

## Research Article

# Tyrosine Is Associated with Insulin Resistance in Longitudinal Metabolomic Profiling of Obese Children

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In obese children, hyperinsulinaemia induces adverse metabolic consequences related to the risk of cardiovascular and other disorders. Branched-chain amino acids (BCAA) and acylcarnitines (Carn), involved in amino acid (AA) degradation, were linked to obesity-associated insulin resistance, but these associations yet have not been studied longitudinally in obese children. We studied 80 obese children before and after a one-year lifestyle intervention programme inducing substantial weight loss >0.5 BMI standard deviation scores in 40 children and no weight loss in another 40 children. At baseline and after the 1-year intervention, we assessed insulin resistance (HOMA index), fasting glucose, HbA1c, 2 h glucose in an oral glucose tolerance test, AA, and Carn. BMI adjusted metabolite levels were associated with clinical markers at baseline and after intervention, and changes with the intervention period were evaluated. Only tyrosine was significantly associated with HOMA ( $p < 0.05$ ) at baseline and end and with change during the intervention ( $p < 0.05$ ). In contrast, ratios depicting BCAA metabolism were negatively associated with HOMA at baseline ( $p < 0.05$ ), but not in the longitudinal profiling. Stratified analysis revealed that the children with substantial weight loss drove this association. We conclude that tyrosine alterations in association with insulin resistance precede alteration in BCAA metabolism. This trial is registered with ClinicalTrials.gov Identifier NCT00435734.

## 1. Introduction

Obesity in childhood is strongly associated with cardiovascular risk factors (CRFs) including dyslipidemia, hyperglycaemia, and hypertension [1]. In obese children hyperinsulinaemia and other CRFs are far more commonly found than in normal weight children and adolescents [2–4]. Most metabolic consequences appear to be mediated through insulin resistance (IR) [5]; therefore improving insulin sensitivity seems even more important than weight loss [6]. “Omics” platforms, such as proteomics, transcriptomics, epigenomics, and metabolomics, provide insights into molecular changes and allow assessing biochemical alterations in the development of obesity and IR [7, 8]. While new targets or potential biomarkers are identified in humans with these approaches [9, 10], the role of known metabolites still needs to be

evaluated. Particularly, the influence of amino acid (AA) metabolism on the onset of IR still needs clarification. Two recent studies have reported on the untargeted metabolomic approach to study the relation of metabolites to IR in older adults [11] and children [12]. Untargeted metabolomics involves an unbiased screening of all metabolites present in a specimen regardless of chemical class. Targeted metabolomic techniques facilitate the profiling of specific metabolites of interest in a given population, to aid in-depth analysis of metabolic processes in the context of preformed findings. Thus, clinical targeted metabolomics platforms are suitable tools to reveal associations between AA and IR. Different studies depicted associations between IR or type 2 diabetes mellitus (T2DM) and branched-chain amino acids (BCAA), aromatic amino acids (AAA), sulphur containing AA, and other AAs as well as short-chain acylcarnitines (Carn)

involved in AA metabolism in adults [13–23]. BCAA were found to be positively associated with homeostasis model assessment (HOMA), an IR index, in nonobese Chinese men [15] and young Finn adults [16]. Mohorko et al. recently reported elevated serum levels of cysteine (Cys) and tyrosine (Tyr) as early biomarkers for metabolic syndrome in young adults [14]. Newgard et al. showed that BCAA and short-chain Carn derived from BCAA contribute to the development of obesity-associated IR [13]. However, the majority of these studies describe relations of clinical markers to metabolites in cross-sectional settings. Furthermore, such associations are susceptible to confounders, like dietary protein intake that was shown to be higher in obese subjects than in normal weight subjects [15]. A few studies describe the prediction potential of BCAA and AAA for the onset of IR [16, 18, 24]. Although metabolomic analyses in children yield the potential to investigate the early onset of metabolic disease, studies on obese children are lacking. Recently, Newbern et al. reported an association of HOMA with a metabolic signature containing BCAA, uric acid, and long-chain Carn in adolescent boys in a cross-sectional study [25]. A combination of BCAA and AAA was associated with HOMA in obese Hispanic children [26], but only BCAA in Korean children [27]. BCAA pattern and androgen hormone pattern were associated with childhood adiposity and cardiometabolic risk, like HOMA, in another recently published cross-sectional study [12]. Longitudinal studies are necessary to explore stronger association between IR and metabolic alterations. To our knowledge, two longitudinal studies in children have been published so far, showing an association of baseline BCAA with HOMA in healthy American children [28] and in Korean children [27].

