Spatial variability of the microbiome in chronic rhinosinusitis is associated with the patients' clinical characteristics but not with sinus ostia occlusion

Running title: Sinus microbiome variability in chronic rhinosinusitis

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Abstract

The sinus microbiome in patients with chronic rhinosinusitis (CRS) is considered homogenous across the sinonasal cavity. The middle nasal meatus is the recommended sampling site for 16S rRNA sequencing. However, individuals with unusually high variability between the middle meatus and the sinuses were identified in previous studies. Patients with CRS present various mechanisms of disease evolution. Their sinuses are frequently separated from the middle meatus by pathological changes. This study aimed to identify which factors determine increased microbial heterogeneity between sampling sites in the sinuses.

In this cross-sectional study samples for 16S rRNA sequencing were obtained from the middle meatus, the maxillary and frontal sinus in 50 patients with CRS. The microbiome diversity between sampling sites was analyzed in relation to the size of the sinus ostia and clinical metadata.

In approximately 15% of study participants, the differences between sampling sites within one patient were greater than between the patient and other individuals. Contrary to a popular hypothesis, obstruction of the sinus ostium resulted in decreased dissimilarity between the sinus and the middle meatus. The dissimilarity between the sampling sites was patient-specific: greater between-sinus differences were associated with greater meatus-sinus differences, regardless of the drainage pathway patency. Decreased spatial variability was observed in patients with nasal polyps and extensive mucosal changes in the sinuses.

Researchers and clinicians should be aware that sampling from the middle meatus is not universally representative of the sinus microbiome. The differences between sites cannot be predicted from the patency of communication pathways between them.

Importance

Chronic rhinosinusitis (CRS) affects up to 10% of the population. The microbiome's importance for its evolution is a subject of intensive research. It is controversial whether the microbiome within an individual is homogenous and whether sampling from one site is representative. Currently, the middle nasal meatus (the drainage pathway from the maxillary, frontal and anterior ethmoid sinuses) is a recommended sampling site. This study assessed the differences between microbial communities in the middle nasal meatus and the sinuses in 50 patients with CRS. In approximately 15% of patients, we noted significant differences between sampling sites that were not associated with their anatomical separation by pathological tissue. The degree of microbiome variability was associated with the patients' clinical characteristics and comorbidities. Our results show that sampling limited to one site can result in incorrect therapeutic decisions and erroneous conclusions in future studies.

Introduction

Chronic rhinosinusitis (CRS) is one of the most prevalent diseases in Western countries, affecting 3-10% of the population. Nevertheless, its pathophysiology is still poorly understood. CRS is a multifactorial inflammatory disease caused by a complicated interaction between the host and the environment (1). The treatment outcomes are also influenced by an interplay of multiple factors (2). The significance of bacteria for the development of CRS remains unclear. Bacteria are believed to cause exacerbations, modify the course of the disease and contribute to its recalcitrance (1, 3, 4). The effectiveness of antimicrobial treatment and reliability of research on the subject depends on the understanding of the microbial ecology of CRS and representative sampling of the sinonasal microbiome.

It is generally assumed that microbiome samples from the middle nasal meatus are representative of the sinuses (1, 5). The middle meatus constitutes a common pathway of physiological mucus drainage from the maxillary, frontal and anterior ethmoid sinuses.

Moreover, it is easily accessible for endoscopic examination, making it a convenient location for specimen collection. Therefore, the samples from the middle meatus are commonly used as a proxy of the whole sinonasal microbiome (1).

In CRS, however, the transport of mucus from the sinuses to the middle meatus is often ineffective due to obstruction of the sinus ostia and impairment of the mucociliary clearance. In many patients, polyps or edematous mucosa cause a complete anatomical separation of the sinuses from the middle meatus. In these cases, the microenvironment in the sinus cavity may promote the development of a distinct microbiome inside the sinus. Previously, we showed that the results of the middle meatus culture in patients with CRS were discordant with maxillary sinus culture in 20% of cases and frontal sinus culture in 34% of cases (6).

In most studies on the spatial organization of the sinonasal microbiome that were conducted using 16S rRNA sequencing, the authors observed small or insignificant differences between the sinuses and the middle meatus (7-10). Nevertheless, several researchers pointed out that larger dissimilarity between sampling sites was apparent in some patients in the study groups (7, 11).

This study aimed to identify the factors that determine the heterogeneity of the sinonasal microbiome and to estimate the prevalence of cases where the middle meatus samples were not representative of the sinus samples. To assess whether the obstruction of drainage pathways affects the sinonasal microbiome composition, samples for 16S rRNA sequencing were obtained from the middle meatus, the maxillary sinus, and the frontal sinus. To evaluate the impact of the anatomical separation of the subsites, we compared three groups of patients: (a) with narrow but patent sinus ostia that allowed for the transport of mucus from the sinus to the middle meatus, (b) with obstructed sinus ostia where the sinus cavity was completely separated from the middle meatus, (c) with wide communication between the sinus cavity and the middle meatus after previous surgery (Fig. 1). Subsequently, we evaluated the relationship between other clinical metadata and microbiome diversity.

Results

Clinical characteristics of the study group

Fifty patients with CRS were enrolled in the study. All of the patients had not improved sufficiently after medical treatment and therefore were scheduled for surgery. Samples were collected during endoscopic sinus surgery from 3 different locations: the middle meatus, maxillary sinus and frontal sinus. The study participants' demographics and clinical characteristics are shown in Table 1.

Table 1. Demographic and clinical characteristics of the 50 study participants.

