# Sputum proteomics and airway cell transcripts of current and ex-smokers with severe asthma in U-BIOPRED: an exploratory analysis

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**Inflammatory, oxidative/ER stress and epithelial barrier pathways are differentially activated in current smoking and ex-smoking severe asthma patients** http://ow.ly/BVv530j3iP3

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ABSTRACT Severe asthma patients with a significant smoking history have airflow obstruction with reported neutrophilia. We hypothesise that multi-omic analysis will enable the definition of smoking and ex-smoking severe asthma molecular phenotypes.

The U-BIOPRED cohort of severe asthma patients, containing current-smokers (CSA), ex-smokers (ESA), nonsmokers and healthy nonsmokers was examined. Blood and sputum cell counts, fractional exhaled nitric oxide and spirometry were obtained. Exploratory proteomic analysis of sputum supernatants and transcriptomic analysis of bronchial brushings, biopsies and sputum cells was performed.

Colony-stimulating factor (CSF)2 protein levels were increased in CSA sputum supernatants, with azurocidin 1, neutrophil elastase and CXCL8 upregulated in ESA. Phagocytosis and innate immune pathways were associated with neutrophilic inflammation in ESA. Gene set variation analysis of bronchial epithelial cell transcriptome from CSA showed enrichment of xenobiotic metabolism, oxidative stress and endoplasmic reticulum stress compared to other groups. CXCL5 and matrix metallopeptidase 12 genes were upregulated in ESA and the epithelial protective genes, mucin 2 and cystatin SN, were downregulated.

Despite little difference in clinical characteristics, CSA were distinguishable from ESA subjects at the sputum proteomic level, with CSA patients having increased CSF2 expression and ESA patients showing sustained loss of epithelial barrier processes.

The transcriptomic data have been deposited in the Gene Expression Omnibus database, www.ncbi.nlm.nih.gov/geo (accession number GSE76225 for gene expression data of bronchial biopsies).

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## Introduction

Severe asthma has been defined as asthma that requires treatment with high-dose inhaled corticosteroids and long-acting $\beta_2$-agonists and often systemic corticosteroids to prevent it from becoming "uncontrolled", or that remains "uncontrolled" despite this therapy [1]. A significant number of patients with asthma are current smokers or ex-smokers [2]. Asthmatic patients who smoke may develop poorly controlled asthma, a poor response to corticosteroid therapy, an accelerated decline in lung function and increased healthcare utilisation [3]. In an analysis of clinical phenotypes of severe asthma of the Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED) cohort based on clinical and physiological features, a phenotype of severe asthma consisting of current and ex-smokers was characterised with late-onset asthma and moderate-to-severe chronic airflow obstruction [4]. This phenotype may represent an asthma–chronic obstructive pulmonary disease (COPD) overlap syndrome, with features of both diseases. In patients who were recruited as COPD patients in the COPDgene cohort, the patients who had a history of asthma before the age of 40 years and who had a smoking history of ⩾10 pack-years with spirometric evidence of severe airflow obstruction had more exacerbations, and a greater airway wall thickness on computed tomographic scans at all degrees of airflow obstruction compared to those with COPD alone [5]. This suggests that asthma may be driving airflow obstruction in concert with cigarette smoking exposure. The mechanisms underlying smoking-associated asthma is unclear but smoking-associated asthma has been considered as a non-T-helper type 2 (Th2) neutrophilic asthma [6].

The U-BIOPRED project recruited patients with severe asthma, including active smokers and ex-smokers [7]. One of the hallmarks of U-BIOPRED is the collection of omics data from blood, bronchial epithelium, bronchial biopsies and sputum cells, the analyses of which have yielded distinct molecular phenotypes of severe asthma [8, 9]. In order to gain insight into the potential mechanisms that could underlie smoking or ex-smoking severe asthma, we examined the differential expression of genes and proteins in various compartments.