We embarked on a longitudinal study on obese children participating in a lifestyle intervention for inducing weight loss to explore the relationship between changes in AA metabolism and IR in the fasting state and after an oral glucose tolerance test (oGTT) in obese European children. Additionally, we analysed the obesity-independent associations of changes during the intervention period in makers of IR, hemoglobin A1c (HbA1c), 2 h glucose in oGTT, and changes of AA and Carn.

## 2. Methods

**2.1. Study.** Written informed consent was obtained from all parents of the participants prior to inclusion in the study. The study has been performed according to the Declaration of Helsinki. The local ethics committee of the University of Witten/Herdecke in Germany approved the study (ClinicalTrials.gov Identifier NCT00435734). We studied 80 obese Caucasian children participating in the one-year lifestyle intervention “Obeldicks,” which has been described in detail elsewhere [29]. Briefly, this outpatient intervention program is based on promoting regular physical activity, nutrition education, and behavior therapy including individual psychological care of the children and their families. The one-year training program was divided into three phases. In the first one, intensive phase (3 months), the children took part in the nutritional course and in the eating-behavior course

in six group-sessions, each lasting for 1.5 hours. Parents were invited to attend six evening classes. In the establishing phase (6 months), individual psychological family therapy was provided (30 minutes/month). In the last phase of the program (accompanying the families back to their everyday lives) (3 months), further individual care was possible, if and when necessary. None of the children in the current study were smokers, took any drugs, or suffered from endocrine disorders or syndromal obesity such as Prader Willi syndrome [30]. Also MC4 receptor mutation was excluded. The children studied were selected at random from the Obeldicks cohort reported previously [30] choosing 40 obese children with substantial weight loss and 40 obese children without weight loss of similar age, gender, pubertal stage, and degree of overweight. We included only children who participated in oGTT both at baseline and after one year. Substantial reduction of overweight was defined by a decrease in standard deviation score of body mass index (BMI-SDS)  $\geq 0.5$  based on previous studies [31], whereas no reduction of overweight was defined by a decrease in BMI-SDS  $< 0.15$ . The metabolomic profile of these children in respect to obesity status and weight loss was previously reported [32].

**2.2. Measurements and Sampling.** Height was measured to the nearest millimeter 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 LMS method, which normalized the BMI skewed distribution and expressed BMI as a standard deviation score (BMI-SDS) [33]. Reference data for German children were used [34]. Waist circumference was measured halfway between lower rib and iliac crest.

For longitudinal analysis, blood samples were collected in the fasting state before the intervention and after 1 year. Furthermore, oGTT were performed according to current guidelines [35]. The glucose load was 1.75 g/kg with a maximum of 75 g. Blood samples were taken at 8 a.m. after overnight fasting for at least 10 hours. Following coagulation at room temperature, blood samples were centrifuged for 10 min at 8000 rpm at room temperature and aliquoted. Glucose (Boehringer, Mannheim, Germany), HbA1c (Germany Tinaquant Hemoglobin A1c Gen), and insulin (Abbott, Wiesbaden, Germany) were measured in serum by using commercially available test kits directly. Intra-assay and interassay CVs of glucose, HbA1c, and insulin were less than 5%. HOMA was used to detect the degree of IR [36]. Furthermore, serum samples were stored at  $-81^\circ\text{C}$  and thawed at room temperature for the metabolomics assay only once.

**2.3. Biochemical Measures.** Metabolites were qualified and quantified with the Absolute IDQ p 150 kit (Biocrates Life Sciences AG, Innsbruck, Austria) as described previously [32]. Briefly, 10  $\mu\text{L}$  of blood serum was analysed with a flow injection tandem mass spectrometer (FIA-MS/MS). An Agilent 1200 SL series high-performance liquid chromatography system (Agilent, Waldbronn, Germany) was coupled to a hybrid quadrupole mass spectrometer (QTRAP 4000, AB Sciex, Darmstadt, Germany). MS/MS analysis was run in

TABLE 1: Characteristics of participating children at start and end point of the 1-year intervention period. Characteristics are shown for all obese children ( $n = 80$ ), children with substantial weight loss (WL,  $n = 40$ ), and children without substantial weight loss (nWL,  $n = 40$ ) as mean  $\pm$  SD unless stated otherwise.

