

# Characterization of LC-MS Based Urine Metabolomics in Healthy Children and Adults

**Xiaoyan Liu**

Chinese Academy of Medical Sciences, Peking Union Medical College

**Xiaoyi Tian**

Beijing Children's Hospital, Capital Medical University, Beijing

**Qinghong Shi**

China-Japan Union Hospital of Jilin University

**Haidan Sun**

Chinese Academy of Medical Sciences, Peking Union Medical College

**Jing Li**

Chinese Academy of Medical Sciences, Peking Union Medical College

**Xiaoyue Tang**

Chinese Academy of Medical Sciences, Peking Union Medical College

**Zhengguang Guo**

Chinese Academy of Medical Sciences, Peking Union Medical College

**Ying Liu**

Beijing Children's Hospital, Capital Medical University, Beijing

**Jie Ma**

Beijing Children's Hospital, Capital Medical University, Beijing

**Na Ren**

Beijing Children's Hospital, Capital Medical University, Beijing

**Fang Jin**

Beijing Children's Hospital, Capital Medical University, Beijing

**Chengyan He** (✉ [chengyanhe469@vip.sina.com](mailto:chengyanhe469@vip.sina.com))

China-Japan Union Hospital of Jilin University

**Wenqi Song**

Beijing Children's Hospital, Capital Medical University, Beijing

**Wei Sun**

Chinese Academy of Medical Sciences, Peking Union Medical College

---

## Research Article

**Keywords:** metabolomics, urine, children, adults, characterization

**Posted Date:** September 29th, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-941914/v1>

**License:**  This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

---

# Abstract

Previous studies reported that gender, age, diets or lifestyles could influence urine metabolomics, which should be considered in biomarker discovery. As a consequence, for the baseline of urine metabolomics characteristics, it becomes critical to avoid confounding effects in clinical cohort studies. In this study, we provided a comprehensive characterization of urine metabolomics in a cohort of 348 healthy children (Aged 1~18) and 315 adults (Aged 20-78) for evaluation gender and age effects. Our results suggested that urine metabolites showed larger gender differences in children than in adults. For both male/boy and female/girl, each age group showed specific metabolic characterization. Especially, the pantothenate and CoA biosynthesis and alanine metabolism pathways were enriched in early life. Androgen and estrogen metabolism showed high activity during adolescence and youth stages. Pyrimidine metabolism was enriched in the old stage. This work could help us understand the baseline of urine metabolism characteristics and contribute to further studies of clinical disease biomarker discovery.

## Introduction

In recent years, urine metabolomics has been widely used in disease biomarker discovery <sup>1,2</sup>. Previous studies have reported that gender and age could influence urine metabolomics, which should be considered during biomarker discovery <sup>3</sup>. Therefore, understanding the baseline of urine metabolomics characteristics would be helpful for better understanding metabolism status under healthy conditions and discovering disease-specific metabolism disorders.

Investigation of urine metabolomics variation in a healthy population has been performed in children and adults using various approaches, including nuclear magnetic resonance (NMR) and mass spectrometry coupled to either gas (GC-MS) or liquid chromatography (LC-MS). In 2016, urinary metabolites from 30 healthy children were assessed at 6 months and 1, 2, 3, and 4 years of age by using NMR spectroscopy. Amino acid metabolism was significantly different between infants aged 6 months and 1 year, whereas carbohydrate metabolism was significantly different between children aged 2 and 3 years <sup>4</sup>. In 2018, a six European centers research on metabolome differences in children aged 6 to 11 were performed using NMR and targeted LC-MS/MS. Both urine and serum metabolomes were found to be associated with age, sex, BMI and dietary habits <sup>5</sup>. For the metabolic phenotypes in adults, in 2015, Etienne A. et al. characterized the urinary metabolomes of 183 healthy subjects using an LC-MS platform. A total of 108 metabolites related to amino acid metabolism, the carnitine shuttle and the TCA cycle were found to be affected by age, gender or BMI <sup>2</sup>. Recently, in 2018, Sili Fan et al. applied metabolomics profiling on urine samples from 60 healthy males and females using GC-TOF-MS. Saturated fatty acids, TCA cycle intermediates, and butyrate were found to be significantly related to the effect of gender <sup>6</sup>. In 2018, we conducted a urine metabolomics study in 203 healthy adults to find age- and gender-dependent urine metabolites. Metabolic pathways, such as tryptophan metabolism, citrate cycle, and pantothenate and CoA biosynthesis, were found to be related to gender and age <sup>7</sup>. Despite the large efforts in characterizing urine metabolomics in healthy subjects, most of the previous studies were focused on the confounding

factors of the urine metabolome. While the metabolic characterization of a population during different age groups was unavailable until the present study.

In the present study, a large sample size including 348 children (aged 1 ~ 18) and 315 adults (aged 20 ~ 78) with balanced gender from China was enrolled. We aimed to characterize urine metabolic features in different age stages for males (boys) and females (girls). HRLC-MS-based metabolic profiling was utilized to characterize the urine metabolome of each individual. In addition to gender- and age-related metabolites and pathways, the key metabolites that played important roles during different gender or age stages were identified and analyzed. Identification of these significant metabolites and metabolite networks would be helpful for understanding the physiological functions during different life stages and would be useful for future disease biomarker discovery.

## Results And Discussion

### Data quality control

The workflow for the present study is shown in Fig. 1. To reduce experimental variations from the sampling process, standard sampling procedures, including sampling time and sampling processing, were performed by a trained professional. Data from the two centers' sample were analyzed separately, since batch effect existed. Metabolite features with RSD (relative standard deviation) < 0.3 were submitted to further analysis. Principal component analysis was performed to evaluate the stability of QCs and variation of samples (Fig S1a). A good cluster of QC samples indicated good stability of the analytical platform.

