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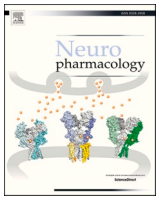

Characterizing the metabolomic signature of attention-deficit hyperactivity disorder in twins

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ABSTRACT

Emerging evidence implicate the gut microbiota as a potential susceptibility factor in attention-deficit hyperactivity disorder (ADHD), a common multifactorial neurodevelopmental condition. However, little is known about the biochemical signature of ADHD, including the metabolic contribution of the microbiota via the gut-brain axis, and the relative contribution of genetics and environmental factors. Here, we perform unbiased metabolomic profiling of urine and fecal samples collected from a well-characterized Swedish twin cohort enriched for ADHD (33 ADHD, 79 non-ADHD), using ^{1}H nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry. Our results highlight sex-specific patterns in the metabolic phenotype of individuals with ADHD. Specifically, the urine profile of males, but not females, with ADHD was characterized by greater excretion of hippurate, a product of microbial-host co-metabolism that can cross the blood-brain-barrier with bioactivity of potential relevance to ADHD. This *trans*-genomic metabolite was also negatively correlated with IQ in males and was significantly correlated with fecal metabolites associated with gut microbial metabolism. The fecal profile of ADHD individuals was characterized by increased excretion of stearoyl-linoleoyl-glycerol, 3,7-dimethylurate, and FAD and lower amounts of glycerol 3-phosphate, thymine, 2(^{1}H)-quinolinone, aspartate, xanthine, hypoxanthine, and orotate. These changes were independent of ADHD medication, age, and BMI. Furthermore, our specific twins' models revealed that many of these gut metabolites had a stronger genetic influence than environmental. These findings suggest that metabolic disturbances in ADHD, involving combined gut microbial and host metabolic processes, may largely derive from gene variants previously linked to behavioral symptoms in this disorder.

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1. Introduction

Attention-Deficit Hyperactivity Disorder (ADHD) is one of the most common neurodevelopmental conditions, defined by impairing symptoms of inattention, impulsivity, and hyperactivity (Posner et al., 2020). Worldwide prevalence of ADHD is estimated to be 3–7% in childhood and adolescence, and around 3% in adulthood with a greater prevalence in boys than girls (2–3:1 ratio). ADHD is associated with an

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increased risk of a range of other mental health issues, including depression, anxiety, oppositional defiant disorder, antisocial behavior, and substance abuse. It is a heterogenous and multifactorial behavioral phenotype, involving genetic susceptibility, environmental risk factors, and gene-environmental interactions. The physiology underlying ADHD is not yet fully understood, but alterations of brain monoaminergic systems and reduced connectivity of brain neural networks have been linked to challenges in executive functioning and reward related processes.

Increasing interest has been devoted to the potential role of the gut microbiome (the trillions of indigenous microbes that inhabit our intestine, along with their accompanying genomes) in the pathogenesis of neurodevelopmental and neuropsychiatric conditions, including ADHD. Over the past decade, studies have revealed a complex bidirectional communication network between the gut microbiota and the brain, known as the microbiota-gut-brain axis. One component of this communication involves the metabolic activity of the microbiota, resulting in the exchange of metabolites between the microbiota and the CNS and alterations in the availability of compounds important to CNS functions. Many microbial-derived metabolites (*e.g,* short-chain fatty acids, indoles, cresols, methylamines, neurotransmitters) and cell-wall components (*e.g.,* peptidoglycans) have been found to possess bioactivity with the potential to impact on brain processes. Indeed, the gut microbiota is now recognized as a key regulator of many aspects of host development and physiology, including the early life programming of brain circuits involved in motor control, emotion regulation, and cognition (for a review, see (Cryan et al., 2019)) as well as a wide-range of neurodevelopmental processes (*e.g.*, blood-brain barrier formation, microglial maturation and function, myelination).

The gut microbiota is being increasingly linked with ADHD. Several studies have identified differences in the gut microbial composition between individuals with ADHD and neurotypical controls, albeit without a clear consensus regarding the specific bacterial taxa or magnitude (Aarts et al., 2017; Szopinska-Tokov et al., 2020). In addition, gastrointestinal (GI) symptoms have been linked to ADHD with an increased prevalence of constipation and fecal incontinence reported in children with ADHD, independent of medication, compared to children without ADHD (Mckeown et al., 2013). Preliminary evidence also suggests that manipulation of dietary components that influence the composition of the gut microbiota, may be beneficial for individuals with ADHD and a history of adverse reactions to food (Stevenson et al., 2014). The causal potential of the microbiota in ADHD has also been suggested from rodent studies transferring the fecal gut microbiota from ADHD individuals into germ-free mice, resulting in changes to brain structure and behavior relevant to ADHD pathology (Tengeler et al., 2020).

As a composite of molecules derived from gut microbial and host metabolic processes, interactions between these systems and with dietary components, the metabolome provides a high-resolution overview of the biochemical events occurring within the holobiont. Metabolomics has been an effective tool for unravelling the metabolic features contributing to the microbiota-gut-brain axis. Using such approaches, Yang et al. recently showed lower plasma concentrations of short-chain fatty acids, produced in the gut during bacterial fermentation of dietary fibers and resistant starch, in children and adults with ADHD (Yang et al., 2022). These differences were partly explained by antibiotic exposure, age, and stimulant medication (Tian et al., 2022). Alterations have also been noted in amino acid (*e.g.*, L-norleucine and citrulline) and fatty acid metabolism (*e.g.,* aminocaproic acid and 3-methylazelaic acid) from the urine profiles of children with ADHD and in fatty acid and kynurenine metabolism from the plasma of adults with ADHD (Aarsland et al., 2015; Bonvicini et al., 2018; Irmisch et al., 2013). These preliminary findings suggest that the biochemical landscape is perturbed in ADHD, potentially including the crosstalk between the intestinal microbiota and the CNS.

