Altered gut microbiota metabolites in patients with AONFH: an integrated omics analysis

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Abstract

Background

Alcohol-induced osteonecrosis of the femoral head (AONFH) is caused by excessive alcohol consumption. The gut microbiota (GM) participates in regulating host health, and its composition can be altered by alcohol. The aim of this study was to improve our understanding of the GM and its metabolites in patients with AONFH.

Methods

The GM of AONFH patients and normal controls (NCs) was characterized by analyzing fecal samples using 16S rDNA and metabolomic sequencing via liquid chromatography-mass spectrometry. To identify whether GM changes at the species level are associated with gut bacteria genes or functions in AONFH patients, metagenomic sequencing of fecal samples was performed.

Results

The abundance of 58 genera differed between the NC group and the AONFH group. *Klebsiella*, *Holdemanella*, *Citrobacter*, and *Lentilactobacillus* were significantly more abundant in the AONFH group than in the NC group. Metagenomic sequencing indicated that most of the species that exhibited significantly different abundance in AONFH subjects belonged to the genus *Pseudomonas*. Fecal metabolomic analysis identified several metabolites that were present at significantly different concentrations in the AONFH group and the NC group; these metabolites were involved in vitamin B6 metabolism, retinol metabolism, pentose and glucuronate interconversions, and glycerophospholipid metabolism. Furthermore, we found that these differences in metabolite levels were associated with altered abundances of specific bacterial species.

Conclusions

Our study provides a comprehensive landscape of the GM and metabolites in AONFH patients and substantial evidence for interplay between the gut microbiome and metabolome in AONFH pathogenesis.

1. Introduction

Osteonecrosis of the femoral head (ONFH) is a common orthopedic disease caused by a decrease in blood supply to the femoral head that is often accompanied by osteocyte necrosis, trabecular bone fracture, and articular surface collapse [1]. Two categories of ONFH have been reported: traumatic ONFH (TONFH) and non-traumatic ONFH (NONFH); the latter includes steroid/corticosteroid-induced ONFH (SONFH), alcohol-induced ONFH (AONFH), and other forms [2]. Excessive alcohol consumption is
commonly recognized as a major risk factor for AONFH. Abnormal alcohol metabolism may contribute to femoral head tissue damage through the production of toxic byproducts such as acetaldehyde, free radicals, and acetaldehyde adducts, as well as regulation of intravascular coagulation and the clotting cascade [3, 4]. However, the pathogenic mechanism of AONFH is not yet completely understood.

The gut microbiota (GM) has been identified as an important symbiotic partner in the maintenance of human health. Recently, a number of studies have suggested that gut microbiome composition and metabolic activity can participate in the regulation of bone homeostasis and affect the development of osteochondral or bone diseases [5, 6], including estrogen deprivation–induced bone loss [7] and bisphosphonate-related osteonecrosis of the jaw [8]. In addition, alcohol can alter GM composition and is closely related to overall health [9]. However, the interactions among alcohol, GM, and GM metabolites, as well as their roles in the development of ONFH, have not been investigated.

Given the regulatory effect of GM on bone, we hypothesized that alcohol-induced gut dysbiosis may play an important role in the development of AONFH. In this study, we performed fecal integrated omics analysis, including 16S rDNA gene sequencing analysis, metagenomics analysis, and metabolomic analysis, to define gut metabolome profiles and metabolic profiles in AONFH patients.

2. Materials and methods

2.1 Sample collection and ethical approval

The study enrolled 98 Chinese men, including 48 healthy adults and 50 AONFH patients. The selected participants experienced the same environmental factors, with similar hygiene status, diet (except alcohol consumption), pollution, and other common lifestyle characteristics. The patients with AONFH were required to meet the following the criteria: (1) age 18 to 80 years old; (2) history of pure ethanol consumption of > 400 mL per week or any type of alcoholic beverage intake > 320 g/week for more than 6 months [10]; (2) AONFH diagnosis within 1 year of alcohol consumption at that level; (3) AONFH diagnosed by clinical examination, X-ray, CT, and MRI; (4) no history of other osteoarticular diseases (such as injury, osteoarthritis, rheumatoid arthritis, gout, or skeletal fluorosis), chronic disease (such as hypertension, diabetes, or coronary heart disease), or bowel disease (such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), colorectal cancer (CRC)) for which they received treatment in the past 6 months. The healthy controls had no musculoskeletal pathologies or recent injuries, had normal bowel habits, and had no history of IBS, IBD, CRC, or other severe gastroenterological disease. Individuals who had used antibiotics, probiotics, prebiotics, or synbiotics in the previous 2 months were excluded. Their general clinical data, including age, educational background, and body mass index were recorded. All participants provided written informed consent before the experiments were performed, and the study protocols followed the Ethical Guidelines of the Declaration of Helsinki. Stool samples were collected by the participants and transported immediately to the laboratory, where they were divided into three portions per sample, packed into three freezer tubes, frozen in liquid nitrogen overnight, and preserved at -80°C for further testing.
2.2 DNA extraction and 16S rDNA gene sequencing

