Stage-specific roles of microbial dysbiosis and metabolic disorders in rheumatoid arthritis

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ABSTRACT
Objective Rheumatoid arthritis (RA) is a progressive disease including four stages, where gut microbiome is associated with pathogenesis. We aimed to investigate stage-specific roles of microbial dysbiosis and metabolic disorders in RA.

Methods We investigated stage-based profiles of faecal metagenome and plasma metabolome of 76 individuals with RA grouped into four stages (stages I–IV) according to 2010 RA classification criteria, 19 individuals with osteoarthritis and 27 healthy individuals. To verify bacterial invasion of joint synovial fluid, 165 rRNA gene sequencing, bacterial isolation and scanning electron microscopy were conducted on another validation cohort of 271 patients from four RA stages.

Results First, depletion of Bacteroides uniformis and Bacteroides plebeius weakened glycosaminoglycan metabolism (p<0.001), continuously hurting articular cartilage across four stages. Second, elevation of Escherichia coli enhanced arginine succinyltransferase pathway in the stage II and stage III (p<0.001), which was correlated with the increase of the rheumatoid factor (p=1.35×10⁻³) and could induce bone loss. Third, abnormally high levels of methoxyacetic acid (p=1.28×10⁻⁸) and cysteine-S-sulfate (p=4.66×10⁻¹²) inhibited osteoblasts in the stage II and enhanced osteoclasts in the stage III, respectively, promoting bone erosion. Fourth, continuous increase of gut permeability may induce gut microbial invasion of the joint synovial fluid in the stage IV.

Conclusions Clinical microbial intervention should consider the RA stage, where microbial dysbiosis and metabolic disorders present distinct patterns and played stage-specific roles. Our work provides a new insight in understanding gut–joint axis from a perspective of stages, which opens up new avenues for RA prognosis and therapy.

INTRODUCTION
Rheumatoid arthritis (RA) affects over tens of millions of people worldwide. RA is recognised clinically as a progressive, inflammatory and autoimmune disease that primarily affects the joints and typically has four stages: (1) In the first stage, the synovium of the joints is inflamed and most people have minor symptoms such as stiffness on awakening; (2) In the second stage, the inflamed synovium has caused damage to the joint cartilage and people begin to feel swelling, and have a restricted range of motion; (3) In the third stage, RA has proceeded to a severe state when bone erosion begins and the cartilage on the surface of the bones has deteriorated, resulting in the bones rubbing against one another and (4) In the fourth stage, certain joints are severely deformed and lose function. To inhibit RA progression, specific therapeutic strategies are necessary for people across different RA stages.

Gut microbial dysbiosis has been implicated in the pathogenesis of RA via a range of mechanisms such as metabolic perturbation and immune response regulation, which is known as the gut–joint axis, for instance, increased abundance of Prevotella and Collinsella in patients with RA are correlated with the production of T17 cell cytokines. Moreover, Gut microbes and their products were likely to be transited to the joint due to the increased gut permeability. Metabolites have also been correlated to immunity regulation in RA: administration of Short-chain fatty acids to mice with collagen-induced arthritis (CIA) can reduce the severity of arthritis by modulation of IL-10. Comprehensive metagenomic and metabolomic analyses could
therefore enhance our understanding about the gut–joint axis. However, the role of the gut–joint axis across successive stages of RA is understudied, where more examinations may provide an alternative approach to ameliorate RA progression.

Here, we aimed to investigate the stage-based profiles and roles of the gut–joint axis in RA pathogenesis, and whether or in which stage gut microbial invasion of the joint synovial fluid happened.

