Untargeted metabolomics identifies a plasma sphingolipid-related signature associated with lifestyle intervention in prepubertal children with obesity

María Jesús Leal-Witt[1,2*], Marta Ramon-Krauel[1,2*], Sara Samino[3,4], Marina Llobet[1,2], Daniel Cuadras[5], Josep C. Jimenez-Chillaron[1,2], Oscar Yanes[3,4], and Carles Lerin[1,2]

[1]Endocrinology Department, Institut de Recerca Sant Joan de Déu, Barcelona 08950, Spain.

[2]Hospital Sant Joan de Déu Barcelona, Barcelona 08950, Spain.

[3]Metabolomics Platform, Department of Electronic Engineering (DEEEA), Universitat Rovira i Virgili, Tarragona 43003, Spain.

[4]Biomedical Research Centre in Diabetes and Associated Metabolic Disorders (CIBERDEM), Madrid 28029, Spain.

[5]Statistics Department, Sant Joan de Déu Research Foundation, Barcelona 08950, Spain.

*These authors contributed equally to this work.

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Corresponding author:

Carles Lerin

Endocrinology department, Institut de Recerca Sant Joan de Déu

c/Santa Rosa 39, 08950 Barcelona (Spain)

Phone: (+34) 936 009 761 / Fax: (+34) 936 009 771 / email: clerin@fsjd.org

Abstract

Objective: Childhood obesity is a strong risk factor for adult obesity and metabolic diseases, including type 2 diabetes and cardiovascular disease. Early lifestyle intervention in children with obesity reduces future disease risk. The objective of this study is to identify metabolic signatures associated with lifestyle intervention in prepubertal children with obesity.

Methods: Thirty-five prepubertal children (7-10 years) with obesity (BMI>2 standard deviations) were enrolled in the study and participated in a 6-month-long lifestyle intervention program. Physiological and biochemical data and blood samples were collected both at baseline and after the intervention. A liquid chromatography-mass spectrometry (LC-MS)-based metabolomics approach was applied to obtain a comprehensive profiling of plasma samples, identifying 2581 distinct metabolite. Principal component analysis (PCA) was performed to consolidate all features into 8 principal components. Associations between metabolites and physiological and biochemical variables were investigated.

Results: The intervention program significantly decreased mean (95% CI) BMI standard deviation score from 3.56 (3.29-3.84) to 3.11 (2.88-3.34) ($p<0.001$). PCA identified one component (PC1) significantly altered by the intervention (Bonferroni adjusted $p=0.008$). A sphingolipid metabolism-related signature was identified as the major contributor to PC1. Sphingolipid metabolites were decreased by the intervention, and included multiple sphingomyelin, ceramide, glycosylsphingosine, and sulfatide species. Changes in several sphingolipid metabolites were associated with intervention-induced improvements in HbA1c levels.

Conclusions: Decreased circulating sphingolipid-related metabolites were associated with lifestyle intervention in prepubertal children with obesity, and correlated to improvements in HbA1c.

Introduction

Despite vast efforts devoted to treatment and prevention, the worldwide prevalence of obesity has increased exponentially during the last decades.[1] Obesity and overweight lead to adverse effects on blood pressure, cholesterol, lipids, and insulin sensitivity, all of which are major risk factors for other metabolic disorders including type 2 diabetes and cardiovascular disease. Lifestyle intervention programs focusing on diet and exercise are currently the main strategy for prevention and treatment of these diseases. However, the implementation of these intervention programs at a population level has proven challenging.[2-4] Obesity in children is of particular concern, as excessive weight gained during childhood can be tracked into later life.[5-7] Notably, prepubertal children show a distinct metabolic profile than pubertal or adolescent subjects, and respond differently to metabolic challenges.[8-10] For instance, oral glucose loading results in lower insulin secretion in prepubertal compared to pubertal children.[10] Furthermore, pubertal development physiologically decreases insulin sensitivity impacting a number of metabolic pathways, including proteolysis, lipid metabolism, and glucose homeostasis.[8] Thus, a deeper understanding of the pathophysiology of obesity specifically in prepubertal children is needed in order to design more efficient therapeutic and preventive strategies.

The advent of metabolomic technologies during the last decade has provided very valuables tools to study metabolic diseases.[11] Metabolomics can be divided into untargeted and targeted methodologies. Untargeted metabolomics aims to obtain a comprehensive profiling of all measurable small molecules in a given sample, including unknown analytes; targeted metabolomics is a hypothesis-driven approach that focuses on measuring specific groups of known metabolites. Taking advantage of these tools, several studies have identified biomarkers associated with obesity, insulin resistance, type 2 diabetes, and cardiovascular disease risk in adult subjects, including branched-chain amino acids (leucine, isoleucine, and valine), phenylalanine, tyrosine, betaine, acylcarnitines, and lysophosphatidylcholines.[12-17] Fewer metabolomic studies have been performed in children. Untargeted metabolomic approaches to study prepubertal and pubertal mixed populations revealed a branched-chain amino acid metabolism pattern, an androgen hormone signature, and altered acylcarnitine levels present in

78 subjects with obesity compared to normal weight controls.[18, 19] Other studies using targeted
79 metabolomics identified alterations in plasma levels of several amino acids,
80 lysophosphatidylcholines, and short- and medium-chain acylcarnitines in populations of
81 prepubertal, pubertal, and adolescent subjects with obesity compared to lean controls.[20, 21] Some
82 of these metabolic alterations, including glutamine, methionine, and lysophosphatidylcholines were
83 reversed after weight-loss interventions, suggesting that metabolite levels were associated with
84 overweight status or changes in lifestyle.[22, 23] Here, we report data from an observational
85 longitudinal study exclusively in prepubertal children with obesity, in which we applied an
86 untargeted metabolomic approach to obtain a comprehensive metabolomic profiling of plasma
87 samples before and after a lifestyle intervention program.

Subjects and Methods

Study participants

This is an observational study of the effects of lifestyle intervention in prepubertal children with obesity. The study was approved by the Hospital's Ethic Committee (Comité Ètic d'Investigació Clínica – CEIC). Pediatric patients with obesity and non-responsive to primary care protocols for treatment are usually referred from the primary care centers to the Obesity Unit at the Hospital Sant Joan de Déu, Barcelona (Spain). The routine therapeutic protocol applied at the Hospital is a family-based lifestyle intervention program. The study initially included 53 children with obesity, defined as BMI standard deviation score (BMI-SDS) greater than two standard deviations for a given age and sex, following the World Health Organization (WHO) standards. Patients were recruited at the Obesity Unit at the Hospital between the months of January 2013 and December 2014. All parents signed an informed consent document. Inclusion criteria were children a) age from 7 to 10-year-old; b) with obesity, defined as BMI-SDS>2; c) prepubertal, defined as Tanner stage I breast development for girls and testicular volume less than 4 ml in boys. Exclusion criteria included any form of endogen obesity, major congenital or chronic disease, drug-induced obesity, use of drugs for weight loss, involvement in another weight-loss program, as well as subjects without a signed informed consent. Finally, subjects with pubertal signs at the 6-month visit were also excluded from the study.

