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NTHi killing activity is reduced in COPD patients and is associated with a differential microbiome

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Abstract

Chronic obstructive pulmonary disease (COPD) is a chronic lung disease characterized by airway obstruction and inflammation. Non-typeable *Haemophilus influenzae* (NTHi) lung infections are common in COPD, promoting frequent exacerbations and accelerated lung function decline. The relationship with immune responses and NTHi are poorly understood. Herein, we comprehensively characterized the respiratory microbiome and mycobiome of patients while investigating microbial dynamics and host immune changes attributable to NTHi killing activity. Mild-to-moderate COPD patients encompassing frequent and infrequent exacerbators and healthy volunteers (HV) were enrolled. Microbial composition, proteomics and NTHi killing activity was analyzed using bronchoalveolar lavage fluid (BALF). In addition, antigen–antibody titers in sera to COPD pathogens were determined using a multiplex assay. Differential abundance analysis revealed an enrichment of *Actinobacteria* and *Bacteroidetes* in the BALF of COPD and HV subjects respectively. Significant differences in the IgA titer response were observed against NTHi antigens in COPD vs. HV. Notably, there was also significantly greater killing activity against NTHi in BALF from COPD vs. HV subjects (OR = 5.64; 95% CI = 1.75–20.20; $p = 0.001$). Stratification of COPD patients by NTHi killing activity identified unique microbial and protein signatures wherein *Firmicutes*, *Actinobacteria* and haptoglobin were enriched in patients with killing activity. We report that differences in host immune responses and NTHi-killing activity are associated with microbiome changes in mild-to-moderate COPD. This is suggestive of a potential link between the respiratory microbiome and immune activity against NTHi in the context of COPD pathogenesis even at this disease stage.

Keywords COPD, Non-typeable *Haemophilus influenzae*, Microbiome, Exacerbations

MICA II Study Group members are listed in Acknowledgement section.

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Introduction

COPD is a chronic inflammatory lung disease characterized by heterogenous pulmonary pathologies and is often associated with systemic comorbidities [1]. Inhalation of cigarette smoke, air pollutants, recurrent bacterial infections, and genetic determinants are among its top reported causes [2, 3]. Despite being one of the leading causes of death worldwide [4], current approaches to therapy are primarily focused on symptom management, for which inhaled therapies are widely employed [5]. Additionally, long-term treatment with antibiotics to treat infectious etiologies is not feasible since it is complicated by the development of antimicrobial resistance and adverse events. Regardless, antibiotics have not proven to be successful in reducing hospitalizations or improving lung function [6]. COPD morbidity is also influenced by the frequency of exacerbations, which are often seasonal, and are characterized by a flare up of disease symptoms usually in association with an infectious event [7]. More frequent exacerbations have been implicated in accelerating the rate of lung function decline in COPD [8].

The respiratory microbiome has emerged as an important contributor to COPD pathogenesis and has also been linked with the onset of exacerbation. Emerging evidence suggests that microbial dysbiosis resulting from the persistence and proliferation of pathogenic bacteria in the lower respiratory tract can outcompete commensals and influence host profiles by triggering a dysregulated immune response. Intuitively, colonization with pathogenic bacteria has been associated with a loss of microbial diversity [9], which in turn leads to further infection susceptibility [10]. Consistent with this hypothesis, changes in microbiome community structure and diversity have been linked to host transcriptomic changes [11] and expression of pro-inflammatory cytokines such as IL-8 in the sputum of COPD patients [12].

In addition, differentially enriched bacterial taxa have been identified in disease in both the sputum and bronchoalveolar lavage fluid (BALF) [12–14], and shifts in respiratory microbiome compositions have been noted during exacerbations. While a universal signature has not yet been identified, persistent colonization in the lower airway with non-typeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* (Mcat) have been associated with an inflammatory response and progressive lung function decline both during stable state and during exacerbations [15]. Notably, *Haemophilus* species have been estimated to be prevalent in up to 70% of COPD patients and have been consistently linked to poorer clinical outcomes [16–19].

Due to the multi-factorial nature of COPD pathogenesis and acute exacerbations, research into novel approaches such as vaccines and long-acting monoclonal antibodies are warranted to treat infectious drivers. Accordingly, NTHi and Mcat have been the focus of therapeutic targeting in COPD, often in combination. Evidence suggests that they aid their mutual existence by counteracting complement-mediated killing and promoting increased resistance to antibiotics [20, 21]. Conserved surface exposed proteins from these pathogens have been investigated as components of an adjuvanted multi-component (three antigens from NTHi and one antigen from Mcat) vaccine designed to reduce the rate of acute exacerbations in moderate to severe COPD in a recent phase II study. The vaccine had an acceptable safety and reactogenicity profile consistent with a prior phase I study [22] and another vaccine study which only used NTHi components [23]. Unfortunately, this vaccine failed to meet its primary efficacy endpoint, further emphasizing the complexity of this space [24, 25].

Herein, we have comprehensively characterized the landscape of the respiratory microbiome and mycobiome in COPD patients vs. healthy volunteers (HV) through 16S rRNA and ITS sequencing of BALF and sputum samples. Furthermore, we studied the impact of microbial dynamics as it relates to immune responses in COPD patients to better understand the role of protective immunity in shaping microbial and clinical outcomes in the disease.