Previous studies have emphasized the heterogeneity in ADHD in terms of etiology, clinical profiles, trajectory, and neurobiological mechanisms. However, there has been a lack of studies deeply phenotyping the participants, at the biochemical and behavioral level, or using twin and sibling designs to control for genetic and environmental confounders. Monozygotic twins discordant for ADHD diagnosis are particularly powerful to control for genetic confounding factors and other biases, such as shared environment, and early family experiences, and can be effective for delineating the genetic influence on the metabolome and behavior from the environmental contributions. In the present study, we applied a ^{1}H nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectrometry (LC-MS)-based unbiased metabolomics approach to characterize the urinary and fecal metabolic signatures of a twin cohort enriched for ADHD.

2. Materials and methods

2.1. RATSS study

Samples were collected from the ongoingRoots of Autism and ADHD Twin Study in Sweden (RATSS) during the recruitment phase between 2011 and 2015. The primary recruitment source for RATSS is the population-based Child and Adolescent Twin Study in Sweden (CATSS) (Anckarsäter et al., 2011; Bölte et al., 2014). Twins with profound intellectual disability (IQ < 35) and any serious psychiatric, neurological, or well-defined genetic disorders were not included in RATSS. The pairs in RATSS have undergone comprehensive phenotypic characterizations and diagnostic evaluation during a 2.5-day visit at a clinical research unit using medical history records, diagnostic interviews and by first choice standardized diagnostic tools (for more detail see Astenvald et al., 2022; Bölte et al., 2014; Willfors et al., 2017). ADHD diagnoses were determined by a group of experienced clinicians during the participants visit to the clinical research unit. Diagnostic instruments to corroborate clinical diagnosis included the Kiddie Schedule for Affective Disorders and Schizophrenia and the Diagnostic Interview for ADHD in adults. During this time, several biological samples were collected. The zygosity of the twin pairs has been estimated using either a panel of single nucleotide polymorphism or a genome-wide SNP array (Hannelius et al., 2007; Stamouli et al., 2018).

For this current study, twins with available urine samples and ADHD diagnosis, including any ICD-10-defined diagnosis of hyperkinetic disorder (F90.0-F90.9) or DSM-5 diagnosis of ADHD (combined, inattentive, or hyperactive-impulsive subtype) were selected. Additionally, co-twins in the pair in which one twin had an ADHD diagnosis and typically developed twin pairs with the approximately same age and sex distribution as for the ADHD twin pairs were selected. This RATSS subsample included 112 participants from 33 monozygotic (MZ) and 13 dizygotic (DZ) pairs (Table 1). A total of 33 participants were diagnosed with

Table 1
Sample characteristics.

Characteristics	ADHD (n = 33)	non-ADHD (n = 79)
Age, mean years (SD) [range]	13.55 (3.01) [8-19]	15.17 (2.99) [8-22]
Sex, n females (%)	14 (42.4%)	37 (46.8%)
MZ:DZ concordant pairs (n)[a]	4:3	25:6
ADHD discordant pairs (n)[a]	8	
MZ	4	
DZ	4	
BMI, mean (SD) [range]	19.061 (2.957) [13–24]	20.835 (2.817) [14–27]
Parental-rated ADHD traits	0.84 (0.38) [0.20–1.700]	0.131 (0.174) [0.000–0.800]
Self-rated ADHD traits, mean (SD) [range]	1.03 (0.40) [0.000–1.889]	0.46 (0.34) [0.000–1.222]
IQ-General Ability Index, mean (SD) [range]	98.82 (12.39) [71–130]	103.5 (12.42) [81–138]
Fecal samples n (%)	26 (72.72%)	66 (83.54%)

[a] Complete twin pairs.

ADHD. We also analyzed available fecal samples from 92 (82%) participants from the sample. All participants and their caregivers provided oral and written informed consent after being fully informed of the study procedures (Ethical permit 2016/1452-31).

2.2. Quantitative phenotype measures

ADHD traits were measured using self-report or parental report from the Achenbach System of Empirically Based Assessment (ASEBA). Depending on the age of the twins, the attention subscale from the parent-rated child behavior checklist (CBCL) or the Adult Behavior Checklist (ABCL), and self-ratings from the Youth Self-Report (YSR) or the Adult Self-Report (ASR) was included. The items are rated on a 3-point Likert-type scale (0 = not true, 1 = somewhat or sometimes true, 2 = very true or often true). Since number of items varies for the different reports, the sum of all values were divided by the number of values, presenting the attention problem subscale as mean scores, both for the parental and self-report. We used the General Ability Index (GAI) as the full-scale IQ estimate. The GAI was calculated from either WAIS-IV in individuals ⩾17 years or the WISC-IV in individuals younger than 17 years, as a composite score that is based on three verbal comprehension and three perceptual reasoning subtests. The score can range between 40 and 160 where GAI scores between 90 and 109 are considered average.

