

RESEARCH PAPER



Altered gut microbiome composition in patients with Vogt-Koyanagi-Harada disease

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ABSTRACT

Background: Vogt-Koyanagi-Harada (VKH) disease is a multisystemic autoimmune disorder characterized by granulomatous panuveitis. Gut microbiome has been considered to play a role in the pathogenesis of this disease but whether the alternation of gut microbiome was involved is unclear. This study was set up to identify abnormalities of gut microbiome composition in VKH disease.

Results: Depleted butyrate-producing bacteria, lactate-producing bacteria and methanogens as well as enriched Gram-negative bacteria were identified in the active VKH patients, as well as in VKH patients of Mix enterotype and *Bacteroides* enterotype. Changes of gut microbiome in the VKH patients were partially restored after an immunosuppressive treatment. The disease susceptibility genotype HLA-DRA was associated with *Bacteroides sp.2.1.33B*, *Paraprevotella clara*, *Alistipes finegoldii* and *Eubacterium eligens*. A microbial marker profile including 40 disease-associated species was established to differentiate patients from controls. Another microbial marker profile including 37 species was found to be associated with the response to treatment. An animal experiment showed that transfer of gut microbiome from VKH patients could significantly exacerbate disease activity clinically and pathologically in the recipient mice.

Conclusion: Our results revealed a distinct gut microbiome signature in VKH patients and showed an exacerbating effect of this gut microbiome on experimental autoimmune uveitis (EAU). We also developed two microbial marker profiles in differentiating VKH patients from healthy controls as well as predicting the effectiveness of treatment.

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Introduction

Vogt-Koyanagi-Harada (VKH) disease is an autoimmune disorder affecting multiple organs such as eye, ear, skin and central nervous system (CNS). Bilateral granulomatous panuveitis is a hallmark of this disease. It is one of the major vision-threatening diseases in pigmented races, such as Asians and Native Americans.^{1,2} Autoimmunity, infection and genetic susceptibility have been proposed as possible etiologic factors of this disease.²⁻⁵ Our previous study revealed an increased expression of Toll like receptor (TLR)-3 and TLR-4 in active VKH patients.⁶ Earlier

experimental data have shown that TLRs are able to recognize ligands from pathogenic microorganisms which may subsequently trigger autoimmune responses.⁷ These observations suggest that the host microbiome might play a role in the pathogenesis of VKH disease.


The gut microbiome has been shown to be involved in the pathogenesis of a number of immune or inflammatory diseases such as Behcet's disease (BD),⁸ rheumatoid arthritis (RA),^{9,10} psoriatic arthritis (PA),¹⁰ inflammatory bowel disease (IBD),¹¹⁻¹³ multiple sclerosis

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(MS),^{14,15} ankylosing spondylitis (AS)¹⁶ and systemic lupus erythematosus (SLE).¹⁷ Recent studies on experimental autoimmune uveitis (EAU) have shown that microorganisms in the gastrointestinal tract play a critical role in driving uveitis in this model.¹⁸ Whether the composition and function of the gut microbiome is altered in VKH disease is not yet known and is the purpose of our study. In this study, we analyzed the phylotype profile and metabolic pathways of the gut microbiome in patients with VKH disease and constructed microbiome-associated gene marker sets that were useful in disease diagnosis and prognosis. Fecal microbiota transplant (FMT) using patients' feces significantly increased the severity of disease in mice undergoing experimental autoimmune uveitis.

Materials & methods

Subjects

A total of 149 Chinese individuals (71 active VKH patients not yet receiving treatment, 11 paired inactive VKH patients after being treated with systemic corticosteroids combined with other immunosuppressive agents and 67 sex-, age- and BMI-matched healthy controls) were enrolled for this study (Table S1 and S2). After taking the stool specimens, the active VKH patients were treated with systemic corticosteroids and immunosuppressive drugs and their response to treatment was monitored over time. Individuals with other diseases such as diabetes, cardiovascular disease and infectious disease and/or taking antibiotics or probiotics within one month of the study were excluded. The diagnosis of VKH disease was made strictly according to the diagnostic criteria revised for VKH disease by an international committee on nomenclature.¹⁹ Cells and flare in the anterior chamber, mutton-fat keratic precipitates, iris nodules and sunset glow fundus were considered as evidence of disease activity. None of the aforementioned signs was identified in inactive patients after treatment except sunset glow fundus. The demographic data (age, gender and BMI), intraocular and extraocular manifestations and treatment information of all participants were recorded.

Ethics, consent and permissions

All procedures followed the tenets of the Declaration of Helsinki and were approved by the Ethics Committee of Chongqing Medical University with written informed consent.

Sample collection and fecal DNA extraction

Fresh fecal samples were collected from all enrolled individuals and then stored at -80°C within two hours. Fecal DNA was extracted from frozen fecal samples using QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration of DNA was measured by NanoDrop (ThermoFisher, Waltham, MA, USA).

Metagenomic sequencing

The metagenomic DNA libraries were constructed with 2 μg genomic DNA according to the Illumina TruSeq DNA Sample Prep v2 Guide (Illumina, Inc; San Diego, CA, USA), with an average of 500 bp insert size. The quality of all libraries was evaluated using an Agilent bioanalyzer (Agilent Technologies, Wokingham, UK) with a DNA LabChip 1000 kit. Whole-genome shotgun sequencing of fecal samples collected was carried out on the Illumina HiSeq 4000 platform with 150 bp paired-end read length.

Raw paired-end reads of metagenomics sequencing were processed and included a quality control using the criteria below: (1) reads with adaptor contamination were removed; (2) reads were trimmed from the 3' end using a quality threshold of 30; (3) reads containing more than 50% bases with low quality (Q30) were removed; (4) reads shorter than 70 bp were removed; (5) reads that mapped to human genome (alignment with SOAPaligner 2.21²⁰) were removed. On average, the proportion of high quality reads was $96.2\% \pm 1.4\%$ in all samples.

De novo assembly and gene catalog construction

Employing the protocol which was used in the construction of the MetaHIT gene catalog,²¹ we performed the assembly and gene prediction from

the high quality reads by using SOAPdenovo²² (version 2.04) and MetaGeneMark²³ (version 3.26). To obtain a non-redundant gene of the human gut microbiome, the predicted ORFs (filtered by length of 100 bp) were clustered at 95% identity and 90% coverage using CD-HIT²⁴ (version 4.5.7), and then the representative genes of each cluster (the longest one) were selected. The final non-redundant gene catalog contained 1,718,179 microbial genes, with an average length of 780 bp.

Taxonomic and gene profiling

Clean reads were mapped by SOAPalign2.21 to the microbial reference genomes downloaded from the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>), which consist of 65,770 bacterial, 898 archaeal, 1,508 fungal and 6,025 viral genomes. We selected the complete over draft genomes for strains with multiple genomes in NCBI. Taxonomic relative abundance profiles were generated with the procedure introduced in Qin.²⁵ Reads mapped to multiple taxa were allocated among them proportionally to reads counts uniquely mapped to these taxa (normalized by genome length). Gene abundance was calculated by the same strategy as used for the abundance profiling of the organisms.

MGS analysis

To cluster genes into Metagenomic Species (MGS), we followed the method described by Le Chatelier²⁶ and Nielsen.²⁷ Firstly, the differentially abundant gene markers were identified by Wilcoxon test with a $P < .05$. Secondly, we clustered the marker genes with a Spearman correlation coefficient (ρ) >0.8 using single-linkage clustering according to their abundance variation across all samples, and then clusters with more than 25 genes were selected as MGS. The taxonomical annotation and abundance profile of MGS were performed according to the taxonomy and the relative abundance of its genes, as previously described.²⁵ Briefly, MGS assignment to a taxonomical level from strain to super kingdom level required $>90\%$ of the genes in this MGS with the best hit to the same phylogenetic group

using blast with $>95\%$ identity and $>90\%$ overlap of query.