Methods

Patient cohort and sample collection

Details about the patient cohort have been previously reported [26–28]. Briefly, mild-to-moderate COPD patients encompassing frequent (P-FE) and infrequent exacerbators (P-IE) and healthy volunteers (HV) comprised of never smokers (HV-NS) and ex-smokers (HV-ES) were recruited into our cohort as part of an AstraZeneca-sponsored study. COPD patients were classified as P-FE if they had a history of ≥2 exacerbations, or P-IE if they had history of ≤1 exacerbation in the preceding year before enrollment, where exacerbations could be moderate (requiring oral steroids or antibiotics or both) or severe (requiring oral steroids or antibiotics or both plus hospital admission). ≥2 exacerbation events have been shown to be a reliable predictor of future exacerbations [29, 30]. Patients reporting an exacerbation event in the one-month preceding enrollment were not included in the cohort. Additionally, subjects using any antibacterial, antiviral or respiratory investigational drug or relevant vaccine up to 30 days prior to the enrollment visit were also not included in the study cohort. The ex-smokers had stopped smoking 6 months prior to enrollment and had at least a 10-pack-years history. All subjects gave written informed consent, and the study was approved by National Research Ethics Service South Central

– Hampshire A and Oxford C Committees (LREC no: 15/SC/0528).

Fiber optic bronchoscopy was used for sampling of Bronchoalveolar lavage fluid (BALF) which has been described in detail previously [26]. BALF samples were collected following enrolment by instilling 100 mL saline into 2 lobes per patient (Fig. S1). A saline blank was recovered prior to the bronchoscopy by flushing 20 mL of the same saline preparation through the bronchoscope. Sputum samples were obtained by spontaneous expectoration or induced by saline inhalation according to standard methods from all patients also at study entry [26, 27, 31].

Expression of recombinant protein antigens for NTHI, *M. Catarrhalis* and *S. pneumoniae*

Sequences for proteins E, F, D P6 and PilA (NTHI), OmpCD, M35 and UspA2 (*M. Catarrhalis*) and *S. pneumoniae* pneumolysin were cloned in pET28a. The production of his-tagged recombinant proteins was performed in *E. coli* after induction with IPTG. *E. coli* was then lysed followed by purification of proteins from the supernatant fraction using nickel affinity columns.

Serological measurement of antibody titers to NTHi, Mcat and *Streptococcus pneumoniae* antigens

Serum levels of IgG, IgA and IgM from healthy subjects and COPD patients subjects against recombinant proteins derived from NTHi, Mcat and *S. pneumoniae* were measured with a customized multiplex assay (Meso Scale Discovery; Meso Scale Diagnostics, Rockville, MD) at Meso Scale Discovery according to the manufacturer's instructions. Briefly, two 10-spot plates were developed upon coating with M35, OMP CD, PilA, Protein D, Protein E, and Protein F or USPA2, P6 and pneumolysin. Empty spots were coated with bovine serum albumin. Plates were first blocked with 150 µL of MSD Blocker A for 1 h at room temperature (RT) under 705 rpm shaking. After 3 washes with PBS/0.1% tween, 50 µL of each sera was incubated at a 1:100 dilution on each plate for 2 h at RT while shaking at 705 rpm. After three washes, plates were incubated with 50 µL of sulfo tag-labelled anti-human IgG (clone Hytest 1G1, 2 µg/mL), sulfo-tag labelled anti-human/nonhuman primates IgA (clone D-20JJ-6, 2 µg/mL) or sulfo-tag labelled human/nonhuman primate IgM (D20JP-6, 1 µg/mL) for 1 h at RT. After 3 washes, specific antibodies were detected with 150 µL of MSD Gold™ Read Buffer A and plates read on a Meso Sector S 600 mm (Meso Scale Discovery). IgG, IgA and IgM were quantified using three human blended sera with arbitrary assigned concentration (AU/mL) for each antigen. To ensure inter-plate consistency we assayed three COPD and healthy control samples across all plates and observed very low levels of variability.

Luminescent high-throughput NTHi killing assay

Bacteria

The *luxCDABE* operon from *Photorhabdus luminescens* was cloned into shuttle vector pACYC184 under the control of the *lac* promoter. The plasmid was electroporated into RD and maintained by chloramphenicol selection. The resulting strain was named NTHi RD-Lux. NTHi RD-Lux strains were grown on brain heart infusion (BHI) agar plates supplemented with 2 µg/mL Nicotinamide adenine dinucleotide (Sigma cat# 43410), 1 µg/mL chloramphenicol (Sigma cat# C0378) and coated with 10 µg/mL of hemin (Sigma cat # 51280) stock (1 mg/mL in triethanolamine; Sigma cat # 90279) and incubated overnight at 37 °C in 5% CO_2. A small population of the plated culture was used to inoculate fresh BHI broth (Criterion Cat# C5141) supplemented with 5% Fildes (Remel cat# 45039) and 1 µg/mL of chloramphenicol (Sigma cat # C0378). The culture was incubated at 37 °C in 5% CO_2, with shaking at 180 RPM until the culture reached an optical density at 600 nM (OD_{600}) of 0.5. One mL of culture was collected and suspended in 1.0 mL of cold assay buffer (RPMI 1640; Gibco cat# 11835-030) + 2.25 g glucose (Sigma cat# G7021) + 1% BSA (Sigma cat# A9576)). The bacterial suspension was further diluted 1:100 in assay buffer.

Preparation of complement source

Twenty-five mL of NTHi RD-Lux, grown to an OD_{600} of 0.5, was centrifuged at 3724 g for 10 min and suspended in 25 mL of baby rabbit serum (Cedarlane cat# CL3441). After a 30-min incubation on ice, the bacteria was collected by centrifugation for 20 min (3724×*g*) at 4 °C. The supernatant was vacuum filter sterilized with a 0.2 µm filter, aliquoted and stored at 80 °C until use. On day of assay the absorbed complement was diluted 1:2 in SBA buffer and kept on ice. Control antibodies were prepared at a four-fold higher concentration than intended for the assay.