### Gender-dependent metabolomics in children and adults

A total of 291 identified metabolites were subjected to further analysis (Table S1). Gender-differences were assessed using PCA and OPLS-DA models. Scatter plots showed that gender differences in the urine metabolites of adults are more apparent than in children (OPLS-DA: children:  $R^2Y = 0.599$ ,  $Q^2 = 0.379$ ; adults:  $R^2Y = 0.928$ ,  $Q^2 = 0.768$ . Fig. S1b and S1c). Metabolites with VIP values above the threshold value 1 and p values below the significance threshold 0.05 were considered gender dependent. Overall 42 metabolites were found to be gender-dependent in children, and 98 metabolites in adults (Fig. 2a and 2b, Tables S2 and S3). A total of 17.9% (21) of these metabolites showed gender differences in both children and adult populations with the same change trend in male/boy and female/girl (Fig. 2c). Guanidoacetic acid, 5-hydroxyindoleacetic acid, dopamine, 5'-methylthioadenosine and indoleacrylic acid showed higher levels in females. The metabolites deoxyinosine, cotinine glucuronide, dopamine glucuronide and L-formylkynurenine showed higher levels in males. Gender differences of these common differential metabolites in the adult population were larger than those in the children population (Fig. 2c). Specifically, the metabolites cortisol, uric acid, 18-hydroxycortisol, deoxycholic acid and glycine conjugate were found to be gender-dependent only in the children population. Metabolites of deoxyuridine, pantothenic acid, riboflavin, and 3-hydroxytetradecanedioic acid were gender-dependent only in the adult population. These metabolites suggested differential metabolic status between children and adults.

Pathway enrichment analysis would provide an overview of gender-dependent metabolism status in children and adults (Fig. 2d and 2e). Consistent with previous studies (Chiu et al., 2016; Fan et al., 2018; Liu et al., 2018), amino acid metabolism, including tryptophan metabolism and arginine and proline metabolism, showed gender differences in both children and adults. In females/girls, tryptophan metabolites, indoleacrylic acid, indolylacryloylglycine and 5-hydroxyindoleacetic acid showed higher levels compared with males/boys. 5-Hydroxyindoleacetic acid is a breakdown product of serotonin that is excreted in the urine and is mainly involved in serotonin degradation and serotonin receptor signaling regulation. The higher level in females could be partly explained by greater precursor, such as tryptophan, availability (Akhmadeev and Kalimullina, 2013). Studies on experimental animals have revealed the effects of gonadal hormones on the indoleamine system, and in the rat brain, 5-hydroxyindoleacetic acid is higher in females than in males (Giulian et al., 1973). These results were consistent with our present urine results. Additionally, children and adults showed different gender-dependent metabolic statuses. In the child population, purine metabolites, steroid metabolites and amino acid metabolites showed specific gender dependence. For example, uric acid, an oxidation product of purine, showed a significantly higher level in boys compared with girls but showed no gender variations in adults. Renal excretion of uric acid in children differs from that in adults. It was reported that the younger the child, the greater the excretion of uric acid (Baldree and Stapleton, 1990). Altered urine uric acid level is an indispensable marker in detecting rare inborn errors of metabolism (Jasinge et al., 2017). The level of uric acid in urine was higher in boys than girls, probably contributing to the higher prevalence of hyperuricemia in boys than in girls (Niegawa et al., 2017). These results indicated the commonness and differences of gender-dependent metabolism characteristics in children and adults, probably resulting from different [physiological characteristics](#), dietary habits or other environmental factors<sup>8</sup>. The detailed gender-associated pathways in each age stage are listed in Table S4.

### Age-dependent metabolomics in children and adults

Age is another important factor in metabolism status. PCA was firstly performed, showing apparent separation of different groups (Fig. S2a). To determine metabolites related to age, PLS-DA modeling was performed on urine metabolite profiles of populations with different age stages. Since the metabolism status of the population was different between sexes, the analyses for age in children and adults were performed separately for males and females.

In the children population, the PLS-DA three-component score plot showed clear associations of metabolite profiles with age in boys ( $R^2Y = 0.719$ ,  $Q^2 = 0.528$ , Fig. 3a, Fig. S2b), indicating significant metabolism differences among different age stages. The same trend was observed in girls ( $R^2Y = 0.78$ ,  $Q^2 = 0.564$ , Fig. 3b, Fig. S2b). In both boys and girls, two clusters of metabolites were found, one cluster with metabolites decreasing with age (Component 1); the second cluster with metabolites showing the highest level in the primary school aging 7 to 12 (Component 2) (Fig. 3c). The first cluster contributes most to age variation in both girls and boys. In the adult population, clear separations were also observed for the three age groups in males and females (PLS-DA-male:  $R^2Y = 0.652$ ,  $Q^2 = 0.17$ ; PLS-DA-female:  $R^2Y = 0.583$ ,  $Q^2 = 0.362$ , Fig. S2b, Fig. S2c and Fig. S2d). Similar to children, metabolites decreasing with

age contributed most to age differences (Fig S2e). According to the significance threshold, a total of 98 and 76 metabolites were selected as age-dependent in boys and girls, respectively. For the adult population, 55 and 80 metabolites were age-dependent in males and females, respectively (Table S5, S6, S7 and S8). These metabolites were further submitted for further pathway analysis.

For children and adults, we found many common age-dependent pathways between boys (males) and girls (females), indicating common metabolism status variations with age in humans. For instance, pantothenate and CoA biosynthesis, fatty acid biosynthesis and tryptophan metabolism were found to be changed with age in both children and adults. These pathways correspond to energy demand changes with aging (Chiu et al., 2016). Interestingly, tryptophan metabolism was only found to be age dependent in boys and males, while no significant changes were found in girls and females. Metabolites of tryptophanol and 5-methoxytryptophan were reported to be associated with increased cellular anti-inflammatory and blood circulation properties (Cheng et al., 2012), probably reflecting age-associated metabolism activity differences between genders. In addition, we found that the pathway of steroid hormone biosynthesis was changed with age in adults and children. As a whole, steroid hormone biosynthesis showed high activity during the adolescent and youth stages, corresponding to sexual development (Steensma et al., 2013).

Additionally, children and adults showed different age-dependent metabolic statuses. Particularly in the children population, the fatty acid biosynthesis pathway was found to be age dependent. The results showed a positive correlation with increasing age. Fatty acids constitute a large energy source for the body. Increased fatty acid metabolism indicated high ATP generation with age in a children population (Wakil and Abu-Elheiga, 2009). In adults, pyrimidine metabolism and caffeine metabolism were found to be age dependent. Pyrimidine metabolism was found to be positively correlated with aging, showing the highest level in the old adults. Deoxyuridine, a naturally occurring nucleoside, is considered to be an antimetabolite that is converted to deoxyuridine triphosphate during DNA synthesis. Disturbance of DNA synthesis may modulate the aging process and contribute to the high incidence of cancer with aging (Kirsh et al., 1986). The detailed age-dependent metabolism pathways for males (boys) and females (girls) are listed in Table S4.

