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CHARACTERIZATION OF THE NASAL MICROBIOTA IN GRANULOMATOSIS WITH POLYANGIITIS

Rennie L. Rhee[1], **Antoine G. Sreih**[1], **Catherine E. Najem**[1], **Peter C. Grayson**[2], **Chunyu Zhao**[3], **Kyle Bittinger**[3], **Ronald G. Collman**[4], and **Peter A. Merkel**[5]

[1]Division of Rheumatology, University of Pennsylvania, Philadelphia, PA

[2]National Institute of Arthritis and Musculoskeletal and Skin Diseases, NIH, Bethesda, MD

[3]Division of Gastroenterology, Hepatology, and Nutrition, The Children's Hospital of Philadelphia, Philadelphia, PA

[4]Division of Pulmonary, Allergy, and Critical Care, University of Pennsylvania, Philadelphia, PA

[5]Division of Rheumatology and the Department of Biostatistics, Epidemiology, and Informatics, University of Pennsylvania, Philadelphia, PA

Abstract

Objectives—Prior studies have suggested a potential link between nasal microbes and granulomatosis with polyangiitis (GPA; Wegener's) but these studies relied on culture-dependent methods. This study comprehensively examined the entire community of nasal microbiota (bacteria and fungi) in participants with GPA compared to healthy controls using deep sequencing methods.

Methods—16S rRNA and ITS gene sequencing were performed on nasal microbial DNA isolated from nasal swabs of 60 participants with GPA and 41 healthy controls. Alpha and beta diversity were assessed as well as the relative abundance of the most abundant bacterial and fungal taxa. The effects of co-variates including disease activity and immunosuppressive therapies on microbial composition were evaluated.

Results—Compared to controls, participants with GPA had a significantly different microbial composition (weighted UniFrac p = 0.04) and lower relative abundance of *P. acnes* and *S. epidermidis* (for both, FDR-corrected p = 0.02). Disease activity in GPA was associated with a lower abundance of fungal order *Malasseziales* compared to participants with GPA in remission (p = 0.04) and controls (p = 0.01). Use of non-glucocorticoid immunosuppressive therapy was associated with "healthy" nasal microbiota while participants with GPA who were off immunosuppressive therapy had more dysbiosis (weighted UniFrac p = 0.01). No difference in the relative abundance of *S. aureus* was observed between GPA and controls.

Corresponding Author: Rennie L. Rhee, MD, MSCE, 3400 Spruce Street, Rheumatology, White Building 5th Floor, Philadelphia, PA 19104, Phone: 215-614-4409, Fax: 215-614-4412, rennie.rhee@uphs.upenn.edu.

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Conclusions—GPA is associated with an altered nasal microbial composition, at both the bacterial and fungal levels. Use of immunosuppressive therapies and disease remission are associated with healthy microbial communities.

Keywords

granulomatosis with polyangiitis; infections; disease activity

INTRODUCTION

Granulomatosis with polyangiitis (GPA; Wegener's) is a systemic vasculitis characterized by granulomatous inflammation and up to 90% of participants will develop sinonasal inflammation during the course of disease[1]. Despite the many therapeutic advances in the management of GPA, relapses remain a significant issue and, in particular, disease activity in the sinuses and nose often persists when a patient is otherwise in clinical remission[2]. It has been speculated that microbes may be involved in the pathogenesis of GPA. A study using culture data found that chronic nasal carriage of *Staphylococcus aureus* is associated with a higher risk of relapse[3] and two randomized clinical trials showed trimethoprim-sulfamethoxazole prevents relapse in GPA[4, 5].

Advances in high-throughput genomic sequencing and the development of new tools for analyzing metagenomic data allow for a better understanding of the dynamic community of microbes (bacteria, fungi, viruses) that inhabit the human body (human microbiome) in a relatively inexpensive and accessible manner. The human microbiota has a large potential to impact many human physiologic functions including immune homeostasis[6]. Dysbiosis, the imbalance or disruption of microbial communities, has been proposed to contribute to the pathophysiology of several autoimmune diseases including inflammatory bowel disease, rheumatoid arthritis, multiple sclerosis, and type 1 diabetes mellitus[7–11]. Furthermore, microbial composition varies across different body sites and, while much attention has been given to the gut microbiome, microbial communities in other body habitats, including the nose, have also been implicated in human health and disease[12–15].

This is the first study to comprehensively examine the entire community of nasal bacteria and fungi in participants with GPA compared to healthy controls using culture-independent sequencing methods. We chose to focus on the nose due to its distinctive involvement in the natural history of GPA and its role as an active component of the immune system[16].

METHODS

Study participants

Participants were recruited through the Penn Vasculitis Center at the University of Pennsylvania. Participants with GPA were eligible if they met the modified American College of Rheumatology classification criteria for GPA[17–19]. Healthy controls were participants without a systemic vasculitis or other inflammatory disorder. Both participants with GPA and controls were excluded if they had another systemic inflammatory disorder, history of intranasal cocaine use within the prior 3 years, known history of Human

Immunodeficiency Virus (HIV) or primary immunodeficiency, lymphoma or other malignancy that mimics AAV. Antibiotic use was not an exclusionary criteria but was incorporated into the analysis. This study was approved by the Institutional Review Board of the University of Pennsylvania and written informed consent was obtained from all participants.

