Title

Species-level, metagenomic and proteomic analysis of microbe-immune interactions in severe asthma *or*

Species-level, integrated metagenomic and proteomic analyses in severe asthma

Authors

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MFJ, TSCH, PHH, AC, PK, IDP contributed to conceptualisation and design of the protocol. MFJ, MT, GD, CB, LCKL, AC, TB, TS, PHH, TSCH contributed to acquisition of study data. Data were analysed by MFJ, NS, EM, TS, CB, AA, TSCH. MFJ, EM, NS have accessed and verified the data. MFJ and TSCH drafted this submission which was approved by all authors.

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Airways infection' is a common treatable trait in severe asthma. It drives neutrophilic inflammation, airway proteases and disrupts regulatory immune responses which may facilitate pathogen persistence and airway remodelling.

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Abstract

Rationale: The airway microbiome in severe asthma has not been characterised at species-level by metagenomic sequencing, nor have the relationships between specific species and mucosal immune responses in 'type-2 low', neutrophilic asthma been defined.

Objectives: Integrate species-level metagenomic data with inflammatory mediators to characterise prevalence of potentially pathogenic organisms and host immune responses.

Methods: Sputum and nasal lavage samples were analysed using long-read metagenomic sequencing with Nanopore and qPCR in two cross-sectional Severe Asthma Cohorts, Wessex (n=66) and Oxford (n=30). We integrated species-level data with clinical parameters and 39 selected airway proteins measured by immunoassay and O-link.

Measurements and Main results: In health and mild asthma sputum microbial diversity was preserved. By contrast, 23% (19/81) of severe asthma participants had microbiomes dominated by a single respiratory pathogen, namely *H. influenzae* (n=10), *M. catarrhalis* (n=4), *S. pneumoniae* (n=4) and *P. aeruginosa* (n=1). Neutrophilic asthma was associated with *H. influenzae, M. catarrhalis, S. pneumoniae* and *T. whipplei* with elevated type-1 cytokines and proteases; eosinophilic asthma with higher *M. catarrhalis*, but lower *H. influenzae*, and *S. pneumoniae* abundance. *H. influenzae* load correlated with TNF and IL-10. *M. catarrhalis* associated with IL-1β, tryptase and YKL40. *R. mucilaginosa* associated negatively with FGF. Bayesian network analysis also revealed close and distinct

relationships of *H. influenzae* and *M. catarrhalis* with type-1 airway inflammation. The microbiomes and cytokine milieu were distinct between upper and lower airways.

Conclusions: This species-level integrated analysis reveals central, but distinct associations between potentially pathogenic bacteria and airways inflammation in severe asthma.

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Keywords

Asthma, bacteria, microbiome, sputum, cytokine

Introduction

Asthma is a complex, heterogenous disease. Recognition of 'treatable traits' ascribed to specific pathophysiological subsets of severe asthma have supported significant therapeutic advances, particularly through biologics targeting interleukin (IL)-4, IL-5 and IL-13 in 'type-2 high' severe asthma[1]. However, 30-50% of severe asthmatics have 'type-2 low' disease refractory to these therapies or systemic corticosteroids[2], constituting an important, poorly-understood, clinical phenotype.

In type-2 low disease, emerging data implicate bacterial airways infection with neutrophilic infiltration driven by innate and adaptive immune responses, and supporting long-term macrolide therapy[2,3]. Recent molecular microbiological approaches have described airway microbial profiles in severe asthma, but early studies were restricted by sequencing approaches unable to assign species level taxonomic identification. These reported reduction in bacterial α-diversity, Proteobacterial enrichment, particularly *Haemophilus spp*. and *Moraxella spp.*[4-6] and relative depletion of *Gemella*, *Porphyromonas* and *Streptococcus* commensals[7]. *H. influenzae* is the commonest pathogen colonising the lower airway[8-10], whose presence is linked to commensal depletion, neutrophilic inflammation and poor asthma outcomes[3]. However, the prevalence of *H. influenzae* and other species is yet to be defined in severe asthma using a metagenomic approach resolved to species level.

We recently demonstrated metagenomic sequencing using Nanopore offers rapid, species-level taxonomic identification from sputum samples[10], providing lower cost library preparation and sequencing, real-time data acquisition, and longer

contiguous reads required for *de novo* genome reconstruction. In our pilot study *H. influenzae* was commonly detected in severe neutrophilic asthma[10]. We thus hypothesised that chronic bacterial airways infection is a common treatable trait in severe asthma, confined to the lower airway and associated with neutrophilic airways inflammation driven by type-1 cytokine responses. We aimed to define the prevalence of airways infection, by inflammatory phenotype, in a large severe asthma population at species level, using Nanopore metagenomic sequencing of induced sputum samples, and to discern inflammatory signals driving refractory airways inflammation through an integrated proteomic analysis. In addition, by comparing the upper and lower airway microbiome and proteome in severe asthma we question the 'one airway, one disease' paradigm. Some of the results of these studies have been previously reported in the form of an abstract[11].

Methods

Clinical cohorts

(1) Oxford Severe Asthma Cohort. Patients with American Thoracic Society/European Respiratory Society definition of severe asthma, on Global Initiative for Asthma (GINA) step 4-5 treatment (n=30) were recruited to the Oxford Airways Study between June 2018 and February 2020 (John Radcliffe Hospital, Oxford, UK) conducted with NHS Research Ethics approval (08/H0406/189). Participants underwent clinical phenotyping, sputum induction and phlebotomy during clinical stability. Hypertonic sputum induction and sputum differential cell counts were performed as previously described[12] and sputum plugs stored at -80°C in sterile Brain Heart Infusion (BHI, Sigma-Aldrich, Dorset, UK) broth containing 10% glycerol.

Sputum plugs were dispersed, and DNA extracted and sequenced (n=30) using established methods[10].

(2) Wessex Severe Asthma Cohort[12]. Participants with severe asthma, mild/moderate asthma and healthy controls were recruited (University Hospital Southampton and Queen Alexandra Hospital Portsmouth). The study was approved by Southampton and South-West Hampshire Research Ethics Committee A (09/H0502/37) and study protocol described elsewhere[13]. Metagenomic sequencing was performed on induced sputum (n=66) and nasal lavage (NL) (n=17), and sequencing data integrated with existing cytokine data from serum, sputum supernatant and nasal lavage[13].

Molecular microbiology

Established methods[10] were used for microbial DNA extraction from samples, and pathogen-specific qPCR and Nanopore sequencing performed and analysed as described in the online supplement.

Measurement of inflammatory mediators

Existing data on 37 inflammatory mediators, 8 metalloproteinases, and total and specific IgE were available from Hinks *et al.*[13], measured by enzyme-linked immunosorbent assays, fluoroenzyme immunoassays and cytokine bead array (Luminex®) (see online supplement).

In addition, O-link Uppsala, Sweden measured 92 inflammation-related proteins on sputum and nasal lavage using in-house Proseek Multiplex Inflammation I panel.

Statistical and bioinformatic analyses

Statistical analysis performed using Rstudio (Version 2022.12.0+353) and GraphPad Prism (Version 9). Data were logarithmically transformed if non-normal distribution. Parametric and nonparametric data are displayed as mean (SEM) and median (range), with comparisons between groups made using unpaired t test or Mann-Whitney test, respectively. For all analyses, 2-tailed P values <0.05 were considered significant. Where indicated, an overall 5% significance level was adjusted for multiple comparisons using the Benjamini-Hochberg procedure. Correlations were tested with the Spearman r statistic. Bioinformatic analyses are described in online supplement and GitLab:
https://gitlab.com/ModernisingMedicalMicrobiology/wessex_analysis.

Data availability

Raw FASTQ data are available on the European Nucleotide Archive with the project accession PRJEB62780.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Baseline participant characteristics are summarised in Table 1 (and Tables S1/S2). Nanopore sequencing was performed on 96 sputum and 17 nasal lavage samples. A further integrated analysis, incorporating clinical and cytokine data, was performed in the 66 participants from the Wessex cohort (Figure 1).

Nanopore sequencing defines a distinct species-level metagenome in severe asthma and identifies patients with airways infection

Z-scores of 30 bacterial species with greatest variability in abundance across health (HC), mild (MA) and severe asthma (SA) are displayed in Figure 2 and Figure S1. Hierarchical clustering revealed a cluster of 19/96 participants, each with severe asthma and highly skewed airway microbiome dominated by a single respiratory pathogen, namely *H. influenzae*, *M. catarrhalis* or *S. pneumoniae*. The remaining participants displayed greater microbial diversity with variable abundance of commensal organisms predominantly of phyla Actinobacteria, Bacteroidetes and Firmicutes.

The most conserved species in HC sputum (HC vs MA and SA, log fold change [LFC] >1, adjusted P<0.05) include *Streptococcus viridans, Streptococcus sp A12, Streptococcus australis* and *Schaalia odontolytica* (Table S3). In MA (MA vs HC and SA) 29 species, predominantly Firmicutes, were similarly identified, of which *Filifactor alocis, Mogibacterium diversum* and *Streptococcus sp. A12* were significantly enriched (Table S4). SA (SA vs HC and MA) is associated with a marked compositional shift and greater abundance of species including *Rothia mucilaginosa, Rothia dentocariosa, H. influenzae* and *M. catarrhalis* (Table S5), and relative depletion of oropharyngeal commensals (Table S5).