### **Metabolic characteristics for gender and age**

According to the above results, the urine metabolites of children and adults showed both gender and age dependent effects. Furthermore, we detailed the metabolic differences affected by both gender and age. The p values of OPLS-DA for gender separation during each stage were used to evaluate the significance of gender difference. The results showed a parabolic trend of gender differences during life span, with less significance in the pre- and primary school stages, high significance during the secondary school, youth and middle stages, and less significance during the old stage (Fig 4).

Second, we examined the main metabolic characteristics marked with metabolites with highest intensity for each age group in males (boys) and females (girls) (Fig 4 and Table 2).

**Metabolomic characteristics during the pre- and primary school stages** During early life in the preschool and primary school stages, gender differences were relatively smaller than in other age stages, as shown in Fig 4. During the preschool stage, pathways of pantothenate and CoA biosynthesis, pyrimidine metabolism, vitamin B6 and alanine metabolism showed high activity in girls and boys. These active pathways were associated with energy and nutrient supply. These metabolic characteristics correspond to the physiological characteristics during this life stage, high metabolism activity for rapid growth and development demands (Chiu et al., 2016). Research on children aged from 2-7 years old suggested that pantothenate and CoA biosynthesis, pyrimidine metabolism and vitamin B6 metabolism were significantly related to autism spectrum disorder (ASD) (Gevi et al., 2016). These results highlight the importance for these pathways to maintain homeostasis during preschool.

During primary school stage, the most active metabolism pathway is tryptophan metabolism, lipid metabolism, nicotinate and nicotinamide metabolism and histidine metabolism. These metabolic features were corresponding to the main physiological characteristic-visual development, blood circulation increasing and rapid metabolism activity during this stage. Although gender differences were small during the primary school stage, metabolites with the highest level in boys and girls showed specific features. In boys, tryptophan metabolites, such as 5-methoxytryptophan, were found to show the highest level in boys. 5-Methoxytryptophan is an endogenous tryptophan metabolite with anti-inflammatory properties (Cheng et al., 2012). In addition, 5-methoxytryptophan was reported to be involved in the cyclic metabolism of the retina (Leino and Airaksinen, 1985), ventricular remodeling and maintaining liver function (Rossignol and Frye, 2011; Lin et al., 2016). In girls, urocanic acid, a breakdown (deamination) product of histidine, showed the highest level. Urocanic acid is one of the essential components of human skin (Wezynfeld et al., 2014). It could accumulate in the epidermis and may be both a UV protectant and an immunoregulator. A higher level of these metabolites in girls may contribute to skin development during this age stage. It was reported that skin disorders are more common among girls than boys aged 6 to 17 years (Sula et al., 2014), which could be affected by the immunomodulatory effects of urocanic acid (Finlay-Jones and Hart, 1997).

**Metabolomic characteristics during adolescence and youth** During adolescence and the youth stage, gender differences become more significant, partly due to changes in hormone and endocrine levels. The main metabolic feature during these stages was fatty acid oxidation and biosynthesis, androgen and estrogen metabolism and steroidogenesis. These metabolic characteristics correspond to pubertal development, neurodevelopmental changes and heightened stress sensitivity during adolescence and youth stages. Cortisol, androstenediol, testosterone, and their glucuronide metabolites showed higher levels during this period. Cortisol is the main glucocorticoid secreted by the adrenal cortex and is involved in the stress response. Synergies between cortisol reactivity and testosterone were reported to influence antisocial behavior in young adolescence. The youth with high diurnal testosterone, combined with high or moderate cortisol reactivity, were significantly higher on antisocial behavior and attention behavior problems (Susman et al., 2017).

In addition to the common metabolic features during adolescence and the youth stage, boys and girls also showed specific metabolic characteristics. During adolescence, spermidine biosynthesis was higher in boys. One of the involved metabolites was 5-methylthioadenosine, a byproduct of polyamine synthesis in DNA turnover cycles that increases with inflammation to modulate cellular stress. It has been shown to influence the regulation of gene expression, proliferation, differentiation, and apoptosis (Avila et al., 2004). Higher serum levels of 5-methylthioadenosine have been reported in youth boys when compared to girls (Perng et al., 2017). A direct association between 5-methylthioadenosine and high metabolic risk was found in boys, possibly driven by proinflammatory pathways (Guasch-Ferre et al., 2016). While in adolescent girls, fatty acid oxidation and biosynthesis showed high activity. Acylcarnitines showed the highest level, indicating high activity of carnitine acetyltransferase in mitochondria (Zammit et al., 2009). These results indicated the preferred metabolic fuel-from fatty acid oxidation in adolescent girls (Zammit et al., 2009).

In the youth males aged 20 to 30 years, linoleic acid metabolites showed higher levels in addition to steroid metabolism. The involved metabolite eicosapentaenoic acid serves as the precursor for prostaglandin-3. It could enhance the production of the cytoprotective prostanoid 15d-PGJ2 (Davidson et al., 2013), which corresponds to the elevated prostaglandin production in youth males (Pace et al., 2017). In contrast to youth males, arachidonic acid metabolites showed higher levels in youth females. It was reported that in females, arachidonic acid metabolism could rescue anti-inflammatory and butyrate-producing microbiota, then upregulate GPR41 and GPR109A and control hypothalamic inflammation (Zhuang et al., 2017).

**Metabolomic characteristics during the middle and old stages** During the middle age stage, males and females showed the most significant gender differences. In males, the main metabolic features were vitamin B6 and purine metabolism. 8-Hydroxy-7-methylguanine, a methylated purine base, showed higher levels in males. High methylated purine bases were found in tumor-bearing patients compared to healthy controls (Morris et al., 1986). Urine alkylated purines were partly derived from covalently bound adducts in DNA formed from exposure to carcinogenic alkylating agents (Choi and Guengerich, 2006). Purine disorders may be associated with serious, sometimes life-threatening consequences. In females, the metabolism pathway of steroidogenesis and caffeine metabolism showed high activity. Menopausal symptoms are an unavoidable problem in females during this period, which could contribute to some metabolic disorders. Caffeine metabolism was reported to be associated with menopausal symptoms, particularly vasomotor symptoms (Faubion et al., 2015).

