Multi-omics links IL-6 trans-signalling with neutrophil extracellular trap formation and *Haemophilus* infection in COPD

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Lung IL-6 trans-signalling driven by *Haemophilus influenzae*-induced NETosis is a pathological feature of COPD patients with chronic *Haemophilus* infection, stable neutrophilic inflammation and uncontrolled disease https://bit.ly/30vhgD5

Cite this article as: Winslow S, Odqvist L, Diver S, *et al*. Multi-omics links IL-6 trans-signalling with neutrophil extracellular trap formation and *Haemophilus* infection in COPD. *Eur Respir J* 2021; 58: 2003312 [DOI: 10.1183/13993003.03312-2020].

This article has supplementary material available from erj.ersjournals.com

This article has an editorial commentary: https://doi.org/10.1183/13993003.02143-2021

Received: 28 Aug 2020
Accepted: 4 March 2021

Abstract

Background: Interleukin (IL)-6 trans-signalling (IL-6TS) is emerging as a pathogenic mechanism in chronic respiratory diseases; however, the drivers of IL-6TS in the airways and the phenotypic characteristic of patients with increased IL-6TS pathway activation remain poorly understood.

Objective: Our aim was to identify and characterise COPD patients with increased airway IL-6TS and to elucidate the biological drivers of IL-6TS pathway activation.

Methods: We used an IL-6TS-specific sputum biomarker profile (soluble IL-6 receptor (sIL-6R), IL-6, IL-1β, IL-8, macrophage inflammatory protein-1β) to stratify sputum data from patients with COPD (n=74; Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbation (BEAT-COPD)) by hierarchical clustering. The IL-6TS signature was related to clinical characteristics and sputum microbiome profiles. The induction of neutrophil extracellular trap formation (NETosis) and IL-6TS by *Haemophilus influenzae* were studied in human neutrophils.

Results: Hierarchical clustering revealed an IL-6TS-high subset (n=24) of COPD patients, who shared phenotypic traits with an IL-6TS-high subset previously identified in asthma. The subset was characterised by increased sputum cell counts (p=0.0001), persistent sputum neutrophilia (p=0.0004), reduced quality of life (Chronic Respiratory Questionnaire total score; p=0.008), and increased levels of pro-inflammatory mediators and matrix metalloproteinases in sputum. IL-6TS-high COPD patients showed an increase in Proteobacteria, with *Haemophilus* as the dominating genus. NETosis induced by *H. influenzae* was identified as a potential mechanism for increased sIL-6R levels. This was supported by a significant positive correlation between sIL-6R and NETosis markers in bronchoalveolar lavage fluid from COPD patients.

Conclusion: IL-6TS pathway activation due to chronic colonisation with *Haemophilus* may be an important disease driver in a subset of COPD patients.

Introduction

COPD is a heterogeneous disease, and an improved understanding of molecular phenotypes characterised by specific inflammatory pathways may help define subsets of COPD and guide targeted therapy [1]. The interleukin (IL)-6-trans signalling (IL-6TS) pathway is implicated in the pathophysiology of COPD, including emphysema, pulmonary fibrosis, epithelial-to-mesenchymal transition, increased epithelial permeability and Toll-like receptor (TLR)-dependent inflammatory responses [2–6]. Stratification of COPD patients based on IL-6TS-associated inflammation may enable identification of a COPD patient subset that benefits from treatments targeting IL-6TS.

The IL-6TS pathway has the capacity to activate cells that do not normally respond to IL-6 due to low expression of the IL-6 receptor (IL-6R), including bronchial epithelial cells and airway smooth muscle cells [2, 7]. In these cells, pathway activation is enabled through interaction of the soluble IL-6 receptor (sIL-6R)/IL-6 complex with the ubiquitously expressed signal-transducing element for the IL-6 family of cytokines, gp130 [8]. IL-6TS leads to phosphorylation of STAT family transcription factors (STAT3 and/or STAT1) by the Janus tyrosine kinase family (JAK1, JAK2, TYK2), and it also causes activation of the mitogen-activated protein kinase, phosphoinositide 3-kinase and mechanistic target of rapamycin (mTOR) signalling cascades [3, 9]. Several studies provide evidence that IL-6TS might be active in the lung and contribute to the pathology of COPD. For instance, higher levels of sIL-6R have been found in sputum of patients with COPD compared to healthy smokers [10] and increased levels of IL-6 in the airways were associated with COPD severity, exacerbations and airway obstruction [11, 12]. In addition, increased levels of sIL-6R and IL-6 in human emphysematous lung tissue showed positive correlation with mTORC1 pathway hyperactivation [3], and STAT3 and STAT1 phosphorylation were shown to be increased in lung tissue of COPD patients compared to nonsmokers [13].

While sIL-6R can be released following alternative splicing of the IL-6R mRNA, the majority of circulating sIL-6R is generated by ADAM10- and ADAM17-mediated shedding of membrane-bound IL-6R (mIL-6R) [14, 15]. At local sites of inflammation, neutrophils have been proposed as the main source of sIL-6R [16], and a recent study showed that neutrophils may be an important source of sIL-6R in the lungs of patients with chronic respiratory diseases [17]. However, the pathophysiological processes that lead to mIL-6R shedding in the lung remain largely unknown.

In a recent study we used cluster analysis of lung epithelium transcriptomics and sputum proteomics data to highlight the association of IL-6TS-specific gene (*TNFAIP6*, *PDE4B*, *IL1R2*, *S100A9*, *S100A8*, *S100A12*, *CHI3L1* and *SPP1*) and protein (IL-6, sIL-6R, macrophage inflammatory protein (MIP)-1β, IL-1β, IL-8, YKL-40 and matrix metalloproteinase (MMP)3) signatures, with a distinct asthma patient phenotype [2]. These signatures were increased in asthma patients with frequent exacerbations, blood eosinophilia, submucosal infiltration of T-cells and macrophages, and it did not overlap with systemic inflammation. Sputum sIL-6R and IL-6 levels correlated with markers of innate immune activation, airway remodelling and increased sputum neutrophils [2].

Based on the increased sIL-6R levels and activation of IL-6TS downstream pathways observed in COPD patients, we hypothesised that our IL-6TS-related signatures detected in asthma would be increased in a subset of COPD patients. Our aims were to explore the existence of such a COPD patient subset, describe its clinical characteristics and elucidate the pathogenic drivers of IL-6TS in COPD. To this end, bioinformatic analysis of multi-omics data from four separate COPD cohorts was complemented with relevant *in vitro* experimental models (figure 1).

Material and methods

Detailed descriptions of patient cohorts, materials and methods can be found in the supplementary material.

Patient cohorts

Patient phenotyping was based on bioinformatic analysis of epithelial brushing transcriptomic data and sputum or bronchoalveolar lavage fluid (BALF) proteomic data from four different cohorts: Southampton cohort (COPD patients, n=38) [18]; Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbations (BEAT-COPD) (COPD patients, n=74) [19]; Manchester cohort 1 (COPD patients, n=23) [20]; and Manchester cohort 2 (COPD patients, n=29; healthy nonsmokers and healthy smokers, n=35) [21].