We defined airways infection – metagenomic dominance by a single airway pathogen – in an individual if a known respiratory pathogen (*H. influenzae*, *M. catarrhalis*, *S. pneumoniae* or *P. aeruginosa,* in this dataset) was the most abundant (%reads/total bacterial reads) species

and the proportion of detected reads (Table 2) and was ≥2-fold of the second ranked abundant species in the sample. Across the two cohorts, airways infection was present during clinical stability in 23% of severe asthmatics (n=19/81). *H. influenzae* (n=10) was most prevalent, followed by *M. catarrhalis* (n=4) and *S. pneumoniae* (n=4). In most (n=18/19) samples sequencing covered ≥1/5th of the reference genome, providing high confidence in species-level classification. *P. aeruginosa* was detected in one individual with a high proportion of total reads (73%) but lower genome coverage (5.1%). For semiquantitative pathogen-specific qPCR see Figure2/ Table 2. Bacterial load was quantified with 16S qPCR (Figure 2) and was higher in Oxford (median 364x10^6, IQR 823x10^6 copies/ml) versus Wessex sputum samples (23x10^6 copies/ml, IQR 83x10^6 copies/ml).

Sputum inflammatory phenotype is predictive of the presence of airway pathogens

Airways infection in severe asthma (visualised with Sankey plots in Figure 3 and Figure S3) was associated with a higher Proteobacteria:Firmicutes ratio (P<0.001, Figure S4) consistent with previous reports[9,14], but no significant differences were observed between SA and HC or MA, or across inflammatory phenotypes. This is consistent with Proteobacterial species *H. influenzae* and *M. catarrhalis* being the most prevalent airway pathogens in severe disease. Airways neutrophilia was associated with dominance of *H. influenzae, M. catarrhalis*, *S. pneumoniae* and *P. aeruginosa* in severe asthma sputum, with higher overall sputum neutrophil count (Figure 4B, P<0.05) and higher prevalence of neutrophilic asthma (Table S2, P<0.05).

Defining bacterial species associated with severe asthma inflammatory phenotypes were sought (LFC>1, adjusted P<0.05). Consistent with the above findings, *H. influenzae, M. catarrhalis, T. whipplei* and *S. pneumoniae* were most strongly associated with neutrophilic asthma, whilst commensal *Streptococcus spp.* (Table S6) including *S. salivarius, S. intermedius, S. oralis* are relatively depleted (LFC< 1, adjusted P<0.05). In paucigranulocytic disease, in contrast, there is lower *M. catarrhalis, H. influenzae* and *T. whipplei* abundance (Table S6). Eosinophilic asthma was characterised by high relative abundance of *M. catarrhalis, S. intermedius* and *V. parvula* and less *H. influenzae, S. pneumoniae* and *Actinomyces pacaensis*, a relatively newly-isolated Gram-positive organism from the human respiratory microbiome[15] (Table S6). No organisms were uniquely defined the mixed inflammatory phenotype.

Integrated cytokine and metagenomic analyses

We integrated sputum metagenomes in the Wessex cohort (n=66) with existing cytokine data[13](Figure 4A). Unsupervised clustering identified healthy controls and mild asthma with higher expression of FGF, IL-1RA, IL-2 and IL-4 relative to severe asthma clusters. Amongst severe asthma two clusters are appreciated. One is associated with predominantly proinflammatory and type 1 cytokines (left) including IL-1α, IL-1β, TNF, IFN-γ, MMP8, MMP9, MPO, YKL40, IL-17, IL-6, elastase, ECP, IL-8, GROα, G-CSF, eotaxin and TIMP-1, typically in presence of airways infection. The second cluster (right) represents a mixture of sputum inflammatory phenotypes, without infection, with high expression of MMPs.

To further dissect the mucosal immune response to individual species we integrated these data, clustering (hierarchical) by species and by cytokine (Figure 5),

selecting the 50 species with greatest variance in abundance across subjects (listed with correlation data in Supplementary file 1). The strongest positive correlations were between *H. influenzae* and both TNF (r=0.4, P≤0.01) and IL-10 (r=0.5, P≤0.01); and between *M. catarrhalis* and IL-1β (r=0.4, P≤0.05), tryptase (r=0.5, P≤0.01) and YKL40 (r=0.4, P≤0.01) implying species-specific immune modulation. Negative correlations between predominantly Gram-positive oropharyngeal/respiratory commensals (top right species cluster Figure 5) and pro-inflammatory cytokines were also identified, exemplified using markers of neutrophilic inflammation – IL-8, YKL40 and MPO, which associated negatively (r<-0.3, P<0.05) with Gram-positives *M. diversum, S. milleri* and *Streptococcus sp. A12* – whereas *Streptococcus sp. LPB0220* correlated negatively most with elastase (r=-0.6, P≤0.001). TIMP-1 and MMP8 correlated negatively with a group of Gram-positive and negative commensals, whose abundance also tended towards association with immunoregulatory molecules IL-1RA and FGF, although statistically significant only with *Streptococcus sp. A12* for both (r=0.4, P<0.05) and *M. diversum* for FGF (r=0.5, P<0.01). Conversely *R. mucilaginosa*, a species with greatest overall detected reads in severe asthma (Table S5), is linked to low FGF levels (r=-0.6, P< 0.001). *Treponema sp. OMZ 838* correlated positively with IL-13 (r=0.4, P<0.05).

Neutrophilic airways infection is accompanied by elevated type-1 cytokine and protease activity, elevated IL-10 and deficiency of IL-1RA

Using the above pragmatic sequencing definition of airways infection, we dichotomised severe asthma by presence or absence of airways infection. Airways infection was associated with sputum neutrophilia (Figure 4B, P<0.05) accompanied by significantly elevated type-1 cytokines and airway proteases, namely: elastase,

TNF, IL-1βα, MIP-1β, MMP8, ECP, YKL40, MPO, MIP-1α, IL-6SR and MMP9 (Figure 4C, P<0.05 and Table S7). Of the immunomodulatory mediators IL-1RA was deficient, whereas TIMP-1 and IL-10 levels were higher in the presence of airways infection (Figure 4D, P<0.05 and Table S7).

Bayesian network analysis in severe asthma

To explore interactions between infection-associated cytokines (n=15), clinical parameters (n=20), and species most differentially-abundant in severe asthma (n=25), we generated a Bayesian network in 51 severe asthmatics (Figure 6), depicting probabilistic dependencies using machine learning. The analysis retained 34/60 parameters in the model (Table S8). Associations are observed between *H. influenzae* or *M. catarrhalis* abundance and infection status, and between cytokines linked to airways infection. In addition, close relationships appear between groups of bacteria, most notably species belonging to phylum Firmicutes, and *Neisseria* spp. The only clinical parameter linked to the microbiome was a non-linear relationship between *M. catarrhalis* and FeNO (Figure S5a).

This approach again highlights associations between sputum IL-10 and *H. influenzae* abundance (Figure S5b, r=0.32, P<0.05), and IL-10 and TNF (Figure S5c, r=0.62, P<0.0001). The macrophage chemoattractant MIP1α is highly connected within the network (Figure 6 and Figure S5) specifically to complementary chemokine MIP1β (r=0.88, P<0.0001), alongside IL-6SR (r=0.65, P<0.0001), TIMP-1 (r=0.65, P<0.0001) and IL-8 (r=0.73, P<0.0001). Sputum YKL40 is strongly linked to MPO (r=0.92, P<0.0001), IL-6SR (r=0.75, P<0.0001) and IL-1β (r=0.63, P<0.0001). Neutrophilic mediators MPO and IL-8 also strongly correlated (r=0.85, P<0.0001).

Distinct microbiomes and cytokine milieus between upper and lower airways in severe asthma

Paired nasal lavage and sputum samples were collected in severe asthma (n=17), with relative abundances of shared bacterial species between the upper and lower airways shown in Figure 7A. Samples and species clustered distinctly by anatomical location. In severe asthma, nasal lavage was enriched in *S. epidermidis* and *S. aureus*, with relatively higher abundance of *D. pigrum*, *M. catarrhalis* and *E. coli* compared to sputum. Sputum was enriched for Firmicutes (predominantly *Streptococcal spp*.) with greater relative abundance of *H. influenzae* and *H. parainfluenzae*.

The cytokine milieu in the upper and lower airway was compared using paired O-link proteomics from nasal lavage and sputum samples (Figure 7B). Proteins were discordant between nasal lavage and sputum compartments (that is, proteins most strongly correlate with others from the same sample type), with the exception of significant associations between sputum cytokines IL-20, FGF5 and IL-2 and specific nasal lavage proteins (described further in Supplementary Results).

