**Original Article**
PEDIATRIC OBESITY

Obesity

# Lifestyle Intervention Decreases Urine Trimethylamine *N*-Oxide Levels in Prepubertal Children with Obesity

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**Objective:** Early lifestyle interventions in children with obesity decrease risk of obesity and metabolic disorders during adulthood. This study aimed to identify metabolic signatures associated with lifestyle intervention in urine samples from prepubertal children with obesity.

**Methods:** Thirty-four prepubertal children with obesity were studied before and after a 6-month lifestyle intervention program, and anthropometric, metabolic, and nutritional variables were collected. A nuclear magnetic resonance approach was applied to obtain the metabolomic profile from urine samples. Partial least squares-discriminant analysis (PLS-DA) was used to achieve group classification and variable importance on projection (VIP) for biomarker selection.

**Results:** The intervention reduced caloric intake by 10% ($P<0.05$) and BMI standard deviation score by 0.47 SD ($P<0.001$). PLS-DA identified trimethylamine *N*-oxide (TMAO, VIP = 2.21) as the metabolite with the highest discrimination properties between groups. Urine TMAO levels were reduced after the intervention ($P<0.05$). TMAO is a biomarker of cardiovascular disease risk and is a product of gut microbiota-dependent metabolism of certain dietary compounds, including choline. Notably, changes in TMAO levels after the intervention did not correlate to differences in choline intake but were inversely associated with fiber intake ($P<0.05$).

**Conclusions:** These results indicate that lifestyle intervention decreases TMAO levels in children with obesity.

*Obesity* (2018) **26**, 1603-1610. doi:10.1002/oby.22271

## Introduction

Worldwide prevalence of obesity in children and adolescents has steadily increased during the past decades (1). Childhood obesity is of special concern because excessive weight and adiposity during early ages are major risk factors for adult obesity and associated metabolic disorders, including type 2 diabetes and cardiovascular disease (2-6). Fortunately, reversing overweight before early adulthood, and especially prior to puberty, significantly decreases future risk of obesity and metabolic disease (5,6). In children with obesity, the prepubertal stage is associated with a more metabolically healthy obesity status, in which cardiovascular risk factors are not overtly impaired (7). Indeed, entering puberty entails important physiological changes, including decreased insulin sensitivity, and is a major driver of metabolic dysregulation in children with obesity (7,8). Thus, lifestyle interventions in prepubertal children with obesity are crucial to decrease risk of disease during adulthood (6).

High-throughput metabolomic technologies developed during the past decade have allowed a comprehensive characterization of metabolic profiles in complex biological systems (9). Plasma and urine are commonly used in metabolomic studies, as they provide a metabolic fingerprint for each individual and can be easily obtained. Several metabolomic studies examining plasma

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**Funding agencies:** This work was supported by a Marie Curie FP7-PEOPLE-2011-CIG to CL.
**Disclosure:** The authors declared no conflict of interest.
**Author contributions:** MJL, JCJ, MR, and CL designed the study and analyzed the data; MJL, ML, and MR implemented the intervention and collected data; SS and OY performed the metabolomics analysis; DC and PC performed the statistical analyses; MJL and CL wrote the manuscript; all authors were involved in editing and had final approval of the submitted version.
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Additional Supporting Information may be found in the online version of this article.
**Received:** 15 March 2018; **Accepted:** 8 July 2018. **Published online** 11 September 2018. doi:**10.1002/oby.22271**

metabolites associated with obesity in children and adolescents have identified increased circulating levels of branched-chain amino acids, acylcarnitines, and steroid-related metabolites in children and adolescents with obesity compared with normal weight controls (10-13). Urine has the advantage that it can be obtained in large quantities in a noninvasive manner. Furthermore, urine accumulates metabolic breakdown products from dietary components, environmental contaminants, and endogenous and bacterial metabolites, providing an excellent ground for disease biomarker discovery. Two recent reports comparing urine samples from children with obesity and normal weight have found several differentially expressed metabolites among groups, including acylcarnitines, amino acids, glycerophospholipids, glucose, and xylitol (14,15). Interestingly, a common finding from all these studies in plasma and urine is the identification of inflammation and oxidative stress biomarkers associated with childhood obesity.

