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Eosinophilic airway inflammation in asthma is associated with an altered airway microbiome

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Abstract

Background

Subjects with asthma have higher microbiome diversity, and an altered composition with more Proteobacteria and less Bacteroidetes compared to healthy controls. Studies comparing airway inflammation and airway microbiome are sparse, especially in subjects not on anti-inflammatory treatment.

Objective

To describe the relationship between the airway microbiome and patterns of airway inflammation in steroid-free subjects with asthma and healthy controls.

Methods

Broncho-alveolar lavage was collected from 23 steroid-free, non-smoking subjects with asthma and 10 healthy controls. Bacterial DNA was extracted from and subjected to Illumina MiSeq sequencing of the 16S rDNA V4 region. Eosinophils and neutrophils in submucosa were quantified by immunohistochemical identification and computerized image analysis. Induced sputum was performed, and Airway hyperresponsiveness to mannitol and Fractional Exhaled Nitric Oxide were measured. Relationships between airway microbial diversity and composition, and inflammatory profiles were analyzed.

Results

In patients with asthma, airway microbial composition was associated with airway eosinophilia and AHR to mannitol, but not airway neutrophilia. The overall composition of the airway microbiome of asthmatics with the lowest levels of eosinophils, but not asthmatics with the highest levels of eosinophils deviated significantly from that of healthy individuals. Asthmatics with the lowest levels of eosinophils had an altered bacterial abundance profile with more *Neisseria*, *Bacteroides* and *Rothia*, and less *Sphingomonas*, *Halomonas* and *Aeribacillus* compared to asthmatics with more eosinophils and healthy controls.

Conclusion

The level of eosinophilic airway inflammation correlates with variations in microbiomes across subjects with asthma, whereas neutrophilic airway inflammation does not. This warrants further investigation on molecular pathways involved in eosinophilic as well as non-eosinophilic asthma.

Clinical Implications:

The level of eosinophilic airway inflammation correlates with variations in microbiomes across subjects with asthma. This warrants further investigation on molecular pathways involved in asthma, and the relation to airway microbiota.

Key words:

Airway inflammation, asthma, bacteria, microbiome, non-eosinophilic asthma, broncho-alveolar lavage

Abbreviations

PCoA: Principal coordinate analysis. A transformation of the beta diversity distance matrix onto a two-dimensional space for visualization.

PERMANOVA: Permutational Multivariate Analysis of Variance.

OUT: Operational Taxonomic Unit - a cluster of similar 16S rDNA sequences which represents a distinct taxonomic unit (species, genus, etc.)

RDP: Ribosomal Database Project

KEGG: Kyoto Encyclopedia of Genes and Genomes

KO: KEGG orthologues

ANOSIM: ANalysis Of SIMilarity

Abundance: The relative representation of a certain OTU in the microbial community

Alpha Diversity: Characterization of the read count distribution of OTUs (expressed via calculation of Shannon index)

Richness: The number of different OTUs represented in the microbial community

Shannon index: A measure of species richness and evenness in a community

Beta diversity: A measure of similarities in species composition between samples

Community: Collection of bacterial OTUs

Bacterial species: Bacteria with less than 97% common nucleotides in 16S rRNA gene.

AHR: Airway hyperresponsiveness

NEA: Non-eosinophilic asthma

FeNO: Fractional exhaled nitric oxide

BAL: Bronchoalveolar lavage

ICS: Inhaled corticosteroids

RDR: Response dose ratio

EG2: Eosinophil cationic protein

MPO: Myeloperoxidase

ACQ: Asthma Control Questionnaire

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Background

The lower airways were until recently considered a sterile environment. However, new culture-independent 16S rDNA sequencing methods have revealed that the lower airways host diverse communities of microbes[1, 2]. In 2010, Hilty and colleagues were the first to show that the microbiome of lower airways of asthmatics differed significantly from that of healthy controls with increased bacterial diversity with more Proteobacteria and less Bacteroidetes[1].

Asthma is defined as a chronic inflammatory disease, and a type 2 immune response characterized by the presence of IL-4, IL-5 and IL-13, with IL-5 driving eosinophilic recruitment, is the most well-described type of inflammation. However, approximately half of all subjects with asthma show no signs of eosinophilic airway inflammation[3], often referred to as non-eosinophilic asthma (NEA). Subjects with NEA also have a Th2-low phenotype of asthma[4], characterized by low expression of IL-13 inducible genes. The disease-driving mechanisms are less well understood in NEA. It has, however, repeatedly been shown that subjects with NEA respond less well to inhaled corticosteroids (ICS)[4, 5].

Only few studies have investigated the relationship between airway microbiota and airway inflammation in asthmatics, and only in patients with severe disease on high doses of ICS that may influence both the type of airway inflammation and the bacterial habitat. However, Green et al.[6] demonstrated that colonization with known pathogenic bacteria like *Moraxella catarrhalis*, *Haemophilus influenzae* or members of the *Streptococcus* genus in sputum was associated with increased numbers of neutrophils and increased levels of IL-8. Positive correlations between Proteobacteria and Th17-associated gene expression have been reported, while airway submucosal eosinophils have been suggested to be negatively associated with the relative abundance of *Moraxellaceae* and *Helicobacteraceae*[7].

Thus, there is a need for studies that systematically describe airway inflammation in relation to the airway microbiome, especially in subjects taking no ICS or other anti-inflammatory treatments that might influence airway inflammation.

Based on the assumption that bacteria can drive a Th1-type inflammation and eventually neutrophil recruitment, we hypothesized that the airway microbiome would differ between steroid-free subjects with asthma depending on their inflammatory status, and that an altered microbiome would be expected especially in those asthmatics with non-eosinophilic or neutrophilic dominated disease. To test this we performed 16S rDNA sequencing on bronchoalveolar lavage (BAL) from steroid-free, non-smoking subjects with asthma and healthy control persons, and compared the microbial composition with inflammatory parameters of induced sputum, BAL and airway mucosa biopsies.

Methods

Study design

This is a cross-sectional study of individuals with asthma and healthy control subjects. All participants attended the clinic four times - a minimum of one day apart and with no more than four weeks between visit 1 and visit 4. Airway hyperresponsiveness (AHR) to mannitol, Fractional exhaled nitric oxide (FeNO), induced sputum and a bronchoscopy with broncho-alveolar lavage and mucosal biopsies were performed. The scientific ethics committee approved the study (H-2-2010-130) and written informed consent was obtained from all individuals before entering the study. Data was originally collected as part of a study exploring airway mast cell involvement in AHR[8].

Participants

Inclusion criteria for asthmatics (n=23): A history of at least one positive objective diagnostic test suggestive of asthma (methacholine test (PD20 < 8 mmol), mannitol test (PD15 < 635 mg), FEV₁ reversibility to beta2-agonist of $\geq 12\%$ and 250 ml), current asthma symptoms (within the last four weeks), non-smoking for at least the past six months (and ≤ 10 pack-years), corticosteroid-free ($\geq$ three months) and no history of other chronic lung diseases. The distribution of objective tests for asthma on which subjects were included is presented in Table 1. No individual was taking long acting β 2-agonists, leukotriene modifiers or cromoglycates. Inclusion criteria for the group of healthy controls were: No history of asthma or other chronic lung diseases, negative objective diagnostic tests suggestive of asthma and non-smoking for at least the past six months (and ≤ 10 pack-years). Controls were not matched with asthmatics on any parameter before inclusion. All participants were without clinical airway infections for at least 6 weeks prior to visit 1 and throughout visit 4, but use of

antibiotics for reasons other than airway infection was not recorded.

To build a profile of microbial composition 16S rDNA V4 region amplicon sequencing was performed on the bacterial community in BAL from 23 asthmatics and 10 healthy controls, and the data was combined with measures of eosinophils and neutrophils in submucosal biopsies and sputum, blood eosinophils, AHR to mannitol, and FeNO.

Lung function, FeNO, AHR to mannitol, atopy and induced sputum

Lung function, FeNO and AHR to mannitol were measured in accordance with international recommendations[8] and manufacturers' guidelines. A positive mannitol test was defined as a decrease in FEV₁ of at least 15% after inhalation of 635 mg of mannitol or less. As an index for the reactivity to inhaled mannitol, the response dose ratio (RDR) was calculated. Skin prick tests were performed, and atopy was defined as a positive skin prick test response to at least one aeroallergen. Induced sputum was performed after bronchodilator pretreatment, and participants inhaled nebulized solutions of sodium chloride 3, 4 and 5%, five minutes for each solution. A minimum of 100 non-squamous cells were counted. See online repository for further details about clinical tests.