To construct the co-occurrence network of MGS, we computed the Spearman correlation coefficient between MGS using their abundances in all samples, and then visualized the co-occurrence network by Cytoscape3.0.2.

KEGG and eggNOG analysis

The metagenomics gene catalog was annotated by aligning gene sequences against the proteins in eggNOG 3.0 database²⁸ and KEGG database (version 20141209)²⁹ using BLASTP (e-value $\leq 1e^{-5}$). A gene was assigned to a OG or KO by the highest scoring annotated hit with at least one HSP (high-scoring segment pair) scoring >60 . For each functional feature (OG in eggNOG or KO in KEGG database), we estimated its abundance by accumulating the relative abundance of all genes belonging to the same family.

Genomic DNA extraction and SNP genotyping

Peripheral blood samples were collected from 61 VKH patients. Genomic DNA was extracted from these blood samples using QIAamp DNA Mini Blood Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five SNPs including rs78377598 for IL-23R-C1orf141, rs442309 and rs224058 for ADO-ZNF365-EGR2, rs3021304 for HLA-DRB1 and rs114800139 for HLA-DRA were performed using Sequenom MassARRAY system (Sequenom Inc.). Rs117633859 typing for IL-23R-C1orf141 was performed using TaqMan SNP genotyping assays (ID: AHVJKBP; Applied Biosystems, CA, USA) on the ABI 7500 Real-Time PCR system (Applied Biosystems).

Species-based classifier

To build the SVM (Support Vector Machine) classifier, extremely low abundant taxa (mean relative abundance less than 0.001) were removed first. To filter the redundant features, the mRMR algorithm (the side Channel Attack R package) and the leave-one-out cross-validation LDA (Linear Discriminant Analysis) (the paleoMAS R package) were applied.

The feature set which has the highest MCC (Matthews Correlation Coefficient) was chosen to build the SVM classifier (the e1071 R package). The ROC curves of both discovery set and validation set were generated by pROC R package.

Fecal microbiota transplant and EAU induction

An antibiotic cocktail containing 1mg/ml ampicillin, 1mg/ml neomycin, 1mg/ml metronidazole and 0.5mg/ml vancomycin (Sigma-Aldrich, St. Louis, Mo, USA) was used to eliminate the indigenous microbiota community of B10RIII mice (Jackson Laboratory, Bar Harbor, ME, USA) as described previously.⁸ Fecal samples from 5 randomly selected active VKH patients without any treatment (3 male, 2 female with an average age of 36.1 years) and 5 sex-, age- and BMI-matched healthy controls (3 male, 2 female with an average age of 35.3 years) were used to colonize the antibiotic-treated mice ($n = 3$ for active VKH patients, $n = 3$ for healthy controls and $n = 3$ for PBS). Each fecal sample (0.2 g) was resuspended in 1ml PBS, and pools were made from equal volumes of donor suspensions. Up to 200 μ l of the pooled suspension was administered by gavage to each antibiotic-treated mouse once a day for 7 days, whereafter EAU was induced (see below).

FMT mice were immunized subcutaneously with 25 μ g human interphotoreceptor retinoid binding protein peptide spanning amino acid residues 161–180 (IRBP161–180, SGIPYIISYLHPGNTILHVD; Shanghai Sangon Biological Engineering Technology & Services Ltd. Co., Shanghai, China) peptide in 100 ml PBS, emulsified 1:1 v/v in complete Freund's adjuvant (CFA; Sigma-Aldrich), supplemented with 1.0 mg/ml *Mycobacterium tuberculosis* (MTB; Sigma-Aldrich). We used a 25 μ g IRBP peptide dose since this dose causes a mild uveitis in the experimental animals (a full blown uveitis is observed when using a 50 μ g dose). The clinical and histological scoring was performed as described previously.³⁰

All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the protocol was approved by the Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

Statistical analyses

To detect significant differences in relative abundance of metagenomics features, the nonparametric Wilcoxon test (`wilcox.test` in R) was performed with false discovery rate (FDR) < 0.1 (Benjamini-Hochberg), and the enrichment group was then determined according to the higher rank-sum. Linear discriminant analysis (LDA) Effect Size (LEfSe) analysis was used to determine the features (organisms, KOs, or OGs) most likely to explain differences between the VKH and healthy controls ($|\text{LDA score}| > 2$). Differentially enriched KO modules were identified according to their reporter scores from the Z-scores of individual KOs.^{31,32} A module with a reporter score of $Z > 1.6$ was defined as differentially enriched module.

Results

Dysbiosis of the gut microbiome in VKH patients

To characterize the gut microbiome in active VKH patients, we performed metagenomic sequencing on 107 fecal samples (55 from active untreated VKH patients and 52 from healthy controls) (Table S1) and generated 6.19 ± 1.92 gigabases (Gb) of cleaned reads per sample (Table S3). Clean reads were aligned to the reference genomes from the National Center for Biological Information (NCBI). The results showed that most of the clean reads could be mapped to bacteria (98.84%), followed by viruses (1.13%) and fungi (0.0029%). No difference was found in the α -diversity (Shannon index, observed species, $P > .05$) or β -diversity (Bray-Curtis distance metric of species abundance; ANOSIM, $P > .05$) of the gut microbiome between the active VKH patients and control group. However, there were apparent differences in the microbial compositions between the two groups. At genus level, we found that *Ramularia*, *Alternaria* and *Rhizophagus* were enriched, whereas *Methanoculleus*, *Candidatus Methanomethylophilus* and *Azospirillum* were depleted in the active VKH patients compared to the control group (Figure 1(a), Table S4). In line with the results at genus level, one *Ramularia* species *R. collo-cygni* and one *Alternaria* species *A. alternata* were identified as VKH-enriched

species and two *Clostridium* spp. (*Clostridium* sp. CAG:813 and *Clostridium* sp. CAG:349), one *Methanoculleus* species (*Methanoculleus* sp. CAG:1088), one *Candidatus Methanomethylophilus* species (*Candidatus Methanomethylophilus alvus*) and one *Azospirillum* species (*Azospirillum* sp. CAG:260) as VKH-depleted species (Figure 1(b), Table S4).

Linear discriminant (LDA) effect size (LEfSe) analysis was performed to further examine the different microbial features between active VKH patients and healthy controls and showed similar findings. Specifically, *Paraprevotella* spp.

(*Paraprevotella clara* CAG116 and *Paraprevotella clara*) were enriched in active VKH patients, whereas *Clostridium* spp. (*Clostridium* sp. CAG:349 and *Clostridium* sp. CAG:813), *Bifidobacterium* spp. (*Bifidobacterium* sp. MSTE12 and *Bifidobacterium dentium*), *Candidatus Methanomethylophilus alvus* and *Methanoculleus* sp. CAG:1088 were depleted (Figure 1(c)).