NTHi killing assay

Assay components were added to a 384-well plate (Greiner white, cat#781,074–25) in the following order: 20 µl of heat inactivated (60 °C for 30 min) BALF or positive and negative controls; 40 µl of adsorbed complement (1:2); and 20 µl of bacteria (1:100 in assay buffer). Plates were sealed with porous adhesive film for culture plates (VWR cat#60941-086) and incubated for 2 h at 37 °C in 5% CO_2. Immediately following incubation, luminescence was read on a luminometer (Tecan Spark). The assay was repeated twice.

16S and ITS sequencing

DNA extraction

BALF samples were treated with 2 U Turbo DNase (Invitrogen) for 30 min at 37 °C to degrade extracellular DNA and the reaction was stopped with 0.5 M EDTA incubated for 10 min at 65 °C. Microbial DNA was extracted using Zymobiomics DNA/RNA Miniprep kit (Zymo Research) following the manufacturer's instructions for DNA purification.

Sputum samples were treated with 1:1 sputolysin (Sigma Aldrich), vortexed for 1 min and incubated for 15 min at room temperature. Homogenized sputum and BALF samples were treated with 2 U Turbo DNase (Invitrogen) for 30 min at 37 °C to degrade extracellular DNA and the reaction was stopped with 0.5 M EDTA incubated for 10 min at 65 °C. Microbial DNA and RNA were extracted in separate fractions using Zymobiomics DNA/RNA Miniprep kit (Zymo Research) following the manufacturer's instructions.

Library preparation and sequencing

Amplicon PCR, Library Preparation and sequencing was performed as outlined for 16S [32] and for ITS [33]. Briefly, V4 16S rRNA gene amplicons were assembled using Accu-Prime Pfx SuperMix (Invitrogen) and dual-index primers. ITS rRNA gene amplicons were generated using Accu-Prime Taq DNA Polymerase High Fidelity (Invitrogen) and dual-indexed primers based on the published fungal ITS primer pair ITS86F/ITS4 [33]. SequalPrep Normalization Kit (Invitrogen) was used for amplicon clean up and normalization.

Final amplicons were pooled, and library quantification was determined by qPCR using the NEBNext Library Quantification kit (NEB). Libraries were mixed with PhiX Control v3 (Illumina) and denatured using fresh NaOH before loading on to the instrument. For 16S, libraries were sequenced on an Illumina MiSeq instrument using a 2×250 base pair reagent Kit v2, whereas for ITS, they were sequenced on an Illumina MiSeq instrument using a 2×300 base pair reagent Kit v3.

Bioinformatics

After appropriate quality filtering, 16S V4 and ITS amplicons were denoised to amplicon sequence variants or ASVs using DADA2 [34]. Taxonomic classifications of ASVs were done using SILVA [35] for 16S and UNITE [36] for ITS. Estimation of alpha and beta diversities were done using QIIME2 [37]. Differential enrichment analyses to compare relative abundances of taxa were done using LEfSe [38].

Proteomics

Proteomics was performed as described [39]. Briefly, peptides were desalted and subjected to TMT (Thermo Fisher Scientific) labelling for 11-plex TMT analysis, according to the manufacturer's instructions. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis was carried out on a Q Exactive HF-X (Thermo Fisher Scientific) mass spectrometer interfaced with a Dionex 3000 RSLCnano. Proteome Discoverer 2.3 (Thermo Fisher Scientific) and Mascot (version 2.6.0) were used for analysis with the latest Uniprot human protein database. Protein quantitation was determined using Perseus software version 1.6.15.0 and abundances were normalized to total protein levels which were consistent across all samples. Annotated proteomics data was used to compute differentially expressed proteins between patients with positive and negative killing capacity of NTHi by Limma [40, 41]. Confirmation of findings was done using random forest (RF) classification, which was trained with 500 trees and 18 features were selected for each split. The number of features for each split was determined based on the least out-of-bag error rate in an iterative manner starting from the default which was the square root of all predictors in the dataset. The overall out-of-bag-error rate for our model was 19.57%, which upon closer examination of the confusion matrix revealed near perfect classification in the category with the higher sample size. Importance of features from the RF model was determined using the mean decrease in gini score.

Results

The median age of subjects across study groups ranged from 67.5 to 72 years, and the distribution of male and female genders was comparable between groups. As expected, all clinical characteristics were significantly different in the patient groups compared to healthy volunteers (Table S1). Also, 57 medication categories were reported by subjects in our cohort (Table S2).

The microbiome and mycobiome were characterized in 130 BAL samples (collected from 65 patients) and 27 sputum samples (Figs. S1–S2, Table S3).

Alpha diversity of both the microbiome and mycobiome were higher in the sputum compared to the BALF (Fig. 1A, Fig. S3A). Upon comparing microbiome composition, we found a significant clustering effect by sample type (Fig. 1B). In contrast, we observed considerable compositional overlap in terms of fungi, perhaps reflective of the low overall diversity of fungal taxa in these samples (Fig. S3B).

These differences in microbiome diversity by sample type were observed when considering all subjects ($n=68$)

