Obesity: exploring neural pathophysiological pathways and improving diagnostic strategies

Groot, C.J. de

Chapter 5

Determinants of advanced bone age in childhood obesity

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ABSTRACT

Background

Childhood obesity is associated with advanced bone age (BA). Previous studies suggest that androgens, oestrogens, sex hormone binding globulin and insulin are responsible for this phenomenon, but results are contradictory and might be biased by confounders. We aim to elucidate this matter by applying a multivariate approach.

Method

We performed a correlation analysis of BA standard deviation score (SDS) with age and sex specific SDS for androgens, oestrogens, and with indicators of insulin secretion derived from oral glucose tolerance testing, in a group of obese children. A multivariate analysis was performed to investigate which parameters were independently predictive of BA SDS.

Results

In this cohort (n=101; mean age 10.9 yrs; mean BA 11.8 yrs; mean BMI SDS 3.3), BMI SDS was significantly correlated to BA SDS ($r=0.55$, $p< 0.001$). In a regression analysis in the total cohort ($B=0.27$, $p<0.001$), as well as in females ($B=0.34$, $p=0.042$), males ($B=0.31$, $p=0.006$) and pubertal children ($B=0.32$, $p=0.046$), DHEAS showed a positive, independent association with BA SDS. No association with indicators of insulin secretion was found.

Conclusion

BMI SDS is highly correlated to BA SDS in obese children. Increased DHEAS has a central role in advanced bone age in obese children.

INTRODUCTION

The worldwide increase in overweight and obese children has led to significant morbidity, including type 2 diabetes, cardiovascular diseases, fatty liver disease, impaired development and psychological problems (1). Furthermore, children with excess weight have been reported to have accelerated sexual maturation and linear growth, often accompanied by an advanced bone age (BA), and a decreased pubertal growth spurt, compared to normal weight children (2-4). The mechanism driving this BA advancement, however, has remained unclear.

Various alterations in hormone levels have been proposed to be responsible for this phenomenon, such as androgens (5-8), oestrogens (5-7, 9) and sex hormone binding globulin (SHBG) (7). Furthermore, two recent studies have indicated that increasing insulin resistance and insulin secretion are associated with BA advancement (10, 11). These studies, however, vary widely in study design and outcome parameters investigated, and led to contradictory results. For example, some studies evaluated the difference between BA and calendar age (CA) (8, 10) whereas others assessed the ratio between these parameters (11), while an age-adjusted indicator would theoretically be superior. Furthermore, some studies included prepubertal children only (6, 10), while others also included pubertal children (5, 7). Additionally, most studies reported on androgen and oestrogen levels as absolute levels, although these vary significantly with age and pubertal staging, making age an important potential confounder in association studies. Finally, various factors are expected to be mutually dependent.

Therefore, we investigated the multivariate relationship between BA standard deviation score (SDS) for age and sex versus age- and sex-adjusted serum concentrations of serum androgens, oestradiol, SHBG (expressed as SDS) and indicators of insulin secretion in a cohort of prepubertal and pubertal obese children.

METHODS

Study cohort

Obese children visiting our obesity clinic between January 2012 and July 2015, in whom BA assessment and an oral glucose tolerance test (OGTT) were performed, were included into this retrospective cohort study. The OGTT, BA assessment and endocrine measurements were, at that time, part of an extensive diagnostic package which we performed as standard care for all obese children. The aims of this diagnostic approach were a) early detection of glucose metabolism abnormalities and other complications of obesity such as polycystic ovary syndrome (PCOS) and b) detection of endocrine or genetic causes of obesity. Exclusion criteria for this study were endocrine disorders (e.g.

hypothyroidism), syndromes known to affect insulin sensitivity or increased skeletal maturation BA (e.g. Bardet-Biedl syndrome or overgrowth syndromes), medication affecting insulin sensitivity or skeletal maturation (e.g. metformin or methylphenidate), missing fasting insulin or unreliable OGTT data (e.g. due to vomiting or problems with i.v. catheter) and missing BA SDS (e.g. outside age reference range of BoneXpert). In this cohort, patients with marked hyperphagia and early onset obesity (onset of obesity < 5 years of age) were tested for genetic causes of obesity by means of a genetic panel developed at the University Medical Centre in Utrecht. It tests for 53 genes known to cause monogenic obesity. Patients with genetic defects indicating monogenic obesity were included in this study, since there is no reason to assume that their BA is affected in any other way than in other obese children. The results for these patients are shown with specific symbols in the figures. The study was approved by the medical ethics committee of the Leiden University Medical Center and conducted within the terms of declaration of Helsinki. Since all participants received standard of care only, subject consent was waived.

Anthropometric data and definitions

At the first visit, height and weight were measured using a stadiometer and calibrated scale, respectively. Obesity and BMI SDS were determined using the International Obesity Taskforce criteria (12). Height SDS was determined based on the Dutch nation-wide growth study performed in 2009 (13). Modified Tanner staging (14) was performed to determine pubertal stage (Tanner stage >G1 in males or >B1 in females were scored as pubertal).

BA evaluation

We used BoneXpert to determine BA and BA SDS on a radiograph of the left hand (15). BoneXpert is a fully automated system based on an extensive database, which determines the Greulich and Pyle BA by analysing 15 bones of the left hand and wrist (15, 16). BoneXpert is validated to determine BA and associated SDS in males of 2.5-17 years old and in females of 2-15 years for different ethnicities (15, 16). We used Caucasian as standard reference, since our cohort was largely Caucasian and the non-Caucasian participants were of north African and middle eastern descent, for which BoneXpert does not provide ethnicity-specific SDS. The radiographs were made on the date of the first visit, or during the visit for oral glucose tolerance testing.

