Branched chain amino acids, androgen hormones, and metabolic risk across early adolescence: a prospective study in Project Viva

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Disclosure: The authors declared no conflict of interest.

Funding: US National Institutes of Health (K24 HD069408, R01 HD 034568, P30 DK092924, P30 DK046200).

Word count: 4163
Tables: 6
References: 43
Supplemental materials: text plus 1 table

Key words: branched-chain amino acids, androgen hormones, puberty, metabolomics, obesity, insulin resistance, triglycerides, metabolic risk

Running title: BCAA, androgens, and metabolic risk in adolescence

Abbreviations:
BCAA – branched-chain amino acid
BMI – body mass index
CI – confidence interval
HOMA-IR – homeostatic model assessment insulin resistance
CRP – high sensitivity C-reactive protein
IL-6 – interleukin-6
mTOR – mammalian target of rapamycin
PCA – principal components analysis

What is already known about this subject?

- Circulating metabolite patterns may serve as early indicators of disease risk.
- In adults, a branched chain amino acid (BCAA) metabolite pattern is detectable over a decade prior to insulin resistance and incident type 2 diabetes.
- The majority of published studies in children have been cross-sectional, thereby precluding inference on temporality between metabolite patterns and conventional metabolic risk factors.

What does this study add?

- The BCAA and androgen hormone metabolite patterns, both of which were previously cross-sectionally associated with excess adiposity and metabolic risk in the study population, were related to change in several metabolic biomarkers during ~5 years of follow-up (baseline age 6-10 years) in a sex-specific manner.
- The BCAA pattern was related to a decrease in fasting glucose in boys, and an increase in triglycerides in girls.
- The androgen hormone pattern was associated with a decrease in leptin and an increase in C-reactive protein in girls.

ABSTRACT

Objective: To examine associations of two obesity-related metabolite patterns with changes in metabolic biomarkers during early adolescence.

Methods: Using multivariable linear regression, we examined associations of a branched chain amino acid (BCAA) and androgen hormone patterns with changes in glycemia (fasting glucose, insulin, HOMA-IR), adipokines (leptin, adiponectin), inflammation (C-reactive protein, interleukin-6), lipid profile, and blood pressure during ~5 years follow-up among 213 children aged 6-10 years at baseline. We adjusted for baseline age, pubertal status, biomarker level, and BMI percentile; and age at follow-up. We also tested for interactions with sex and baseline BMI percentile.

Results: Median age at baseline was 7.7 years; 48.8% were boys. In adjusted models, each 1 unit of the BCAA pattern corresponded with a 4.82 (95% CI: 0.92, 8.71) mg/dL decrease in fasting glucose in boys. In girls, the BCAA pattern was associated with an increase in triglycerides (4.17 [0.03, 8.32] mg/dL). The androgen pattern was associated with decreased leptin (-2.35 [-4.34, -0.35] ng/dL) and increased CRP (0.28 [0.03, 0.54] mg/dL) in girls. These relationships did not differ by baseline BMI percentile.

Conclusions: The BCAA and androgen hormone metabolite patterns are related to changes in metabolic parameters in a sex-specific manner during early adolescence.

INTRODUCTION

In the last two decades, prevalence of type 2 diabetes increased by almost a third among children and adolescents in the U.S. (1). These trends are alarming, as youth with chronic conditions present treatment challenges and will enter adulthood with several years of disease duration, and greater risk of early complications (2). Despite population-based efforts to reduce risk of metabolic disease via obesity prevention and lifestyle modifications, the need to pair this strategy with targeted approaches for high-risk individuals and subgroups was recently acknowledged by the Institute of Medicine (3).

Profiling of circulating metabolites ("metabolomics") shows promise as one route to identifying specific targets for primary prevention. Studies in adults have unveiled distinct differences in plasma metabolite composition of persons who are obese vs. lean (4), some of which precede development of insulin resistance and type 2 diabetes by over a decade, independently of weight status (5). These findings suggest that metabolite patterns have higher discriminative capacity than weight or traditional biomarkers to identify persons at risk of type 2 diabetes earlier on the disease continuum, and that some metabolite patterns may signal risk even among individuals who are not overweight/obese.

Less is known of these relationships earlier in the life course. Despite a handful of recent analyses exploring cross-sectional associations of circulating metabolites with conventional biomarkers of glycemia in children and adolescents (6-9), only three studies interrogated this relationship prospectively: one study followed 17 adolescents in Boston over the course of 18 months (9), another was a two-year study of 102 Korean boys (10), and a third followed 16 non-diabetic adolescents from an obesity clinic for 2.3 years (11). The scant literature in youth is

problematic given the importance of understanding determinants and etiology of worsening metabolic health during early-life for effective prevention.

Here, we used data from a cohort of children aged 6-10 years at baseline to attain a better understanding of biological pathways underlying worsening metabolic health during adolescence. We investigated associations of two previously-characterized metabolite patterns related to metabolic risk during mid-childhood (a branched chain amino acid [BCAA] and an androgen steroid hormone metabolite pattern (8)) with change in several metabolic biomarkers during 5 years of follow-up. We also aimed to identify and characterize differences in the relationships of interest by baseline weight and pubertal status.

METHODS

Study population

This study includes participants of Project Viva, a pre-birth cohort recruited from a multi-specialty group practice in Massachusetts (Atrius Harvard Vanguard Medical Associates). Details on study design and recruitment are reported elsewhere (12). Children in this analysis are a subset of participants in a pilot study that characterized serum metabolites associated with obesity and metabolic risk during mid-childhood (age 6-10 years) (8). Of the 262 participants in the pilot study, we considered 253 who attended the early teen research visit (age 11-15 years) and provided fasting blood. The final analytic sample included 213 children with data on change in any of the metabolic biomarkers of interest. The Institutional Review Board of Harvard Pilgrim Health Care approved all study protocols. All mothers provided written informed consent and children provided verbal assent.

Blood collection

At the mid-childhood (baseline) and early teen (follow-up) research visits, trained research assistants (RAs) collected an 8-hour fasting blood sample from the antecubital vein. All samples were refrigerated immediately, processed within 24 hours, and stored at -80^{o}C until time of analysis.

Exposure: plasma metabolites

We carried out untargeted metabolomic profiling in fasting plasma collected during mid-childhood via a multi-platform mass spectroscopy (MS)-based technique (13-15). Details regarding sample preparation and analysis for this population have been published (13-15) and are in the **Supplemental Material**.

For statistical analysis, we examined metabolites in the form of a principal component analysis (PCA) factor score, henceforth referred to as the branched chain amino acid (BCAA) and androgen pattern, both of which were cross-sectionally associated with excess adiposity and metabolic risk in this population during mid-childhood (8). We also examined key metabolites within each metabolite pattern (i.e., those with a factor loading >|0.5|) as a z-score, centered at the median, and scaled to 1 mean absolute deviation (8).

Outcome: change in metabolic biomarkers during follow-up

At both the mid-childhood and early teen visits, we used fasting blood to measure plasma glucose, insulin, leptin, adiponectin, C-reactive protein (CRP), and interleukin-6 (IL-6); and serum total cholesterol, triglycerides, and high-density lipoprotein (HDL). We calculated low-density lipoprotein (LDL) using the following equation: LDL = total cholesterol–HDL–(triglycerides/5), and estimated insulin resistance using the homeostasis model assessment for insulin resistance [HOMA-IR= (glucose *mg/dL* x insulin *μIU/mL*)/405]. Details on assays for laboratory analyses are in the **Supplemental Material**. We measured systolic (SBP) and diastolic blood pressure (SBP) using biannually-calibrated automated oscillometric monitors (Dinamap Pro100, Tampa, Florida). RAs recorded BP on the child's upper arm up to five times at one-minute intervals. We used the average of the five measurements for the statistical analysis.