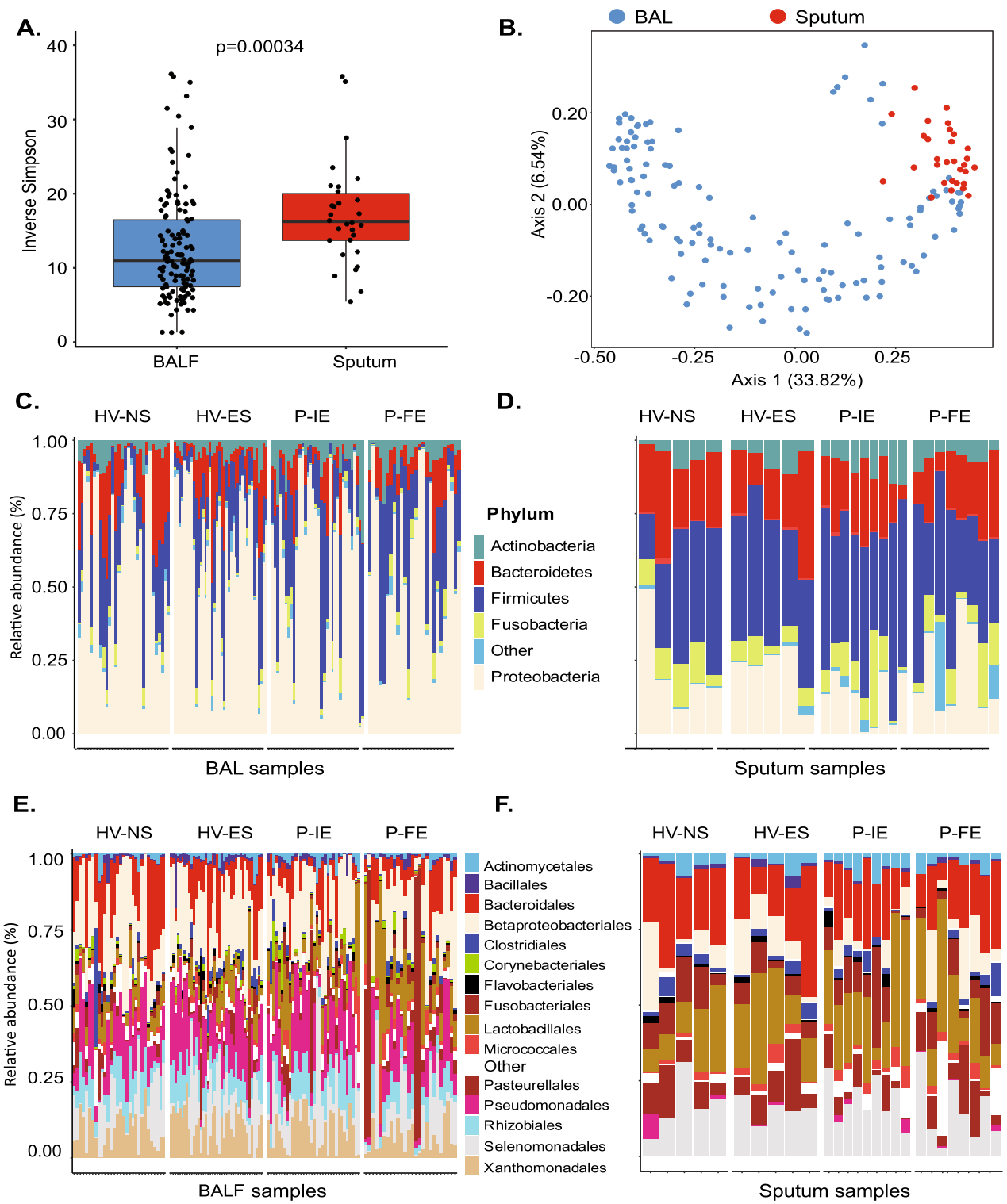

Fig. 1 Characterizing the microbial landscape. **A** Comparison of alpha-diversity (measured using the Inverse Simpson index) in sputum ($n=30$) vs. BALF ($n=65$ patients, 130 samples) samples. p-value by Wilcoxon ranksum test. **B** Ordination of beta-diversity (Bray–curtis) distances by principal coordinate analysis ($p=0.001$). p-value by ANOSIM 999 permutations. Taxonomic landscape at the phylum level in **C** BALF ($n=65$ patients, 130 samples) and **D** sputum ($n=27$ patients). Taxonomic landscape at the order level in **E** BALF ($n=65$ patients, 130 samples) and **F** sputum ($n=27$ patients)

and only those who contributed both sample types ($n=27$) (Fig. S4). This suggests that the bacterial community structure in BALF is distinct from that of sputum and that microbiome readouts from these two sample types should be considered independently.

Of note, there were no differences in bacterial alpha- or beta- diversity by patient groups by either BAL (Fig. S5 A, C) or sputum (Fig. S6). There were also no differences in bacterial diversity by lobe of the lung from which BALF was sampled (Fig. S5 B, D).

Next, we characterized the taxonomic landscape (both bacteria and fungi) across all samples. The BAL samples were dominated by Proteobacteria with very little Bacteroidetes in all subject groups, whereas the sputum samples had lesser abundance of Proteobacteria and greater abundance of Firmicutes and Bacteroidetes (Fig. 1C, D). At the order level, Betaproteobacteriales and Pseudomonadales were prevalent across all groups; Lactobacillales was more abundant in P compared to HV ($p=0.014$) (Fig. 1E, F). In terms of fungi, both BAL and sputum samples were predominated by Ascomycota and Basidiomycota, whereas at lower levels the fungal niche was primarily composed of low abundance taxa, that were collapsed into the 'other' category (Fig. S7).

To evaluate microbiome signatures by patient groups, differential abundance analyses were performed using LEfSe to compare the relative abundances of microbial taxa. We found more differences in the BAL samples (Fig. 2A), (as opposed to sputum; Fig. 2B) of COPD patients vs. HV. Bacteria belonging to the Actinobacteria and Bacilli phyla; and specifically, those belonging to the Micrococcales and Lactobacillales orders were more abundant in BALF of COPD patients, whereas members of the Bacteroidetes phylum were more abundant in HV (Fig. 2A). Therefore, in the setting of less-severe COPD disease, while the overall microbiome composition in the BALF was comparable between groups (Fig. S5C), differences were more apparent when comparing the abundances of individual bacterial taxa. There were also limited differences when comparing BAL samples for COPD patients who were frequent (P-FE) vs. infrequent (P-IE) exacerbators (Fig. S8).

