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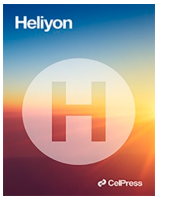

Research article

Integrative multiomics analysis of infant gut microbiome and serum metabolome reveals key molecular biomarkers of early onset childhood obesity

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ABSTRACT

Evidence supports a complex interplay of gut microbiome and host metabolism as regulators of obesity. The metabolic phenotype and microbial metabolism of host diet may also contribute to greater obesity risk in children early in life. This study aimed to identify features that discriminated overweight/obese from normal weight infants by integrating gut microbiome and serum metabolome profiles. This prospective analysis included 50 South Asian children living in Canada, selected from the SouTh Asian biRth cohorT (START). Serum metabolites were measured by multisegment injection-capillary electrophoresis-mass spectrometry and the relative abundance of bacterial 16S rRNA gene amplicon sequence variant was evaluated at 1 year. Cumulative body mass index (BMI_{AUC}) and skinfold thickness (SSF_{AUC}) scores were calculated from birth to 3 years as the total area under the growth curve (AUC). BMI_{AUC} and/or SSF_{AUC} >85th percentile was used to define overweight/obesity. Data Integration Analysis for Biomarker discovery using Latent cOmponent (DIABLO) was used to identify discriminant features associated with childhood overweight/obesity. The associations between identified features and anthropometric measures were examined using logistic regression. Circulating metabolites including glutamic acid, acetylcarnitine, carnitine, and threonine were positively, whereas γ-aminobutyric acid (GABA), symmetric dimethylarginine (SDMA), and asymmetric dimethylarginine (ADMA) were negatively

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associated with childhood overweight/obesity. The abundance of the *Pseudobutyrivibrio* and *Lactobacillus* genera were positively, and *Clostridium sensu* stricto 1 and *Akkermansia* were negatively associated with childhood overweight/obesity. Integrative analysis revealed that *Akkermansia* was positively whereas *Lactobacillus* was inversely correlated with GABA and SDMA, and *Pseudobutyrivibrio* was inversely correlated with GABA. This study provides insights into metabolic and microbial signatures which may regulate satiety, energy metabolism, inflammatory processes, and/or gut barrier function, and therefore, obesity trajectories in childhood. Understanding the functional capacity of these molecular features and potentially modifiable risk factors such as dietary exposures early in life may offer a novel approach for preventing childhood obesity.

1. Introduction

The pathophysiological mechanisms leading to excess adiposity early in life remains poorly understood despite an alarming increase in childhood obesity worldwide [1]. Recent advances in –omics technologies and approaches have allowed for a comprehensive characterization of metabolic networks to decipher underlying biological responses that contribute to the contemporary obesogenic environment [2]. For instance, the distribution of bacterial communities found in the intestine (i.e., the microbiome) of children who are overweight or obese differs from those who are of normal weight [3]. Perturbations in the gut microbiota therefore may underlie the phenotypic expression of obesity and corresponding changes in other –omics markers [4]. For instance, people with high levels of adipose tissue have also lower gut microbial diversity than normal weight controls [5], as well as elevated serum branched-chain amino acids (BCAAs) and biomarkers of inflammation, such as C-reactive protein [6]. Although host-derived factors are genetically hardwired, the microbiome can be regulated by environmental exposures, such as habitual diet, medication use, and hygiene [7]. However, it is unclear whether imbalances in the microbiome composition (i.e., dysbiosis) associated with disease and accompanying changes in the metabolome are a cause or consequence of childhood adiposity.

The colonization of the gut microbiota starts from birth and alterations in maturation during infancy is a potential contributor to obesity and metabolic traits [3]. Alterations in intestinal microbiota composition, specifically a higher *Firmicutes*-to-*Bacteroidetes* ratio and lower microbiota diversity are shown to be associated with obesity in children as young as 7 years of age [8], and can lead to disruption in energy acquisition and regulation [9]. Moreover, elevated *Firmicutes*-to-*Bacteroidetes* ratio can lead to more efficient hydrolysis and fermentation of the indigestible dietary polysaccharides to generate short-chain fatty acids (SCFAs), which can increase the host's ability to extract energy from the food components entering the gastrointestinal tract and activate the lipogenic pathways [10]. However, the structure and composition of infant microbiota is generally unstable and still developing, thus differences at the genus level and specific metabolites may be more directly related to childhood obesity [11].

Almost 10% of all circulating metabolites are derived from gut microbiota activity and participate in metabolic pathways [12]. Fecal metabolome studies have showed that obesity incidence is associated with higher levels of BCAAs, including leucine, isoleucine, and valine, and aromatic amino acids (AAs), including phenylalanine, tryptophan, and tyrosine [13]. Interestingly, the composition of intestinal microbiota, specifically the abundance of Bacteroides spp., may improve the efficiency of BCAA degradation [14]. Furthermore, trimethylamine *N*-oxide (TMAO), derived from gut microbial metabolism and dietary nutrients has been linked to pediatric obesity [13]. These findings support the role of certain metabolites that can be endogenously produced or derived in response to diet exposure and gut microbiota activity, which may contribute to a metabolic phenotype of excessive fat storage and low-grade inflammation. Integrative multi-omics analyses can be used to characterize molecular changes that accompany childhood obesity and augment a mechanistic understanding of complex interactions involving host metabolism, dietary exposures, and microbiome activity relevant to the developmental origins of adiposity.

Obesity and its complications are disproportionately more prevalent in non-white populations [15]. Children of South Asian ancestry have an increased cardiometabolic risk at lower body mass index (BMI) than other ethnic groups which has been attributed to lower lean mass and higher abdominal fat mass at the same BMI [16]. However, studies of metabolome or microbiome in childhood obesity have been performed primarily in white Europeans, and with sparse data reported in other ethnic populations, such as South Asians [13]. Another research gap is the integration of metabolomics with microbiome, which is of great interest in pediatric obesity research. To address these knowledge gaps, we employed a multi-omics approach of serum metabolomics together with amplicon sequence variants (ASVs) of 16S rRNA genes to identify integrated molecular features that characterize risk of obesity in young children. In addition to BMI, we included skinfold thickness as a measure of obesity as evidence suggests that fat mass is better associated with metabolic risk factors in children with obesity [17].

2. Methods

2.1. Data source and participants

The South Asian Birth Cohort (START) is a prospective birth cohort designed to study the influence of diverse environmental exposures and genetics on early life adiposity, growth trajectory and cardiometabolic health of South Asians living in Canada. START enrolled 1,012 South Asian mother–child (people who originate from the Indian sub-continent: India, Pakistan, Sri Lanka, or Bangladesh) pairs from the Peel Region of Ontario. Ancestral origin of both the woman, her partner, and both offspring's grandparents

were required to be classified as South Asian. Participants were recruited through physician referrals between 2011 and 2015, and followed up at 1-, 2-, and 3-years. Details on the START design and methodology have been described previously [18]. Of the 182 infants who provided fecal samples for microbiome analyses, our analytic dataset includes 50 infants who provided complete data on microbiome and serum metabolome at 1-year, and anthropometric measures at birth, 1, 2, and 3 years (Fig. S1). A primary caregiver of all enrolled participants provided full informed consent. The study was approved by the McMaster Hamilton Integrated Research Ethics Board [START (HiREB #10–640)].

