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TITLE PAGE

Title: Metabolomics reveals sex-specific pathways associated with changes in adiposity and muscle mass in a cohort of Mexican adolescents

Running Title: Metabolites related to body composition change

Authorship:

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Competing Interests Statement:

The authors declare that they have no competing interest.

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ABSTRACT

Background: Alterations in body composition during adolescence relates to future metabolic risk, yet underlying mechanisms remain unclear.

Objectives: To assess the association between the metabolome with changes in adiposity (BMI, waist circumference [WC], triceps skinfold [TS], and fat percentage [BF%]) and muscle mass (MM).

Methods: In Mexican adolescents (n=352), untargeted serum metabolomics was profiled at baseline and data was reduced by pairing hierarchical clustering with confirmatory factor analysis, yielding 30 clusters with 51 singleton metabolites. At the baseline and follow-up visits (1.6-3.5 years apart), anthropometry was collected to identify associations between baseline metabolite clusters and change in body composition (Δ) using seemingly unrelated and linear regression.

Results: Between visits, MM increased in boys and adiposity increased in girls. Sex differences were observed between metabolite clusters and changes in body composition. In boys, aromatic amino acids (AAA), branched chain amino acids (BCAA) and fatty acid oxidation metabolites were associated with increases in ΔBMI, and ΔBF%. Phospholipids were associated with decreases in ΔTS and ΔMM. Negative associations were observed for ΔMM in boys with a cluster including AAA and BCAA, whereas positive associations were found for a cluster containing tryptophan metabolites. Few associations were observed between metabolites and body composition change in girls, with one cluster comprising methionine, proline, and lipids associated with decreases in ΔBMI, ΔWC, and ΔMM.

Conclusion: Sex-specific associations between the metabolome and change in body composition were observed, highlighting metabolic pathways underlying adolescent physical growth.

Abbreviations**:** AA, amino acids; AAA, aromatic amino acids; AC, acylcarnitine; AICAR, 1,5-phosphoribosyl-5-amino-4-imidazolecarboxamide; BC, body composition; BCAA, branched chain amino acids; BF%, body fat percentage; BMI, body mass index; BMIz, BMI z-score; CFA, confirmatory factor analysis; CMD, cardiometabolic disease; DiC-FA, dicarboxylic fatty acid; ELEMENT, Early Life Exposures in Mexico to ENvironmental Toxicants; FFM, fat free mass; FA, fatty acid; HCA, hierarchical cluster analysis; IOTF, International Obesity Task Force; IQR, interquartile range; IR, insulin resistance; LC-MS, liquid chromatography mass spectrometry; LOESS, locally estimated scatterplot smoothing regression; LPC, lysophosphatidylcholine; LV, latent variable; MLR, multiple linear regressions; MM, muscle mass; PA, physical activity; Phe, phenylalanine; SES, socioeconomic status; SUR, seemingly unrelated regression; TEI, total energy intake; Thr, threonine; TS, triceps skinfold; WC, waist circumference; WHO, World Health Organization

INTRODUCTION

Adolescence is characterized by rapid changes in body composition (BC) that are sex-specific and reflect physical growth and sexual maturation. Boys tend to become leaner and gain muscle mass during puberty, while girls increase levels of adiposity.[1] Variations in BC changes among adolescents may have implications for development of obesity and cardiometabolic disease (CMD). Higher adiposity changes, specifically during puberty, are associated with mortality and cardiovascular disease, especially among men[2-5]. Nevertheless, there is limited understanding of metabolic pathways related to body composition change (ΔBC) during adolescence and their implications for later CMD.

Cross-sectional studies have identified metabolic pathways associated with body mass index (BMI) and CMD in adolescents.[6-9] Correlating metabolites with clinical and phenotypic outcomes can provide biomarkers of development of CMD, potentially identifying future disease risk.[10] Lipids, including diacylglycerols, have been related to BMI z-score and a metabolic risk score, with a larger effect size among girls.[11,12] Branched chain amino acids (BCAA) and aromatic amino acids (AAA) were associated with BMI z-score (BMIz) in boys,[11] aligning with studies in adolescents with obesity.[6,8] Consistent relationships between BCAA and AAA with insulin resistance (IR) have not been demonstrated in adolescents.[7,8,10,11] For example, Perng et al[13] observed that BCAAs were inversely associated with change in IR measured by C-peptide in adolescents; opposing findings in adults.[14]

These findings suggest that cross-sectional studies may obscure the direction of the relationships during adolescence, e.g., whether adiposity alters the metabolome leading to metabolic dysfunction or metabolic dysfunction alters the metabolome leading to increased fat storage and weight gain. Nevertheless, few studies have examined how the metabolome is related to

prospective ΔBC. Wahl et al.[15] observed that lipids predicted change in BMI in children with obesity undergoing weight loss. To our knowledge, no reports have considered how the metabolome is associated with ΔBC including changes in both lean and fat mass in the same adolescents. Further, metabolite and lipid profiles are dependent on sex,[16] emphasizing the importance of examining sex differences during this developmental period. Our aim was to examine baseline metabolomic profiles in relation to changes in BC within a cohort of adolescents. Our results may generate biological hypotheses on the sexual dysmorphism involved in BC changes.

METHODS

Setting and Subjects

Subjects included adolescent offspring of women participating in the Early Life Exposures in Mexico to ENvironmental Toxicants (ELEMENT) project. ELEMENT comprises three birth cohorts originally recruited over a ten-year period (1994-2004).[17] Subsequently, 622 children from cohorts 2 and 3 were re-recruited in 2008-2012 for a study of fetal lead exposure and long-term cognitive outcomes. Among these, 554 youth (ages 9 to 18 years) were re-recruited in 2015-2016 (baseline visit) and 519 adolescents (ages 11 to 20 years) returned for the 2016-2019 follow-up visit. Trained research staff administered interviews including sociodemographic and lifestyle behaviors; fasting blood samples for metabolomics were collected at baseline and anthropometry and BC were measured at both time points. A total of 404 youth had untargeted metabolomics measured at baseline. The final analytic sample included 352 participants (182 girls and 171 boys) with complete information on metabolomics, BC, and covariates. Compared to all adolescents at the baseline visit (n=554), the final analytic sample was younger (13.8±1.9 vs 15.8±2.0 years; $p<0.0001$) and had lower muscle mass (20.6±4.9 vs 23.4±5.0 kg; $p=0.016$); no other differences were found for total energy intake (TEI), physical activity (PA), socioeconomic status (SES) and BC.

