Metabolomic analysis of gut metabolites in patients with colorectal cancer: Association with disease development and outcome

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Abstract. Colorectal cancer (CRC) is one of the leading global malignancies with low 5-year survival and high mortality rates. Despite extensive research, the precise role of gut metabolites in CRC development and clinical outcomes remains unclear, while its elucidation may aid the development of improved clinical diagnosis and treatment options. In the present study, targeted metabolomic analysis was conducted on fecal samples from 35 patients with CRC, 37 patients with colorectal adenoma and 30 healthy controls (HC) to identify metabolite biomarkers. Using orthogonal partial least squares discriminant analysis, metabolomic features distinguishing the three groups were identified. Receiver operating characteristic (ROC) curve analysis was used to assess diagnostic utility for distinguishing CRC from HC. The association of gut metabolites with survival in patients with CRC was also analyzed by comparing short-term survivors (STS) and long-term survivors (LTS), and the prognostic ability of metabolites was predicted using Cox regression and Kaplan-Meier analysis. The results of the current study showed that the enriched pathways in CRC included 'caffeine metabolism', 'thiamine metabolism', 'phenylalanine, tyrosine and tryptophan biosynthesis' and 'phenylalanine metabolism'. ROC analysis found that 9,10-dihydroxy-12-octadecenoic acid, cholesterol ester (18:2) and lipoxinA4 distinguished CRC from HC. Joint quantification of these three metabolites resulted in an area under the ROC curve of 0.969 in the diagnosis of CRC. The analysis of the current study also showed that the expression of metabolites involved in 'sphingolipid metabolism' was mainly dysregulated in LTS and STS, while N-acetylmannosamine and 2,5-dihydroxybenzaldehyde were associated with better overall survival. In conclusion, the present study provided preliminary insight into the metabolic changes associated with CRC and may have important implications for the development of future diagnostic and treatment strategies.

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Introduction

Colorectal cancer (CRC) is a significant global health concern, accounting for ~10% of all new cancer cases worldwide and ranking as the second leading cause of cancer-related mortality (1). In 2020, there were an estimated 1.9 million new cases of CRC worldwide, leading to ~935,000 deaths globally (2). The majority of CRCs arise from pre-existing colorectal adenomas (CRAs) (3). CRC development often follows the adenoma-carcinoma sequence, a process that can take several years. Therefore, early detection and surgery are critical in controlling this disease. However, the majority of patients present with advanced disease and have a poor prognosis, and even with surgery the recurrence rate remains high. Overall, <20% of patients diagnosed with advanced CRC survive beyond 5 years (4). CRC is thought to be triggered by various factors, including genetic predisposition. For instance, mutations in genes such as APC, TP53 and KRAS have been implicated in increasing the risk of CRC (5). In addition, certain environmental factors, such as smoking,

obesity, sedentary lifestyle and exposure to certain chemicals or toxins, have also been associated with an increased risk of CRC development. For instance, long-term alcohol abuse may cause damage to the intestinal mucosa and increase the risk of colorectal cancer (6). A previous study has shown that the gut microbiota has a significant impact on human health and disease (7). Alterations in the intestinal microecosystem have been identified as a key factor influencing the development of CRC (8). Such alterations may cause ecological dysbiosis, an imbalance in microbial composition that disturbs the host-microbiota interactions and may drive the development of CRC.

In a previous study, the contribution of gut microbiota to the development and progression of CRC was examined using macro-genomic analysis (9). The phyla Bacteroides, Firmicutes and Proteobacteria were found to be abundant in patients with CRC compared with healthy individuals. Certain microorganisms reach a relatively stable abundance or composition when cancer progresses to an advanced stage, supporting their role in cancer progression. Recent evidence also suggests the role of the gut microenvironment in CRC development. For example, alterations in the gut microbiota, such as *Bacteroides fragilis* and *Escherichia coli*, can promote excessive colon cell proliferation and drive cancer development (10). *Fusobacterium nucleatum*, a common organism that is abundant in CRC, has been shown to induce host cell epigenetic modifications and microsatellite instability (11). While fecal coliforms are normally not associated with disease, a previous study has shown a higher abundance of fecal coliforms in stool samples from patients with CRC compared with healthy controls (HC). A higher abundance of fecal coliforms has also been observed in tumor tissue samples and adjacent mucosa of patients with CRC compared with tissues of HC, confirming the association between CRC and fecal coliforms (12). In addition, intestinal bacteria produce a series of metabolites during the reproduction process that can directly or indirectly affect the metabolism of the host (13). For instance, *Escherichia coli* is another microorganism that is abundant in CRC, which has been reported to induce CRC-associated DNA methylation by producing trimethylamine in the intestine, thereby enhancing carcinogenicity (14). Methylphenidate sulfate is another genotoxic metabolite secreted by bacteria that affects cell cycle kinetics and induces DNA damage in colonic epithelial cells (15).

Furthermore, bacterial metabolites are of increasing interest in CRC research. Yachida *et al* (16) compared the abundance of microbial metabolic genes in the feces of HC and patients at different stages of CRC development. In patients with precancerous polyps, an increase in the abundance of genes involved in amino acid and sulfur metabolism and a decrease in the abundance of genes involved in methane metabolism was observed compared to the HC group. Furthermore, patients with CRC also exhibited a higher abundance of amino acid-related genes compared to the HC group. This is consistent with the long-standing hypothesis that a gut microbial environment that favors protein hydrolysis over glycolysis may increase the risk of CRC (17). As bacteria convert dietary intake into metabolic byproducts, diet contributes significantly to the metabolites secreted by bacteria (18). However, to date, the involvement of fecal metabolites in CRC has still not been fully elucidated. Furthermore, data on intestinal metabolites have not been associated with potential clinical benefits in patients with colorectal cancer, to the best of our knowledge. Therefore, elucidating the macro-metabolome of feces may aid in the understanding of CRC development.

The present study conducted a metabolomics analysis on fecal samples using liquid chromatography-mass spectrometry (LC-MS), with an aim to identify differential fecal metabolites among patients in the CRC, CRA, and HC groups. Subsequently, profiling of the gut metabolome of a group of patients with CRC was performed, comparing long-term survivors (LTS) vs. short-term survivors (STS). The objective of the current study was to determine whether certain metabolites were associated with improved survival by analyzing clinical data alongside gut metabolite data.

Materials and methods

Human fecal sample collection. Fecal samples were collected prospectively from patients who underwent colonoscopy and histopathological examination at the Department of Gastroenterology, Tianyou Hospital, Wuhan University of Science and Technology (Wuhan, China), between January and December 2017. The subjects were divided into three groups: i) Patients with colorectal adenocarcinoma (n=35) were classified into the CRC group; ii) patients with CRA (n=37) were classified into the adenoma group; and iii) patients without colorectal pathology (n=30) were used as the HC group. Participants who had taken antibiotics or microecological drugs within 2 months prior to enrollment were excluded, as were subjects with bowel infections, gastrointestinal symptoms, hypertension, heart disease, diabetes or a history of colonoscopy, adjuvant radiotherapy or surgical treatment prior to sampling. The present study was approved by the Ethics Committee of Tianyou Hospital, Wuhan University of Science and Technology (Wuhan, China), and all subjects provided written informed consent. Patients with CRC were followed up, and their survival data were collected until August 2022 during the follow-up period.