Measurements in sputum

BEAT-COPD sputum samples were analysed for bacteria (using standard routine culture) and processed to produce cytospins for cell analysis and supernatant for fluid phase measurements. A broad panel of serum

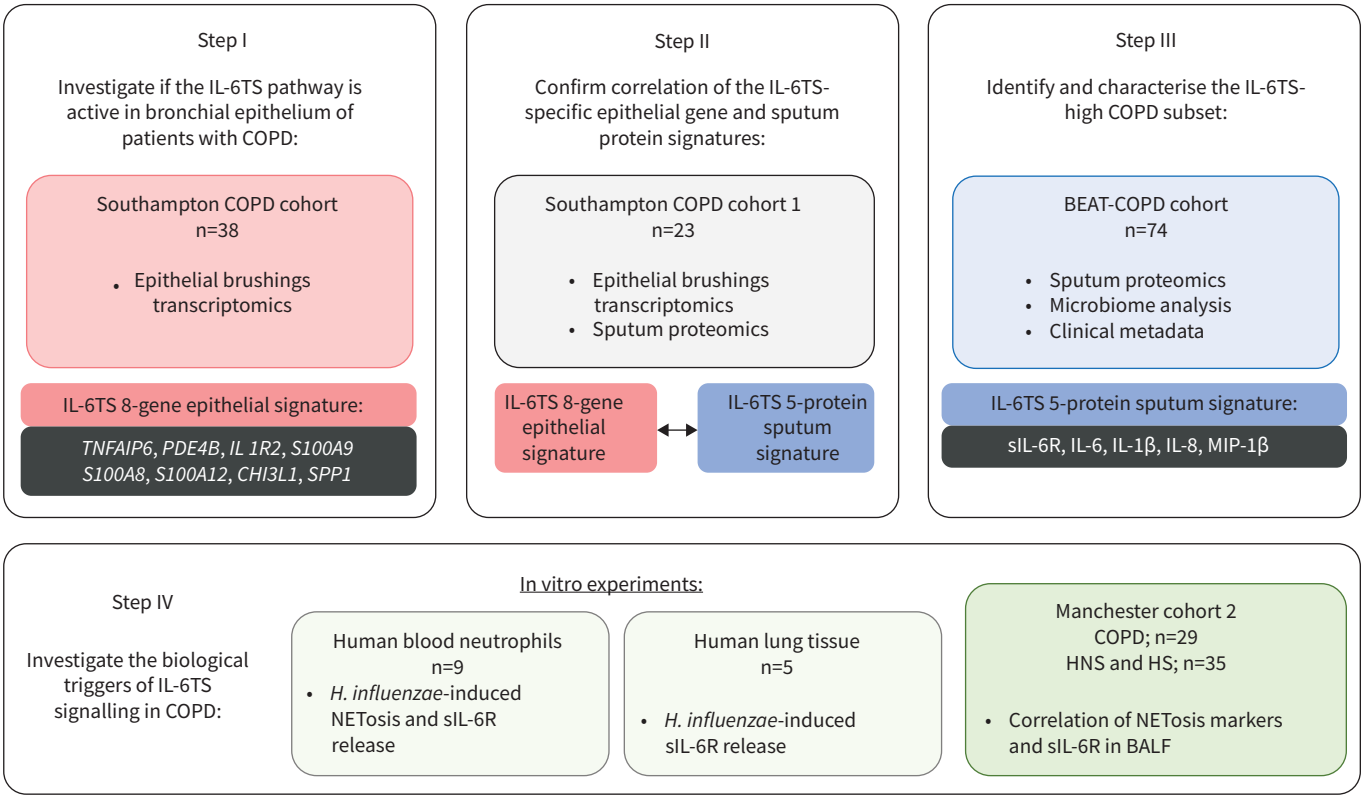

FIGURE 1 Study flow diagram. IL-6TS: interleukin (IL)-6 trans-signalling; BEAT-COPD: Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbation; *H. influenzae*: *Haemophilus influenzae*; NETosis: neutrophil extracellular trap formation; HNS: healthy nonsmokers; HS: healthy smokers; BALF: bronchoalveolar lavage fluid.

and sputum biomarkers were measured using the Meso-Scale-Discovery and single ELISA at stable and exacerbation visits [19]. Bacterial genomic DNA was extracted from sputum samples and 16S rRNA gene sequencing was performed as described previously [22, 23] and summarised in the supplementary material.

Unsupervised hierarchical clustering

Hierarchical clustering of gene expression data and sputum proteomic data was performed using the average linkage and Euclidean metric methods, with each variable normalised to mean 0 and variance 1, using Qlucore Omics Explorer 3 (Qlucore, Lund, Sweden). Results were visualised as dendrogram heat maps where the colour scale corresponds to a range from −2.0 (blue), *via* 0.0 (grey) to +2.0 (red).

NETosis

Human blood neutrophils were treated with *H. influenzae* for 3 h or with 4 µM ionomycin and 2 mM calcium chloride ($CaCl_2$) for 1 h. The extracellular DNA associated with NETosis was measured by adding the cell impermeable SYTOX green nucleic acid stain (ThermoFisher Scientific) to the live neutrophil culture at the time of *H. influenzae* or ionomycin/$CaCl_2$ challenge. To assess NETosis by the expression of citrullinated histone H3 (H3cit) the cells were fixed and analysed by immunofluorescence staining. sIL-6R levels were measured using Human IL-6R alpha Quantikine ELISA Kit (R&D).

Measurements in BALF

The levels of surrogate NETosis markers and sIL-6R were analysed in BALF from COPD patients and healthy volunteers from Manchester cohort 2. Cell-free (cf)DNA was measured using PicoGreen Quant-It assay (Invitrogen P7589). Myeloperoxidase (MPO) and sIL-6R levels were measured using the Human Myeloperoxidase Kit (MSD K151EEC) and Human IL-6R alpha Quantikine ELISA Kit, respectively.

Statistical analyses

Gene expression and sputum biomarker data was log_2-transformed and analysed using general linear model based statistical tests, adjusting for age and sex, using Qlucore Omics Explorer 3.3. Benjamini–Hochberg

multiple correction was used for gene expression data to control for rate of false positives (referred to as q-value). Statistical analysis of clinical variables and biomarker data was performed with Kruskal–Wallis tests, Mann–Whitney test or Chi-squared test. Proportions of bacteria were considered not normally distributed and were analysed by Mann–Whitney test. Statistical analyses of *in vitro* data were performed using one-way ANOVA (Tukey's multiple comparisons) and t-tests. All data analyses except analysis of gene expression data were considered hypothesis based and significance reached if $p \leqslant 0.05$ (q for gene expression data). Prism 6.0 (GraphPad Software) was used for data analysis and graphical representation.

Results

An IL-6TS-specific gene signature in bronchial epithelium defines a subset of COPD patients with increased markers of innate inflammation

Our previously described IL-6TS eight-gene signature (*TNFAIP6*, *PDE4B*, *IL1R2*, *S100A9*, *S100A8*, *S100A12*, *CHI3L1* and *SPP1*), derived from IL-6TS-stimulated primary human bronchial epithelial cells [2] was used to investigate whether the IL-6TS pathway is active in bronchial epithelium of patients with COPD (Southampton cohort; n=38) [18]. Hierarchical clustering identified a subset of patients (IL-6TS-high, n=12, 31.6%) with increased expression of the IL-6TS eight-gene epithelial signature (figure 2a). The IL-6TS-high subset showed a significantly increased expression of *SOCS3*, the main STAT3-inducible gene [24], linking the subset with JAK/STAT3 pathway activation (figure 2b). Furthermore, the IL-6TS-high patients displayed an increased expression of TLR genes and other genes associated with innate inflammation, including *CCL4*, *IL1B* and *IL8* (figure 1c and d). This IL-6TS related bronchial epithelial gene expression profile supports MIP-1β (*CCL4*), IL-1β and IL-8, together with the pathway triggers IL-6 and sIL-6R, as protein biomarkers of IL-6TS pathway activation in COPD patients, herein referred to as "IL-6TS five-protein sputum signature". A modest, but significant positive correlation