Lifestyle intervention program

Subjects were recruited at the Obesity Unit by the pediatric endocrine physician. At the first visit, parents signed the informed consent and all relevant clinical and anthropometric data was obtained. Blood sampling and first interview with the nutritionist (with at least one of the parents present) was performed within the next 10 days (baseline time point). We used motivational interviewing primarily focused on behavioral changes to improve lifestyle of the child and all family members. In order to achieve sustainable results, counseling by the dieticians was individualized according to patient and family needs. Counseling followed the recommendations of the Department of Health of the autonomous government of Catalonia (Spain), based on the Mediterranean diet and in agreement with the WHO. Such diet consists in 55% of kcal from

carbohydrates (less than 10% of sugars), 15% of kcal from protein, and 30% of kcal from lipids (less than 10% saturated fat). We used visual laminated support material, including food models and plates, to educate on portion size. We helped patients and families designing food menus emphasizing the importance of variety and quality, as well as the cooking method. Additionally, participants were encouraged to choose healthy nutrition options and to incorporate a minimum of 30 min of physical exercise per day into their lives. Subjects had follow-up interviews with the nutritionist 2 weeks and 3 months after the initial interview, to review changes made and set new goals. The 6-month visit, which included blood sampling, was scheduled with the pediatric endocrinologist who gave feed-back to the family on clinical outcomes (6m time point). Finally, participants came to a follow-up visit 1 year after the end of the intervention program (18-month time point) when anthropometric data was collected.

Physiological and biochemical analysis

All data and samples were obtained at baseline and after the intervention program (6-month time point). We measured weight (kg) and height (mt) with light clothing in a calibrated scale and rigid stadiometer. Body mass index (BMI) was calculated, and BMI-SDS for a given age and sex was obtained by using “Anthro Plus” software (WHO). Blood pressure was measure in the right arm using an automated system with the appropriate sleeve size for the arm diameter. Waist circumference was determined as middle point between the last rib and iliac crest. Blood samples were taken after 8 to 10 hours of overnight fast in tubes containing EDTA, and plasma was immediately separated, aliquoted, and stored at -80ºC until further use. Glucose, insulin, glycated hemoglobin (HbA1c), lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides), and liver enzymes (AST, ALT) were measured using standard protocols at the Hospital’s clinical laboratory. Nutritional data was evaluated at baseline and 6-month time point by using the qualitative KidMed questionnaire that measures adherence to Mediterranean Diet.[24] The KidMed test is a 15-item scale scored as following: 0-3 points is considered poor adherence, 4-7 points medium adherence, and ≥ 8 points high adherence to Mediterranean diet.

Plasma metabolomic analysis

Plasma aliquots (25 µL) were thawed at 4ºC and briefly vortex-mixed. Proteins were precipitated by the addition of 475 µL cold methanol/water (8:1 vol/vol) followed by 3 min of ultrasonication and 10 s of vortex-mixing. Samples were subsequently maintained on ice for 10 min. After centrifugation (10 min, 19.000 g, 4ºC), 100 µL of supernatant were transferred to a LC autosampler vial. Samples were then injected into an UHPLC system (1290 Agilent) coupled to a quadrupole time of flight (QTOF) mass spectrometer (6550 Agilent Technologies) operated in positive electrospray ionization (ESI+) mode. Metabolites were separated using HILIC (ACQUITY UPLC BEH 1.7 µm, Waters) chromatography at a flow rate of 0.4 mL/min. The solvent system was A = 50 mM NH_4OAc in water, and B = ACN. The linear gradient elution started at 95% B (time 0–2 min) and finished at 55% B (6 min). The injection volume was 2 µL. ESI conditions were gas temperature, 225ºC; drying gas, 13 L/min; nebulizer, 20 psig; fragmentor, 125 V; and skimmer, 65 V. The instrument was set to acquire over the *m*/*z* range 80–1200 with an acquisition rate of 4 spectra per second. Quality control samples (QC), consisting of pooled plasma samples from all patients, were injected before the first study sample, and then periodically after five study samples. Samples were randomized to reduce systematic error associated with instrumental drift. LC-MS (HILIC ESI+ mode) data were processed using the XCMS software (version 1.38.0) to detect and align features.[25] A feature is defined as a molecular entity with a unique m/z and a specific retention time. XCMS analysis of these data provided a matrix containing the retention time, m/z value, and integrated peak area of greater than 7400 features. Only the integrated areas of those metabolite features above 5,000 spectral counts in at least one of the groups were considered for quantification. The tab-separated text files containing LC-MS data were imported into Rstudio (version 3.0.2) where QC samples were used to filter analytical variation as previously described[26] From the resulting 2647 features, 66 showed below detectable level readings in at least one sample, and were eliminated from the analysis. The resulting matrix of 2581 features was used for principal component analysis (PCA). Metabolic features selected by the PCA model were identified using the HMDB[27] and Metlin database. Identified metabolites were then used as input in MBRole 2.0 (ref. [28]) to perform pathway enrichment (KEGG pathways). To validate metabolic features, MS/MS was performed in targeted mode, with the instrument set to acquire over the m/z

range 40–950, with a default iso width of 1.4 m/z. Collision energy was fixed at 20 V. Metabolites were identified conforming to Level 2, as specified by the Metabolomics Standards Initiative[29] (i.e., putatively annotated compound) since their accurate mass and experimental MS/MS spectra coincide with the expected fragmentation pattern of lipid families or by comparison with chemical standards from the METLIN database. All spectra data have been deposited at the EMBL-EBI MetaboLights database (https://www.ebi.ac.uk/metabolights/, accession number MTBLS423).

Statistical analysis

Unless otherwise stated, normally distributed variables are described by mean and standard deviation (SD), and non-normally distributed variables by median and interquantile range (IQR). Data were compared between baseline and 6-month time point by two-tail paired t-test for normal distribution, or Wilcoxon matched pairs signed-rank test for non-normal distribution. Principal component analysis with minimum residuals as factoring method was performed on the log2-transformed metabolomic matrix, assessed on each subject at baseline and 6-month time point. Eight factors, accounting for a cumulative variance of 58%, were extracted with the Varimax rotation method to produce interpretable components. Wilcoxon matched pairs signed-rank test was used to compare baseline and 6-month factor scores, and Bonferroni correction was applied to adjust for multiple comparisons. Metabolic features with loadings > |0.75| were considered to significantly contribute to a given factor. To assess differences in individual metabolites between baseline and 6-month, concentrations were evaluated as z scores (centered at 0 and standardized) after log2 transformation. Partial Spearman correlations adjusted for sex, age, and baseline BMI-SDS were used to measure the dependence between numerical variables. R 3.3.2 (2016, R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical calculations. P-values less than 0.05 were considered significant.