Subsequent analyses on microbial genes revealed that a total of 11,720 microbial genes were enriched or depleted in the active VKH patients. These differential genes were clustered

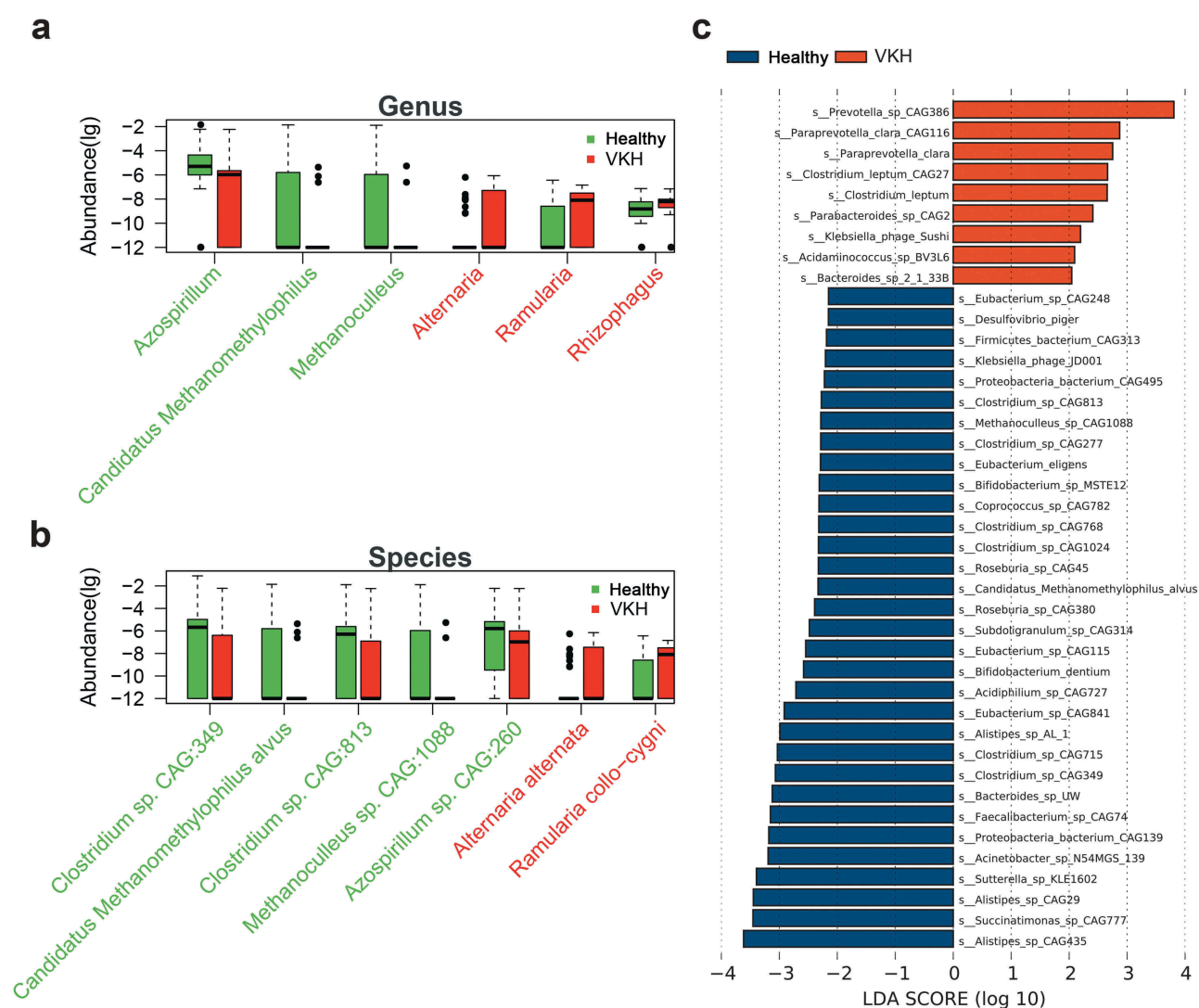


Figure 1. Differentially abundant taxa between the VKH and healthy controls.

(a, b) Differentially abundant genera (a), species (b) were identified by Wilcoxon rank sum test (adjusted P values < 0.1 , corrected by the Benjamini and Hochberg method). The relative abundance is shown by boxplot. Boxes represent the inter quartile ranges, lines inside the boxes denote medians. (c) Linear discrimination analysis (LDA) Effect Size (LEfSe) analysis results comparing the VKH and healthy controls. The LDA scores (log₁₀) > 2 are listed.

into 10 metagenomic species (MGSs) according to the abundance profiles (Fig. S1a, Table S5). We also analyzed the relationship between these VKH-associated MGSs based on their co-abundance. The cluster including *Bacteroides*, *Paraprevotella* and *Eubacterium*, which were positively correlated with each other, was enriched in the active VKH patients. In addition, another cluster with some MGSs including *Faecalibacterium prausnitzii*, *Ruminococcus bromii* and *Alistipes* was enriched in the control group (Fig. S1c).

Dysbiosis of the gut microbiome in different enterotypes in VKH patients

To investigate whether the dysbiosis of the gut microbiome in VKH patients was also present in subpopulations of enterotypes, we first identified the top 20 most abundant genera in active VKH patients and healthy controls (Fig. S2a-b). According to the relative abundance of the top genera, three enterotypes were identified both in VKH patients and healthy controls by hierarchical clustering analysis. Cluster 1 was characterized by a mix of genera including *Bacteroides*, *Prevotella*, *Alistipes* and *Parabacteroides*. Cluster 2 was characterized by a high relative abundance of *Bacteroides* but a low abundance of *Prevotella*. The third cluster was characterized by a high relative abundance of *Prevotella* but a low abundance of *Bacteroides*. Partitioning around medoids (PAM) clustering method using Jensen-Shannon distances confirmed the presence of these three enterotypes (Fig. S2c-d). The results showed that there was no difference between VKH patients and controls concerning the sample distributions across the three subgroups ($P = .34$, Fisher's exact test).

LEfSe analysis was performed to identify the differences of microbial composition in three Clusters between VKH patients and controls. The VKH-depleted species, which were identified in all VKH patients, including *Clostridium* spp. (*Clostridium* sp. CAG:349 and *Clostridium* sp. CAG:813), *Bifidobacterium* spp. (*Bifidobacterium* sp. MSTE12 and *Bifidobacterium dentium*), *Candidatus Methanomethylophilus alvus* and *Methanoculleus* sp. CAG:1088 were also depleted in patients from Cluster 1, the Mix enterotype. In

Cluster 2, the *Bacteroides* enterotype, we identified an enriched *Parabacteroides* sp. CAG2 and a depleted *Alistipes* spp. (*Alistipes* sp. AL.1 and *Alistipes* sp. CAG:29), *Proteobacteria bacterium* CAG:495, *Eubacterium* sp. CAG:115 and *Roseburia* sp. CAG:380 in active VKH patients, which were also in line with our LEfSe results in all patients. We also found some *Parabacteroides* spp. (*Parabacteroides* sp.2.1.7 and *Parabacteroides* sp.D25) and *Bacteroides* spp. (*Bacteroides coprocola* CAG:162 and *Bacteroides thetaiotaomicron* CAG:40) were enriched in VKH patients in Cluster 3, *Prevotella* enterotype (Figure 2).

Dysfunction of the gut microbiome in VKH patients

The aforementioned data showed differences in microbiome composition between the active VKH patients and controls. To further investigate the functional role of the gut microbiome in VKH disease, we constructed a gene set of 1,718,179 non-redundant genes from VKH patients and healthy controls and then functionally annotated them using KEGG (Kyoto Encyclopedia of Genes and Genomes database) and eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups database). LEfSe analysis showed that 6 KEGG orthologues (KO) and 6 eggNOG orthologues (OG) were significantly different between the active VKH patients and healthy controls (Table S6). At the pathway level, 10 metabolic pathways including that of oxidative phosphorylation (ko00190) and lipopolysaccharide biosynthesis (ko00540) were enriched in active VKH patients (Table S7). In addition, 54 modules including lipopolysaccharide biosynthesis (M00060), type VI secretion system (M00334), and reductive acetyl-CoA pathway (M00377) were found to be more abundant in active VKH patients (Figure 3, Table S8).

