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Nasal microbiome research in ANCA-associated vasculitis: Strengths, limitations, and future directions

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ABSTRACT

The human nasal microbiome is characterized by biodiversity and undergoes changes during the span of life. In granulomatosis with polyangiitis (GPA), the persistent nasal colonization by *Staphylococcus aureus* (*S. aureus*) assessed by culture-based detection methods has been associated with increased relapse frequency. Different research groups have characterized the nasal microbiome in patients with GPA and found that patients have a distinct nasal microbiome compared to controls, but the reported results between studies differed. In order to increase comparability, there is a need to standardize patient selection, sample preparation, and analytical methodology; particularly as low biomass samples like those obtained by nasal swabbing are impacted by reagent contamination. Optimization in obtaining a sample and processing with the inclusion of critical controls is needed for consistent comparative studies. Ongoing studies will analyze the nasal microbiome in GPA in a longitudinal way and the results will inform whether or not targeted antimicrobial management in a clinical trial should be pursued or not. This review focuses on the proposed role of *S. aureus* in GPA, the (healthy) nasal microbiome, findings in the first pilot studies in GPA, and will discuss future strategies.

Contents

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1. Introduction

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis is a small vessel vasculitis, characterized by few or absent immune deposits (negativity or low-grade positivity for complement or immunoglobulins) in the affected organs (referred to as "pauci-immune"). The diseases within this disease spectrum are histologically presenting with necrotizing or granulomatous inflammation. In most cases with systemic disease, serological detection of ANCA is positive [1]. Three different entities exist: granulomatosis with polyangiitis (GPA), microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA).

In cases with GPA (formerly Wegener's granulomatosis), a cytoplasmic pattern of ANCA is detected in most cases (c-ANCA), with the respective target antigen, proteinase 3 (PR3) [2]. GPA is a necrotizing granulomatous disease usually involving the respiratory tract (most often the ear, nose and throat (ENT)-tract and to a lesser degree the lower respiratory tract) [3]. More than half of the patients with GPA present with PR3-ANCA/c-ANCA positivity [2]. ANCA-negativity does not exclude a diagnosis of GPA and localized antibody production, that is not detectable in circulation, has been suggested to play a role in these cases [4]. These cases often present with ENT-limited disease with a particularly severe nasal involvement, which is often responsible for a delay in diagnosis and an accrual of damage, including saddle nose deformity [1,5]. The development of a serologic test to allow for early diagnosis would be of particular clinical utility, but no such biomarker exists at the moment. ENT involvement during active disease is characterized by bloody rhinorrhea, and rhinoscopy is a sensitive tool to investigate disease activity, but lacks specificity as infections may resemble active vasculitis [6].

The etiopathogenesis of GPA remains obscure, but a multifactorial mechanism of disease onset is discussed. Besides genetic associations [7], a central role of the environment and associated factors has been reported [8]. Microbial factors are among the environmental factors thought to play a role, and a direct relation to the presence of *Staphylococcus aureus (S. aureus)* in the nose has been proposed [9]. Up to date, it is unclear whether dysbiosis is causative in the development of autoimmunity, or is an effect of immunosuppressive therapy or damage related to vasculitic processes of the affected organs providing an altered niche.

This review will focus on the nasal microbiome in health and particularly in GPA, strengths of such an analysis, and associated limitations. A special focus will lie on potential strategies to overcome these limitations, how these efforts should be driven forward, and future directions of microbiome research in the field of ANCA-associated vasculitis.

2. Granulomatosis with polyangiitis (GPA) – the *Staphylococcus aureus* nasal carriage theory – clinical impact and possible explanations

2.1. S. aureus positivity in GPA and impact on disease outcome

S. aureus represents an important commensal pathogen which is permanently colonizing 20–30% of the general population and approximately 60% intermittently, though a sizable subset seem never to carry it [10]. *S. aureus* carriage is associated with the presence of autoimmune diseases, not only in GPA but also discussed to be more prevalent in rheumatoid arthritis and psoriasis/psoriatic arthritis. Several factors may contribute to the virulence of *S. aureus* including surface structure, the production of exotoxins, and exoenzymes [11]. In patients with GPA, presence of chronic *S. aureus* colonization is an independent risk factor for relapse when compared to non-carriers [12]. Persistent carriage in GPA patients is reported to be 60–70% in several independent investigations [12,13]. Of importance, patients with chronic nasal *S. aureus* carriage had higher endoscopically proven endonasal activity. These patients had their initial manifestation of GPA more often in the ENT tract [13]. Though, more recent studies of the nasal microbiome in GPA have questioned whether *S. aureus* carriage is as prevalent/abundant as historically reported using culture-based methods [14,15]. One of these studies, conducted by the French Vasculitis Study Group (FVSG), however, has not performed enrichment (i.e., with a *Staphylococcus* spp. agar) and did not perform swab analysis on fresh samples [14].

