Gut Microbiota Composition and Fecal Metabolic Phenotype in Patients With Acute Anterior Uveitis

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PURPOSE. To investigate gut microbiota composition and fecal metabolic phenotype in patients with acute anterior uveitis.

METHODS. Fecal DNA was extracted from 78 fecal samples (38 acute anterior uveitis (AAU) patients and 40 family members of patients or sex- and age-matched healthy controls) and then sequenced by high-throughput 16S rDNA analysis. Gas chromatographic mass spectrometry (GC-MS) based metabolomics was performed on 60 fecal samples (30 AAU patients and 30 healthy controls).

RESULTS. A significant difference was observed in beta diversity between AAU patients and healthy controls. Eight genera including Roseburia were reduced in AAU patients, and Veillonella was increased in AAU patients as compared with healthy controls. Significance was however lost after false discovery rate (FDR) correction. The expression of seven fecal metabolites including 6-deoxy-D-glucose 1, linoleic acid, N-Acetyl-beta-D-mannosamine 3, shikimic acid, azelaic acid, isomalto 1 and palmitoleic acid was increased in AAU patients. Linoleic acid showed a significant correlation with Roseburia and Veillonella according to Spearman correlation analysis.

CONCLUSIONS. Our results did not reveal a difference in gut microbiota composition, but did show that the fecal metabolic phenotype in AAU patients was significantly different from healthy controls.

Keywords: acute anterior uveitis, gut microbiota, fecal metabolic phenotype

Uveitis is one of the primary causes of visual impairment and blindness in the world. Acute anterior uveitis is the most common uveitis entity, which is often accompanied with other systemic diseases such as ankylosing spondylitis (AS) and spondyloarthropathies (SpA). Previous studies have shown that undiagnosed SpA occurs in about 40% of patients with idiopathic AAU. The human leucocyte antigen B27 (HLA-B27) has been reported to have a strong association with AAU. A significant number of independent genetic associations of AAU and SpA, which cluster in several immunologic pathways such as the IL-17 and -23 pathways, has been revealed by recent genome-wide association studies (GWAS). Recent studies have also shown that an imbalance of Th17 and Treg cells is thought to contribute to the development of AAU. The human gut microbiota and its effects on the metabolic phenotype in the host have been shown to play a critical role in maintaining immune homeostasis. Microbial components have been thought to be involved in the regulation of the immune response such as activating IL-17-producing T cells in the gut environment. The presence of Collinsella, for instance, showed a strong correlation with alpha-aminoadipic acid and asparagine and was able to promote the production of the proinflammatory cytokine IL-17A. Previous studies furthermore showed that gut bacteria, such as Yersinia Enterocolitica and Salmonella typhimurium, are involved in the pathogenesis of SpA. Significantly decreased genera such as Roseburia and butyrate production have been observed in Behçet patients as compared with healthy controls. Commensal bacteria are thought to be a potential trigger and a factor which can influence the development of disease in experimental autoimmune uveitis (EAU). Antibiotics, such as metronidazole or vancomycin, can increase the abundance of some bacteria including Dorea and Clostridium and reduce the mean uveitis score in EAU. Considering the possible role of microbiota and metabolites in the development of immune diseases, we decided that it would be worthwhile to study whether the composition of gut microbiota and metabolites is different in AAU as compared to controls.

In the present study, we utilized the 16S rDNA sequencing and GC-MS based metabolomics to investigate gut microbiota composition and the fecal metabolic phenotype in patients with AAU.