Study protocols were reviewed and approved by the Research, Research Ethics and Biosafety Committees of the National Institute of Public Health of Mexico and the Institutional Review Board of the University of Michigan (HUM00034344). Subjects who were ≥ 18 years provided informed written consent. Subjects between 9 and 18 years old provided maternal consent and child assent.

Body composition

Multiple measures of BC allowed for comprehensive phenotyping including BMI, waist circumference (WC); an indicator of abdominal obesity, triceps skinfold (TS); a measure of peripheral fat reserves, body fat percentage (BF%); a measure of overall adiposity, and skeletal muscle mass (MM).[18,19] Participants wore a clinical examination gown and were asked to remove hair ornaments, shoes and socks according to the ELEMENT study protocol. Research assistants measured height to the nearest 0.5 cm with a BAME Model 420; Catálogo Médico, Tokyo, Japan with height rod (Model WB-3000m), weight to the nearest kg (InBody 270, Biospace, California, USA), WC to the nearest 0.1 cm at the iliac crest using a non-stretchable measuring tape (QM2000 QuickMedical; SECA model 201, Hamburg, Germany), and TS in mm (Lange calipers; Beta Technology, CA, USA) using standard anthropometry procedures.[20] BF% and MM were estimated using bioelectrical impedance equipment (InBody 270, Biospace, CA, USA). Staff obtained duplicate measures for height, WC and TS and the average of two measures was used for analysis. Changes in BC parameters were calculated by subtracting the follow-up value from the baseline, denoted by Δ.

Metabolomics

The untargeted metabolome was profiled in fasting serum samples using liquid chromatography mass spectrometry (LC-MS) with an 1290 Infinity Binary LC with a Waters Acquity HSS T3 1.8μm column and a 6530 quadrupole Time-of-Flight MS (Agilent Technologies, Inc., Santa Clara, CA).[21] Samples (100μL) were isolated using an extraction solvent (400 μL) containing methanol : acetonitrile : acetone (1:1:1). Chromatography run time was 20 minutes, using a varying methanol : water solvent gradient across the run. Mass spectrometry used an electron ion source mass detector. Mass spectrometry was performed by electrospray ionization with an Agilent Jetstream ion source, with full-scan mass spectra acquired over the *m*/*z* range 50–1500

Da. Positive and negative electrospray ionization modes were run. Raw data peak processing was performed using Agilent software (Agilent MassHunter Qualitative Analysis and Profinder, Santa Clara, CA), identifying the MS ion counts for each feature. The application of Binner[22] allowed for the visualization of feature relationships and the removal of redundant features. Annotated metabolites were identified using Post-Processor, a metabolomics naming library, via comparing their MS/MS spectra to internal or external standards run on the same instrument. Data normalization methods[23] used "pooled" reference samples were run in each batch.[24] Peak intensities were adjusted for batch drift using locally estimated scatterplot smoothing regression (LOESS) and between batches using a feature global median[25]. Missing peak intensities were imputed using K-nearest neighbor (K = 5) in features with ≥70% detection across samples (R package "impute"); features with <70% detection were removed. Metabolites were natural log-transformed and normalized to normal distribution with mean of 0 and variance of 1. The final metabolomics dataset contained 336 annotated metabolites grouped in nine metabolite classes: amino acids (AA; 12%), carbohydrates (2%), cofactors and vitamins (2%), energy metabolites (1%), exogenous metabolites (3%), lipids (70%), nucleotides (3%), peptides (5%), and xenobiotics (2%). Lipids are reported with the nomenclature as X:Y, where X is the length of the carbon chain and Y is the number of double bonds.

Covariates

Baseline covariates included age, TEI, sedentary time, moderate/vigorous activity, and household SES. Interviewers administered a semi-quantitative food frequency questionnaire, validated in a Mexican population,[26] to obtain adolescents' habitual dietary intake over the past seven days. TEI was calculated using food composition tables.[26] PA was estimated from accelerometers worn for 7 days[27] using Chandler's vector magnitude cutoffs,[28] classified as

average sedentary and moderate/vigorous (min/day). Household SES was reported using the Mexican Association of Marketing Research and Public Opinion Agencies questionnaire, consisting of 7 categories ranging from A/B (highest SES) to E (lowest SES).[29,30] Tanner stages for secondary sexual characteristics (e.g., pubic hair and genital development in boys; menarche and pubic hair and breast development in girls[31,32]) may be associated with the metabolome and BC, thus be considered potential confounders. Nevertheless, we did not include Tanner stages as covariates since pubertal progression coincides with and influences BC and likely mediates these associations.

Statistical Analysis

At baseline and follow-up, descriptive statistics were computed for measures of BC, ΔBC, and sociodemographic characteristics, stratified by sex. Sex-differences were evaluated using Student's t or Wilcoxon tests for normal and non-normally distributed continuous variables, respectively, and Fisher exact tests were used for categorical variables. The relationship between ΔBC and covariates were evaluated stratified by sex using ANOVA and Kruskal Wallis tests for normal and non-normally distributed variables, respectively. Proportion tests were performed to compare the change in the prevalence of obesity, using BMI for age according to the World Health Organization (WHO) (>3 Z-score)[33] and the International Obesity Task Force (IOTF) (sex and age-specific centiles corresponding to ≥ 30 kg/m^2 at 18 years)[34] criteria between visits by sex. For descriptive statistics, non-normally distributed variables were reported as medians (Q1, Q3); while normally distributed variables were reported as means±SD.

