Steroid Metabolomic Disease Signature of Nonsyndromic Childhood Obesity

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Context: The profile of urinary steroids as measured by gas chromatography-mass spectrometry defines a subject's "steroidal fingerprint."

Objective: Here, we clustered steroidal fingerprints to characterize patients with nonsyndromic childhood obesity by "steroid metabolomic signatures."

Hypothesis: Nonsyndromic obesity is a symptom of different diseases and conditions, some of them will have their own signature.

Design: A total of 31 steroid metabolites were quantified by gas chromatography-mass spectrometry, and their excretion rates were z-transformed. Using MetaboAnalyst 3.0, we divided the subjects into 5 distinctive groups by k-means clustering. Steroidal fingerprints and clinical/biochemical data of patients in each cluster were analyzed.

Patients: A total of 87 obese children (44 females), aged 8.5–17.9 years, were clinically characterized, and their 24-hour urine was collected.

Results: Cluster 1 (n = 39, 21 females) had normal steroid profile. Cluster 2 (n = 20, 11 females) showed mild, nonspecific elevation of C19 and C21 steroids, females' resistance to polycystic ovary morphology, and hirsutism. Cluster 3 (n = 7 female), with relative 21-hydroxylase insufficiency, was characterized by partial or full polycystic ovary syndrome. Cluster 4 (n = 4 males), showed markedly elevated C21 steroids and imbalance in the 11β-hydroxysteroid dehydrogenase system, higher insulin, increased frequency of glucose/insulin index more than 0.3, γ-glutamyl transpeptidase activity, systolic blood pressure, and tendency to liver steatosis. Cluster 5 (n = 17, 5 females) had elevated dehydroepiandrosterone and 17-OH-pregnenolone metabolites, suggesting 3β-hydroxysteroid dehydrogenase insufficiency but no clinically unique phenotype. Z-score body mass index values were not significantly different between the clusters.

Conclusions: We defined a novel concept of disease-specific steroid metabolomic signature based on urinary steroidal gas chromatography-mass spectrometry. Clustering by software designed for metabolic data analysis reclassified childhood obesity into 5 groups with distinctive signatures; groups require further definition and may require cluster-specific therapeutic strategies. **(*J Clin Endocrinol Metab* 101: 4329–4337, 2016)**

Childhood obesity is a complex disease stemming from and affecting multiple mechanisms. The question of whether obesity is a "disease" or a "symptom" has significant connotations for the approach one takes to its understanding and treatment. Here, we regard childhood obesity as a symptom of several entities and consider its classification from a viewpoint of steroid metabolism, for which we use our previous definition of childhood obesity as a "steroid-related complex disorder" (1) and study gas chromatography-mass spectrometry (GC-MS)-generated steroid profiles of children with obesity.

Steroid hormones are catabolized by a series of reductions and hydroxylations and are finally conjugated with glucuronide or sulfate groups. About 90% of these inac-

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Abbreviations: BMI, body mass index; BP, blood pressure; fT_4, free T_4; GC-MS, gas chromatography-mass spectrometry; DHEA, dehydroepiandrosterone; GGTP, γ-glutamyl transpeptidase; GLU, glucose; HOMA-IR, homeostatic model assessment of INS resistance; HSD, hydroxysteroid dehydrogenase; INS, insulin; PCOM, polycystic ovary morphology; PCOS, polycystic ovary syndrome.

tive conjugated metabolites are excreted by the kidneys as water-soluble compounds. Thus, the urinary steroid metabolome reflects the body's steroid metabolism (1–3).

GC-MS is the tool of choice for characterizing an individual's quantitative steroid metabolome, because it allows for the most comprehensive steroid profile currently obtainable. An individual urinary steroid metabolite profile represents a subject's unique metabolic fingerprint and offers a means of phenotyping individuals at a chemical level (2, 4, 5). Each individual has a unique "steroidal fingerprint." A cluster of similar steroidal fingerprints related to a disease might be regarded as a "steroid metabolomic disease signature" (1). The signature represents the impact of the disease in people who differ in their phenotypes or have other health problems.

Habitually, we look at the steroid metabolome through the prism of the clinical presentation. We have previously identified a specific metabolic profile for childhood obesity: a general decrease of glucocorticoid and mineralocorticoid metabolites, increased androgens, up-regulation of 17,20-lyase, 17-OHase, and 11β-hydroxysteroid dehydrogenase (HSD)1 activity and down-regulation of 21-OHase enzymatic activity (1). Here, we hypothesize that obesity is a symptom of different diseases and conditions, some of them may have their own steroid metabolomic disease signature. By using powerful state-of-the-art analysis methods, we clustered individual steroidal fingerprints to classify childhood obesity by steroid metabolomic disease signatures.

Subjects and Methods

Between March 2012 and August 2013, we examined a consecutive series of 117 obese Caucasians children and adolescents (body mass index [BMI] >97th percentile) (6). They were recruited from the patients referred to the Department of Pediatric Endocrinology, Medical University of Silesia. After exclusion of younger participants (<8 y), syndromic obesity, chronic diseases, pharmacotherapy (also metformin), or precocious puberty, we included the remaining 87 patients (44 girls), aged 8.5–18.0 years (mean age, 14.4; SD, 2.33; median, 14.5 y).

Clinical phenotype of obese children

All patients underwent a clinical assessment and diagnostic procedures that included general physical examination, anthropometric measurements (height, weight, waist and hip circumference), and puberty assessment. Height was measured to the nearest 0.1 cm with a calibrated Harpenden stadiometer. Weight was measured in fasting state in light underwear on a calibrated scale accurate to 0.1 kg. Puberty was assessed according to the Tanner scale (7). The Ferriman-Gallwey score for excessive hair growth in girls was used (8).

The BMI was calculated as weight (kg)/squared height (m), and values exceeding 97%, according to chart were considered obese. The z-score BMI was calculated using Cole's international childhood BMI cut-offs (International Obesity Task Force) (9, 10). The waist and hip circumferences were measured with nonelastic flexible tape by the same investigator, and the waist to hip ratio was calculated.

