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The intestinal microbiota associated with cardiac valve calcification differs from that of coronary artery disease

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HIGHLIGHTS

- Cardiac valve calcification and coronary artery disease patients suffered from different gut microbial dysbiosis.
- *Prevotella copri* [OTU568118] might be a potential therapeutic target for cardiac valve calcification.
- *Collinsella aerofaciens* serve as an important operational taxonomic units "hub" in coronary artery disease.

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ABSTRACT

Background and aims: Although most risk factors for cardiac valve calcification (VC) are similar to those for coronary artery disease (CAD), they differ regarding lesions and clinical symptoms. Recently, increasing evidence suggests that intestinal bacteria play essential roles in cardiovascular disease (CVD). It is plausible that the gut microbiota is linked to the occurrence of different CVDs under similar risk factors. Thus, we aimed to explore the gut microbiomes in patients with VC or CAD and determine their underlying connections.

Methods: We collected samples from 119 subjects and performed 16S rRNA gene sequencing to analyze the gut microbiomes in VC and CAD patients and in control volunteers.

Results: The gut microbiomes of VC and CAD patients were significantly different in terms of beta-diversity. Bacteria from *Veillonella dispar*, *Bacteroides plebeius* and *Fusobacterium* were enriched in the VC group, while members of *Collinsella aerofaciens*, *Megamonas*, *Enterococcus*, *Megasphaera*, *Dorea* and *Blautia* were decreased. According to the association with dyslipidemia, seven operational taxonomic units (OTUs), including *Parabacteroides distasonis, Megamonas*, *Fusobacterium*, *Bacteroides* sp., *Bacteroides plebeius, Lactobacillus* and *Prevotella copri*, were regarded as potential pathogens for CVDs. Additionally, *Prevotella copri* might be a keystone of CVDs, especially in VC patients, while *Collinsella aerofaciens* is a possible keystone of CAD, based on the multi-correlations of these bacteria with other OTUs in microbial communities.

Conclusions: Patients with VC and CAD suffer from different gut microbial dysbiosis. The gut microbiomes are associated with the clinical characteristics in these diseases and might be potential therapeutic targets.

1. Introduction

Recently, the gut microbiota has been proposed to play crucial roles in the formation and progression of coronary artery disease (CAD). Traditionally, CAD is strongly associated with obesity, diabetes, hyperlipidemia and hypertension [1,2], and these risk factors are regarded

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as being almost identical to those of cardiac valve calcification (VC). Therefore, it is plausible that the gut microbial composition in VC and CAD patients is similar.

However, it is unclear why some CAD patients fail to exhibit VC in clinical practice and *vice versa*. Whether VC randomly emerges regardless of the occurrence of CAD or is caused by a particular mechanism remains unknown. A possible explanation for why CAD patients fail to exhibit VC is because they are not old enough. Recent studies suggest that calcific aortic stenosis can be divided into two phases, including an early stage similar to atherosclerosis and a subsequent stage involving pro-calcification [3,4]. Nonetheless, except for the factors of age, infection and dialysis, the promoting factors of VC are remarkably similar to those of CAD. Combining these findings, it is possible that the intestinal microbiota is involved in the progression of cardiac VC. Additionally, our previous studies determined that inflammation and immunity are closely linked to the osteogenic responses by lipopolysaccharide (LPS) stimulation in valvular interstitial cells [5,6], and most of the LPS was generated by microbiota from the gut. Thus, shifting focus to the gut microbiome may provide some insights into these interesting issues.

2. Materials and methods

2.1. Ethics statement

Clinical investigations were performed according to the Declaration of Helsinki. The present study was reviewed and approved by the review boards at Nanfang Hospital of Southern Medical University. All enrolled patients and volunteers provided written informed consent.

2.2. Study population and sample collection

Fecal samples were obtained from volunteers and inpatients at Nanfang Hospital of the Southern Medical University. To alleviate confounding effects of dietary consumption and contact with hospital environments, fecal samples were collected within 24 h of patients' admittance to the hospital and were stored at −80 °C until DNA extraction. All enrolled patients underwent coronary artery angiography or coronary computed tomography angioplasty to aid in the differential diagnosis of CAD, while cardiac ultrasounds were performed to verify the diagnosis of cardiac VC. We excluded patients who had psychiatric or mental diseases that precluding informed consent, tricuspid VC, kidney dialysis, acute infection, inflammatory bowel disease or other inflammatory diseases, gastrointestinal diseases, cancer, autoimmune diseases or treatments with antibiotics or probiotics within one month. We categorized subjects by their final clinical diagnosis. The control individual samples were obtained from healthy people and patients without cardiometabolic diseases and atherosclerosis.

2.3. DNA isolation, 16S rRNA gene amplification, bioinformatics and statistical analysis

Fecal samples were processed with a Fecal Total DNA EXTRACT kit (BioEAsy, Shenzhen) for the extraction of bacterial genomic DNA. The process was strictly performed according to the manufacturer's protocol, and the details have been described previously [7]. Deposition of the sequences is in progress. The *16S* rRNA V4-V5 hypervariable region was amplified and sequenced by Illumina MiSeq PE300 sequencing (primer: V4-V5 515F 5′-GTGCCAGCMGCCGCGGTAA-3′ and V4-V5 907R 5′-CCGTCAATTCMTTTRAGTTT-3′).

Raw sequences were processed using the BIPES pipeline [8]. First, we used FastQC to perform quality control of the raw data, and barcode primers were deleted. In order to obtain a longer sequence, the analysis required joining the sequences of Reads 1 (R1) and Reads 2 (R2) by matching overlapping regions in their tail ends. The likelihood of successfully joining the sequence ends was increased by removing low-quality bases from the end of each sequence. Therefore, we cut off 70 bp from the tail ends of R1 and R2. According to the overlap of R1 and R2, the minimum overlapping sequence length and the ratio of minimum mismatches were 6% and 8%, respectively. However, sequence validation was based on the joined data and low-quality filtration. Chimeras affect the accuracy of classification, which may result in an error sequence or classification as a new species. Thus, we also perform Chimera filtration.

