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Integrated multi-omics revealed that dysregulated lipid metabolism played an important role in RA patients with metabolic diseases

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Abstract

Objectives Patients with rheumatoid arthritis (RA) commonly experience a high prevalence of multiple metabolic diseases (MD), leading to higher morbidity and premature mortality. Here, we aimed to investigate the pathogenesis of MD in RA patients (RA_MD) through an integrated multi-omics approach.

Methods Fecal and blood samples were collected from a total of 181 subjects in this study for multi-omics analyses, including 16S rRNA and internally transcribed spacer (ITS) gene sequencing, metabolomics, transcriptomics, proteomics and phosphoproteomics. Spearman's correlation and protein-protein interaction networks were used to assess the multi-omics data correlations. The Least Absolute Shrinkage and Selection Operator (LASSO) machine learning algorithm were used to identify disease-specific biomarkers for RA_MD diagnosis.

Results Our results found that RA_MD was associated with differential abundance of gut microbiota such as *Turicibacter* and *Neocosmospora*, metabolites including decreased unsaturated fatty acid, genes related to linoleic acid metabolism and arachidonic acid metabolism, as well as downregulation of proteins and phosphoproteins involved in cholesterol metabolism. Furthermore, a multi-omics classifier differentiated RA_MD from RA with high accuracy (AUC: 0.958). Compared to gouty arthritis and systemic lupus erythematosus, dysregulation of lipid metabolism showed disease-specificity in RA_MD.

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Conclusions The integration of multi-omics data demonstrates that lipid metabolic pathways play a crucial role in RA_MD, providing the basis and direction for the prevention and early diagnosis of MD, as well as new insights to complement clinical treatment options.

Keywords Rheumatoid arthritis, Multi-omics, Metabolic diseases, Lipid metabolism

Introduction

Rheumatoid arthritis (RA) is a systemic, chronic and autoimmune inflammatory disorder that affects approximately 1% of the population worldwide [1, 2]. The prevalence of multiple metabolic diseases (MD), including metabolic syndrome, hyperlipidemia, diabetes, atherosclerosis, is higher in rheumatic diseases with RA, gouty arthritis (GA), systemic lupus erythematosus (SLE), and ankylosing spondylitis, ranging from 14 to 62.8% [3–8]. This not only impacts the disease treatment but also increases the risk for cardiovascular disease, leading to higher morbidity and premature mortality [9–11]. Due to the lack of comprehensive, efficient, and systematic study, the pathogenesis of RA with MD (RA_MD) remains elusive.

In recent years, the development of high-throughput sequencing technology has provided researchers with crucial tools for understanding disease pathogenesis, discovering new biomarkers and therapeutic targets across various levels, including genes, proteins, metabolites, and microbiomes [12]. Notably, the integration of multi-omics data has gained significant attention due to its ability to deeply excavate potential pathogenic factors and provides valuable information for early disease warning, diagnosis, and treatment, both theoretically and practically, while avoiding errors and deviations that may arise from a single omics technique [13–15]. A multi-omics study whose analytical approach included metabolomics, proteomics and peptide analysis found that six different MDs exhibited significant molecular and clinical differences in glucose and lipid metabolism [16]. Moreover, regarding rheumatic diseases such as RA, SLE, primary Sjögren syndrome, a study reveals that aberrant regulation of megakaryocyte expansion may contribute to the pathogenesis of rheumatic diseases through transcriptomic and proteomic analyses [16]. However, few multi-omics studies of RA_MD have been reported, and most of them have focused on observational studies of clinical characteristics and drug responses [17, 18].

Here, we integrated microbiomics, metabolomics, transcriptomics, proteomics, phosphoproteomics and clinical information to unveil the latent molecular characteristics and functional pathways that contribute to the development of MD complications in RA patients. Furthermore, we also aimed to identify several biomarkers to distinguish between RA and RA_MD patients by using LASSO (Least Absolute Shrinkage and Selection Operator) analysis. Then, we validate the disease-specific gut microbiota and plasma metabolites in different rheumatic diseases. Ultimately, our research held the latent capacity to bring new insights into the pathogenesis and personalized treatment strategies for patients with RA combined with MD.

Materials and methods

Study participant

A cross-sectional study was used to recruit participants from the Department of Rheumatology and Immunology, Dazhou Central Hospital, between November 2017 and July 2020. All patients were diagnosed with RA or GA or SLE based on the 2010 or 2015 American College of Rheumatology (ACR)- European League Against Rheumatism (EULAR) classification criteria for diagnosis [19, 20]. At the same time, their clinical information including age, female, disease duration, disease activity score of 28 joints (DAS28), pharmacological information and laboratory test parameters such as rheumatoid factor (RF), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), interleukin 6 (IL-6), total cholesterol (TC), triglyceride (TG), HDL (high density lipoprotein), LDL (low-density lipoprotein) and glucose (GLU) were collected. All the laboratory measurements were performed by the Department of Laboratory Medicine of Dazhou Central Hospital, and described in Supplementary Methods 1. We also recruited a group of control subjects, who were precisely matched by age and gender, from May 2020 to July 2020 and underwent medical examinations at Dazhou Central Hospital. Supplementary Tables 1–3 present detailed information about the characteristics of all subjects. It should be noted that all enrolled participants provided informed consent. The enrollment was followed the particular inclusion and exclusion criteria, which were as follows.

The inclusion criteria included: (1) Participants must be older than 18 to be eligible. (2) RA_MD subjects were RA patients diagnosed with MD complications (type 2 diabetes, hyperlipidemia, and atherosclerosis). (3) Control subjects without MD and rheumatic diseases. The exclusion criteria include: history of cancer, organ transplantation, or other infectious diseases.

Study design

120 faecal, 110 plasma and 24 PBMCs (Peripheral Blood Mononuclear Cells) samples were collected from a total of 172 subjects, consisting of 40 control, 32 RA, 32 RA_MD, 40 GA and 28 SLE, for analyses by 16 S rRNA, ITS

(internal transcribed spacer) gene sequencing (n=120) and metabolomics (n=110), transcriptomics (n=18), and proteomics (n=6) and phosphorylated proteomics (n=4). The composition of RA_MD includes RA_T2D (RA with type 2 diabetes), RA_HLP (RA with hyperlipidaemia) and RA_AS (RA with atherosclerosis). Detailed samples and corresponding participant information can be found in the Supplementary Tables 1–3. Then, spearman's correlation, protein-protein interaction (PPI) networks and shared pathway analyses were used to assess the multi-omics data correlations, and a machine learning model was built to identify disease-specific biomarkers for RA_MD diagnosis. Finally, the differences in the pathogenesis of MD among three rheumatic diseases, namely RA, GA and SLE, were explored (Fig. 1).

Analysis of gut microbiome profiling (16 S/ITS)

Total DNA of the microbial community was extracted from fecal samples using E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, USA). The primers 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the bacteria 16 S rRNA gene fragments (V3–V4), and fungal internally transcribed spacer (ITS) fragments were amplified with primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3'). Illumina MiSeq PE30 sequencing platform (Illumina, San Diego, CA, USA) was used to sequence the foregoing PCR amplicons. The Amplicon Sequence Variants (ASVs) were obtained through the QIIME2 (Quantitative Insights Into Microbial Ecology 2) process and analyzed taxonomically based on the SILVA 16 S rRNA (v138) and ITS databases. Subsequently, the alpha and beta diversity, community composition, species variation, and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) prediction analyses were performed using the online platform of Majorbio Cloud Platform (www.majorbio.com). Detailed sequencing and analyses methods are shown in Supplementary Methods 2.1.