Age	19-83 (mean 49)
Gender	female: 25, male: 25
Previous endoscopic sinus surgery	24
CRS with nasal polyps	21
Computed tomography – Lund-Mackay score (0-24, greater values indicate greater extension of the sinus opacification)	2-24 (mean 13)
Comorbidities	asthma: 20 aspirin-exacerbated respiratory disease: 9 allergy: 24

	gastroesophageal reflux: 15

Variability between sampling sites

The variability between samples from the middle meatus, the maxillary sinus and the frontal sinus was explored through beta diversity analyses. In every patient, the Bray-Curtis and weighted UniFrac distances between the following pairs of samples were calculated:

(a) middle meatus-maxillary sinus distance (between samples from both sides of the maxillary ostium);

(b) middle meatus-frontal sinus distance (between samples from both sides of the frontal ostium);

(c) maxillary sinus-frontal sinus distance.

The distribution of the beta-diversity indexes was not normal. Therefore, nonparametric tests were used to compare the distances within each patient and to explore the relationships between the distances and clinical metadata. The values of the weighted UniFrac and Bray-Curtis dissimilarity are shown in Table 2. Although the median values of dissimilarity measures were 0.28-0.34 for the weighted UniFrac and 0.22-0.26 for the Bray-Curtis metrics, in some patients we observed much higher values of the indexes. For example, the maximal values of the Bray-Curtis metric reached 0.75 for the middle meatus-maxillary sinus distance and 0.92 for the middle meatus-frontal sinus. The closest similarity was noted between the samples from the middle meatus and the maxillary sinus. However, the differences between meatus-sinus and sinus-sinus distances were small and not statistically significant (Wilcoxon signed-rank test).

Table 2. The values of the weighted UniFrac and Bray-Curtis dissimilarity between sampling sites.

	distance	median	min	max	interquartile range
weighted UniFrac	middle meatus-frontal sinus	0.34	0.05	1.05	0.40
	middle meatus-maxillary sinus	0.28	0.07	1.16	0.34
	frontal sinus-maxillary sinus	0.31	0.04	1.63	0.47
Bray Curtis	middle meatus-frontal sinus	0.24	0.09	0.92	0.26
	middle meatus-maxillary sinus	0.22	0.10	0.74	0.22
	frontal sinus-maxillary sinus	0.26	0.07	0.96	0.31

We observed statistically significant positive correlations between all intra-individual distances within a patient. Greater disparity between the middle meatus and the maxillary sinus correlated with greater disparity between the middle meatus and the frontal sinus and between both sinus

cavities in the same individual (Bray-Curtis: Spearman's rho 0.55-0.71, weighted UniFrac: Spearman's rho 0.58-0.66; $p < 0.05$).

The effect of the maxillary ostium size on the microbiome continuity

To assess whether the large middle meatus-maxillary sinus differences noted in some patients were caused by the anatomical separation between the middle meatus and the maxillary sinus cavity, we divided the patients into three groups according to the size of the maxillary ostium (blocked, narrow or wide). We identified 23 patients with ostium closed by pathological mucosal changes, where the drainage of the mucus from the maxillary sinus to the middle meatus was blocked, 14 patients with narrow ostium and 12 patients with very wide communication between the maxillary sinus and the middle meatus created during previous surgery.

We compared the middle meatus-maxillary sinus beta diversity values between the three groups of patients. We found that obstruction of the drainage pathway did not cause larger differences between the microbial communities on both sides of the obstructed ostium. On the contrary, we found that the middle meatus-maxillary sinus distances were smaller in patients with blocked ostia than in patients with narrow or wide ostia. The differences were not statistically significant. However, the difference between the Bray-Curtis metrics in the groups with narrow and blocked ostia was close to the significance cutoff (Mann-Whitney-Wilcoxon test; $p = 0.06$) with closer middle meatus-maxillary sinus similarity if the ostium was blocked than in case of patent narrow ostium (Fig. 2A).

A similar but stronger relationship with the maxillary ostium size was noted for the middle meatus-frontal sinus beta diversity measures (Fig. 2B). The middle meatus-frontal sinus Bray-Curtis distance in patients with narrow maxillary ostium was significantly greater than in patients with blocked maxillary ostium (Mann-Whitney-Wilcoxon test; $p = 0.03$) and insignificantly greater than in patients with wide ostium ($p = 0.08$).

Due to the fact, that only 3 patients in the study group had a patent frontal ostium, analogical computations for frontal ostium did not yield statistically significant results.

Relationships between intra-individual beta diversity and clinical metadata

To further explore the possible sources of increased spatial differences between sampling sites observed in some study participants, we investigated the relationships between the patients' clinical characteristics and intra-patient beta diversity measures. The distance between the middle meatus and the frontal sinus was significantly smaller in patients with nasal polyps than in patients without nasal polyps (Mann-Whitney-Wilcoxon test; Bray-Curtis distance: $p = 0.016$, weighted UniFrac distance: $p = 0.025$). The middle meatus-frontal sinus distance was also negatively correlated with the Lund-Mackay radiologic score: the dissimilarity between the middle meatus and the frontal sinus was less pronounced in patients with more extensive pathological changes in the sinuses (Spearman's rho -0.35; $p < 0.05$). Analogical correlations were not noted for the middle meatus-maxillary sinus distance. Other clinical metadata did not correlate significantly with the beta diversity measures between sampling sites.