Forty-eight NC samples and fifty AONFH samples were subjected to 16S rDNA gene sequencing analysis. DNA was extracted using the E.Z.N.A.® Stool DNA Kit (D4015, Omega, Inc., USA) according to the manufacturer's instructions. The active reagent in this kit has been shown to effectively extract DNA from most bacteria. Nuclease-free water was used as the negative control. Total DNA from each sample was eluted in 50 µl of elution buffer and stored at -80°C until PCR was performed.

The V3-V4 region of the prokaryotic (bacterial and archaeal) small subunit (16S) rDNA gene was amplified with the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). The 5′ ends of the primers were tagged with specific barcodes for each sample and were sequenced with universal primers. PCR amplification was performed in a reaction mixture with a total volume of 25 µL containing 25 ng of template DNA, 12.5 µL PCR premix, 2.5 µL of each primer, and PCR-grade water to adjust the volume. The PCR conditions used to amplify the prokaryotic 16S fragments were as follows: an initial denaturation step at 98°C for 30 seconds; 32 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 45 seconds; and then final extension at 72°C for 10 minutes. The PCR products were confirmed with 2% agarose gel electrophoresis. Throughout the DNA extraction process, ultrapure water was used instead of sample solution as a negative control to exclude the possibility of false-positive PCR results. The PCR products were purified using AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon library were assessed on an Agilent 2100 Bioanalyzer (Agilent, USA) and using the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. The libraries were sequenced on a NovaSeq PE250 platform.

Samples were sequenced on an Illumina NovaSeq platform according to the manufacturer's recommendations (LC-Bio). Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH. Quality filtering of the raw reads were performed under specific filtering conditions to obtain high-quality clean tags using fqtrim (v0.94). Chimeric sequences were filtered using Vsearch software (v2.3.4). Dereplication with DADA2 generated a feature table and feature sequence. Alpha diversity and beta diversity were calculated by QIIME2, for which the same number of sequences was extracted randomly by reducing the number of sequences to the minimum for some samples, and the relative abundance was used to determine the bacterial taxonomy. Alpha diversity and beta diversity figures were drawn using the R package (v3.5.2). Blast was used for sequence alignment, and each representative feature sequence was annotated using the SILVA database.

2.3 Fecal metagenomics analysis

Forty-seven NC samples and fifty AONFH samples were subjected to metagenomics analysis. The DNA library was constructed using a TruSeq Nano DNA LT Library Preparation Kit (FC-121-4001). DNA was fragmented with dsDNA Fragmentase (NEB, M0348S) by incubating at 37°C for 30 min, and the library
was constructed from the fragmented cDNA. Blunt-end DNA fragments were generated using a combination of fill-in reactions and exonuclease activity, and size selection was performed with the provided sample purification beads. An A-base was then added to the blunt ends of each strand, preparing them for ligation to the indexed adapters. Each adapter contained a T-base overhang for ligating the adapter to the A-tailed fragmented DNA. These adapters contained the full complement of sequencing primer hybridization sites for single, paired-end, and indexed reads. Single- or dual-index adapters were ligated to the fragments, and the ligated products were amplified by PCR using the following conditions: initial denaturation at 95°C for 3 min; eight cycles of denaturation at 98°C for 15 sec, annealing at 60°C for 15 sec, and extension at 72°C for 30 sec; and then final extension at 72°C for 5 min.

Raw sequencing reads were processed to obtain valid reads for further analysis. First, sequencing adapters were removed from sequencing reads using cutadapt v1.9. Second, low quality reads were trimmed by fqtrim v0.94 using a sliding-window algorithm. Third, reads were aligned to the host genome using bowtie2 v2.2.0 to remove host DNA contamination. Once quality-filtered reads were obtained, they were de novo assembled to construct the metagenome for each sample using IDBA-UD v1.1.1. All coding regions (CDS) within the metagenomic contigs were predicted by MetaGeneMark v3.26. CDS sequences from all samples were clustered using CD-HIT v4.6.1 to obtain unigenes. Unigene abundances for individual samples were estimated by TPM based on the number of aligned reads using bowtie2 v2.2.0. The lowest common taxonomic ancestors of the unigenes were obtained by aligning them against the NCBI NR database using DIAMOND v 0.9.14. Functional annotation of the unigenes was also performed. Finally, differentially expressed unigenes were identified at the taxonomic, functional, or gene level by Fisher’s exact test based on the taxonomic annotation, functional annotation, and abundance profiles, respectively.