Oral Glucose Tolerance Test procedure

OGTT was performed after an overnight fast with a minimum of 10 hours. A standardized dose of oral glucose of 1.75 gram/kg, with a maximum of 75 gram, was administered at the beginning of the test. An intravenous catheter was used to collect the blood samples

at t = 0, 30, 60, and 120 minutes. These samples were analysed for insulin and glucose concentrations. An extra sample to measure concentrations of oestradiol (E2), testosterone (T), androstenedione (Adione), dehydroepiandrosterone sulphate (DHEAS), and sex hormone binding globuline (SHBG) was obtained at t=0.

Laboratory measurements

Blood samples were analysed in the clinical laboratory of the Leiden University Medical Center (LUMC, the Netherlands). Immulite 2000 XPi (Siemens Healthcare Diagnostics, Tarrytown NY, USA) immunoassays were used to determine the serum concentrations of insulin (mU/l), SHBG (nmol/l), and DHEAS (umol/l). T (nmol/l) was analysed by immunoassay (ECLIA) on a Roche Modular E170 immunoanalyser, Adione (nmol/l) was analysed using a radioimmunoassay of Beckman Coulter (formerly DSL, Woerden, the Netherlands). Glucose was analysed in serum using a hexokinase method on Roche Modular P800 chemistry analyser. Two different but compatible methods, the automated ECLIA assay of Roche and the Orion ultra-sensitive RIA, were used to measure the E2 levels (pmol/l). Concentrations of the Orion RIA method were converted to ECLIA by a conversion factor. Due to the retrospective nature of this study, using data obtained in standard care, no mass spectrometry measurements were available for estradiol and testosterone. The Roche testosterone (generation 2) and estradiol generation 2 assays are state of the art immunoassays with limits of detection of 0.09 nmol/l and 18.4 pmol/l respectively. Both assays have been standardized against international reference methods (ID-GCMS). The Orion ultrasensitive RIA for estradiol had a similar limit of detection with excellent correlation in comparison with the Roche assay.

Concentrations of measured outcomes under the detection limit were defined as the mean between the lower detection limit of the test and zero. The Homeostatic Model Assessment of insulin resistance (HOMA-IR) was calculated using the formula: T0 glucose (mmol/l) x T0 insulin (mU/l) / 22.5 (17). We calculated the area under the curve for insulin levels during the OGTT using the trapezoid method**.**

Conversion of serum steroid levels to standard deviation scores

In order to estimate the possible influence of serum SHBG, E2 and steroid levels on BA advance, we converted patients' serum levels to SDS, based on published reference values using the same assays. Since the age distribution is skewed, for each age interval separate SD values were calculated above and below the mean. Values for +1 SD and -1 SD were estimated by dividing the difference between P97.5 and P50 and the difference between P50 and P2.5 by 1.96, respectively, as previously described (18). For DHEAS and SHBG we used the age and sex specific centiles provided by Elmlinger et al. (19).

Reference data for T and E2 were derived from the Caliper database (20). For these parameters, we calculated SDS only for children ≥9.0 years of age, since the reference

values for children below 9 years of age were largely below the detection limit. For children ≥9.0 years with plasma concentrations below the detection limit we imputed the data by dividing the lower detection limit by 2. We used the data from the Caliper database to directly calculate 1SD and -1SD scores in different age groups.

For DHEAS, SHBG, T and E2 smoothed-fit lines of the -1SD, P50 and 1SD data points were created, providing an equation to calculate age- and sex-adjusted SDS for these hormones: (serum concentration – age specific P50)/(age specific -1 or +1SD); SDS(X)=([X]-P50)/SD. There were no reference data applicable for our assay for serum insulin and Adione, so these concentrations in our patients could not be expressed as SDS. The results of smooth-fitting and plots of SDS scores in our cohort are summarized in the supplementary figures; SHBG SDS (supplementary figure 1), DHEAS SDS (supplementary figure 2), T SDS (supplementary figure 3), E2 SDS (supplementary figure 4).

Statistical analysis

All analyses were performed using IBM 23.0 SPSS Statistics. We performed analyses for the total cohort, as well as for subgroups based on sex and puberty. Normality was tested using the Kolmogorov-Smirnov and the Shapiro-Wilk Test ($p > 0.05$ was considered normally distributed). We report on mean and SD and median with interquartile range (IQR) for Gaussian and non-Gaussian distributed data, respectively. For the various SD-scores we investigated whether they significantly differed from zero using one-sample t-tests or a one sample Kolmogorov-Smirnov test. Correlation analyses were performed using Pearson and Spearman correlations depending on normality.

First, we performed correlation analyses exploring the possible effect of age and BMI SDS on the various parameters possibly influencing BA. We then investigated the correlation of these parameters with BA SDS. In both analyses, we report on significant correlations ($p<0.05$).

As a last step, we investigated which parameters were independently associated with BA SDS using backward regression analyses, using the pairwise exclusion option in SPSS. In a model with BA SDS as the dependent variable we entered age, sex, DHEAS SDS, SHBG SDS, fasting insulin, HOMA-IR and AUC insulin and investigated independent relationships to BA SDS in the total cohort and subgroups split on sex and puberty. E2 SDS and T SDS were only entered in the model for the pubertal subgroup, since they were unavailable for most prepubertal subjects. We tested the assumptions of each model by checking the independence of the residuals (Durbin-Watson test), inspecting their homogeneity (inspection of the scatterplot) and testing their normality (Kolmogorov-Smirnov test > 0.05).