Intra-individual versus inter-individual variability

Despite the high values of beta diversity noted in some patients, PERMANOVA tests (adonis) indicated that in the whole study group the variation between the community structure in different sampling sites was not statistically significant (Bray-Curtis: $p = 0.596$, weighted UniFrac: $p = 0.281$). On the contrary, variation caused by differences between patients was significant in both metrics (Bray-Curtis: $p = 0.041$, weighted UniFrac: $p = 0.03$). This result indicates that overall the highly pronounced variability between subjects outweighs intra-individual variation of the sinonasal microbiota.

Figure 3 shows that the middle meatus-maxillary sinus and middle meatus-frontal sinus distances within each patient were significantly smaller than distances between the same sites among the study participants. However, the difference between intra-individual maxillary sinus-frontal sinus distances and inter-individual distances was insignificant.

In several study participants, between-patient Bray-Curtis distances were greater than within-patient distances (Fig. 4). The middle meatus-maxillary sinus differences within a patient were greater than distances between the patient's middle meatus and the maxillary sinuses of other participants in 7 individuals. The same relationship was observed in 8 patients for middle meatus-frontal sinus distances. A trend towards increased intra-individual variability was observed in patients who also presented higher beta diversity distances from other study participants.

Inter-individual functional variability

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was used to predict the functional potential of the bacterial communities in the samples (12). PERMANOVA analysis of the results revealed statistically significant differences between sampling sites (Bray Curtis dissimilarity based on KO predictions $F=2.86$, $p=0.01$; similar values were observed for EC and MetaCyc predictions). The impact of the sinus ostium size on the functional predictions was not significant.

Alpha diversity in the sinonasal samples

Faith's PD was used to measure the alpha diversity in the samples. We found no significant differences in alpha diversity between samples from the middle meatus, the maxillary sinus and the frontal sinus (data not shown). Moreover, there were no differences between alpha diversity in the samples from the maxillary sinus between patients with wide, narrow and blocked maxillary ostium. The taxonomic composition of the samples is presented in Fig. 5 and Fig. 6.

The relationships between alpha diversity and clinical metadata were not statistically significant. Still, we observed decreased alpha diversity in patients with nasal polyps, history of recurrent exacerbations or comorbidities such as aspirin-exacerbated respiratory disease, gastroesophageal reflux or asthma. Recent steroid use was almost significantly associated with lower alpha diversity (t-Student test, $p = 0.052$).

Discussion

In this study, we explored the topographical differences between the microbial communities in several locations of the sinonasal cavity. The study's primary goal was to improve the understanding of the microbial ecology of the paranasal sinuses. We tested whether the sinonasal cavity could be considered a homogenous habitat or a system of communicating but distinct microenvironments.

Most studies on the subject suggested that the samples from the sinus cavities and the middle nasal meatus were generally similar (7, 8). This observation leads to the conclusion that samples from the middle nasal meatus are representative of the sinuses as a whole. The assumption agreed with current recommendations for researchers and clinicians to obtain samples from the middle nasal meatus where the mucus is transported from the largest sinuses in physiological conditions (1).

In CRS, the drainage into the middle meatus is frequently impaired. Therefore, we decided to question this paradigm. It has been postulated that the hypoxic microenvironment inside an occluded sinus may promote the development of a distinct microbiome while the wide opening of the ostium may decrease the concentration of nitric oxide that controls bacterial growth (7, 13-15).

Moreover, our previous study that utilized microbial cultures showed that the middle meatus swabs missed pathogens found in the sinuses in 24% of patients (6). Interestingly, the disparities were not limited to patients with blocked sinus ostia. We also found it worth attention that in many studies of the sinonasal microbiome some patients presented significant differences between sampling sites (7, 11).

These controversies motivated us to explore whether individuals with marked heterogeneity within the sinonasal microbiome are frequently encountered in the population of patients with CRS. Our goal was to find out whether the topographical differences were simply induced by obstruction of the sinus ostia or associated with other factors. For this purpose, we conducted a detailed analysis of the sequencing data results in the context of the individual patterns of drainage pathway disruption and clinical metadata of the patients.

The microbiome is not universally homogenous across the sinonasal cavity

Differences between samples are quantified by beta diversity measures. However, there is no consensus on what values of these metrics indicate truly meaningful differences. Joss *et al.* assumed that if the weighted UniFrac distance between samples obtained from two sampling sites in the sinuses did not exceed 0.2 then sampling from one site could be considered representative of the other. According to this interpretation, such samples shared more than 80% of the community structure (11). In our study, the median beta diversity values between sampling sites exceeded 0.2. Values of weighted UniFrac above 0.2 were noted in 66% of patients for the middle meatus-maxillary sinus distances and 73% of patients for the middle meatus-frontal sinus distances. Therefore, according to the definition suggested above, the middle meatus samples were not representative of the sinus samples in the majority of patients. In comparison, Joss *et al.* noted between-site weighted UniFrac distances that exceeded 0.2 in 38% of patients and concluded that collecting specimens from a single sinus may not provide representative data. De Boeck *et al.* studied the differences between sampling sites in 190 patients with CRS and reported the median Bray-Curtis distance of 0.27 between the maxillary sinus and the ethmoid sinus that is anatomically close to the middle meatus (10). This result is comparable to our observations. In our study, the median Bray-Curtis distance between the maxillary sinus and the middle meatus equalled 0.22. Of note, the maximal Bray-Curtis value noted in our study reached 0.75 for the middle meatus-maxillary sinus distance and 0.92 for the middle meatus-frontal sinus distance.