2.4 Metabolomics analysis and data analysis

Forty-eight NC samples and forty-nine AONFH samples were subjected to metabolomics analysis. The metabolites were extracted from fecal samples with 50% methanol buffer and incubated at 24°C for 10 min. The extraction mixture was then stored overnight at -20°C. After centrifugation at 4,000 g for 20 min, the supernatants were transferred to 96-well plates and stored at -80°C prior to being subjected to liquid chromatography mass spectrometry (LC-MS) analysis to identify the metabolites. Pooled QC samples were prepared by combining 10 µL of each extraction mixture. Chromatographic separation was performed using a Thermo Scientific UltiMate 3000 HPLC. An ACQUITY UPLC BEH C18 column (100mm×2.1mm, 1.8µm, Waters, UK) was used for the reversed phase separation. The column temperature was maintained at 35°C. The flow rate was 0.4 ml/min, and the mobile phase consisted of solvent A (water, 0.1% formic acid) and solvent B (Acetonitrile, 0.1% formic acid). The gradient elution conditions were as follows: 0-0.5 min, 5% B; 0.5-7 min, 5–100% B; 7–8 min, 100% B; 8-8.1 min, 100–5% B; 8.1–10 min, 5%B. The injection volume for each sample was 4 µl.

A high-resolution Triple TOF 5600 Plus tandem mass spectrometer (SCIEX, UK) was operated in both PIM and NIM to detect metabolites eluted from the column. The curtain gas was set to 30 PSI, Ion source
gas1 was set 60 PSI, Ion source gas2 was set 60 PSI, and the interface heater temperature was set to 650°C. For positive ion mode, the Ionspray voltage was set at 5000 V. For negative ion mode, the Ionspray voltage was set at -4500V. The mass spectrometry data were acquired in IDA mode. The TOF mass range was from 60 to 1200 Da. The survey scans were acquired in 150 ms, and as many as 12 product ion scans were collected if exceeding a threshold of 100 counts per second (counts/s) and with a 1 + charge-state. The total cycle time was fixed at 0.56 s. Four time bins were summed for each scan at a pulser frequency value of 11 kHz through monitoring the 40 GHz multichannel TDC detector with four-anode/channel detection. Dynamic exclusion was set at 4 s. During acquisition, the mass accuracy was calibrated every 20 samples. Furthermore, to evaluate the stability of the LC-MS procedure throughout acquisition, a quality control sample (pooled sample) was processed after every 10 samples.

The acquired MS data were pretreated by peak picking, peak grouping, retention time correction, second peak grouping, and annotation of isotopes and adducts using XCMS software. LC-MS raw data files were converted into mzXML format and then processed using XCMS, CAMERA, and the metaX toolbox implemented with R software. Each ion was identified by combining retention time (RT) and m/z data. The intensity of each peak was recorded, and a three dimensional matrix containing arbitrarily assigned peak indices (retention time-m/z pairs), sample names (observations), and ion intensity information (variables) was generated.

The online KEGG and HMDB databases were used to annotate the metabolites by matching the exact molecular mass data (m/z) of samples with those from the database. If the mass difference between the observed and database values was less than 10 ppm, the metabolite was annotated, and the molecular formula of the metabolite was further identified and validated by isotopic distribution measurements. We also used an in-house metabolite fragment spectrum library to validate the identified metabolites.

Peak intensity data were further preprocessed using metaX. Features that were detected in less than 50% of QC samples or 80% of biological samples were removed, and the remaining peaks with missing values were imputed using the k-nearest neighbor algorithm to further improve data quality. PCA was performed for outlier detection and batch effect evaluation using the pre-processed dataset. Quality control–based robust LOESS signal correction was fitted to the QC data with respect to the order of injection to minimize signal intensity drift over time. In addition, the relative standard deviations of the metabolic features were calculated across all QC samples, and any that were > 30% were removed.

Student t-tests were conducted to detect differences in metabolite concentrations between the two groups. The P value was adjusted for multiple tests using an FDR (Benjamini–Hochberg). Supervised PLS-DA was conducted using metaX to discriminate between the different variables, and XCMS software was used to pretreat the acquired MS data. The raw LC-MS raw data files were processed with metaX using the XCMS package for peak detection and the CAMERA package for peak annotation, all based on R. Each ion was identified by combining the retention time and m/z data. The KEGG and HMDB databases were used to annotate the metabolites using the exact molecular mass data (m/z) of the samples. Student’s t-test was used to detect the differences in metabolite concentrations between the two
groups. The P value was adjusted for multiple tests using an FDR (Benjamini-Hochberg). Supervised PLS-DA was conducted using metaX to discriminate the variables between the different groups. The VIP was calculated, and a VIP cut-off value of 1.0 was used to select important features.

