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Original research

Alternations in the gut microbiota and metabolome with newly diagnosed unstable angina

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ABSTRACT

Gut microbiota plays an important role in coronary heart disease, but its compositional and functional changes in unstable angina (UA) remain unexplored. We performed metagenomic sequencing of 133 newly diagnosed UA patients and 133 sex- and age-matched controls, and profiled the fecal and plasma metabolomes in thirty case-control pairs. The alpha diversity of gut microbiota was increased in UA patients: the adjusted odds ratios (ORs) per standard deviation increase in Shannon and Simpson indices were 1.30 (95% confidence interval, 1.01−1.70) and 1.36 (1.05−1.81), respectively. Two common species (depleted *Klebsiella pneumoniae* and enriched *Streptococcus parasanguinis*; $P \leq 0.002$) and three rare species (depleted *Weissella confusa*, enriched *Granulicatella adiacens* and *Erysipelotrichaceae bacterium* 6_1_45; $P \leq 0.005$) were associated with UA. The UA-associated gut microbiota was depleted in the pathway of *L*-phenylalanine degradation ($P = 0.001$), primarily contributed by *Klebsiella pneumoniae*. Consistently, we found increased circulating phenylalanine in UA patients (OR = 2.76 [1.17−8.16]). Moreover, *Streptococcus parasanguinis* was negatively correlated with fecal citrulline (Spearman's $r_s = -0.470$, $P = 0.009$), a metabolite depleted in UA patients (OR = 0.26 [0.08−0.63]). These findings are informative to help understand the metabolic connection between gut microbiota and UA.

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Introduction

Coronary heart disease (CHD) is the leading cause of mortality and disability worldwide (GBD 2017 Causes of Death Collaborators, 2018; GBD 2017 DALYs and HALE Collaborators, 2018). Unstable angina (UA), together with myocardial infarction (MI), is termed acute coronary syndrome. UA represents a severe and life-threatening manifestation of CHD, whose pathogenesis, clinical presentation, and prognosis are different from stable angina (Agewall, 2008). In addition to their different extent of coronary atherosclerosis, UA and stable angina are considered as two different diseases because of their differences in plaque vulnerability. UA is caused by the disruption or erosion of an atherosclerotic plaque, resulting in thrombosis and thus myocardial ischemia, while stable angina is caused by stable anatomical atherosclerotic plaque (Anderson et al., 2013; Montalescot et al., 2013). In contrast to stable angina, which has a prognosis of ~5% death or non-fatal MI in four years (Karakas et al., 2017), UA is an emergent medical condition requiring immediate hospitalization and has a much worse prognosis, with >10% of patients experiencing death or non-fatal MI in a year (Benjamin et al., 2019; Puelacher et al., 2019).

The gut microbiota, which is the largest and most complex host-related microbial community, plays an important role in human health

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and disease (Fan and Pedersen, 2021). Several studies have linked the alteration in the composition of gut microbiota to CHD, possibly by modulating inflammation and metabolism in the host (Emoto et al., 2016; Cui et al., 2017; Jie et al., 2017; Zhu et al., 2018; Yoshida et al., 2018; Brown and Hazen, 2018; Liu et al., 2019; Tang et al., 2019). The bacteria or the metabolites produced by the gut microbiota might reach distant locations through blood circulation. It has been reported that two anti-inflammatory species *Bacteroides vulgatus* and *Bacteroides dorei* could effectively suppress pro-inflammatory immune responses and thus attenuate atherosclerotic lesion (Yoshida et al., 2018). Gut microbiota can also metabolize dietary nutrients to various metabolites, such as trimethylamine (TMA) and short-chain fatty acids (SCFAs) (Brown and Hazen, 2018; Tang et al., 2019). TMA is further oxidized in the liver to trimethylamine *N*-oxide (TMAO), which can enhance platelet hyperreactivity, promote atherosclerosis, and ultimately lead to an increased risk of cardiovascular disease (CVD) (Wang et al., 2011; Koeth et al., 2013; Zhu et al., 2016; Schiattarella et al., 2017). On the other hand, circulating SCFAs, especially butyrate produced by *Roseburia* and *Faecalibacterium*, were associated with lower CVD risk (Kurilshikov et al., 2019). These microbiome studies, most of which were based on 16S ribosomal RNA (rRNA) sequencing, have demonstrated the importance of gut microbiota in CVD (Emoto et al., 2016; Cui et al., 2017; Zhu et al., 2018; Yoshida et al., 2018; Liu et al., 2019).

Compared to 16S rRNA sequencing, metagenome-wide shotgun sequencing allows for functional profiling and has a much higher resolution for taxonomic identification (Tang et al., 2019), which is of particular importance given the species-specific functions of bacteria (Zhao, 2013; De Filippis et al., 2016). Furthermore, 16S rRNA sequencing analyses based on the different hypervariable regions often generated inconsistent results (Tang et al., 2019). To date, there has been only one metagenome-wide association study on CHD, in which the majority of the cases were patients with stable angina (Jie et al., 2017). While a recent 16S rRNA sequencing study has shown distinct gut microbial composition across different clinical subtypes of CHD (Liu et al., 2019), the compositional and functional changes of gut microbiota in UA patients remain largely unexplored.

In this study, we performed metagenomic sequencing of 133 newly diagnosed UA cases and 133 age- and sex-matched controls (Fig. S1). We conducted a metagenome-wide association study to identify gut microbial species and functional pathways associated with UA. In addition, we performed untargeted metabolomic profiling in fecal and plasma samples collected from 30 pairs of matched cases and controls randomly selected from the whole sample (Fig. S2). By integrative analysis of metagenomic and metabolomic data, we explored the potential metabolic mechanism of gut microbiota related to UA.