Results

Physiological and biochemical effects of the lifestyle intervention program

Fifty-three subjects with obesity (BMI-SDS>2) were initially enrolled in the intervention program. Among participants, five voluntarily withdraw from the program, six showed pubertal status at the 6-month time point, and two refused to provide a blood sample at the post-intervention visit. Furthermore, two showed altered C-reactive protein levels suggesting concomitant infection and were therefore excluded from the metabolomics analysis. Finally, three samples could not be analyzed in the metabolomics platform for technical reasons resulting in subject exclusion from the analysis. Thus, we analyzed paired samples (baseline and 6-month) from 35 exclusively prepubertal subjects with obesity. Baseline and post-intervention anthropometric and biochemical characteristics of all subjects are reported in Table 1. Lifestyle intervention significantly decreased BMI-SDS by 0.45 units ($p<0.001$, Table 1), and reduced waist circumference by 1.8 cm ($p<0.001$, Table 1). Notably, improvements in BMI-SDS levels were maintained one year after the end of the intervention program (Table S1). Subjects showed a modest increase in fasting glucose, triglyceride, and cholesterol levels after the intervention, while insulin levels and HOMA-IR were not modified (Table 1). However, HbA1c levels were significantly lowered by lifestyle intervention ($p<0.001$, Table 1). To assess the impact of the intervention program on participants' diet, we performed a qualitative dietary study before and after the intervention by applying the KidMed test. The lifestyle intervention program increased the adherence to the Mediterranean diet, as shown by the increase in the number of subjects with a higher score after the intervention (Table S2).

Effects of lifestyle intervention on the plasma metabolome

We applied an unbiased approach using LC-MS to obtain a comprehensive metabolic profiling from fasting plasma samples at baseline and at the 6-month time point. This untargeted metabolomics approach identified a total of 2581 distinct metabolite features. Peak intensity values, mass, and retention times for all features are included in Table S3. We applied unsupervised principal component analysis (PCA) to consolidate metabolite features into 8 factors, which explained 58% of total variance. Figure 1A shows the score plot of the first 2 components, accounting for 26.1% of cumulative variance; Figure 1B shows the loadings for the 2581 metabolic

features in PC1 and PC2. We then performed a paired analysis of the principal components between baseline and the 6-month time point, and observed a decrease in principal component 1 (PC1) after adjusting for multiple comparisons (adj. p=0.008, Figure 1C). None of the other components significantly differed among groups (Figure 1C). Metabolic features highly contributing to PC1 (those with loadings > |0.75|) are shown in Table S4. These selected features were then identified using the HMDB[27] and Metlin databases. Notably, PC1 was mostly comprised of a combination of sphingomyelin, ceramide, and glycosphingolipid species, and to a lesser extent phosphatidylcholine, phosphatidylethanolamine, diacylglycerol, and triacylglycerol molecules (Table S4). Indeed, pathway enrichment analysis of the PC1-contributing features identified the sphingolipid metabolism pathway as the main contributor to this component (FDR q=2.19E-11, Table S5). Multiple metabolic features from PC1 were further identified using LC-MS/MS (Table S4). Validated metabolites from the sphingolipid metabolism pathway included a number of sphingomyelin, ceramide, monoglycosylceramide (glucosyl- or galactosylceramide), diglycosylceramide (galabiosyl- or lactosylceramide), and sufatide (3-o-sulfogalactosylceramide) species. All these metabolites were significantly decreased after the intervention compared to baseline levels (Figures 2A-2D).

Associations between sphingolipid-related metabolites and physiological parameters

Sphingolipids have been consistently associated with obesity, insulin resistance, and type 2 diabetes in human subjects.[30-32] Thus, we next analyzed whether lifestyle intervention-induced differences in sphingolipid levels were associated with changes in physiological parameters, including BMI-SDS, HOMA-IR, and HbA1c. The decrease in sphingolipid levels induced by the intervention was not associated with changes from baseline to the 6-month time point in BMI-SDS or HOMA-IR (Table 2). Remarkably, the improvement in HbA1c levels from baseline to the 6-month time point was directly associated with the decrease in several sphingolipids, including sphingomyelin, ceramide, glycosphingolipid, and sulfatide species, even after adjusting for gender, age, and baseline BMI-SDS (Table 2).

Discussion

Prepubertal children are metabolically distinct than pubertal or adolescent subjects, with pubertal development physiologically decreasing insulin sensitivity[8] and accelerating metabolic dysregulation in patients with obesity.[9] Thus, early interventions in prepubertal individuals are crucial to decrease future risk of disease. Lifestyle interventions mainly based on a healthy diet and physical activity are the current strategy for childhood obesity treatment.[33] However, such interventions in young children are complex, often achieving only a temporary and modest reduction in BMI. A decrease of 0.25 units in BMI-SDS is considered sufficient to improve metabolic health parameters in children and adolescents.[34] The lifestyle intervention program resulted in a mean decrease of 0.45 units in BMI-SDS and of 1.8 cm in waist circumference in our cohort of prepubertal children. Importantly, the improvement in BMI-SDS was maintained up to one year after the end of the intervention.

Despite the significant decrease in BMI-SDS and waist circumference, we observed no improvements in several metabolic parameters, including fasting glucose, triglyceride, or cholesterol levels. Successful interventions in adult individuals with obesity frequently lead to improvements in these variables. However, adult subjects with obesity often show impaired baseline metabolic characteristics, while in our prepubertal population these variables were mostly in the normal clinical reference range for their age, and not overtly impaired. These data are in line with a report by Reinher et al. describing that the prepubertal stage is associated with a more "metabolic healthy obese" phenotype.[9] This report and other studies have found only minor or no effects of lifestyle intervention on glucose, HOMA-IR, triglyceride, and cholesterol levels in children with obesity.[9, 35, 36] Despite the lack of effect on fasting glucose and insulin levels in our study, HbA1c levels were significantly reduced after the intervention (from 5.4% to 5.2%). Interestingly, Blüher et al. reported a similar decrease in HbA1c (from 5.47% to 5.22%) associated with improved glucose tolerance after a 1-year lifestyle intervention program in children and adolescents, while fasting glucose and insulin levels remained stable throughout the study.[35] Based on these data, our results suggest that the intervention in prepubertal children improved glycemic control. Finally, the prepubertal patients enrolled in our study showed a high degree of

obesity at baseline (BMI-SDS of 3.56), and despite the significant reduction in BMI-SDS, still had a notable level of obesity after the intervention (6-month BMI-SDS of 3.11). Thus, we cannot exclude the possibility that a much further decrease in BMI-SDS would have a bigger impact on glucose and lipid systemic metabolism in this prepubertal population.

