Characterization and Analysis of The Nasal Microbiota and Plasmatic Extracellular Vesicles in Allergic Rhinitis: A Case-Control Study

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Abstract

Background: Increasing evidence suggests a possible link between the bacterial nasal microbiota (bNM) to allergic diseases such as allergic Rhinitis (AR), as it might modulate the allergic response by acting on the onset and progression of allergic inflammation. In this case-control study, we aimed to compare the bNM between 25 AR patients and 25 matched healthy subjects (HS) as well as to evaluate possible modifications within the host-microbiota cross-talk, investigating the release pattern of both bacterial- and host-Extracellular Vesicles (EVs).

Results: bNM analysis showed that Actinobacteria (AR: 2.5%-83.5%; HS: 18.6%-92.7%), Firmicutes (AR: 6.1%-63.5%; HS: 6.4%-72.2%) and Proteobacteria (AR: 0.6%-89.6%; HS 0.7%-38%) were the most abundant phyla in the study population. Diversity reduction in AR patient bNM was pointed out compared to the HS group bNM (Observed OTUs: $p = 0.018$; PD whole tree: $p = 0.014$). Moreover, the distribution of the weighted normalized intra-group UniFrac distances analysis showed a less uniform bacterial community within AR patients rather than HS group (nonparametric test $p = 0.01$). Since EVs may have a central role in the cross-talk between bNM and the host, we evaluated their size and concentration in the plasma of the study subjects. AR patients were characterized by a higher concentration of EVs ranging between 130-231 nm, 257-287 nm and 323-348 nm ($p < 0.05$). We further characterized plasmatic EVs investigating both bacterial-EVs and host-EVs subpopulations and observed that all EV subtypes mean concentrations were higher in AR patients compared to those of HS group, except for EpCAM+ EVs.

Conclusion: Our results suggest that AR patients are characterized by a less diverse and wide intra-group variable bNM compared to that of HS, as well as an altered host-microbiota EV communication network. Further studies are needed to disentangle the relationship between the host and the nasal bacterial community during pathological conditions and health.

Introduction

The occurrence of respiratory diseases has risen dramatically in recent decades turning respiratory conditions, including Allergic Rhinitis (AR), into an “epidemic” [1, 2]. AR is a systemic airway inflammatory condition induced by inhaled allergens in sensitized subjects. It is characterized by immunoglobulin E (IgE) production and a modified mucosal immune homeostasis [3], which induces an accumulation of eosinophils and mast cells as well as alterations of the nasal epithelium [4]. Symptoms can range from mild to moderate-severe and AR can result in a significant worsening of quality of life [5, 6]. Depending on geographic location, the incidence of AR has been estimated to affect from 10–20% of worldwide population [1, 2].

To explain the high prevalence of AR, one possible explanation is linked to the “hygiene hypothesis”, correlating the increasing incidence of allergic diseases to urbanization, westernized diet and antibiotic overuse [7]. According to this hypothesis, reducing the exposure to microbes and environmental antigens might cause an improper development of the host immune system, finally leading to an imbalanced

antigen response [8]. Several actors could have a role in this altered immune response to allergens and environmental agents, including the innate and the adaptive immune system, the nasal nervous system and the nasal epithelium [1, 3].

A wide range of evidence has indicated the relevance of the lower respiratory tract microbiota to lung homeostasis and disease [9–11]. Nonetheless, the critical role of the bacterial nasal microbiota (bNM) in modulating the allergic response has been recently postulated, as a significant association between microbial composition and the onset and progression of allergic inflammation was consistently reported [7, 12–14]. The nasal epithelial surface is usually colonized by niche-specific bacteria, which includes a variety of commensal and pathobiont bacteria, belonging to the Actinobacteria, Firmicutes and Proteobacteria phyla, dominated by *Corynebacterium spp., Propionibacterium spp.*, *Staphylococcus spp.*, *Streptococcus spp., Finegoldia sp., Peptoniphilus sp.*, and *Moraxella spp.* genera respectively [14, 15]. Forming an interface between the external environment and the nasal epithelium, the bNM can be considered as a gatekeeper of respiratory health, regulating the host immunity and reducing the likelihood of pathobionts from establishing an infection [16, 17].

When functional or compositional modifications (i.e. dysbiosis) occur, the protecting role of the microbiota in maintaining a stable and suitable environment may be altered, influencing the immune system activity, thus favoring the expansion of pathobionts [18–20]. Dysbiosis has been detected in the inferior turbinate mucosa of AR patients, showing a decreased microbiome diversity and an anaerobic taxa enrichment [21]. In addition, modifications in the bNM were also identified in respiratory conditions such as chronic rhinosinusitis, cystic fibrosis and asthma [14, 22–24].

The host and bacterial community need to maintain an equilibrium. Among the possible factors that allow the communication of these two compartments, plasmatic extracellular vesicles (EV) may have a central role as they can mediate the conflict that can occur between host and microbiota [25–28]. Indeed, EV release, represent a universally conserved and shared mechanism between domains of life [29].

EVs are spherical structures, delimitated by a lipid bilayer, secreted by cells, and travelling in biological fluids [30]. They can transfer their cargo of bioactive molecules to specific recipient cells.

In addition to a broad range of studies highlighting the role of human-EVs (hEVs) in human health and disease processes [31, 32], it has also been demostrated that bacterial-EVs (bEV) might influence the host cells [33]; in particular, they could modulate cellular mechanisms that control proliferation [34], apoptosis [35] and, above all, the immune response [36].

Moreover, to additionally support the choice of EVs as markers of communication between the host and the bacterial community when the bNM is considered, it was recently reported that an unbalanced nasal bacterial membership, characterized in that case by an over-abundance of *Moraxella spp.* can be considered as an effective modifier of the association between particulate matter exposures, a well-known source of airways and systemic inflammation, and EV release modifications [37]

Due to increasing evidence, linking the respiratory microbiota to allergic disease the present study sought to characterize the bacterial nasal community of allergic rhinitis (AR) and healthy (HS) subjects and consequently evaluate possible modifications within the host-microbiota cross-talk, investigating possible fluctuations in both hEVs and bEVs release pattern.

Material And Methods

Subject recruitment and characteristics

This study included 25 patients with AR and 25 healthy subjects matched for age, gender and BMI, recruited between February and June 2019 at the Allergology Unit, Fondazione IRCCS Ca' Granda - Ospedale Maggiore Policlinico, Milan, Italy. These subjects underwent a standard battery of skin prick test and/or eosinophil counts, in order to confirm or rebut AR diagnosis. Exclusion criteria included diabetes, hypertension, autoimmune diseases, cancer or other major chronic health condition, pregnancy, history of illicit drug use. After signing a detailed informed consent form, all participants were asked to donate blood and nasal swab samples. In addition, each participant gave his/her written informed consent to participate to the study and filled in a standardized questionnaire about demographics and lifestyle information (e.g. smoking habits, alcohol consumption, and diet). Clinical history was also collected for each of the AR patient.