2.5 Statistical analyses

Significant differences in clinical characteristics were evaluated with Pearson's chi-square test or Fisher's exact test. Spearman's correlation analysis was conducted to calculate the correlation between species and metabolites. Differences were considered significant when P < 0.05. All data were analyzed with GraphPad Prism 6 software (GraphPad Software, Inc., San Diego, California, USA), R version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria), and Microsoft Excel (Microsoft Corporation, Seattle, WA, USA).

3. Results

3.1 Clinical characteristics of the study population

The demographic characteristics of the two groups were generally matched, suggesting that there were no confounding factors that could have influenced the results. A total of 50 patients with AONFH and 48 healthy adults were recruited; individuals with comparable eating habits were selected to exclude dietary differences (Table 1).

Table 1
Characteristics of participants in this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ANFH</th>
<th>NC</th>
<th>P value</th>
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<td>Subjects (n)</td>
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<td>48</td>
<td>-</td>
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<td>Age (mean), years</td>
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<td>Height, cm</td>
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<td>Weight, kg</td>
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<td>71.19 ± 11.48</td>
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</tr>
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<td>BMI</td>
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<td>23.35 ± 3.54</td>
<td>0.20</td>
</tr>
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</tr>
<tr>
<td>Smoking</td>
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<td>0</td>
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</table>

3.2 Gut microbiome changes in AONFH patients

To identify changes in the gut microbiome in AONFH patients, 16S rRNA metagenomics analysis was conducted of 98 fecal samples, including 48 samples from the NC group and 50 samples from the AONFH group. After quality control, at least 21 million valid bases were obtained for each sample. Detailed information about the 16S rDNA data obtained from all of the samples is provided in Supplementary Table S1.
The rRNA sequences were grouped into operational taxonomic units (OTUs) based on sequence similarity to classify microbial diversity in terms of bacterial strains. Performing a 97% similarity cluster analysis identified 4966 OTUs in the NC group and 4208 OTUs in the AONFH group and showed that 1697 OTUs were shared between the two groups (Fig. 1a).

Alpha diversity analysis was used to analyze the complexity of species diversity in each sample using several indices, including the observed OTUs, Chao1, Shannon, and Simpson indices. The richness and diversity rarefaction curve in the two groups tended to be flat or reach a plateau, demonstrating satisfactory sequencing depth (Supplementary Fig. 1). Alpha diversity analysis showed no significant differences in observed OTUs, Chao1, Shannon, and Simpson indices between the NC group and the AONFH group (Supplementary Fig. 2).

Principle coordinate analysis (PCoA) and analysis of similarities (ANOSIM) testing for beta diversity revealed a significant difference in GM composition and abundance between the two groups (unweighted Unifrac P = 0.005 and Jaccard P = 0.003) (Fig. 1b and c).

### 3.3 AONFH-Related changes in gut microbiome composition

Taxon-dependent analysis (Fig. 1d) identified 31 phyla in both groups, with *Firmicutes, Actinobacteriota, Proteobacteria, Bacteroidota*, and *Verrucomicrobiota* being the most dominant phyla. *Firmicutes* was the most predominant phylum, accounting for 55.23% and 48.19% of the GM in the NC group and the AONFH group, respectively. Further analyses revealed that, at the phylum level, *Campylobacterota* (P = 0.0048) and *Chloroflexi* (P = 0.0392) were significantly more abundant in the NC group, whereas *Planctomycetota* (P = 0.0099) and *Proteobacteria* (P = 0.0438) were significantly more abundant in the AONFH group (Fig. 1e).

At the genus level, the abundance of 58 genera was significantly different between the NC group and the AONFH group. Among them, *UCG-002* (P = 0.0241), *Pseudomonas* (P = 0.0342), *Firmicutes* (P = 0.0496), *UCG-005* (P = 0.0436), and *Incertae-Sedis* (P = 0.0208) were significantly more abundant in the NC group, whereas *Klebsiella* (P = 0.0232), *Holdemanella* (P = 0.0229), *Citrobacter* (P = 0.0468), and *Lentilactobacillus* (P = 0.0093) were significantly more abundant in the AONFH group (Fig. 1f).

Next, we performed linear discriminant analysis (LDA) integrated with effect size (LEfSe) to generate a cladogram to identify the specific bacteria involved in AONFH (Fig. 2a). We found significant differences in 21 OTUs (LDA > 3), including *Pseudomonas, Pseudomonadaceae, Oscillospiraceae, UCG-002, Firmicutes,* and *Streptococcus*, which were more abundant in the NC group relative to the AONFH group. In contrast, *Burkholderiaceae, Buekholderiales, Holdemanella, Erysipelotrichaceae, Klebsiella-pneumoniae, Klebsiella, Gammaproteobacteria, Proteobacteria,* and *Enterobacterales* were more abundant in the AONFH group (Fig. 2b).