A confirmatory factor analysis (CFA) model was fit for all the metabolites within each cluster, with the clusters generated by hierarchical cluster analysis (HCA) using Spearman's rank correlation coefficients. To determine the final clusters from the HCA, we considered the

following criteria: a) ≥5 metabolites per cluster and b) an average pairwise correlation of $r \geq 0.15$, resulting in a dendrogram height of 3.5. A total of 30 clusters were generated, leaving 51 single metabolites (Figure 1; Table S1). Therefore, 30 CFA models were fit separately. CFA models aim to study the degree to which vectors of observed random variables can be used to assign values to one or more unobserved variables, which we call latent variables (LV). The investigation is largely accomplished by estimating and evaluating the loading of each observed random variable used to mine aspects of the unobserved LV. The CFA models were built up using the R package "lavaan" with the formulation as follows:

$$f = \sim m_1 + m_2 + \cdots + m_n,$$

where f is the unobserved LV we want to estimate and $\{m_1, \ldots, m_n\}$ are the n number of metabolites within one cluster under consideration. After model fitting, the predicted LV was extracted to be included as a covariate into seemingly unrelated regression (SUR). The main objective of CFA is to summarize the information contained in the n number of metabolites within one cluster by one single vector of predicted LV, thus, to reduce the number of covariates that need to be included into the SUR model. Details on the 30 clusters can be found in **Table S1**.

Prior to modeling this association, we determined if our ΔBC measures were correlated, stratified by sex (**Table S2**). Using Pearson's correlations, the four adiposity measures, ΔBMI, ΔWC, ΔBF% and ΔTS, displayed positive correlations (r) ranging between 0.616-0.906 in boys and 0.515-0.803 in girls. In both boys and girls, ΔMM was weakly correlated with ΔBMI, ΔWC, ΔBF% and ΔTS. We chose SUR (R package '*systemfit'*) to jointly analyze ΔBMI, ΔWC, ΔBF% and ΔTS. We ran multiple linear regressions (MLR) for ΔMM separately, due to its weak correlation with adiposity measures.

SUR is a multi-dimensional linear regression model consisting of several multiple regression models for correlated multiple outcomes, each having its own dependent variable and potentially different sets of exogenous explanatory variables.[35] The choice of this model for the present analysis is to utilize the correlation between adiposity measures to increase statistical power and to examine if clusters are simultaneously associated with a set of outcomes.

Using SUR, we evaluated the relationship between the baseline metabolome (30 clusters and 51 singletons) and change in adiposity measures (ΔBMI, ΔWC, ΔBF% and ΔTS) in sex-stratified models, while adjusting for baseline age, baseline outcome, TEI, SES, moderate and vigorous PA, and sedentary time. The nested models are used to evaluate the relative importance of each cluster sequentially in terms of goodness-of-fit via the residual sum of squares (**Table S4**).

The relationship between the metabolome (30 clusters and 51 singletons) and ΔMM was evaluated using MLR, while adjusting for the same variables. All clusters and singletons were included in the sex-stratified models. Like the SUR analysis, we examined the relative importance of each cluster and singleton metabolite to the model (**Table S5**). A sensitivity analysis was performed adjusting for Δage from baseline to follow-up. We found no statistically significant differences in the length of the follow up interval between boys and girls (p=0.147). In models with and without Δage, the same LVs were selected, and coefficients were minimally different, thus we reported the results from models without Δage (data available upon request). Statistical analyses were performed using the R statistical software.

RESULTS

Subject characteristics

Baseline and follow-up characteristics were collected over an interval of 1.6 to 3.5 years for 352 adolescents (**Table 1**). At baseline, adolescents were on average 13.8 years and approximately half had low to medium SES (45%), with no significant differences in distribution of household SES by sex (p=0.103). The median TEI was higher in boys (2399 kcal/d [interquartile range, IQR=1235 kcal/d] vs 1928 kcal/d [IQR=895 kcal/d], p<0.0001) and boys were more sedentary than girls (593.9±74.4 min/day vs 573.2±74.1 min/day, p=0.009). Girls had higher TS and BF% and boys had more MM (p<0.0001). At follow-up, adolescents were at average 15.8 years and similar sex differences were observed in BC measures, with higher WC, TS, and BF% in girls and higher MM in boys (**Table S6**).

Between baseline and follow-up, the prevalence of overweight decreased (WHO: 5.7%, p=0.007; IOTF: 2.3%, p=0.616), while the prevalence of obesity increased (WHO: 6.5%, p=0.002; IOTF: 2.3%, p=0.471). The prevalence of obesity increased more in girls than in boys (WHO: 8.8% vs 4.2, p=0.082; IOTF: 2.8% vs 1.7%, p=0.489, respectively) (**Table 1** and **Table S6**).

Increases in adiposity were observed in both sexes, with larger increases in girls compared to boys in ΔBMI (1.6±1.5 vs 1.0±1.8 kg/m^2, p<0.001), ΔWC (8.0±4.8 vs 4.8±5.5 cm, p<0.0001), and ΔTS (3.0±4.3 vs 1.1±5.7 mm, p<0.001). Girls increased ΔBF% while boys decreased (2.8±3.6 vs -1.6±5.4%, p=0.0001). Boys had a larger increase in ΔMM than girls (4.0±2.8 vs 1.6±1.4 kg, p<0.0001). Associations between the ΔBC and covariates are presented in **Table S7**, stratified by sex.

Association between metabolome and change in adiposity

Using sex-stratified SUR, the relationship between the baseline metabolome and change in adiposity measures (ΔBMI, ΔWC, ΔBF% and ΔTS) was evaluated. Metabolite cluster ID names are found in **Table 2**.

Metabolome and change in adiposity in boys

In boys, clusters 12 (ID: dicarboxylic fatty acids [DiC-FA]), 20 (ID: phospholipids), and 29 (ID: BCAA, AAA, and glucose) and singleton metabolites mannitol, dipeptide (Phe, Thr) and lysophosphatidylcholine (LPC) 16:0 were selected in the SUR model (**Figure 2a**). The dicarboxylic fatty acid cluster (cluster 12) was positively associated with ΔBMI (β= 3.1, p=0.038). The BCAA, AAA, and glucose cluster (cluster 29) was positively associated with ΔBF% (β=5.3, p=0.003). The phospholipid cluster (cluster 20) was inversely associated with ΔTS (β= -7.5, p=0.010). Several singleton metabolites were associated with ΔBF% measures. Lenticin was inversely associated with ΔBF% (β= -1.1, p=0.026) and LPC 16:0 was inversely associated with ΔBMI (β= -0.5, p=0.042), and ΔWC (β= -1.5, p=0.050). Dipeptide (Phe-Thr) was positively associated with all adiposity measures (ΔBMI: β= 0.6, p=0.048; ΔWC: β= 2.4, p=0.008; ΔTS: β= 2.3, p=0.008, and ΔBF: β= 1.6, p=0.038), and mannitol was positively associated with ΔTS (β= 1.8, p=0.017) and ΔBF% (β= 1.4, p=0.026), with a trending positive association with ΔBMI (β= 0.5, p=0.053).