Sample preparation. The fecal samples were stored at -80˚C until required and then thawed on ice prior to extraction. A total of 400 μl of methanol was added to 20 mg of each sample, and each sample was homogenized in a blender for 3 min at 25˚C. The homogenized samples were then centrifuged at 7,900 x g for 10 min at 4˚C. The supernatant of each sample was collected and subjected to a second centrifugation at 7,900 x g for 3 min at 4˚C. The supernatant obtained after the second centrifugation was used for ultra-high performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis.

Metabolite analysis by UPLC-MS/MS. The analytical conditions were as follows: UPLC was performed using a Waters ACQUITY UPLC™ HSS T3 C18 column (1.8 μm, 2.1x100.0 mm) by SCIEX Corp., with a solvent system consisting of water (0.04% acetic acid) and acetonitrile (0.04% acetic acid) and a gradient program of 100:0 v/v at 0 min, 5:95 v/v at 10.0 min, 5:95 v/v at 11.0 min, 95:5 v/v at 11.0 min and 95:5 v/v at 15.0 min. The flow rate was 0.00035 l/min,

the temperature was 40˚C, and the injection volume was 5 μl. The effluents were then alternatively connected to an electrospray ionization (ESI)-triple quadrupole-linear ion trap (QTRAP)-MS/MS. Linear ion trap (LIT) and triple quadrupole (QQQ) scans were obtained using an API 4500 QTRAP LC/MS/MS system equipped with an ESI turbo ion-spray interface by SCIEX Corp. The ionisation mode used was both negative and positive, with the other parameters set the same. The ESI source operating parameters were as follows: Ion source, turbojet; source temperature, 550˚C; ion injection voltage, 5,500 V; ion source gas I, gas II and curtain gas were set to 55, 60 and 25.0 psi, respectively; and collision gas was set to high. Instrument tuning and mass calibration were performed in QQQ and LIT modes using 10 and 100 μmol/l polypropylene glycol solutions, respectively. QQQ scans were obtained as multiple reaction monitoring (MRM) experiments, and collision gas (nitrogen) was set to 5 psi. Further optimization was performed for declustering potential and collision energy of individual MRM conversions by determining the initial settings. A specific set of MRM transitions was monitored based on the metabolites eluted within each period.

Quality control (QC) of samples. The first and second order spectra detected by MS were qualitatively analyzed using the Metware database (https://cloud.metware.cn/) and Human Metabolome database (HMDB; https://hmdb.ca/). After obtaining MS analysis data from different samples, the peak areas of all MS peaks were integrated and the peaks of the same metabolites in different samples were corrected using Analyst 1.6.3 software (SCIEX Corp.). The peak area of each chromatographic peak represents the relative content of the corresponding metabolite. For fecal samples, aliquots of each individual sample were combined and extractions were performed as aforementioned. A mixed QC sample was included every 10 test samples during instrumental analysis to monitor the reproducibility of the analysis. The stability of the samples was evaluated by measuring the total ion current (TIC) as part of the QC procedure. The mixed QC samples showed a stable TIC within a specific range, indicating that no decomposition or other adverse reactions occurred during the analysis. The high overlap of the curves for the detection of metabolites' TIC met the QC criteria. The percentage of substances with a coefficient of variation (CV)< the reference value was analyzed using the empirical cumulative distribution function (ECDF). The proportion of substances with a coefficient of CV less than 0.5 was >85%, indicating that the experimental data were relatively stable.

Statistical analysis. Unsupervised multivariate principal component analysis (PCA) was performed on the fecal samples to obtain a preliminary understanding of the overall metabolic differences between the three groups (CRC, CRA and HC) (19). Orthogonal partial least squares discriminant analysis (OPLS-DA) was used to confirm the differentiation of metabolite profiles between the groups (20). The OPLS-DA model was validated using a random permutation test. This involved randomly permuting the sample labels 200 times and recalculating the model's evaluation metrics (21). R2Y, Q2 and R2X are important evaluation metrics used in the validation of the OPLS-DA model. A variable importance in projection (VIP) value ≥1 was used to identify metabolites for further analysis. Univariate analysis of metabolite differences was conducted for each metabolite using Cox proportional hazards models and fold change (FC) values were calculated to determine significance. Metabolites with VIP ≥1 and $|\log_2 FC| > 1$ were considered significantly different. Venn analysis was performed to determine the overlapping differentially abundant metabolites. Participants were stratified into subgroups based on smoking status, alcohol consumption, and red meat intake for stratified analysis. The identified metabolites were annotated using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/) and Metabolite Set Enrichment Analysis (MSEA; https://www.msea.com.cn/). The diagnostic value was assessed using receiver operating characteristic (ROC) curves. Overall survival (OS) was defined as the time from the first diagnosis of CRC to the date of death from any cause, and was used as the clinical endpoint. Patients with no events/death were reviewed at the last follow-up date (August 2022). We stratified CRC patients into two subgroups based on their OS: Those with an OS ≤5 years and those with an OS >5 years. This allowed us to perform a stratified analysis based on the duration of survival in patients with CRC. Univariate Cox regression and Kaplan-Meier analysis were used to predict the prognostic ability of patients with CRC. Patients were categorized into high and low groups based on the median relative abundance of metabolites for Cox regression and Kaplan-Meier analysis. The P-values were obtained using the Wald test for Cox regression and the log-rank test for Kaplan-Meier analysis. The relative abundance data of metabolites were normalized using unit variance scaling. PCA and OP-LSDA analyses were performed using R 3.5.1. Statistical analysis was performed using SPSS 26 (IBM Corp.) and P-values were two-tailed. The differences in baseline data, such as age, gender, area, among the three groups (CRC, CRA and HC), were analyzed using the chi-square test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics. The present study enrolled a total of 35 patients with CRC, 37 patients with CRA and 30 HC. Among the patients with CRC, there were 19 males (54.3%) and 16 females (45.7%), with a median age of 57 years (range, 37-81 years). In the CRA group, there were 23 males (62.2%) and 14 females (37.8%), with a median age of 52 years (range, 30-75 years). In the HC group, there were 12 males (40%) and 18 females (60%), with a median age of 45 years (range, 23-67 years). There were no significant differences in baseline characteristics such as age, gender and area among the three groups. The baseline data are presented in Table I. For the 35 patients with CRC, baseline characteristics and survival information were collected during the follow-up period. Among the patients followed up, 17 individuals had a total survival period of >5 years, accounting for 48.6%. The characteristics of the study cohort and their survival information are summarized in Table II.

QC. To evaluate the repeatability of metabolite extraction and detection, overlapping display analysis of TIC plots was performed on MS/MS data of different QC samples

Table I. Baseline characteristics.

