RESEARCH

Higher steroid sulfation is linked to successful weight loss in obese children

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Abstract

Objective: Little information is available on the steroid sulfates profile in obese children. Therefore, we examined whether sulfated steroids are linked with weight status and associated comorbidities in obese children.

Methods: We analyzed 66 obese children (mean age 10.5 ± 2.5 years, 57.6% female, 53.9% prepubertal, mean BMI 27.0 ± 4.6 kg/m², 50% with BMI-SDS reduction >0.5 , 50% without BMI-SDS reduction) who participated in an outpatient 1-year intervention program based on exercise, behavior and nutrition therapy. We measured intact sulfated steroids (cholesterol sulfate (CS), pregnenolone sulfate (PregS), 17 α OH pregnenolone sulfate (17OH-PregS), 16 α OH dehydroepiandrosterone sulfate (16OH-DHEAS), DHEAS, androstenediol-3-sulfate, androsterone sulfate and epiandrosterone sulfate) by LC–MS/MS, and insulin resistance index HOMA, lipids, blood pressure at baseline and 1 year later.

Results: All sulfated steroids except 17OH-PregS, 16OH-DHEAS, androsterone sulfate and epiandrosterone sulfate were higher in boys compared to girls. Concentrations of CS before intervention were higher in children who lost weight. After 1 year of treatment, both groups showed increased levels of DHEAS, 16OH-DHEAS and androstenediol-3-sulfate, but PregS was only increased in children with weight loss. None of the steroid sulfates was significantly related to cardiovascular risk factors or HOMA except 17OH-PregS, which was associated with systolic blood pressure both in cross-sectional (β -coefficient: 0.09 ± 0.07 , $P=0.020$) and longitudinal analyses (β -coefficient: 0.06 ± 0.04 , $P=0.013$) in multiple linear regression analyses.

Conclusions: Since higher steroid sulfation capacity was associated with successful weight intervention in children disruption of sulfation may be associated with difficulties to lose weight. Future studies are necessary to prove this hypothesis.

Key Words

- lifestyle intervention
- weight loss
- sulfated steroids
- cholesterol sulfate
- children

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Introduction

Obesity is a complex condition associated with changes in many steroid hormones also including androgens: concentrations of testosterone and DHEAS and their precursors are increased in children (1, 2) and obese women (3), while obese men demonstrated decreased testosterone levels (4). Interestingly, obese women with

increased androgens and obese men with low testosterone concentrations are more prone to metabolic disturbances such as insulin resistance, type 2 diabetes mellitus, lipid abnormalities and hypertension and may therefore be at particular risk of developing atherosclerotic complications (2, 5, 6, 7, 8, 9). Furthermore, increased androgens are

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related to polycystic ovarian syndrome (PCOS) in females (7, 10). In children, a high androgenic activity is discussed to be associated with precocious puberty, premature adrenarche and accelerated bone age with relatively tall stature (11, 12).

Of importance, the great majorities of studies analyzed unconjugated steroids, but recent research suggests that also sulfated steroids may be involved in these processes linking steroids to comorbidities of obesity. For example, a relationship between the concentration of sulfated steroids and glucose tolerance has been reported in mice (13). Sulfated steroids are the most abundant fraction of steroids in human blood. Sulfated steroids are originally synthesized from the unconjugated steroid by sulfation. This reaction, which belongs to phase II metabolism, requires the action of steroid sulfotransferases (SULTs) (14). Disruption of sulfation due to inactivating mutations in the human gene encoding PAPSS2, a crucial cofactor of sulfotransferases, has been shown to result in increased androgen activation and PCOS phenotype in both homozygous and heterozygous individuals (15, 16).

Sulfated steroids cannot interact directly with steroid receptors (17). However, there is growing evidence suggesting that sulfated steroids can access different tissues of the human body by circulation and transport into cells, where they can be activated by the action of steroid sulfatase (STS) (18). In absolute STS deficiency (STSD), most sulfated steroids in serum are elevated compared to healthy controls (19). STS is not the only enzyme that exhibits sulfatase activity for sulfated steroids, at least *in vitro*. Recently, we reported that 3 β -hydroxysteroid dehydrogenase type 2 (3 β HSD2) can cleave the sulfate group of some sulfated steroids to produce unconjugated steroids, i.e. PregS produced progesterone and androstenedione was synthesized from DHEAS (19).