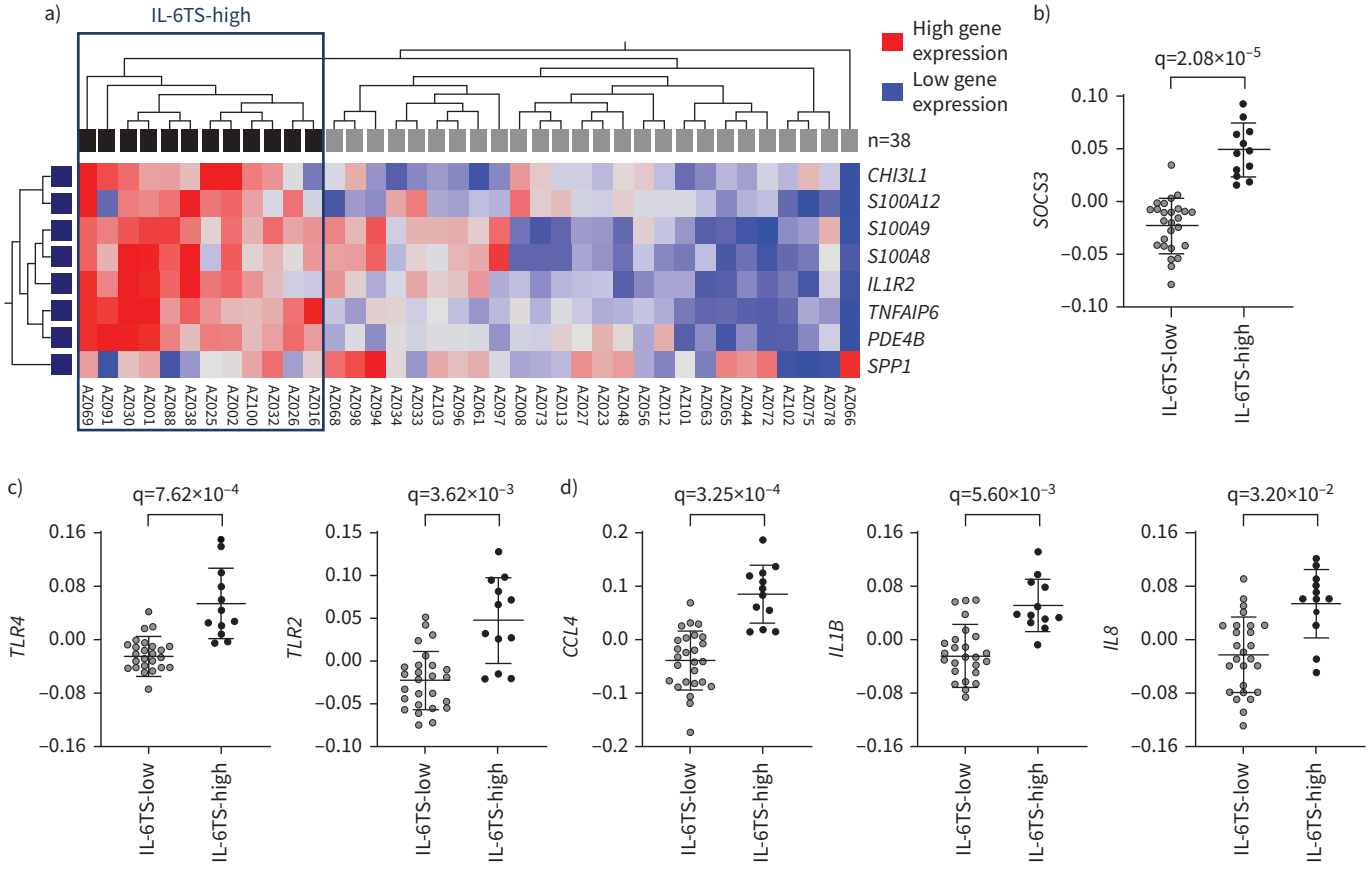

FIGURE 2 Clustering of COPD patients based on interleukin (IL)-6 trans-signalling (IL-6TS) eight-gene epithelial signature in the Southampton cohort. a) Hierarchical clustering by using the IL-6TS eight-gene epithelial signature (*TNFAIP6*, *PDE4B*, *IL1R2*, *S100A9*, *S100A8*, *S100A12*, *CHI3L1* and *SPP1*). The subset of patients with increased IL-6TS eight-gene epithelial signature (IL-6TS-high) is highlighted. b–d) Differential gene expression between IL-6TS-high (n=12) and IL-6TS-low (n=26) patients. Normalised and zero-centred gene expression values are shown. Benjamini–Hochberg test was used for q-values. Lines represent means±SD.

(r=0.49; p=0.017) between the IL-6TS eight-gene epithelial signature and the IL-6TS five-protein sputum signature was confirmed in an additional COPD cohort (Manchester cohort 1; n=23) [20] where paired bronchial epithelial brushings and sputum samples were available (supplementary figure S1).

IL-6TS five-protein sputum signature IL-6, sIL-6, MIP-1β, IL-8 and IL-1β identifies a neutrophilic subset of poorly controlled COPD patients

To further explore the role of the IL-6TS pathway in COPD, we investigated the existence of an IL-6TS-associated phenotype in the BEAT-COPD cohort (n=74), a clinically well-characterised cohort with sputum proteomic and microbiome data [19]. Stratification of patients based on the IL-6TS five-protein sputum signature identified an IL-6TS-high subset of a similar size (n=24, 32.4%) to the one in the Southampton cohort (figure 3a). There were no significant differences in age, sex, smoking status, pack-year history, body mass index, frequency of exacerbations, corticosteroid dose or lung function between the IL-6TS-high and -low BEAT-COPD subsets (table 1). However, the IL-6TS-high subset exhibited an increase in total sputum cell counts (p=0.0001; figures 2 and 3b) with a significantly

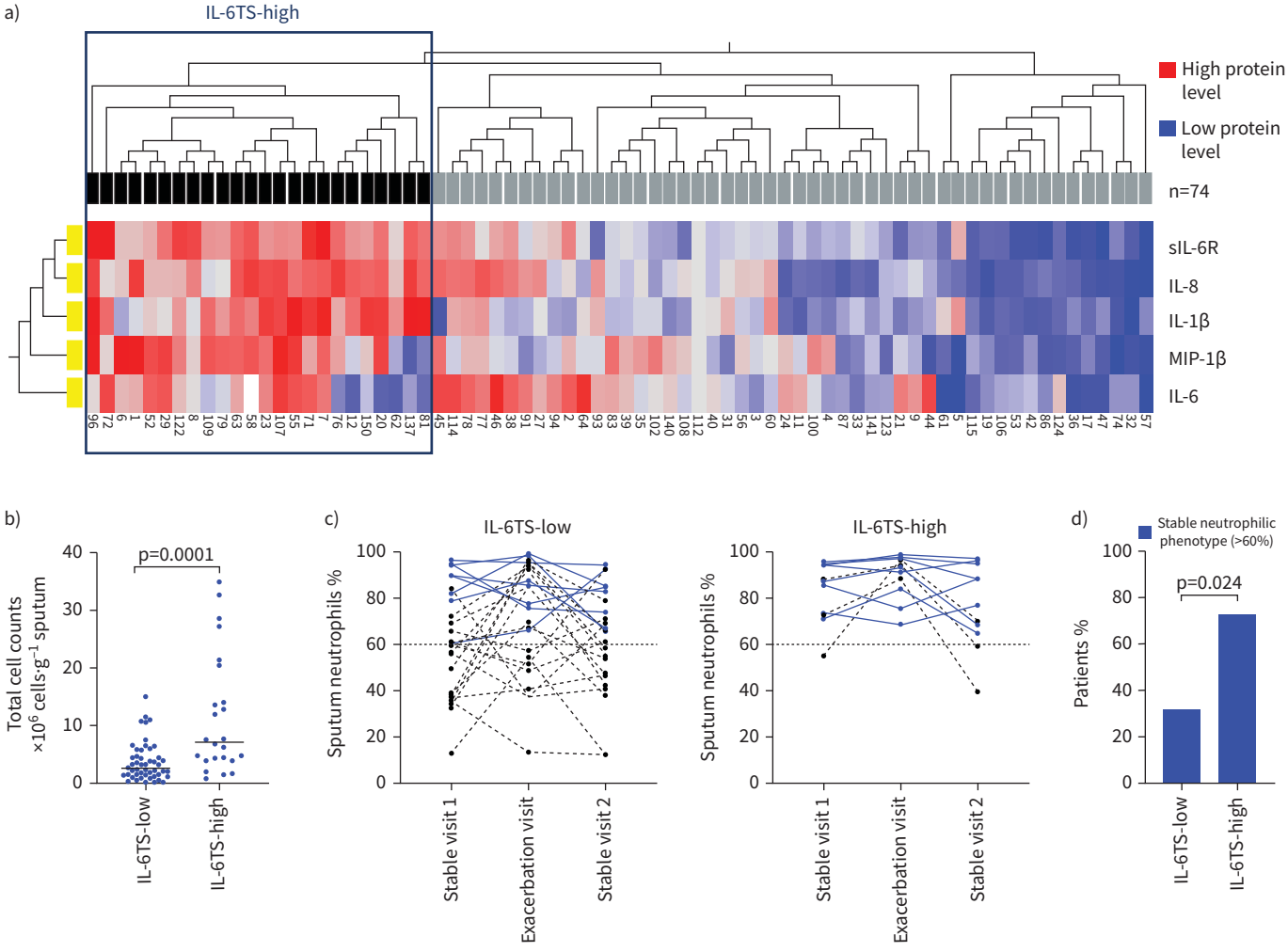

FIGURE 3 Clustering of COPD patients based on the interleukin (IL)-6 trans-signalling (IL-6TS) five-protein sputum signature in the BEAT-COPD (Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbation) cohort and association with sputum neutrophils. a) Hierarchical clustering by using the IL-6TS five-protein sputum signature (IL-6, soluble (s)IL-6, macrophage inflammatory protein (MIP)-1β, IL-8 and IL-1β). High protein levels are denoted by red and low levels by blue. The subset of patients with increased IL-6TS protein signature (IL-6TS-high) is highlighted. b) Total cell counts in the sputum of IL-6TS-high (n=24) are compared to the rest of COPD patients (IL-6TS-low; n=50). Kruskal–Wallis test was used for p-value. c) Proportion of sputum neutrophils (IL-6TS-high n=11, IL-6TS-low n=25) at baseline visit (stable visit 1), at exacerbation (exacerbation visit), and 6 weeks post-exacerbation (stable visit 2). Dotted line represents a cut-off for the neutrophilic phenotype (>60% sputum neutrophils). Patients maintaining a stable neutrophilic phenotype, *i.e.* >60% sputum neutrophils throughout all three visits, are highlighted in blue. d) Percentage of patients with a stable neutrophilic phenotype. Chi-squared test was used for p-value.