Variable	Number of cases			χ^2-value	P-value
	Colorectal cancer	Colorectal adenoma	Healthy control		
Age, years					
<60	21	28	24	3.657	0.161
≥60	14	9	6		
Sex				3.305	0.192
Male	19	23	12		
Female	16	14	18		
Body mass index, kg/m^2				2.302	0.316
<18.5	6	10	10		
≥18.5	29	27	20		
Area				0.970	0.616
Urban	15	19	12		
Rural	20	18	18		
Marital status				0.208	0.901
No	7	9	7		
Yes	28	28	23		
Smoking				0.113	0.945
Heavy	15	16	14		
Light	20	21	16		
Alcohol consumption				0.148	0.929
Heavy	16	17	15		
Light	19	20	15		
Vegetable intake				5.547	0.062
High	22	13	15		
Low	13	24	15		
Red meat intake				0.805	0.669
High	14	18	15		
Low	21	19	15		
White meat intake				1.848	0.397
High	18	15	17		
Low	17	22	13		
Processed meat intake				1.769	0.413
High	20	16	13		
Low	15	21	17		
Exercise				1.390	0.499
Regular	20	16	15		
Irregular	15	21	15		

(Fig. 1A and B). TIC measures the total intensity of all ions detected at each retention time, and the TICs of all QC samples were compared to ensure their consistency. ECDF analysis revealed that the percentage of substances with CV values <0.5 in QC samples was >85% (Fig. 1C). These results confirmed the reproducibility and stability of the proposed method, indicating that the significant differences observed between the two groups using multivariate statistical analysis were more likely to be caused by genuine metabolite changes rather than technical errors.

A comprehensive targeted metabolomics analysis of the collected stool samples was conducted using a targeted UPLC-MS/MS method, resulting in the detection of 1,641 metabolites. Unsupervised multivariate PCA was utilized to assess trends among all groups in the initial cohort and potential outliers in the data. PCA results demonstrated no clear trend of separation among the CRC group and the other two groups (Fig. 1D). However, the mixed samples used for QC were gathered at one point, further confirming the reliability of the assay.

Table II. Characteristics of patients with colorectal cancer.

Variables	Number of cases (%)
Sex	
Male	19 (54.3)
Female	16 (45.7)
Age, years	
<60	12 (34.3)
≥60	23 (65.7)
CA 19-9, U/ml	
≥27	21 (60.0)
<27	14 (40.0)
CEA, ng/ml	
≥5	20 (57.1)
<5	15 (42.9)
Degree of differentiation	
Low	17 (51.4)
High	18 (48.6)
Lymph node metastasis	
Yes	15 (42.9)
No	20 (57.1)
Distant metastasis	
Yes	11 (31.4)
No	24 (68.6)
Type	
Colon	23 (65.7)
Rectal	12 (34.3)
Overall survival, years	
≤5	18 (51.4)
>5	17 (48.6)
Treatment options	
Surgery and chemotherapy	10 (28.6)
Surgery only	8 (22.9)
Chemotherapy only	12 (34.3)
Palliative care	5 (14.3)

CA 19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen.

OPLS-DA model and validation. To identify metabolites that may significantly contribute to CRC development, two-by-two comparisons were performed among the three groups. Metabolomics analysis was carried out using the OPLS-DA model, which maximizes the separation between samples. OPLS-DA successfully differentiated the CRC vs. HC group, CRC vs. CRA group and CRA vs. HC group (Fig. 2A-C). The discriminatory ability of the model was confirmed by constructing cross-validated OPLS-DA models. The 200-time permutation test revealed Q^2 values of 0.738, 0.728 and 0.699, respectively, indicating that these models were not overfitting (Fig. 2D-F).

Metabolite profiling. The aim of the present study was to identify metabolites that may significantly affect CRC development. To achieve this, metabolomics analysis was carried out and two-by-two comparisons were performed using the OPLS-DA model, the results of which confirmed the maximum separation between specimens. Differential metabolites were discovered using a VIP threshold ≥1 and $|\log_2 FC| > 1$.

The results of the current study showed that when comparing HC with CRC (Table SI), 245 differential metabolites were identified, including 121 upregulated and 124 downregulated metabolites (Fig. 3A). Sphingomyelin (SM; d18:1/22:1) and 2-aminoethylphosphonate were the top two most increased metabolites in patients with CRC compared with the HC group. By contrast, SM (d18:1/19:1), prostaglandin E2, theobromine and ribosyl adenosine were among the metabolites significantly downregulated in patients with CRC.

When comparing CRA with CRC (Table SII), 350 metabolites were found to be differentially produced, while certain metabolites, such as ceramide, carnitine, amino acids and their metabolites and small peptides, were decreased in patients with CRC compared with those with CRA. Certain glycerophospholipids, such as triglyceride (10:0_12:0_14:0), were found to be increased in patients with CRC compared to those with CRA (Fig. 3B). A total of 406 metabolites were found to be significantly altered in patients with CRA compared with those in the HC group (Table SIII; Fig. 3C). Patients with CRA showed an increase in 2-aminoethylphosphonate, 5-hydroxyeicosatetraenoic acid glycochenodeoxycholic acid, phenethylamine and 4-ethoxyphenyl. By contrast, (R)-equol was the most decreased of the identified metabolites in patients with CRA compared with the HC group. Fig. 3D-F illustrates the top 10 upregulated and top 10 downregulated metabolites between each of the two groups compared.

Upon further analysis of the significantly altered metabolites using Venn analysis, the same differential metabolites from these three comparisons were found (Fig. 4A). Details of the 20 metabolites that were significantly changed in all three comparisons are provided in Table SIV. These metabolites may potentially contribute to the progression of colon tumorigenesis, characterizing CRC tumors from a metabolic perspective.

In addition, stratified analysis based on smoking status, alcohol consumption and red meat intake was performed to better understand the association between intestinal metabolites and colon cancer. SM (d18:1/19:1) was found significantly enriched in the heavy smoking subgroup among patients with CRC compared with the HC group. Furthermore, 3-sulfocatechol was significantly elevated in the heavy alcohol consumption subgroup among patients with CRC compared with the HC group. In the high red-meat intake subgroup, significant elevations in multiple saturated and unsaturated fatty acids such as free fatty acid (34:6) and α-carboxy-α-methylbutyrylhydroxamic acid were observed among patients with CRC compared with the HC group (Table SV).

