Original Article

Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

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Abstract
Objective: The human serum metabolite profile is reflective of metabolic processes, including pathophysiological changes characteristic of diseases. Therefore, investigation of serum metabolite concentrations in obese children might give new insights into biological mechanisms associated with childhood obesity. ***Methods:*** Serum samples of 80 obese and 40 normal-weight children between 6 and 15 years of age were analyzed using a mass spectrometry-based metabolomics approach targeting 163 metabolites. Metabolite concentrations and metabolite ratios were compared between obese and normal-weight children as well as between children of different pubertal stages. ***Results:*** Metabolite concentration profiles of obese children could be distinguished from those of normal-weight children. After correction for multiple testing, we observed 14 metabolites (glutamine, methionine, proline, nine phospholipids, and two acylcarnitines, $p < 3.8 \times 10^{-4}$) and 69 metabolite ratios ($p < 6.0 \times 10^{-6}$) to be significantly altered in obese children. The identified metabolite markers are indicative of oxidative stress and of changes in sphingomyelin metabolism, in β-oxidation, and in pathways associated with energy expenditure. In contrast, pubertal stage was not associated with

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KARGER

Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

metabolite concentration differences. **Conclusion:** Our study shows that childhood obesity influences the composition of the serum metabolome. If replicated in larger studies, the altered metabolites might be considered as potential biomarkers in the generation of new hypotheses on the biological mechanisms behind obesity.

Introduction

The prevalence of childhood obesity has been increasing dramatically worldwide over the past three decades [1]. The underlying cause is a complex interaction between predisposing genetic factors and the change of environmental factors such as nutritional habits [2, 3]. This trend is of great concern, considering the manifold consequences of obesity on children's health. These include metabolic disturbances such as insulin resistance, dyslipidemia, and hypertension, which in turn promote the development of cardiovascular diseases in childhood as well as in later life [2, 4].

For the development of appropriate treatment strategies, a good understanding of obesity-related pathophysiological mechanisms is inevitable. In recent years, advanced laboratory techniques and data processing tools have been developed, allowing for large-scale, simultaneous investigation of a large number of metabolites in human biofluids or tissues [5–7]. The human serum metabolite profile is reflective of metabolic processes including disease-related changes [8]. Therefore, investigation of serum metabolite concentrations in obese children might give new insights into biological mechanism associated with childhood obesity. Few studies have already examined obesity-associated changes in the serum metabolome in adults [9–12] and adolescents [13].

In the present study, we applied a targeted metabolomics approach to identify serum metabolites associated with childhood obesity. The foremost aims of this study were i) to investigate whether the concentration profile of serum metabolites differed between obese and normal-weight children, ii) to identify potential obesity-associated metabolite biomarkers, and iii) to analyze the association of pubertal stage with the metabolite profile.

Material and Methods

Study Population and Anthropometric Measures

In the years 2008–2009, 80 obese children and adolescents aged 6–15 years from the outpatient clinic for obesity in the Vestische Kinder- und Jugendklinik Datteln, Germany, and 40 normal-weight children with a similar age and sex distribution were examined. All children were born in Germany. Children with syndromal obesity as well as psychiatric or endocrine disorders including type 2 diabetes mellitus were excluded. The Ethics Committee of the University of Witten/Herdecke approved this study. Written informed consent was obtained from all subjects and their parents.

Height was measured to the nearest centimeter using a rigid stadiometer. Undressed weight was measured to the nearest 0.1 kg using a calibrated balance scale. Obesity was defined as BMI above the 97th percentile of German population-specific data [14]. All obese children met obesity criteria of the International Task Force of Obesity, being above the population-, age-, and sex-specific percentile translating to a BMI of 30 kg/m^2 at the age of 18 [15]. BMI percentiles and standard deviation scores (SDS-BMI) were calculated based on Cole's LMS method [16]. Pubertal stage was assessed according to Marshall and Tanner [17, 18] and categorized into three stages based on pubic hair and genital stages: prepubertal = boys/girls with pubic hair stage I and gonadal/breast stage I, pubertal = boys/girls with pubic hair stage ≥ II and gonadal/breast stage ≥ II and late/postpubertal = boys with change of voice and girls with menarche.

Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

Sampling and Metabolite Measurement

Blood samples were taken at 8 a.m. after overnight fasting for at least 10 h. Following coagulation at room temperature, blood samples were centrifuged for 10 min at 8,000 rpm. Aliquoted serum samples were stored at –80 °C and thawed at room temperature for the metabolomics assay.

We used the metabolomics Absolute*IDQ*™ kit p150 (Biocrates Life Sciences AG, Innsbruck, Austria) for the quantification of 163 metabolites, following the instructions described in the manufacturer's manual. Liquid handling of serum samples was performed with a Hamilton Micro Lab Star robot (Hamilton Bonaduz AG, Bonaduz, Switzerland). Samples were analyzed on an API4000 LC/MS/MS System (AB Sciex Deutschland GmbH, Darmstadt, Germany) equipped with an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an electrospray ionization (ESI) source which was used in both positive and negative mode. MS/MS analysis was run in Multiple Reaction Monitoring (MRM) mode. The entire analytical process was controlled by the Analyst 1.4 software and the MetIQ™ software package, the latter being an integral part of the Absolute*IDQ* kit. Metabolite concentrations were determined with the MetIQ software.

Measurements took place in two separate batches. For data quality assessment, 43 of the samples of the first batch were remeasured in the second one. Also, a reference sample was measured five times on each of the two batches. We excluded metabolites from the analysis that failed to meet two or more of the following quality criteria:

i)   The concentration of the metabolite should be above the limit of detection (LOD) specified by the manufacturer in at least 60% of the samples.

ii)   The Pearson's correlation coefficient of the metabolite concentrations in the 43 repeatedly measured samples should be at least 0.5 between the two batches.

iii)   For each batch, the coefficient of variation (CV) for the metabolite concentration in the reference samples should not be higher than 0.2.

In total, 130 metabolites passed the quality control. Most of the 33 excluded metabolites were characterized by concentrations below or marginally above the LOD. As a consequence, measurement stability was assumably affected in these cases. In accordance with the kit instruction all measurements were multiplied by a metabolite- and batch-specific correction factor to further correct for a potential batch effect.

The metabolite spectrum targeted by the used kit is shown in supplementary table 1 (see supplementary material). The 130 metabolites included in our analysis comprised 24 conjugated carnitines (acylcarnitines (Cx:y), hydroxylacylcarnitines (C(OH)x:y) and dicarboxylacylcarnitines (Cx:y-DC), free carnitine (C0), 14 amino acids, hexose (H1), 32 diacyl phosphatidylcholines (PC aa Cx:y), 35 acyl-alkyl phosphatidylcholines (PC ae Cx:y), 9 lysophosphatidylcholines (LPC a Cx:y) as well as 14 sphingomyelins (SM Cx:y) and hydroxysphingomyelins (SM (OH) Cx:y), where Cx:y abbreviates the lipid side chain composition, x and y denoting the number of carbons and double bonds, respectively. As a point to note, the analytical technique applied here is not capable of determining the precise position of the double bonds and – in the case of PCs – the distribution of carbon atoms between the two fatty acid side chains. We report all metabolite concentrations in μmol/l. In the following, we refer to metabolite concentrations simply as metabolites, and to metabolite concentration ratios as metabolite ratios.

Statistical Analysis

All calculations were performed in the statistical environment *R*, version 2.10.1 [19]. Anthropometric characteristics of obese and normal-weight children were compared using Wilcoxon signed-rank and Pearson's chi-square tests.

To examine the degree to which the serum metabolite profiles of obese and normal-weight children and of children of different pubertal stages were separated, Partial Least Squares (PLS) analysis with 10-fold cross-validation was conducted on the scaled and centered matrix of metabolite concentrations using the *R* package *pls* [20]. Data were visualized by plotting the cross-validated scores of the first two components in a scores plot where each point represents an individual serum sample.