Nasal mucosa was sampled by swabbing the middle meatus with a sterile flocked specimen collection swab (Copan Diagnostics) which was transferred to a −80°C freezer. To control for environmental contamination, negative controls (swab exposed to ambient air) were obtained with each participant sampling and processed in parallel.

Clinical data was collected at time of sampling and included demographics and medication use within prior 6 months (antibiotics, systemic glucocorticoids, and non-glucocorticoid immunosuppressive therapy such as cyclophosphamide, rituximab, azathioprine, methotrexate, etc). For participants with GPA, a detailed disease history was obtained including antineutrophil cytoplasmic antibody (ANCA) type by ELISA (proteinase-3 [PR3], myeloperoxidase [MPO], or negative), disease status determined by the Birmingham Vasculitis Activity Score for Wegener's Granulomatosis[20] (BVAS/WG; BVAS/WG > 0 indicates active disease and BVAS/WG = 0 indicates disease remission), and sinonasal damage according to the ear, nose, and throat items on the Vasculitis Damage Index (VDI)[21].

Microbiota profiling using DNA sequencing

DNA was extracted using QIAamp ultraclean production pathogen mini kit. For bacterial profiling, PCR amplification targeting the V1-V2 region of the 16S rRNA gene was performed. For analyses of fungal communities (mycobiome), the internal transcribed spacer (ITS1) region was targeted, using a fungal-specific primer set[22]. Amplicons were purified and sequenced by the sequencing core of the Penn-CHOP Microbiome program on an Illumina MiSeq instrument, yielding 250bp paired-end sequence reads. Environmental and reagent control samples, consisting of air-exposed swabs, DNA free water, and empty wells were processed alongside participant samples[23].

Sequencing data analysis was performed by QIIME 1.9.1[24]. Paired-end reads were quality filtered and joined, clustered into operational taxonomic units (OTUs) with 97% sequence similarity using UCLUST[25]. Taxonomic assignments were generated by alignment to the Greengenes r13_8 reference databases[26]. Taking into consideration the various lengths of the ITS1 regions, we used high quality forward reads (R1) for analysis, clustered into OTUs with 95% sequence similarity, and taxonomy assignments generated by comparison to the nt reference database using the consensus method implemented in BROCC software[27].

16S reads assigned as Archaea, Mitochondria or Chloroplast were removed. Potential contaminant OTUs showing a strong negative correlation with the amplicon concentration (FDR < 0.05) were also removed[28] (Supplementary Table 1). Samples with more than 10,000 read counts for 16S analysis and 4,000 read counts for ITS analysis were retained. To reduce the influence of low-abundant fungi which are potential contaminants, a PicoGreen-

corrected OTU abundance was determined by multiplying the relative abundance of fungal taxa by the post-PCR ITS DNA concentration as previously described and validated[29].

Taxonomic heatmap was generated at the genus level to depict all taxa with a relative abundance of over 1% in at least one sample. The most abundant OTUs within taxa of interest were also identified for secondary analyses.

Analytical approach

The main comparison of interest was between GPA and controls. Outcome measures included alpha and beta diversity and relative abundance of individual taxa. We measured alpha diversity, or within-sample diversity, using the Shannon diversity index which accounts for evenness and abundance of OTUs within a sample. Beta diversity, which compares dissimilarity of microbial composition between samples, was measured by UniFrac distance which estimates the fraction of a sample's phylogenetic tree that differs from another sample, with (weighted) or without (unweighted) accounting for the relative abundance of OTUs[30, 31]. UniFrac distances were visualized using principal coordinate analysis (PCoA). To determine if specific bacterial or fungal taxa were more common or more rare, we evaluated relative abundance (which refers to the percent composition of a taxa relative to the total number of taxa) of the 5 most abundant genera and 10 most abundant OTUs (here on referred to as species). We focused on these relatively abundant bacteria because they are more likely to represent "true" nasal commensals in these low microbial biomass samples. Furthermore, these taxa together represent 92% of all sequences obtained, thus reflecting the dominant microbiota present. In exploratory analyses, differential relative abundance was also investigated between subgroups of GPA according to ANCA type, sinonasal damage, disease activity, and medications.

Detailed profiling of the nasal mycobiota or fungal communities included examination of total fungal abundance and was initially planned to evaluate the relative abundance of the 10 most abundant taxa; however, due to the predominance of the order *Malasseziales* and the small number of other fungal OTUs detected and their sparse distribution among samples, further investigation of relative abundance focused only on *Malasseziales*.

Statistical analyses

Categorical variables were compared between groups using chi-square test; continuous variables were compared using the Wilcoxon rank sum test. One-way ANOVA was used to test for differences in alpha diversity (Shannon index) between groups. Beta diversity (UniFrac) was analyzed using permutational analysis of variance (PERMANOVA)[32]. Relative abundance values were log-transformed and compared using the Wilcoxon rank sum test. Potential fungal-bacterial interactions were assessed by examining associations between abundance of total fungi as well as *Malasseziales* and the following: 1) UniFrac distance using PERMANOVA, and 2) abundance of 5 most abundant bacterial genera and 10 most abundant species using Wilcoxon rank sum test adjusting for study group (GPA vs control).