### 3.4 Prediction of gene function in the GM
Next, we used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) to compare gut microbial gene functions across Clusters of Orthologous Genes (COGs), Kyoto Encyclopedia of Genes and Genomes (KEGG), and KEGG orthology (KO) functional orthologues between the AONFH and NC groups. The top 30 functions identified by each database are shown in Supplementary Fig. 3. KEGG pathway analysis identified important functions such as CDP-diacylglycerol biosynthesis I/II, L-histidine biosynthesis, and the L-serine and glycine biosynthesis I superpathway (Fig. 2c), and COG database analysis highlighted Flp pilus assembly protein TadG, lipid-binding SYLF domain, and CBS-domain-containing membrane proteins.

**Metagenomic sequencing revealed significant differences between the AONFH and NC groups**

We then performed metagenomic sequencing on fecal samples from 50 AONFH patients and 47 healthy subjects. A total of 317,243 genes were identified (Supplementary Fig. 4a-c). The samples from the NC group contained 3177 specific genes that were not detected in the AONFH samples (Supplementary Fig. 4d). Compared with the NC group, 20,823 unigenes were differentially expressed in the AONFH group (10,171 upregulated and 10652 downregulated).

The alpha diversity was lower in the AONFH group than in the NC group, as measured by the observed species and Chao1 indices, while the Shannon, and Simpson indices did not detect any significant difference in alpha diversity between the groups (Supplementary Fig. 5).

PCoA and ANOSIM testing for beta diversity revealed no significant difference in microbial composition between the AONFH and NC groups at the species level (Bray-Curtis Unifrac P = 0.06) (Fig. 3a). Comparing the profiles of the AONFH and NC groups showed that *Pseudomonas, Pseudomonas-fluorescens, Pseudomonas-sp.-TMW-2.1634, Pseudomonas-weihenstephanensis, and Pseudomonas-fragi* were significantly less abundant in the AONFH group compared with the NC group (Fig. 3b and Supplementary Table S2)

### 3.5 The potential role of GM biomarkers in AONFH risk assessment

Next we constructed a random forest model based on the genera with significantly different abundances to identify potential diagnostic biomarkers that could be used to predict AONFH. The optimal model that provided the best discriminatory power utilized 20 genera (Fig. 3c). According to the analysis described earlier, there are significant differences in the composition of the microbial community between AONFH and NC subjects. Thus, to determine the ability of the identified bacterial biomarkers to discriminate between the two groups, we produced receiver operating characteristic (ROC) curves and computed values for the area under the curve (AUC). The top five AUC values were for *Enhygromyxa_salina* (89.47%), *Hyphomonas_beringensis* (87.84%), *Thermococcus_profundus* (87.41%), *Syncephalastrum_racemosum* (86.93%), and *Roseovarius_nitratireducens* (86.91%) (Fig. 3d).
3.6 Functional analysis of differentially expressed genes in the AONFH group identified by metagenomic sequencing

We selected the top 10 GO items for the three types of definitions provided by the GO database. The results of the GO functional classification analysis of differentially expressed unigenes between the two groups are shown in Supplementary Fig. 6a. Next, GO enrichment analysis of differentially expressed unigenes between the two groups was performed; and the top 20 GO terms are shown in Supplementary Fig. 6b. Finally, KEGG analysis of differentially expressed unigenes was performed to identify the metabolic pathways that differed most significantly between the groups. The expression of genes related to starch and sucrose metabolism, RNA degradation, pentose and glucuronate interconversions, glutathione metabolism, flagellar assembly, and bacterial chemotaxis differed most significantly between the AONFH and NC groups (Supplementary Figure S6c and Supplementary Table S3).

3.7 Metabolomic analysis revealed abnormal metabolic alterations in patients with AONFH

To identify changes in the gut microbiome in AONFH patients, metabolomic analysis was performed of 97 fecal samples, including 48 samples from the NC group and 49 samples from the AONFH group.

Partial least squares discriminant analysis (PLS-DA) was performed to identify discriminant metabolites in the fecal samples from these two groups. Using negative ion mode (NIM), there was an apparent trend toward separation of the metabolic features of the fecal samples from AONFH patients and healthy individuals (Fig. 4a). The combined explained variance of PC1 and PC2 was 18.84%; $R^2 = (0.6568)$, $Q^2 = (0.3303)$ (Fig. 4b). In positive ion mode (PIM), there was also an apparent trend toward separation between the metabolic features of the fecal samples from AONFH patients and healthy individuals (Fig. 4c). The combined explained variance of PC1 and PC2 was 18.41%; $R^2 = (0.6386)$, $Q^2 = (0.3213)$ (Fig. 4d).

