Serum metabolites and childhood-diagnosed ADHD: Prospective Cohort Study and Mendelian Randomization Analysis

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Article

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Abstract

ADHD, a neurological condition that onset in childhood is now an important part of the disease burden in the global population. ADHD is usually diagnosed at school age, and there are no authoritative research to articulate whether ADHD in adult shares a common pathogenic mechanism with ADHD in children. Previous studies have elucidated metabolic profiles as functional mediators, and the present study is the first to combine metabolomics and Mendelian randomization(MR) to elucidate the causal relationship between serum metabolites and ADHD diagnosed in children. A metabolomic study of childhood-diagnosed ADHD and normal children in a prospective cohort of preschoolers. Metabolomic results of preschool children enrolled in the cohort study identified 112 differential metabolites, with 69 metabolites upregulated and 43 metabolites downregulated. For MR studies, single nucleotide polymorphisms associated with childhood-diagnosed ADHD were identified from metabolite-wide association studies for IVW analysis. MR results revealed that the IVW approach revealed a total of 15 significant pathogenic association profiles from 486 metabolites, including 10 known metabolites and 5 unknown metabolites. Combining the results of MR analyses from metabolomic studies and cohort studies, arginine and α-tocopherol were two important metabolites affecting the diagnosis of ADHD in childhood. The metabolic pathways of primary bile acid biosynthesis and arginine/proline metabolism were the overlapping metabolic pathways in both studies.

Introduction

Attention deficit hyperactivity disorder (ADHD) is a neurological disorder that emerges in childhood and is characterized by age-appropriate motor hyperactivity and impulsivity, dyspraxia, and inattention, a condition that often leads to persistent-diagnosed ADHD(1). According to studies, such psychiatric disorders affect about 5-6% of school-age children and about 3% of adults(2, 3). The rapid transformation of global economic, demographic, and epidemiological conditions has made psychiatric disorders a major contributor to overall morbidity and disability in recent decades(4, 5). ADHD is usually diagnosed during school age and can also be diagnosed in adulthood. There are no authoritative research to articulate whether adult-diagnosed ADHD shares common pathogenic mechanisms with childhood ADHD, or whether there are distinct pathogenic causes that lead to delayed diagnosis or even adult-diagnosed ADHD (6). Identifying the characteristics of ADHD in childhood can help play a crucial role in intervening in the progression of the disease.

Currently, the diagnosis of ADHD relies on behavioral analysis, especially in children who need to rely on their guardians. Objective laboratory biomarkers for the diagnosis of ADHD must be investigated (7). Previous studies have elucidated metabolic features as regulatory mediators to elucidate the influence of genetic factors on psychiatric disorders (8-10). Metabolites, as products of the exchange of substances between the organism and the environment, are important products for the maintenance of body functions. (11). The calibration of serum metabolites can reflect human health and provide fresh perspectives on the effects of diet, environment and disease. Recent work identified multiple potential serum biomarkers, such as mono/polyunsaturated fatty acid pathways, in patients with ADHD,

suggesting a potential link between metabolic profiles and the pathophysiology of ADHD.(12-15). However, observational studies mostly hinder causal inference and comprehensive and novel analyses are needed to determine the causal relationship between genetic variants and metabolite interactions and ADHD.

Mendelian randomization (MR) is a causal research method that strengthens causal inferences about exposure-outcome associations by using genetic variation as an instrumental variable for exposure. By using a genetic variation, such as single nucleotide polymorphisms (SNPs), as modifiable disease risk factors or instrumental variables (IVs) for exposure, MR designs can strengthen causal inferences about exposure-outcome associations. According to Mendelian laws of inheritance, genetic variants are less susceptible to confounding factors because they are randomly assigned during gamete formation(16, 17). In addition, confounding factors and reverse causality can be minimized because genotypes cannot be altered by disease progression.

In this project, we intended to elucidate whether metabolites are relevant to childhood-diagnosed ADHD, which involved a multi-stage prospective cohort study. In addition, we conducted a 2-sample MR study to explore whether metabolites are causally associated with ADHD diagnosed in children.

Methods

Cohort

All participants 39–78 months of age were enrolled voluntarily through the school. Authorized consent was acquired from the legally authorized representative of each child. Each child's legally authorized representative completed the SNAP-IV Attention Deficit Hyperactivity Disorder Rating Scale for inclusion in ADHD (18). An experienced child psychiatrist performed a neurocognitive assessment and physical examination to exclude any neurological disorder other than ADHD. The first 220 preschoolers were recruited for this study, and 21 children with assessed ADHD and 10 healthy preschoolers were selected for the study. The study was approved by the Ethics Committee of Jiangsu Provincial Center for Disease Prevention and Control **(**JSJK202I-B009-01**).**

Blood sample collection and pre-processing

Participants were urged to fast for 8 hours prior to blood collection to prevent direct effects of diet on metabolic status. Venous blood was extracted into non-heparinized tubes, rested at room temperature for 30 minutes, and centrifuged at 1800×g for 10 minutes. Serum samples were stored in a -80°C refrigerator. Serum samples were prepared according to the procedure described in a previous study(19, 20).