In the 43 adolescent girls, a transabdominal pelvic ultrasound examination was performed with 5-MHz convex transducer (Acuson Antares 5.0; Siemens Medical Solution USA, Inc), and volume and structure of the ovaries were evaluated. Ovaries were considered polycystic (polycystic ovary morphology [PCOM]) if 12 or more cysts of 2–9 mm in diameter were present at least in 1 ovary or if increased ovarian volume (>10 mL) occurred. PCOM and hirsutism were used as the criteria of polycystic ovary syndrome (PCOS) (11). Abdomen ultrasonography to evaluate the liver for hepatic steatosis features was performed with 5-MHz convex transducer (Acuson Antares 5.0; Siemens Medical Solution USA, Inc or Logiq; GE Healthcare GmbH). Liver echogenicity was judged by comparison with kidney and spleen. Increased liver echogenicity in relation to the right kidney was defined as steatosis.

Chemical phenotype

Morning fasting venous blood samples were collected to measure lipids, TSH, free T_4 (fT_4), and aminotransferases. Plasma total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were analyzed enzymatically (Beckman Coulter). Serum concentrations of fT_4 and TSH were measured with a chemiluminescent immunometric assay (Immulite 2000 Free T_4, Immulite 2000 Third Generation TSH; Siemens). γ-Glutamyl transpeptidase (GGTP) and alanine and aspartate aminotransferases activity in the serum were assessed according to International Federation in Clinical Chemistry (Beckman Coulter). Cortisol was measured in the morning (8 AM) and midnight using chemiluminescent immunoassay by Immulite 2000 analyzer (DPC).

Glucose (GLU) and insulin (INS) levels were measured in an oral GLU tolerance test (1.75 g/kg, max 75 g). Fasting INS (mIU/L) to GLU (mg/dL) ratio (INS resistance >0.3) and homeostatic model assessment of INS resistance (HOMA-IR) (fasting GLU [mmol/L] × fasting INS [mIU/L]/22.5) were calculated as indices of INS resistance (12). INS was determined using a chemiluminescence immunoassay on Immulite 2000 analyzer (DPC). Enzymatic test (hexokinase method) was used for the quantitative determination of GLU (Beckman Coulter).

Gas chromatography-mass spectrometry (13, 14)

The urinary collections were carried out under standardized conditions (no stress, no infection, normal diet, no drugs). Five milliliters of urine of a 24-hour urine collection were extracted with Sep-Pak C18 cartridges (Waters). After evaporation of the solvent under a stream of nitrogen, the extract was taken up in 0.1M acetate buffer (pH 5.0), and 75 U of sulfatase from helix pomatia type H-1 (Sigma-Aldrich Chemie) were added. The samples were stirred and kept at 37°C for 48 hours for hydrolization.

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Then, the resulting free steroids were reextracted using Sep-Pak C18 cartridges, dried, and taken up in methanol. An internal standard mixture (5α-androstane-3α,17α-diol; Paesel+Lorei), stigmasterol, and cholesteryl butyrate (Sigma-Aldrich Chemie) were added. For derivatization, the mixture was dried, 100 μL of 2% methoxyamine hydrochloride (Sigma-Aldrich Chemie) in pyridine were added and incubated for 1.5 hours at 60°C. Then, the pyridine was blown off, 100 μL of trimethylsilyimidazole (Macherey-Nagel) were added and the samples kept at 100°C for 18 hours. The derivatized extract was purified by gel chromatography on a Lipidx-5000 column (PerkinElmer) with cyclohexane (LGC Standards). After evaporation of the eluate, the sample was taken up in 500 μL of isooctane/pyridine/hexamethyldisilazane (Sigma-Aldrich Chemie) (98:1:1, vol/vol/vol). Solvents except of cyclohexane were purchased from Merck. For quantification 1 μL of the solution was injected into the GC-MS system. GC was run on an Optima-1 MS fused silica column (length, 25 m; film thickness, 0.1 μm; inner diameter, 0.2 mm; Macherey-Nagel) housed in an Agilent Technologies 6890 series GC that was directly interfaced to an Agilent Technologies 5975 inert XL mass selective detector. The MS was run in the selected ion monitoring mode (ions as well as retention times for each steroid, see Table 1).

Statistical analysis of metabolomic data

Steroid metabolites quantities were z-transformed based on sex and age-adjusted normal reference groups (1). MetaboAnalyst 3.0 (www.metaboanalyst.ca), a web-based software tool designed for metabolomic data analysis, was used to generate the clusters of patients (15) and subsequent statistical analysis. MetaboAnalyst 3.0 has generated 5 unique clusters by taking as an input steroid metabolites excretion profiles and by invoking the k-means clustering algorithm, where k = 5 had been chosen by visual inspection of the heatmap (Figure 1A) with the MetaboAnalyst 3.0 default 2-way hierarchical clustering. A subsequent principal component analysis has been performed. It showed that 51.5% of metabolite levels variation are explained by the first, and 11.6% by the second PC (Figure 1B). The steroid metabolomic disease signatures of the generated clusters are shown in Figure 2, and their descriptive data are presented in Table 2.

