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Changes in the composition of the upper respiratory tract microbial community in granulomatosis with polyangiitis

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ABSTRACT

Dysbiosis, *i.e.* changes in microbial composition at a mucosal interface, is implicated in the pathogenesis of many chronic inflammatory and autoimmune diseases. To assess the composition of the microbial upper respiratory tract (URT) community in patients with granulomatosis with polyangiitis (GPA), we used culture-independent high-throughput methods. In this prospective clinical study, nasal swabs were collected from patients with GPA, patients with rheumatoid arthritis (RA, disease control), and healthy controls. Nasal bacterial taxa were assessed using V3–V4 region 16S rRNA amplicon sequencing. *Staphylococcus aureus, Haemophilus influenza*, and entero- and rhinoviruses were detected using qPCR. Unbiased metagenomic RNA sequencing (UMERS) was performed in a subset of samples to determine the relative abundance of bacterial, fungal, and viral species. A trend toward reduced microbiome diversity was detected in GPA samples compared with healthy controls. The abundance of bacterial taxa and microbial richness were significantly decreased in GPA samples compared with RA samples. The relative abundance of bacterial families shifted, with increased *Planococcaceae* and decreased *Moraxellaceae, Tissierellaceae*, *Staphylococcaceae*, and *Propionibacteriaceae* in GPA and RA. Further, decreased abundance of *Corynebacteriaceae,* and *Aerococcaceae* was observed in GPA samples. Significantly more colonization of *S. aureus* was seen in the nasal microbiome of GPA compared with RA and healthy control samples. *H. influenzae* colonization was also observed in GPA samples. UMERS detected the presence of rhinoviral sequences in some GPA samples. Thus, our study uncovered changes in the URT microbial composition in patients with GPA and RA, suggesting that both immunosuppression and disease background affect the URT microbiome. Complex alterations of host-microbiome interactions in the URT could influence chronic endonasal inflammation in GPA.

1. Introduction

Granulomatosis with polyangiitis (GPA) is a rare systemic autoimmune disorder strongly associated with anti-neutrophil cytoplasmic autoantibodies targeting neutrophil- and monocyte-derived lysosomal enzyme proteinase 3 (PR3-ANCA) [1]. GPA presents with necrotizing autoimmune vasculitis that preferentially affects small-to medium-sized pulmonary and renal vessels and extravascular necrotizing neutrophilic granulomatous inflammation usually involving the upper and/or lower respiratory tract. Owing to its systemic nature, the vasculitis can affect any organ [1–3]. The most frequent presenting features in patients with GPA are concomitant respiratory tract manifestations and flu-like symptoms. In large cohorts, virtually all patients experience these symptoms during follow-up [4–6]. Furthermore, patients frequently experience “grumbling disease,” a condition with persistent endo- and paranasal disease activity that affects patients who are otherwise in

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clinical remission and despite the use of immunosuppressive treatments [7,8].

GPA is a prototypical adulthood autoimmune disease with a multifactorial etiology. The disease is initiated and maintained by the combination of predisposing genetic and environmental factors [2,3,8,9]. In GPA, chronic nasal carriage of *Staphylococcus aureus*, an environmental factor, has been reported to be associated with inflammatory endonasal activity and increased relapse rate [10–14]. Treatment with the antibiotic cotrimoxazole reduces the rate of respiratory tract infections and risk of relapse, especially in relapses involving the upper respiratory tract (URT) [15]. Nasal *S. aureus* carriage is detected more frequently in patients with GPA compared with healthy individuals and patients with chronic rhinosinusitis [10,13–15].

Studies have shown that less than 1% of microorganisms in an environment can be cultured. Thus, studies of microorganisms based on culturing are not representative of actual microbial diversity. By contrast, high-throughput sequencing technologies allow culture-independent characterization of entire bacterial, viral, and fungal communities [16,17]. 16S rRNA gene sequencing is commonly used for characterization of bacterial diversity. Metagenomic sequencing is applied for an unbiased analysis of entire bacterial, fungal, and viral communities in a disease sample [18,19]. Most studies analyzing nasal *S. aureus* carriage in GPA to date were based on cultivation methods. Because of this technical limitation, they could not analyze entire microbial communities [11–14,20]. To assess microbial community diversity in the nasal cavities of patients with GPA using a comprehensive culture-independent methodology, we performed 16S rRNA amplicon sequencing of samples from the nasal cavities of patients with GPA and healthy controls. Samples were also collected from patients with rheumatoid arthritis (RA) for use as a disease control, *i.e.* a different autoimmune disorder treated with immunosuppressants. Samples were tested for the presence of *S. aureus*, *Haemophilus influenza*, and respiratory viruses, *e.g.*, entero- and rhinoviruses using a highly sensitive routine diagnostic qPCR. We also performed unbiased metagenomic RNA sequencing (UMERS) [21–23] on a subset of samples from patients with GPA and controls to rule out the presence of unexpected pathogens.

2. Materials and methods

2.1. Study design and patient population

This prospective clinical study was conducted between August 2016 and March 2017. Sterile bilateral nasal swabs were collected from a total of 77 individuals, including patients with GPA (n = 29) and RA (n = 21) as well as healthy controls (n = 27). Supplementary Tables 1–5 provide individual-level patient information, details of the analyses methods applied to each sample (*i.e.*, V3–V4 region 16S rRNA amplicon sequencing, quantitative PCR, UMERS), and the next-generation sequencing data obtained from UMERS. Sequence data have been submitted to the BioProject database/project ID PRJNA494384. Patients and controls were followed at the Department of Otorhinolaryngology, Christian-Albrechts-University Kiel and the Department of Rheumatology and Clinical Immunology, University of Lübeck. All patients met the American College of Rheumatology criteria and the Chapel Hill Consensus Conference definition for GPA [1,24]. Disease activity was evaluated using the clinical composite Birmingham Vasculitis Activity Score (BVAS) V3.0 for patients with GPA and the modified Disease Activity Score (DAS) 28 for those with RA [25,26]. GPA activity in the URT was estimated using the Ear Nose Throat Activity Score (ENTAS) [7]. The characteristics of patients with GPA and controls are summarized in Table 1. None of the swabbed individuals were currently on antibiotic therapy, and all had been off antibiotic therapy for at least 3 weeks. All patients and controls provided written informed consent. This study was approved by the ethics committees at the Christian-Albrechts-University Kiel (Az A101/07) and the University of Lübeck (Az 16-199).

Table 1
Overview of subjects included in the study.