Discussion

Using Nanopore sequencing we provide the first large-scale, species-level definition of the airway microbiome in severe asthma. We identified presence of 'airways infection', characterised by dominance of a single pathogenic bacterial species, and associated mucosal inflammation, in 20-30% of individuals during stable disease. Such infection occurs with a limited repertoire of species: *H. influenzae* being the most prevalent, followed by *M. catarrhalis* and *S. pneumoniae*. Whilst these

metagenomic measures do not imply tissue invasion by bacteria, we identified strong correlation between such dominance of potentially pathogenic bacteria and evidence of airway neutrophilia and mucosal inflammation beyond that expected by simple commensal colonisation. Using novel supervised and unsupervised approaches our integration of species-level metagenomics with cytokine and protein datasets shows, for the first time, consistent evidence of type-1 inflammatory responses accompanied by neutrophilia in airways infection. This coincides with commensal depletion (largely Firmicutes), previously linked to worse asthma outcomes[3]. Finally, we demonstrate that the lower airway microbiome remains distinct from the upper airways in severe asthma, with anatomical compartmentalisation of the local cytokine milieu.

Just three species dominate the airway microbiome in severe asthma, and their presence observed in both neutrophilic and non-neutrophilic phenotypes likely explains the striking clinical efficacy of long-term, low dose azithromycin therapy in both phenotypes[16], but most notably in those with greatest *H. influenzae* abundance, measured by qPCR[17]. Mechanistically, whilst azithromycin is primarily antibacterial, additional anti-viral and anti-inflammatory effects have been reported. However, antimicrobial resistance with widespread use of azithromycin is a significant concern[18], and if the antibacterial effect is the dominant mechanism, this could be addressed through targeting therapy to those with airways infection identified through molecular techniques. We have previously reported that Nanopore sequencing identifies clinically-relevant airway pathogens and shows agreement with sputum culture and semi-quantitative pathogen specific qPCR[10].

In this present study similar consistency is seen between airways infection identified by Nanopore sequencing and a positive pathogen specific qPCR result for

354 *H. influenzae*, *S. pneumoniae*, *M. catarrhalis* and *P. aeruginosa* in samples from the
355 Oxford cohort. The lower, 73% concordance between sequencing and qPCR results
356 in the Wessex samples may be attributable to age-related sample degradation in this
357 historical cohort, as total 16S load was >15-fold lower in Wessex than Oxford
358 samples. Overall, for operational reasons, qPCR on selected pathogens may offer
359 the most accessible and accurate means of identifying airways infection for targeted
360 therapy in routine clinical practice.

361 Nanopore sequencing provides further insight into microbiome of distinct
362 sputum inflammatory phenotypes. In addition to *H. influenzae, M. catarrhalis* and *S.*
363 *pneumoniae*, *T. whipplei* was strongly associated with neutrophilic asthma. This
364 predominantly intracellular bacterium causes chronic multi-system inflammation in
365 Whipple's disease and may play a role in airways disease. *T. whipplei* has been
366 detected in up to 40% of severe asthmatics, linked to both eosinophilic[8] and
367 corticosteroid resistant inflammation[19] albeit in smaller cohorts. Its ability to grow
368 within acidic vacuoles, induce M2 macrophage polarisation and apoptosis are
369 virulence traits capable of promoting chronic infection[20]. The asthmatic airway is
370 susceptible to persistent infection with intracellular bacteria, most commonly non-
371 typeable strains of *H. influenzae*[2] (NTHi) which facultatively enter macrophages and
372 airway epithelial cells, and the high dose ICS exposure may also render mucosa
373 susceptible to *T. whipplei*. Further research is needed to determine if specific innate
374 immune deficiencies promote this intracellular infection. The microbiome in
375 eosinophilic airways inflammation varies with disease severity and in severe asthma
376 displays high relative abundance of Firmicutes[14]. Interestingly, the Proteobacteria *M.*
377 *catarrhalis* was a defining species of eosinophilic asthma in this study, alongside

Firmicutes *S. intermedius* and *V. parvula*. BNA linked *M. catarrhalis* abundance to FeNO level (Figure S5), which has been shown to correlate negatively with Proteobacteria abundance[14]. Integrated metagenome and cytokine analyses showed *M. catarrhalis* strongly correlated with IL-1β, YKL40 and tryptase in keeping with studies demonstrating a TLR2 and NF-κB dependent neutrophilic inflammatory response[21] and mast cell induction[22] following *M. catarrhalis* infection, suggesting this pathogen may drive concurrent type-1/2 responses, besides innate eosinophil and mast cell responses.

In SA, notably in absence of airways infection, there is greater abundance of *Rothia* spp., previously detected in mild non-eosinophilic asthma[23] and bronchiectasis, where abundance negatively correlated with IL-8, IL-1β, MMP-1, MMP-8 and MMP-9 in sputum[24]. Of these species, *R. mucilaginosa* is most often detected in chronic lung diseases and can inhibit NF-κB activation in lung epithelium *in vitro*[24]. We saw no relationship between *R. mucilaginosa* and pro-inflammatory markers, but a strong negative correlation was evident with FGF which also correlated positively with oropharyngeal commensals. FGFs are a diverse group of growth factors exerting pleiotropic effects via tyrosine kinase receptors. FGF-2 promotes bronchial smooth muscle hyperplasia, shows higher expression in asthma, and together with FGF-1 stimulates MAPK dependent VEGF production, inhibited by azithromycin and dexamethasone[25]. Thus, microbial composition could influence airway remodelling processes further manipulated by corticosteroids and macrolides.

Integration of metagenome and inflammatory mediators, taken together with the BNA offer additional novel insights. Overall, a pattern emerges linking airways infection and type-1 inflammation (IL-1β, TNF) with neutrophilic infiltration (IL-8,

YKL40), release of neutrophil granule proteins and proteases (MMP8, MMP9, MPO and elastase), counter regulatory mechanisms (TIMP-1, IL-10), and macrophage recruitment (MIP1α and MIP1β) which could be facilitated through IL-6 signalling (IL-6SR). *H. influenzae* strongly correlated with TNF and the anti-inflammatory cytokine IL-10, consistent with data in COPD[26]. Co-production of IL-10/TNF, induced via MyD88 dependent TLR activation following NTHi infection, may offer a regulatory response, and *H. influenzae* could harness excess IL-10 secretion as a virulence strategy. This could be explored further in murine studies.

IL-1RA and TIMP-1 were differentially regulated in airways infection. IL-1RA, an antagonist to IL-1β and its receptor, was reduced in airways infection, consistent with reduced IL-1RA:IL-1β ratio reported in neutrophilic asthma[27], potentially representing IL-1β quenching during infection. IL-1RA is upregulated in steroid-naïve atopic asthma[28], with IL-1RA polymorphisms increasing asthma risk, whilst ICS dampens IL-1RA production, as supported by relatively higher concentrations in healthy/mild asthmatic sputum (Figure 4A). TIMP-1, also elevated in airways infection, possesses antiprotease activity, and MMP-9:TIMP-1 ratio is reduced in asthma and chronic bronchitis[29], correlating inversely with airflow obstruction, with TIMP-1 polymorphisms associated with female asthma[30]. Thus, whilst TIMP-1 can block proteolytic activity, in excess it might reduce extracellular matrix turnover resulting in remodelling. IL-10, IL-1RA and TIMP-1 are thereby integral to complex regulatory networks with likely differing roles dependant on the wider inflammatory landscape. This could be better characterised through dedicated transcriptomic and proteomic studies in well phenotyped severe asthmatics.

We have shown that the 'one airway, one disease' hypothesis does not apply to the airway microbiome in severe asthma where distinct microbial niches are maintained in the nasopharynx and lower airway. This is consistent with mild atopic asthma and health. The nasal microbiome can be informative and has been shown to predict exacerbation risk in paediatric asthma when dominated by *Moraxella* species, but nasopharyngeal samples should not be used as a replacement for lower airway sampling in stable state or exacerbation[31]. We found the cytokines IL-20[32] and IL-2[33] in sputum, which have been linked to airway remodelling and allergic inflammation in asthma respectively, to correlate with nasal proteins involved in diverse cellular responses. The importance of cross-signalling between these compartments is not known but of interest given the burden of comorbid sinonasal disease in severe asthma.

This study had some limitations. Samples from the Wessex cohort are from a pre-biologic era with widespread maintenance oral corticosteroid use which may confound detection of sputum inflammatory mediators. Sample collection and storage protocols can affect quality of metagenomic data, particularly in low biomass samples, and we suspect some storage-related DNA degradation in Wessex samples. This study would be strengthened by longitudinal data spanning exacerbations. Sequencing depth was inadequate for reliable inferences about antimicrobial resistance patterns or specific strains, though this could be achieved by probe-based enrichment for pathogen specific sequences enabling deeper sequencing.

In conclusion, we show that airways infection is a common treatable trait in severe asthma, present in 20-30% of patients, most often with *H. influenzae*. It is

accompanied by neutrophilic inflammation, elevated airway proteases and disruption of counterregulatory immune responses which could facilitate pathogen persistence and airway remodelling.

Data sharing

Raw FASTQ data are available on the European Nucleotide Archive with the project accession PRJEB62780. Remaining data analysed and presented in this study are available from the corresponding author on reasonable request, providing the request meets local ethical and research governance criteria after publication. Patient-level data will be anonymised.