As only sparse data exist on the sulfates of androgens and their precursors in children, we analyzed intact sulfated steroids using state-of-the-art liquid chromatography–tandem mass spectrometry (LC–MS/MS) (20). This method can quantify 11 sulfated steroids simultaneously, providing a powerful tool to understand the sulfated steroidome in human blood (21). We hypothesized that sulfated steroids concentrations are linked with weight status in obese children since substantial weight loss in obese children is associated with variations of unconjugated steroids like androgens and corticoids (1, 2, 6). In addition, we analyzed whether cardiovascular risk factors linked to obesity, including insulin resistance, are associated with steroid sulfates.

Subjects and methods

Subjects

Written informed consent was obtained from all children and their parents. The study was approved by the Local Ethics Committee of the University of Witten/Herdecke in Germany.

We examined 66 obese Caucasian children (mean age 10.5 ± 2.5 years, 57.6% female, 53.9% prepubertal, mean BMI $27.0 \pm 4.6 \text{ kg/m}^2$). We choose 33 children with substantial BMI-SDS reduction of >0.5 and 33 age-, gender- and pubertal stage-matched children without BMI-SDS reduction. This classification was used because a reduction of >0.5 SDS-BMI leads to an improvement of insulin resistance as well as cardiovascular risk factors and normalized hormones like leptin, cortisol or adiponectin (6, 22, 23, 24).

All children participated in the lifestyle intervention ‘Obeldicks’, which has been described in detail elsewhere (25). Briefly, this outpatient intervention program for obese children is based on physical exercise, nutrition education and behavior therapy including the individual psychological care of the child and his or her family. The nutritional course is based on a fat and sugar-reduced diet as compared to the every-day nutrition of German children.

Exclusion criteria

None of the children in the current study suffered from endocrine disorders, premature adrenarche or syndromal obesity. None of the obese children entered into puberty during the study period.

Measurements

We analyzed BMI, blood pressure (BP), lipids, the insulin resistance index HOMA and sulfated steroids in children (cholesterol sulfate (CS), pregnenolone sulfate (PregS), 17OH-PregS, DHEAS, 16OH-DHEAS, androstenediol-3-sulfate (A-3-S), androsterone sulfate (AS) and epiandrosterone sulfate (ES)) at baseline and at the end of the 1-year lifestyle intervention ‘Obeldicks’.

Height was measured to the nearest centimeter using a rigid stadiometer. Weight was measured unclothed to the nearest 0.1 kg using a calibrated balance scale. BMI was calculated as weight in kilograms (kg) divided by the square of height in meters (m^2). The degree of overweight was quantified using Cole’s least mean square method,

which normalized the BMI skewed distribution and expressed BMI as a standard deviation score (BMI-SDS) (26). Reference data for German children were used (27). All children in the study were obese according to the definition of the International Obesity Task Force (28).

Pubertal stage was determined by well-trained physicians according to Marshall and Tanner. Pubertal developmental stage was categorized into two groups based on breast and genital stages (prepubertal: boys with genital stage I, girls with breast stage I, pubertal: boys with genital stage $\geq$ II; girls with breast stage $\geq$ II).

BP was measured using a validated protocol (29). Systolic and diastolic BP were measured at the right arm twice after a 10-min rest in the supine position by using a calibrated sphygmomanometer and averaged. The cuff size was based on the length and circumference of the upper arm and was as large as possible without having the elbow skin crease obstructing the stethoscope (29). The intra- and inter-operator variability was $<5\%$ for systolic and diastolic BP.

Blood sampling was performed in the fasting state at 08:00h. After clotting, blood samples were centrifuged for 10 min at 5150g. Serum was stored at -81°C for later determination of steroid hormones sulfates and insulin. All samples were thawed only once. Serum triglyceride, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol and total cholesterol concentrations were measured using commercially available test kits (LDL-C- and HDL-C-Plus, Roche Diagnostics; Vitros analyzer, Ortho Clinical Diagnostics, Neckargemuend, Germany; MEIA, Abbott). Intra- and inter-assay variations for the concentrations (CV) of these variables were less than 5%. Insulin concentrations were measured by microparticle enhanced immunometric assay (MEIA, Abbott). Glucose levels were determined by colorimetric test using a Vitros analyzer (Ortho Clinical Diagnostics). Homeostasis model assessment (HOMA) was used to detect the degree of insulin resistance using the formula: resistance (HOMA) = (insulin (mU/L) $\times$ glucose (mmol/L))/22.5 (30).