Group 1: healthy control, n = 27	
Age, years (median, range)	55, 18–81
Sex (f/m)	14/13
Group 2: GPA, n = 29	
Age, years (median, range)	49, 21–83
Sex (f/m)	20/9
Disease duration, months (median, range)	64, 18–216
Localized/generalized GPA (n/n)	3/26
C-ANCA/P-ANCA positive (n/n)	23/2
PR3-ANCA/MPO-ANCA positive (n/n)	24/1
Active disease with BVAS V3.0 ≥ 1 (n; median, range)	6; 7.5; 1–12
Remission, BVAS V3.0 = 0 (n)	23
ENTAS (n of grade none, mild, moderate, mild-moderate, and high)	19, 6, 1, 3, 0
Positive histology (n)	17
Organ involvement (n of ENT/lung/kidney)[a]	29/17/11
Prednisolone dosage, mg (n; median, range)	28; 5, 2–30
CYC/RTX/AZA/MTX/LEF/MMF/CTR (n)	0/10/5/13/3/1/0; (32)
Group 3: RA, n = 21	
Age years (median, range)	60, 29–73
Sex (f/m)	12/9
Disease duration, months (median, range)	84, 16–714
RF/Anti-CCP positive (n/n)	11/11
Active disease, DAS 28 ≥ 2.6 (n; median, range)	12; 3.55; 2.93–7.78
Remission, DAS 28 < 2.6 (n; median, range)	9; 1.65; 0.91–2.17
Organ involvement (n of lung)	1
Prednisolone dosage, mg (n; median, range)	17; 4, 2–10
RTX/MTX/LEF/aTNF/TOZ/ABC (n)	0/11/3/6/4/1; (25)

Abbreviations: ABC, abatacept; ANCA, anti-neutrophil cytoplasmic autoantibody; Anti-CCP, anti-cyclic citrullinated peptide antibody; aTNF, anti-tumor necrosis factor; AZA, azathioprine; BVAS, Birmingham vasculitis activity index, version V3.0 [26]; CTR, cotrimoxazole; CYC, cyclophosphamide; DAS 28, Disease Activity Score 28 [25]; ENTAS, ENT activity score [7]; GPA, granulomatosis with polyangiitis; LEF, leflunomide; MMF, mycophenolate mofetil; MPO, myeloperoxidase; MTX, methotrexate; PR3, proteinase 3; RA, rheumatoid arthritis; RF, rheumatoid factor; RTX, rituximab; TOZ, tocilizumab.

[a] Further description of organ involvement in GPA is provided in Supplemental Table 1.

2.2. DNA/RNA extraction

Swabs were soaked in 500 µl PBS for 1 h at room temperature. Nucleic acid (DNA and RNA) was extracted from 200 µl of sample using an automated extraction system, Qiasymphony (Quiagen, Hilden), according to the manufacturer's instructions with one modification. Because samples were also being used for UMERS, carrier RNA was omitted from the extraction protocol. Samples were eluted in 60 µl volumes.

2.3. Library preparation, high-throughput sequencing and bioinformatics, de novo contig assembly, and taxonomic classification

An Illumina library was prepared from the RNA using a modified version of the SCRIPT SEQ™ v2 RNA Seq Kit (Illumina, San Diego) protocol, described previously [22,23]. Briefly, total RNA concentration was measured using Qubit (Thermo Fisher Scientific, Carlsbad), and 15 ng was used for size fragmentation, cDNA synthesis, and the addition of a terminal-tagged oligonucleotide. Di-tagged cDNA was purified using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis) and amplified for 12 cycles. The size of the library was visualized using

a BioAnalyzer High Sensitivity LabChip (Agilent, Santa Clara). Diluted libraries (2 nM) were multiplex-sequenced on a HiSeq 2500 instrument (2 × 150 bp paired-end run, estimated 50 million reads/sample) (Illumina, San Diego).

To subtract reads originating from the host, reads were first aligned to a human reference assembly (NCBI 37.2) using Bowtie2 (v2.1.0). Trinity version r2013-02-25 was used to assemble contigs from reads without significant host alignment. Contigs shorter than 400 bp or of mammalian origin were excluded from further analysis. To estimate abundance, all filtered reads were aligned to filtered contigs using Bowtie2. Reads without significant alignments at this step were also aligned to sequences of known plasmids and viruses. In both cases, putative PCR duplicates were excluded from abundance estimations.

For taxonomic classification, filtered contigs were aligned to the NCBI nt database using the blast + package (v2.2.30). The first round of alignments was performed using megablast. All sequences lacking significant megablast hits (E-value cutoff of 0.01) were included in a second round of alignment using blasting. Contigs that still failed to produce alignments with an E-value ≤ 0.01 were classified as "unknown sequences." For each of the remaining contigs, all blast hits with a maximum bitscore difference of 7 (corresponding to a maximum difference of p-values < 0.01) relative to the hit with the highest observed bitscore were retained. To determine the level of taxonomic ambiguity, for each contig sequence, the lowest shared taxonomic ancestor was determined by moving up the taxonomic tree until all retained blast hits had an unambiguous assignment. For downstream analysis of a given taxonomic level, only contigs with an unambiguous assignment at or below the selected taxonomic level was used.

To avoid taxonomic assignments of doubtful significance, for example due to sequences that have nucleotide homology across a minor fraction of the entire contig length, contigs were defined as principally classifiable if they had at least one blast hit that extended over at least 80% of the entire contig length and exhibited at least 80% nucleotide identity. These contigs were retained for downstream analysis.

2.4. Real-time PCR

DNA and RNA was extracted using a Qiasymphony (Qiagen, Hilden) with a low-concentration DNA spike (Qiagen, Hilden) as internal control (IC) to detect possible PCR inhibition. The eluate (5 μl) was evaluated for RNA viruses using the QuantiFast pathogen RT-PCR Kit + IC (Qiagen, Hilden) and for DNA viruses and bacteria using the QuantiFast pathogen PCR Kit + IC (Qiagen, Hilden) containing primers and probes at 400 nM and 200 nM concentrations, respectively [27,28]. Real-time PCR was performed using a LightCycler 480 II (Roche Mannheim Germany) with the following cycling conditions 10 min 95 °C, 45 cycles of 95 °C 15 s, 60 °C 30 s and data acquisition on the FAM and VIC channel during the 60 °C step. Enterovirus and rhinovirus sequences were identified using a recently published protocol [29,30]. *S. aureus* and *H. influenzae* were detected using a real-time PCR Taqman assay targeting the glutamate synthase gene [28] and the omp P6 gene [27], respectively.