AUCHIME (implemented in USEARCH, version 6.1) analysis was performed to screen out and remove chimeras under the de novo mode (using –minchunk 20 –xn 7 –noskipgaps 2). All the following analysis were performed by QIIME (1.9.1) as described previously [7]. Briefly, USEARCH with default parameters (USERACH61) was applied for the clustering of sequences to an operational taxonomic unit (OTU). Based on the threshold distance, when the similarity between two 16S rRNA sequences was more than 97%, the sequences were classified as the same OTU. QIIME-based alignments of representative sequences were performed by PyNAST, and the Greengenes 13_8 database was used as the template file. The Ribosome Database project (RDP) algorithm was applied to classify the representative sequences into specific taxa using the default database. The Shannon and PD_whole_tree indices were used to analyze alpha diversity, and a principal coordinate analysis (PCoA) analysis was performed using QIIME based on the UniFrac distance. Beta-diversity analyses were performed based on the UniFrac distance, and Adonis was used to estimate the amount of dissimilarity in microbial compositions between groups and to describe the association between variation in the gut microbiome and the results of clinical detection. OTU selection for data analysis was conducted according to the relative abundance of the median in any group larger than 0.3%. The relationship between discriminated OTUs and clinical features was determined by Spearman analysis, and outliers larger than the mean plus two times the standard deviation were deleted.

Redundancy analysis (RDA) was performed by using Canoco 5.0 to assess the relationship between blood lipid indices or body mass index (BMI) and different groups. The linear discriminant analysis effect size (LEfSe) method was used to identify features that differed between the groups. The threshold of the logarithmic LDA score for discriminative features was set to 3.0. Co-abundance analysis was performed using the Spearmen correlation, and network analysis was performed with Cytoscape 3.5.1 software [9]. All statistical analyses were performed using SPSS 20.0, Canoco 5.0 and R version 3.5.1.

3. Results

3.1. Clinical data from volunteers and patients included in the study

Clinical features of patients are shown in Table 1. Samples from 119 individuals were collected, and after sequencing, deletion of low-quality samples resulted in a final enrollment of 104 subjects.

3.2. Bacterial community composition profile in different groups

A total of 104 fecal samples were analyzed from volunteers and patients with cardiac VC or CAD. The samples were divided into four groups: control (Ctrl), cardiac VC, cardiac VC accompanied by CAD (VC + CAD) and CAD groups. From the taxonomic composition plots at the phylum level, we found that Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria represented more than 80% of the total bacteria community in the gut (Fig. 1A), while at the OTU level, *Bacteroides* spp., *Prevotella copri*, *Bacteroides fragilis* and *Faecalibacterium prausnitzii* accounted for a high proportion of the community (Fig. 1B). Moreover, no significant differences in alpha diversity were observed among these groups.

Table 1
Demographic and clinical characteristics of patients and volunteers.

	Ctrl	VC	VC + CAD	CAD	*p* value
Variables	(n = 17)	(n = 19)	(n = 21)	(n = 46)	
Demographicsro					
Age, years	36.64 ± 3.53	62.21 ± 2.67[a]	71.81 ± 2.07[ab]	58.84 ± 1.52[ac]	< 0.001
Gender (male/female)	10/7	10/9	14/7	36/10	0.384
Admission physical examination					
Systolic BP, mmHg	121.88 ± 3.45	134.53 ± 9.03	130.38 ± 5.54	133.41 ± 2.93	0.383
Diastolic BP, mmHg	73.12 ± 2.55	72.74 ± 4.88	68.57 ± 2.37	77.09 ± 2.34	0.209
BMI, kg/m^2	20.45 ± 0.91	24.23 ± 1.00[a]	25.15 ± 0.58[a]	24.59 ± 0.75[a]	0.017
Laboratory values					
Cholesterol, mmol/L	4.22 ± 0.21	4.31 ± 0.29	4.28 ± 0.25	4.61 ± 0.17	0.567
HDL-c, mmol/L	1.26 ± 0.11	0.96 ± 0.06[a]	0.92 ± 0.06[a]	1.00 ± 0.03[a]	0.009
LDL-c, mmol/L	2.43 ± 0.16	2.62 ± 0.21	2.82 ± 0.23	2.86 ± 0.14	0.545
VLDL-c, mmol/L	0.53 ± 0.05	0.72 ± 0.09	0.77 ± 0.08	0.75 ± 0.05	0.327
Triglyceride, mmol/L	1.00 ± 0.11	1.72 ± 0.32	2.35 ± 0.55[a]	1.89 ± 0.17	0.178
C reactive protein, mg/L	1.00 ± 0.53	26.07 ± 10.72	31.18 ± 12.15	7.73 ± 2.22[a]	0.022
White blood cell, 10^9/L	7.38 ± 0.93	7.94 ± 0.66	8.73 ± 0.71	7.89 ± 0.33	0.532
Neutrophilic granulocyte, %	59.78 ± 3.09	63.12 ± 2.90	70.26 ± 2.61[a]	64.12 ± 2.06	0.119
Platelet, 10^9/L	251.60 ± 21.94	225.78 ± 16.94	220.43 ± 16.93	230.04 ± 9.40	0.695
Hemoglobin, g/L	129.36 ± 6.75	121.11 ± 3.82	123.29 ± 4.64	132.47 ± 5.60	0.473
Hematocrit, L/L	0.39 ± 0.02	0.37 ± 0.01	0.36 ± 0.01	0.41 ± 0.01[bc]	0.001
Creatine kinase-MB, ng/mL	18.27 ± 8.71	20.63 ± 32.0	15.84 ± 2.41	27.47 ± 5.17	0.490
Cardiac troponin I, ng/mL	0.00 ± 0.00	0.04 ± 0.02	0.98 ± 0.72	4.82 ± 1.93	0.175
NT-pro-BNP, pg/mL	94.57 ± 59.00	1716.06 ± 567.88	4786.38 ± 2090.38	733.42 ± 242.53	0.017
Serum calcium, mmol/L	2.31 ± 0.04	2.16 ± 0.12	2.23 ± 0.03	2.29 ± 0.02	0.250
Serum phosphorus, mmol/L	1.19 ± 0.04	1.14 ± 0.04	1.04 ± 0. 053[a]	1.13 ± 0.02	0.099
Total bilirubin, μmol/L	11.66 ± 1.47	13.96 ± 1.99	19.75 ± 5.00	11.75 ± 0.80	0.094
Indirect bilirubin, μmol/L	7.40 ± 1.08	8.33 ± 1.21	8.65 ± 1.41	7.31 ± 0.53	0.681
Direct bilirubin, μmol/L	4.26 ± 0.42	5.63 ± 0.83	11.10 ± 3.95	4.44 ± 0.33	0.039
ALT, U/L	12.67 ± 2.49	19.67 ± 3.47	59.62 ± 138.60	36.98 ± 12.15	0.561
AST, U/L	16.91 ± 1.28	24.28 ± 2.84	56.62 ± 28.04	54.38 ± 18.86	0.567
Serum creatinine, μmol/L	72.91 ± 4.74	89.28 ± 11.63	105.71 ± 11.91	97.11 ± 9.96	0.462
Serum uric acid, mg/dL	344.64 ± 36.75	395.75 ± 36.49	450.38 ± 33.58[a]	399.71 ± 16.92	0.188
Serum cystatin C, mg/L	0.88 ± 0.07	1.16 ± 0.07	1.60 ± 0.18[ab]	1.20 ± 0.10[c]	0.007
PT,s	12.42 ± 0.27	15.04 ± 1.50	14.218 ± 1.23	12.21 ± 0.19	0.042
APTT,s	30.61 ± 1.05	30.69 ± 1.72	31.33 ± 2.75	28.92 ± 0.88	0.660
INR	1.11 ± 0.02	1.33 ± 0.13	1.26 ± 0.11	1.09 ± 0.02	0.045
D-dimer, mg/L	0.33 ± 0.12	0.66 ± 0. 15	1.34 ± 0.32[a]	0.65 ± 0.13	0.025
Fibrinogen, g/L	2.76 ± 0.26	3.19 ± 0.23	3.17 ± 0.23	3.34 ± 0.17	0.455