Enrichment analysis for metabolites. To gain insight into the functional roles of significantly altered metabolites in each group, MSEA was performed. Significant differences were observed in several pathways related to metabolism among the three groups analyzed. Specifically, the differential metabolites between the CRC and HC groups were found to be enriched in pathways related to 'caffeine metabolism', 'thiamine metabolism', 'phenylalanine metabolism' and 'phenylalanine, tyrosine

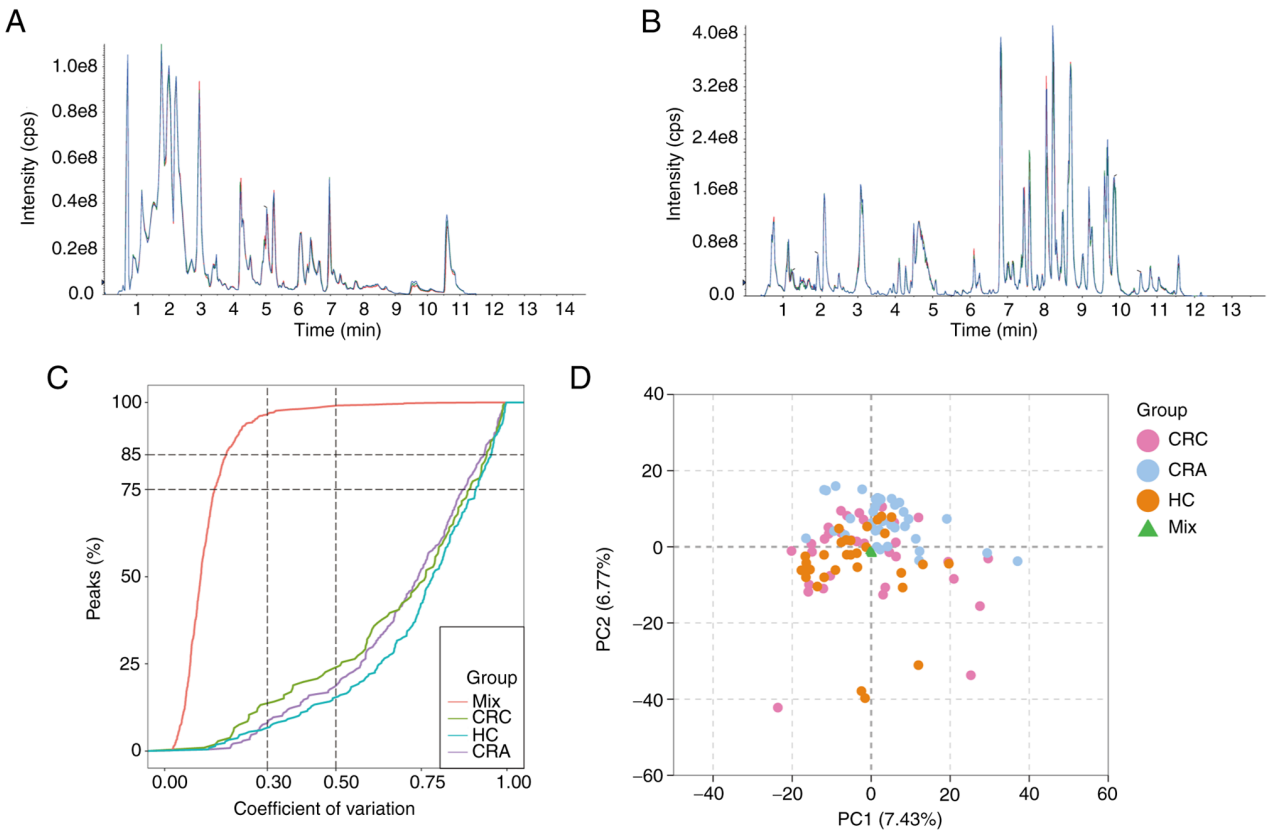

Figure 1. Metabolomic data profiles. (A) Representative total ion chromatogram of mixed fecal samples in positive ion mode using UPLC-MS/MS for quality control. (B) Representative total ion chromatogram of mixed fecal samples in negative ion mode using UPLC-MS/MS for quality control. The Y-axis represents the ion current intensity detected (intensity units in cps). (C) Distribution of coefficient of variation for each group of samples. (D) PC analysis for the HC, CRA and CRC groups. MS, mass spectrometry; PC, principal component; CRC, colorectal cancer; CRA, colorectal adenoma; HC, healthy control; cps, counts per second.

and tryptophan biosynthesis' (Fig. 4B). The significantly enriched metabolites between the CRC and CRA groups were related to 'primary bile acid biosynthesis', 'glycine, serine and threonine metabolism', 'steroid biosynthesis' and 'porphyrin and chlorophyll metabolism' (Fig. 4C). These results suggested that significant changes in metabolic pathway patterns were observed during the ongoing tumor progression.

Diagnostic value of metabolites. To assess the potential diagnostic value of gut-associated metabolites in CRC, ROC analysis was conducted to evaluate the sensitivity, specificity and the area under the curve (AUC), thereby providing a quantitative measurement of the diagnostic performance of differential metabolites. Differential metabolites with high AUC values can effectively differentiate between the CRC and HC groups. Notably, the top three metabolites 9,10-dihydroxy-12-octadecenoic acid, cholesterol ester (18:2) and lipoxinA4 had AUC values of 0.900, 0.891 and 0.860, respectively. Furthermore, the combined quantification of these three metabolites showed an AUC of 0.969 for CRC diagnosis (Fig. 4D). These findings demonstrated the potential of the combined diagnostic group as a non-invasive approach for the early detection of CRC.

Gut metabolite composition for patients stratified by survival. To investigate the role of gut metabolites in modulating clinical outcomes (particularly OS) in patients with CRC, a cohort of 18 STS who survived ≤5 years was compared with 17 LTS who survived >5 years. The differences in gut metabolites were evaluated and significant alterations in metabolites between the two groups were identified. Unsupervised OPLS-DA was conducted to further differentiate the metabolite profiles and screen for key metabolites. As shown in the score chart and validation plot (Fig. 5A and B), the LTS group was clearly separated from the STS group, indicating that metabolic changes were STS- or LTS-specific. Volcano plots and bar graphs showed 45 significantly upregulated and 161 significantly downregulated metabolites in the STS compared with the LTS group and revealed a potential metabolic discrimination of top metabolites between these two groups (Fig. 5C and D). These differential metabolites characterized LTS and STS from a metabolic perspective, particularly the dysregulation of hexosylceramide (d16:1/22:0) (Table SVI).

Based on the differential metabolites between the LTS group and STS group, KEGG pathway enrichment analysis was performed (Fig. 5E), highlighting several pathways, including 'ubiquinone and other terpenoid - quinone biosynthesis', 'steroid biosynthesis', 'sphingolipid signaling pathway', 'rheumatoid arthritis', 'pyrimidine metabolism' and 'purine metabolism'. These pathways may be involved in the survival of patients with CRC. The results suggested that the gut metabolic composition determines the differential enrichment of metabolic functional pathways between the LTS and STS groups, which may ultimately affect patient survival.