Table 1. The List of the Urinary Steroid Metabolites Analyzed in the Study

Abbreviation	Urinary Steroid Metabolites	Origin of Urinary Steroid	m/z	RT (min)
AN	5α-androstane-3α-ol-17-on (androsterone)	DHEA, androstenedione, testosterone	270.3	19.8
ET	5β-androstane-3α-ol-17-on (etiocholanolone)	DHEA, androstenedione, testosterone	270.3	20.2
DHEA	5-androstene-3β-ol-17-on (dehydroepiandrosterone)	DHEA-sulfate	268.3	21.3
16α-OH-DHEA	5-androstene-3β,16α-diol-17-one	DHEA-sulfate	446.3	24.9
A5–3β,17α	5-androstene-3β,17α-diol	DHEA	239.3	20.8
A5–3β,17β	5-androstene-3β,17β-diol (androstenediol-17β)	DHEA	239.3	21.8
A5T-16a	5-androstene-3β,16α,17β-triol (androstenetriol-16α)	DHEA-sulfate	432.3	27.4
PD	5β-pregnane-3α,20α-diol (pregnanediol)	Progesterone	269.3	25.7
PT	5β-pregnane-3α,17α,20α-triol (pregnanetriol)	17-hydroxyprogesterone	255.2	26.4
P5D	5-pregnene-3β, 20α-diol (pregnenediol)	Pregnenolone	372.4	27.0
P5T-17α	5-pregnene-3β,17α,20α-triol (pregnenetriol-17α)	17-hydroxypregnenolone	433.4	30.0
Po-5β,3α	5β-pregnane-3α,17α-diol-20-one (17a-OH-pregnanolone)	17-hydroxyprogesterone	476.4	24.1
Po-5α,3α	5α-pregnane-3α,17α-diol-20-one	17-hydroxyprogesterone	476.4	24.8
F	4-pregnene-11β,17α,21-triol-3,20-dione (cortisol)	Cortisol	605.4	37.3
THE	5β-pregnane-3α,17 α,21-triol-11,20-dione	Cortisone	578.4	30.3
THF	5β-pregnane-3α,11β,17α,21-tetrol-20-one	Cortisol	652.5	31.7
αTHF	5α-pregnane-3α,11β,17α,21-tetrol-20-one	Cortisol	652.5	32.0
α-Cl	5β-pregnane-3α,17α,20α,21-tetrol-11-one (α-cortolone)	Cortisone	449.3	32.3
β-Cl	5β-pregnane-3α,17α,20β,21-tetrol-11-one (β-cortolone)	Cortisone	449.3	33.0
α-C	5β-pregnane-3α,11β,17α,20α, 21-pentol (α-cortol)	Cortisol	523.4	34.0
β-C	5β-pregnane-3a,11b,17a,20β,21-pentol (β-cortol)	Cortisol	523.4	32.9
6β-OH-F	4-pregnene-6β,11β,17α,21-tetrol-3,20-dione (6β-hydroxycortisol)	Cortisol	693.4	38.2
20α-DHF	4-pregnene-11β,17α,20α,21-tetrol-3-one (20α-dihydrocortisol)	Cortisol	296.3	39.0
11-OH-AN	5α-androstane-3α,11β-diol-17-one (11-hydroxy-androsterone)	Cortisol, 11-hydroxyandrostenedione	448.4	24.1
11-O-AN	5α-androstane-3α-ol-11,17-dione (11-oxo-androsterone)	Cortisol, 11-hydroxyandrostenedione	374.4	22.2
11-OH-ET	5β-androstane-3α,11β-diol-17-one (11-hydroxy-etiocholanolone)	Cortisol, 11-hydroxyandrostenedione	448.4	24.5
11-O-PT	5β-pregnane-3α,17α,20α-triol-11-one (11-oxo-pregnanetriol)	21-deoxycortisol	449.4	29.0
THA	5β-pregnane-3α,21-diol-11,20-dione (tetrahydro-11-dehydro-corticsterone)	Corticosterone	400.3	30.7
THB	5β-pregnane-3α,11β,21-triol-20-one (TH-corticosteron)	Corticosterone	474.4	31.1
α-THB	5α-pregnane-3α,11β,21-triol-20-one (allo-TH-corticosteron)	Corticosterone	474.4	31.5
THS	5β-pregnane-3α,17α,21-triol-20-one (tetrahydro-11-deoxycortisol)	11-deoxycortisol	564.4	27.9