Correction for multiple comparisons was performed by the Benjamini-Hochberg false discovery rate (FDR) procedure for all analyses except for exploratory analyses performed within GPA subgroups of interest[33]. All statistical analyses were conducted with R 3.4.1.

RESULTS

Participant characteristics

There were 101 participants enrolled in this study: 60 participants with GPA and 41 healthy controls. Characteristics at time of sampling are shown in Table 1. Demographics were similar between the 2 groups. Participants with GPA were more likely to have received antibiotics within the prior 6 months (48% vs 29%). Among participants with GPA, 25% had active disease and a majority of participants had received immunosuppressive agents within the prior 6 months.

Bacterial composition and diversity in nasal cavities of participants with GPA and healthy controls

A total of 9,235,460 raw sequence reads were generated from all participant samples and, after quality filtering and removal of contaminants, a total of 9,213,337 high-quality reads were used for analysis (mean reads per sample 91,221 ± 56,533). *Corynebacterium*, *Staphylococcus,* and *Propionibacterium* featured as prominent bacterial taxa in the nasal cavities of the cohort, consistent with previous descriptions of the nasal microbial composition in healthy individuals[34] (Figure 1 and Supplementary Figure 1 for heatmap). Within these genera, the most abundant species identified were *Corynebacterium tuberculostearicum, Propionibacterium acnes*, *Staphylococcus aureus*, and *S. epidermidis*.

To compare overall composition of bacterial communities, alpha and beta diversity were measured. The Shannon diversity index (alpha diversity) was not significantly different between participants with GPA and healthy controls (p = 0.51). The analysis of beta diversity calculated on UniFrac distances revealed a borderline difference by unweighted UniFrac (i.e., based on the identify of community members) (p=0.05), and a significant difference in weighted UniFrac (i.e., accounting for both community membership and abundances), between participants with GPA and healthy controls, suggesting that microbial composition between the 2 groups is different (p = 0.04) (Figure 2A). We then compared the relative abundances of individual bacterial genera between groups and found that participants with GPA had a lower relative abundance of *Propionibacterium* (FDR-corrected p = 0.03) compared to healthy controls. When we examined the most abundant species, both *P. acnes* and *S. epidermidis* had a significantly lower abundance in GPA compared to controls (for both species, FDR-corrected p = 0.02). No difference was seen in the relative abundance of *S. aureus* (FDR-corrected p = 0.49).

Use of non-glucocorticoid immunosuppressive agents is associated with “healthy” nasal bacteria in GPA

When evaluating the association of co-variates such as medications and disease characteristics with microbial communities in GPA and controls, we found that the nasal microbial communities in participants with GPA receiving non-glucocorticoid

immunosuppressive therapies either currently or within the prior 6 months were similar to healthy controls, whereas participants with GPA not receiving non-glucocorticoid immunosuppressive therapies were significantly different from controls. Specifically, there was a significant difference in weighted UniFrac distance between participants with GPA off immunosuppressive therapies compared to controls ($p = 0.01$) while no difference was observed between GPA on immunosuppressive therapies versus controls ($p = 0.16$) (Figure 2B). No significant difference in unweighted UniFrac distance was found according to use of immunosuppressive therapies.

When we evaluated the relative abundance of *Propionibacterium* and then stratified participants with GPA based on their use of non-glucocorticoid immunosuppressive therapy, we found that data from the participants with GPA who were off immunosuppressive therapy were driving the differences observed between GPA and controls; specifically, participants off immunosuppressive therapy had a significantly lower abundance of *Propionibacterium* compared to controls ($p < 0.01$) while participants with GPA on immunosuppression were similar to controls ($p = 0.68$) (Figure 3). Analysis of the most abundant bacterial species revealed similar differences in relative abundance between groups for *P. acnes, P. granulosum,* and *S. epidermidis* (Figure 4). Relative abundances for each sample and OTU are available in Supplementary Table 2.

When examining the association between disease activity in GPA and microbial communities, univariate and multivariate analyses found no relationship between disease activity and UniFrac distances, either weighted or unweighted, or relative abundance of bacterial taxa. Similarly, use of antibiotics or prednisone was not associated with UniFrac distance or relative abundance. Additional exploratory analyses within clinically-distinct subgroups of GPA are shown in the Supplementary Text 1.