Interestingly, upon examining the abundance of the *Haemophilus* genus, which has been previously implicated in COPD patients, we noted that it tended to have the highest abundance in the P-FE group (Fig. S9). Lastly, there were fewer differences with the mycobiome, with trends for taxa such as *Penicillium* and Tremellomycetes to be higher in the BALF of COPD patients (Fig. S10).

Building on these observed microbiome differences, we sought to investigate any potential links with immune activity in the context of COPD. For this, we developed a multiplex assay with Meso Scale Discovery (Rockville, MD) to evaluate antibody responses in serum to antigens derived from bacteria that are known contributors of COPD pathogenesis, namely NTHi, Mcat and *Streptococcus pneumoniae*. In these studies, we observed a higher trend towards reactivity in COPD patients for ubiquitous NTHi derived antigens only (Fig. 3A, Fig. S11). When evaluating individual antibody isotypes, significant differences were observed for IgA titers against P6, Protein D and Protein F when comparing all four subject groups with the highest titers observed in P-IE (unadjusted $p=0.03$, 0.03, $p=0.01$ for P6, Protein D and Protein F, respectively, Fig. 3B–D). Pairwise comparisons using the Dunn's test further revealed that the P-IE group was driving the significant differences between the groups.

Lastly, we determined killing activity against NTHi from BALF of subjects in this cohort using a high throughput NTHi killing assay. Consistent with our finding of enhanced immune activity in COPD patients, we found that the odds of observing killing activity was 5.64 times more likely in BALF obtained from COPD patients ($n=30$ patients) when compared to HV ($n=36$ patients) (OR = 5.64, 95% CI = 1.75–20.20; $p=0.0012$, Fig. 4A, Fig S12). When we stratified COPD patients based on NTHi killing activity, we noted a differential microbiome signature wherein Firmicutes and Actinobacteria were more abundant in patients with observable killing activity while Proteobacteria were more abundant in patients with no killing activity (Fig. 4B–C).

In addition, we also noted differences in host characteristics, specifically the proteome, when we compared BAL samples from COPD patients with NTHi killing activity vs. those without. The protein haptoglobin (HP), which is synthesized by the lung epithelial cells and alveolar macrophages and has been linked to increased disease severity in COPD, was found to be significantly enriched in patients with observable NTHi killing activity in BALF using both differential enrichment analysis and random forest classification (Fig. 4D–E, Fig S13).

Interestingly, HP was also significantly inversely correlated with certain lung function parameters such as post-FEV1% and post-FEV1/FVC (Fig S14 A–B), while there was no apparent correlation with HRCT-LAA% and HRCT E/I MLD (Fig S14 C–D) and Gammaproteobacteria abundance (Fig. S14E). Additionally, upon examining specific genera that have been previously implicated in respiratory disease, we noted a trend for *Haemophilus* abundance to be low in COPD patients with high haptoglobin (Fig. S15). Taken together, these data point to ongoing host immune activity against NTHi in this patient population despite the low-severity of disease seen in most participants, and is therefore suggestive of a potential link between microbial dynamics and host immune responses.