2.3. ^{1}H nuclear magnetic resonance (NMR) spectroscopy of urine samples

A total of 112 urine samples were collected for untargeted ^{1}H nuclear magnetic resonance (NMR) spectroscopy-based metabolomics analysis. Each urine sample (630 μl) was combined with 70 μl of phosphate buffer solution (pH 7.4, 100% D_2O) containing 1 mM of the internal standard 3-trimethylsilyl-1-[2,2,3,3-2H4] propionate (TSP). Samples were mixed by vortex, spun (12,000 *g* at 4 °C for 10 min) to remove particulates, and transferred (600 μl) to 5 mm NMR tubes. One dimensional ^{1}H NMR spectra were acquired on a Bruker 600 MHz NMR spectrometer equipped with a SampleJet autosampler with a cooling rack held at 6 °C (Bruker Biospin GmbH, Rheinstetten, Germany). A standard one-dimensional solvent suppression pulse sequence was used for spectral acquisition (relaxation delay, 90° pulse, 4 μs delay, 90° pulse, mixing time, 90° pulse, acquire FID) with 32 scans and 4 dummy scans per sample. Each spectrum was acquired with a total of 64K domain points, a spectral width of 20 ppm, a relaxation delay of 4 s, a mixing time of 10 ms, and an acquisition time of 2.73 s. Spectra were automatically phased, baseline corrected, and calibrated to the TSP singlet (δ 0.0) in Topspin 3.2 (Bruker Biospin GmbH, Rheinstetten, Germany). Spectra were imported into Matlab (Version 2018a, Mathworks Inc), manually aligned, and normalized using a probabilistic quotient-based approach. Prior to normalization, redundant spectral signals from water, TSP and urea were removed.

2.4. Ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) of fecal samples

Metabolic profiling of the fecal samples (n = 94; 66 non-ADHD, 28 ADHD) using UPLC-MS was performed by Metabolon (Durham, NC, US). Stool samples were prepared using the automated MicroLab STAR® system from the Hamilton Company. Recovery standards were added to the fecal samples prior to protein precipitation using methanol with vigorous shaking for 2 min followed by centrifugation. The resulting extract was separated into four aliquots and the organic solvent was removed using a TurboVap® (Zymark). Sample extracts were stored overnight under nitrogen before preparation for analysis. Each aliquot was used for a different UPLC-MS method including two separate reverse phase UPLC-MS/MS methods with positive mode electrospray ionization (ESI), one reverse phase UPLC-MS/MS method with negative ion mode ESI, and one HILIC-UPLC-MS/MS method with negative ion mode ESI. All methods were performed on a Waters Acquity ultra-performance liquid chromatography system coupled to a Thermo Scientific Q-Exactive mass spectrometer interfaced with a heated electrospray ionization (HESI-II) source and Orbitrap mass analyzer. Each reconstitution solvent contained a series of standards at fixed concentrations to ensure injection and chromatographic consistency.

One aliquot was analyzed using acidic positive ion conditions, chromatographically optimized for hydrophilic conditions. This extract was gradient eluted from a C18 column (Waters UPLC BEH C18–2.1 × 100 mm, 1.7 μm) using water and methanol, containing 0.05% perfluoropentanoic acid (PFPA) and 0.1% formic acid (FA). Another aliquot was also analyzed using acidic positive ion conditions, optimized for more hydrophobic compounds. This extract was gradient eluted from a C18 column using methanol, acetonitrile, water, 0.05% PFPA and 0.01% FA and was operated at an overall higher organic content. The third aliquot was analyzed using basic negative ion optimized conditions using a C18 column. The basic extracts were gradient eluted using methanol and water, however with 6.5 mM ammonium bicarbonate at pH 8. The final aliquot was measured using negative ionization following elution from a HILIC column (Waters UPLC BEH Amide 2.1 × 150 mm, 1.7 μm) using a gradient consisting of water and acetonitrile with 10 mM ammonium formate, pH 10.8. The MS analysis alternated between MS and data-dependent MS^n scans using dynamic exclusion. The scan range varied between methods but covered 70–1000 m/z. Data curation was performed following the in-house pipeline of Metabolon using their metabolite library. From this combined analysis, a total of 625 metabolites were measured in each fecal sample. Features with >80% of values below the limit of detection (LOD) were removed from the analysis. The remaining missing values (<LOD) were replaced with the lowest intensity value for that metabolite divided by the square root of 2.

2.5. Multivariate statistical analysis of the metabolic phenotypes

Principal components analysis was performed on the urinary and fecal metabolic profiles to identify outliers in the dataset. Untargeted NMR spectral data was analyzed in Matlab using orthogonal projection to latent structures-discriminant analysis (OPLS-DA) models. Here, pairwise models were constructed using the urinary metabolic signatures as the X matrix and diagnosis (ADHD versus no ADHD) as the response (Y) vector. The predictive ability (Q^2Y) of the model was assessed using 7-fold cross-validation and model significance was evaluated through permutation testing (1000 permutations, threshold $p < 0.05$). OPLS-DA coefficients plots were produced to identify metabolic features significantly correlated with diagnosis. Datapoints with a correlation significance to $Y < 0.05$ are plotted with a color scale and those >0.05 are plotted in black. Models were built for the complete dataset and the individual models for the female and male participants.

Metabolomic profiles are high-dimensional and often have non-linear and complex interactions between metabolites. Thus, we applied random forest-based machine learning models to identify complex interaction dependency patterns within the fecal metabolites related to the phenotype (ADHD vs non-ADHD). This random forest approach is fully non-parametric and allows such patterns to be extracted from the data providing variable importance measures (VIMs) to identify relevant features. Mean minimal depth was calculated based on the position of the features in the decision tree (Ishwaran et al., 2012). The random forest models and the minimal depth distribution were calculated using the "ranger" and "randomForestExplainer" R packages, respectively. To facilitate model interpretation, Shapley Additive exPlanations (SHAP) scores were calculated by leveraging the internal structure of the random forest models (Lundberg et al., 2020). SHAP values determine the importance of a specific value in a specific feature by comparing the model prediction with and without the feature for each individual. SHAP values were calculated using the R package "treeshap" and plotted with "SHAPforXGBoost". As VIMs in random

forests are not directly related to statistical significance, an all-relevant machine learning selection strategy based on the Boruta algorithm was used (Kursa and Rudnicki, 2010). All models were constructed using 5000 trees and the number of features (ntree) randomly sampled at each split was given by the rounded down square root of the number of features. For the model containing all participants, 5000 iterations were used and a confidence level cut-off of 0.005 for the Bonferroni adjusted *p* values, whereas the sex-specific models used 1000 iterations.