Measurement of steroid sulfates by mass spectrometry was performed as previously described in detail (21). Briefly, 300 μL of each serum sample were incubated during 15 min with a mix of internal standards (50 μL). All internal standards had a concentration of 1 $\mu\text{g}/\text{mL}$, with the exception of CS, which was 6 $\mu\text{g}/\text{mL}$. Next, 1 mL of acetonitrile- ZnSO_4 (89 g/L, 4:1 (v/v)) was added to precipitate the proteins and the mixture was incubated again for 15 min. After incubation, the samples were centrifuged for 10 min at 14,500g.

The supernatant was collected in a glass tube and 3 mL of water were added. Solid phase extraction of the samples was performed with SepPak C18 cartridges, which were conditioned with 2 mL of methanol followed by 2 mL of water. Each sample was loaded onto the cartridge and washed with different solvents: first 3 mL of water, followed by 3 mL of hexane, 4 mL of chloroform and 4 mL of methanol. Sulfated steroids were eluted with the methanolic fraction. This final fraction was evaporated with nitrogen at 40°C and reconstituted with 250 μL of a solution of 79.75% water, 10% MeOH, 10% acetonitrile and 0.25% ammonium hydroxide. Finally, each reconstitution solution was centrifuged and 10 μL were injected in the LC-MS/MS system. Liquid chromatography was performed with a Accucore Phenyl-X column (100 $\times$ 2.1 mm, 2.6 μm) from Thermo Fisher Scientific, connected to a HPLC system (Agilent 1200SL). MS/MS quantification was performed with a triple quadrupole mass spectrometer (TSQ, Quantum Ultra; Thermo Fisher Scientific).

Statistics

Statistical analyses were performed using the Winstat software package (R. Fitch Software, Bad Krozingen, Germany). Normal distribution was tested by the Kolmogorov-Smirnov test. Changes of steroid hormone sulfates in the 1-year follow-up were correlated to changes of insulin, HOMA, lipids and changes of BP by Spearman correlation. To compare variables at baseline or in the course of 1 year, Fisher exact test and Student's *t*-test for paired and unpaired observations, Wilcoxon and Mann-Whitney *U* test were used as appropriate. In all significant associations in univariate correlation analyses, backward multiple linear regression analyses were performed adjusted for age, gender, pubertal stage and degree of overweight at baseline as well as changes of BMI-SDS and pubertal stage in longitudinal analyses.

A *P*-value <0.05 was considered as significant. Data were presented as mean and standard deviation for normally distributed variables and median and interquartile range (IQR) for not normally distributed variables.

Results

All sulfated steroids except CS were significantly associated to age (Table 1). Furthermore, we found significant correlations between the concentrations of some sulfated steroids (Table 2).

Table 1 Associations between steroid sulfates and anthropometrics and cardiovascular risk factors at baseline (Spearman correlation).

	CS	PregS	17 α OH PregS	16 α OH DHEAS	DHEAS	A-3-S	AS	ES
Age	0.07	0.22*	0.24*	0.43***	0.63***	0.55***	0.46***	0.54***
BMI	0.05	0.19	0.16	0.31**	0.51***	0.49***	0.38***	0.44***
Systolic blood pressure	0.19	0.19	0.27*	0.15	0.16	0.23*	0.15	0.22*
Diastolic blood pressure	0.05	0.07	0.08	0.19	0.23*	0.16	0.29**	0.32**
Fasting glucose	-0.02	0.21	0.16	0.26*	0.25*	0.26*	0.10	0.18
HOMA	-0.09	0.28*	0.29**	0.42***	0.30**	0.28**	0.21*	0.29**
Cholesterol	0.52***	0.03	0.06	-0.24*	-0.13	-0.07	-0.25*	-0.22*
HDL-cholesterol	0.10	0.05	-0.02	-0.06	0.02	0.02	-0.03	-0.06
LDL-cholesterol	0.45***	0.13	0.15	-0.17	-0.04	0.01	-0.19	-0.17
Triglycerides	0.25*	-0.18	-0.11	-0.24*	-0.28*	-0.26*	-0.10	-0.11

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A-3-S, androstenediol-3-sulfate; AS, androsterone sulfate; CS, cholesterol sulfate; DHEAS, dehydroepiandrosterone sulfate; ES, epiandrosterone sulfate; HDL, high-density lipoprotein; LDL, low density lipoprotein; PregS, pregnenolone sulfate.

Comparing boys and girls who did not differ significantly in their pubertal stage, we demonstrated that CS, DHEAS and A-3-S concentrations were significantly higher in boys, while 17OH-PregS, 16OH-DHEAS, AS and ES did not present significant differences (Table 3).