Parameter	All start (%)	All end (%)	WL start (%)	WL end (%)	nWL start (%)	nWL end (%)
Sex, male	36 (45%)		18 (45%)		18 (45%)	
Age (years)	11.5 $\pm$ 2.42	12.5 $\pm$ 2.42	10.6 $\pm$ 2.54	11.6 $\pm$ 2.54	12.4 $\pm$ 1.9	13.4 $\pm$ 1.9
Prepubertal	34 (42%)	26 (32%)	23 (58%)	18 (45%)	11 (28%)	8 (20%)
Early pubertal	42 (52%)	44 (55%)	17 (42%)	22 (55%)	25 (62%)	22 (55%)
Postpubertal	4 (5%)	10 (12%)	—	—	4 (10%)	10 (25%)
BMI-SDS	2.4 $\pm$ 0.45	2.1 $\pm$ 0.63*	2.4 $\pm$ 0.44	1.7 $\pm$ 0.58*	2.4 $\pm$ 0.46	2.4 $\pm$ 0.47*
Waist circumference (cm)	91.7 $\pm$ 14	89.3 $\pm$ 13.89*	87 $\pm$ 13.59	81 $\pm$ 10.99*	96.5 $\pm$ 12.88	97.3 $\pm$ 11.55
Insulin (mU/L)	19.9 $\pm$ 15.01	17 $\pm$ 12.39	18 $\pm$ 12.01	9.3 $\pm$ 3.87*	21.9 $\pm$ 17.44	25.1 $\pm$ 13.14*
Fasting glucose (mg/dL)	86.3 $\pm$ 7.38	87.1 $\pm$ 6.39	84.8 $\pm$ 7.05	85.5 $\pm$ 5.76	87.8 $\pm$ 7.47	88.8 $\pm$ 6.65
2-hour oGTT glucose (mg/dL)	132.7 $\pm$ 25.02	113.9 $\pm$ 23.94*	133.9 $\pm$ 24.26	98.9 $\pm$ 10.42*	131.4 $\pm$ 25.99	128.9 $\pm$ 24.28
HbA1C (mmol/mol Hb)	373.43 $\pm$ 32.92	372.81 $\pm$ 37.98	364.16 $\pm$ 30.12	363.77 $\pm$ 30.81	382.95 $\pm$ 33.32	382.61 $\pm$ 42.78
HOMA	4.29 $\pm$ 3.1	3.79 $\pm$ 3.2	4.01 $\pm$ 3	1.96 $\pm$ 0.81*	4.58 $\pm$ 3.21	5.67 $\pm$ 3.64*

\*Significant different means between start and end point ( $p < 0.05$ , paired Wilcoxon rank sum test).

Multiple Reaction Monitoring mode with electrospray ionization used in both positive and negative modes. Data acquisition on the mass spectrometer was controlled by Analyst 1.5 software (AB Sciex, Darmstadt, Germany). For raw data processing, peak integration, isotope correction, calibration, and quality control, the Met IQ software package (Biocrates Life Sciences AG, Innsbruck, Austria) was used, which is an integral part of the Absolute IDQ kit quantifying a total of 163 metabolites. Middle- and long-chain Carn, sphingomyelins (SM), acyl-linked phosphatidylcholines, ether-linked phosphatidylcholines, and lysophosphatidylcholines were not used for the data analysis of this work, since the presented study focused on alterations in AA metabolism with respect to IR. For the presented work, we analyzed 14 short-chain Carn (Cx:y, hydroxyl acylcarnitines Cx:y-OH, oxoacylcarnitines Cx:y-oxo, and dicarboxylacylcarnitines Cx:y-DC), free carnitine (Carn C0), and 14 AA. Cx:y abbreviates the lipid side chain composition,  $x$  and  $y$  denoting the number of carbons and double bonds, respectively. The sum of leucine (Leu) and isoleucine (Ile) is expressed as xLeu. Samples were integrated with the Met IQ software by automated calculation of metabolite concentrations. For the data analysis performed here, only short-chain Carn, Carn C0, and AA are used. The sum of xLeu and valine (Val) is expressed as BCAA sum. The sum of phenylalanine (Phe), tryptophan (Trp), and Tyr is expressed as AAA sum. We report all metabolite concentrations in  $\mu\text{mol/L}$ . In addition to the 29 metabolite concentrations and two sum parameters, eleven metabolite ratios were calculated resulting in a total of 42 metabolites and metabolite ratios.