TABLE 1 Characteristics of interleukin (IL)-6 trans-signalling (IL-6TS)-high subjects with COPD (BEAT-COPD (Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbation) cohort)

	IL-6TS-high	IL-6TS-low	p-value
Subjects n	24	50	
Age years	69.0±1.8	67.8±1.4	0.60
Female	33.3	28.0	0.75#
BMI kg·m^{-2}	25.70±0.96	25.04±0.63	0.32
Current and ex-smokers	95.83	96.00	0.97#
Pre-FEV$_1$ L	1.27±0.12	1.14±0.07	0.26
Post-FEV$_1$ L	1.29±0.12	1.19±0.08	0.46
Exacerbations in past year	3 (1.5–4.0) (n=19)	2 (2.0–3.0) (n=37)	0.61
Daily ICS dose µg·day^{-1}	1000 (500–1000) (n=18)	1000 (800–1000) (n=35)	0.75
Maintenance prednisolone	10.53 (n=19)	10.81 (n=37)	0.97#
CRQ total score	13.64±0.98	16.72±0.63	**0.008**
CRQ mastery	4.07±0.29	4.87±0.21	**0.021**
CRQ fatigue	2.84±0.26	3.64±0.18	**0.049**
CRQ emotion	3.74±0.32	4.61±0.18	0.088
CRQ dyspnoea	2.64±0.29	3.06±0.19	0.26
Blood eosinophils ×1000·µL^{-1}	0.24 (0.17–0.37)	0.22 (0.13–0.39)	0.32
Blood neutrophils ×1000·µL^{-1}	5.35 (4.48–5.90)	4.74 (4.15–5.78)	0.24
Serum CRP mg·L^{-1}	6.77±2.22	4.55±1.10	0.088
Serum IL-6 pg·mL^{-1}	17.42±7.18	12.64±3.14	**0.024**
TCC ×10^6 cells·g^{-1} sputum	7.14 (4.2–15.6)	2.62 (1.4–4.6) (n=49)	**0.0001**
Sputum eosinophils %	1.00 (0.3–2.5)	1.75 (0.5–4.3) (n=48)	0.13
Sputum neutrophils %	87.13 (72.6–92.6)	66.25 (44.4–81.7) (n=48)	**0.0004**
Sputum macrophages %	10.11 (5.3–19.7)	25.00 (12.7–36.4) (n=48)	**0.002**
Sputum TNF-α pg·mL^{-1}	46.02±56.36	2.11±3.11	**<0.00001**
Sputum MIP-1α pg·mL^{-1}	153.80±52.27	44.63±0.37	**<0.00001**
Sputum RANTES pg·mL^{-1}	7.41±2.34	2.28±0.58	**<0.00001**
Sputum MMP9 ng·mL^{-1}	1008.79±279.40	201.32±49.47	**<0.00001**
Sputum MMP8 ng·mL^{-1}	736.57±229.86	127.32±372.61	**<0.00001**
Sputum MMP2 ng·mL^{-1}	3.11±0.54	0.00±0.40	**0.005**

Data are presented as geometric mean±SE, % or median (interquartile range), unless otherwise stated. Bold type represents statistical significance ($p \leq 0.05$). p-values by Kruskal–Wallis test. BMI: body mass index; FEV$_1$: forced expiratory volume in 1 s; ICS: inhaled corticosteroid; CRQ: Chronic Respiratory Questionnaire; CRP: C-reactive protein; TCC: total sputum cell count; TNF: tumour necrosis factor; MIP: macrophage inflammatory protein; MMP: matrix metalloproteinase. #: Chi-squared test.

increased proportion of neutrophils (p=0.0004) and increased levels of pro-inflammatory mediators and MMPs in sputum (table 1). Notably, the IL-6TS-high patient subset was associated with a lower quality of life as assessed by Chronic Respiratory Questionnaire (CRQ; total score 13.64±0.98 compared to 16.72±0.63 in the IL-6TS-low subset; p=0.008), with significantly lower scores for mastery (p=0.021) and fatigue (p=0.049; table 1). In contrast to the IL-6TS-low patients, most IL-6TS-high patients maintained the distinctive neutrophilic phenotype (>60% sputum neutrophils) observed at baseline visit at exacerbation and 6 weeks post-exacerbation (figure 3c and d).

The IL-6TS-high COPD subset is characterised by infection with Haemophilus

The sputum microbiome from patients in the BEAT-COPD cohort was assessed by 16S rRNA gene sequencing at stable state and at exacerbations (n=39). Comparison of the microbiome profiles between IL-6TS subsets showed a significantly decreased relative abundance of the phylum Firmicutes and increased abundance of Proteobacteria, with *Haemophilus* as the most represented genus in the IL-6TS-high subset at stable state (figure 4a and b). A significantly increased relative abundance of Proteobacteria and *Haemophilus* (figure 4a and b) and an increased Proteobacteria:Firmicutes (P:F) proportion ratio (figure 4c), were maintained at exacerbations in IL-6TS-high patients. The percentage of patients maintaining high relative proportions (*i.e.* >0.4) of Proteobacteria and *Haemophilus* throughout both visits was significantly increased in the IL-6TS-high subset (supplementary figure S2). Analysis of bacterial growth from the sputum samples revealed that significantly more patients in the IL-6TS-high subset (73.7% *versus* 22.5%; p=0.0002; figure 5a) were positive for pathogenic microorganisms (*H. influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*), in particular *H. influenzae* (42.1% *versus* 7.5%; p=0.001; figure 5b).

