Title: Specific microbiome profile in Takayasu Arteritis (TAK) and Giant Cell Arteritis (GCA).

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Contributions

AC. Desbois: designing research studies, conducting experiments, acquiring data, analyzing data, and writing the manuscript.

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Abstract

Objectives: There is increasing evidence of a close link between microbiota and inflammatory diseases. Microbiota has never been studied in large vessel vasculitis (LVV). We aimed to analyse the blood microbiome profile of patients with LVV [Takayasu arteritis (TAK) or giant cell arteritis (GCA)] and healthy donors (HD).

Methods: We studied blood samples of 13 patients with TAK (20 samples), 9 (11 samples) with GCA and 15 HD. The blood microbiome profile was assessed by sequencing of the 16S rDNA blood bacterial DNA. Linear Discriminant Analysis (LDA) coupled with effect size measurement (LEfSe) was used to analyse the differences in the blood microbiome profile between the groups.

Results: Samples of TAK patients showed an increase in the levels of Clostridia, Cytophagia and Deltaproteobacteria and a decrease in Bacilli at the class level as compared to HD (LDA>2, $p<0.05$). Active compared to inactive TAK patients had significantly lower levels of Staphylococcus. Samples of GCA patients showed an increased abundance of Rhodococcus and an unidentified member of the Cytophagaceae family. Microbiota of TAK compared to GCA patients showed higher levels of Candidatus Aquiluna and Cloacibacterium (LDA>2; $p<0.05$). Differences in blood microbiome were also associated with a shift of bacterial predicted metabolic functions in TAK compared to HD. Similar results were also found in active compared to inactive TAK patients.

In conclusion, TAK patients showed a specific blood microbiome profile as compared to healthy controls and GCA patients. Among TAK patients, significant changes of blood microbiome profile were associated with specific metabolic functions.

Introduction

Large vessel vasculitis (LVV) belong to the group of systemic vasculitis and mainly include giant cell arteritis (GCA) and Takayasu arteritis (TAK). LVV may lead to segmental stenosis, occlusion, dilatation and/or aneurysm formation in the aorta and/or its main branches (1). The pathogenesis of LVV is not well understood. LVV are characterized by an inflammatory infiltrate located in arterial wall but the mechanisms leading to such lesions remain unclear. Weyand and al. have demonstrated that inhibitory signals by which dendritic cells provide stop signal to T cells (through PDL1 and PD1 interactions) were defective in GCA, emphasizing the regulatory importance of arterial dendritic cells in GCA pathogenesis (1). Specific toll like receptors (TLR), involved in pathogen associated molecular patterns recognition have also been shown to be implicated in LVV pathogenesis (2). These data emphasize the importance of interactions between antigen presenting cells (APC) and T cells and suggest the role of antigenic triggers promoting an uncontrolled immune response. Although interactions between pathogen agents and dysfunction of immune cells seem likely, there is not currently strong data for this hypothesis.

The tight relation between gut dysbiosis and altered immune response has been well established in recent studies and such alterations may be involved, at least in part, in the pathogenesis of auto-immune diseases (3). It has long been thought that blood is a sterile environment. However, recent sequence-based studies have revealed that changes in blood microbiota are associated with various diseases (4,5). Studying blood microbiota is particularly interesting because it is a reflexion of various microbiota (gut, oral, nasal) and it allows thus to determine the presence of bacteria or bacterial DNA that have passed the mucosal barrier. Such bacteria may have direct interactions with the immune cells present within the vessels leading to the activation of immune response.

In the present study, we aimed to evaluate blood microbiota of patients with LVV as compared to the microbiota of healthy donors (HD).

Methods

Blood samples of consecutive patients with LVV fulfilling the criteria for GCA or TAK and healthy blood donors (HD) were collected using dry tubes (30 mL). The study was approved by our institutional ethics review board and was performed according to the Helsinki declaration. Patients gave informed consent.