For the stage aged above 50 years, the gender difference decreased. During this period, organ metabolism activity gradually slows down. Energy-supply metabolism pathways, such as fatty acids and amino acids, showed low levels. In contrast, steroidogenesis, caffeine and pyrimidine metabolism showed high levels. Higher levels of pyrimidine metabolites in the old stage have adverse effects on health (Choi and Guengerich, 2006). Additionally, several cognitive impairment-related metabolites, including acetylhistidine and steroid hormones, were found to be higher in the old population, partly contributing to the high incidence of cognitive impairment diseases at older ages (Bressler et al., 2017).

In conclusion, we provide a comprehensive view of human metabolism status across the life span. To our knowledge, this is the first systematic study to analyze metabolism characterization based on population across a considerable age range. This study showed that gender differences existed from early life stages, and these differences were much smaller than those in adults. Age is another recognized confounder. Metabolism characteristics for each age group could reflect the metabolism status during different life stages and possibly contribute to some age-dependent disease incidences. Our present study would be helpful to understand the age- and gender-dependent metabolism differences, which will be a critical component for the development of metabolomics-based systems biology as a population screening and precision medicine research.

Additionally, several limitations still exist and need to be further validated in the future. First, urine samples of children and adults were collected from two different hospitals. Although sampling operation is strictly controlled, batch effects resulting from center differences could not be completely eliminated. Considering batch effects, data from children and adults were first analyzed separately and then a cross comparison was performed, resulting in an age gap between second school and the youth stages. Second, in the children population, each stage could show specific features for their rapid development. However, due to sample size limitations, we provide an overview of the average metabolism status over a period of 6 years. Third, the influences of diets and circadian rhythm on urine metabolomics could not be completely eliminated, though all subjects were from the same region. For future validation analysis, these influences should be systematically evaluated and analyzed.

## Methods

### Study sample population

Our study enrolled 348 children aged 1 to 18 years from Beijing Children's Hospital and 315 adults aged 20 to 70 years from 3rd Clinical Hospital, Jilin University (Table 1). According to physiological developmental characteristics, the enrolled population was divided into 6 age stages for males (boys) and females (girls): preschool (aged 1-6), primary school (aged 7-12), secondary school (aged 13-18), youth (aged 20-30), middle (aged 31-50) and old (aged >50). Participants were checked and examined by trained nurses according to standard operating procedures. All physical examination indexes were in the normal range. On the day of the examinations, urine samples were collected at approximately 7:00 and 9:00 a.m. on an empty stomach. This study was approved by the Ethics Committee of Peking Union Medical College. A doctor informed the eligible participants about the nature of the study and invited them to participate. All human subjects provided informed consent before participating in this study. The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki). and institutional review board of Peking Union Medical College has approved the study.

### Urine sample preparation

Urine samples were processed according to our previous protocols. Briefly, proteins from urine samples (200  $\mu$ L) were precipitated using acetonitrile (400  $\mu$ L). The mixture was vortexed for 30 sec and

centrifuged at 14,000 g for 10 min. The supernatant was dried under vacuum and then reconstituted with 200  $\mu$ L 2% acetonitrile/water. Quality control (QC) samples were prepared by mixing aliquots of all samples across different groups to be analyzed. All samples were randomly injected into the LC-MS system within a single analysis (within 12 days). QC sample was injected every ten or twelve samples.

### **Urine metabolite LC-MS measurements**

HRLC-MS was selected for urinary metabolite detection due to its high sensitivity and reproducibility. Urine metabolite separation and analysis was conducted using a Waters ACQUITY H-class LC system (Waters, Milford Massachusetts, USA) coupled with a LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, MA). The following 18-min gradient on a Waters HSS C18 column (3.0  $\times$  100 mm, 1.7  $\mu$ m) at a flow rate of 0.5 mL/min was used: 0-1 min, 2% solvent B (mobile phase A: 0.1% formic acid in H<sub>2</sub>O; mobile phase B: acetonitrile); 1-3 min, 2-55% solvent B; 3-8 min, 55-100% solvent B; 8-13 min, 100% solvent B; 13-13.1 min, 100-2% solvent B; 13.1-18 min, 2% solvent B. The column temperature was set at 45°C.

All samples were untargeted scanned from 100 to 1000 m/z at a resolution of 60 K at the positive ESI mode. The automatic gain control (AGC) target was  $1 \times 10^6$ , and the maximum injection time (IT) was 100 ms. The extracted MS features were divided into several targeted lists and imported to MS2 method for targeted data dependent analysis. MS/MS fragment acquisition was acquired at a resolution of 15 K with an AGC target of  $5 \times 10^5$ . Collision energy was optimized as 20, 40 or 60 for each targeted list with higher-energy collisional dissociation (HCD) fragmentation. The injection order of urine samples was randomized to reduce any experimental bias. The QC sample was injected regularly to monitor system stability.

### **Statistical analysis**

Raw data files were processed by Progenesis QI (Waters, Milford, MA) software based on a previously published identification strategy, which included sample alignment, peak picking, peak grouping, normalization to total compound and final information export<sup>9</sup>. The exported data were further preprocessed by MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca>), which included missing value estimation, log transformation and Pareto scaling. Variables that were missed in 50% or greater of the samples were removed from further statistical analysis.

Nonparametric tests (Wilcoxon rank-sum tests and Kruskal-Wallis tests) were used to evaluate the significance of variables related to gender and age using MetaboAnalyst 3.0. Benjamini-Hochberg correction was applied throughout to account for multiple test comparisons. Cutoff of FDR 0.05 was applied. Pattern recognition analysis (principal component analysis [PCA]; partial least squares discrimination analysis [PLS-DA]; and orthogonal partial least square analysis [OPLS-DA]) was carried out using SIMCA 14.0 software (Umetrics, Sweden) to visualize group classification and select significant features. 100 permutation tests were used to validate the OPLS-DA and PLS-DA model to avoid over-fitting of the model. Significantly differential metabolites were chosen according to the following criteria:

(i) adjusted  $p < 0.05$ ; (ii) fold change between two groups  $> 2$ ; and (iii) the variable importance plot (VIP) value obtained from OPLS-DA was above 1. Heat map virtualization and metabolic pathway enrichment analysis was performed by MetaboAnalyst 3.0, and the enrichment results were visualized using R based on previous methods <sup>10</sup>.