Our untargeted metabolic profiling identified a strong sphingolipid metabolism-related signature associated with lifestyle intervention in prepubertal children with obesity. Sphingolipids, which include sphingomyelins, ceramides, and glycosphingolipids,[37] are basic constituents of the plasma membrane lipid bilayer, where they help maintain a stable structure to protect the cellular membrane from environmental factors. In addition to their important role in plasma membrane structure, sphingolipids also function as crucial signaling molecules in a wide array of biological processes, including apoptosis, proliferation, inflammation, autophagy, and differentiation. Notably, obesity and insulin resistance have been consistently associated with altered sphingolipid metabolism and increased circulating ceramide levels in humans.[30-32] In children and adolescents, targeted metabolomics identified altered sphingomyelin/phosphatidylcholine ratios associated with obesity (age range 6-15 year-old) compared to normal weight controls,[20] suggesting impaired sphingolipid metabolism. Experimental animal models have demonstrated that increased sphingolipid levels contribute to the development of obesity and related metabolic disorders, including insulin resistance and cardiovascular disease.[38, 39] Thus, modulating sphingolipid metabolism is being actively investigated as a target for therapeutic strategies. Indeed, targeted genetic deletions or pharmacological inhibition of ceramide, glycosphingolipid, or sphingomyelin synthesis in mouse models significantly improves glucose tolerance, insulin sensitivity, and atherosclerosis.[40-44] Therefore, our data suggest that decreasing circulating ceramides in prepubertal children might be an important mechanism to reduce future metabolic and cardiovascular risk. In this context, the decrease in multiple circulating sphingolipid species induced by the intervention was associated with improvements in HbA1c levels, suggesting that decreasing ceramide levels has a long-term effect on glycemic control. Further studies will be required to determine whether targeting sphingolipid metabolism or ceramide levels during early

childhood improve HbA1c levels and can be beneficial to decrease future metabolic risk during adolescence and adulthood.

A number of weight-loss intervention studies in adult subjects or adolescents have shown an impact in certain sphingolipid-related molecules. To our knowledge, this is the first study reporting a global effect of lifestyle intervention on sphingolipid metabolism in a prepubertal population of children with obesity. A study in adolescents showed a decrease in two sphingomyelin species (C26:0 and C26:1) after laparoscopic sleeve gastrectomy surgery.[45] In adult subjects, gastric bypass surgery resulted in decreased ceramide levels correlating to the extent of weight loss.[46] These studies suggest obesity-related alterations in sphingolipid metabolism. However, changes in sphingolipid metabolites were not associated with the decrease in BMI-SDS in our study with prepubertal children. On the other hand, dietary interventions aimed at decreasing risk of type 2 diabetes and cardiovascular disease in adult subjects lowered plasma sphingolipid levels without modifying body weight.[47, 48] Specifically, higher unsaturated fat content decreased ceramide levels compared to a saturated fat-rich diet in adult subjects.[47] Also, a healthy “Nordic diet”, characterize by higher fiber and unsaturated fat content was shown to modulate ceramide levels compared to a control diet.[48] Given that sphingolipid-related metabolite levels were not associated with the reduction in BMI-SDS in our cohort, these data suggest that dietary changes induced by the lifestyle intervention program contributed to the decrease in circulating sphingolipid levels independently of the effects on BMI-SDS.

Metabolites from the BCAA pathway and acylcarnitine species have been consistently associated with obesity and insulin resistance in both adult and children subjects compared to normal weight controls.[12, 15, 18, 20, 49] Weight-loss interventions in adults, including gastric by-pass and lifestyle interventions, reversed theses changes in BCAA and acylcarnitine levels.[50-52] Notably, BCAA-related metabolites and acyl-carnitines were measured in the untargeted metabolomics analysis, but not identified as modified by lifestyle intervention in our study with prepubertal children. Disturbances in these metabolites have been also linked to insulin resistance in prepubertal children with obesity.[49] Furthermore, adjustment for clinical and biochemical measures attenuate

the associations between BCAA levels and diabetes risk in adult subjects,[14] suggesting that increased BCAA levels may be the consequence of a combination of different factors, including obesity, fasting glucose levels, and insulin resistance. Since participants in the present study did not show changes in HOMA-IR, these data suggest that alterations in BCAA metabolism might be more linked to insulin sensitivity rather than to BMI itself, at least in a prepubertal population. Moreover, despite the notable reduction in BMI-SDS, participants in the present study still showed a high degree of obesity after the intervention. Thus, it seems rather plausible that BCAA and acyl-carnitine levels still reflect the obesity status.

Limitations of this study include the modest sample size. Subjects showing signs of puberty at baseline or the 6-month visit were excluded from the study, substantially decreasing the potential sample size. A main strength of this study, intimately linked to the limitation in sample size, is that participants are exclusively in a prepubertal stage, avoiding potential confounding factors related to entering puberty.[9] Further strengths include the unbiased approach, both by the use of untargeted metabolomic techniques and unsupervised principal component analysis applied to identify metabolic signatures associated with lifestyle intervention.

In summary, our data indicate that lifestyle intervention induced a sphingolipid metabolism-related signature in prepubertal children with obesity. Since sphingolipid and ceramide levels are associated with risk for insulin resistance and cardiovascular disease, our data suggest that decreasing circulating ceramides in prepubertal children might be an important mechanism to reduce future metabolic and cardiovascular risk. Further studies are warranted to determine whether targeting sphingolipid metabolism in prepubertal children with obesity can provide a valid strategy to decrease future risk of metabolic disease.

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Author contributions: MJL, JCJ, MR, and CL designed the study, analyzed the data, and wrote the manuscript; MJL, ML, and MR implemented the intervention and collected data; OY and SS performed the metabolomics analysis; DC performed the statistical analysis; all authors were involved in editing the paper and had final approval of the submitted version. MJL, MR, and CL had full access to the data in the study and final responsibility for the decision to submit for publication.

References

1. Ng M, Fleming T, Robinson M, Thomson B, Graetz N, Margono C *et al.* Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* 2014; **384**(9945)**:** 766-781.

2. Forster M, Veerman JL, Barendregt JJ, Vos T. Cost-effectiveness of diet and exercise interventions to reduce overweight and obesity. *Int J Obes (Lond)* 2011; **35**(8)**:** 1071-1078.

3. Gluckman PD, Hanson M, Zimmet P, Forrester T. Losing the war against obesity: the need for a developmental perspective. *Sci Transl Med* 2011; **3**(93)**:** 93cm19.

4. Kahn R, Davidson MB. The reality of type 2 diabetes prevention. *Diabetes Care* 2014; **37**(4)**:** 943-949.

5. Singh AS, Mulder C, Twisk JW, van Mechelen W, Chinapaw MJ. Tracking of childhood overweight into adulthood: a systematic review of the literature. *Obes Rev* 2008; **9**(5)**:** 474-488.

6. Juonala M, Magnussen CG, Berenson GS, Venn A, Burns TL, Sabin MA *et al.* Childhood adiposity, adult adiposity, and cardiovascular risk factors. *N Engl J Med* 2011; **365**(20)**:** 1876-1885.