2.5. 16S rRNA amplicon library preparation, MiSeq sequencing, and bioinformatic analysis

V3–V4 region 16S rRNA amplicons were generated using previously published degenerate primers that contain the Illumina adapter consensus sequence F: (5′- TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and R: (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGG-TATCTAATCC-3′) [31]. A detailed description of the protocol has been published by Illumina [32]. Samples were multiplexed using the Illumina Nextera XT Index Kit to construct barcoded libraries. The libraries were multiplexed and sequenced by 2 × 500 bp PE sequencing on the MiSeq platform. FastQC (Babraham Bioinformatics, Babraham Institute, UK) was used to determine the average quality scores of each sample before and after paired reads. The paired ends in each sample were joined, and all sequences less than 250 bp and/or with a Phred score < 33 were discarded. Quality filtering was applied using QIIME 53 (at Phred ≥ Q20). We performed operational taxonomic unit (OTU) clustering and alpha- and beta-diversity analysis using QIIME version 1.7. A chimera filter was applied using USEARCH 8.1. All sequences were clustered based on 97% similarity to reference sequences. The reads that did not meet the 97% similarity criteria were clustered de novo. Taxonomy levels of representative sequences in the OTU table were assigned at 95% similarity based on the SILVA database. We calculated alpha diversity based on the Shannon diversity index.

Analysis of beta diversity statistics (analysis of similarities, ANOSIM) was performed to determine if differences between the distributions of microbiota profiles from the five datasets were significant.

3. Results

3.1. Diversity of nasal microbiota

To investigate microbial diversity using a culture-independent approach, we analyzed the nasal bacterial community using V3–V4 region 16S rRNA amplicon sequencing in patients with GPA and RA and healthy controls. Principal component analysis comparing the inter-group bacterial taxonomy (beta diversity) and inter-individual variation within groups (alpha diversity) showed no clear clustering of individual groups, reflecting high intra-group variation in the alpha and beta diversity of the groups (Fig. 1A). We used different measures of alpha diversity to evaluate abundance, taxonomic evenness (Shannon index, OTUs), and the full richness of samples, *i.e.* accounting for rare organisms that could have been missed because of under sampling (Chao1 index). Based on the Shannon index, a slight but not statistically significant trend toward a reduced diversity of the nasal microbiome was observed in GPA samples compared with controls. Moreover, taxonomic abundance and microbial richness were significantly decreased in the GPA samples compared with RA samples based on OTU and Chao1 analysis, respectively (Fig. 1B).

Alpha diversity was slightly, but not significantly reduced in GPA patients with active disease (BVAS V3.0 ≥ 1) compared with those in remission (BVAS V3.0 = 0) (Supplementary Fig. 1A). By contrast, no major difference in alpha diversity was observed in patients with ENT activity compared to those without (Supplementary Fig. 1B). No clear alpha diversity differences were observed between RA patients with active disease (DAS 28 ≥ 2.6) and those in remission (DAS 28 < 2.6) (Supplementary Fig. 1C).

3.2. Bacterial community composition

Variations in the bacterial composition (relative abundance) in the nasal cavity were determined for each group. The nasal microbiome was composed of mainly three phyla: *Proteobacteria, Firmicutes* (classes *Clostridia* and *Bacilli*), and *Actinobacteria*. Few sequences (< 1.5%) were assigned to phyla *Bacteroides* and *Tenericutes*. There were no differences in the phylum levels observed between samples from healthy controls, GPA patients, and RA patients (Fig. 2A and B). However, significant differences in the bacterial composition were observed at the class level. *Bacilli* was significantly increased in GPA samples compared with control samples (Fig. 2C and D). Notably, there were prominent differences at the family level (Fig. 2E). The relative abundance of the bacterial family *Planococcaceae* was increased in samples from patients with GPA and RA compared with controls. An increase in bacterial species assigned to the families *Streptococcaceae, Pasteurellaceae,* and *Prevotellaceae* was observed in the GPA group. Conversely, the GPA group had a decrease in species assigned to the families *Corynebacteriaceae, Moraxellaceae, Tissierellaceae*, *Staphylococcaceae*, and *Propionibacteriaceae*, and the RA group had a decrease in species assigned to the