METHODS

Patients

In the present study, a total of 38 AAU patients (14 women and 24 men) and 40 controls, with no personal history of uveitis,
including family members of patients (n = 17) or age-, sex-, and ethnicity-matched healthy individuals (n = 23) were enrolled. All procedures in the study followed the Declaration of Helsinki. The mean ± SD age was 33.87 ± 8.77 years (range, 20–65 years; see Table 1). AAU was diagnosed according to the symptoms (red eyes, eye pain, photophobia); clinical examinations (keratic precipitates, anterior chamber cells); and instrumental exams (anterior segment image and ultrasound biomicroscopy) as described before. The uveitis patients who had AS were diagnosed by our rheumatology department according to the modified New York criteria 1984. Exclusion criteria of each group were as follows: recent (<3 months prior to the sample collection) treatment with probiotics, antibiotics, corticosteroids, gastrointestinal tract surgery; or having a history of diseases including malignancies, obesity, hypertension, diabetes, psoriasis, psoriatic arthritis, or inflammatory bowel disease. Individuals enrolled in our study should have a normal diet and had regular bowel movements and didn’t have prolonged diarrhea or chronic constipation.

### Fecal Sample Collection and DNA Extraction

Fecal samples were collected at patients’ preferred location during an active episode of AAU. The samples were kept at 4°C until sending to our laboratory within 6 hours after production and then immediately transferred to −80°C for storage until being used. Microbial DNA from stool samples was isolated with a commercial DNA kit (QIAamp Fast DNA Stool Mini Kit; Qiagen, Hilden, Germany) according to the manufacturer’s requirements. The total DNA quality was measured by a spectrophotometer (NanoDrop 2000 UV; Thermo Fisher Scientific, Waltham, MA, USA) and 1% agarose gel electrophoresis.

### 16S rDNA Sequencing

The 16S rDNA high-throughput sequencing procedure was performed by using commercial equipment (Illumina HiSeq PE250; Illumina, San Diego, CA, USA). The V3-V4 16S rDNA region was amplified, using universal primers 341F (CCTACGGGGRSGGCAGCAG) and 806R (GGACTACVGGGTATC TAATCC). The specific primers were designed by adding the index sequence and adapter sequence suitable for high-sequencing system (HiSeq2500 PE250; Illumina, CA, USA) sequencing at the 5′ end of the universal primer.

### Sequence Analysis

Pandaseq software was used to construct clean reads. Then the Clean Reads with the same sequence were sorted according to their abundance. Singletons were filtered out, and the reads clustered using UPARSE in Usearch 7.0. After filtering the chimera sequences by UCHIME, we clustered the sequences and grouped them as operational taxonomic units (OTUs) with a similarity of 97%. After filtering the chimeric sequences by UCHIME, we clustered the sequences and grouped them as operational taxonomic units (OTUs). QIIME software was used to summarize the OTU data and the OTUs were further subjected to the taxonomy assignment using the Ribosomal Database Project (RDP; provided in the public domain, http://rdp.cme.msu.edu/).

QIIME software was also used to calculate microbial alpha and beta diversity for each sample. Alpha diversity is an analysis of the diversity within a community, including chao1 index and Shannon index. Rank sum test analysis was used to analyze significant differences concerning alpha diversity. Beta diversity, which is defined as the distance between communities, was calculated with Unweighted and Weighted UniFrac by QIIME software. Principal coordinates analysis (PCoA) was performed by the ade4 function from the stats R package to visualize the distance matrix among all the samples. Multiple response permutation procedure (MRPP) group-difference analysis, which was performed by the statistical R package, was used to determine significant differences between these two groups in PCoA.

A linear discriminant analysis (LDA) of effect size (LEfSe), which was performed by the LEfSe tool (provided in the public domain, http://huttenhower.sph.harvard.edu/lefse/), and Kruskal-Wallis test, which was performed by the wilcox.test function from the stats R package, were used to estimate the effect of abundance of each sample on the effect of differences, and to identify the bacterial taxa that have significant differences in their demarcation. Only those taxa that showed a P value < 0.05 and a log LDA score ≥2 were ultimately considered. Multiple test corrections were based on the false discovery rate (FDR). An FDR value of 0.05 was used as a statistically significant cutoff.

