Hidden link in gut–joint axis: gut microbes promote rheumatoid arthritis at early stage by enhancing ascorbate degradation

With great interest, we read the review article by Agus et al, which suggested that gut microbiome alterations could affect metabolic homeostasis. Moreover, gut microbiome alterations in concert with metabolites perturbation could contribute to the early development of rheumatoid arthritis (RA). We thus conducted a three-pronged association study on multiomics datasets to detect the potential microbiome–metabolites–arthritis link.

We integrated multiomics datasets including gut metagenomics, clinical phenotypes and metabolites of blood and knee-joint synovial fluid from 122 participants in the healthy group (n=27), osteoarthritis (OA) group (n=19) and RA group (n=76), using a three-pronged association framework (figure 1, online supplemental material). Metagenomic genes were collapsed into metagenomic species (MGS) and grouped into KEGG functional modules (figure 1A). Additionally, the co-abundant metabolites were categorised into metabolite clusters using WGCNA framework (figure 1A). The functional modules associated with clinical phenotypes (eg, types of arthritis and levels of cytokines) were further identified and the cross-domain associations between these modules and metabolite clusters were assessed (figure 1B). Furthermore, the leave-one-out analysis was performed to determine the MGS that particularly contributed to the observed linkage between functional modules and clinical phenotypes (figure 1C).

We found that gut microbial functionality in ascorbate degradation (KEGG module: M00550) was positively correlated with the types of arthritis (healthy=0, OA=1, RA=2), pWilk=2.15×10−4 and the levels of proinflammatory cytokines TNF-α (tumour necrosis factor-α), pWilk=6.59×10−4 and IL-6 (interleukin-6), pWilk=1.12×10−4). Ascorbate (vitamin C) was previously reported to prevent the development of inflammatory arthritis, possibly through facilitating collagen synthesis, moderating autoimmune responses and ameliorating inflammation. Additionally, the patients with RA are usually ascorbate deficient and require high-dose supplementation to maintain an acceptable plasma level of ascorbate. In this study, the functional module of ascorbate degradation was observed to positively correlate with the blood metabolite cluster MB02 (pWilk=6.90×10−3), which was represented by the level of palmitic acid (kME (eigengene-based connectivity) = 0.911, kDN (intramodular connectivity) = 3.46, online supplemental table 1) that acts as a proinflammatory factor, upregulating IL-6 secretion by human chondrocytes and fibroblast-like synovial cells in inflammatory arthritis. Furthermore, we found that

Figure 1 Overview of the three-pronged association framework integrating multiomics datasets. (A) Metabolites are summarised as co-abundance clusters, and microbial genes are grouped into KEGG modules and MGS, which are further filtered for statistically positive or negative associations (based on spearman correlation) with the clinical phenotypes. The association analyses were divided by using healthy, OA and RA samples for arthritis types and using OA and RA samples for cytokine levels. The number in brackets represent the number of metabolites/microbial genes/KEGG modules/MGS in each analytical module. (B) The filtered features are further used for cross-domain association analyses. For each analysis, the left panel shows the significant associations (Mann-Whitney U test FDR<0.1) between KEGG modules and clinical phenotypes, and colour indicates significantly positive association (red), significantly negative association (blue) or insignificant association (grey). The right panel shows the associations between KEGG modules and metabolite clusters, and the colour represents the median Spearman correlation coefficient (SCC) of metabolite clusters with KEGG orthologies (KO) in KEGG module minus those with KO not in KEGG module. Mann-Whitney U test FDRs are denoted: *FDR<0.1; **FDR<0.01; ***FDR<0.001. (C) The MGS that particularly contributed to the observed linkage between functional modules and clinical phenotypes. Three density plots: Dashed line represents the median SCC of the phenotypes with KOs in M00550 (red) and all other KOs (blue). Density plot shows the median SCC of the phenotypes with KOs in M00550, when a given MGS (indicated by short vertical lines) has been excluded from the analysis. The bottom-left dot plots show the mean±SEM of the top three driving MGS abundances among patients at each stage of disease development, with the four RA stages connected to display the variance. FDR, false discovery rate; IL-6, interleukin-6; MGS, metagenomic species; OA, osteoarthritis; RA, rheumatoid arthritis; TNF-α, tumour necrosis factor-α.
Escherichia coli and Streptococcus bovis were the driving species for the observed linkage between ascorbate degradation and the arthritis types or the cytokines levels of TNF-α and IL-6 (figure 1C). Subsequently, we grouped patients with RA by four stages according to the comprehensive scores in rheumatoid diagnostic criteria, as RASI: 6–7, RASII: 8, RASIII: 9 and RASIV: 10 (online supplemental table 2). We observed that both E. coli and S. bovis were prevalent at RA stage I (RASI), while S. bovis was depleted after RASI or in the OA group. It suggested S. bovis mainly functioned at the early stage of RA, while E. coli might be crucial throughout the entire developmental stages of RA and OA. Taken together, we speculate that E. coli and S. bovis could facilitate ascorbate degradation and thus promote proinflammatory responses that facilitate the development of inflammatory arthritis.