Results

Clinical characteristics and medication history of study participants are presented in Table 1 and Table S1, respectively. Compared to controls, UA cases had higher body mass index (BMI) and higher systolic blood pressure (SBP), and were more likely to have diabetes and take antidiabetic medications. There were no significant differences in the status of smoking, drinking, hypertension, hyperlipidemia, and antihypertensive and lipid-lowering medications between UA cases and controls.

After quality control (QC) for metagenomic sequencing data, we obtained a total of 10.5 billion high-quality reads with an average depth of 39.3 million reads per sample. There was no significant difference in the sequencing depth between UA cases and controls (*t*-test, $P = 0.659$). We constructed a phylogenetic tree with 17, 27, 38, 73, 172, and 480 microbial taxonomies at phylum, class, order, family, genus, and species levels, respectively (Fig. S3). Rarefaction curves of species richness plateaued before the minimum sequencing depth of 14.0 million reads across all samples (Fig. S4), suggesting that our sequencing depth was sufficient to detect most common and rare species present in each sample. When exploring the association of gut microbial diversity with UA, we found that UA was associated with increased Shannon and Simpson indices (Table 2). The odds ratios (ORs) and the corresponding 95% confidence interval (CI) for UA per standard deviation (SD) increment of Shannon and Simpson indices were 1.34 (1.05−1.73) and 1.38 (1.07−1.82), respectively. These associations remained largely unchanged after adjusting for age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes (adjusted OR [95% CI], 1.30 [1.01−1.70] for Shannon index, 1.36 [1.05−1.81] for Simpson index). However, there were no significant changes in either species richness or gene richness, suggesting that the gut microbial differences between UA patients and controls might be primarily driven by the changes in relative abundances. The significant global compositional dissimilarity of gut microbiota between UA cases and controls was further confirmed by permutational multivariate analysis of variance (PERMANOVA) tests on the beta diversity distance matrices (Omnibus $P = 0.009$; Table S2), despite no clear separation between cases and controls in the principal coordinate analysis (PCoA) plots (Fig. S5). We noted that both the weighted and unweighted UniFrac distances yielded significant results ($P = 0.008$ and $P = 0.019$, respectively), indicating that both common and rare species might contribute to the compositional shifts of gut microbiota in UA patients.

We next performed metagenomic association analyses at taxonomic levels from phylum to species to pinpoint UA-related microbial taxa. We found six common taxa significantly associated with UA (false discovery rate [FDR] < 0.2; Table 3), including one family (*Clostridiaceae*), three genera (*Clostridium*, *Enterobacter*, and *Klebsiella*), and two species (*Klebsiella pneumoniae* [*K. pneumoniae*] and *Streptococcus parasanguinis* [*S. parasanguinis*]). In particular, *K. pneumoniae* (adjusted OR = 0.65 [95% CI, 0.48−0.85], FDR = 0.126) and the corresponding genus *Klebsiella* (adjusted OR = 0.63 [95% CI, 0.47−0.83], FDR = 0.112) were significantly depleted in UA cases, while *S. parasanguinis* (adjusted OR = 1.58 [95% CI, 1.21−2.09], FDR = 0.112) was significantly enriched in UA cases. No significant interactions were observed between these two common species (*K. pneumoniae* and *S. parasanguinis*) and known risk factors (Table S3). We identified three rare species significantly associated with UA (FDR <0.2; Table 3), which were *Weissella confusa* (*W. confusa,* adjusted OR = 0.27 [95% CI, 0.09−0.68], FDR = 0.163), *Granulicatella adiacens* (*G. adiacens*, adjusted OR = 25.22 [95% CI, 3.06−3288.70], FDR = 0.054), and

Table 1
Clinical characteristics of participants in the metagenome-wide association study.

Characteristic	Controls ($n = 133$)	UA cases ($n = 133$)	*P*
Age (years)	65.2 ± 9.1	64.6 ± 9.6	0.563
Female, *n* (%)	51 (38.3)	51 (38.3)	1.000
BMI (kg/m^2)	23.9 ± 3.1	25.0 ± 3.1	0.005
SBP (mmHg)	127.7 ± 18.4	140.6 ± 19.7	<0.001
Diastolic blood pressure (mmHg)	78.1 ± 11.3	80.0 ± 11.1	0.168
Smoking, *n* (%)			0.720
Never smoker	77 (57.9)	71 (53.4)	
Former smoker	27 (20.3)	28 (21.1)	
Current smoker	29 (21.8)	34 (25.6)	
Current drinker, *n* (%)	32 (24.1)	28 (21.1)	0.660
Hypertension, *n* (%)	88 (66.2)	96 (72.2)	0.353
Hyperlipidemia, *n* (%)	55 (41.4)	52 (39.1)	0.803
Diabetes, *n* (%)	23 (17.3)	47 (35.3)	0.001

Data are shown as mean ± SD or *n* (%). *P* values were derived using Student's *t*-tests or Chi-squared tests.

Table 2
Association between alpha diversity indices of gut microbiota and UA.

Diversity index	Controls[a]	UA cases[a]	Model 1[c]		Model 2[d]	
	(n = 133)	(n = 133)	OR (95% CI)[b]	P	OR (95% CI)[b]	P
Shannon index	2.25 ± 0.68	2.43 ± 0.56	1.34 (1.05−1.73)	0.022	1.30 (1.01−1.70)	0.047
Simpson index	0.76 ± 0.19	0.81 ± 0.13	1.38 (1.07−1.82)	0.015	1.36 (1.05−1.81)	0.025
Species richness	75.82 ± 18.17	77.13 ± 19.57	1.07 (0.84−1.37)	0.571	1.06 (0.82−1.37)	0.651
Gene richness	196397.79 ± 58871.88	196461.23 ± 56835.52	1.00 (0.79−1.27)	0.993	1.00 (0.78−1.29)	0.990

[a] Data are shown as mean ± SD.
[b] Alpha diversity indices were calculated based on the rarefied data, and were standardized to z scores. ORs (95% CI) for UA per SD increase in alpha diversity indices were estimated by logistic regression models.
[c] Univariate logistic model.
[d] Multivariate logistic model adjusting for age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes.