### GC-MS Analysis

The GC-MS analysis in our study concerning the metabolite composition in the gut, was done by the BIOTREE company in Shanghai, China. Feces (100 mg) were placed in 2-ML EP tubes; 0.5 mL methanol extraction liquid and 20 μL of L-2-Chlorophenylalanine, which served as an internal standard, were added to the samples. Samples were then vortexed for 30 seconds and homogenized in a ball mill for 4 minutes at 45 Hz; treated with ultrasound for 5 minutes (incubated in ice water); and then centrifuged at 16,200g, (4°C) for 15 minutes. The supernatant was transferred to fresh 2 mL GC/MS glass vials. We took 5 μL from each sample and collected as QC samples. Drying in a vacuum concentrator without heating and 80 μL of methoxylated hydrochloride and 100 μL of BSTFA regent were added respectively. We added 10 μL of FAME (standard solution of fatty acid methyl ester) to QC samples at room temperature. Samples were thoroughly mixed prior to GC-MS analysis. GC/TOFMS analysis was performed using a gas chromatograph (7890B GC System; Agilent, Santa Clara, CA, USA) and a time-of-flight mass spectrometer (Pegasus HT; LECO Corp., St. Joseph, MI, USA). Helium was used as the carrier gas. The initial temperature was maintained at 50°C for 1 minute and then raised to 310°C at a rate of 10°C per 1 minute, then held at 310°C for 13 minutes. The injection, delivery lines, and ion source temperatures were 280, 270, and 220°C, respectively.
In the electronic shock mode, the energy is –70eV. After a solvent delay for 455 seconds, the mass spectrometry data were obtained in full scan mode with 50 to 500 m/z range at a rate of 20 spectra per second.

Data Analysis

Raw peaks and peak identification were performed using commercial software of and database (Chroma TOF 4.3X and LECO-Fiehn Rtx5; LECO Corp.). Metabolic data, which were only detected in <50% of QC samples, were removed.

Multivariate Analysis of GC–MS Data

The processed data set was then entered in the software package (SIMCA14, version 14.1; MKS Data Analytics Solutions, Umeå, Sweden) for principal component analysis (PCA), which is an unsupervised method to gain an overview of multivariate data and could help to show the distribution of original data. Orthogonal projections to latent structures-discriminate analysis (OPLS-DA), was used to obtain a higher level of group separation and to get a better understanding of variables responsible for classification. The OPLS-DA model was validated using a permutation test. To identify the variables responsible for this large separation, the first principal component of variable importance in the projection (VIP) was obtained. The VIP values, which exceed 1.0, were chosen as altered metabolites. Student’s t-test was performed to assess the remaining variables. Multiple test corrections were based on the false discovery rate (FDR). An FDR value of 0.05 was used as a statistically significant cutoff.

Statistical Analyses

Spearman correlation between the level of fecal metabolites and the relative abundance of genera was performed by the cor.test function from the stats R package. We only performed the correlation in those genera (P < 0.2, FDR corrected) and metabolites (P < 0.05, FDR corrected), which were found to be statistically significant between groups.

In all statistical tests, a value of P < 0.05 was considered as significant.

RESULTS

Diversity of Microbiota in AAU Patients

A total of 4,579,162 high-quality clean reads were obtained from 30 AAU patients and 30 healthy controls, with a mean of 58,707.21 ± 4,408.41 reads (range, 39,597–64,520) per subject. Their mean ± SD average length was 417.56 ± 2.92 bp (range, 408–423 bp). A total of 2249 operational taxonomic units (OTUs) were clustered by reads at 97% of identity. No significant difference of alpha diversity was observed between AAU patients and healthy controls (See Supplementary Fig. S1). The PCoA could distinguish AAU patients from healthy controls, with a significant value when unweighted UniFrac distance metrics was used (P = 0.027), suggesting that the composition of gut microbiota in AAU patients was significantly different from those observed in healthy controls (Fig. 1).