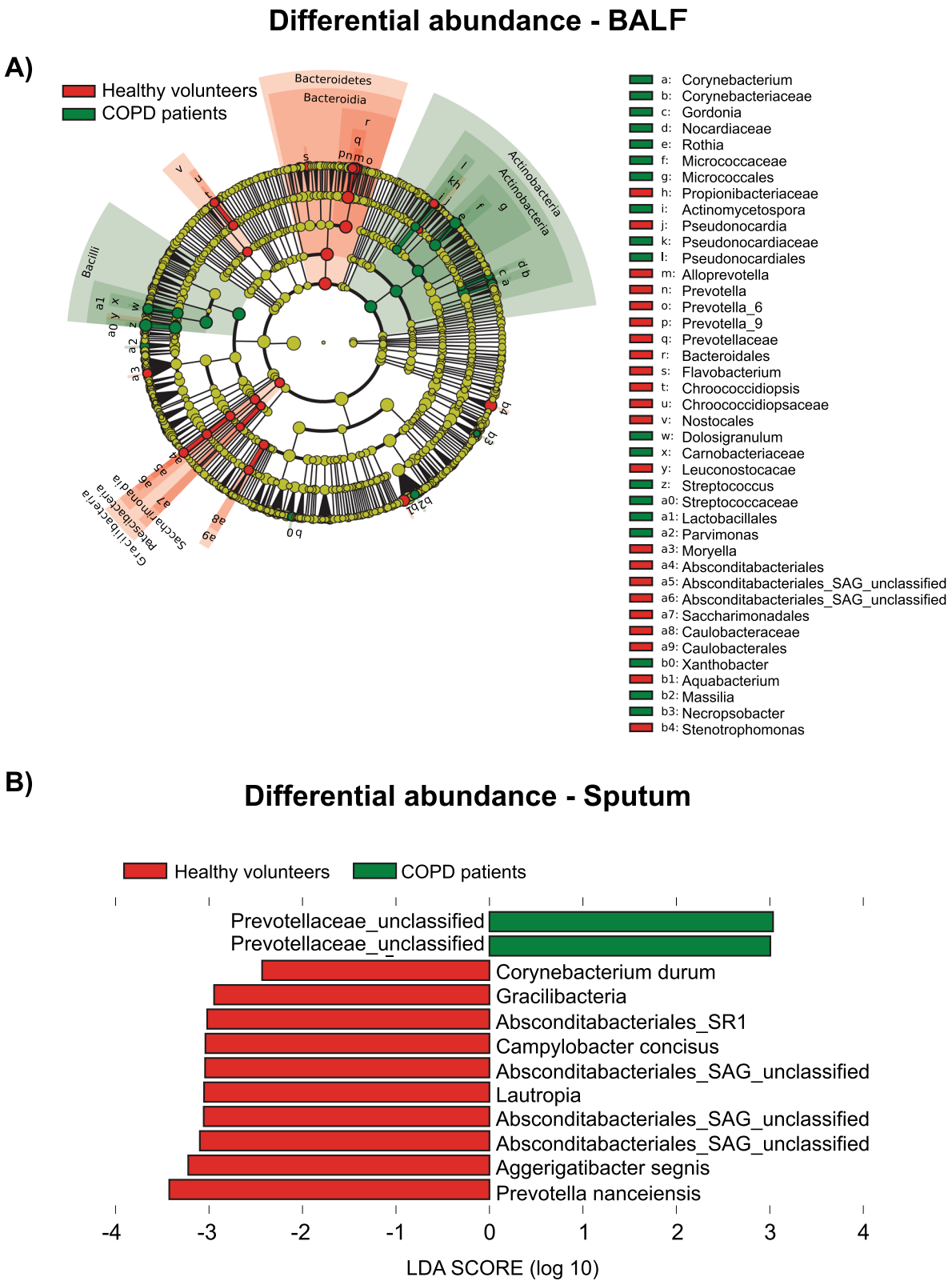

Fig. 2 Differential abundance comparisons. **A** Taxonomic cladogram and **B** LDA score plot by LEfSe (LDA score minimum = 2.0, $p = 0.05$) displaying differential taxa in COPD patients in **A** BAL (P; $n = 30$ patients, 60 samples) vs. healthy volunteers (HV; $n = 35$ patients, 70 samples), **B** sputum (P; $n = 17$) vs. healthy volunteers (HV; $n = 10$)

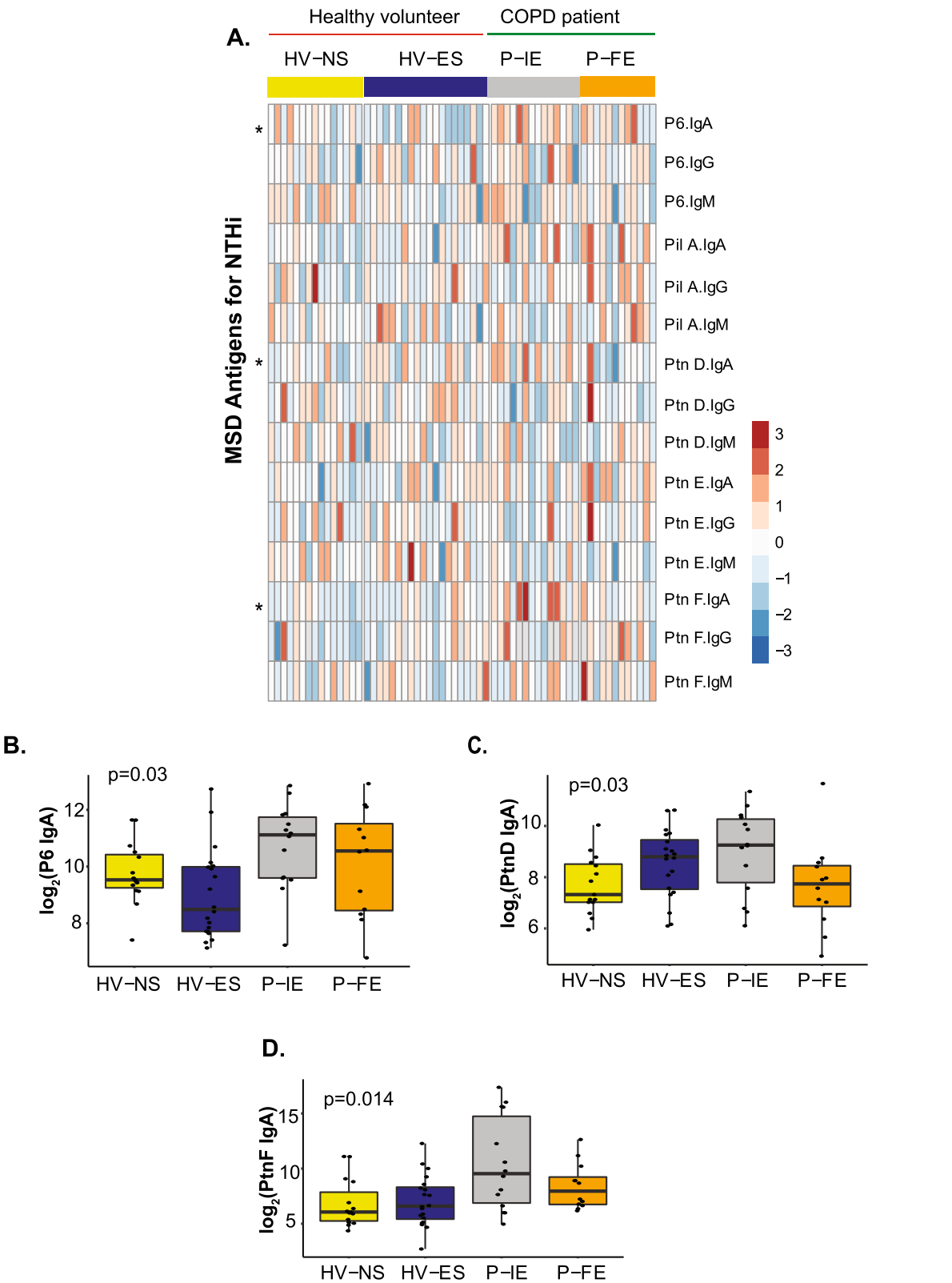

Fig. 3 Serum immunological response to NTHi. **A** Unsupervised hierarchical clustering heatmap of antibody titers to NTHi antigens. Significantly different antibody titers are marked with an asterix. Comparison of the IgA titers (log_2) of **B** P6, **C** Protein D and **D** Protein F by subject group. *p*-value by Kruskal–Wallis test. HV-NS $n = 15$; HV-ES $n = 20$; P-IE n = 14; P-FE $n = 12$. Antigen titers are z-scaled

Discussion

Herein, we have comprehensively characterized the microbiome and mycobiome in a unique cohort of ex-smoker COPD patients vs. healthy volunteers. We also report a higher trend towards reactivity to NTHi antigens driven by infrequent exacerbators in the serum, and significantly enhanced NTHi killing activity in the BALF of COPD patients compared to healthy volunteers. Consistent with others [42, 43], we also report a demonstrable immunological response to known COPD pathogens in this observational cohort. However, in addition, we also report unique microbiome and proteomic signatures associated with NTHi killing, thereby suggesting a close interplay between functional activity against NTHi, microbial dynamics and immunological responses in the host.