Comparison of the gut microbiome between VKH and Behcet's disease patients

Our recent study showed that BD,⁸ another uveitis entity common in China, had a characteristic composition of the gut

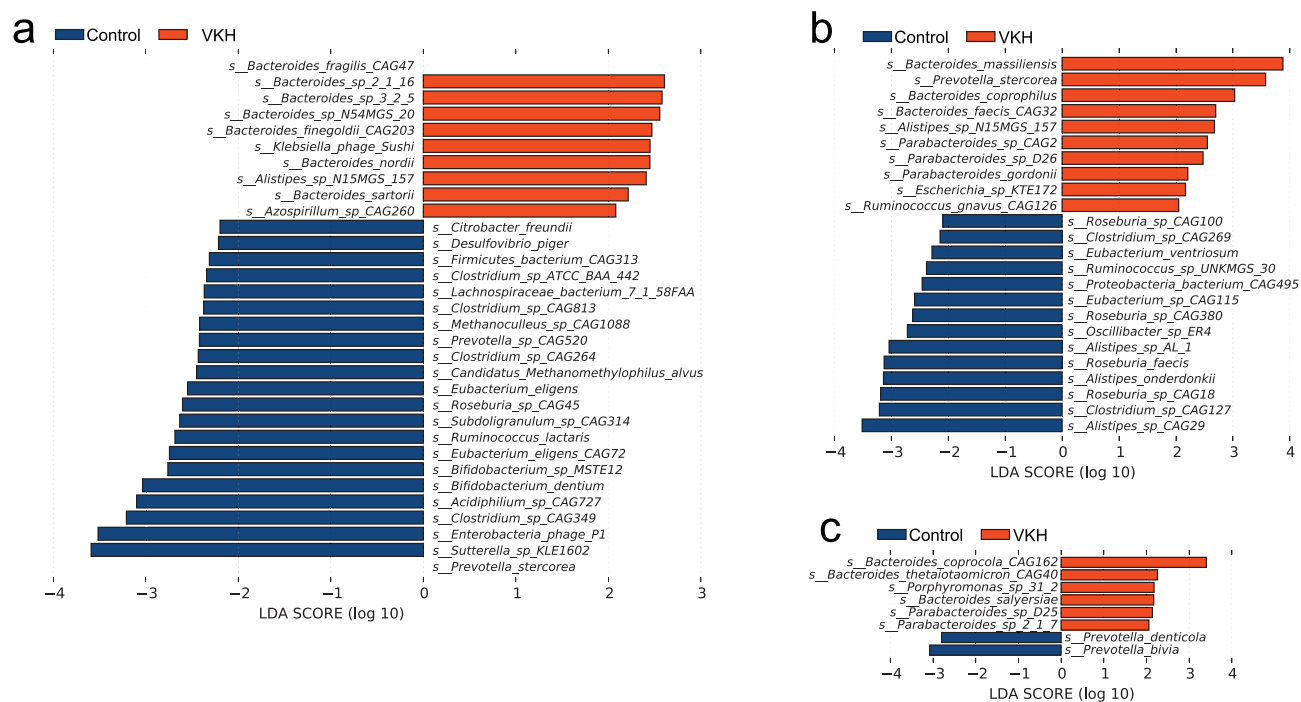


Figure 2. LefSe analysis results comparing the VKH patients and healthy controls for 3 enterotype clusters. The LDA scores (\log_{10}) > 2 are listed. (a) Cluster 1; (b) Cluster 2; (c) Cluster 3.

microbiome. To investigate whether there are differences and similarities in the gut microbiome composition between BD and VKH, we compared the disease-associated species between these two uveitis entities. As shown in Fig. S3a, 9 species were found to be associated with both BD and VKH disease. Only *Paraprevotella clara* CAG:116 was enriched in both diseases. Three *Clostridium* spp., *Acidiphilium* sp. CAG: 727, *Subdoligranulum* sp. CAG:314, *Candidatus Methanomethylophilus alvus*, *Methanoculleus* sp. CAG:1088 and *Coprococcus* sp. CAG:782 were all depleted in both VKH patients and BD patients (Fig. S3b).

Altered gut microbiome composition is associated with HLA-DRA in VKH patients

Using Genome-wide association analysis (GWAS), our previous study identified three susceptibility loci in VKH disease including IL-23R-C1orf141, ADO-ZNF365-EGR2 and HLA-DRA/HLA-DRB1.³³ To investigate whether there was any correlation between these susceptibility genotypes and gut microbiome components, we genotyped 54 active VKH patients

according to the six major SNPs for these three susceptibility loci (rs78377598 and rs117633859 for IL-23R-C1orf141, rs3021304 for HLA-DRB1, rs114800139 for HLA-DRA, rs442309 and rs224058 for ADO-ZNF365-EGR2). The VKH patients were divided into groups with and without the risk allele for each susceptibility locus. The microbiome was then compared between patients with and without a given susceptibility locus by LefSe analysis. The results showed that HLA-DRA risk alleles were positively correlated with 2 VKH-enriched species (*Bacteroides* sp.2.1.33B and *Paraprevotella clara*) but negatively correlated with 2 VKH-depleted species (*Alistipes finegoldii* and *Eubacterium eligens*) (Fig. S1d). We did not observe a detectable correlation between any gut microbiome component and IL-23R-C1orf141, HLA-DRB1 or ADO-ZNF365-EGR2.

Immunosuppressive treatment partly changes the gut microbiome in VKH patients

Immunosuppressive agents are used in the treatment of VKH disease and have been shown to effectively control the ocular inflammation.²