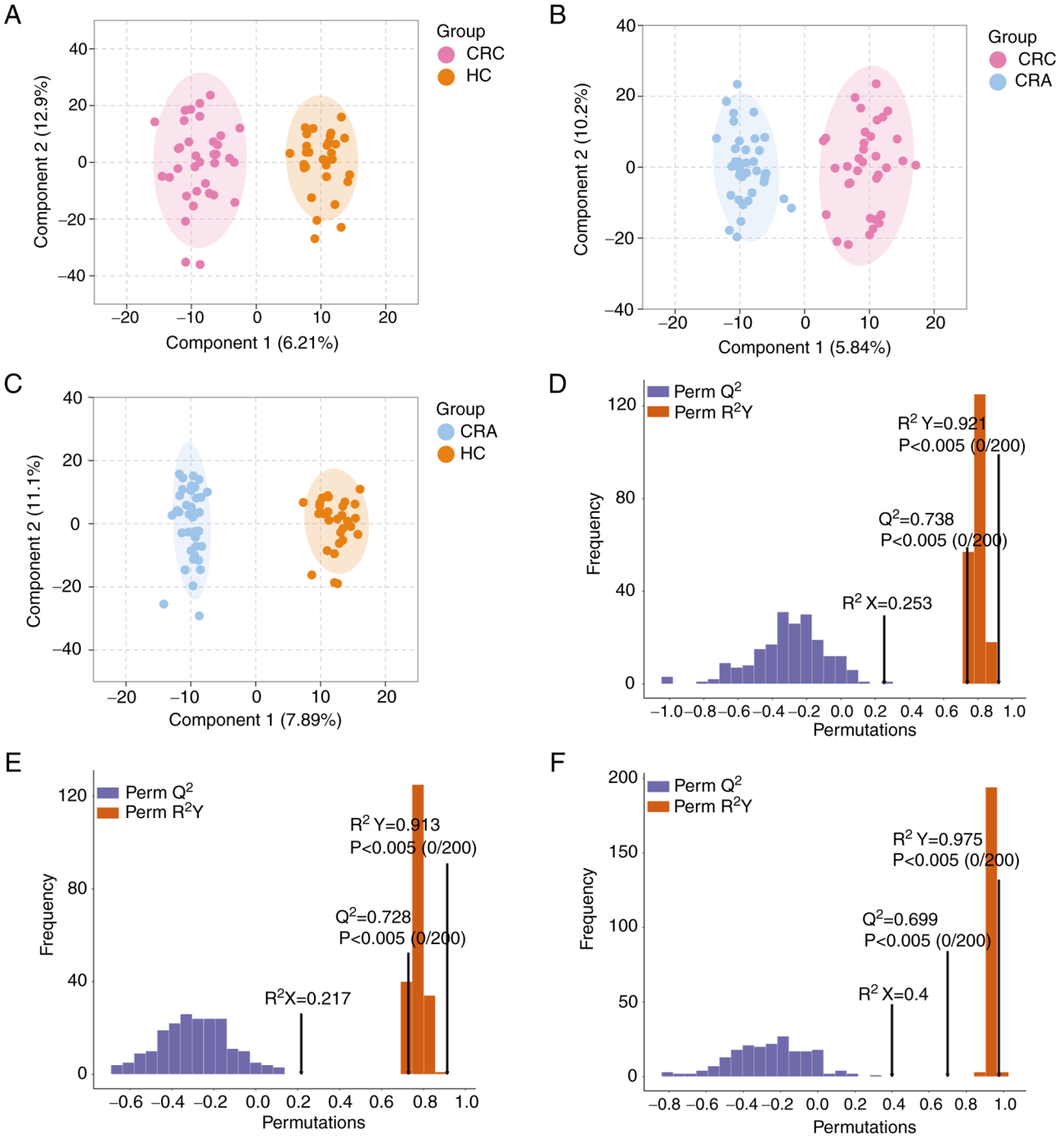

Figure 2. OPLS-DA model and validation. Pairwise OPLS-DA analysis for (A) CRC vs. HC group, (B) CRC vs. CRA group and (C) CRA vs. HC group. OPLS-DA model validation for (D) CRC vs. HC, (E) CRC vs. CRA and (F) CRA vs. HC. R^2X, R^2Y and Q^2 are the prediction parameters of the evaluation model. The closer these three indices are to 1, the more stable and reliable is the model. A value of $Q^2>0.5$ is considered to indicate a valid model. OPLS-DA, orthogonal partial least squares discriminant analysis; CRC, colorectal cancer; CRA, colorectal adenoma; HC, healthy control; perm, permutation.

Prognostic ability of metabolites. Subsequently, the association between metabolites and OS in the CRC cohort was investigated by dividing patients into two groups based on their relative metabolite content. As expected, N-acetylmannosamine [hazard ratio (HR), 0.381; 95% confidence interval (CI), 0.147-0.985] and 2,5-dihydroxybenzaldehyde (HR, 0.336; 95% CI, 0.126-0.895) had significant clinical prognostic value, according to univariate Cox proportional hazards models (Table III). Potential confounders, including gender, age, carbohydrate antigen 19-9 (CA19-9), carcinoembryonic antigen (CEA), differentiated degree, lymph node metastasis, distant metastasis and type, were evaluated, but significant associations with CRC were not found (Table III). Importantly, an increased abundance of N-acetylmannosamine and 2,5-dihydroxybenzaldehyde was associated with better OS (Fig. 6A and B). The findings from the present study suggested that gut metabolites may serve as predictors of survival outcomes in patients with CRC, indicating the potential relevance of the metabolic composition in mediating CRC progression.