Metabolome and change in adiposity in girls.

In girls, cluster 24 (ID: amino acid [methionine and proline] and lipids) and singleton metabolites hydroxy-fatty acid 10:0, 24:2 DiC-FA, mannitol, ursodiol and 2-piperidinone were selected in the SUR model (**Figure 2b**), observing an overlap with mannitol in boys' SUR model. The methionine, proline, and lipid cluster (cluster 24) was negatively associated with ΔBMI (β= -2.7, p=0.049), and ΔWC (β= -9.0, p=0.048). Singleton metabolites ursodiol (β= -0.9,

p=0.016), and hydroxy-FA 10:0 and β= -0.984, *p*=0.024) displayed significant inverse associations with ΔBF%. DiC-FA 24:2 was negatively associated with ΔBMI (β= -0.4, *p*=0.035), and mannitol was negatively associated with ΔTS (β= -1.2, *p*=0.014). 2-piperidinone was positively associated with ΔTS in girls (β= 0.9, *p*=0.029).

In boys and girls, all associations between metabolite clusters and change in adiposity are reported in **Table S8**.

Association between metabolome and change in muscle mass

Metabolome and change in muscle mass in boys.

In boys, clusters 21 (ID: phospholipids), 24 (ID: amino acid [methionine and proline] and lipids), 27 (ID: AAA and bile acids), and 29 (ID: BCAA, AAA, and glucose), and singleton metabolite 1,5-phosphoribosyl-5-amino-4-imidazolecarboxamide (AICAR) were significantly related to ΔMM (**Figure 3a**). The phospholipid cluster (cluster 21) was negatively associated with ΔMM (β= -0.7, *p*=0.047). The methionine, proline, and lipid cluster (cluster 24) and the BCAA, AAA, and glucose cluster (cluster 29) inversely associated with ΔMM (β= -3.3, *p*=0.026; β= -2.2, *p*=0.001, respectively). The AAA and bile acid cluster (cluster 27) was positively associated with ΔMM (β= 0.6, *p*=0.047). A positive association was observed between ΔMM and AICAR (β= -0.6, *p*=0.036).

Metabolome and change in muscle mass in girls.

In girls, clusters 13 (ID: long chain acylcarnitines and nucleotides) and 24 (ID: amino acid [methionine and proline] and lipids) were significantly associated with ΔMM (**Figure 3b**). The methionine, proline, and lipids cluster (cluster 24) was inversely associated with ΔMM (β= -2.4, *p*=0.031), in alignment with results in boys. The long-chain acylcarnitine (AC) and nucleotide

cluster (cluster 13) was positively associated with ΔMM (β= 140.8, *p*=0.041). In boys and girls, all associations between metabolite clusters and ΔMM are reported in **Table S9**.

DISCUSSION

In this longitudinal study, we quantified and assessed the baseline untargeted metabolome to predict Δadiposity and ΔMM in adolescents. Our analysis distinguished between fat tissue, using BF%, WC, TS, and BMI, and lean tissue, using MM. Furthermore, boys and girls diverge in type of ΔBC during adolescence; boys demonstrate marked decreases in TS with concomitant increases in MM, whereas girls show greater increases in total central and peripheral adiposity[36]. Our results demonstrate sex-specific associations between the metabolome and changes in body composition, highlighting metabolic pathways involved in adolescent development.

Branched chain and aromatic amino acids associated with body composition change

BCAA and AAA cluster displayed statistically significant associations with Δadiposity measures in boys only (cluster 29; **Figure 2a**), with higher baseline levels of these metabolites being associated with increases in BMI, WC, and TS between baseline and follow-up. These results align with previous cross-sectional studies[6,8] and elicit strong evidence for sex-differences in these essential AA's associations with adiposity.[11] Interestingly, two clusters containing BCAA and AAA exhibited opposite associations with ΔMM in boys, with significant positive associations with cluster 27 and inverse associations with cluster 29 (**Figure 3**). The amino acids in cluster 29 are leucine and phenylalanine and the amino acids in cluster 27 are tryptophan metabolites.

Overall, these results may suggest two different biological pathways driving the associations with essential AA, as ΔMM is not correlated with Δadiposity measurements in boys (**Table S2**). The first proposed pathway suggests that higher baseline essential AA are predictive of increasing adipose tissue in boys. Beginning over half a century ago,[37] strong evidence has been found for the association between BCAA and the AAAs phenylalanine and tyrosine with

obesity. The biological implications of these associations have been suggested by hypotheses of alterations in BCAA catabolism in white adipocytes and hepatocytes in individuals with obesity, (reviewed by Adams et al[38]), and the signaling role of BCAA, in particular leucine, in controlling metabolism (e.g. activation of mTOR signaling pathway) (reviewed by Zhang et al[39]). Our results may provide a biomarker of AA metabolism dysregulation (e.g., leucine in cluster 29), potentially associated with future metabolic health risks in boys. Although we did not measure insulin levels at the follow-up visit, elevations in BCAAs, tyrosine, and phenylalanine are predictive of type 2 diabetes incidence,[40] further illustrating the importance of these metabolites. The second proposed pathway suggests higher baseline essential AA are predictive of increasing MM in boys. The concept that dietary essential AA levels stimulate muscle protein synthesis is inconclusive,[41] potentially indicating that their elevation may reflect, rather than cause, changes in MM. Pubertal development is associated with increases in insulin-like growth factor 1 (IGF-1),[11] responsible for stimulating muscle growth and, in turn, increasing circulating AA. These associations may be more apparent in boys due to their larger gain of MM and earlier stages of puberty compared to girls (**Table 1**).