Participants with active GPA harbor an altered nasal mycobiota

We found a non-significant trend towards lower total fungi abundance in participants with GPA compared to controls ($p = 0.06$). However, we found that participants receiving prednisone had a lower total abundance of nasal fungi ($p = 0.02$) compared to healthy controls, and those receiving other immunosuppressive therapies had a significantly higher abundance of fungi ($p = 0.03$), independent of disease activity, antibiotics, ANCA type, and sinonasal damage. When we investigated the nasal fungal community composition, unclassified *Malasseziales* was the most abundant fungal taxa present, followed by the genera *Penicillium* and *Aspergillus*. Evaluation of *Malasseziales* demonstrated a significantly lower abundance in GPA compared to controls ($p = 0.04$). In particular, participants with GPA with active disease appeared to be driving these differences, as they had the lowest abundance compared to participants in remission ($p = 0.04$) and healthy controls ($p = 0.01$), while participants with GPA in remission were similar to controls ($p = 0.19$) (Figure 5). Significant correlations were also found between the 5 most abundant bacterial species and all fungi (Supplementary Figures 2 and 3) as well as just *Malasseziales* (Supplementary Text 2).

DISCUSSION

This study used deep sequencing methods to comprehensively define the entire community of nasal bacteria and fungi in GPA, and found significant differences in nasal microbial composition between participants with GPA and healthy controls. Furthermore, being off immunosuppressive therapy and disease activity were associated with bacterial and fungal dysbiosis, respectively.

For many decades, there has been speculation that infections may have a major influence on disease activity in GPA. With a growing understanding of the human microbiome, a shift occurred within the field of microbiology from focusing only on individual disease-causing organisms to studying the effects of endogenous microbes or commensals as communities. In this study we examined the relationship between microbes and GPA from a different perspective than prior studies, by characterizing the entire community of resident bacteria and fungi in the nasal cavity, an active site of immunity and a known reservoir for pathobionts such as *S. aureus*. We found that participants with GPA have dysbiosis in the nose and, in particular, a lower abundance of *P. acnes* and *S. epidermidis*, both of which have been suggested to be negative competitors of *S. aureus*[14, 35]. Most interestingly, differences in microbial composition were primarily driven by participants with GPA who were off non-glucocorticoid immunosuppressive therapy independent of disease activity, suggesting that immunosuppressive therapy is associated with a healthy nasal microbiome in GPA (and conversely, absence of immunosuppressive therapy is associated with nasal dysbiosis). This was an unexpected finding since we had anticipated immunosuppressive therapy would cause more dysbiosis, not less, based on prior studies[36]. Similar observations have been made in rheumatoid arthritis[37].

We postulate there are 2 potential explanations for this association between immunosuppressive use and improvements in nasal dysbiosis: 1) immunosuppressive therapy has direct benefits on the nasal microbiota, or 2) subclinical disease activity, not identified using standard methods of assessment, was present in participants who were off immunosuppressive therapy, which in turn is associated with an altered nasal microbiota. These results suggest either that the manipulation of the nasal microbiome may be a novel therapeutic target and/or that the nasal microbiome may be a more sensitive, non-invasive biomarker of disease activity.

Unlike prior studies, no difference was found in the abundance of *S. aureus* between GPA and controls, although differences were seen among GPA subgroups (however, these differences were no longer significant after correction for multiple comparisons)[38, 39]. Several differences in methodology may explain this discrepancy. The current study used sequencing data to identify bacteria which may be more sensitive than culture data for identifying bacteria present; alternatively, sequencing data does not discriminate between viable versus non-viable bacteria. The location within the nasal cavity where swabbing was performed also differed: we sampled the middle meatus to study a mucosal surface whereas prior studies swabbed the anterior nares which is lined with skin-like squamous epithelium. Despite their proximity, spatial variation in microbiota composition (in particular *S. aureus*

colonization) has been found within the nasal cavity, particularly between the middle meatus and anterior nares[15].

To our knowledge, only 2 prior studies have examined the nasal mycobiome using culture-independent methods and this is the first to examine the mycobiome in GPA[40, 41]. *Malasseziales*, a fungal taxa which has previously been demonstrated to be abundant in the nose, mouth, and skin, was the most abundant fungal taxa in the nasal cavity in this cohort[40, 42, 43]. Furthermore, *Malasseziales* was significantly lower in abundance in GPA compared to controls and this difference was most pronounced in participants with active disease. The abundance of *Malasseziales* correlated with the abundance of several bacteria, suggesting interactions between bacteria and fungi occur in the nose. Prior studies also have suggested that interactions between fungi and bacteria may impact microbial composition and specifically the possibility has been raised that fungi may stabilize microbial community organization[44]. Future studies further exploring the role of fungi in GPA are warranted.

Limitations to this study include the cross-sectional design which restricts investigations of temporal dynamics and is unable to assess for inter-individual heterogeneity. Given the variability in the microbiome between individuals, a future longitudinal study would have the advantage of controlling for potential confounders through repeated sampling within individuals. Furthermore, causal relationships cannot be determined and confounders such as inflammation itself can perturb the nasal microbiota. Validation of these results in other cohorts along with mechanistic studies are needed to interpret these findings. Although the sample size was adequate to evaluate differences between GPA and controls, the ability to explore differences between GPA subgroups may have been underpowered. Lastly, measurement of disease activity relied on a validated disease activity measure, the BVAS/WG, which may not be sensitive enough to identify subclinical disease activity.