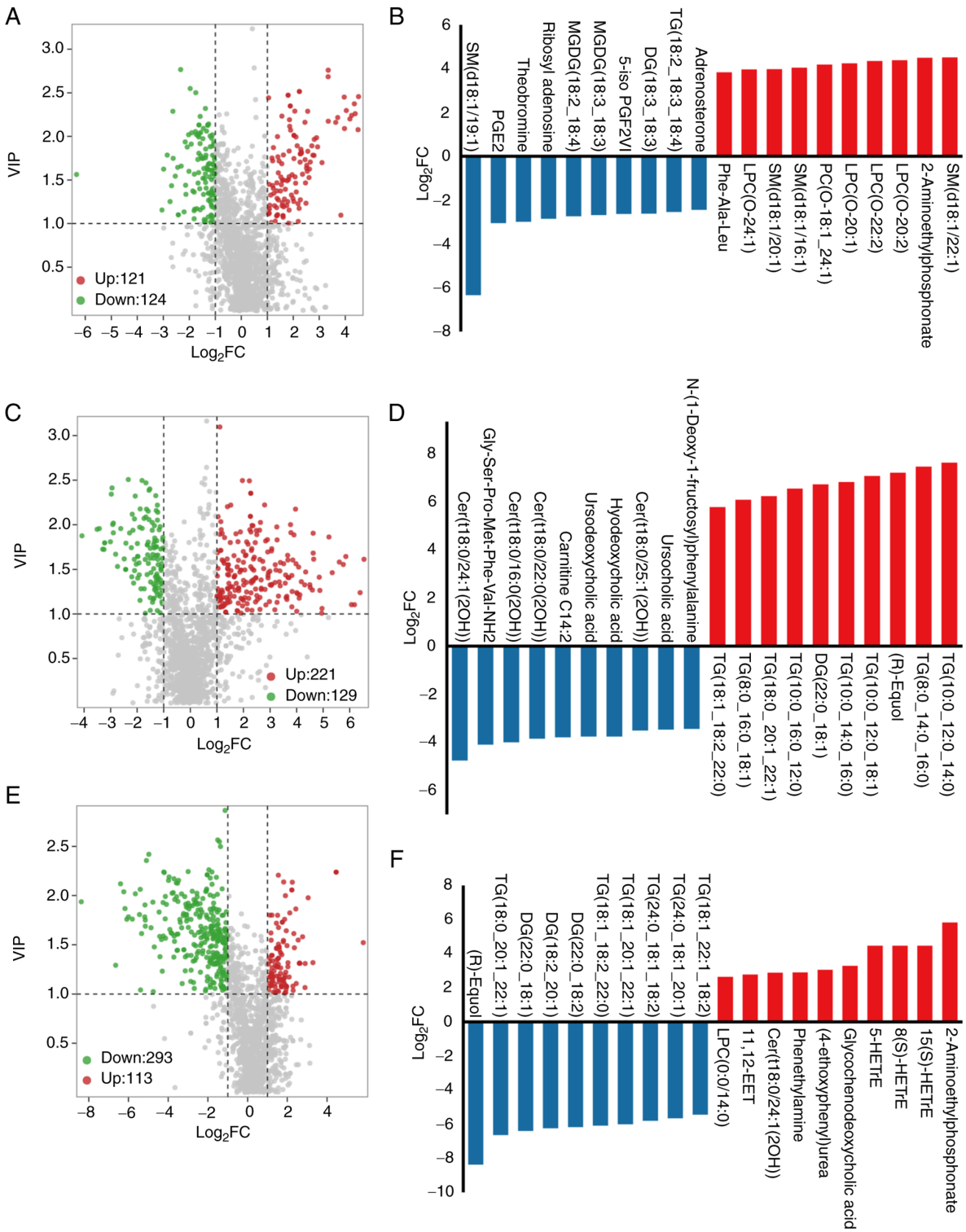

Figure 3. Identification of significantly differentially expressed metabolites. (A) Significantly altered metabolites were determined in the CRC vs. HC group using VIP score and FC value. (B) The top 10 upregulated (red) and top 10 downregulated (blue) metabolites were identified in the CRC vs. HC group. (C) Significantly altered metabolites were determined in the CRC vs. CRA group. (D) The top 10 upregulated and top 10 downregulated metabolites were identified in the CRC vs. CRA group. (E) Significantly altered metabolites were determined in the CRA vs. HC group. (F) The top 10 upregulated and top 10 downregulated metabolites were identified in the CRA vs. HC group. VIP, variable importance in projection; FC, fold change; CRC, colorectal cancer; CRA, colorectal adenoma; HC, healthy control.

Discussion

The high prevalence of CRC is a growing challenge in China, and specific biomarkers are needed, especially in the early stages of the disease, to enable early detection and improve patient outcomes (22). Gut microecology dysregulation has been associated with the development of CRC (23). Proximal feces, located in the colorectal mucosa, can represent structural and metabolic alterations associated with disease progression (24). Elucidating the association between the intestinal metabolome

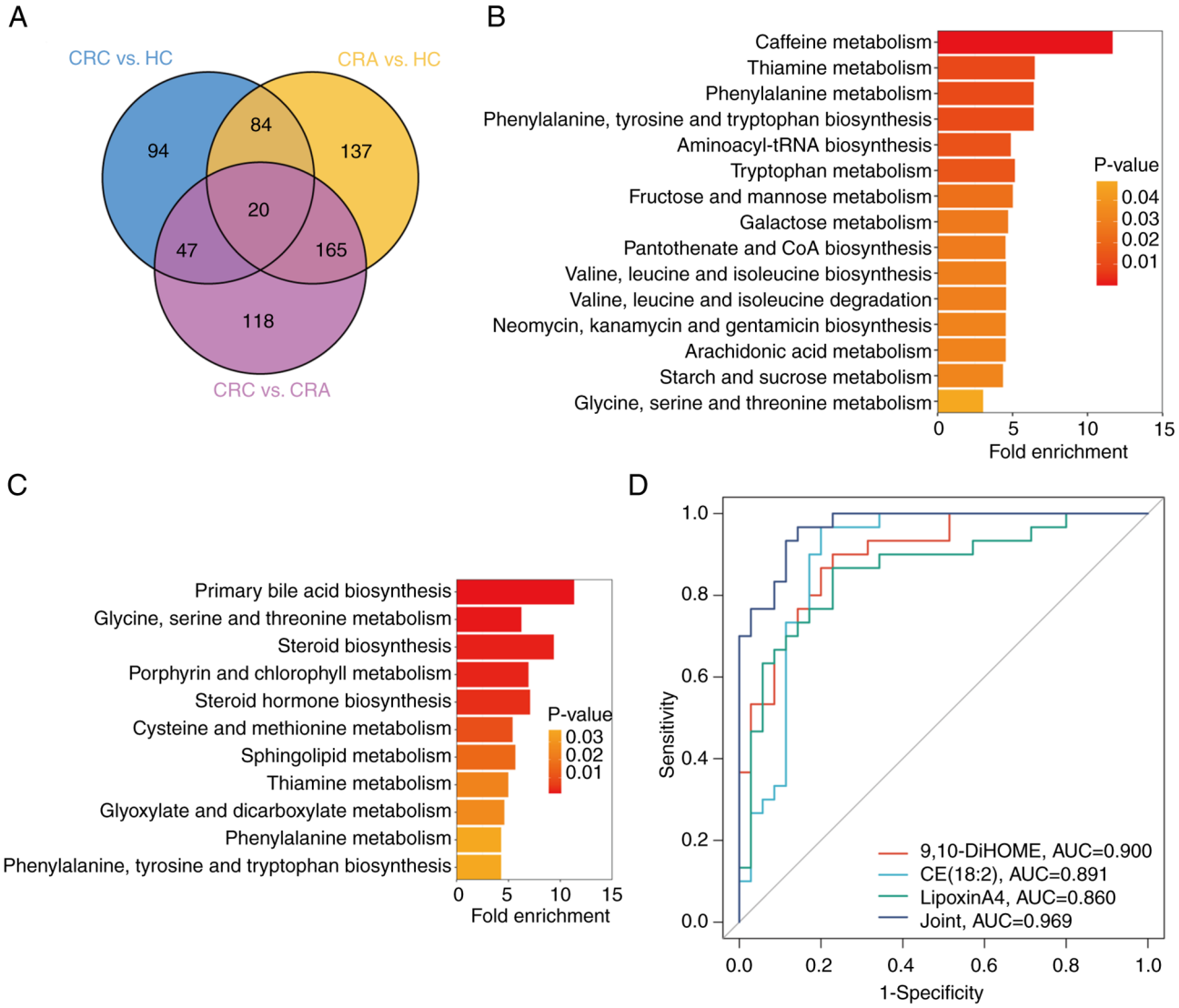

Figure 4. Kyoto Encyclopedia of Genes and Genomes pathway and ROC analysis of differential metabolites. (A) Venn diagram of significantly differentially expressed metabolites in the three groups. Metabolite Set Enrichment Analysis in (B) CRC vs. HC group and (C) CRC vs. CRA group. (D) ROC curves for the diagnosis of gut metabolites between CRC and HC cases. CRC, colorectal cancer; CRA, colorectal adenoma; HC, healthy control; ROC, receiver operating characteristic curve; AUC, area under the ROC curve; 9,10-DiHOME, 9,10-dihydroxy-12-octadecenoic acid, CE, cholesterol ester.

and the molecular characteristics of tumors in individuals with CRC is necessary to understand the role of the metabolome in colorectal carcinogenesis. The aim of the present study was to identify potential biomarkers by analyzing fecal metabolites from patients with CRC and comparing them with those of pre-cancerous patients with CRA and healthy subjects, and to explore the impact of metabolites on the ongoing progression of CRC.