3.8 Fecal metabolomic changes in AONFH patients

Metabolomic analysis identified 21,486 features and 11,723 metabolites in PIM and 14,155 and 7576 metabolites in NIM. The obtained data were used as the batch query against the human metabolome database (HMDB) for single-stage mass spectrometry analysis, which annotated 5098 and 3689 individual samples with the features identified in PIM and NIM, respectively. In the HMDB superclass analysis, the top most abundant metabolites were lipid molecules and organic acids and derivatives (Fig. 4e).

Comparative metabolomic analysis showed clear differences in fecal metabolite profiles between AONFH patients and healthy individuals. A volcano plot showing the upregulated and downregulated metabolites is shown in Fig. 4f. We identified 483 significantly upregulated and 396 significantly downregulated metabolites in positive-ion mode and 358 significantly upregulated and 296 significantly downregulated
metabolites in negative-ion mode in the AONFH group vs. the control group (Supplementary Table S4). These metabolites can be regarded as the elements of the signaling pathway network underlying AONFH occurrence.

### 3.9 Metabolite profiling and AONFH-related pathways

Pathway analysis showed the detailed impact of AONFH-related alterations in metabolic networks (Fig. 4g). The most influential metabolic pathway had a pathway impact > 0.05 and log(p) > 0.3. Four metabolic pathways were identified as being disturbed in the fecal profiles of AONFH patients, including the vitamin B6 metabolism, retinol metabolism, pentose and glucuronate interconversions, and glycerophospholipid metabolism pathways. KEGG enrichment analysis identified the most abundant metabolic pathways in the two groups (Fig. 4h). Based on P values, pathway impact, and enrichment ratios, the top two metabolic pathways were the vitamin B6 metabolism and retinol metabolism pathways.

A correlation matrix was created using Spearman correlation and correlation network analyses to explore the potential relationships between changes in the gut microbiome and changes in metabolic product concentrations (Fig. 4i, Supplementary Table S5). The levels of ptilosteroid b, 16-methyl-6z,9z,12z-heptadecatrienoic acid, allylestrenol, heptabarbital, n-benzoylaspartic acid, ezetimibe, and vanilloylglycine were positively correlated with the abundance of the genera Holophagae_noname, Schizosaccharomyces, Oceanicella, Basidiobolus, Mortierella, Roseovarius, and more. The levels of kanzonol e, sophoracoumestan a, dicaffeoylputrescine, neobavaisoflavone, pentyllbenzene, sophorapterocarpan a, pyrazine, ropivacaine, o-methylpongamol, erythrinin c, and semilicioisoflavone b were negatively correlated with the abundance of the genera Eukaryota_unclassified, Phanerochaete, Ceratobasidium, Candidatus_Hodgkinia, Smittium, Schizosaccharomyces, Syncephalis, and more.

### 4. Discussion

The GM is an important symbiotic partner that helps maintain animal and human health. As the prime pathogenic factor contributing to AONFH, alcohol is largely metabolized within the gastrointestinal tract, and it can impact gut microbiome composition, the gut immune system and downstream systemic immune communications with other organs. High levels of alcohol intake can cause malabsorption and nutrient deficiencies (including vitamin D), alter the gut microbiome and gut metabolites, affect the expression of bone metabolism-regulating hormones, induce osteoclast activation, and influence GM composition [11]. In addition, glucocorticoids can induce the loss of Lactobacillus animalis and its extracellular vesicles from the gut, which is associated with the pathogenesis of osteonecrosis GC-induced ONFH [12]. Thus, it can be inferred that GM plays an important role in ONFH. However, to date no study has systematically investigated the role of GM and gut metabolites in the development of ONFH.

The results from our study show that AONFH patients have gut dysbiosis at the phylum, genus, and species levels, suggesting that alcohol participates in AONFH pathogenesis through altering GM composition. The 16S rDNA gene sequencing results showed that Pseudomonas, Pseudomonadaceae,
Oscillospiraceae, Firmicutes, and Streptococcus were more abundant in the NC group than in the AONFH group. In contrast, Burkholderiaceae, Buekholderiales, Holdemanella, Erysipelotrichaceae, Klebsiella-pneumoniae, Klebsiella, Proteobacteria, and Enterobacteriales were more abundant in the AONFH group than in the NC group. The metagenomics analysis results showed that Pseudomonas was significantly less abundant in the AONFH group than in the NC group.

In studies of clinical alcohol use disorder (AUD), the associated dysbiosis is characterized by lower abundances of Bacteroidetes and Akkermansia muciniphila [13]. In animal models of high-dose alcohol consumption, a decrease in bacterial diversity is observed, along with less Bacteroidetes, more Proteobacter, more Actinobacter [14], less Firmicutes, and more Bacteroidetes [15].