Erysipelotrichaceae bacterium 6_1_45 (*E. bacterium* 6_1_45, adjusted OR = 3.30 [95% CI, 1.41−8.36], FDR = 0.163). Notably, *G. adiacens* was only present in UA cases (7.5%). In addition, as shown in Table S4, we detected significant interactions between *W. confusa* and age (adjusted OR = 0.04 for people aged <65 versus 0.66 for those aged ≥65; $P_{interaction}$ = 0.019), and between *E. bacterium* 6_1_45 and BMI (adjusted OR = 7.08 for people with BMI <24 versus 1.01 for those with BMI ≥24; $P_{interaction}$ = 0.038), smoking status (adjusted OR = 5.99 for non-smokers versus 0.70 for current smokers; $P_{interaction}$ = 0.021), and hyperlipidemia (adjusted OR = 6.04 for people free of hyperlipidemia versus 0.69 for those with hyperlipidemia; $P_{interaction}$ = 0.028). When excluding participants with hypertension, the associations between the five microbial species with UA had the same trends, but the P values became insignificant due to a substantially reduced sample size (45 controls and 37 UA cases after excluding participants with hypertension; Tables S3 and S4). On the other hand, associations between the five gut microbial species with UA were largely unchanged after excluding participants with diabetes or antidiabetic medications (Tables S3−S5).

To further explore potential metabolic function associated with changes in the gut microbiota, we reconstructed 356 metabolic pathways and identified 5 pathways significantly associated with UA (FDR <0.2; Table 4). Specifically, the relative abundance of one biosynthesis pathway, *L*-histidine biosynthesis, was significantly increased in UA cases. Conversely, the relative abundances of four degradation pathways were significantly decreased in UA cases, including three pathways involved in the degradation of catechol or its derivatives (catechol degradation I, protocatechuate degradation II, and 4-methylcatechol degradation). The other significant degradation pathway was *L*-phenylalanine degradation IV belonging to amino acid degradation. Notably, we found that the overall relative abundances of *L*-phenylalanine degradation IV, catechol degradation I, and protocatechuate degradation II were primarily contributed by *K. pneumoniae* (Fig. 1; Table S6), suggesting an important role of *K. pneumoniae* in the degradation of related metabolites.

We found significant global metabolomic difference between thirty pairs of matched UA cases and controls in plasma samples (P < 0.001, PERMANOVA), but not in fecal samples (P = 0.758, PERMANOVA; Table S7). While the top components from partial least squares-discriminant analysis (PLS-DA) on circulating metabolites and fecal metabolites seemed to separate UA cases and controls well (Fig. S6), the separation was likely due to overfitting, which was verified by leave-one-out cross-validation. A minimum misclassification rate was achieved at 13.3% using top four components of PLS-DA on circulating metabolites (Fig. S7). In contrast, the minimum misclassification rate was as high as 46.7% when using the first component of PLS-DA on fecal metabolites (Fig. S7). We further pinpointed 83 circulating metabolites in significant association with UA (FDR <0.2; Table S8). Consistent with a previous study (Feng et al., 2016), we found significant depletion of free fatty acids and lysophosphatidylcholines in UA patients, probably due to lipid-lowering medications. We found no significant association between individual fecal metabolites and UA.

Finally, we analyzed data of thirty-nine metabolites identified in both fecal and plasma samples, with a focus on exploring potential connections with the two UA-related common gut microbial species, *K. pneumoniae* and *S. parasanguinis* (Fig. 2; Tables S9−S11). Overall, the fecal and circulating metabolites were weakly correlated; only seven out of thirty-nine metabolites had Spearman correlation r_s > 0.2, including three bile acids (Table S9). A group of free fatty acids and three lysophosphatidylcholines were significantly depleted in the plasma of UA patients but not in the stool. In addition, three circulating amino acids, including phenylalanine, tryptophan, and

Table 3
Gut microbial taxa significantly associated with UA.

Taxon	Taxonomic level	OR (95% CI)	P	*FDR*
Common[a]				
Clostridiaceae	Family	1.56 (1.19−2.09)	0.002	0.112
Clostridium	Genus	1.56 (1.19−2.08)	0.002	0.112
Enterobacter	Genus	0.67 (0.50−0.88)	0.004	0.181
Klebsiella	Genus	0.63 (0.47−0.83)	0.002	0.112
Klebsiella pneumoniae	Species	0.65 (0.48−0.85)	0.002	0.126
Streptococcus parasanguinis	Species	1.58 (1.21−2.09)	0.001	0.112
Rare[b]				
Erysipelotrichaceae bacterium 6_1_45	Species	3.30 (1.41−8.36)	0.005	0.163
Granulicatella adiacens	Species	25.22 (3.06−3288.70)	0.001	0.054
Weissella confusa	Species	0.27 (0.09−0.68)	0.004	0.163

[a] ORs (95% CIs) for UA related to common taxa were estimated by multivariate logistic regression models adjusting for sequencing depth (log10 scale), age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes.
[b] Rarefied data of rare species were dichotomized to absence (0) or presence (1). ORs (95% CI) for UA related to rare gut microbial species were estimated by multivariate firth logistic regression models adjusting for age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes. Significance level was defined as FDR < 0.2.