2.2. Anthropometrics

A standard protocol was used to obtain child anthropometric measurements [18]. Length was measured using the O'Leary Pediatric Length Board at birth, 1-, and 2-years, and height after 2 years was measured using a stadiometer. Infant birth weight was obtained from the birth delivery reports, and weight at each follow-up visit was measured using an electronic scale. BMI was calculated as weight in kilograms divided by squared height or length in meters. The skinfold thickness of triceps and subscapular sites were measured in triplicate using calipers (Holtain Tanner/Whitehouse, UK) to the nearest 0.2 mm, and summed to create "sum of skinfolds" (SSF) [18].

2.3. Area under the curve (AUC) of BMI and SSF

For each child, we calculated area under the curve (AUC) of BMI_{AUC} and SSF_{AUC} from birth to 3-years as a cumulative exposure to summarize the duration and degree of body mass. The BMI_{AUC} and SSF_{AUC} were calculated separately using the following formula:

AUC = Average (BMI or SSF at age 1, BMI or SSF at birth) x (Age at 1–0) +

Average (BMI or SSF at age 2, BMI or SSF at age 1) x (Age at 2 – Age at 1) +

Average (BMI or SSF at age 3, BMI or SSF at age 2) x (Age at 3 – Age at 2)

In the analysis, children with BMI_{AUC} and/or SSF_{AUC} at or above internally derived 85th percentile were classified as being overweight/obese and those with BMI_{AUC} and SSF_{AUC} below 85th percentile were classified as normal weight [19]. A comparison of the classification of overweight/obesity using the BMI_{AUC} and SSF_{AUC} is provided in Table S1.

2.4. Serum metabolome analyses

A validated multiplexed separation platform based on multisegment injection-capillary-electrophoresis-mass spectrometry (MSI-CE-MS) was used for targeted and nontargeted analyses of 73 polar ionic metabolites measured in serum filtrate samples with stringent quality control (QC) [20]. A standardized protocol for identification and quantification of circulating serum metabolites under positive and negative ion mode detection is described in detail elsewhere [20]. Briefly, an iterative data workflow was used to effectively filter out spurious signals, redundant peaks, and background ions, when performing targeted and nontargeted metabolite profiling based on analysis of a pooled serum sample that served as a QC sample to monitor technical precision [21]. Serum metabolites were reported if they were detected in majority of the individual samples ($\geq$75%) with an acceptable technical precision (CV $<$30%) based on repeated analysis of QC samples to minimize false discoveries and data overfitting. Missing values (below method detection limit) were set as half of the lowest detected value for each metabolite. Unambiguous identification of most serum metabolites (level 1) in this work was achieved after spiking a pooled serum sample with authentic standards based on their co-migration and accurate mass with low mass error ($<$5 ppm). These authenticated metabolites were quantified in terms of their absolute concentration (μmol/L) using external calibration curves, where the ion response for each compound was normalized to an internal standard (i.e., relative peak area). Unknown serum metabolites were otherwise annotated based on their relative migration time, accurate mass and most likely molecular formula when chemical standards were lacking.

2.5. Microbiome data acquisition

A fecal sample was collected from infants at the 1-year visit. Mothers were instructed to collect stool sample from a regular diaper and record the time and date of the sample and place it in a sterile bag in the freezer until their scheduled appointment. Upon arrival, the stool samples were divided into four pre-labeled cryovials and transferred to the lab in a cooler, weighed, and stored at −80 °C. Sample storage, DNA extraction, 16S rRNA gene sequencing, and analysis has been described in detail previously [22]. In brief, the V3 region of 16S rRNA gene (150 base pairs) was sequenced in the McMaster Genomics Facility with paired-end 250-base pair sequencing on the MiSeq sequencer (Illumina, Inc.). Adapter, primer and barcode sequences were trimmed from sequencing reads using cutadapt (v1.2.1) [23], and ASVs were inferred using the Divisive Amplicon Denoising Algorithm 2 (DADA2) package in R [24]. The naive Bayesian classifier method in DADA2 was used to assign taxonomy using the SILVA 16S rRNA gene reference file.

2.6. Statistical analysis

Data Integration Analysis for Biomarker discovery using Latent cOmponent (DIABLO) was used to integrate 73 serum metabolites

and 55 bacterial ASV data to identify discriminant features between children with overweight/obesity and those with normal weight [25]. DIABLO is a supervised learning approach based on partial least squares (PLS) that builds on sparse Generalized Canonical Correlation Analysis (sGCCA) and aims to maximize covariance between linear combination of variables (latent component scores) and a response variable. Before proceeding with data integration, individual sparse-PLS-discriminant analysis (sPLS-DA) was used to understand major sources of variation in each dataset and guide the integration process (Figs. S2–3). A 10% prevalence filter was used to remove low-prevalence ASVs and then transformed using the Centered Log Ratio (CLR) with the ALDEx2 package in R [26]. Serum metabolome data were transformed using natural logarithm. First, the *block.splsda* function was used to determine the optimum number of components based on the performance of the model considering the centroid distance technique and lowest balanced error rate with a 5-fold cross-validation (repeated 500 times). One component was selected for use in the final model based on the lowest balanced error rate of 39% with a centroid distance metric. Next, a *tune.block.splsda* function was applied to choose the optimal number of variables from each data on each component. Furthermore, a *plotDiablo* function was used to generate a plot to show the overall correlation between the most discriminant ASVs and metabolites, and *circosPlot* to visualize correlations greater than 0.4 between them. Finally, the *plot.loadings* function is used to visualize the set of loading vectors assigned to each selected variables in each component. For discriminant analysis, the magnitude of the median value corresponds to the importance of each variable and the colour corresponds to the outcome group (overweight/obese and normal weight) in which the variable is most abundant.

Logistic regression models were also used to examine the association of the identified discriminant ASVs and metabolites with overweight/obesity. We estimated overweight/obesity per standard deviation increase in log-transformed serum metabolite concentration. Odds ratios (OR), 95% confidence intervals (95% CI), and p-values were reported. All analyses were carried out using R software, version 1.2.5.

3. Results

3.1. Descriptive statistics

The distribution of demographic characteristics in the overall cohort and by pediatric adiposity status are presented in Table 1. A total of 11 (22.0%) children were classified as overweight/obese and about 36% of these children were males. The overweight/obesity classification in our study were comparable to the World Health Organization Child Growth Standards (Fig. S4, Table S2). Children with overweight/obesity compared to those with normal weight had a lower mean social disadvantage index (0.91 vs. 2.06, p = 0.0146), and they were more likely to have been born preterm (18.2% vs. 7.7%, p = 0.3012, corresponding to mean gestational age of 35.52 vs. 39.52 months). Overall, 96% of infants were either currently being breastfed or had been previously but were no longer at the time of assessment.

Table 1
Descriptive statistics of maternal and infant characteristics overall and by overweight/obesity status of children in START cohort.