The present study provided evidence that intestinal metabolites are closely associated with CRC progression. As colorectal tumors progress along the adenoma-carcinoma sequence, gut metabolite homeostasis is disrupted, and key metabolic pathways are altered in CRC pathogenesis. Disruption of intestinal metabolic pathways plays an important role in colorectal tumorigenesis (25). Cholesterol metabolites and sphingolipids were the most highly upregulated metabolites and significantly associated with CRC. Furthermore, fat-rich diets and increased amounts of cholesterol are associated with the development of CRC (26). It has been shown that reprogramming of amino acid and lipid metabolism in the tumor microenvironment and metabolic crosstalk between pathogens and host cells contribute to the rapid growth of cancer cells and tumor formation (27). Enhanced lipid synthesis or uptake leads to uncontrolled tumor development (28).

The metabolomic analysis of the present study revealed that several metabolites, such as 2-aminoethylphosphonate, were enriched in the CRA and CRC groups compared with the HC group. 2-Aminoethylphosphonate is a potentially nutritionally active phosphatidyl sphingolipid that significantly increases the content of hydroxy ceramides, which play an important role in the expression of genes related to its biosynthesis-related genes in the skin (29). Amino acids play an important role in several steps of protein biosynthesis, where they maintain redox homeostasis as both electron donors and acceptors and act as energy sources (30). The abundance of amino acid derivatives is essential to promote cancer cell proliferation. They provide crucial building blocks for protein synthesis and contribute to energy generation and nucleotide synthesis (31). By contrast,

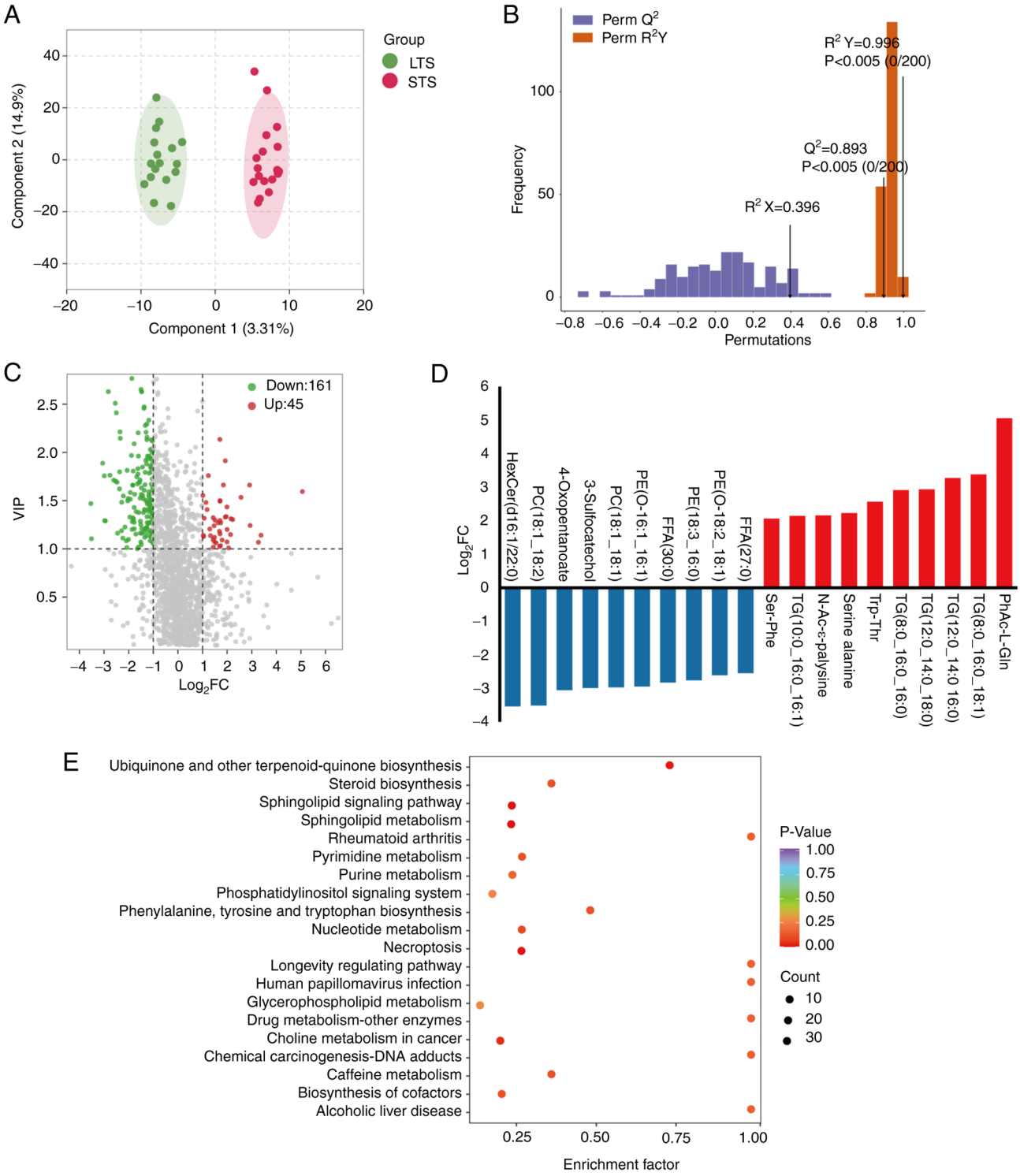

Figure 5. Metabolic profiling in LTS and STS groups. (A) Orthogonal partial least squares discriminant analysis and (B) model validation. (C) Significantly differentially expressed metabolites were identified according to survival subgroup comparisons between LTS and STS and (D) the corresponding top 10 upregulated and top 10 downregulated metabolites are indicated in red and blue, respectively. (E) Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the differential metabolites between LTS and STS. LTS, long-term survivors; STS, short-term survivors; VIP, variable importance in projection; FC, fold change; perm, permutation.

L-carnitine was reported to ameliorate symptoms of cachexia in tumor patients, which may be a point of ambiguity in the function of the complex network of intestinal metabolites (32).

The present study suggested that fecal metabolites have the potential to aid in the non-invasive diagnosis of CRC, as indicated from the set of metabolomic markers that showed the potential to accurately differentiate between patients with CRC and HC. Microbiome alterations associated with CRC have been considered a promising source of diagnostic biomarkers, with several studies focusing on this aspect. For instance, Zeller *et al* (33) provided evidence for the potential of such biomarkers by developing highly predictive and accurate

Table III. Cox regression analysis of overall survival.