The metabolites identified to be significantly related to ADHD diagnosis were further investigated using generalized estimating equations (GEE) models to utilize fully the twin design, as well as test if quantitative measures related to the diagnosis were also associated with the same features. Statistics were calculated in R version 4.2.1. First, we computed linear regression models across all individuals adjusted for the non-independence of twins with metabolite abundance as outcomes and ADHD diagnosis controlling for sex, age at sample collection and BMI using the "drgee" package (Zetterqvist and Sjölander, 2015). ADHD medication was included as a covariate in the fecal metabolite analyses. Thereafter, we employed conditional models to estimate the within-pair effects, adjusting for all shared factors in twin pairs. In this within-pair analysis, the difference in the exposure variable within a pair is correlated with the difference in the outcome variable within the same pair. Furthermore, models with the quantitative phenotypes (GAI-IQ and Self/Parental reported Attention problem measure score) were calculated. Sex and zygosity (MZ/DZ)-specific models were computed separately. For the fecal metabolite models, a Bonferroni adjusted p-value threshold of 0.005 was used to account for the 10 metabolites assessed.

3. Results

3.1. Urinary hippurate is increased in males with ADHD

Principal Components Analysis (PCA) was performed to assess the presence of outliers in the urinary metabolic dataset (Supplementary Fig. S1A). No outliers were observed in the model comparing all urine samples. An OPLSDA model comparing the urinary metabolic profiles of all ADHD participants to the controls was not found to be significant. However, when considering only the male participants, 2 outliers were identified from the PCA model (1 male non-ADHD, 1 male ADHD; Supplementary Fig. S2). These outliers were due to high amounts of creatinine (ADHD male) and acetone (non-ADHD male) and were excluded from the subsequent OPLSDA analysis. A significant OPLS-DA model was obtained comparing the urinary profiles of ADHD and non-ADHD males ($Q^2Y = 0.113$; $p = 0.007$). ADHD males were found to excrete higher amounts of hippurate compared to the non-ADHD participants (Fig. 1). The OPLSDA model comparing the ADHD to non-ADHD females was not significant.

The association of hippurate with general IQ, ADHD diagnosis and parental and self-reported ADHD condition was further investigated using GEE models utilizing the twin design. ADHD medication was not associated with hippurate (Supplementary Table S1) and was not adjusted for in the models. For ADHD diagnosis, a significant positive association with hippurate was present in males as expected ($p = 0.003$; Fig. 1B) when adjusting for IQ, age, and BMI; however, no significant association was seen within the male twin pairs. No significant association between hippurate and ADHD diagnosis was observed when separating the sample for MZ and DZ and analyzing the association within twin pairs (Supplementary file 'GEE_results.csv'). When the association between parental and self-reported ADHD problems and

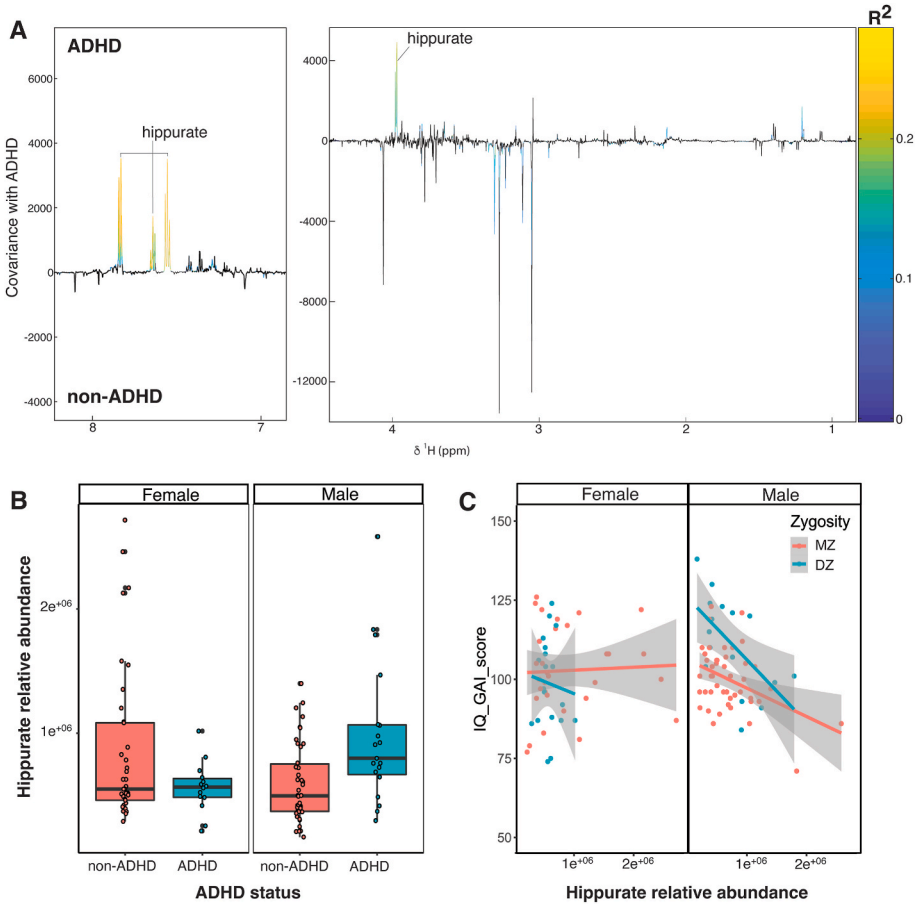

Fig. 1. Urinary hippurate is positively associated with ADHD diagnosis. A) Orthogonal projection to latent structures-discriminant analysis (OPLS-DA) model comparing the urinary ^{1}H NMR spectral profiles of male ADHD and non-ADHD participants (seven-fold cross validation, $Q^2Y = 0.113$; 1000 permutations. $p = 0.007$). Coefficients plot highlighting the urinary metabolites associated with ADHD status. Data points are colored by their correlation significance with ADHD, data points with $p < 0.05$ plotted with color scale and those with $p > 0.05$ are black). B) Difference in urinary hippurate excretion in females and males with and without ADHD diagnosis. C) Correlation between IQ and hippurate excretion separated by females and males and showing linear model for monozygotic (MZ) and dizygotic (DZ) separately.

