

# Metabolomics analysis of gut metabolites in patients with colorectal polyps and in healthy individuals

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**Abstract.** The aim of the present study was to characterize the metabolomic signatures associated with colorectal polyps (CPs) in the gut. A metabolomics analysis was conducted on fecal samples collected from patients diagnosed with CPs as well as from healthy participants. A total of 60 participants were selected for analysis, including 30 patients diagnosed with CPs (CP group) and 30 healthy individuals serving as controls [healthy control (HC) group]. Fecal metabolomes were analyzed using ultra-high performance liquid chromatography and mass spectrometry. Metabolomics analyses revealed a higher abundance of phosphatidylcholine (PC; 16:0/18:2) in the CP group compared with that in the HC group. By contrast, 6-keto-decanoylcarnitine, trimethadione, aalsolinol-1-carboxylic acid, histidyl-proline, norethindrone, gibberellin A12 aldehyde, 6-isopentenyladenine-9- $\beta$ -D-glucopyranoside, prostaglandin E2, yucalexin B5 and 5,6-dihydroxyprostaglandin F1a were less abundant in the CP group. Moreover, the CP group showed significant Kyoto Encyclopedia of Genes and Genomes pathway alterations compared with the HC group, involving 'neuroactive ligand-receptor interaction', 'aminoacyl-tRNA biosynthesis', 'central carbon metabolism in cancer', and 'phenylalanine, tyrosine and tryptophan biosynthesis'. Notably, PC (16:0/18:2) and prostaglandin A1 could predict CPs, with an area under

the curve of 1. The current study showed that fecal metabolites can be used for non-invasive diagnosis of CPs. Moreover, the findings of the present study suggested significant involvement of the aminoacyl-tRNA and aromatic amino acid metabolomic pathways in the development of CPs.

## Introduction

Colorectal cancer (CRC) is a major global public health concern, responsible for high rates of both mortality and morbidity (1). Colorectal polyps (CPs) are widely recognized as the predominant precursors of CRC (2), due to their propensity to undergo malignant transformation (3). Modern medical research has confirmed several risk factors associated with CPs, including aging, high protein consumption (particularly red meat), a diet rich in fats and deficient in fiber, smoking and excessive alcohol consumption (4,5).

Notably, the intestinal microbiota has gained recognition as an important contributor that mechanistically links various risk factors to the development of CRC (6-8). More specifically, the intestinal microbiota and its metabolites have been demonstrated to induce epigenetic modifications in host cells, with these metabolites playing an important role as signaling molecules in intercellular communication (9). *Fusobacterium nucleatum*, a commonly found microorganism prevalent in CRC, has been demonstrated to increase gene methylation levels and induce microsatellite instability (10,11). Furthermore, trimethylamine is primarily produced by *Escherichia coli* and induces DNA methylation, which is closely linked to CRC (12). *Bilophila wadsworthia* and *Pyramidobacter* spp. are additional examples of microorganisms enriched in CRC, which have been reported to promote carcinogenesis through the production of genotoxic hydrogen sulfide within the gastrointestinal tract (13-15). Conversely, specific gut bacteria including *Faecalibacterium*, *Bifidobacterium*, *Roseburia*, *Lactobacillus* and *Eubacterium* can ferment dietary fibers into short-chain fatty acids (SCFAs). These SCFAs possess gut-protective properties and are inversely correlated with CRC; SCFAs such as acetate, propionate and butyrate provide protection against CRC by regulating gut inflammation and the immune system through multiple mechanisms (15). Moreover, both butyrate and acetate can function as histone deacetylase

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inhibitors, exerting an influence on the epigenetic alterations that regulate the development of CRC (16).

Although colonoscopy is still considered the standard procedure for diagnosing CRC, its invasive nature and high cost limits its use in widespread screening (17). The progress in metabolomic profiling technology has enabled several studies to showcase the potential of fecal metabolites in noninvasive CRC diagnosis (18,19). Our previous study revealed a structural imbalance in the gut microbiota composition of patients with CPs compared with that in healthy individuals, characterized by decreased beneficial bacteria and increased harmful species (20). However, the signatures of fecal metabolites in patients with CPs and healthy individuals have yet to be fully understood.

In the present study, the gut metabolome was profiled in patients diagnosed with CPs and was compared with that of healthy individuals. The objective was to define fecal metabolic profiles that could differentiate patients with CPs from healthy individuals.