LC-MS Metabolomic Analyses

The procedure for setting up and using the LC/MS instrument is described in the previous article(21). Briefly, the sample was eluted in positive and negative mode using a Hypesil Gold column with a flow rate

of 0.2 mL/min. The eluent gradient was controlled and the elution procedure lasted for 36.6 min. QExactive

Data Processing

CompoundDiscoverer 3.1 is used to manipulate raw metabolite data and perform peak processing and quantification(21). After normalization, it is matched against the corresponding database to obtain metabolite names and relative quantification results.

Characterization and metabolite identification

KEGG, HMDB databases were used to interpret the corresponding metabolite attribution and metabolic pathways. Principal component analysis (PCA) was used to distinguish differences between data groups, and partial least squares discriminant analysis (PLS-DA) was applied for effective separation of data. (22). Metabolites were considered differential if their VIP score exceeded 1, their *P*-value was less than 0.05, and their fold change was equal to or greater than 2, or less than or equal to 0.5. The R language was used to visualize and cluster the differential metabolites using the ggplot2/Pheatmap package and to plot the corresponding images. A *P*-value less than 0.05 is considered statistically significant.

MR study design and data sources

The procedure of this MR study is demonstrated in Fig. 1. The instrumental variables used in MR analysis must satisfy three assumptions: (1) IV must be related to exposure (serum metabolomics); (2) IV must only be related to the outcome (childhood-diagnosed ADHD) through exposure (serum metabolomics); (3) IV must be independent of any confounding factors(17). The study utilized the most extensive report of genetic loci for human metabolism up to date, which encompassed 7,824 adult individuals from two population studies in Europe. (23). After strict quality control, a total of 486 metabolites were used for genetic analysis, including 309 known metabolites and 177 unknown metabolites. The summary statistics of GWAS can be accessed by the public through the following resource: http://metabolomics.helmholtz-muenchen.de/gwas/'s metabolomics GWAS server.

Selection of Instrumental Variables for the 486 Metabolites

For each metabolite, we calibrated SNPs with an association of $P < 3\times10-6$. The clumping procedure was performed using the R language for the calibrated snps, specifically with an $r^2 < 0.5$, 5000kb setting. This study screens whether IVs are representative of metabolite levels based on explained variance (R^2) and F-statistical parameters. We calibrated instrumental variables with $F > 10$ to be eligible for this study.

GWAS of childhood-diagnosed ADHD

Conducted a GWAS meta-analysis using the latest available data from Psychiatric Genomics Consortium (PGC) website(https://ipsych.dk/en/research/downloads*)* to identify genetic variants associated with

childhood-diagnosed ADHD. Our study included children diagnosed with ADHD (n = 14,878) and 45,398 controls.

Metabolic pathway analysis

Metabolites that were identified as significant by IVW analysis ($P_{IVW}< 0.05$) were selected for metabolic pathway analysis.

MR analyses

"TwoSampleMR" packages using R4.21 for MR analysis.MR Egger, Weighted median, Inverse variance weighted (IVW), Simple mode, Weighted mode A total of five methods are used to infer causality between Serum metabolomics and childhood-diagnosed ADHD. In addition, we used the MR-Egger method to assess MR pleiotropy (24).MR-PRESSO is used to detect heterogeneity in IVW analysis. Cochrane's Q test was used to detect heterogeneity among the selected SNPs ($P< 0.05$). In cases of significant heterogeneity, a random-effects IVW test was used to provide more conservative and robust estimates(25). We also assessed and corrected for the influence of directional pleiotropy using the intercept obtained from the MR-Egger regression model. In addition, we conducted sensitivity analyses by systematically removing individual SNPs to assess the robustness of the results. The findings were visualized using a forest plot.

Result

Characteristics of the Cohort Study Participants

The first 220 preschoolers were recruited for the cohort study and 21 children evaluated for ADHD and 10 healthy preschoolers were selected for the metabolomics study. The clinical information of the recruited subjects showed no significant difference in height, weight, age, male, and waist circumference ($P > 0.05$) (Table 1).