Fatty acid (FA) oxidation intermediates and phospholipids associated with body composition change

A long-chain DiC-FA cluster (cluster 12) was positively associated with ΔBMI in boys (**Table 2**). DiC-FA are lipids derived from FAs or fatty acyl-CoA esters during omega-oxidation[42] and are suggested to be a compensatory measure to maintain the tricarboxylic acid cycle due to inadequate glycolysis.[43] Although we did not quantify insulin resistance, boys within this age range are typically going through puberty, and therefore, are more insulin resistant. There is an association with increased extra-mitochondrial FA oxidation and insulin resistance, as evidenced

in girls with polycystic ovary syndrome[44]. These results may highlight alterations in fatty acids metabolism during puberty in boys with increasing BMI, evidenced by increased production of DiC-FA from omega-oxidation. In boys, Cluster 20 was inversely associated with ΔTS and Cluster 21 was inversely associated with ΔMM. Interestingly, these clusters both contain phospholipids and lysophospholipids of similar FA chain length and saturation. The main difference in their composition was the presence of alpha-tocopherol in Cluster 21. In a sensitivity analysis, alpha-tocopherol was removed from cluster 21, and the significance between Cluster 21 and ΔMM was lost, potentially suggesting the importance of this metabolite in the association.

Limited associations with metabolites and body composition change in girls

Few associations were observed between the metabolome and ΔBC in girls. The methionine, proline, and lipid cluster (cluster 24) was negatively associated with ΔBMI, ΔWC, and ΔMM in girls. Positive Pearson's correlations exist between ΔBMI and ΔMM (r=0.496) and ΔWC and ΔMM (r=0.395) in girls (Supplemental Table 2). BMI and WC measurements capture subcutaneous fat, visceral fat, bone, and muscle mass, suggesting that these measures may not reflect solely fat-mass[45]. Higher levels of metabolites within cluster 24 at baseline may be reflect of decreases in mass (MM) in girls, although the relationship between methionine and proline with muscle mass in girls is uncertain. Furthermore, the long chain AC and nucleotides cluster (cluster 13) was positively associated with ΔMM in girls (**Figure 3b**). The coefficient values from the CFA analysis of Cluster 13 varied greatly (**Supplemental Table 3**), potentially due to collinearity of metabolites, and duplicate metabolites (e.g. FA 18:1) within the cluster.

Sex differences in body composition changes during adolescence

Analyses were sex-stratified to account for the sexual dysmorphisms known to drive ΔBC during adolescence. Given the mean age of 13.5 years at baseline and 15.8 years at follow-up, boys were undergoing the pubertal transition during the study period, whereas most girls would had completed puberty (evidenced by 79.3% of girls having experienced menarche, a later milestone of puberty, before the baseline visit). Pubertal development in both boys and girls is associated with transient IR,[46] which could affect circulating metabolites,[47] especially around menarche and peak height velocity; a milestone that girls reach around 12 years and boys reach around 14 years. Thus, the higher number of associations between metabolites with Δadiposity and ΔMM observed among boys may represent being in the midst of the dynamic changes that are characteristic of puberty. The Fels Longitudinal Study provides evidence of a sexual dimorphism in the timing of ΔBC including that fat free mass (FFM) increased until age 15, then stabilized in girls while it continued to increase until 18 years in boys.[48] It could be argued that the differences in findings between boys and girls reflect pubertal tempo rather than sex differences *per se*. Confounding factors including pubertal changes in sex steroids could precede changes in metabolites and adiposity and lean mass. Future research on these participants after they reach full sexual maturity may elucidate these relationships.

Findings must be interpreted in light of strengths and limitations of this study. This study utilized comprehensive measurements of body composition during adolescence, enhancing our ability to consider associations of metabolites with changes in both adiposity and lean mass. The well characterized ELEMENT cohorts offer rich data on covariates that could confound associations, including sociodemographic characteristics, dietary intake, and physical activity. Utilizing a data-driven approach, such as HCA, allowed for a reduction of comparisons, paired with a sophisticated analytic approach using SUR to account for correlations between measures of

adiposity and lean mass. The complexity and diversity in the metabolites within clusters underscores the interconnectivity between metabolic pathways from AA, lipid, carbohydrate, and nucleotides; but it is challenging to interpret the biological implications for these groups of metabolites.

While accounting for fat and lean mass is a strength, our BC measures used BIA, which could have underestimated the fat mass in both sexes, introducing non-differential measurement error of BF%[49]. All participants live in Mexico City, potentially limiting generalizability of results, including to populations with different racial/ethnic composition.

Overall, our results demonstrated sex-specific associations between the metabolome and change in body composition measures, highlighting important metabolic pathways involved in adolescent development, in particular BCAAs, AAAs, and phospholipids with Δadiposity and ΔMM measurements in boys. Through profiling the untargeted metabolome, our results may provide impetus for future studies to assess if baseline levels of essential AA, FA oxidation intermediates, and phospholipids are causing or reflecting changes in body composition. The results of this study warrant future work in the ELEMENT cohort assessing the sex-specific relationship between IR during puberty and the metabolome to further elucidate the relationship between metabolites and cardiometabolic health during adolescent development.

Competing Interests: The authors declare that they have no competing interests.

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Data accessibility statement: The supporting data of this study is available from the ELEMENT team on reasonable request at: https://sph.umich.edu/cehc/element/access_data.html

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Figure Legends

Figure 1. Hierarchical clustering of metabolome. Dendrogram depicts tightness of metabolites (n=336) with the cut-off 3.5 for cluster designation determined by the inclusion of ≥ 5 metabolites, and Spearman correlation coefficient (r> 0.15). These criteria resulted in the creating of 30 metabolite clusters and 51 singletons metabolites. Color depicts the strength and direction of the Spearman correlations.