Bronchoscopy

BAL and mucosa biopsies were collected under local anesthesia and sedation with intravenous administered Midazolam. BAL was performed as the first thing in the left upper lobe (segment 4+5), installing 2 x 60ml of NaCl. Biopsies (n=4–8 / patient) were taken from right middle and lower lobes, immediately immersed in fixative (4% formaldehyde) and then processed for paraffin embedding. BAL was centrifuged at 4000 rpm for 10 minutes, and the pellet was used for further 16S sequencing.

Immunohistochemistry

Eosinophils and neutrophils were detected by immunohistochemistry using antibodies (eosinophil cationic protein (EG2) and Myeloperoxidase (MPO), respectively) previously validated for use on paraffin-embedded tissue sections[9, 10]. Immunohistochemistry was performed identically and simultaneously for all individuals in an automated immunohistochemistry robot operating at room temperature (Autostainer LV-1; Dako A/S, Glostrup, Denmark). Sections stained for periostin were digitalized in an automated digital slide-scanning robot as specified above. The generated high-resolution images were then analysed in ImageScope (v. 10.0.36.1805; Aperio Technologies Inc, Vista, CA). See online material for further details.

Quantification of eosinophils, neutrophils and periostin

Stained sections were digitalized in an automated digital slide-scanning robot (Scanscope CS, Aperio Technologies Inc, Vista, CA). The generated high-resolution images were then analyzed in Visiopharm Integrator System (v. 4.4.4.0; Visiopharm A/S, Hoersholm, Denmark). The program detected the area of tissue (as revealed by hematoxylin background staining) and areas of positive immunostaining. For each leukocyte marker a fixed threshold value for positive immunoreactivity was used to detect and quantify chromogen colour and staining intensity.

A positive staining recognition algorithm was used to detect periostin staining and separate it from background staining. Periostin staining was measured as previously done by others[11], i.e. by measuring the mean percent immunoreactivity of periostin in the subepithelial region, defined as the region between the epithelium and muscularis mucosa or submucosal gland tissue.

16S rDNA Sequencing

Bacterial DNA from BAL pellets was extracted using the NucleoSpin soil kit from Macherey-Nagel according to manufacturer's instructions. DNA yield and integrity were assessed using a Nanodrop and

agarose gel electrophoresis, respectively. The PCR-based library formation was performed using 10 ng bacterial DNA, 0.2 μ M of each barcoded forward and reverse primer, 0.2 mM dNTPs and 0.5 units Phusion high fidelity DNA polymerase (Thermo Scientific) in a total volume of 25 μ l. Specific primers and detailed sequencing methods are described in the online material and [12].

Bioinformatics

The generated Fastq files were demultiplexed by the MiSeq Controller Software and trimmed for diversity spacers using biopieces[biopieces.org], mate-paired using usearch v7.0.1090[13] and filtered using usearch -maxee 0.5. UPARSE[14] for Operational Taxonomic Unit (OTU) clustering as recommended. Representative sequences were classified using Mothur v.1.25.0 wang() function[15] at 0.8 confidence threshold. There was an average of $37,570 \pm 13,016$ (SD) sequences per sample before, and $36,923 \pm 12,915$ (SD) sequences per sample after filtering.

Subsequent analyses were performed in R v3.1.0 using the metagenomeSeq[16], phyloSeq[17], Vegan[18] and ggplot2[19] packages. Data was filtered for low-abundant OTUs by removal of OTUs present in fewer than 2 of the samples and with a relative abundance across all samples $\leq 0.005\%$ for all but alpha diversity analysis. The Shannon diversity index (alpha-diversity), which accounts for species richness and evenness, was computed with the phyloSeq package plot_richness() function. The Whittaker index (beta-diversity), which is a measure of the dissimilarity between communities at different sites, was used to calculate the beta diversity within the Vegan package.

Statistical analysis

Eosinophilia and neutrophilia are expressed as percentage of immunoreactivity to EG2 or MPO, respectively (i.e. the area of positive immunostaining divided by the combined area of tissue and

positive immunostaining). All biopsy measures are expressed as median (IQR). Based on the median EG2 and MPO values, asthmatics were divided into 'eosinophil high' and 'eosinophil low', and 'neutrophil high' and 'neutrophil low'. Basic characteristics were calculated in SPSS version 22 (SPSS, Inc, Chicago, Ill). GraphPad Prism6 was used to plot and calculate significant differences in alpha and beta diversity using Kruskal-Wallis test followed by Dunn's corrections for multiple comparisons. Correlations between eosinophils and bacterial OTUs were calculated using `cor.test()` in R with spearman correlations. Normalized counts for the significant different abundant bacteria between 'eosinophil high' and 'eosinophil low' were also plotted using GraphPad Prism6 and re-tested for significance using Mann-Whitney tests.

Results

Data on airway microbiota and airway inflammation in submucosa was available in 23 subjects with asthma and 10 healthy controls. Based on the median values of EG2 and MPO submucosal staining activity, respectively, asthmatics were sub-divided into ‘eosinophil low’ and ‘eosinophil high’, and ‘neutrophil low’ and ‘neutrophil high’. Asthmatics in the ‘eosinophil high’ group had more sputum and BAL eosinophils, higher levels of FeNO, more AHR to mannitol and a higher ACQ-score compared to asthmatics in the ‘eosinophil low’ group (Table 1). Correlations between measures of eosinophilia have previously been reported elsewhere[8].

Measures of periostin staining-activity in submucosa were significantly higher in ‘eosinophil high’ asthmatics compared to healthy controls, but not between ‘eosinophil high’ vs. ‘eosinophil low’ asthma and ‘eosinophil low’ vs. healthy controls, respectively (Table 1).

Submucosal eosinophilia, but not neutrophilia, is correlated with diversity and community composition in the lower airways microbiome

The alpha-diversity in the BAL microbiome in patients with ‘eosinophil low’ asthma compared to healthy controls was higher, indicating patients with ‘eosinophil low’ asthma host more different bacteria in BAL compared to healthy. Beta diversity was lower in subjects with ‘eosinophil low’ asthma compared to both healthy subjects and subjects with ‘eosinophil high’ asthma (Figure 1A), which indicate the abundance profile is more uniform in patients with ‘eosinophil low’ asthma. There was no difference in alpha diversity between groups stratified for neutrophils in submucosa, whereas asthmatics with ‘neutrophil high’ asthma had lower beta diversity compared to asthmatics with ‘neutrophil low’ asthma and healthy controls (Figure 1B).

The differences in microbiome composition between subjects with ‘eosinophil low’ and

'eosinophil high' asthma were visualized with Principal coordinate analysis plots (PCoA plots) (Figure 2A and 2B), and confirmed with PERMANOVA. A separation between the healthy subjects and subjects with 'eosinophil low' asthma ($p < 0.05$) as well as between subjects with 'eosinophil high' and 'eosinophil low' asthma ($p < 0.01$) but not healthy subjects and subjects with 'eosinophil high' asthma ($p = 0.10$) was observed (Figure 2A). An environmental fit of EG2 and MPO levels in submucosa, RDR to mannitol and the 5% most extreme bacterial vectors are shown in Figure 2A. No separation of the samples was observed when MPO levels in submucosa were used as an explanatory variable (Figure E1).

When plotting only asthmatic subjects, a separation of the samples could be observed in relation to the level of EG2 staining activity in submucosa (Figure 2B, $p < 0.01$) and mannitol reactivity ($p = 0.01$), but not to the level of MPO staining activity in submucosa ($p = 0.12$). An environmental fit of the variables confirmed that level of EG2 ($p = 0.001$), mannitol reactivity ($p < 0.01$), sputum and BAL eosinophils ($p < 0.01$ and $p < 0.05$) and FeNO ($p < 0.05$), but not submucosal, BAL or sputum neutrophils ($p = 0.11$, $p = 0.64$ and $p = 0.11$) significantly explained separation of microbiota.

Relative abundances of bacteria in lower airways in healthy subjects and steroid-free subjects with 'eosinophil high' versus 'eosinophil low' asthma

Taxa summary plots of the 20 most abundant bacterial genera in healthy controls and subjects with asthma are presented in Figure 3. Among bacterial genera with a prevalence of 100% in the samples, 14 bacterial genera differed significantly in relative abundance between healthy subjects and asthmatics with either 'eosinophil low' or 'eosinophil high' disease (Table 2). Eight of these (*Aeribacillus*, *Halomonas*, *Neisseria*, *Nesterenkonia*, *Rothia*, *Shewanella*, *Sphingomonas*, and *Virgibacillus*) exhibited significantly different relative abundances between both healthy subjects versus subjects with 'eosinophil low' asthma, and subjects with 'eosinophil high' versus subjects with 'eosinophil low'

asthma. Only 2 bacterial genera differed significantly in relative abundance between healthy subjects and subjects with ‘eosinophil high’ asthma (*Blautia* and *Lachnospiracea* Incerta Sedis).