### MATERIALS AND METHODS

#### Study design and sample collection

Data collection for this multimomics study was conducted in The First Affiliated Hospital of Shandong First Medical University (Jinan, Shandong, China), which was a provincial-level large-scale comprehensive tertiary first-class hospital and had tens of thousands of outpatients with arthritis per year. A total of 122 faecal and 122 plasma samples were collected from 122 outpatients of the The First Affiliated Hospital of Shandong First Medical University from 2017 to 2020. These outpatients included 76 patients with RA, 19 patients with OA and 27 healthy individuals (table 1). Patients with RA were grouped into four RA stages including RAS1 (n=15), RAS2 (n=21), RAS3 (n=18) and RAS4 (n=22) according to the rheumatoid diagnostic score, where RAS1, RAS2, RAS3 and RAS4 has a score of 6–7, 8, 9 and 10, respectively. The score was evaluated by the sum of four categories as summarised in the 2010 RA classification criteria.

#### Metagenomic sequencing and processing to analyse the faecal microbiome

Whole-genome shotgun sequencing and processing of faecal samples, non-redundant gene catalogue construction, identification of metagenomic species (MGS), functional annotation to Kyoto Encyclopaedia of Genes and Genomes (KEGG) were performed (details in online supplemental text). Two parallel processes were used for gut metagenomic data analysis: One was based on 4 million non-redundant genes and investigated the functional composition across RA stages and OA, as well as the MGS that most drove the correlation of these microbial functions with RA or OA and (2) The other reported the 232 classified microbial species composition across RA stages and OA, profiled by MetaPhlAn2 (V.2.7.8).

#### UHPLC-QTOF-mass spectrometry analysis of plasma metabolites

Untargeted plasma metabolome was examined by ultra-performance liquid chromatography-quadrupole time-of-flight (UHPLC-QTOF) mass spectrometry: liquid chromatography with tandem mass spectrometry on an UHPLC system (1290, Agilent Technologies) with a UPLC BEH Amide column (1.7 μm, 2.1×100 mm, Waters) coupled to TripleTOF 6600 (Q-TOF, AB Sciex) and QTOF 6550 (Agilent) (details in online supplemental text).  

#### 16S rRNA gene sequencing and processing to analyse the synovial fluid microbiota

Bacterial DNA was extracted from 271 5 mL synovial fluid samples. The tube containing PBS serves as environmental control.
control. Only a total of 86 synovial fluid samples from patients in RAS4 had enough bacterial DNA content (≥10 ng) (Bacterial DNA Kit, TIANGEN) for bacteria 16S rRNA gene high-throughput sequencing. The V1/V2 hypervariable regions of the 16S ribosomal RNA gene were sequenced using the Illumina HiSeq platform. The 16S sequence paired-end data set was joined and quality filtered using the FLASH as previously described. Taxonomic annotation was then performed (details in online supplemental text).

**Bacterial isolation and scanning electron microscopy**

Six synovial fluid samples (1 mL) per RA stage were used for bacteria isolation, and the obtained isolated colonies were identified using 16S rRNA gene sequencing (details in online supplemental text). For the samples from which bacterial can be isolated, synovial fluid samples (1 mL) of the same individuals were then filtered and imaged with scanning electron microscope (ZEISS Sigma 300, details in online supplemental text).

**Statistical analysis**

Samples were divided into three groups including the healthy group, the OA group and the RA group. Samples of the RA group were further divided into four subgroups including RAS1, RAS2, RAS3, RAS4. For comparisons of vectors across groups or subgroups, such as microbial species abundance, KO abundance, metabolite intensity. Mann-Whitney-Wilcoxon test (p values) with Benjamini and Hochberg correction (q values) was used to test the significance. A threshold for statistical significance was p<0.05, and for multiple testing the threshold was p<0.05 and q<0.1.

For correlations between KEGG modules and clinical phenotypes including arthritis (healthy=0, OA=1, RA=2), cytokine levels and rheumatic factor level, owing to that a KEGG module contained multiple KOs, Spearman correlation coefficients (SCC) between abundances of KOs and clinical phenotypes were first calculated. Subsequently, Mann-Whitney-Wilcoxon test (p values) with Benjamini and Hochberg correction (q values) was used to test if SCC between the KOs in a given KEGG module and phenotypes were different from that between all the other KOs out of the KEGG module and phenotypes. In this process, the KEGG module with statistical significance was viewed as significantly correlated with the clinical phenotypes. A threshold for statistical significance was p<0.05 and q<0.1. Considering that sex and age might have potential effects on gut microbiome, partial SCCs with age and gender adjusted were also calculated and compared, and a threshold for statistical significance was p_{partial}<0.05 and q_{partial}<0.1.