Variable	Overall n = 50	Overweight/Obese n = 11	Normal Weight n = 39
Sex (Male), n (%)	25 (50.0)	4 (36.36)	21 (53.85)
Maternal age (years), mean (SD)	30.64 (3.88)	31.18 (2.82)	30.49 (4.15)
Gestational age (weeks), mean (SD)	39.09 (1.54)	38.49 (2.23)	39.26 (1.28)
Gestational weight gain (kg), mean (SD)	13.92 (6.52)	14.72 (8.33)	13.68 (8.33)
Pre-pregnancy BMI (kg/m^2), mean (SD)	24.77 (4.78)	24.82 (4.46)	24.76 (4.92)
Gestational diabetes (GDM), n (%)[a]	21 (42.0)	7 (63.64)	22 (56.41)
Preterm birth (Yes), n (%)	5 (10.0)	2 (18.18)	3 (7.69)
Mode of delivery, n (%)			
Vaginal	34 (68.0)	9 (81.82)	25 (35.90)
Caesarean section (planned or emergency)	16 (32.0)	2 (18.18)	14 (35.90)
Antibiotic use in labour (Yes), n (%)	26 (53.06)	7 (70.0)	19 (48.72)
Breastfeeding status at 1-year, n (%)			
Yes, and child is still being breast fed	22 (44.0)	4 (36.36)	18 (46.15)
Yes, child was breast fed but now stopped	26 (52.0)	6 (54.55)	20 (51.28)
Child was never breast fed	2 (4.0)	1 (9.09)	1 (2.56)
Time of solid food introduction (months), mean (SD)	6.02 (1.36)	6.0 (0.89)	6.03 (1.48)
Maternal physical activity (min per day), mean (SD)	11.94 (18.05)	13.64 (17.04)	11.45 (18.52)
Social disadvantage index, mean (SD)[b]	1.77 (1.38)	0.91 (0.94)	2.06 (1.39)
Total fibre intake at 1 year (grams), mean (SD)	18.35 (8.36)	18.06 (10.44)	18.43 (7.83)
Energy Intake at 1 year (kcal), mean (SD)	24.07 (12.05)	25.23 (16.98)	23.75 (10.53)
Birthweight (kg), mean (SD)	3.23 (0.46)	3.23 (0.71)	3.23 (0.38)
BMI_{AUC}, mean (SD)	48.47 (5.06)	54.57 (5.37)	46.74 (3.41)
SSF_{AUC}, mean (SD)	52.17 (8.90)	63.33 (9.10)	49.02 (5.84)

BMI = Body mass index, SSF = Sum of skinfold, AUC = Area under the curve.
[a] GDM was defined based on the Born in Bradford oral glucose tolerance test criteria, self-reported GDM, and insulin use in pregnancy.
[b] The maximum social disadvantage index was five, and the lowest possible score was zero, reflecting the least social disadvantage.

3.2. Integrative analysis of the gut microbiome and serum metabolome

The DIABLO analysis revealed a weak correlation (r = 0.28) between discriminant bacterial ASV and circulating serum metabolites (Fig. 1A). The optimal feature panel consisted of 9 ASVs and 10 serum metabolites, which produced the highest correlations across the datasets and discriminated children with overweight/obesity from those with normal weight. The contribution of each selected feature based on its loading weights is shown in Fig. 1B. The most important serum metabolites associated with overweight/obesity in this cohort were glutamic acid (Glu), acetylcarnitine, threonine, carnitine, tryptophan, and asparagine, and the most important bacteria were ASVs from the genera *Pseudobutyrivibrio*, *Lactobacillus*, *Rothia*, and *Lachnospira*. In contrast, the most important serum metabolites

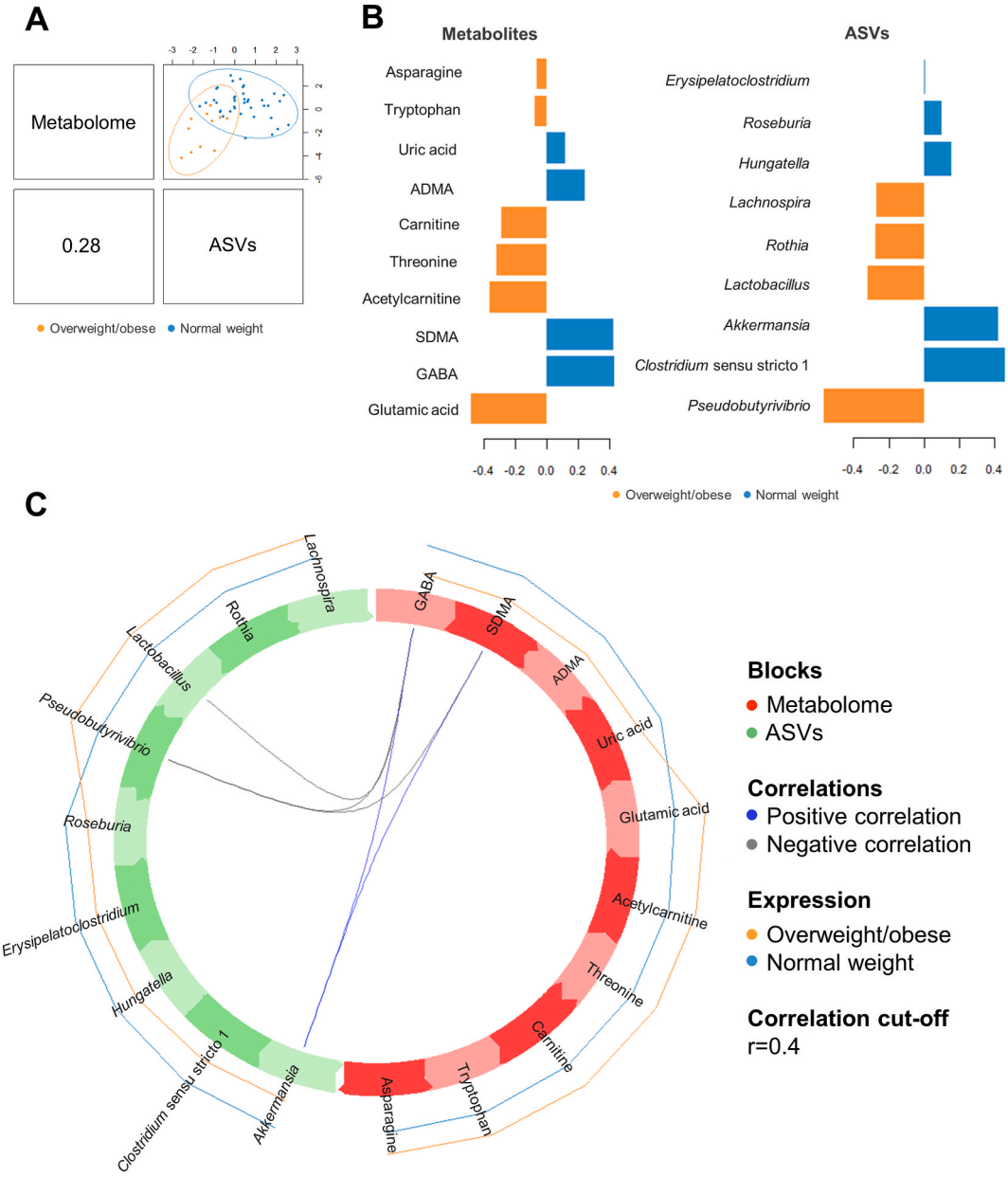

Fig. 1. DIABLO integrative analysis of metabolome and ASVs discriminatory between overweight/obese and normal weight groups. (A) Matrix scatter plot shows the clustering of samples based on the first component in each dataset and the correlation between the datasets. (B) Loading weights of the selected discriminant metabolites and ASVs. Colours indicate the group in which the median relative abundance is maximum, and values indicate the contribution to the first component. (C) Circos plot showing correlations between the most discriminatory metabolites and ASVs. Positive correlations are displayed using blue line-connectors. γ-aminobutyric acid (GABA); Symmetric dimethylarginine (SDMA); Asymmetric dimethylarginine (ADMA). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

associated with normal weight relative to children with overweight/obesity were γ-aminobutyric acid (GABA), symmetric dimethylarginine (SDMA), asymmetric dimethylarginine (ADMA), and uric acid, whereas ASV were members of the genera *Clostridium* sensu stricto 1, *Akkermansia*, *Hungatella*, *Roseburia*, and *Erysipelatoclostridium*. A circos plot displays correlated features between selected ASVs and serum metabolites using a minimum cut-off value of r = 0.40 (Fig. 1C). In this case, ASVs belonging to the genera *Pseudobutyrivibrio* and *Lactobacillus* were inversely correlated with GABA (r = −0.43 and r = −0.41, respectively) and SDMA (r = −0.42 and r = −0.40, respectively), and an ASV from the genus *Akkermansia* was positively correlated with GABA (r = 0.43) and SDMA (r = 0.41). Pearson correlation and their significance between the discriminatory serum metabolites and stool derived bacterial ASVs are depicted in Figs. 2–3.