In addition to examining individual biomarkers, we derived a metabolic syndrome z-score (MetS z-score) calculated the mean of five age- and sex-specific internal z-scores for waist circumference, inverted HDL, natural log (ln)-transformed triglycerides (due to non-normal distribution of the original variable), ln-transformed HOMA-IR, and systolic blood pressure. This score is modified version proposed by Viitasalo et al. (16). Specifically, we used HOMA-IR in lieu of fasting glucose and insulin, as this index has been shown to be a better assessment of

glycemic homeostasis in children than glucose or insulin alone (17-19); included HDL and triglycerides individually rather than as a ratio given evidence of limited utility of this ratio in children (20); and used SBP instead of the average of SBP and DBP since SBP is more reliably measured in children and is a stronger predictor of future health (21).

In the analysis, we focused on change in each biomarker and MetS z-score between the two research visits.

Covariates

Assessment of covariates, including perinatal and sociodemographic characteristics, and child anthropometry and pubertal status are in the **Supplemental Material**.

Data analysis

Step 1. We examined bivariate associations of the BCAA and androgen metabolite pattern factor scores (see Perng et al. (8) for details on factor creation) with background and sociodemographic characteristics. This step, in conjunction with our knowledge of determinants of metabolic health, informed covariate selection.

Step 2. We investigated relations of the two metabolite patterns, separately, with change in each of the biomarkers using multivariable linear regression models that accounted for the child's age, pubertal status, and the biomarker of interest at baseline; and age at follow-up (**Model 1**). We also further accounted for BMI percentile at baseline (**Model 2**), since weight status is a determinant of future metabolic risk (22). Because tests for interactions indicated effect modification by sex (P-interaction<0.05), we ran all models separately for boys and girls. We used complete case analysis, which resulted in a decreasing sample size with the addition of

covariates. To assess for potential bias due to missing data, we examined background characteristics across each of the subsamples (**Table S1**).

Step 3. To gain insight into biochemical pathways underlying associations of the BCAA and androgen patterns with the conventional biomarkers, we further investigated relations of individual metabolites with a factor loading >|0.50| in each of the patterns with change in biomarkers that were predicted by this pattern in Step 2. In other words, if we detected a significant association (P<0.05) between a metabolite pattern and change in a biomarker, we further explored associations of individual metabolites within the metabolite pattern with this biomarker.

In all models, we tested for an interaction between the metabolite pattern and baseline BMI percentile (continuous) and pubertal status (ordinal summary score). We observed evidence of interactions between the BCAA pattern and pubertal status for one of the biomarkers in boys, so we evaluated puberty-stratified associations for this biomarker (pre-pubertal vs. pubertal). We also carried out sensitivity analyses to assess the impact of further adjustment for covariates that were associated with the metabolite patterns in bivariate analysis.

Because the relationships of interest involve correlated exposures (metabolites on related pathways) and outcomes that cluster and track over time (metabolic biomarkers), we did not account for multiple comparisons. Instead, we focus on the magnitude and direction of associations rather than statistical significance when interpreting results. Additionally, although tempo of maturation during follow-up could impact findings, we controlled only for pubertal status at baseline and interpreted the estimates as the total effect of the metabolite patterns on metabolic risk during follow-up given that change in pubertal status could be on the causal pathway, and thus, adjusting for it could introduce bias (23).

All models met standard assumptions for multivariable linear regression (linearity of exposure/outcome relationship, homoscedasticity of error, normality of residuals). All analyses were performed using SAS 9.4 (Cary, NC).

RESULTS

Median age of the participants at the baseline mid-childhood visit was 7.7 years (range: 6.7 to 10.6); 48.8% (*n*=104) were boys. Descriptive statistics on the factor scores, age, BMI, and the metabolic biomarkers at the mid-childhood and early teen visits are presented in **Table 1**.

Table 2 shows bivariate associations of the BCAA and androgen factor scores with perinatal and sociodemographic characteristics. Children from households with an annual income <$70K vs. >$70K had a higher BCAA score (β=0.43, 95% CI: 0.01, 0.85). As we have previously shown (8), children of obese women had a higher BCAA score than those whose mothers were overweight (β=0.64, 95% CI: 0.06, 1.22) or normal weight (β=0.74, 95% CI: 0.23, 1.24) prior to pregnancy. Children who were obese at baseline exhibited a higher BCAA score than their overweight (β=0.93, 95% CI: 0.29, 1.56) and normal weight (β=1.14, 95% CI: 0.72, 1.55) counterparts. We observed similar associations for the androgen factor score (**Table 2**). Additionally, children classified as pubertal had a higher score for this metabolite pattern than pre-pubertal participants (β=0.60, 95% CI: 0.14, 1.06).

Table 3 shows associations of the BCAA pattern at baseline (mid-childhood) with changes in the metabolic biomarkers during follow-up (difference between the early teen and mid-childhood values). A higher score for the BCAA pattern was associated with a decrease in fasting glucose in boys, even after adjusting for age, pubertal status, and fasting glucose at baseline; and age at follow-up. Specifically, each 1 unit increment in the BCAA score corresponded with a 4.70 (95% CI: 0.93, 8.47) mg/dL decrease in fasting glucose (**Model 1**). This association did not materially change after accounting for baseline BMI percentile (**Model 2**: β=-4.97, 95% CI: -8.83, -1.11 mg/dL per 1 unit BCAA score), suggesting that the BCAA pattern predicts change in fasting glucose even after accounting for glucose levels and adiposity

at baseline. In girls, each 1 unit increment in the BCAA score was associated with a 4.38 (95% CI: 0.40, 8.37) mg/dL increase in serum triglycerides levels in **Model 1**. Adjustment for baseline BMI percentile in **Model 2** attenuated the estimate by <10% (β= 3.99, 95% CI: -0.13, 8.11 mg/dL per 1 unit BCAA score).

We did not observe any associations of the androgen pattern with change in the metabolic biomarkers among boys (**Table 4**). In girls, a higher score for the androgen pattern corresponded with a decrease in leptin (β=-2.39, 95% CI: 4.76, -0.02 ng/dL per 1 unit factor score in **Model 1**), and an increase in CRP (β=0.28, 95% CI: 0.03, 0.54 mg/dL per 1 unit factor score in **Model 1**). These estimates were essentially unchanged after adjustment for baseline BMI percentile in **Model 2**.

To obtain a more granular understanding of specific compounds driving the associations detected with respect to the factor scores, we further examined relations of key metabolites within the BCAA pattern with change in fasting glucose among boys, and with serum triglycerides among girls, based on results shown in Table 3 (**Table 5**), Among boys, all metabolites were associated with a decrease in fasting glucose in boys, with the strongest association for propionylcarnitine (β= -4.38, 95% CI: -7.79, -0.97 mg/dL per 1 z-score) and isobutyrylcarnitine (β=-3.54, 95% CI: -6.18, -0.90 mg/dL per 1 z-score). Among girls, the majority of compounds exhibited a positive relationship with change in triglycerides, with the strongest association detected for kynurenine (β=3.65, 95% CI: 0.10, 7.21 mg/dL per 1 z-score) and gamma-glutamylleucine (β=4.44, 95% CI: 1.10, 7.79 mg/dL per 1 z-score).