Table 4
Five metabolic pathways of gut microbiota significantly associated with UA.

Pathway	OR (95% CI)[a]	*P*	FDR
Biosynthesis			
L-histidine biosynthesis	1.58 (1.19−2.14)	0.002	0.154
Degradation			
L-phenylalanine degradation IV	0.43 (0.24−0.68)	0.001	0.154
Catechol degradation I	0.61 (0.46−0.81)	0.001	0.154
Protocatechuate degradation II	0.65 (0.49−0.85)	0.002	0.154
4-Methylcatechol degradation	0.65 (0.49−0.85)	0.002	0.154

[a] ORs and 95% CIs for UA in relation to metabolic pathways were estimated by multivariate logistic regression models adjusting for sequencing depth (log10 scale), age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes. Significance level was defined as FDR < 0.2.

ornithine, were significantly enriched in UA cases (adjusted ORs per SD [95% CI]: 2.76 [1.17−8.16], 3.07 [1.40−8.17], and 3.54 [1.43−10.76], respectively). Levels of these three amino acids were also enriched in stool but the effect sizes were attenuated (adjusted ORs per SD [95% CI]: 1.57 [0.82−3.27], 2.21 [1.12−4.99], and 2.07 [1.09−4.57], respectively). The increased phenylalanine level was consistent with the depletion of the *L*-phenylalanine degradation IV pathway, which was primarily contributed by *K. pneumoniae* in the metagenomic analysis. Nevertheless, the correlation between *K. pneumoniae* and phenylalanine was weak in both plasma and stool. Finally, we found that citrulline and carnitine were significantly depleted in the stool of UA patients (adjusted ORs per SD [95% CI]: 0.26 [0.08−0.63] and 0.46 [0.21−0.92], respectively). Citrulline, a known protective metabolite for CVD (Allerton et al., 2018), was also found to be negatively correlated with *S. parasanguinis* in the stool ($r_s = -0.470$, $P = 0.009$), indicating that *S. parasanguinis* might affect UA by modulating the metabolic capability of citrulline.

Discussion

Our study represents the first metagenome-wide association study to systematically investigate the association between gut microbiota and UA. In addition, we identified potential metabolites connecting microbial species and UA by untargeted metabolomic profiling in both fecal and plasma samples from a subset of samples. A key strength of this study relied on the careful study design and sample collection, as well as confounding adjustment. To minimize potential confounding effects resulting from the duration of disease or medications, all of our cases were newly diagnosed UA patients without a prior history of CHD, from whom, fecal and blood samples were collected after hospitalization and before medication use of heparin and contrast agents for coronary interventions. All the controls were recruited from the same city and were selected to match the cases by age and sex. We adjusted for traditional CHD risk factors in all our statistical analyses, with the objective to identify gut microbiota changes that could affect UA independently from traditional risk factors.

We observed an increased alpha diversity (Shannon and Simpson indices) in the gut microbiota of UA patients, which is contradictory to the conventional perception that healthy gut microbiota generally has higher diversity (Turnbaugh et al., 2009; Kostic et al., 2014; Forslund et al., 2015). Consistent with our result, several 16S rRNA studies also reported an increased alpha diversity in severe conditions of CHD, such as UA cases (Liu et al., 2019) and severe CHD cases who had undergone coronary artery bypass graft or percutaneous coronary intervention (Cui et al., 2017). However, no significant difference in the alpha diversity between stable CHD and controls was reported (Jie et al., 2017; Liu et al., 2019). Conversely, another 16S rRNA study reported a decreased alpha diversity in CHD patients, who were diagnosed by coronary angiography but had unknown clinical subtypes (Zhu et al., 2018). These inconsistent results might reflect genuine differences in the gut microbiota between different clinical subtypes of CHD. In addition, UA is an emergent medical condition requiring immediate hospitalization (Benjamin et al., 2019), whereas stable CHD is a chronic condition with a long duration from illness to hospitalization. Thus, the inconsistent changing direction of the diversity of gut microbiota between UA and stable CHD might be attributed to medication treatments or changes in lifestyles (such as smoking, drinking, and diet) during the chronic condition of stable CHD (Ryan et al., 2017). Similarly, studies that reported a decreased diversity in the gut microbiota of patients were mostly cross-sectional studies of chronic diseases such as type 2 diabetes (Forslund et al., 2015) and obesity (Turnbaugh et al., 2009), for which the direction of causality is unclear. In fact, it is controversial to draw conclusions on health effects simply based on the diversity without further examining changes in specific microbial species and the underlying biological mechanism (Shade, 2017).

We identified two common species (*K. pneumoniae* and *S. parasanguinis*) and three rare species (*G. adiacens*, *E. bacterium* 6_1_45, and *W. confusa*) in association with UA. *K. pneumoniae* is a well-known pathogen to cause lung infection if aspirated. Surprisingly, we found that its relative abundance was decreased in UA patients. By mapping metagenomic sequencing data to functional pathways,