3.3. Associations of selected metabolites and bacterial ASVs with overweight/obesity

The associations between serum metabolites and ASVs as a function of pediatric adiposity status are presented in Table 2. For metabolites involved in glutamate metabolic pathway, higher serum Glu was positively associated with the odds of childhood adiposity (OR per SD = 2.9; 95% CI = 1.3, 7.4), whereas higher serum GABA was negatively associated (OR per SD = 0.5; 95% CI = 0.2, 0.8) with the odds of childhood adiposity compared to children with normal weight. We examined the association between the ratio of the Glu/GABA with overweight/obesity. In comparison to single serum metabolites (Glu or GABA, AUC = 0.77 and 0.72, respectively),

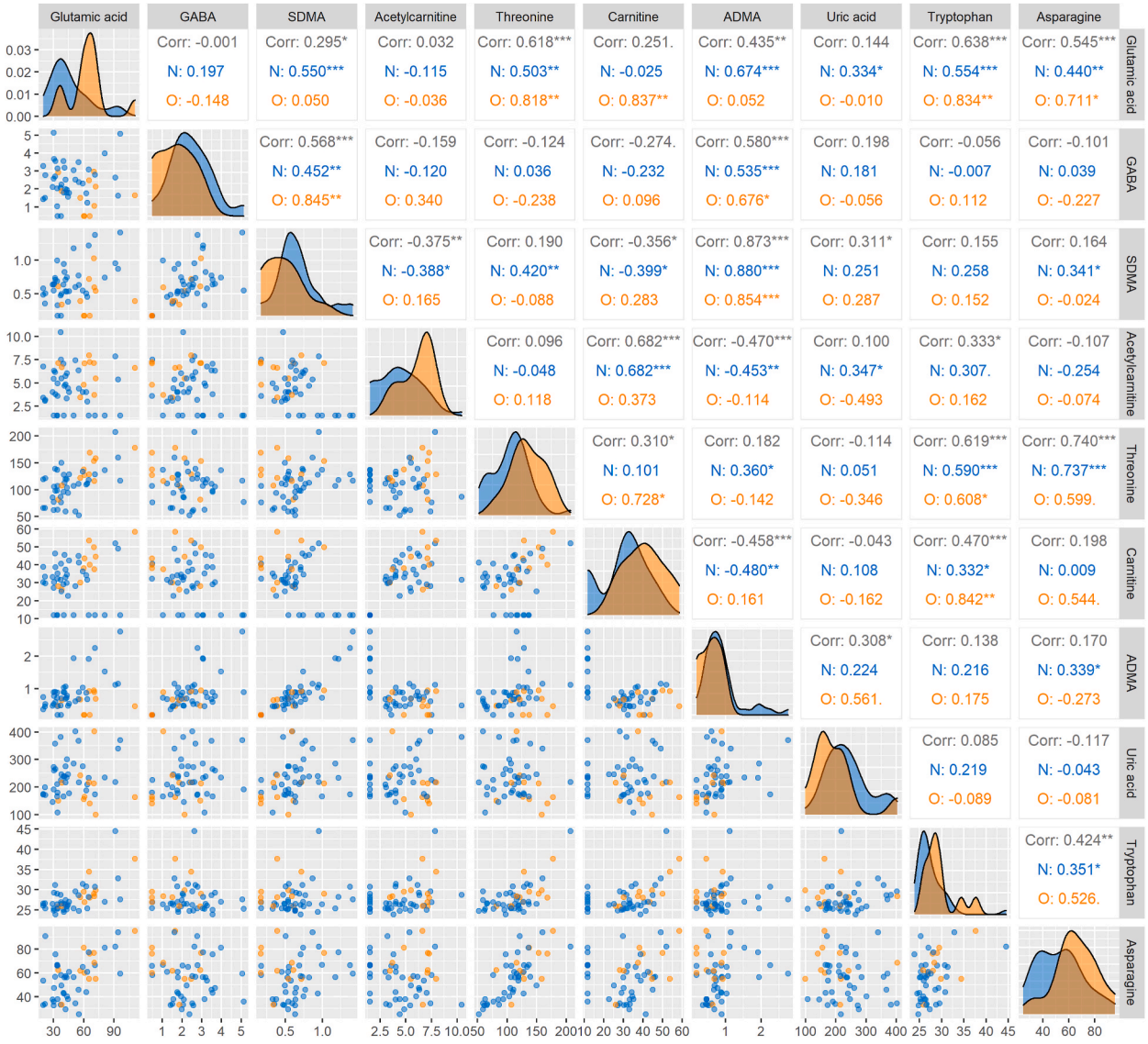

Fig. 2. Correlation between most discriminatory metabolites in participants overall and by overweight/obese (O) and normal weight (N) groups. γ-aminobutyric acid (GABA); Symmetric dimethylarginine (SDMA); Asymmetric dimethylarginine (ADMA).