Table 1
Baseline characteristics of children

	ADHD	CONTROL	P
Height (cm)	105.2 ± 4.07	107.1 ± 4.41	0.293
Age (months)	46.9 ± 4.04	46.7 ± 3.65	0.885
Male (%)	50	40	0.243
Waist circumference(cm)	44.50 ± 5.96	38.57 ± 4.40	0.688
Body weight(kg)	17.4 ± 2.45	18.0 ± 3.05	0.606

Multivariate Data Analysis of Serum Metabolites

This study uses the supervised statistical method of pattern recognition, OPLS-DA, to reduce information unrelated to classification and improve the effectiveness of the model. The OPLS-DA model and OPLS-DA permutation test showed significant separation of metabolic spectra between the ADHD group and the Control group, with R2Y values of 0.96 and 0.95, respectively, indicating that the model has good discrimination between samples and high aggregation of samples within the same group (Fig. 2a–d). The VIP and *P* values were used to reveal the importance of metabolites. Differential metabolites were screened based on the standard with VIP > 1 and $P < 0.05$. Through differential metabolism analysis, 112 differential metabolites were found, 69 metabolites showed up-regulation, and 43 metabolites showed down-regulation **(Appendix S1: Table S1)**. The volcano plot and clustering heat map shows the overall distribution of differential metabolites (Fig. 2e-f,3a-b). Differential metabolite correlation analysis elucidated synergistic or mutually exclusive relationships between different metabolites (Fig. 3c-d).

Metabolic Pathway Analysis

Metabolic pathway enrichment analysis was performed using the KEGG database. We found that 20 metabolic pathways were affected. The major Steroid hormone biosynthesis, Vitamin digestion and absorption, and Serotonergic synapse pathways were detected to be affected in positive ion mode, while the metabolic pathways observed in negative ion mode included Pyrimidine metabolism and Bile secretion pathways (Fig. 4a-b).

Strength of the instrumental variables

Two-sample Mendelian randomization study to determine the causal effect of genetically determined metabolites in blood on childhood-diagnosed ADHD. 486 metabolites had VIs ranging from 1 to 113, and these generated IVs could explain 0.3058- 46.20% **(Appendix S2: Table S2)**. In addition, the minimum F-statistic for these IVs was 21.75, indicating that all IVs were sufficiently valid for MR analysis of the 486 metabolites (F-statistic > 10) with no weak instrumental variables.

Genetically determined metabolite causation of ADHD

From the 486 metabolites, the IVW approach revealed the identification of a total of 15 significant pathogenic association features, including 10 known metabolites and 5 unknown metabolites (Fig. 5). P-values from the Cochran Q test indicated no detectable heterogeneity. In addition, the MR-Egger intercept term indicated no horizontal pleiotropy for any of them (Table 2). Therefore, these metabolites were identified as potential candidate metabolites involved in the pathogenesis of childhood-diagnosed ADHD for the next analysis.

Table 2
Heterogeneity and horizontal pleiotropy of genetically determined metabolites

Metabolites	Heterogeneity test	Pleiotropy test
Threonate	0.27	-
Inosine	0.40	-
X-10506	0.85	0.70
Androsterone sulfate	0.37	0.28
Decanoylcarnitine	0.44	0.37
X-12217	0.13	0.89
Arginine	0.88	0.89
Cis-4-decenoyl carnitine	0.93	0.65
X-06226	0.84	0.93
Alpha-tocopherol	0.72	0.52
Deoxycholate	0.73	0.71
Propionylcarnitine	0.44	0.59
X-01911	0.79	0.42
X-12039	0.66	0.52
Succinylcarnitine	0.91	0.57

Metabolite co-analysis

Combining the results of metabolomic studies and MR analysis from the cohort study identified Arginine and Alpha-tocopherol as two important metabolites affecting childhood-diagnosed ADHD (Fig. 6). The metabolic pathways of primary bile acid biosynthesis and arginine and proline were the overlapping metabolic pathways of the two studies.

Discussion

This study used blood metabolomics and MR of preschool enrolled in cohort studies to assess the association between serum metabolites and childhood-diagnosed ADHD. A total of 112 differentiated metabolites were identified in the metabolomics results of the cohort study, with 69 metabolites showing upregulation and 43 metabolites showing downregulation. Fifteen genetically determined metabolites were found to be causally associated with childhood-diagnosed ADHD using more rigorous MR analysis

criteria, with arginine and α-tocopherols overlapping with metabolomic differential metabolites in cohort studies. In addition, pathway enrichment analysis identified two important metabolic pathways, the "Biliary secretion" pathway and the "metabolic pathways of arginine and proline" pathway.

According to previous findings, this is the first study of childhood-diagnosed ADHD that combines cohort studies, MR studies, and metabolomics. Here, the results identified a cluster of metabolites in serum associated with childhood-diagnosed ADHD, with Alpha-tocopherol having a potent effect on childhood-diagnosed ADHD. In a recent study, it was elucidated that alpha-tocopherol has a positive effect as a non-enzymatic antioxidant against depression and anxiety (26–28). Several studies have shown that alpha-tocopherol antioxidant supplementation therapy can effectively enhance the oxidative defense function of the body (29–31). Also, a case-control study suggested that mild oxidative stress and immune disorders may affect ADHD (32). Thus α-tocopherol may play an important role in the neurodevelopment of childhood-diagnosed ADHD.