Longitudinal studies evaluating the metabolic effects of lifestyle intervention programs in children with obesity are scarce. Plasma levels of tyrosine, glutamine, methionine, phosphatidylcholine, and sphingomyelin species have been shown to normalize after lifestyle or bariatric surgery interventions in children and adolescents (16-18). Furthermore, our laboratory recently identified a plasma sphingolipid metabolism signature associated with lifestyle intervention in a cohort of prepubertal children with obesity (19). In the present study, we aimed to characterize the effects of lifestyle intervention on the urine metabolome from prepubertal children with obesity. For this purpose, we performed a nuclear magnetic resonance (NMR)-based metabolomic analysis of urine samples in a subset of subjects from this cohort of prepubertal children and analyzed associations between potential biomarkers and changes in nutritional and physiological parameters.

# Methods

## Study participants

This is an observational study of the impact of lifestyle intervention on the urine metabolome from prepubertal children with obesity. Subjects in this study are a subset from a previously reported cohort recruited at the Hospital Sant Joan de Déu in Barcelona, Spain (19). Pediatric patients with obesity referred to our hospital usually show very high BMI standard deviation scores (BMI-SDS) and have failed to respond to treatment protocols at primary care centers. The study was approved by the hospital's ethic committee (identification code PIC-21-12), and signed informed consent was obtained from all parents. Inclusion criteria were prepubertal children (7 to 10 years old) with obesity, defined as BMI-SDS > 2 SD for a given age and sex, using the World Health Organization (WHO) reference; prepubertal stage was defined as Tanner stage I breast development for girls and testicular volume less than 4 mL in boys. Fifty-three children were originally included in the study (19). From the 43 children who provided urine samples at baseline and 6 months, 6 had pubertal status at the end of the intervention and three had altered C-reactive protein levels (> 50 μg/mL), suggesting concomitant infection; these subjects were excluded from the analysis. Therefore, paired samples from 34 subjects at both baseline and 6 months were included in the study and analyzed.

## Lifestyle intervention program

The program is the routine protocol for children with obesity at the hospital and has been described in detail elsewhere (19). Briefly, it consists of a 6-month intervention based on nutritional counseling following the Mediterranean diet and WHO recommendations. A normocaloric diet based on normal weight for height adjusted for age and sex was designed for each participant, consisting of 55% kcal from carbohydrates (less than 10% from sugars), 15% kcal from protein, and 30% kcal from lipids (less than 10% saturated fat), promoting intake of fruits and vegetables. Counseling on increasing physical activity was also provided to the participants.

## Data and sample collection

Anthropometric data and blood and urine samples were obtained at baseline and after the lifestyle intervention program (6-month time point). BMI-SDS was obtained using "Anthro Plus" software (version 1.0.4; WHO) for a given age and sex. Blood and urine samples were taken in the morning after 8 to 10 hours of overnight fasting. Urine samples were aliquoted and stored at −80ºC until further analysis.

## Dietary assessment

Nutritional data were evaluated at baseline and 6-month time points by using a 4-day food record (3 consecutive weekdays and 1 holiday). Directions for completing the food records were provided during the screening visit, and parents were instructed to complete them the week before the study visits (both baseline and 6-month). Records were then validated by a nutritionist in a personal interview during the study visits with the parents and children. Validation of portion size was achieved by showing images used in our clinical practice. Thirty participants provided the food records at both the baseline and 6-month time points. Food records were analyzed with DIAL software (version 3.3.0.0, Alce Ingeniería, S.L., Madrid, Spain) to determine total caloric intake, nutrient composition, and food group classification.

## Urine NMR spectroscopy

For NMR measurement, 200 μL of buffer phosphate (1.5mM $Na_2HPO_4/NaH_2PO_4$ in $D_2O$, pH = 7.2) containing 0.62mM 3-trimethyl-silyl[2,2,3,3-d4] propionate (TSP) as internal reference was added to 400 μL urine (adjusted pH = 7.2), and the resulting mixture was subsequently transferred to a 5-mm NMR tube. $^1$H-NMR spectra were recorded at 298K on a Bruker AVANCE III 600 spectrometer (Bruker, Billerica, Massachusetts) operating at a proton frequency of 600.20 MHz using a 5-mm CPTCI triple resonance ($^1$H, $^{13}$C, $^{31}$P) gradient cryoprobe (Bruker). One-dimensional $^1$H pulse experiments were carried out using the nuclear Overhauser effect spectroscopy (NOESY)-presaturation sequence (RD-90º–t1–90º–tm–90º ACQ) to suppress the residual water peak. t1 time was set to 4 μs, tm (mixing time) was 100 milliseconds, and recycling delay time was 7 s. The 90° pulse length was calibrated for each sample varying from 16.3 to 18.9 μs. Spectral width was 12.000 Hz (20 ppm), and a total of 64 transients were collected into 64k data points for each spectrum. The acquired spectra were phased, baseline-corrected, and referenced to a TSP signal at δ (0.00 ppm). Several database engines (BBioref AMIX database

[Bruker], Chenomx, and the Human Metabolome Database) (20) were used for $^1$D-resonances assignment and metabolite identification. Variables were normalized by creatinine content and log2-transformed.