**2.4. Statistics.** All statistical analyses were performed using the statistical software R (3.0.2) [37]. In a first step, we graphically screened for outliers and normality. An absolute metabolite concentration that lay greater than 1 standard deviation (SD) away from its nearest neighbor was considered to be an outlier and this measurement was excluded from the analysis. Principal component analysis score plots were used

as a complementary tool to ensure that no outliers remained undetected.

Differences in clinical parameters between baseline and follow-up were calculated using the paired Wilcoxon rank test. Associations between markers for insulin and glucohomeostasis were quantified using Spearman rank correlation coefficients.

The changes in the clinical markers, metabolite concentrations, and metabolite ratios over the one-year intervention are expressed as the relative difference of baseline and follow-up measurements (with the baseline values being the reference). For each time point (baseline and follow-up) as well as for the relative change, we calculated the following model to assess the association between the metabolites and the clinical parameters: (1) firstly, in order to account for the effect of obesity status on the metabolite level, we fitted age and sex adjusted robust regression models of the BMI on the metabolite using the M-estimator with Huber bisquare weighting (R package MASS); (2) subsequently, we regressed the obtained metabolite residuals on markers for IR with robust regression models using the M-estimator with Huber bisquare weighting (R package MASS).  $p$  values and estimates are taken as proxies for the strengths and directions of the associations. Results of selected clinical outcomes are represented graphically in Manhattan plots, where the  $\log_{10}(p)$  values are plotted and the sign is used to indicate the direction of the relationship, as assessed by the robust regression model. Due to the small sample size and in order not to veil differences in  $p$  values, we will report the raw (unadjusted)  $p$  values. The significance level was thus set at  $p < 0.05$ . Bonferroni corrected  $p$  values can be obtained by multiplying the reported  $p$  values with the factor 42 (number of analytes tested). The Bonferroni corrected significance level is 0.0012.

### 3. Results

**3.1. Population Characteristics.** Characteristics of participating children are presented in Table 1. In all obese children,



TABLE 3: Estimates and  $p$  values ( $p$ ) of changes in metabolite concentrations which are significantly associated with changes in HOMA in at least one (sub)group (All, WL, and nWL). Change is defined as the relative change over the one-year intervention. Estimates are given with 95% confidence interval (CI). Estimates, confidence intervals, and  $p$  values were calculated with robust regression models. WL, children with substantial weight loss; nWL, children without substantial weight loss; AAA, aromatic amino acids; Carn, acylcarnitine; Pro, proline; Trp, tryptophan; Tyr, tyrosine; Val, valine, xLeu, sum of leucine and isoleucine.

Analyte	All ( $n = 80$ )		WL ( $n = 40$ )		nWL ( $n = 40$ )	
	Estimate [95% CI]	$p$	Estimate [95% CI]	$p$	Estimate [95% CI]	$p$
AAA sum	0.79 [-0.04; 1.60]	0.059	1.04 [0.29; 1.80]	0.009	0.27 [-1.40; 1.90]	0.742
Carn C0	1.10 [0.29; 1.90]	0.008	1.71 [0.88; 2.50]	<0.001	0.24 [-1.30; 1.70]	0.751
Carn C3	0.34 [-0.16; 0.83]	0.174	0.49 [0.03; 0.96]	0.036	0.24 [-0.72; 1.20]	0.615
Carn C6:1-DC	-0.33 [-0.59; -0.06]	0.015	-0.29 [-0.47; -0.10]	0.003	-0.37 [-1.10; 0.38]	0.342
Carn C6-oxo	-0.24 [-0.43; -0.05]	0.014	-0.21 [-0.35; -0.08]	0.003	-0.21 [-0.73; 0.31]	0.438
Pro	0.81 [0.19; 1.40]	0.011	0.72 [0.11; 1.30]	0.023	0.88 [-0.08; 1.80]	0.067
Ratio of Carn C4/Carn C5-oxo	0.23 [-0.09; 0.55]	0.177	0.48 [0.18; 0.77]	0.003	-0.21 [-0.86; 0.45]	0.530
Ratio of Carn C5/Carn C6-oxo	0.24 [0.07; 0.41]	0.007	0.22 [0.08; 0.35]	0.002	0.29 [0.02; 0.57]	0.041
Ratio of Carn C6:1-DC/Carn C5:1	-0.27 [-0.54; 0.01]	0.055	-0.22 [-0.41; -0.03]	0.024	-0.39 [-1.10; 0.37]	0.321
Ratio of Carn C6-oxo/xLeu	-0.19 [-0.34; -0.03]	0.016	-0.15 [-0.25; -0.04]	0.007	-0.24 [-0.84; 0.37]	0.442
Trp	0.93 [-0.02; 1.90]	0.056	1.13 [0.14; 2.10]	0.027	0.44 [-1.30; 2.20]	0.613
Tyr	0.79 [0.17; 1.40]	0.015	1.09 [0.51; 1.70]	0.001	0.28 [-0.99; 1.50]	0.651
Val	0.65 [-0.11; 1.40]	0.090	0.73 [0.07; 1.40]	0.033	0.48 [-1.10; 2.10]	0.544