Even though beta diversity metrics are stable within a single experiment and saturate quickly with sequencing depth, they still retain variance dependent upon experiment design (i.e. primer selection) (16), sequencing parameters (i.e. sequencing depth) (17) and data preprocessing pipeline (i.e. denoising method, trimming and rarefaction parameters) (18). The additional layer of complexity is added by a nonlinear nature of common beta diversity measures. All these factors restrict the ability to interpret raw numbers. To alleviate this problem, in our analysis we compare beta diversity between different sampling sites within one patient and beta diversity of the same sampling sites across all patients, utilizing the latter as a point of reference.

In several studies, the sinuses were more similar to one another than the middle meatus was to the sinuses (7, 9). In contrast, in our study, the maxillary sinus samples were closest to the middle meatus samples. This result seems plausible because the maxillary sinus is anatomically closer to the middle meatus than the frontal sinus. However, the differences between the calculated distances were not statistically significant.

In a minority of patients, the intrapersonal differences outweigh the interpersonal variation

Although the values of beta-diversity measures suggested that the middle meatus was not a representative sampling site of the sinuses, PERMANOVA analysis did not demonstrate statistically significant differences between sampling sites. The adonis test indicated that interpersonal differences were a significant source of variation, but not the sampling site. There were also no significant differences in alpha diversity between the three locations. These findings remain in agreement with the observations of other authors. The results of most studies show that the interpersonal variation significantly outweighs the intrapersonal variation (7, 9-11, 19). As a result, it has been assumed that every individual harbors a characteristic sinonasal microbial community that may vary from one anatomical subsite to another, but the distance between subsites is usually much lower than the distance to other individuals. Therefore, for certain types of studies requiring only general characterization of the participants' microbiome, sampling from the middle meatus is considered sufficient to represent the composition of the whole sinonasal microbial community (7). De Boeck, who studied a larger group of 190 patients with CRS, observed that the sampling site explained a small (2.2%) but statistically significant proportion of microbial variation (10). However, differences were observed between the sinuses and the nasopharynx or the anterior nares. The maxillary sinus and the ethmoid sinus, which is close to the middle meatus, were not significantly different.

Nevertheless, in our study, we showed that in approximately 15% of patients the intrapatient middle meatus-sinus distances were greater than the differences between the patient's middle meatus and the sinuses of other study participants. (Fig. 3) These observations prove that the apparent intrapersonal variation in some individuals is not captured by statistical analyses that comprise the whole group of CRS patients.

Despite the lack of statistically significant taxonomic differences between sampling sites in the whole study group, PERMANOVA analysis of PICRUSt2 results proved that the functional potential of the bacterial communities differed significantly between various locations within one patient. This observation sheds new light on the sinonasal microbial ecology. It indicates that the microenvironments of the sinuses are heterogeneous and the metabolic activity of

bacteria in various niches is distinct. These differences could be explored in detail by deep shotgun sequencing.

Differences between sampling sites are not related to their anatomical separation

The observation that patients with CRS differ in the degree of microbiome heterogeneity motivated us to investigate whether the disparities were caused by the anatomical separation of the sampling sites. It is important to note that CRS patients present various patterns of drainage pathway obstruction. In the most severe cases, all sinuses are blocked. However, in the majority of patients, some sinuses are occluded while other sinuses maintain effective drainage to the middle meatus. We analyzed the individual configuration of ostia occlusion in each patient and compared the differences between samples from various sites (Fig. 2). We expected that blockage of the drainage pathway between the sinus and the middle meatus would result in increased differences between the two sites. We also hypothesized that wide communication between a sinus and the middle meatus created during surgery should reduce these differences. Our observations did not support these predictions.

The largest beta diversity distances between the maxillary sinus and the middle meatus were noted in patients with a narrow maxillary ostium which is similar to its configuration in healthy individuals. Blocked maxillary ostia were associated with smaller beta diversity between the maxillary sinus and the middle meatus (Fig. 2). This observation seems counterintuitive because a blocked maxillary ostium means anatomical separation of the maxillary sinus cavity from the middle meatus. Narrow maxillary ostia were also associated with greater distances between the frontal sinus and the middle meatus than blocked or wide maxillary ostia. It suggests that a more divergent sinonasal microbiome was observed in patients whose sinus anatomy was not significantly altered by mucosal changes and polyps (blocked ostia) or by previous surgery (wide ostia). This result was unexpected because other authors reported greater meatus-sinus differences in CRS patients than in healthy individuals and concluded that microbial communities in the sinuses diverge during CRS (9, 19). However, the differences noted in these studies were not statistically significant and require verification in larger study groups.

The results of our study did not support the hypothesis that the microenvironment in an occluded sinus that is depleted in oxygen stimulates the development of a distinct microbial community. The analysis of alpha-diversity and PICRUSt2 predictions of the metabolic

potential of the microbiome did not reveal any significant microbiome differences between blocked and well-ventilated sinuses. However, the predictions provided by PICRUSt2 are only an approximation of metagenomic functional potential and this phenomenon requires further investigation with the use of deep shotgun sequencing and untargeted metabolomics.

The degree of sinonasal microbiome variability is characteristic of an individual and not of the topography of the sinuses

Our study showed that between-site beta diversity distances within a patient were positively correlated. Patients with greater meatus-maxillary distances also tended to have greater meatus-frontal distances regardless of the size of the maxillary and frontal ostia. This observation indicates that a tendency for an increased or decreased between-site variability is a feature that characterizes an individual's sinonasal microbiome rather than local drainage pathway obstruction between certain sites. Some patients tend to host microbial communities that are more disparate across the sinonasal area even if the mucus transport pathways remain patent.