7. Cunningham SA, Kramer MR, Narayan KM. Incidence of childhood obesity in the United States. *N Engl J Med* 2014; **370**(5)**:** 403-411.

8. Hannon TS, Janosky J, Arslanian SA. Longitudinal study of physiologic insulin resistance and metabolic changes of puberty. *Pediatr Res* 2006; **60**(6)**:** 759-763.

9. Reinehr T, Wolters B, Knop C, Lass N, Holl RW. Strong effect of pubertal status on metabolic health in obese children: a longitudinal study. *J Clin Endocrinol Metab* 2015; **100**(1)**:** 301-308.

10. Ball GDC, Huang TTK, Gower BA, Cruz ML, Shaibi GQ, Weigensberg MJ *et al.* Longitudinal Changes in Insulin Sensitivity, Insulin Secretion, and β-Cell Function During Puberty. *The Journal of Pediatrics* 2006; **148**(1)**:** 16-22.

11. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. *Nat Rev Mol Cell Biol* 2012; **13**(4)**:** 263-269.

12. Lerin C, Goldfine AB, Boes T, Liu M, Kasif S, Dreyfuss JM *et al.* Defects in muscle branched-chain amino acid oxidation contribute to impaired lipid metabolism. *Mol Metab* 2016; **5**(10)**:** 926-936.

13. Ejaz A, Martinez-Guino L, Goldfine AB, Ribas-Aulinas F, De Nigris V, Ribo S *et al.* Dietary Betaine Supplementation Increases Fgf21 Levels to Improve Glucose Homeostasis and Reduce Hepatic Lipid Accumulation in Mice. *Diabetes* 2016; **65**(4)**:** 902-912.

14. Walford GA, Ma Y, Clish C, Florez JC, Wang TJ, Gerszten RE *et al.* Metabolite Profiles of Diabetes Incidence and Intervention Response in the Diabetes Prevention Program. *Diabetes* 2016; **65**(5)**:** 1424-1433.

15. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF *et al.* A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* 2009; **9**(4)**:** 311-326.

16. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA *et al.* Relationships between circulating metabolic intermediates and insulin action in overweight to obese, inactive men and women. *Diabetes Care* 2009; **32**(9)**:** 1678-1683.

17. Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. *PLoS One* 2010; **5**(12)**:** e15234.

18. Perng W, Gillman MW, Fleisch AF, Michalek RD, Watkins SM, Isganaitis E *et al.* Metabolomic profiles and childhood obesity. *Obesity (Silver Spring)* 2014; **22**(12)**:** 2570-2578.

19. Butte NF, Liu Y, Zakeri IF, Mohney RP, Mehta N, Voruganti VS *et al.* Global metabolomic profiling targeting childhood obesity in the Hispanic population. *Am J Clin Nutr* 2015; **102**(2)**:** 256-267.

20. Wahl S, Yu Z, Kleber M, Singmann P, Holzapfel C, He Y *et al.* Childhood obesity is associated with changes in the serum metabolite profile. *Obes Facts* 2012; **5**(5)**:** 660-670.

21. Mihalik SJ, Michaliszyn SF, de las Heras J, Bacha F, Lee S, Chace DH *et al.* Metabolomic profiling of fatty acid and amino acid metabolism in youth with obesity and type 2 diabetes: evidence for enhanced mitochondrial oxidation. *Diabetes Care* 2012; **35**(3)**:** 605-611.

22. Reinehr T, Wolters B, Knop C, Lass N, Hellmuth C, Harder U *et al.* Changes in the serum metabolite profile in obese children with weight loss. *Eur J Nutr* 2015; **54**(2)**:** 173-181.

23. Hellmuth C, Kirchberg FF, Lass N, Harder U, Peissner W, Koletzko B *et al.* Tyrosine Is Associated with Insulin Resistance in Longitudinal Metabolomic Profiling of Obese Children. *J Diabetes Res* 2016; **2016:** 2108909.

24. Serra-Majem L, Ribas L, Ngo J, Ortega RM, García A, Pérez-Rodrigo C *et al.* Food, youth and the Mediterranean diet in Spain. Development of KIDMED, Mediterranean Diet Quality Index in children and adolescents. *Public Health Nutrition* 2004; **7:** 931-935.

25. Smith CA, Want EJ, O'Maille G, Abagyan R, Siuzdak G. XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal Chem* 2006; **78**(3)**:** 779-787.

26. Vinaixa M, Samino S, Saez I, Duran J, Guinovart JJ, Yanes O. A Guideline to Univariate Statistical Analysis for LC/MS-Based Untargeted Metabolomics-Derived Data. *Metabolites* 2012; **2**(4)**:** 775-795.

27. Wishart DS, Jewison T, Guo AC, Wilson M, Knox C, Liu Y *et al.* HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res* 2013; **41:** D801-807.

28. Lopez-Ibanez J, Pazos F, Chagoyen M. MBROLE 2.0-functional enrichment of chemical compounds. *Nucleic Acids Res* 2016; **44**(W1)**:** W201-204.

29. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA *et al.* Proposed minimum reporting standards for chemical analysis Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). *Metabolomics* 2007; **3**(3)**:** 211-221.

30. Bergman BC, Brozinick JT, Strauss A, Bacon S, Kerege A, Bui HH *et al.* Serum sphingolipids: relationships to insulin sensitivity and changes with exercise in humans. *Am J Physiol Endocrinol Metab* 2015; **309**(4)**:** E398-408.

31. Haus JM, Kashyap SR, Kasumov T, Zhang R, Kelly KR, DeFronzo RA *et al.* Plasma Ceramides Are Elevated in Obese Subjects With Type 2 Diabetes and Correlate With the Severity of Insulin Resistance. *Diabetes* 2009; **58**(2)**:** 337-343.

32. Lopez X, Goldfine AB, Holland WL, Gordillo R, Scherer PE. Plasma ceramides are elevated in female children and adolescents with type 2 diabetes. *Journal of Pediatric Endocrinology and Metabolism* 2013; **26**(9-10)**:** 995.

33. Eloranta AM, Lindi V, Schwab U, Tompuri T, Kiiskinen S, Lakka HM *et al.* Dietary factors associated with overweight and body adiposity in Finnish children aged 6-8 years: the PANIC Study. *Int J Obes* 2012; **36**(7)**:** 950-955.

34. Reinehr T, Lass N, Toschke C, Rothermel J, Lanzinger S, Holl RW. Which Amount of BMI-SDS Reduction Is Necessary to Improve Cardiovascular Risk Factors in Overweight Children? *J Clin Endocrinol Metab* 2016; **101**(8)**:** 3171-3179.

35. Blüher S, Petroff D, Wagner A, Warich K, Gausche R, Klemm T *et al.* The one year exercise and lifestyle intervention program KLAKS: Effects on anthropometric parameters, cardiometabolic risk factors and glycemic control in childhood obesity. *Metabolism* 2014; **63**(3)**:** 422-430.