To determine whether single metabolite concentrations differed between obese and normal-weight children, we performed logistic regression analyses with weight status (obese vs. normal-weight) as the dependent variable and the natural logarithm of single metabolite concentrations and the covariates age, sex, and pubertal stage as the independent variables. A similar analysis was performed to investigate associations between pubertal stage (prepubertal vs. pubertal/postpubertal) and metabolite concentrations, adjusted for age and sex and for sex only.

Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

Table 1. Anthropometric characteristics of normal-weight and obese children[a]

Parameter	Normal-weight (n = 40)	Obese (n = 80)	p value
Age, years	11.1 ± 2.8	10.9 ± 2.1	0.700
Sex (% male/female)	62.5/37.5	52.5/47.5	0.338
Pubertal stage (% prepubertal/pubertal/postpubertal)	47.5/35.0/17.5	51.3/36.3/12.5	0.706
Weight, kg	36.5 ± 14.2	65.2 ± 17.6	<0.001
BMI, kg/m^2	17.2 ± 2.1	27.7 ± 4.0	<0.001
SDS-BMI[b]	-0.4 ± 0.8	2.4 ± 0.4	<0.001

[a]All values are presented as mean ± SD if not indicated otherwise.
[b]Standard deviation score of BMI.

In a subsequent step, we repeated the analysis for obesity with ratios of all possible pairs of metabolites, allowing for the discovery of differences between obese and normal-weight children that are otherwise masked by a large intra-group variability in the single metabolite concentrations [21, 22]. In addition, changes in metabolite ratios might reflect biological situations such as changes in enzyme activities or imbalances in metabolic pathways. For all regression analyses, we considered an experiment-wise type I error of 0.05 as statistically significant. Correction for multiple testing using the Bonferroni correction resulted in a significance level of $p = 0.05/130 = 3.8 \times 10^{-4}$ and $p = 0.05/8 385 = 6.0 \times 10^{-6}$ for single metabolites and metabolite ratios, respectively. In the case of metabolite ratios, we calculated the p gain, defined as the minimum of the two p values for the single metabolite analyses divided by the p value for the metabolite ratio [21]. The higher the p gain for a metabolite ratio, the more a low p value for the metabolite ratio is due to a change in the ratio rather than to a strong change in one of the single metabolites alone. In this study, we focused on metabolite ratios with a p gain of at least 1.0×10^3.

Results

Characteristics of the Study Population

Anthropometric characteristics of the children are presented in table 1. Obese and normal-weight children did not differ significantly in terms of sex, age, and pubertal stage distribution. By design, body weight, BMI, and SDS-BMI were significantly higher in the group of obese children (p < 0.001).

The Serum Metabolite Profile in Obesity

The associations of obesity and pubertal stage with the metabolite profile were first investigated by means of PLS analysis. We observed a distinct separation of obese and normal-weight children by the first two components, which accounted for 22.1% and 29.7% of the total variance in the metabolite data, respectively (fig. 1). In contrast, we could not observe a separation of children of different pubertal stages.

After correction for multiple testing, concentrations of 14 metabolites differed significantly between obese and normal-weight children (table 2). Concentrations of two acylcarnitines (C12:1 and C16:1) were significantly increased in obese compared to normal-weight children, while concentrations of the remaining 12 metabolites (glutamine, methionine, proline, PC ae C34:1, C34:2, C34:3, C36:2, C36:3 and C38:2, LPC a C18:1, C18:2 and C20:4) were significantly decreased in obese children. Logistic regression results for all 130 metabolites are shown in supplementary table 2 (see supplementary material). Performing a similar analysis for pubertal stage, we did not identify any significant associations with metabolite concentrations, neither with nor without adjustment for age (data not shown).