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Figure legends

Table 1: Clinical characteristics of participants

Table 2: Airways infection in severe asthma

Figure 1 Study design (Created with BioRender.com)

Figure 2 Relative abundance of species in sputum: Heatmap of relative abundance of species from induced sputum sequenced samples using ONT; Z-scores, denoted by shade, represent the number of standard deviations above the mean number of reads per taxon for each sample. Cohorts include Wessex (WES; Healthy [H]: n=5, Mild [M]: n=10 and Severe asthma [S]: n=51) and Oxford (OX; Severe asthma[S]: n=30). Clustering by species (rows) and individuals (columns) using Euclidian distance performed independently on samples and most differentially abundant bacterial species. Sputum inflammatory phenotypes are eosinophilic (E), neutrophilic (N), mixed granulocytic (M) and paucigranulocytic (P). Positive pathogen specific PCR results (>1x106copies/ml) are indicated in black: S. aureus (Saur), P. aeruginosa (PsA), S. pneumoniae (Spneu), M. catarrhalis (Mcat) and H. influenzae (Hinf). Total eubacteria (16S) PCR load is denoted by shade (x106 copies/ml).

Figure 3 Taxonomic profiles in sputum in severe asthma: Sankey visualisation of taxonomic profiles in severe asthma in the absence or presence of airways infection with a single dominant pathogen using Pavian; Kraken reports combined from patients with no single dominant organism on metagenomic sequencing from (A)

Wessex (n=40) and (E) Oxford (n=22) cohorts for visualisation. Skewed taxonomic profiles in the presence of airways infection dominated by (B)+(F) H. influenzae ([B] Wessex, n= 6; [F] Oxford, n=4), (C) M. catarrhalis (Wessex, n=3) and S. pneumoniae (Wessex, n=2) have been generated using the same method

Figure 4 Integrated analyses of microbiome and inflammatory mediators in sputum; Integrated cytokine analysis (A) Heatmap of scaled and centralised sputum cytokines in health, mild and severe asthma. Z-score centralised cytokine measurements denoted by shade. Independent clustering performed by subject (columns) and cytokine (rows). Clustering distances shown are representative of correlation distances. In severe asthma, presence of a dominant pathogenic organism on metagenomic sequencing is indicated as HI (H. inf), MC (M. cat) and SP (S. pneu); Airways infection is associated with (B) sputum neutrophilia, (C) elevated type-1 cytokines and (D) reduced anti-inflammatory mediators in sputum supernatant. Median/IQR shown –/+ Airways infection ([C] Mann-Whitney test, [D]/[E] unpaired t-test, adjusted for multiple comparisons with Benjamini–Hochberg procedure [FDR 0.05]. *P≤0.05, **P≤0.01).

Figure 5 Heatmap displaying correlations between integrated sputum microbiome and cytokine data; The 50 most variable bacterial species across all subjects were selected (listed in Supplementary file 1). Correlation coefficients between species and cytokines denoted by shade. Independent clustering performed by cytokine (columns) and species (rows).

Figure 6 Integrated Bayesian Network Analysis: Bayesian network showing strongest interactions between most differentially abundant bacterial species, inflammatory mediators, and clinical features in severe asthma (WES, n=51). Nodes are coloured by class: bacterial species (blue), infection status (red), inflammatory mediators (green) and clinical features (orange). Nodes without strong interactions have been excluded. Line thickness represents strength of interaction (Euclidean distance).

Figure 7 Upper and lower airway microbiome and cytokines; (A) Heatmap of relative abundances of bacterial species on metagenomic sequencing of paired nasal lavage (NL) and sputum (SP) samples from severe asthmatics (n=17). Z-score denoted by shade. Independent clustering by samples (columns) and 23 most variable species (rows) using Euclidian distance. (B) Correlation matrix of the most significantly correlated (P<<0.001) proteins in paired nasal lavage (NAS) and sputum (SPU) cytokines (O-link). Significantly correlated pairs displayed (p<<0.001, adjusted for multiple comparisons with Benjamini–Hochberg procedure [FDR 0.05]).

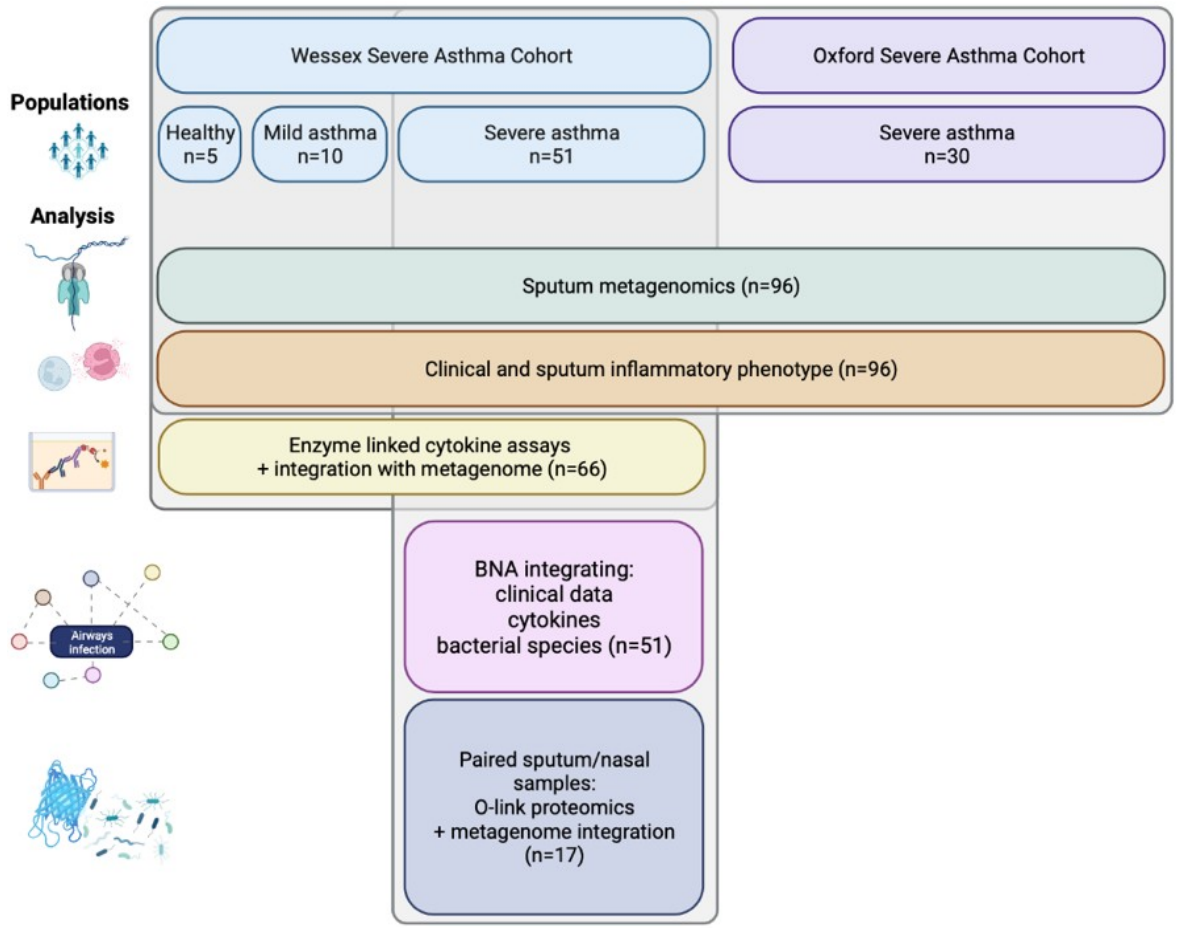
Footnotes

Table 1: BMI, body mass index; FEV1, Forced expiratory volume in 1s; FVC, Forced vital capacity; FeNO, exhaled nitric oxide; Inflammatory phenotypes: eosinophilic >3% sputum eosinophils, neutrophilic >61% sputum neutrophils and <3% eosinophils, mixed granulocytic >61% sputum neutrophils and >3% eosinophils, paucigranulocytic <61% sputum neutrophils and <3% eosinophils; SD, standard deviation; Q1, quartile 1; Q3, quartile3; na, not applicable; nd, no data

Table 2: *Patients with severe asthma from Wessex and Oxford cohorts demonstrating dominance of single pathogenic organism using Nanopore. †Proportion of total bacterial reads occupied by indicated pathogenic organism (represented as bar chart). ‡Coverage breadth (percentage of reference genome covered, represented as bar chart). §Average coverage depth (at positions with ≥1 read). ||Clinically significant (≥1x10^6 copies/ml) PCR result shown in bold. Inflammatory phenotypes: eosinophilic >3% sputum eosinophils, neutrophilic >61% sputum neutrophils and <3% eosinophils, mixed granulocytic >61% sputum neutrophils and >3% eosinophils, paucigranulocytic <61% sputum neutrophils and <3% eosinophils. ND, no data (not performed)