### Statistical analysis

Unless otherwise stated, variables are described by mean and 95% confidence intervals (CI) and compared between baseline and 6-month time points by two-tailed paired *t* test. Normality was tested with the Kolmogorov-Smirnov test. Partial least squares-discriminant analysis (PLS-DA) was performed to achieve discrimination between baseline and 6-month groups. Metabolites with variable importance for the projection (VIP) values higher than 1.50 were considered to contribute to group separation. Multivariate linear regression adjusted for sex, age, baseline BMI-SDS, and change in caloric intake was applied to assess potential associations between variables. Statistical methods were implemented in R 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria). $P < 0.05$ was considered significant.

**TABLE 1** Demographic and clinical characteristics of study participants

| | Baseline | 6 Months | *P* |
|---|---|---|---|
| Subjects (F/M) | n = 34 (15/19) | | – |
| Age (y) | 9.0 (8.7-9.4) | 9.6 (9.2-9.9) | – |
| Weight (kg) | 55.0 (50.2-59.7) | 55.1 (50.3-60.0) | 0.770 |
| Height (m) | 1.40 (1.37-1.43) | 1.43 (1.40-1.47) | **<0.001** |
| BMI-SDS | 3.56 (3.27-3.84) | 3.09 (2.85-3.33) | **<0.001** |
| Waist circumference (cm) | 83.6 (80.2-86.9) | 81.8 (78.2-85.5) | **0.005** |
| HOMA-IR | 2.81 (2.36-3.40) | 2.81 (2.28-3.34) | **0.960** |
| HbA1c (%) | 5.4 (5.3-5.4) | 5.2 (5.1-5.3) | **<0.001** |
| Total cholesterol (mg/dL) | 166 (157-175) | 180 (169-190) | **0.002** |
| LDL-cholesterol (mg/dL) | 107 (97-115) | 115 (107-124) | **0.020** |
| HDL-cholesterol (mg/dL) | 43 (40-47) | 45 (41-48) | 0.363 |
| Triglycerides (mg/dL)[a] | 69 [60-95] | 80 [65-119] | **0.011** |
| SBP (mmHg) | 113 (110-115) | 111 (108-114) | 0.437 |
| DBP (mmHg) | 69 (67-72) | 69 (67-71) | 0.939 |
| Creatinine (mg/dL) | 0.57 (0.44-1.29) | 0.57 (0.55-0.59) | 0.970 |
| ALT (UI/L) | 20.9 (18.5-23.3) | 20.8 (18.2-23.5) | 0.881 |
| AST (UI/L) | 21.6 (20.0-23.3) | 22.6 (20.7-24.4) | 0.492 |

Subjects were 79% Caucasian ($n = 27$), 12% Hispanic ($n = 4$), 6% North African ($n = 2$), and 3% Asian ($n = 1$). Values presented as mean (95% CI), and significance assessed by two-tailed paired Student *t* test Bold font indicates $P < 0.05$.
[a]Non-normal distributed variables are presented as median [IQR], and significance assessed by Wilcoxon matched paired signed rank test.
BMI-SDS, BMI standard deviation score; HOMA-IR, homeostatic model assessment of insulin resistance; HbA1c, hemoglobin A1c; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TAG ALT, alanine transaminase; AST, aspartate transaminase; DBP, diastolic blood pressure; SBP, systolic blood pressure.

## Results

### Physiological and nutritional impact of lifestyle intervention in prepubertal children with obesity

Demographic and physiological characteristics for the original cohort have been previously described (19). Data for the 34 subjects included in the present study are shown in Table 1. As previously reported (19), the lifestyle intervention program resulted in a significant decrease in BMI-SDS, waist circumference, and hemoglobin $A_{1c}$, while no improvements in other variables, includinghomeostatic model assessment of insulin resistance, and even a modest increase in lipid profile parameters were observed (Table 1). Blood pressure, creatinine levels, and hepatic markers, including aspartate transaminase and alanine transaminase, were all in the normal clinical range at baseline and did not significantly change after the intervention. Notably, the decrease in BMI-SDS observed in this cohort of prepubertal children was sustained 1 year after the intervention program (19).