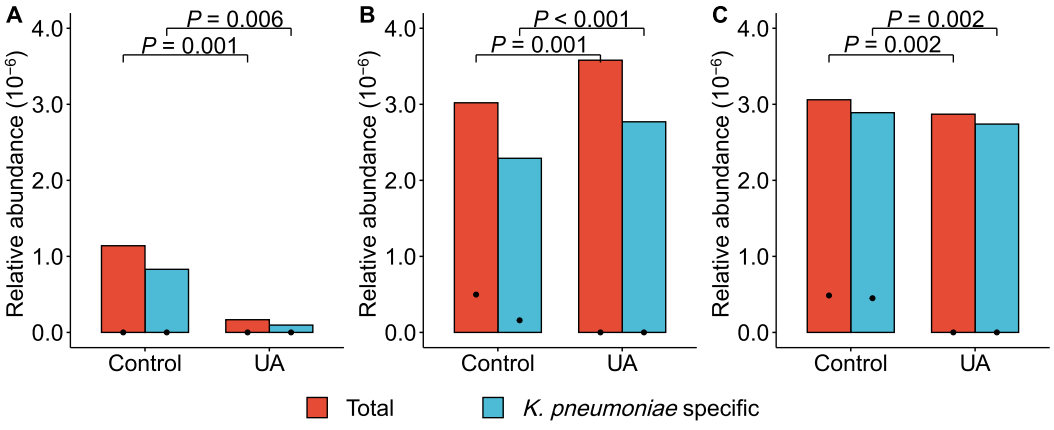

Fig. 1. Relative metagenomic abundances of three UA-related metabolic pathways primarily contributed by *K. pneumonia.* **A**: *L*-phenylalanine degradation IV. **B**: Catechol degradation I. **C**: Protocatechuate degradation II. The height of each bar indicates the average relative abundance. Solid dot represents the median relative abundance. *P* values were calculated using multivariate logistic regression adjusting for sequencing depth (log10 scale), age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes.

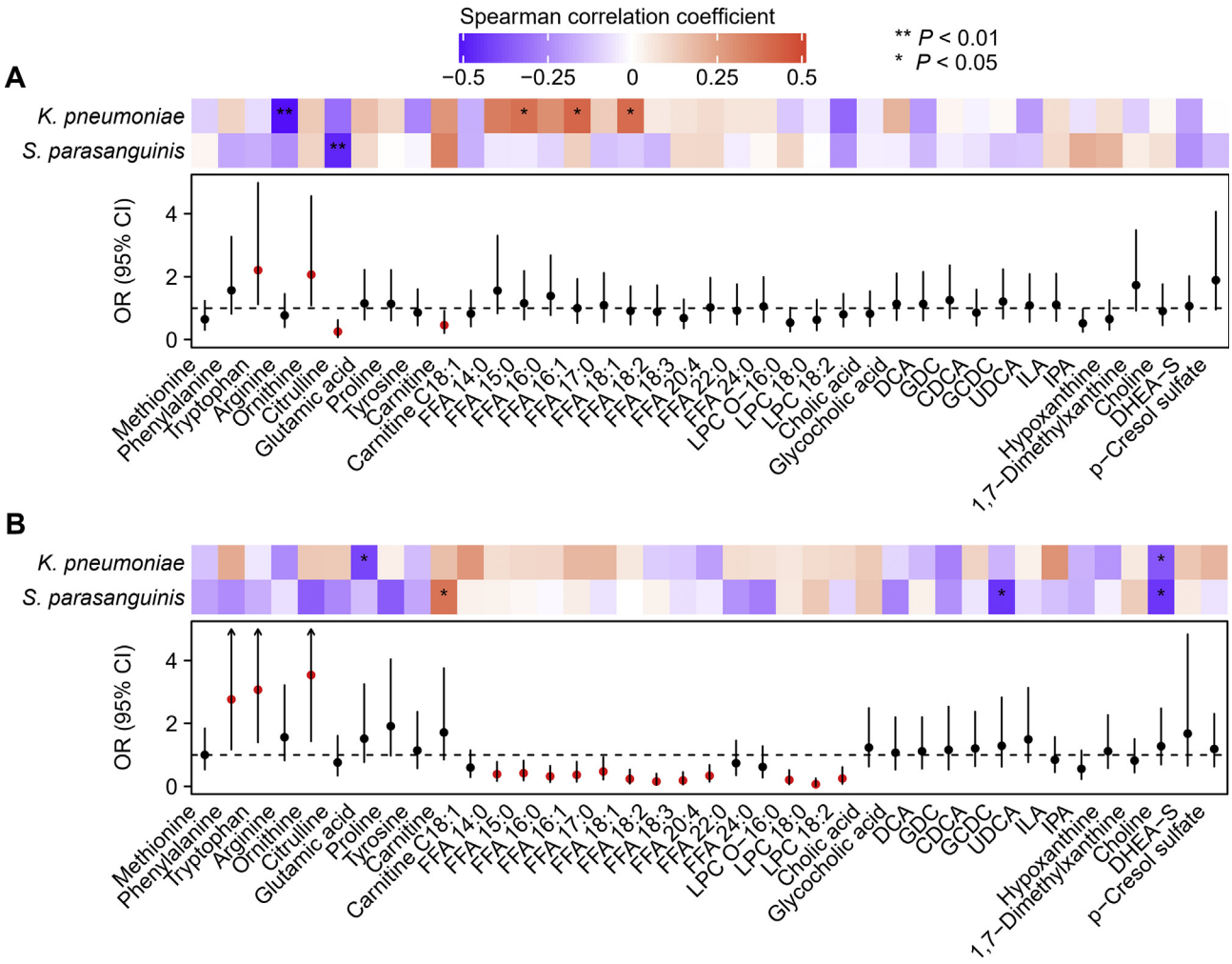

Fig. 2. Metabolomic associations with UA-related common gut microbial species and risk of UA. **A**: Fecal metabolites. **B**: Circulating metabolites. The intensity of each metabolite was log transformed and followed by z-score standardization. Heatmaps show the Spearman correlations between metabolites (column) and UA-related common gut microbial species (row). Each cell is colored by the Spearman correlation coefficient and marked by the corresponding *P* value. Forest plots below the heatmaps show the ORs (95% CI) for UA per SD increase in metabolite levels. ORs (95% CI) were calculated using multivariate logistic regression models adjusting for age, sex, BMI, smoking, hypertension, hyperlipidemia, and diabetes. Each dot represents the point estimate of OR and is colored red if $P < 0.05$. Each segment represents the corresponding 95% CI. The segment with an arrow indicates the upper 95% CI exceeds the maximum axis value. FFA, free fatty acid; LPC, lysophosphatidylcholine; DCA, deoxycholic acid; GDC, glycodeoxycholate; CDCA, chenodeoxycholic acid; GCDC, glycochenodeoxycholate; UDCA, ursodeoxycholic acid; ILA, indole-3-lactic acid; IPA, indole-3-propionic acid; DHEA-S, dehydroepiandrosterone sulfate.