Sample collection and processing

Nasal swabs were collected from each of the participant through nasal swab following WHO guidelines (https://goo.gl/pMzSrT) and stored at -80°. DNA was extracted using QIAamp® UCP Pathogen Mini (QIAGen, Hilden, Germany) following the manufacturers guidelines. The extracted DNA was stored at -20°C and later shipped to the sequencing service facility Personal Genomics Srl (Verona, Italy) to perform qualitative and quantitative checks, PCR amplification and second-generation sequencing analysis. Bacterial communities were investigated through amplicon sequencing analysis of the 16S rRNA gene hypervariable regions V3-V4, amplified with the primer pair Pro341F (5'-CCTACGGGNBGCASCAG-3') and Pro805R (5'-GACTACNVGGGTATCTAATCC-3'), and then sequenced through the Illumina MiSeq platform using a paired-end library of 300bp insert size. Blood samples were collected in ethylenediamine tetra-acetic acid (EDTA) tubes and processed within 2-hours from the phlebotomy. The Minimal information for studies of extracellular vesicles (MISEV) 2018 guidelines were followed performing EV purification, isolation and characterization [38] and summarized in supplemental Table S1. Briefly, EDTA-treated blood was centrifuged at 1200 × g for 15 min at room temperature to obtain platelet-free blood plasma and then further centrifuged following a three-step centrifugation protocol (1000, 2000, and 3000 × g for 15 min at 4 °C), and finally ultracentrifuged to obtain an EV-rich pellet (110,000 × g for 75 min at 4 °C) as previously described [39].

Upstream analyses and operational taxonomic units (OTUs) clustering

Raw reads quality and statistics were checked using FastQC v0.11.2 and then trimmed at the 3' end using Trimmomatic v0.32 to improve the following read joining. Fastq-join.py tool were applied to join the raw reads and quality filtered using a minimum base quality 20 (Phred-scale) over 5 bases sliding window and then analysed using the default settings for QIIME 1.9.1[40]. After chimeric reads removal, the resulting reads were clustered using 97% of similarity applying the open-reference OTUs pipeline using USEARCH61[41]. Taxonomic assignment was carried out with the RDP classifier [42] through the comparison of representative reads against the Greengenes v13.8 database using standard options. PyNast method and default settings suggested in QIIME pipeline were applied to align sequences [43, 44]. The resulting OTU table was successively filtered, removing singleton and low abundance OTUs to performer downstream and statistical analysis.

Downstream analysis

Downstream analyses were carried out using QIIME v1.9.1 parsing the above-described OTUs table. Taxonomic values within each sample and group was assigned to each OTU from the phylum to the genus level. OTUs, which fails genus attribution, were tagged as "Unassigned" followed the specific family label. To assess significant differences between the OTU abundance in the AR and HS groups a nonparametric t-test (999 Monte Carlo permutations), was applied. Before diversity analysis, all samples were rarefied to 10,000 sequences with a seed of 10, in order to avoid the influence of different sequencing depths. Alpha-diversity richness, evenness and genetic distance were calculated using observed OTUs, Shannon and PD_whole_tree indices. In addition, a nonparametric two sample t-test was applied to assess differences between groups of samples, with Monte Carlo permutations (999). Beta-diversity was examined applying the Weighted_Normalized UniFrac distance measure. To compare the tightness of clustering distance within all samples in a group comparing the state of disease, a two-sample t-test was performed (999 MonteCarlo permutations). Furthermore, to visualize and interpret the result of the applied distance measure, Principal Coordinate Analysis (PCoA) were performed on the produced distance matrix and plotted using Emperor and the adonis function in the R Vegan package was used to test significance in dissimilarity matrices between AR and HS groups.

Extracellular vesicles analysis: distribution and immunophenotyping

The EV enriched pellet obtained after the ultracentrifugation step was resuspended in 500-μL triple-membrane filtered phosphate-buffered saline PBS to perform flow-cytometry and nanoparticle tracking analyses (NTA).

NTA analysis was carried out with the Malvern NanoSight NS300 system (Malvern Panalytical Ltd., Malvern, UK), used to visualize the EVs by laser light scattering. For each sample five 30-sec records were registered. NTA output was then analysed with integrated NTA software (Malvern Panalytical Ltd.), providing high-resolution particle size distribution profiles and EV concentration measurements. NTA EV data were expressed as 10^6 for 1 ml of plasma.

To determine EV cellular origins, immunophenotyping was achieved with the MACSQuant Analyser flow cytometer (Miltenyi Biotec, Bergish Gladbach, Germany) following the customer-provided protocol (https://bit.ly/2z2s69i). The Fluoresbrite Carboxylate Size Range Kit I (0.2, 0.5, 0.75, and 1 μm) was used to set the calibration gate on the MACSQuant Analyser system. To evaluate integrity and to highlight the hEV subset, 60-μL sample aliquots were stained with 0.02 μM 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) at 37 °C for 20 min in the dark. CFSE is a vital dye non-fluorescent molecule, able to enter into MV, where intracellular esterase enzymes remove the acetate group and convert the molecule into the fluorescent ester form. To characterize and count hEVs, the following panel of antibodies was used: AbCD177 (neutrophils), AbCD14 (monocytes), AbCD61 (platelets), EpCAM (epithelium), AbCD62E (activated epithelium), AbCD203C (mast cells), AbCD294 (eosinophils). In addition, two different 60-μL sample aliquots for each sample were also incubated with specific antibodies to recognize the bEVs: LPS and a primary unconjugated Lipoteichoic acid combined with a secondary IgG-antimouse to distinguish EVs derived from gram-negative or gram-positive bacteria from the whole subset. Before use, each antibody was centrifuged at 17,000×*g* for 30 min at 4°C to eliminate aggregates. A stained PBS blank sample was used to detect auto-fluorescence of the antibody. Quantitative multiparameter analysis of flow cytometry data (expressed as 10^3 for 1ml of plasma) was run using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Statistical Analysis

Descriptive statistics of the study participant characteristics were performed on all variables. Categorical data are presented as frequencies and percentages. Continuous variables are expressed as the mean ± standard deviation (SD). Multivariable negative binomial regression models were applied to evaluate marginal means of EVs and cell origins in AR and HS participants. Each model was adjusted for age, gender and smoking habits. Statistical analyses were carried out with SAS software (version 9.4; SAS Institute Inc., Cary, NC, USA).

