Steroid Metabolomic Signature of Insulin Resistance in Childhood Obesity

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Diabetes Care 2020;43:405–410 | https://doi.org/10.2337/dc19-1189

OBJECTIVE

On the basis of urinary steroidal gas chromatography-mass spectrometry (GC-MS), we previously defined a novel concept of a disease-specific "steroid metabolomic signature" and reclassified childhood obesity into five groups with distinctive signatures. The objective of the current study was to delineate the steroidal signature of insulin resistance (IR) in obese children.

RESEARCH DESIGN AND METHODS

Urinary samples of 87 children (44 girls) aged 8.5–17.9 years with obesity (BMI >97th percentile) were quantified for 31 steroid metabolites by GC-MS. Defined as HOMA-IR >95th percentile and fasting glucose-to-insulin ratio >0.3, IR was diagnosed in 20 (of 87 [23%]) of the examined patients. The steroidal fingerprints of subjects with IR were compared with those of obese children without IR (non-IR). The steroidal signature of IR was created from the product of IR − non-IR for each of the 31 steroids.

RESULTS

IR and non-IR groups of children had comparable mean age (13.7 ± 1.9 and 14.6 ± 2.4 years, respectively) and *z* score BMI (2.7 ± 0.5 and 2.7 ± 0.5, respectively). The steroidal signature of IR was characterized by high adrenal androgens, glucocorticoids, and mineralocorticoid metabolites; higher 5α-reductase (An/Et) ($P = 0.007$) and 21-hydroxylase [(THE + THF + αTHF)/PT] activity ($P = 0.006$); and lower 11βHSD1 [(THF + αTHF)/THE] activity ($P = 0.012$).

CONCLUSIONS

The steroidal metabolomic signature of IR in obese children is characterized by enhanced secretion of steroids from all three adrenal pathways. As only the fasciculata and reticularis are stimulated by ACTH, these findings suggest that IR directly affects the adrenals. We suggest a vicious cycle model, whereby glucocorticoids induce IR, which could further stimulate steroidogenesis, even directly. We do not know whether obese children with IR and the new signature may benefit from amelioration of their hyperadrenalism.

Nonsyndromic childhood obesity is associated with "insulin resistance (IR)," which denotes a decreased metabolic response to insulin at the cellular level or, at the whole-organism level, a diminished lowering effect of insulin on blood glucose (1). However, many individuals with obesity, mostly those with subcutaneous rather than visceral adipose tissue, seem to be protected from IR and adverse metabolic responses (2). Indeed, human omental adipocytes display an approximately twofold higher

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Received 16 June 2019 and accepted 24 October 2019

glucose uptake rate compared with subcutaneous adipocytes, and this could be explained by a higher GLUT4 expression. A marked suppression is exerted by glucocorticoids on glucose uptake and on the expression of insulin signaling proteins in omental but not in subcutaneous adipocytes (3). These findings may be of relevance for the bidirectional interaction between endogenous glucocorticoids and visceral fat in the development of IR.

We have previously shown that the steroid metabolomic signature of a subtype of childhood obesity reflects the derangements of steroid metabolism in obesity (4) and in nonalcoholic fatty liver disease (5) that include enhanced glucocorticoids and deranged androgen and mineralocorticoids as well as IR. Here, we used our previously reported concept to argue that an individual's urinary steroid metabolite profile represents his or her unique metabolic fingerprint and offers a means of metabolomic phenotyping at the individual level. Thus, each individual has a unique steroidal fingerprint. A cluster of similar steroidal fingerprints related to a disease would be regarded as a steroid metabolomic disease signature (4), which represents the impact of a disease in people who differ in their phenotypes or have other health problems.

Here, we analyzed the clinical data of a group of 87 patients with well-phenotyped nonsyndromic childhood obesity and defined those affected and those unaffected by IR. We not only generated steroidal disease signatures of the two groups and suggest that they might help in clinical diagnosis but also shed light on steroid-related metabolic sequelae of IR in childhood obesity.