BP blood pressure, BMI body mass index, NYHA class New York Heart Association, HDL-c high-density lipoprotein cholesterol, LDL-c low-density lipoprotein cholesterol, VLDL-c very low-density lipoprotein cholesterol, NT-proBNP N-terminal pro-brain natriuretic peptide, ALT alanine aminotransferase, AST aspartate aminotransferase, PT prothrombin time, APTT activated partial prothrombin time, INR international standardization rate.
Values are the mean ± SEM; [a] $p < 0.05$ *vs.* Ctrl, [b] $p < 0.05$ *vs.* VC, [c] $p < 0.05$ *vs.* VC + CA.

3.3. Beta-diversity differences among the four groups

To explore the differences in beta-diversity between patient groups, unweighted UniFrac distance analysis was performed. The analysis revealed significant differences in microbial composition between the Ctrl and CAD (R [2] = 0.042, $p < 0.001$, Fig. 1D), Ctrl and VC (R [2] = 0.085, $p < 0.001$, Fig. 1E), VC and CAD (R [2] = 0.048, $p < 0.001$, Fig. 1F) and VC and VC + CAD (R [2] = 0.035, $p < 0.05$, Fig. 1G) groups. In addition, PCoA analysis (Fig. 1C) showed that the CAD group overlapped with the Ctrl group, while VC and VC + CAD groups did not overlap with the Ctrl group, These findings imply that VC and VC + CAD patients might harbor a more unique gut microbiota structure than CAD patients. Additionally, the PCoA also exhibited overlaps between the VC and VC + CAD groups, though with significant differences. These findings might suggest that the intestinal microbiota in VC + CAD patients is more similar to that of VC patients than that of CAD patients. Taken together, these results suggested that VC and CAD patients harbor distinct gut microbiome dysbioses, and the microbial composition of the VC + CAD group was similar to that of the VC group rather than that of the CAD group.

3.4. Impact of age, BMI, blood lipids and other clinical features on bacterial community composition

Age, BMI and blood lipids were regarded as factors that influence both CVDs and the gut microbiota. To test what extent age, BMI and blood lipids as well as different groups contributed to explaining the observed variation in bacterial community composition, RDA was applied using data on the relative abundance of OTUs. RDA analysis showed that BMI and age significantly influenced the gut microbiota in disease groups relative to the Ctrl group, while the influence of these factors on gut microbial composition showed no significance between the VC and CAD groups (Fig. 2A). We also found that HDL was highly positively associated with the gut microbiota of the Ctrl group, and triglycerides (TG), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and cholesterol (CHOL) were negatively correlated with the Ctrl group, while the VC, VAD and VC + CAD groups showed an opposite trend (Fig. 2B). Additionally, the gut microbial composition between the VC and CAD groups did not seem to be influenced by these blood lipid indices (Fig. 2B), just as indicated by the ANOVA analysis in Table 1. Therefore, gut microbiota differences between the VC and CAD groups are more likely related to the disease itself rather than the