A randomized, placebo-controlled study to evaluate the efficacy of trimethoprim-sulfamethoxazole twice daily for 24 months revealed a significant reduction of disease relapses in patients with GPA, particularly driven by reduced ENT-related disease recurrences [16]. While the authors did not state if treatment led to the eradication of *S. aureus* in their patients' nostrils, antibiotic treatment might lead to the restoration of the nasal microbiome with a reduction of pathogenic bacterial strains which might be of importance to prevent disease activity. A smaller randomized, controlled trial recruited 31 patients in disease remission, of whom 16 received 960 mg trimethoprim-sulfamethoxazole thrice weekly. The use of trimethoprim-sulfamethoxazole and PR3-ANCA positivity at inclusion were associated with relapse-free survival. Chronic *S. aureus* carriage was associated with disease recurrence [17].

The retrospective analysis by the FVSG focused on differences between patients receiving antibiotic prophylaxis or no prophylaxis. Low-dose trimethoprim-sulfamethoxazole (480 mg a day) reduced the number of persistent *S. aureus* carriers, and nasal carriage was more frequent in patients with GPA who did not receive trimethoprim-sulfamethoxazole compared to controls. A subset of patients had a follow-up period of four years, and nasal *S. aureus* carriage was not significantly associated with disease relapse. No effect on relapse rate was found in trimethoprim-sulfamethoxazole users [14].

2.2. Toxic shock syndrome toxin-1-producing S. aureus and relapse

Several mechanisms have been proposed to be implicated in persistent carriage and pathogenicity. The pathogenicity of *S. aureus* in GPA may be attributed to the production of pyrogenic toxins. Diverse antigens of *S. aureus* have been identified in patients with GPA. Among these superantigens (SAg), the toxic shock syndrome toxin-1 (tsst-1) is one of the most potent. A high proportion of GPA patients (> 70%) carry strains that harbor at least one *S. aureus* SAg [18]. In a single study the likelihood of isolating tsst-1 positive *S. aureus* strains was higher in individuals with GPA (36%, in comparison to 5% expected in the general population), and was associated with an increase of disease relapses [18].

2.3. Immunologic aspects

T-cell expansion was present at a higher rate in patients with GPA than in healthy individuals, but was neither associated with the presence of *S. aureus* nor its SAg [19]. *S. aureus*-restricted T-cell clones were of the αβ-TCR+CD4+ phenotype and HLA-DR restricted. Of particular interest, seven of the *S. aureus*-reactive T-cell clones were capable of recognizing the PR3 antigen [20]. In order to elucidate further pathogenetic consequences of *S. aureus* presence, staphylococcal acid phosphatase (SAcP) and its binding ability was studied in human umbilical vein endothelial cells. It was demonstrated that SAcP is capable of binding to endothelial cells in a concentration-dependent manner. Moreover, endothelial cell-bound SAcP was recognized by sera of patients with GPA [21].

Examination of nasal epithelial cells obtained from patients with GPA indicated an up-regulation of granulocyte-colony

stimulating factor [22]. After stimulation with supernatants of *S. aureus*, patients displayed a lower interleukin-8 secretion and a diminished dynamic range of response towards the stimulus, which may help explain the higher carriage rates of patients with GPA [22]. Antimicrobial peptides from patients with either *S. aureus* colonization or negative controls were assessed by ELISA. In patients with colonization, significant higher levels of LL-37 could be detected. After stimulation with *S. aureus* higher levels of LL-37 and human ß-defensin 3 could be detected in the supernatant of nasal epithelial cells of GPA patients [23]. A transcriptomic approach revealed differential expression of 10 transcripts, including antimicrobial transcripts such as human ß-defensin 1, lysozyme and human ß-defensin 4 [24].

More recently, lower anti-staphylococcal IgG levels against 59 *S. aureus* antigens in GPA patients were reported in comparison to healthy controls despite similar overall IgG levels [25]. In the same study the authors reported an increased frequency of *S. aureus* strains resistant to trimethoprim-sulfamethoxazole or ciprofloxacin, which seemed to coincide with the increased treatment of patients with trimethoprim-sulfamethoxazole [25]. PR3-ANCA vasculitis patients carry similar *S. aureus* types as observed in the general population [25]. There are differences in the distribution of *S. aureus* clonal complexes (CCs) amongst PR3-ANCA and MPO-ANCA vasculitis [26]. Several genetic loci were found to be associated with either PR3-ANCA or MPO-ANCA vasculitis, and investigations found a possible role of leukocidins in PR3-ANCA vasculitis [26]. However, both these studies [25,26] only used small numbers of isolates, which are not sufficient to reach genome-wide significance, and no replication studies have been conducted so far to confirm these findings. Recent studies investigated the role of tissue-resident memory T (T_{RM}) cells in autoimmunity, which are induced by *S. aureus* and other bacteria, and found that *S. aureus* infection induced T_{RM} cells with a T_H17 signature in the kidney. Kidney-resident $T_{RM}17$ cells reinforced the local inflammatory response by production of IL-17A, leading to an exacerbation of the observed renal pathology [27]. Further studies should address if immunologic changes in the nostrils resemble changes observed in kidneys of humans and in experimental models.