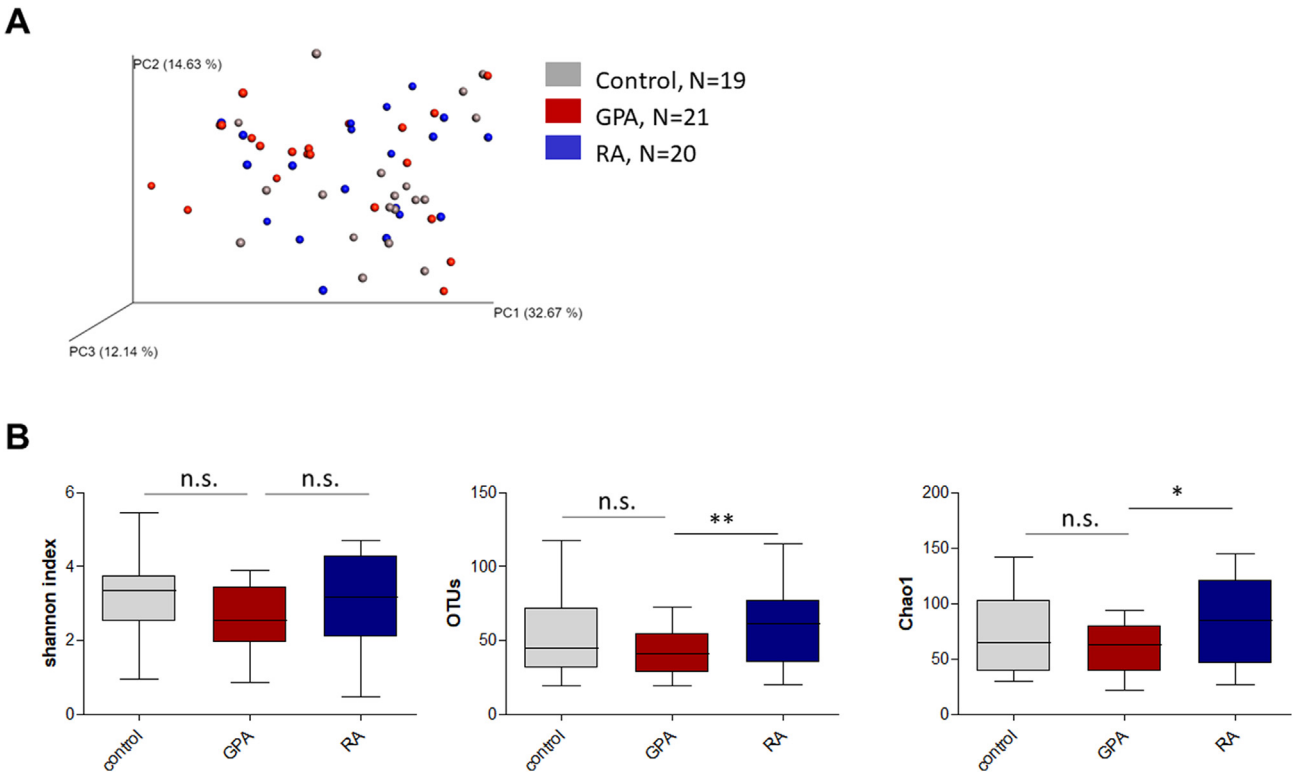

Fig. 1. Alpha and beta diversity of nasal samples from patients with GPA and RA and healthy controls. (A) Principal component analysis was used to compare bacterial taxonomy between groups and inter-individual variation within groups. **(B)** Alpha diversity is evaluated using various diversity metrics. Biodiversity is determined using the Shannon index, operational taxonomic units (OTUs) represent the number of standardized OTUs. Microbial richness is determined using the Chao1 metric, which primarily depends on the number of rare OTUs.

families *Moraxellaceae, Tissierellaceae*, *Staphylococcaceae,* and *Propionibacteriaceae*. The relative abundance of *Neisseriaceae* and *Aerococcaceae* was increased in the RA group compared with GPA and healthy controls (Figs. 2E and 3). While the relative abundance of *Porphyromonas* was detected at very low levels in samples from some RA patients and healthy controls, it was not detected in those from GPA patients (data not shown).

Among samples from patients with GPA, the bacterial composition at the phylum, class, and family level was largely similar in patients with active disease (BVAS V3.0 ≥ 1) compared with those in remission (BVAS = 0) except for the family *Staphylococcaceae*, which were more abundant during remission (Fig. 4A, Supplementary Fig. 2A). However, in patients with GPA, the bacterial composition varied depending on ENT activity, with families *Streptococcaceae* and *Planococcaceae* showing an increase in patients with ENT activity and a decrease in those without. By contrast, abundance of *Corynebacteriaceae* was decreased in patients with ENT activity (Fig. 4B, Supplementary Fig. 2B). In RA, no major differences in bacterial composition were shown between patients with active disease (DAS 28 ≥ 2.6) compared with those in remission (DAS 28 < 2.6) (Fig. 4C, Supplementary Fig. 2C).

The differences in the relative abundances of bacterial species at the family level, determined by V3–V4 region 16S rRNA amplicon sequencing, are provided in Table 2. *Streptococcaceae* and *Pasteurellaceae* were more abundant and *Aerococcaceae* was less abundant in the GPA group compared with the RA group and healthy controls. *Planococcaceae* were more abundant in both the GPA and RA groups, whereas *Moraxellaceae*, *Tissierellaceae*, and *Staphylococcaceae* were less abundant in both groups compared with healthy controls. Finally, *Neisseriaceae*, *Aerococcaceae,* and *Veillonellaceae* were more abundant in the RA group compared with the GPA group and healthy controls (Table 2).

3.3. Detection of S. aureus and H. influenzae

In addition to analyzing the phylum-, class-, and family-level bacterial composition using 16S rRNA sequencing, genus-level testing of samples for the presence of *S. aureus* and *H. influenzae* was performed using qPCR. *S. aureus* was detected in a significantly higher proportion of samples from GPA patients compared with RA patients and healthy controls. *H. influenzae* was numerically more frequent in samples from the GPA group compared with the RA group and healthy controls; however, this difference was not statistically significant (Table 3). Samples were also tested for the presence of enteroviruses and rhinoviruses using quantitative RT-PCR. Neither enteroviruses nor rhinoviruses were detected in samples from any of the evaluated groups (data not shown).

3.4. Unbiased metagenomic sequencing of RNA from GPA and control patients

UMERS is a novel method that can detect known and distantly related or novel pathogens of viral, bacterial, fungal, or parasitic origin [22,23]. We performed UMERS on a subset of nasal swabs (not included in qPCR) from nine patients with GPA and four healthy controls. The relative abundance of contig reads mapping to bacterial, fungal, and viral species was displayed as a heatmap. Two GPA samples had reads with significant sequence homology to *H. influenzae*; two GPA samples had reads corresponding to Rhinovirus A sequences, with one samples showing a co-infection of both pathogens. Furthermore, one GPA sample contained reads with significant sequence homology to *S. aureus*, and two GPA samples and one control sample had reads for *Moraxella catarrhalis*. None of the reads from control samples had sequence homologies to *H. influenzae* or *rhinoviruses*. However, one control sample contained a high abundance of sequences homologous to *Corynebacterium aurimucosum*, a known commensal bacteria of the nasal