To assess the effects of the intervention on the participants' diet, we performed a comprehensive nutritional analysis at baseline and at the 6-month time point by analyzing 4-day food records. The nutritional analysis showed that caloric intake was reduced by 10% after the intervention ($P < 0.05$; Table 2); this decrease was mainly explained by lower intake of sugar and saturated fat ($P < 0.05$ for both; Table 2). Macronutrient distribution, fiber intake, and micronutrients, including total folic acid, choline, iron, and vitamin B6 and B12, were not significantly modified by the intervention (Table 2). We next performed a nutritional analysis by comparing the intake of different food groups. In agreement with the lower sugar intake shown in Table 1, a significant decrease in the sweets and added sugar group was observed after the intervention (Supporting Information Figure S1). Except for a trend to decreased meat intake ($P = 0.08$), no major changes in other main food groups were detected, including grains, legumes, vegetables, fruits, fish, dairy and eggs, and fats and oils (Supporting Information Figure S1).

### Effects of lifestyle intervention on the urine metabolome

We applied an NMR-based strategy to obtain the urine metabolome at baseline and postintervention time points. NMR profiling identified 32 distinct metabolites present in all urine samples. A representative $^1$H-NMR spectrum of one of the urine samples with the metabolite assignment is shown in Figure 1. Data for all subjects and metabolites are included in Supporting Information Table S1. We then performed PLS-DA to separate baseline and 6-month time point groups (Figure 2A), and VIP scores were used to identify metabolites contributing to group classification (Figure 2B). Trimethylamine *N*-oxide (TMAO) was the strongest metabolic classifier (VIP = 2.21) and was significantly decreased by the intervention (log fold change −0.66, $P < 0.05$; Figure 2C and Supporting Information Table S1). Other high-ranking metabolites (VIP > 1.50) that were significantly altered after the intervention (paired *t* test, $P < 0.05$) included xanthosine (log fold change 0.11, $P < 0.05$), 3-hydroxyisovalerate (log fold change −0.20, $P < 0.05$), and dimethylglycine (log fold change −0.35, $P < 0.05$) (see Supporting Information Table S1). Valine was also selected by the PLS-DA model (VIP = 2.05), although the difference between groups did not reach significance in a paired analysis ($P = 0.154$; Supporting Information Table S1).

**TABLE 2** Dietary assessment at baseline and 6-month time points

| | Baseline | 6-Month | *P* |
|---|---|---|---|
| Subjects (F/M) | *n* = 30 (14/16) | | |
| Energy intake (kcal) | 1,777 (1,650-1,904) | 1,614 (1,500-1,728) | **0.026** |
| Protein (g) | 77 (72-82) | 73 (68-79) | 0.286 |
| (%) | 17.7 (16.4-18.9) | 18.4 (17.4-19.5) | 0.292 |
| Carbohydrates (g) | 201 (180-222) | 182 (165-199) | 0.106 |
| (%) | 45.1 (42.5-47.6) | 44.9 (42.6-47.3) | 0.920 |
| Sugars (g) | 34 (24-43) | 23 (18-29) | **0.016** |
| (%) | 7.3 (5.4-9.2) | 5.9 (4.5-7.2) | 0.126 |
| Lipids (g) | 70 (62-78) | 63 (57-69) | 0.102 |
| (%) | 35.2 (32.7-37.7) | 35.0 (32.6-37.3) | 0.849 |
| Saturated (g) | 22 (19-24) | 19 (16-21) | **0.046** |
| (%) | 10.8 (9.9-11.7) | 10.3 (9.5-11.0) | 0.264 |
| Monounsaturated (g) | 33 (29-38) | 30 (27-34) | 0.248 |
| (%) | 16.7 (15.0-18.3) | 17.0 (15.6-18.3) | 0.725 |
| Polyunsaturated (g) | 9 (8-10) | 8 (7-9) | 0.110 |
| (%) | 4.7 (4.1-5.2) | 4.6 (4.2-4.9) | 0.743 |
| Fiber (g) | 17 (16-19) | 16 (14-18) | 0.344 |
| Calcium (mg) | 774 (679-869) | 786 (704-868) | 0.670 |
| Iron (mg) | 11.4 (10.2-12.6) | 11.3 (9.9-12.6) | 0.868 |
| Zinc (mg) | 8.4 (7.9-9.0) | 8.3 (7.6-8.9) | 0.696 |
| Sodium (mg) | 1,801 (1,636-1,965) | 1,670 (1,510-1,831) | 0.101 |
| Vitamin B6 (mg) | 2.0 (1.8-2.3) | 2.1 (1.8-2.3) | 0.804 |
| Vitamin B12 (μg) | 4.4 (3.7-5.1) | 5.0 (4.1-6.0) | 0.185 |
| Folic acid (μg) | 242 (213-271) | 243 (214-273) | 0.949 |
| Choline (mg) | 129 (112-145) | 135 (118-151) | 0.617 |