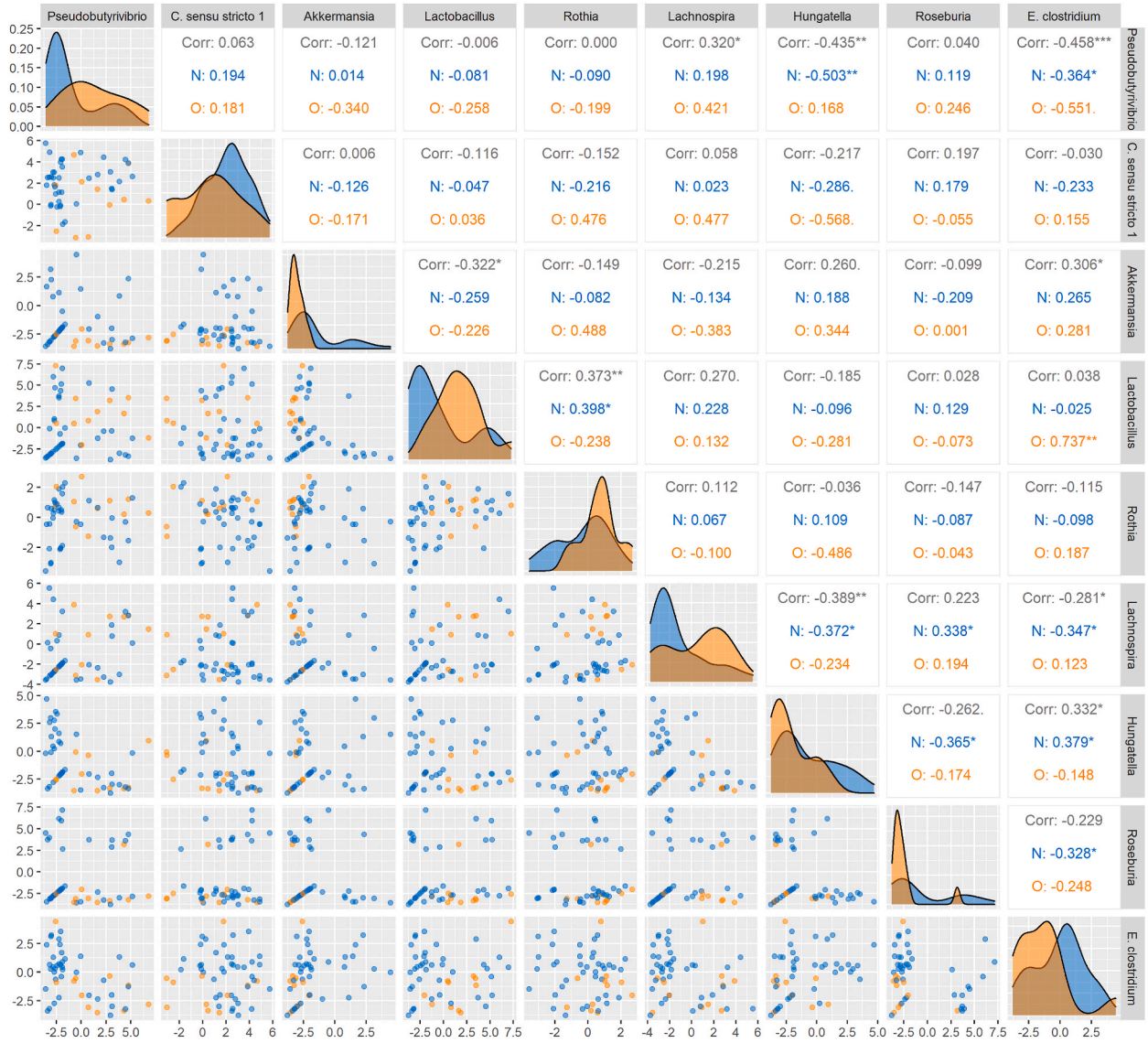

Fig. 3. Correlation between most discriminatory ASVs in participants overall and by overweight/obese (O) and normal weight (N) groups.

the discrimination is slightly more improved as a ratio (AUC = 0.81; 95% CI = 0.7, 0.9) with a significant difference using *t*-test (p = 0.0029) and a median fold-change of 2 (Fig. 4). Two main metabolites involved in carnitine metabolism, namely carnitine (OR per SD = 5.0; 95% CI = 1.4, 32.1) and acetylcarnitine (OR per SD = 3.6; 95% CI = 1.3, 14.6), were both also positively associated with pediatric adiposity. Serum concentration for SDMA (OR per SD = 0.4; 95% CI = 0.2, 0.8) and ADMA (OR per SD = 0.46; 95% CI = 0.2, 0.9), both isomers generated via methylation of arginine, were negatively associated with childhood adiposity. Threonine, related to glycine, serine, and threonine metabolic pathway, was positively associated with childhood adiposity. However, tryptophan, uric acid, and asparagine were not statistically associated with childhood anthropometric measures in this study.

The relative abundance of ASV from the genera *Pseudobutyrivibrio* (OR = 1.3; 95% CI = 1.0, 1.7) and *Lactobacillus* (OR = 1.2; 95% CI = 1.0, 1.5) were positively associated and the abundance of *Clostridium* sensu stricto 1 (OR = 0.7; 95% CI = 0.5, 0.9) and *Akkermansia* ASV (OR = 0.2; 95% CI = 0.1, 0.7) was inversely associated with children with overweight/obesity compared to normal weight children. ASV assigned as *Rothia*, *Lachnospira*, *Hungatella*, *Roseburia*, and *Erysipelatoclostridium* were not statistically associated with overweight/obesity. Table S3 reports the distribution of discriminatory serum metabolite concentration identified in DIABLO and Fig. 5 shows the distribution of statistically significant discriminatory serum metabolites and ASVs by overweight/obesity status.

4. Discussion

This study aimed to identify multi-omic molecular features that discriminated overweight/obese children from normal weight

Table 2
Results from logistic regression models examining the association of discriminatory metabolites (concentrations) and ASVs with overweight/obesity among children in the START cohort.

	Odds ratio[a] (95% CI)	p-value		
Metabolites (μmol/L)			**Sub-Pathway**	**Super-Pathway**
Glutamic acid	2.90 (1.33, 7.57)	0.0144	Glutamate metabolism	Amino Acid
GABA	0.45 (0.21, 0.86)	0.0204	Glutamate metabolism	Amino acid
Symmetric dimethylarginine	0.43 (0.20, 0.85)	0.0217	Urea cycle; arginine and proline metabolism	Amino acid
Acetylcarnitine	3.60 (1.34, 14.63)	0.0326	Carnitine metabolism	Lipid
Threonine	3.10 (1.29, 9.89)	0.0266	Glycine, serine and threonine metabolism	Amino acid
Carnitine	4.45 (1.42, 23.09)	0.0370	Carnitine metabolism	Lipid
Asymmetric dimethylarginine	0.46 (0.20, 0.92)	0.0378	Urea cycle; arginine and proline metabolism	Amino acid
Uric acid	0.46 (0.19, 0.96)	0.0536	Purine metabolism	Purine derivative
Tryptophan	1.69 (0.9, 3.46)	0.1113	Tryptophan metabolism	Amino acid
Asparagine	2.27 (1.06, 5.79)	0.0538	Alanine and aspartate metabolism	Amino Acid
ASVs			**Phylum**	**Family**
Pseudobutyrivibrio	1.30 (1.04, 1.67)	0.0258	Firmicutes	Lachnospiraceae
Clostridium sensu stricto 1	0.69 (0.47, 0.96)	0.0344	Firmicutes	Clostridiaceae 1
Akkermansia	0.23 (0.05, 0.67)	0.0301	Verrucomicrobia	Akkermansiaceae
Lactobacillus	1.23 (1.01, 1.54)	0.0497	Firmicutes	Lactobacillaceae
Rothia	1.79 (1.06, 3.53)	0.0534	Actinobacteria	Micrococcaceae
Lachnospira	1.27 (0.98, 1.67)	0.0684	Firmicutes	Lachnospiraceae
Hungatella	0.70 (0.43, 1.0)	0.0831	Firmicutes	Lachnospiraceae
Roseburia	0.75 (0.45, 1.00)	0.1237	Firmicutes	Lachnospiraceae
Erysipelatoclostridium	0.73 (0.49, 1.03)	0.0920	Firmicutes	Erysipelotrichaceae

[a] ORs are estimated per standard deviation (SD) increase in log-transformed metabolite levels.

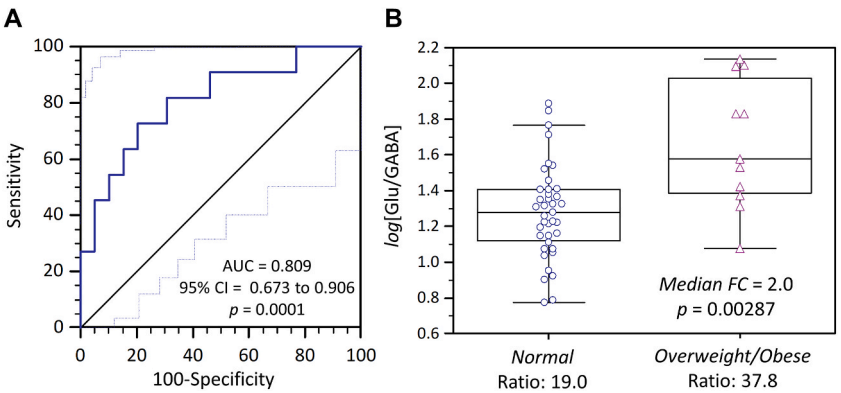

Fig. 4. (A) Receiver operating characteristic (ROC) curve and (B) boxplot for the serum Glu/GABA ratio illustrate the differentiation of overweight/obese (n = 11) from normal weight (n = 39) South Asian infants. Glutamic acid (Glu); γ-aminobutyric acid (GABA).

children. The results showed that children with higher adiposity as classified by BMI or SSF had higher circulating concentrations of Glu, acetylcarnitine, carnitine, and threonine, and lower levels of GABA, SDMA, and ADMA compared to normal weight children at 1-year of age. Furthermore, higher abundance of members of the genera *Pseudobutyrivibrio* and *Lactobacillus*, and lower abundance of members of the genera *Clostridium* sensu stricto 1 and *Akkermansia* were observed in the feces of children with excess adiposity. An *Akkermansia* ASV was positively correlated with GABA and SDMA, whereas an ASVs from the genera *Lactobacillus* was inversely correlated with GABA while ASVs from the genera *Pseudobutyrivibrio* was inversely correlated with both, GABA and SDMA.