Overall, we demonstrate that gut microbiota could promote RA progression via enhancing ascorbate degradation and provide a potential approach to prevent the development of arthritis through interfering gut–joint axis. The results of this study could be prospected in following contexts: First, our study provides a reservoir of the potential microbiome–metabolites–arthritis links as a reference of gut–joint axis for future studies. Second, the findings supplement the potential mechanisms related to metabolic perturbation through which gut microbiome promotes arthritis. Third, considering the inflammatory pathways of arthritis were revisited in COVID-19, it deserves further investigations whether microbiome–ascorbate–inflammation link of this study could contribute to the treatment of COVID-19.

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Contributors YZ and MC designed the study, conducted the data analysis and wrote the manuscript. YZ, MC, LZ, LY, CZ, YZ and XC collected the samples, conducted the experiments and participated in data analysis. LZ, KN and JH supervised the study and revised the manuscript.

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Supplementary information

A hidden link in gut-joint axis: Gut microbes promote rheumatoid arthritis at early stage by enhancing ascorbate degradation

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Sample description

A total of 122 fecal and 122 serum samples were collected from 122 outpatients from the Shandong Provincial Qianfoshan Hospital (Jinan, Shandong, China). These outpatients included 27 healthy individuals, 19 patients with osteoarthritis (OA), and 76 patients with rheumatoid arthritis (RA). Subsequently, the fecal samples were sequenced and the serum samples were used to examine serum metabolites and inflammatory cytokines. Serum inflammatory cytokines TNF-α and IL-6 were quantified by the MESO SCALE DISCOVERY (MSD®) Quick Plex S600MM multiplex assay. The cytokine levels of healthy individuals were extremely low and not available. In addition, 95 knee-joint synovial fluid samples were collected from the RA and OA patients to examine synovial fluid metabolites. Both serum and synovial fluid metabolites were examined by UHPLC-MS/MS.

All of the participants were at fasting status during sample collection in the morning. The participants were recruited in this study following the standards shown below:

1. Healthy individuals in good health condition with no gastrointestinal diseases, such as diarrhea, constipation, and hematochezia, in the recent one month, no hepatobiliary system diseases, no history of gastrointestinal tumors or inflammatory diseases, no serious heart, liver, kidney, lung, brain or other organ disorders, no infections, chronic diseases, or antibiotic treatment;
2. Healthy individuals had not taken any acid inhibitors, gastrointestinal motility drugs, antibiotics, or living bacteria products such as yogurt in the recent one month;
3. Healthy individuals with no history or family history of mental illness, and no history of gastrointestinal surgery;
4. RA/OA individuals with no other co-morbidity.

Metagenome sequencing and data processing

Whole-genome shot-gun sequencing of fecal samples were carried out on the Illumina Hiseq X Ten. All samples were paired-end sequenced with a 150-bp read length. After
quality control, the paired-end reads were assembled into contigs using MEGAHIT (version 1.2.6) with the minimum contig length set at 500 bp. The open reading frames (ORFs) were predicted from the assembled contigs using Prodigal (version 2.6.3) with default parameters. The ORFs of <100 bp were removed. The ORFs were then clustered to remove redundancy using Cd-hit (version 4.6.6) with a sequence identity threshold set at 0.95 and the alignment coverage set at 0.9, which resulted in a catalog of 4,047,645 non-redundant genes. The non-redundant genes were then collapsed into metagenomic species (MGS) and grouped into KEGG functional modules.