Thirty participants completed 4-day food records at both time points. Values presented as mean (95% CI), and significance assessed by two-tailed paired *t* test. Bold font indicates $P < 0.05$.

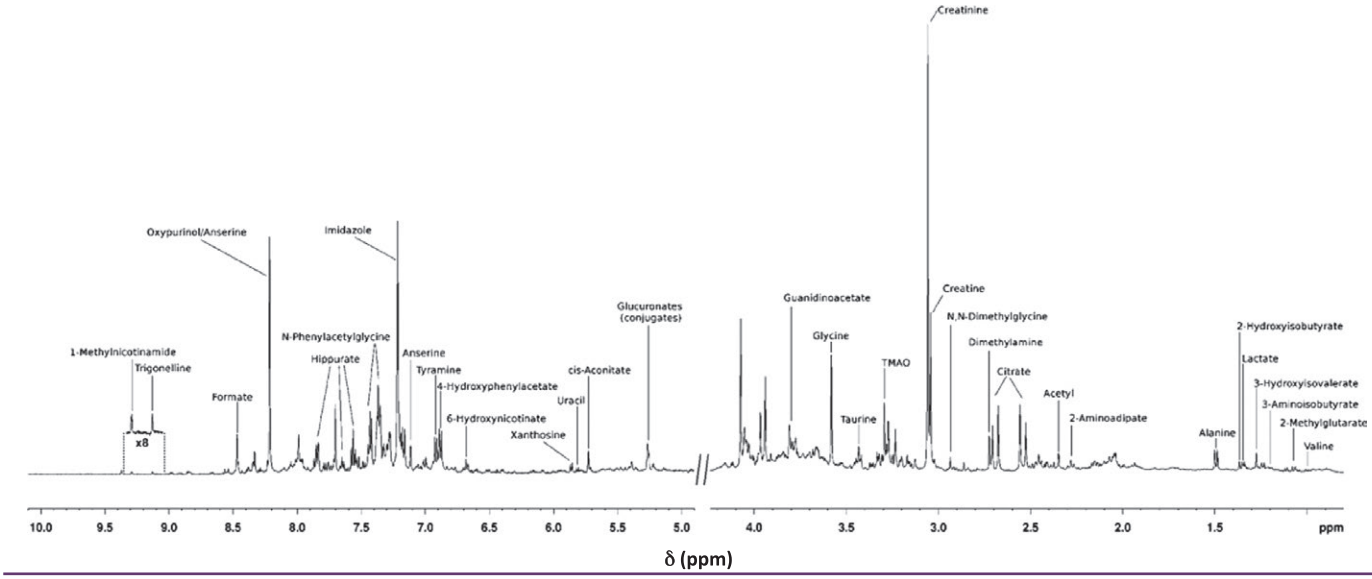


**Figure 1** Representative 600-MHz $^1$H-NMR spectrum of a urine sample including metabolite assignment.

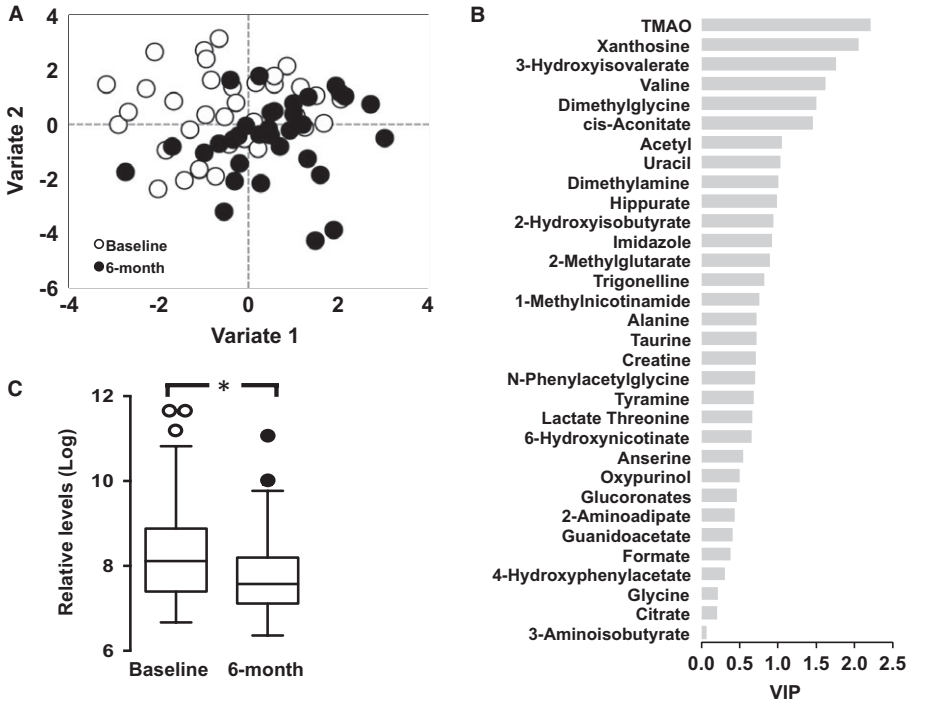


**Figure 2** Partial least squares-discriminant analysis identifies TMAO as a metabolic classifier between baseline and 6-month time points. (**A**) Subject score plot derived from the PLS-DA model; white and black circles indicate baseline and 6-month time points ($n=34$), respectively. (**B**) Variable importance on projection (VIP) values for all metabolites from the PLS-DA model. (**C**) Box plot of TMAO levels at baseline and 6 months. The *y* axis shows log-transformed values; whiskers represent the entire spread of the data points, excluding extreme points (higher or lower than the median ±1.5 times the interquartile distance), indicated with white (baseline) or black (6 months) circles. *Two-tailed paired *t* test, $P<0.05$.

**TABLE 3** Correlations between changes in TMAO levels and physiological and nutritional variables

| | | Unadjusted | | Model 1[a] | | Model 2[b] | |
|---|---|---|---|---|---|---|---|
| | *n* | *r* | *P* | ßstd | *P* | ßstd | *P* |
| Physiological | | | | | | | |
| BMI-SDS | 34 | 0.21 | 0.228 | 0.22 | 0.161 | 0.18 | 0.342 |