Results

Characteristics of the enrolled subjects

The main characteristics of the study population are listed in Table 1. The mean age of AR patients was 39.3 years (± 13.1 years), and the mean age of HS was 42.7 (± 16.1 years). The mean BMI was equal in the two groups and gender was equally distributed. Approximately 60% of the study participants were non-smokers. The percentage of current smokers was higher in healthy subjects (24%) than in AR patients (4%).

Table 1
Characteristics of the study participants (N = 50)

	AR	HS
	(N = 25)	(N = 25)
Age, years	39.3 ± 13.1	42.7 ± 16.1
Gender, n(%)		
Males	13 (50.0%)	13 (50.0%)
Females	12 (50.0%)	12 (50.0%)
Smoking, n(%)		
Yes	1 (4.0%)	6 (24.0%)
No	16 (64.0%)	15 (60.0%)
Former	8 (32.0%)	4 (16.0%)
BMI, kg/m^2	23.8 ± 3.1	23.7 ± 3.6
Continuous variables expressed as mean ± standard deviation (SD).		

General compositional overview of the Nasal Microbiota between AR and HS participants

Among the 50 subjects, a sufficient yield for amplicon-based analysis was obtained in 23 AR patients and 23 healthy subjects' samples (92%). Bacterial community composition analysis was thus conducted, from phylum to genus level, in 46 of the total enrolled subjects. Considering the entire study population as a whole, the bNM was dominated by the Actinobacteria (abundance range AR: 2.5%-83.5%; abundance range HS: 18.6%-92.7%), Firmicutes (AR: 6.1%-63.5%; HS: 6.4%-72.2%) and Proteobacteria (AR: 0.6%-89.6%; HS 0.7%-38%) phyla. Comparison of the two groups in terms of mean relative abundance of each phylum showed that the bNM of AR patients showed an increased abundance for the Proteobacteria phylum compared to the HS, which was mostly represented by Actinobacteria and the Firmicutes (Fig. 1A). In addition, focusing on the community structure of each AR patient, six subjects were characterized by a very high presence of the Proteobacteria phylum. The remaining 17 AR patients seem to exhibit a more heterogeneous composition with high relative abundances for the Actinobacteria and the Firmicutes phyla (Fig. 1B). No difference was observed in terms of clinical, demographics and lifestyle information between the six AR patients with high prevalence of Proteobacteria phylum and the remaining 17 AR patients.

Examining the composition of the bacterial communities in more detail, we identified 81 genera represented in the study population. The most represented taxa were *Corynebacterium* (AR: 2.3%-71.3%; HS: 6.9%-90.9%), *Staphylococcus* (AR: 0.2%-54.9%; HS: 1.7%-53.5%), *Moraxella* (AR: 0.006%-88.9%; HS: 0-34.4%), *Propionibacterium* (AR: 0.14%-44.1%; HS: 0.73%-17.8%), *Alloiococcus* (AR: 0.005%-46.2%; HS:

0.015%-59.6%), *Unassigned_Neisseriaceae* (AR: 0.003%-62.8%; HS: 0-33.4%), *Streptococcus* (AR: 0.005%-37%; HS: 0.1%-22.6%), *Anaerococcus* (AR: 0.01%-5.5%; HS: 0.006%-11%), *Peptoniphilus* (AR: 0.001%-9.24%; HS: 0.005%-4.9%), *and Pseudomonas* (AR: 0.002%-11.56%; HS: 0.006%-10.9%) genera. The AR group displayed an increased relative abundance for both *Moraxella* and a genus belonging to the *Nesseriaceae* family with a consequently marked reduction in the *Corynebacterium* and *Staphylococcus* genera abundances, which had higher abundance values in the HS group (Fig. 2A), although no significant differences between groups were observed by nonparametric t-test. Considering the individual AR patients' composition, within the group enriched by Proteobacteria phylum, five of them were dominated by *Moraxella* and one by reads classified as *Unassigned Neisseriaceae* genus. The remaining AR patients showed a pattern broadly characterized by *Corynebacterium* genus, even if in some cases high proportion of *Alloiococcus*, *Propionibacterium* and *Streptococcus* genera were seen (Fig. 2B).

Diversity evaluation and feature comparison of the Nasal Microbiota across AR and HS groups

To inspect compositional diversity within the study groups, we first focused on alpha diversity evaluation between the AR patients and HS group. Analysing the applied index, the AR group was characterized by less diversity compared to the HS group (Fig. 3). In particular, statistical analysis performed on the chosen diversity indices highlighted that bNM of the AR patients expressed a reduced mean richness and phylogenetic diversity compared to the HS group (Observed OTUs: p = 0.018; PD whole tree: p = 0.014) (Fig. 3b-c). Even if no statistical significance was identified for the Shannon index, the values showed consistency with the other two applied diversity measures illustrating that the AR bNM was characterized by a moderately less evenly distributed community (Shannon: p = 0.39) (Fig. 3a). Means, standard deviations (SD) and p-value for the tested alpha diversity indices are shown in Table 2.

Table 2
Alpha diversity means, SD and t-test p-values for each evaluated index in AR and HS groups.

		AR		HS		
		Mean	SD	Mean	SD	P-value
A)	*Shannon*	2.98	0.96	3.23	0.91	0.39
B)	*Observed_OTUs*	81.9	24.0	98.2	18.9	**0.018**
C)	*PD_Whole_tree*	6.36	1.38	7.35	1.22	**0.014**

SD: standard deviation. Bold p-values denote a significant difference ($p < 0.05$).

As a next step, distances between groups of subjects were inspected parsing the Weighted Normalized Unifrac distance metric. Analysis performed pairing the AR and HS intra-group distances suggested that their distribution was significantly greater for the AR patients rather than HS group (nonparametric test = 3.77, p = 0.01), thus indicating that the bNM of the AR patients was characterized by a less similar and evenly distributed bacterial community membership than within the HS group (Fig. 4).

To visualize the intra-group distance variability of the AR patients and HS group, a PCoA plot was generated (Fig. 5). The first two axes, PC1 and PC2, explain the 66.9% of the bNM variability across the study population. Moreover, the graphic visualization confirmed the greater dispersion of the subjects that constitute the AR group in contrast to the ones of the HS group. Through the PCoA plot, the six AR patients with the higher distribution of the Proteobacteria phyla were separated from the rest of the AR

patients, along the PC2 axis. However, the Adonis test failed to describe composition and abundance related variations when the disease status was considered, and no clusters were identified ($R^2 = 0.045$ $p = 0.1$).