Six bacterial genera were significantly more abundant in ‘eosinophil low’ compared to ‘eosinophil high’ asthma (Figure 4A), while eight bacterial genera were significantly less abundant (Figure 4B). The full phylogeny of these bacterial genera and their candidate species are shown in Table 3.

Within the group of gram-negative bacteria the most abundant genera were *Neisseria*, which exhibited significantly higher, and *Halomonas*, which exhibited significantly lower relative abundance in ‘eosinophil low’ compared to ‘eosinophil high’ asthma. The representative sequence of the *Neisseria* OTU was identified as *N. subflava* by building a phylogenetic tree with *Neisseria* sequences from the RDP database. For *Halomonas* it was not possible to unequivocally suggest a candidate species among *Halomonas korensis*, *Halomonas diaqiaonensis* and *Halomonas cerina* using the V4 16S rDNA region.

Of the most abundant gram-positive bacteria, *Aeribacillus* was present in significantly lower, while *Actinomyces* and *Rothia* were found in significantly higher relative abundance in ‘eosinophil low’ compared to ‘eosinophil high’ asthma. The candidate species for *Aeribacillus*, *Aeribacillus pallidus*, is the only known species in this genus.

A similar analysis was carried out for samples stratified by neutrophils in submucosa, which revealed very few significant differences in the abundance of bacteria, and none of these showed 100% prevalence among the samples (Online Supplementary: Table E1+E2).

Metagenome prediction from 16S rDNA data reveals functional differences in ‘eosinophil low’ and ‘eosinophil high’ asthma

16S rDNA amplicon data were supplemented with genomic data using PiCrust. Briefly, a repository of

full genome sequenced bacteria was searched by the representative 16S rDNA sequences and the most likely genome content of each bacteria found in the sample was selected and multiplied by the original abundance to give a quantitative estimate of the gene content of the sample. Genes from different bacteria likely to perform the same function have been grouped into KEGG orthologues (KO) by the Kyoto Encyclopedia of Genes and Genomes. Differentially abundant KOs were screened using Bonferroni-corrected Wilcoxon's ranked sum test for differences between subjects with 'eosinophil high' and 'eosinophil low' asthma revealing 12 different KOs (Figure E2). No differences were found between subjects with 'neutrophil high' and 'neutrophil low' asthma after adjustment for multiple testing.

Of particular interest were Nitric oxide synthase (K00491, $P < 0.05$). Linear regression of FeNO against the predicted abundance of NO synthase showed a statistically significant correlation that could explain 25% of the variation in the FeNO measurements (Figure E3). Adjusting for blood eosinophils still gave a significant increase in FeNO ($p < 0.05$), with a ratio of F scores (eosinophils/NO synthase) at 2.24, suggesting that blood eosinophils explain roughly twice the amount of variance as compared to NO synthase linked to airway microbiota.

Other potential interesting enzymes that came out significantly in the analysis were protein disulfide-isomerase and glutamate racemase, the latter involved in peptidoglycan production, suggesting differences in the gram positive/negative ratio. The fraction of gram positive bacteria was found to be positively correlated with eosinophils in submucosa (PCC: 0.49, $p < 0.05$). Other genes seemed to be merely household genes, such as DNA polymerase.

Sensitivity analysis

To test the robustness of the association between eosinophilic airway inflammation and airway microbiota we did a number of additional analyses. Figure E4 (a-d) show p-values for varying diversity

cut-points in subjects stratified on EG2 or MPO-levels, which confirm that beta-diversity (and to a lesser extent alpha-diversity) significantly differs over a range of cut-points in subjects stratified based on EG2, but not MPO. The findings were further substantiated by using sputum eosinophils instead of EG2, confirming a lower beta-diversity in asthmatics with the lowest levels of sputum eosinophils compared to both asthmatics with the highest levels of eosinophils and healthy controls (Figure E5). A similar separation of subjects in a PCoA-plot was also seen using sputum eosinophils instead of EG2 (data not shown).

Since most of the variation (62.7%) in microbiota was explained on the primary axis of the PCoA-plot in Figure 2A (but with three potential outliers in the group of healthy controls), we wanted to determine which bacteria explained most of the variation along the axis. The same bacteria that were identified in Table 2 were also the ones that contributed the most in explaining the variation along the primary axis (Figure 2A), and remained to be so considering only the asthmatics (Figure 2B). To examine if disease status (asthma vs. healthy controls) contributed in explaining the variation along the primary axis, disease status as well as eosinophils were fitted against the primary axis. Whereas disease status did not contribute to the separation along the primary axis (goodness of fit 0.07, $p=0.13$), the level of EG2 did contribute to the separation (goodness of fit 0.32, $p<0.01$).

Finally, to further characterize the association between a type-2 inflammatory response and airway microbiota, we subdivided asthmatics into two equal groups based on the median level of periostin staining activity in submucosa (primarily representing an IL-13 pathway) as opposed to eosinophilia (representing an IL-5 pathway). No separation of the samples was observed when submucosal periostin was used as explanatory variable in a PCoA plot looking at differences in airway microbiome (Figure E6). No difference was found in alpha-diversity, whereas the group of asthmatics with the lowest levels of periostin had lower beta-diversity (Figure E7).

Discussion

In this study of steroid-free subjects with asthma and healthy controls, the microbiome in BAL samples differed in accordance with eosinophilic, but not neutrophilic, airway inflammation. Subjects with 'eosinophil low' asthma compared to subjects with 'eosinophil high' asthma and healthy controls had a lower beta-diversity, and a higher alpha-diversity compared to healthy subjects, which means the lung microbial community in 'eosinophil low' asthmatics harbours a more diverse microbiota, but with less variation between individuals. Differences in relative abundance of specific bacteria were identified between both healthy and subjects with 'eosinophil low' as well as 'eosinophil high' asthma, but also between subjects with either 'eosinophil low' or 'eosinophil high' asthma. The results substantiate previous findings linking asthma with microbiota diversity, and suggest that microbial diversity is associated with eosinophilic airway inflammation.

Existing data on on airway microbiome and airway inflammation are inconsistent. Green et al.[6] identified increased levels of neutrophils and IL-8 in severe asthmatics colonized with the genera *Haemophilus* or *Streptococcus* in sputum, whereas Huang et al.[2] collected airway microbiota from subjects with suboptimally controlled asthma, but found no association with neither eosinophils nor neutrophils in sputum. Another study by Huang et al.[7] examined the microbiome in brush biopsies from 40 patients with severe asthma and found that airway submucosal eosinophils were predominantly negatively associated with the relative abundance of bacteria such as *Moraxellaceae* and *Helicobacteraceae*, whereas no association with measures of neutrophilia was identified. Bacterial diversity and specific strains of bacteria have also been related to inflammatory and clinical markers in other respiratory diseases like COPD and Cystic Fibrosis[20-22], and at the moment, it seems that an increased proportion of proteobacteria in subject with disease is the consistent finding in most studies, whereas the specific genera and species identified in each study vary. The inconsistencies in these

reports may be a result of differences in sampling site (sputum vs. brush biopsies vs. BAL), measures of inflammation (sputum vs. submucosa), but also the level and type of anti-inflammatory treatment in the study populations.

Knowledge of functional properties of specific bacterial species in relation to airway inflammation is sparse. Larsen et al.[23] showed in rodents that Proteobacteria (*H. Influenzae* and *M. Catarrhalis*) as compared to Bacteroidetes (*Prevotella spp*) were able to induce higher levels of pro-inflammatory cytokines (such as IL-8, TNF- α and TSLP) and a higher airway neutrophil recruitment (but with no detectable eosinophil recruitment). Conversely, bacteria may also play a critical role for ensuring normal cellular maturation and control of allergic airway inflammation[24]. The precise role and identity of bacteria involved in airway inflammation still remains to be established, and it even seems that phylogenetically closely related bacteria exhibit both anti-inflammatory and proinflammatory actions[25]. We identified a number of Proteobacteria that differed in abundance with the level of eosinophilia, but with opposite correlations for different genera. Thus, *Neisseria spp.* was negatively associated with EG2-levels in submucosa, whereas *Halomonas* and *Sphingomonas* were positively correlated. This emphasizes the complex relationships between airway inflammation and microbiota, and calls for intervention studies that allow conclusions on possible causal relations.