Gut Microbiota Differences in AAU Patients Compared to Controls

To study which bacterial taxa were different in AAU patients as compared with healthy controls, LEfSe analysis was performed. Results showed that 14 bacterial taxa were enriched in AAU patients, while 39 bacterial taxa were enriched in healthy controls (Fig. 2). The cladograms at six different levels (from kingdom to genus) were obtained by the LEfSe analysis method. In AAU patients, the class Negativicutes and the order Selenomonadales had a large effect size, the order Oceanospirillales and the family Halomonadaceae as well as the family Clostridiales_IncertaeSedisXI showed large effect sizes (Fig. 3).

Furthermore, the relative abundance of microbiota was compared by Kruskal-Wallis test and log LDA scores to detect significantly different bacterial taxa between AAU patients and healthy controls by. At the genus level, eight genera were reduced in AAU patients including Roseburia, Veillonella was the only increased genus in AAU patients as compared with healthy controls. However, the significance at genus level between AAU patients and controls was lost after FDR correction. At taxonomic classification levels, abundance of several microbists, including the family Lachnospiraceae, were different between AAU patients and healthy controls. However, the significance was also lost after FDR correction (see Supplementary Table S1).

To identify possible gut biomarkers, a volcano plot was made between both patients groups and controls or between AAU patients as compared with healthy controls. However, the significance was lost after FDR correction either between AAU patients and healthy controls. The genus Veillonella was increased and Odoribacter, Dorea were decreased in AAU patients and controls. A reduction of Roseburia, Clostridium XI, Blautia, Clostridium sensu stricto, Lachnospiracea_IncertaeSedes, and Barnesiella was observed in AAU patients as compared with controls. However, the significance was lost after FDR correction either between AAU patients and healthy controls. (See Supplementary Tables S2, S3) We also compared the composition of gut microbiota in HLA-B27−/B27+ patients with HLA-B27−/B27+ patients. The genus Bifidobacterium, Blautia and Collinsella were decreased in HLA-B27+ patients compared with HLA-B27− patients. Unfortunately, there was no statistically significant difference after FDR correction (See Supplementary Table S4).

Diversity of Metabolites in AAU Patients

To investigate the metabolic phenotype of AAU patients, GC–MS was performed on fecal samples from 30 AAU and 30 healthy controls. The PCA score plots revealed a clear discrimination between AAU patients and healthy controls (Fig. 4A). In the OPLS-DA model, there was a significant difference in the metabolic phenotype between AAU patients and healthy controls (Fig. 4B). After the randomization (n = 200), we obtained R2 = 0.74 and Q2 = −1.05, indicating that the model was predictive and reliable (Fig. 4C).

Metabolic Phenotype in AAU Patients

To identify possible gut biomarkers, a volcano plot was made to describe the variation tendencies. The results showed that 7 metabolites including 6-deoxy-D-glucose, linoleic acid, N-Acetyl-beta-D-mannosamine, shikimic acid, azelaic acid, Isomaltose 1, and palmitolac acid were significantly increased (P < 0.01, FDR corrected). Two metabolites were decreased in AAU patients compared with healthy controls. However, this significance was lost after FDR correction (see Supplementary Fig. S2, Table 2).

We further compared the metabolic phenotype either between both patients groups and controls or between AAU+/− patients and AAU−/− patients to find whether
metabolite level was associated with AS. We found 15 metabolites to be enriched in AAU $^{\text{+}}$ AS $^{\text{+}}$ patients compared with healthy controls. However, only azelaic acid was found to be significantly different between AAU $^{\text{+}}$ AS $^{\text{+}}$ patients and controls after FDR correction ($P = 0.017$, FDR corrected; see Supplementary Table S5). Although an increase of five metabolites was observed in AAU $^{\text{+}}$ AS $^{\text{+}}$ patients, the significance was lost after FDR correction (see Supplementary Table S6). There was an increase of eight metabolites in AAU $^{\text{+}}$ AS $^{\text{+}}$ patients compared with AAU $^{\text{+}}$ AS $^{\text{+}}$ patients. However, the significance was lost after FDR correction (see Supplementary Table S7). We also compared the metabolic phenotype in HLA-B27 $^{\text{+}}$ patients and HLA-B27 $^{\text{+}}$ patients. Five metabolites were decreased in HLA-B27 $^{\text{+}}$ patients compared with HLA-B27 $^{\text{+}}$ cases. However, only inosine was found to be significant difference between HLA-B27 $^{\text{+}}$ patients and HLA-B27 $^{\text{+}}$ patients after FDR correction ($P = 0.013$, FDR corrected; see Supplementary Table S8).