	Wessex			Oxford
Characteristic	**Healthy (n=5)**	**Mild Asthma (n=10)**	**Severe Asthma (n=51)**	**Severe asthma (n=30)**
Male sex, n (%)	3 (60)	5 (50)	22 (43)	14 (46.7)
Age (years), Mean (SD)	29 (12.3)	44.1 (15.9)	49.1 (14.7)	59.5 (15.0)
BMI (kg/m^2), Mean (SD)	25 (4.4)	27.5 (6.0)	32.2 (8.1)	29.2 (6.2)
Presence of atopy, n (%)	0 (0)	5 (50)	35 (69)	6 (20.0)
Presence of nasal polyps, n (%)	0 (0)	0 (0)	6 (12)	10 (33.3)
Smoking status, n (%)				
Never	4 (80)	5 (50)	27 (53)	22 (73.3)
Current	0 (0)	1 (10)	17 (33)	1 (3.3)
Ex-smoker	1 (20)	4 (40)	7 (14)	7 (23.4)
Pack years, Mean (SD)	7.5 –	1 (1,2)	8.4 (13.0)	12 (10)
Basline inhaled corticosteroid use (BDP eq., mcg/d), Median (Q1,Q3)	na na	300 (0, 400)	2080 (1800, 3575)	2000 (1600, 2000)
Maintenance oral corticosteroid, n (%)	na na	0 (0)	19 (37)	4 (13.3)
Unscheduled GP/ hospital visits in 12 months, Median (Q1,Q3)	na na	0 (0, 0)	3 (0, 7)	2 (0,6)
FEV1 (%predicted), Mean (SD)	96 (9.8)	107 (12.8)	73.6 (24.5)	73.8 (20.6)
FEV1/FVC, Mean (SD)	1 (0.2)	0.8 (0.2)	0.6 (0.1)	0.6 (0.1)
FeNO (ppb), Median (Q1,Q3)	11 (8, 11)	31 (16, 45)	10 (7, 17)	38 (18, 64)
Blood eosinophils (x10^9/L), Median (Q1,Q2)	nd nd	0.4 (0.2, 0.4)	0.2 (0.1, 0.3)	0.4 (0.2, 0.7)
Sputum eosinophils (%), Median (Q1,Q3)	1.1 (0.9, 3.3)	2.2 (1.2, 3.1)	1 (0.3, 7.7)	4.5 (1.0, 42.2)
Sputum neutrophils (%), Median (Q1,Q3)	31 (20.2, 37.3)	23.9 (16.7, 31.7)	51.8 (28.5, 76.1)	38.1 (17.0, 86.0)
Sputum Inflammatory phenotype, n (%)				
Eosinophilic	1 (20)	1 (10)	13 (25.5)	11 (36.7)
Neutrophilic	0 (0)	0 (0)	13 (25.5)	6 (20.0)
Mixed granulocytic	0 (0)	0 (0)	3 (6)	1 (3.3)
Paucigranulocytic	2 (40)	7 (70)	20 (39)	4 (13.3)
No data	2 (40)	2 (20)	2 (4)	8 (26.7)

Cohort	PID*	Dominant Pathogen	%Pathogen/ bacterial reads†	Length of reference genome (bases)	Coverage breadth of reference genome (%)‡	Average coverage depth§	Pathogen specific PCR (x10^6 copies/ml)‖	Sputum inflammatory phenotype	Clinical sputum culture result
Oxford	OAS1076	*Haemophilus influenzae*	77	1.83E+06	92.9	11.0	**179.9**	Neutrophilic	*H. influenzae*
Oxford	OAS1021	*Haemophilus influenzae*	93	1.83E+06	74.1	2.1	**175.4**	Neutrophilic	URT flora
Oxford	OAS1102	*Haemophilus influenzae*	92	1.83E+06	89.9	8.0	**135.6**	Neutrophilic	*H. influenzae*
Oxford	OAS1145	*Haemophilus influenzae*	22	1.83E+06	88.6	4.5	**83.9**	ND	ND
Oxford	OAS1041	*Moraxella catarrhalis*	59	1.86E+06	19.1	3.8	**4.3**	Neutrophilic	URT flora
Oxford	OAS1047	*Streptococcus pneumoniae*	57	2.04E+06	83.0	2.6	**28.3**	Neutrophilic	*S. pneumoniae*
Oxford	OAS1065	*Streptococcus pneumoniae*	63	2.04E+06	56.5	1.7	**15.9**	ND	*S. pneumoniae*
Oxford	OAS1017	*Pseudomonas aeruginosa*	73	6.26E+06	5.1	1.0	**4.6**	ND	URT flora
Wessex	MRCSAC0142	*Haemophilus influenzae*	78	1.83E+06	92.6	14.5	**6.2**	Neutrophilic	ND
Wessex	MRCSAC0115	*Haemophilus influenzae*	54	1.83E+06	52.8	1.5	**5.7**	Neutrophilic	ND
Wessex	MRCSAC0130	*Haemophilus influenzae*	17	1.83E+06	65.5	2.0	**4.8**	Mixed	ND
Wessex	MRCSAC0098	*Haemophilus influenzae*	93	1.83E+06	88.8	3.5	**2.1**	Neutrophilic	ND
Wessex	MRCSAC0151	*Haemophilus influenzae*	25	1.83E+06	47.3	1.5	0.8	Eosinophilic	ND
Wessex	MRCSAC0150	*Haemophilus influenzae*	72	1.83E+06	20.0	1.2	0.5	Eosinophilic	ND
Wessex	MRCSAC0101	*Moraxella catarrhalis*	79	1.86E+06	95.2	4.3	**33.8**	Eosinophilic	ND
Wessex	MRCSAC0132	*Moraxella catarrhalis*	92	1.86E+06	92.6	3.7	**18.8**	Neutrophilic	ND
Wessex	MRCSACC001	*Moraxella catarrhalis*	28	1.86E+06	23.5	1.2	**18.6**	Neutrophilic	ND
Wessex	MRCSAC0135	*Streptococcus pneumoniae*	93	2.04E+06	92.8	4.3	**18.7**	Paucigranulocytic	ND
Wessex	MRCSAC0104	*Streptococcus pneumoniae*	79	2.04E+06	79.0	2.4	0.7	Neutrophilic	ND