RESEARCH DESIGN AND METHODS

A consecutive series of 117 obese Caucasian children and adolescents (BMI >97th percentile) who were patients referred to the Department of Pediatric Endocrinology, Medical University of Silesia, were examined. After exclusion for syndromic obesity, chronic diseases, pharmacotherapy (also metformin), immobilization, and young age (<8 years), 87 patients (44 girls) aged 8.5–17.9 years (mean age 14.4 years, SD 2.3 years) were included for further analysis. Their data were previously used to classify five different steroid metabolomic signatures in patients with childhood obesity (4).

All patients underwent a clinical assessment and diagnostic procedures that included a general physical examination, anthropometric measurements of height and weight (to calculate BMI and *z* score BMI), waist and hip circumference (to calculate waist-to-hip ratio [WHR] and waist-to-height ratio [WHtR]) and puberty assessment, as previously described (4). None of the patients were on a special elimination diet. During hospitalization, they followed a regular hospital diet. Morning fasting venous blood samples were collected to measure glucose, insulin, lipids, thyroid-stimulating hormone (TSH), cortisol, and aminotransferases. Plasma total cholesterol, HDL cholesterol, and triglyceride levels were analyzed enzymatically (Beckman Coulter, Brea, CA). Cortisol was measured in the morning (8:00 A.M.) and at midnight using chemiluminescent immunoassay by IMMULITE 2000 analyzer (DPC, Atlanta, GA). Serum concentrations of TSH were measured with a chemiluminescent immunometric assay (IMMULITE 2000 Free T4, IMMULITE 2000 Third Generation TSH; Siemens, Malvern, PA). γ-Glutamyl transpeptidase, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) activity in the serum were assessed according to the International Federation in Clinical Chemistry and Laboratory Medicine using Beckman Coulter instrumentation.

IR Indices

Glucose and insulin levels were also measured with an oral glucose tolerance test (1.75 g/kg, maximum 75 g). An enzymatic test (hexokinase method) was used for the quantitative determination of glucose (Beckman Coulter). Insulin was determined using a chemiluminescence immunoassay on an IMMULITE 2000 analyzer. Fasting insulin-to-glucose ratio and HOMA of insulin resistance (HOMA-IR) (fasting glucose [mmol/L] × fasting insulin [mIU/L] / 22.5) were calculated as indices of IR (6). IR was defined as HOMA-IR >95th percentile and fasting insulin-to-glucose ratio >0.3 present at the same time (7,8).

Gas Chromatography–Mass Spectrometry of Urinary Steroids

Steroid metabolites in 24-h urine samples were analyzed by quantitative targeted gas chromatography (GC)–mass spectrometry (4,9,10). In brief, free and conjugated urinary steroids were extracted by solid phase extraction; conjugates were enzymatically hydrolyzed; and after addition of known amounts of internal standards (5α-androstane-3α,17α-diol, stigmasterol), methyloxime-trimethylsilyl ethers were formed. GC was performed using an Optima-1 fused silica column (MACHEREY-NAGEL, Düren, Germany) housed in an Agilent 6890 series GC that was directly interfaced to an Agilent 5975 series Inert XL Mass Selective Detector. Two characteristic ions (quantifier and qualifier) were measured per analyte. Sensitivity ranged from 1.6 μg/L for 11-oxo-pregnanetriol (11-O-PT) and 25 μg/L for 6β-hydrocortisol (6β-OH-F). Intra-assay precision ($n = 6$) varied between 1.7% (for 17β-adiol) and 6.2% (for DHEA) and interassay precision ($n = 6$) between 1.1% (α-cortol [αC]) and 9.2% (DHEA).

The ratios of steroid metabolites defined in our previous study (4) were used to calculate the activity of the enzymes: 17-hydroxylase/17,20-lyase, 17α-hydroxylase, 17,20-lyase activity, 11β-hydroxylase, 3β-hydroxysteroid dehydrogenase (3βHSD), 21-hydroxylase, 11βHSD type 1 (11βHSD1), and 5α-reductase.

The study was conducted according to the Declaration of Helsinki and approved by the ethics committee of the Medical University of Silesia. Informed consent was obtained from each patient aged >16 years, a parent, or a legal guardian after full explanation of the purpose and nature of all procedures.