36. Pedrosa C, Oliveira BMPM, Albuquerque I, Simões-Pereira C, Vaz-de-Almeida MD, Correia F. Markers of metabolic syndrome in obese children before and after 1-year lifestyle intervention program. *European Journal of Nutrition* 2011; **50**(6)**:** 391-400.

37. Meikle PJ, Summers SA. Sphingolipids and phospholipids in insulin resistance and related metabolic disorders. *Nat Rev Endocrinol* 2017; **13**(2)**:** 79-91.

38. Zhang QJ, Holland WL, Wilson L, Tanner JM, Kearns D, Cahoon JM *et al.* Ceramide mediates vascular dysfunction in diet-induced obesity by PP2A-mediated dephosphorylation of the eNOS-Akt complex. *Diabetes* 2012; **61**(7)**:** 1848-1859.

39. Camerer E, Regard JB, Cornelissen I, Srinivasan Y, Duong DN, Palmer D *et al.* Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. *J Clin Invest* 2009; **119**(7)**:** 1871-1879.

40. Ussher JR, Koves TR, Cadete VJ, Zhang L, Jaswal JS, Swyrd SJ *et al.* Inhibition of de novo ceramide synthesis reverses diet-induced insulin resistance and enhances whole-body oxygen consumption. *Diabetes* 2010; **59**(10)**:** 2453-2464.

41. Chatterjee S, Bedja D, Mishra S, Amuzie C, Avolio A, Kass DA *et al.* Inhibition of glycosphingolipid synthesis ameliorates atherosclerosis and arterial stiffness in apolipoprotein E-/- mice and rabbits fed a high-fat and -cholesterol diet. *Circulation* 2014; **129**(23)**:** 2403-2413.

42. Xia JY, Holland WL, Kusminski CM, Sun K, Sharma AX, Pearson MJ *et al.* Targeted Induction of Ceramide Degradation Leads to Improved Systemic Metabolism and Reduced Hepatic Steatosis. *Cell Metab* 2015; **22**(2)**:** 266-278.

43. Chaurasia B, Kaddai VA, Lancaster GI, Henstridge DC, Sriram S, Galam DL *et al.* Adipocyte Ceramides Regulate Subcutaneous Adipose Browning, Inflammation, and Metabolism. *Cell Metab* 2016; **24**(6)**:** 820-834.

44. Holland WL, Brozinick JT, Wang LP, Hawkins ED, Sargent KM, Liu Y *et al.* Inhibition of ceramide synthesis ameliorates glucocorticoid-, saturated-fat-, and obesity-induced insulin resistance. *Cell Metab* 2007; **5**(3)**:** 167-179.

45. Oberbach A, von Bergen M, Bluher S, Lehmann S, Till H. Combined serum proteomic and metabonomic profiling after laparoscopic sleeve gastrectomy in children and adolescents. *J Laparoendosc Adv Surg Tech A* 2012; **22**(2)**:** 184-188.

46. Huang H, Kasumov T, Gatmaitan P, Heneghan HM, Kashyap SR, Schauer PR *et al.* Gastric bypass surgery reduces plasma ceramide subspecies and improves insulin sensitivity in severely obese patients. *Obesity (Silver Spring, Md.)* 2011; **19:** 2235-2240.

47. Kien CL, Bunn JY, Poynter ME, Stevens R, Bain J, Ikayeva O *et al.* A lipidomics analysis of the relationship between dietary fatty acid composition and insulin sensitivity in young adults. *Diabetes* 2013; **62**(4)**:** 1054-1063.

48. Lankinen M, Schwab U, Kolehmainen M, Paananen J, Nygren H, Seppanen-Laakso T *et al.* A Healthy Nordic Diet Alters the Plasma Lipidomic Profile in Adults with Features of Metabolic Syndrome in a Multicenter Randomized Dietary Intervention. *J Nutr* 2016.

49. Mastrangelo A, Martos-Moreno GA, Garcia A, Barrios V, Ruperez FJ, Chowen JA *et al.* Insulin resistance in prepubertal obese children correlates with sex-dependent early onset metabolomic alterations. *Int J Obes (Lond)* 2016; **40**(10)**:** 1494-1502.

50. Laferrere B, Reilly D, Arias S, Swerdlow N, Gorroochurn P, Bawa B *et al.* Differential metabolic impact of gastric bypass surgery versus dietary intervention in obese diabetic subjects despite identical weight loss. *Sci Transl Med* 2011; **3**(80)**:** 80re82.

51. Glynn EL, Piner LW, Huffman KM, Slentz CA, Elliot-Penry L, AbouAssi H *et al.* Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. *Diabetologia* 2015; **58**(10)**:** 2324-2335.

52. Shah SH, Crosslin DR, Haynes CS, Nelson S, Turer CB, Stevens RD *et al.* Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss. *Diabetologia* 2012; **55**(2)**:** 321-330.

Figure Legends

Figure 1. Principal component analysis identifies PC1 as decreased after lifestyle intervention.

A) Score plot of principal components 1 and 2 accounting for 15.4% and 10.7% of total variance. White and black circles represent baseline and the 6-month time point data, respectively. **B**) Loading plot of the 2581 metabolic features in PC1 (x-axis) and PC2 (y-axis). **C**) Comparison of PC scores between baseline and 6 months. Wilcoxon matched-pairs sign rank was applied to determine significance. 95% CIs and p values reflect Bonferroni's multiple comparison correction.

Figure 2. Lifestyle intervention decreases circulating sphingolipid levels.

Concentrations of validated sphingolipid-related metabolites contributing to PC1 were evaluated as z-scores. Differences between baseline and the 6-month time point for **A**) N-acylsphigosines (ceramides), **B**) sphingomyelins, **C**) glycosyl-N-acylsphingosines, and **D**) sulfatides are reported in the box plots. Top and bottom of the box represent the 75th and 25th percentile, respectively. Whiskers represent the entire spread of the data points, excluding extreme points (higher or lower than the median ± 1.5 times the interquartile range), which are indicated with circles. GlcCer, glucosylceramide; GalCer, galactosylceramide; diGalCer, galabiosylceramide; LacCer, lactosylceramide. Wilcoxon matched-pairs sign rank was applied to determine significance, and p values are reported. [a]Alternative structures d18:1/20:1 or d18:2/20:0, unresolved by MS/MS. [b]Acyl chain unresolved by MS/MS.

Table legends

Table 1. Demographic and metabolic characteristics of subjects before and after the intervention program.

Subjects were 76% (n=26) caucassian, 12% (n=4) hispanic, 9% (n=3), north African 3% (n=1), and Asian 3% (n=1), roughly representing the distribution of the resident population of the Hospital's influence area. Normally distributed values are presented as mean (95% CI), and significance assessed by two-tail paired Student *t* test. [a]Non-normal distributed variables are presented as median [IQR], and significance assessed by Wilcoxon matched paired signed-rank test. Bold font indicates $p < 0.05$.