Evaluation and differences of the bNM-host EV network between AR and HS groups

EVs may have a central role in transferring information between bNM and the host. Thus, we measured the size and concentration of plasma circulating EVs in the study subjects. We compared the total number of EV means between the two groups (Table 3). AR patients were characterized by a greater amount of EVs released in the blood stream (EV mean AR: 1315×10^6/ml, 95%CI 1098–1576; HS: 1167×10^6/ml, 95%CI 992–1374), although no statistical significance was reached. We further compared the AR patients and HS group in terms of distribution of mean vesicle concentrations for each size (Fig. 6). In the upper part of the figure, we reported for each EV size (from 30 to 700 nm) the mean concentration calculated in each group. The lower part of the plot reports the p-values and q-values obtained of the EVs distribution in the enrolled groups. As highlighted in Fig. 6, AR patients were characterized by a higher concentration of EVs ranging between 130–231 nm, 257–287 nm and 323–348 nm ($p < 0.05$; $q < 0.01$), with a peak at 130 nm.

Table 3
NTA and CF analyses EVs mean concentrations of AR and HS groups

		AR		HS		P-value
		Mean*	95% IC	Mean*	95% IC	
NTA analysis	*EVs (total)*	1315	(1098–1576)	1167	(992–1374)	0.306
	Gram-positive	20	(14–29)	10	(7–14)	**0.006**
	Gram-negative	228	(155–337)	125	(87–180)	**0.024**
	EpCAM	12	(10–16)	11	(9–13)	0.303
Flow cytometry analysis	*CD294*	28	(21–37)	15	(11–19)	**0.001**
	CD203C	20	(15–26)	8	(6–10)	**< 0.001**
	CD177	33	(24–44)	19	(14–26)	**0.012**
	CD62E	41	(31–55)	20	(15–26)	**< 0.001**
	CD61	792	(463–1356)	212	(130–343)	**0.001**
	CD14	60	(39–93)	30	(20–43)	**0.015**

*Means are adjusted for age, gender, smoking. Bold p-values denote a significant difference (p-value < 0.005)

We further characterized plasmatic EVs investigating both bEVs and hEVs subpopulations by flow-cytometry analysis. Consistent with the NTA results, all EV subtypes mean concentrations were higher in AR patients compared to those of HS group, except for EpCAM + EVs (Table 3). It is noteworthy that the mean concentrations of both Gram-positive ($p = 0.006$) and Gram-negative ($p = 0.024$) bEVs were increased approximately two-fold in AR patients compared to the HS group. Focusing on the hEVs subpopulations, CD61 + EVs were increased almost four-fold in AR compared with HS and the strongest supported statistical differences ($p \leq 0.001$) between the two groups were detected for CD294+, CD203C+, and CD62E + EVs.

Discussion

In this study, we investigated the nasal bacterial composition and plasmatic EV release in both a group of patients with allergic rhinitis and healthy subjects.

Observing the bacterial community structure in the whole population from the phylum level, the nasal microbiota resulted dominated by the Actinobacteria, Proteobacteria and Firmicutes phyla. The structure we observed is in agreement with other studies on the nasal bacterial community, where Actinobacteria was the most represented taxon followed by Firmicutes and Proteobacteria, which is usually found in less abundant concentration [15, 45, 46]. Although no statistical difference was identified between groups of taxa, the mean proportion of the Proteobacteria phylum in the AR patients outcompeted the one found in the HS group. Interestingly, a higher Proteobacteria concentration has been linked to respiratory diseases such as chronic obstructive pulmonary disease (COPD), tuberculosis and asthma, where the presence of numerous Proteobacteria species was positively associated with the severity of bronchial hyperresponsiveness [47–49]. This greater concentration of Proteobacteria phylum is representative of a higher abundance of *Moraxella* genus in the AR patients. *Moraxella* has previously been reported as an important pathobiont found in the respiratory microbiota of patients with asthma as well as in chronic rhinosinusitis (CR) patients, especially considering those with a coexistent cystic fibrosis or asthmatic condition [50, 51]. In particular, the *Moraxella* genus is known to increase the host inflammatory response [52, 53]. Indeed, the relative abundance of nasal *Moraxella* genus was positively correlated with systemic and airway eosinophil counts, tumor-necrosis factor alpha (TNF-α) and interleukin (IL-)7, suggesting a possible pro-inflammatory role of this genus in the bNM. On the contrary, the *Corynebacterium* genus, found in greater abundance in the analysed HS group, has been negatively correlated with atopic markers such as IL-6, IL-7 and IL-21 as well as with total eosinophil counts [54]. In agreement with our finding, it is known that exaggerated immune response along with a dysfunctional immune barrier and an inflamed mucosal epithelium can promote the overgrowth of certain microorganisms and dysbiosis in AR, asthmatic and CR patients [55].

Consequently, the modifications exerted by disease status over the bNM composition may be linked to the less diverse and to the greater intra-group diversity found in the AR patients. Indeed, the AR patients' bNM was less rich in terms of residential bacteria and associated with a reduced bacterial phylogenetic diversity compared to the HS group. Interestingly, reduced diversity in the bNM was identified also in other respiratory diseases such as in patients with Chronic Rhinosinusitis (CRS) [56, 57], in patients with asthma [58], and in patients affected by granulomatosis with polyangiitis [59]. The importance of a diverse microbiota has been widely documented as pivotal in maintaining the mucosa integrity and an effective immune system [16, 45, 60]. Moreover, the difficulty in defining clusters between AR patients and HS group, when the disease status was considered, could be attributed to the greater intra-group distances found in the AR patients compared to the ones observed in the HS group. The large variation within the AR patients' bNM could be linked to a more unstable structural behaviour of the nasal bacterial community during AR. Similar to this study, a large intra-group divergence has previously been observed in asthmatic CRS patients compared to controls [50]. Longitudinal studies to compare bNM in AR subjects and HS may help inform about stability of the bNM and further, whether changes in bNM within AR precede clinical manifestations of the condition.