RESULTS

Study cohort characteristics

Out of the 184 children that visited the Willem-Alexander Children's Hospital 101 children met the inclusion criteria for this study. Figure 1 summarizes the reasons for exclusion of the remaining subjects. Baseline characteristics are presented in table 1. The cohort had a mean age of 10.9 years and a mean BA of 11.8 years resulting in a mean BA SDS of 1.2; 57 % of the children were pubertal, and 47% female. Mean height SDS was 0.6 and mean BMI SDS 3.3. Mean BA SDS and DHEAS SDS were increased (both $p<0.001$), while T SDS and SHBG SDS were decreased versus age references ($p= 0.032$ and 0.003, respectively).

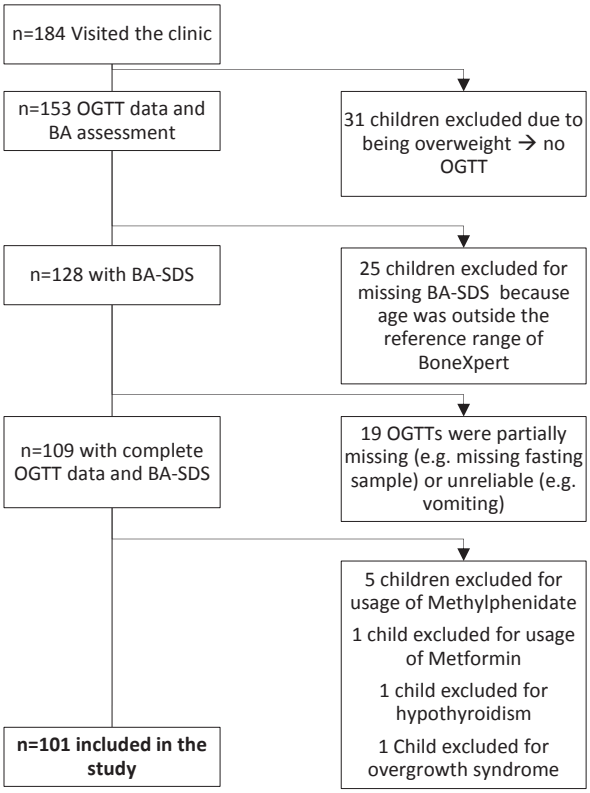

Figure 1. *Flowchart with reasons of exclusion.*

Table 1. Baseline characteristics, expressed as mean (standard deviation) unless otherwise stated

	Total cohort		Subgroups by sex				Subgroups by puberty			
	n	(n=101)	n	Female (n=47)	n	Male (n=54)	n	Prepubertal (n=43)	n	Pubertal (n=57)
Age, yrs	101	10.9 (3.1)	47	10.3 (2.9)	54	11.4 (3.1)	43	8.4 (1.9)	57	12.8 (2.3)
BA, yrs	101	11.8 (2.1)	47	10.9 (2.9)	54	12.6 (2.9)	43	9.6 (2.0)	57	13.5 (2.5)
BA SDS	101	1.2 (1.1)*	47	0.8 (1.2)*	54	1.4 (1.0)*	43	1.6 (1.1)*	57	0.8 (1.1)*
Caucasian[a]	101	64 (63.4)	47	27 (57.4)	54	37 (68.5)	43	19 (44.2)	57	44 (77.2)
Height SDS	101	0.6 (1.0)*	47	0.4 (1.0)*	54	0.7 (1.1)*	43	0.8 (1.0)*	57	0.4 (1.0)*
BMI SDS	101	3.4 (0.6)*	47	3.1 (0.5)*	54	3.6 (0.7)*	43	3.6 (0.7)*	57	3.2 (0.5)*
Fasting insulin mU/l[b]	101	12 (6/18)	47	11 (6/19)	54	12 (7/16)	43	7 (4/11)	57	16 (10/26)
HOMA-IR[b]	101	2.1 (1.3/3.7)	47	2.1 (1.3/3.9)	54	2.3 (1.3/3.5)	43	1.3 (0,8/2.0)	57	2.4 (2.1/4.8)
AUC insulin mU/l[b]	94	305 (202/468)	43	267 (186/ 454)	51	309 (210/482)	40	211 (159/397)	53	362 (238/544)
Oestradiol SDS	50	0.0 (1.4)	21	-0.2 (1.1)	29	0.1 (1.5)		-	39	0.0 (1.3)
Testosterone SDS[b]	64	-0.6 (-1.2/0.3)*	28	-0.7 (-2.7/1.6)	36	-0.6(-1.1/0.0)*		-	51	-0.7 (-1.3/0.4)*
DHEAS SDS[b]	96	0.4 (0.0/1.0)*	45	0.3 (-0.8/0.7)	51	0.4 (0.1/1.0)*	41	0.4 (-0.3/1.1)	54	0.3 (0.0/0.9)*
***SHBG SDS**[b]	96	-1.9 (-2.4/-1.1)*	46	-1.9 (-1.2/-2.2)*	50	-1.9 (-2.4/-1.1)*	40	-1.5 (-2.2/-0.7)*	55	-2.0 (-2.4/-1.4)*
Androstenedione nmol/l[b]	100	2.25 (1.30/3.60)	47	2.40 (1.20/4.10)	53	2.20 (1.30/3.30)	42	1.25 (0.78/2.23)	57	3.00 (2.15/4.50)

Table 1. *Data on oestradiol SDS and testosterone SDS are on age group ≥ 9 years.* [a] *n (%)* [b] *Median (interquartile range) * SDS p < 0.05. Abbreviations: SDS, standard deviation score; BA, bone age; BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin-resistance; AUC, area under the curve; DHEAS, dehydroepiandrosterone sulphate; SHBG, sex hormone binding globulin.*

In five subjects of the final cohort, a genetic mutation was found. Two male subjects showed a heterozygous *MC4R* mutation (Cys293Tyr and Ile251Leu) and one male subject showed a heterozygous mutation in *WDPCP* (Leu379Ser). Furthermore, one female subject showed a heterozygous, mutation in *BBS7* (Gln365Leu), while another female subject was found to have two heterozygous variants in *CEP290* (Ile1059fs) and *MKKS* (Ala242Ser).