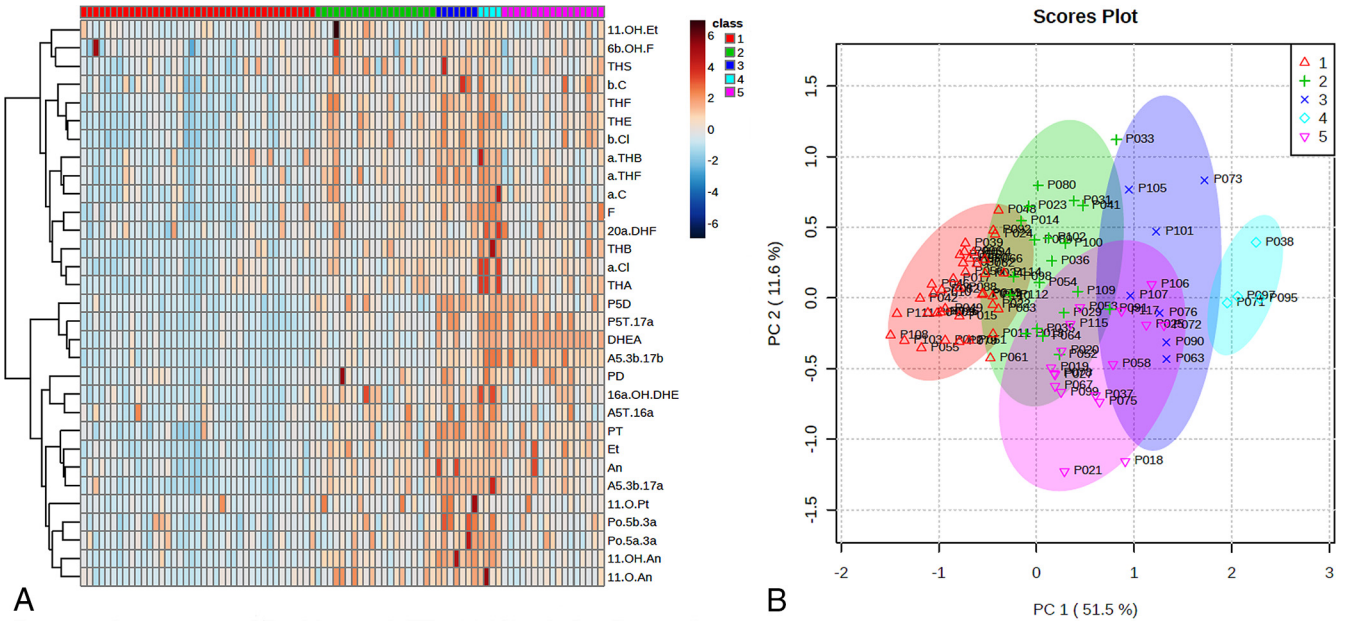

Figure 1. Urine steroid metabolites excretion analysis by MetaboAnalyst 3.0. A, Heatmap and metabolites clusters. The columns represent the patients and the rows the z-transformed metabolite concentrations; the colors in the heatmap are red to blue, indicating high to low concentration of the metabolites. Colors at the top (first row) indicate the clusters assigned by MetaboAnalyst 3.0. B, Principal component analysis (PCA). Each dot represents 1 of the 87 samples projected on the principal plane formed by the first and second principal axes. The dots are colored semitransparent according to the subject's classification group.

Z-transformed quantities of 31 steroid metabolites were used as features for MetaboAnalyst 3.0 analysis.

Steroidal signatures and clinical data of patients in each cluster were analyzed, and ANOVA was used to assess the clinical and chemical differences between clusters. Subsequently, per each populated cluster, each one of the metabolites has been tested in a 1-sample 2-sided Student's *t* test vs the 0, which represents the mean metabolite z-transformed reference of normal children.

The *P* values were computed by the standard R-function *t* test (https://www.r-project.org/) and Bonferroni corrected for multiple comparisons. $P < .05$ was considered statistically significant.

The study was conducted according to Helsinki declaration and approved by the Ethics Committee of the Medical University of Silesia. Informed consent was obtained from each subject over age 16, a parent or a legal guardian.

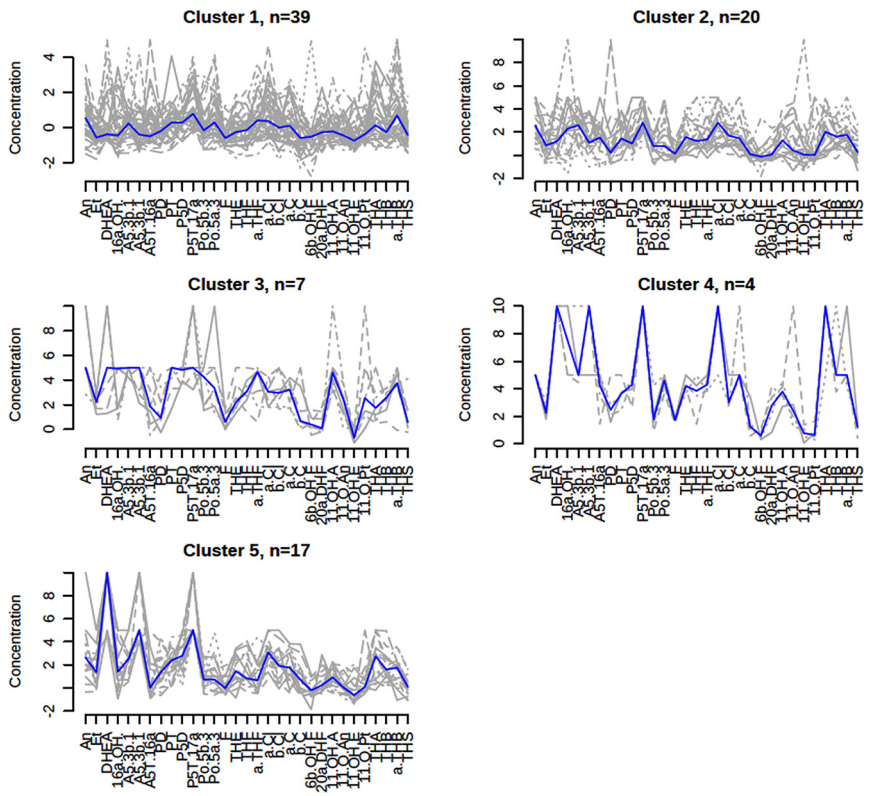

Figure 2. Disease signature by clustering metabolomics data by MetaboAnalyst 3.0. The x-axis includes 31 steroid metabolites. The y-axis indicates the z-transformed 24 urinary quantities of steroid metabolites. Median values of subjects in each cluster are connected by blue lines.

Results

Clinical and chemical phenotype data according to clusters' division are presented in Table 3. Z-transformed concentrations of steroid metabolites are shown in Table 4. Concentrations of steroid metabolites (raw data) and reference data are presented in Supplemental Tables 1 and 2.

The mean age, BMI, and z-score BMI values were not significantly different between the clusters (ANOVA $P > .05$).

Table 2. Distinctive Metabolomic Characteristics of the 5 Groups of Children With Obesity

Cluster	n	Distinctive Metabolomic Characteristics (Comments)
Cluster 1	39	No specific abnormality in the steroid metabolome
Cluster 2	20	Mild general elevation of C19 and C21 steroids, no specific pattern
Cluster 3	7	Dominance of metabolites of 17-OH-progesterone and 21-deoxycortisol, steroid excretion generally elevated
Cluster 4	4	The highest elevation of all steroid metabolites including cortisol- (mostly α-cortolone), corticosterone, and androgen metabolites
Cluster 5	17	Elevations of DHEA and 17-OH-pregnenolone metabolite with a general mildly elevated steroid metabolite excretion

See also Figure 2.