Identification of MGS

High-quality reads were mapped to the catalog of non-redundant genes using Bowtie 2 (version 2.2.9) with default parameters. The abundance profile for each catalogue gene was calculated as the sum of uniquely mapped sequence reads, using 19M sequence reads per sample (downsized). The co-abundance clustering of the 4,047,645 genes was performed using canopy algorithm (http://git.dworzynski.eu/mgs-canopy-algorithm), and 553 gene clusters that met the previously described criteria and contained more than 700 genes were referred to as MGS. MGS present in at least 4 samples were used for the following analysis. The abundance profiles of MGS were determined as the medium gene abundance throughout the samples. MGS were taxonomically annotated as described by Nielsen et al. and each MGS gene was annotated by sequence similarity to NCBI bacterial genome (BLASTN, E-value < 0.001)

Annotation of KEGG modules

The catalog of the non-redundant genes was functionally annotated to KEGG database (release 94.0) by KofamKOALA (version 1.3.0). The produced KEGG Orthologies (KOs) were mapped to the KEGG modules annotation downloaded on August 1, 2020 from the KEGG BRITE database. KOs present in at least 4 samples were used for the following analysis. The KO abundance profile was calculated by summing the
abundances of genes that were annotated to each KO.

**Clustering of co-abundant metabolites**

Co-abundant metabolites in serum or synovial fluid were identified using the R package WGCNA\(^9\). As recommended by Pedersen *et al.*\(^4\), a signed network and biweighted mid-correlation were used for clustering with the soft threshold $\beta = 8$ for both serum and synovial fluid metabolites. The minimum cluster size was set as 3. Similar clusters were subsequently merged if the biweight mid-correlation between the cluster's eigen vectors exceeded 0.8 for both serum and synovial fluid metabolites. The kIN of a metabolite was calculated by summing connectivity with all other metabolites in the given metabolite cluster. The kME was determined by the bicor-correlation between the metabolite profile and module eigenvector. Both kIN and kME were used to measure the intramodular hub-metabolite status.

**Cross-domain association analyses**

The clinical phenotypes, including types of arthritis (Healthy = 0, OA = 1, RA = 2) and the levels of pro-inflammatory cytokines TNF-$\alpha$ and IL-6, were used in the association analysis. TNF-$\alpha$ and IL-6 were selected based on their potentials to act as the therapeutic targets for RA treatment.\(^ {10,11} \) The associations between clinical phenotypes and KEGG modules/metabolites clusters were determined through evaluating if the Spearman correlations of the phenotype with the abundances of KOs/metabolites in the given KEGG module/metabolite clusters were significantly higher or lower (Mann–Whitney U-test FDR < 0.1) than with the abundances of all other KOs/metabolites. The phenotypes adjusted by age and gender were also tested. Moreover, the union set of the significant associations between KEGG modules and phenotypes/phenotypes adjusted by age and gender, and the intersect set of the significant associations between metabolites clusters and phenotypes/phenotypes adjusted by age and gender, were used for the following association analysis. The associations between metabolite clusters and
KEGG modules were determined through evaluating if the Spearman correlations of
the eigen vectors of the metabolite clusters with the abundances of KOs in the given
KEGG module were significantly higher or lower (Mann–Whitney U-test FDR < 0.1)
than with the abundances of all other KOs/metabolites.

**Leave-one-out analysis**

Leave-one-out analysis was used to identify the specific MGS driving the observed
associations between KEGG module M00550 and the clinical phenotypes, including
the types of arthritis or the levels of pro-inflammatory cytokines TNF-α and IL-6. The
calculation of the KO abundance was iterated excluding the genes from a different MGS,
in each iteration. The effect of a given MGS on a specified association was defined as
the change in median Spearman correlation coefficient between KOs and clinical
phenotypes when genes from the respective MGS were left out, as previously
described.4 12

**Taxonomic identity of differentially present microbes across conditions**

MetaPhlAn213 was used to generate species profiles. Species that were present in less
than 10% samples were excluded. Supplementary Figure 1 displays the union set of the
species (n=15) with significantly different abundances (Mann–Whitney U-test FDR <
0.05) between the healthy and RA groups or between the healthy and OA groups.
Supplementary figure 1 Taxonomic identity of differentially present microbes across conditions. Each row represents a species with significantly different abundances (Mann–Whitney U-test FDR < 0.05) between the healthy and RA groups or between the healthy and OA groups. Each column represents a sample from one of the groups including the healthy, RAP1, RAP2, RAP3, RAP4, and OA groups. Color of each heatmap unit represents the scaled abundance of a certain species in a specific sample. Species are colored for significantly elevation (red) or depletion (green) in the arthritis groups, in comparison with the healthy groups.

Data accession

Whole-genome shot-gun sequencing data are available in the Genome Sequence Archive (GSA) section of National Genomics Data Center (project accession number CRA004348) at https://bigd.big.ac.cn/gsa/browse/CRA004348.
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