One of the genera that was closely associated with the level of EG2 was the gram-negative *Neisseria* (subflava) that were significantly more abundant in ‘eosinophil low’ compared to ‘eosinophil high’ asthma. Hilty et al.[1] were the first to identify *Neisseria spp.* to be more abundant in asthmatics, but did not report measures of airway inflammation. It has previously been shown that *Neisseria spp.* have immunomodulatory properties via membrane porines that have been established as pathogen-associated molecular pattern recognized by Toll Like Receptor 2 on dendritic cells, causing increased levels of IL-

6[26]. *Neisseria subflava* has specifically been shown to significantly increase after rhinovirus infection[27], which further stresses the importance of evaluating not only the microbiome but also the virome in future studies.

Another genus of potential interest is *Sphingomonas* (also gram-negative) that was less abundant in 'eosinophil low' compared to 'eosinophil high' asthma. *Sphingomonas* has previously been associated with increased AHR to methacholine[2]. Glycolipid antigens from *Sphingomonas* are believed to induce AHR in mice through activation of invariant natural killer T cells in an IL33 dependent manner[28, 29]. In this present study, AHR was significantly more prevalent in subjects with 'eosinophil high' asthma, in whom *Sphingomonas* was also more abundant. Further, independent of airway eosinophilia, AHR to mannitol was found to explain a significant part of the variation in airway microbiome. Prospective studies investigating the possible mechanisms whereby bacterial glycolipids lead to AHR would be of interest in relation to the development of improved therapies for asthma.

The subjects in this study with 'eosinophil low' asthma resemble in many ways subjects previously defined as having paucigranulocytic asthma or non-eosinophilic asthma[30]. Non-eosinophilic asthma constitutes up to 50% of the asthma population[3], but opposed to eosinophilic asthma, the causes of non-eosinophilic asthma are poorly understood, and few treatments are targeted this group of patients. None of the subjects with 'eosinophil low' asthma had sputum eosinophils $\geq 2\%$ (whereas this was the case for 8 out of 11 subjects in the 'eosinophil high' group). Only few subjects (three in the 'eosinophil low' group and four in the 'eosinophil high' group) had significant neutrophilia ($\geq 61\%$) in sputum. It is possible that this may reflect the relatively mild disease in the subjects studied, as opposed to subjects with severe disease and a more pronounced neutrophilic inflammatory response. Finally it needs to be stressed that the cut-point between subjects with 'high' and 'low' degree of inflammation in this paper

was solely based on the median values of EG2 and MPO in submucosa, making the cut-points clinically arbitrary. This was primarily due to the exploratory study design, but also the limited number of subjects. The results therefore cannot be related directly to specific asthma phenotypes.

Even though the overall composition of airway microbiota showed a more clear separation between subjects with 'eosinophil low' asthma and healthy controls as compared to subjects with 'eosinophil high' asthma and healthy controls, significant differences at the genus level were identified between all groups. Based on the results we can only speculate on any causal relation between microbiota and airway inflammation, but as stated above, the interrelations are undoubtedly complex with indices of bacteria being able to work both pro- and anti-inflammatory dependent on the disease state of the subject and the overall microbial composition (including vira).

In conclusion, in steroid-free subjects with asthma and healthy controls the microbiome in BAL differed in accordance with eosinophilic, but not neutrophilic, airway inflammation. Asthmatics with the lowest levels of EG2 in submucosa differed in microbial diversity (had higher alpha diversity and lower beta diversity) compared to asthmatics with the highest levels of EG2 in submucosa and healthy controls. This was mainly due to changes in bacterial abundances rather than replacement of certain taxa. Both groups of asthmatics ('eosinophil low' and 'eosinophil high') exhibited specific alteration at the genus level, which calls for further studies that look into the causal relationship between airway microbiota and alterations of molecular pathways in eosinophilic and non-eosinophilic airway inflammation.

Table 1. Baseline characteristics of healthy controls and subjects with ‘eosinophil high’ and ‘eosinophil low’ asthma.

	Healthy controls, n=10	Asthma, eosinophil high, n=11	Asthma, eosinophil low, n=12
Age, median (min; max)	23 (20; 25)	27 (20; 36)	25 (20; 40)
Sex, female n (%)	5 (50)	6 (55)	7 (58)
FEV1 %pred., mean (95CI)	97.6 (86.3-109.0)	85.9 (79.6-92.2)	94.0 (84.9-103.1)
FEV1/FVC, mean (95CI)	0.82 (0.77-0.86)	0.76 (0.69-0.82)	0.76 (0.73-0.79)
Mannitol positive, n (%)	0 (0)	7 (64)*	2 (17)
RDR, median (IQR)	0.007 (0.001-0.010)##	0.080 (0.016-0.378)**	0.016 (0.011-0.020)
Sputum eos. %, median (IQR)	0.0 (0.0-0.0)	2.0 (1.8-7.4)**	0.0 (0.0-0.2)
BAL eos. %, median (IQR)	0.0 (0.0-0.3)#	3.3 (2.3-7.9)*	1.0 (0.3-2.6)
Blood eosinophils (n*10 ⁹ /L), median (IQR)	0.12 (0.04-0.17)□	0.36 (0.14-0.48)	0.15 (0.08-0.25)
Sputum neutrophils (%), median (IQR)	49.8 (19.8-65.8)	56.0 (41.4-71.4)	30.0 (14.9-58.6)
BAL neutrophils (%), median (IQR)	14.0 (4.6-26.4)	13.9 (11.1-24.4)	10.3 (7.1-29.8)
MPO in submucosa, median (IQR)	1.75 (1.02-3.27)	0.82 (0.51-2.95)	1.01 (0.83-1.34)
Periostin in submucosa, median (IQR)	4.6 (2.1-7.1)□	10.8 (9.4-14.6)	8.3 (4.0-15.2)
Atopy, n (%)	3 (30)	8 (73)	7 (58)
FeNO ppb, median (IQR)	18.3 (13.8-23.2)	35.7 (23.1-92.3)*	15.0 (10.6-27.4)
ACQ-6, median (IQR)	-	1.7 (1.0-2.0)**	0.8 (0.3-1.1)
EG2 in submucosa, median (IQR)	0.31 (0.18-0.43)	0.93 (0.45-2.20)**	0.22 (0.17-0.30)
Asthma diagnosis initially based on:			
PEF variability, n (%)	-	0 (0)	4 (33)
Beta2-reversibility, n (%)	-	4 (36)	3 (25)
AHR, n (%)	-	7 (64)	5 (42)

FEV₁: Forced expiratory volume in 1 second; FVC: Forced vital capacity; RDR: Response Dose Ratio; FeNO: Fractional exhaled nitric oxide; ACQ: asthma control questionnaire; PEF: Peak expiratory flow; AHR: Airway Hyperresponsiveness; EG2: percentage of immunoreactivity to EG2 in submucosa. MPO: percentage of immunoreactivity to myeloperoxidase in submucosa. Eosinophil high vs. eosinophil low: * p<0.05. ** p<0.01. Healthy vs. ‘eosinophil low’: # p<0.05. ## p<0.01. Healthy vs. ‘eosinophil high’: □ p<0.01.

Table 2:

Genera that differ in abundance in BAL from healthy controls and subjects with 'eosinophil high' and 'eosinophil low' asthma. NS=not significant.

		Adjusted p-values			Total counts			Correlation with eosinophils
	Genus	Healthy vs. 'eosinophil low'	'eosinophil low' vs. 'eosinophil high'	Healthy vs. 'eosinophil high'	Healthy	'eosinophil high'	'eosinophil low'	
Different between Healthy and 'Eosinophil low' and Different between 'Eosinophil low' and 'Eosinophil high'	Aeribacillus	<0.05	<0.05	NS	21821	19490	3700	0.69
	Halomonas	<0.05	<0.05	NS	46880	43292	10398	0.66
	Neisseria	<0.05	<0.05	NS	10725	7708	27341	-0.65
	Nesterenkonia	<0.05	<0.05	NS	14189	10891	1839	0.71
	Rothia	<0.05	<0.05	NS	2114	3393	8413	
	Shewanella	<0.05	<0.05	NS	1011	704	191	0.71
	Sphingomonas	<0.05	<0.05	NS	20109	12569	2487	0.66
	Virgibacillus	<0.05	<0.05	NS	3726	2802	608	0.62
Only different between 'Eosinophil low' and 'Eosinophil high'	Actinomyces	NS	<0.05	NS		3674	7610	
	Bacteroides	NS	<0.01	NS		1836	5651	
Only different between Healthy and 'Eosinophil low'	Burkholderia	<0.05	NS	NS	1198		126	0.68
	Caldalkalibacillus	<0.05	NS	NS	9707		1786	0.65
Different between Healthy and 'Eosinophil high'	Blautia	NS	0.001	<0.05	1402	535	1770	
	Lachnospiraceae_incertae_sedis	NS	NS	<0.05	607	250		

Table 3.