Statistical Analysis and Visualization of Metabolomic Data

Steroid metabolite quantities were *z* transformed on the basis of sex- and age-adjusted normal reference groups, as described elsewhere (4). Per each of 31 *z*-transformed steroid metabolites and per non-IR and IR groups, means were computed. The standard R function matplot (11) was used to depict the steroidal signature of IR as the difference between the mean of IR and mean of non-IR.

Clinical and chemical data as well as steroid metabolite concentration ratios of patients in each group were analyzed, and Student *t* test and *t* test with separate variance estimation were used to assess the difference between groups. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical and Biochemical Phenotype

Of 87 obese children, 20 (23%) were diagnosed with IR. The comparison of clinical phenotype of the IR and non-IR groups is presented in Table 1.

The mean age, BMI, *z* score BMI, and blood pressure values were not significantly different between patients

Table 1—Comparison of clinical and biochemical phenotype of non-IR and IR groups

	Non-IR (n = 67)		IR (n = 20)		
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	P value
Clinical phenotype					
Sex (female/male)	36/31		8/12		NS
Age (years)	14.6 ± 2.4	14.9 (8.5–17.9)	13.7 ± 1.9	14.2 (10.7–17.0)	NS
BMI (kg/m^2)	32.5 ± 5.4	31.6 (24.1–45.3)	31.6 ± 4.2	30.8 (26.0–43.1)	NS
z score BMI*	2.72 ± 0.5	2.71 (1.46–3.84)	2.68 ± 0.5	2.76 (1.75–3.63)	NS
WHR	0.93 ± 0.1	0.95 (0.7–1.13)	1.00 ± 0.1	0.98 (0.93–1.13)	0.029
WHtR	0.61 ± 0.05	0.61 (0.51–0.73)	0.63 ± 0.08	0.62 (0.55–0.80)	NS
BP systolic (mmHg)	125 ± 10	125 (100–150)	130 ± 13	130 (110–160)	NS
BP diastolic (mmHg)	77 ± 8	80 (60–100)	77 ± 10	80 (50–90)	NS
Biochemical phenotype					
TSH (μIU/mL)	2.74 ± 1.3	2.61 (0.73–8.23)	2.97 ± 1.2	2.83 (1.54–6.79)	NS
Cortisol at 8:00 A.M. (μg/dL)	17.6 ± 6.1	17.8 (0.74–30.7)	19.1 ± 6.3	17.6 (7.57–31.2)	NS
Cortisol at midnight (μg/dL)	3.0 ± 3.5	1.68 (1.0–19.3)	2.8 ± 3.1	1.5 (1.0–12.5)	NS
Total cholesterol (mg/dL)	173 ± 36	172 (19.3–271)	168 ± 29	167 (127–234)	NS
HDL-C (mg/dL)	49.9 ± 10.6	48.8 (27.5–85.6)	45.7 ± 8.6	43.7 (3.1–65.1)	NS
TG (mg/dL)	138.9 ± 67.5	119 (51–367)	172.1 ± 58.9	159 (76–295)	NS
GLU 0 min (mg/dL)	90 ± 9	89 (73–129)	92 ± 10	90 (78–119)	NS
GLU 120 min (mg/dL)	113 ± 19	115 (64–155)	126 ± 24)	125 (88–164)	0.011
INS 0 min (μIU/mL)	14.5 ± 6.3	13.4 (2.0–35.1)	42.8 ± 19.8	34.5 (24.7–97.8)	<0.00001
INS 120 min (μIU/mL)	78.5 ± 48.9	66.5 (14.5–276)	205.6 ± 110.8	173.5 (60.9–309.5)	<0.00001
ALT (units/L)	28 ± 19	23 (8–131)	41 ± 24	33 (16–124)	0.018
AST (units/L)	27 ± 10	26 (11–69)	30 ± 11	28 (19–70)	NS
GGTP (units/L)	23 ± 11	21 (7–56)	32 ± 11.0	30 (16–52)	0.026

Significance by Student t test. BP, blood pressure; GGTP, γ-glutamyl transpeptidase; GLU, glucose; HDL-C, HDL cholesterol; INS, insulin; NS, not significant; TG, triglycerides. *The z score BMI is according to the International Obesity Task Force.

with and without IR. The WHR, but not WHtR, was significantly higher in the IR group than in the non-IR group (Table 1). At the biochemical level, patients with IR versus those without IR presented higher mean values of fasting insulin (but not glucose), ALT, AST, and postprandial glucose and insulin (Table 1).