In conclusion, this study found an altered nasal microbial composition associated with GPA, both at the bacterial and fungal levels, and suggests that immunosuppressive therapies and inactive disease status are associated with healthy microbial communities. These findings justify further investigation of host-microbe interactions in GPA, a potentially exciting new dimension to our understanding of the disease, and may be the first step to elucidating critical components of the pathophysiology of disease and identification of novel therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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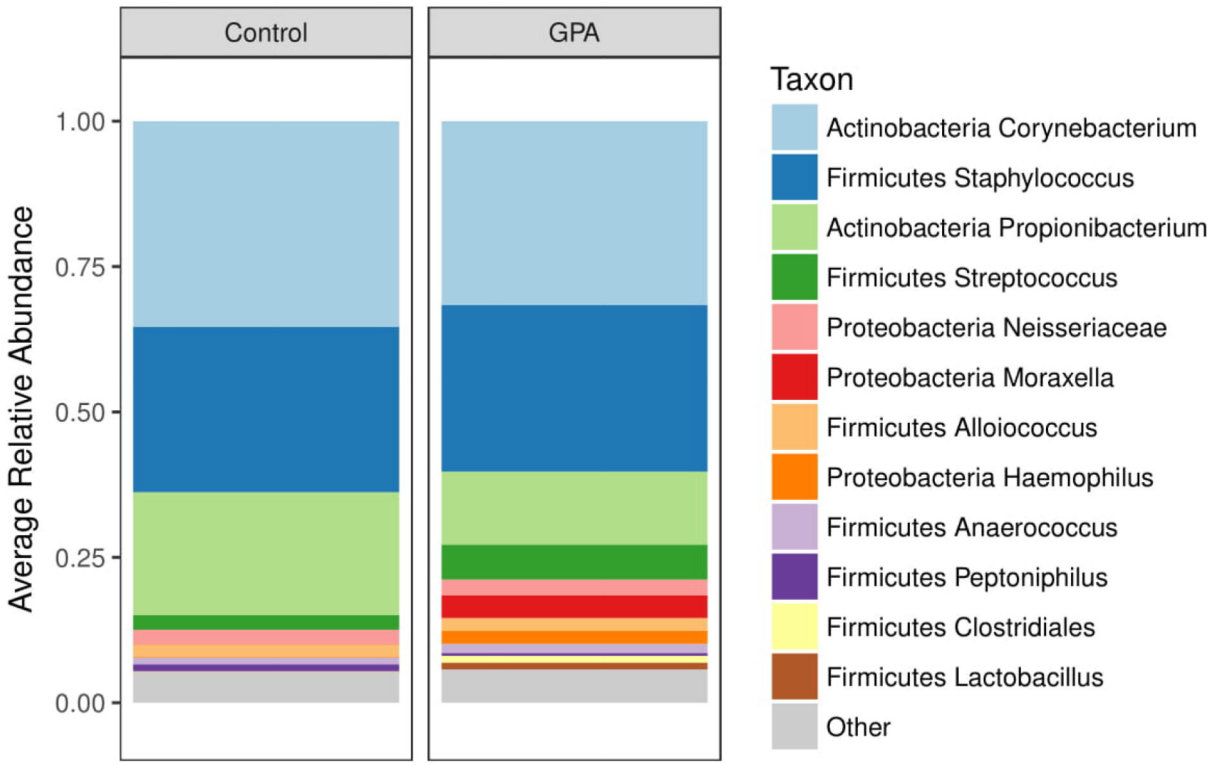

Figure 1:

The 12 most abundant bacteria that are present in the samples are depicted in a stacked barplot. *Corynebacterium*, *Staphylococcus*, and *Propionibacterium* featured as most abundant genera, similar to previously published studies of nasal microbial composition in healthy individuals.