At the clinical level, patients of cluster 1 presented the lowest blood pressure (BP) and hirsutism incidence but the highest prevalence of gynecomastia among boys. Their biochemical phenotype showed the lowest concentration of TSH, basal INS, and HOMA-IR values (Table 3). From 31 analyzed z-transformed steroid metabolites, the mean values of 17 were different from normal references; none of them was higher or lower than ± 2 (Table 4). After Bonferroni correction, *P* value was significantly different only in 5 metabolites, which did not produce any specific steroid profiles (Figure 2).

Patients of cluster 2 presented with low frequency of hepatic steatosis, features of PCOM in ultrasonography and a relatively high proportion of hirsutism in girls (45.5% of the girls). Comparing with sex and age-adjusted normal reference group, cluster 2 showed significantly higher concentrations of 25 of the 31 analyzed steroid metabolites, but only AN, 16α-OH-DHEA, A5–3β,17α, P5T-17α, α-Cl, and THA were higher than +2 of z-transformed values (Table 4).

In cluster 3, which included 7 females only, 6/7 showed features of PCOS. The fasting GLU level and aminotransferases activity tended to be low ($P < .1$). Patients from cluster 3 presented with significantly higher concentration of 27 steroid metabolites in the urine than in healthy pop-

Table 3. Clinical and Chemical Phenotype of 87 Children With Obesity According to Cluster Division

	Cluster 1 (n = 39)	Cluster 2 (n = 20)	Cluster 3 (n = 7)	Cluster 4 (n = 4)	Cluster 5 (n = 17)	*P* Value
Clinical phenotype						
Sex (F/M)	21/18	11/9	7/0	0/4	5/12	
Age (y)	14.18 (2.53)	14.46 (2.26)	15.58 (1.38)	13.33 (1.25)	14.52 (2.42)	.565
BMI (kg/m^2)	30.81 (4.5)	33.26 (5.65)	32.58 (4.07)	34.05 (6.08)	33.97 (5.59)	.151
Z-score BMI	2.59 (0.49)	2.77 (0.60)	2.57 (0.47)	3.02 (0.44)	2.92 (0.48)	.113
WHR	0.94 (0.09)	0.96 (0.08)	0.92 (0.07)	1.03 (0.08)	0.94 (0.09)	.344
BP systolic (mm Hg)	124.5 (9.7)	125.0 (10.65)	132.2 (9.9)	143.3 (15.3)	127.8 (10.9)	***.027***$^{C4>C1,2,5}$
BP diastolic (mm Hg)	76.5 (9.3)	76.6 (8.7)	80.7 (6.1)	81.7 (10.4)	77.8 (6.2)	.694
Hepatic steatosis (%)	21.6	15.0	14.3	50.0	17.6	.599
PCOM (%)	24.0	9.0	42.9	—	40	.569
Hirsutism (%)	14.3	45.5	57.1	—	40	.105
Gynecomastia (%)	27.8	11.1	—	0.0	16.7	.517
Chemical phenotype						
TSH (μIU/mL)	2.68 (0.82)	2.97 (1.71)	3.13 (1.67)	3.00 (1.03)	2.71 (1.32)	.819
Cortisol 8.00 (nmol/L)	496.52 (173.05)	554.21 (167.26)	431.94 (199.55)	529.92 (231.01)	447.67 (124.75)	.298
Cortisol 24.00 (nmol/L)	79.76 (85.00)	83.35 (121.44)	116.75 (107.36)	137.72 (163.94)	64.86 (55.48)	.853
T Chol (mmol/L)	4.50 (0.77)	4.32 (1.08)	4.16 (0.38)	4.42 (0.55)	4.62 (1.08)	.848
HDL-Chol (mmol/L)	1.25 (0.16)	1.28 (0.35)	1.30 (0.40)	1.27 (0.23)	1.27 (0.32)	.882
TG (mnol/L)	1.63 (0.69)	1.81 (0.74)	1.71 (1.20)	1.49 (0.61)	1.55 (0.81)	.862
GLU 0′ (mmol/L)	5.25 (0.47)	5.18 (0.61)	4.86 (0.33)	5.24 (0.68)	5.00 (0.58)	.247
GLU 120′ (mmol/L)	6.51 (1.04)	6.67 (1.25)	6.57 (1.09)	7.30 (1.62)	6.63 (1.53)	.811
INS 0′ (μIU/mL)	16.18 (9.83)	25.91 (20.45)	31.46 (31.67)	34.48 (15.84)	19.49 (9.73)	***.042***$^{C\ 4>C1,2,5}$
INS 120′ (μIU/mL)	79.75 (56.02)	102.38 (69.82)	110.30 (99.44)	195.38 (91.62)	117.46 (73.27)	.127
INS/GLU 0′ (% >0.3)	7.7	35.0	42.9	75.0	17.6	***.009***$^{C4\&3>C1}$
HOMA-IR	3.74 (2.65)	5.57 (4.66)	6.77 (7.08)	8.15 (4.77)	4.26 (2.30)	.126
ALT/GPT (U/L)	30.18 (19.97)	35.05 (29.55)	21.71 (8.16)	44.00 (17.06)	30.00 (14.17)	.447
AST/SGOT (U/L)	28.44 (10.11)	28.11 (13.74)	21.86 (6.79)	29.00 (10.68)	29.00 (9.36)	.650
GGTP (U/L)	22.38 (11.11)	30.00 (11.46)	14.67 (7.51)	46.33 (6.66)	24.08 (7.05)	***.001***$^{C4>C\ 1,3,5}$

Significance by ANOVA. Values are mean, and SD is shown in parentheses. ALT/GPT, alanine aminotransferases; AST/SGOT; aspartate aminotransferases; BP, blood pressure; BMI, body mass index; GGTP, glutamyl transpeptidase; C, cluster; T Chol, total cholesterol; HDL-Chol, high-density lipoprotein cholesterol; INS, insulin; TG, triglyceride; PCOM, polycystic ovary morphology; WHR, waist to hip ratio. Italics and boldface indicate statistical significance.