Overall, these findings suggest an impaired inflammatory response, reduced response to a microbial stimulation leading to alterations of the microbial composition of the nostrils, and differences in the distribution of specific *S. aureus* strains among patients with PR3-ANCA vasculitis and comparators.

3. The (healthy) nasal microbiome and influences on diversity

The healthy human nasal microbiome contains a low number of observed genera, and is dominated by a limited number of bacterial genera [28]. Localized factors such as temperature, nutrient availability and humidity may shape the nasal microbiome. The biodiversity seems to be lower in the anterior nares in comparison to the middle meatus and sphenoethmoidal recesses [29]. The microbiome of the anterior nares is dominated by *Propionibacterium*, *Corynebacterium*, *Staphylococcus* and *Moraxella* [30]. *S. aureus*, *Staphylococcus epidermidis* and *Propionibacterium acnes* are the most prevalent and abundant microorganisms detected in the middle meatus [31]. Over the span of human life, the composition of the nasal microbiome undergoes significant changes. Puberty has a major impact on its composition, with Proteobacteria (*Moraxella*, *Haemophilus*, and *Neisseria*) and Firmicutes (*Streptococcus*, *Dolosigranulum*, *Gemella*, and *Granulicatella*) overrepresented in prepubertal children [32]. In contrast, the nasal microbiome of healthy adults is dominated by Actinobacteria (*Corynebacterium*, *Propionibacterium*, and *Turicella*) and Firmicutes (*Staphylococcus* spp.), and to a variable degree Proteobacteria. Overall, analysis of the nasal cavity identified that among Actinobacteria, the most prevalent families were Corynebacteriaceae (ranging from 1.5% to 62.8%) and Propionibacteriaceae (ranging from 0.4% to 42.4%). Staphylococcaceae were the most prevalent family amongst Firmicutes and the overall percentage of the community ranged from 2.2% to 55.0% [33]. Together, these three bacterial phyla account for over 80% of the colonizing species [34]. Insights into the shape of the adult nasal microbiome can be generated by analysis of residents living in different healthcare institutes, as different environmental factors might influence the composition (i.e., cleaning processes, disinfection of indoor air, or the source of ventilated air). A study from Taiwan found that the nasal microbiome more strongly relates to the environmental surface microbiome, and this was the case when participants from three healthcare institutes were analyzed [35]. A particular influence of the surface microbiome was observed in participants who have been hospitalized in the year prior to study enrollment, with a significantly higher proportion of microbes derived from surfaces. Notably, the average age of residents in both groups was over 70 years, with a significant amount of co-morbidities, which might have influenced the individual susceptibility to a change of the microbial composition [35].

The diversity of the healthy adult microbiome can be explained by adaption processes executed by bacteria. In habitats with limited nutrient supply such as the nostrils, bacteria are under strong selective pressure to increase their fitness to compete with other competing species. Such competition can occur in a direct and indirect manner. Direct competition may be exerted by the production of antimicrobials that selectively kill competing susceptible species (i.e. Esp-secreting *S. epidermidis* correlates with the absence of *S. aureus* [36] or secretion of hydrogen peroxide by *Streptococcus pneumoniae* [37]), whereas indirect competition may be related to modified living conditions or competition for nutrients [38]. In our pilot study investigating the nasal microbiome in patients with GPA, we found that in most cases with presence of *Staphylococcus pseudintermedius* the antagonistic relationship between *S. aureus* and *S. epidermidis* is broken, indicating that all three occupy the same niche [39].

Other studies investigated the effect of specific techniques to obtain a robust sampling of the nasal microbiome. One large study investigated the effect of three different swabs, and found that the species diversity was comparable when flocked nylon, rayon or polyurethane tipped swabs are used [40]. Further analyses revealed that there was no difference in relative abundance of selected genera when swabs were self-taken at home or collected by designated staff, with the exception of a higher abundance of *Lactobacillus* spp. in home-collected samples. Collection by nasal brushes should be the preferred method, as investigations have shown a significant reduction in phylogenetic diversity by nasal washes [41]. Procession of nasal swabs after standard postage of study participants may not influence the yield of specific bacteria (*S. aureus*) in comparison to immediate procession [42]. Other investigations found that storage at room temperature for 14 days led to an undesired *S. aureus* growth. As storage at room temperature may impact the (relative) abundance of genera, a storage at 4 °C is advised until analyses are performed [43].

Notably, treatment with antibiotics is altering the microbiome. A study investigating the role of trimethoprim-sulfamethoxazole on the gut microbiome found that the Shannon diversity at a phylum level declined by 17.95%, and especially patients with high baseline diversity were more likely to lose diversity. Long-term treatment with trimethoprim-sulfamethoxazole might exhibit detrimental effects on the commensal microbiome, fueling the inflammatory response in some cases. During treatment with trimethoprim-sulfamethoxazole, an increase in *Proteus vulgaris*, *Actinomyces meyeri* and *Acinetobacter lwoffii* was observed [44].