hippurate was examined, no significant associations were observed for the total sample, males or MZ/DZ subsamples. Surprisingly, a negative association between self-reported ADHD problems and hippurate excretion was observed in females both across the twins ($p = 0.019$) and between twins ($p = 0.026$). In addition, hippurate excretion was found to be negatively associated with IQ across all twins ($p = 0.038$) and males ($p = 0.002$) when adjusting for BMI and age (Fig. 1C, Supplementary file 'GEE_results.csv'). Analyzing the association within twin pairs and separating to MZ and DZ twins, found only significant associations for the DZ twin pairs ($p = 0.045$), suggesting genetic influences on the association between hippurate excretion and IQ (Supplementary file 'GEE_results.csv'; Fig. 1C).

3.2. Fecal metabolic alterations associated with ADHD

Comparing the fecal metabolic profiles of all individuals (ADHD vs non-ADHD) using a variable selection random forests-based machine learning approach, the ADHD individuals excreted lower amounts of glycerol-3-phosphate, thymine, 1H-quinolin-2-one, aspartate, xanthine, hypoxanthine, and orotate, and greater amounts of flavin-adenine-dinucleotide (FAD), stearoyl-linoleoyl-glycerol (18:0/18:2), and 3,7-dimethylurate (Fig. 2A). SHAP values for these metabolic associations are presented in Fig. 2B. The model comparing only the female participants identified that those with ADHD excreted higher amounts of adenine, 11-ketoetiocholanolone sulfate, and L-urobilinogen and lower

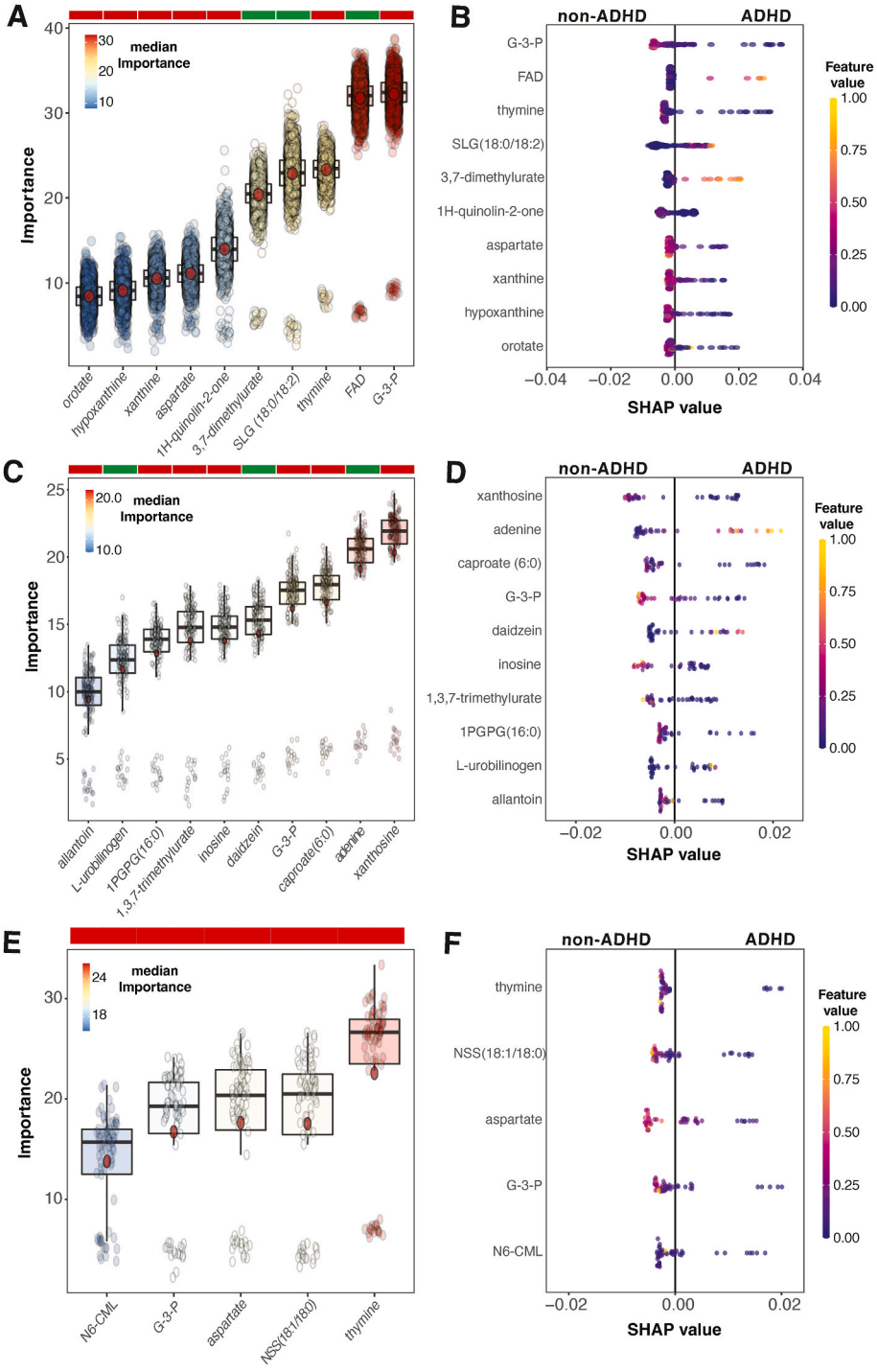

Fig. 2. Machine learning results comparing the fecal metabolites of ADHD and non-ADHD participants. Features selected by the Boruta algorithm for the comparison between ADHD and non-ADHD profiles and their SHAP summary plots for the models comparing all (A–B), female (C–D), and male (E–F) participants. FAD, flavin-adenine-dinucleotide; G-3-P, glycerol-3-phosphate; N6-CML, N6-carboxymethyl lysine; NSS(18:1/18:0), stearoyl-linoleoyl-glycerol (18:0/18:2). The bar above the Boruta plots indicates the correlation between the metabolites and the phenotype, with green and red indicating positive and negative correlations, respectively.