Table 4. Z-Transformed Concentrations of Steroid Metabolites of 87 Children With Obesity According to Cluster Division

Steroid Metabolites	Cluster 1 (n = 39)	Cluster 2 (n = 20)	Cluster 3 (n = 7)	Cluster 4 (n = 4)	Cluster 5 (n = 17)	*P* Value
AN	0.54 ± 1.19 (.007)	2.78 ± 1.59 (<.001)[a]	6.12 ± 2.76 (.001)	4.99 ± 0.01	2.82 ± 2.35 (<.001)[a]	NS
ET	−0.48 ± 0.61 (<.001)[a]	1.11 ± 1.35 (.002)	2.07 ± 0.53 (<.001)[a]	2.32 ± 0.57	1.54 ± 1.51 (.001)	NS
DHEA	0.27 ± 1.44 (<.001)[a]	1.71 ± 2.10 (.002)	5.25 ± 3.55 (.008)	10.00 ± 0.00	9.09 ± 2.03 (<.001)[a]	NS
16α-OH-DHEA	−0.08 ± 1.31 (.004)	2.53 ± 2.67 (<.001)	3.45 ± 1.94 (.003)	7.50 ± 2.89	1.78 ± 1.97 (.002)	NS
A5–3β,17α	0.35 ± 1.07 (.050)	2.53 ± 1.36 (<.001)[a]	4.79 ± 0.36 (<.001)[a]	6.12 ± 2.60	2.46 ± 1.20 (<.001)[a]	NS
A5–3β,17β	**−0.16 ± 1.04 (NS)**	**1.58 ± 1.43 (<.001)**[a]	**4.02 ± 1.26 (<.001)**[a]	**8.75 ± 2.50**	**5.78 ± 2.03 (<.001)**[a]	**<.05**
A5T-16α	**0.04 ± 1.24 (NS)**	**1.44 ± 1.54 (.001)**	**2.36 ± 2.13 (.026)**	**3.74 ± 1.68**	**0.69 ± 1.62 (NS)**	**<.05**
PD	**−0.12 ± 0.72 (NS)**	**0.87 ± 2.35 (NS)**	**1.75 ± 1.69 (.033)**	**2.88 ± 1.50**	**1.55 ± 1.46 (<.001)**	**<.05**
PT	**0.28 ± 0.98 (NS)**	**1.60 ± 1.25 (<.001)**[a]	**4.16 ± 1.28 (<.001)**[a]	**3.69 ± 1.05**	**2.14 ± 1.23 (<.001)**[a]	**<.05**
P5D	0.39 ± 0.67 (.001)	1.60 ± 1.53 (<.001)[a]	4.36 ± 0.77 (<.001)[a]	3.98 ± 0.96	2.87 ± 1.41 (<.001)[a]	NS
P5T-17α	1.14 ± 1.23 (<.001)[a]	3.14 ± 1.32 (<.001)[a]	6.82 ± 3.03 (.001)	10.00 ± 0.00	6.47 ± 2.35 (<.001)[a]	NS
Po-5β,3α	**0.06 ± 0.96 (NS)**	**0.84 ± 0.94 (.001)**	**3.43 ± 1.58 (.001)**	**2.17 ± 1.45**	**0.81 ± 0.94 (.003)**	**<.05**
Po-5α,3α	0.57 ± 1.28 (.008)	1.51 ± 1.62 (.001)	4.26 ± 2.89 (.008)	4.47 ± 0.66	1.07 ± 1.40 (.006)	NS
F	**−0.52 ± 0.56 (<.001)**[a]	**0.18 ± 0.52 (NS)**	**0.71 ± 0.73 (.041)**	**1.70 ± 0.19**	**0.16 ± 0.69 (NS)**	**<.05**
THE	**−0.14 ± 0.84 (NS)**	**1.78 ± 1.35 (<.001)**[a]	**2.62 ± 1.28 (.002)**	**4.28 ± 0.55**	**1.52 ± 1.23 (<.001)**[a]	**<.05**
THF	**−0.14 ± 0.83 (NS)**	**1.77 ± 1.49 (<.001)**[a]	**3.15 ± 1.16 (<.001)**[a]	**3.53 ± 1.52**	**1.59 ± 1.38 (<.001)**[a]	**<.05**
αTHF	0.40 ± 1.11 (.029)	1.82 ± 1.46 (<.001)[a]	3.55 ± 1.74 (.002)	4.36 ± 0.60	1.07 ± 1.36 (.005)	NS
α-Cl	0.72 ± 1.29 (.001)	2.63 ± 1.71 (<.001)[a]	2.98 ± 1.07 (<.001)[a]	8.75 ± 2.50	3.00 ± 1.15 (<.001)[a]	NS
β-Cl	**0.04 ± 0.93 (NS)**	**1.89 ± 1.15 (<.001)**[a]	**3.16 ± 1.38 (.001)**	**3.40 ± 1.09**	**2.02 ± 1.43 (<.001)**[a]	**<.05**
α-C	**0.34 ± 1.14 (NS)**	**1.73 ± 1.47 (<.001)**[a]	**2.96 ± 1.01 (<.001)**[a]	**6.18 ± 2.55**	**1.90 ± 1.37 (<.001)**[a]	**<.05**
β-C	**−0.48 ± 0.93 (.002)**	**0.33 ± 0.69 (.049)**	**1.65 ± 1.89 (NS)**	**1.62 ± 1.25**	**0.79 ± 1.29 (.021)**	**<.05**
6β-OH-F	**−0.51 ± 1.09 (.006)**	**0.01 ± 0.96 (NS)**	**0.47 ± 0.64 (NS)**	**0.66 ± 0.36**	**−0.21 ± 0.70 (NS)**	**<.05**
20α-DHF	**−0.12 ± 0.79 (NS)**	**0.36 ± 0.78 (.050)**	**0.53 ± 0.85 (NS)**	**2.64 ± 1.45**	**0.62 ± 1.02 (.024)**	**<.05**
11-OH-AN	**−0.04 ± 0.76 (NS)**	**1.49 ± 1.36 (<.001)**[a]	**5.11 ± 2.25 (.001)**	**3.74 ± 0.82**	**0.89 ± 0.92 (.001)**	**<.05**
11-O-AN	**−0.23 ± 0.71 (NS)**	**1.32 ± 1.82 (.004)**	**2.22 ± 1.33 (.005)**	**4.02 ± 4.04**	**0.19 ± 0.76 (NS)**	**<.05**
11-OH-ET	**−0.53 ± 0.83 (<.001)**[a]	**0.57 ± 2.46 (NS)**	**−0.54 ± 0.46 (.022)**	**0.76 ± 0.60**	**−0.25 ± 1.12 (NS)**	**<.05**
11-O-PT	**−0.08 ± 1.03 (NS)**	**0.68 ± 1.75 (NS)**	**3.60 ± 3.39 (.031)**	**0.80 ± 0.58**	**0.54 ± 1.32 (NS)**	**<.05**
THA	0.46 ± 1.28 (.030)	2.21 ± 1.66 (<.001)[a]	2.06 ± 1.59 (.001)	8.75 ± 2.50	2.45 ± 1.83 (<.001)[a]	NS
THB	**0.04 ± 0.97 (NS)**	**1.65 ± 1.16 (<.001)**[a]	**2.37 ± 0.99 (.001)**	**5.94 ± 2.77**	**1.70 ± 1.28 (<.001)**[a]	**<.05**
α-THB	0.88 ± 1.63 (.002)	1.90 ± 1.27 (<.001)[a]	3.63 ± 1.74 (.001)	6.05 ± 2.66	1.48 ± 1.28 (<.001)[a]	NS
THS	**−0.36 ± 0.72 (.004)**	**0.46 ± 1.08 (NS)**	**1.14 ± 1.46 (NS)**	**1.10 ± 0.48**	**0.10 ± 0.80 (NS)**	**<.05**