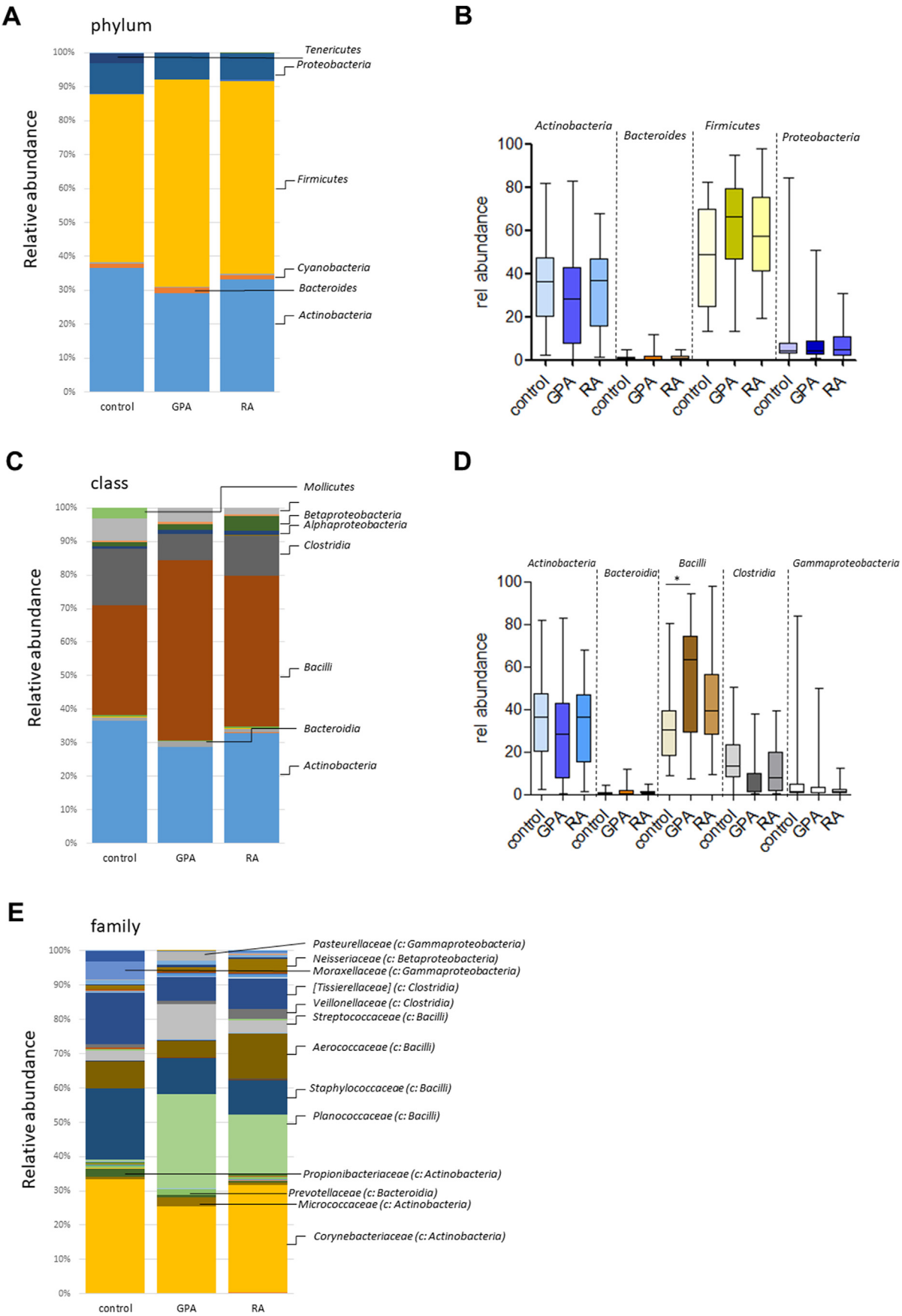

Fig. 2. Overview of the bacterial community composition of the URT in patients with GPA and RA and healthy controls. Sequences from patients with GPA (n = 21) and RA (n = 20) and healthy controls (n = 19) were classified. Shown are the relative abundances of sequences classified to major taxonomic phylum **(A, B)**, taxonomic classes **(C, D)**, and taxonomic families **(E).**

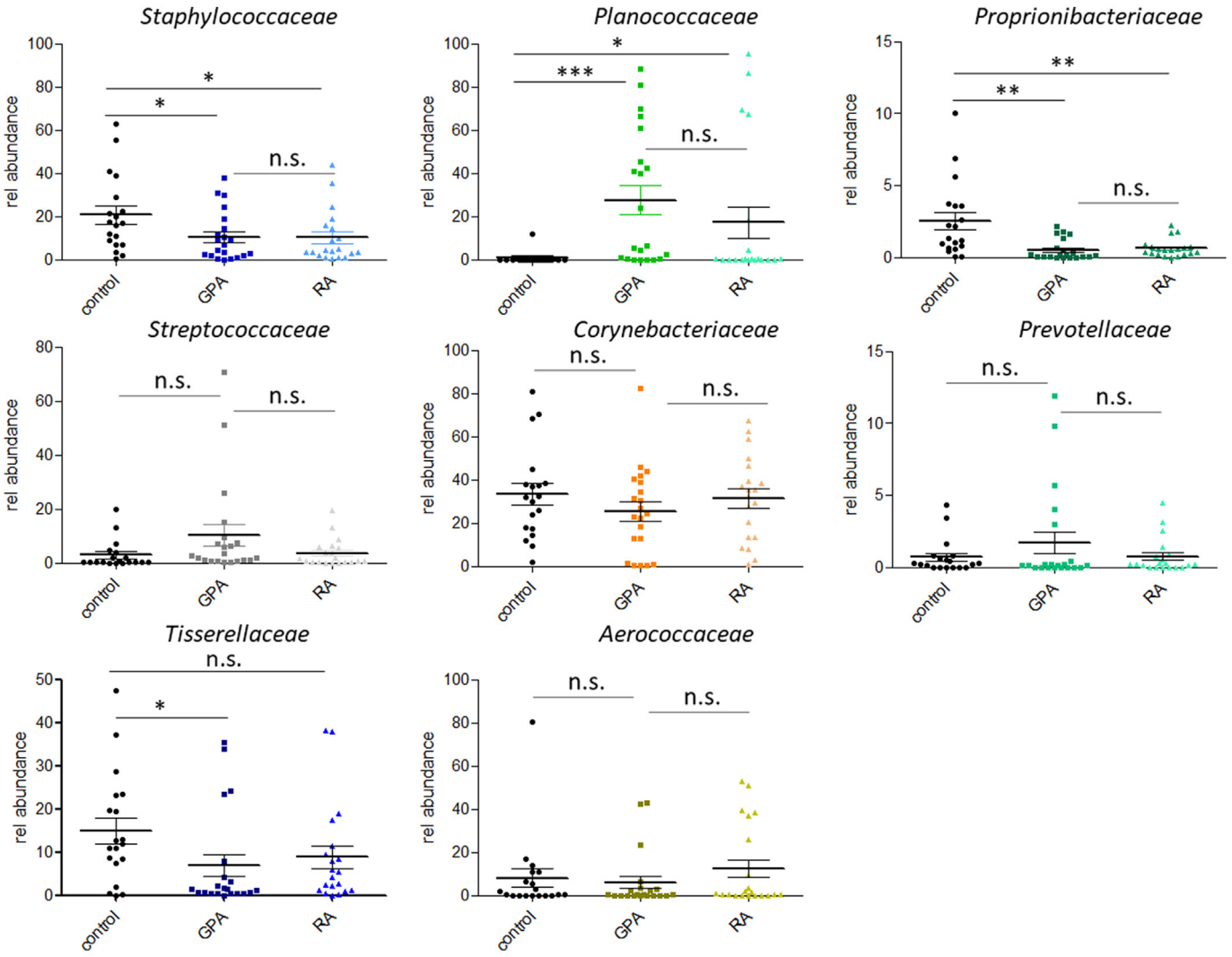

Fig. 3. Bacterial community composition of selected families within the URT in patients with GPA and RA and healthy controls. Sequences from patients with GPA (n = 21) and RA (n = 20) and healthy controls (n = 19) were classified. Shown are the relative abundances of different families.

cavity in adults (Fig. 5). No sequences corresponding to unknown viruses or viruses not described were identified in respiratory samples.