Analysis of plasma metabolite profiling

The collected blood samples were processed with reference to the previous methods to obtain plasma samples

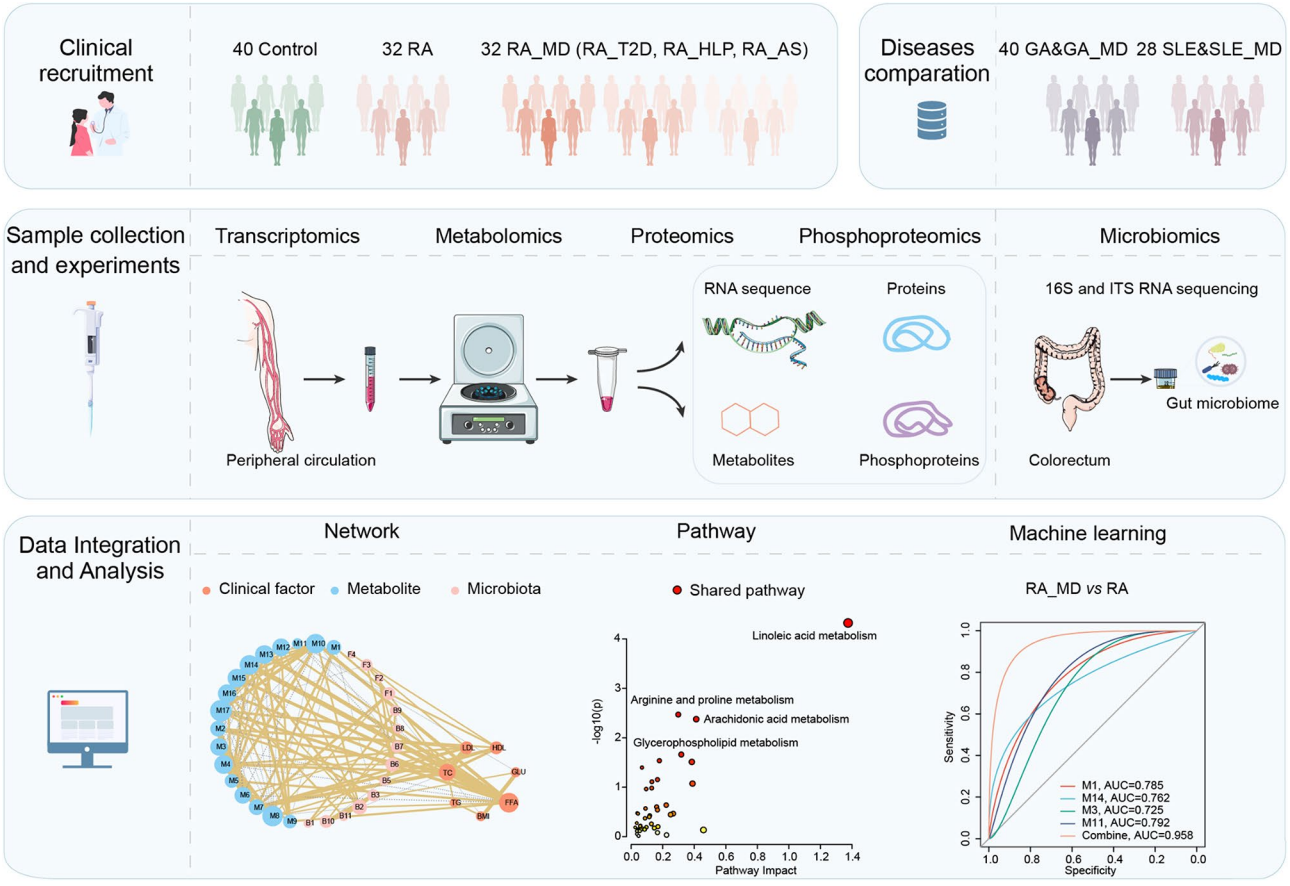

Fig. 1 The flowchart of our study design. RA, rheumatoid arthritis, RA_MD, RA patients with metabolic diseases, RA_T2D, RA patients with type 2 diabetes, RA_HLP, RA patients with hyperlipidaemia, RA_AS, RA patients with atherosclerosis, GA, gouty arthritis, GA_MD, GA patients with metabolic diseases, SLE, systemic lupus erythematosus, SLE_MD, SLE patients with metabolic diseases

[21, 22], and the metabolites extracted therefrom were subsequently sequenced by non-targeted metabolomics. The metabolite information was obtained by matched with the online Human Metabolome Database (HMDB) (https://hmdb.ca/) and Metlin (https: //metlin.scripps.edu/) database. Then, the above pre-processed data were uploaded onto the Majorbio Cloud Platform (www.majorbio.com) for multivariate statistical analysis such as Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), student's *t*-test, unpaired Wilcoxon test, and fold change analysis setting a Variable Importance in Projection (VIP) threshold≥1, | log2(fold change) | >1, and $p<0.05$. Metabolic pathway annotation was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.kegg.jp/) to determine the pathways in which the different metabolites were involved. For detailed information, see Supplementary Methods 2.2, 2.3.

Gene expression data analysis

PBMCs were extracted from the collected blood subjects with reference to the methods of previous studies see Supplementary Methods 2.4 [21–23]. Details regarding RNA extraction and detection, PCR amplification and library construction, sequencing and quality control are described in the Supplementary Methods 2.5. Subsequently, bioinformatics analysis was performed on the expression matrices obtained from the above procedure. Gene Set Enrichment Analysis (GSEA) based on KEGG pathway database was carried out using an online platform for data analysis and visualization (https://www.bioinformatics.com.cn). DEseq2 package of the language R was employed to examine the differential expression of the transcriptome, and the Benjamini & Hochberg method was used to adjust p-values. A threshold of $p<0.05$ & | log2(fold change) | > 1 was set for significant differential expression, and a volcano plot was drawn using ggplot2.Using David (https://david.ncifcrf.gov/) for differential gene function annotation, Gene Ontology (GO) function enrichment analysis and KEGG pathway enrichment analysis.

DIA (data-independent acquisition) based proteomics and phosphoproteomics analysis

DIA based quantitative proteomic and phosphoproteomic analyses of PBMC proteins were done at a biological company (Norogene, Beijing, China). The data was searched against the human Uniprot fasta protein database using the library search software Spectronaut-PulsarX 14.0 (Biognosys, Zurich, Switzerland) in the DDA scanning mode. Meanwhile, to improve the quality of analysis results, Spectronaut-Pulsar software further filtered the search results. The DIA data was imported into the Spectronaut software, and the ion-pair chromatographic peaks were extracted according to the pulsar constructed DDA database, and the subion matching and peak area calculation were performed to realize the simultaneous characterization and quantification of the peptides. The retention time was corrected using the iRT kit (Biognosys, Zurich, Switzerland) added in the samples, and the Qvalue cutoff value of precursor ions was set at 0.01. The statistical analysis of the protein quantification results was performed by the *t*-test method, and those proteins with significant quantitative differences between RA and RA_MD groups ($p<0.05$, | log2(fold change) | > 1) were classified as significantly differentially expressed proteins. Detailed methods are described in the Supplementary Methods 2.6.