## Feature annotation and metabolite identification

MS1 features were divided into several targeted lists and imported to MS method for targeted data dependent analysis. The MS/MS spectra were further imported to Progenesis QI for database searching (HMDB: <http://www.hmdb.ca/>) and MS/MS spectra matching using “MetFrag” algorithm <sup>11</sup>. Detailed compound identification information (.csv file) included compound ID, adducts, formula, score, MS/MS score, mass error (in ppm), isotope similarity, theoretical isotope distribution, web link, and m/z values. Confirmation of the differential compounds was performed by the parameters, including Score, Fragmentation score, and Isotope similarity given by Progenesis QI. Score ranging from 0 to 60, is used to quantify the reliability of each identity. According to the score results of the reference standards, the threshold was set at 35.0. Isotope similarity is calculated by comparison of the measured isotope distribution of a precursor ion with the theoretical. The compound identification is more reliable if the higher the values obtained. For metabolites with biological significance, the annotations were further manually validated by standards MS2 spectra or MS2 spectra database (Masbank: <http://www.massbank.jp/>), combined with fragmentation explanation.

## Abbreviations

LC-HRMS: liquid chromatography coupled with high resolution mass spectrometry; NMR: nuclear magnetic resonance; GC-TOF-MS: gas chromatography-time-of-flight mass spectrometry; TCA: tricarboxylic acid; CoA: coenzyme; QC: quality control; PCA: principal component analysis; PLS-DA: partial least squares discrimination analysis; OPLS-DA: orthogonal partial least square discrimination analysis; VIP: variable importance plot; HMDB, human metabolome database

## Declarations

### Availability of data and material

The datasets generated and/or analysed during the current study are available in the iProx repository: <https://www.iprox.cn/page/PSV023.html?url=1624348624443Gjgs>. Password: JuPW.

### Compliance and ethics

The authors have declared no competing interests.

This study conformed with the Helsinki Declaration of 1975 (as revised in 2008) concerning Human Rights, and followed out policy concerning Informed Consent as shown on Springer.com.

## Acknowledgments

This work was supported by the National Key Research and Development Program of China (No. 2016 YFC 1306300, 2018YFC0910202), National Natural Science Foundation of China (No. 30970650, 31200614, 31400669, 81371515, 81170665, 81560121, 81572082), Beijing Natural Science Foundation (No. 7173264, 7172076), Beijing cooperative construction project (No.110651103), Beijing Science Program for the Top Young (No.2015000021223TD04), Beijing Normal University (No.11100704), Beijing Municipal Administration of Hospitals' Youth Program (QML20171205), Beijing Children's Hospital Young Investigator Program Fund (BCHYIPB-2016-01), and Biologic Medicine Information Center of China, National Scientific Data Sharing Platform for Population and Health. The funding bodies played no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

## References

- 1 Chen, Z. & Kim, J. Urinary proteomics and metabolomics studies to monitor bladder health and urological diseases. *BMC Urol* **16**, 11, doi:10.1186/s12894-016-0129-7 (2016).
- 2 Thevenot, E. A., Roux, A., Xu, Y., Ezan, E. & Junot, C. Analysis of the Human Adult Urinary Metabolome Variations with Age, Body Mass Index, and Gender by Implementing a Comprehensive Workflow for Univariate and OPLS Statistical Analyses. *Journal of proteome research* **14**, 3322-3335, doi:10.1021/acs.jproteome.5b00354 (2015).
- 3 Reusch, J. E. B., Kumar, T. R., Regensteiner, J. G. & Zeitler, P. S. Identifying the Critical Gaps in Research on Sex Differences in Metabolism Across the Life Span. *Endocrinology* **159**, 9-19, doi:10.1210/en.2017-03019 (2018).
- 4 Chiu, C. Y. *et al.* Metabolomics Reveals Dynamic Metabolic Changes Associated with Age in Early Childhood. *PloS one* **11**, e0149823, doi:10.1371/journal.pone.0149823 (2016).
- 5 Lau, C. E. *et al.* Determinants of the urinary and serum metabolome in children from six European populations. *BMC medicine* **16**, 202, doi:10.1186/s12916-018-1190-8 (2018).
- 6 Fan, S. *et al.* Sex-associated differences in baseline urinary metabolites of healthy adults. *Scientific reports* **8**, 11883, doi:10.1038/s41598-018-29592-3 (2018).
- 7 Liu, X. *et al.* Investigation of the urinary metabolic variations and the application in bladder cancer biomarker discovery. *International journal of cancer* **143**, 408-418, doi:10.1002/ijc.31323 (2018).
- 8 de Prado, D. M., Dias, R. G. & Trombetta, I. C. Cardiovascular, ventilatory, and metabolic parameters during exercise: differences between children and adults. *Arquivos brasileiros de cardiologia* **87**, e149-155, doi:10.1590/s0066-782x2006001700035 (2006).

- 9 Zhang, J. *et al.* An intelligentized strategy for endogenous small molecules characterization and quality evaluation of earthworm from two geographic origins by ultra-high performance HILIC/QTOF MS(E) and Progenesis Q1. *Analytical and bioanalytical chemistry* **408**, 3881-3890, doi:10.1007/s00216-016-9482-3 (2016).
- 10 Wang, S., Zheng, W., Hu, L., Gong, M. & Yang, H. MixProTool: A Powerful and Comprehensive Web Tool for Analyzing and Visualizing Multigroup Proteomics Data. *Journal of computational biology : a journal of computational molecular cell biology* **25**, 1123-1127, doi:10.1089/cmb.2018.0050 (2018).
- 11 Allen, F., Pon, A., Wilson, M., Greiner, R. & Wishart, D. CFM-ID: a web server for annotation, spectrum prediction and metabolite identification from tandem mass spectra. *Nucleic acids research* **42**, W94-99, doi:10.1093/nar/gku436 (2014).