Patients with nasal polyps tended to have significantly less dissimilar microbial communities in the middle meatus and the frontal sinus than patients without nasal polyps. Similarly, more extensive mucosal changes in the sinuses correlated with smaller beta diversity distances between the middle meatus and the frontal sinus. Massive sinus opacification and nasal polyps are frequently encountered with type 2 inflammation (1). The alpha-diversity was also not significantly associated with the sampling site or the patency of the sinus ostium. Still, it tended to be lower in patients who presented certain clinical features (recent steroid use, nasal polyps, comorbidities such as asthma, aspirin-exacerbated respiratory disease, gastroesophageal reflux). Therefore, it is probable that the spatial heterogeneity of the microbiome depends on individual characteristics of the patient's mucosa and the type of its inflammatory reaction rather than the pattern of ostia occlusion.

Conclusions

In most patients with CRS, the differences between sampling sites in the sinuses are insignificant. However, in approximately 15% of patients, the sinus microbiome is not homogenous and the intra-individual differences outweigh inter-individual differences. The microbiome variability in the sinonasal complex is not associated with the anatomical separation of the cavities. The degree of between-site variation is patient-specific. Some individuals present greater heterogeneity between all sampling sites even if the communication between the sites is not anatomically impaired. Blockage of the sinus ostia, presence of nasal polyps and extensive mucosal changes in the sinuses are associated with decreased microbiome variability. Researchers and clinicians should be aware that in a minority of patients sampling from the middle meatus may not correctly reflect the sinus microbiome.

Further investigations should focus more on the use of deep shotgun metagenomic sequencing and untargeted metabolomics to be able to more exhaustively address the hypothesis that the microenvironment in an occluded sinus stimulates the development of a distinct microbial community adapted to an anaerobic environment.

Methods

Sample collection

In this cross-sectional observational study samples were collected from patients with CRS during endoscopic sinus surgery between October 2018 and June 2019 in the Otolaryngology Clinical Department of the University Hospital in Krakow. CRS was defined according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 (20). All of the participants had failed to respond to medical treatment and were scheduled for surgery. Patients who received antibiotics one month before the surgery were excluded. Clinical data collected for the patients included the patient's age, time since CRS onset, nasal polyps, disease duration, comorbidities (asthma, aspirin-exacerbated respiratory disease, gastroesophageal reflux, allergy), recent steroid use, history of recurrent exacerbations, number of previous polypectomies and endoscopic sinus surgeries, radiological staging (Lund-Mackay score (21)) and self-evaluation of the severity of symptoms on the visual analogue scale (0-10).

The samples were collected under endoscopic guidance from 3 sites in each patient which provided a total of 150 samples. The first swab was taken from the middle nasal meatus at the beginning of the procedure. The second and third swabs were taken from the maxillary and frontal sinus on the same side. If the sinus was blocked, the swab was collected immediately after the surgical opening of its ostium. Contact with the nasal vestibule or other sites in the nasal cavity was carefully avoided and in case of suspected contamination, the samples were discarded. The samples were transported on ice to the laboratory where they were stored at −80◦C. The study was approved by the Jagiellonian University Bioethics Committee (1072.6120.78.2018, 20.04.2018).

DNA isolation, library preparation and sequencing

The swabs were thawed and vigorously shaken in 1 mL of saline. Afterwards, the samples were treated with lysozyme (1 mg/mL) and lysostaphin (0.1 mg/mL) enzymes (Sigma-Aldrich, Poznań, Poland) at 37 °C for 20 min to digest the bacterial cell walls. Further, the samples were subjected to DNA extraction using a Mini Genomic DNA isolation kit (A&A Biotechnology, Gdynia, Poland). The concentration and purity of DNA isolates were determined spectrophotometrically for A260 nm and A260nm / 280nm ratio using NanoDrop (Thermofisher, Waltham, MA USA).

Libraries were prepared strictly according to Illumina's protocol (San Diego, CA, USA) and Kowalska-Duplaga *et al.* (22, 23).

Primers (Genomed, Warsaw, Poland) specific to the V3 and V4 16S rRNA sequences of bacteria were used: (F) 5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG3′ (R)5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC 3′

After purification and concentration measurement, libraries were pooled and sequenced using the MiSeq sequencer (Illumina, San Diego, CA, USA).

In one sample from the middle meatus, amplifying the library was impossible due to too low concentration of DNA - it was excluded from further analysis.

Data and statistical analysis

The sequencing results were processed using a pipeline available within QIIME 2 (24). Truncation was performed at 290 bp length for forward reads and at 250 bp length for reverse reads to avoid the technical quality drop with the increased base position. Paired-end sequences were denoised using DADA2 via q2-dada2 and merged with minimal overlap of 12 base pairs. The phylogenetic tree was constructed using q2-fragment-insertion with the SEPP reference database based on SILVA 128. After analysis of rarefaction curves, the sampling depth of 20,504 reads per sample was chosen. The rarefaction procedure resulted in the exclusion of additional 14 samples from analysis (7 from middle meatus, 5 from maxillary sinus, and 2 from frontal sinus).

The measure of microbiome diversity within each sample used in this study was Faith's phylogenetic diversity (Faith’s PD) which incorporates phylogenetic differences between the taxa identified in the sample.

The disparities in microbiome composition between pairs of samples were measured using two dissimilarity metrics: Bray-Curtis and weighted UniFrac (wUniFrac). Bray-Curtis dissimilarity quantifies compositional differences between biological communities based on counts at each site. The values of the metrics range from 0 to 1, where 0 indicates that the samples are identical and 1 means that the two samples do not share any species. The wUniFrac distance additionally incorporates information on the phylogenetic distances between observed organisms. The lowest wUniFrac value is 0, indicating that the communities do not differ. Larger values indicate greater differences and the maximal value may exceed 1 (25).