DHEAS, 16OH-DHEAS, A-3-S and ES were significantly higher in pubertal children, while CS, PregS and 17OH-PregS had no significant differences between prepubertal and pubertal children (Table 4).

All steroid sulfates increased significantly in the observation period with independence of change of weight status, with the exception of CS and 17OH-PregS. CS levels before intervention were higher in children with substantial weight loss (Table 5). After 1-year intervention, PregS concentrations increased only in children with substantial decrease of BMI-SDS (Table 5). In addition, the increase of DHEAS was higher in children with substantial weight loss compared to the children without substantial decrease of BMI-SDS (Table 5).

Analysis of only prepubertal children, who remained prepubertal in the 1-year intervention period, demonstrated the same findings with one exception

(Table 6): 17OH-PregS decreased in the children with substantial weight loss and increased in the children without substantial weight loss.

CS was significantly and positively associated with cholesterol and triglycerides both at baseline as well as to changes of overweight in the 1-year follow-up period (Tables 1 and 7). A-3-S was significantly positively associated with baseline BMI and HOMA both cross-sectionally and longitudinally. 17OH-PregS were significantly positively associated with systolic BP both at baseline as well as to its changes in the 1-year follow-up period. There were no further significant correlations between steroid hormone sulfates and anthropometrics, lipids or BP that were present in both cross-sectional and longitudinal analyses.

In multiple linear regression analyses adjusted to age, gender and pubertal stage, baseline 17OH-PregS significantly associated with baseline systolic BP (β -coefficient 0.09 ± 0.07 ; $P = 0.020$), and baseline 16 α OH DHEAS was significantly associated with baseline HOMA (β -coefficient 6.1 ± 6.0 ; $P = 0.049$), while no further significant associations could be observed.

Table 2 Associations between steroid sulfates at baseline (Spearman correlation).

	CS	PregS	17 α OH PregS	16 α OH DHEAS	DHEAS	A-3-S	AS	ES
CS	–	-0.04	0.11	-0.13	0.01	0.02	-0.10	-0.09
PregS	-0.04	–	0.84***	0.37**	0.53***	0.52***	0.08	0.18
17 α OH PregS	0.11	0.84***	–	0.42***	0.55***	0.50***	0.16	0.25*
16 α OH DHEAS	-0.13	0.37**	0.42***	–	0.70***	0.61***	0.54***	0.58***
DHEAS	0.01	0.53***	0.55***	0.70***	–	0.91***	0.54***	0.91***
A-3-S	0.02	0.52***	0.50***	0.61***	0.91***	–	0.41***	0.46***
AS	-0.10	0.08	0.16	0.54***	0.54***	0.41***	–	0.90***
ES	-0.09	0.18	0.25*	0.58***	0.91***	0.46***	0.90***	–

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A-3-S, androstenediol-3-sulfate; AS, androsterone sulfate; CS, cholesterol sulfate; DHEAS, dehydroepiandrosterone sulfate; ES, epiandrosterone sulfate; PregS, pregnenolone sulfate.

Table 3 Comparison of age, pubertal stage and steroid sulfates between boys and girls.

	Boys	Girls	P-Value
Number	28	38	
Age (years)	12 (IQR 10–13)	11 (IQR 8–13)	0.111
Prepubertal (%)	17 (61)	18 (47)	0.151
CS (μM)	1087 (IQR 882–1304)	903 (IQR 704–1000)	0.003
PregS (μM)	35 (IQR 28–54)	25 (IQR 14–43)	0.049
17OH-PregS (μM)	5 (IQR 3–8)	4 (IQR 1–7)	0.173
16OH-DHEAS (μM)	78 (IQR 55–172)	76 (IQR 59–145)	0.989
DHEAS (μM)	875 (IQR 477–1080)	551 (IQR 284–881)	0.009
A-3-S (μM)	45 (IQR 26–58)	25 (IQR 13–35)	<0.001
AS (μM)	305 (IQR 200–392)	433 (IQR 220–581)	0.111
ES (μM)	106 (IQR 67–149)	152 (IQR 66–178)	0.143

Data as median and interquartile range (IQR), *P*-value derived from Fisher exact test or Mann–Whitney *U* test.

A-3-S, androstenediol-3-sulfate; AS, androsterone sulfate; CS, cholesterol sulfate; DHEAS, dehydroepiandrosterone sulfate; ES, epiandrosterone sulfate; PregS, pregnenolone sulfate.