When testing for effect modification by pubertal status, we found evidence of an interaction with respect to SBP in boys, so we ran these models within strata of baseline pubertal status (**Table 6**). The BCAA pattern was inversely related to change in SBP in pre-pubertal boys

(*n*=79; **Model 1**: β=-0.86, 95% CI: -2.06, 0.33 mmHg per 1 unit BCAA score), but was positively associated with change in SBP in pubertal boys (*n*=19; **Model 1**: β=1.67, 95% CI: -0.82, 4.16 mmHg per 1 unit BCAA score). Further adjustment for baseline BMI percentile did not change these findings (pre-pubertal boys: β=-0.88, 95% CI: -2.09, 0.33; pubertal boys: β=2.48, 95% CI: -0.45, 5.40 mmHg per 1 unit BCAA score). We examined these relationships with individual metabolites, and identified associations with respect to tryptophan (β=2.59, 95% CI: 0.03, 5.15 mmHg per 1 z-score of the metabolite) and kynurenine (β=2.53, 95% CI: 0.51, 4.55 mmHg per 1 metabolite z-score; **Table 6**).

Table 7 shows associations of metabolites in the androgen pattern with the change in leptin and CRP in girls (outcomes selected based on findings from Table 4). All individual metabolites were consistently inversely associated with change in leptin, with the strongest associations for 4-androsten-3beta,17beta-diol disulfate 2 (β=-1.78, 95% CI: -3.35, -0.20 ng/dL per 1 z-score) and pregn steroid monosulfate (β=-2.50, 95% CI: -4.87, -0.13 ng/dL per 1 z-score). The majority of metabolites were positively associated with change in CRP, but none of the estimates were statistically significant.

None of the relationships assessed differed by baseline BMI percentile. In sensitivity analyses, we evaluated the impact of adjustment for background characteristics associated with the metabolite patterns in bivariate analysis – namely, pre-pregnancy BMI, and annual household income, and child's race/ethnicity. Inclusion of these variables did not change the results, so we did not include them for the sake of parsimony. An assessment of background characteristics of each of the subsamples analyzed in multivariable analyses yielded no notable differences, thus indicating that risk of bias due to missing data is likely low (**Table S1**).

DISCUSSION

In this prospective study of 253 children 6-10 years of age at baseline, we examined associations of two previously-derived metabolite patterns – a branched chain amino acid (BCAA) metabolite pattern and an androgen hormone pattern (8) – with change in conventional metabolic biomarkers during ~5 years of follow-up. Counter to findings from adults (5, 24), the BCAA pattern was not associated with worsening metabolic health in this cohort of adolescents, as the majority of the relationships we examined were null. However, we did find an inverse association of the BCAA pattern with change in fasting glucose in boys, and a direct relation of this pattern with change in serum triglycerides in girls. The androgen hormone pattern, which, to our knowledge has not been evaluated in relation to metabolic risk in other populations, was related to decreased leptin and increased CRP during follow-up in girls.

BCAA metabolite pattern

During mid-childhood, the BCAA pattern was associated with obesity, as well as higher fasting glucose, insulin, HOMA-IR, leptin, CRP, and IL-6. When we examined associations with change in the biomarkers during follow-up, the majority of estimates were null, with the exceptions of an inverse relationship between the BCAA pattern and fasting glucose in boys, and a direct association with serum triglycerides in girls.

BCAA pattern and change in fasting glucose in boys

This finding was counter to what we expected based on findings in adults (5, 24), as well as two prospective studies in adolescents that did not account for glycemia at baseline (9, 10). However, our findings align with results of two cross-sectional analyses in similarly-aged youth. In a study of 139 adolescents ~13 years of age, Michaliszyn et al. found that several compounds

in the BCAA pattern, including leucine, isoleucine, valine, phenylalanine, and propionylcarnitine, were associated with higher insulin sensitivity (7). In the same population, Mihalik et al. unveiled evidence that these metabolites were also associated with enhanced fatty acid oxidation (6). The investigators hypothesized that the discrepancy in direction of association in adults vs. adolescents may be due to an adaptive increase in mitochondrial function (and accordingly, an improvement in glycemia) during early life that eventually wanes with age and continued metabolic dysregulation (25). Another explanation for our finding relates to the nutrient-signaling properties of BCAAs. For example, leucine activates the mammalian target of rapamycin (mTOR), a nutrient sensor involved in the neurocircuitry of energy balance, food intake, and glycemic regulation (26), that has been shown to improve glucose tolerance in mice (27). The fact that we observed positive, albeit non-significant, associations with fasting insulin and HOMA-IR supports this mechanism.

Of note, a recent study of 16 children 8-13 years of age who were obese but non-diabetic at baseline found that although circulating BCAAs were associated with worse glycemia throughout the course of an oral glucose tolerance test (OGTT) at baseline, these metabolites were not associated with glucose control or indices of insulin secretion/sensitivity over 2.3 years of follow-up (11). These findings are generally in line with those of the present analysis, given our predominantly null results with respect to other glycemia biomarkers (fasting insulin, HOMA-IR).

When we examined associations of individual metabolites within the BCAA pattern with glucose during follow-up in boys, we found that propionylcarnitine and isobutyrylcarnitine were the strongest determinants of decreasing glucose during follow-up. Both compounds are downstream intermediates of BCAA catabolism, and the latter of which (isobutyrylcarnitine) is

also product of acyl coenzyme A (acyl-CoA) beta oxidation – a process that feeds into the citric acid cycle for energy production (28). Although we were not able to locate any published studies on isobutyrylcarnitine in relation to glycemia, a pilot trial of 24 patients who were obese and had type 2 diabetes found that intravenous administration of propionylcarnitine improved glycemic control (29). Proposed mechanisms include propionylcarnitine's involvement in muscle metabolism, endothelial function, and carbohydrate oxidation (30, 31).

The BCAA pattern was also associated with increasing SBP among pubertal, but not pre-pubertal boys. Specific metabolites driving this relationship were tryptophan and its downstream metabolite, kynurenine. These results support findings of Wolf et al., who demonstrated that intraperitoneal tryptophan administration reduced blood pressure in hypertensive rodents, but increased blood pressure in normotensive animals (32). The investigators speculated that the duality of tryptophan's effect on blood pressure is complex and regulated by numerous physiological processes involved in control of blood pressure, like brain monoamines and catecholamines (32). In the present study population, the majority of children were normotensive at baseline (98.8%), therefore the positive relationship between tryptophan and SBP corroborates results from rodent models. The fact that this relationship was only observed among pubertal boys may reflect the sex-specific differences in blood pressure change during puberty (33).

BCAA pattern and change in serum triglycerides in girls

In girls, a higher score for the BCAA pattern was associated with increased serum triglycerides. When we examined component metabolites of the BCAA pattern, we detected the strongest relations with respect to kynurenine and gamma-glutamylleucine.

Although there have not been any publications specifically relating kynurenine to triglycerides, growing evidence suggests that deregulation of the tryptophan-to-kynurenine pathway is involved in atherosclerotic cardiovascular disease, possibly through perturbations in immune pathways (34). Despite an equally scanty literature on gamma-glutamylleucine in relation to lipid profile, the positive association between this compound and triglycerides may reflect defective hepatic lipid metabolism, as gamma-glutamylleucine has been associated with non-alcoholic fatty liver disease – a condition characterized by accumulation of triglycerides in the liver, and elevated circulating triglycerides (35, 36). Because blood lipid levels exhibit high within-person variability until after puberty (37), whether or not these compounds serve as early indicators of dyslipidemia deserves further investigation beyond adolescence.