## Materials and methods

**Participants.** The study cohort used in the present study included patients described in our previous study (20). The present study involved conducting untargeted metabolomics analysis on fecal samples obtained from patients with CPs and healthy individuals at the Shanghai Jiading District Hospital of Chinese Medicine (Shanghai, China) between March and August 2024. All participants were enrolled consecutively upon providing written informed consent. Patients undergoing colonoscopy and diagnosed with CPs and healthy individuals without CPs were included. A total of 30 patients with CPs were classified as the CP group, whereas 30 healthy individuals were classified as the healthy control (HC) group. Participants in each group were matched based on age, sex and body weight during enrollment. The CP group was selected according to the following inclusion criteria: Aged >18 years, and a diagnosis of CP confirmed by colonoscopy and histopathology. The exclusion criteria for the CP group were as follows: i) Use of antibiotics or probiotics within the past 2 months; ii) a previous history of CRC. The inclusion criteria of the HC group was: Aged >18 years without CP confirmed by colonoscopy examination. The exclusion criteria for the HC group were as follows: i) Presence of any underlying diseases, including autoimmune diseases such as ankylosing spondylitis and systemic lupus erythematosus, infectious diseases, cachexia, organ failure, respiratory diseases and cardiovascular diseases; ii) history of surgery or chronic drug use; iii) use of antibiotics or probiotics within the past 2 months.

**Sample processing.** Fecal samples were collected by the participants after enrolling in the study. Immediately after collection, the samples were placed on dry ice and stored at -80°C until further use. Subsequently, the samples were sent to Shanghai Bioprofile Biotechnology Co., Ltd. for untargeted metabolomics analysis. These samples were mixed with 400  $\mu$ l cold (4°C) methanol acetonitrile (v/v; 4:1) and homogenized with a tissue crusher. The mixture was then sonicated (53 kHz) for 20 min in an ice bath. Following this, the mixture was incubated at -20°C for 1 h, and centrifuged at 4°C for 20 min

at 16,000 x g. Next, the supernatants were harvested and were vacuum dried.

**Ultra-high performance liquid chromatography (UHPLC)-mass spectrometry (MS)/MS analysis.** UHPLC (LC-30AD; Shimadzu Corporation) coupled with Q-Exactive™ Plus (Thermo Fisher Scientific, Inc.) was used for metabolomics profiling analysis.

Samples were placed in a 4°C autosampler and underwent liquid chromatography separation using an ACQUITY UPLC® HSS T3 column (2.1x100 mm; 1.8  $\mu$ m; Waters Corporation). The injection volume was 4  $\mu$ l and the column temperature was 40°C. The flow rate was set at 0.3 ml/min, and the mobile phase consisted of two components: i) A (0.1% formic acid in water); and ii) B (0.1% formic acid in acetonitrile). The gradient started at 0% buffer B for 2 min, increased linearly to 48% over 4 min, was further increased to 100% over 4 min and held for 2 min, then decreased to 0% buffer B within 0.1 min, followed by a 3 min re-equilibration period.

Electrospray ionization (ESI) in both positive and negative modes was used for separate acquisition of MS data. The heated-ESI source was configured with the following conditions: i) Spray voltage at 3.8 kV (positive) and 3.2 kV (negative); ii) capillary temperature at 320°C; iii) sheath gas (nitrogen) flow at 30 arbitrary units (AU) with temperature at 300°C and with nebulizer pressure at 100 pounds per square inch; iv) aux gas flow at 5 AU; v) probe heater temperature at 350°C; and vi) S-Lens radio frequency level at 50. The instrument was set to acquire full MS data over the m/z range of 70-1,050 Da. The full MS scans were performed at a resolution of 70,000 at m/z 200 and 17,500 at m/z 200 for MS/MS scan. The maximum injection time was set at 100 msec for MS and 50 msec for MS/MS. The isolation window for MS/MS was set to 2 m/z and the normalized collision energy (stepped) was set to 20, 30 and 40 V for fragmentation.