Populations
Analysis
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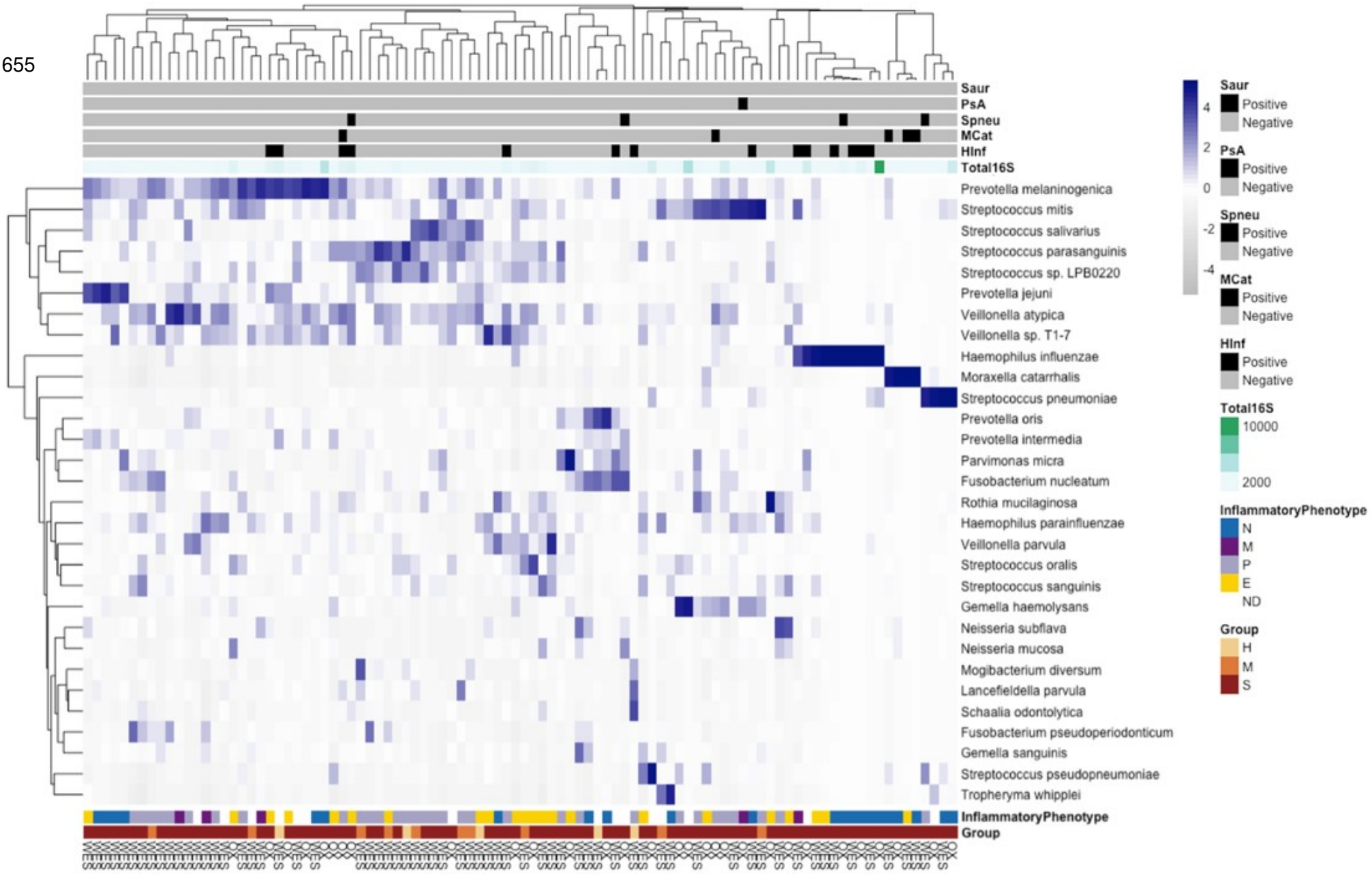
Oxford Severe Asthma Cohort
Healthy n=5
Mild asthma n=10
Severe asthma n=51
Severe asthma n=30
Sputum metagenomics (n=96)
Clinical and sputum inflammatory phenotype (n=96)
Enzyme linked cytokine assays + integration with metagenome (n=66)
BNA integrating: clinical data cytokines bacterial species (n=51)
Airways infection
Paired sputum/nasal samples: O-link proteomics + metagenome integration (n=17)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Spneu
MCat
HInf
Total16S
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Prevotella jejuni
Veillonella atypica
Veillonella sp. T1-7
Haemophilus influenzae
Moraxella catarrhalis
Streptococcus pneumoniae
Prevotella oris
Prevotella intermedia
Parvimonas micra
Fusobacterium nucleatum
Rothia mucilaginosa
Haemophilus parainfluenzae
Veillonella parvula
Streptococcus oralis
Streptococcus sanguinis
Gemella haemolysans
Neisseria subflava
Neisseria mucosa
Mogibacterium diversum
Lancefieldella parvula
Schaalia odontolytica
Fusobacterium pseudoperiodonticum
Gemella sanguinis
Streptococcus pseudopneumoniae
Tropheryma whipplei
InflammatoryPhenotype
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-4
Saur
Positive
Negative
PsA
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Negative
HInf
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P
E
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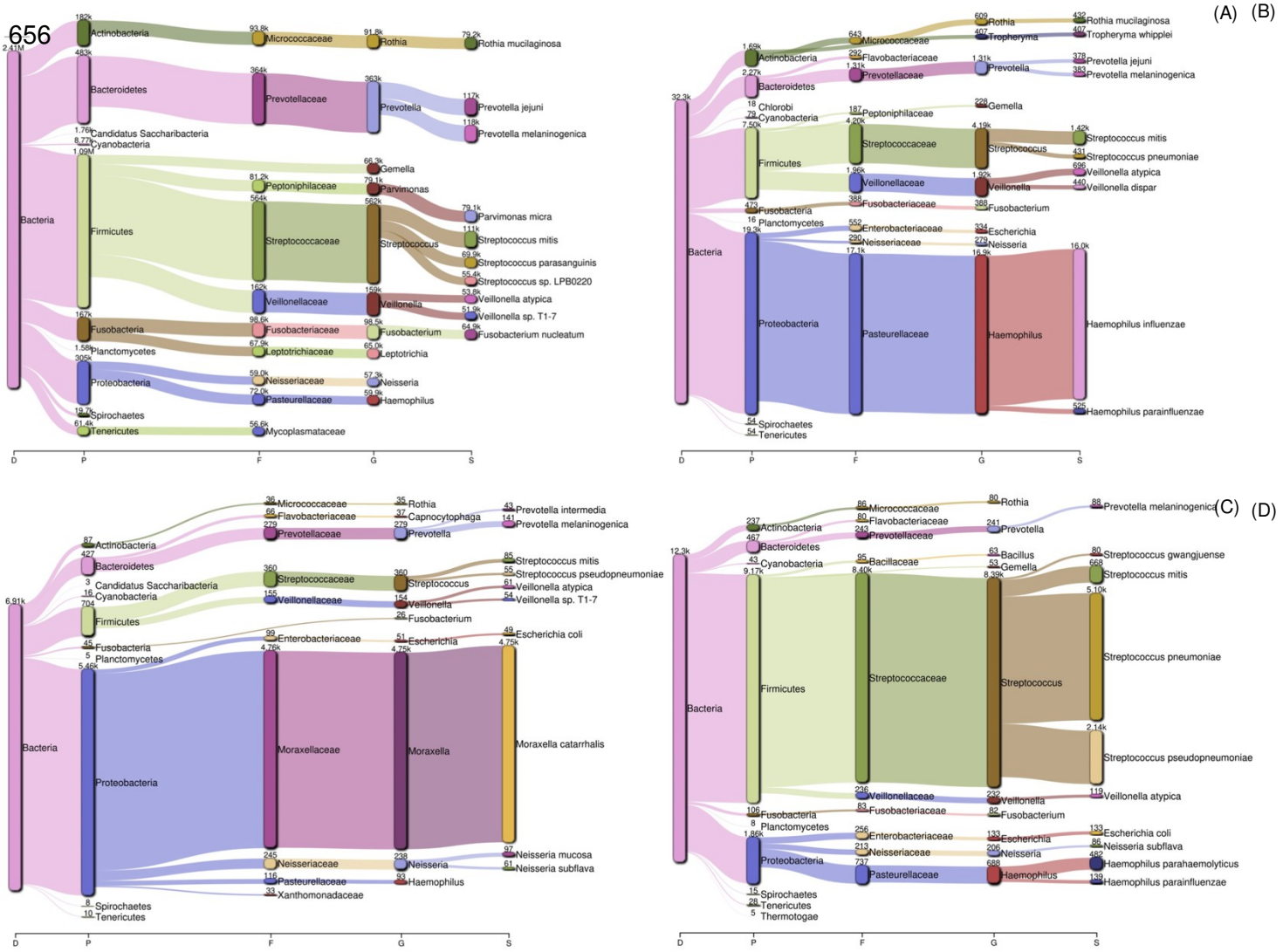
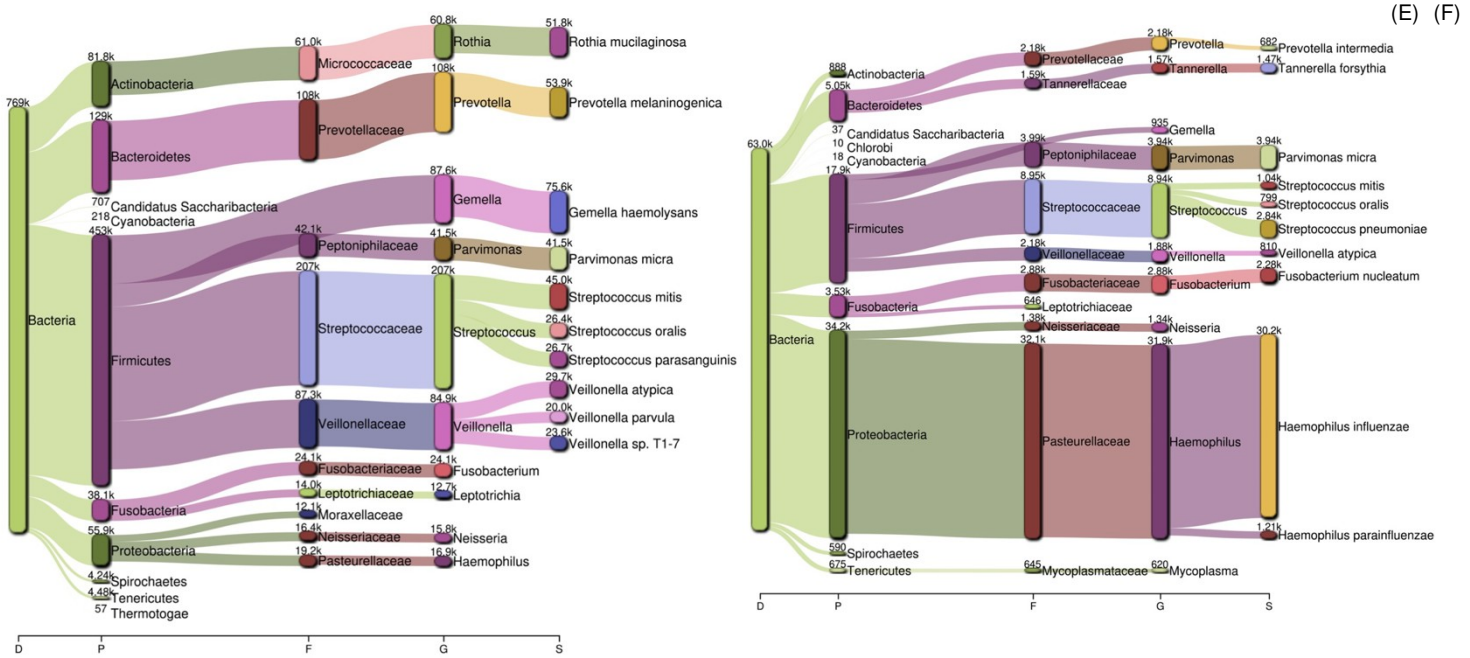
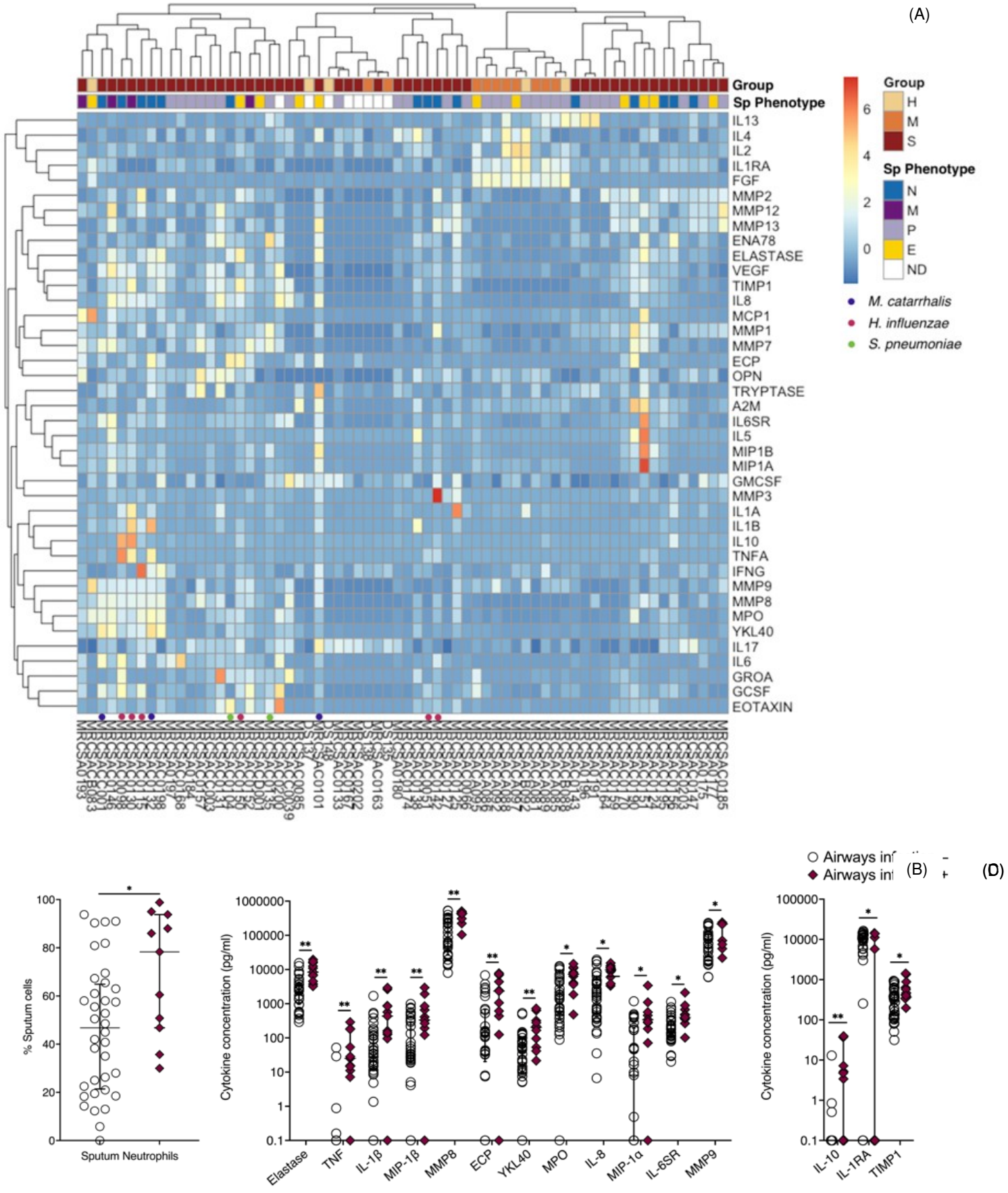
Group
H
M
S