Proteobacteria is one of the most abundant phyla in the human GM and is often overrepresented in several diseases, mostly those associated with an inflammatory phenotype [16]. In studies of clinical alcohol use disorder (AUD), the dysbiosis is characterized by higher Proteobacteria abundance [17]. We found that Proteobacteria was more abundant in AONFH patients, which may be attributable to alcohol consumption. Pseudomonas is a member of the Proteobacteria phylum that has been shown to be associated with alcohol-related diseases and ONFH. Pseudomonas was found to be prevalent in the intestine of rats with alcohol-related liver injury, and its abundance could be decreased by transplantation with fecal filtrate from a healthy rat [18]. Liu et al. suspected that Pseudomonas aeruginosa and Pseudomonas putida may be two of pathogens in the patients with ONFH [19]. However, we found decreased Pseudomonas abundance in AONFH patients, indicating that Pseudomonas abundance was decreased by alcohol consumption. Klebsiella and Streptococcus are another two members of the Proteobacteria phylum. Individuals who abuse alcohol have increased susceptibility to lung infection by Streptococcus pneumoniae and Klebsiella pneumoniae [20]. Yuan et al. reported that up to 60% of individuals with nonalcoholic fatty liver disease in a Chinese cohort were infected with Klebsiella pneumonia, a bacterial strain that produces alcohol as a byproduct [21]. Taken together, these studies indicate that alcohol consumption increases susceptibility to infection with Klebsiella pneumonia, which leads to the excess production of endogenous alcohol because of gut microbiome alteration. In our study, we found that Klebsiella-pneumoniae and Klebsiella were more abundant in the AONFH group. We therefore speculate that alcohol consumption increases Klebsiella-pneumoniae abundance, which may result in the excess production of endogenous alcohol and promote AONFH pathogenesis.

Firmicutes and Bacteroidetes are two major phyla in the normal human GM that are involved in colonic metabolism via a complex metabolic energy-harvesting mechanism based on cross-feeding and co-metabolism [22]. The Firmicutes/Bacteroidetes ratio has been implicated in predisposition to disease states [23]. Wang et al. found that patients with Kashin-Beck disease were characterized by lower Firmicutes levels and a significantly higher Firmicutes/Bacteroidetes ratio [24]. In addition, Firmicutes abundance was positively correlated with calcium absorption [25] and was significantly decreased in the presence of alcohol [26], which was verified by Cheng et al. [11]. In our study, we verified that Firmicutes levels were decreased in AONFH patients, which indicates that alcohol consumption participates in AONFH pathogenesis by decreasing Firmicutes abundance.
ONFH develops because of pathological changes that occur in or result from micro-necrosis in the local micro-environment and involve disorders of multiple metabolic processes, including lipid metabolism, endovascular coagulation, intravascular fat embolism, and inhibition of angiogenesis, apoptosis. It can therefore be inferred that alterations in some metabolic molecular markers should be evident in the bloodstream at early stages of the ONFH pathological process. In our study, several important gut microbial gene functions were identified, such as CDP-diacylglycerol biosynthesis I/II, L-histidine biosynthesis, and the L-serine and glycine biosynthesis I superpathway.

CDP-diacylglycerol is a critical intermediate in lipid metabolism, including in the synthesis of phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylinositol (PI). CDP-diacylglycerol synthase (CDS) produces CDP-diacylglycerol from phosphatidic acid (PA) and cytidine triphosphate (CTP) \(^27\). CDS has been reported to play an important role in various biological effects, including mitochondrial function, signal transduction, membrane trafficking, secretion, and cytoskeletal rearrangements \(^28\). In our study, CDP-diacylglycerol biosynthesis was identified as one of the most important gut microbial gene functions altered in AONFH. Thus, CDP-diacylglycerol and CDS may play key roles in AONFH pathogenesis, and further studies are needed to investigate the detailed mechanism of this process.

Bioinformatics analysis performed by Yang et al. indicated that histidine, cysteine, and methionine metabolism are associated with the ONFH pathogenic progress, and a metabolic pathway analysis performed as part of the same study revealed that L-histidine was hit in the histidine metabolism and L-serine was hit in the cysteine and methionine metabolism \(^1\). Histidine is an essential amino acid in mammals, and could regulate gene expression, the biological activity of proteins, and signal transduction \(^29\). L-serine has been shown to promote osteoclast formation, and thereby induce bone resorption \(^30\). These studies agree with our gut microbial gene function prediction results and suggest that L-histidine and L-serine play vital regulatory roles in the pathology of AONFH.

Betaine is a trimethyl derivative of glycine and an important human nutrient, which regulates a series of vital biological processes, including oxidative stress, inflammatory responses, osteoblast differentiation, and cellular apoptosis \(^31\text{–}33\). Yang et al. reported that betaine is a potential pharmacotherapy for alcohol-induced ONFH in vivo, as it plays a protective role against ethanol-induced suppression of osteogenesis and mineralization of hBMSCs \(^34\).