Z-transformed concentrations of steroid metabolites ± SD. Data are mean ± SD. In Parentheses, *P* value with respect to norms. Boldface data indicate statistical significance.

[a] Statistically significant Bonferroni adjusted *P* value.

ulation; most of them were higher than +2Z. The quantities of PT, Po-5β,3α, β-Cl, α-C, 11-OH-AN and 11-O-PT were significantly higher comparing with both norms and the other clusters (Tables 3 and 4).

Cluster 4 patients presented with significantly higher systolic BP ($P = .027$). Comparing their carbohydrate parameters, they had higher fasting INS concentration ($P = .042$) and increased frequency of GLU/INS index elevated above 0.3 ($P = .009$), as compared with the other clusters. These patients presented also the highest GGTP activities ($P = .001$) and in half of them hepatic steatosis features were found in ultrasound examination. Serum levels of lipids, TSH, fT_4, and morning cortisol were statistically comparable between the clusters; however, midnight cortisol levels were highest in cluster 4 (Table 3). The small size of cluster 4 prevents the comparison of steroid metabolites excretion with normal references.

Cluster 5 patients had no specific clinical phenotype; however, they showed the lowest concentration of midnight cortisol. Twenty-four steroid metabolites were significantly different comparing with reference population, but only mean z values of AN, DHEA, A5–3β,17α, A5–3β,17β, PT, P5D, P5T-17α, α-Cl, β-Cl, and THA were higher than +2 (Tables 3 and 4).

Comparing the z-transformed values of steroid metabolites between the clusters, significant differences were found for A5–3β,17β, A5T-16α, PD, F, THE, THF, β-Cl, α-C, 6β-OH-F, 20α-DHF, 11-O-AN, 11-OH-ET, THB (highest means in cluster 4), and PT, Po-5β,3α, β-C, 11-OH-AN, 11-O-PT, THS (highest means in cluster 3) (Table 4). Calculating 5α reductase activity from the ratios ET to AN, THF to aTHF, and THB to aTHB showed no difference between the clusters (ANOVA).

Discussion

We have previously defined the concept of individual steroidal fingerprint and steroid metabolomic disease signature based on quantitative urinary steroidal GC-MS data (1). Here, we take this concept a step further and report clustering of nonsyndromic childhood obesity by a software designed for metabolic data analysis into 5 groups with their characteristic "steroid metabolomic signatures." Further analysis focuses on the comparison of both

clinical and biochemical phenotype parameters between the clusters. The mean age as well as values of BMI and z-score BMI were comparable.

Almost 45% of obese children belonged to cluster 1, with no specific abnormality in the steroid metabolome. They presented with the highest incidence of gynecomastia in boys, however, comparable or even lower than in the general healthy population; during puberty, its prevalence ranges between 4% and 69% (16, 17). An imbalance of estradiol and testosterone in overweight and obese adolescents may be related to an increase of peripheral conversion of androgens to estrogens (18); however, the GC-MS method that we used does not identify estrogen metabolites. Not all studies confirm that adipose tissue positively correlates with the breast tissue hypertrophy in boys (19).

Cluster 2 presented with an unspecific mild general elevation of C19 and C21 steroids in both boys and girls. Clinically half of the females from cluster 2 presented with hirsutism but no other symptoms of hyperandrogenism. The association of obesity with elevated C19 androgens and C21 glucocorticoids is well documented (1, 20), and the mechanisms are multiple. It was considered a secondary effect of obesity, but we now show that this is a unique feature of a subgroup of cluster 2 that includes some 23% (20/87) of the obese mostly adolescents that do not differ in their age, puberty, or degree of obesity. It is interesting that such females have hirsutism as the only virilization symptom and that their 5α reductase activity was not enhanced.

Urinary steroid profiles in 7 girls from cluster 3 were characterized by dominance of metabolites of 17-OH-progesterone and 21-deoxycortisol. This could point to a relative 21-hydroxylase "weakness." More than 80% of them presented with partial or full PCOS features. Indeed, it has been reported that PCOS patients show relative weakness of the 21-hydroxylase (21, 22), but current results suggest that this is a feature of a selected subgroup of obese children. This association requires further consideration in terms of mechanism and eventual personalized management.