we found that the relative abundance of *L*-phenylalanine degradation IV pathway was primarily contributed by *K. pneumoniae*. Consistent with the depletion of *K. pneumoniae*, we found an increased level of circulating phenylalanine in UA patients. Our results were consistent with a large prospective study of three population-based cohorts, which showed that a higher level of serum phenylalanine was associated with an increased risk of CVD events (hazard ratio per SD [95% CI] = 1.18 [1.12−1.24]) (Wurtz et al., 2015). Conversely, a number of *K. pneumoniae* related metagenomic linkage groups were reported to enrich in patients with stable angina (Jie et al., 2017). Further prospective studies are needed to identify the causative role of *K. pneumoniae* in UA.

The other four significant species, including *S. parasanguinis*, *G. adiacens*, *E. bacterium* 6_1_45, and *W. confusa*, belong to the *Firmicutes* phylum, which was reported to enrich in CVD patients (Emoto et al., 2016; Cui et al., 2017). Our finding that *S. parasanguinis* was enriched in UA patients was consistent with the previous findings of enrichment at higher taxonomic levels of genus (*Streptococcus*) and family (*Streptococcaceae*) in CHD patients (Jie et al., 2017; Liu et al., 2019). Metabolomic analysis suggested that the positive association of *S. parasanguinis* with UA was related to a lower level of fecal citrulline, for which the biological mechanism was reviewed in details by a recent paper (Allerton et al., 2018). Briefly, citrulline is a substrate for arginine recycling, while arginine is the precursor for the synthesis of nitric oxide, which can protect the development and progression of CVD (Allerton et al., 2018). Our findings on *S. parasanguinis* demonstrated the strength of our study on pinpointing the species-level association with UA and connecting the microbial association to established metabolic function responsible for the disease.

Due to the small sample size of our metabolomic data, we were not able to identify significant associations between metabolites and rare species (*G. adiacens*, *E. bacterium* 6_1_45, and *W. confusa*). Through extensive literature review, however, we found that *G. adiacens* and *E. bacterium* 6_1_45 might contribute to UA by inducing inflammation. *G. adiacens* could induce the production of high levels of the pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α (Bhardwaj et al., 2018), and has been linked to a variety of diseases characterized by inflammation, such as infective endocarditis, metabolic syndrome, and obesity (Si et al., 2017; Tellez et al., 2018; Wu et al., 2018). Similarly, *Erysipelotrichaceae*, which is the genus that *Erysipelotrichaceae bacterium*

belongs to, has been linked to several inflammation-related disorders, such as impaired fasting glucose and abnormal lipid metabolism (Kaakoush, 2015; Lippert et al., 2017). These inflammatory diseases are known risk factors for CVD. We reviewed the blood test results on high-sensitivity C-reactive protein (hs-CRP), a biomarker of inflammation, among our UA cases. We found that UA cases positive for *G. adiacens* had a significantly higher level of serum hs-CRP than those without *G. adiacens* and *E. bacterium* 6_1_45 ($P = 0.010$, Wilcoxon rank-sum test; Fig. S8), supporting the pro-inflammatory role of *G. adiacens*. Nevertheless, we observed no significant difference between those with and without *E. bacterium* 6_1_45 ($P = 0.697$, Wilcoxon rank-sum test; Fig. S8). Data on other inflammation biomarkers, if available, might help confirm the pro-inflammatory role of *E. bacterium* 6_1_45. In contrast, we found that the rare species *W. confusa* was protective for UA, which was consistent with the health benefits of *W. confusa* for irritable bowel syndrome and celiac disease (Ponnusamy et al., 2011; Nistal et al., 2012). *W. confusa* is ubiquitous in the gut of healthy individuals across different age groups (Aakko et al., 2015; Pan et al., 2016), and has been served as probiotic bacteria included in the fermentation of a variety of foods and beverages (Sturino, 2018).

Interestingly, we identified elevated tryptophan levels in stool and plasma of UA patients. Tryptophan can be metabolized by the host through kynurenine and serotonin pathways, and the gut microbiota through indole pathway (Metghalchi et al., 2015; Agus et al., 2018). Kynurenic acid, a downstream host metabolite of tryptophan, has been reported to be involved in atherosclerosis (Metghalchi et al., 2015), while indole-3-lactic acid, a gut microbiota-dependent metabolite of tryptophan, has shown to be beneficial to hypertension by reducing TH17 polarization (Wilck et al., 2017). Nevertheless, we did not observe significant association between UA and circulating kynurenine (adjusted OR per SD [95% CI]: 1.10 [0.56−2.24], $P = 0.779$), circulating serotonin (1.38 [0.61−3.58], $P = 0.469$), or the indole derivatives (Fig. S9; Table S12).