In order to disentangle the complex cross-talk between the host and the microbiota, we analysed the amount and the origin of EVs released in the blood stream by the host and the bacterial community, both

in AR patients and the HS group. The analysis on EV distribution underlined a greater and statistically different concentration in the AR groups especially for those vesicles ranging between 130 and 348 nm. Interestingly, this EV range, which falls in the category of microvesicles [61], has been linked to proinflammatory activation [62–65] The greater abundance of bEVs along with that of hEVs, may further underline alterations in the host-microbiota cross talk in AR patients compared to the HS group. Indeed, associations between EVs derived from specific cellular types and various diseases have been identified in the past few years [31, 32, 66]. The greatest differences of EVs subpopulations between AR patients and HS group were identified for platelets, endothelium, mastocytes and eosinophil derived EVs. Indeed, during a proinflammatory stimulus an increased amount of EVs released by both platelets and endothelium has been already identified as well as for mastocytes derived EVs, specifically found in greater concentration during an IgE based immune response, a specific signature of allergic conditions [67–69]. The effects of EVs released by mastocytes was also linked to the maturation of the dendritic cells, critical players and sentinels of the immune system in the nasal mucosa during AR [70, 71]. Consistent with the proinflammatory-linked release for the abovementioned EVs, *in-vitro* studies showed that EVs derived from eosinophils were found in a greater amount after TNF-α stimulation and were also linked to both tissue remodelling and the disease exacerbation during allergic asthma condition [72, 73].

Focusing on the bEV proportion, *in-vitro* studies pointed out that bEVs derived from gram-negative bacteria can trigger inflammation through the activation of the toll-like receptors 2 and 4 [74, 75] as well as EVs derived from *Moraxella spp.* have been linked to contribute to sinusitis development through the stimulation of the immune response [76]. Consistently, Gram-positive bEVs were associated with an exacerbated immune response principally through the presence of toxins on bEVs surface. In particular, those released by *Staphylococcus aureus* were linked both to respiratory neutrophilic activation and atopic dermatitis like skin inflammation [33, 77–79].

Merging and speculating upon these findings, two different hypotheses can be suggested: firstly, the altered composition of bNM and the modified EV release observed in AR patients could be attributed to an excessive reaction of the immune system toward the different types of sensitizing allergens. On the other hand, environmental exposure, such as air pollution, could alter bNM composition, favouring a local aberrant immune response. In both conditions, the generation of a differential selective pressure in the bNM can affects the fitness of different bacterial taxa able to explain the huge discrepancies of the bNM within the AR group.

We acknowledge two main limitations of the study: first, the obtained results derived from the analysis of 46 subjects, which consequently may be not sufficient to comprehensively cover the intrinsic bNM variation between AR patients and HS group. However, the consistency of our findings with the literature make us confident that they were not found by chance. Second, we observed strong alterations of plasmatic bEVs and hEVs network, aware that the analysed EVs are derived from different regions of the body. However, through this approach we were able to reconstruct an overall overview of some of the component involved in the systemic effects exerted by AR throughout the body.

Conclusions

Our results suggest that AR patients were characterized by a less diverse and wide intra-group variable bacterial community compared to that of healthy participants, as well as an altered host-microbiota EV communication network. To the best of our knowledge, this is the first study that has attempted to dissect the differences of the bNM and the communication mechanisms between the host and the bacterial community in AR. Further studies should be carried out to better characterize the differences of the bNM as well as the relationship between the host and the nasal bacterial community between pathological conditions and health, also considering environmental exposures as effect modifiers.

Declarations

AVAILABILITY OF DATA AND MATERIALS AVAILABILITY

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Additional data related to this paper may be requested to the authors.

AUTHOR CONTRIBUTION

JM performed microbiota analyses, contributed to the conceptualization of the study and wrote the manuscript; SI performed statistical and formal analyses; LC performed Extracellular Vesicles analysis; PM and EC recruited patients and collected samples; RI contributed to the interpretation of the results; PAB contributed to the interpretation of the results; MSL contributed to the conceptualization of the study and to the interpretation of the results; RPH contributed to the conceptualization of the study, to the interpretation of the results and supervised manuscript preparation; VB conceptualized and supervised the study, supervised the manuscript preparation; LF conceptualized and supervised the study, contributed to the writing and supervised the manuscript preparation.

ETHICAL APPROVAL AND CONSENT TO PARTECIPATE

Each participant signed a written informed consent, approved by the Comitato Etico – Milano Area 2” of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, 20122 Milan, Italy (approval number 157_2019bis), in accordance with the Helsinki Declaration principles.

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COMPETING INTERESTS

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AUTHORS’ INFORMATION

Not available.