| SBP (mmHg) | 34 | 0.20 | 0.252 | 0.21 | 0.253 | 0.22 | 0.280 |
| DBP (mmHg) | 34 | 0.23 | 0.188 | 0.25 | 0.355 | 0.27 | 0.151 |
| Cholesterol (mg/dL) | 34 | **0.36** | **0.044** | **0.40** | **0.025** | **0.53** | **0.004** |
| LDL-Cho (mg/dL) | 34 | 0.29 | 0.105 | 0.32 | 0.091 | **0.46** | **0.023** |
| HDL-Cho (mg/dL) | 34 | 0.20 | 0.278 | 0.24 | 0.190 | 0.27 | 0.184 |
| Triglycerides (mg/dL) | 34 | 0.08 | 0.676 | 0.09 | 0.633 | 0.08 | 0.708 |
| Nutritional | | | | | | | |
| Protein (g) | 30 | −0.15 | 0.435 | −0.13 | 0.503 | −0.06 | 0.731 |
| Carbohydrates (g) | 30 | −0.06 | 0.749 | −0.05 | 0.800 | 0.07 | 0.583 |
| Fat (g) | 30 | −0.08 | 0.660 | −0.10 | 0.613 | 0.00 | 0.982 |
| Fiber (g) | 30 | **−0.42** | **0.020** | **−0.39** | **0.029** | **−0.34** | **0.044** |
| Choline (mg) | 30 | −0.01 | 0.957 | −0.03 | 0.897 | 0.00 | 0.992 |

Multivariate linear regression applied to assess potential associations between intervention-induced changes in TMAO levels and changes in physiological and dietary parameters (calculated as difference between 6-month and baseline values). Bold font indicates $P<0.05$.
[a]Model 1, adjusted for sex, age, and baseline BMI-SDS.
[b]Model 2, model 1 further adjusted for change in caloric intake.
ßstd, standardized beta coefficient from the regression model; BM-SDS, BMI standard deviation score; SBP, systolic blood pressure; diastolic blood pressure; LDL-Cho, low-density lipoprotein cholesterol; HDL-Cho, high-density lipoprotein cholesterol.