## Tables

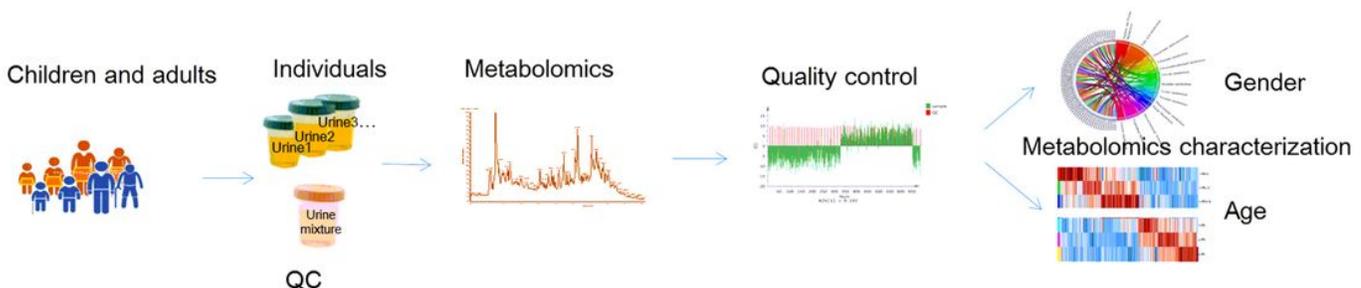
**Table 1. Basic characteristics of the participants in this study**

Age stage	Female	Male	Total
1-6	54	59	113
7-12	59	57	116
13-18	59	60	119
20-30	38	43	81
31-50	72	68	140
>50	54	40	94
Total	336	327	663

**Table 2 Metabolic characteristics in males (boys) and females (girls)**

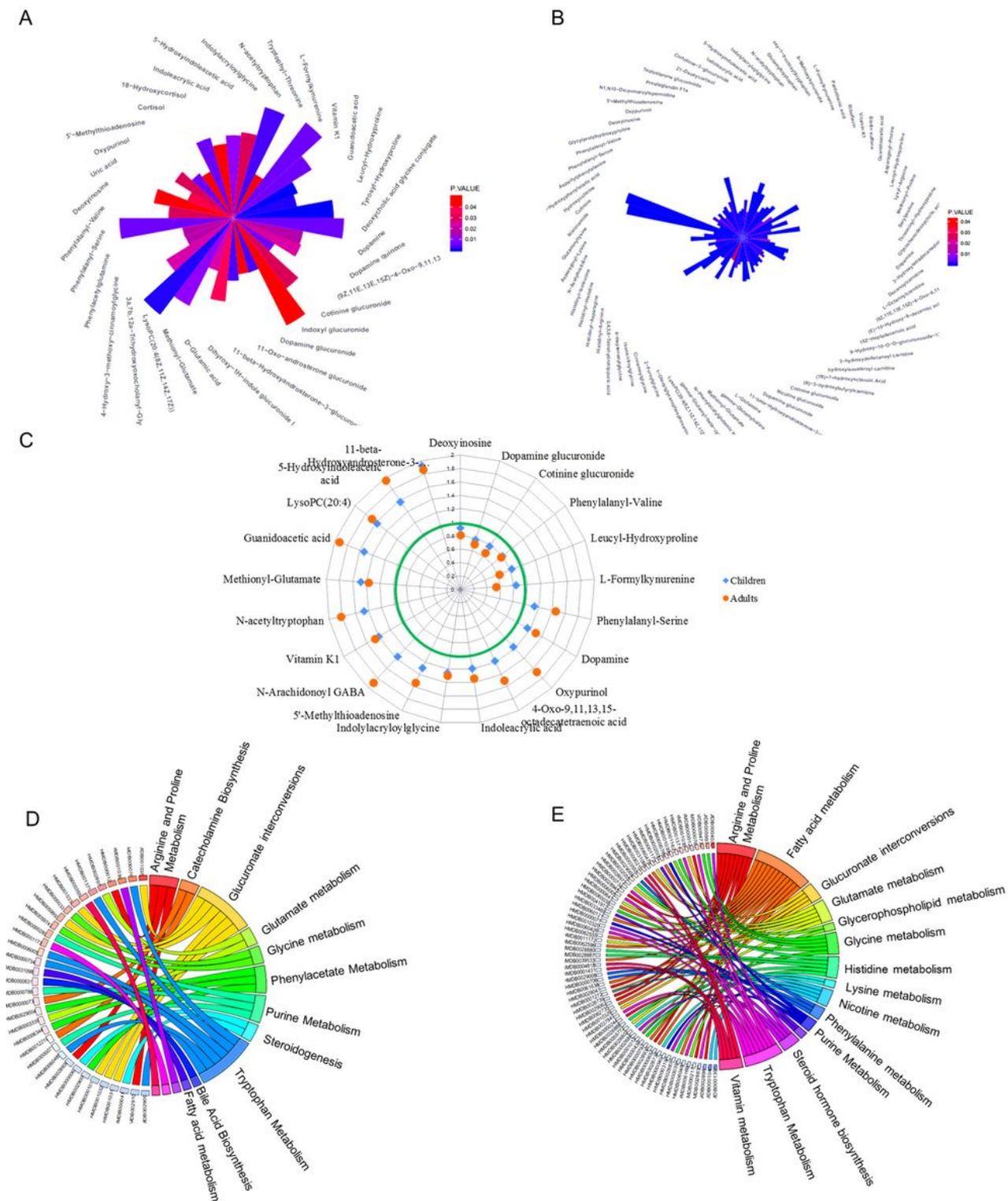
	<b>Males (boys)</b>	<b>Females (girls)</b>
<b>1-6</b>	Pantothenate and CoA biosynthesis Alanine metabolism Pyrimidine metabolism	Pantothenate and CoA biosynthesis Alanine metabolism Vitamin B6 metabolism
<b>7-12</b>	Tryptophan metabolism	Ether lipid metabolism Nicotinate and nicotinamide metabolism Histidine metabolism
<b>13-18</b>	Fatty Acid oxidation and biosynthesis Spermidine and spermine biosynthesis Riboflavin metabolism	Fatty acid oxidation and biosynthesis
<b>20-30</b>	Androgen and estrogen metabolism Steroidogenesis Linoleic acid metabolism	Androgen and estrogen metabolism Steroidogenesis Arachidonic acid metabolism
<b>30-50</b>	Vitamin B6 metabolism Purine metabolism	Steroidogenesis Caffeine metabolism
<b>&gt;50</b>	Vitamin K metabolism Steroidogenesis Caffeine metabolism	Vitamin K metabolism Pyrimidine metabolism