Changes of 17OH-PregS were significantly positively associated with changes of systolic BP (β -coefficient 0.059 ± 0.04 ; $P=0.013$) and changes of DHEAS were significantly negatively associated with changes of BMI-SDS (β -coefficient -150 ± 109 ; $P=0.008$), while no further significant associations could be observed in multiple linear regression analyses adjusted to age, gender, pubertal stage and changes of HOMA and changes of pubertal stage.

The children with and without substantial BMI-SDS reduction did not differ significantly according to age, gender, pubertal stage, cardiovascular risk factors or steroid sulfates at baseline except CS, which, as previously mentioned, was higher in children with BMI-SDS reduction.

Weight loss in 33 obese children (mean BMI-SDS -0.75 ± 0.25) was associated with significant decreases of HOMA, lipids and BP. In the obese 33 children with weight gain (mean change of BMI-SDS $+0.25 \pm 0.17$), triglycerides and systolic BP increased significantly,

while all other cardiovascular risk factors did not change significantly (Table 5).

Discussion

To the best of our knowledge, this is the first study analyzing the longitudinal relationships of the sulfated steroidome in obese children participating in a lifestyle intervention. Our data show that concentrations of CS before intervention are higher in children who finally lost weight, which points to a higher capacity to sulfate cholesterol. This fact can be due to increased SULTs activity, to a decreased activity of sulfatases or to a combined effect of all enzymes.

The finding that children with higher CS concentrations loose more weight than those with lower CS concentrations are in line with the observation that obese girls with PCOS (a disease which has been reported to be associated with a disruption of sulfation (15,16))

Table 4 Comparison of steroid sulfates between prepubertal and pubertal children.

	Prepubertal	Pubertal	P-Value
Number	35	31	
Gender	17 (49%) boys	11 (35%) boys	0.287
CS (μM)	945 (IQR 755–1149)	939 (IQR 801–1211)	0.782
PregS (μM)	31 (IQR 17–41)	29 (IQR 20–56)	0.508
17OH-PregS (μM)	5 (IQR 2–6)	5 (IQR 2–9)	0.407
16OH-DHEAS (μM)	64 (IQR 37–100)	95 (IQR 67–204)	0.003
DHEAS (μM)	529 (IQR 299–789)	901 (IQR 601–1160)	0.002
A-3-S (μM)	24 (IQR 14–41)	37 (IQR 25–53)	0.023
AS (μM)	250 (IQR 165–393)	IQR 341–619	<0.001
ES (μM)	77 (IQR 57–142)	160 (IQR 116–204)	<0.001

Data as median and interquartile range (IQR), *P*-value derived from Fisher exact test or Mann–Whitney *U* test.

A-3-S, androstenediol-3-sulfate; AS, androsterone sulfate; CS, cholesterol sulfate; DHEAS, dehydroepiandrosterone sulfate; ES, epiandrosterone sulfate; PregS, pregnenolone sulfate.

Table 5 Age, gender, weight status and steroid hormones in 66 obese children stratified to change of weight status.