Blood was afterwards centrifuged at 4°C immediately after sampling during 5 minutes at 2500 g. Serum was aliquoted into separate polypropylene tubes that were immediately stored at -80°C until analysis. Bacterial DNA was extracted from 300 µL serum from fasting specimens collected in the morning, as previously described (4,6).The concentration of 16S rRNA gene copies normalized to 1 mL of serum in each sample was determined by real-time qPCR using primers EUBF 50-TCCTACGGGAGGCAGCAGT-30 and EUBR 50-GGACTACCAGGGTATCTAATCCTGTT-30 As many reagents required in the qPCR and sequencing pipeline contain bacterial DNA which can be misinterpreted as present in the samples, numerous combinations of reagents were tested to minimize bacterial contaminants. The protocol was adapted to increase the yield of amplification of the bacterial DNA present in the blood. Numerous controls were performed both *in vitro* and *in silico* to ensure the absence of artefacts (such as bacterial DNA contaminants from reagents or nonspecific amplification of eukaryotic DNA) as previously described (6). The V3-V4 hypervariable regions of the 16S rDNA were amplified and quantified by qPCR, sequenced with MiSeq technology (Vaiomer, Labège, France). The sequences were processed using the quantitative insights into microbial ecology (QIIME v1.9.0) pipeline, using its default parameters (7). Sequences were then clustered into operational taxonomic units (OTUs) displaying at least

97.0% sequence similarity, by a closed reference-based picking approach in UCLUST software applied to the Greengenes 13.8 database of bacterial 16S rDNA sequences. The mean number of quality-controlled reads was 38,039 ± 3,945 (mean ± SD) per sample.

After rarefaction at 30,000 reads per sample, the bacterial alpha diversity (species richness or number of taxa within a sample) was estimated based on the observed species, Faith's PD_Whole_Tree and Shannon's index. OTUs with a prevalence < 5% were removed from the analysis to decrease the probability of including OTUs generated by sequencing errors. The beta diversity (diversity of microbial communities between different categories) was assessed using weighted and unweighted UniFrac distances. The weighted Unifrac metric is weighted by the difference in the abundance of OTUs from each community, whereas unweighted UniFrac considers only the absence/presence of OTUs, providing different information. Both are phylogenetic beta diversity metrics. We investigated the OTUs not identified by QIIME further, using the Basic Local Alignment Search Tool (BLASTN program, vBLAST+ 2.6.0) from NCBI Blast, against the NCBI 16S Microbial database.

Inferred metagenomics

The functional composition of the intestinal metagenome was predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (8). This is a computational approach that accurately predicts the abundance of gene families in the microbiota and thus provides information about the functional composition of the microbial community. A total of 6,909 KEGG orthologs were assigned using the complete 16S sequence dataset, corresponding to 328 modules. Among these, 146 modules were assigned to metabolic pathways and the rest of the analysis was focused on the metabolic pathways.

Statistical analysis

The results are expressed as means ± SEM. Alpha diversity comparisons were performed with nonparametric Student's *t*-tests and Monte Carlo permutations in QIIME. Individual

comparisons were performed at all the levels of classification or taxonomic rank (phylum, class, order, family and genus). Taxa were compared using Mann–Whitney U-tests and the ANOSIM test with 999 permutations was used to compare distance matrices (weighted and unweighted UNIFRAC) in QIIME. The Benjamini–Hochberg false discovery rate (FDR) correction was used to correct for multiple hypothesis testing, when applicable.

Linear discriminative analysis (LDA) effect size (LEfSe) analysis was performed to identify the taxa displaying the largest differences in abundance in the microbiota between groups. Briefly, it consists of a Kruskall-Wallis test that analyzes whether the values in different classes are differentially distributed and a pairwise Wilcoxon test that assesses whether all pairwise comparisons between subclasses within different classes significantly agree with the class level trend. Finally, the resulting subset of vectors is used to build a LDA model, from which the relative difference among classes is used to rank the features according to the effect size with which they differentiate classes. The final output consists of a list of features that are discriminative with respect to the classes, consistent with the subclass grouping within classes, and ranked according to the effect size with which they differentiate classes (9). Only taxa with an LDA score >2 and a significance of $\alpha < 0.05$, as determined in Wilcoxon signed-rank tests, are reported. The size of the circles in the cladogram plot is proportional to bacterial abundance. LEfSe and Picrust were accessed online (http://huttenhower.sph.harvard.edu/galaxy/).