Androgen hormone metabolite pattern

During mid-childhood, the androgen hormone metabolite pattern, which we speculated was an indicator of pubarchal/pubertal advancement, was associated with higher HOMA-IR, and marginally higher serum triglycerides, leptin, and IL-6, and lower adiponectin (8). When we examined these relationships prospectively, we found that this metabolite pattern corresponded with a decrease in leptin and an increase in CRP in girls. The androgen hormone pattern was not associated with change in any of the biomarkers in boys.

Androgen pattern and change in leptin in girls

A higher score for this pattern at baseline corresponded with a decrease in leptin in girls, which was unexpected given that this metabolite pattern is associated with more advanced pubarchal/pubertal status at baseline (8) and the fact that leptin levels increase throughout

adolescence in females (38, 39). While this finding could be due to variability in tempo of maturation during follow-up (a variable that we did not control for because it could be on the causal pathway), additional studies are warranted to confirm our results. When we examined associations with specific metabolites, we detected the strongest relations with the testosterone precursor 4-androsten-3beta,17beta-diol disulfate 1, and pregn steroid monosulfate which is an intermediate in the steroidogenesis of androgen hormones from cholesterol.

Androgen pattern and change in CRP in girls

The androgen hormone pattern, but not the individual metabolites, was also related to increasing CRP in girls. This finding aligns with published data on the precipitous increase in CRP in females, but not males, during adolescence – particularly during the later stages of puberty (40). Future investigations are warranted to ascertain whether this relationship persists beyond puberty.

Strengths & weaknesses

This study has several weaknesses. First, assessment of plasma metabolites at a single point in time precludes our ability to infer on upregulation vs. downregulation of specific pathways. Second, we did not implement challenge testing (e.g., OGTT), nor did we measure glycated hemoglobin, and thus were not able to evaluate the entire range of glycemia. Third, use of complete case analysis is subject to missing data bias; however, a comparison of background characteristics among each of the subsamples provided no indication of differences in background characteristics of participants within the subsamples. Fourth, there may be residual confounding from variation in the tempo of sexual maturation during follow-up. Finally, as is the case with most analyses of high-dimensional 'omics data, we cannot rule out the possibility of

false positive associations, although we aimed to examine and compare associations (many of which are on correlated biochemical pathways) in order to gain insight on etiology, rather than to predict outcomes.

Strengths of this investigation include our ability to examine the relationship of two metabolite patterns with prospective change in multiple metabolic biomarkers in a cohort of multi-ethnic youth. Each of these elements are key improvements upon published studies, which have mostly been cross-sectional (6-9), of smaller sample size and shorter duration of follow-up (n=17 for 18 months (9); n=102 for 2 years (10)), and conducted in rather specific populations (e.g., Korean boys (10)).

Conclusions

We detected sex-specific associations of the BCAA and androgen hormone metabolite patterns with change in conventional metabolic biomarkers during ~5 years of follow-up in this population of youth aged 6-10 years at baseline. Specifically, the BCAA pattern corresponded with decreasing fasting glucose in boys, and increasing serum triglycerides in girls. However, given that the majority of associations were null (which may be an artefact of small sample sizes), the BCAA metabolite pattern does not appear to be an indicator of worsening metabolic health during early adolescence. The androgen hormone pattern – which, to our knowledge, has not been reported in other populations – was associated with a decrease in leptin and an increase in CRP in girls; these finding are likely related to timing of sexual maturation.

Given that puberty is a time of rapid physiological change, future studies are required to evaluate these relationships beyond the adolescence, and to interrogate the capacity of the these metabolite patterns to predict metabolic disease progression beyond that of conventional

biomarkers. Nevertheless, given that many metabolic risk factors, including blood pressure (41, 42), lipid profile (42, 43), and glycemia (42), track from late childhood/early adolescence into adulthood, a better understanding of early biomarkers of metabolic risk is a crucial step towards identification of targets for primary prevention.

ACKNOWLEDGEMENTS: We are indebted to past and present Project Viva participants and research assistants.

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Table 1 Descriptive statistics (mean ± SD) for the branched chain amino acid (BCAA) factor score, age, and metabolic biomarkers of 104 boys and 109 girls in the Project Viva cohort during mid-childhood (baseline) and the early teens (follow-up).

	Boys (*n* = 104)				Girls (*n* = 109)			
	N	Baseline	Follow-up	*P*[a]	*N*	Baseline	Follow-up	*P*[a]
Factor 4 (*BCAA pattern)*		-0.02 ± 1.51				0.02 ± 1.51		
Factor 9 (*Androgen pattern*)		-0.02 ± 1.56				-0.02 ± 1.56		
Age (years)	104	7.9 ± 0.8	13.0 ± 0.7	0.0007	109	7.9 ± 0.8	13.1 ± 0.7	
BMI (kg/m^2)	104	17.8 ± 3.3	21.3 ± 5.0	<0.0001	109	19.2 ± 4.2	23.5 ± 5.7	<0.0001
BMI percentile[b]	104	66.7 ± 27.3	63.2 ± 30.7	0.04	109	71.0 ± 31.0	72.3 ± 28.7	0.37
Insulin (μU/mL)	78	8.1 ± 5.8	17.4 ± 14.8	<0.0001	85	11.2 ± 7.6	18.7 ± 11.6	<0.0001
Glucose (mg/dL)	66	102.0 ± 17.8	96.2 ± 27.5	0.08	79	96.0 ± 14.4	93.7 ± 14.5	0.33
HOMA-IR	63	2.1 ± 2.0	3.9 ± 3.7	0.0007	77	2.5 ± 1.6	4.3 ± 3.1	<0.0001
Leptin (ng/mL)	81	5.9 ± 7.4	9.9 ± 14.6	0.001	86	10.2 ± 10.9	22.3 ± 18.7	<0.0001
Adiponectin (ng/mL)	81	14.7 ± 8.3	6.0 ± 2.6	<0.0001	86	15.5 ± 10.5	6.3 ± 2.8	<0.0001
CRP (mg/L)	82	0.97 ± 2.90	0.93 ± 1.97	0.88	83	1.73 ± 4.98	1.19 ± 2.08	0.28
IL-6 (pg/mL)	80	0.92 ± 1.10	1.13 ± 1.45	0.25	86	1.15 ± 1.55	1.44 ± 2.03	0.24
Total cholesterol (mg/dL)	83	163.5 ± 26.9	155.3 ± 29.4	0.005	87	164.6 ± 22.5	157.2 ± 27.7	0.003
HDL (mg/dL)	83	59.9 ± 22.7	55.4 ± 15.3	0.0006	87	55.5 ± 12.3	55.1 ± 13.4	0.74
LDL (mg/dL)	83	92.3 ± 25.0	86.2 ± 24.5	0.02	87	97.0 ± 20.7	89.2 ± 22.6	0.0001
Triglycerides (mg/dL)	83	56.9 ± 22.7	68.7 ± 31.2	0.0008	87	60.3 ± 25.5	64.2 ± 29.5	0.26
SBP (mmHg)	103	96.0 ± 9.0	110.0 ± 8.9	<0.0001	108	95.8 ± 9.0	105.8 ± 9.4	<0.0001
DBP (mmHg)	103	54.9 ± 5.3	61.3 ± 7.4	<0.0001	108	54.3 ± 5.4	62.7 ± 6.7	<0.0001
MetS z-score[c]	62	0.17 ± 0.61	0.21 ± 0.72	0.57	76	0.17 ± 0.68	0.05 ± 0.63	0.12

HOMA-IR: homeostatic model assessment of insulin resistance; **HDL:** high-density lipoprotein; LDL: low-density lipoprotein; **SBP:** systolic blood pressure; **DBP:** diastolic blood pressure; **MetS z-score**: metabolic syndrome z-score; **CRP:** c-reactive protein; **IL-6**: interleukin-6; BMI: body mass index.

a From a paired t-test.

b According to the CDC growth reference for children 2-19 years of age.

c Calculated as the average of 5 internally-standardized sex-specific z-scores for inverted HDL, waist circumference, ln-transformed HOMA-IR, ln-transformed triglycerides, and SBP.