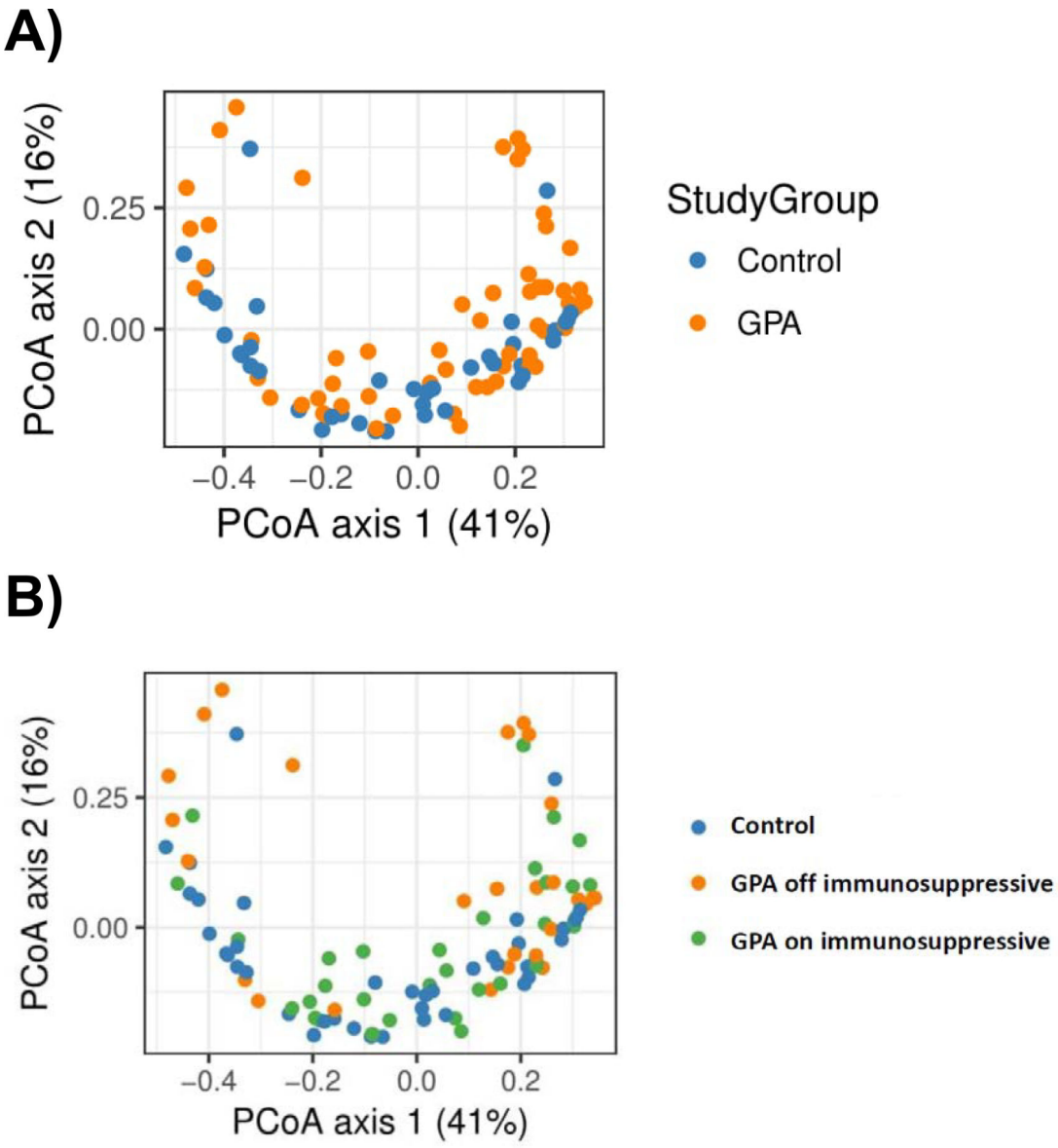

Figure 2:

Difference in overall microbial composition between (A) controls and granulomatosis with polyangiitis (GPA), and (B) controls and GPA stratified by use of non-glucocorticoid immunosuppressive medications. The principal coordinates analysis (PCoA) plots depict differences in beta diversity based on weighted UniFrac distance, which accounts for phylogenetic differences as well as abundance. (A) There is a significant difference in microbial composition between controls (blue) and participants with GPA (orange) ($p = 0.04$). (B) Differences in microbial composition are primarily driven by participants with GPA off non-glucocorticoid immunosuppressive therapy (orange) who had a significantly different weighted UniFrac from controls in blue ($p = 0.01$) while those on immunosuppressive therapies (green) are similar to controls ($p = 0.16$).

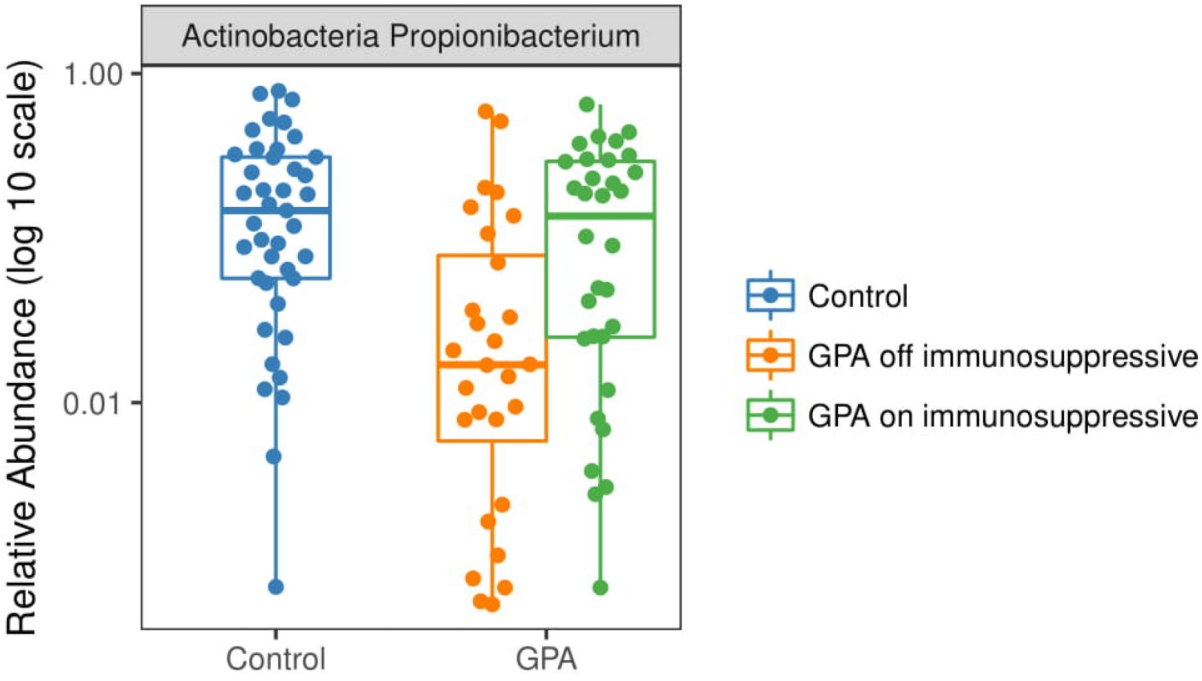

Figure 3:

Differential abundance of *Propionibacterium* between controls and participants with granulomatosis with polyangiitis (GPA) stratified by use of non-glucocorticoid immunosuppressive therapy. Participants off immunosuppressive therapy (orange) had a significantly lower abundance of *Propionibacterium* compared to controls (blue) (FDR-corrected $p < 0.01$). No difference was observed between participants with GPA on immunosuppression (green) and controls (FDR-corrected $p = 0.68$) or participants with GPA off immunosuppression (FDR-corrected $p = 0.06$).

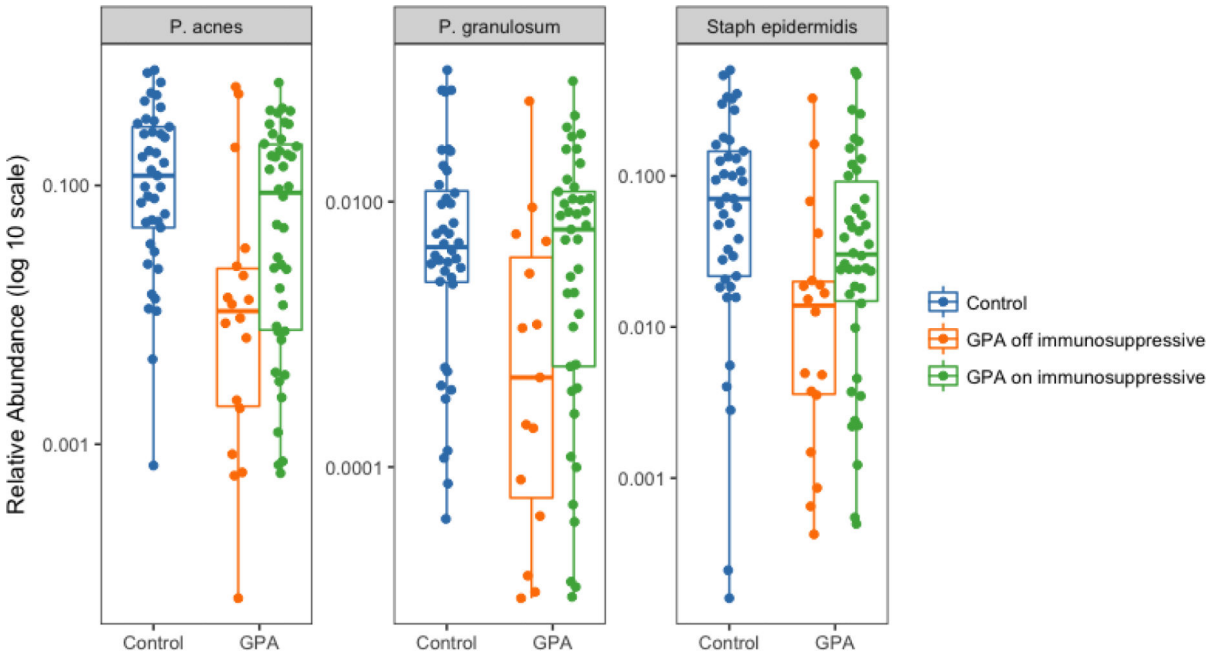

Figure 4:

Differential abundance of *Propionibacterium acnes, P. granulosum,* and *Staphylococcus epidermidis* between control and participants with granulomatosis with polyangiitis (GPA) stratified by use of non-glucocorticoid immunosuppressive therapy. Participants with GPA who are off immunosuppressives had a significantly lower abundance of *P. acnes*, *P. granulosum,* and *S. epidermidis* (for all, FDR-corrected $p < 0.01$) compared to controls and compared to participants on immunosuppression (FDR-corrected $p = 0.03$, $p = 0.01$, and $p = 0.03$, respectively), while participants with GPA on immunosuppression were similar to controls ($p > 0.05$).