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Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

Table 2. Metabolites with concentrations differing significantly between normal-weight and obese children

Metabolite	Normal-weight (n = 40)[a]	Obese (n = 80)[a]	Δ Obese – normal-weight[b]	OR (95% CI)[c]	p value
Acylcarnitines					
C12:1	0.10 ± 0.03	0.13 ± 0.04	32.3%	3.62 (2.09, 6.98)	2.6×10^{-5}
C16:1	0.03 ± 0.01	0.04 ± 0.01	20.8%	2.74 (1.64, 4.98)	3.3×10^{-4}
Amino acids					
Glutamine	638.62 ± 109.94	509.43 ± 114.91	–20.2%	0.20 (0.09, 0.38)	7.0×10^{-6}
Methionine	35.09 ± 8.76	27.55 ± 6.81	–21.5%	0.28 (0.15, 0.49)	3.4×10^{-5}
Proline	258.91 ± 91	180.88 ± 63.23	–30.1%	0.27 (0.14, 0.46)	1.3×10^{-5}
Acyl-alkyl phosphatidylcholines					
PC ae C34:1	10.26 ± 3.20	8.26 ± 2.07	–19.5%	0.34 (0.18, 0.59)	3.0×10^{-4}
PC ae C34:2	12.70 ± 4.07	9.32 ± 2.69	–26.6%	0.29 (0.15, 0.50)	4.1×10^{-5}
PC ae C34:3	9.22 ± 3.31	6.95 ± 2.02	–24.6%	0.39 (0.23, 0.62)	2.3×10^{-4}
PC ae C36:2	13.63 ± 3.69	10.25 ± 2.55	–24.8%	0.24 (0.12, 0.43)	1.1×10^{-5}
PC ae C36:3	8.51 ± 2.52	6.61 ± 1.86	–22.2%	0.33 (0.17, 0.56)	2.0×10^{-4}
PC ae C38:2	1.96 ± 0.51	1.55 ± 0.48	–21.0%	0.36 (0.20, 0.59)	1.8×10^{-4}
Lysophosphatidylcholines					
LPC a C18:1	17.13 ± 4.59	12.73 ± 3.96	–25.7%	0.29 (0.15, 0.49)	1.8×10^{-5}
LPC a C18:2	36.37 ± 11.44	18.09 ± 5.07	–50.3%	0.05 (0.01, 0.13)	5.7×10^{-8}
LPC a C20:4	5.82 ± 1.77	4.4 ± 1.21	–24.4%	0.36 (0.20, 0.58)	1.2×10^{-4}

Cx:y = acyl-group with chain length x and y double bonds; PC ae = acyl-alkyl phosphatidylcholine; LPC a = lysophosphatidylcholine with acyl chain.

[a]All values are presented as mean ± SD of serum metabolite concentration in μmol/l.

[b]Percentage (%) difference between values for obese and normal-weight children.

[c]OR of logistic regression analysis with respective 95 % CI; age, sex and pubertal stage were included as covariates; only metabolites whose concentrations differed significantly after adjustment for multiple testing ($p \leq 3.8 \times 10^{-4}$) were included.
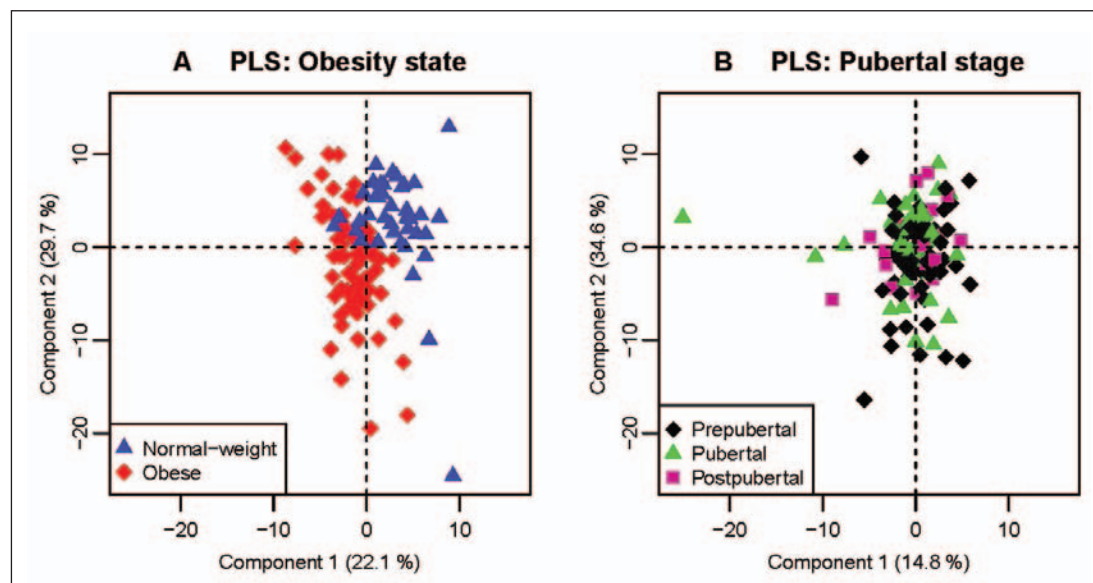