Taxonomic distribution of genera in the BAL microbiome that differ between ‘eosinophil high’ and ‘eosinophil low’ asthma, and candidate species within each bacterial genus. The 16S V4 region sequence of the most abundant OTU within an identified genus was aligned using MUSCLE to other known sequences within that genus downloaded from the Ribosomal Database Project (RDP). Phylogenetic tree building was performed in MEGA6 using the Maximum Likelihood method based on the Tamura-Nei model to identify species candidates.

Gram stain	Phylum	Class	Order	Family	Genus	Number of OTUs	Most abundant	Species candidate(s)	P-value ‘eosino phil high’ vs ‘eosino phil low’	Difference
Pos itiv e	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces	5	OTU17	Actinomyces massiliensis Actinomyces israelii Actinomyces gerencseriae	<0.05	More abundant in low
				Micrococcaceae	Nesterenkonia	1		Nesterenkonia flava	<0.001	Less abundant in low
					Rothia	8	OTU38	Rothia mucilaginosa Rothia amarae	<0.05	More abundant in low
	Firmicutes	Bacilli	Bacillales	Bacillaceae 1	Aeribacillus	2	OTU6	Aeribacillus pallidus	<0.001	Less abundant in low
				Bacillaceae 2	Virgibacillus	1		Virgibacillus campisalis	<0.01	Less abundant in low
				Bacillales incertae sedis	Caldalkalibacillus	1		Caldalkalibacillus uzonensis Caldalkalibacillus therarum	<0.01	Less abundant in low
		Clostridia	Clostridiales	Lachnospiraceae	Blautia	10	OTU48	Blautia wexlerae	<0.01	More abundant in low
					Lachnospiraceae Incertae sedis	5	OTU269	Eubacterium hallii	<0.01	More abundant in low
Ne gati ve	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	5	OTU9	Sphingomonas oligophenolica	<0.001	Less abundant in low
		Betaproteobacteria	Burkholderiales	Burkholderiaceae	Burkholderia	3	OTU158	Burkholderia ferrariae	<0.01	Less abundant in low
			Neisseriales	Neisseriaceae	Neisseria	8	OTU5	Neisseria subflava	0.001	More abundant in low
		Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas	3	OTU2	Halomonas korlensis Halomonas diaquionensis Halomonas cerina	<0.001	Less abundant in low
			Alteromonadales	Shewanellaceae	Shewanella	1		Shewanella algae Shewanella haliotis Shewanella indica	<0.001	Less abundant in low
	Bacteroidetes	Bacteroidia	Bacteroidales	Bacteroidaceae	Bacteroides	20	OTU41	Bacteroides dorei	<0.01	More abundant in low

Figure Legends

Figure 1. Alpha and B) beta diversity in BAL microbiomes

A) Alpha and B) beta diversity in the BAL microbiome as stratified by healthy (green) or asthma with either 'eosinophil high' or 'neutrophil high' (blue), or 'eosinophil low' or 'neutrophil low' (red) in submucosa. Alpha diversity was calculated as Shannon index (a measure of species richness and evenness) and beta diversity was calculated using the Whittaker index (a measure of the dissimilarity between communities). Significant differences between the groups were tested using a Kruskal-Wallis test with Dunn's corrections for multiple comparisons and indicated by a horizontal bar between the two different columns with stars denoting the p-values. Corrected p-values without significance are given for comparisons where the p-value was close to 0.05. ★, ★★, ★★★ denote $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Figure 2. Principal Coordinate Analysis (PCoA) plots using Weighted UniFrac distances in the BAL microbiome.

A) PCoA plot with healthy controls, and 'eosinophil high' and 'eosinophil low' asthma. Analysis of similarity (ANOSIM) conducted on each pairwise comparison and Holm-corrected for the number of pairwise comparisons showed significant difference in microbiome composition between Healthy vs. 'eosinophil low' asthma ($p < 0.05$) and 'eosinophil high' vs. 'eosinophil low' asthma ($p < 0.01$), but not between healthy controls and 'eosinophil high' asthma ($p = 0.10$). Ellipses represent the 0.95 confidence level. Environmental variables and individual bacterial abundances were fitted onto the ordination and displayed as vectors. The degree of submucosal eosinophilia ($p < 0.01$, $R^2 = 0.26$) and mannitol reactivity ($p = 0.01$, $R^2 = 0.20$) were major drivers explaining the separation. Submucosal neutrophilia ($p = 0.12$, $R^2 = 0.12$) was not significant, but is shown in the plot. For the bacterial vectors only the 5% most extreme correlations are shown. The length and direction of a vector represents the correlation between the vector and the microbiome data.

B) PCoA plot with asthmatics only, colored according to submucosal eosinophilia. ANOSIM analysis confirmed the significant difference in microbiome composition between ‘eosinophil high’ and ‘eosinophil low’ asthma ($p < 0.01$). Ellipses represent the 0.95 confidence level. Environmental variables and individual bacterial abundances were fitted onto the ordination and displayed as vectors. For analysis of the asthmatics, the degree of submucosal eosinophilia ($p < 0.05$, $R^2 = 0.28$) and mannitol reactivity ($p < 0.01$, $R^2 = 0.38$) were major drivers of separation. The length of the vector represents the correlation between the vector and the microbiome data. Submucosal neutrophilia ($p = 0.51$, $R^2 = 0.07$) was not significant, but is shown in the plot. For the bacterial vectors only the 5% most extreme vectors are shown. The length and direction of a vector represents the correlation between the vector and the microbiome data.

Figure 3. Taxa summary plots

Taxa summary plots of the 20 most abundant bacterial genera in healthy controls ($n = 10$) and subjects with asthma ($n = 23$) stratified according to the level of EG2 or MPO in submucosa.

A) EG2

B) MPO

Figure 4. Abundance of bacterial genera in BAL from subjects with ‘eosinophil high’ or ‘eosinophil low’ asthma.

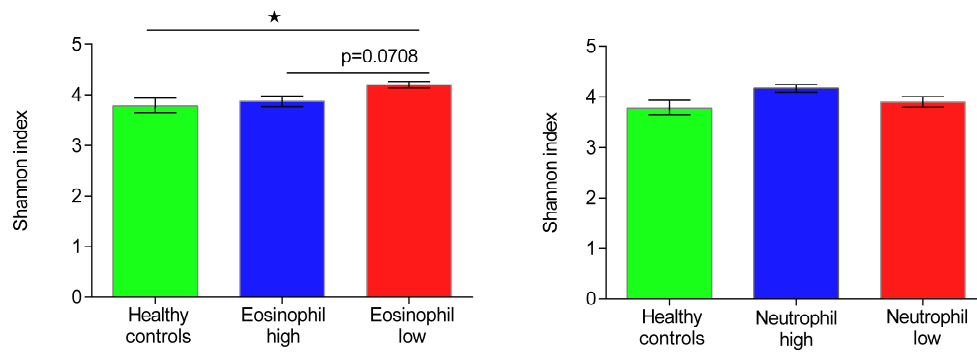
The Expectation-Maximization algorithm was run on normalized count data for all OTUs in each sample to identify genera that differed significantly in abundance in BAL from asthmatics with either high or low submucosal eosinophilia. Only genera with 100% prevalence in both groups are shown. Normalized counts of all OTUs belonging to a specific bacterial genus were summed within each sample and the average and SEM for all samples in each group (high or low) were divided into bacterial genera either A) significantly more abundant or B) significantly less abundant in asthmatics with ‘eosinophil low’ compared to ‘eosinophil high’ asthma. Horizontal bars between the different columns represent significant findings with stars denoting the p-values ★, ★★, ★★★ ($p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively).