### Correlations of changes in urine TMAO levels with cardiometabolic and nutritional variables

TMAO levels are strongly associated with cardiovascular risk and atherosclerosis in adult humans (21-24). Even though cardiometabolic risk parameters in our cohort of prepubertal children were within the normal clinical reference range, including blood pressure and lipid profile parameters, we studied potential associations between changes in TMAO and these variables. While we observed no correlation between differences in TMAO concentration and BMI-SDS or blood pressure, there was a direct association with changes in total cholesterol levels and, to a lesser extent, with low-density lipoprotein cholesterol (Table 3).

TMAO is mostly derived from specific dietary components, including choline-containing compounds and L-carnitine, which are converted into trimethylamine (TMA) by intestinal bacteria and further transformed to TMAO in the liver (for review, see (25)). The fact that TMAO sources mostly consist of dietary components prompted us to assess potential correlations between changes in urine TMAO levels and nutritional variables. As observed in Table 3, the decrease in TMAO levels was not associated with improvements in macronutrient intake. Interestingly, we observed a significant inverse correlation between changes in TMAO levels and fiber intake, even after adjusting for sex, age, baseline BMI-SDS, and change in caloric intake (Table 3). No association with total choline intake was found (Table 3). Other dietary sources of TMAO include fish (high content of TMAO/TMA) and meat (high content of L-carnitine); thus, we next assessed potential correlations between TMAO levels and the different food groups. Interestingly, the decrease in TMAO concentration after lifestyle intervention was not associated with differences in fish or meat intake (Supporting Information Table S2), suggesting that changes in intake of L-carnitine or TMAO/TMA were not contributing to the decrease in TMAO levels. We observed an inverse correlation between the change in TMAO levels and legume consumption (Supporting Information Table S2), in agreement with the high fiber content of this food group and the significant association observed with fiber intake (Table 3).

## Discussion

Childhood obesity predicts adult obesity and associated complications, and thus early interventions in children are crucial to decrease future disease risk. As we have previously described (19), children in our cohort were exclusively prepubertal and had significantly decreased BMI-SDS and waist circumference after the intervention. These subjects showed a very high degree of obesity at baseline (BMI-SDS = 3.56) and, despite the significant decrease in BMI-SDS, still had a high level of obesity after the intervention (6-month BMI-SDS = 3.09). Fasting glucose, lipid profile parameters, and blood pressure values at baseline were within the normal clinical reference range for their age, and no improvements were observed for these variables after the intervention. Several studies in prepubertal children with obesity have also found only minor effects on glucose and lipid metabolism after lifestyle intervention (7,26,27). Prepubertal children have a more distinct metabolic profile than pubertal or adolescent individuals (7), highlighting the importance of interventions during this early stage.

Our metabolomic study in urine samples from prepubertal children with obesity identified several metabolic classifiers between baseline and the 6-month time point, including TMAO, 3-hydroxyisovalerate, xanthosine, dimethylglycine, and valine. Among them, TMAO was the top-ranking metabolite in the model, showing the highest VIP value and the highest fold change in the group comparison. TMAO levels were significantly decreased after lifestyle intervention. TMAO is strongly associated with cardiovascular risk in humans (21-24), and it has been mechanistically linked to the development of atherosclerosis in mice (21,22,28,29). Atherosclerosis is a progressive and cumulative process and a major pathophysiologic factor in cardiovascular disease. Thus, lowering TMAO is considered a potential therapeutic strategy for atherosclerosis (30) and might be of special relevance in children to decrease long-term cardiovascular risk. In this regard, and even though cholesterol levels in children from our cohort were within the normal reference range, we observed a direct association between changes in TMAO and cholesterol levels, further supporting the potential link between TMAO and cardiovascular risk observed in adult subjects. However, a causal mechanistic link between TMAO and cardiovascular disease in humans is yet to be demonstrated (for review, see (25)), and further studies will be required to determine whether decreasing TMAO might be useful to attenuate future atherosclerosis and cardiovascular risk in prepubertal children with obesity.