LASSO classifier

Datasets were divided 70%/30% into training and test sets and LASSO classifiers were constructed using the glmnet R package, which was then subjected to stratified 5-fold cross-validation to distinguish between RA_MD and RA. The accuracy of the generated classifiers was determined by calculating the Area Under the Curve (AUC) of receiver operating characteristic using test data.

Statistical analysis

All statistical analyses were performed in IBM SPSS Statistics 25 and GraphPad Prism 8. In order to perform statistical comparisons, student's unpaired *t* test, chi-square test, Fisher's exact test, Wilcoxon rank sum test, and Mann-Whitney U test were used as appropriate. Clustering correlation heatmap with signs and volcano plot were performed using the OmicStudio tools (https://www.omicstudio.cn). The visualization of bubble charts was executed by an online platform (https://www.bioinformatics.com.cn). In addition, the correlation network was mapped using string protein interaction database (https://string-db.org/) and cytoscape software.

Results

Baseline participant characteristics

The demographic characteristics of the study were summarized in Supplementary Tables 1–3. We found that in Supplementary Table 1, compared with the RA group, the TC, number of users of leflunomide and LDL of the RA_MD group were significantly increased, while there was no significant difference in other clinical indicators between the RA_MD and RA groups. Meanwhile, in Supplementary Tables 2–3, compared with the group without MD, both TC and TG were significantly elevated in the combined MD group. It was worth noting that CRP and ESR were common markers of inflammation. GA itself was an inflammatory disease, and patients with GA who did not have comorbid MD may be in a more acute or severe inflammatory response, resulting in elevated

CRP and ESR values. Conversely, GA_MD patients may be in a controlled state of chronic metabolic disease with a lower inflammatory response.

Neocosmospora and *Turicibacte* r were positively correlated with TC in RA_MD patients

We evaluated the alpha diversity, and found that the RA_MD group was characterized by an increase in fungal Chao richness and Shannon diversity, in contrast to a decrease in bacterial Chao richness and Shannon diversity compared to the RA group (Fig. 2A, B and Supplementary Fig. 1A, B). Principal coordinate analysis (PCoA) revealed that bacterial community composition among the RA_MD, RA and control groups formed significantly different clusters (Fig. 2C) while, fungi community composition formed significant overlapping clusters (Supplementary Fig. 1C). Among the three groups, dominant fungal phyla were *Ascomycota*, *Basidiomycota*, and *Mucoromycota* (Fig. 2D). The principal bacterial phyla were embraced on *Firmicutes*, *Actinobacteriota*, *Proteobacteria* and *Bacteroidota* (Fig. 2F). Regarding the genus differences, the RA and RA_MD groups had higher abundance of *Candida* and *Blautia* (Fig. 2E), while the abundance of *Aspergillus* and *Faecalibacterium* lower than control (Fig. 2G). In addition, Venn diagrams illustrated more overlapping numbers of fungal and bacterial genus among the three groups (Supplementary Fig. 1D, E). Further, by analyzing differences between RA_MD and RA groups, we identified 4 and 12 significantly differential fungal and bacterial genus respectively (Fig. 2H, I and Supplementary Table 4). Specifically, *Neocosmospora*, *Sebacina*, *Romboutsia* and *Turicibacter* were significantly increased in the RA_MD group compared to the RA group, while *Lycoperdon* and *Veillonella* were significantly decreased. And what's more, 12 functionally predicted metabolic pathways (Supplementary Fig. 1F) were obtained based on the PICRUSt2 tool and correlation clustering analysis was performed with the 12 significantly different bacteria in Fig. 2J. Subsequently, we found that *Veillonella* had the strongest positive correlation with both alpha-Linolenic acid metabolism (ko00592) and lysine degradation (ko00310) pathways. In addition, both *Romboutsia* and *Turicibacter* were significantly positively correlated with the primary bile acid biosynthesis (ko00120) pathway, while *Turicibacter* was also significantly positively correlated with galactose metabolism (ko00052) and starch and sucrose metabolism (ko00500) pathways. Meanwhile, *Turicibacter* and *Neocosmospora* were positively correlated with TC and LDL (Supplementary Fig. 1G). Overall, our findings suggested that the abundance of gut microbiota regulating host TC, cholesterol, and fatty acid metabolism was elevated in RA_MD patients.

Lower levels of unsaturated fatty acids (UFAs) in RA_MD patients

With the nontargeted metabolomics analysis, the plasma metabolome showed a clear separation between RA_MD and RA in OPLS-DA score scatter plots (Fig. 3A, B). We detected 179, 176 and 175 annotated cationic metabolites in the control, RA, and RA_MD groups, respectively, with 224 anionic metabolites in all three groups. Then, we identified 42 significantly differentially expressed metabolites (DEMs) between RA_MD and RA (Supplementary Table 5), 28 upregulated and 14 downregulated (Fig. 3C). Afterwards, KEGG enrichment analysis of this 42 DEMs yielded 18 significant pathways, including 6 metabolic pathways such as synthesis and degradation of ketone bodies, linoleic acid metabolism, fatty acid biosynthesis, glycine, serine and threonine metabolism, glycerophospholipid metabolism and biosynthesis of unsaturated fatty acids (Fig. 3D). Interestingly, among the three-group comparative analyses of control, RA and RA_MD, we observed that there were 6 DEMs that are currently not annotated on any KEGG pathway, of which galactonic acid expression was sequentially elevated, and hexadecanedioic acid, 9-OxoODE, all cis-(6,9,12)-Linolenic acid, N6-Methyl-L-lysine and (4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10, 13,1 6,19-Docosahexaenoic acid (DHA) expressions were sequentially decreased and significantly different (Fig. 3E-H and Supplementary Fig. 2A, B). Therefore, we combined this 6 unannotated DEMs with 11 DEMs from the 6 metabolic pathways for correlation cluster analysis with clinical indicators (Fig. 3I). Moreover, we also found that FFA (Free Fatty Acid) showed significantly positive correlations with UFAs, including oleic acid, 20-HETE, 9-OxoODE ci−9–Palmitoleic acid, erucic acid, all cis-(6,9,12)-Linolenic acid and DHA, as well as significantly negative correlations with L-tryptophan and L-threonine. It was important to note that only a small number of UFAs, such as linoleic acid and trans-2-Hydroxycinnamic acid, were found to be enriched in RA_MD patients. Our findings suggested that the depletion of UFAs (Supplementary Table 5) and N6-Methyl-L-lysine may have a significant impact on the development and regulation of MD in RA patients.