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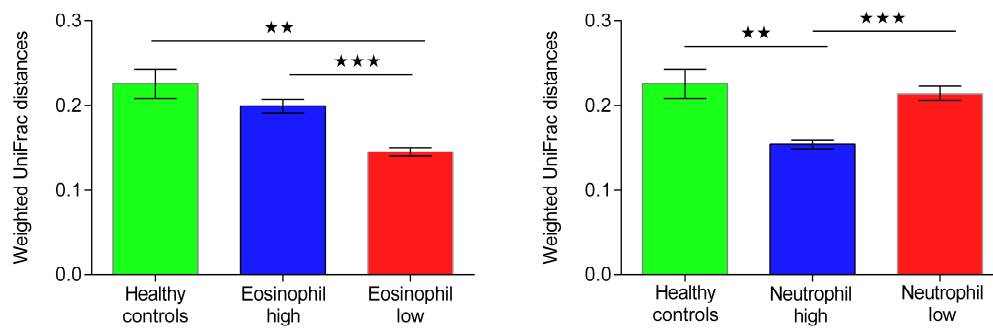
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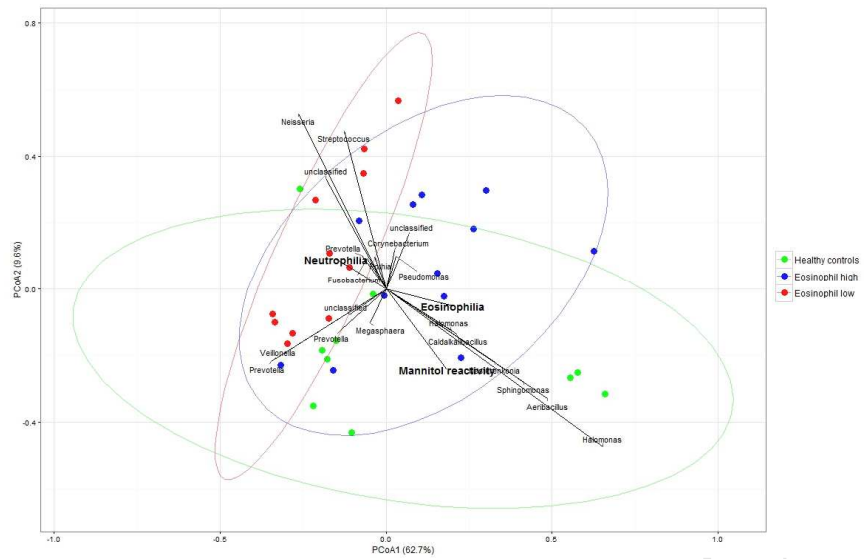
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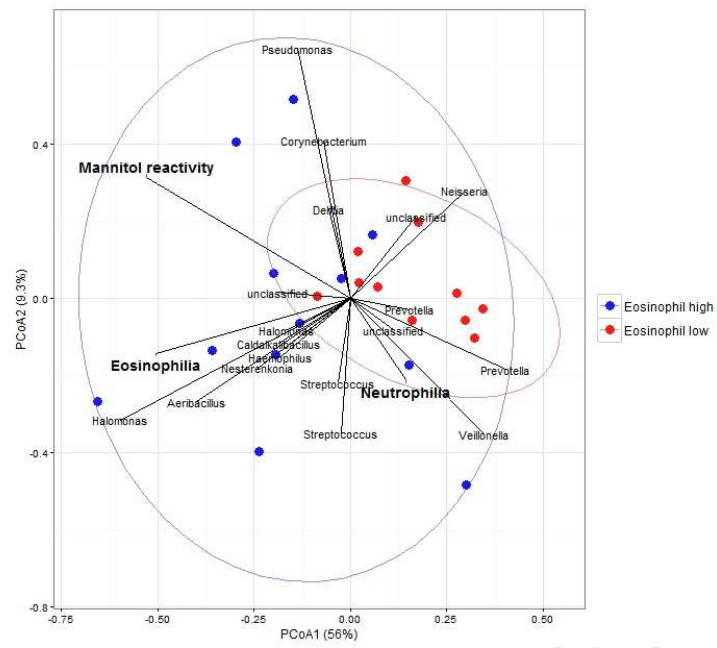
A) Alpha diversity