Table 2. Correlations of intervention-induced changes in sphingolipid levels to physiological measures. Partial Spearman correlations between baseline to 6-month changes in validated sphingolipid metabolites to changes in BMI-SDS, HOMA-IR, and HbA1c. Correlations are adjusted for child sex, age, and baseline BMI-SDS. GlcCer, glucosylceramide; GalCer, galactosylceramide; diGalCer, galabiosylceramide; LacCer, lactosylceramide. [a]Alternative structures d18:1/20:1 or d18:2/20:0, unresolved by MS/MS. [b]Acyl chain unresolved by MS/MS. Bold font indicates $p < 0.05$.

Figure 1

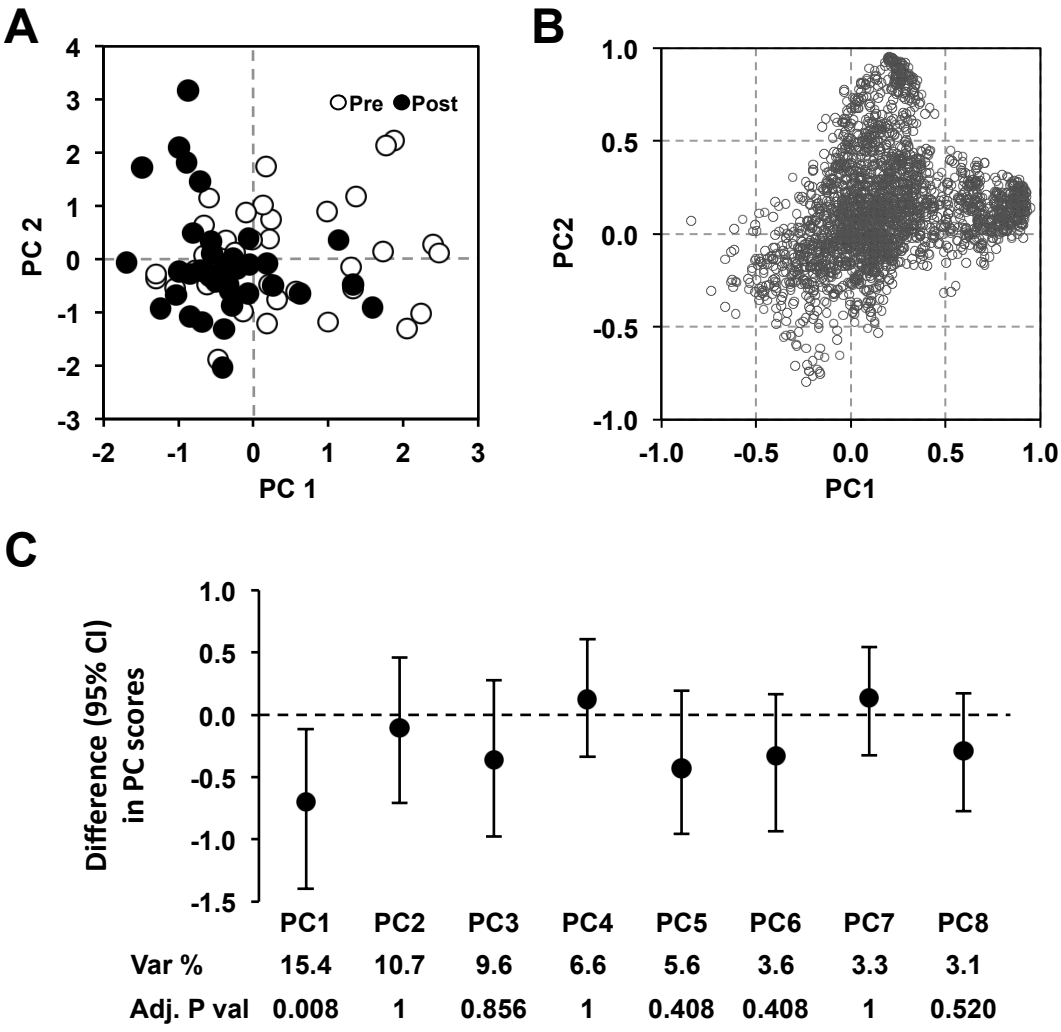
A
Pre
Post
PC 2
PC 1
B
PC2
PC1
C
Difference (95% CI) in PC scores
PC1 PC2 PC3 PC4 PC5 PC6 PC7 PC8
Var % 15.4 10.7 9.6 6.6 5.6 3.6 3.3 3.1
Adj. P val 0.008 1 0.856 1 0.408 0.408 1 0.520