amounts of xanthosine and caproate(6:0) than their neurotypical counterparts. Males with ADHD excreted lower amounts thymine, N-stearoyl-sphingosine(18:1/18:0), aspartate, glycerol-3-phosphate, and N6-carboxymethyllysine compared to their non-ADHD equivalents.

We also examined the associations of these discriminatory fecal metabolites using GEE models to add the twin comparison. As ADHD medication (atomoxetine and methylphenidate) was significantly associated with most of the metabolites (Supplementary Table S1), it was adjusted for in the models investigating the association with IQ, ADHD diagnosis and traits. None of the metabolites had a significant association with general IQ across or within all twin pairs (Supplementary file "*GEE_results.csv*"). Fecal thymine abundance had a negative association with IQ levels within ($p = 0.001$) male and MZ twin pairs ($p = 0.001$), of which the later association indicates a putative non-shared environmental influence for the association.

Assessing each metabolite independently across and within all twin pairs, no significant associations were observed when accounting for the multiple comparisons (Supplementary file "*GEE_results.csv*"). Within male twin pairs, two fecal metabolites (stearoyl-linoleoyl-glycerol(18:0/18:2)[2] and glycerol-3-phosphate) were significantly associated with ADHD-diagnosis. Two out of the ten metabolites (orotate and thymine) had significant associations with ADHD diagnosis within DZ twin pairs when adjusting for IQ and BMI, again suggesting genetic influences on the associations. Orotate was also the only metabolite significantly associated with parental-reported ADHD traits in addition to the ADHD diagnosis within the DZ twin pairs.

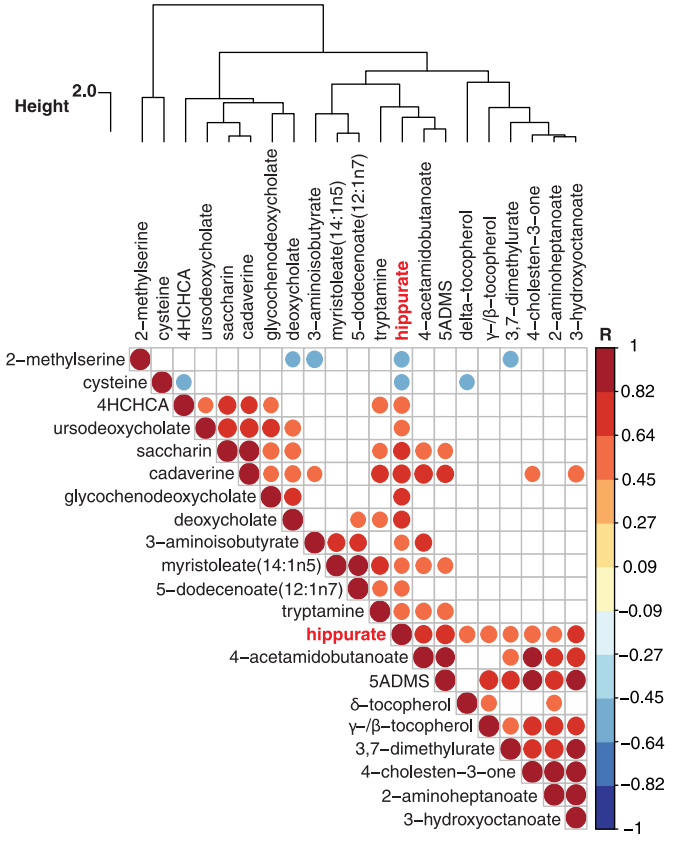

Fig. 3. Correlation analysis between urinary hippurate and fecal metabolites in the male participants with ADHD. Significant Pearson correlations ($p < 0.05$) shown between metabolites. Color and size of point indicate the strength of correlation. 4HCHCA, 4-hydroxycyclohexylcarboxylic acid; 5ADMS, 5α-androstan-3α, 17β-diolmonosulfate.