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was used for the prediction of the functional potential of the microbial communities. This computational tool allows for the prediction of metagenomic functional profiles from 16 rRNA sequencing data via hidden state prediction on a constructed phylogenetic tree. Although prediction accuracy is varied depending on the microbial environment, PICRUSt2 was shown to be highly efficient within the human organism (12).

The normality of distribution was assessed using the Shapiro-Wilk normality test. For biodiversity measures that had normal distribution, a t-Student test was used to compare subgroups of patients and Pearson correlation was used to explore their relationships with quantitative clinical metadata. For biodiversity measures that did not have normal distribution Wilcoxon signed-rank test was used to compare sampling sites within one patient, Mann-Whitney-Wilcoxon and Kruskal-Wallis tests were used for comparisons between two or more subgroups of patients, respectively, and Spearman's rank correlation was used to measure the associations between the biodiversity measures and quantitative clinical variables.

The differences in microbiome composition between subgroups of samples were further explored using permutational multivariate analysis of variance (PERMANOVA) using the adonis function.

Data is available at the European Nucleotide Archive (ENA): https://www.ebi.ac.uk/ena/browser/view/PRJEB55924

The Strengthening The Organization and Reporting of Microbiome Studies (STORMS) Checklist can be found at https://zenodo.org/record/7092029#.YzF6CuxBx6o

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Figures

Fig. 1. Schematic pictures and computed tomography scans showing the anatomical relationships between the sampling sites in the 3 groups of patients: (a) narrow but patent sinus ostia – physiological communication between the sinuses and the middle nasal meatus, (b) sinus ostia obstructed by pathological tissue - sinus cavities anatomically separated from the middle meatus, (c) wide communication between the sinuses and the middle meatus created during previous surgery. The arrows show the directions of mucus transport.

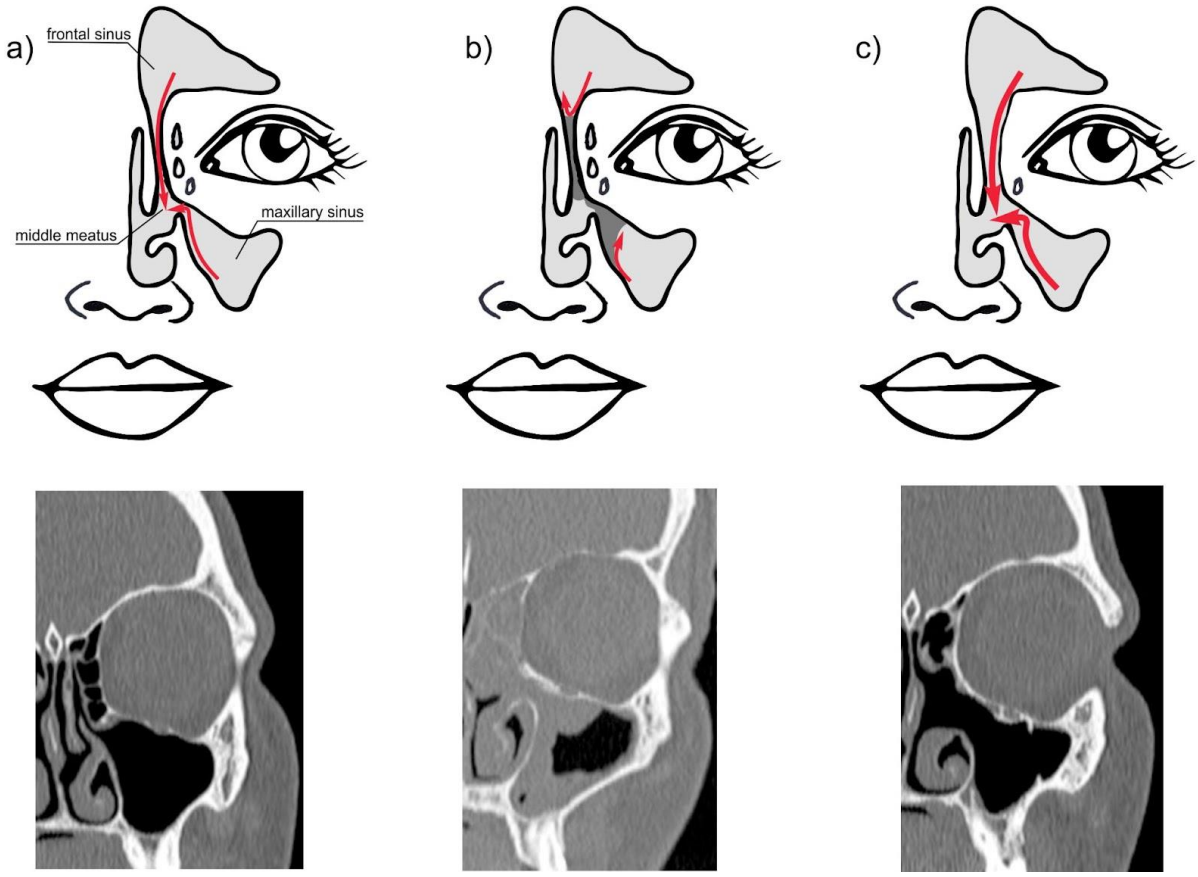

Fig. 2. The Bray-Curtis distances between the middle meatus and (A) the maxillary sinus, (B) the frontal sinus in the three subgroups of patients (with wide, narrow and blocked maxillary ostium). Larger metrics values mean greater differences between the sampling sites. M.W.W. - Mann-Whitney-Wilcoxon test.