**Data preprocessing and filtering.** The raw MS data underwent processing using MS-DIAL (version 4.9) (21) to align peaks, correct retention time and extract peak areas. Metabolites were identified based on accurate mass (with a mass tolerance of <10 parts per million) and MS/MS data (with a mass tolerance of <0.01 Da) through matching against The Human Metabolome Database (<https://hmdb.ca/>), MassBank (<https://massbank.eu/MassBank>), Global Natural Product Social Molecular Networking (<https://gnps.ucsd.edu>) library databases and Baipu Metabolite Standard Library (in-house database of Shanghai Bioprofile Biotechnology Co., Ltd.). Only variables with >50% non-zero measurement values in at least one group were retained from the extracted-ion features.

**Metabolomics data analysis.** R programming language (v4.0.3) and R packages were used for all multivariate data analyses and modeling (<https://www.r-project.org/>). Metabolomics data were mean-centered using Pareto scaling (22). Models were constructed using principal component analysis (PCA), orthogonal partial least squares (OPLS)-discriminant analysis (DA) and partial least squares (PLS)-DA. All models were evaluated and tested for overfitting using permutation tests. The descriptive performance of the models was assessed based on the cumulative R<sup>2</sup>X (with a

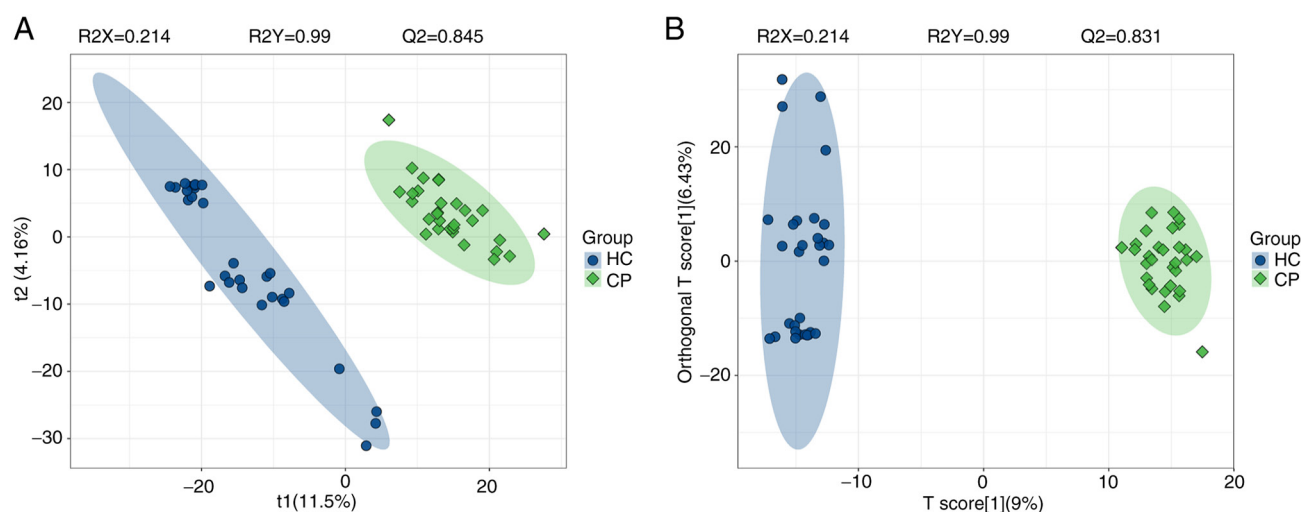


Figure 1. PLS-DA and OPLS-DA demonstrating the separation of patients with CPs and healthy individuals. (A) PLS-DA for CPs and HCs. (B) OPLS-DA analysis for CPs and HCs.  $t_1$  and  $t_2$  denote the first and second components, respectively.  $R^2$  is the percentage of the variance explained by the model.  $R^2X$  and  $R^2Y$  represent the explanatory power of the model for the X matrix and the Y matrix, respectively.  $Q^2$  is a measure of predictive ability. CP, colorectal polyp; HC, healthy control; PLS-DA, partial least squares-discriminant analysis; OPLS-DA, orthogonal partial least squares-discriminant analysis.

perfect model having  $R^2X$ , 1) and  $R^2Y$  (with a perfect model having  $R^2Y$ , 1) values, while their predictive performance was evaluated using cumulative  $Q^2$  (with a perfect model having  $Q^2$ , 1) and a permutation test ( $n=200$ ). A permuted model should be unable to predict the classes: The  $R^2$  and  $Q^2$  values at the Y-axis intercept should be lower than those of the non-permuted model.