Vitamin A is vital for many bodily functions, including but not limited to gene expression, reproduction, embryonic development, and even immune function \(^35\). Too little vitamin A intake has many adverse effects, including low bone density, but too much vitamin A can also cause bone loss and a higher risk of fracture, leaving a narrow optimal dosage range \(^36\). As the biologically active form of vitamin A, retinol can enhance osteoblast proliferation as well as hinder osteoclast resorption activity \(^37\). It has been reported that chronic alcohol consumption has adverse effects on vitamin A metabolism, which is directly linked with the development of alcohol-induced disease \(^38\). In our study, retinol metabolism was identified as one of the most important AONFH-related pathways. Thus, we can infer that alcohol disturbs
retinol metabolism, which may be a vital part of AONFH pathogenesis. Vitamin B6 deficiency is common in alcoholics [39].

In conclusion, our study revealed that AONFH patients have gut dysbiosis at the phylum, genus, and species levels that is associated with metabolite alterations. The altered GM profile and metabolites are potential diagnostic markers for AONFH. In particular, the analysis of the interactions among alcohol, GM, metabolites, and AONFH discussed here could enhance our understanding of the mechanisms underlying AONFH pathogenesis. The findings from this study regarding GM and metabolite changes could also point to novel therapeutic targets.

**Declarations**

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**Authorship contributions**

Conceived and designed the study: Chen Yue, Youwen Liu, and Bin Xu. Study conduct: Chen Yue, Maoxiao Ma, Jiayi Guo and Hongjun Li. Data collection: Maoxiao Ma, Hongjun Li. Data analysis: Yuxia Yang and Youwen Liu. Chen Yue and Bin Xu interpreted data and drafted the manuscript. Drafting manuscript: Chen Yue, Maoxiao Ma, Youwen Liu and Bin Xu. All authors approved the final manuscript.

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**Ethics statement**

This study was approved by the ethics committee of Luoyang Orthopedic-Traumatological Hospital of Henan Province (KY2021-007-01), and the study was performed in accordance with the Declaration of Helsinki.

**Competing interests**

The authors declare that they have no competing interests.

**Consent for publication**

All authors confirmed that this work can be published. The content of this manuscript is original and has not yet been accepted or published elsewhere.
References


Figures
Figure 1

Gut microbiome diversity and structure analysis. (a) Venn diagram of the observed features in the AONFH and NC groups. (b), (c) Principal coordinate analysis (PCoA) of the microbiota based on unweighted UniFrac (ANOSIM, $R = 0.058$, $P = 0.005$) and Jaccard (ANOSIM, $R = -0.060$, $P = 0.003$) distance matrices for the AONFH and NC groups. (d) Proportions of different bacterial phyla in each group; (e), (f)
Statistically significant differences in bacterial abundance at the phylum and genus level between the AONFH and NC groups.

Figure 2

Gut microbiome composition and functional analyses. (a) Cladogram indicating the phylogenetic distribution of the microbiota in the AONFH and NC group groups; (b) Linear discriminant analysis (LDA)
integrated with effect size (LEfSe) indicating differences in abundance between the AONFH and NC groups; (c) Predicted function of gut microbiota based on KEGG pathway analysis. The extended error bar plot shows the significantly different KEGG pathways between the AONFH and NC groups.

Figure 3
Differences in the gut microbiota in the AONFH and NC groups based on the metagenomic sequencing data. (a) PCoA analysis based on the Bray-Curtis distance matrix between the AONFH and NC groups at the species level (ANOSIM, R=0.02; P=0.06); (b) Relative abundance of the top 20 species enriched in the AONFH and NC groups; the box represents the interquartile ranges, and the line denotes the median; (c) Performance of a random forest model classification as assessed using the R random forest package; (d) ROC curve displaying the top five biomarkers for distinguishing between the AONFH and NC groups. AUC, area under curve.
Figure 4

Differing metabolic patterns between the AONFH and NC groups. (a), (b) PLS-DA score plot and permutation plot of the AONFH and NC groups based on LC-MS analysis in negative ion mode; (c), (d) PLS-DA score plot and permutation plot of the AONFH and NC groups based on LC-MS in positive ion mode; (e) The gut metabolites were identified by HMDB superclass analysis; (f) Volcano plot showing the number of dysregulated metabolites in the feces of AONFH patients compared with healthy adults; (g) Plots depicting the computed metabolic pathways as a function of P value and the pathway impacts of the key metabolites that were differentially expressed between the AONFH and NC groups; (h) The most abundant metabolic pathway in the two groups as identified by KEGG enrichment analysis; (i) Correlations between genera and metabolites. The top 25 genera were defined based on the metagenomics data. The correlation effect is indicated by a color gradient from blue (negative correlation) to red (positive correlation).

Supplementary Files

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