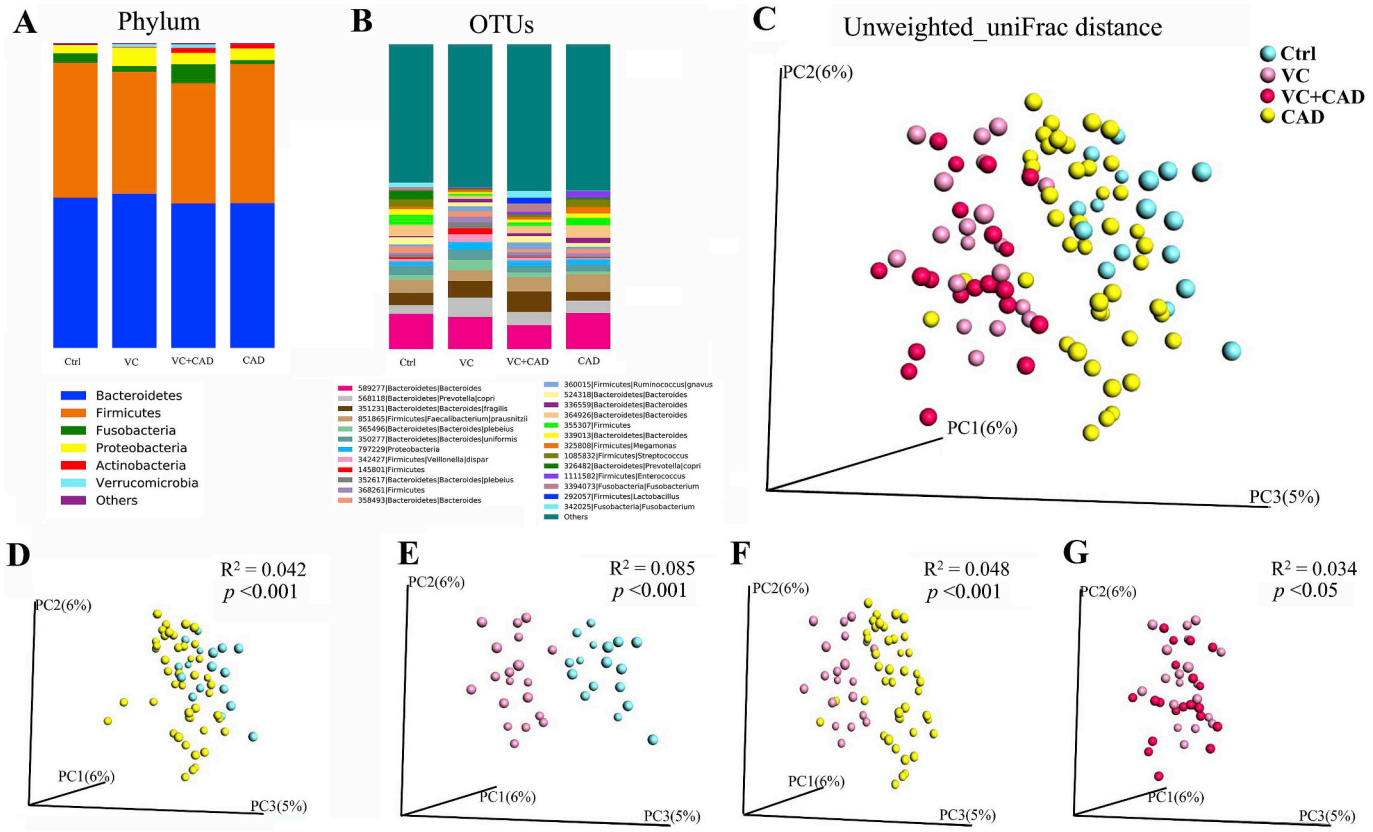

Fig. 1. Gut microbiota composition of control, VC, VC + CAD and CAD groups.
Taxonomic composition of all taxonomically assignable bacteria at the (A) phylum and (B) OTU levels for control (Ctrl), valve calcification (VC), VC accompanied by coronary artery disease (VC + CAD) and CAD groups. (C-G) Analysis of unweighted UniFrac distance demonstrates clear segregation and overlap among the four groups mentioned above.

influence of age, BMI or blood lipids. Thus, the gut microbiota might be an essential factor in distinguishing the VC and CAD groups.

In addition, Adonis analysis of abund_jaccard distance demonstrated that NT-pro-BNP, DBIL, APTT, INR, PT, TBIL, CysC, NYHA grade and HCT ($p < 0.05$, FDR < 0.1) were significantly correlated with the gut microbiota. Together, these results suggest that the gut microbiota probably affects clinical indices involved in CVDs, and these effects might be the origin of different diseases.

3.5. Discriminative OTUs by LEfSe analysis that correlated with clinical features in lipidology

We next further explored the relationship between discriminative bacteria picked by LEfSe (Fig. 3A and B) and blood lipid indices. Seven OTUs were probably pathogens, as demonstrated by the fact that they positively correlated with CVD risk factors, including LDL, TG, CHOL, and BMI. Among these, *Fusobacterium* [OTU 572889] and *Megamonas*

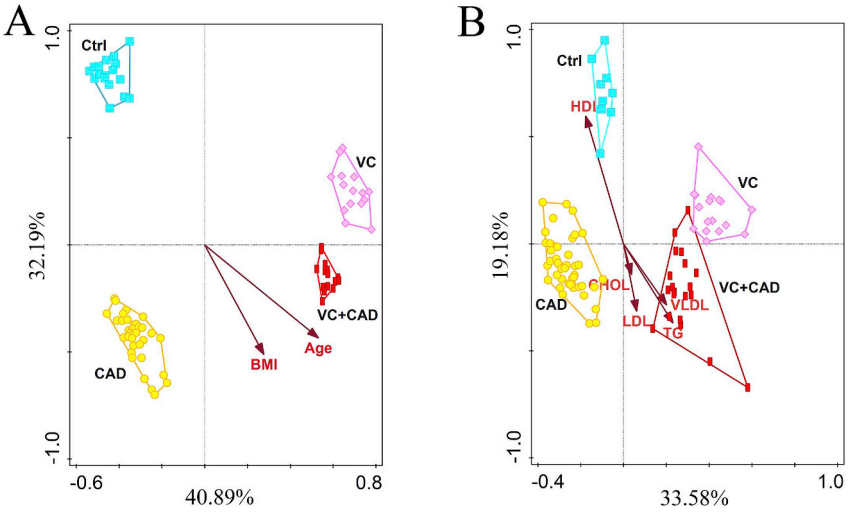

Fig. 2. Redundancy analysis among gut microbiome, BMI, age and blood lipids.
Redundancy analysis (RDA) triplot showing the relationships among age, BMI and lipid indices, variations in which are associated with diseases. The canonical axes are labeled with the percentage of total variance explained (%).