The remaining comparisons were performed with R software v2.14.1 or GraphPad v7.01 (Graphpad Prism, Graphpad Software Inc, La Jolla, California, USA). Unpaired t-tests or Mann–Whitney U-tests were used to compare continuous data between groups, depending on the data distribution. Chi2 or Fisher's exact tests were used to compare discrete parameters between groups.

Results

Patient Characteristics

A total of 13 patients with TAK (20 blood samples), 9 with GCA (11 blood samples) and 15 HD were analysed. TAK and GCA patients had a mean age 45 (23.1; 70.6) and 74.5 (58; 84) years, and 54.5% and 85% were females, respectively. Among samples of TAK patients, 10 were performed when the disease was active disease and 10 when inactive; among GCA patients, 6 were active and 4 inactive. Among TAK samples, 7 were performed without treatment at inclusion and 13 were under treatments including low dose corticosteroids (n=2), methotrexate (n=6), azathioprine (n=1) or biotherapy (n=4). Among GCA samples, 2 were performed without treatment and the others were under low dose corticosteroids (n=4), corticosteroids >10 mg/day (n=4) and tocilizumab (n=1).

Blood microbiota signature of LVV patients

There was no difference in the absolute quantity of 16s DNA bacterial measured in the samples in the different groups (**Fig 1A**). We found no difference in terms of alpha and beta diversity or in term of phyla composition between the groups (**Fig 1B**). Using linear discriminant analysis effective size, we identified changes in the blood microbiome between LVV and HD patients at smallest taxonomic levels. LVV patients showed an increased abundance of Cytophagia and Clostridia at the class level as compared to HD patients (**Figure 1C**). At the genus level, LVV showed an increase in an unidentified taxa from the Cytophagaceae family and a decrease in Zoogloea and Staphylococcus compared to HD patients ($p<0.05$) (**Figure 1C**). Concerning the predicted metagenomic functions of the blood microbiota, LVV patients showed enrichment of "P450 Cytochrom" and a decrease in

"ubiquinone and other terpenoid quinone biosynthesis" pathways as compared to HD (**Fig 1D**).

Blood microbiota signature of TAK patients

Compared to HD, the blood microbiome of TAK patients showed an increased abundance of Clostridia, Cytophagia and Deltaproteobacteria and a decrease in Bacili, at the class level. At the genus level, there was an increase in Bdellovibrio and three unidentified taxa from the Cytophagaceae (identified as *Pseudarcella Hirudinis* using BLAST), Clostridiaceae family (identified as *Clostridium saudiense* using BLAST) and Sphingomonadales ordre (identified as *Bdellovibrio bacteriovorus* using BLAST) and a decrease in *Staphylococcus* and *Hyphomicrobium* ($p<0.05$) (**Figure 2A**). Compared to active TAK patients, inactive patients had an increased abundance of Staphylococcus. Concerning the predicted bacterial metagenomic functions of the blood microbiota, TAK patients compared to HD showed enrichment in the “porphyrin and chlorophyll pathways” and a decrease in the “toluene degradation pathway”. Active TAK patients showed enrichment of porphyrin and chlorophyll pathways compared to TAK patients with inactive disease.

Blood microbiota signature of GCA patients

Compared to HD, the blood microbiota of GCA patients showed an increased abundance of Rhodococcus and an unidentified member of the Cytophagaceae family ($p<0.05$) (**Figure 2B**). For the predicted bacterial metagenomic functions of the blood microbiota, GCA patients compared to HD showed a decrease in the “Ubiquinone and other terpenoid_quinone biosynthesis” and the “C5_Branched dibasic acid metabolism” pathways. Predicted bacterial metagenomic functions were similar in TAK and GCA patients. Compared to TAK patients, the blood microbiota of GCA patients had an increase in the relative abundance of *Rhodococcus* and an unidentified member of the Hyphomonadaceae family and a decrease in

Candidatus Aquiluna and *Coacibacterium* at the genus level, as compared to TAK patients ($p<0.05$) (**Fig 2C**).