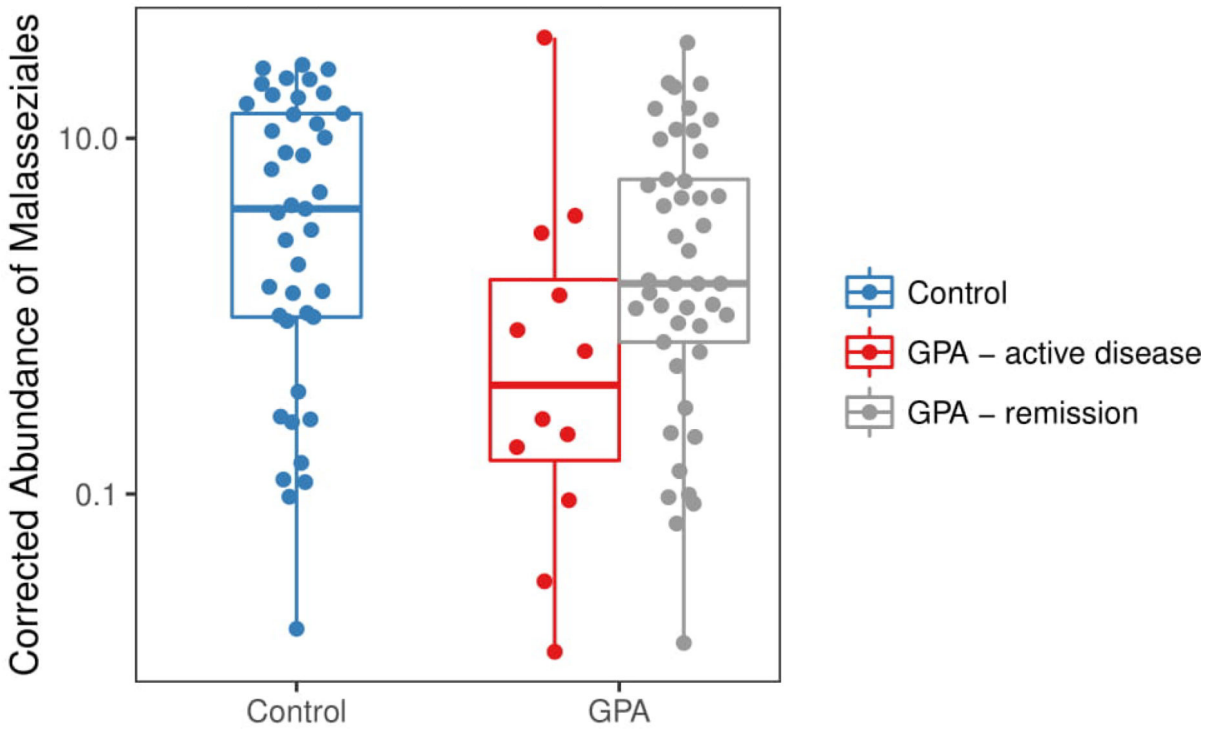

Figure 5:

Differential abundance of *Malasseziales* between controls and participants with granulomatosis with polyangiitis (GPA) stratified by disease activity. PicoGreen-corrected abundance of *Malasseziales* was significantly lower in participants with GPA with active disease (red) compared to controls (blue) ($p = 0.01$) and participants with GPA in remission (gray) ($p = 0.04$). No difference was seen between participants with GPA in remission and controls ($p = 0.19$).

Table 1:

Participant characteristics at time of nasal sampling

	GPA N = 60	Control N = 41	P-value
Age	55 (37, 66)	61 (47, 68)	0.06
Female	35 (58%)	24 (59%)	0.98
White race	57 (95%)	36 (88%)	0.18
Ever smoker	19 (32%)	19 (48%)	0.11
Current allergies	14 (23%)	15 (37%)	0.15
GPA manifestations			
ANCA type (ever)		N/A	--
Proteinase-3	36 (60%)		
Myeloperoxidase	15 (25%)		
Negative	9 (15%)		
Disease duration, years	4 (2, 7)	N/A	--
Newly diagnosed (within 30 days)	5 (8%)	N/A	--
Current disease status		N/A	--
Remission	45 (75%)		
Severe flare	2 (3%)		
Limited flare	10 (17%)		
Persistent disease	3 (5%)		
Currently active disease of sinonasal area	10 (10%)	N/A	--
Ever had flare involving sinonasal area	26 (43%)	N/A	--
Vasculitis Damage Index			
Any sinonasal item	28 (47%)	N/A	--
Nasal blockade/chronic crusting	21 (35%)		
Nasal bridge collapse	11 (18%)		
Chronic sinusitis	10 (17%)		
Medication use			
Systemic glucocorticoid			
Current	25 (42%)	0	< 0.01
Current or in past 6 months	31 (52%)	3 (7%)	< 0.01
Other immunosuppressive drug			
Current	32 (53%)	0	< 0.01
Current or in past 6 months	42 (70%)	0	< 0.01
Current intranasal glucocorticoid	12 (20%)	5 (12%)	0.30
Antibiotic			
Current	15 (25%)	6 (15%)	0.21
Current or in past 6 months	29 (48%)	12 (29%)	0.06
Current trimethoprim-sulfamethoxazole			
Full dose *	0	0	--
Low dose **	10 (17%)	0	< 0.01

Values expressed as median (inter-quartile range) or percentage

*Full dose is trimethoprim-sulfamethoxazole 160–800mg twice daily.

**Low dose refers to doses used for prophylaxis of *Pneumocystis jirovecii* pneumonia (trimethoprim-sulfamethoxazole 160–800mg three times a week or trimethoprim-sulfamethoxazole 80–400mg daily).