Colonization of *S. aureus* plays an important role in health and disease. Several factors are discussed to affect the composition of the nasal microbiome, including environmental factors [34,45] and host genetic polymorphisms [34]. Seasonal variations were observed including temperature changes, humidity, pollen or dust levels, and smoking negatively correlated with *S. aureus* nasal colonization. Altered nasal colonization risk with *S. aureus* has been associated with genetic polymorphisms in the genes encoding the glucocorticoid receptor, C-reactive protein or ß-defensin 1 [34]. Importantly, direct or indirect mechanisms are involved in the highly competitive colonization of the nasal cavity, including the binding of bacterial adhesions to epithelial ligands, with *S. aureus* producing different adhesins for different epithelia and thus colonizing various anatomical regions of the nose in a similar manner. Selection of the nasal microbiome is further characterized by competition for nutrients, competition by antibiosis which are produced by certain bacterial strains, and competition by the induction of host defense mechanisms. *Staphylococcus* spp. produce antimicrobial substances at a high frequency (86%), which seem to be less harmful to *S. aureus*. Moreover, *S. aureus* is actively inducing inflammatory host responses to make survival for commensals within the nasal cavity more difficult [34]. These implications explain in part the complexity of understanding the diverse composition of the healthy nasal microbiome and specifically the colonization by *S. aureus*, and highlight that certain bacterial strains co-occur with *S. aureus* (such as *Corynebacterium accolens*), and others show a competitive interaction (such as *C. pseudodiphtheriticum*) [46].

The healthy nasal microbiome undergoes changes from infancy to adulthood. Different sites of the nasal cavity may exhibit a different spectrum of microorganisms, and these differences need to be taken into account when analyzing the microbiome in health and disease states. There are no single "healthy microbiome" as significant inter-individual differences have been reported. Several factors including adaption processes, locoregional changes, and influences by recent drug prescriptions (i.e., antibiotics) are attributable. Sampling should be performed by nasal swabs rather than nasal washes. Recruited individuals may collect nasal swabs at home, as there are no significant changes in the main selected genera between staff- or home-collected nasal swabs.

4. Granulomatosis with polyangiitis – microbiome research

Three independent studies [39,47,48] have investigated the role of the nasal microbiome in GPA. Differences in geographic background, disease activity status, and more importantly the analysis paths (i.e., storage) lead to difficulties in direct comparison. Table 1 summarizes key demographics, laboratory findings (i.e., ANCA status), and key results of these studies.

The nasal microbiome of 60 GPA patients (60% with a positive PR3-ANCA test, 25% with active disease) and 41 healthy controls was analyzed by 16S rRNA gene sequencing [47]. The Shannon

Table 1
Summary of three independent studies of the nasal microbiome in patients with GPA. Geographical differences, differences in disease activity status (especially in the ENT tract), treatment differences (i.e., antibiotics), storage, and analysis paths limit comparability of results.

	Rhee et al. [47]	Lamprecht et al. [48]	Wagner et al. [39]
GPA patients (number)	60	29	56
Country	USA	Germany	UK
ANCA type	PR3 (60%), MPO (25%), negative (15%)	PR3 (83%), MPO (3%), negative (14%)	Not reported
New diagnosis	5 (8%)	0%	Not reported
Disease status	Remission (75%), severe flare (3%), limited flare (17%), persistent disease (5%)	Remission (79%), active disease (21%)	Remission (79%), active disease (21%)
Current ENT disease activity	10%	21%	21%
Antibiotic treatment	48% (in the past 6 months)	No recent prescription	17.9% (in the past months)
Storage	Freezer (swab; −80 °C)	Immediately processed (swab)	Immediately processed (swab)
Main findings	GPA patients had a lower relative abundance of *Propionibacterium* (*P. acnes*) and *S. epidermidis* compared to controls	GPA patients had an increase in bacterial species assigned to the families *Streptococcaceae*, *Pasteurellaceae*, and *Prevotellaceae*; and a decrease in *Corynebacteriaceae*, *Moraxellaceae*, *Tissierellaceae*, *Staphylococcaceae*, and *Propionibacteriaceae*	16S: GPA patients (grouped together) had a distinct microbiome composition compared to healthy controls WGSS: active GPA group was different to the healthy and diseased controls
S. aureus	No difference in the relative abundance of *S. aureus* between patients and controls	*S. aureus* was detected in a significantly higher proportion of samples from GPA patients (compared with RA patients and healthy controls)	Culture: *S. aureus* detection active group (67%), inactive group (34%) 16S: no statistical association between mean abundance and disease outcome WGSS: *S. aureus* was detected at statistically higher abundance in active GPA patients compared with healthy and diseased controls
Strengths	Additional analysis of the fungal community composition; investigations focusing on non-immunosuppression users versus patients on immunosuppression; well characterized patients	Comparison of GPA patients with a diseased comparison group (RA); in depth analyses of the bacterial community composition; the use of UMERS as a novel method that can detect distantly related microorganisms; well characterized patients	First study to perform whole genome shotgun sequencing from nasal swabs including study of critical pathways involved; report of significant findings with higher relative abundance; well characterized patients; Remission patients as comparators
Limitations	Cross-sectional; small sample size; no immediate processing of samples; no information on current smoking status, patients on immunosuppression (>50%) and antibiotics (25%) at the time of nasal swab	Cross-sectional, small sample size, patients on immunosuppression (28/29 on steroids; and a majority on additional measures)	Cross-sectional; small sample size; no information on current smoking status, steroid prescription (75% active group, 47.6% remission group), additional immunosuppression (75% active group, 57.1% inactive group)