Identification and functional analysis of differentially expressed genes profiles

GSEA revealed that lipid metabolic pathways, namely arachidonic acid metabolism, linoleic acid metabolism and primary bile acid biosynthesis, were significantly upregulated in the RA_MD group (Fig. 4A, B and Supplementary Fig. 3A). In total, 355 differential expressed genes (DEGs) were identified between RA_MD and RA, of which 68 DEGs were up-regulated and 287 DEGs were down-regulated (Supplementary Fig. 3B and

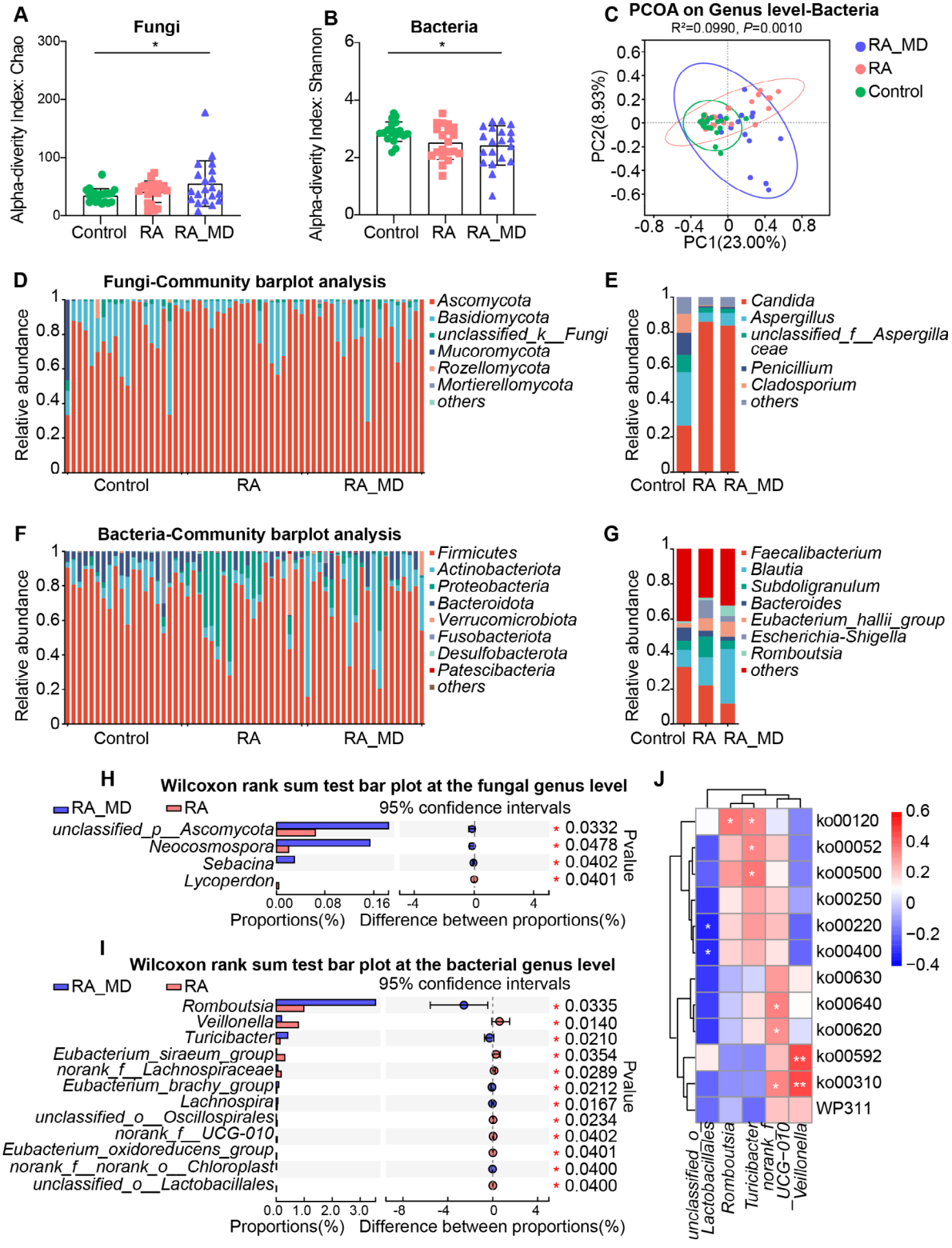

Fig. 2 The alteration of the structural composition and diversity of microbial communities among control, RA and RA_MD. The differences of α-diversity by Kruskal-Wallis H test for Chao index in fungi (**A**) and Shannon index in bacteria (**B**). * $p < 0.05$. (**C**) The Beta diversity of bacteria shown by principal co-ordinates analysis (PCOA) based on Bray Curtis distance analysis. Relative abundance of fungi at phylum (**D**) and genus(**E**) levels. Relative abundance of bacteria at phylum (**F**) and genus(**G**) levels. The Wilcoxon rank-sum test bar plot between RA and RA_MD at the fungal (**H**) and bacterial (**I**) genus levels. Values represented mean and standard error. (**J**) Spearman's correlation heatmap of Ko pathways and significant bacteria at genus level

