Pitfalls in sampling and analyzing low-biomass human nasal microbiome samples

To the Editor:

We read with great interest the original article by Liang et al on alterations of the microbiome in eosinophilic chronic rhinosinusitis with nasal polyps (eCRSwNP).[1] Unlike the authors of previous reports who did not observe any changes or reduced α-diversity in patients with CRS versus in healthy controls, Liang et al[1] report a higher α-diversity in patients with eCRSwNP and suggest using the nasal microbiome as a novel diagnostic classifier for eCRSwNP. Despite these interesting findings, we would like to raise some concerns.

First, the group of patients with eCRSwNP may have been biased toward patients with severe type 2 disease. The cutoff value of an absolute tissue count of 55 eosinophils per hpf used by Liang et al[1] has been evaluated for predicting the recurrence of polyps in eCRSwNP[2] but is much higher than the European Position Paper on Rhinosinusitis and Nasal Polyps–based cutoff of 10 eosinophils per hpf defining eosinophilic chronic rhinosinusitis.[3] Additionally, the population of individuals without CRSwNP used by Liang et al[1] also shows signs of type 2 inflammation (eg, ≤500 eosinophils/μL blood).[3]

Furthermore, we would like to voice our concerns with regard to the quality of the presented microbiome data. Liang et al[1] do not clarify whether any controls were performed during sampling, DNA extraction, and 16S rRNA gene amplicon preparation and sequencing, which is a critical step during microbiome profiling of low-biomass samples.[4] Even if such controls were performed, they were neither deposited in the Sequence Read Archive alongside the sample data (PRJNA785109) nor appropriately documented in the article's Methods section. Although the aforementioned information would be crucial to comprehensively evaluate the data set, we would like to point out 2 further considerations. First, a number of the taxa discussed in the context of differential abundance and correlation with clinical parameters are most probably not native to the nasal mucosa. On the basis of the ecology of these microorganisms, they are most likely cross-contaminations from distinct sample types (ie, anaerobes abundant in human gut samples [*Akkermansia*, *Blautia*, *Desulfovibrio*, and *Sutterella*]).[4] Such contaminations may have inflated α-diversity measures (see Fig 1, *A* in Liang et al[1]), which here are atypically high compared with those of other nasal microbiome data sets.[5] Second, the β-diversity presented in Fig 1, *B* in Liang et al[1] displays a clustering in 3 groups not consistent with the patient groups, which is a common feature observed with sequencing batch effects. To evaluate this further, it would be necessary to disclose in the Methods section whether samples were sequenced in a single run or as multiple batches. Third, although the relative abundance for significantly differentially abundant taxa (see Fig 2 in Liang et al[1]) or taxa displaying correlations with clinical factors (see Fig 3 in Liang et al[1]) is not explicitly discussed, on the basis of the coarse taxonomic profile, it has to be assumed that they are predominantly relatively low-abundant and sporadically occurring (see Fig 1, *C* in Liang et al[1]). This is problematic if these low-abundant sporadic taxa occur because of contamination and sample cross talk, as may partly be the case in the study by Liang et al.[1]

In conclusion, the article by Liang et al[1] clearly raises the urgent need for stringent and uniform guidelines for sampling and processing low-biomass human microbiome samples to achieve comparable data and explore the diagnostic potential of the human microbiome further.

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Reply

To the Editor:

We thank Pjevac et al[1] for their interest in our article "Alterations of nasal microbiome in eosinophilic chronic rhinosinusitis."[2] From the clinician's perspective, the eosinophilic chronic rhinosinusitis with nasal polyps (eCRSwNP) endotype is frequently associated with poorer treatment effectiveness and higher polyp recurrence rates. Identifying specific risk factors for polyp recurrence may provide valuable guidance for treatment selection and appropriate management of nasal polyps. Therefore, the cutoff value of mucosal eosinophils, which can accurately identify polyp recurrence, is of more interest to clinicians. However, mucosal eosinophilia has shown significant geographic and ethnic differences. The effect of mucosal eosinophilia on polyp recurrence may vary with different sites and conditions.[3] At present, the European Position Paper on Rhinosinusitis and Nasal Polyps guidelines only suggest the value of eosinophil in the diagnosis of eosinophilic chronic rhinosinusitis, which is not a diagnostic criterion.[4] From the perspective of clinicians, if this standard is used, it will inevitably increase the experimental error. Instead, we chose to group based on geographic and ethnic similarity. Therefore, our results are more consistent with clinical practice. In addition, through bulk clinical data analysis, Pan et al found that blood and tissue eosinophilic inflammation is not always consistent in patients with