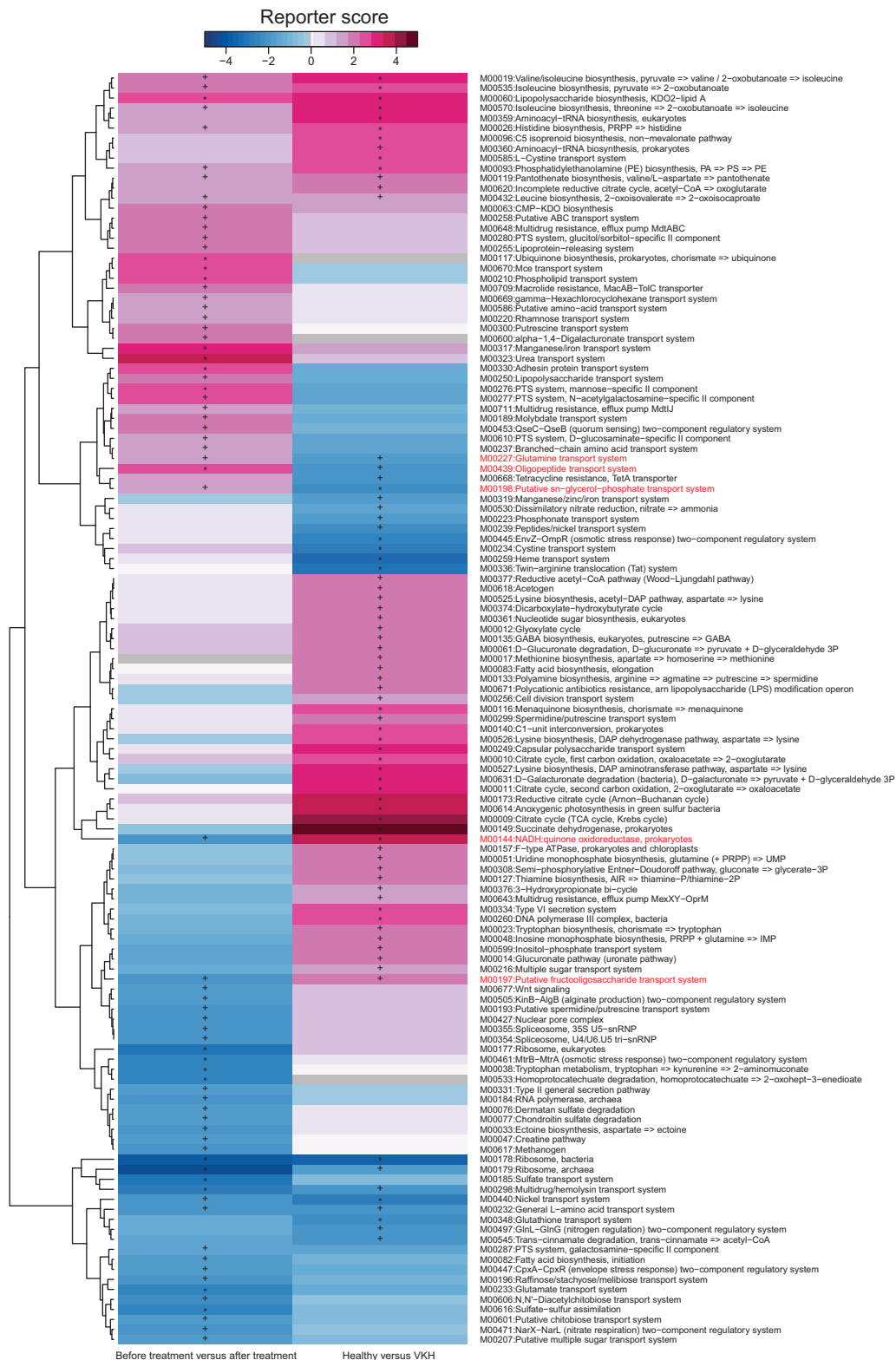


Figure 3. KO modules overrepresented between the VKH and healthy controls, or patients before and after treatment.

The relative abundances of KO modules were compared between control ($N = 52$) and VKH ($N = 55$), or 11 VKH patients before treatment with active ocular inflammation and the same patients without ocular inflammation after treatment with systemic corticosteroids combined with cyclosporine A. The modules with a significant difference in reporter score (> 2.3 or < -2.3) in any of the two comparisons are shown. Score < -1.6 represents enriched in former and > 1.6 represents enriched in latter. '+', reporter score < -1.6 or > 1.6 ; '*', reporter score < -2.3 or > 2.3 . Blue, enriched in patients before treatment (left) or healthy (right); red, enriched in patients after treatment (left) or VKH patients (right).

LEfSe analysis was designed to investigate the influence of immunosuppressive treatment on the gut microbiome composition in a group of 11 VKH patients before (with active intraocular inflammation) and after treatment with systemic corticosteroids combined with cyclosporine A. This treatment was effective since patients were subsequently free of intraocular inflammation. The results showed that 9 genera and 27 species were enriched after the treatment when compared with before treatment, whereas 15 species were decreased (Fig. S4). Of these differential features, a VKH-enriched species *Acidaminococcus sp.BV3L6* was significantly decreased after the treatment, whereas 3 VKH-depleted species *Proteobacteria bacterium CAG495*, *Azospirillum sp.CAG260* and *Alistipes sp.CAG435* were remarkably increased (Fig. S4). MGS analysis showed that 5 MGSs were enriched in VKH patients before the treatment and 3 MGSs including *Faecalibacterium spp.* were enriched in VKH patients after the treatment (Fig. S1b, Table S9). A further analysis was made to investigate the functional profile of the microbiome alterations following the treatment. Up to 36 modules were enriched and 35 modules were depleted in VKH patients after the treatment as compared to the profile found before treatment. Of these modules that exhibited significant differences between these patients before and after treatment levels, 3 VKH-depleted modules were increased and 2 VKH-enriched modules were reduced (Figure 3, Table S10); in other words, their responses to the treatment mirrored their VKH association.

To examine whether the gut microbiome is associated with the response to treatment, we reviewed 50 active VKH patients' clinical data after treatment and then compared the gut microbiome between the patients with inactive ocular inflammation (30 patients) and those with a relapse ocular inflammation after treatment (20 patients). LEfSe analysis identified 70 species to be different between these two groups (Table S11). Of these, 5 *Alistipes spp.* and *Bacteroides sp. UW* were associated with a good response to treatment (Figure 5(a), Table S11).

Development of a gut microbial marker profile for VKH disease

To develop a marker profile of the gut microbiome in VKH disease, a training set that included 55 active VKH patient and 52 healthy controls was used. Firstly, we removed the extremely low abundant species (mean relative abundance < 0.001) and then filtered the redundant features with the mRMR algorithm and leave-one-out cross-validation LDA (Linear Discriminant Analysis). The final feature set with the highest MCC (Matthews Correlation Coefficient) was chosen to build the SVM (Support Vector Machine) classifier, which consists of 40 species. The area under the receiver operating characteristic curve (AUC) of the classifier was 89.16% and the 95% CI was 83.37%-94.95% (Figure 4(b)). The classifier was also validated in 16 VKH patients and 15 controls. The result showed an AUC value of 83.75% (95% CI: 69.74%-97.76%), demonstrating the usefulness of these markers in differentiating VKH patients from healthy controls (Figure 4(c)).

Another microbial marker profile based on 37 inflammation prognosis-associated species (Figure 5(b)) was selected using the SVM classifier. Results showed that the classifier was able to distinguish the patients with a good prognosis from their counterparts showing relapse (AUC = 93.65%, 95% CI = 86.87%-100%) (Figure 5(c)).

Fecal transfer of VKH patient microbiota exacerbates experimental autoimmune uveitis (EAU)

To investigate whether gut microbiome composition in VKH patients contributes to the development of this disease, we examined the effect of fecal microbiota transplantation (FMT) on the course of EAU, an animal model which has many features resembling human uveitis including VKH disease.³⁴ Three groups of antibiotic-treated B10RIII mice, each containing three animals, were colonized with a pooled fecal sample from five randomly selected active VKH patients or a pooled fecal sample from five randomly selected healthy controls or were treated with PBS,

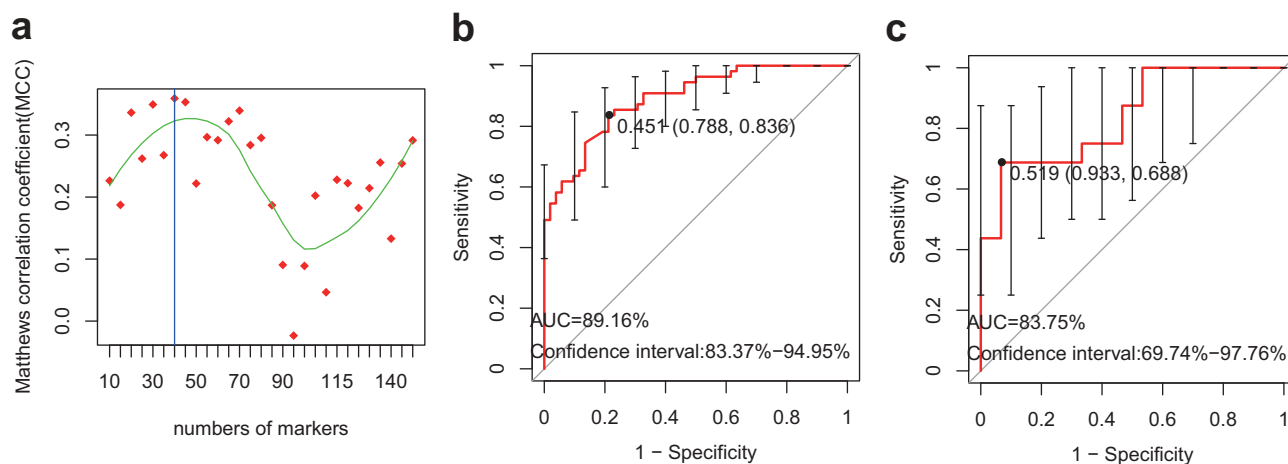


Figure 4. The classifier used to distinguish VKH patients from healthy controls.