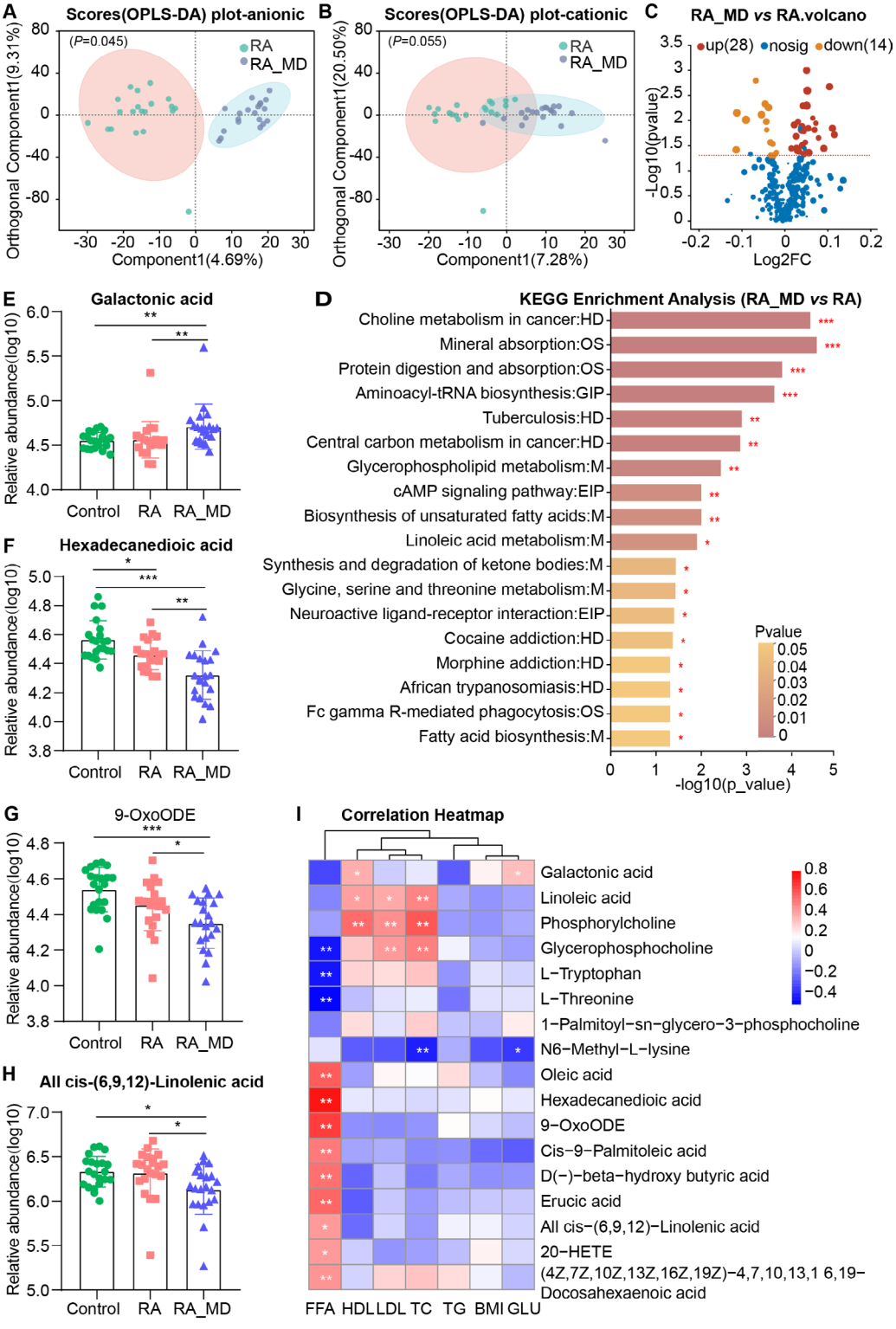

Fig. 3 Down-regulation of unsaturated fatty acids (UFAs) in RA_MD patients. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) displayed the differential metabolites of anionic (**A**) and cationic (**B**) modes between RA_MD and RA. (**C**) Volcano plot showed that the differentially expressed metabolites between RA and RA_MD. (**D**) The KEGG enrichment analysis of 42 significant metabolites between RA_MD and RA. (**E-H**) The relative abundance of galactonic acid, 20-HETE, 9-OxoDE and all cis-(6, 9, 12)-linolenic acid among control, RA and RA_MD. (**I**) Spearman's correlation heatmap of differential metabolites and clinical characteristics

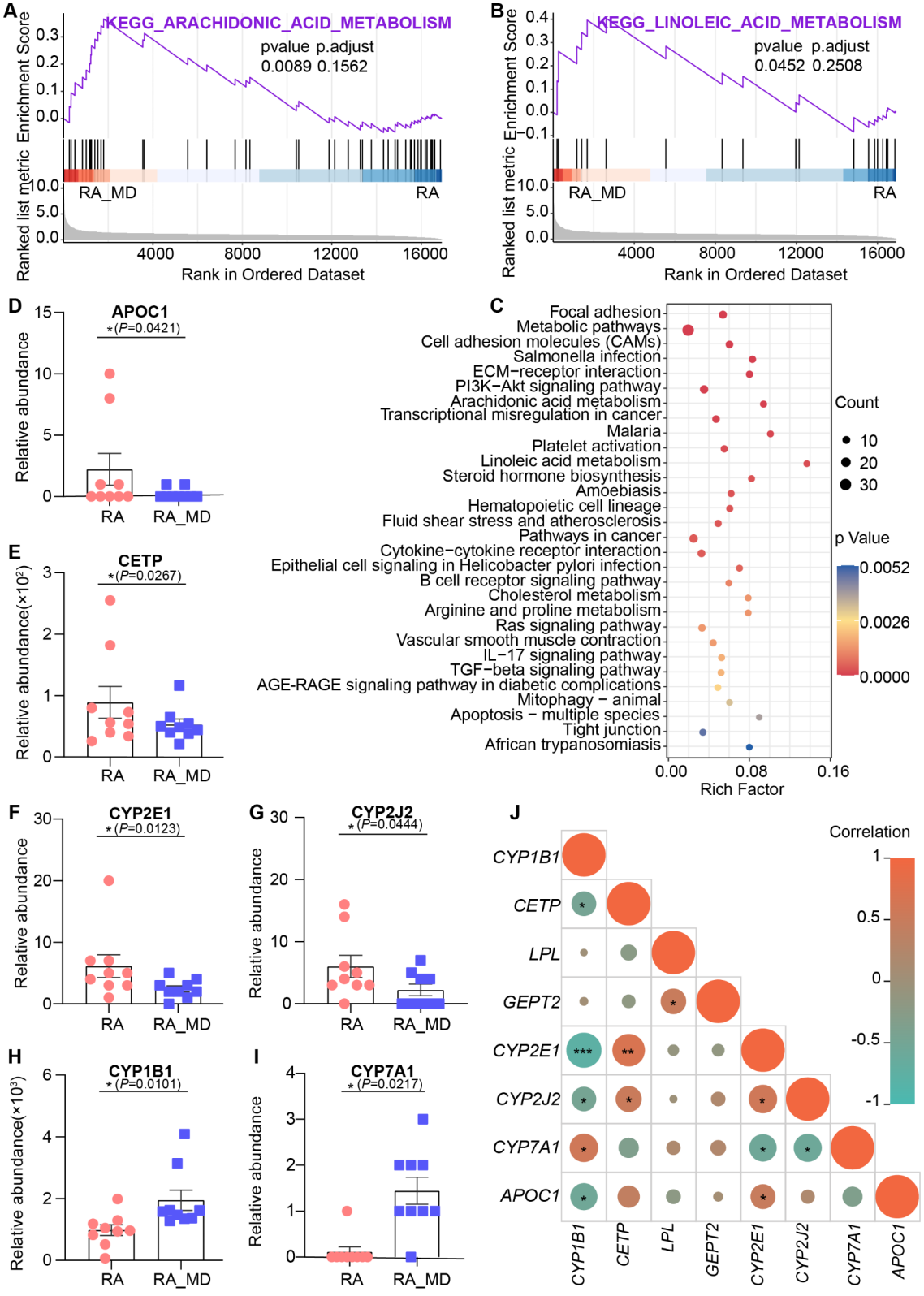

Fig. 4 Identification and functional analysis of differentially expressed genes profiles. (**A, B**) The Gene Set Enrichment Analysis. (**C**) Bubble plot depicted KEGG enrichment analysis of 355 differentially expressed genes. (**D-I**) The relative abundance of 6 significantly expressed genes. (**J**) The correlated relationship of 8 different genes that were associated with metabolic diseases

Supplementary Table 6). Then, KEGG pathway analysis revealed a significant enrichment of genes involved in metabolic pathways, such as arachidonic acid metabolism, linoleic acid metabolism and cholesterol metabolism, which were highly relevant to MD (Fig. 4C). Based on the above metabolic pathway, we identified 8 significantly key DEGs between RA_MD and RA, of which APOC1, CETP, CYP2E1, and CYP2J2 were down-regulated and CYP1B1, CYP7A1, LPL, and GFPT2 were up-regulated in RA_MD (Fig. 4D-I and Supplementary Fig. 3D, E). Additionally, GO enrichment analysis displayed the top 20 functional terms, and we found that the biological functions of the 355 DEGs was also enriched in the metabolic process, namely, long−chain fatty acid biosynthetic process, linoleic acid metabolic process and cholesterol metabolic process (Supplementary Fig. 3C). Correlation analysis of these 8 DEGs revealed a significant correlation between CYP1B1 and the other genes (Fig. 4J). In addition, only APOC1 had a significant positive correlation with CRP (Supplementary Fig. 3F). These results revealed that dysregulation of metabolic pathways, especially lipid metabolic pathways and cholesterol metabolic pathway, play an indispensable role in RA_MD patients.