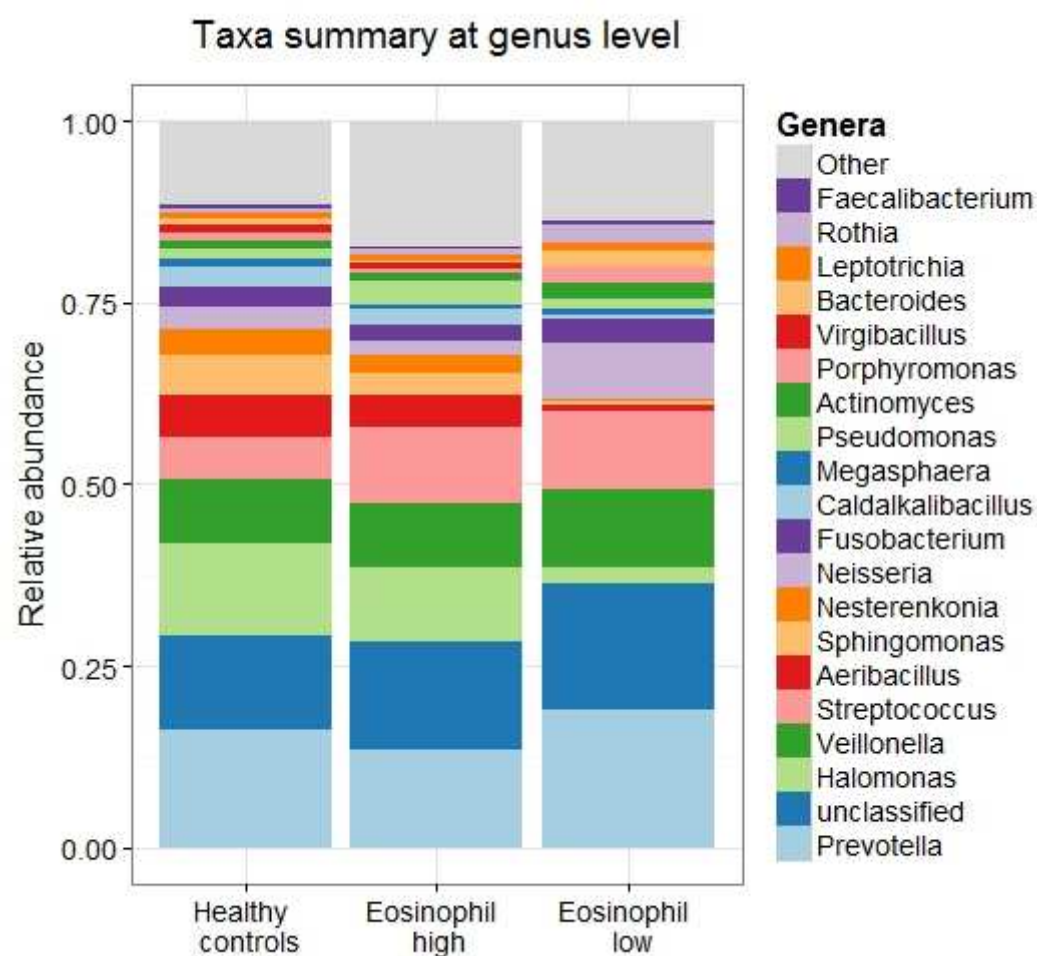


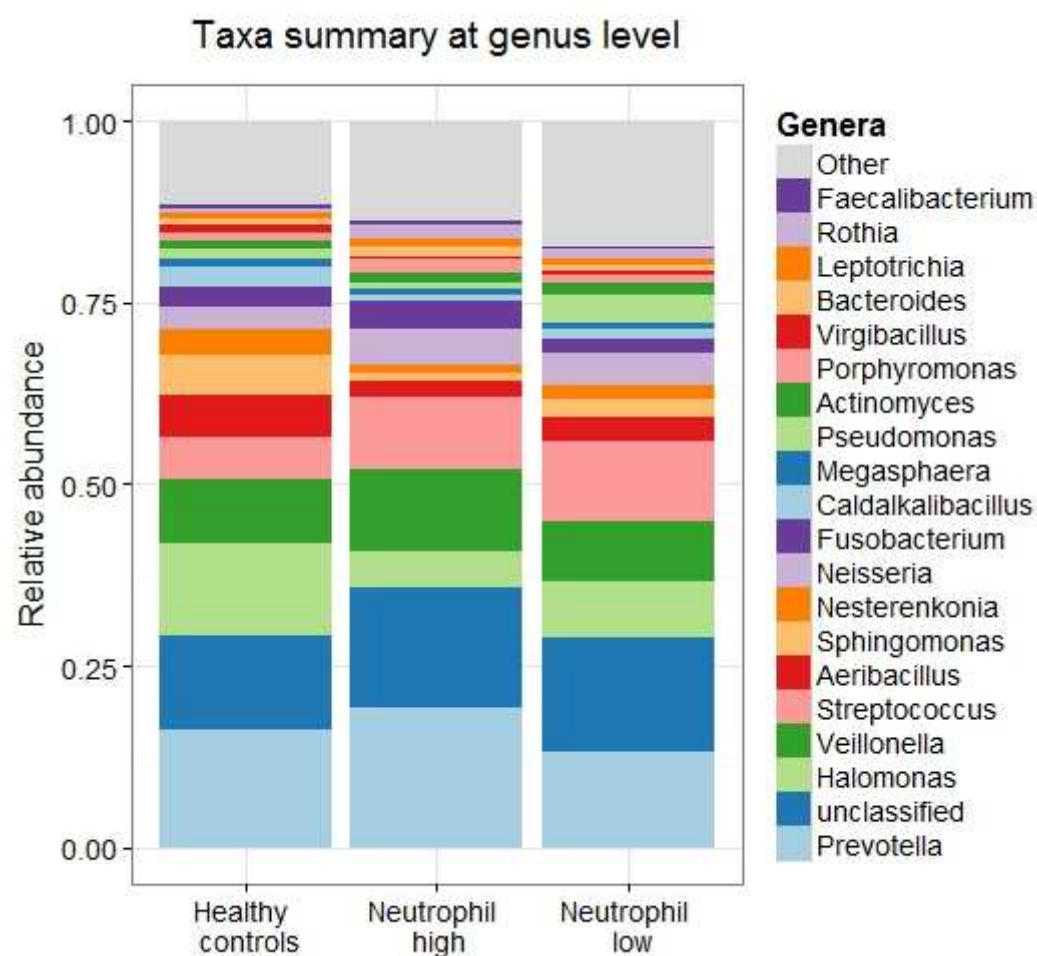
B) Beta diversity

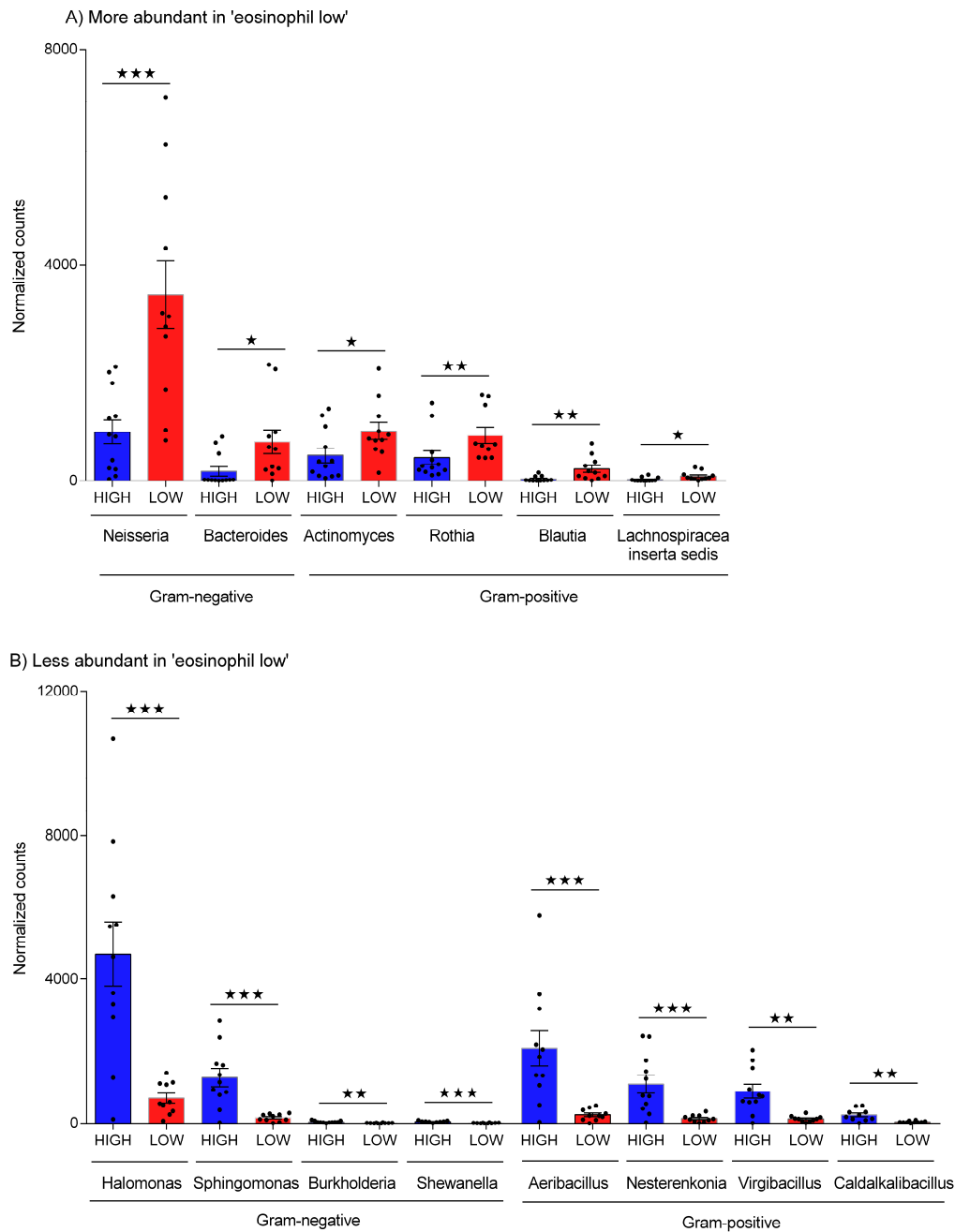












Online repository material

Methods

Lung function, FeNO, skin prick test

Lung function and FeNO were assessed according to international recommendations. Lung function was measured using EasyOne™ (nidd Medical Technologies, Inc) according to ERS recommendations[1] and FEV₁ reversibility was recorded 15 minutes after the administration of two inhalations of 0.5 mg Terbutaline Sulfate. FeNO levels were measured online (rate, 0.05 L/s) with the Nitric Oxide Analyzer (NIOX; Aerocrine AB, Solna, Sweden) and according to American Thoracic Society guidelines [2]. A skin prick test to 10 aeroallergens (birch [Betula species], grass [Phleum pratense] mugwort, horse, dog, cat [Felis domesticus], house dust mite [Der p1 and Der f 2], and fungi [Alternaria and Cladosporium species; ALK-Abello', Hørsholm, Denmark]) was performed. Atopy was defined as a positive skin prick test response (> 3 mm) to at least 1 of these 10 aeroallergens.

Bronchial provocation tests

Dry-powder, inhaled mannitol (Osmohale™; Pharmaxis LTd, Frenchs Forest, NSW Australia) was administered in increasing doses one minute apart (0, 5, 10, 20, 40, 80, 160, 160, 160 mg) and FEV₁ was recorded after each dose[3]. The challenge was stopped at a decrease in FEV₁ of 15% or greater from baseline values (0 mg placebo capsule) or when the maximum cumulative dose of 635 mg had been administered. A positive test was defined as a decrease in FEV₁ of at least 15% after inhalation of 635 mg of mannitol or less. Short acting beta₂-agonists were paused at least 8 hours before the two challenges.

As an index for the reactivity to inhaled mannitol and methacholine, the response dose ratio (RDR) was calculated. This is defined as the percentage decrease in FEV₁ after the last dose administered divided by the final cumulative dose in milligrams or micromoles. RDR values were log transformed (individuals with no reduction or a negative reduction in FEV₁ after a BPT were set to an arbitrary 0.1 reduction in FEV₁).

Immunohistochemistry

Simultaneous detection of eosinophils and neutrophils was done as previously described[4,5], using a double staining kit (K5361, Dako A/S) according to the manufacturer's instructions. Prior to automated immunohistochemistry, the sections were first incubated at 60 °C for 30 minutes and then pretreated (deparaffinized, rehydrated and antigen retrieved) in pH 6.0 using a PT Link (Dako A/S.) according to the manufacturer's instructions. Immunohistochemical staining was then performed by firstly quenching endogenous peroxidase and alkaline phosphatase using a dual endogenous enzyme-blocking reagent (K5361, Dako A/S) for 10 minutes. Next, sections were treated with antibodies to detect chymase (CC1, Novocastra Ltd, Newcastle upon Tyne, UK; concentration 1:100) or eosinophils (Eosinophil cationic protein, EG2; Pharmacia-Upjohn Diagnostics AB, Sweden; concentration 1:1000) for 60 minutes. Sections were then incubated with a dextran polymer reagent containing both secondary antimouse and antirabbit antibodies and peroxidase molecules (K5361, Dako A/S) for 30 minutes. After this step, sections were incubated with non-permeable chromogen DAB (K5361, Dako A/S) for 10 minutes. Next, a double staining blocking solution (K5361, Dako A/S) was added (5 min) to denature residual secondary antibodies on the dextran polymer in order to avoid cross-reactivity with the second primary antibody. Following this, sections were treated with antibodies to detect tryptase (G3, Chemicon Inc, Temecula, CA; concentration 1:10000) or neutrophils (Myeloperoxidase,

A0398, Dako A/S; concentration 1:20000) for 60 minutes. Next, a dextran polymer reagent containing secondary antimouse and antirabbit antibodies (K5361, Dako A/S) was added (10 minutes) followed by incubation with a polymer reagent containing alkaline phosphatase (K5361, Dako A/S) for 30 minutes. Lastly, sections were incubated with a permanent red chromogen solution (K5361, Dako A/S) for 10 minutes. Between the aforementioned steps, sections were washed with buffer solution (S3006, Dako A/S) or distilled water according to the manufacturer's recommendations. Background staining was visualized with hematoxylin and the sections were mounted with Pertex mounting medium (Histolab Products AB, Gothenburg, Sweden).