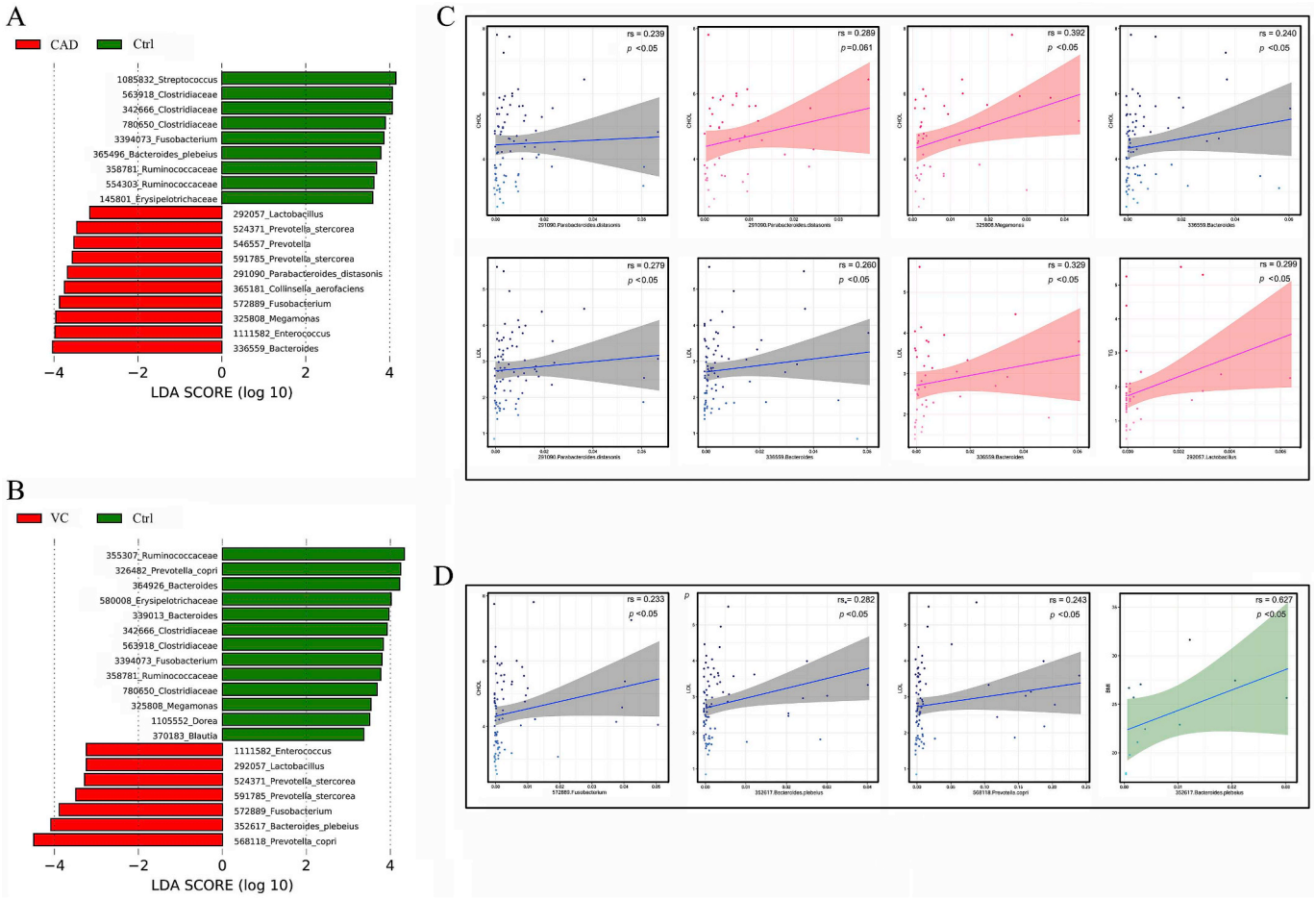

Fig. 3. The correlation between clinical features and discriminative OTUs identified by LEfSe.
OTUs significantly enriched or depleted in relative abundance in (A) CAD *vs.* Ctrl, (B) VC *vs.* Ctrl. Bacteria with significant correlations with clinical indices are shown in (C) and (D), blue, red and green dots, denoting all enrolled patients, CAD and VC patients, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

[OTU 325808] were positively correlated with CHOL, *Bacteroides* [OTU336559] and *Parabacteroides distaso* [OTU 291090] were positively correlated with CHOL and LDL, *Lactobacillus* [OTU 292057] was positively correlated with TG, and *Bacteroides plebeius* [OTU 352617] and *Prevotella copri* [OTU 568118] were positively correlated with LDL (Fig. 3C and D). These results suggested that several OTUs are involved in metabolic disorders and also supported the fact that CVDs are affected by dyslipidemia.

3.6. Gut microbiota differences between VC and CAD patients

To further explore discriminative features between CAD and VC groups, LEfSe was performed, and 22 distinct OTUs between the VC and CAD groups were found (Fig. 4A). In addition, we compared the eight most important OTUs among the four groups (Fig. 4B–D): *Collinsella aerofaciens* [OTU 365181], *Enterococcus* [OTU 1111582], *Megamonas* [OTU 325808] and *Megasphaera* [OTU 266210] were significantly increased in the CAD group; *Bacteroides plebeius* [OTU 352617], Enterobacteriaceae [OTU 819999], *Veillonella dispar* [OTU 342427] and *Prevotella copri* [OTU 568118] were mostly associated with the VC group (Fig. 4B–C). We also showed the average relative abundances of the above-mentioned OTUs for these groups (Fig. 4D). These results support the possibility that VC and CAD patients have different intestinal bacteria and that these differences might be involved in their pathogenesis.