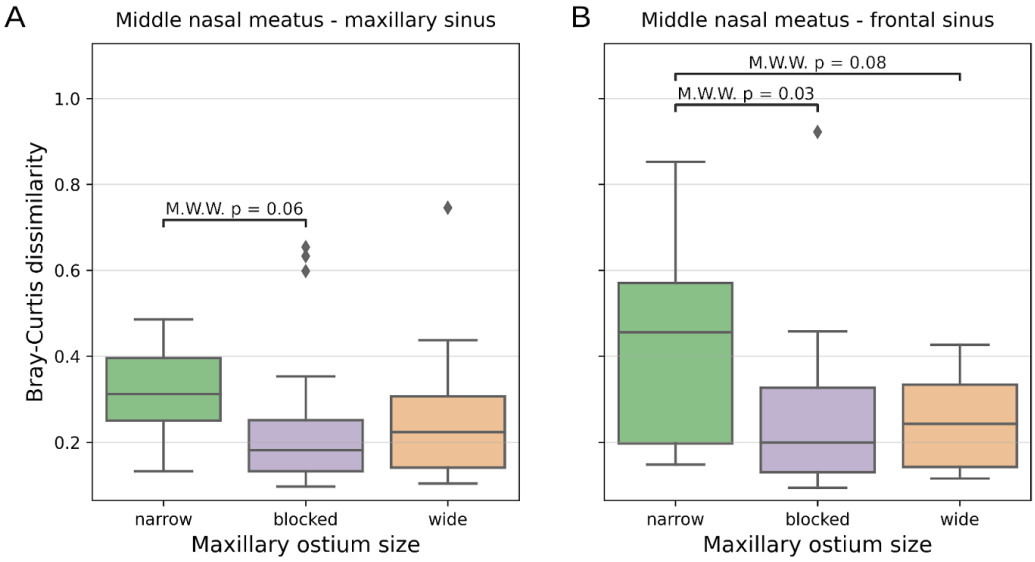

Fig. 3. Comparisons of beta-diversity distances within and between patients. The "within patient" distances were calculated between two sites for each study participant (50 distances). The "between patient" distances were calculated between one site in each patient and the second site in all other patients (50*49=2450 distances). A. Bray-Curtis distances. B. weighted UniFrac distances. M.W.W. - Mann-Whitney-Wilcoxon test.

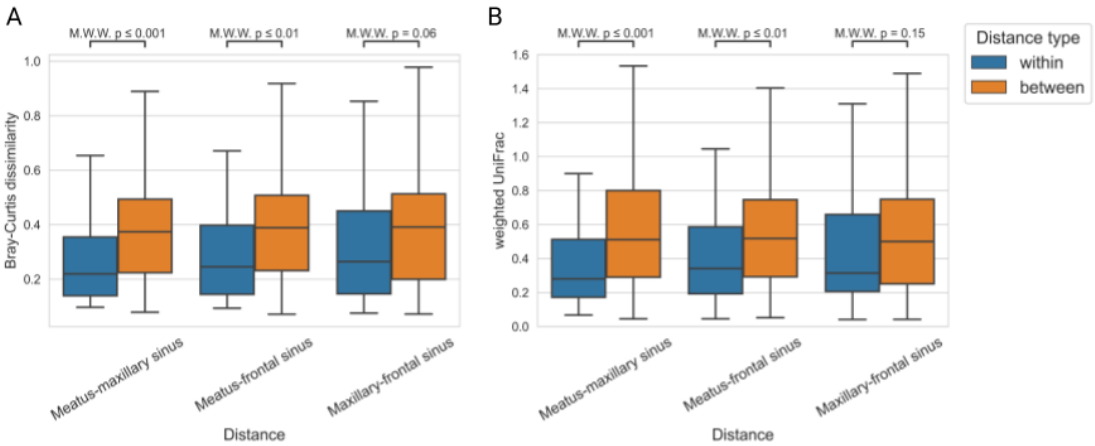

Fig. 4. Bray-Curtis beta-diversity distances within and between patients presented for each patient separately. The "within patient" distances were calculated between two sites for each study participant. The "between patient" distances were calculated between one site in each patient and the second site in all other patients. The points below the dotted line indicate patients who presented higher variability between sites than between the patient and other study participants.