Discriminating metabolites that differentiate patients with CPs from HCs were identified utilizing a statistically significant threshold of the variable influence on projection (VIP) values derived from the OPLS-DA model, along with two-tailed unpaired Student's t-test (P-value) applied to the normalized raw data at the univariate analysis level. The VIP score represents the contribution of the variable to discriminating between sample classes, calculated as the weighted sum of squares of PLS weights for each variable. The mean VIP value is 1, and VIP values  $>1$  and  $P<0.05$  are considered significant, indicating a strong discriminatory ability and serving as criteria for biomarker selection. Fold change was determined as the logarithm ratio of the average mass response (area) between two arbitrary classes. Additionally, the identified differential metabolites were subjected to cluster analyses using R package (v4.0.3) and a heatmap was generated. Receiver operating characteristic (ROC) analysis was performed and the area under the curve (AUC) was calculated to assess the predictive capability of potential bacterial markers for distinguishing between the CP and HC groups.

**Statistical analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.** To identify the disrupted biological pathways, KEGG pathway analysis was conducted on the differential metabolite data using the KEGG database (<http://www.kegg.jp>). KEGG enrichment analyses were performed using Fisher's exact test, and multiple testing correction was applied using the false discovery rate method. Pathways enriched at  $P<0.05$  level were considered statistically significant. The correlations between change in metabolites

were estimated with Pearson correlation tests using R package (v4.0.3).

## Results

**Demographic and clinical characteristics of study participants.** No significant differences were found between the CP and HC groups in terms of age, sex, height, weight or BMI, as previously shown (20). The histological types of CPs comprised the following: i) Tubular adenoma (63.3%); ii) tubular adenoma/serrated polyps (3.3%); iii) tubular adenoma/hyperplastic polyps (16.7%); iv) tubular adenoma/hyperplastic polyps/serrated polyps (3.3%); v) serrated adenoma (3.3%); and vi) hyperplastic polyps (10.0%).

**Metabolomics analysis.** After filtering and pre-processing data, a total of 62,209 peaks were normalized and utilized for subsequent analyses. The unsupervised PCA revealed that the quality control study pools formed a distinct cluster at the center of the corresponding study samples (Fig. S1). PLS-DA, a supervised multivariate analysis, effectively distinguished CP and HC samples into well-defined groups using the untargeted data, supported by strong model statistics ( $R^2Y$ , 0.990;  $R^2X$ , 0.214;  $Q^2$ , 0.845; Fig. 1A). Similarly, supervised multivariate analysis using OPLS-DA also discriminated CP and HC groups with good model statistics ( $R^2Y$ , 0.990;  $R^2X$ , 0.214;  $Q^2$ , 0.831; Fig. 1B).

Next, differential abundance analysis of metabolites was performed, and a total of 300 metabolites were identified (Table SI). Fig. 2A displays the top 30 metabolites with statistically significant differences (based on P-value) between the CP and HC groups, with a VIP score  $>1$  and  $P<0.001$  (Table SII). Specifically, the  $\log_2$  fold change, VIP and P-value for prostaglandin A1 were -7.453520969, 2.161393135 and  $4.88 \times 10^{-8}$ , respectively (Table SI). Correlation analysis showed a positive correlation between these identified metabolites (Fig. S2; Table SIII). Specifically, 6-keto-decanoylcarnitine, trimethadione,