Abbreviations used: ANCA (anti-neutrophil cytoplasmic antibody), ENT (ear, nose and throat), GPA (granulomatosis with polyangiitis), MPO (myeloperoxidase), PR3 (proteinase 3), RA (rheumatoid arthritis), UK (United Kingdom), USA (United States of America), WGSS (whole genome shotgun sequencing).

Diversity Index was not different between both cohorts, while analysis of beta diversity calculated on UniFrac distances revealed a significant difference in weighted UniFrac between individuals with GPA and healthy controls. A relative lower abundance of *Propionibacterium* (especially *P. acnes*) and *S. epidermidis* in the cohort of GPA accounted for the difference observed in this study [47]. Thus far, no study performed in-depth analyses of glucocorticoid prescription on nasal microbiome alterations. It is tempting to speculate that glucocorticoids exert similar effects as observed on the gut microbiome [49,50], with a proposed involvement of direct and indirect alterations, the latter potentially reducing the pro-inflammatory local milieu caused by certain bacterial strains. When assessing the impact of non-glucocorticoid immunosuppression, a difference between participants with GPA off immunosuppression and controls was found, while no difference was observed between those receiving immunosuppression versus controls. These changes were mainly driven by a significantly lower abundance of *Propionibacterium* in GPA patients off immunosuppression versus controls. Glucocorticoid treatment led to a lower abundance of fungi, and these alterations were particularly seen in cases with active disease, harboring the lowest abundance compared to those in remission and controls. This study indicates that immunosuppression is capable to alter the nasal microbiome. On the other hand, a cross-sectional study in this field with a low sample size is unlikely to report specific changes implicated in relapse risk of an individual patient. A follow-up time including periods off immunosuppression and sequential sampling is necessary to predict the impact of the microbiome on relapse risk.

In a prospective clinical study, 29 subjects with GPA, 21 with rheumatoid arthritis (RA) and 27 healthy controls were included [48]. 16S rRNA amplicon sequencing and unbiased metagenomic RNA sequencing (UMERS) were performed. Differences were observed among patients with GPA during active disease (n = 6) and remission (n = 23), with a higher abundance of *Staphylococcaceae* during remission. Differences in the diversity of the microbial composition was observed based on ENT activity, with the families *Streptococcaceae* and *Planococcaceae* being found at higher abundance in patients with active ENT disease, while a lower abundance for *Corynebacteriaceae* was observed. Amongst the three different groups, significant differences were reported, with a higher abundance of *Streptococcaceae* and *Pasteurellaceae* and a lower abundance of *Aerococcaceae* in patients with GPA compared to the RA group. *Planococcaceae* were more abundant in both, the GPA and RA group, while a lower abundance was found for *Moraxellaceae*, *Tissierellaceae*, and *Staphylococcaceae* compared to healthy controls. 16S analyses detected *S. aureus* in a significantly higher proportion in individuals with GPA compared to RA patients and healthy controls. UMERS, a novel method to detect known and distantly related or novel pathogens of viral, bacteria, fungal, or parasitic origin, was performed in a proportion of included patients (nine patients with GPA and four healthy controls). Sequence reads from patients with GPA displayed sequence homology to *Haemophilus influenzae*, Rhinovirus A, *S. aureus* and *Moraxella catarrhalis*, indicating that patients had acute respiratory tract infection at the time of sampling and presence of *S. aureus* and *M. catarrhalis* indicated colonization. This study found that with the use of 16S rRNA gene sequencing, *S. aureus* is more abundant in patients with GPA compared to controls (either RA patients or healthy controls), again highlighting a significant role of *S. aureus* in the pathogenesis of GPA.

In another case-control study including 12 patients with active GPA, 44 with remission, 13 diseased controls (either MPA or EGPA) and healthy controls, culture-dependent analyses revealed that more patients with active GPA were tested positive for *S. aureus* [39]. Multivariate ordination analysis using bacterial 16S sequence data indicated that samples from the healthy control group clustered differently compared to patients with either active or inactive GPA. When individuals with GPA were clustered together, a significant different microbiome compared to healthy controls was found. There was a non-significant higher abundance of *S. aureus* in patients with relapsing GPA compared to those with long-lasting remission using bacterial 16S sequence data. A proportion of recruited individuals were followed up longitudinally and swabbed again. While an inter-individual difference was observed, the intra-individual profile remained consistent over time (within 3 months).