3.7. Co-abundance networks revealing risk-related "hub" OTUs in the VC and CAD groups

As the "guild" in the microbiome likely affects diseases together [10], we further performed co-abundance analysis by 33 discriminative OTUs between the CAD and VC groups to further investigate "hub" OTUs in these groups (Fig. 5). In VC patients, three OTUs from *Prevotella copri* were significantly associated with most other OTUs, of which eight OTUs were positively correlated with *Prevotella copri*, while three OTUs were negatively correlated with *Prevotella copri* (Spearman analysis, $p < 0.05$). In CAD patients, four OTUs from *Prevotella copri* were also linked with most other OTUs, of which 11 OTUs were positively correlated with *Prevotella copri*, while ten other OTUs were negatively correlated with *Prevotella copri* (Spearman analysis, $p < 0.05$). Additionally, an OTU from *Collinsella aerofaciens* also showed multi-correlations with other OTUs, specifically positive correlations with four OTUs and negative correlations with another four OTUs (Spearman analysis, $p < 0.05$). Therefore, *Prevotella copri* is possible a keystone of CVD, especially in the VC group, while *Collinsella aerofaciens* is important in the CAD group, based on multi-correlations with other bacterial OTUs in their microbial communities. Together, *Prevotella copri* and *Collinsella aerofaciens* might be key factors that trigger different clinical consequences.

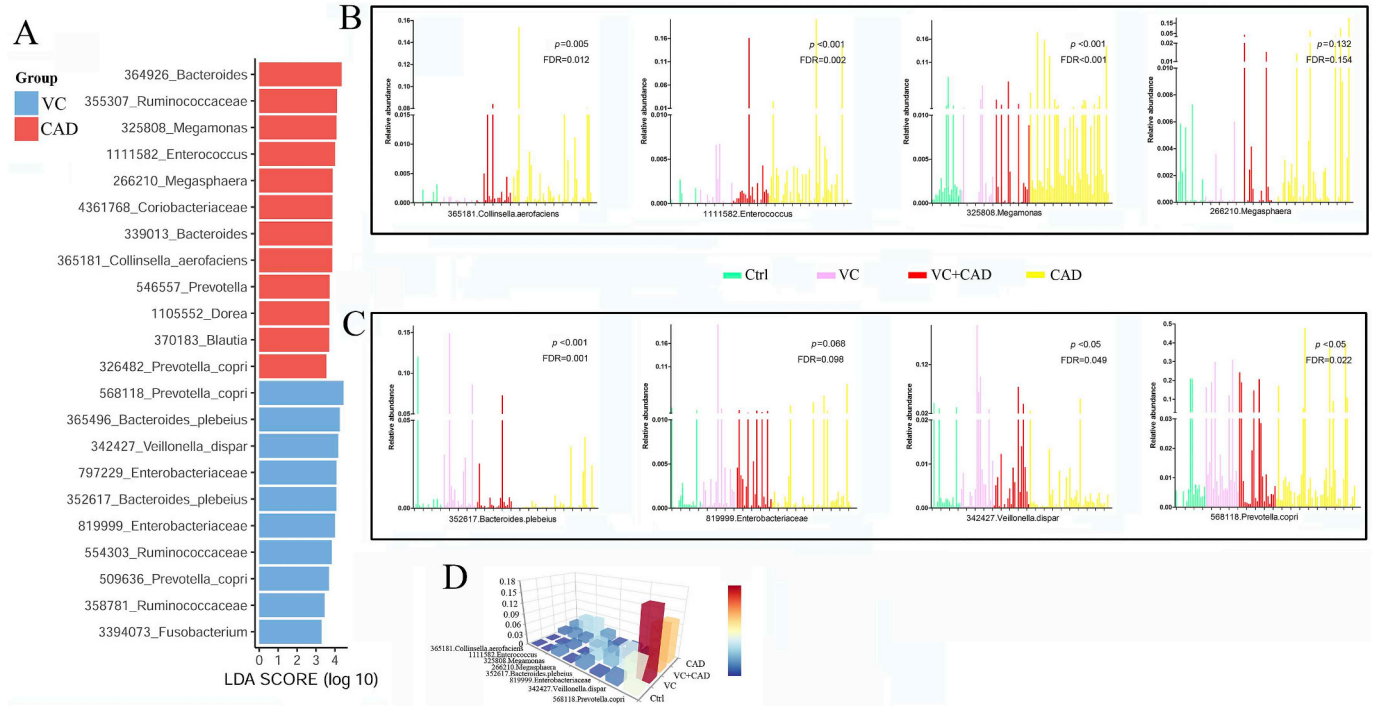

Fig. 4. LEfSe-based discriminating features between the VC and CAD groups.
(A) Names of the OTUs in discriminating VC and CAD patients are listed on the left side of the Fig. Only significant LDA thresholds greater than 3 are shown. (B and C) Bar plots showing the distribution of each sample among the Ctrl, VC, VC + CAD and CAD groups. (D) The bar plot shows the average levels of important bacteria in each group.