Leave-one-out analysis was used to test which MGS was driving the observed correlations between KEGG modules and arthritis. Owing to that one MGS contained multiple genes that were mapped to KOs, if one MGS was excluded in the dataset, the overall profiles of the KO abundance would change, resulting in the change of the correlations between KEGG modules and arthritis. Therefore, to determine the driving effects of each of MGS, the calculation of the KO abundance was iterated excluding the genes from a different MGS in each iteration, and the correlations between each KEGG module and arthritis were recalculated. Finally, the driving effects of a given MGS on a specified correlation was defined as the change in median SCC between KOs and arthritis when genes from the respective MGS were left out.

To determine the diagnostic potential of RA stages using multomics features, random forest algorithm was performed on 6,224 KOs, 232 microbial species and 277 plasma metabolites, using the R package ‘randomForest’. Function ‘trainControl’ in R package ‘caret’ was used to perform 10 repeats of 10-fold cross-validation for each data set. Function ‘train’ in R package ‘caret’ was used to fit models over different tuning parameters to determine the ‘mtry’ for random forest algorithm. Gini coefficients were used to measure how each variable contributed to the homogeneity of the nodes and leaves in the resulting random forest.

**RESULTS**

**Stage-specific microbial taxonomic profiles**

We obtained a total of 231 classified microbial species from metagenomic data, and tested their alterations in each stage of RA, as compared with healthy controls (see online supplemental figure S1, table S1–S5). The elevated species in RA progression were mostly from the phyla Firmicutes and Actinobacteria, while the depleted species were predominantly from the phylum Bacteroides (q<0.1). We found certain microbes did not remain altered across RA stages, as compared with healthy controls. Bifidobacterium dentium, for instance, was reported to be associated with the development of dental caries and periodontal disease, both of which were particularly prevalent in patients with RA. Compared with healthy controls, it remained elevated across RA stages except for RAS1 (RAS2: p=7.16×10⁻³, RAS3: p=3.70×10⁻³, RAS4: p=9.15×10⁻⁴). Moreover, we noticed that 29 species that were altered exclusively in a specific stage (see online supplemental table S1–S5). We found that Collinsella aerofaciens was elevated exclusively in RAS1 (p=0.043). C. aerofaciens was previously reported to generate severe arthritis when inoculated into CIA-susceptible mice, and in vitro experiment showed that C. aerofaciens could increase gut permeability and induce IL-17A expression, a key cytokine involved in RA pathogenesis. The elevation of C. aerofaciens in RAS1 might contribute to the early breach in gut barrier integrity, through which the translocation of microbial products would then trigger the subsequent clinical arthritis. Moreover, Veillonella parvula, whose infection could cause osteomyelitis, was found elevated exclusively in RAS3 (p=0.027). Eggerthella lenta (p=0.018) and Bifidobacterium longum (p=0.022) were found elevated exclusively in RAS4. The gavage of E. lenta were reported to increase gut permeability and produce proinflammatory cytokines. We also recognised species altered exclusively in OA, such as elevated Dialister invisus (p=0.041) that was positively correlated with spondyloarthritis severity. These stage-specific altered species had the potential to serve as the targets for intervention in a given RA stage.

**Stage-specific microbial functional profiles**

Next, we sought to detect the microbial dysfunction across stages of RA. We grouped 4047645 metagenomic genes into 6,224 KOs and 404 KEGG modules. We identified 12 KEGG modules that were significantly correlated with RA or OA (q<0.1 or q_{par-}tal <0.1, see online supplemental figure S2) and presented their variation across stages (figure 1A). We then used leave-one-out analysis to identify the MGS that most drove the correlations of these KEGG modules with RA or OA (figure 1B, online supplemental figure S3).