Univariate analysis	Hazard ratio (95% confidence interval)	P-value
Sex (female vs. male)	0.643 (0.170-2.438)	0.516
Age (≥60 vs. <60 years)	1.118 (0.355-3.521)	0.849
CA 19-9 (≥27 vs.< 27 U/ml)	0.725 (0.231-2.275)	0.581
CEA (≥5 vs. <5 ng/ml)	2.790 (0.599-13.002)	0.191
Differentiated degree (high vs. low)	1.864 (0.462-7.516)	0.382
Lymph node metastasis (yes vs. no)	0.887 (0.193-4.083)	0.877
Distant metastasis (yes vs. no)	2.500 (0.636-9.828)	0.189
Type (colon vs. rectal)	0.357 (0.105-1.207)	0.097
N-Acetylmannosamine (high vs. low)	0.381 (0.147-0.985)	0.046[a]
2,5-Dihydroxybenzaldehyde (high vs. low)	0.336 (0.126-0.895)	0.029[a]

[a]P<0.05. CA 19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen.

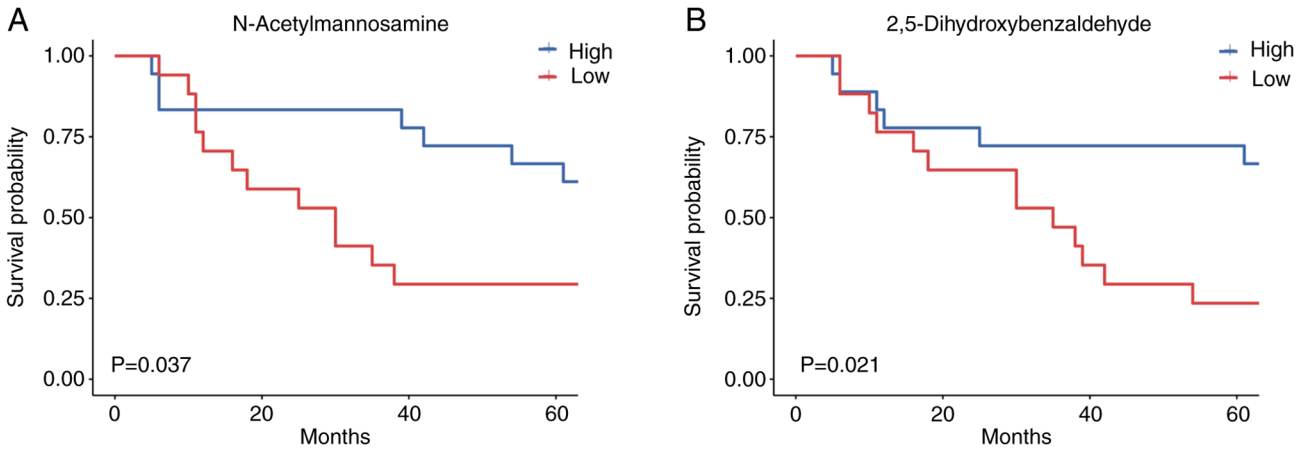

Figure 6. Kaplan-Meier analysis of the association between gut metabolites and survival in patients with colorectal cancer. (A) N-Acetylmannosamine and (B) 2,5-dihydroxybenzaldehyde were related to better overall survival.

models using up to 22 CRC-associated microbial taxa and validating their findings in multiple independent cohorts from different countries. Combining metabolomics and microbiome data can help determine the metabolic alterations that contribute to CRC progression. Previous studies have used such combined data to infer potential metabolic alterations in CRC (34). For example, Yachida *et al* (16) reported an increase in methane metabolism in the gut of CRC patients compared to HC.

We conducted a prospective analysis of metabolomics data in patients with CRC explore the association between gut ecology and survival in patients with cancer. This sets our study apart from others as most studies have been cross-sectional in nature (35,36). Several investigators have analyzed cancer progression and the distribution of bacterial abundance, demonstrating an association between the increase in specific flora and favorable clinical outcomes. For instance, in 43 patients with advanced melanoma, an increase in *Clostridium perfringens* was associated with a favorable response to anti-PD-1 therapy (37). Similarly, the association between *Agrobacterium tumefaciens* and the clinical response to anti-PD-1 therapy was validated in a Japanese cohort of patients with advanced non-small lung cancer (NSCLC), indicating a lack of beneficial effect (38).

The present study utilized comprehensive macro-metabolomic analysis to demonstrate higher levels of phenylacetyl-L-glutamine, triglyceride (8:0_16:0_18:1), tryptophan-threonine and serine-alanine in STS. By contrast, hexosylceramide (d16:1/22:0), phosphatidylcholine (18:1_18:2), 4-oxopentanoate and 3-sulfocatechol were more abundant in the feces of LTS. Sphingolipids are being investigated for their role in carcinogenesis and cancer therapy, and have emerged as a topic of interest for anticancer therapies (39,40). Gut microbial metabolites are believed to associate the gut microbiome with systemic activity. For example, *Agrobacterium tumefaciens* and *Barnesiella intestinihominis* have been shown to promote therapeutic immune modulation induced by cyclophosphamide (41). A crucial metabolic function of the gut microbiome involves the conversion of dietary fiber and mucopolysaccharides ingested by the host into short-chain fatty acids (42). Short-chain fatty acids have been shown to exhibit protective effects in animal

models of colitis and colitis-induced CRC, as well as exerting anti-proliferative effects on cancer cells (43).

A recent study examining plasma tryptophan metabolites in patients with NSCLC treated with immunotherapy found that low levels of 3-hydroxyaminobenzoic acid were significantly associated with longer median progression-free survival (44). Furthermore, patients with pancreatic ductal adenocarcinoma and a rare LTS phenotype had significantly higher tumor bacterial diversity than patients with more typical shorter survival. The diversity of the microbiome was also found to have a significant impact on the survival of tumor patients, supporting a causal role for the gut microbiome in shaping tumor progression (45). However, certain metabolites, including 2-pentanone and tridecane, have been reported to adversely affect therapeutic efficacy in CRC patients (46). The current study identified N-acetylmannosamine and 2,5-dihydroxybenzaldehyde as potential prognostic markers for patients with CRC. While the association between these metabolites and CRC progression remains unclear, the findings of the current study suggest that they may have value in predicting CRC prognosis.

It is important to note that the current study was limited by its small sample size and single-center design, and larger trials are needed to confirm the present results.

In summary, metabolomic data were utilized to track the dynamic changes in metabolites during the progression of CRC. The findings of the present suggest that gut metabolites may play a critical role in CRC development and clinical outcomes; however, more extensive studies are required to validate these observations. These outcomes could have significant implications for the development of future diagnostic and treatment strategies for CRC.

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Availability of data and materials

The raw metabolomics data generated and/or analyzed during the current study are available in the MetaboLights repository under accession no. MTBLS7833 (https://www.ebi.ac.uk/metabolights/MTBLS7833). All other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HLo, QWu and ZX designed the study and enrolled patients. RZ, XH, FY, SJ and QH collected patient information and analyzed the data. DW, HLi, QWa and ZX confirmed the authenticity of the raw data. ZX, RZ, DW, HLi and QWa wrote the manuscript and evaluated the results. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All patients signed written informed consents. The collection and use of samples were approved by the Ethics Committee of Tianyou Hospital, Wuhan University of Science and Technology (Wuhan, China). All aspects of the study complied with The Declaration of Helsinki.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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