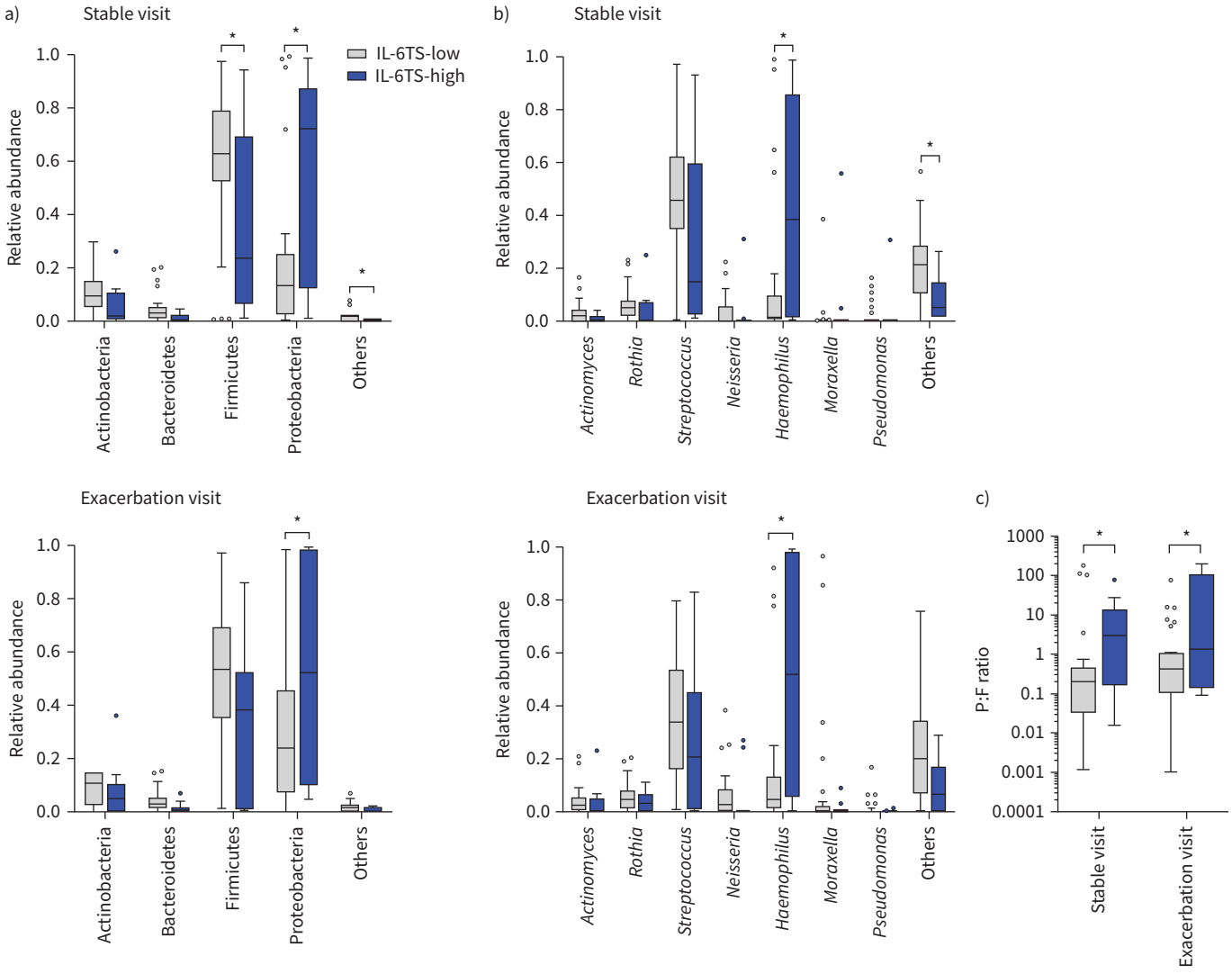

FIGURE 4 Sputum bacterial profiling of the interleukin (IL)-6 trans-signalling (IL-6TS)-high patients in the BEAT-COPD (Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbation) cohort. Relative abundances of the most abundant sputum bacterial a) phyla and b) genera in clinically stable disease and at exacerbation (IL-6TS-high n=11, IL-6TS-low n=28). c) Proteobacteria:Firmicutes (P:F) proportions ratio at stable and exacerbation visit. Data are presented as Tukey's boxplots. *: p<0.05 (Mann–Whitney).

H. influenzae-*induced NETosis leads to sIL-6R release from primary human neutrophils*

NETosis is a process whereby neutrophils release chromatin filaments coated with citrullinated histones and antibacterial proteins in order to trap and kill bacteria [25, 26]. Neutrophil extracellular traps (NETs) have been observed in the airways of patients with COPD infected by *Haemophilus* species [27]. The association of the IL-6TS-high COPD subset with persistent lung neutrophilia and colonisation with *H. influenzae* suggested that NETosis induced by *H. influenzae* may be a driver of sIL-6R release from primary human neutrophils. Citrullination of histones by the enzyme peptidyl arginine deiminase (PAD)4 is a requirement for NETosis [28, 29], and we have used a novel small molecule PAD4-inhibitor (PAD4i), developed by AstraZeneca and described in the supplementary methods and supplementary figure S3) to specifically block NETosis. *H. influenzae* efficiently induced NETosis of fresh human blood neutrophils, as shown by increased accumulation of extracellular DNA and expression of H3cit, a characteristic marker of PAD4-dependent NET formation [25, 26, 30], and these processes were efficiently inhibited by PAD4i (figure 6a and b). Increased expression of extracellular H3cit and colocalisation with extracellular DNA positive NETs were confirmed in *H. influenzae*-infected neutrophils (figure 6c). Ionomycin, a known and widely used inducer of NETosis, was included as a positive control [25]. Induction of NETosis by *H. influenzae* was consistently associated with increased sIL-6R release across different neutrophil donors

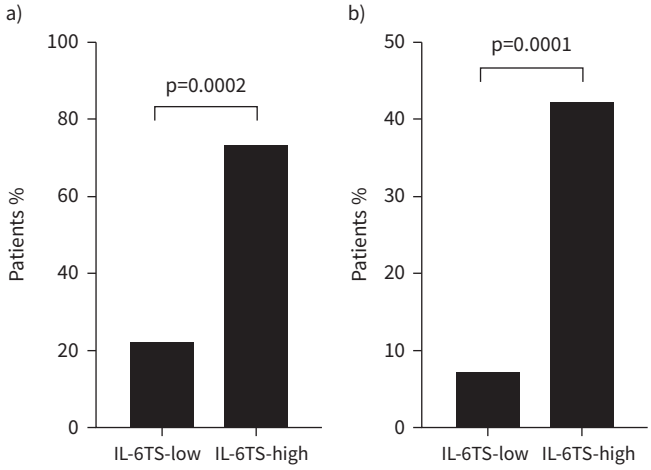

FIGURE 5 Sputum bacterial growth in the interleukin (IL)-6 trans-signalling (IL-6TS)-high patients in the BEAT-COPD (Biomarkers to Target Antibiotic and Systemic Corticosteroid Therapy in COPD Exacerbation) cohort. Percentages of patients (IL-6TS-high n=19, IL-6TS-low n=39) with significant growth of a) pathogenic micro-organisms (*Haemophilus influenzae, Moraxella catarrhalis*, *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*) and b) *H. influenzae* evaluated according to United Kingdom Health Protection Agency standard operating procedures. Chi-squared test was used for p-values.

(n=9; figure 6d). Blocking NETosis by PAD4i significantly reduced the levels of sIL-6R (figure 6e). A potential functional link between colonisation with *H. influenzae* and increased sIL-6R release in the lungs was confirmed in fresh human lung tissue infected with *H. influenzae* (supplementary figure S4). Infection with *H. influenzae* resulted in a significant increase of sIL-6R in the surrounding medium. The bacterial concentrations inducing the highest levels of sIL-6R differed between the donors, and the levels of sIL-6R were reduced after reaching a peak, potentially due to excessive proteolytic activity induced by higher bacterial loads.

The levels of sIL-6R positively correlate with surrogate NETosis markers in BALF from COPD patients

The levels of surrogate NETosis markers, including cfDNA and MPO were increased in BALF from COPD patients (n=29; infrequent exacerbators n=16 and frequent exacerbators n=13) compared to healthy volunteers (n=35; healthy nonsmokers n=27 and healthy smokers n=8) from the Manchester cohort 2 (figure 7a), while sIL-6R was not significantly different between the groups (supplementary figure S5). cfDNA and MPO were significantly increased in COPD patients with high levels of sIL-6R (upper quartile; >165 pg·mL^{-1}), compared to patients with low sIL-6R (lower quartile; <84 pg·mL^{-1}) (figure 7b). In addition, the levels of sIL-6R positively correlated with cfDNA (r=0.67; p<0.0001) and MPO (r=0.66; p=0.0001) (figure 7c). A nearly perfect correlation was shown for cfDNA and MPO (r=0.99; p<0.0001), suggesting we were detecting cfDNA–MPO complexes which are specific NET components. This observation strengthens our hypothesis that sIL-6R release is a NETosis-driven process. The positive correlations of sIL-6R with cfDNA and MPO was more prominent in COPD patients with frequent exacerbations than in patients with infrequent exacerbations (supplementary figure S5).

Discussion

While there is an established link between IL-6TS and the pathophysiology of COPD [2–6], little is known regarding the pathological drivers of IL-6TS in the airways and the phenotypic characteristics of COPD patients with increased IL-6TS pathway activation. We show that increased expression of IL-6TS-related biomarkers overlaps with persistent neutrophilic airway inflammation and infection with Proteobacteria dominated by the genus *Haemophilus* in patients with COPD. Furthermore, we provide evidence for a direct connection between infection of lung tissue with *H. influenzae* and increased release of sIL-6R. Consistent with previous studies [17], we found that neutrophils represent a source of sIL-6R, and we show that sIL-6R is released from neutrophils during *H. influenzae*-induced NETosis. Providing novel insights into the heterogeneity of COPD, by identifying a new patient subset characterised by IL-6TS and by elucidating the underlying pathological mechanisms, will empower future development of specific treatments and precision medicine approaches.