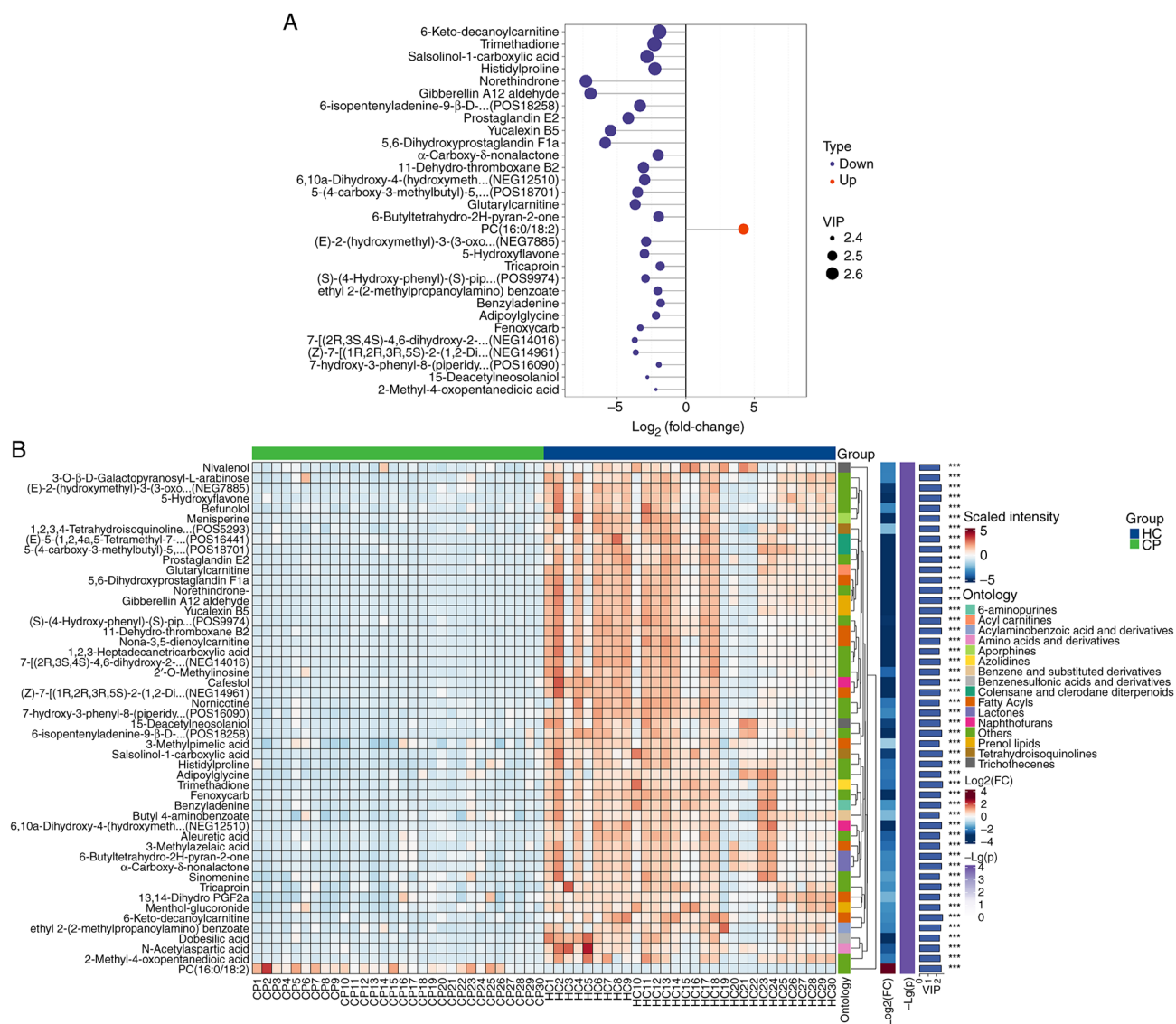


Figure 2. Investigation of gut microbiome-associated fecal metabolites that are significantly altered in patients with CPs by fecal metabolome analyses. (A) Top 30 differentially abundant metabolites identified with VIP >1 and P<0.05. Red circles denote metabolites that are less abundant in the CP group compared with in the HC group; blue circles denote metabolites that are more abundant in the CP group compared with in the HC group. (B) Hierarchical clustering heatmap showing altered metabolites between the CP group (green) and the HC group (blue). The rows display the metabolites, and the columns display the samples. CP, colorectal polyp; HC, healthy control; VIP, variable influence on projection.

salsolinol-1-carboxylic acid, histidyl-proline, norethindrone, gibberellin A12 aldehyde, 6-isopentenyladenine-9-β-D-glucopyranoside, prostaglandin E2, yucalexin B5 and 5,6-dihydroxyprostaglandin F1a were significantly less abundant in the CP group compared with in the HC group (Fig. 2A). By contrast, phosphatidylcholine (PC; 16:0/18:2) exhibited a significantly greater abundance in the CP group compared with in the HC group (Fig. 2A). The hierarchical clustering heatmap (Fig. 2B) visually illustrates the distribution of metabolites that significantly differ between the CP (green) and HC (blue) groups.