Table 2 Mean ± SD of the BCAA and androgen metabolite pattern factor scores according to background characteristics of 213 Project Viva mother-child pairs

	N	BCAA pattern factor score	*P*[a]	Androgen pattern factor score	*P*[a]
Maternal & perinatal characteristics					
Annual household income			0.05		0.02
≤$70 k	81	0.25 ± 1.49		0.27 ± 1.69	
>$70 k	115	-0.17 ± 1.57		-0.25 ± 1.42	
Smoking habits			0.21		0.23
Never	141	-0.15 ± 1.56		0.09 ± 1.55	
Former	47	0.20 ± 1.43		-0.36 ± 1.62	
Smoked in early pregnancy	25	0.29 ± 1.27		0.04 ± 1.52	
Pre-pregnancy BMI[b]			0.008		0.25
Normal weight	114	-0.20 ± 1.54		-0.05 ± 1.56	
Overweight	53	-0.11 ± 1.31		-0.28 ± 1.41	
Obese	46	0.53 ± 1.53		0.36 ± 1.71	
Gestational weight gain			0.18		0.27
Inadequate	23	-0.15 ± 1.95		-0.31 ± 1.30	
Adequate	65	-0.24 ± 1.59		-0.08 ± 1.47	
Excessive	125	0.12 ± 1.36		0.07 ± 1.66	
Gestational glucose tolerance			0.68		0.53
Normoglycemic	163	0.00 ± 1.52		-0.06 ± 1.61	
Isolated hyperglycemia	28	-0.18 ± 1.44		0.18 ± 1.20	
Impaired glucose tolerance	6	1.25 ± 1.28		-0.85 ± 2.21	
Gestational diabetes	17	-0.39 ± 1.42		0.32 ± 1.41	
Duration of any breastfeeding			0.52		0.20
<1 months	33	-0.05 ± 1.58		0.30 ± 1.34	
1-<7 months	71	0.27 ± 1.32		-0.20 ± 1.80	
7-<12 months	40	-0.74 ± 1.53		0.25 ± 1.60	
≥12 months	47	0.11 ± 1.48		-0.38 ± 1.31	
Child's characteristics at baseline (mid-childhood visit)					
Age			0.73		0.03
<7 years	12	-0.14 ± 1.18		-0.39 ± 1.49	
7 to <8 years	129	-0.03 ± 1.56		-0.16 ± 1.41	
≥8 years	72	0.02 ± 1.47		0.30 ± 1.79	
Race/ethnicity			0.76		0.003
White	125	-0.03 ± 1.51		-0.25 ± 1.49	
Black	46	0.15 ± 1.36		0.71 ± 1.77	
Hispanic	15	-0.27 ± 1.55		0.15 ± 1.29	
Other	28	-0.13 ± 1.72		-0.27 ± 1.36	
Sex			0.36		0.80
Male	104	-0.12 ± 1.49		-0.05 ± 1.60	
Female	109	0.07 ± 1.52		0.01 ± 1.53	
Weight status[c]			<0.0001		0.0004
Normal weight	122	-0.48 ± 1.46		-0.29 ± 1.44	

Overweight	23	0.49 ± 1.26		-0.38 ± 1.50	
Obese	68	0.68 ± 1.38		0.59 ± 1.65	
Pubertal status			0.93		0.01
Pre-pubertal	144	-0.02 ± 1.51		-0.22 ± 1.53	
Pubertal	60	0.00 ± 1.48		0.38 ± 1.56	

a Represents a test for linear trend for ordinal variables, and a type 3 test for difference for dichotomous and categorical (race/ethnicity and smoking habits)

b According to the World Health Organization (WHO) and Centers for Disease Control and Prevention (CDC) adult weight status classification. "Normal weight" includes 4 women classified as "underweight."

c According to the CDC 2000 age- and sex-specific reference data. "Normal weight" includes 4 children classified as underweight (BMI <5th percentile).

Table 3 Associations of the BCAA metabolite pattern factor score with change in metabolic biomarkers between mid-childhood (~7 years) and the early teens (~12 years) among boys and girls in Project Viva

	β (95% CI) per 1 unit BCAA pattern factor score[a]					
	Boys (*n* = 104)			Girls (*n* = 109)		
	Unadjusted	Model 1	Model 2	Unadjusted	Model 1	Model 2
Change in metabolic biomarkers						
Insulin (μU/mL)	-0.26 (-2.40, 1.88)	1.64 (-0.35, 3.64)	1.51 (-0.49, 3.51)	0.57 (-1.26, 2.41)	1.04 (-0.67, 2.75)	0.59 (-1.14, 2.32)
Glucose (mg/dL)	-3.42 (-7.63, 0.79)	**-4.70 (-8.47, -0.93)**	**-4.97 (-8.83, -1.11)**	2.58 (-0.69, 5.86)	2.12 (-0.22, 4.45)	1.60 (-0.78, 3.98)
HOMA-IR	-0.48 (-1.12, 0.15)	0.31 (-0.17, 0.79)	0.26 (-0.22, 0.73)	0.35 (-0.16, 0.85)	0.39 (-0.11, 0.89)	0.24 (-0.26, 0.75)
Leptin (ng/mL)	0.53 (-0.99, 2.05)	0.23 (-1.34, 1.80)	0.06 (-1.54, 1.65)	0.23 (-2.21, 2.68)	0.35 (-2.18, 2.89)	-1.04 (-3.39, 1.31)
Adiponectin (ng/mL)	-0.29 (-1.43, 0.84)	-0.13 (-0.49, 0.23)	-0.04 (-0.40, 0.33)	-0.21 (-1.54, 1.11)	-0.01 (-0.35, 0.33)	0.03 (-0.33, 0.38)
CRP (mg/L)	0.07 (-0.23, 0.37)	0.03 (-0.17, 0.23)	0.03 (-0.17, 0.24)	-0.16 (-0.82, 0.51)	0.07 (-0.21, 0.36)	0.09 (-0.21, 0.39)
IL-6 (pg/mL)	0.15 (-0.08, 0.38)	0.13 (-0.08, 0.33)	0.11 (-0.11, 0.32)	-0.08 (-0.40, 0.24)	-0.04 (-0.34, 0.25)	0.05 (-0.25, 0.35)
Total cholesterol (mg/dL)	-2.29 (-5.80, 1.22)	-2.78 (-5.96, 0.39)	-2.87 (-6.13, 0.38)	-0.94 (-4.13, 2.25)	-0.99 (-4.16, 2.19)	-0.97 (-4.30, 2.35)
HDL (mg/dL)	-1.06 (-2.62, 0.51)	-1.21 (-2.71, 0.30)	-1.02 (-2.56, 0.51)	0.62 (-0.91, 2.15)	0.06 (-1.44, 1.55)	0.49 (-1.02, 1.99)
LDL (mg/dL)	-1.54 (-4.61, 1.53)	-1.85 (-4.56, 0.86)	-2.15 (-4.92, 0.61)	-2.00 (-4.54, 0.54)	-1.61 (-4.14, 0.92)	-1.99 (-4.62, 0.65)
Triglycerides (mg/dL)	1.55 (-2.70, 5.79)	1.40 (-2.70, 5.49)	1.13 (-3.07, 5.33)	2.19 (-2.31, 6.69)	**4.38 (0.40, 8.37)**	3.99 (-0.13, 8.11)
SBP (mmHg)	-0.96 (-2.27, 0.35)	-0.49 (-1.58, 0.60)	-0.51 (-1.63, 0.62)	0.33 (-0.95, 1.62)	0.87 (-0.24, 1.98)	1.03 (-0.14, 2.20)
DBP (mmHg)	0.28 (-0.68, 1.24)	0.21 (-0.72, 1.14)	-0.03 (-0.98, 0.92)	0.47 (-0.37, 1.32)	0.46 (-0.36, 1.29)	0.56 (-0.30, 1.42)
MetS z-score[b]	-0.03 (-0.12, 0.07)	0.01 (-0.08, 0.11)	0.00 (-0.10, 0.09)	0.02 (-0.08, 0.13)	0.06 (-0.02, 0.15)	0.04 (-0.04, 0.12)