The same group also conducted deep shotgun metagenomic sequencing in addition to bacterial 16S gene profiling. The analyses focused on the retrieved *Staphylococcus* taxa since it was the most abundantly observed. Multivariate ordination analysis and permutational multivariate analysis of variance revealed that the differences among the patient and control groups were significant. Individuals with active GPA had a different composition of *Staphylococci* compared to healthy and diseased controls. The healthy controls were different to GPA patients in remission. *S. epidermidis* was detected at higher abundance in healthy controls compared with active GPA patients. Contrary to this finding, *S. aureus* was detected at statistically higher abundance in active GPA compared with healthy and diseased controls. Another interesting finding from the shotgun metagenomic sequencing was the detection of *S. pseudintermedius*, which was observed in high abundances in all groups. In most cases with presence of *S. pseudintermedius*, an antagonistic relationship between *S. aureus* and *S. epidermidis* was broken, with both species present at lower levels, suggesting that *S. pseudintermedius* occupies the same niche [39]. In a single patient, *S. pseudintermedius* was cultured in serial swabs [51]. Again, this study was performed in a cross-sectional manner and only some patients were subsequently swabbed within a short period of follow-up. Within 1–3 months, the individual microbiome of patients was stable over time. More investigations are needed to investigate the influence of potential changes on relevant disease outcomes (i.e., infectious risk or relapse risk).

Taken together, cross-sectional studies in the field of GPA focused on the differences in microbial composition. The few studies and the inconsistencies between patient recruitment, laboratories, and analysis pipeline, together with environmental and reagent contamination issues, make it a challenging task to directly compare and combine data from these studies.

5. (Nasal) microbiome analysis: impact of low biomass and contamination

Despite the benefits of culture-independent analysis of the microbial composition of a specimen, this method has key limitations, especially when working with samples containing low biomass (low DNA). The non-proportional target amplification and the presence of laboratory and reagent contaminations have been identified as potentially misleading taxonomic classifications in many culture-independent low-biomass sequence analysis [52].

There are tools addressing the non-proportional target amplification, but thus far no systematic requirement to report irregularities or report contamination have been implemented as a standard requirement. Many sources may lead to contaminant DNA despite particular care in sample collection and preparation. These potential sources include sampling and laboratory environments, researchers, plastic consumables, nucleic acid extraction kits, laboratory reagents including polymerase chain reaction (PCR) master mixes, and cross-contamination (most frequently due to transfer of primary sample DNA from neighboring wells or tubes) from other samples or sequencing runs [53].

A variable degree of the impact of contaminant DNA and cross-contamination has been reported, largely depending on the level of microbial biomass. Measuring the microbial DNA in samples and comparison to the amount of DNA extraction blank controls may help to identify samples with low biomass, which are likely to be influenced by contaminant DNA when undergoing further sequencing [53]. The presence of contaminant DNA was demonstrated in an elegant study using up to five rounds of serial ten-fold dilutions of a pure *Salmonella bongori* culture. In the undiluted samples, *S. bongori* was the sole organism identified, while with subsequent dilutions a range of contaminating bacterial groups increased and the *S. bongori* reads concurrently decreased. Further analyses highlighted that sixty-three taxa were absent from all PCR blank controls but present at >0.1% proportional abundance in one or more serially diluted *S. bongori* samples, which suggests that these taxa were introduced at the DNA extraction stage. Some of the taxa were only abundant in samples processed by one or two sites, possibly indicative of variation in contaminants between different batches of the same type of DNA extraction kit. This study elegantly illustrated that bacterial DNA contamination of extraction kits and laboratory reagents can significantly alter results of microbiome studies, particularly when low biomass samples are treated [54].

To overcome limitations of contaminant DNA extraction and PCR master mix, introduction of commercially available dsDNase treatment to decontaminate the PCR master mix might be used. A study demonstrated that large amounts of contamination was derived from the PCR master mix and after treatment with dsDNase this contamination was almost completely eliminated, yielding a 99% reduction in contaminating bacterial reads [55]. Batch effects in DNA isolation may be identified by nonparametric correlation analysis per batch on the different microbial species. Another option is combination of unsupervised hierarchical clustering analyses of microbial groups and samples in a heatmap that indicates the relative abundance of these microbial groups. In the case of nasal shotgun metagenomics, reagent-derived species were present especially in samples which failed by 16S rRNA gene sequencing. A further approach is measurement the correlation between input DNA quantity and output species abundance [56]. Tools to identify the presence of contaminants have been established, i.e. decontam which is an open-source R package and showed that it effectively improves the quality of marker-gene and metagenomics sequencing by removing contaminant DNA sequences [57].