Combined multi-omics analysis revealed dysregulation of lipid metabolism

Given that all multi-omics data and clinical parameters demonstrated alterations in RA_MD compared to RA, we then assessed whether there were potential associations across multiple data types. We first combined gut microbiota, metabolites and clinical indicators in a correlation network analysis, which showed that linoleic acid (M2) and glycerophosphocholine (M4) were significantly positively correlated with *Romboutsia* (B5) and *Turicibacter* (B6), respectively. While, *Lachnospira* (B7) was negatively correlated with N6-Methyl-L-lysine and oleic acid (M10) that was substantially correlated with several UFAs as well as amino acids (Fig. 5A and Supplementary Table 7). Based on the shared metabolic pathways, we found that both transcriptomics and metabolomics profiles were mainly enriched in lipid metabolic pathways (Fig. 5B). In addition, our LASSO analyses combining microbial and metabolite data yielded less favorable results than analyses using metabolite data only, and we ultimately used 17 DEMs data and identified 4 metabolite biomarkers (M1: 1-Palmitoyl-sn-glycero-3-phosphocholine, M14: hexadecanedioic acid, M3: L−Tryptophan, M11: N6−Methyl−L–lysine), whose diagnostic performance was generally above 0.7 (AUC) and united AUC up to 0.958 (Fig. 5C and Supplementary Fig. 4). After that, we combined proteins, phosphoproteins and genes involved in cholesterol metabolism to perform the PPI network analysis, in which the phosphoprotein APOL1, where the phosphorylation occurs on serine 4 (S4), as well as the APOB protein, played a central role in the network analysis (Fig. 5D and Supplementary Fig. 5). Finally, the molecules obtained from multi-omics and RA_MD subgroups analyses were mainly focused on lipid metabolic pathways, followed by cholesterol metabolism pathways, amino acid metabolism pathways and carbohydrate metabolism pathways (Fig. 5E, Supplementary Fig. 6 and Supplementary Table 8). Our findings indicated that dysregulation of lipid metabolism was predominant in RA_MD and involved in gut microbiota, genes and proteins associated with TC, cholesterol and fatty acid metabolism.

Validation of the specificity of gut microbiota and plasma metabolites in SLE and GA comorbidity groups

To further validate the disease-specific gut microbiota and plasma metabolites in our study of RA_MD vs. RA (G1), we integrated two additional groups of the same complication but different diseases, which comprised GA_MD vs. GA (G2) and SLE_MD vs. SLE (G3). We found that fungal Chao richness remained reduced in the SLE_MD and GA_MD groups and increased in the RA_MD group compared to the groups without MD and control, respectively. At the same time, bacterial Shannon diversity was significantly reduced in the RA_MD, SLE_MD and GA_MD groups compared to the groups without MD and control (Figs. 2A and B and 6A and B). Moreover, the significantly different gut microbiota among theG1, G2 and G3 groups showed smaller overlaps, while, most of the bacterial genera, such as *Peptostreptococcus*, *Intestinimonas* and *Turicibacter*, were from the *Firmicutes*, which was the main producer of short-chain fatty acids (Fig. 6C and Supplementary Fig. 7). At the level of plasma metabolomics, metabolites such as lipids and lipid-like molecules predominated in the G1 and G2 groups, followed by organic acids and derivatives (Fig. 6D). In addition, dysregulation of lipid metabolic pathways showed unique disease-specificity in the G1 group (Fig. 6E).

Discussion

In this study, we demonstrated the significant alterations in gut microbes, plasma metabolite profiles, gene expression profiles, protein and phosphoprotein profiles between RA and RA_MD patients. Meanwhile, combining the multi-omics and clinical information revealed that the key role of lipid metabolic pathway dysregulation in the pathogenesis of RA_MD patients. Finally, the disease uniqueness of lipid metabolic pathway dysregulation in RA_MD was reconfirmed by the comparative analysis of three rheumatic diseases.

It is widely recognized that the gut microbiota is a factor in metabolic homeostasis and the immune system [24,