a Model 1: Estimates are adjusted for pubertal status and the biomarker of interest at the mid-childhood visit, and age at mid-childhood and early teen visits. **Model 2**: Model 1 + BMI percentile at baseline. Bolded values indicate statistical significance at alpha <0.05.

b Calculated as the average of 5 internally-standardized sex-specific z-scores for inverted HDL, waist circumference, ln-transformed HOMA-IR, ln-transformed triglycerides, and SBP.

Table 4 Associations of the androgen metabolite pattern factor score with change in metabolic biomarkers between mid-childhood (~7 years) and the early teens (~12 years) among boys and girls in Project Viva

	β (95% CI) per 1 unit androgen factor score[a]					
	Boys (*n* = 104)			Girls (*n* = 109)		
	Unadjusted	Model 1	Model 2	Unadjusted	Model 1	Model 2
Change in metabolic biomarkers						
Insulin (μU/mL)	1.04 (-1.09, 3.18)	-0.19 (-2.15, 1.78)	-0.48 (-2.47, 1.52)	-1.35 (-2.96, 0.25)	-1.12 (-2.66, 0.43)	-1.14 (-2.64, 0.36)
Glucose (mg/dL)	2.53 (-1.56, 6.62)	1.90 (-1.88, 5.68)	1.97 (-1.90, 5.85)	-0.63 (-3.38, 2.13)	1.27 (-0.78, 3.32)	1.26 (-0.74, 3.25)
HOMA-IR	0.43 (-0.17, 1.03)	-0.09 (-0.52, 0.35)	-0.21 (-0.65, 0.23)	-0.32 (-0.73, 0.10)	-0.22 (-0.66, 0.22)	-0.21 (-0.64, 0.22)
Leptin (ng/mL)	0.37 (-1.17, 1.91)	-0.12 (-1.75, 1.52)	-0.25 (-1.89, 1.39)	-1.85 (-4.09, 0.40)	**-2.39 (-4.76, -0.02)**	**-2.50 (-4.61, -0.39)**
Adiponectin (ng/mL)	0.46 (-0.68, 1.61)	-0.01 (-0.38, 0.38)	0.07 (-0.31, 0.45)	1.04 (-0.18, 2.26)	0.00 (-0.32, 0.33)	0.01 (-0.32, 0.33)
CRP (mg/L)	-0.23 (-0.52, 0.05)	0.12 (-0.09, 0.32)	0.12 (-0.09, 0.33)	0.06 (-0.54, 0.66)	**0.28 (0.03, 0.54)**	**0.28 (0.03, 0.54)**
IL-6 (pg/mL)	0.08 (-0.15, 0.31)	0.20 (-0.02, 0.41)	0.18 (-0.03, 0.40)	0.06 (-0.23, 0.36)	0.26 (-0.02, 0.55)	0.26 (-0.02, 0.54)
Total cholesterol (mg/dL)	-1.97 (-5.46, 1.52)	-1.07 (-4.38, 2.24)	-1.06 (-4.42, 2.30)	-0.43 (-3.38, 2.52)	-0.61 (-3.69, 2.46)	-0.60 (-3.68, 2.48)
HDL (mg/dL)	-0.71 (-2.28, 0.85)	-1.02 (-2.61, 0.58)	-0.87 (-2.47, 0.72)	0.02 (-1.39, 1.44)	-0.05 (-1.45, 1.36)	0.01 (-1.36, 1.39)
LDL (mg/dL)	-1.25 (-4.30, 1.81)	0.30 (-2.51, 3.11)	0.17 (-2.67, 3.01)	-0.28 (-2.66, 2.09)	-0.56 (-2.99, 1.87)	-0.58 (-3.01, 1.84)
Triglycerides (mg/dL)	-0.07 (-4.30, 4.16)	0.12 (-4.09, 4.32)	-0.12 (-4.37, 4.13)	-0.85 (-5.02, 3.32)	1.73 (-2.11, 5.57)	1.71 (-2.10, 5.52)
SBP (mmHg)	-0.81 (-2.02, 0.40)	0.16 (-0.91, 1.22)	0.16 (-0.90, 1.22)	**-1.32 (-2.57, -0.07)**	-0.52 (-1.63, 0.59)	-0.52 (-1.63, 0.59)
DBP (mmHg)	-0.17 (-1.05, 0.72)	0.19 (-0.71, 1.08)	0.06 (-0.83, 0.95)	-0.17 (-1.01, 0.68)	0.14 (-0.68, 0.96)	0.15 (-0.67, 0.96)
MetS z-score[b]	-0.03 (-0.12, 0.06)	-0.01 (-0.11, 0.08)	-0.02 (-0.11, 0.07)	**-0.10 (-0.18, -0.02)**	-0.04 (-0.12, 0.03)	-0.04 (-0.11, 0.03)

a Model 1: Estimates are adjusted for pubertal status and the biomarker of interest at the mid-childhood visit, and age at mid-childhood and early teen visits. **Model 2**: Model 1 + BMI percentile at baseline. Bolded values indicate statistical significance at alpha <0.05.

b Calculated as the average of 5 internally-standardized sex-specific z-scores for inverted HDL, waist circumference, ln-transformed HOMA-IR, ln-transformed triglycerides, and SBP.

Table 5 Associations of metabolites within the BCAA pattern with change in fasting glucose in boys and change in serum triglycerides in girls.