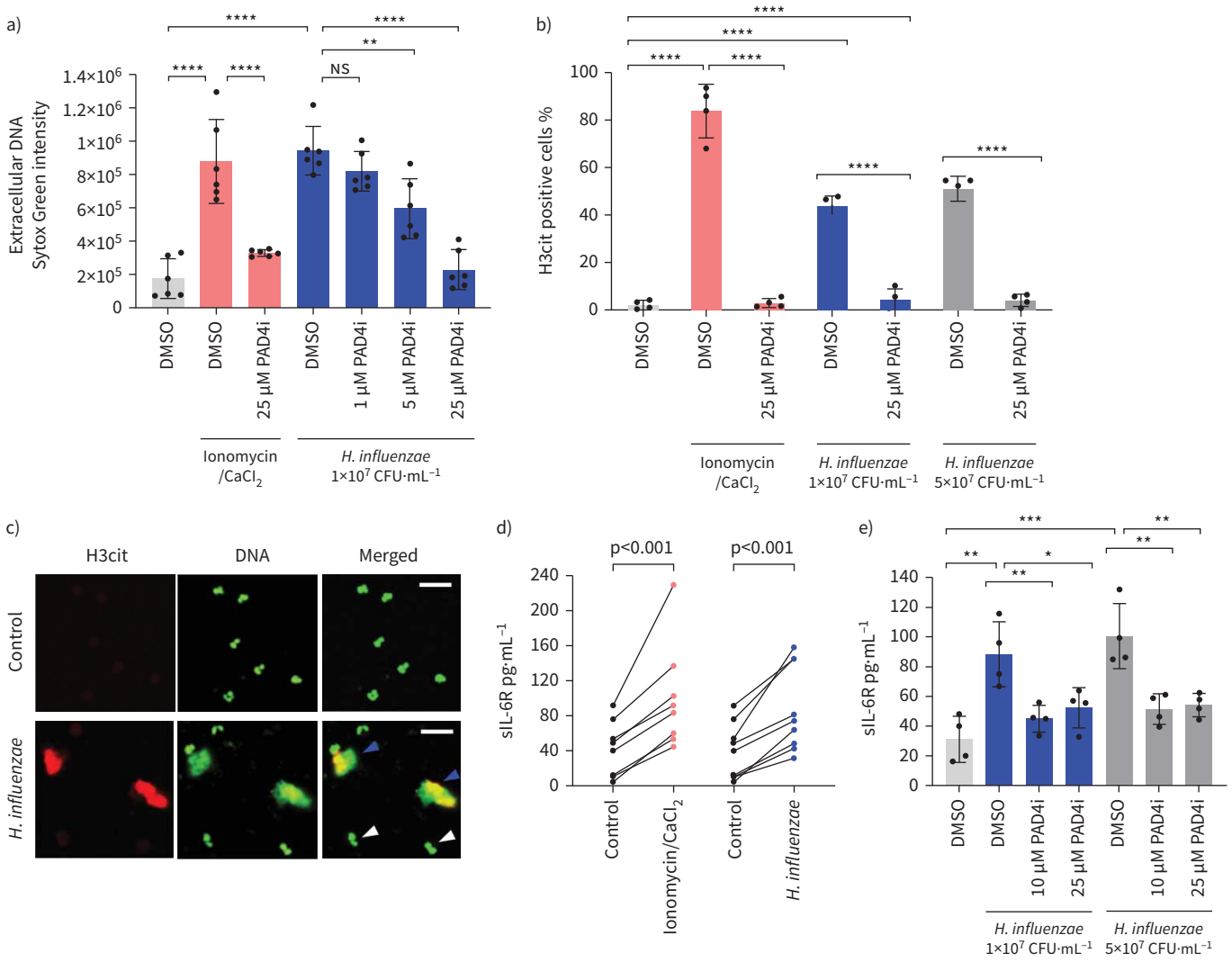

FIGURE 6 Increased release of soluble interleukin-6 receptor (sIL-6R) from human neutrophils during *Haemophilus influenzae*-induced neutrophil extracellular trap (NET) formation (NETosis). Fresh human blood neutrophils were infected with *H. influenzae* or stimulated with ionomycin/calcium chloride ($CaCl_2$) and analysed for the accumulation of extracellular DNA, expression of citrullinated H3 (H3cit), NET formation and the release of sIL-6R. Peptidyl arginine deiminase inhibitor (PAD4i) was used to specifically block NETosis. a) The accumulation of extracellular DNA (six donors) and b) the percentage of H3cit-positive cells (four donors) were analysed. **: p<0.01, ****: p<0.0001 (one-way ANOVA). c) Representative images of H3cit and DNA-positive NETs in *H. influenzae*-infected cells (blue arrowheads) and intact neutrophils (white arrowheads). Scale bars=25 µm. d) Release of sIL-6R into the surrounding medium following stimulation with ionomycin/$CaCl_2$ and *H. influenzae* (1×10^8 CFU·mL^{-1}), respectively (nine donors). Paired t-test was used for p-values. e) sIL-6R release was significantly inhibited by PAD4i (four donors). *: p<0.05, **: p<0.01, ***: p<0.001 (one-way ANOVA). NS: nonsignificant; DMSO: dimethyl sulfoxide.

In a recent study we identified an IL-6TS eight-gene epithelial signature (*TNFAIP6*, *PDE4B*, *IL1R2*, *S100A9*, *S100A8*, *S100A12*, *CHI3L1* and *SPP1*) in patients with asthma and proposed that it corresponds to a set of IL-6TS-related sputum protein biomarkers, including IL-6, sIL-6R, MIP-1β, IL-8, IL-1β, YKL-40 and MMP3 [2]. This led us to the identification of a novel IL-6TS-high subset in asthma, characterised by lung epithelial IL-6TS pathway activation in notable absence of systemic IL-6 inflammation. In this study, we confirmed a positive correlation between the IL-6TS eight-gene and five-protein (IL-6, sIL-6, MIP-1β, IL-8 and IL-1β) signatures within the same patients in a smaller COPD cohort with paired epithelial and sputum samples (supplementary figure S1). This supports the potential of the IL-6TS eight-gene epithelial and five-protein sputum signatures to identify comparable IL-6TS-high subsets of COPD patients.