This joint conjoint analysis also identified arginine (Arg) as an important genetically determined essential metabolite both inside and outside the RI. Arginine is a semi-essential amino acid and the Arg pathway is associated with cardiovascular, renal, neurological, and immune system disorders (33, 34). the Arg pathway is significantly different in pediatric patients with congenital metabolic disorders, type I diabetes, or ADHD matched to healthy age (35–39), and it is worth mentioning that our results are in agreement with the above results, emphasizing the importance of arginine in the progression of mental disorders.

In this study, metabolic pathway analysis showed that the "primary bile acid biosynthesis" and "Arginine and proline metabolism" pathways were primarily associated with childhood-diagnosed ADHD. The study found that primary bile acids are not associated with ADHD. It was found that primary bile acid metabolites/pathways are involved in metabolic functions related to brain health and play an important role in several psychiatric disorders: depression and anxiety disorders, and these results are consistent with those of the present study (40–43). Similarly, the arginine and proline metabolic pathways are involved in the progression of diseases such as irritable bowel syndrome and amyotrophic lateral sclerosis (44, 45). In conclusion, it is likely that these two important metabolic pathways play an important role in childhood-diagnosed ADHD.

Of course, our study has some limitations. The small sample size of our cohort study is to be followed up by continued sample collection and enrollment for validation of a large sample. Of course, we are already working on the inclusion of cohort members in a large sample and are also focusing on whether changes in the participants' gut flora metabolites are related to serum metabolites. In addition, the questionnaires for ADHD in this cohort were sourced from their guardians, and the presence of information bias cannot be excluded. Also, the accuracy of the MR analysis depends on the interpretation of the instrumental variables of exposure. Further expanded sample sizes and multiple pedigree studies that are not limited to individuals of European ancestry may more accurately assess genetic effects on metabolites.

Conclusion

In conclusion, the results of the joint analysis showed a causal association between genetically predicted serum metabolites and childhood-diagnosed ADHD. Arginine and α-tocopherols were important overlapping metabolites in both studies. In addition, genetic susceptibility to ADHD is inversely correlated with levels of serum arginine and α-tocopherol. Arginine and α-tocopherols can be core metabolites that can be further studied by mechanistic.

Declarations

Author Contributions

LG and ZY designed the study and drafted the article. ZWJ, ZLL, and WJM conducted data acquisition. CLM, WYK, MQQ, and HX performed data analysis and manuscript revision. All authors read and approved the final manuscript.

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Data Availability Statement

The original contributions presented in the study are included in the article/Supplementary Materials.

Ethics

Authorized consent was acquired from the legally authorized representative of each child. The study was approved by the Ethics Committee of Jiangsu Provincial Center for Disease Prevention and Control (JSJK202l-B009-01).