Wessex Severe Asthma Cohort



Oxford Severe Asthma Cohort

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MMP13
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VEGF
TIMP1
IL8
MCP1
MMP1
MMP7
ECP
OPN
TRYPTASE
A2M
IL6SR
IL5
MIP1B
MIP1A
GMCSF
MMP3
IL1A
IL1B
IL10
TNFA
IFNG
MMP9
MMP8
MPO
YKL40
IL17
IL6
GROA
GCSF
EOTAXIN
Group
H
M
S
Sp Phenotype
N
M
P
E
ND
M. catarrhalis
H. influenzae
S. pneumoniae
(B)
(D)
% Sputum cells
Sputum Neutrophils
Cytokine concentration (pg/ml)
Elastase
TNF
IL-1β
MIP-1β
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ECP
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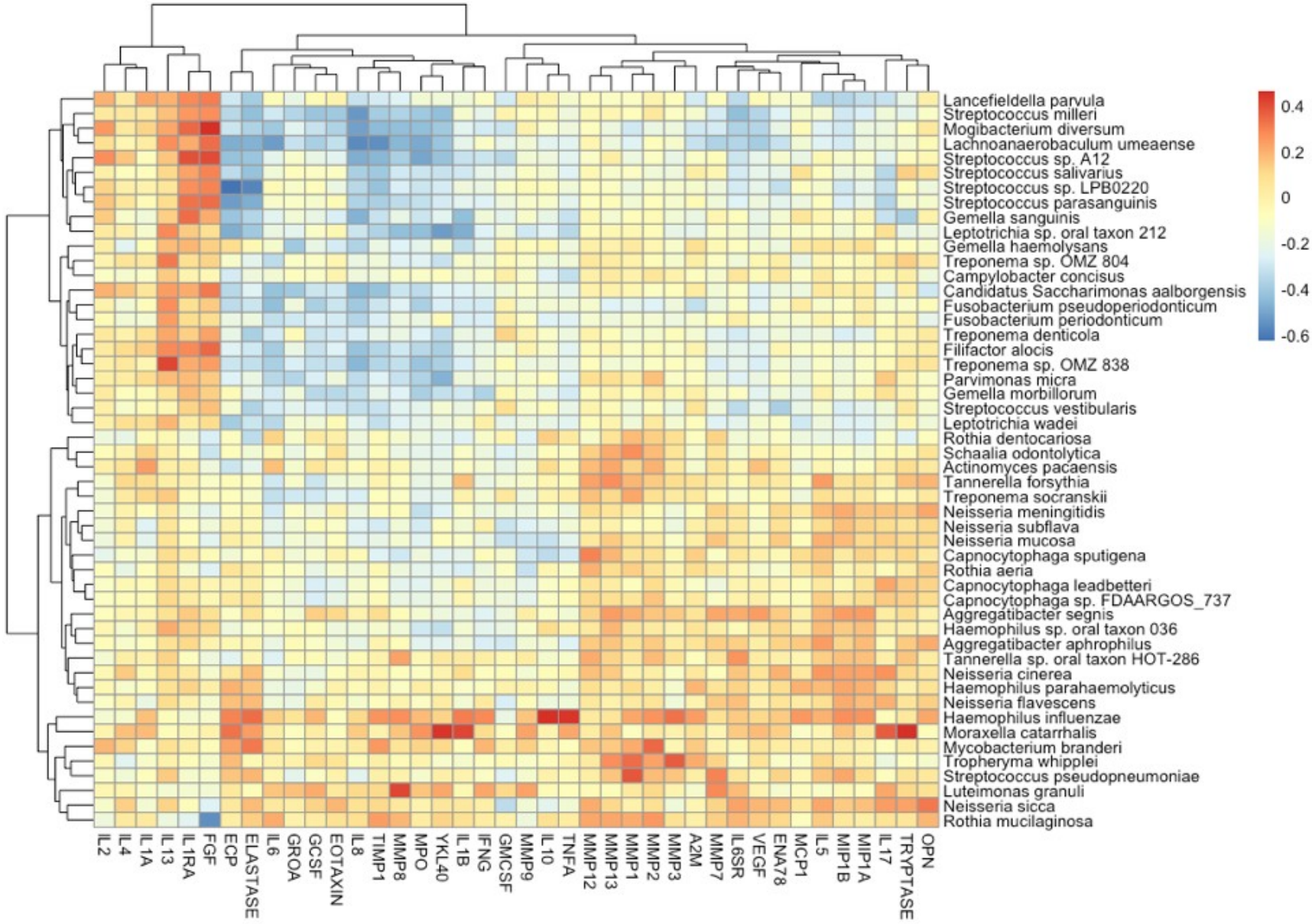
IL-10
IL-1RA
TIMP1