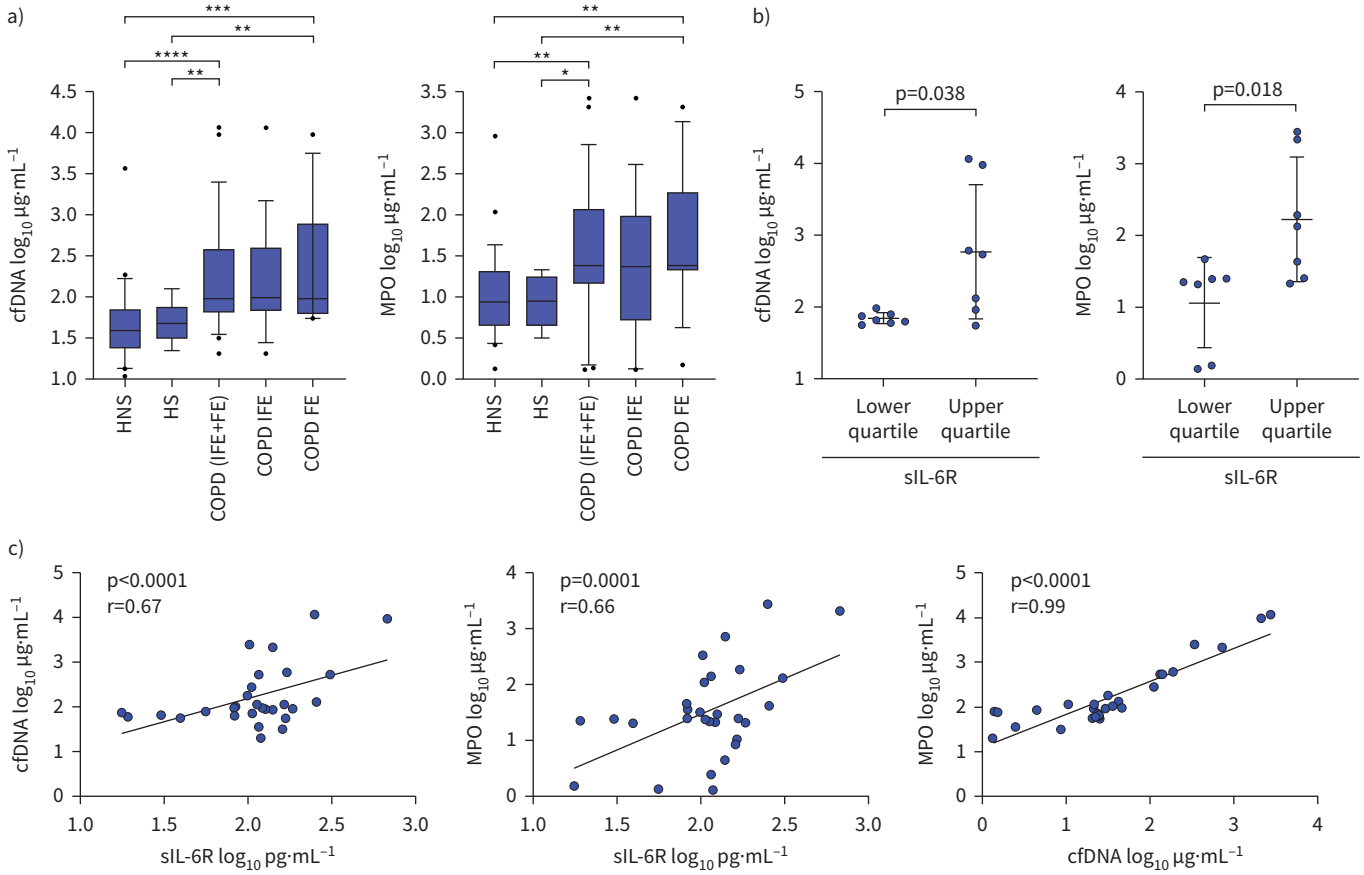

FIGURE 7 Correlation of soluble interleukin-6 receptor (sIL-6R) and surrogate neutrophil extracellular trap (NET) formation (NETosis) markers in bronchoalveolar lavage fluid (BALF) from COPD patients. a) The levels of surrogate NETosis markers, including cell-free DNA (cfDNA) and myeloperoxidase (MPO) were analysed in BALF from COPD patients (infrequent exacerbators (IFE) n=16, frequent exacerbators (FE) n=13) compared to healthy volunteers (healthy nonsmokers (HNS) n=27, healthy smokers (HS) n=8) from Manchester cohort 2. Data are presented as 10th to 90th percentile boxplots. b) The levels of surrogate NETosis markers were increased in COPD patients (IFE+FE) with high levels of BALF sIL-6R (upper quartile; >165 pg·mL^{-1}) compared to low levels of sIL-6R (lower quartile; <84 pg·mL^{-1}). *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001 (Mann–Whitney). c) Correlations of BALF sIL-6R, cfDNA and MPO in COPD patients (IFE+FE; n=29) were examined using Pearson's correlation test. The line represents a linear regression fit.

The IL-6TS eight-gene epithelial signature was increased in a subset of COPD patients from the Southampton lung epithelial brushing cohort. The IL-6TS-high COPD patients showed significantly higher lung epithelium expression of TLR2 and TLR4, replicating the findings from the IL-6TS-high subset in asthma, where high IL-6TS signature overlapped with augmented markers of TLR pathway activation [2]. Several lines of evidence suggest a positive interplay between IL-6TS and TLR pathways. IL-6TS has been shown to enhance TLR4-dependent inflammatory responses *via* STAT3, and specific inhibition of IL-6TS completely protected mice from lipopolysaccharide/TLR4-mediated septic shock [31]. Similarly, hyperactivation of STAT3 upregulated TLR2 gene expression in gastric epithelial cells [32]. Furthermore, IL-6TS significantly amplified TLR ligand induced production of inflammatory mediators (IL-1β, IL-8, tumour necrosis factor (TNF)-α, monocyte chemoattractant protein-1) by stromal and innate immune cells [6]. Conversely, activation of TLR2 in human monocytes induced IL-6TS by promoting the secretion of IL-6 and the generation of sIL-6R [33], suggesting cross-talk between the IL-6TS/JAK/STAT and TLR pathways as a broader mechanism that augments the severity of inflammatory responses in the IL-6TS-high phenotype.

The IL-6TS five-protein sputum signature was upregulated in a subset of stable COPD patients in the BEAT-COPD cohort correlating with increased total sputum cell counts and a higher percentage of sputum neutrophils. Importantly, high levels of sputum IL-6 did not necessarily overlap with the IL-6TS-high

subset. This implies that the IL-6TS subset identified in this study is distinct from a COPD subset that would be identified by IL-6 alone. In contrast to IL-6TS-low patients, the majority of the IL-6TS-high patients maintained a stable neutrophilic phenotype over time, including during exacerbation. Similar to the IL-6TS-high subset in asthma, the IL-6TS signature was associated with increased levels of airway remodelling biomarkers (MMP9, MMP8) and pro-inflammatory mediators (TNF-α, MIP-1α), suggesting a similar molecular phenotype, characterised by increased innate inflammatory responses. Unlike the IL-6TS phenotype in asthma, the IL-6TS-high COPD patients did not exhibit increased blood eosinophils and did not have a tendency towards increased exacerbations, indicating there might be different clinical manifestations of the IL-6TS-driven pathology in asthma and COPD. Instead, the IL-6TS-high COPD subset was characterised by a significantly lower quality of life as assessed by the CRQ compared to the rest of the patients.

The IL-6TS-high patients were characterised by an increased abundance of Proteobacteria, specifically the genus *Haemophilus*, and reduced Firmicutes. This replicates a previous finding, where an increased Proteobacteria:Firmicutes ratio was observed in a cluster of exacerbating COPD and asthma patients with neutrophilic inflammation and increased pro-inflammatory mediators in sputum [34]. The lung microbiome in our subset maintained a similar composition in the clinically stable state as at the onset of an exacerbation, suggesting that the microbial profile in the IL-6TS-high subset is longitudinally stable and possibly involved in maintaining chronicity of the host inflammatory responses, including IL-6TS. This hypothesis is supported by studies of cultured human bronchial epithelial cells incubated in the presence of purified endotoxin preparations from *H. influenzae*, which have demonstrated that these endotoxins lead to significantly increased expression and release of IL-6 [35, 36]. In addition, *H. influenzae* strongly induced IL-6 production by alveolar macrophages from COPD patients [37], and sputum IL-6 levels were found to be higher in COPD patients with bacterial colonisation of the lower airways with *H. influenzae* as the most frequently isolated pathogen compared with patients without bacterial colonisation or healthy controls [38]. To demonstrate a direct mechanistic link between the IL-6TS pathway and lung colonisation with *H. influenzae*, human lung tissue explants were infected with *H. influenzae*, which led to increased release of sIL-6R. A notable limitation of our experimental model was a high interdonor variability of the doses of *H. influenzae* that triggered sIL-6R release, presumably due to different cellular composition of the tested lung tissue (*i.e.* different levels of immune cells representing the main source of sIL-6R).

Human neutrophils express high levels of mIL-6R on their surface and are considered a major source of sIL-6R, released in response to inflammatory [17] and apoptotic stimuli [39]. In the present study we show that infection of neutrophils with *H. influenzae* induced NETosis coinciding with increased release of sIL-6R. Here, we used a novel inhibitor that was able to specifically block NETosis by targeting the known NETosis driver PAD4 [28, 29] to confirm the role of *H. influenzae*-induced NETosis in sIL-6R release. NETosis is more common in the airways of patients with neutrophilic asthma and COPD [40, 41], and it is associated with increased levels of *Haemophilus* species [27]. These findings, together with our new data, suggest that *Haemophilus* may be a main driver of airway IL-6TS pathway activation by triggering sIL-6R release from neutrophils during the process of NETosis. Importantly, we confirmed that the levels of sIL-6R positively correlate with the levels of surrogate NETosis markers cfDNA and MPO in BALF from patients with COPD. Even though the proteases responsible for NETosis-mediated mIL-6R shedding are not revealed in this study, the major protease activity associated with NETs has been attributed to neutrophil elastase, cathepsin G and proteinase 3 [42], implicating these serine proteases as likely candidates. Of these, cathepsin G, but not neutrophil elastase or proteinase 3, has previously been shown to release sIL-6R at sites of inflammation [43].