Staining of periostin

Sections were first incubated at 60 °C and pretreated (deparaffinized, rehydrated, and antigen retrieved) at pH 6.0 as described above. Immunohistochemical staining was then performed by using a single-staining kit from Dako (K8010, Dako). In brief, endogenous peroxidase activity was first quenched by using a peroxidase blocking solution (K8010, Dako). Next, sections were incubated with a rabbit polyclonal antibody against periostin (Ab79946, Abcam Inc, Cambridge, MA) at a concentration of 1:15000 for 60 minutes. Sections were then treated with a dextran polymer reagent containing secondary antimouse and antirabbit antibodies and peroxidase molecules (K8010, Dako) for 30 minutes. After this, sections were incubated with non-permeable chromogen DAB (K8010, Dako) for 10 minutes. Between the aforementioned steps, sections were washed with buffer solution (K8807) or distilled water according to manufacturer's recommendations. Background staining was visualized with hematoxylin and sections were mounted with Pertex mounting medium (Histolab Products).

16S Sequencing

To target the 16S rRNA gene's variable region 4 (V4) a forward primer 515F (5' AATGATACGGCGACCACCGAGATCTACACNNNNNNNNNTATGGTAATTGTGTGCCAGCMGCCGCGG TAA 3') and a reverse primer 806R (5' CAAGCAGAAGACGGCATACGAGAT NNNNNNNNNNNNAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT 3', where "N" indicates the nucleotides of the barcode sequence) were used, both with Illumina adaptor sequences in the 5' end[6]. Cycling conditions were as follows: 98°C for 30 seconds followed by 35 cycles of 98°C for 5 s, 56°C for 20 s and 70°C for 20 s. PCR products were purified and normalized to 1–2 ng/μl using the SequalPrep Normalization Plate kit (Life Technologies Europe). Subsequently, samples were pooled (2 μl of each sample) and quantified using a KAPA Library Quantification Kit (KAPA Biosystems) on a Stratagene Mx3000 (Agilent Technologies Denmark). 6.65 pM library and 0.35 pM PhiX Control v3 (Illumina) was sequenced using an Illumina MiSeq V2 PE500 cartridge (500 cycles) on an Illumina MiSeq.

Bioinformatics

Environmental fit of variables was performed in the Vegan package by fitting environmental factors and vectors onto a Bray dissimilarity ordination of the abundance data. Variables were considered significantly correlated with axes at $p < 0.05$.

Principal Coordinate Analysis (PCoA) was performed in phyloSeq using weighted UniFrac distances as similarity index, projecting similarities into a 2-dimensional visualization. The result was visualized with ggplot2 stat_ellipse () with ellipses representing the 95% confidence level of the multivariate students t-distribution. Significant separation of

the sample groups was tested using ANOSIM and Holm-adjusted for the number of pairwise comparisons[7].

For identification of significantly different bacterial genera metagenomeSeq was used. The eff option was set to 0.5 to exclude OTUs with low effective sample sizes.

Phylogenetic trees for candidate species identification were built in MEGA6[8], using type strains from the Ribosomal Database Project[9] from the same genus with MUSCLE sequence-alignment[10] and phylogenetic tree building using the Maximum Likelihood method based on the Tamura-Nei model[11] performed in MEGA v6.06.

Online Repository Material – Tables and Figures

Table E1.

Genera that differ in abundance in BAL between healthy controls and subjects with ‘neutrophil high’ and ‘neutrophil low’ asthma.

Different between healthy and low		Prevalence			Total counts	
	Genus	Healthy	Low	Adjusted p-values	Healthy	Low
	Flavobacterium	5/10	8/12	<0.05	138	643
	Brevundimonas	10/10	11/12	<0.05	455	2380
	Phenylobacterium	8/10	10/12	<0.05	358	140
Different between healthy and high		Prevalence			Total counts	
		Healthy	High	Adjusted Pvalues	Healthy	High
	Bradyrhizobium	8/10	9/11	<0.01	455	67
	Sediminibacterium	9/10	6/11	<0.01	490	53
	Gemella	9/10	11/11	<0.01	948	4009

Table E2.

Taxonomic distribution of the genera and candidate species within each bacterial genus in the BAL microbiome that differ between healthy controls, and subjects with 'neutrophil high' and 'neutrophil low' asthma.

Gram stain	Phylum	Class	Order	Family	Genus	Number of OTUs	Most abundant	Species candidate(s)	P-value 'neutrophil high' vs 'neutrophil low'	Difference
Positive	Firmicutes	Bacilli	Bacillales	Bacillales incerta sedis XI	Gemella	1	OTU29	Gemella haemolysans Gemella morbillorum Gemmella sanguinis	<0.01	More abundant in high than healthy
Negative	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	3	OTU108	Brevundimonas faecalis Brevundimonas abyssalis Brevundimonas diminuta Brevundimonas vancouveriensis Brevundimonas neajanganensis	0.15	More abundant in low than healthy
							OTU118	Brevundimonas halotolerans Brevundimonas bullata	0.07	
							Both		<0.01	

Figure E1

A) PCoA plot with healthy controls, and 'neutrophil high' and 'neutrophil low' asthma. ANOSIM for the neutrophilic plot shows no significant difference between any of the groups (Healthy controls vs. 'neutrophil low' asthma ($p=0.27$), 'neutrophil high' vs. 'neutrophil low' asthma ($p=0.27$), Healthy controls vs. 'neutrophil high' ($p=0.19$). Ellipses represent the 0.95 confidence level.

B) PCoA plot with asthmatics only, colored due to 'neutrophil high' or 'neutrophil low'. ANOSIM analysis showed no separation of the samples due to submucosal neutrophilia ($p=0.12$). Ellipses represent the 0.95 confidence level.

Figure E2

KEGG orthologues that differed in abundance between subjects with 'eosinophil high' and 'eosinophil low' asthma.

Figure E3

Correlation between measured FeNO and normalized abundance of the Nitric Oxide synthase KEGG Orthologue

Figure E4

Assessment of robustness of the chosen cut-off points on the conclusions. The y-axis show the nominal Kruskal-Wallis p-value of the variable tested against the cut-off on the x-axis. Analysis from figure 1a (alpha diversity) and figure 1b (beta diversity) was performed on an equally spaced grid of 20 cut-offs from 0 to 1. As in figures 1a and 1b the hypothesis testing was performed against Eosinophil counts measured in submucosal tissue by EG2 stain and neutrophil counts from submucosal tissue stained by MPO.

Figure E5

Alpha and beta diversity in the BAL microbiomes according to sputum eosinophilic and neutrophilic inflammation

Alpha and beta diversity in the BAL microbiome as stratified by healthy (green) or asthmatic with 'eosinophil high' (blue), or 'eosinophil low' (red) in sputum. Alpha diversity was calculated as Shannon index (a measure of species richness and evenness) and beta diversity was calculated using the Whittaker index (a measure of the dissimilarity between communities). Significant differences between the groups were tested using a Kruskal-Wallis test with Dunn's corrections for multiple comparisons and indicated by a horizontal bar between the two different columns with stars denoting the p-values. Corrected p-values without significance are given for comparisons where the p-value was close to 0.05. ★, ★★, ★★★ denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively.

Figure E6

PCoA plot with asthmatics only, colored according to submucosal periostin staining intensity. ANOSIM analysis showed no separation of the samples due to submucosal periostin ($p=0.26$). Ellipses represent the 0.95 confidence level.

Figure E7. Alpha and beta diversity in the BAL microbiomes according to the level of periostin staining activity in submucosa

Alpha and beta diversity in the BAL microbiome as stratified by healthy (green) or asthmatic with 'periostin high' (blue), or 'periostin low' (red) in sputum. Alpha diversity was calculated as Shannon index (a measure of species richness and evenness) and beta diversity was calculated using the Whittaker index (a measure of the dissimilarity between communities). Significant differences between the groups were tested using a Kruskal-Wallis test with Dunn's corrections for multiple

comparisons and indicated by a horizontal bar between the two different columns with stars denoting the p-values. Corrected p-values without significance are given for comparisons where the p-value was close to 0.05. ★, ★★, ★★★ denotes $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

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