4. Discussion

In GPA, URT involvement is associated with reduced epithelial ciliary motility, impaired response of the inducible anti-microbial peptide human beta defensin-3, and an alteration in the transcriptional profile of nasal mucosa with downregulation of the pattern-recognition receptor Toll-like receptor 4 [33–35]. The formation of ectopic lymphoid structures within granulomatous lesions of the URT suggest antigen-driven inflammation [36–38]. However, the consequences of this alteration in mucociliary clearance and the associated respiratory immune response cannot be evaluated experimentally because animal models do not adequately recapitulate the URT pathology seen in GPA [39]. Notably, barrier dysfunction may play a role in dysbiosis (*i.e.* changes in microbial composition at a mucosal interface) and chronic URT inflammation in patients with GPA [3]. Previous studies based on cultivation methods observed an increased frequency of nasal *S. aureus* carriage in patients with GPA [10–15]. In our study, we found a trend toward lower alpha diversity in the nasal microbiome of GPA samples compared with control samples. This observation suggests fewer bacterial species dominate the nasal microbiome [18,40]. There were no major compositional differences in the bacterial community at the phylum and class level between samples from GPA patients, RA patients, and healthy controls. However, at the family level, patients with GPA and RA displayed similarities (*e.g.* increased abundance of *Planococcaceae*) and differences (*e.g.* increase abundance of *Prevotellaceae* in GPA but not RA) in microbial composition, suggesting that both immunosuppression and the disease background affect the URT microbiome composition. Consistent with this hypothesis, different microbial composition changes have been reported for other conditions of the nasal cavity, such as chronic rhinosinusitis [41,42]. Furthermore, we showed differences in the bacterial composition at the family level regarding the relative abundance of *Staphylococcaceae* depending on disease activity measured by the clinical composite score BVAS and regarding *Streptococcaceae*, *Planococcaceae*, and *Corynebacteriacea* depending on ENT activity scored by ENTAS. In case the trend we observed will be confirmed by further studies, changes in the bacterial composition of the nasal microbiome might become a non-invasive surrogate biomarker of disease activity. By contrast, no major differences with regard to bacterial composition of the nasal microbiome were found in active RA as compared with RA in remission. Notably, well-controlled studies showing changes in the microbial composition of the URT under immunosuppressive treatment similar to that in GPA, *e.g.* in organ transplantation, are missing so far. In RA, periodontitis with *Porphyromonas gingivalis* plays a role in autoantibody production [43]. In the present study, low levels of the genus *Porphyromonas* were

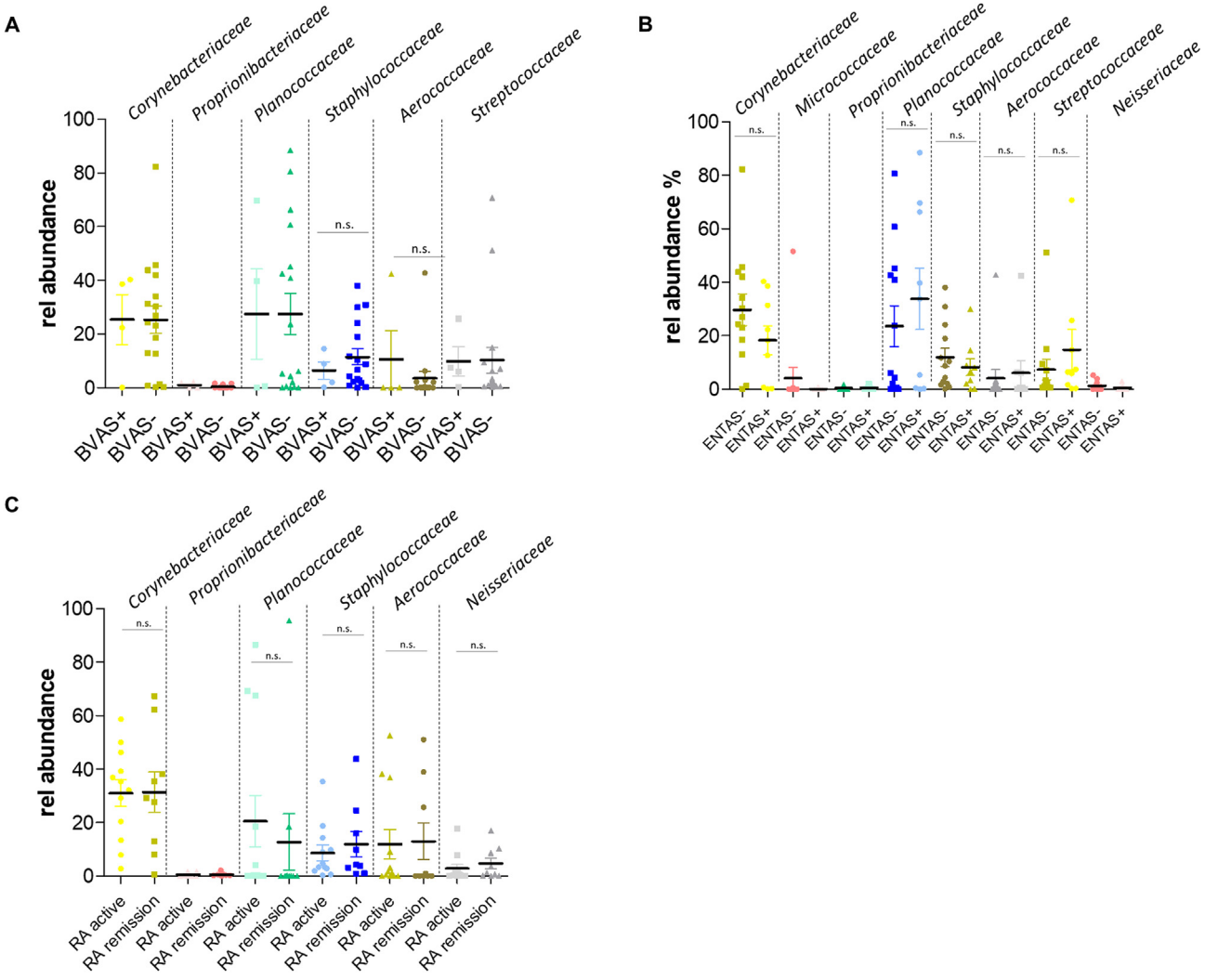

Fig. 4. Community composition of selected bacterial families within the URT in patients with GPA and RA and healthy controls. Sequences from patients with GPA (n = 21) and RA (n = 20) and healthy controls (n = 19) were classified. Shown are the relative abundances of different families.