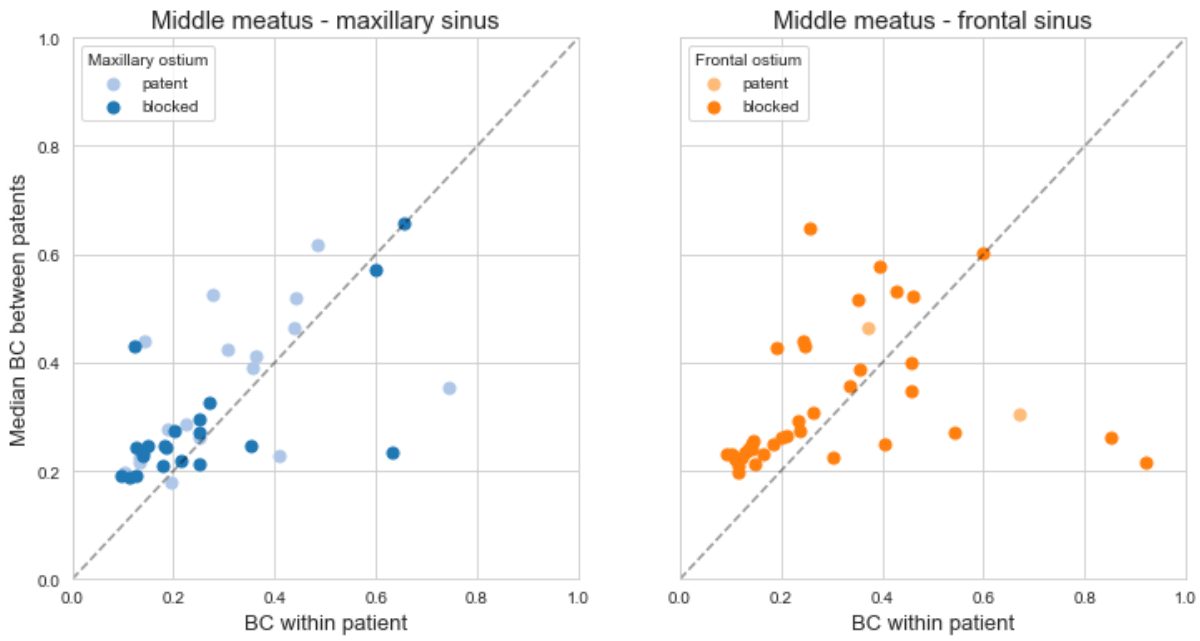

Fig. 5. The taxonomic composition of the samples from the frontal sinus, middle nasal meatus and maxillary sinus.

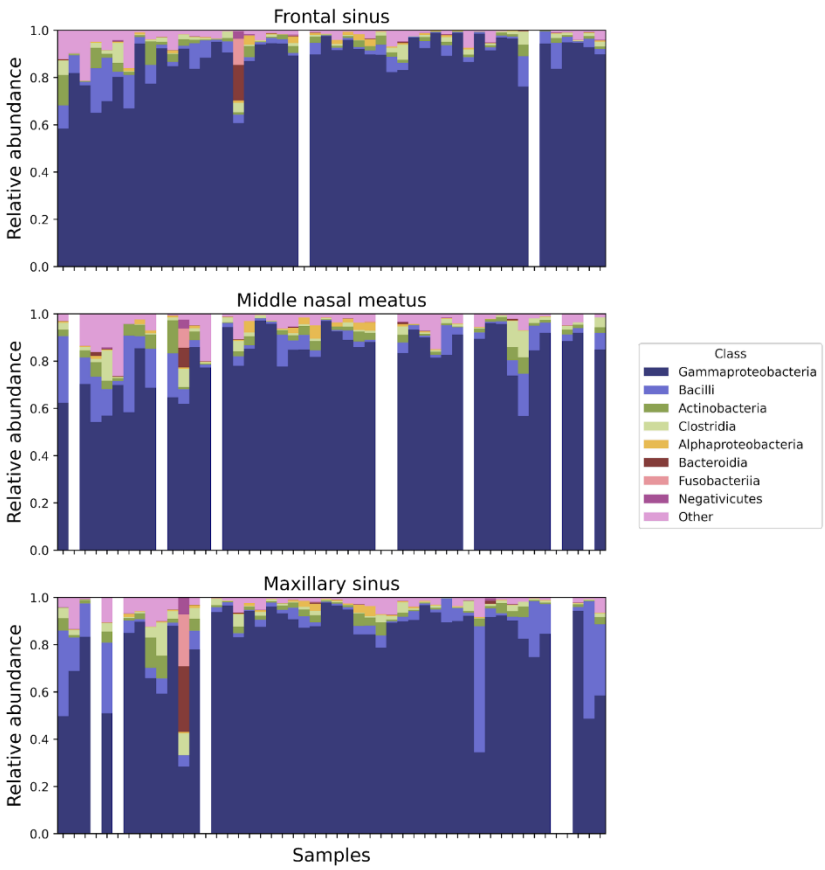
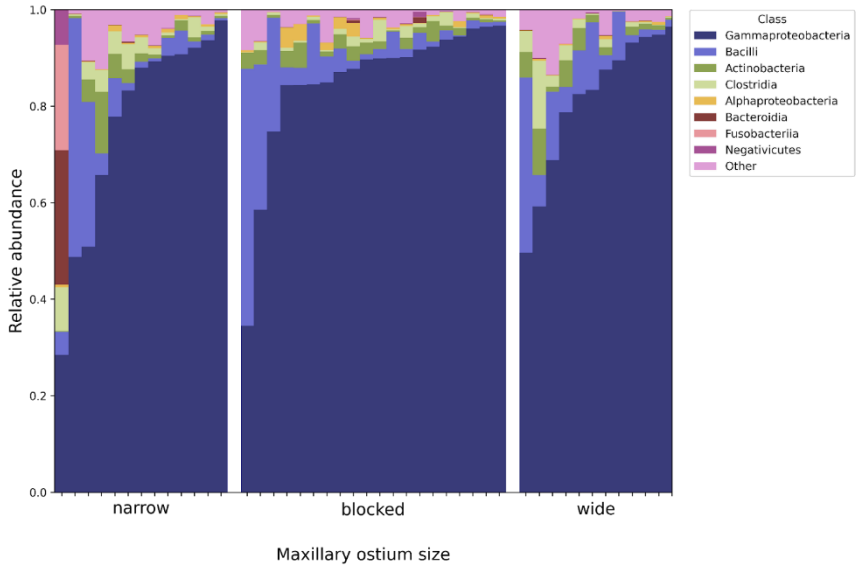
Fig. 6. The taxonomic composition of the samples from the maxillary sinus in the groups with wide, narrow and blocked maxillary ostium.