## Figures



**Figure 1**

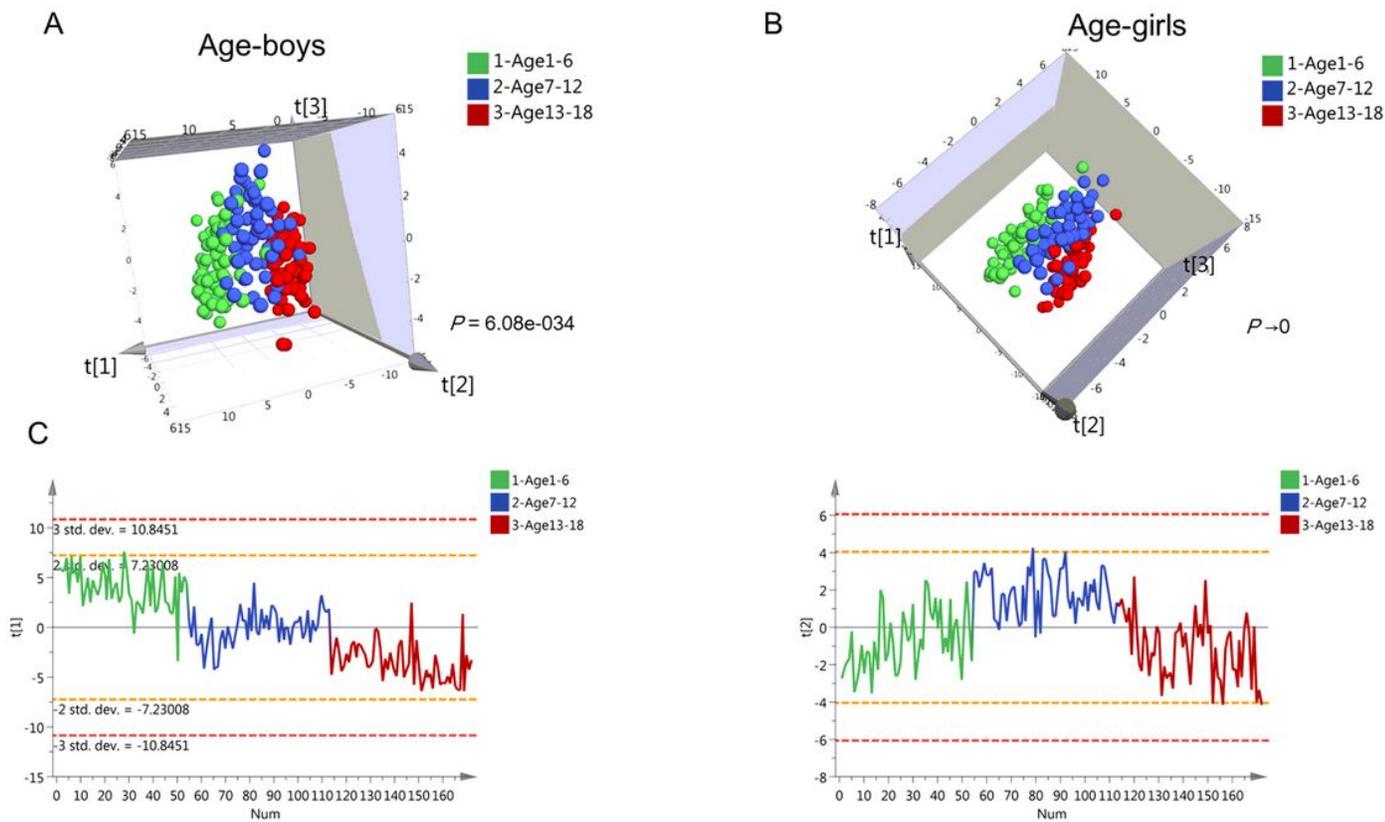
The workflow of this study.



**Figure 2**

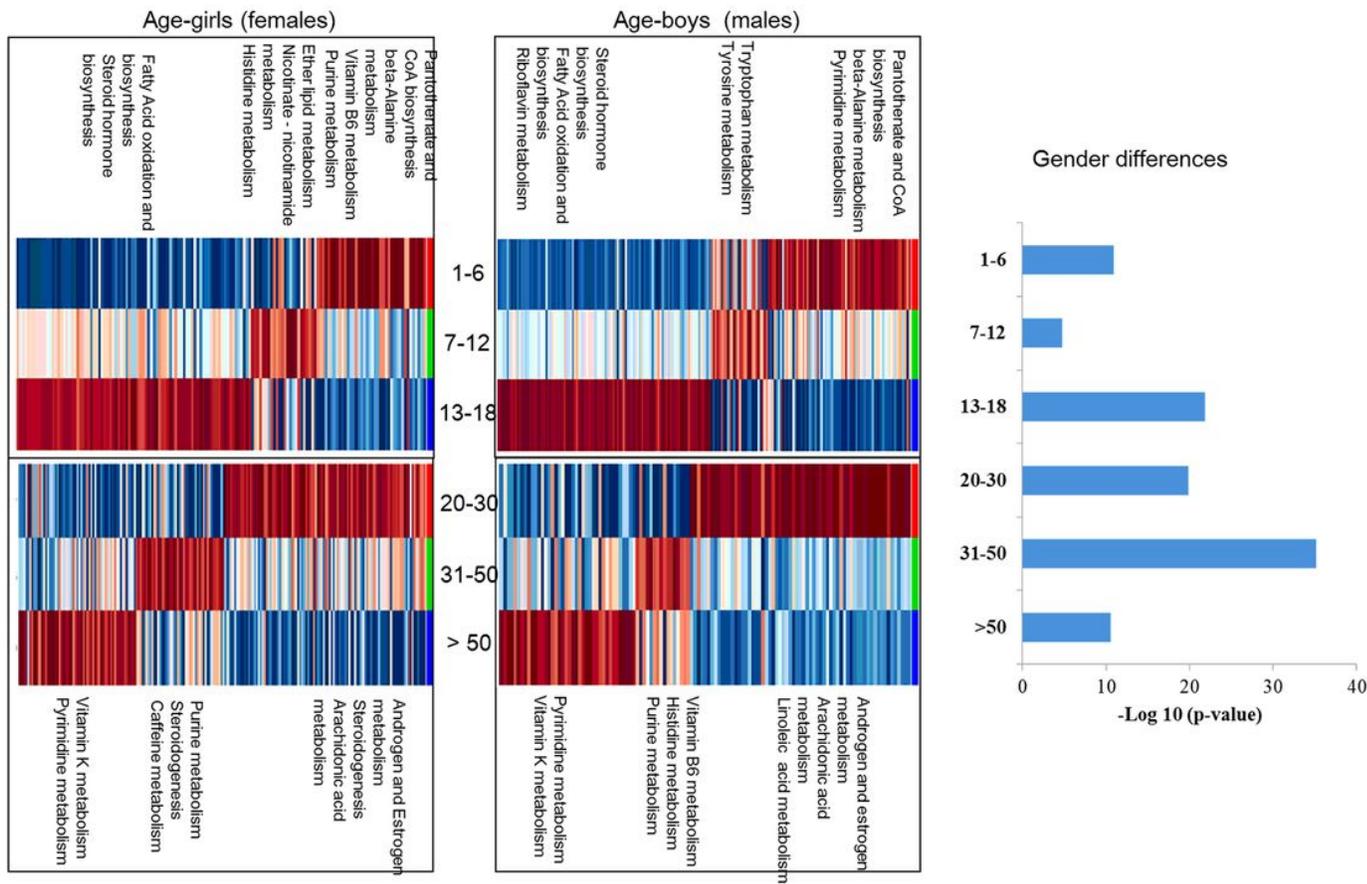
Representative gender-differential metabolites and metabolism pathways in children and adults. a. polar plot for fold change (Female/Male) and p value of gender-dependent metabolites in children. The length of the column indicates the fold change, and the color indicates the p value. b. Polar plot for fold change (Female/Male) and p value of gender differential metabolites in adults. c. Fold change value of 50 common gender-dependent metabolites in children and adults. Values inside the green circle represent