The clinical phenotype of cluster 4 was characterized by the highest systolic BP, fasting INS concentration, GGTP activity, and a high incidence of ultrasonographic hepatic steatosis. The syndrome of obesity hypertension, hyperinsulinemia, and hepatic steatosis has been designated as the metabolic syndrome. The association is not surprising given the pathophysiologic pathways thought to be essential in their genesis (23). The results of the present study show that the metabolic syndrome is associated with, and may be caused by, increased hypothalamic-pituitary-adrenal axis activity. Cluster 4 was characterized by the highest cortisol and, as a unique finding, corticosterone metabolites. This association was previously suggested (24–28). Steroid hormones are metabolized and conjugated in the liver. In patients with nonalcoholic fatty liver disease, which represents a spectrum of liver diseases, changes in cortisol metabolism were previously described; in steatosis decreased hepatic 11β-HSD1 activity and increased 5α-reductase, in steato-hepatitis contrary increased 11β-HSD1 activity were found. However, nonalcoholic fatty liver disease may also influence metabolism through multiple metabolic effects by nondetoxified metabolites (29). The disease signature of cluster 4 includes also increased androgen metabolites (DHEA and its end metabolites). The increase in cortisol metabolites, 17-deoxygenated corticosterone metabolites and DHEA metabolites may suggest a common stimulatory mechanism, eg, increased hypothalamic activity and ACTH release stimulating adrenal cortex activity.

Female patients of cluster 5 showed a high incidence of hirsutism and PCOM, and its signature was characterized by elevation of DHEA and 17-OH-pregnenolone metabolites within a mild general steroid metabolite elevation. This could indicate a relative weakness of 3β-HSD. Indeed, congenital adrenal hyperplasia due to 21-hydroxylase activity can result in PCOS-like phenotype; yet, the exact mechanism of this association is unclear (30). Circulating androstenedione and DHEA primarily act as prehormones. DHEA by hepatic and peripheral conversion is a prehormone for androstenedione (31, 32). Kumar et al suggested that in PCOS there is a generalized up-regulation of adrenal precursor androgens (33). Obesity by hyperinsulinemia or/and the effect of adipocyte-generated adipocytokines may stimulate the secretion of cortisol, androstenedione and DHEA in response to ACTH and predispose to PCOS (30). We now show that this combination manifests in a cluster of its own in 20% (17/87) of the subjects.

Two different steroid metabolomic disease signatures but comparable clinical presentations in clusters 3 and 5 suggest the necessity of precision/personalized medicine. Nevertheless, the question rises why exactly these groups of obese patients are the most predisposed to specific steroidal fingerprint leading to PCOS-like phenotype. Is it a consequence or rather the reason for obesity? The 5 clusters did not differ in age and severity of obesity; however, cluster 4, with 4 boys only, was characterized by the highest risk of cardio-metabolic consequences. It presented with a steroid metabolomic signature that was characterized by a general elevation of all steroid metabolites including cortisol metabolites (especially α-cortolone), corticosterone metabolites, and androgens. This may indicate a general stimulation of the adrenals, eg, by ACTH and a

shift in the equilibrium of 11β-HSD1 towards the active 11-oxidized products (34). Indeed, cortisol has been implicated as a pathophysiological mediator in idiopathic obesity, but circulating cortisol concentrations are not consistently elevated (35). In obese Zucker rats, 11β-HSD1 activity is reduced in the liver but enhanced in adipose tissue (36). In humans, higher BMI was associated with increased total cortisol metabolite excretion, but lower plasma cortisol after dexamethasone, and no difference in response to CRH (37). Obese men excreted a greater proportion of cortisone than cortisol metabolites and converted less cortisone to cortisol after oral administration, suggesting impaired hepatic 11β-HSD1 activity (38).

The steroid profile in the 24-hour urine collection is recognized as a stable and reproducible method for steroid metabolites assessment. Regarding day to day variation, a low mean variation of 12% for glucocorticoids was found (39). Imbalances in steroid metabolism can reliably be characterized by one-time assessment of urinary steroids (40). Some limitations concerning the sample size and statistical analysis need to be mentioned. Clustering of 87 subjects generate poorly stable clusters. We therefore had to resort to vector quantization (k-means) for cluster analysis. We used version 3.0 of MetaboAnalyst, a web server designed to permit comprehensive metabolomic data analysis. First introduced in 2009, MetaboAnalyst has experienced more than a 50× growth in user and 3 versions in that short period; k-means algorithm are not changed by the new versions, but results might change when run by another version due to the heuristic and partially probabilistic k-means algorithms, regardless of the software used. Version 3.0 will most probably be improved in the near future challenging the reproducibility of our results. Recognizing the sample size limitation, we do not claim this model to have a predictive value. We hope that we and others would expand the data to allow it in the future.

In conclusion, we present childhood obesity from the perspective of steroidal individual fingerprints and disease signature. Clustering by software designed for metabolic data analysis, we reclassified childhood obesity into 5 clusters with their steroid metabolomic signatures, which require further definition and may need cluster-precision therapy. Current clinical practice relies on data that have been averaged for a given disease or population. This traditional approach complies with most patients, but misdiagnosis, treatment failure, and adverse drug reaction are common in marginal cases that lie away from average as part of individual variation or missclassification. Here, we contemplate the contributions that steroid metabolomics have made, and can potentially still make, towards an approach that we now call personalized medicine. Classifying a child as "obese" is only a small part of the phenotype. The steroid profile was used here as a biomarker to add precision to the term obese. Identifying a cluster signature in a given child would then be used to develop personalized prognostic, diagnostic, and therapeutic approaches and can also be applied to the monitoring of disease evolution and progression, treatment choices and efficacy, predisposition to drug-related side effects, and potential relapse.

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