Steroidal Signature of IR

Patients with and without IR presented comparable diurnal cortisol profiles (i.e., morning and midnight plasma cortisol concentrations were not different). Comparing the z-transformed values of steroid metabolites, significantly higher concentrations of DHEA metabolite 5-androstene-3β,17α-diol; cortisol metabolites cortisol (F), 5α-pregnane-3α,17α-diol-20-one (Po-5α,3α), 5β-pregnane-3α,11β,17α,21-tetrol-20-one (THF), 5α-pregnane-3α,11β,17α,21-tetrol-20-one (5α-THF), and 6β-OH-F; cortisone metabolites 5β-pregnane-3α,17 α,21-triol-11,20-dione (THE), α-cortolone (αCl), and β-cortolone (βCl); and corticosterone metabolites tetrahydro-11-dehydrocorticosterone (THA) and TH-corticosterone (THB) were found in the IR group (Fig. 1). The sum of major cortisol metabolites (5α-THF + THF + THE) and overall cortisol metabolite secretion (5α-THF + THF + THE + αC + β-cortol [βC] + αCl + βCl) was higher in the IR than in the non-IR group (11,376.5 ± 5,568 vs. 7,822.9 ± 3,506 μg/day [$P < 0.001$] and 16,706.9 ± 7,850 vs. 11,741.8 ± 5,032 μg/day [$P = 0.013$], respectively).

Patients with IR have shown significantly enhanced 5α-reductase and 21-hydroxylase activity and lower activity of 11βHSD1 than patients without IR (Table 2). The ratios of steroid metabolites to calculate the activity of 17-hydroxylase/17,20-lyase, 17α-hydroxylase, 17,20-lyase, 11β-hydroxylase, and 3βHSD were not significantly different ($P > 0.05$) between the groups.

Steroid metabolomics signature of IR in childhood obesity is presented as the difference between mean z-transformed concentrations of steroid metabolites in patients with and without IR (Fig. 2).

CONCLUSIONS

On the basis of our previous definition of steroid metabolomic disease signature by quantitative urinary steroidal GC–mass spectrometry data (4), we define the steroidal signature of IR in nonsyndromic childhood obesity. At the clinical level, children with and without IR had comparable mean age and z score BMI. However, fat distribution was different with visceral fat, measured as waist-to-hip circumference, and greater in the IR group, as described by others (12). As previously reported by others and us, liver enzymes were higher in the IR group, both in the adult and in the pediatric obese population (13,14).

The most common contributor to IR is central obesity, although primary IR in normal-weight individuals is also possible. Excess abdominal adipose tissue has been shown to release increased amounts of free fatty acids, which directly affect insulin signaling, diminish glucose uptake in muscle, drive exaggerated triglyceride synthesis, and induce gluconeogenesis in the liver. The main characteristics of IR are disinhibited lipolysis in adipose tissue, impaired uptake of glucose by muscle, and disinhibited gluconeogenesis (15). The results published by Kinyua et al. (16) suggested that insulin upregulates steroidogenic factor-1 (transcriptional factor SF-1) and the steroidogenetic genes directly, independent of the CRH-ACTH-MC2R-PKA pathway, increasing the generation of adrenal gland hormones.