Fig. 1. PLS analysis results. Cross-validated scores plot of the first two components. Outcome was A, obesity, and B, pubertal stage. Percentages in parentheses represent the % variance in the metabolite data accounted for by the respective component.

KARGER

Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

Table 3. Selection of metabolite concentration ratios differing significantly between normal-weight and obese children

Metabolite ratio	OR (95 % CI)[a]	p value	p gain
Saturated / unsaturated lysophosphatidylcholine ratios			
LPC a C16:0 / LPC a C20:3	6.0 (3.3, 12.2)	6.3×10^{-8}	1.3×10^{5}
LPC a C18:0 / LPC a C20:3	9.6 (4.5, 24.6)	1.3×10^{-7}	6.1×10^{4}
LPC a C16:0 / LPC a C20:4	6.5 (3.5, 13.8)	1.0×10^{-7}	1.1×10^{3}
LPC a C18:0 / LPC a C20:4	8.4 (4.1, 20.1)	1.1×10^{-7}	1.1×10^{3}
Lysophosphatidylcholine / phosphatidylcholine ratios			
LPC a C18:0 / PC aa C36:2	8.3 (4.2, 19.6)	6.5×10^{-8}	1.2×10^{5}
LPC a C18:0 / PC aa C36:1	5.7 (3.1, 12.0)	4.4×10^{-7}	2.0×10^{4}
LPC a C16:0 / PC aa C32:0	5.3 (2.9, 10.7)	3.3×10^{-7}	2.5×10^{3}
LPC a C16:0 / PC aa C36:2	3.8 (2.2, 6.9)	3.3×10^{-6}	2.3×10^{3}
Sphingomyelin / phosphatidylcholine ratios			
SM (OH) C22:1 / PC ae C38:4	5.1 (2.9, 10.3)	4.8×10^{-7}	1.1×10^{4}
SM C24:0 / PC aa C36:2	4.2 (2.4, 7.8)	1.3×10^{-6}	6.0×10^{3}
SM C16:1 / PC ae C32:1	3.7 (2.2, 6.7)	3.2×10^{-6}	4.9×10^{3}
SM C18:1 / PC ae C38:4	5.8 (3.0, 13.0)	1.6×10^{-6}	3.2×10^{3}

Cx:y = acyl-group with chain length x and y double bonds; LPC a = lysophosphatidylcholine with acyl chain; PC aa = diacyl phosphatidylcholine; PC ae = acyl-alkyl phosphatidylcholine; SM = sphingomyelin; SM (OH) = hydroxysphingomyelin.

[a]OR of logistic regression analysis with respective 95 % CI; age, sex and pubertal stage were included as covariates; only metabolites pairs whose concentration ratios differed significantly after adjustment for multiple testing (p ≤ 6.0×10^{-6}) and had a p-gain ≥ 1.0×10^{3} were included in the table.