We found an evident decrease in glycosaminoglycan (CAG) metabolism across four RA stages and OA. It was mainly reflected by the significant decrease in K01197 (hyaluronoglucosaminidase) of dermatan sulfate (DS) degradation and the significant decrease in K10532 (heparan-alpha-glucosaminide
N-acetyltransferase) of heparan sulfate (HS) degradation (p<0.05, figure 1A). Chondroitin 4-sulfate is a major component of the extracellular matrix of many connective tissues, such as cartilage, bone and skin. We found that the significant depletion of DS degradation would inhibit the production of chondroitin 4-sulfate (figure 1B), which might hurt the mechanical properties of the articular cartilage. Moreover, the significant depletion of HS degradation might be a potential cause of the higher plasma level of HS observed in RA and OA patients, which could promote arthritis progression by regulating protease activity. The most driving species of DS degradation and HS degradation were MGS Bacteroides uniformis and MGS Bacteroides plebeius, respectively. The genes of MGS B. uniformis related to K01197 were found most depleted in RAS2, while the genes of MGS B. plebeius related to K10532 were found most depleted in RAS3 and RAS4 (figure 1B). These results indicated that the depleted microbial function in DS degradation and HS degradation driven by B. uniformis and B. plebeius.
plebeius, respectively, could promote RA and OA in a way of hurting articular cartilage.

We also identified elevated microbial functions that were related to inflammation such as the previously reported ascorbate degradation. Here, we found most of the KOs related to ascorbate degradation retained a higher level across RA stages and OA, especially in RAS2 and RAS3 (p<0.05, figure 1A). Genes of K02821 (phosphotransferase system) in RAS1, K03475 (phosphotransferase system), K03476 (L-ascorbate 6-phosphate lactonase), and K03479 (L-ribulose-5-phosphate 3-epimerase) were mostly driven by MGS Escherichia coli (figure 1B). The enhanced ascorbate degradation might contribute to the deficiency of the ascorbate reported in patients with RA and enhanced ascorbate degradation might contribute to the deficiency of the ascorbate reported in patients with RA and OA.

Moreover, we could recognize Enterococcus gallinarum strain, and three were identified as Clostridium sporogenes strain, and three were identified as Enterococcus gallinarum strain, and one was identified as Enterococcus faecalis strain (see online supplemental table S8). Interestingly, Enterococcus gallinarum and Citrobacter freundii could also be detected in faecal metagenomic data of 18% of patients with RA. We subsequently observed the corresponding synovial fluid samples, using scanning electron microscopy, and found substances shaped like bacteria in rod-like or spherical forms (figure 2). Taken together, this multifaceted investigation has provided unprecedented evidence to support the existence of microbial invasion of the joints in the fourth stage of RA.

Microbial invasion of the joint synovial fluid
Next, we investigated whether or in which stage microbial invasion of the joint synovial fluid happened. Enhanced gut permeability may render it possible for microbes and their products to translocate, triggering an immune response. For other elevated microbial functions, the trans-cinnamate degradation driven by MGS E. coli, where most KOs were notably elevated in RAS2, was also correlated with multiple cytokines (q<0.1 or qpartial <0.1, see online supplemental table S6), such as IL-1β (p=5.44×10^{-5}), TNF-α (p=6.59×10^{-5}) and IL-6 (p=1.12×10^{-5}). Moreover, to confirm the effects of ascorbate on RA progression, we examined the plasma TNF-α level and IL-6 level, bone CT scans, and bone density of (1) normal DBA/1 mice, (2) DBA/1 mice with CIA and (3) DBA/1 mice with CIA and gavage of ascorbate. We found that the 3-month gavage of ascorbate to CIA mice can prevent the increase of TNF-α and IL-6 levels by half, inhibit bone destruction, and maintain bone density (1.58±0.0034 g/cm³), as compared with the CIA mice without ascorbate (1.53±0.013 g/cm³), and the normal group (1.61±0.021 g/cm³, see online supplemental figure S4).