Glu is a non-essential α-amino acid necessary for the biosynthesis of protein that also functions as a major neurotransmitter, but it is also acquired from foods common to omnivore diets (e.g., meats, poultry, fish, eggs, and dairy products) and a widely used flavoring additive in processed foods (e.g., monosodium glutamate). Overall, serum Glu was identified to have the greatest discriminatory power with higher circulating concentrations measured in overweight/obese than normal weight children. Hyperglutamataemia has earlier been implicated in childhood obesity that may be toxic to neuronal cells and disrupt the hypothalamic signalling cascade of leptin, thereby impairing the regulation of appetite with reduced satiety [27,28]. Previous metabolomic studies have independently reported that elevated glutamate is associated with obesity in children [29,30] and is also a proposed indicator of future risk of cardiometabolic disorders [31]. Moreover, it is proposed that elevated Glu concentrations increase the transamination of pyruvate to alanine, which can lead to the development of obesity-related insulin resistance [32]. We observed a moderate correlation between Glu and alanine ($r = 0.57$, $p < 0.001$), and on average higher concentrations of alanine in overweight/obese compared to those with normal weight children

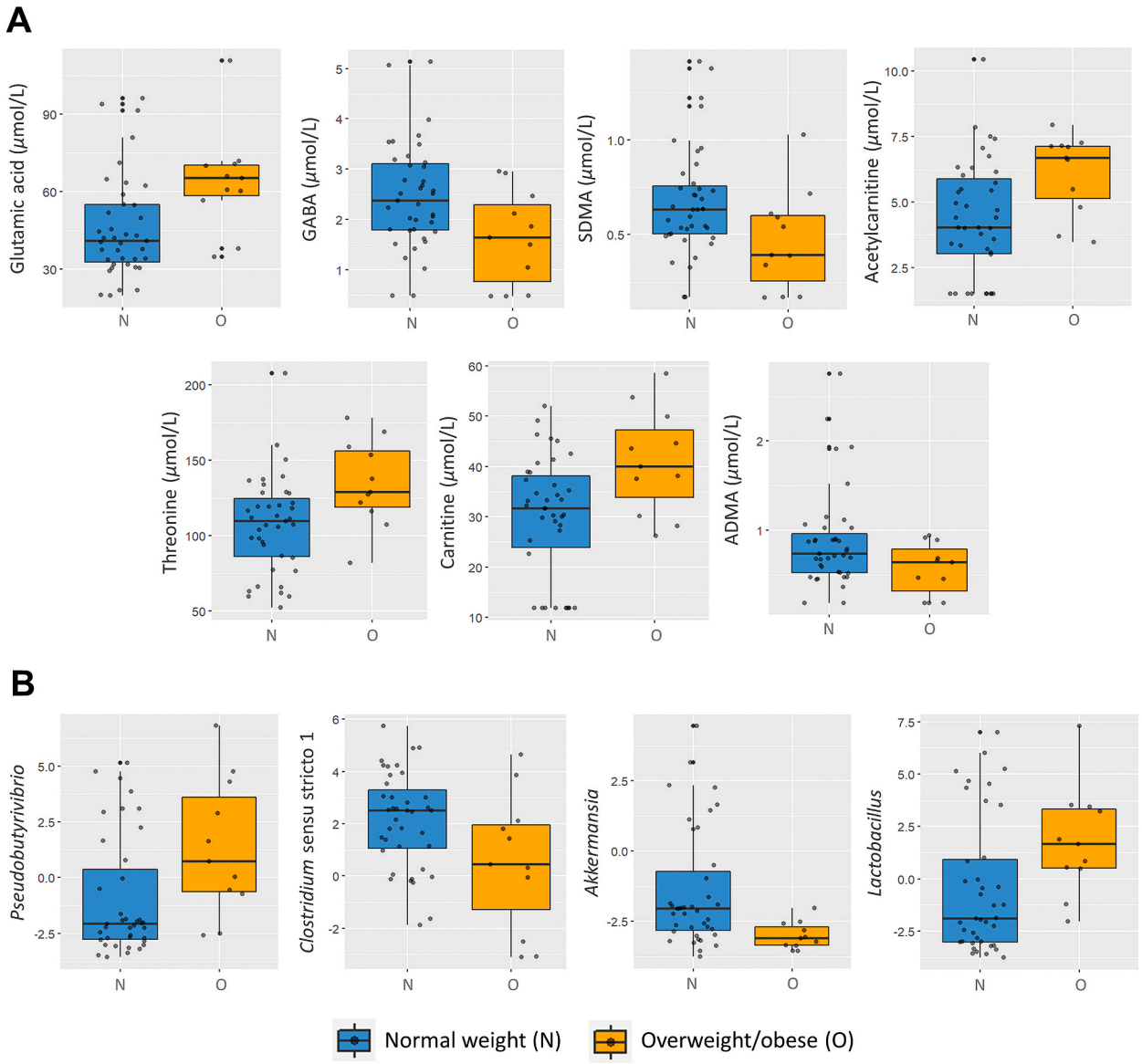

Fig. 5. Distribution of significantly different (A) metabolites (concentration) and (B) ASV between children who were overweight/obese (O) and normal weight (N). ASV counts were transformed using CLR-transformation.

(mean = 413 vs. 337 μmol/L, p = 0.0331). Several factors likely contribute to the variation in circulating levels of Glu, including dietary habits, psychological factors, genetic variation, and gut microbiota. We used maternal food-frequency data from pregnancy to compare serum levels of Glu and its associated foods. Higher Glu was positively correlated with maternal consumption of red or processed meat (r = 0.37, p = 0.0085) and eggs (r = 0.38, p = 0.0066) based on Pearson correlation analysis, but it did not correlate with any other protein-based dietary factor (Fig. S5).