Metabolite Ratios

Comparing metabolite ratios between obese and normal-weight children in logistic regression analyses revealed significant differences in 69 ratios after correction for multiple testing (p ≤ 6.0×10^{-6}) and applying a p gain cutoff at ≥ 1.0×10^{3} (supplementary table 3; see supplementary material). Results for selected representative ratios are shown in table 3. Ratios between saturated and unsaturated LPCs, between saturated LPCs and PCs and between SMs and PCs were increased in obese compared to normal-weight children. As an example, the odds ratio for being in the obese group of the LPC a C18:0 / LPC a C20:4 ratio was 8.4 (4.1, 20.1) with a p value of 1.1×10^{-7} and a p gain of 1.1×10^{3}.

Discussion

This study compared serum metabolite concentrations of obese and normal-weight children in a systematic metabolomics approach. 14 metabolites and 69 metabolite ratios were significantly different in obese compared to normal-weight children.

Metabolite Changes Associated with Obesity

The observed higher concentrations of the acylcarnitines C12:1 and C16:1 in obese compared to normal-weight children are consistent with findings in adults. In a study comparing acylcarnitine species of 12 lean and 14 obese adults, serum concentrations of several medium- to long-chain acylcarnitines were increased in the obese compared to the non-obese participants [9]. Acylcarnitines are formed from acyl-coenzyme A (CoA) by the outer mitochondrial enzyme carnitine palmitoyltransferase 1 (CPT1) as a prerequisite to

Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

acyl transport into mitochondria where β-oxidation takes place [23]. Elevated free fatty acid (FFA) concentrations, as have been observed in the serum of obese children [24], may exceed the capacity of β-oxidation and downstream pathways such as the mitochondrial electron transport chain, the activities of which have been shown to be reduced in obese subjects [25, 26]. As a consequence, incompletely oxidized fatty acid products such as acyl-carnitines might accumulate, exit the cell, and are found increased in the blood [9, 27]. A recent study in obese and normal-weight adolescents reported unchanged longer-chain acylcarnitines and decreased concentrations of medium- to short-chain acylcarnitine species [13]. They explain the inconsistency with findings in adults with the existence of adaptive mitochondrial mechanisms in youth. Our results do not support such a hypothesis.

We found significantly reduced glutamine, methionine and proline concentrations in obese compared to normal-weight children. Thus, we replicated the findings of an earlier study reporting reduced levels of methionine and proline in extremely obese children [28]. To our knowledge, no study on obese children has shown a reduction of glutamine. However, findings of other studies point towards an obesity-associated activation of the hexosamine pathway [29–31]. Glucosamine and hexosamines are known to contribute to the development of insulin resistance [29, 32]. In addition, activation of the hexosamine pathway reduces energy expenditure [30].

Our study confirmed that acyl-alkyl PC concentrations are significantly lower in the obese state [10]. A subclass of acyl-alkyl PCs are the so-called plasmalogens which have anti-oxidant properties [33]. The observed decrease in acyl-alkyl PCs in obese children may reflect a consumption of plasmalogens during oxidative stress, which is known to play a role in childhood obesity [34].

We observed significantly increased ratios between saturated LPCs (C16:0, C18:0) and PCs in obese children. Saturated LPC concentrations were also marginally increased. LPCs are derived from PCs during LDL oxidation via either the lecithin-cholesterol acyltransferase (LCAT) or the lipoprotein-associated phospholipase A2 (LpPLA2) pathway [35]. In fact, LpPLA2 activity has been reported to be increased in obese children [36]. As a major component of oxidized LDL, saturated LPCs exert pro-atherogenic and pro-inflammatory effects and impair insulin signaling [35, 37].