	Substantial decrease of BMI-SDS			Stable or increase of BMI-SDS			P-Value ²	P-Value ³
	Baseline	1 year later	P-Value ¹	Baseline	1 year later	P-Value ¹		
Number	33				33			
Age (years)	10.7 ± 2.5				10.9 ± 2.4		0.680	
Gender	42.4% male				42.4% male		0.999	
Prepubertal	51.5%	45.5%	0.243	54.5%	42.4%	0.172	0.688	0.881
BMI (kg/m ²)	26.7 ± 4.1	23.9 ± 2.9	<0.001	27.3 ± 5.1	30.3 ± 5.6	<0.001	0.582	<0.001
BMI-SDS	2.29 ± 0.46	1.54 ± 0.50	<0.001	2.24 ± 0.46	2.50 ± 0.46	<0.001	0.706	<0.001
CS (μM)	1013 (IQR 850–1243)	1028 (IQR 894–1201)	0.648	895 (669–999)	821 (IQR 726–1044)	0.851	0.006	0.631
PregS (μM)	28 (IQR 17–37)	35 (IQR 23–50)	<0.001	35 (IQR 19–60)	37 (IQR 22–59)	0.241	0.132	0.125
17αOH PregS (μM)	5.5 (IQR 2.8–5.5)	4.6 (IQR 2.8–7.0)	0.893	4.1 (IQR 1.8–8.2)	5.7 (IQR 2.7–8.5)	0.098	0.383	0.221
16αOH DHEAS (μM)	85 (IQR 55–183)	150 (IQR 70–254)	<0.001	75 (IQR 58–111)	130 (IQR 80–173)	<0.001	0.654	0.892
DHEAS (μM)	636 (IQR 345–962)	984 (IQR 562–1464)	<0.001	702 (IQR 421–985)	781 (IQR 487–1150)	<0.001	0.863	0.003
A-3-S (μM)	29 (IQR 18–45)	32 (IQR 20–58)	0.026	28 (IQR 19–44)	39 (IQR 17–55)	<0.001	0.898	0.235
AS (μM)	391 (IQR 227–563)	417 (IQR 278–777)	<0.001	328 (IQR 193–525)	446 (IQR 267–517)	0.114	0.477	0.094
ES (μM)	143 (IQR 64–143)	156 (IQR 92–248)	0.001	116 (IQR 69–159)	140 (IQR 93–186)	0.020	0.577	0.075
Total cholesterol (mg/dL)	171 ± 31	160 ± 28	0.019	171 ± 31	170 ± 30	0.647	0.956	0.005
HDL-cholesterol (mg/dL)	51 ± 9	53 ± 7	0.015	50 ± 13	53 ± 16	0.353	0.968	0.097
LDL-cholesterol (mg/dL)	101 ± 33	92 ± 27	0.016	101 ± 25	102 ± 26	0.809	0.990	0.017
Triglycerides (mg/dL)	113 ± 59	81 ± 17	0.002	97 ± 47	110 ± 46	0.029	0.225	<0.001
Fasting glucose	87 ± 8	86 ± 7	0.445	86 ± 6	86 ± 6	0.427	0.468	0.216
HOMA	2.8 (IQR 1.7–4.1)	2.0 (IQR 1.4–2.9)	0.005	2.6 (IQR 1.4–3.9)	3.2 (IQR 3.2–5.1)	0.010	0.299	<0.001
Systolic BP (mmHg)	120 ± 13	110 ± 10	0.003	118 ± 14	120 ± 9	0.011	0.274	<0.001
Diastolic BP (mmHg)	70 ± 8	65 ± 78	0.026	69 ± 10	71 ± 10	0.351	0.850	0.026

Data as mean and standard deviation (±) or median and interquartile range (IQR), P-value derived from Fisher exact test or Student's t-test for paired and unpaired observations, Wilcoxon and Mann-Whitney U test as appropriate, 1: baseline compared to 1 year later, 2: baseline compared between children with and without substantial weight loss, 3: changes compared between children with and without substantial weight loss; significant changes plotted in bold.

A-3-S, androstenediol-3-sulfate; AS, androstosterone sulfate; BP, blood pressure; CS, cholesterol sulfate; DHEAS, dehydroepiandrosterone sulfate; ES, epiandrosterone sulfate; HDL, high-density lipoprotein; LDL, low density lipoprotein; PregS, pregnenolone sulfate.

Table 6 Age, gender, weight status and steroid hormones in 14 obese prepubertal children remaining prepubertal in the observation time with weight loss (stratified to change of weight status).

	Weight loss		Stable or increase of BMI-SDS		P-Value ²	P-Value ³
	Baseline	1 year later	Baseline	1 year later		
Number	Substantial decrease of BMI-SDS		12			
Age (years)	8.5 ± 1.6		8.6 ± 1.5		0.901	
Gender	42.9% male		33.3% male		0.999	
BMI (kg/m ²)	24.7 ± 2.3	22.5 ± 2.6	22.7 ± 2.4	24.7 ± 3.1	<0.001	<0.001
BMI-SDS	2.40 ± 0.37	1.61 ± 0.54	1.96 ± 0.45	2.13 ± 0.46	<0.001	<0.001
CS (µM)	1149 (IQR 944–1290)	1065 (IQR 997–1210)	883 (644–992)	831 (IQR 691–1121)	0.875	0.381
PregS (µM)	27 (IQR 17–32)	28 (IQR 22–42)	37 (IQR 18–46)	30 (IQR 18–58)	0.582	0.237
17αOH PregS (µM)	5.2 (IQR 1.9–6.3)	3.2 (IQR 2.2–5.0)	4.2 (IQR 1.2–6.1)	5.0 (IQR 1.9–8.3)	0.049	0.019
16αOH DHEAS (µM)	59 (IQR 29–88)	85 (IQR 33–151)	62 (IQR 32–94)	75 (IQR 51–128)	0.084	0.837
DHEAS (µM)	488 (IQR 249–789)	740 (IQR 382–1264)	439 (IQR 176–529)	513 (IQR 306–698)	0.084	0.006
A-3-S (µM)	22 (IQR 11–42)	26 (IQR 13–48)	20 (IQR 6–30)	19 (IQR 13–40)	0.051	0.396
AS (µM)	246 (IQR 123–300)	404 (IQR 283–823)	204 (IQR 116–388)	305 (IQR 161–469)	0.059	0.959
ES (µM)	64 (IQR 37–105)	136 (IQR 94–288)	72 (IQR 39–133)	111 (IQR 43–145)	0.136	0.355