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

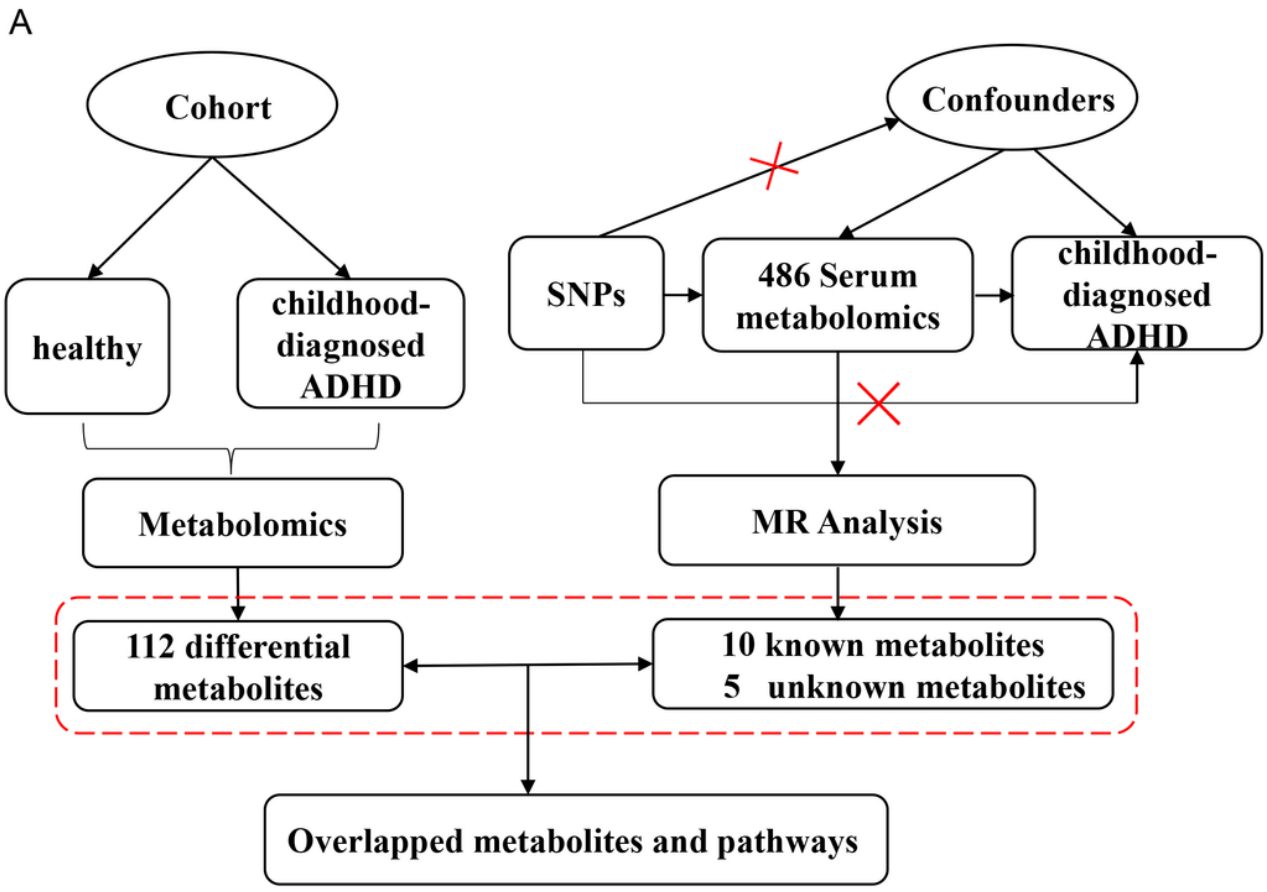

Figure 1

Cohort study and MR design diagram

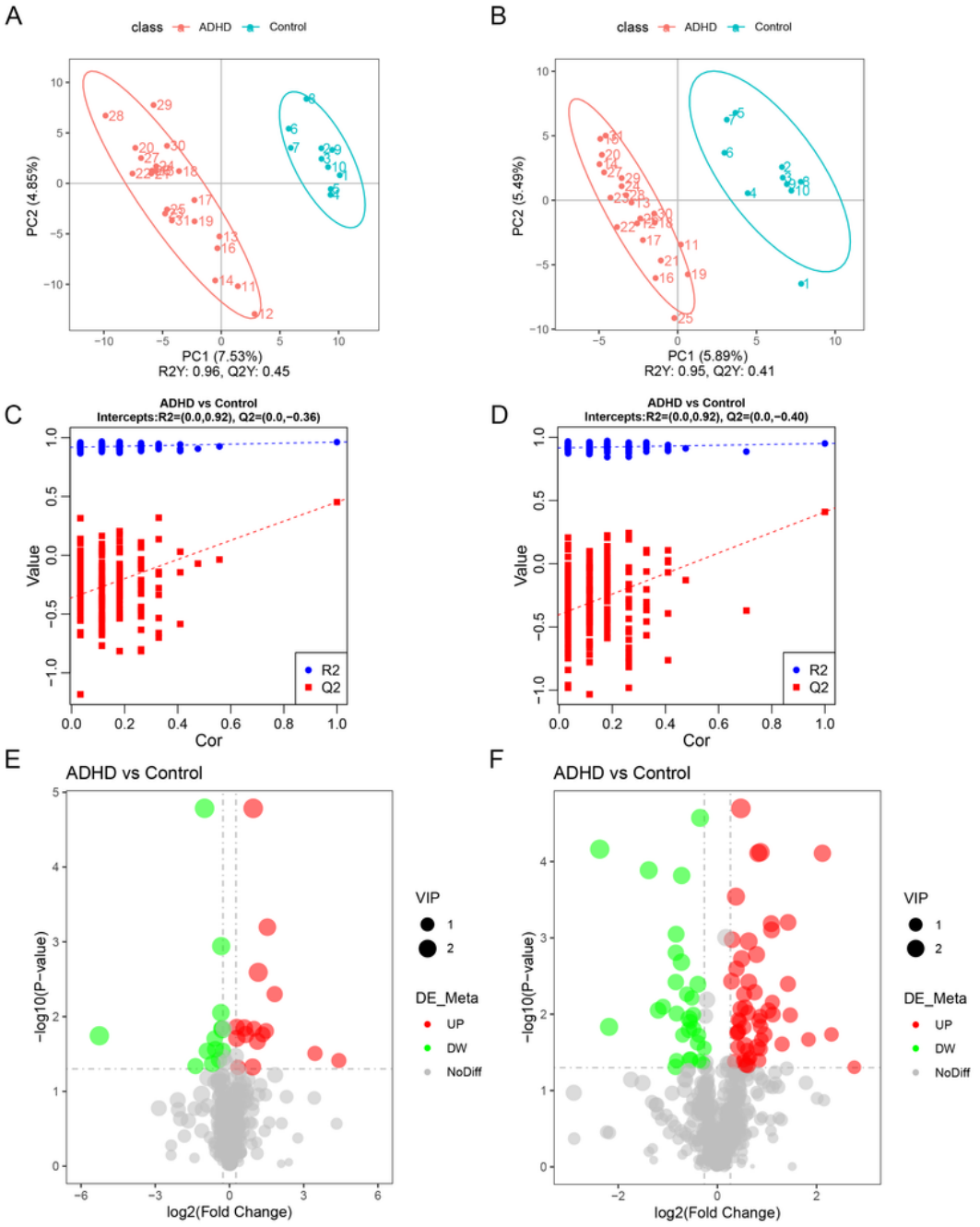

Figure 2

OPLS-DA model and Volcano plots. (A) positive ion mode OPLS-DA score (B) negative ion mode OPLS-DA score (C) positive ion mode OPLS-DA permutation test(D) negative ion mode OPLS-DA permutation test(E) positive ion mode volcanic plots of serum metabolic profiling (F) negative ion mode volcanic plots of serum metabolic profiling