Discussion

Recent studies on microbiome provide evidence of strong relationship between microbiome and immune system regulation. The microbiome is able to induce regulatory immune response and participate to immunological tolerance. It is now well demonstrated that an inappropriate intestinal immune response impairs the intestinal homeostasis, leads to gut dysbiosis, and contributes to local and systemic inflammation and metabolic dysfunction. Altered microbiome composition has been shown to be associated with inflammatory diseases such as inflammatory intestinal disease [Crohn disease (CD) and ulcerative colitis (UC)], rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (3).

In large vessel vasculitis, such as TAK and GCA, there is no published data on the composition of microbiome. To our knowledge, the present study is the first evaluating the blood microbiome in LVV patients. Particularly in TAK patients, we found an imbalanced blood microbiota characterized by an increase abundance of Clostridia (Firmicutes phylum), Cytophagia (Bacteroidetes phylum) and Deltaproteobacteria (Proteobacteria phylum) while we found a decrease in Bacilli (Firmicutes phylum) at the class level. Expansion in the Proteobacteria phylum was associated with inflammatory conditions such as CD (10). Consistently, some studies have suggested that changes in the gut microbiome, especially a Proteobacteria–dominated community, predispose genetically susceptible mice to chronic colitis (10). Altogether, these data provide evidence that expansion of Proteobacteria may trigger inflammatory responses (10). Consistent with our findings, it has been reported an increase of mice faecal Firmicutes and Proteobacteria during the immune priming phase of RA (11). A lower Firmicutes/Bacteroidetes ratio and an increased frequency of *Prevotella* and *Klebsiella* have also been described in SLE individuals (12). In RA patients, a correlation between antibodies against *Porphyromonas gingivalis* and anti-citrullinated protein antibodies was found. This latter bacteria was shown to be implicated with periodontal disease and to be

able to induce citrullination, through a protein containing citrullin that is recognized by anti-citrullinated protein antibodies (13).

Of note, in autoimmune and inflammatory diseases, most data come from studies performed on gut microbiota. We found some similarities between our findings and those reported in gut microbiome in other diseases (3,10,11). We can speculate that some bacteria or bacterial products such as bacterial DNA may translocate from gut or other mucous membranes (mouth), then interact with immune system present within the vascular wall, leading to activation of the immune process and thus participating to LVV pathogenesis.

We acknowledge some limitations of the present study. The low number of patients reflects the rarity of LVV diseases. One major concern is related to the origin of the bacterial DNA detected in blood: free bacteria, bacterial DNA resulting from immune degradation truly present in blood or contamination. The significant differences found in LVV patients compared to HD and the high number of bacterial cells (10^6 to 10^7 genomes/mL) are not consistent with contamination. In the technique we used, the abundance of 16S ribosomal RNA genes is 1000-fold lower in negative controls as compared to blood samples. The blood samples have also higher genus richness and different composition suggesting that the technical contamination had no significant impact. The presence of a specific blood microbiome has already been demonstrated in several diseases such as chronic kidney diseases, liver fibrosis, diabetes mellitus or cardiovascular events (5,14,15).

In summary, we found for the first time, specific alterations of blood microbiome in LVV compared to healthy donors, and between LVV (i.e. TAK compared to GCA). These alterations were associated with enrichment of specific metabolic pathways, which may be implicated in LVV pathogenesis. Some of these alterations have already been described in other inflammatory diseases and were associated to disease activity and to a potential effect

on immune regulation. Further studies should include larger group of LVV patients and assess the impact of these bacteria on activation and differentiation of T cells of LVV patients.

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Figure 1: Circulating microbiome profile in patients with large vessels vasculitis (LVV).

A. Absolute quantity of bacterial 16s DNA measured in the samples in the different groups. **B**. Histograms of the circulating microbiome composition at the phyla level in HD (n=15), GCA (n=11) and TAK (n=20). **C**. LDA effect size (LEfSe) cladograms showing the taxa most differentially associated with LVV (n=26, green) or HD (n=15, red) (Wilcoxon rank-sum test, $p<0.05$). Circle sizes in the cladogram plot are proportional to bacterial abundance. The circles represent, going from the inner to outer circle: phyla, genus, class, order, and family. **D**. KEGG pathway contributions of predicted metagenomic data in LVV and HD patients (Wilcoxon rank-sum test, $p<0.05$).
GCA, giant cell arteritis; TA, Takayasu arteritis; HD, healthy blood donors.