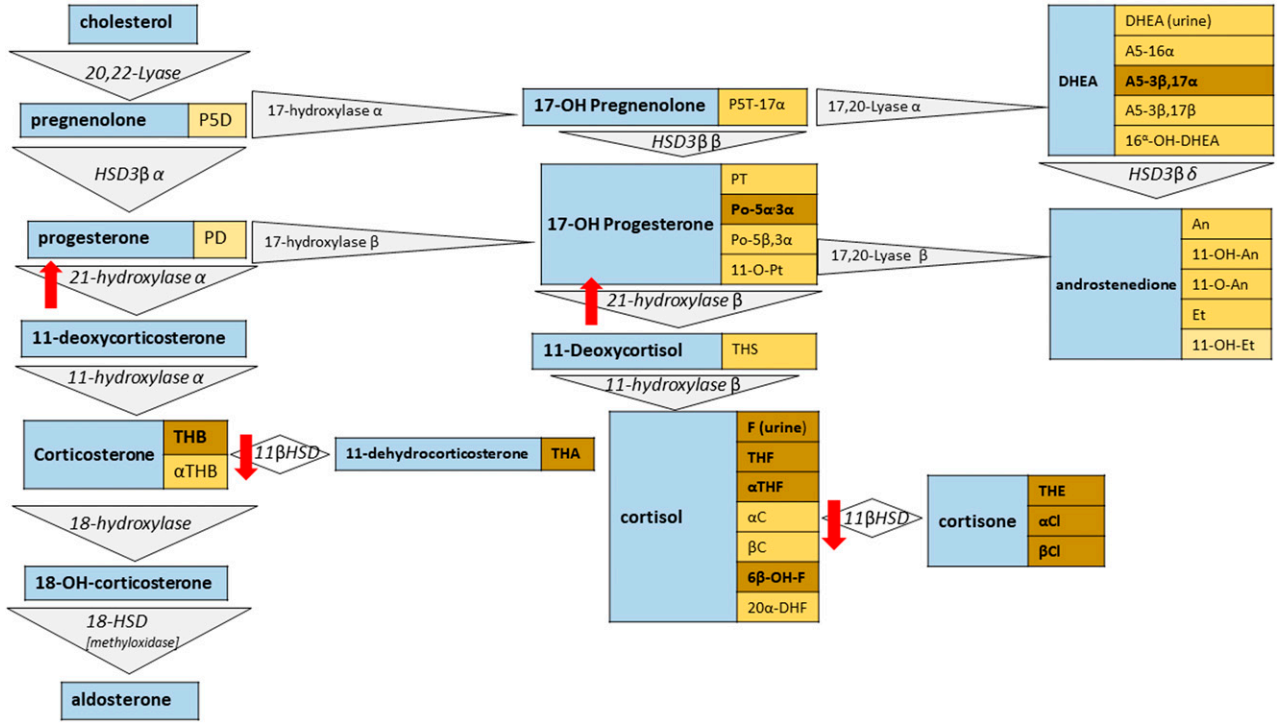

Figure 1—Steroidogenesis in the IR group compared with the non-IR group with obesity. Blood metabolite (highlighted in blue) and urinary end products (highlighted in tan) of the expanded steroid pathway. Triangles and rhombi correspond, respectively, to uni- and bidirectional blood enzymatic reactions. Color saturation of urinary end products (from light to dark tan color) corresponds to the concentrations of steroid metabolites, with darker meaning significantly higher concentrations in the IR group than in the non-IR group. The direction of the arrows reflects the enzyme activity in IR. DHF, dihydrocortisol; THS, tetrahydro-11-deoxycortisol.

The steroidal signature of IR presented here seems to be in line with this suggestion because it is characterized by high adrenal androgens, glucocorticoids, and mineralocorticoid metabolites. The former has been reported in conjunction with polycystic ovary syndrome, as previously reviewed (17). There is a postbinding defect in receptor signaling that is likely due to increased receptor and insulin receptor substrate 1 serine phosphorylation that selectively affects metabolic but not mitogenic pathways in classic insulin target tissues and the ovary. Constitutive activation of serine kinases in the MAPK-ERK pathway may contribute to resistance to insulin's metabolic actions in skeletal muscle.

It has been previously observed that obesity correlates with higher aldosterone concentrations (18,19). Goodfriend et al. (18) suggested that visceral fat stimulates adrenal steroidogenesis. Their study confirmed that in humans, plasma aldosterone correlates with measures of visceral obesity and IR. Moreover, they showed that certain fatty acids stimulate aldosterone production in vitro by rat adrenal cells incubated with rat hepatocytes but not by adrenal cells alone. These results suggested that fatty acids from visceral adipocytes induce hepatic formation of an adrenal secretagogue/aldosterone-stimulating factor, which may explain the correlation between plasma steroids and visceral obesity. An Italian study confirmed that in patients with and without hypertension, insulin is related not only to BMI but also to plasma aldosterone (19).