	Factor loading	Boys (n = 63)	Girls (n = 83)
		β (95% CI) for change in fasting glucose (mg/dL)[a]	β (95% CI) for change in serum triglycerides (mg/dL)[a]
Valine	0.83	-2.91 (-6.83, 1.02)	1.72 (-1.88, 5.31)
Leucine	0.76	-2.12 (-5.48, 1.24)	1.72 (-1.56, 4.99)
Phenylalanine	0.72	-3.35 (-6.70, 0.01)	0.78 (-2.87, 4.44)
Isoleucine	0.71	-3.10 (-7.07, 0.87)	2.26 (-1.16, 5.67)
Propionylcarnitine (C3)	0.66	**-4.38 (-7.79, -0.97)**	2.01 (-2.31, 6.33)
2-methylbutyrylcarnitine (C5)	0.63	-2.01 (-6.13, 2.10)	2.74 (-0.69, 6.16)
Isovalerylcarnitine	0.30	-2.18 (-5.13, 0.76)	-0.46 (-3.78, 2.86)
Isobutyrylcarnitine	0.56	**-3.54 (-6.18, -0.90)**	-0.22 (-3.40, 2.95)
Tryptophan	0.54	-2.65 (-6.29, 0.98)	-1.81 (-5.79, 2.16)
3-methyl-2-oxovalerate (KMV)	0.52	-0.81 (-4.46, 2.85)	1.13 (-2.99, 5.25)
Kynurenine	0.52	-1.39 (-5.06, 2.28)	**3.65 (0.10, 7.21)**
Tyrosine	0.51	-3.70 (-8.35, 0.94)	-2.38 (-6.14, 1.38)
Gamma-glutamylleucine	0.51	-2.92 (-6.72, 0.88)	**4.44 (1.10, 7.79)**
4-methyl-2-oxopentanoate (KIC)	0.51	-0.63 (-4.06, 2.80)	-0.19 (-4.23, 3.85)

a Estimates are adjusted for age, pubertal status, BMI percentile, and biomarker level at the mid-childhood visit, and age at the early teen visit. Bolded text indicates statistical significance at alpha<0.05.

Table 6 Associations of metabolites within the BCAA pattern with change (Δ) in SBP in 98 boys, stratified by pubertal status at baseline (mid-childhood).

	Factor loading	β (95% CI) for Δ in SBP (mmHg)[a]	
		Pre-pubertal (*n*= 79)	**Pubertal (*n* = 19)**
BCAA factor score		-0.88 (-2.09, 0.33)	2.48 (-0.45, 5.40)
Individual metabolites			
Valine	0.83	-0.44 (-1.40, 0.52)	0.91 (-1.77, 3.59)
Leucine	0.76	-0.05 (-0.91, 0.81)	1.48 (-0.67, 3.63)
Phenylalanine	0.72	0.19 (-0.70, 1.07)	1.79 (-0.23, 3.81)
Isoleucine	0.71	-0.32 (-1.26, 0.62)	1.32 (-1.08, 3.73)
Propionylcarnitine (C3)	0.66	-0.77 (-1.80, 0.26)	-0.54 (-2.71, 1.41)
2-methylbutyrylcarnitine (C5)	0.63	-0.17 (-1.32, 0.98)	0.65 (-2.19, 3.50)
Isovalerylcarnitine	0.30	-0.17 (-1.14, 0.79)	-0.25 (-1.71, 1.21)
Isobutyrylcarnitine	0.56	-0.50 (-1.32, 0.33)	-0.95 (-2.72, 0.82)
Tryptophan	0.54	-0.80 (-1.64, 0.04)	**2.59 (0.03, 5.15)**
3-methyl-2-oxovalerate (KMV)	0.52	-0.33 (-1.35, 0.68)	2.32 (-0.06, 4.71)
Kynurenine	0.52	-0.38 (-1.39, 0.62)	**2.53 (0.51, 4.55)**
Tyrosine	0.51	-0.21 (-1.44, 1.02)	-0.17 (-3.12, 2.77)
Gamma-glutamylleucine	0.51	-0.08 (-1.27, 1.10)	1.03 (-0.78, 2.84)
4-methyl-2-oxopentanoate (KIC)	0.51	-0.04 (-1.02, 0.94)	2.31 (-0.08, 4.70)

a Estimates are adjusted for age, pubertal status as an ordinal variable, BMI percentile, biomarker level at the mid-childhood visit, and age at the early teen visit. Bolded text indicates statistical significance at alpha<0.05.

Table 7 Associations of metabolites within the androgen pattern with change in leptin and CRP in girls.

	Factor loading	β (95% CI)[a]	
		Change in leptin (ng/dL)	Change in CRP (mg/dL)
		***n* = 82**	***n* = 79**
4-Androsten-3beta,17beta-diol disulfate 1	0.86	-0.92 (-2.95, 1.11)	0.18 (-0.07, 0.42)
Dehydroisoandrosterone sulfate (DHEA-S)	0.84	-1.68 (-4.47, 1.11)	0.05 (-0.29, 0.39)
Epiandrosterone sulfate	0.79	-0.40 (-2.33, 1.52)	0.13 (-0.11, 0.37)
Androsterone sulfate	0.79	-1.56 (-3.83, 0.71)	0.10 (-0.17, 0.37)
4-androsten-3beta,17beta-diol disulfate 2*	0.78	**-1.78 (-3.35, -0.20)**	0.04 (-0.16, 0.23)
Pregn steroid monosulfate*	0.76	**-2.50 (-4.87, -0.13)**	0.02 (-0.27, 0.31)
Pregnen-diol disulfate*	0.70	-1.94 (-4.11, 0.23)	-0.02 (-0.29, 0.25)
Pregnenolone sulfate	0.65	-0.64 (-2.66, 1.39)	0.05 (-0.20, 0.30)
Andro steroid monosulfate 2*	0.61	-1.09 (-3.14, 0.97)	-0.02 (-0.28, 0.24)

a Estimates are adjusted for age, pubertal status, BMI percentile, and biomarker level at the mid-childhood visit, and age at the early teen visit. Bolded text indicates statistical significance at alpha<0.05. Bolded text indicates statistical significance at alpha<0.05.
* Indicates tier 2 identification in which no commercially available authentic standards could be found, however annotated based on accurate mass, spectral and chromatographic similarity to tier 1 identified compounds

SUPPLEMENTAL MATERIAL

Assays, laboratory techniques, and instrumentation for measurement of conventional metabolic biomarkers

At both the mid-childhood and early teen visits, we used fasting blood to measure plasma glucose enzymatically, and insulin using an electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, IN). Serum total cholesterol, triglycerides, and high-density lipoprotein (HDL) were measured enzymatically with correction for endogenous glycerol. We measured plasma leptin and adiponectin concentrations with a radioimmunoassay (Linco Research, St Charles, MO). We used an immunoturbidimetric high-sensitivity assay on a Hitachi 911 analyzer to determine C-reactive protein (CRP) concentrations (Roche Diagnostics, Indianapolis, IN). Plasma interleukin-6 (IL-6) was measured by ultrasensitive enzyme-linked immunosorbent assay (ELISA).

Assessment of covariates

At enrollment, mothers reported their pre-pregnancy weight and height, from which we calculated pre-pregnancy BMI. We used standard criteria to categorize BMI as normal (defined as 18.5-<25 kg/m^2, but we also included 4 women with BMI <18.5) overweight (25-<30 kg/m^2), and obese (≥30 kg/m^2) (1). Using interviews and questionnaires, we collected information on maternal race/ethnicity, age, household income; and child race/ethnicity. We determined gestational weight gain (GWG) as the difference between the last clinically-measured weight within 4 weeks prior to delivery and self-reported pre-pregnancy weight, and categorized it according to current IOM guidelines (2). Obstetric clinicians screened women for gestational diabetes mellitus (GDM) at 26–28 weeks of gestation with a non-fasting 50-g oral glucose

challenge, followed by a 100-g 3-hour oral glucose test for women with abnormal results. Based on results of the 2-stage testing, we categorized women as having GDM, impaired glucose tolerance, isolated hyperglycemia, or normoglycemia (3). We obtained birth characteristics, including sex and delivery date, from medical records. Mothers reported on breastfeeding duration in postpartum questionnaires.