Correlation between outcome parameters and age or BMI SDS

The correlation analysis of the outcome parameters with age and BMI SDS are presented in table 2 and scatterplots are shown in Figure 2. The data are presented as Pearson correlation or Spearman's ρ, where applicable. BMI SDS was negatively correlated with age in prepubertal children and positively in pubertal children, showing a U-shape over the whole age range (Fig. 2A).

The insulin parameters in the total cohort as well as in subgroups according to sex and puberty showed a positive correlation with age. T SDS showed a significant negative correlation with age (ρ = -0.35) in males. In the prepubertal subgroup, SHBG SDS

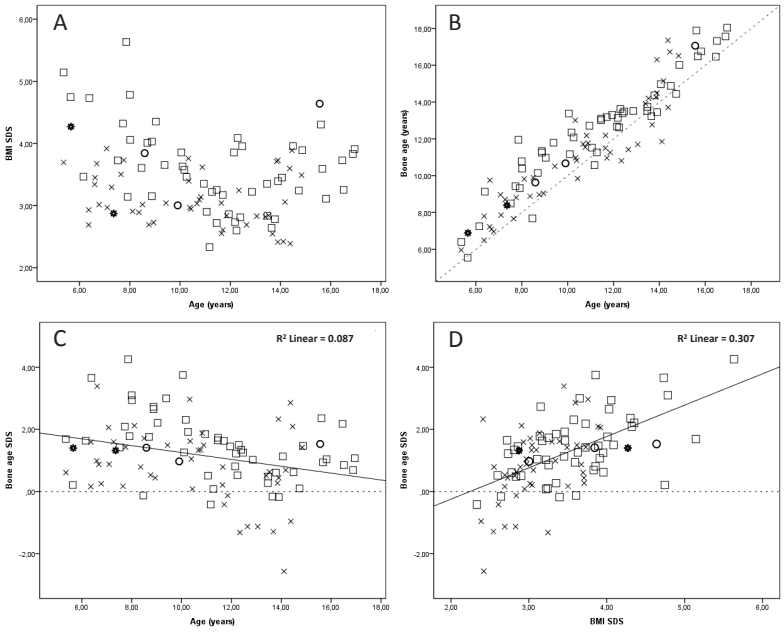

Figure 2. *A: association between BMI SDS and age (years); B: association between bone age (years) and chronological age (years); C: association between bone age SDS and age; D: association between bone age SDS and BMI SDS. Abbreviations: SDS: standard deviation score; BMI: body mass index; R^2: Coefficient of deviation. Squares represent males, x represent females, bold circles represents males with monogenetic obesity, bold stars represent females with monogenetic obesity.*

Table 2. Correlations between outcome parameters and age or BMI SDS

		BMI-SDS[a]	Fasting insulin	HOMA-IR	Insulin AUC	Oestradiol SDS_a	Testosterone SDS	DHEAS SDS	SHBG SDS
Age	**Total cohort**	-0.22*	0.65***	0.65***	0.48***	0.19	-0.17	0.05	-0.12
	Female	-0.26	0.68***	0.69***	0.58***	0.29	-0.09	0.00	0.02
	Male	-0.35**	0.63***	0.62***	0.40**	0.15	-0.35*	0.03	-0.20
	Prepubertal	-0.36*	0.59***	0.61***	0.53***	-	-	0.35*	-0.38*
	Pubertal	0.28 *	0.33*	0.30*	0.24	0.28	-0.10	-0.03	0.19
BMI SDS	**Total cohort**	-	-0.08	-0.10	0.11	0.19	0.14	0.20	-0.17
	Female	-	-0.11	0.11	0.06	0.28	0.36	0.22	-0.15
	Male	-	-0.13	-0.15	0.08	0.13	-0.16	0.07	-0.14
	Prepubertal	-	-0.19	-0.25	-0.05	-	-	0.13	-0.08
	Pubertal	-	0.28*	0.28*	0.42**	0.23	0.07	0.27	-0.39**

Table 2. *Correlations are shown as Spearman rho's unless otherwise stated. Correlations for oestradiol SDS and testosterone SDS are calculated on age group ≥ 9 years. Abbreviations: BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; insulin AUC, area under the curve of insulin during oral glucose tolerance test; SDS, standard deviation score; DHEAS, dihydroepiandrosterone sulphate; SHBG, sex hormone binding globulin.* [a] *Pearson correlation; *p < 0.05; **p <0.01; *** p<0.001.*

negatively correlated with age (ρ = -0.38), in contrast to a positive correlation with age (ρ = 0.35) for DHEAS SDS.

Fasting insulin (ρ = 0.28), HOMA-IR (ρ = 0.28) and AUC insulin (ρ = 0.42) showed a significant positive correlation with BMI SDS in the pubertal subgroup. BMI SDS showed a trend toward a positive correlation with DHEAS SDS in the whole cohort and pubertal subgroup. In contrast, there was a trend toward a negative correlation with SHBG SDS in the whole cohort, which reached statistical significance in the pubertal subgroup.

Correlation between BA SDS versus clinical and biochemical parameters

Figure 2B shows that in the great majority of patients BA is advanced. As shown in table 3 and Fig 2C, BA SDS is relatively more advanced in young children: there was a significant negative correlation between BA SDS and age in the total cohort (*r* = -0.29) as well as in subgroups split on sex (female r = -0.31, male r = -0.41). BMI SDS showed a strongly significant correlation with BA SDS in the total cohort (ρ = 0.55) (figure 2D), as well as in female (ρ = 0.49), male (ρ = 0.55), prepubertal (ρ = 0.52) and pubertal (ρ = 0.51) subgroups.