NTHi is an airway commensal that can become a pathobiont although the mechanisms by which it does so is unclear. The Proteobacteria phylum (of which NTHi is a member) is a chronic colonizer of the COPD airway. Its detection is increased in exacerbations [19] and is associated with increased COPD mortality [44]. In this study, although Proteobacteria was the most abundant phylum detected in the BAL samples, we did not observe any difference in its abundance between COPD patients and controls. This may reflect a difference between COPD microbiome studies that use sputum and those that use BAL with all the various caveats this difference implies, not least increased upper respiratory tract contamination in sputum [45]. However, there is little difference in the predominant bacteria seen between those studies that have used sputum and those that use BAL [46]. A further explanation might be that many previous COPD microbiome studies included current smokers [19, 47–49], whereas our cohort are all ex-smokers, although a recent study demonstrated no significant differences at the phylum and genus level in sputum derived from COPD ex-smokers and current smokers [50]. Changes in the COPD microbiome have been shown to change as disease severity increases [9]. For safety reasons, we sampled a mild-moderate patient cohort and this difference in disease severity may be a further factor in the lack of detection of NTHi in our cohort. Another potential reason for this miss could be the lack of sensitivity of 16S rRNA sequencing with regards to NTHi species detection.

Despite this inability to detect NTHi, there is a clear echo of prior airway infection with NTHi in the increased serum IgA titres against NTHi-associated proteins (Fig. 4), which is in line with a previous study demonstrating increased concentrations of both IgA and sIgA in BAL from COPD patients [51]. In further support of such prior NTHi infection, BAL samples derived from COPD patients demonstrated increased NTHi-killing ability (Fig. 4). Moreover, there was greater detection of Gammaproteobacteria in those samples that exhibited no ability to kill NTHi (Fig. 4B, C). This association may be a result of either no prior NTHi exposure or a defect in the ability of the individual to mount an effective immune response to NTHi. If the latter, it is tempting to speculate that the lower IgA titers to NTHi targets in frequent exacerbators could be a reflection of such a defect and could be an indicator for future colonization and exacerbation. However, such supposition requires further investigation in a longitudinal cohort to prospectively investigate an association with exacerbations.

We have also identified decreased pulmonary haptoglobin as a further potential biomarker of decreased NTHi killing and detection of Gammaproteobacteria. Haptoglobin is an acute phase protein that complexes with haemoglobin facilitating haemoglobin uptake by macrophages via the scavenger receptor, CD163 [52]. Thus a reduction in haptoglobin might result in the increased free iron observed in the COPD airway [53], promoting overgrowth of pathogenic bacteria, such as NTHi, for which we saw a trend in our dataset (Fig. S15). It has long been observed that haptoglobin is a natural bacteriostat that can limit the availability of extracellular heme-derived iron for iron binding proteins in adventitious bacteria [54, 55]. This is particularly relevant for *Haemophilus* species which are known heme auxotrophs that lack the enzymes to secrete their own heme and therefore rely heavily on extracellular iron from heme and employ several sophisticated mechanisms to access it [56, 57].Additionally, this reduction in haptoglobin may be a function of the individual genotype of the volunteer, as those individuals with a haptoglobin genotype of 2–1 or 2–2 had lower haptoglobin concentrations in their

(See figure on next page.)

Fig. 4 NTHi killing activity in BALF. **A** Contingency plot of killing activity between COPD patients and healthy volunteers. **B** Taxonomic landscape at the family level by killing activity (with killing activity: $n = 22$ patients, 44 samples; no killing activity: $n = 7$ patients, 14 samples). **C** LEfSe LDA score plot by NTHi killing activity (LDA score minimum = 2.0, $p = 0.05$; with killing activity: $n = 22$ patients, 44 samples; no killing activity: $n = 7$ patients, 14 samples). **D** A bubble plot depicting top and bottom 20 differentially expressed proteins ranked by the log fold-changes (with killing activity: $n = 19$ patients, 33 samples; No killing activity: $n = 7$ patients, 13 samples). **E** Comparison of Haptoglobin protein expression by killing activity (with killing activity: $n = 19$ patients, 33 samples; no killing activity: $n = 7$ patients, 13 samples)