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Figures

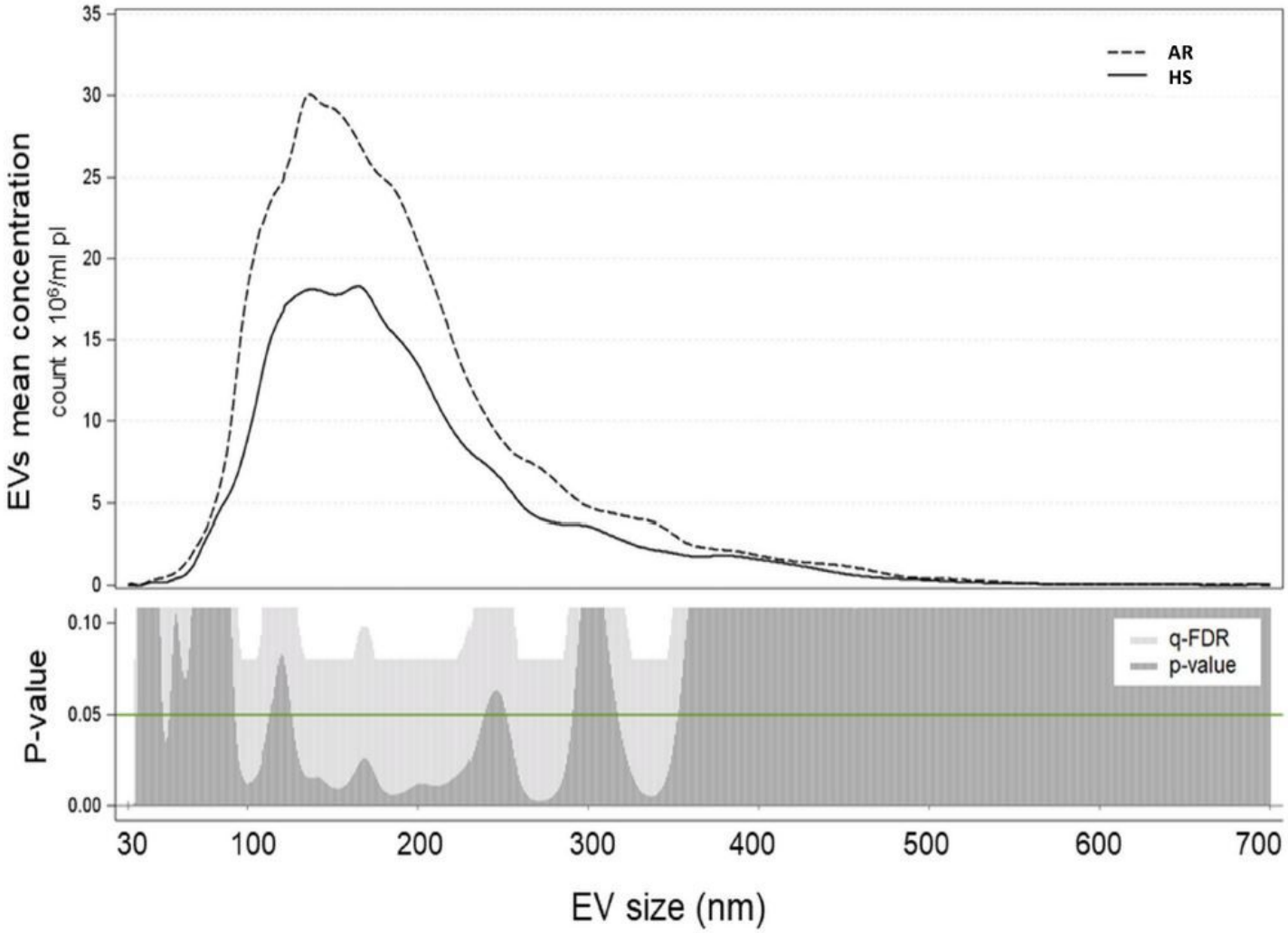

Figure 1

NTA plasma EV size profiling. The upper panel of the figure reports EV concentration for each size for AR (dashed line) and HS (solid line) groups. The lower panel reports the p- and q- values of comparisons of each size EV for the entire 30–700 nm size range (AR vs HS).

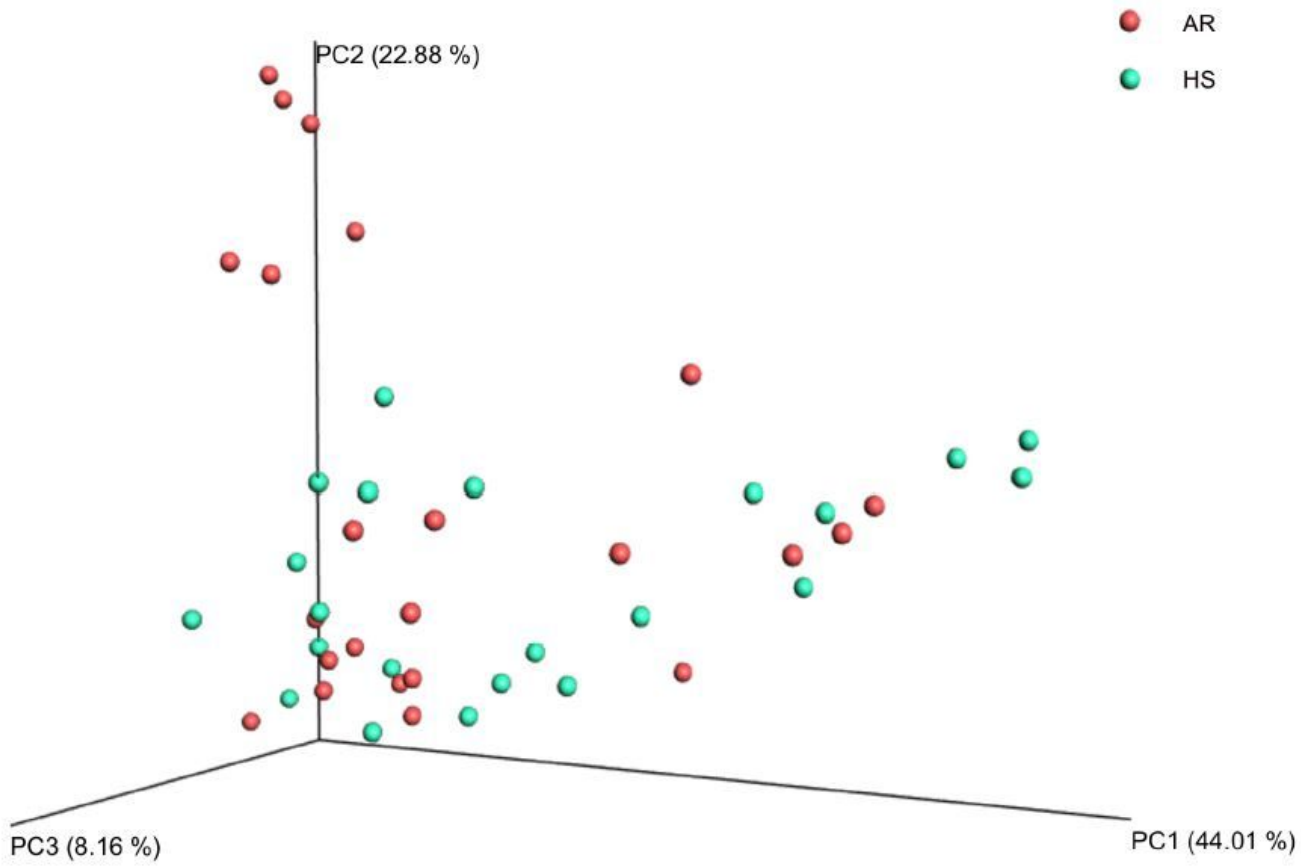

Figure 2

Principal coordinate analyses (PCoA) plot made using the normalized Weighted UniFrac distance metric. Each dot corresponds to a single subject belonging either to AR (red dot) or HS (green dot) groups. The variance explained by each axis is given in parentheses.