Stage-specific metabolomic profiles
We then introduced metabolomic data, and performed a random forest algorithm on 232 microbiome species, 6224 KOs and 277 metabolites to test their diagnostic potential for each stage of RA and OA (figure 3A–E). Metabolites exhibited the best area under the receiver operating characteristic curve (AUROC) in discriminant samples of four RA stages or OA from healthy samples, with AUROC ranging from 0.974 to 0.998. Other characteristics at the species and KO levels exhibited weaker discriminant ability, with AUROC ranging from 0.760 to 0.838 and from 0.799 to 0.852, respectively. The most prominent changes in metabolites were the significant increase of DL-lactate and gly-glu in RAS1 (p=2.15×10^{-4}, p=4.70×10^{-4}), the decrease of...
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N,N-dimethylaniline and the increase of methoxyacetic acid in RAS2 (p=4.60×10^{-8}, p=1.28×10^{-8}) in RAS3, the increase of galactinol and 3α-mannobiose in RAS4 (p=5.71×10^{-5}, p=5.68×10^{-4}), and the decrease of N,N-dimethylaniline and increase of gly-glu (p=2.00×10^{-7}, p=1.74×10^{-4}) in OA, as compared with a healthy state. The predominant metabolic disorders implicated a critical involvement in pathogenesis and a great diagnostic potential for RA stages.

Moreover, metabolic disorders could distinguish a given RA stage from not just healthy controls but also other RA stages or OA (figure 3F): Methoxyacetic acid in RAS2 (p=1.68×10^{-4}) or cysteine-S-sulfate in RAS3 (p=2.42×10^{-4}) or Galactinol and 3α-mannobiose in RAS4 (p=9.37×10^{-4}, p=4.89×10^{-3}), respectively, was higher than that in all the other RA stages and OA. Methoxyacetic acid was reported to have inhibitory effects on osteoblasts and could cause reductions in bone marrow cellularity. Additionally, cysteine-S-sulfate was a structural analogue of glutamate, acting as an agonist of N-methyl-D-aspartate receptor (NMDA-R) whose expression and function in osteoclasts engaged in bone resorption. Therefore, notable elevations of methoxyacetic acid in RAS2 might hinder osteoblasts, whereas notable elevations of cysteine-S-sulfate in RAS3 might encourage osteoclasts. The imbalance between osteoblasts and osteoclasts would promote the bone erosion that occurred clinically in the third stage of RA. Moreover, DL-lactate in OA was less than that in all RA stages (p=0.037), which might improve clinical differentiation of early RA from OA.

DISCUSSION

Our findings reveal dynamic shifts in gut microbiome and plasma metabolome, and their continuous roles in pathogenesis of RA across four successive stages (figure 4). Moreover, we demonstrate that microbial invasion of the joint synovial fluid happens in the fourth stage of RA.
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The samples used for this study can fairly represent gut microbiome of each RA stage. Our hospital had tens of thousands of outpatients with arthritis per year and we have kept collecting samples from patients diagnosed with each stage of RA from 2017 to 2020. Considering the potential effects of clinical intervention on gut microbiome, in this study, we only recruited samples of those patients who had not received treatment within 1 month and were in the active period. Therefore, the microbial dysbiosis and metabolic disorders depicted here could serve as a profound reference for future studies in each stage of RA.

Clinical microbial intervention should take into account the stage of RA. We found each RA stage had its special elevated or depleted microbes that played a role in RA pathogenesis (figure 4A). Hence, it may not be adequate for clinical guidance to generally report microbial alterations in RA without information of the stage, as many studies have done. For instance, early inhibition of C. aerofaciens that was elevated exclusively in the first stage could help prevent increasing of gut permeability. Additionally, inhibition of E. coli in the second and third stage could help maintain the content of L-arginine that acted as an inhibitor of bone lose, as well as the content of anti-inflammatory ascorbate. Moreover, certain species may need intervention across stages owing to its depletion during the whole RA progression. A cross-stages restoration of B. uniformis could help maintain the content of chondroitin 4-sulfate to keep mechanical properties of the articular cartilage.