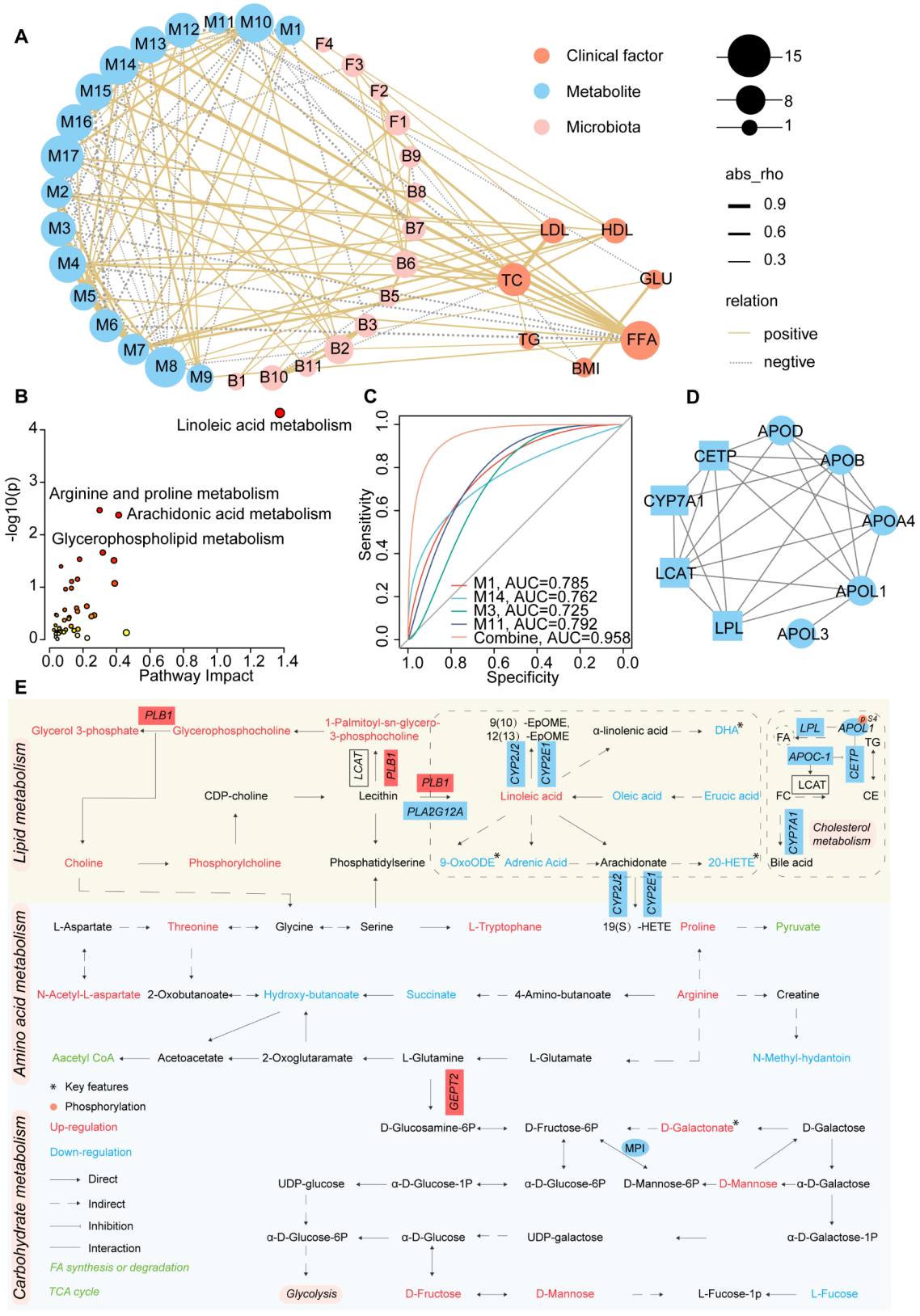

Fig. 5 Combined multi-omics analysis revealed dysregulation of lipid metabolism. (**A**) Correlation network among differential metabolites, gut microbiota and clinical indicators. (**B**) The shared metabolic pathways between metabolomics and transcriptomics. (**C**) The Roc curve of significantly expressed gut microbiota and metabolites. (**D**) Protein-protein interaction (PPI) network map among differential genes, proteins and phosphoproteins involved in the cholesterol metabolism. (**E**) 3 types of metabolism pathways displayed the connections among metabolites, genes, proteins and phosphoproteins

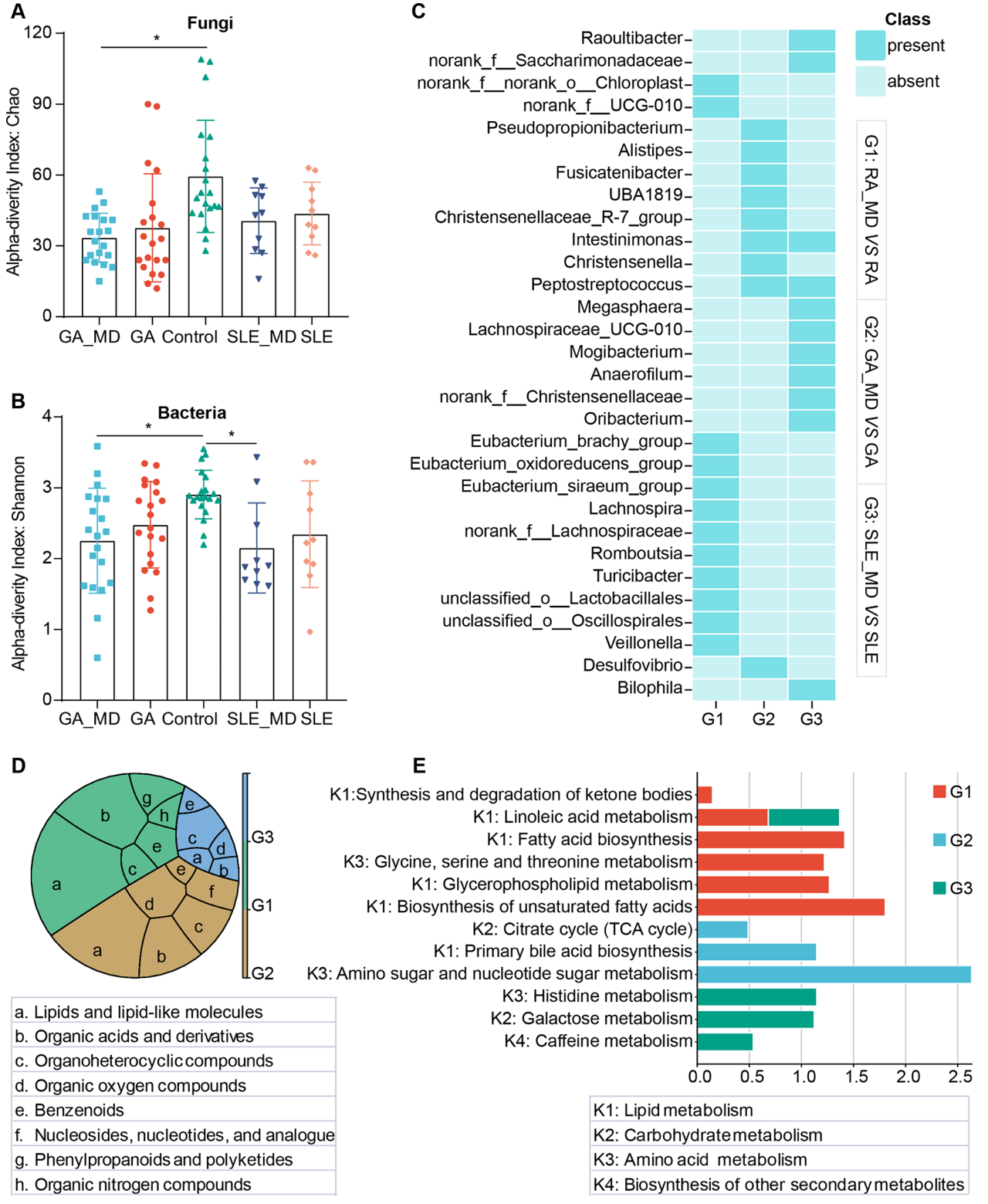

Fig. 6 Specific alterations in gut microbiota and plasma metabolites among GA, SLE and RA. The differences of α-diversity by Kruskal-Wallis H test for Shannon index in fungi (**A**) and Chao in bacteria (**B**) index among control, GA, GA_MD, SLE and SLE_MD groups. (**C**) The plot depicted the composition of the differential bacterial genera in the three types disease comparison groups. G1, RA_MD vs. RA, G2, GA_MD vs. GA, G3, SLE_MD vs. SLE, (**D**) The classification of differential metabolites in G1, G2 and G3 groups. (**E**) Stacked bar charts showed metabolism pathways and classification of differential metabolites enrichment in three groups

25]. The researchers have indicated that the increased abundance of *Candida* and decreased abundance of *Aspergillus* in the feces of RA patients, whose outcome aligns with our own study [26]. It is also reported that the abundance of the probiotics *Faecalibacterium* was decreased in the RA patients compared with control [27]. These results reinforce the notion that alterations in the gut microbiota could be associated with RA.

Notably, *Veillonella*, a producer of lipopolysaccharide and propionic acid that modulates host joint inflammation [28], which has been reported to be significantly more abundant in RA [29], as well as gestational diabetes mellitus with hyperlipidaemia [30], and atherosclerosis patients [31], respectively. In contrast, in our results, *Veillonella* was significantly lower in the RA_MD group, possibly due to differences in dosing profiles [32]. Additionally, we also found that *Turicibacter*, which could modify host bile acids and lipid metabolism [33], was positively correlated with TC, in accordance with a previous study [34]. In fact, our PICRUSt2 analysis demonstrated that *Turicibacter* was positively correlated not only with primary bile acid biosynthesis pathway, but also with galactose metabolism & starch and sucrose metabolism pathways.