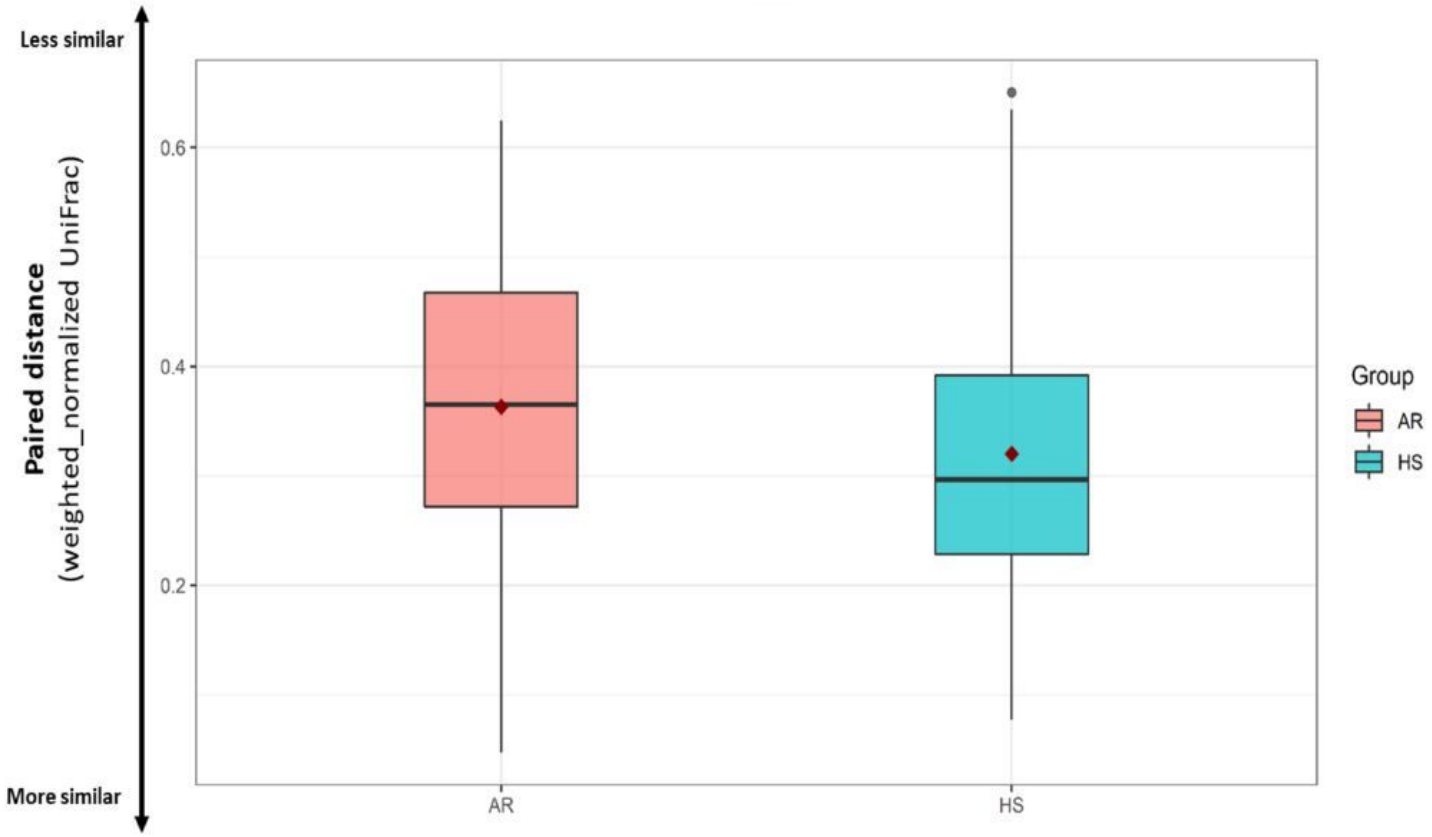

Figure 3

Intra-subject paired distance of the AR and HS group.

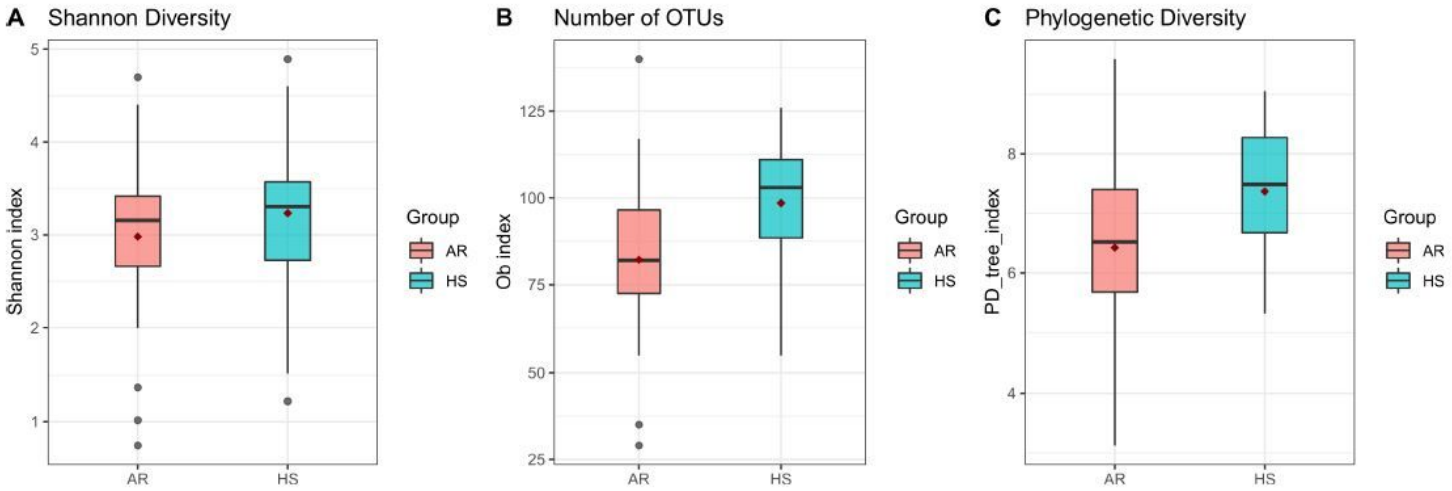

Figure 4

Comparison of bNM alpha diversity between AR and HS groups. Values for each group were measured by: Shannon (A) Observed_OTUs (B) and PD_whole_tree (C) indices.