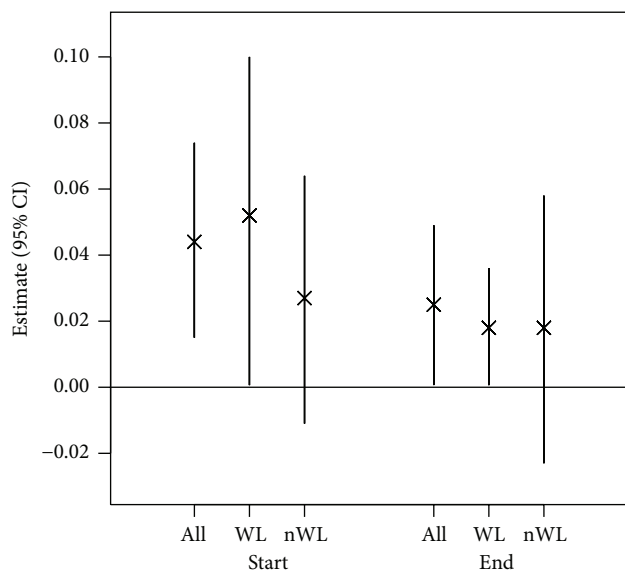


FIGURE 2: Estimates (95% CI) for associations of tyrosine with HOMA. Associations were calculated at baseline (Start) and after the intervention (End). Associations were calculated for all obese children ( $n = 80$ ), children with substantial weight loss (WL,  $n = 40$ ), and children without substantial weight loss (nWL,  $n = 40$ ). 95% CI: 95% confidence interval.

negative), serine (Ser,  $p = 0.043$ , negative), and Carn C4 ( $p = 0.049$ , positive) were associated with 2-hour oGTT glucose in children with substantial weight loss, while children without substantial weight loss showed only positive associations between arginine (Arg) and 2-hour oGTT glucose at baseline. After the intervention, 2-hour oGTT glucose was associated negatively with Ser ( $p = 0.048$ ) and Orn ( $p = 0.039$ ) in children with substantial weight loss and with glutamine

(Gln,  $p = 0.011$ ) and Carn C3-OH ( $p = 0.012$ ) in children without substantial weight loss. Interestingly, changes of 2-hour oGTT glucose during the one-year intervention period were not significantly associated with changes of any of the measured metabolites, ratios, or sums in any of the groups.

#### 4. Discussion

To our knowledge, this is the first longitudinal study analysing the relationships between metabolites and markers of glucose metabolism in obese children. Tyr is the only metabolite which was significantly associated with HOMA at baseline and after intervention. Changes of Tyr over time were also positively associated with changes of HOMA in our obesity-independent model. Thus, Tyr, rather than BCAA, seems to be associated with IR. This is in accordance with a recent study where Tyr was identified as the most important metabolite in a random forest analysis in obese children [26]. In the same study, a combination of BCAA and AAA was most strongly related to HOMA. Furthermore, Tyr was found to be a strong predictor for diabetes in South Asian men [21]. Tyr is biosynthesised endogenously by hydroxylation of Phe by phenylalanine hydroxylase in mammals [38]. Since Phe was not associated with HOMA in any of the groups at any time point, we assume that there is no confounding dietary effect on Tyr levels. Tyr stimulates insulin secretion, but other AAs are more effective in stimulating insulin release [39]. In contrast, Michaliszyn et al. showed a positive association of  $\beta$ -cell function and all AAs except for Tyr and citrulline in adolescents [40]. The same group reported lower AA plasma levels, except for Tyr, in diabetic adolescents [41], which is in contrast to the role of elevated AA in IR [42]. However, the effect on insulin secretion should not be driving the relation between Tyr and HOMA. An influence on Tyr metabolism appears more likely. Insulin is known to increase

