</b>
Fat mass (%)*	42.7 (39.3 – 46.0)	19.7 (15.5 – 23.9)	<b>&lt;0.001</b>
Android fat (%)	53.4 (47.2 – 56.7)	23.4 (13.6 – 26.7)	<b>&lt;0.001</b>
Gynoid fat (%)	45.9 (39.2 – 50.2)	23.6 (19.6 – 28.3)	<b>&lt;0.001</b>
Physical activity (min/day)	34.0 (9.1 – 49.9)	55.8 (29.4 – 81.5)	0.098

IQR, inter-quartile range; BMI, body-mass index; FSH, follicle stimulating hormone; LH, luteinizing hormone; SHBG, sex hormone-binding globulin; INSL3, insulin-like 3; AMH, anti-müllerian hormone; BA, bone area; BMD, bone mineral density. \*, mean (95% confidence interval), n, cases vs. controls, 18 vs. 21.

Table 2 Correlations between bone parameters and serum free testosterone, estradiol, and INSL3 concentrations controlled for age and BMI in the case-control cohort of males with severe childhood-onset obesity and healthy age-matched controls. For each given correlation, the upper values indicate correlation coefficients and lower values represent p values. Correlations with statistical significance ( $p < 0.05$ ) are indicated in bold.

<b>Partial correlations controlled for age and BMI</b>						
Correlation coefficient P-value	<b>Free testosterone</b>		<b>Estradiol</b>		<b>INSL3</b>	
	Cases	Controls	Cases	Controls	Cases	Controls
WB BA (cm <sup>2</sup> )	<b>0.738</b> <b>0.004</b>	0.003 >0.9	0.281 0.4	-0.106 0.7	-0.078 0.8	-0.133 0.7
WB BMD (g/cm <sup>2</sup> )	0.248 0.4	0.372 0.1	0.229 0.5	0.443 0.058	0.533 0.074	-0.075 0.8
LS BA (cm <sup>2</sup> )	<b>0.669</b> <b>0.012</b>	0.089 0.7	0.247 0.4	0.166 0.5	-0.106 0.7	-0.260 0.4
LS BMD (g/cm <sup>2</sup> )	0.305 0.3	0.318 0.2	0.195 0.5	0.366 0.1	<b>0.634</b> <b>0.027</b>	-0.389 0.2
FN BA (cm <sup>2</sup> )	<b>0.799</b> <b>0.001</b>	-0.166 0.5	0.181 0.6	0.065 0.8	<b>-0.625</b> <b>0.030</b>	-0.114 0.7
FN BMD (g/cm <sup>2</sup> )	0.268 0.4	0.401 0.089	<0.001 >0.9	0.396 0.093	0.418 0.2	-0.171 0.6
Muscle mass (g)	<b>0.798</b> <b>0.001</b>	0.310 0.2	0.221 0.5	0.395 0.094	-0.145 0.7	0.142 0.6

WB, whole body; BA, bone area; BMD, bone mineral density; LS, lumbar spine; FN femoral neck.

Figure 1 Correlations between BMI and free testosterone, estradiol and INSL3 concentrations in the case-control cohort of males with severe childhood-onset obesity and healthy age-matched controls. Cases are indicated with closed circles and controls with open circles. A: BMI and free testosterone concentration; cases,  $\tau = -0.516$ ,  $P=0.003$ ; controls,  $\tau = 0.019$ ,  $P=0.9$ ; all,  $\tau = -0.414$ ,  $P<0.001$ . B: BMI and serum estradiol concentration; cases,  $\tau = 0.388$ ,  $P=0.025$ ; controls,  $\tau = 0.320$ ,  $P=0.043$ ; all,  $\tau = 0.107$ ,  $P=0.3$ . C: BMI and serum INSL3 concentration; cases,  $\tau = -0.229$ ,  $P=0.2$ ; controls,  $\tau = -0.133$ ,  $P=0.5$ ; all,  $\tau = -0.281$ ,  $P=0.022$ .