TMAO production is intimately linked to gut microorganism metabolism (22-24,28). Certain commensal bacteria can metabolize specific nutrients (mainly choline, choline-containing compounds, and L-carnitine), producing TMA; TMA is then absorbed into portal circulation, converted into TMAO by the hepatic enzymes FMO1/FMO3 (flavin containing monooxygenase 1 and 3) (21), and finally excreted into urine (25). Thus, TMAO concentration could be modulated at multiple levels, including intake of precursors, bacterial and hepatic metabolism, and urine excretion. First, TMAO, choline, and L-carnitine intake modulates circulating TMAO levels (22,23). Our multivariate analysis showed no correlation between the changes in TMAO levels and total choline intake, indicating that the decrease in TMAO is not a direct effect of lower choline intake. The software used in this study did not provide quantification for L-carnitine or TMAO intake. Given that main dietary sources of these nutrients include meat (especially rich in L-carnitine) and fish (rich in TMAO) (25), we analyzed intake of these two food groups. Although meat consumption tended to decrease after the intervention, we observed no significant association between changes in TMAO concentration and differences in meat or fish consumption, suggesting a minor role for dietary L-carnitine and TMAO/TMA intake in lowering TMAO levels. Second, differential hepatic FMO1/FMO3 activity can modulate TMA conversion into TMAO (21). Participants in our study showed normal liver function at baseline, assessed by circulating aspartate transaminase and alanine transaminase activities, and there were no changes with the intervention. Unfortunately, we could not directly assess FMO1/FMO3 levels or activity, as liver biopsies from this cohort were unavailable. Third, excretion into urine can also be a factor in determining TMAO levels. Participants showed plasma creatinine levels within the normal range at baseline and 6 months, suggesting that renal function did not significantly contribute to modulating urinary TMAO levels.

Finally, another possibility is that changes in specific gut bacteria could alter precursor metabolism, modulating TMA synthesis and consequently affecting TMAO production. For instance, directly inhibiting microbial TMA synthesis in mice was shown to lower TMAO levels, decreasing atherosclerosis (29). The intestinal microbiome has recently emerged as an important contributor to metabolic disease (31). Diet is a main determinant of intestinal bacteria composition (32,33), and dietary interventions can modulate gut microbiota composition in human subjects (34,35). Unfortunately, fecal samples from subjects in our cohort were not available, and we could not determine the impact of the intervention in the gut microbiome. However, we observed that the decrease in TMAO levels was inversely associated with legume intake, a food group characterized by high fiber content, and with fiber intake itself. Fiber provides substrates for bacterial fermentation, regulates intestinal transit time, and is able to shape microbiota composition (35,36). These potential differences induced by dietary fiber in the gut microbiome could be modulating choline and L-carnitine metabolism and consequently modifying TMAO production. This hypothesis is supported by a study demonstrating that a 30-day intensive intervention with a high-fiber diet modulated gut microbiota in children and adolescents with obesity, leading to lower urine TMAO levels (35). Thus, we can speculate that changes in TMAO levels might reflect differences in gut microbiota composition, possibly related to fiber intake. However, we cannot exclude the possibility that other dietary components could contribute to decreasing TMA production, ultimately affecting TMAO content.

A strength of this study is the exclusively prepubertal population, which eliminates potential confounding factors related to puberty (7,8). Limitations include the relatively modest number of subjects, the fact that dietary registries are parental reported and some meals might not have been accurately assessed, and the potential underreporting of energy intake inherent to collecting dietary intake data using food records. Finally, children with obesity attending our hospital had failed to respond to primary care protocols, and a control group with no intervention was not ethically justified for this observational study; thus, we cannot exclude the possibility that some changes could occur independently of the intervention program.

In summary, our data indicate that urine TMAO levels decreased after lifestyle intervention in prepubertal children and suggest that changes in the gut microbiome could at least in part mediate this response. Given that TMAO is a strong biomarker of cardiovascular risk, our results also suggest that the reduction in TMAO might contribute to the beneficial effects of lifestyle intervention on long-term cardiovascular disease risk in children. However, further studies are required to demonstrate whether decreasing TMAO concentration can provide a strategy to decrease future cardiovascular risk in prepubertal children with obesity.O

## Acknowledgments

We are indebted to the families and children who voluntarily participated in the study. We thank the "Biobanc de l'Hospital Infantil Sant Joan de Déu per a la Investigació" integrated in the Spanish Biobank Network of Instituto de Salud Carlos III for sample procurement. We also thank Miguel A. Rodriguez from the Center for Omic Sciences (Reus, Spain) for assistance in NMR measurements.



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