The renin-angiotensin-aldosterone system and mineralocorticoid receptor antagonism implicate excessive serine phosphorylation and proteosomal degradation of the docking protein, insulin

Table 2—Ratio of steroid metabolites (enzyme activity): differences between the non-IR and IR groups

Ratio of steroid metabolites*	Enzyme activity	Non-IR (n = 67)	IR (n = 20)	P value
An/Et	5α-reductase	2.1 ± 0.8	2.7 ± 0.9	0.007
(THF + αTHF)/THE	11βHSD1	0.9 ± 0.3	0.7 ± 0.2	0.012
(THE + THF + αTHF)/PT	21-hydroxylase	10.2 ± 4.3	13.3 ± 4.5	0.006
(11-O-PT + PT + Po-5β3α + Po-5α3α)/(5α-THF + THF + THE)#		0.14 ± 0.1	0.11 ± 0.0	0.021
(PT + Po-5β3α + Po-5α3α)/(5α-THF + THF + THE)#		0.14 ± 0.1	0.11 ± 0.0	0.019

Data are mean ± SD. Only ratios that are significantly different are shown. Significance by Student t test. *Calculated on the basis of steroid metabolite concentrations. #Ratio of relative 21-hydroxylase deficiency.

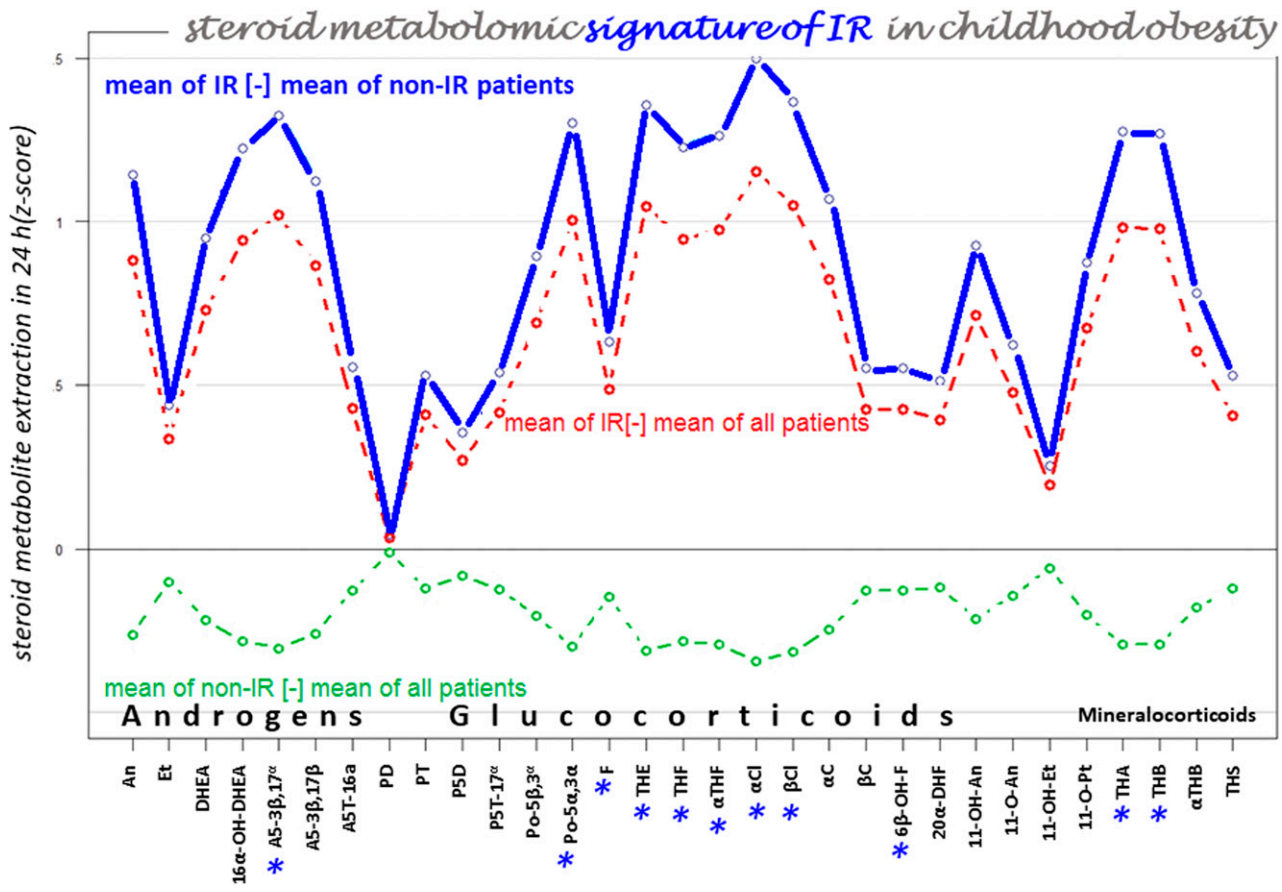

Figure 2—Steroid metabolomics signature of IR in childhood obesity presented as the difference between mean *z*-transformed concentrations of steroid metabolites in non-IR and IR groups (blue). [-], minus; THS, tetrahydro-11-deoxycortisol. *Differences between IR and non-IR (*z* score) $P < 0.05$.

receptor substrate, and enhanced signaling through hybrid insulin/IGF-I receptor as important mechanisms underlying aldosterone-mediated interruption of downstream insulin signaling (20). In turn, animal studies by Huby and colleagues (21,22) pointed to leptin as an adipocyte-derived, aldosterone-secreting factor, which was in conflict with other reports (23–25).

Cortisol/glucocorticoid metabolism changes depending on altered production, different peripheral clearance, and, notably, tissue-specific 11βHSD1 activity. In obese patients, increased cortisol production is observed, which in our study population is expressed as higher urinary cortisol metabolites.

Animal models of obesity and type 2 diabetes not only have shown elevated steroid hormone concentrations but also have confirmed increased expression of genes of steroidogenic enzymes in adrenals but not in adipose tissue (24,25). More specifically, IR in childhood obesity is characterized by higher 5α-reductase (An/Et) (26) and 21-hydoxylase (27) activity and lower 11βHSD1 (28,29). Similar findings were simultaneously observed in