3.8. Distinct members of the gut microbiota are associated with different clinical features

To explore the relationship between clinical features and important lineages, we performed a corrected Spearman correlation analysis between clinical features and bacteria from the order to genus taxonomic levels according to the 33 discriminative OTUs identified by LEfSe (Fig. S1). Five lineages (primarily from Clostridiales, Lactobacillales, Bacteroidales, Fusobacteriales and Coriobacteriales) (blue nodes) from the gut microbiota significantly correlated with clinical features. Clostridiales and Lactobacillales appear to be important in cardiovascular disease, as five genera from three families of Clostridiales and three genera from three families of Lactobacillales had relationships with clinical indices. *Enterococcus* would probably serve as a potential biomarker of cardiac dysfunction, as it positively correlated with cTnI (Cardiac troponin I). In addition, *Streptococcus* and *Enterococcus*, which belong to Lactobacillales, were positively correlated with alanine transaminase (ALT), a marker of hepatic dysfunction that also increases in some CVDs such as heart failure. Another Lactobacillales genus (*Lactobacillus*) showed a negative correlation with diastolic blood pressure and may therefore be important in coronary disease. It is plausible that antiplatelet drugs influence the gut microbiota. However, it is also possible that it was accompanied with the appearance of coronary disease. Additionally, a correlation was observed between members of Veillonellaceae and taking antiplatelet drugs; three genera of this family significantly correlated with taking antiplatelet drugs, as *Megamonas* and *Megasphaera* were positively correlated with drug and *Veillonella* was negatively correlated with drug. Clostridiaceae may be connected with the severity of cardiac injury, since it showed a significant negative correlation with the cardiac-type isoenzyme creatine phosphokinase (CK-MB). Additionally, *Parabacteroides* was positively correlated with CK-MB. However, some controversial findings were still observed. For instance, *Blautia* was positively correlated with CHOL and LDL, while *Dorea* was negatively correlated with NT-proBNP, although all of these genera belonged to Lachnospiraceae. Furthermore, *Dorea* showed a negative correlation with CysC.

4. Discussion

In this study, we provide evidence that VC and CAD patients suffer from distinct gut microbiota dysbioses, which imply that intestinal bacteria might be a factor determining the origin of different CVDs.

First, we observed that the gut microbial compositions of the Ctrl and CAD groups, as well as the Ctrl and VC groups, were significantly different in unweighted UniFrac distance. In addition, cardiac VC, an age-related disease, probably harbored a more distinct composition than CAD relative to the Ctrl group; PCoA plots indicated that the CAD and Ctrl groups had more overlaps than the VC and Ctrl groups. Additionally, the microbial communities of the VC and VC + CAD groups had significant differences; these communities appeared to overlap with each other in the PCoA, and their differences were not as obvious as those of the VC and CAD groups. It seems that VC + CAD patients harbored a gut microbiome phenotype that was more similar to that of the VC group. A possible explanation is that the coronary artery lesion is not as severe in VC + CAD patients as that observed in CAD patients, resulting in a more similar gut microbiota to that of VC patients. Although CTnI showed positively correlates with *Enterococcus*, it had no significance in the Adonis analysis. Thus, we postulated that some particular pathogens in VC patients may be more robust than those in CAD patients, and these pathogens drive the gut microbiota to induce the VC phenotype. This result may support shifting of the gut microbial composition in cardiac VC patients, and VC patients might have a more unique microbiome.

To be more specific, we excluded patients with rheumatic heart disease (RHD), a disease involving valve injury that is generally triggered by streptococcal infection, before conducting the analysis to avoid the confounding factors of bacterial infection and its related VC, while the traditional type of VC and stenosis that appears with aging is termed degenerative valve disease. Recently, some studies have examined the distinct features of the gut microbiota in CAD or