At the mid-childhood and early teen visits, RAs measured children's weight (kg) using an electronic scale (Tanita Corporation of America, Inc., Arlington Heights, IL) and height (cm) with a calibrated stadiometer (Shorr Productions, Olney, MD). We used these values to calculate BMI, standardized it as percentile using the Centers for Disease Control growth reference (4), and categorized participants as normal weight (5^{th} to $<85^{th}$ percentile, but also included 4 children with BMI $<5^{th}$ percentile), overweight (85^{th} to $<95^{th}$ percentile), and obese ($\geq 95^{th}$ percentile). At the mid-childhood research visit, mothers reported on pubarchal/pubertal phenotype based on appearance of body hair, breast development for girls, and body hair, facial hair, and deepening of voice for boys on a scale of 1 (no development) to 4 (full development) (5). For the analysis, we combined the characteristics as an ordinal summary score of breast development and body hair for girls, and the mean of deepening of voice, facial hair, and body hair for boys for use as a covariate in multivariable models, as well as dichotomized as pre-pubertal (puberty score=1) vs. pubertal (puberty score>1) for stratified analyses.

Untargeted metabolomics profiling

For untargeted metabolomics profiling, we sent fasting plasma samples collected at the mid-childhood visit to Metabolon Inc. (Durham, NC, USA). Samples were prepared using the automated MicroLab STAR® liquid handling machine from Hamilton Robotics, which employs

an aqueous methanol extraction (with recovery standards to monitor extraction efficiency) to precipitate proteins fraction while allowing maximum recovery of small molecules. The extract was then divided into four fractions: one each for analysis on four different columns on ultrahigh performance liquid chromatography (UPLC)/MS/MS (2 for positive ions, 2 for negative ions) and mixed the samples for 5 minutes on a Geno/Grinder 2000 (Glen Mills, Inc.), followed by brief placement on a TurboVap® (Zymark) to remove the organic solvent.

Next, sample extraction and ultrahigh performance liquid chromatography (UPLC) was carried out as previously described (6)**.** The liquid chromatography (LC)/MS of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan linear trap quadrupole mass spectrometer, which consisted of an electrospray ionization source and linear ion-trap mass analyzer. The sample extract was reconstituted in acidic or basic LC-compatible solvents, each of which contained 8 or more injection standards at fixed concentrations to ensure injection and chromatographic consistency. One aliquot each was analyzed using a reverse-phase positive ion method for polar compounds, a reverse-phase positive ion method for lipid compounds, a reverse-phase negative ion method, and a negative ion method for hydrophilic compounds. The MS analysis alternated between MS and data-dependent MS/MS scans using dynamic exclusion. Raw data files are archived and extracted as described below.

For quality assurance/quality control (QA/QC) purposes, extracts of a pool created from a small aliquot of the experimental samples and process blanks were included with each day's analysis. The QC samples were spaced evenly among the injections and all experimental samples, and randomly distributed them throughout the run. A selection of QC compounds were added to every sample for chromatographic alignment, including those under test. These

compounds were carefully chosen so as not to interfere with the measurement of the endogenous compounds.

Finally, the raw data were extracted and compound peaks were identified using Metabolon's hardware and software. Compounds were identified via comparison to library entries of purified standards or recurrent unknown entities. More than 4000 commercially available purified standard compounds have been acquired and registered into Laboratory Information Management System (LIMS) for distribution to both the LC and GC platforms for determination of their analytical characteristics.

Table S1 Background characteristics of Project Viva participants within each of the analytical subsamples.

	Boys			Girls			
	Overall	**Table 5** (Δglucose)	**Table 6** (ΔSBP)	**Overall**	**Table 5** (Δtriglycerides)	**Table 7** (Δleptin)	**Table 7** (ΔCRP)
	***n* = 104**	***n* = 63**	***n* = 98**	***n* = 109**	***n* = 83**	***n* = 82**	***n* = 79**
Maternal & perinatal characteristics							
Annual household income ≤$70 k	61.9%	70.5%	62.6%	55.6%	53.3%	52.7%	52.1%
Smoked during pregnancy	10.6%	12.7%	11.2%	12.8%	13.3%	13.4%	13.9%
Pre-pregnancy BMI (kg/m^2)	25.3 ± 5.2	25.2 ± 5.5	25.2 ± 5.3	26.6 ± 6.5	26.5 ± 6.0	26.6 ± 6.0	26.6 ± 6.1
Gestational weight gain[a]							
Inadequate	7.7%	9.5%	8.2%	13.8%	14.5%	14.6%	15.2%
Adequate	28.9%	23.8%	25.5%	32.1%	32.5%	31.7%	32.9%
Excessive	63.5%	66.7%	66.3%	54.1%	53.0%	53.7%	51.9%
Gestational glucose tolerance							
Normoglycemic	79.8%	85.7%	81.6%	73.4%	74.7%	74.4%	73.4%
Isolated hyperglycemia	9.6%	9.5%	9.2%	15.6%	16.9%	17.1%	17.7%
Impaired glucose tolerance	2.9%	1.6%	3.1%	2.8%	2.4%	2.4%	2.5%
Gestational diabetes	7.7%	3.2%	6.1%	8.3%	6.0%	6.1%	6.3%
Duration of any breastfeeding							
<1 months	19.6%	18.3%	19.2%	14.9%	14.3%	14.5%	13.6%
1-<7 months	33.0%	30.0%	34.0%	41.5%	42.9%	42.0%	42.4%
7-<12 months	21.7%	23.3%	21.3%	20.2%	18.6%	18.8%	18.2%
≥12 months	25.8%	28.3%	25.5%	23.4%	24.3%	24.6%	25.8%
Child's characteristics at baseline							
Age (years)	7.9 ± 0.8	7.7 ± 0.7	7.9 ± 0.8	8.0 ± 0.8	8.0 ± 0.9	8.0 ± 0.9	8.0 ± 0.9
Race/ethnicity							
White	61.5%	68.3%	63.3%	55.1%	53.0%	52.4%	53.2%
Black	20.2%	15.9%	19.4%	22.9%	22.9%	23.2%	21.5%
Hispanic	5.8%	4.8%	6.1%	8.3%	9.6%	9.8%	10.1%
Other	12.5%	11.1%	11.2%	13.8%	14.5%	14.6%	15.2%
BMI percentile[b]	66.6 ± 27.2	66.8 ± 28.8	66.1 ± 27.5	71.0 ± 31.0	72.2 ± 30.8	72.9 ± 30.3	72.9 ± 30.3
Pubertal status							
Pre-pubertal	80.8%	82.5%	80.6%	61.0%	59.0%	58.5%	58.2%
Pubertal	19.2%	17.5%	19.4%	39.1%	41.0%	41.5%	41.8%

a According to the 2009 Institute of Medicine recommendations.

b Age- and sex-specific BMI percentile based on CDC 2000 reference data.

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