our study, and together, they comprise the steroid metabolomic disease signature. Decreased activity of 11βHSD1, in obesity observed in liver tissue (30) but not in visceral fat (31), leads to higher concentrations of urinary cortisone metabolites, as observed in our study. It could be compensated by activation of the hypothalamus-pituitary-adrenal axis and be a second trigger for glucocorticoid and androgen production.

Finally, on the basis of our steroidal signature of IR in nonsyndromic childhood obesity and the literature, we propose a vicious cycle model whereby glucocorticoids induce IR, which further, even directly, stimulates steroidogenesis. Undoubtedly, reduction of IR/improvement of insulin sensitivity by weight loss (through nutritional therapy and/or lifestyle adjustment) will be advantageous because it can interrupt this cycle. The pharmacotherapy effect to influence expression and activity of 11βHSD1 in this context still remains unclear (32). In the context of a personalized approach to each obese patient with the risk of metabolic consequences, more studies are needed to verify our findings.

Acknowledgments. The authors thank all the patient-volunteers who participated in the clinical study.

Funding. This work was supported by Śląski Uniwersytet Medyczny funds for statutory works of Medical University of Silesia (KNW-1-146/K/6/K, KNW-1-015/N/8/K). Justus Liebig University Giessen financed the steroid analysis by the Steroid Research and Mass Spectrometry Unit of Justus Liebig University Giessen.

Duality of Interest. Z.H. is a recipient of a research grant from Agilent Technologies. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. A.M. contributed to the project administration, funding acquisition, investigation, resources, and data curation. A.M.G. and M.S. contributed the software and to

validation and conceptualization. A.M.G., M.S., M.F.H., S.A.W., and Z.H. contributed to the methodology, drafting and reviewing and editing of the manuscript, and supervision. A.M.G. and Z.H. contributed to the formal analysis. A.M.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Prior Presentation. Parts of this study were presented in abstract form at the 10th International Meeting of Pediatric Endocrinology, Washington, DC, 14–17 September 2017.

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