Our COPD patient phenotyping relies on patient cohorts with overlapping proteomic, transcriptomic and microbiome data. However, the relatively small number of patients in the multi-omics COPD cohorts available to us represents a main limitation of this study, especially for the purpose of linking a molecular phenotype to clinical presentation. Additionally, we had limited information about comorbidities that could impact the quality of life score assessed by CRQ. Larger studies are needed for deep clinical characterisation of the COPD patient subset associated with increased IL-6TS, neutrophilic airway inflammation and *Haemophilus* infection. Interestingly, consistent with our findings, a recent larger study involving 253 COPD patients established an association between Proteobacteria (predominantly *Haemophilus*) dominance and more frequent exacerbations, lower forced expiratory volume in 1 s and increased mortality [44].

In conclusion, we show that chronic IL-6TS is a hallmark of COPD patients with persistent neutrophilic inflammation, and it is potentially implicated in amplifying host inflammatory responses and airway remodelling. Moreover, our data suggest that *H. influenzae* can drive IL-6TS in the lungs by triggering

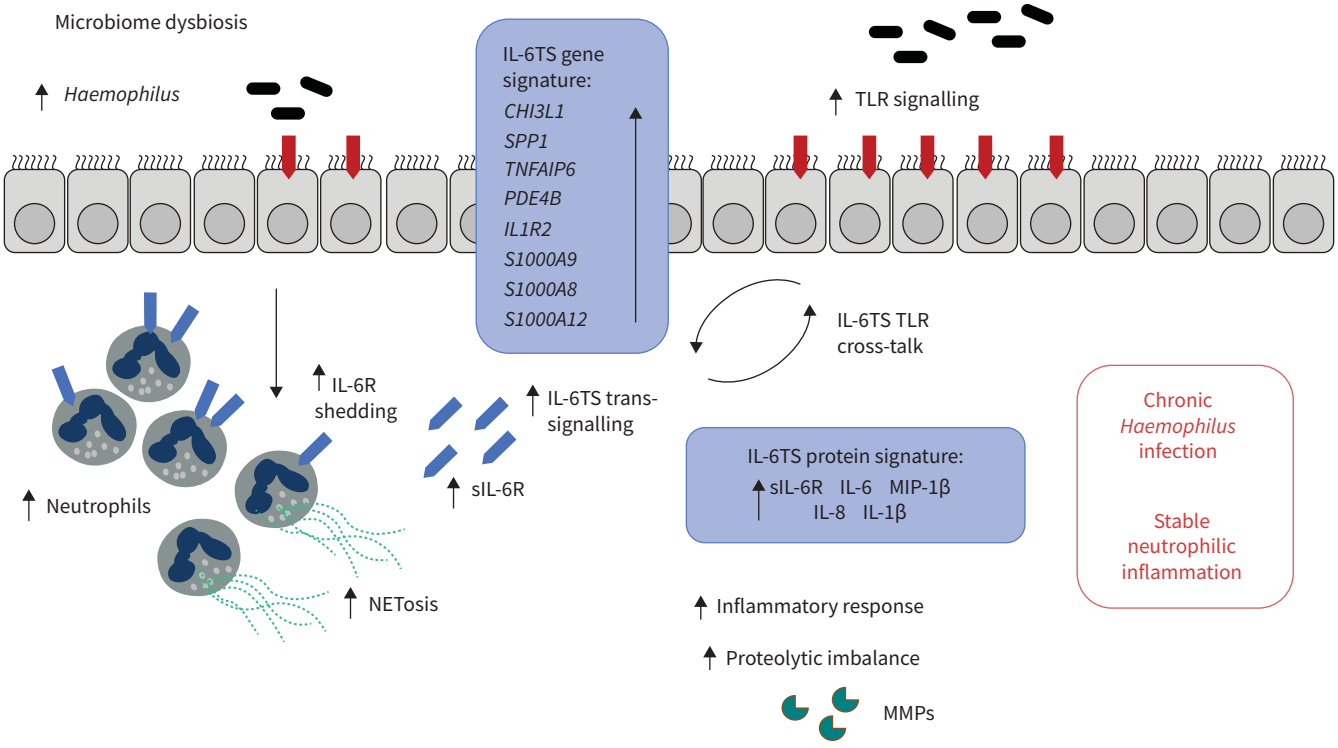

FIGURE 8 Schematic overview of the multi-omics identification of the interleukin (IL)-6 trans-signalling (IL-6TS)-high COPD patients and the underlying pathology of the subset. TLR: Toll-like receptor; MMP: matrix metalloproteinase.

sIL-6R release from neutrophils during the process of NETosis (figure 8). This furthers our understanding of the cross-talk between the microbiome and the airways, opening potential new avenues for the discovery of new biomarkers and respiratory therapeutics.

Acknowledgements: We are grateful to all the lung tissue donors. We thank the surgeons and nurses from the Thoraxkliniken, Sahlgrenska University Hospital and Gothenburg University, Gothenburg, Sweden for lung sample collection. We thank the students M. Marande and R. Dolgos (Faculty of Science and Technology of Nancy, Nancy, France) for their help with the lung tissue experiment. Special thanks also to the occupational health specialist K. Vesterlund (AstraZeneca, Gothenburg, Sweden) for blood sample collection. We thank L. Öberg, P. Konings, E. Mohamed and N. Van Zuydam (AstraZeneca, Gothenburg) for their help with bioinformatic and statistical analyses.

Conflict of interest: S. Winslow is an employee and a share/stockholder of AstraZeneca. L. Odqvist is an employee and a share/stockholder of AstraZeneca. S. Diver has nothing to disclose. R. Riise is an employee and a share/stockholder of AstraZeneca. S. Abdillahi is an employee and a share/stockholder of AstraZeneca. C. Wingren is an employee and a share/stockholder of AstraZeneca. H. Lindmark is an employee and a share/stockholder of AstraZeneca. A. Wellner is an employee and a share/stockholder of AstraZeneca. S. Lundin is an employee and a share/stockholder of AstraZeneca. L. Yrlid is an employee and a share/stockholder of AstraZeneca. E. Ax is an employee and a share/stockholder of AstraZeneca. R. Djukanovic reports personal fees for lectures and consultancy from TEVA and Novartis, personal fees for consultancy from Synairgen, personal fees for meeting participation from GlaxoSmithKline, outside the submitted work. S. Sridhar is an employee and a share/stockholder of AstraZeneca. A. Higham has nothing to disclose. D. Singh reports personal fees from AstraZeneca, Boehringer Ingelheim, Chiesi, Cipla, Genentech, GlaxoSmithKline, Glenmark, Gossamerbio, Menarini, Mundipharma, Novartis, Peptinnovate, Pfizer, Pulmatrix, Theravance, Verona, outside the submitted work. T. Southworth has nothing to disclose. C.E. Brightling reports personal fees from GSK, AstraZeneca/MedImmune, Boehringer Ingelheim, Novartis, Roche and Chiesi, outside the submitted work. H.K. Olsson is an employee and a share/stockholder of AstraZeneca. Z. Jevnikar is an employee and a share/stockholder of AstraZeneca.

Support statement: This study was funded by AstraZeneca. Employees from AstraZeneca participated in the study design, data collection, data analysis, data interpretation and the writing of the manuscript. AstraZeneca reviewed

the publication, without influencing the opinions of the authors, to ensure medical and scientific accuracy and the protection of intellectual property. The corresponding author had access to all data and had the final responsibility for the decision to submit the manuscript for publication. The other funding bodies were not involved in any parts of the design, analysis or interpretation of the study. E. Ax was also supported by the Swedish Foundation for Strategic Research (SSF). C.E. Brightling was supported by MedImmune, Medical Research Council, National Institute for Health Research Leicester Biomedical Research Centre (UK) and AirPROM (FP7–270194). D. Singh was supported by the National Institute for Health Research (NIHR) Manchester Biomedical Research Centre (BRC), North West Lung Centre Charity, Manchester, UK and by AstraZeneca, Gaithersburg, MD, USA. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

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