Moreover, metabolic alterations kept considerable throughout RA progression, in spite of which we found that certain of these metabolites need a higher priority of intervention in a specific stage. In the second stage of RA, the aberrant elevation of methoxyacetic acid might have inhibitory effects on osteoblasts and cause reductions in bone marrow cellularity (figure 4B). The inhibited osteoblasts then drew the foreshadowing for the bone erosion that happened in the next stage. In the third stage, the considerable elevation of cysteine-S-sulfate might enhance the osteoclasts by NMDA-R interaction. The imbalance between osteoblasts and osteoclasts would then promote bone erosion that happened in the third stage and persisted in the late RA stages. Thus, methoxyacetic acid may be a targeted metabolite for treatment to patients in the second stage of RA and serve as a precaution against the upcoming third stage.

Our findings suggested that bacterial invasion of joint synovial fluid happened in the fourth stage of RA (figure 4B). Joint synovial fluid was generally considered sterile, and indeed, we failed to either extract enough DNA or isolate bacteria from the synovial fluid in the first three stages. However, in the

Figure 4 Potential pathogenesis across successive RA stages from multiomics perspective. (A) Potential mechanisms by which gut microbial dysbiosis play roles in RA pathogenesis through hurting bone tissue and increasing inflammation. The driving species, microbial dysfunction and related metabolites were extracted from figure 1B. The red or blue box of metabolites represents their speculated elevation or depletion according to the KEGG map. The dotted line represents the speculated effects of microbial and metabolic variation on arthritis pathogenesis. (B) The most representative effects of microbial dysbiosis and metabolic disorders on RA progression across successive stages. KEGG, Kyoto Encyclopaedia of Genes and Genomes; RA, rheumatoid arthritis.
fourth stage, we succeeded to obtain bacterial 16S reads, isolate bacteria and observe substances shaped like bacteria in rod-like or spherical forms under scanning electron microscopy. Moreover, in the multomics cohort, we found two facal microbes elevated exclusively in the fourth stage of RA, E. lenta and B. longum, and their existence in the joint synovial fluid was validated by the other cohort. It might due to the buildup of the continuous damages in gut barrier and microbes and microbial metabolites would then be transferred to the joints via blood.5
Hence, for patients in the fourth stage of RA, in addition to routine medical therapies, specific treatments to the microbes in the joint synovial fluid may ameliorate the joint micro-environment to decrease synovial inflammation and inhibit potential bacterial effects.

This study also has limitations and prospects. First, a long-term follow-up investigation on a single individual throughout his/her RA development may reinforce the conclusions of this study. Second, it remains unclear how bacterial genetic materials his/her RA development may reinforce the conclusions of this study. Secondly, it remains unclear how bacterial genetic materials his/her RA development may reinforce the conclusions of this study. Additionally, researches into the synovial fluid microbiome and metabolome may clarify the potential role of the joint microbiome to decrease synovial inflammation and inhibit potential bacterial effects.

In conclusion, this study demonstrates microbial and metabolic roles in RA pathogenesis across four successive stages. A stage-specific intervention of microbial dysbiosis and microbial metabolites is warranted for prognosis and prevention of RA.

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Data availability statement Data are available in a public, open access repository. Whole-genome shotgun sequencing data are available in the Genome Sequence Archive (GSA) section of the National Genomics Data Center (project accession number CRA004348). 16S rRNA gene sequencing data are available in the Genome Sequence Archive (GSA) section of the National Genomics Data Center (project accession number CRA055811). Plasma metabolomic data are available in the MetaboLights (project accession number MTBLS5297).

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