Obese children demonstrated significantly decreased concentrations of the unsaturated LPCs C18:1, C18:2 and C20:4. Furthermore, ratios between saturated and unsaturated LPCs were significantly increased. Our findings are in line with a recent metabolomics study in overweight/obese and normal-weight men showing significantly reduced concentrations of LPC C18:1 and increased concentrations of LPC C18:0 along with a reduced ratio of dietary polyunsaturated to saturated fatty acid intake [12]. The arising assumption that fatty acid composition of serum (lyso-)phospholipids partly reflects an individual's medium-term dietary fatty acid intake is supported by the reported correlations between dietary and plasma phospholipid fatty acids [38, 39]. So far, there is little evidence for a role of specific dietary characteristics, including fatty acid balance, in childhood obesity risk [40].

Our observation that SM/PC ratios were significantly increased in obese children may reflect an increased rate of the sphingomyelin synthase (SMS) reaction [41]. We did not observe a significant increase in single SM concentrations. This may depict the equilibrium of SM synthesis and metabolism. Synthesized SMs are immediately subject to hydrolysis by sphingomyelinase (SMase), yielding ceramides, which have been shown to play a role in the development of insulin resistance and cardiovascular disorders [42, 43].

Pubertal Stage Subanalysis

In our study, pubertal stage was not associated with serum metabolite concentrations. There are, to our knowledge, no studies published which have investigated the association

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Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

between metabolites and pubertal stage in a similar way, making a direct comparison to our results difficult. Both cross-sectional and longitudinal studies of pubertal stage indicate a decrease in insulin sensitivity during pubertal development, which is accompanied by increased fatty acid oxidation [51, 52]. Further, decreased proteolysis and protein oxidation during puberty as compared to prepuberty was reported [53]. The fact that we did not observe pubertal stage-related changes in serum acylcarnitine or amino acid concentrations might indicate that these changes occur within a physiological and compensatable range, or are not captured by the metabolite panel targeted in this study.

Comparison with Studies in Adults

Data from adults and children are difficult to compare, since growth might have an impact on metabolism. Still, it is interesting to see that there is consistency as well as inconsistency concerning metabolomic changes related to obesity. Our findings of obesity-associated changes in acylcarnitine, acyl-alkyl PC, LPC and SM concentrations are largely in concordance with earlier studies in adults [9, 10, 12, 44, 45]. In contrast, the results concerning amino acid concentrations in our study of obese children deviate from findings in adults. For instance, branched-chain amino acids (BCAAs) and related metabolites have been consistently found increased in obese compared to lean adults [11, 12, 46–48], whereas we as well as Mihalik et al. [13] did not observe the same in children. In adults, one of the proposed mechanisms for the increased BCAA levels in obese and insulin-resistant subjects is reduced adipose tissue BCAA oxidation [49, 50]. Arguing in line with Mihalik et al. [13], children may exhibit early adaptive mechanisms preserving a normal BCAA metabolism in the presence of obesity and disturbed insulin sensitivity.

Strengths and Limitations

Our study has some potential limitations. Its sample size was only moderate so that the study was underpowered to detect smaller effects. Furthermore, as a cross-sectional study, it does not allow us to conclude causality from the observed differences between the weight groups. In this regard, longitudinal and interventional studies might provide further insights. Moreover, we lack information concerning diet and physical activity of the children. However, in contrast to using questionnaires, which have a very high measurement error, serum metabolite concentrations are possibly more reliable surrogates for dietary intake.

A strength of our study is that we applied a systematic metabolomics approach to compare the serum metabolome of obese children to that of normal-weight children. Furthermore, the study of metabolomics in children might lead to larger and in particular more 'true' effects than in adults, as children usually do not suffer from other diseases, take medications or smoke – factors that could influence the metabolite concentration profile [5].

In conclusion, our data provides evidence for obesity-related changes of the serum metabolite profile in children. If replicated in larger studies, the identified metabolites and metabolite ratios might be considered as potential biomarkers in the generation of new hypotheses on obesity-related pathophysiological processes.

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Disclosure Statement

All authors declare that there is no conflict of interest.

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Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

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Wahl et al.: Childhood Obesity Is Associated with Changes in the Serum Metabolite Profile

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