In contrast to glutamic acid, GABA, a primary inhibitory neurotransmitter synthesized from glutamate by glutamic acid decarboxylase, was present in higher concentrations in the normal weight sample, and this finding is in agreement with a previous study involving American Indian adolescents [33]. Animal studies have previously demonstrated that GABA in the frontal cortex and hippocampus is involved in the regulation of food intake and body weight that can be lowered via consumption of a high-fat diet [34]. We observed higher infant dietary fat (grams) to be only weakly negatively correlated with GABA concentration (r = −0.25, p = 0.0762). Also, hepatic GABA synthesis may modulate insulin and glucagon secretion, homeostatic model assessment for insulin resistance (HOMA-IR), type 2 diabetes, and BMI [35]. GABA in the central nervous system has been implicated in regulation of systemic metabolism. Activation of lateral hypothalamus GABA type A receptors suppresses eating and reduces body weight, and suppression of this signaling pathway results in increased food intake [36]. Alterations in gut microbiome can influence changes in plasma concentration of Glu and GABA levels [37]. Evidence showed that fecal transplants from lean to obese individuals resulted in increased

GABA levels in plasma [38]. GABA is not restricted to production by host neurons as a major inhibitory neurotransmitter, but can be generated by certain gut microbiota [39] and promote brown adipose tissue dysfunction [40]. In our study, GABA was positively correlated with member of the genus *Akkermansia*, and negatively correlated with members of the genera *Pseudobutyrivibrio* and *Lactobacillus*. In fact, higher abundance of *Akkermansia* has been shown to impact the net production capacity of GABA [41]. Also, GABA has been shown to serve as an acid-resistance mechanism for few bacterial species including *Lactobacillus* [42]. Although there is no evidence in children, increased abundance of *Akkermansia* has been inversely associated with higher fasting glucose, waist-to-hip ratio, and subcutaneous adipocyte diameter in adults [43,44]. Meanwhile, *Pseudobutyrivibrio* [11] and *Lactobacillus* [45] have been found in higher abundance in feces of obese children, and higher abundance of *Lactobacillus* was correlated with plasma inflammatory marker C-reactive protein [45]. Previously, *Lactobacillus* species have been identified in microbiota of breast milk [46]. Our data confirms this finding where children who were breastfed until 1-year had a higher abundance of members of the genus *Lactobacillus* compared to those who were breastfed less than 1 year or never breastfed ($p = 0.0488$). Although these results are intriguing and largely consistent with previous studies, the mechanisms involved are not clear, thus further studies with greater study power are required to better decipher the potential causal relationship between these ASVs and circulating metabolites in early onset childhood obesity.

We also observed higher concentrations of serum carnitine and acetylcarnitine in infants who were overweight/obese. Carnitine is largely acquired from breast milk and formula milk in newborns but can also be synthesized endogenously from two essential amino acids, lysine and methionine [47]. Although our data showed a strong correlation between lysine and methionine ($r = 0.80$, $p < 0.001$), we did not find any correlation of carnitine concentrations with lysine and methionine suggesting an exogenous contribution from (maternal) diet to infant carnitine status. Carnitine is essential for the transport of long-chain fatty acids from cytoplasm into mitochondria for β-oxidation and energy production, and therefore it has a vital regulatory role in lipid metabolism and body composition [48]. Supplementation with carnitine can increase fat oxidation in individuals with overweight/obesity, and therefore has been widely studied for weight loss [49]. Thus, the positive effect of carnitine supplementation on body composition conversely suggests that higher body fat maybe related to mitochondrial dysfunction and impairments in energy generation [50]. Additionally, greater body fat may overload β-oxidation of fatty acids and lead to higher short- or medium-chain-acylcarnitines [51]. Short-chain acylcarnitines such as acetylcarnitine and carnitine are associated with higher BMI in children [29]. Furthermore, both carnitine and acetylcarnitine have been linked to protein-rich diets [47]. However, we could not confirm these associations due to the low consumption of meats in our South Asian cohort (Fig. S5).

Another metabolite, threonine, was shown to be present in higher level in children who were overweight/obese, which is consistent with a previous study [52]. Dietary threonine restriction may protect against metabolic alterations associated with obesity and improve metabolism via regulation of liver-derived hormone fibroblast growth factor 21 [52]. Threonine restriction induces fibroblast growth factor 21 and deletion of this hormone in mice blocks adaptive metabolic responses to protein restriction [53]. Also, fibroblast growth factor 21 signaling is essential for increase energy expenditure and resistance to diet-induced obesity [54]. The role of threonine deficiency and its association with lower intestinal immunity and greater inflammation has been examined in various animal models [55] given its key role as a nutrient immunomodulator impacting mucosal integrity and barrier function [56]. However, its underlying pathophysiology is speculative in humans and further research needs to elucidate the relevance of circulating threonine on early-onset obesity in children, including its impact on gut barrier function [57]. We observed threonine to be positively correlated with Glu, tryptophan, and asparagine ($r > 0.68$, $p < 0.0001$). Several studies have shown that restriction of essential and non-essential amino acids can improve lipid metabolism and resist obesity through several pathways [58,59].

In addition to GABA (discussed above), SDMA [60,61] and its structural isomer ADMA was present in higher concentrations in normal weight compared to overweight/obese infants. Both SDMA and ADMA are dimethylarginine isomers generated from turnover of histone proteins that function as inhibitors of endothelial nitric oxide biosynthesis by competitive binding to nitric oxide synthases, thus it has been traditionally linked to endothelial dysfunction [62]. However, higher levels of urinary ADMA/creatinine ratio concentration were found to be protective against cardiac deaths [63], and results from the Framingham Offspring Study found no association between plasma ADMA and cardiovascular death [64]. While dimethylarginine dimethylaminohydrolase (DDAH) can metabolize ADMA, alanine-glyoxylate aminotransferase 2 (AGXT2), a mitochondrial aminotransferase expressed primarily in the kidney can metabolize both ADMA and SDMA [65]. Higher AGXT2 is suggested to be related to elevated systemic ADMA and SDMA due to decreased renal excretion and have been associated with worse clinical outcomes [66]. Thus, given the strong correlation between ADMA and SDMA in our study ($r = 0.90$), higher AGXT2 activity could possibly explain lower levels of ADMA and SDMA in children with obesity in our study. Although the underlying cause of the inverse association with obesity is unclear, studies have attributed this to increased cellular uptake and hepatic extraction of SDMA, where both mechanisms have been related to increased insulin levels associated with obesity-induced insulin resistance [67,68].

Tryptophan, an essential aromatic amino acid, acquired from certain whole foods (such as oats, poultry, fish, eggs, and milk) was present in higher levels in the overweight/obese samples in the DIABLO analysis, but was not statistically significant in the regression analysis, although the effect size supports a potential association (OR = 1.9; $p = 0.0667$). Our data shows that higher tryptophan levels were correlated with consumption of red meat ($r = 0.29$, $p = 0.0382$) and eggs ($r = 0.34$, $p = 0.0151$). Overnutrition may lead to excess tryptophan uptake and availability [69]. Approximately 90–95% of tryptophan is metabolized by the kynurenine pathway in the liver via tryptophan-2,3-dioxygenase (IDO) into co-enzyme nicotinamide adenine dinucleotide (NAD+) and other bioactive metabolites; and residual tryptophan is largely used for serotonin synthesis [70]. Tryptophan catabolism is shifted towards the kynurenine pathway in human obesity induced by inflammatory biomarkers (TNFα and IL-6) and oxidative stress [71,72]. Elevated tryptophan [73], IDO activity [73], and kynurenine levels [73] and reduced serotonin production [74] has been shown to be associated with obesity and related metabolic diseases. Further, alterations in tryptophan metabolism may also be driven by the gut microbiota, as previously

shown to be disrupted at the compositional and functional level in individuals with obesity [75]. Taken together, the evidence suggests that obesity may induce concomitant alterations of host (kynurenine pathway) and microbial (indole) tryptophan metabolic pathways, both of which are associated with obesity-related inflammation [74].