3.3. Urinary hippurate correlation analysis with the fecal metabolome

Correlation analysis was performed to identify fecal metabolites associated with the relative abundance of urinary hippurate (Fig. 3; Supplementary Table S2). Based on the urinary and fecal profiles of all individuals (92 matched profiles), urinary hippurate was significantly positively correlated with 18 fecal metabolites and negatively correlated with 46 compounds. This included positive correlations with the hippurate precursors 3,4-dihydroxybenzoate, a major metabolite of antioxidant polyphenols found in green tea and the coffee flower and 3-(3-hydroxyphenyl)propanoic acid, a gut microbial metabolite of caffeic acid (phenolic compound found in coffee and tea) and proanthocyanidins. Hippurate can also arise from the gut microbial reduction of phenylalanine to phenylpropionic acid followed by β-oxidation in the host via medium chain acyl-CoA dehydrogenase (MCAD)(Pruss et al., 2023). Consistently, urinary hippurate was negatively correlated with the dipeptides phenylalanylalanine (phenylalanine-alanine) and threonylphenylalanine (threonine-phenylalanine) and positively correlated with 3-(2-hydroxyphenyl)propionate and 3-(3-hydroxyphenyl)propionate. In addition, hippurate was positively associated with caffeine and its metabolites, theophylline, 1,3-dimethylurate, 1,3,7-trimethylurate, 4-acetylamino-6-amino-3-methyluracil, and trigonelline. Interestingly, caffeine was detected in 31/66 (46.97%) neurotypical stools and 13/26 (50%) ADHD stools with no statistical differences in abundance between the two groups (males only: 20/37 (54.05%) neurotypical; 8/15 (53.33%) ADHD ($p = 0.543$)). Hippurate was associated with several metabolites related to gut microbial metabolism including positive relationships with salsolinol and glycodeoxycholic acid, and negative associations with *N*6-carboxyethyllysine, and 4-hydroxyphenylpyruvic acid (4-HPPA). Urinary hippurate was also negatively correlated with several fecal amino acids including glycine, glutamate, and proline, previously implicated in the gut-brain axis, as well as beta-alanine, and lysine. Correlation analysis performed on the metabolic signatures of only the male ADHD participants found 18 positive and 2 negative associations between the fecal metabolites and urinary hippurate (Supplementary Table S3. This included a positive relationship with the caffeine metabolite 3,7-dimethylurate, the microbial metabolites tryptamine and cadaverine, and the microbial-host bile acids, deoxycholic acid, ursodeoxycholic acid, and glycochenodeoxycholic acid.)

4. Discussion

In this study we characterized the urinary and fecal metabolomic signatures of ADHD using a combination of ^{1}H NMR spectroscopy and LCMS-based techniques followed with detailed statistical analyses utilizing a twin design. First, our untargeted approach allowed the unbiased assessment of biochemical variation related to ADHD in the human superorganism, measuring features from the genome, microbiome, and environment, including those derived from the diet, and interactions between these components. Interestingly, we observed sex-specific patterns in these metabolic profiles related to the presence of ADHD suggesting differences in the underlying biochemistry associated with ADHD in males and females. This is consistent with sex-dependent variation in objective and subjective measures of ADHD, with girls having more inattention problems than boys, who had higher levels of impulsivity (Slobodin and Davidovitch, 2019). Furthermore, we show by using powerful twin design statistics, that there is a genetic influence in the association of some of the metabolites identified, including those derived from the gut microbiota, and ADHD associations.

From the urine profiles, males with ADHD excreted greater amounts of hippurate than their neurotypical equivalents. GEE analysis confirmed that urinary hippurate was positively associated with ADHD diagnosis and negatively correlated with IQ in males, independent of ADHD medication (including atomoxetine and methylphenidate), age and BMI. Consistently, 4-aminohippurate, a derivate of hippurate, has been previously observed to be higher in the plasma of individuals with

ADHD compared to non-ADHD controls. While hippurate was not observed to be related to ADHD diagnosis in the females, it was identified by the GEE analysis to be negatively associated with self-reported ADHD problems in these participants. Sex-differences in the urinary excretion of hippurate has been previously observed, being lower in females(Chong et al., 2020). It is currently unclear whether this is driven by microbial or host variation, but sexually dimorphic development of the microbiota-gut-brain axis has been previously described with hormonal variation implicated, emerging during puberty (Jašarević et al., 2016). In the feces, comparing all ADHD and neurotypical individuals identified that those with ADHD excreted greater amounts of stearoyl-linoleoyl-glycerol (18:0/18:2), 3,7-dimethylurate, and FAD and lower amounts of glycerol 3-phosphate, thymine, 2(^{1}H)-quinolinone, aspartate, xanthine, hypoxanthine, and orotate. From these discriminatory molecules, 2(^{1}H)-quinolinone, orotate, aspartate, and hypoxanthine are fecal metabolites that can be derived from the diet or produced by the microbiota, while urinary hippurate is the product of microbial-host co-metabolism.

Hippurate is formed in the liver and kidneys from the conjugation of benzoate with glycine by host glycine N-acyltransferase. Benzoate can be obtained from the diet directly as sodium benzoate, a preservative often used in carbonated drinks, sauces, and fruit preserves, or it can be produced by the gut microbiota following the metabolism of polyphenolic compounds often present in fruits, vegetables, tea, and coffee. In this study, urinary hippurate was positively correlated with fecal metabolites derived from the gut microbial metabolism of polyphenols, including 3,4-dihydroxybenzoate (protocatechuic acid), a bacterial metabolite of anthocyanin components and 3-(3-hydroxyphenyl)propanoic acid (3-3-PPA), a bacterial derivate of caffeic acid and *trans*-cinnamic acid. These associations suggest that gut bacterial activity underlies this observation rather than increased intake of sodium benzoate. Coffee is a major source of polyphenols for the microbiota and caffeine and several of its metabolites were also positively correlated with hippurate excretion when all study participants were considered. Specifically, hippurate excretion has been associated with the fecal abundance of Clostridiales and consistently, the *Clostridium coccoides-Eubacterium rectale* group has been found to increase when human fecal samples were incubated with coffee (Mills et al., 2015). However, variation in coffee/caffeine intake is unlikely to drive the disparities in hippurate excretion in this study as no differences were noted in the excretion of caffeine or its related metabolites between the groups. As such, variation in the metabolic activity of the microbiota is more likely to underpin these findings rather than dietary variation. This is supported by the correlation between urinary hippurate in the ADHD males and fecal metabolites derived from gut microbial activity unrelated to the intake of hippurate precursors, namely tryptamine, cadaverine and several bile acids. While the absence of detailed dietary information for these participants is a limitation of the current study, our untargeted urine and fecal metabolomics approach measures a broad range of diet-related molecules providing a powerful tool for the objective assessment of dietary intake. Indeed, this approach is being increasingly used in the field of nutrition. In this study, minimal variation was noted in diet-related components of the urine and fecal metabolomes between ADHD and non-ADHD individuals further indicating comparable dietary intake between the two groups. Nevertheless, future studies should further characterize this diet-microbiota-host relationship and its contribution to ADHD.