metabolites higher in males (boys), and values outside the green circle represent metabolites higher in females (girls). d. Gender-dependent metabolism pathways in children. Metabolites involved in some pathway were connected using lines. e. Gender-dependent metabolism pathways in adults. Metabolites involved in some pathway were connected using lines.



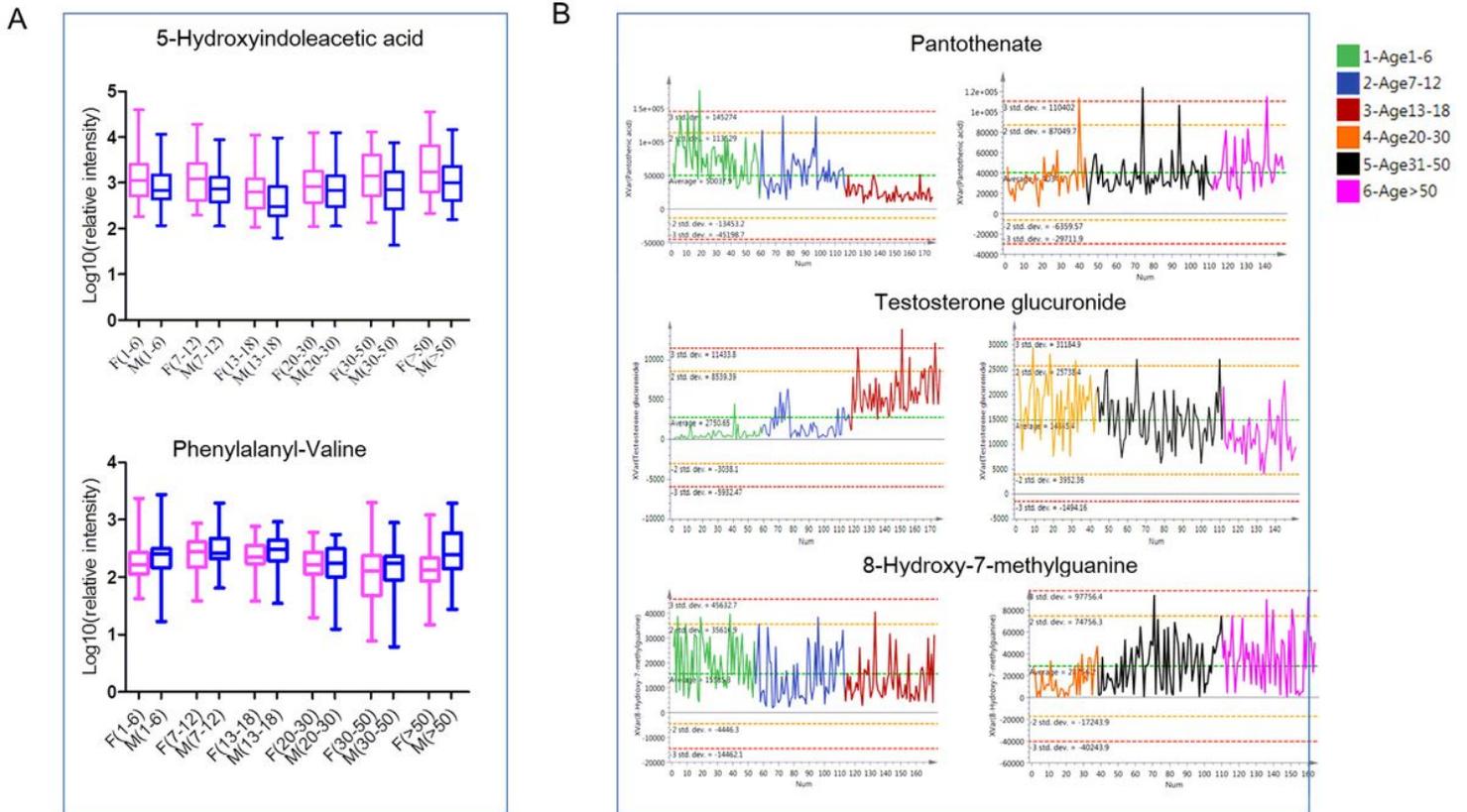
**Figure 3**

Urine metabolomics variation with age in children. a. PLS-DA score plot of urine metabolomics of different ages in boys. b. PLS-DA score plot of urine metabolomics of different ages in girls. c. Change trends of the first and second principal components of PLS-DA in children; these two principal components contribute most to the age separation in children.



**Figure 4**

Metabolic characteristics for different gender and age stages. Age-dependent metabolic pathways were enriched based on metabolites with the highest level in each age group. The KEGG database was the background pathway database. The significance of gender differences was evaluated using the p value of OPLS-DA models. The cutoff for the p value was 0.05.



**Figure 5**

Key metabolite variation with gender and age. a. Relative intensity of gender-differential metabolites, 5-hydroxyindoleacetic acid and phenylalanyl-valine in male (boy) and female (girl) during different age stage. b. Relative intensity change of age differential metabolites, pantothenate, testosterone glucuronide and 8-hydroxy-7-methylguanaine with age in males.

## Supplementary Files

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

- [supplementarymethod.docx](#)
- [Supplementaryfigures.docx](#)
- [SupplementaryTables.docx](#)