Overall, our findings in the South Asian children are mainly consistent with those reported in white Europeans. However, our study did not detect any statistically significant difference in expression of circulating concentrations of BCAAs (leucine, isoleucine, and valine) and aromatic AAs (phenylalanine and tyrosine), which have been proposed candidate biomarkers of childhood obesity [13]. This discrepancy could be in part explained by differences in genetic background and/or lifestyle factors between South Asians and other ethnic groups (mainly Europeans) included in previous studies. Infants present several differences than older children in terms of metabolism, diet, and lifestyle, all of which may contribute to differences in their metabolic phenotypes. Moreover, our study examined very early obesity onset using serum metabolites at 1 year, and two studies that predicted children weight status using plasma sample collected in infancy did not identify BCAAs and aromatic AAs (phenylalanine and tyrosine) as predictors of excessive weight gain. Nevertheless, our data demonstrated a significant positive correlation between BCAAs and Glu, which is produced during the transamination reaction (first step) in BCCA catabolism (Fig. S6). Higher BCAAs have been shown to disrupt the balance of essential amino acids including tryptophan and threonine (both were correlated with BCAAs in our data), which we observed in higher levels in children with overweight/obesity. Given this data, future studies need to address this discrepancy in a larger cohort in different ethnicities to verify their clinical utility and overall generalizability.

GABA is produced by gut microbes from Glu and known to regulate appetite via the gut–brain axis. Our work revealed for the first time the significance of two abundant neurotransmitters associated with childhood obesity given their roles in regulating food satiety and behavior. In fact, the ratio of Glu to GABA was a stronger discriminant of overweight/obesity and normal weight infants than these two metabolites individually (Fig. 4). However, a larger cohort (notably in overweight/obese sub-group) and repeated measures of serum metabolites at 2, 3, and 5 years are required for further validation. Furthermore, carnitine/acetylcarnitine accumulation can reflect underlying mitochondrial dysfunction with incomplete or reduced mitochondrial fatty acid oxidation, whereas threonine is necessary for gut inflammatory responses and intestinal barrier function, and elevated SDMA and ADMA may increase systemic inflammation.

The gut microbiome provides essential capacities for fermentation of non-digestible substrates such as complex plant carbohydrates (dietary fibre) [76]. Differences in gut microbiota composition can influence an individual's capacity to extract more energy from diet which in turn can activate lipogenic pathways [77]. Several studies have shown children with obesity to have higher levels of bacteria in the *Firmicutes* phylum and lower in the *Bacteroidetes* phylum [78], and it is proposed that *Firmicutes* are more efficient at extracting energy from dietary fiber than *Bacteroidetes* [79]. Given that South Asians have a carbohydrate rich diet, it is possible that they have an elevated risk of obesity as their microbiome is enriched with bacteria that is more efficient at extracting energy and absorbing more calories, which increases the risk of cardiometabolic diseases. However, it must be acknowledged that exposure during fetal development such as mode of delivery (vaginal or Caesarian section), breastfed or formula-fed, and antibiotics use were not accounted for in this study but may contribute to the observed link between microbiome and obesity in South Asians.

5. Conclusions

Our study suggests the potential role of integrated molecular analysis for identifying biomarkers that discriminate between children who are overweight/obese and those who were of normal weight to unravel the pathophysiology of childhood obesity early in infancy. Notable differences were found in serum metabolome, and between specific metabolites and ASVs associated with overweight/obesity. Several correlated pairs of bioactive metabolites within distinct biochemical pathways, including Glu and GABA, acetylcarnitine and carnitine, SDMA and ADMA, and threonine were altered in overweight/obese infants. These findings suggest that eating behaviors and energy metabolism may be programmed early in childhood as reflected by differences in neurotransmitter expression and carnitine status. Furthermore, early signatures of poor gut barrier function and systemic inflammation in overweight/obese as compared to normal weight infants, reflected by differences in serum concentrations of threonine, and SDMA/ADMA, respectively. The abundance of the *Pseudobutyrivibrio* and *Lactobacillus* genera were positively, and *Clostridium sensu* stricto 1 and *Akkermansia* were negatively associated with childhood overweight/obesity. Additionally, by integrating the metabolites and bacterial genus, we found that *Akkermansia* was positively and *Pseudobutyrivibrio* was negatively correlated with both GABA and SDMA, and *Lactobacillus* was inversely correlated with GABA. While correlative, these findings suggest that the serum metabolic phenotype and bacterial fecal microbiome of overweight/obese South Asian children are distinct from those who are normal weight. Our analysis confirmed previously identified biomarkers and revealed significant associations between the multi-omics biomarkers. Future prospective studies in a larger cohort of children from different ethnicities and dietary habits are required to establish the causal relationships among these biomarkers and their overall clinical utility when linked to other well-established measures of immune function, inflammation and gut health. Understanding the functional capacity of these biomarkers and potential modifiable risk factors (e.g., diet, microbiome) early in life may lead to targeted early-life screening and therapeutic interventions, thereby offer a novel approach for prevention of the increasing prevalence of childhood obesity worldwide.

5.1. Limitations of the study

This study has some limitations that should be considered when interpreting the results. Although the study had a smaller sample size with a modest number of overweight/obese infants, it is consistent with a number of previous studies in this context [80,81]. The choice of -omic platforms and biological sample can influence the performance of data integration and comparison, and our study

lacked the analysis of serum lipids. Further, the number of features per dataset may determine the integration process and classification performance. This may explain why we did not observe a significant overlap between the two -omics datasets. Another likely explanation for the identification of small number of ASVs is due to the high between-subject variation in gut microbiome, which is well-known to confound studies with smaller sample size. Finally, we were unable to adjust for covariates in the regression analysis and therefore causal association cannot be established. Nevertheless, a major strength of this study is the integration of the metabolomic and microbiome data in early infancy to understand their impact on childhood obesity, an area that is not extensively studied in the literature, notably among South Asians.

Author contribution statement

Talha Rafiq: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.
Jennifer C. Stearns: Conceived and designed the experiments. Analyzed and interpreted the data.
Meera Shanmuganathan: Performed the experiments; Contributed reagents, materials, analysis tools or data.
Sandi M. Azab: Performed the experiments; Contributed reagents, materials, analysis tools or data.
Sonia S. Anand: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Lehana Thabane: Analyzed and interpreted the data.
Joseph Beyene: Analyzed and interpreted the data.
Natalie C. Williams: Contributed reagents, materials, analysis tools or data.
Katherine M. Morrison: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Koon K. Teo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.
Philip Britz-McKibbin: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.
Russell J. de Souza: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. RJ de Souza has served as an external resource person to the World Health Organization's Nutrition Guidelines Advisory Group on *trans* fats, saturated fats, and polyunsaturated fats. The WHO paid for his travel and accommodation to attend meetings from 2012-2017 to present and discuss this work. He has presented updates of this work to the WHO in 2022. He has also done contract research for the Canadian Institutes of Health Research's Institute of Nutrition, Metabolism, and Diabetes, Health Canada, and the World Health Organization for which he received remuneration. He has received speaker's fees from the University of Toronto, and McMaster Children's Hospital. He has served as an independent director of the Helderleigh Foundation (Canada). He serves as a member of the Nutrition Science Advisory Committee to Health Canada (Government of Canada), co-chair of the Method working group of the ADA/EASD Precision Medicine in Diabetes group, and is a co-opted member of the Scientific Advisory Committee on Nutrition (SACN) Subgroup on the Framework for the Evaluation of Evidence (Public Health England). He has held grants from the Canadian Institutes of Health Research, Canadian Foundation for Dietetic Research, Population Health Research Institute, and Hamilton Health Sciences Corporation as a principal investigator, and is a co-investigator on several funded team grants from the Canadian Institutes of Health Research.

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Appendix A. Supplementary data

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