Figure 2. Sex differences in the relationship between the metabolome and change in adiposity measures. In seemingly unrelated regression models, the association between all 30 metabolite clusters and 51 singletons, the predictor, and change in adiposity measures (ΔBMI, ΔWC, ΔTS, and ΔBF%) was evaluated in **(a)** boys and **(b)** girls, including the covariates age, energy intake, socioeconomic status, moderate and vigorous physical activity, and sedentary time. Colors depict direction of association with numbers within the heatmap describing the estimated beta coefficients of the selected clusters. Significance of association denoted by asterisks ("*", *p*-value<0.05; "**", *p*-value <0.01; "***", *p*-value <0.001). *Abbreviations:* AAA, aromatic amino acids; BCAA, branched chain amino acids; BF%, body fat percentage; BMI, body mass index; DiC, dicarboxylate; FA, fatty acid; LPC, lysophosphotidylcholine; Met, Methionine; MM, muscle mass; OH, hydroxyl; Phe, phenylalanine; Pro, proline; Thr, Threonine; TS, triceps skinfold; WC, waist circumference.

Figure 3. Sex differences in the association between the metabolome and change in muscle mass. Sex-specific multiple linear regressions classified how the 30 metabolite clusters and 51 singletons were associated with change in muscle mass (ΔMM) in **(a)** boys and **(b)** girls, adjusting for age, energy intake, socioeconomic status, moderate and vigorous physical activity,

and sedentary time. Significant clusters are represented. Estimated beta coefficients plotted with significance of association denoted by asterisks ("*", *p*-value<0.05; "**", *p*-value <0.01).

Abbreviations: AAA, aromatic amino acids; AC, acylcarnitine; AICAR, 1,5-phosphoribosyl-5-amino-4-imidazolecarboxamide; BCAA, branched-chain amino acids; Met, Methionine; Pro, proline.

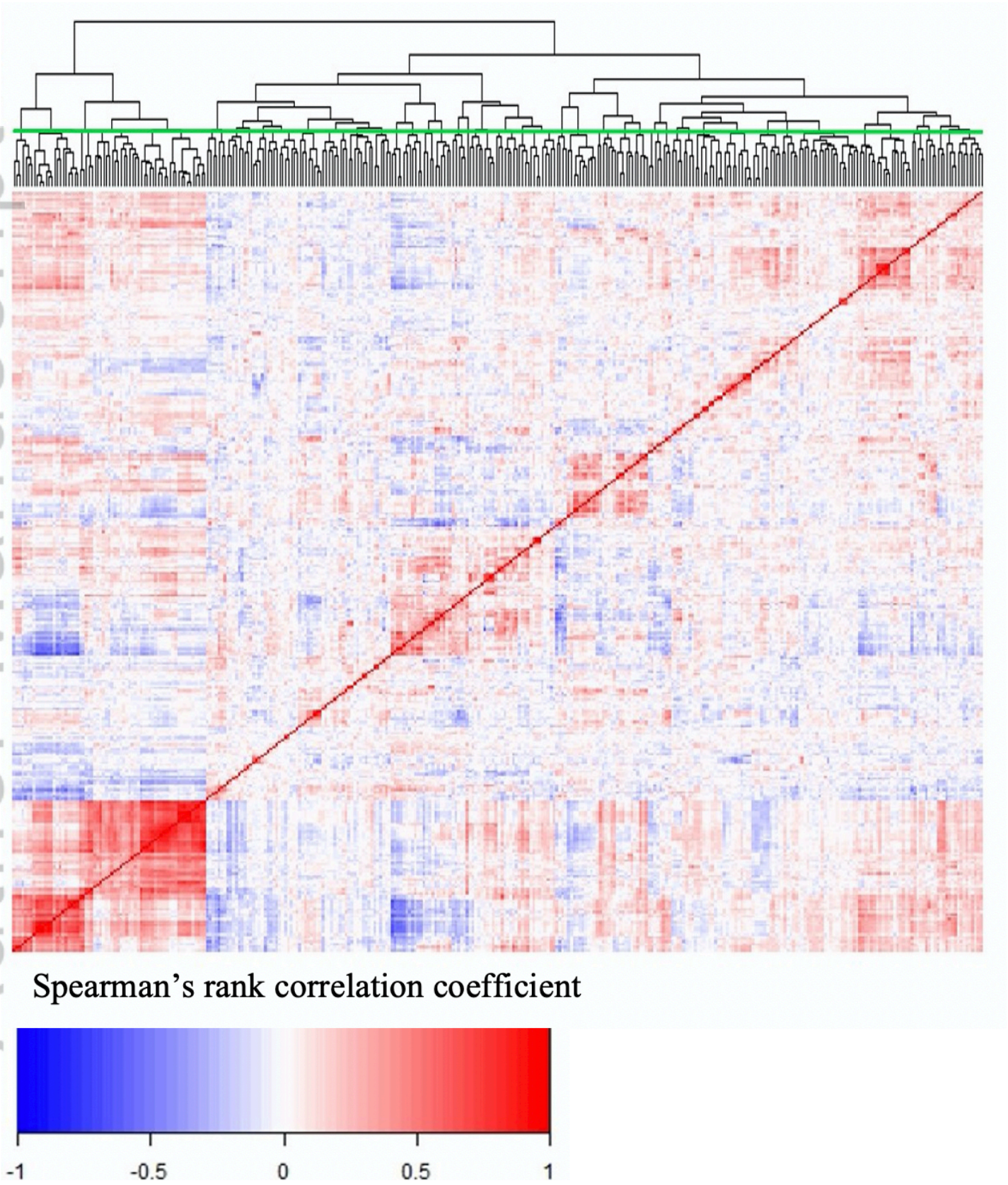
Table 1. Subject characteristics and change in body composition, stratified by sex.

[a]Normal distribution. Presented as mean ± SD. Statistical significance between sex assessed using Student's t-test. [b] Non-normal distribution. Presented as median (Q1, Q3). Statistical significance between sex assessed using Wilcoxon tests. [c] Categorical variables presented as n (%). Statistical significance between sex assessed using Fisher's Exact test.

Abbreviations: BMI, body mass index; IOTF, International Obesity Task Force; PA, physical activity; SD, standard deviation; SES, socioeconomic status; WHO, World Health Organization.

Table 2. Description of metabolites within the 30 clusters.

Abbreviations: BCAA, branched-chain amino acid.