658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Campylobacter concisus
Candidatus Saccharimonas aalborgensis
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Fusobacterium periodonticum
Treponema denticola
Filifactor alocis
Treponema sp. OMZ 838
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Gemella morbillorum
Streptococcus vestibularis
Leptotrichia wadei
Rothia dentocariosa
Schaalia odontolytica
Actinomyces pacaensis
Tannerella forsythia
Treponema socranskii
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Neisseria subflava
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Rothia aeria
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Haemophilus influenzae
Moraxella catarrhalis
Mycobacterium branderi
Tropheryma whipplei
Streptococcus pseudopneumoniae
Luteimonas granuli
Neisseria sicca
Rothia mucilaginosa
IL2
IL4
IL1A
IL13
IL1RA
FGF
ECP
ELASTASE
IL6
GROA
GCSF
EOTAXIN
IL8
TIMP1
MMP8
MPO
YKL40
IL1B
IFNG
GMCSF
MMP9
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IL5
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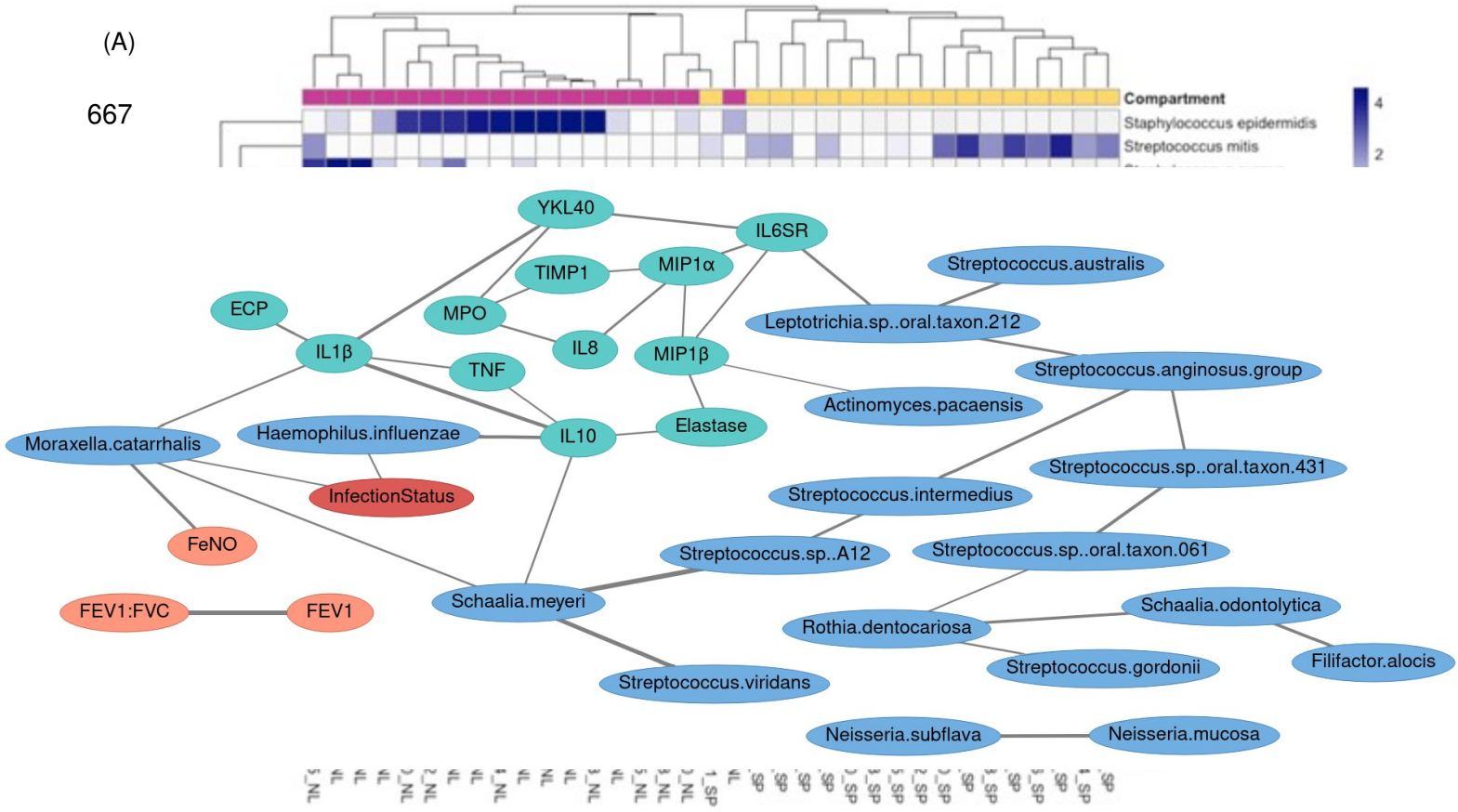
(A)

667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IL8
MIP1β
Streptococcus.anginosus.group
TNF
Actinomyces.pacaensis
Moraxella.catarrhalis
Haemophilus.influenzae
IL10
Elastase
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InfectionStatus
Streptococcus.intermedius
FeNO
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FEV1
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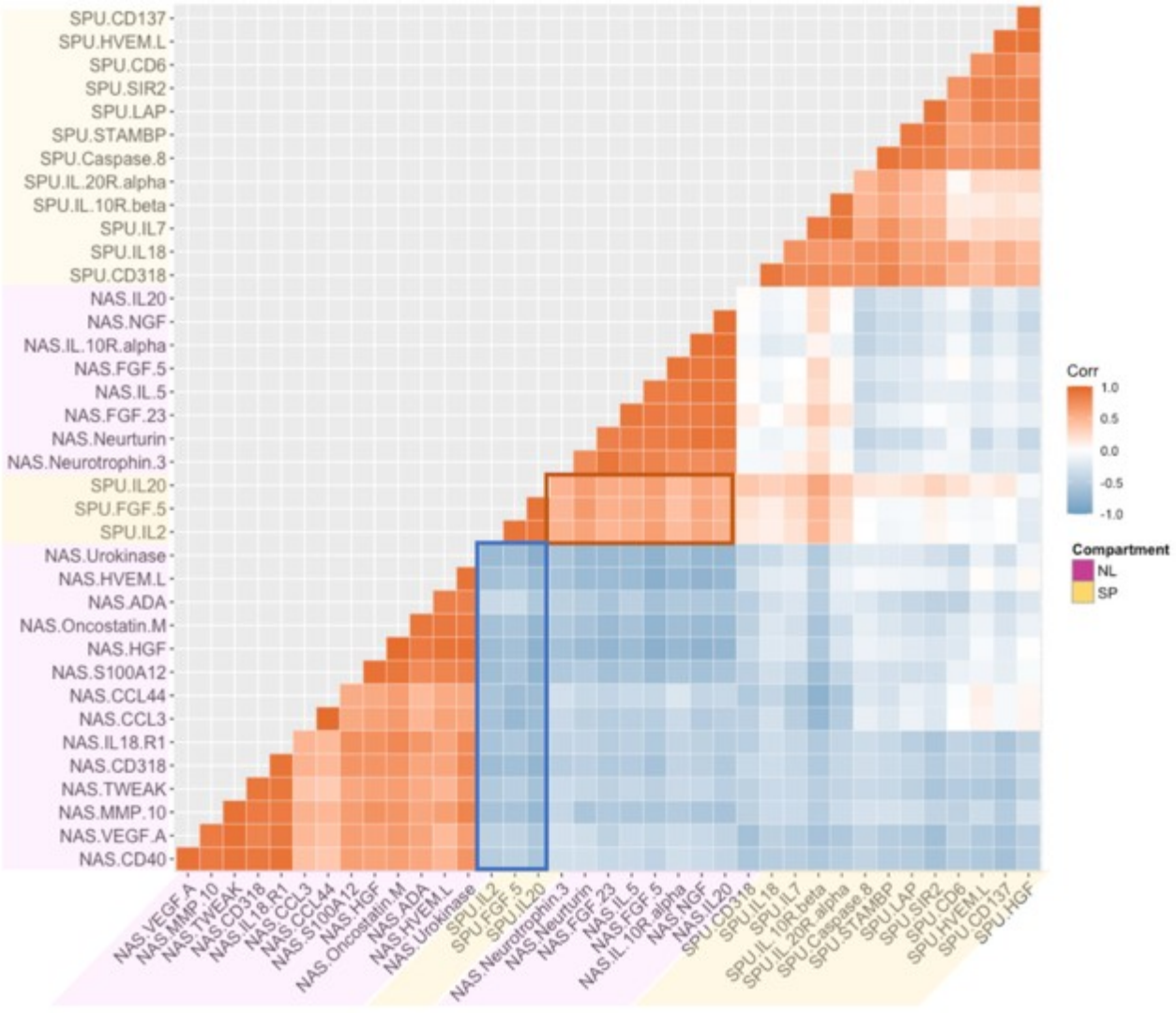
(B)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SPU.Caspase.8
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SPU.IL.10R.beta
SPU.IL7
SPU.IL18
SPU.CD318
NAS.IL20
NAS.NGF
NAS.IL.10R.alpha
NAS.FGF.5
NAS.IL.5
NAS.FGF.23
NAS.Neurturin
NAS.Neurotrophin.3
SPU.IL20
SPU.FGF.5
SPU.IL2
NAS.Urokinase
NAS.HVEM.L
NAS.ADA
NAS.Oncostatin.M
NAS.HGF
NAS.S100A12
NAS.CCL44
NAS.CCL3
NAS.IL18.R1
NAS.CD318
NAS.TWEAK
NAS.MMP.10
NAS.VEGF.A
NAS.CD40
Corr
1.0
0.5
0.0
-0.5
-1.0
Compartment
NL
SP