(a) The mRMR method was used to identify the VKH-associated markers. Sequential subsets were generated at five-species intervals. For each subset, the error rate was estimated using a leave-one-out cross-validation of a linear discrimination classifier. The optimum (highest value of the Matthews correlation coefficient) subset contains 40 species markers. (b) Receiver operating characteristic curves (ROC) for discovery sets comprising samples from 55 active VKH patients without treatment and 52 healthy controls, the 95% confidence intervals (CIs) are shown as shaded areas. (c) ROC for validation sets comprising samples from 17 active VKH patients without treatment and 15 healthy controls.

respectively. To investigate whether the indigenous microbiota community was adequately knocked-down by antibiotic treatment, we performed 16S rRNA gene sequence analysis on the fecal samples from antibiotic-treated mice and human fecal-colonized mice first. Shannon diversity index (Figure 6(a)) and PCoA plot analysis (Figure 6(b)) showed that the diversity of antibiotic-treated mice was extremely low and the gut microbial phenotypes of human fecal-colonized mice were totally different from antibiotic-treated mice. Furthermore, the VKH-depleted genus *Alistipes* was also found to be depleted in the VKH-recipient group when compared with the control-recipient group (Table S12).

EAU was successfully induced in the VKH-recipient group following immunization with the retinal protein peptide IRBP₁₆₁₋₁₈₀ combined with complete Freund's adjuvant (CFA), evidenced by conjunctival hyperemia, ciliary injection, corneal edema, posterior synechiae, aqueous flare and cells (clinical scores: 3.5 to 5) (Figure 6(c)). A minor case of uveitis manifested by conjunctival hyperemia was observed in the control-recipient group (clinical scores: 0 to 1.5) (Figure 6(e)) and PBS-treated group (clinical scores: 0 to 0.5) (Figure 6(g)). Histological analysis also showed severe uveitis symptoms in the VKH-recipient group,

evidenced by destruction of the retinal architecture with severe folding and detachment, inflammatory cells throughout the retinal and choroid and intensive retinal vasculitis (histological scores: 2 to 4) (Figure 6(d)). However, in the control-recipient group (Figure 6(f)) and PBS-treated group (Figure 6(h)), none or minimal derangement of the retinal architecture was observed (histological scores: 0 to 1). Both our clinical and histological results showed that the scores in the VKH-recipient group was significantly higher than that in the control-recipient group as well as PBS treated-group (Figure 6(i-j)).

Discussion

In the present study, we showed that the gut microbiome composition was different (both microbial composition and function) either between VKH patients with active intraocular inflammation and healthy controls or among the three enterotypes, namely Mix enterotype, *Bacteroides* enterotype and *Prevotella* enterotype. Up to 4 VKH-associated species were found to be associated with HLA-DRA, a disease susceptibility genotype. We also found that immunosuppressive treatment in the VKH patients led to partial restoration of the gut microbiome

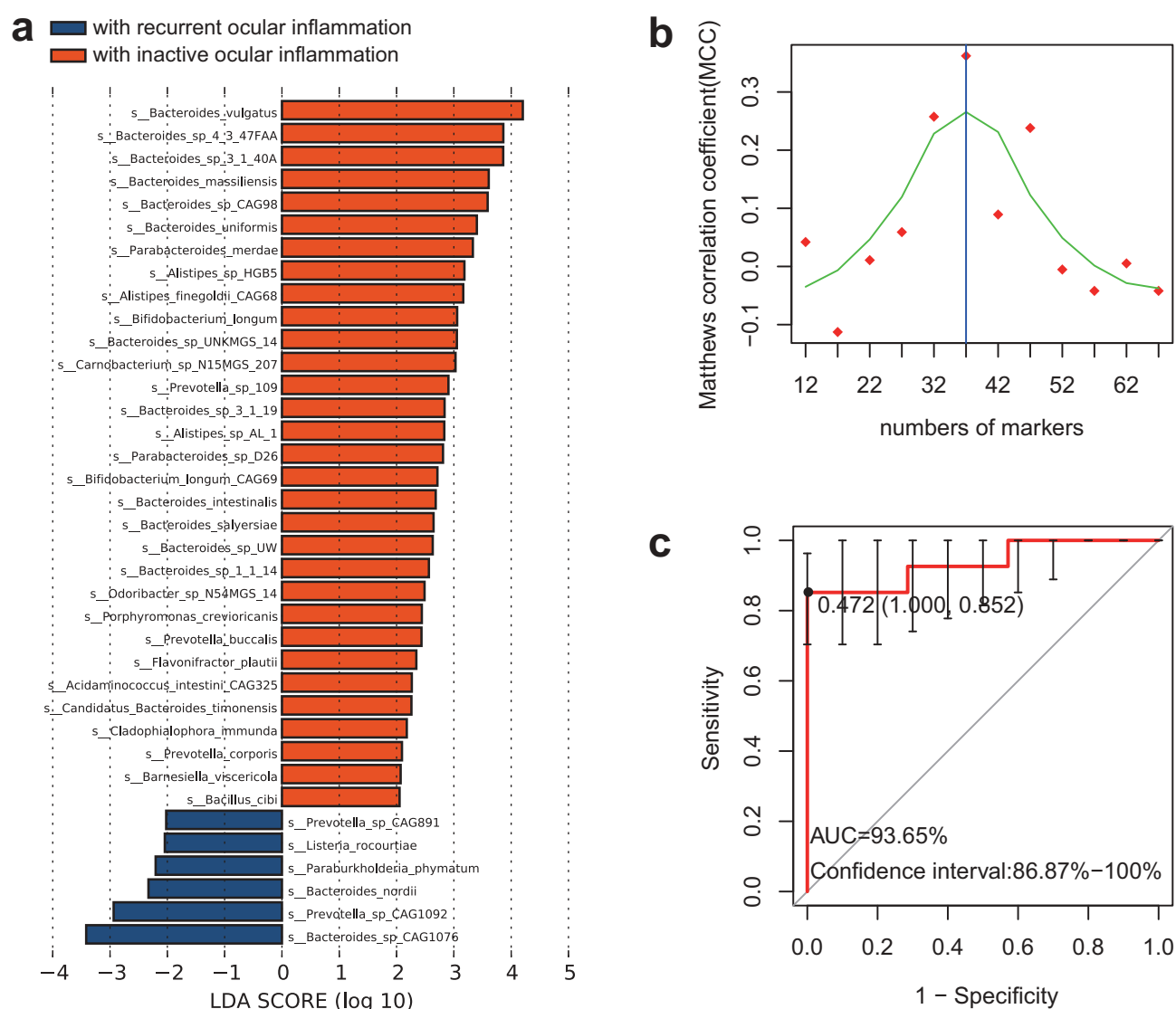


Figure 5. Species used to predict the prognosis.