KEGG pathway analysis identified multiple perturbed metabolic pathways between the CP and HC groups. The perturbed pathways (Fig. 3) encompassed ‘neuroactive ligand-receptor interaction’, ‘aminoacyl-tRNA biosynthesis’, ‘central carbon metabolism in cancer’, and ‘phenylalanine, tyrosine and tryptophan biosynthesis’. These findings indicated that metabolic

pathways, as well as individual metabolites, may be altered in patients with CPs.

**ROC analysis.** All 300 metabolites with differential abundance underwent ROC analysis. Fig. 4 summarizes the ROC analysis results for six significantly abundant metabolites. 6-Keto-decanoylcarnitine, trimethadione, salsolinol-1-carboxylic acid and 6-isopentenyladenine-9-β-D-glucopyranoside were ranked in the top 30 by VIP score and with AUC values in the top 5% ( $\geq 0.967$ ), indicating accurate performance for the CP status. In addition, PC (16:0/18:2) and prostaglandin A1 achieved an AUC of 1 indicating perfect discrimination.

## Discussion

Accumulating evidence has suggested that the gut microbiota and its metabolites have important implications in the

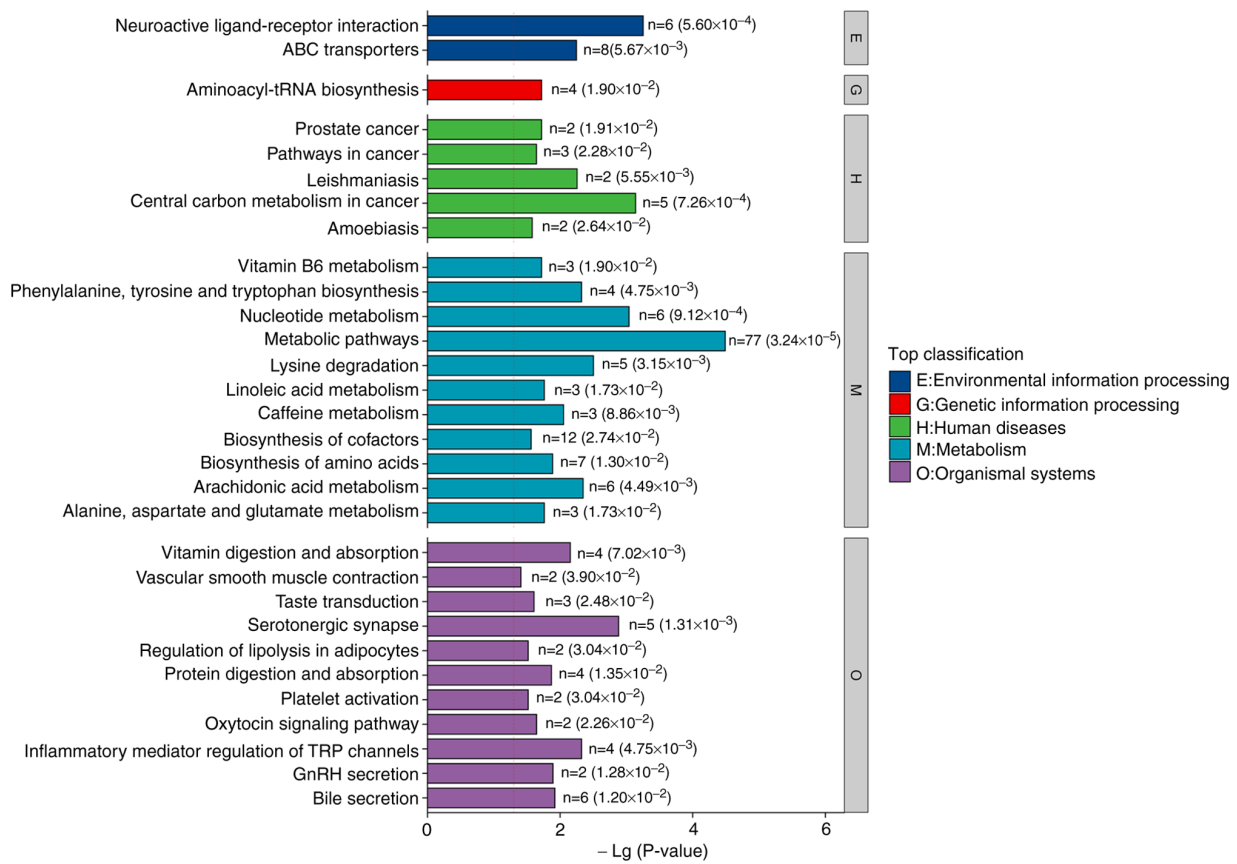


Figure 3. Metabolomic pathway enrichment analysis of significantly altered metabolites between the colorectal polyp group and the healthy control group.