Correlations between BA SDS and biochemical variables are presented in table 3. In females, T SDS showed a positive correlation with BA SDS (ρ = 0.44). In the total cohort, as well as in the male and both puberty subgroups, DHEAS SDS showed a positive correlation with BA SDS. SHBG SDS was negatively associated with BA SDS, particularly in pubertal children (ρ = -0.31). The insulin parameters and E2 SDS did not show significant correlations with BA SDS in the total cohort, nor in any subgroup.

Table 3. Correlation between bone age SDS and clinical and biochemical variables

		Sex		Puberty	
	Total cohort	**Female**	**Male**	**Prepubertal**	**Pubertal**
Age in years[a]	-0.29**	-0.31*	-0.41**	-0.15	-0.07
BMI SDS[a]	0.55 ***	0.49***	0.55***	0.52***	0.51***
Fasting insulin	-0.14	-0.22	-0.15	0.02	0.09
HOMA-IR	-0.14	-0.21	-0.14	-0.03	0.12
AUC insulin	0.07	-0.06	0.13	0.13	0.22
Oestradiol SDS[a]	0.14	0.13	0.13	-	0.10
Testosterone SDS	0.24	0.44*	-0.06	-	0.18
DHEAS SDS	0.29**	0.18	0.33*	0.32*	0.28*
SHBG SDS	-0.17	-0.10	-0.22	-0.24	-0.31*

Table 3. *Correlations are expressed as Spearman's rho (p-value) unless otherwise stated. [a] Pearson correlate (p-value). Correlations for oestradiol SDS and testosterone SDS are calculated on age group ≥ 9 years. Abbreviations: SDS, standard deviation score; BA, bone age; BMI, body mass index; HOMA-IR, homeostatic model assessment for insulin-resistance; AUC, area under the curve; DHEAS, dehydroepiandrosterone sulphate; SHBG, sex hormone binding globulin. *p < 0.05; **p <0.01; *** p<0.001.*

Regression analysis for BA SDS

The results of backward regression analysis are summarized in table 4. In the total cohort, backward regression analysis resulted in a model including sex, DHEAS SDS and age, explaining 27% of the total variance in BA SDS (overall fit of the regression model F=10.55, p<0.001). In the female subgroup we found a model explaining 21% of the vari-

Table 4. Backward regression analysis of bone age SDS

		Coefficiënt	CI 95%	R^2	p-value
Total cohort (n=88)	Constant	2.17	1.39/2.96		<0.001
	Sex male	0.62	0.18/1.06		0.006
	DHEAS SDS	0.27	0.09/0.44		<0.001
	Age	-0.13	-0.20/-0.06		0.036
	Model			0.27	<0.001
Female (n=52)	Constant	2.68	1.25/4.11		0.001
	DHEAS SDS	0.34	0.01/0.66		0.042
	SHBG SDS	0.29	-0.04/0.62		0.086
	Age	-0.14	-0.26/-0.02		0.024
	Model			0.21	0.030
Male (n=46)	Constant	2.81	1.80/3.81		<0.001
	DHEAS SDS	0.31	0.09/0.52		0.006
	Age	-0.14	-0.22/-0.05		0.002
	Model			0.30	<0.001
Prepubertal (n=36)	Constant	2.26	1.17/4.02		0.001
	Sex male	0.98	0.30/1.67		0.006
	SHBG SDS	-0.41	-0.76/-0.09		0.013
	Age	-0.27	-0.46/-0.07		0.009
	Model			0.31	0.006
Pubertal (n=37)	Constant	0.70	0.32/1.08		0.001
	DHEAS SDS	0.43	0.01/0.85		0.046
	Model			0.11	0.046

Table 4. *Backward linear regression analysis of bone age SDS. Variables included in all model: age, fasting insulin, HOMA-IR, AUC-insulin, DHEAS SDS, SHBG SDS. In the total cohort and pubertal subgroups, sex was added as an independent variable. In the pubertal subgroup only, oestradiol SDS and testosterone SDS were added as independent variables. Abbreviations: HOMA-IR, homeostatic model assessment of insulin-resistance; SHBG, sex hormone binding globulin; DHEAS, Dihydroepiandrosterone sulphate; SDS, standard deviation score.*

ance in BA SDS including DHEAS SDS, SHBG SDS and age (F=3.33, p=0.030). In contrast, in males the model did not include SHBG SDS, but only contained DHEAS SDS and age (F=9.50, p<0.001), explaining 30% of the variance. For the subgroups split on puberty, regression analysis showed a model explaining 31% of variance in BA SDS, including sex, SHBG SDS and age in prepubertal subjects (F=4.88, p=0.006) and a model explaining 11% of variance only including DHEAS SDS (F=4.27, p=0.046) in pubertal subjects.

DISCUSSION

The results of this study show that the mechanisms driving BA advancement in obese children are complex. In multiple regression analysis we have shown that DHEAS levels positively associate with BA SDS and SHBG levels negatively. Results are variable, however, across subgroups according to sex and pubertal status. Furthermore, we were able to explain only a limited percentage of the variance in BA SDS (with a maximum of 31% in prepubertal children), indicating that some factors driving bone age advancement were not included in this analysis.