Data as mean and standard deviation (±) or median and interquartile range (IQR), P-value derived from Student's t-test for paired and unpaired observations, Wilcoxon and Mann-Whitney U test as appropriate; 1: baseline compared to 1 year later; 2: baseline compared between children with and without substantial weight loss; 3: changes compared between children with and without substantial weight loss; significant changes plotted in bold.

A-3-S, androstenediol-3-sulfate; AS, androstosterone sulfate; CS, cholesterol sulfate; DHEAS, dehydroepiandrosterone sulfate; ES, epiandrosterone sulfate; PregS, pregnenolone sulfate.

have greater difficulties to lose weight compared to girls without PCOS (31). The underlying mechanisms are unclear so far and cannot be analyzed by our study. An impact of insulin resistance seems unlikely since CS was not related to HOMA in our study. Furthermore, it has been reported that CS and SULTs are important regulators of glucose metabolism inhibiting hepatic gluconeogenesis (32): In mice, CS alleviated insulin resistance and lowered the expression of liver gluconeogenic gene.

CS can also give rise to PregS – a key molecule to control steroidogenesis (33). Children who lost weight showed a significant rise in the concentration of this compound after 1 year, in contrast to children who did not lose weight. Higher concentrations of PregS decrease the amounts of free unconjugated pregnenolone, the starting metabolite for the synthesis of androgens and corticoids. Similarly, DHEAS increased more in children with substantial BMI-SDS reduction compared to children without BMI-SDS reduction in the 1-year observation period. Similarly, this decreases the availability of DHEA and has an impact on steroid biosynthesis. Such an effect can be clearly observed in STSD patients, with higher DHEAS concentrations but lower DHEA levels than controls (19, 34).

Therefore, our results pointing to a higher capacity to synthesize steroid sulfates in obese children with weight loss are coherent with previous reports in which substantial weight loss in obese children was associated with a decrease of unconjugated androgens or corticoids (1, 2, 6). A general scheme for these ideas is depicted in Fig. 1.

The persistently elevated androgen sulfates after weight loss are in line with previous studies demonstrating elevated DHEAS after weight loss in long-term follow-up studies (1, 2). Our findings could suggest an irreversible maturation of the zona reticularis and the formation of sulfated androgens in obese children or shifted balance of STS and SULTs. Once the developmental path of adrenarche has been initiated, it continues irreversibly as reflected by the increasing sulfated androgen concentrations during the study period even after successful weight loss. We cannot decipher, however, whether obesity has been the first event which has only secondarily initiated exaggerated adrenarche or whether exaggerated adrenarche has actually been the first hit promoting development of obesity as the secondary event, which has also been suggested by others (12, 35). One potential mechanism explaining the increased levels of most sulfated steroids could be a lower activity of STS or of 3βHSD2, since 3βHSD2 could also have sulfatase

Table 7 Associations between changes (Δ) of steroid sulfates and changes (Δ) of anthropometrics and cardiovascular risk factors at baseline (Spearman correlation).

	Δ CS	Δ PregS	Δ 17 α OH PregS	Δ 16 α OH DHEAS	Δ DHEAS	Δ A-3-S	Δ AS	Δ ES
Δ BMI-SDS	0.11	-0.16	0.17	0.07	-0.26*	0.026*	-0.15	-0.11
Δ Systolic blood pressure	0.09	0.07	0.24*	0.07	-0.31**	0.04	-0.09	-0.01
Δ Diastolic blood pressure	0.16	-0.14	0.03	-0.10	-0.19	-0.02	-0.14	-0.09
Δ Fasting glucose	-0.13	-0.11	-0.07	-0.16	-0.22*	-0.21*	0.02	0.02
Δ HOMA	-0.04	-0.01	0.04	0.05	-0.37***	0.22*	-0.09	0.02
Δ Cholesterol	0.36**	-0.27*	-0.07	-0.33**	-0.18	0.03	-0.21*	-0.30**
Δ HDL-cholesterol	0.04	-0.11	-0.17	0.01	0.01	0.03	-0.21*	-0.30**
Δ LDL-cholesterol	0.22	-0.18	-0.23	-0.22*	-0.19	0.04	-0.04	-0.11
Δ Triglycerides	0.21*	-0.07	0.08	-0.11	-0.16	0.11	-0.24*	-0.37**

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

A-3-S, androstenediol-3-sulfate; AS, androsterone sulfate; CS, cholesterol sulfate; DHEAS, dehydroepiandrosterone sulfate; ES, epiandrosterone sulfate; HDL, high-density lipoprotein; LDL, low density lipoprotein; PregS, pregnenolone sulfate.

activity (19). Interestingly, adrenarche is accompanied by a lower expression of 3 β HSD2 (36).