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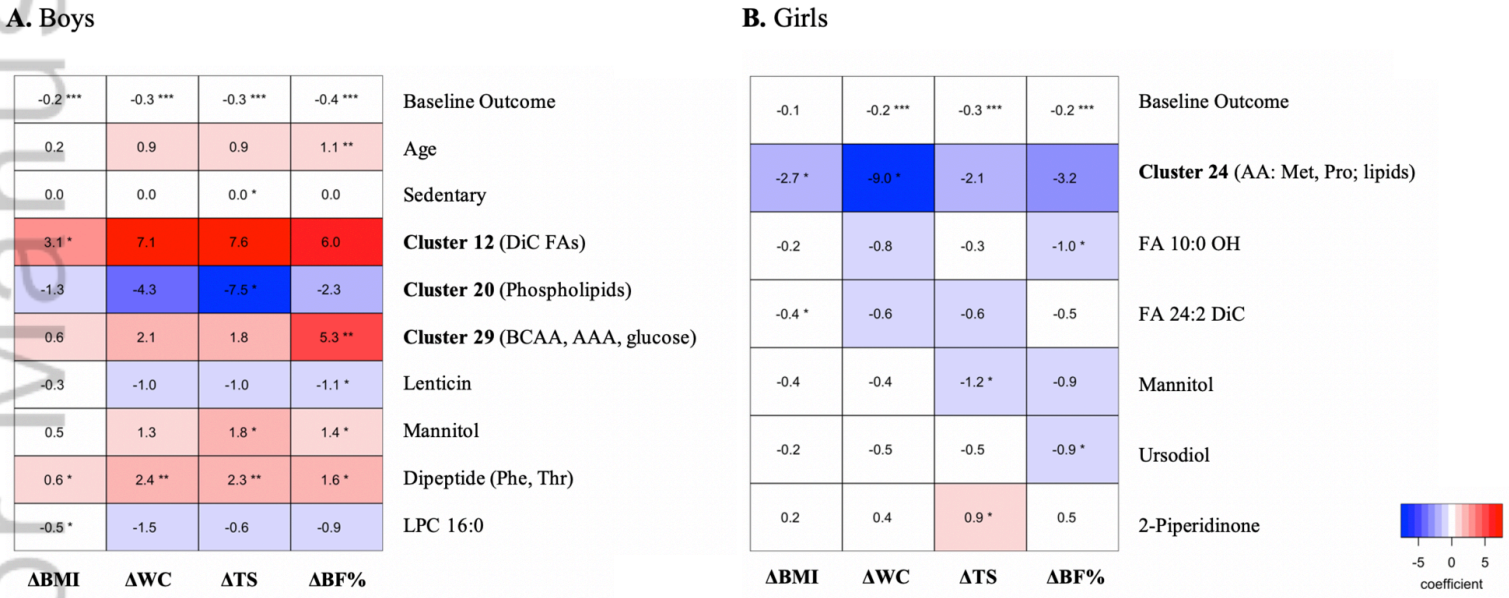

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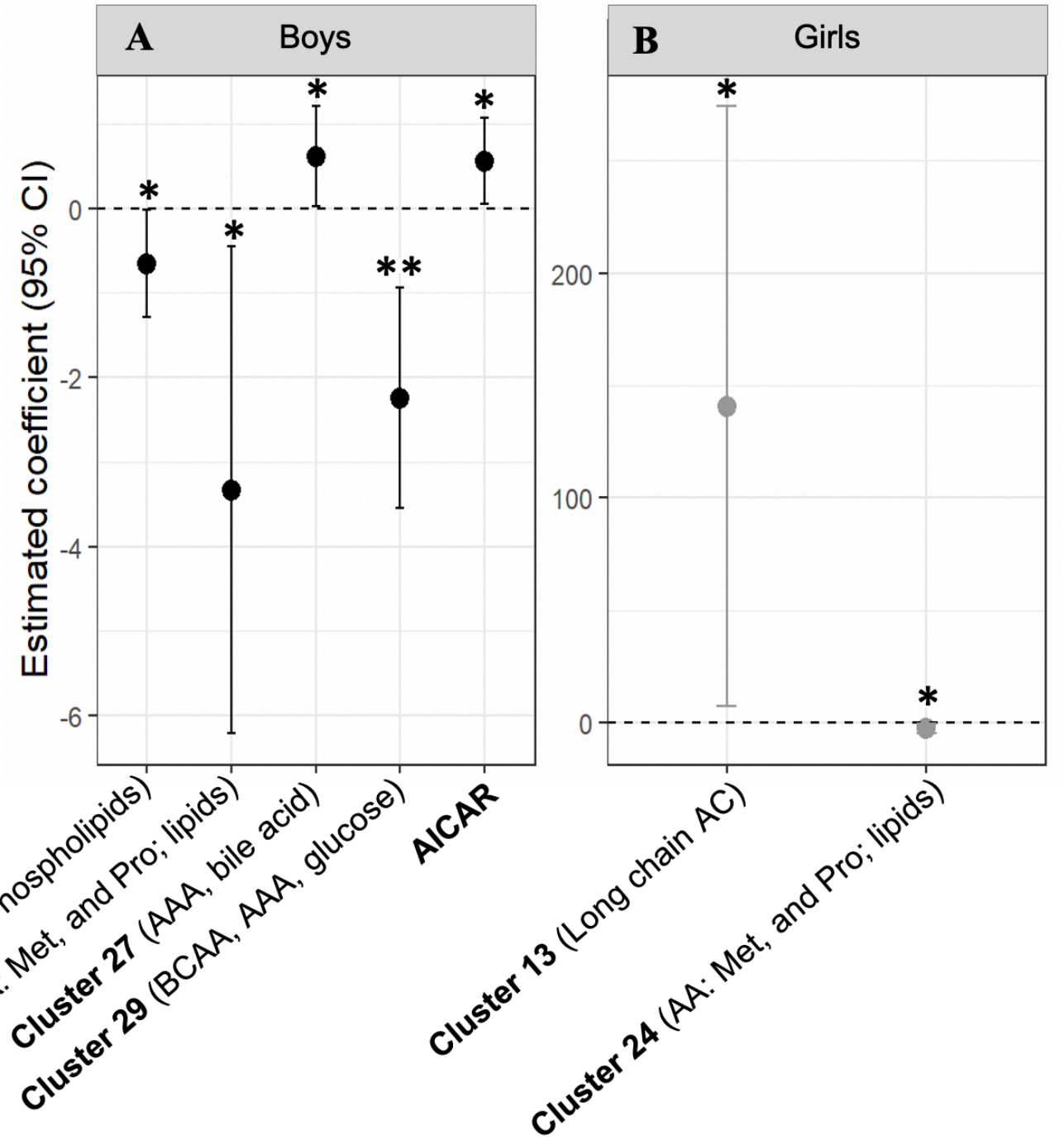

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Table 1. Subject characteristics and change in body composition, stratified by sex.