(a) LefSe analysis identified 70 species as different microbial features between the VKH patients with inactive ocular inflammation ($N = 30$) and with relapse ocular inflammation ($N = 20$) after treatment. (b) The mRMR analysis showed the optimum (highest value of the Matthews correlation coefficient) markers set for classifier to predict the prognosis contains 37 species. (c) ROC for the classifier, the 95% confidence intervals (CIs) are shown as shaded areas.

alterations. Gut microbiome-based markers were constructed and showed a high specificity for VKH, as well as for disease prognosis. Fecal transplants into mice subjected to EAU significantly worsened the severity of intraocular inflammation in these animals.

The gut microbiome, as an environmental factor, has been proposed to be involved in host innate and adaptive immune responses and the pathogenesis of a number of immune mediated diseases.³⁵ In this study, we investigated whether the gut microbiome was implicated in the

development of VKH disease. Since the microbiome may be influenced by various factors such as medication, dietary habits, environment and diseases, we made the following efforts to eliminate possible confounding factors. Firstly, VKH disease was strictly diagnosed according to the diagnostic criteria revised for VKH disease that was issued by an international committee on nomenclature.¹⁹ The enrolled VKH patients and controls did not have any underlying diseases. Secondly, only patients with active ocular inflammation who did not receive any medication

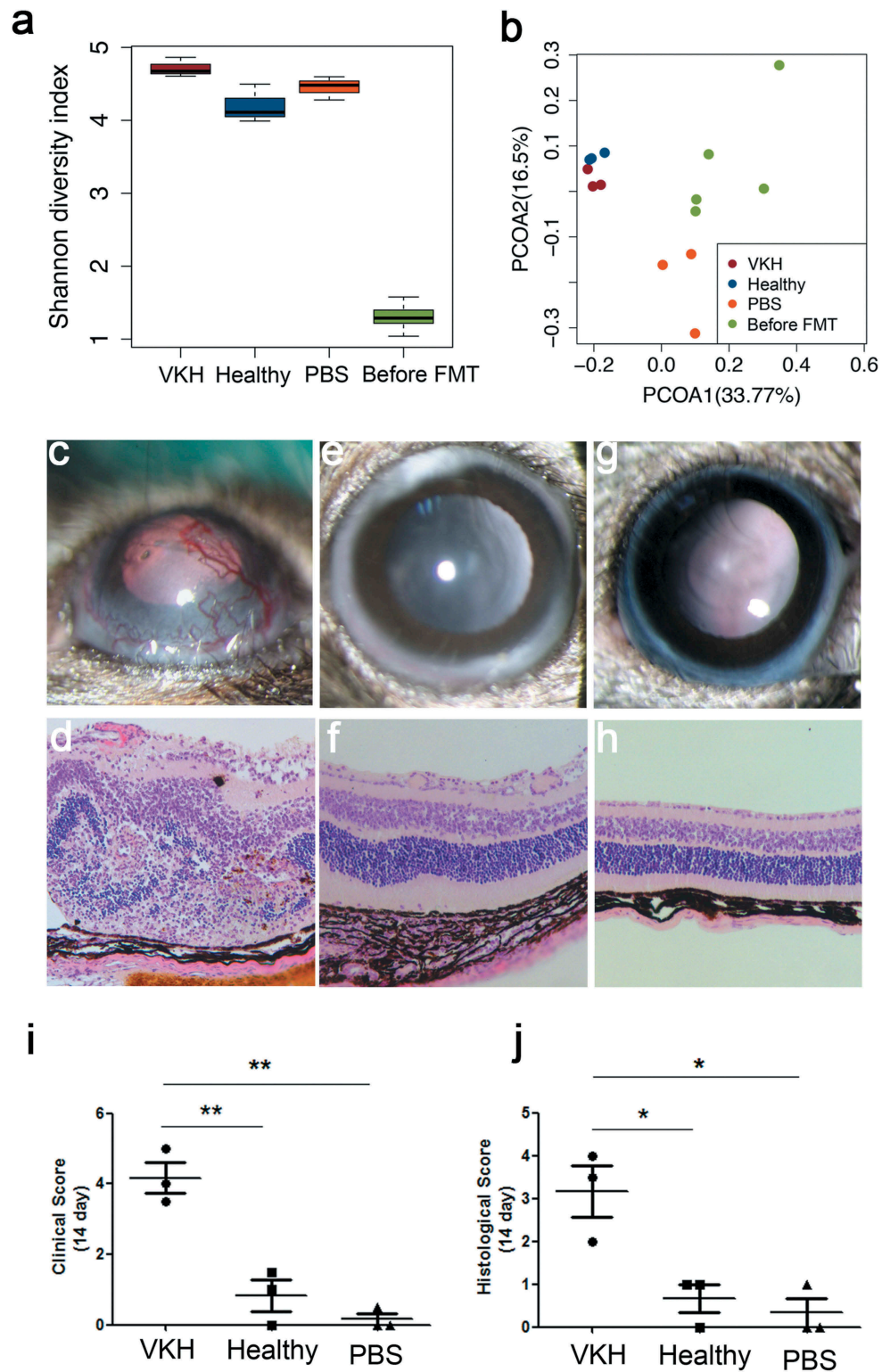


Figure 6. Effect of FMT using active VKH patient microbiota on EAU.

Mice were colonized with pooled feces from active VKH patients ($n = 3$), healthy controls ($n = 3$) or PBS ($n = 3$) and then induced EAU by immunization with IRBP161-180. (a) Shannon diversity index of mice before FMT (Green box) and after FMT (Red box: FMT with active VKH patient microbiota; Blue box: FMT with healthy control microbiota; Orange box: PBS treatment) at genus level. Boxes represent the inter quartile ranges, lines inside the boxes denote medians and circles are outliers. (b) The indigenous microbiota communities clustered based on PCoA of unweighted unifracs matrix. Each point represents a mouse indigenous microbiota community. Images of clinical and histological analysis of active VKH patients' colonized group (c-d), healthy controls' colonized group (e-f) and PBS-treated group (g-h). Statistical results of clinical (i) and histological (j) score were shown on day 14 following the immunization with the retinal peptide IRBP. Each point represents an individual mouse. The horizontal bars denote the average scores of each group.

including antibiotics and/or probiotics for at least one month before the start of the project were enrolled for our study. Finally, healthy controls were strictly matched with the enrolled patients based on similarity in dietary habits, living conditions, age, sex and body mass index (BMI).

Gut microbial gene repertoire of VKH disease was established by metagenomic analysis. VKH patients were enriched in Gram-negative bacteria in their gut such as *Bacteroides spp.*, *Paraprevotella spp.*, *Prevotella spp.* and *Parabacteroides spp.* but depleted with butyrate-producing bacteria (BPB, such as *Clostridium spp.* and *Roseburia spp.*), lactate-producing bacteria (LPB, such as *Bifidobacterium spp.*) and methanogens (such as *Candidatus Methanomethylophilus spp.* and *Methanoculleus spp.*). These results were partially consistent with those reported recently in BD,⁸ showing that BPB and methanogens were depleted in patients with the two inflammatory eye diseases, although most VKH-associated species were specific for this uveitis entity. These results indicate that the similar clinical manifestations, each disease has a unique microbiome composition.

Previous studies revealed that the gut microbiome can be classified into different clusters termed “enterotypes” based on the dominant taxa.³⁶ Different enterotypes were shown to be associated with different dietary habits^{37,38} and life style.^{39,40} Recent studies on Type II diabetes,⁴¹ neuro-Behcet’s and multiple sclerosis⁴² both confirmed that enterotype analysis provided crucial biological insight. In the present study, we identified three enterotypes, namely Mix enterotype, *Bacteroides* enterotype and *Prevotella* enterotype, in VKH patients and healthy controls. Subsequent analysis showed enrichment of Gram-negative bacteria and depletion of BPB, LPB and methanogens in both the Mix enterotype and *Bacteroid* enterotype, which was in line with our whole-cohort analysis. The subcluster stratified analysis results suggest that the disease state may be the only reason in explaining the differences observed in the VKH gut microbiome.