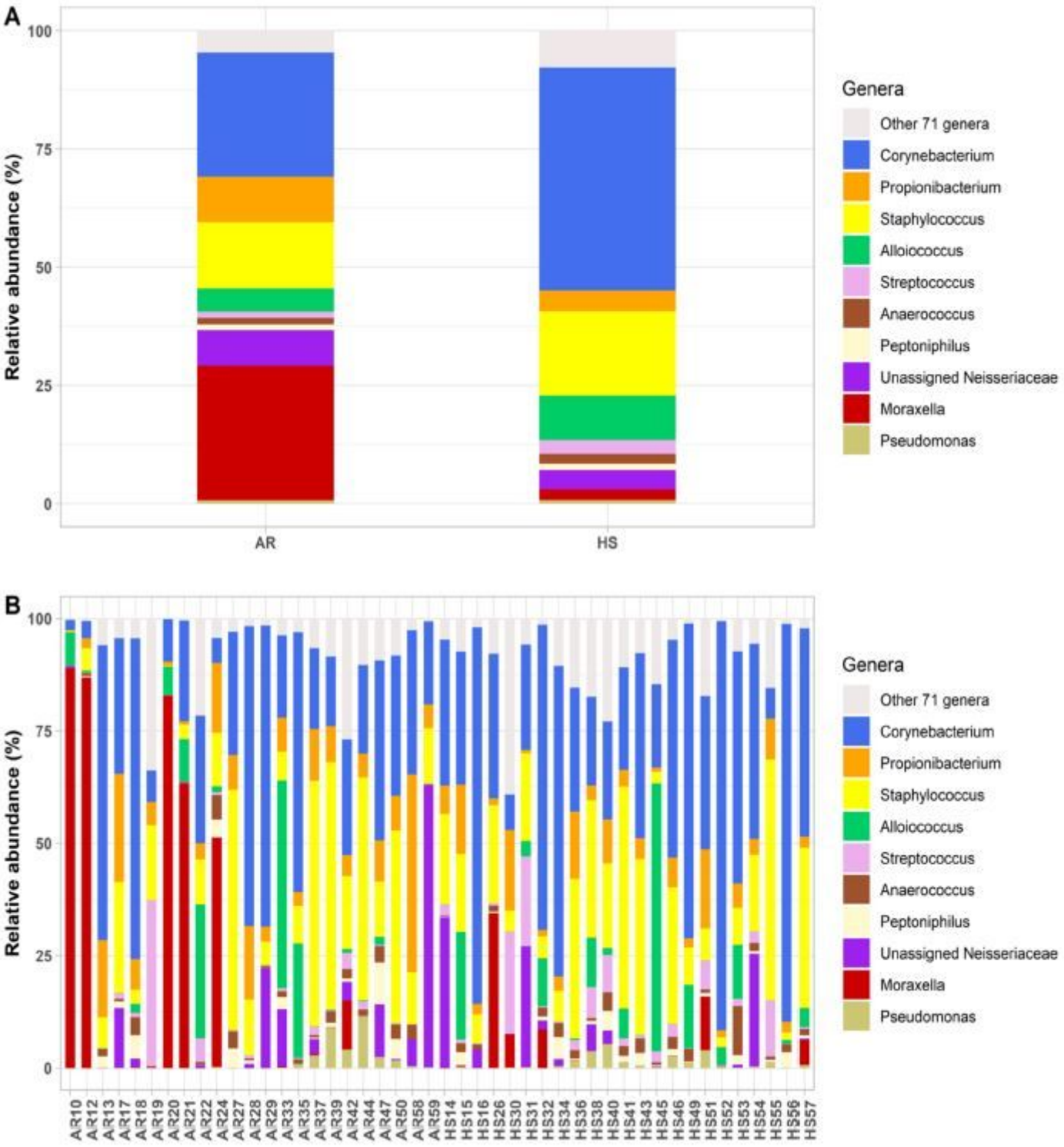

Figure 5

bNM genus-profile structure A)the average relative abundance in the AR and HS group, B) genus-profile structure of the bNM for each recruited subjects

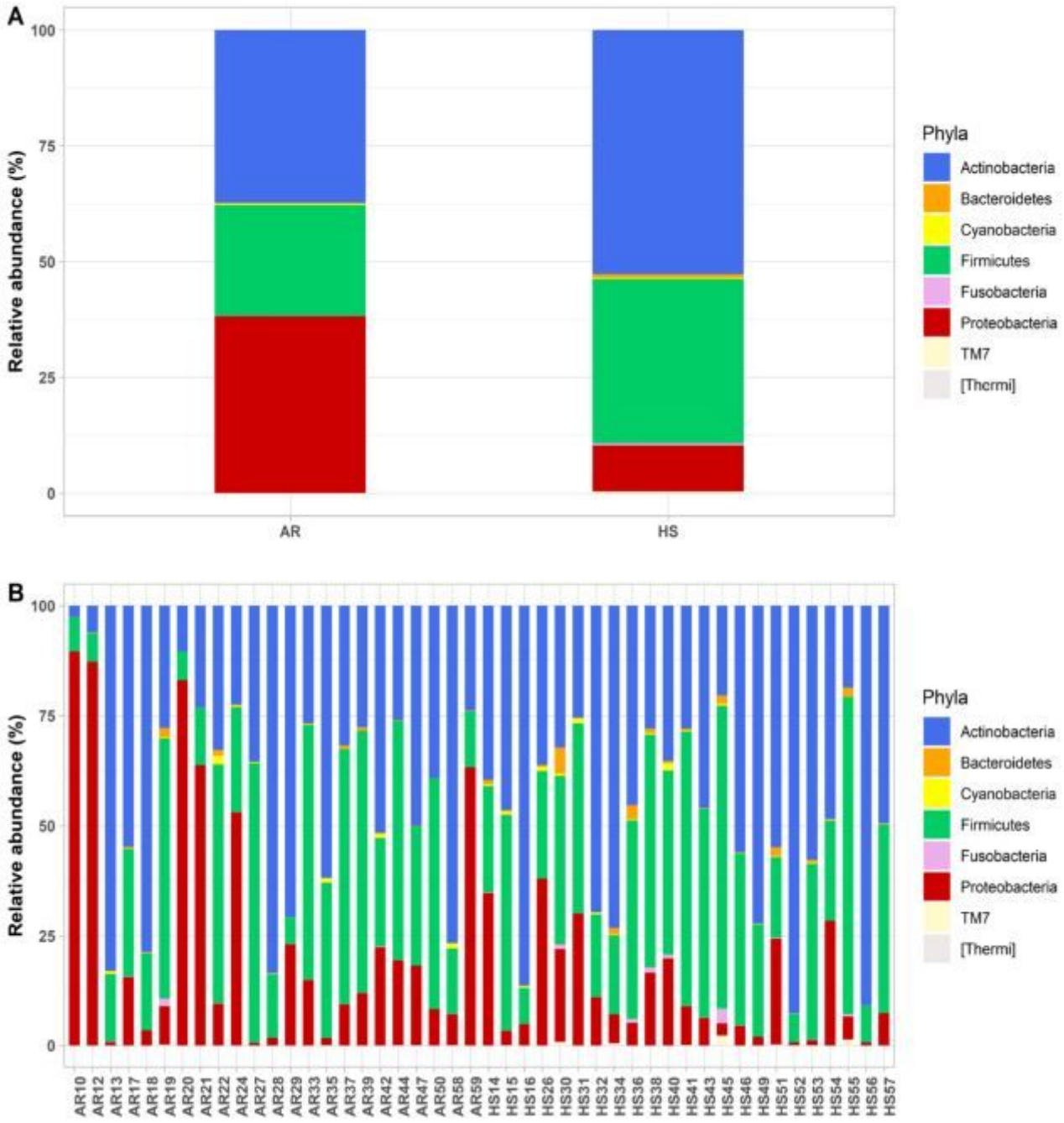

Figure 6

bNM phylum-profile composition. A) Average relative abundance of the bNM structure in the AR and HS groups, B) relative abundance of the bNM of each enrolled subject