**Correlation of Gut Microbiota and Metabolic Phenotype in AAU Patients**

To investigate whether gut microbiota composition is associated with a fecal metabolic phenotype in AAU patients, Spearman correlation analysis was performed. The results showed that *Roseburia* was negatively correlated with linoleic acid ($P = 0.043$, $r = -0.272$) and *Veillonella* was positively correlated with linoleic acid ($P = 0.021$, $r = 0.189$) and palmitoleic acid ($P = 4.45 \times 10^{-5}$, $r = 0.093$).
DISCUSSION

In the present study, we found that AAU patients had a characteristic fecal metabolite profile, irrespective of the presence of AS or their HLA-B27 status. Although the alpha analysis or abundance of gut microbiota between patients and controls failed to show any significant differences, we revealed significant alterations in microbiota composition in AAU patients compared with healthy controls following analysis of PCoA plots of unweighted UniFrac distances. Our findings are, by and large, in agreement with recent observations showing that the development of ocular inflammation during experimental autoimmune uveitis in mice is dependent on the presence of certain bacteria in the gut of these animals.14 To our knowledge this is the first report concerning the analysis of gut microbiota composition and fecal metabolite profile in clinical AAU, although we are aware of the fact that due to the various limitations of our study, one should see this report as a preliminary observation in an important field. One of the limitations of the study is that the techniques used, generate an abundance of data. Although many trends concerning gut microbiota composition were seen in AAU, the differences lost statistical significance after correction for multiple comparisons. Fecal metabolite composition differences; however, maintained statistical significance even after correction. The limitations mentioned above can be addressed by studying larger groups or performing experiments to test specific hypotheses about specific bacteria. An additional component is the heterogeneity of the sample population and heterogeneity of the gut microbiota across individuals. Environmental factors, genetic factors, dietary habits, age, BMI, sex, and disease status may cause a change in the gut microbiota and may cause overlaps in PCoA plots. These difficulties can be addressed by studying different subtypes of AAU such as active and inactive disease or by using statistical methods that control for important covariates of the study population (such as age, BMI, sex).

Recent studies have revealed that a dysbiosis of gut microbiota may play an important role in the development of several autoimmune diseases. 44,45 The family Lachnospiraceae, belonging to Firmicutes, was reported to be involved in inflammatory bowel disease (IBD) and type 1 diabetes (T1D).46,47 It was found that Lachnospiraceae could protect against T1D by decreasing the number of regulatory FoxP3+ Treg cells.47 In our present study, four genera in this family were found to be decreased in AAU patients although the significance was lost after FDR correction. This increased trend of these four genera is expected to be clarified by using a larger sample size. A recent study showed a significant increase in the genus of Dialister in SpA patients compared with healthy controls.48 In our study, we failed to find a similar result in our AAU+AS group. One of the reasons could be because AS is a subtype of SpA and its pathogenesis may be different from that of SpA. Another possibility is that the samples used in the latter study are different from those we used. In the SpA study, colonic and ileac samples were used, whereas stool samples were used in our study. More studies are needed to address this issue. A recent study by Lin et al.49 has shown an increase of Prevotella spp. and a decrease of Rikenellaceae in HLA-B27 transgenic rats. In our current study, we neither found a difference of these two genera nor did we observe a difference of other genera tested between our HLA-B27+ and HLA-B27− patients. This could be attributed to differences between humans and rats.