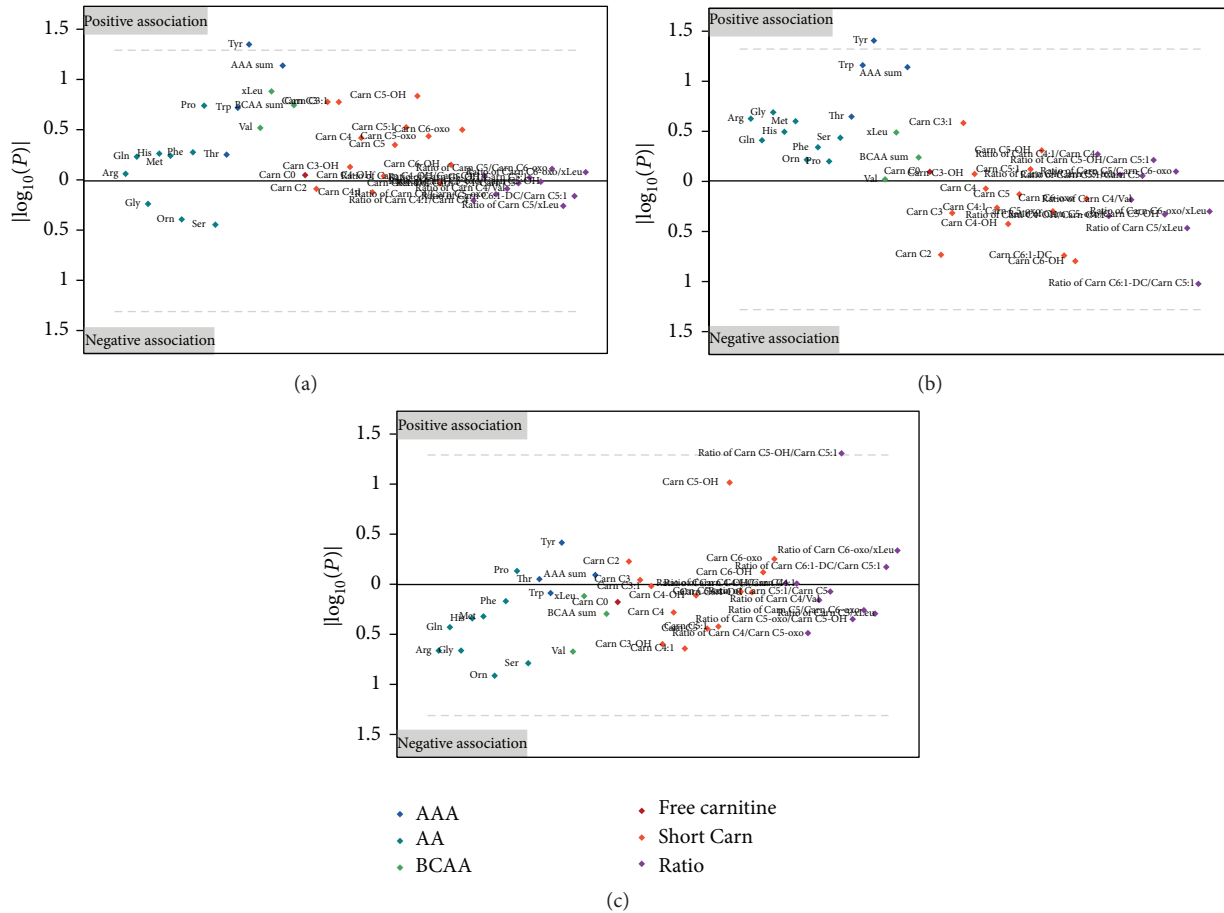


FIGURE 3: Manhattan plot for associations of amino acids (AA) and acylcarnitines (Carn) with HOMA after the one-year lifestyle intervention for all obese children (a), children with substantial weight loss (b), and children without substantial weight loss (c). Plotted are the  $\log_{10}(p)$  values and the sign is used to indicate the direction of the relationship, as assessed by the robust regression model. The area below or above the dashed lines contains metabolites that are significantly related to HOMA ( $p$  values  $< 0.05$ ). AAA, aromatic amino acids; BCAA, branched-chain amino acids.