Except 17OH-PregS, none of the sulfated androgens were associated to cardiovascular risk factors including insulin resistance. This finding is in contrast to unconjugated androgens, which are related to parameters of the metabolic syndrome (2, 5, 6, 7, 8, 9). However, systolic BP was significantly related to 17OH-PregS both in cross-sectional and longitudinal analyses. Further studies are necessary to prove whether this relationship is relevant and to analyze the underlying mechanisms.

Most sulfated androgens and their precursors were higher in boys compared to girls, with the exception of AS, ES, 16OH-DHEAS and 17OH-PregS. We anticipated this difference previously for AS and ES based on the differences in STS activity between males and females (19). As previously described, DHEAS and CS had the highest concentrations of all analyzed steroid sulfates (19,21). DHEAS and A-3-S production normally starts to increase at the age of 6–8 (adrenarche) and reaches its

highest concentrations in blood at around 20–30 years of age in males and females (37, 38). The relationship between blood concentrations of sulfated steroids and age is well known (39). The increase of sulfated steroids with age could also be observed in our study, with the exception of CS, which showed similar levels in all children after one year of intervention. CS is the only measured sulfated steroid, which is not of adrenal origin and can be synthesized in other tissues (21).

Of interest, we found strong correlations between the concentrations of many sulfated steroids. These correlations are similar to the ones reported before in adult males (19). Synthesis of sulfated steroids is not only possible by a sulfation reaction of unconjugated steroids. Enzymatic conversion of some sulfated steroids into others is also a well-known physiological reaction. For instance, DHEAS can be converted into A-3-S, and PregS can be hydroxylated to produce 17OH-PregS (33, 40, 41). As a consequence, steroid biosynthesis in humans is a complex set of interconnected pathways (19).

Figure 1

Overview of the steroidogenesis in obese children during weight loss. The increase of sulfated steroids affects the concentration of unconjugated steroid precursors and therefore of hormonal steroids.

Strengths and limitations of the study

The strengths of this study are its longitudinal design, the analyses of a comprehensive profile of intact steroid sulfates by state-of-the-art LC-MS/MS (21) and the study of a homogenous cohort of obese children naive to drugs. However, our study presents some potential limitations. First, BMI percentiles were used to classify overweight. Although BMI is a good measure for overweight, one needs to be aware of its limitations as an indirect measurement of fat mass. Second, we did not have a control group of normal weight children. Third, our study sample was too small to study gender differences on the changes of sulfated steroids in weight loss. Finally, weight loss may be too small to measure effects on sulfated steroids. On the other hand, a reduction of ≥ 0.5 BMI-SDS, as achieved in our children with substantial weight loss, is reported to normalize many hormonal and metabolic changes in childhood obesity like insulin sensitivity, the cardiovascular risk factor profile, hormones including unconjugated androgens and adipocytokines (1, 2, 6, 23, 24).

In summary, most sulfated androgens and their precursors were higher in boys compared to girls. Furthermore, there is an increase in the levels of sulfated steroids, which can be associated to age in both groups, but in addition, children with weight loss showed higher capacity to sulfate steroids. This capacity was present before the intervention, with higher CS blood levels. Sulfated androgens were not related to cardiovascular risk factors except 17OH-PregS, which was related to systolic BP. There is still a great need for further research on the alterations of sulfated steroids in obesity also to understand how the activity of enzymes related to the synthesis of these compounds, including SULTs, STS and probably β HSD2, can be altered in obesity. This study also points to the importance of quantifying sulfated steroids at different ages, in order to establish ranges for some compounds like CS and PregS. This could provide additional information previous to medical intervention in the treatment of obesity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T R and S A W developed the study design, T R and N L performed the anthropometrical measurements and A S-G performed the laboratory measurements. All authors discussed the findings and participated in writing of the paper. T R and A S-G analyzed the data and wrote the first draft of the manuscript. T R, A S-G and S A W were the leading writers of the paper.

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