Increased tryptophan catabolism is a common metabolic event in chronic inflammatory diseases and may directly affect systemic immunity [35], although plasma tryptophan levels are elevated in patients with type 2 diabetes mellitus [36], elevated tryptophan is enough to delay the increase in postprandial glucose [37]. Thus, lower tryptophan levels in our study may be associated with both rheumatoid arthritis and metabolic diseases. Moreover, studies have reported that fatty acids are associated with chronic inflammation and autoimmune diseases [38, 39]. In particular, UFAs, including oleic acid, 20-HETE and DHA plays an important role in lipid metabolism and anti-inflammation, it is important in the prevention of atherosclerosis, dementia, rheumatoid arthritis, and Alzheimer's disease [40–43]. Previous studies indicate that increased consumption of omega-3 fatty acids (FAs), specially eicosapentaenoic acid (EPA) and DHA, may have a beneficial effect on human health by decreasing pain, disease activity and TG in patients with RA [44, 45]. In addition, Lu et al. [46] found that supplementation with fish oil containing DHA and EPA significantly reduced high triglyceride levels in patients with type 2 diabetes. At the same time, a higher proportion of DHA in the blood is negatively correlated with the prevalence of diabetes mellitus [47] and reduces the risk of cardiovascular disease in patients with type 2 diabetes mellitus [48]. In our findings, compared to RA patients, further reductions of UFAs may be relevant to RA_MD.

In addition, we found that transcriptomics, proteomics, and phosphoproteomics collectively focused on the cholesterol metabolism pathway with downregulated genes like CETP, LPL, APOC1 and CYP7A1, which was associated with atherosclerosis and hypercholesterolemia through regulating the metabolism of TC and TG [49]. Meanwhile, it has reported that CETP expression levels are reduced in RA [50] and diabetic patients [51], and the results of changes in expression levels are consistent with our results and are associated with an increased risk of cardiovascular disease [52]. Thus, our results also suggested that dysregulation of cholesterol metabolism pathway was associated with the development of MD in RA.

Lipid metabolism is an important and complex biological process that includes the digestion, absorption, catabolism and metabolism of lipids such as TG, cholesterol and fatty acids. Long-term TNF inhibitors or methotrexate treatment were associated with increased levels of TC, TG, and APOB/A decreased in RA patients [53, 54]. In our results, the elevated TC and the presence of metabolic disease comorbidities in the RA_MD group may be associated with altered expression levels of DHA, *Turicibacter*, *Neocosmospora*, CETP, LPL, APOB and APOL1 by analysing multi-omics and clinical data.

Since metabolic diseases have a high prevalence in diverse rheumatic diseases [7]. Our study compared three types of rheumatic diseases, RA, SLE, and GA, and found that the significantly differentially expressed gut microbiota identified from RA and RA_MD groups, GA and GA_MD groups, and SLE and SLE_MD groups showed a high disease-specificity. In addition, the proportion of lipid differential metabolites was highest in both RA and GA, but multiple lipid metabolic pathways were significantly enriched only in RA and RA_MD groups, further suggesting that dysregulated of lipid metabolism played a crucial role in RA_MD groups.

It is necessary to acknowledge the limitations of this study. Because this study aimed to investigate the metabolic pathway disturbance in RA_MD groups, we had less explored the potential role of other types of pathways in RA_MD groups, which may neglect several minor pathogenic mechanisms. Additionally, the influence of individual differences including a few confounding factors such as extreme dietary habits and non-rheumatic drugs remains unavoidable. Finally, as a cross-sectional study, the relatively limited sample size of this study may affect the generalizability of this work. Future work will require multi-centre approaches and large sample sizes as well as mechanistic experiments to validate our findings.

Conclusions

In conclusion, we combined a multi-omics analysis approach to reveal the alterations in the gut microbiota, plasma metabolites, mRNA genes, proteins and phosphoproteins of RA_MD patients. This study suggested that down-regulation of the metabolites of UFAs, genes

related to cholesterol metabolism, proteins, and phosphoproteins expression, as well as up-regulation of expression of gut microbiota related to lipid metabolism, were associated with RA_MD patients. Our findings provide a foundation and direction for the prevention and early diagnosis of MD, as well as new insights to complement clinical treatment options.

Abbreviations

RA	Rheumatoid arthritis
MD	Metabolic diseases
RA_MD	Rheumatoid arthritis with metabolic diseases
ITS	Internally transcribed spacer
LASSO	Least Absolute Shrinkage and Selection Operator
AUC	Area under the curve
GA	Gouty arthritis
SLE	Systemic lupus erythematosus
ACR	American College of Rheumatology
EULAR	European League Against Rheumatism
DAS28	Disease activity score of 28 joints
RF	Rheumatoid factor
CRP	C-reactive protein
ESR	Erythrocyte sedimentation rate
IL-6	Interleukin 6
TC	Total cholesterol
TG	Triglyceride
LDL	Low density lipoproteins
HDL	High-density lipoproteins
GLU	Glucose
RA_T2D	Rheumatoid arthritis with type 2 diabetes
RA_HLP	Rheumatoid arthritis with hyperlipidaemia
RA_AS	Rheumatoid arthritis with atherosclerosis
PPI	Protein-protein interaction
QIIME2	Quantitative Insights Into Microbial Ecology 2
PICRUSt2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2
HMDB	Human Metabolome Database
OPLS-DA	Orthogonal Partial Least Squares Discriminant Analysis
VIP	Variable Importance in Projection
KEGG	Kyoto Encyclopedia of Genes and Genomes
PBMCs	Peripheral Blood Mononuclear Cells
GSEA	Gene Set Enrichment Analysis
GO	Gene Ontology
DIA	Data-independent acquisition
PCoA	Principal coordinate analysis
DEMs	Differentially expressed metabolites
DEGs	Differential expressed genes
UFAs	Unsaturated Fatty Acids
FFA	Free Fatty Acid
DHA	(4Z,7Z,10Z,13Z,16Z,19Z)-4,7,10, 13,1 6,19-Docosahexaenoic acid
GA_MD	Gouty arthritis with metabolic diseases
SLE_MD	Systemic lupus erythematosus with metabolic diseases

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Supplementary Material 6

Supplementary Material 7

Supplementary Material 8

Supplementary Material 9

Supplementary Material 10

Supplementary Material 11

Supplementary Material 12

Supplementary Material 13

Supplementary Material 14

Supplementary Material 15

Supplementary Material 16

Supplementary Material 17

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Author contributions
Study conception and design were performed by F. Z, J. Z. and Q. Z. The first draft of the manuscript, data analysis and visualization implemented by X. Z., W.L. and J. Z. and all authors commented on previous versions of the manuscript. C. J., J. C., J.H., J.Z., S.L., L.W., Y.C., J. W. and T.W. carried out material preparation, participant recruitment and data collection. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was conducted in compliance with all relevant national regulations and institutional policies and was approved by the Medical Ethics Review Committee of Dazhou Central Hospital (2021-022). Furthermore, informed consent forms were signed by all participants to ensure their voluntary participation in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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