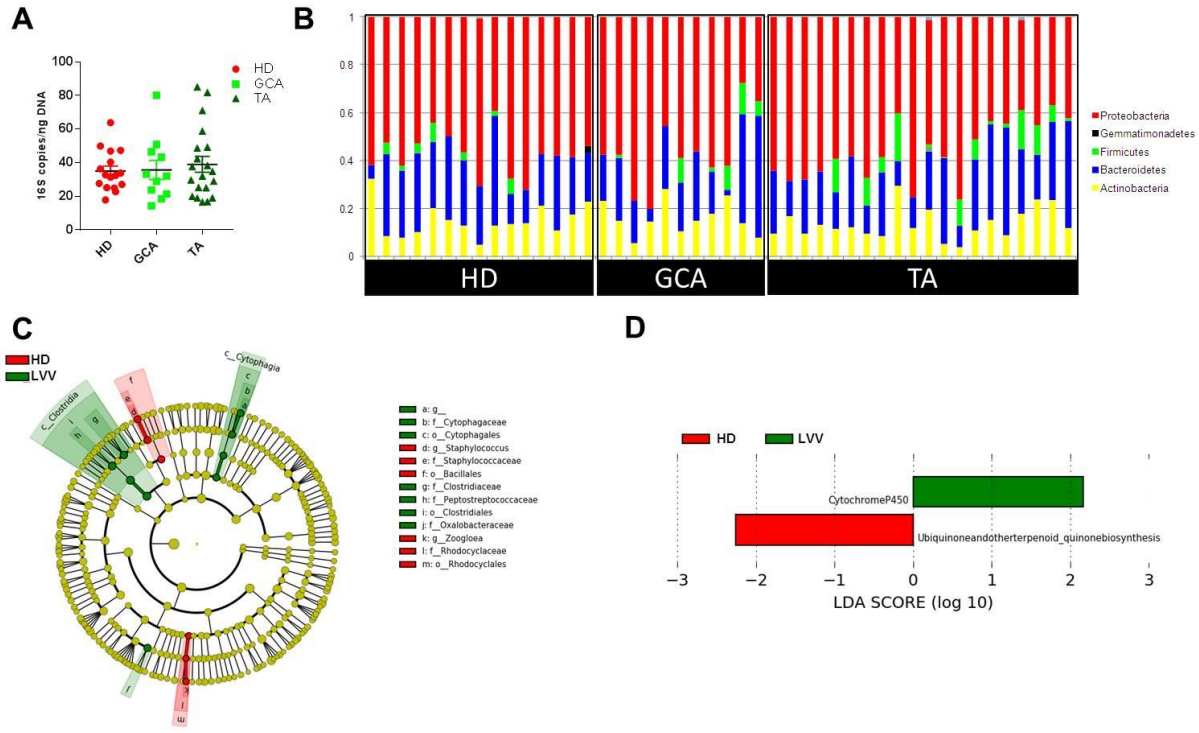

Figure 2: Circulating microbiome profile and predicted metagenomic function in patients with large vessels vasculitis depending of their phenotype.
A. **Upper panel**. LDA effect size (LEfSe) cladograms showing the taxa most differentially associated with TAK patients (n=20) compared to HD (n=15). **Lower panel**. Specific changes in bacterial and fungal relative abundance between the two groups using LEFSe. **B**. LDA effect size (LEfSe) cladograms showing the taxa most differentially associated with GCA (n=11) patients compared to HD (n=15). **C**. LDA effect size (LEfSe) cladograms showing the taxa most differentially associated with GCA patients compared to TAK patients. LDA: Linear discriminant analysis. ($p<0.05$).
GCA, giant cell arteritis; TA, Takayasu arteritis; HD, healthy blood donors.