[a] Normal distribution. Presented as mean ± SD. Statistical significance between sex assessed using Student's t-test.

[b] Non-normal distribution. Presented as median (Q1, Q3). Statistical significance between sex assessed using Wilcoxon tests.

[c] Categorical variables presented as n (%). Statistical significance between sex assessed using Fisher's Exact test.

Abbreviations: BMI, body mass index; IOTF, International Obesity Task Force; PA, physical activity; Q1, first quartile (25th percentile); Q3, third quartile (75th percentile); SD, standard deviation; SES, socioeconomic status; WHO, World Health Organization.

	Total (n= 352)	Girls (n=182)	Boys (n=170)	p-value
Baseline				
Age (years) [a]	13.8 ± 1.9	13.7 ± 2.0	13.9 ± 1.9	0.300
Lifestyle				
Energy intake (kcal/day) [b]	2155 (1682, 2754)	1928 (1496, 2391)	2399 (1909, 3144)	<0.0001
Moderate and vigorous PA (min/day) [b]	77.8 (61.0, 97.8)	78.9 (66.0, 97.9)	75.9 (57.6, 95.0)	0.165
Sedentary time (min/day) [a]	583.2 ± 74.8	573.2 ± 74.1	593.9 ± 74.4	0.009
Body composition				
BMI (kg/m^2) [b]	21.0 (18.2, 23.8)	21.3 (18.3, 24.3)	20.7 (17.9, 23.6)	0.259
Waist circumference (cm) [b]	77.1 (69.9, 86.5)	78.0 (71.1, 87.4)	76.5 (69.0, 85.1)	0.221
Triceps skinfold (mm) [b]	18.5 (14.0, 23.0)	20.0 (16.0, 24.5)	16.0 (10.0, 21.0)	<0.0001
Body fat (%) [b]	27.1 (19.7, 34.0)	30.5 (25.5, 36.1)	21.1 (14.8, 28.6)	<0.0001
Muscle mass (kg) [b]	19.8 (17.2, 24.0)	18.9 (16.7, 20.8)	23.0 (18.6, 26.6)	<0.0001
SES [c]				
A/B	24 (6.8)	11 (6.0)	13 (7.7)	0.103
C+	63 (17.9)	35 (19.2)	28 (16.5)	
C	106 (30.1)	46 (25.3)	60 (35.3)	
D+	108 (30.7)	57 (31.3)	51 (30.0)	
D	15 (4.3)	12 (6.6)	3 (1.8)	
E	36 (10.2)	21 (11.5)	15 (8.8)	
BMI for age WHO-classification [c]				
Underweight/Normal	216 (61.3)	114 (62.7)	102 (60)	0.953
Overweight	89 (25.3)	43 (23.6)	46 (27.1)	
Obesity	47 (13.4)	25 (13.7)	22 (12.9)	
BMI for age IOTF-classification [c]				
Underweight/Normal	231 (65.6)	120 (66.0)	111 (65.3)	0.424
Overweight	90 (25.6)	43 (23.6)	47 (27.6)	
Obesity	31 (8.8)	19 (10.4)	12 (7.1)	
Body composition change				
Δ BMI (kg/m^2) [a]	1.3 ± 1.7	1.6 ± 1.5	1.0 ± 1.8	0.0003
Δ Waist circumference (cm) [a]	6.4 ± 5.4	8.0 ± 4.8	4.8 ± 5.5	<0.0001
Δ Triceps skinfold (mm) [a]	2.1 ± 5.1	3.0 ± 4.3	1.1 ± 5.7	0.0006
Δ Body fat (%) [a]	0.7 ± 5.1	2.8 ± 3.6	-1.6 ± 5.4	<0.0001
Δ Muscle mass (kg) [a]	2.8 ± 2.5	1.6 ± 1.4	4.0 ± 2.8	<0.0001

Table 2. Metabolite classes within Clusters. Pairing hierarchical clustering with a dendrogram height of 3.5, 30 metabolite clusters were identified. Clusters were named based on the primary metabolites within. *Abbreviations*: BCAA, branched-chain amino acids.

Cluster ID	Cluster Name
1	Lysophospholipids
2	Lipids, sterols
3	Fatty acid intermediates, long chain
4	Phospholipids, nucleotides
5	Lipids, nucleotides
6	Acylcarnitines
7	Amino acids (isoleucine), urate
8	Xanthine Metabolism
9	Fatty acids, hydroxyl fatty acids
10	Long-chain and very-long chain fatty acids, hydroxyl fatty acids
11	Polyunsaturated very-long chain fatty acids, fatty acid intermediates
12	Dicarboxylic fatty acids
13	Long chain acylcarnitines, nucleotides
14	Lipids, fatty acid oxidation intermediates, glycerol backbones
15	Dicarboxylic and amine fatty acids
16	Amino acids (serine and histidine)
17	Amino acids (BCAA metabolites and aromatic amino acids)
18	Amino acids (aromatic amino acids), amine fatty acids
19	Phospholipids
20	Phospholipids
21	Phospholipids
22	Lysophospholipids, fatty acid intermediates
23	Amino acid (lysine), diacylglycerols
24	Amino acid (methionine and proline), lipids
25	Polyunsaturated phospholipids, long-chain acylcarnitines
26	Carbohydrate (lactose), dipeptides
27	Amino acid (aromatic amino acid), bile acid
28	Monoacylglycerol
29	Amino acid (BCAA, aromatic amino acid), glucose
30	Very-long chain fatty acids