Several recommendations taking into account considerations as stated above have been issued and are summarized in Table 2 [56,58]. Steps from sample collection to final analysis are highlighted in Fig. 1.

6. Summary and outlook

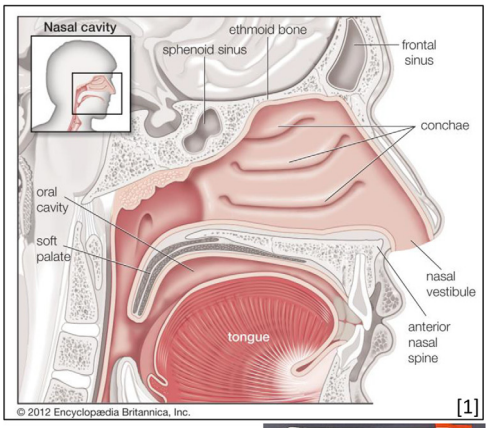
Microbiome research with the use of low biomass samples has its strengths and limitations. The unsupervised analysis of samples allows for identification of a broad spectrum of genera which might be critically involved in disease pathogenesis. Nonetheless, the nasal microbiome even in a healthy state is characterized by a large biodiversity, undergoes significant changes during the span of life, and might be influenced by the environment, host genetics, and factors produced by bacteria leading to direct or indirect inhibition of other genera. In the near future, large-scale studies with the aim to further characterize the healthy microbiome are needed. Influences such as recent systemic or local antibiotic treatment, hospitalization, smoking status need to be excluded in such study. In GPA, several different working groups have performed microbiome studies. No study could corroborate the high frequency of *S. aureus* positivity as reported by culture-dependent investigations [39,47,48], while the three studies published [39,47,48] have demonstrated different results. This can be explained by patient selection, differences in the analysis paths and might in part be attributable to the background of the patients (genetic differences, "other" background microbiome related to geographical differences).

There is a clear need to standardize the reporting of microbiome research, as generalizable conclusions are largely absent and physi-

Table 2
Overlapping and stand-alone recommendations/guidelines have been issued to control for several lines of contamination. Importantly, studies should include negative and positive controls which help to identify batch effects and contamination between different suppliers of DNA extraction kits. Results should be critically discussed in terms of ecological plausibility. Sample time points and essential confounders such as the use of antibiotics within a time period of a few weeks before sampling need to be recorded. Uniform sample collection and processing (i.e., storage) are necessary to guarantee reproducibility of the results. The validity of the results is increased when a separate discovery and validation cohorts are recruited.

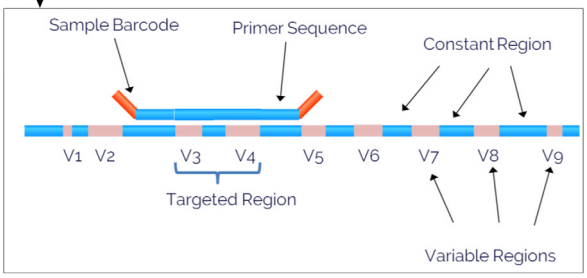
Recommendations for the design and execution of microbiome studies
Confounding factors: antibiotic use, age, sex, diet, geography, and pet ownership
Animal studies: cage effects (each condition must be studied in multiple cages)
Sample storage: at −80 °C immediately after collection for most accurate results
Sample time point: importance to assess the relationship of possible longitudinal dynamics
Negative controls: create and analyze negative controls – DNA extraction kits usually contain contaminants, and contamination may vary between suppliers and batches of the same kit
Positive controls: for each batch of samples; place controls asymmetrically in purification plates
Low biomass samples: quantify the microbial load; different methods to measure DNA can be used; when all sequence data reflects contamination only, the idea might be rejected
Statistical methods: control for multiple comparisons
Cohort selection: consider to study a separate discovery and validation cohorts
Cohort selection: history of daily medication intake (i.e., oral contraceptives, immunosuppressive medication) and account for environmental factors associated with changes of the respiratory microbiome (i.e., smoking)
Contamination identification guidelines
Negative controls: every step of the process need to be covered
Positive controls: biomass and contamination levels should be assessed
Batch effects: create batch effects not by accident
Statistics: to control between and within batch variation patterns
Bioinformatics: increase identification resolution
Reproducibility: two different kits to isolate DNA should be used
Reproducibility: non-sequencing methods should be used to confirm results
Ecological plausibility: check the literature (what you should/can expect)
Use all available lines of defense
Standardized questionnaire

1.1 Collection of nasal sample for sequencing



1) collect nasal swab samples with sterile collection swabs

2) Extract nasal DNA in the laboratory under sterile condition to avoid environmental and laboratory contaminations



3) Bacterial 16S rRNA gene library preparation with primers targeting a specific bacterial 16S variable region e.g., V3 and V4 https://support.illumina.com/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

4) Sequencing on a next generation sequencing platform, most commonly platform for bacterial 16S sequencing is Illumina MiSeq