Our study has several limitations, including lack of independent replication of the metagenomic associations, small sample size of the metabolomic data, and technical limitation of liquid chromatography-mass spectrometry in profiling small molecule compounds such as TMAO and SCFAs. Future metagenomic association studies of UA and metabolic profiling with a larger sample size and multiple experimental techniques, such as the gas chromatography-mass spectrometry, can lead to a more comprehensive understanding of the metabolic mechanism related to UA. In addition, we did not collect dietary information from the participants, which could be a potential confounding factor. Nevertheless, we expect our cases and controls to have similar dietary patterns because they were recruited from the same city and were matched by age and sex. Finally, we could not clarify the causal relationship between gut microbiota and UA due to the case-control design. Despite these limitations, our findings provide important evidence for future prospective and experimental studies to elucidate the causal effects of the gut microbiota on UA, which might lead to novel prevention and therapeutic strategies for UA given the modifiable nature of gut microbiota.

Material and methods

The Ethics Committee of Tongji Medical College approved this study, and all participants provided written informed consent. Data, analytic methods, and study materials are available upon reasonable requests to the corresponding authors.

Study design and participants

The study population and design are illustrated in Figs. S1 and S2. More details can be found in the *Detailed method* section in the Supplemental Materials. Patients, who were diagnosed with UA for the first time without a prior history of CHD following the international guidelines (Anderson et al., 2013), were consecutively recruited from the Dongfeng General Hospital (Shiyan, Hubei, China). Community-based controls with ≥30 years of age were recruited from the same city. Trained interviewers administered a semi-structured epidemiology questionnaire to collect information on demographic characteristics (age and sex), lifestyles (smoking and drinking), and history of diseases, surgeries, and medications. For medication history, in addition to medications related to hypertension, hyperlipidemia, and diabetes (Table S1), each participant was specifically asked about whether they had taken antibiotics, probiotics or prokinetic agents in the previous six months, including time, frequencies, and types. All participants completed a range of physical (height, weight, and blood pressure) and laboratory examinations (blood lipids and fasting glucose). To reduce the potential impact of medication on UA patients, we collected a fresh fecal sample from the first bowel movement after hospitalization and a fasting blood sample before medication use of heparin and contrast agents for coronary interventions. Plasma samples were immediately prepared by centrifugation after collection of the blood samples. Both fecal and plasma samples were then immediately stored at −80 °C until analysis. Fecal and plasma samples from controls were collected on the day of the medical examination using the same protocols. We excluded participants who had a history of peptic ulcer, inflammatory bowel disease, gastrointestinal tumor or gastrointestinal surgery, and those who took any antibiotics, probiotics or prokinetic agents within three months before fecal sample collection. We also excluded controls who had a history of CHD, stroke, peripheral arterial disease, congenital heart disease, acute infection, cancer, or abnormal electrocardiogram with ST-segment changes. We randomly matched one control to each UA case by age (±3 years) and sex. In total, 133 case-control pairs with sufficient DNA quantities for library preparation were included in the metagenome-wide association study. For 114 out of 133 cases, we retrieved data on the serum hs-CRP from blood test records at hospitalization. In addition, we randomly selected a subset of thirty case-control pairs for untargeted metabolomic profiling.

Assessment of gut microbiota

The compositional and functional (gene and pathway) profiles of gut microbiota were determined by metagenomic shotgun sequencing. Briefly, a frozen aliquot (200 mg) of each fecal sample was processed for pair-end sequencing (2 × 125 base pairs) on Illumina HiSeq 2500 platform. After removing adapter sequences, low-quality reads, and human reads, we profiled the composition and relative abundances of gut microbiota using MetaPhlAn2, which relies on unique clade-specific marker genes identified from ~17,000 reference genomes (~13,500 bacterial and archaeal, ~3500 viral, and ~110 eukaryotic) (Segata et al., 2012). In addition, we used HUMAnN2 to profile functional gene families and reconstruct metabolic pathways (Franzosa et al., 2018). The relative abundances of genes and pathways were profiled for the whole-community ("overall") and each species ("species-level"). Please refer to the *Detailed method* section in the Supplemental Materials for more technical information.

Metabolomic profiling

For metabolomic profiling in fecal and plasma samples, we prepared each test sample by spiking a mixture of multiple internal standards. We also prepared a QC sample by pooling all test samples with equal volume, which was used after each set of ten test samples during the metabolomic profiling by ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS). Data from positive (m/z range 70−1000 for stool and 80−1200 for plasma) and

negative (m/z range 70−1000 for stool and 70−1100 for plasma) ion modes, including retention time, m/z, intensity, and MS/MS fragmentation, were collected and subsequently mapped to an in-house standard database containing ~2000 metabolites for metabolite identification using an in-house software program OSI/SMMS (Zhao et al., 2018).

For plasma samples, we normalized the intensity of each ion peak to the average intensity of two nearby QC samples (Luo et al., 2016). For fecal samples, considering the large variation across samples in features such as the water content, we instead normalized the intensity of each ion peak to the total intensity of all detected ion peaks in each sample. Relative standard deviation (RSD) of each ion peak in QC samples was calculated. We included ion peaks with an RSD <30%, and present in >80% samples in either UA or control group. Ion peaks with zero intensity were replaced with half of the minimum value across all samples. After merging data from positive and negative ion modes, we obtained 1902 (including 78 identified metabolites) and 2981 (including 221 identified metabolites) ion peaks in fecal and plasma samples, respectively. A total of thirty-nine metabolites were identified in both fecal and plasma samples (Fig. S2).

Statistical analysis

Clinical characteristics between UA patients and controls were compared using Student's *t*-tests for continuous variables and Chi-squared tests or Fisher's exact tests for categorical variables. A two-tailed $P < 0.05$ was considered statistically significant unless otherwise stated. All statistical analyses were performed in the R 3.5.1 environment.