tyrosine aminotransferase (TAT) activity in rat liver [43], probably by selectively slowing down the rate of degradation of TAT [44]. TAT catalyses the transamination of Tyr to p-hydroxyphenylpyruvate. This should result in lower Tyr levels. A possible inhibition of TAT may occur due to higher Cys levels. Cys is an inhibitor of TAT [19, 45]. In a recent study, only Cys and Tyr were found to be increased in nonobese adults who had one symptom of the metabolic syndrome [14]. With further progression of the metabolic syndrome, BCAA and Phe were enhanced in subjects with two or more symptoms of the metabolic syndrome. It seems that alterations in Cys and Tyr metabolism precede changes in BCAA metabolism. Thus, Tyr is a potential early marker for the onset of IR. Higher insulin levels in the IR state may still cover demands to ensure adequate glucose metabolism, but Tyr may be affected and Tyr may present an early biomarker for the onset of IR in obese children. This predictive value of Tyr was shown in previous studies in adults, along with BCAA in young adults [16, 18]. In the presented study, we could not investigate Tyr as predictor for later IR, since the “Obeldicks” study has an interventional design not a prospective one.

Contrarily, Lee et al. found BCAA, and not AAA, as predictive marker for IR in Korean children [27]. Thus, further prospective, longitudinal studies are required to unravel associations between AA and IR with respect to sex and ethnicity.

Furthermore, Tyr can affect BCAA levels, since BCAA and AA compete for the same neutral AA transporter for cellular uptake [46]. Thus, prolonged elevated Tyr levels may also result in elevated BCAA level. Many studies found altered levels of BCAA when studying obesity in adults [9], but also when looking at IR and T2DM in adults [13, 15–18, 42]. Similar results were found in a few cross-sectional studies in children [12, 25]. We found no association between HOMA and BCAA, neither at baseline nor after the intervention. A previous cross-sectional metabolomic analysis of the presented population did not find different BCAA levels between obese and normal weight children [47]. Levels of BCAA, being essential AAs, are mainly defined by the diet. Thus, higher BCAA levels later in life may result from competition for the neutral AAs transport [46], higher protein intake, and/or disturbed BCAA clearance [48]. During BCAA degradation, Val, Leu, and Ile are first degraded to the  $\alpha$ -keto acids

C5-oxo and C6-oxo by the branched-chain amino transferase (BCAT). These keto acids are subsequently reduced by branched-chain  $\alpha$ -keto acid dehydrogenase (BCKDH) in the rate limiting step [49]. To identify alterations of BCAA metabolism in obesity and IR, we calculated the ratios of the different steps in the BCAA degradation pathway. We found that the ratio of C6-oxo to C5 was positively associated with HOMA at baseline and in the change during the intervention period in all three groups. The ratio of C5-oxo to C4 was positively associated with HOMA only in children with substantial weight loss only. Both ratios are markers for the second, rate limiting degradation step of BCAA which is regulated by BCAA itself to keep BCAA concentrations at a constant level. Thus, this pathway may have been upregulated in our study resulting in BCAA levels not associated with HOMA. In contrast, all other ratios showed no significant association with HOMA or were negatively associated with HOMA, particularly the first step of xLeu degradation to methylketopentanoate (C6-oxo) by BCAT. It was shown recently that IR subjects have a significant reduction in BCAT expression and other enzymes involved in BCAA metabolism in the adipose tissue compared to non-IR subjects [50]. Additionally, BCAT2 (mitochondrial) was significantly downregulated, whereas BCAT1 (cytosolic) was significantly upregulated in the adipose tissue of obese subjects [51]. Other mitochondrial genes of BCAA metabolism were also downregulated in adipose tissue, but not in liver or muscle tissue. Our study showed that the reduction of BCAA degradation seems to precede elevated BCAA levels in IR state or obesity in children, since no elevated BCAA were found but alterations in BCAA metabolite ratios. Thus, insulin negatively alters BCAA metabolism resulting in higher plasma BCAA in later life, when BCAA concentrations overcome their own degradation and contribute to the vicious circle of IR [52]. The analysis of metabolite profiles in children allowed us to study this early development effect of IR, but far more studies in children and early adulthood are needed to investigate the molecular changes in the early state of obesity and IR. However, BCAA and BCAA metabolism are more affected by protein-rich diet than other AAs [53], and thus diet is a known confounder, especially in obese patients with higher protein intake [15, 22]. After the intervention, in a state of homogenous lifestyle and diet, the BCAA ratios were no longer significantly associated with HOMA, except for the ratio of Carn C5-OH to Carn C5:I, which was positively associated with HOMA in children without substantial weight loss. Thus, lifestyle may have strong effect on BCAA metabolism, and the results found at baseline were hidden by the homogenous lifestyle of the studied cohort. This possible confounding effect of diet and lifestyle on BCAA metabolism has to be investigated in further studies. The nonsignificant association of HOMA and BCAA could also be result of less power of our study. Tai et al. depicted the same challenge when they found an association of IR with BCAA in a large group of Chinese men, but not in a smaller group of Indian Asian men [15]. Two-hour oGTT glucose showed different associations with AA and AA derivatives compared to HOMA. Particularly, Ser was negatively associated with 2-hour oGTT glucose. Since fewer studies exist on relations of