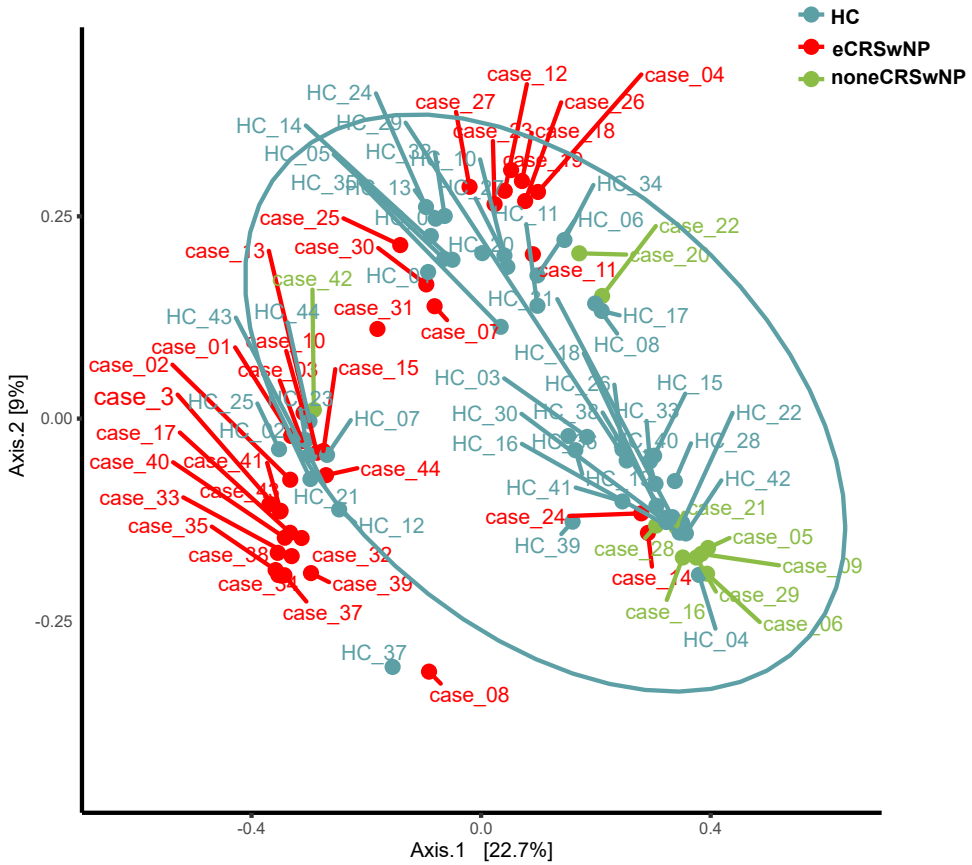

FIG 1. Comparison of β-diversity among patients with eCRSwNP, patients without CRSwNP (non-eCRSwNP), and healthy controls (HCs). Two batches were evenly distributed, and there was no obvious batch effect (the small label number is the first sample delivery).

CRSwNP, in which patients with nasal polyps which have increased single blood eosinophilia had low T_H2 cell counts and eosinophilic inflammation.[5]

We agree with Pjevac et al[1] that quality control is important in low-biomass human nasal microbiome samples, and thus, we acknowledged the limitations in our article.[2] As mentioned in the article's Methods section, we adopted the sampling and sequencing methods of the previous studies.[6] The sampling and sequencing process was conducted in a sterile environment and as quickly as possible. Given the significance of our findings, these limitations can be ignored. In future studies, we will set a negative control group to further strict quality control.

In our study,[2] we described for the first time the characteristics of nasal microbiome in patients with diffuse primary CRSwNP. Previous studies have found differences in the composition of the microbial community between patients with chronic rhinosinusitis (CRS) and healthy controls. Nonetheless, the results of these studies are variable and discrepant. There are 3 reasons for the aforementioned phenomenon. First, many studies did not distinguish between CRSwNP and CRS without polyps. Second, some studies have not been able to separate patients with different risk factors for CRSwNP, such as local pathology, mechanical factors, and inflammatory factors. Finally, the vast majority of studies did not distinguish between local and diffuse types of CRSwNP. All of these factors might cause bias for inflammatory types or histologic discordance among different types of CRS, which has been demonstrated by many previous studies. Thus, the microbiome might vary depending on the endotype of the disease. To reduce the influence of different pathogenic factors on the results of our study's,[2] patients with diffuse primary CRSwNP were selected as the subjects. In addition, the Lund-Mackay computed tomography score in our study was high, suggesting extensive sinus mucosal involvement. Extensive sinus mucosal lesions create an anaerobic environment for the sinuses. Samples were sequenced in 2 batches. The samples labels were displayed in Fig 1.[2] We found that the samples of the 2 batches were evenly distributed and there was no obvious batch effect (the small label number is the first sample delivery).

Our study is the first to characterize the nasal microbiome of patients with eCRSwNP and lead to new ideas for therapeutic intervention. Although the concerns of Pjevac et al[1] do exist, the novelty of our findings cannot be ignored. An opportunity to validate these findings to determine a nasal microbiome diagnosis of eCRSwNP patients exists and warrants further study.

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