To estimate the association of the gut microbial diversity with UA, we first performed rarefaction analysis, in which we generated rarefied data of species and genes by setting the cutoff value as the minimum sequencing depth among all samples (function *rrarefy* in R package "vegan") (Heck et al., 1975). From the rarefied data, we computed within-sample diversity (alpha diversity), including Shannon index, Simpson index, species richness, and gene richness. The alpha diversity indices were z-score transformed. ORs and the corresponding 95% CI for UA per SD increment of alpha diversity indices were calculated using logistic models, with and without adjustment for age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes. Furthermore, we estimated between-sample diversity (beta diversity) using the function *distance* in R package "phyloseq", including Jaccard distance, Bray-Curtis distance, and unweighted and weighted UniFrac distances of the gut microbiota (Lozupone and Knight, 2005). To calculate the unweighted and weighted UniFrac distances, we constructed a phylogenetic tree from the rarefied metagenomic data using the *ExpressionSet2phyloseq* function in R package "curatedMetagenomicData" (Pasolli et al., 2017). We applied PERMANOVA with 9999 permutations on four beta diversity indices to test the association of overall gut microbial composition with UA, adjusting for the same covariates described above (McArdle and Anderson, 2001). An Omnibus *P* value was obtained by taking the minimum *P* values for individual distance matrices and assessing the statistical significance through permutations (function *PermanovaG* in R package "GUniFrac") (Chen et al., 2012). We performed PCoA (function *ordinate* in R package "phyloseq") based on each of the beta diversity distance matrices (McMurdie and Holmes, 2013).

To evaluate the associations between gut microbial taxa and UA, we defined microbial taxa with an average relative abundance >0.01% and present in >10% of the total samples as common taxa (8 phyla, 13 classes, 15 orders, 29 families, 56 genera, and 132 species), and the rest as rare taxa. The relative abundances of common taxa were pre-processed following Morgan et al. (2012). Briefly, we removed outliers more than three times of the interquartile range (IQR) away from the median, and imputed missing values with the average relative abundance of the taxa across all samples. We transformed the processed relative abundances of common taxa using arcsine square-root transformation followed by z-score standardization. From the standardized relative abundances, we evaluated the associations of common taxa with UA using logistic regression, adjusting for sequencing depth (log10 scale), age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes. In addition, we conducted analyses for the rare species present in ≥10 samples ($n = 91$). Considering that the presence of rare species might be sensitive to sequencing depth, we analyzed rare species using the rarefied data described above, and dichotomized the relative abundances to absence (0) or presence (1). We used Firth logistic regression (Heinze, 2006), which could handle the (quasi-) complete separation issue for rare events, to estimate the association between rare species and UA after adjustment of age, sex, BMI, smoking, drinking, hypertension, hyperlipidemia, and diabetes. Statistical significance was defined by a FDR <0.2 based on the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Here, we chose a lenient cutoff of FDR <0.2 because signals in the gut microbiome of clinical samples were often attenuated and a lenient cutoff would not incur drastically a greater number of false discoveries. To test the robustness of our results, we further performed subgroup analyses for those significant species stratified by age (<65, ≥65 years), sex, BMI (<24, ≥24 kg/m^2), or the status of current smoking, current drinking, hypertension, hyperlipidemia, and diabetes. Additionally, we tested for interaction between each significant species and each stratified variable by including an interaction term in the full model. Given the potential influence of antidiabetic medications on gut microbiota (Forslund et al., 2015; Maier et al., 2018), we performed sensitivity analyses for the association between significant species and UA after excluding individuals on antidiabetic medications. We explored the associations with UA for gut microbial pathways (whole-community level unless otherwise stated) present in >10% of the total samples, using the same methods for data processing and association analysis of common taxa.

Finally, we investigated the association between fecal and circulating metabolites, UA status, and common microbial species. We transformed the intensity of each metabolite using natural-log transformation followed by z-score standardization. To detect overall metabolomic differences between UA cases and controls, we performed PERMANOVA (function *adonis* in R package "vegan") and PLS-DA (function *plsda* in R package "mixOmics") based on the annotated metabolites of fecal ($n = 78$) and plasma ($n = 221$) samples, respectively. We performed leave-one-out cross-validation to evaluate the classification performance of PLS-DA on the basis of maximum distance (function *perf* in R package "mixOmics"). To estimate the association of each metabolic feature with UA status, we estimated ORs (95% CI) for UA per SD increment of metabolites using logistic regression, adjusting for age, sex, BMI, smoking, hypertension, hyperlipidemia, and diabetes. We calculated Spearman correlations between fecal and circulating metabolites, and between metabolites and common microbial species among controls ($n = 30$).

Credit authorship contribution statement

Xuezhen Liu: Formal analysis, Data curation, Visualization, Investigation, Writing - Original draft, Writing - Reviewing & Editing. **Miaoyan Shen**: Visualization, Investigation. **Pinpin Long**: Writing - Reviewing & Editing. **Han Yan**, **Haijing Jiang**, **Yizhi Zhang**, **Lue Zhou**, **Meian He** and **Xiaomin Zhang**: Investigation. **Kuai Yu**, **Gaokun Qiu** and **Hyungwon Choi**: Formal analysis. **Handong Yang**, **Xiulou Li** and **Xinwen Min**: Investigation, Resources. **Chaolong Wang**: Conceptualization, Formal analysis, Visualization, Writing -

Reviewing & Editing, Supervision. **Tangchun Wu**: Conceptualization, Visualization, Supervision, Resources, Project administration, Writing - Reviewing & Editing, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interests.

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Supplementary data

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