Figure 2

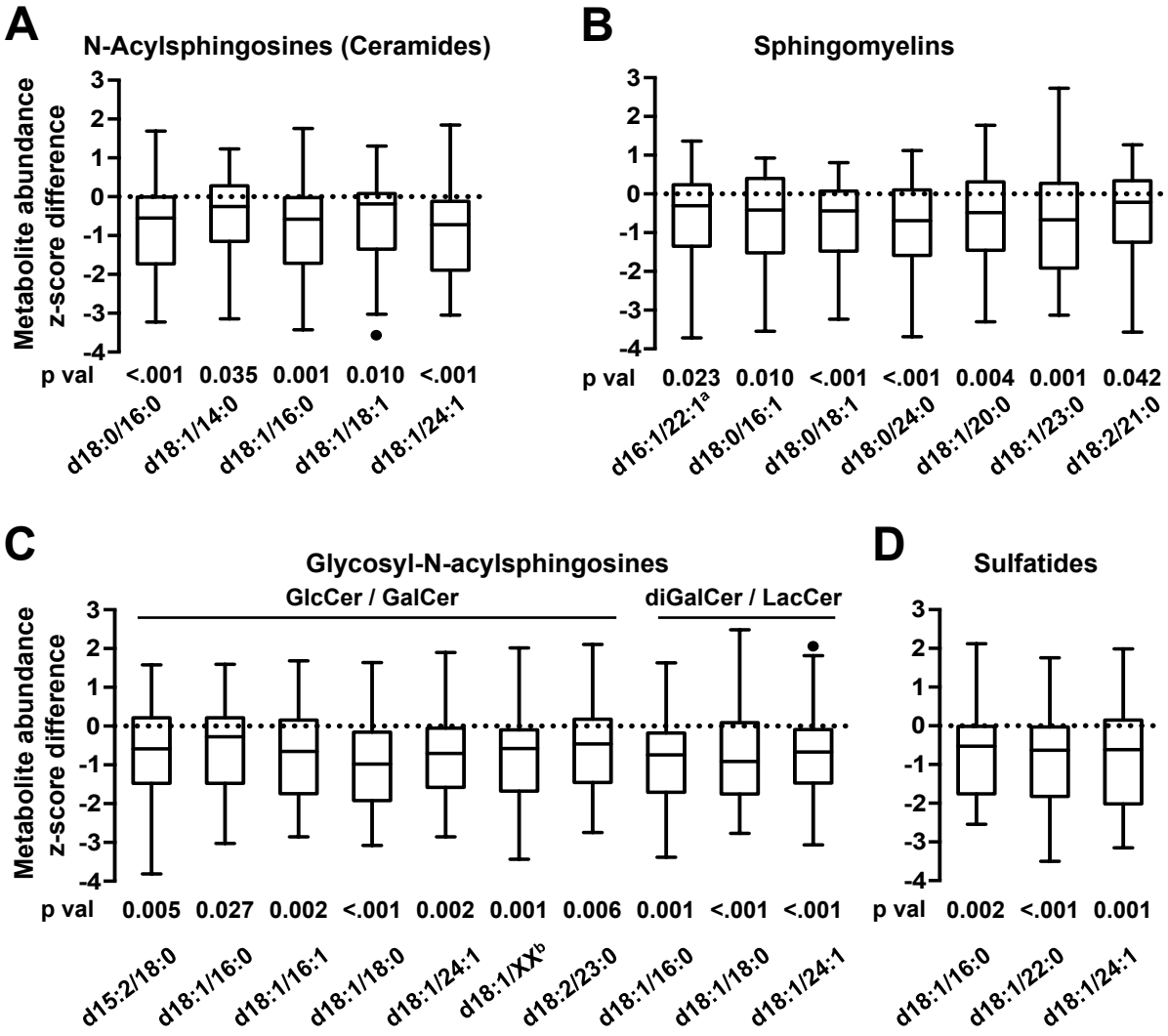
A
N-Acylsphingosines (Ceramides)
Metabolite abundance z-score difference
p val <.001 0.035 0.001 0.010 <.001
d18:0/16:0
d18:1/14:0
d18:1/16:0
d18:1/18:1
d18:1/24:1
B
Sphingomyelins
p val 0.023 0.010 <.001 <.001 0.004 0.001 0.042
d16:1/22:1[a]
d18:0/16:1
d18:0/18:1
d18:0/24:0
d18:1/20:0
d18:1/23:0
d18:2/21:0
C
Glycosyl-N-acylsphingosines
GlcCer / GalCer
diGalCer / LacCer
Metabolite abundance z-score difference
p val 0.005 0.027 0.002 <.001 0.002 0.001 0.006 0.001 <.001 <.001
d15:2/18:0
d18:1/16:0
d18:1/16:1
d18:1/18:0
d18:1/24:1
d18:1/XX[b]
d18:2/23:0
d18:1/16:0
d18:1/18:0
d18:1/24:1
D
Sulfatides
p val 0.002 <.001 0.001
d18:1/16:0
d18:1/22:0
d18:1/24:1

Table 1. Demographic and metabolic characteristics of subjects before and after the intervention program.

	Baseline	6-month	p val
Subjects	n = 35		
Sex (F/M)	17/18		
Age (years)	8.9 (8.6-9.3)		
Weight (kg)	54.8 (50.1-59.4)	55.0 (50.4-59.7)	0.627
BMI-SDS	3.56 (3.29-3.84)	3.11 (2.88-3.34)	**<0.001**
Waist circumference (cm)	83.4 (80.3-86.6)	81.7 (78.3-85.0)	**0.001**
Systolic Pressure (mmHg)	112 (109-115)	110 (107-113)	0.332
Diastolic Pressure (mmHg)	70 (67-72)	69 (67-71)	0.552
Fasting Glycemia (mg/dL)	85 (82-88)	89 (87-91)	**0.003**
Fasting Insulin (µU/mL)	13.2 (10.9-15.6)	13.4 (11-15.7)	0.838
HOMA-IR	2.80 (2.29-3.31)	2.96 (2.42-3.5)	0.657
HbA1c (%)	5.4 (5.3-5.4)	5.2 (5.2-5.3)	**<0.001**
Total Cholesterol (mg/dL)	164 (155-174)	178 (169-188)	**<0.001**
LDL-Cholesterol (mg/dL)	105 (96-114)	114 (105-122)	**0.010**
HDL-Cholesterol (mg/dL)	43 (40-47)	45 (42-48)	0.266
TAG (mg/dL)[a]	67 [60-95]	80 [65-119]	**0.006**
ALT (UI/L)	20.7 (18.4-23.1)	20.9 (18.5-23.4)	0.801
AST (UI/L)	22.1 (20.6-23.5)	22.7 (20.9-24.5)	0.358

Table 2. Correlations of intervention-induced changes in sphingolipid levels to physiologic measures.

	BMI-SDS		HOMA-IR		HbA1c	
Metabolite	**r**	**p val**	**r**	**p val**	**r**	**p val**
N-Acylsphingosines (Ceramides)						
d18:0/16:0	-0.09	0.617	0.15	0.418	0.24	0.200
d18:1/14:0	-0.17	0.359	0.21	0.242	0.12	0.503
d18:1/16:0	-0.09	0.635	0.16	0.377	0.27	0.139
d18:1/18:1	-0.09	0.621	0.20	0.283	0.03	0.856
d18:1/24:1	-0.01	0.962	0.06	0.745	**0.41**	**0.022**
Sphingomyelins						
d16:1/22:1[a]	-0.08	0.673	0.17	0.362	0.03	0.887
d18:0/16:1	-0.08	0.671	0.14	0.453	0.16	0.390
d18:0/18:1	0.05	0.804	0.22	0.226	0.19	0.309
d18:0/24:0	-0.19	0.300	0.08	0.677	0.07	0.717
d18:1/20:0	-0.04	0.809	0.12	0.690	0.25	0.174
d18:1/23:0	-0.04	0.808	0.05	0.773	**0.40**	**0.027**
d18:2/21:0	-0.10	0.586	0.16	0.370	0.08	0.667
GlcCer / GalCer						
d15:2/18:0	-0.02	0.901	0.15	0.423	0.25	0.168
d18:1/16:0	0.01	0.944	0.13	0.493	0.29	0.113
d18:1/18:0	0.08	0.662	0.17	0.344	**0.36**	**0.046**
d18:1/24:1	0.01	0.936	0.11	0.551	**0.43**	**0.016**
d18:1/16:1	-0.06	0.744	0.07	0.713	0.20	0.277
d18:1/XX[b]	-0.13	0.462	-0.03	0.883	**0.42**	**0.020**
d18:2/23:0	0.02	0.921	0.14	0.447	0.23	0.207
diGalCer / LacCer						
d18:1/16:0	0.04	0.810	0.17	0.355	0.29	0.114
d18:1/18:0	0.05	0.794	0.11	0.539	**0.38**	**0.036**
d18:1/24:1	0.01	0.952	0.01	0.970	**0.54**	**0.002**
Sulfatides						
d18:1/16:0	-0.11	0.545	0.09	0.608	0.29	0.115
d18:1/22:0	0.01	0.946	0.16	0.395	0.26	0.153
d18:1/24:1	-0.01	0.973	0.10	0.577	**0.41**	**0.024**