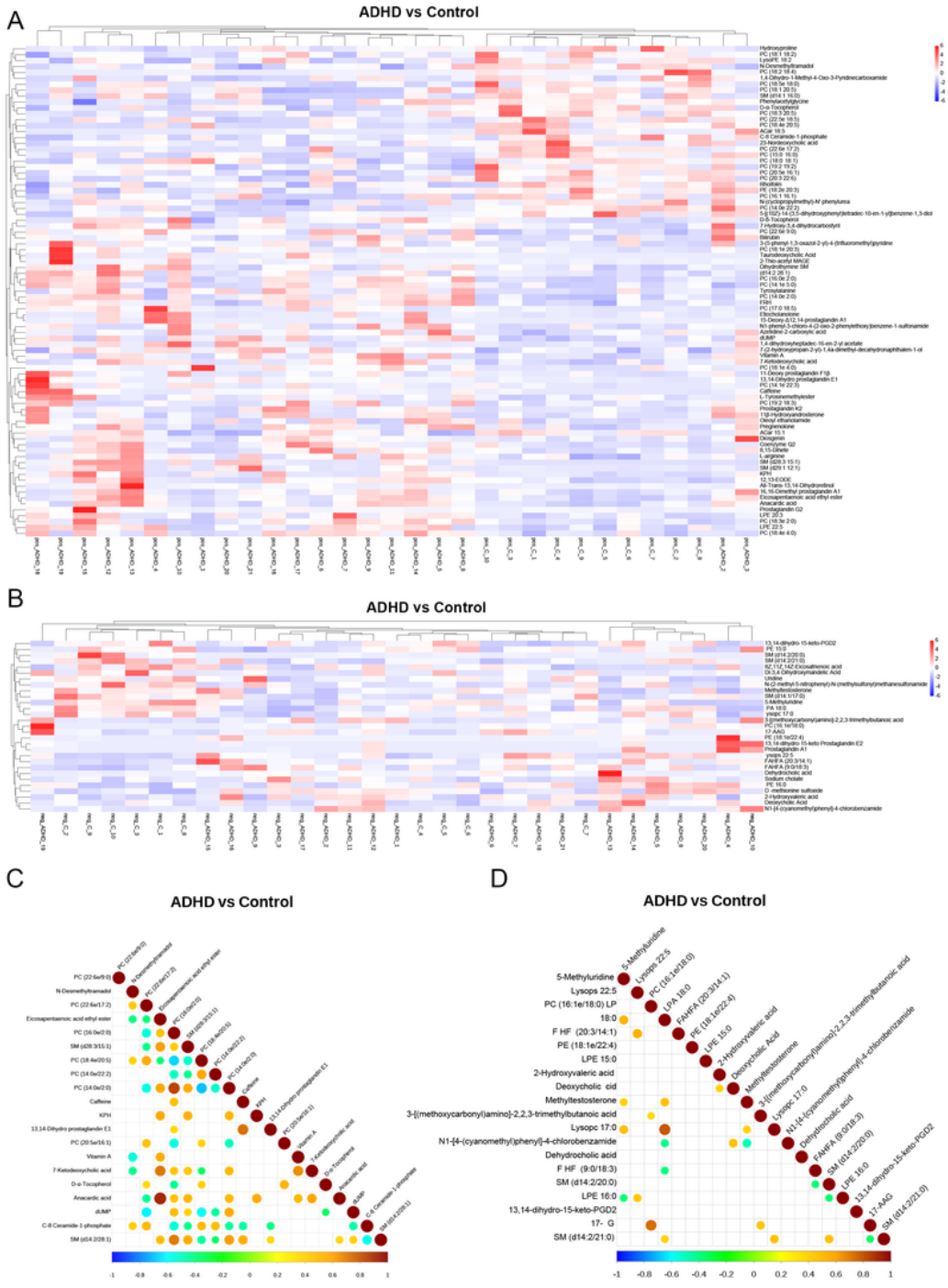

Figure 3

Heat map and correlation analysis of differential metabolites. (A) positive ion mode Cluster heat map of differential metabolites (B) negative ion mode Cluster heat map of differential metabolites (C) positive ion mode correlation analysis of differential metabolites (D) negative ion mode correlation analysis of differential metabolites