2-hour oGTT glucose to metabolites, we can only speculate about the underlying mechanisms. Ser and glycine (Gly) were found to be decreased in obese Hispanic children [26] and in obese Korean children [27]. The concentrations of Gly and Ser were found to be lower in diabetic than in fasted normal rats [54]. The authors concluded that the contribution of Ser to gluconeogenesis becomes proportionally higher in diabetes. Thus, an increased gluconeogenesis rate in diabetic or prediabetic patients most likely leads to decreased Ser levels. In this case, Ser seems to be the AA which is first affected by higher gluconeogenesis rate. But to unravel this relation, further studies are needed. Another explanation is the use of consumed Ser for SM synthesis, since SM are elevated in IR state [55]. Furthermore, we have to recall the relatively low reliability of 2h glucose in oGTT [56]. Additionally, elevated 2h glucose levels in obese children tend to normalize in follow-up even without weight loss as also demonstrated in our study [57, 58].

All the described relations between metabolites and HOMA or 2-hour oGTT glucose were driven by children with substantial weight loss. Children without substantial weight loss showed fewer and different associations. Thus, it is plausible that different metabolomic changes are associated with different types of IR. However, HOMA and 2-hour oGTT glucose did not change significantly in children without substantial weight loss during the intervention period, and thus the information in these parameters may be too little to depict associations between metabolites and clinical parameters in this group. Nevertheless, estimates for the associations between metabolites and HOMA were different at baseline, in change, and at follow-up, assuming different metabolic pattern which could be related to IR, the intervention, or weight loss. Thus, further prospective studies should focus on the relation of IR to different metabolomic patterns.

However, we were not able to differentiate the effect of diet, increased physical exercise, and weight loss on metabolites and their ratio concentrations due to our study protocol. Unfortunately, we could not perform stratified analyses to the influence of pubertal status on the associations of metabolites with IR. An explorative statistical approach showed no influence on the association of IR with Tyr, but on Carn. Further studies with larger sample number are required to determine differences in IR development with respect to puberty status. A limitation of our study is that BMI percentiles were used to classify overweight. Although BMI is a good measure for overweight, it is not a precise measure of body fat mass. Furthermore, the degree of obesity was relatively homogeneous in our obese children. Additionally, the HOMA model is only an assessment of IR [59]. Clamp studies are actually the gold standard for analysing IR. In addition to the small sample number, these facts reduced the odds to detect associations between metabolites and clinical outcomes. The relatively low sample number also reduced the statistical power of the presented data analysis, which kept us from correction for multiple testing. Among the strengths of our study are the longitudinal design and the analysed children that were naïve to drugs and other diseases and had similar lifestyle during the one-year intervention. The additional focus on ratios allowed for a closer insight

into degradation pathways associated with obesity-related IR. Thus, the influence of diet and physical activity on changes of metabolite levels should be limited.

## 5. Conclusions

This study provides novel insights into the longitudinal interrelations of IR and obesity markers to metabolites and generates possible questions for further mechanistic studies of IR in obese children. Our cross-sectional and longitudinal analyses confirm a relationship between the Tyr and HOMA in obese children. So, Tyr and the Tyr metabolism should be focused more on in studies searching for early biomarkers and predictors in the switch from obesity to IR. In contrast, BCAA levels were negatively related to IR in cross-sectional analyses, while there was no significant association in the longitudinal analysis, which does not support a causal role of BCAA in inducing IR. Furthermore, responders to the intervention showed different associations between HOMA and AA compared to nonresponders, which appears to reflect different mechanisms for the development of obesity-induced IR. Further studies should also explore other analytes which were not determined in our study, such as p-hydroxyphenylpyruvate, fumarate, or acetoacetate that are involved in Tyr metabolism and sulfur containing AA.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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