In line with the observation that Gram-negative bacteria were more abundant in VKH, increased KEGG pathways and modules in lipopolysaccharide (LPS) biosynthesis, capsular polysaccharide transport system and bacterial

secretion system (type VI) were enriched in patients. Interaction of LPS from gram-negative bacteria and the Toll-like receptor 4 (TLR4) can trigger strong inflammatory responses in host immune cells.⁶ In a previous study we reported an over activation of the LPS/TLR4 pathway in monocyte-derived macrophages from active VKH patients.⁶

Bifidobacterium spp. are considered beneficial bacteria and many strains in the genus are commonly used as probiotics. Previous studies have shown that *Bifidobacterium spp.* plays a role in the treatment or prevention of IBD,⁴³ inhibits bacterial translocation,⁴⁴ exerts antimicrobial activity,⁴⁵ and enhances the intestinal epithelial barrier (IEC) function.⁴⁶ Taken together, these results suggest that *Bifidobacterium spp.*, which were significantly depleted in active VKH patients, are beneficial in maintaining the health of the gastrointestinal tract. Butyrate also promotes gut health and has a role in controlling the permeability of the IEC.⁴⁷ In the present study, the abundance of BPB *Clostridium spp.* was significantly decreased in active VKH patients, which is in line with earlier observations in other autoimmune or autoinflammatory diseases such as BD⁸ and IBD.^{48,49} The depleted *Clostridium spp.* in VKH patients is in agreement with the findings concerning purine metabolism modules, showing the role of these species in purine ribonucleoside degradation.⁵⁰ *Bifidobacterium spp.*, *Clostridium spp.* and methanogens, which are characterized by their ability to produce methane, were also significantly depleted in active VKH patients. Earlier studies in animal models have confirmed the regulatory effect of methane on oxidative stress damage as well as on the control of inflammation.⁵¹ In the present study, we observed an overabundance of the oxidation phosphorylation pathway in active VKH patients, which has also been observed in patients with systemic lupus erythematosus (SLE), and is thought to be related to abnormal oxidative stress in the digestive tract.⁵² These observations collectively suggest that the patients with VKH disease might have a more “harmful” gut environment compared to healthy individuals. Whether this is the cause or effect of the disease is subject of further investigation.

Numerous studies have shown that host genetic factors, especially HLA polymorphism, are associated with VKH disease.^{5,53,54} Our study found that gut microbiome and the disease susceptibility genotype HLA-DRA, which are two possible factors associated with VKH disease, also showed a correlation. These results support the hypothesis that gut microbiome composition could be dependent on host genotype, which is supported by earlier studies on IBD.^{55,56} How interactions between host genes and gut microbiome contribute to the development of VKH disease is still unknown. One hypothesis is that a susceptible genotype may influence the gut micro-ecology, leading to the alteration of the gut microbiome, which ultimately leads to disease development. Alternatively, individuals with a susceptible genotype may develop disease, which is followed by alteration of their gut microbiome to generate a disease specific pro-inflammatory profile.

Immunosuppressive agents are the first choice in the treatment of VKH disease. Our results showed that the altered gut microbiome in VKH could be partially restored after immunosuppressive treatment, which coincides with resolution of intraocular inflammation. An increased abundance of bacteria negatively associated with VKH such as *Alistipes* sp. CAG435, *Azospirillum* sp. CAG260 and Proteobacteria bacterium CAG495 was observed after the treatment. A beneficial alteration seems to occur in the gut environment after the immunosuppressive treatment, although the exact mechanism is still unknown.

Previous studies have shown the potential of gut microbiome composition in disease classification.^{9,25,41} Our results confirmed that a disease-associated microbiome marker profile could distinguish the VKH patients from the healthy control group and that the use of another specific microbiome profile might facilitate the prediction of treatment effects. Further study is needed to investigate whether gut microbiome composition can be useful in disease prevention, diagnosis or prognosis in a large cohort.

FMT was performed in the present study to determine whether the gut microbiome composition might play a causal role in the pathogenesis of VKH disease. Transplantation of feces from active VKH patients to mice undergoing EAU

resulted in a more severe manifestation of the intraocular inflammation in these animals. These results are similar to our recent study on BD, where we showed that the gut microbiome from BD patients could also exacerbate disease activity in mice receiving a fecal transplant.⁸ While the exact mechanism involved is still unknown, these results together indicated that the gut microbiome from uveitis patients might play a role in the pathogenesis of the intraocular inflammatory disease.

The present study still has several limitations. Recruitment of all VKH patients and healthy controls was limited to a Chinese Han population. Our findings should therefore be confirmed in other ethnic populations. Furthermore, only a limited alteration of the gut microbiome was identified in *Prevotella*-dominant VKH patients. This might be due to the relatively small cohort size (number of *Prevotella* enterotype: VKH = 11, N = 7) and further study on more patients after enterotype stratification should be performed.

In conclusion, we found changes in the gut microbial composition and function in active VKH patients, as well as in VKH patients of different enterotypes. These changes could partly be restored after immunosuppressive treatment. Differences in the gut microbiome were correlated with disease susceptibility genotypes. Microbial-associated gene profiles could be used to differentiate VKH patients from controls and could also be used to predict response to treatment. Moreover, an animal experiment suggests that gut microbiome composition might be a causal factor in the development of this disease.

Author contributions

Z. Y., C. W., N. Z., N.Q. and P. Y. conceived and directed the study; N.Q., C.W. and X.X. analyzed the data; P. Y. made clinical diagnoses and performed treatment; N. Z., X. H., J. T., Q. C., L. D., F. L. and C. Z. collected the samples; Z. Y., X. H., J. T., and Q. W. extracted the fecal DNA; Z. Y., X. H. and J. T. extracted the genomic DNA and contributed the SNP genotyping; Q. X. and X. X. performed the 16S rRNA and Metagenomic sequencing; Z. Y., Q. W. and X. H. performed the animal experiment; Z. Y., C. W., and N. Z. drafted the manuscript; P. Y., N. Q., and

A. K. reviewed data analysis and edited the manuscript. All authors reviewed the manuscript.

Availability of data and materials

Metagenomic sequencing data for all samples have been deposited in NCBI with accession number of PRJNA356225.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Ethics approval and consent to participate

All procedures followed the tenets of the Declaration of Helsinki and were approved by the Ethics Committee of Chongqing Medical University with written informed consent.

Abbreviations

| | |
|--------|---|
| AS | Ankylosing spondylitis |
| AUC | Area under the receiver operating characteristic curve |
| BMI | Body mass index |
| CNS | Central nervous system |
| EAU | Experimental autoimmune uveitis |
| eggNOG | Evolutionary genealogy of genes: Non-supervised Orthologous Groups database |
| Gb | Gigabases |
| GWAS | Genome-wide association analysis |
| IBD | Inflammatory bowel disease |
| KEGG | Kyoto Encyclopedia of Genes and Genomes database |
| KO | KEGG orthologues |
| LPS | Lipopolysaccharide |
| MGS | Metagenomic Species |
| MS | Multiple sclerosis |
| NCBI | National Center for Biological Information |
| OG | EggNOG orthologues |
| ORFs | Non-redundant open reading frames |
| OUT | Operational taxonomic unit |

| | |
|------|------------------------------|
| PA | Psoriatic arthritis |
| PCoA | Principal component analysis |
| RA | rheumatoid arthritis |
| SLE | systemic lupus erythematosus |
| VKH | Vogt-Koyanagi-Harada |

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