1.2 Sequence analysis using bioinformatics

```
AGCCTTGTCATCCGTCTC-TTTCAA----
AGCCTTGTCATCCGTCTC-TTTCA------
- GCCTTGTCATCCGTCTC-TTTCAACG-
- - CCTTGTCATCCGTCTC-TTTCAA-----
```

1) Sequence quality filtering using bioinformatics program such as MOTHUR [2] and Dada2 [3]

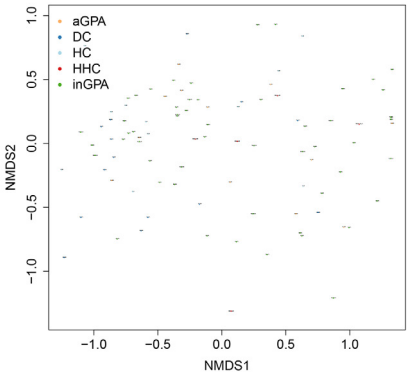
2) Remove chimeras. Different programs are available e.g. chimera uchime and chimera search. Chimera programs are usually a part of other program suites such as MOTHUR and Dada2

3) Clustering bacterial 16S sequences using various clustering methods. E.g., conventional OTU clustering in MOTHUR, oligotyping using Minimum Entropy Decomposition [4], or Dada2

4) High quality chimera free bacterial 16S sequences are then used fort taxonomic classification against bacterial 16S databases. E.g. Silva database [5], Ribosomal Database Project [6], ARB analysis [5] and blast or Diamond [7] search follow by analysis using MEGAN [8]. MEGAN can also be used for shot gun sequenced metagenomics analysis of nasal microbiome

5) Recognizing the contaminating microbiome and removing it. Extracted DNA from nasal swab samples are low biomass samples and as such are prone to environmental contamination [9-10]. Techniques for removing contaminations have been reported [11].

6) The exciting part is the analysis of high quality and contamination free nasal bacterial sequences by using different analytical methods, e.g., ordination plots, taxonomic abundance figures, heatmap annotation etc.



Differences in shotgun sequenced nasal Staphylococcus species were visualised using non-metric multi-dimensional scaling. aGPA = active granulomatosis with polyangiitis inGPA = inactive GPA, DC = disease controls, HC = healthy controls, HHC = healthy household controls
Taken from [12]

[1] Human Nasal Cavity by courtesy of Encyclopaedia Britannica, Inc. URL: https://www.britannica.com/science/nose#/media/1/420420/70985 Access Date: 24th November 2020
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[4] Murat et al, The ISME Journal, 2015, 9:968-979
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Fig. 1. The figure (1.1.) was provided by courtesy of Encyclopaedia Britannica, Inc.

cians treating patients with GPA have difficulties to understand the complexity of the findings. However, standardizing the reporting of microbiome research is also associated with limitations and does not take into account the fast-paced changes in sequencing methodology and analysis pipeline. For example, the best bacterial sequence region depends on the source of the sample to be ana-

lyzed. One suboptimal solution would be to use full-length bacterial 16S sequencing with long read sequence technology. But more importantly is to provide all the details for the analysis pipeline to ensure reproducibility and to implement a thorough pipeline to remove contamination which is particularly important for low biomass samples such as the nasal microbiome.

Further light needs to be shed on the role of *S. aureus* in disease onset, progression (in localized forms), and relapse of GPA. Research on the microbiome in the field of vasculitis needs to be associated with these clinically meaningful endpoints and long-term studies are necessary to understand the impact of different genera. Further efforts to expand our understanding of the impact of prescribed immunosuppressive measures (steroids, rituximab, and others) on the shape of the microbiome and to underline critical pathways either restored or induced by these commonly used drugs are needed. Currently ongoing studies will investigate whether or not there is a change in the microbial composition before relapse and if interventional trials (i.e., localized or systemic antibiotics) make sense to improve the management of patients with ANCA-associated vasculitis. A randomized controlled trial showed that treatment with trimethoprim-sulfamethoxazole in a therapeutic dosage is able to prevent disease relapses, especially those related to the ENT-tract, in patients with GPA [16]. A positive effect of prophylactic trimethoprim-sulfamethoxazole on relapse risk was confirmed in a small study from Poland [17]. The question remains how a 'microbiome-based' study can be conducted, but a certain threshold of *S. aureus* abundance might be helpful to identify patients at risk of ENT-relapse. Efforts of such a sophisticated trial design need to be driven forward by multi-national consortia. Taken together, microbiome research with low biomass samples (i.e., the nasal cavity) is still in its infancy. More efforts are needed to understand the composition of the nasal microbiome in health and disease, and if targeting the microbiome of the nostrils will be a therapeutic approach in the management of GPA or not.

CRediT authorship contribution statement

Andreas Kronbichler: Conceptualization, Writing - original draft. **Ewan M. Harrison:** Conceptualization, Writing - original draft. **Josef Wagner:** Conceptualization, Writing - original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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