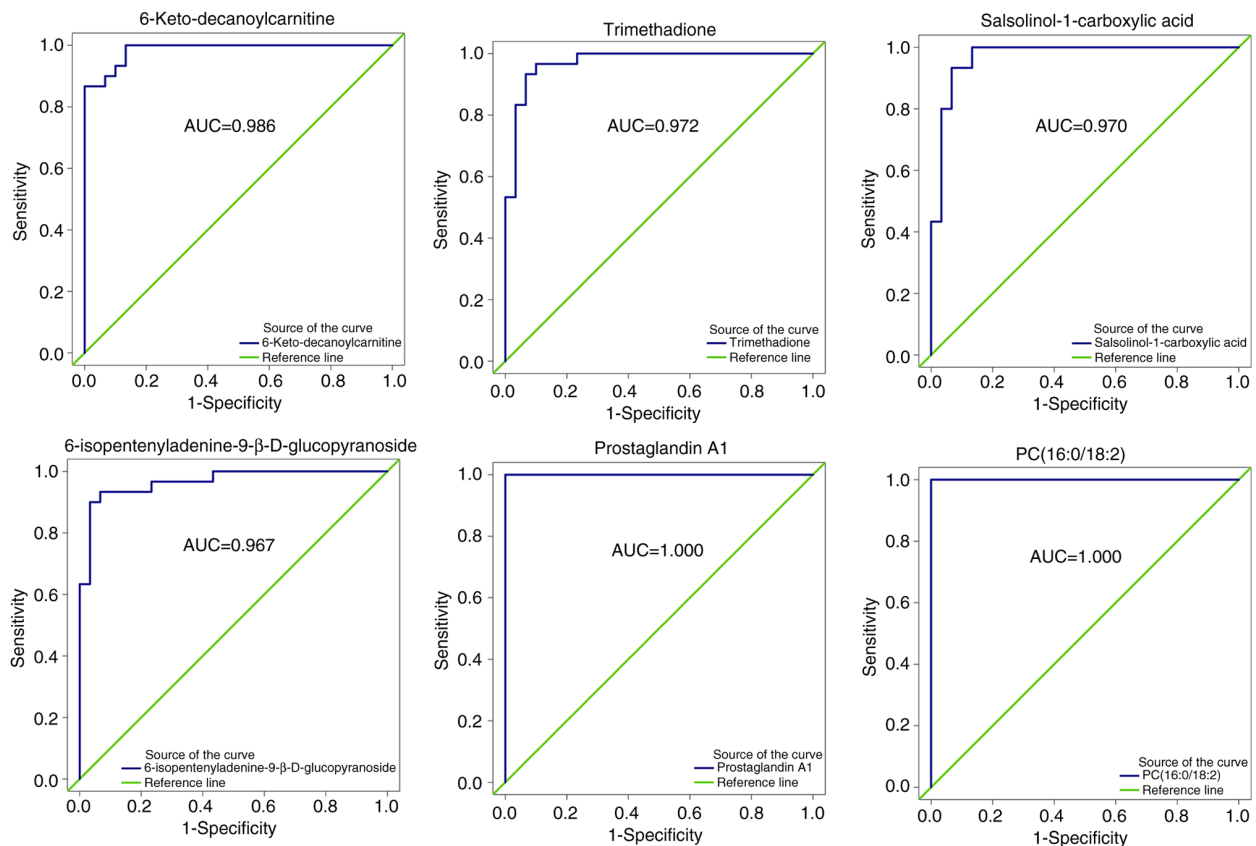


Figure 4. Receiver operating characteristic analysis of the metabolite markers discriminating the colorectal polyp group from the healthy control group. AUC, area under the curve.

development of CRC (23,24). Consequently, exploring the potential of using the gut microbiome and its metabolites as screening tools for early CRC detection holds great promise. Notably, the fecal metabolome reflects the complex interactions among genetic, dietary and environmental factors (25). Thus, conducting metabolomics research using fecal samples may enable the identification of metabolic biomarkers linked to CRC. In the present study, metabolic analysis of fecal samples obtained from patients with CPs and healthy individuals was conducted to identify metabolites associated with pathophysiological changes. These findings highlight the potential of fecal metabolites as non-invasive biomarkers for the early detection of CPs.