As expected, in this cohort of obese children, mean height SDS was above average for the population, and BA was advanced compared to chronological age (CA). Furthermore, BA SDS and BMI SDS were strongly correlated. This is in line with studies reporting advanced linear growth and skeletal maturation in children with excess weight (2-4, 21). In addition, we have confirmed previous studies (3, 7, 22) showing that obese children have high DHEAS levels compared to a reference population. Our observation that DHEAS SDS is associated with BA SDS, especially in pubertal children, independent of various confounders, is in accordance with the results of a study by Sopher et al., which showed that, in a group of obese children, the highest tertile of the ratio between BA and CA was associated with high DHEAS levels (6). These authors posed that high DHEAS levels indicate high levels of androgens, leading to increased levels of E2 by peripheral conversion, which in turn leads to advanced bone maturation. The absence of an association between E2 and BA SDS in our cohort might be caused by the fact that our E2 assay lacks sensitivity in the lower ranges. Consistent with this explanation is a study by Klein et al. showing that E2 levels correlated with bone age in obese and lean children when using a more sensitive assay (5). Alternatively, it has been suggested that the production of E2 takes place at tissue level (6), so that no rise in circulating E2 levels can be detected, thereby explaining the lack of association between E2 and BA SDS in our cohort. Furthermore, our findings are in line with the work of DeSalvo et al. who showed that, in a cohort of children with premature adrenarche, the subgroup of children with BA advancement > 2 years had higher BMI and higher DHEAS levels than the subgroup of children with BA advancement < 1 year. This might suggest an overlap between the

pathophysiological mechanisms leading to BA advancement in patients with premature adrenarche and patients with obesity (23).

Although the pathophysiological mechanism remains uncertain, the results of our study show an independent association between DHEAS SDS and BA SDS in the total cohort as well as in males, females and pubertal children, indicating a central role for DHEAS in the BA advancement found in obese children. The scientific implications of the results of our study are that insulin is an unlikely cause of bone advancement in obese children, while DHEAS secretion can now be viewed as at least one of the intermediary factors. A possible clinical implication of our findings could be that it would be useful to measure DHEAS in obese children with substantial bone age advancement and/or increased statural growth. If available, it would also be useful to measure serum oestradiol with an ultrasensitive assay. In case of high concentrations, these could be accepted as causes of the clinical phenotype, so that the clinician can consider abstaining from further diagnostic workup of the patient.

Our finding of decreased plasma SHBG levels in obese children compared to reference intervals, based on lean children, is in accordance with previous reports (3, 7, 23) and has been reported to be caused by hyperinsulinemia, related to insulin resistance and low grade inflammation (24). Using sensitive E2 assays, it was also shown that obese adolescents have increased E2 levels, combined with decreased SHBG levels, possibly resulting in high levels of free E2 (23), which in turn might lead to increased bone maturation (24).

In addition to the generally decreased SHBG in obese children, we found a negative association in the regression analysis of SHBG SDS with BA SDS in prepubertal children. Decreased SHBG is associated with the increase of andrenal androgens during puberty (25), which in there turn, can stimulate bone maturation by locally increasing oestrogen levels via expression of aromatase (26). In contrast, a trend toward a positive association between SHBG SDS and BA SDS was found in regression analysis in the female subgroup, possibly reflecting increased gonadal oestrogen production during puberty, stimulating SHBG in girls. This association, however, did not reach significance ($p=0.086$), possibly because it is obscured by lack of assay sensitivity or the combination of the results of two oestrogen immunoassays.

It is of interest that we did not find an association between any of the insulin parameters with bone age advancement, neither in correlation analyses, nor in regression analyses. In the literature, contradictory results on the association between hyperinsulinemia and advanced bone age have been reported. No association between insulin resistance and the ratio between BA and CA was found in prepubertal children in a study by Sopher et al. (6) whereas Klein et al. found an association between insulin levels and the top tertile of this ratio in a cohort aged 3-18 years (5). Furthermore, Pinhas-Hamiel et al. showed that overweight children aged 4-13 years with a fasting insulin > 30 mU/l had a 6.8 fold increased risk of falling into the top tertile of the ratio between BA and CA,

independent of degree of obesity (11). Lee et al. investigated the relation between insulin resistance and bone age in prepubertal obese children and found an independent, positive correlation between HOMA-IR and the difference between BA and CA using multiple regression analysis (10). None of these three studies, however, corrected for the possible confounding effects of androgens and oestrogens, which might bias these results, and the outcome parameter of bone advance was not adjusted for age and sex. Furthermore, there was considerable variability in ethnicity between studies, which might in part explain the differences in outcome. In addition, the positive association between insulin secretion and age could lead to bias too. Another possible explanation for the lack of association between bone age SDS and insulin parameters in this cohort might be that a large number of the subjects in this cohort is already insulin resistant. Possibly the effects of insulin on bone age are more pronounced in children in the early stages of developing insulin resistance.

The finding of independent effects of sex in the multiple regression analysis is remarkable. It suggests that male and female subjects are differentially affected by increased BMI in their advanced bone maturation. This is in agreement with the findings of Crocker et al. who have recently shown that pubertal development is differentially affected in obese male and female subjects. They showed that, in female subjects, progressive Tanner staging correlated with advanced BA, while in boys BA advancement was independent of testicular development. Furthermore, insulin resistance correlated positively with breast development in girls while it was negatively correlated with testicular size in boys (27). This underlines the sexual dimorphism in the way obesity affects maturation.

As shown in our regression models, the maximum percentage of variance explained by a model was 31%, suggesting that factors not included in this study might contribute to BA advancement. It has been suggested that leptin (28) and IGF-1 (8) might contribute to bone age advancement in obesity, although recent work by Sopher et al. showed no association between these parameters and BA advancement (6). Future studies in larger cohorts should include these parameters to clarify the role these factors play in this matter.

A major strength of our study is the use of an automated method for BA assessment, which results in a reduced inter-subject, and an absent inter-observer variance (29). The use of BoneXpert also enabled the calculation of a reliable BA SDS from a representative population reference. Furthermore, where possible, we used age and sex specific SDS to investigate the relationship between hormone levels and BA SDS, thereby correcting for variance in these hormones caused by age and sex. Furthermore, we corrected for multiple confounders using regression analysis, which makes a causal relationship between the observed factors associated with advanced BA more plausible.