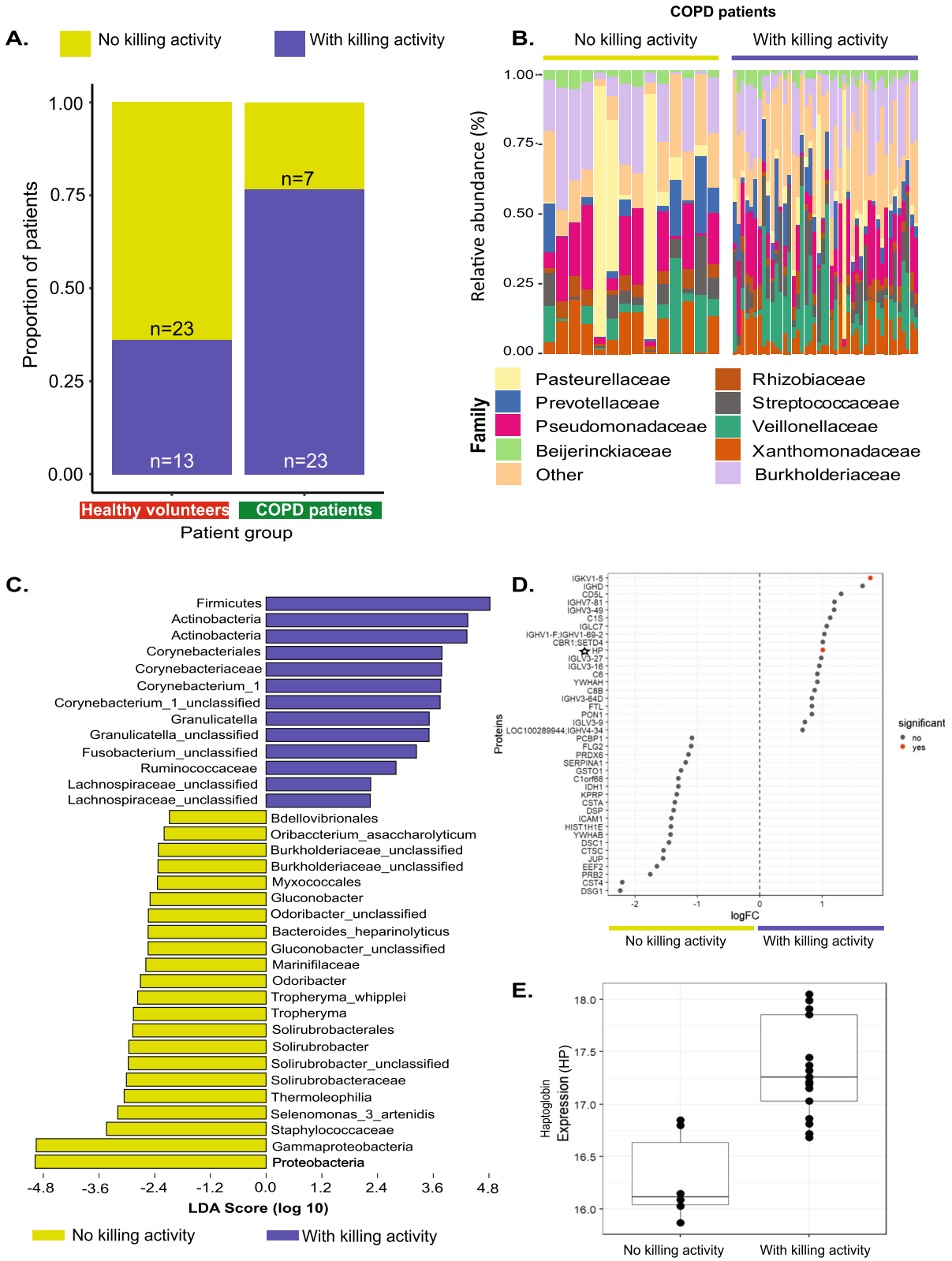

Fig. 4 (See legend on previous page.)

sputum than individuals with a 1–1 genotype [58]. Further work to confirm this possible link between airway free iron, haptoglobin levels, bacterial colonization and COPD exacerbations is therefore warranted.

We recognize that this study is not without its limitations. Due to the deep characterization of subjects and intensive sampling, the cohort is small, and the study captured only cross-sectional measures, providing no insight into changes in BAL microbiome or proteome over time and disease states (e.g. stable vs. exacerbation). We thus cannot fully rule out that lack of differences between healthy volunteers and COPD patients is not due to cohort size. Relatedly, this also precluded us from performing adjustment for multiple hypothesis testing in several of our statistical comparisons. Furthermore, exacerbation history was determined retrospectively at enrollment and largely based on patient recall rather than documented events. Sputum data was not available from all subjects making direct comparisons between BAL and sputum difficult, and due to limited sample availability, we were also not able to determine the factors responsible for increased NTHi killing activity from COPD BALF. Lastly, our study included mild-moderate COPD patients and gives insights about earlier disease. Comparison of our findings with those in more severe disease in future studies is warranted as microbiome diversity is known to decrease with disease severity [9].

Our study demonstrates a clear increase in NTHi-killing in COPD compared to health in BAL. Furthermore, we demonstrate that this killing ability was associated with decreased detection of Proteobacteria. We have identified haptoglobin as a differentially expressed protein that associated with this NTHi-killing ability and the presence of Gammaproteobacteria. Further investigation of how these observations can be combined to enable better phenotyping of COPD patients to allow optimal targeting of existing (e.g. antibiotics) and novel (e.g. vaccines, monoclonal antibodies) therapies to modify the outcomes of this important condition is warranted.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12931-025-03113-z.

Additional file 1.

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Author contributions

AD, TMAW, KS, designed the study. VG, BS, CM, JB, AD, TMAW, KS, BRS planned and conducted the gut microbiome analyses. LO, BA, JHK, VG and JW planned and conducted the proteomics analysis. AD, JB, JHK, MK, TK, CC, PW, VAH, SW, HS, AB, AR, SH, CT, BRS planned and performed the experiments and conducted subsequent data analysis. CM, AP, DM, KO, MGB conceptualized parts of the project, analysis, supervised the project, and contributed to writing of the manuscript. VG, AD, JHK and BS prepared all figures. VG and AD drafted the first version of the manuscript. All authors contributed to data interpretation, and critical review of the manuscript and gave final approval of the version to be published.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

All subjects gave written informed consent. The study was approved by National Research Ethics Service South Central – Hampshire A and Oxford C Committees (LREC no: 15/SC/0528) and complied with the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

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