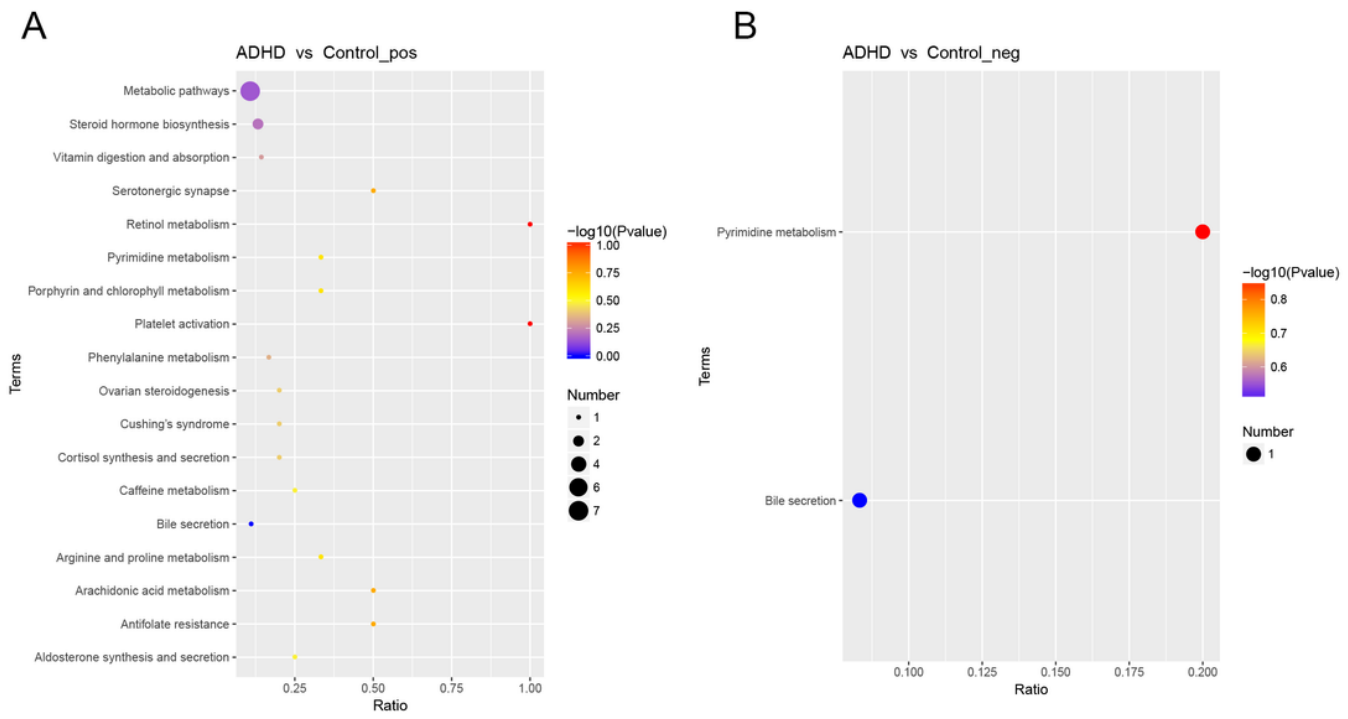

Figure 4

Pathways obtained by metabolic pathway enrichment analysis. (A)metabolic pathways obtained by enrichment analysis in the positive ion mode (B)metabolic pathways obtained by enrichment analysis in negative ion mode

Methods	Methods	SNPs	Pval	Hazard Ratio(95%CI)
Deoxycholate	IVW	4	0.003	0.502(0.319-0.788)
Inosine	IVW	2	0.015	1.468(1.078-1.999)
Alpha-tocopherol	IVW	4	0.027	2.507(1.108-5.672)
Arginine	IVW	3	0.014	4.849(1.374-17.118)
X-06226	IVW	9	0.036	0.388(0.161-0.938)
X-10506	IVW	3	0.041	0.189(0.038-0.933)
Threonate	IVW	2	0.0451	0.307(0.097-0.975)
Androsterone sulfate	IVW	5	0.046	1.168(1.003-1.359)
Propionylcarnitine	IVW	18	0.031	1.702(1.049-2.76)
X-01911	IVW	6	0.015	0.653(0.464-0.92)
X-12039	IVW	7	0.006	0.698(0.54-0.903)
X-12217	IVW	4	0.034	2.701(1.076-6.78)
Decanoylcarnitine	IVW	7	0.005	1.604(1.156-2.225)
Succinylcarnitine	IVW	18	0.028	1.73(1.06-2.824)
Cis-4-decenoyl carnitine	IVW	6	0.037	1.502(1.024-2.204)

Figure 5

Mendelian randomization associations between serum metabolites and childhood-diagnosed ADHD based on inverse-variance weighted (IVW) method.