A limitation of our study is the fact that BoneXpert only supports the BA assessment of boys between 2.5 – 17 years and girls between 2.0 – 15 years (15, 16). However, older ad-

olescents have usually reached near adult height by this age, and we pose that therefore they are a clinically less relevant study group. Secondly, BoneXpert contains reference data for standard deviation scores of Caucasian, Asian, Hispanic, or Afro-Americans (16) but not from children of Turkish or Moroccan background. Therefore, we used Caucasian references as the standard for all children. The majority of the cohort, however, is Caucasian. A third limitation is that the assays for oestradiol has a limited sensitivity, which might obscure its association with BA SDS in prepubertal children. Finally, due to the large number of potential confounders included in the regression models, our sample size was too small to investigate sex effects separately in the prepubertal and pubertal age group. In addition, the small sample size in some subgroups (e.g. prepubertal), may have led to false negative results in the multiple regression analysis. Future research should therefore include larger cohorts, allowing for adjusting for multiple confounders in the regression analysis. Furthermore, longitudinal designs could help to gain additional insights into the mechanisms driving accelerated bone maturation in obesity. In addition, future studies would benefit from age and sex specific SDS for Adione and insulin, and should include leptin and IGF-1.

In conclusion, using multiple regression analysis, we have shown that increased DHEAS levels, reflecting adrenal androgen production, play a central role in BA advancement in obese children and adolescents and that decreased SHBG levels may further contribute to this phenomenon, though this finding needs further investigation.

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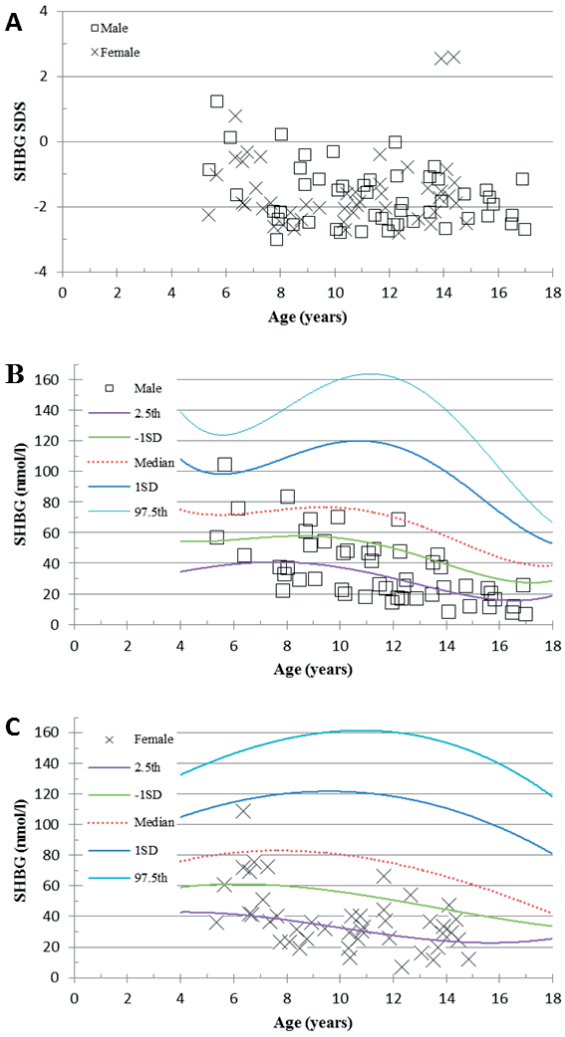
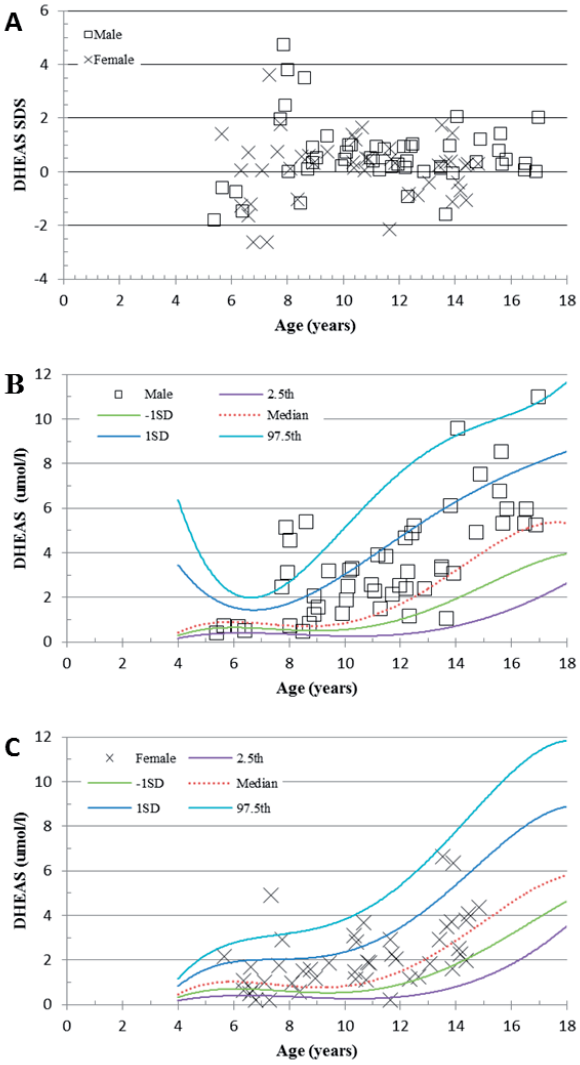
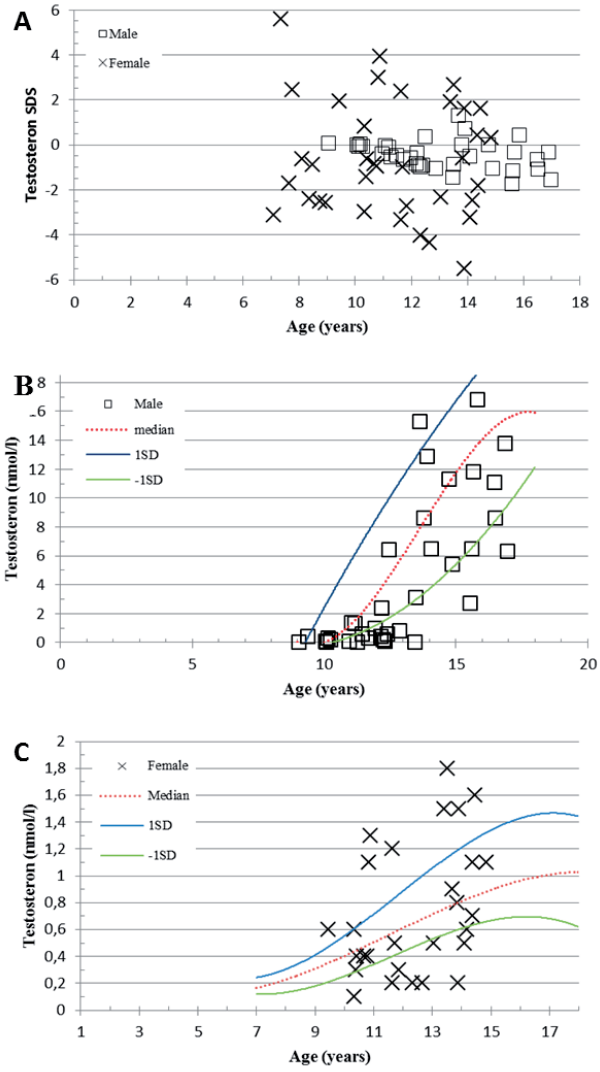
SUPLEMENTARY FIGURES