Table 2
Relative abundances of bacterial species.

	Control (%)	GPA (%)	RA (%)
Pasteurellaceae	0.32	*2.7*	0.28
Neisseriaceae	1.1	1.0	3.8
Moraxellaceae	5.6	**0.14**	**0.5**
Tissierellaceae	15.0	**6.9**	**8.8**
Veillonellaceae	1.1	0.9	2.7
Streptococcaceae	2.9	*10.2*	3.6
Aerococcaceae	7.9	**4.9**	**13.0**
Staphylococcaceae	20.6	**10.5**	**10.1**
Planococcaceae	0.7	**27.5**	**17.2**

The taxonomic family level was determined by V3–V4 region 16S rRNA sequencing. Changes in GPA samples compared with healthy control samples and RA samples are shown in italics. Changes observed both in the GPA and RA groups compared with healthy controls are in bold. Abbreviations: GPA, granulomatosis with polyangiitis; RA, rheumatoid arthritis.

observed in a few patients with RA and healthy controls but not those with GPA, suggesting that the URT is not a relevant niche for *Porphyromonas* in either disease.

Recently, *Rhee* et al. [44] showed alterations in nasal bacterial and fungal composition in GPA patients compared with healthy controls. Their study was the first to investigate nasal samples of patients with GPA using culture-independent sequencing technology, *i.e.*, 16*S* rRNA sequencing and amplicon sequencing targeting the internal transcribed spacer (ITS1) region of fungi. The majority of the patients had received immunosuppressive therapy within the past 6 months. Notably, 25% of patients with GPA and 15% of controls were being treated with antibiotics at the time the study was performed [44]. By contrast, in our study, patients and controls were not currently receiving or had not recently undergone antibiotic therapy. The study by *Rhee* et al. [44] also lacked a disease control group, whereas we included RA patients as disease controls in our study. RA shares a number of predisposing genetic risk factors and immunological features with GPA, *e.g.* the association with the *PTPN22 R620W* polymorphism and expansion of circulating and tissue-resident $CD4^+CD28^-$ T-cells with anomalous NK-receptor expression facilitating cell proliferation and mediating endothelial cytotoxicity. Association studies and transcriptome analysis suggest pathogen- and inflammation-driven alteration of the peripheral T-cell compartment in RA and GPA, respectively [3,20]. Moreover, RA patients are similarly treated with immunosuppressants [10]. In *Rhee* et al. [44], patients with GPA had lower relative abundances of *Propionibacterium acnes* and *Staphylococcus epidermidis*, whereas no difference in the relative abundance of *S. aureus* was observed. In our study, the abundance of *Propionibacteriaceae* and *Staphylococcaceae* was lower at the family level. However, RA patients showed a similar reduction in abundance, suggesting that this observation is not specific for GPA.

Table 3
Summary of qPCR results, n (%) of positive samples.

	S. aureus	Chi^2 test	*H. influenzae*	Chi^2 test
Control, n = 23	1/23 (4.35%)	Control *vs.* GPA $p < 0.0005$	4/23 (17.39%)	Control *vs.* GPA n.s.
GPA, n = 19	14/19 (73.68%)	GPA *vs.* RA $p = 0.0046$	6/19 (31.58%)	GPA *vs.* RA n.s.
RA, n = 21	5/21 (23.81%)		4/21 (19.04%)	

All samples were also tested for the presence of enteroviruses and rhinoviruses; all samples were negative for both. Statistical significance: $p < 0.05$. Abbreviations: GPA, granulomatosis with polyangiitis; n.s., not significant; RA, rheumatoid arthritis.