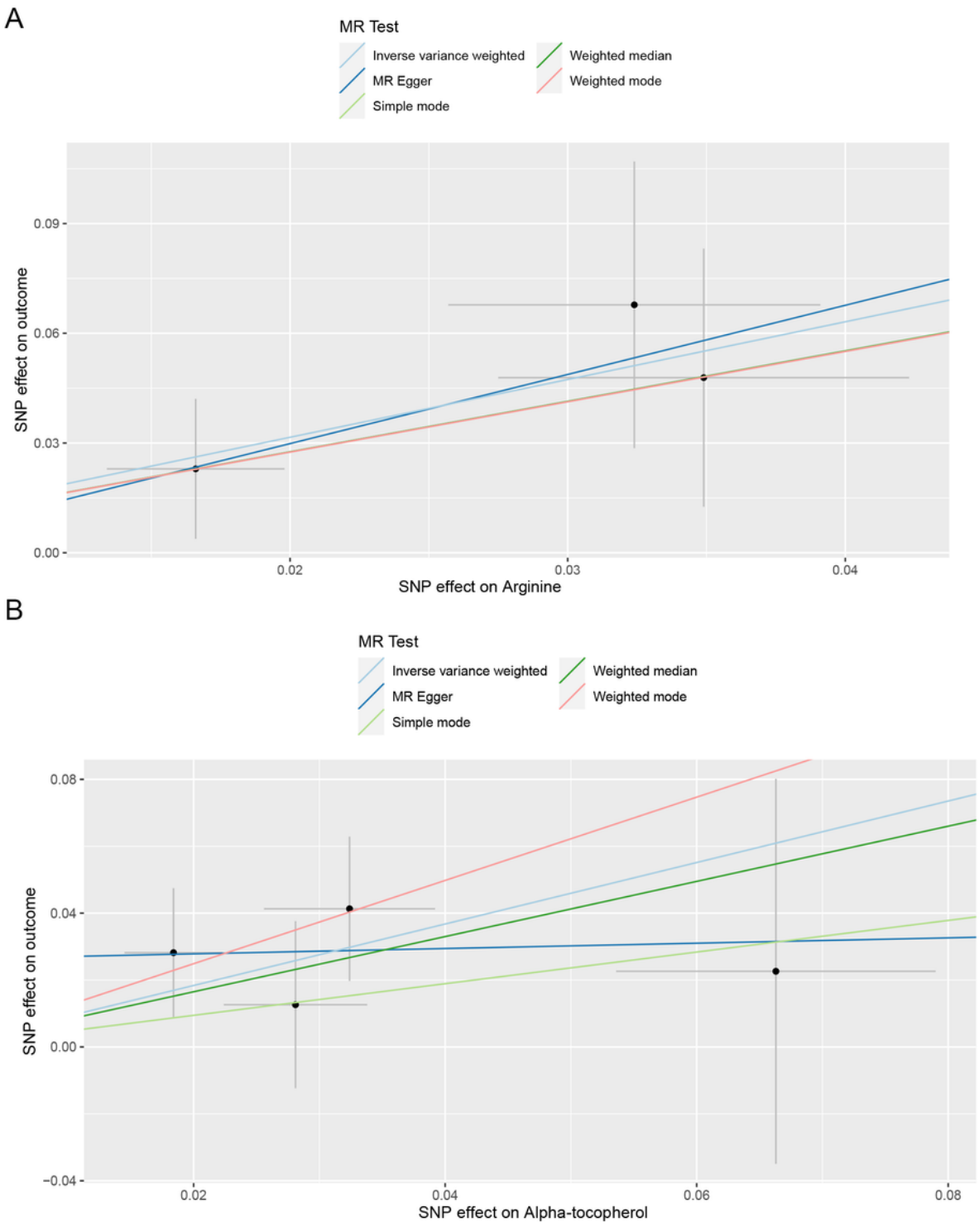

Figure 6

Genetic associations of arginine and α-tocopherol with childhood-diagnosed ADHD. (A) Genetic effect of arginine on childhood-diagnosed ADHD. (B) Genetic effect of α-tocopherol on childhood-diagnosed ADHD. Each of the single nucleotide polymorphisms (SNPs) associated with metabolite are represented by a black dot with the error bar depicting the SE of its association with metabolite (horizontal) and childhood-diagnosed ADHD (vertical). The slopes of each line represent the causal association for each method.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AppendixS1.xlsx
- AppendixS2.xlsx