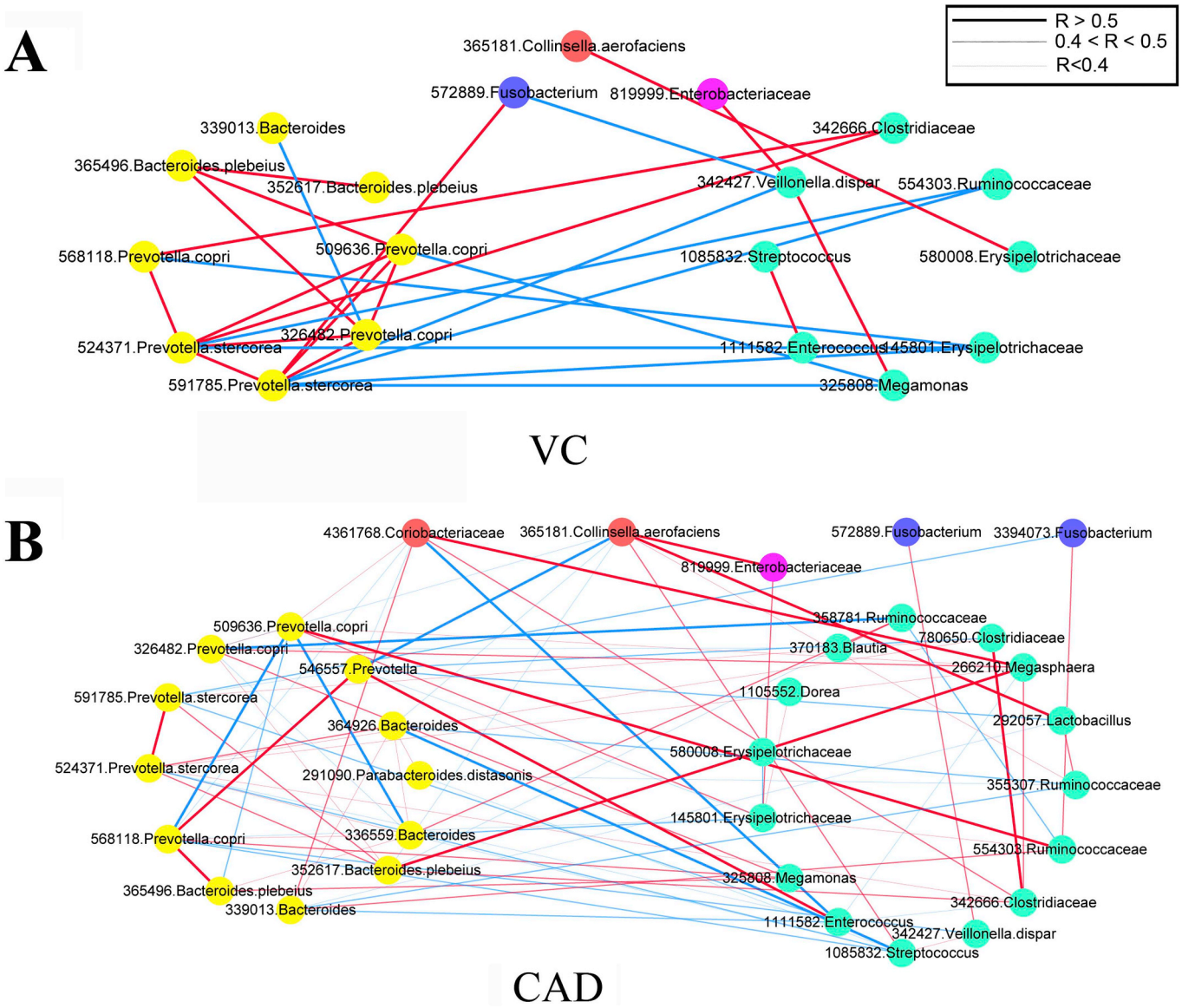

Fig. 5. Co-abundance analysis revealing "hub" OTUs in the gut microbial communities of the VC and CAD groups.
Co-abundance networks of OTUs in (A) the VC and (B) CAD groups. Red and blue edges represent positive and negative correlations, respectively. Red, yellow, cyan, blue and pink notes represent OTUs from Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria, respectively. All of the pairs presented are significant, with $p < 0.05$ and FDR < 0.1 for each pair. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cardiometabolic diseases [11,12], though the concerns of VC were neglected. Another study focusing on atherosclerosis risk factors indicated that hypercholesterolemic patients were enriched with *Prevotella* and decreased in *Clostridium* [13]. These findings partly support our results regarding the altered microbial composition of CVDs patients, as we observed three OTUs from *Clostridium* that were decreased and three OTUs from *Prevotella* that increased in the VC group compared with the Ctrl group, while CAD patients also showed a similar trend for *Clostridium* and *Prevotella*. In addition, Clostridiaceae was negatively correlated with CK-MB, an index of cardiac injury, and *Prevotella* was positively associated with LDL. Thus, *Prevotella* and *Clostridium* might be potential therapeutic targets in patients with CVDs.

Additionally, compared with Ctrl individuals, CAD patients harbored significantly elevated abundances of OTUs from *Enterococcus*, *Megamonas*, *Collinsella aerofaciens* and *Fusobacterium*, most of which have been shown to be connected with human diseases [11,14–16]. Furthermore, CAD patients harbored fewer commensal or beneficial bacteria, such as OTUs from Ruminococcaceae and Clostridiaceae, than Ctrl individuals. Yin et al. reported that stroke patients have elevated levels of *Enterobacter*, *Megasphaera*, *Oscillibacter* and *Desulfovibrio* [17], which partly supports our results. Moreover, an OTU from *Megamonas* that was important in distinguishing Ctrl and CAD patients was positively correlated with CHOL, suggesting that this species might be harmful to CAD patients. However, VC patients harbored more opportunistic pathogens, such as OTUs from *Prevotella copri* and *Fusobacterium*. A recent study indicated that *Prevotella copri* is immune-relevant, as it is involved in rheumatoid arthritis pathogenesis [18]. Controversially, another study concluded that *Prevotella copri* produced succinate and exhibited metabolic benefits [19]. Nevertheless, we found that *Prevotella copri* positively correlated with LDL, which probably supports its role in pro-inflammation. Thus, due to its roles in immunity and inflammation, *Prevotella copri* is a possible critical pathogen involved in CVD.

Intriguingly, comparing the VC and CAD groups also reveal a significant difference despite the fact that these groups shared common clinical risk factors. We observed that *Collinsella aerofaciens* was

increased in the CAD group relative to the VC group and served as a "hub" OTU. In addition, Fredrik et al. reported that the genus *Collinsella* was enriched in patients with symptomatic atherosclerosis [20], indicating that this genus, even in different races, might be problematic in CAD. Furthermore, *Blautia*, a short-chain fatty acid (SCFA)-producing bacteria that has been associated with the anti-inflammatory response [21], was reduced in VC group and has been shown to be reduced in patients with heart failure [22], was negatively associated with NT-proBNP and positively correlated with LDL. These results suggest that inflammation might be more essential in VC patients, which was reflected by their unique microbiota. Nevertheless, an epidemiological study indicated that degenerative valvular disease is highly associated with aged population [23]; however, the VC and CAD groups showed no significance in terms of age level in our study. Therefore, the microbial dissimilarities between the VC and CAD groups were probably caused by the disease itself, or the differences in gut microbial composition originally contributed to the formation of these diseases.

Another interesting point is that the concept of the "guild" in the gut microbiome was proposed to describe microorganism mutualistic symbiosis and interplay among gut microorganisms [24]. Some "hub" bacteria are potential key pathogens, which might suppress symbiotic bacteria and promote other pathogens. These findings suggest that disturbance of the native microbiota in VC and CAD is not induced by a single specific microbe, and these events are presumably caused by multiple microbial groups.

There are still some limitations in this study. The Ctrl group was younger than the disease groups, though age is an independent risk factor of cardiac VC. However, age showed no significant difference between the CAD and VC groups, and the difference between these two groups is of primary concern. Additionally, germ-free animals are needed to further verify causes and effects among the discriminated bacteria.

In summary, our study showed that CAD and VC patients harbored distinct gut microbiota compositions and revealed that *Collinsella aerofaciens* and *Prevotella copri* are possible keystones in CAD and VC, providing some possible therapeutic targets for CVDs.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Q.C.Z., D.L.X, H.R., L.H.A. and X.Z.M. designed and coordinated the study. Z.H.L., J.Y.L. and H.Y.L. designed and performed the experiments. Z.H.L. and H.Y.L. analyzed the data and wrote the paper. Y.T., Q.Z. and W.Y.L. collected fecal samples and clinical features.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2018.11.038.

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