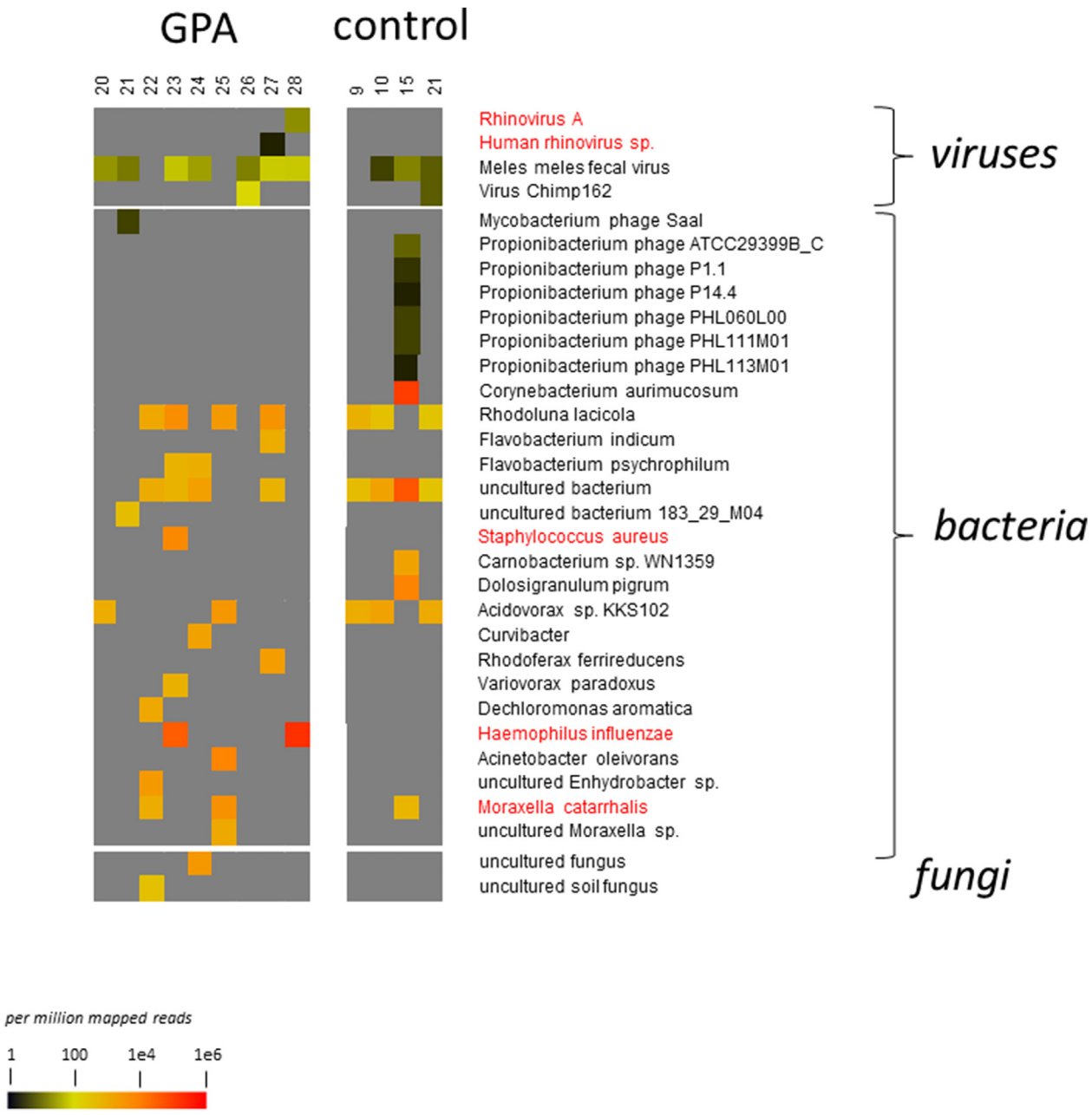

Fig. 5. Unbiased metagenomic sequencing of RNA (UMERS) isolated from nasal swab samples. Shown are data from a BLAST analysis of de novo-assembled sequence contigs [22,23]. The relative abundance of contig reads mapping to bacterial, fungal, or viral species is indicated by the heat map (legend in the lower left corner). A grey rectangle indicates that no reads were detected. Viral or bacterial species involved in respiratory diseases are shown in red.

Notably, we detected *S. aureus* in a significantly higher proportion of samples from GPA patients compared with RA patients and healthy controls by qPCR. Moreover, *Rhee* et al. [44] reported that the nasal microbial composition of patients with GPA undergoing immunosuppressive therapy was similar to healthy controls, whereas patients with GPA not undergoing immunosuppressive therapy displayed dysbiosis. The authors speculated this observation was due to subclinical disease activity altering the nasal microbiome of patients not receiving immunosuppressive therapy, suggesting that immunosuppressant therapy may have a beneficial effect on URT

dysbiosis in GPA [44]. Because all patients included in our study were undergoing immunosuppressive treatment, we could not perform a similar comparison. In the present study, we found the bacterial composition to be largely similar in GPA patients with active disease and those in remission based on the clinical composite BVAS V3.0 score with the exception of *Staphylococcaceae* as outlined above. Unlike *Rhee* et al. [44], the GPA patients in our study were not currently receiving or had not recently received antibiotic treatment, which potentially could impact URT dysbiosis. In our study, ENT activity, determined based on ENTAS score, was associated with changes in the URT microbial composition in GPA patients with an increase in the abundance of *Streptococcaceae* and *Planococcaceae* observed with ENT activity and a decrease in the absence of ENT activity.

As mentioned above, previous studies using cultivation methods reported an increased frequency of nasal *S. aureus* carriage [10–15]. Consistent with these reports, we detected a significantly higher proportion of *S. aureus* in nasal cavity samples from GPA patients compared with those from RA patients and healthy controls using qPCR. The dominant role of *S. aureus* in URT dysbiosis could be due to reduced epithelial inducible human beta defensin-3 secretion (hBD3) in patients with GPA. HBD3 is an antimicrobial peptide displaying high activity against *S. aureus* [33]. Dysbiosis is implicated in the pathogenesis of many chronic inflammatory and autoimmune diseases [20]. The best evidence for the impact of host-microbiome interactions on immune response regulation comes from clinical and experimental studies of the intestinal microbiome [20,45–49]. In addition to testing for the presence of *S. aureus*, we evaluated samples for the presence of *H. influenzae* and entero- and rhinoviruses using qPCR. While not statistically significant, we found *H. influenzae* was numerically more frequently detected in samples from patients with GPA compared with RA patients and healthy controls. Entero- and rhinoviruses were not detected our samples. Additionally, we used UMERS to analyze the relative abundance of bacterial, fungal, and viral species in a subset of GPA patients and healthy controls [22,23]. Our UMERS analysis uncovered RNA transcripts of pathogens with a relative contig read abundance suggestive of acute respiratory tract infection in three GPA patients. Specifically, we found Rhinovirus A in one patient, a co-infection of Rhinovirus and *H. influenzae* in another, and *H. influenzae* in a third. Moreover, the UMERS analysis suggested colonization with *S. aureus* and *M. catarrhalis* in another three patients with GPA. In another study, we found pathogen-related signatures, including a *S. aureus*-induced signature, in differentially expressed transcripts from the T-cells of GPA patients [50]. This observation is consistent with the present data showing that multiple bacterial and viral taxa impact GPA patients. Moreover, in that study, we detected circulating T-cells specific for cytomegalovirus, Epstein Barr virus, influenza A virus, metapneumovirus, and respiratory syncytial virus as well as PR3-specific T-cells in individual GPA patients using peptide/MHC class I dextramer staining of T-cell populations [50].

5. Conclusions

In conclusion, we found evidence of changes in the microbial composition of the nasal cavity in patients with GPA. Specifically, we found a decrease in alpha diversity compared with patients RA and a trend toward a decrease compared with healthy controls. Changes in the relative abundance of bacterial families were observed in GPA patients, primarily a significant increase in the taxonomic class *Bacilli*. We detected *S. aureus* more frequently in GPA samples using qPCR. Moreover, *H. influenza* was detected more frequently in GPA samples compared with healthy control and RA samples, although this observation was not statistically significant. We excluded the presence of unknown or previously undescribed pathogens in the URT using UMERS analysis of select GPA and healthy control samples. Taken together, our results suggest changes in the microbial composition of the URT in patients with GPA, resulting in microbial dysbiosis. These data suggest complex alterations in host-microbiome interactions of the URT rather than *S. aureus* carriage alone affect endonasal disease activity and shape the immune response, predisposing patients with GPA to chronic inflammation and autoreactivity.

Competing financial interest statement

None of the authors received financial support or other benefits from commercial sources for the work reported on in the manuscript. None of the authors has any other financial interests, which could create a potential conflict of interest or the appearance of a conflict of interest with regard to the work.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2018.10.005.

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