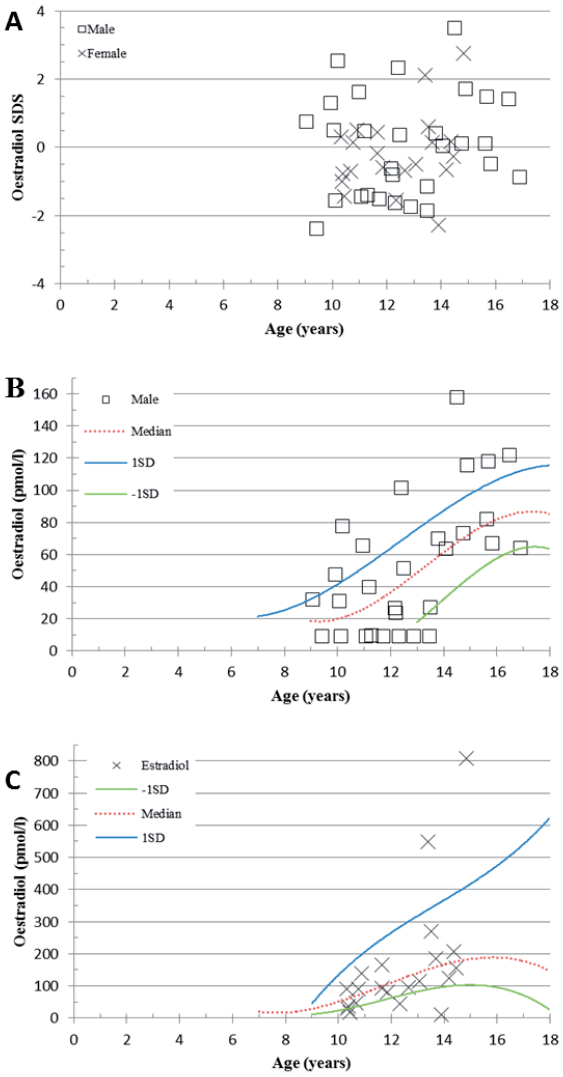
Supplementary figure 1. *A: scatterplot of SHBG SDS versus age. B: SHBG (nmol/l) versus age in males plotted on the age reference range. C: SHBG (nmol/l) versus age in females, plotted on the age reference range. Abbreviations: SDS, standard deviation score; SHBG, sex hormone binging globulin. The coloured lines represent references values of normal weight children from Elmlinger et. al. (19).*



Supplementary figure 2. *A: scatterplot of DHEAS SDS versus age. B: DHEAS (µmol/l) versus age in males plotted on the age reference range. C: DHEAS (µmol/l) versus age in females, plotted on the age reference range. Abbreviations: SDS, standard deviation score; DHEAS, dehydroepiandrosterone sulphate. The coloured lines represent references values of normal weight children from Elmlinger et. al. [19].*



Supplementary figure 3. *A: scatterplot of testosterone SDS versus age. B: testesterone (nmol/l) versus age in males plotted on mean ± 1SD lines based on a reference population. C: testosterone (nmol/l) versus age in females plotted on mean ± 1SD lines based on a reference population. Abbreviations: SDS, standard deviation score. The coloured lines represent references values of normal weight children from Konforte et. al. [20].*



Supplementary figure 4. *A: scatterplot of oestradiol SDS versus age. B: oestradiol (pmol/l) versus age in males plotted on mean ± 1SD lines based on a reference population . C: oestradiol (pmol/l) versus age in females plotted on mean ± 1SD lines based on a reference population . Abbreviations: SDS, standard deviation score. The coloured lines represent references values of normal weight children from Konfronte et. al. [20].*