It has been shown that metabolic phenotype correlates with gut microbiota composition and that this may contribute to the development of several diseases, such as IBD, Crohn disease and obesity.50,51 In the present study, we examined
whether there were alternations of metabolites of gut microbiota in AAU patients. We found alternations of three metabolites including linoleic acid, azelaic acid, and inosine in AAU patients as well as in the HLA-B27\textsuperscript{+} patients. An increased linoleic acid was also found to be positively correlated with Roseburia and Veillonella. It has been shown that linoleic acid is able to stimulate the production of proinflammatory mediators in IBD,\textsuperscript{52} cause more oxidative damage and mediates selective loss of intrahepatic CD4\textsuperscript{+} T lymphocytes in nonalcoholic fatty liver disease (NAFLD).\textsuperscript{53} Azelaic acid has been shown to be involved in the development of chronic depression through modulating autoimmune response.\textsuperscript{54} Inosine has been shown to inhibit the production of IL-17 and -14 and to stimulate the release of adenosine A2A receptor (A2AR) in experimental autoimmune encephalomyelitis (EAE) models.\textsuperscript{55} The role of increased linoleic acid and azelaic acid in AAU patients or inosine in HLA-B27\textsuperscript{+} patients is unclear and deserves further study. It has been shown that short chain fatty acids (SCFAs) could promote the differentiation of Treg cells and are found to be implicated in several autoimmune diseases.\textsuperscript{51} Unfortunately, we failed to find any difference concerning SCFAs between AAU patients and controls. More studies using a large patient sample size are needed to clarify whether SCFAs play a role in the development of AAU as well as in the pathogenesis of HLA-B27-associated disease.

**FIGURE 3.** The cladograms of six different taxonomic levels (from kingdom to genus) were also constructed. *Orange circles* and shadings show the significantly enriched bacterial taxa were obtained in healthy controls. *Blue circles* and shadings show the significantly enriched bacterial taxa obtained in AAU patients.

<table>
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<tr>
<th>Metabolite</th>
<th>VIP</th>
<th>Mean AAU</th>
<th>Mean N</th>
<th>P Value\textsuperscript{a}</th>
<th>Q Value\textsuperscript{b}</th>
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<tr>
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<td>0.00</td>
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<td>0.00</td>
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<td>Shikimic acid</td>
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<td>0.00</td>
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<tr>
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<td>0.00</td>
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<td>0.03</td>
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<td>0.348</td>
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</table>

\textsuperscript{a} P values were determined using Student’s t-test.
\textsuperscript{b} Q values were determined using the FDR.
FIGURE 4. Alteration of metabolites between AAU patients and healthy controls. (A) PCA score plots of fecal samples from AAU patients (blue circle) and healthy controls (orange square). (B) OPLS-DA of fecal samples from AAU patients (blue circle) and healthy controls (orange square). (C) Validation of OPLS-DA model (using 200 random permutations).
In our study, we used the modified New York criteria (1984) to diagnose AS. These criteria are still widely used in clinical studies but may be replaced by the newer ASAS criteria that use novel imaging techniques and have been shown to be useful to diagnose early disease. In some of our AAU AS patients (n = 15), we used MRI; but none fulfilled the ASAS criteria for AS. Because of the expensive cost of MRI and patients’ own wishes not to have to make extra appointments in the hospital, we decided not to use the ASAS criteria for AS. Patients were also contacted by phone, to ensure that they had not developed AS during their follow-up in local hospitals. One should keep in mind, however, that some of our patients with AAU, currently classified as AS-negative, may ultimately develop AS in future.

Taken together, we show that AAU patients have a unique metabolic phenotype in their gut but do not have a different microbiota composition when compared with healthy controls.

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