The observed increase in PC (16:0/18:2) in the CP group highlighted the role of phospholipids in CRC development; PCs are essential components of cell membranes and are involved in various signaling pathways, such as the CDP-choline pathway and the phosphatidylethanolamine methylation pathway (26). Elevated levels of specific PC species, such as PC (16:0/16:1), have been reported in CRC tissues, suggesting that these lipids may serve as biomarkers for early detection (27). The findings in the present study further supported the notion that dysregulation of lipid metabolism, particularly in the context of PCs, may be a key feature of CPs and could potentially contribute to their progression to malignancy. By contrast, the reduced abundance of metabolites such as 6-keto-decanoylcarnitine, trimethadione and prostaglandin E2 in the CP group suggests a disruption in inflammatory pathways. Prostaglandin E2 in particular is known to play a dual role in cancer biology, acting both as a proinflammatory mediator and a regulator of immune responses (28,29). The decreased levels of prostaglandin E2 in the current study may reflect an altered inflammatory and immune response in the gut microenvironment of patients with CP, which may influence the progression of CPs to CRC.

Pathway enrichment analysis revealed significant alterations in aminoacyl-tRNA biosynthesis and aromatic amino acid metabolism in the CP group. Aminoacyl-tRNA synthetases, which are critical for protein synthesis, have been implicated in various types of cancer, including CRC (30); the dysregulation of these enzymes in patients with CP suggests that translational control mechanisms may be perturbed early in the carcinogenic process. Furthermore, the observed changes in aromatic amino acid metabolism, particularly phenylalanine, tyrosine and tryptophan, are noteworthy. These amino acids are not only essential for protein synthesis, but also serve as precursors for various signaling molecules, including neurotransmitters and immune modulators (31). The dysregulation of these pathways may contribute to the disruption of the gut barrier function and immune homeostasis, both of which are critical factors in CRC development (32). The potential of fecal metabolites as non-invasive tools for the detection of CPs is further supported by the performance of the identified biomarkers in ROC analysis, with AUC values >0.96. Notably, PC (16:0/18:2) and prostaglandin A1 achieved perfect discrimination (AUC, 1), highlighting their potential utility in clinical settings. Nonetheless, a pending further validation study in larger cohorts is necessary to validate the candidate biomarker.

The current study has several limitations. Firstly, the single-center design and relatively small sample size may limit

the generalizability of the findings. Multicenter studies with larger cohorts are needed to validate these results. Secondly, the complex interplay between diet, gut microbiota and host metabolism poses a challenge in distinguishing between host- and microbial-derived metabolites. Future studies incorporating dietary assessments and isotope labeling techniques may elucidate the origins of these metabolites and their role in CP pathogenesis. Furthermore, functional validation is necessary in future studies to confirm the biological relevance of the metabolic alterations identified.

In conclusion, the present study provides compelling evidence that fecal metabolomics profiling can serve as a non-invasive tool for the detection of CPs. The identified metabolic alterations, particularly in lipid and amino acid metabolism, offer new insights into the molecular mechanisms underlying CP development. These findings pave the way for further research into the role of gut metabolites in CRC pathogenesis and their potential as therapeutic targets.

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### Availability of data and materials

The UHPLC-MS/MS data generated in the present study may be found in the National Genomics Data Center repository under BioProject number: PRJCA040951 and accession number: OMIX010385 or at the following URL: [ngdc.cncb.ac.cn/omix/release/OMIX010385](http://ngdc.cncb.ac.cn/omix/release/OMIX010385).

### Authors' contributions

DD, ZG, and YJ conceived and designed the study. DD, YY, HZ, and HS interpreted the analysis results and wrote the manuscript. DD, YY, YX, HW, and LZ collected the samples, and contributed to the acquisition and analysis of data. DD, ZG and YJ confirm the authenticity of all the raw data. All authors revised the manuscript, and read and approved the final manuscript.

### Ethics approval and consent to participate

Ethics approval was obtained from the Ethics Committee of the Shanghai Jiading District Hospital of Chinese Medicine (approval no. 2022-002; Shanghai, China) and written informed consent was obtained from all participants.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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