We have previously shown in mice that hippurate can reach the brain and cross the blood-brain barrier (Swann et al., 2020). Microglial activation has been implicated in the neurophysiological mechanism of ADHD. This can be inhibited by the binding of G-protein coupled receptor 109A (GPR109A; hydroxycarboxylic acid receptor 2 [HCAR2]) expressed on these cells by β-hydroxybutyrate (Chen et al., 2021). Interestingly, hippurate can inhibit HCAR2 expression, blunting this effect (Fu et al., 2015). Moreover, the hippurate precursor benzoate can also cross the blood-brain barrier, and has been shown to increase hyperactive behavior in children and has been implicated in ADHD-related symptoms (Bateman et al., 2004; Beezhold et al., 2014; Zhao et al., 2018a). Benzoate can inhibit D-amino acid oxidase (DAAO) in the brain, which is the enzyme responsible for degrading D-amino acids, particularly D-serine (Lane et al., 2013; Lin et al., 2014). Benzoate-induced inhibition of DAAO may therefore result in the accumulation of D-serine, an endogenous co-agonist for the N-methyl-D-aspartate receptor (NMDAR), a receptor implicated in synaptic plasticity, memory, and learning (Kolodney et al., 2016). A recent study using the juvenile stroke-prone spontaneously hypertensive rat (SHRSP/Ezo) model identified a role for NMDA dysfunction in ADHD, although these authors found DAAO inhibition improved ADHD-like behaviors (Shindo et al., 2022). Furthermore, in zebrafish, sodium benzoate exposure induced neurotoxic effects in developing larvae, reducing dopaminergic neurons, and dopamine transporters in these neurons. This is noteworthy given the association between ADHD symptoms and the dysregulation of dopamine neurotransmission.

This work highlights another microbial-derived metabolite that contributes to the pan-kingdom communication network that has co-evolved between the intestinal microbiota and the host CNS. Known producers of benzoic acid include *Enterococcus faecalis, Staphylococcus aureus*, *Bifidobacterium breve*, and *Faecalibacterium prausnitzii*, with microbial carboxylesterases involved in its production (Pallister et al., 2017; Zhao et al., 2018b). Bacterial taxa found to be statistically associated with hippurate in humans include *Actinomyces* and *Ruminococcus* species including *Ruminococcus gnavus* (Pallister et al., 2017). In addition, Pruss et al. have demonstrated that hippurate can arise from the microbial conversion of dietary phenylalanine to phenylpropionate via the *fldC* gene cluster (Pruss et al., 2023). Phenylpropionate then undergoes β-oxidation by host medium-chain acyl-CoA dehydrogenase (MCAD) to form benzoic acid, which is subsequently conjugated with glycine to form hippurate. In this study, urinary hippurate was negatively correlated with phenylalanine containing dipeptides and positively associated with phenylpropionate-related molecules in the stools suggesting this microbial activity was increased in the ADHD males. These alterations may have consequences for host neurotransmission reducing the availability of the inhibitory neurotransmitter, glycine and dietary phenylalanine, a dopamine precursor (Badenhorst et al., 2014).

Hippurate has been reported to be a positive marker of gut microbiome diversity however, most studies have observed no differences in the alpha diversity of the gut microbiota between ADHD and neurotypical individuals (Aarts et al., 2017; Jiang et al., 2018; Szopinska-Tokov et al., 2020). Studies investigating the compositional variation in the microbiota between ADHD and non-ADHD individuals has also been inconsistent. This is likely to be a combination of medication effects, geographical and dietary variation, as well as differences in the age, BMI, and sex profiles of the studied populations. In the current study, the lack of information on the microbial profiles of these participants represents another limitation, preventing direct microbial-function-metabolite linkages from being made. Nonetheless, these results reveal functional variation in the microbiota with ADHD, exposing ADHD males to greater amounts of microbial-derived molecules with the bioactive potential to modify the CNS.

These findings highlight that ADHD males are exposed to greater amounts of hippurate than their neurotypical equivalents. This is a *trans*-genomic metabolite that can reach the brain arising from the combinatorial metabolism of the gut microbiota and host on dietary components. Given the existing data suggesting that hippurate, and its precursor benzoate, have potential to modify central processes that may contribute to ADHD features, this result warrants further investigation to establish causality. This observation has potential to connect genetic and expression status, with gut microbial composition and dietary intake. For example, an individual may possess a microbiome with enhanced efficiency for processing polyphenols and phenylalanine, as well as a greater inherent MCAD activity, resulting in higher exposure to benzoic acid. When additional dietary inputs are consumed a threshold

may be breached resulting in behavioral changes such as hyperactivity. In this context, the diet and the microbiome are attractive targets to ameliorate the symptoms of ADHD as they are both accessible and modifiable. Systematic reviews and meta-analyses studying dietary patterns and ADHD have concluded that while the current evidence is weak, a healthy diet characterized by greater amounts of fruits and vegetables could protect against ADHD or hyperactivity (Shareghfarid et al., 2020; Del-Ponte et al., 2019). However, such diets will be rich in hippurate precursors emphasizing the importance of understanding the gut microbial contribution to ADHD and its interactions with dietary intake. A range of microbial modulators are now available for targeting the gut microbiota, including pre-, pro-, syn-, and postbiotics, microbial enzyme inhibitors, metabolite binders, and more extreme methods such as antibiotics and fecal microbial transplants (Letertre et al., 2022; Salminen et al., 2021). As we better understand the influence of the microbiota-gut-brain axis in neurological disorders, the microbiota will become an increasingly attractive target for neuropharmacological interventions.

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Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2023.109562.

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