## Materials and methods

### *Clinical data*

We enrolled 374 severe asthma patients in the U-BIOPRED cohort, divided into three groups by smoking status: current smokers with severe asthma (CSA), ex-smokers with severe asthma (ESA) and nonsmokers with severe asthma (NSA). We narrowed down the NSA to those who had never smoked (0 pack-years), although the original NSA group in the U-BIOPRED cohort contained patients whose smoking history was <5 pack-years. In addition, 81 nonsmoking healthy volunteers (NH) with 0 pack-years were enrolled. Differential blood and induced sputum cell counts, serum total immunoglobulin E and skin prick tests, serum periostin and exhaled nitric oxide fraction ($F_{ENO}$) and pre- and post-bronchodilator spirometry were obtained [8, 9]. Bronchial biopsies, bronchial brushings and sputum were obtained, as previously described [8]. Because of the bronchoscopy exclusion criteria (online supplementary material), only 95 bronchial brushings and 69 bronchial biopsies were obtained. The number of sputum samples for proteomic analysis was 88. All subjects whose samples were adequate and underwent omics analyses are

shown in online supplementary figure S1. The study was approved by the ethics committees of each of the 16 clinical recruiting centres. All subjects gave written and signed informed consent.

### *Transcriptomic microarray analysis*

Sputum plugs were obtained and separated into cells and supernatants [7]. Cell pellets were used to prepare RNA using the miRNeasy mini kit (Qiagen, Valencia, CA, USA). Sputum samples with >30% squamous cells were excluded from microarray analysis. Bronchial brushings and biopsy samples were immediately placed in TRIzol reagent (Invitrogen, ThermoFisher Scientific, Paisley, UK) and preserved at −80°C. Expression profiling of transcriptome was performed using GeneChip® Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) as previously described [8, 9]. Pathway analysis, enrichment analysis and functional clustering of differentially expressed genes (DEGs) were performed as described previously [8, 9] and protein interaction analysis using annotated protein-coding genes was performed using STRING (version 10.0; STRING Consortium 2016, www.string-db.org) [10].

### *SomaLogic proteomic technique*

The SOMAscan proteomic assay of sputum supernatants performed by SomaLogic (Boulder, CO, USA) was used [9].

### *Gene set variation analysis*

Gene set variation analysis (GSVA) was performed in R using the Bioconductor GSVA package for estimating variation of gene set enrichment [11]. Gene sets were obtained from the Molecular Signatures Database v5.2 (MSigDB) (http://software.broadinstitute.org/gsea/msigdb) or from published papers (online supplementary table S1). We used Immunomap graphics ( Johnson & Johnson, New Brunswick, NJ, USA) for visualisation.

### *Statistical analysis*

All datasets were quality controlled and normalised, followed by adjustment of batch effects using ComBat tools and uploaded into tranSMART, an open-source knowledge management platform for sharing research data supported by European Translational Information and Knowledge Management Services (eTRIKS) [8, 9]. All categorical variables were analysed using Fisher's exact test. Continuous variables were analysed using Kruskal–Wallis testing. Gene and protein expression data were analysed using multivariate ANOVA; age, sex and systemic corticosteroids use were analysed as covariates. A p-value <0.05 was considered significant. A linear model for microarray data (Bioconductor limma package for R) with Benjamini–Hochberg false discovery rate (FDR) correction was used in the analysis of the DEGs and for GSVA. Fold change ⩾1.5 and FDR <0.05 was considered statistically significant in transcriptomic and proteomic analyses. When using GSVA, FDR <0.05 was considered statistically significant. Statistical analyses were performed using R version 3.3.1 (R Core Team, 2016; www.r-project.org).

## Results

### *Clinical characteristics of subjects with sputum SomaLogic data*

Table 1 shows the characteristics of subjects who provided sputum samples for SomaLogic analysis. $F_{eNO}$ levels of CSA subjects were lower than in the other severe asthma groups. Although there were numerical differences in blood eosinophil counts (CSA 259 cells·μL$^{-1}$, ESA 296 cells·μL$^{-1}$ and NSA 407 cells·μL$^{-1}$), sputum eosinophils (CSA 7.2%, ESA 14.8% and NSA 18.8%) and the proportion of those on oral corticosteroids (CSA 30.0%, ESA 63.6% and NSA 45.7%), these were not statistically significant. NSA patients had the highest blood and sputum eosinophil counts. No differences were seen between the three severe asthma groups in terms of pulmonary function, airway reversibility, clinical (asthma control test (ACQ)-7)) and quality of life (asthma quality of life questionnaire (AQLQ)) measures or in exacerbations in the previous year.

### *Comparison of differentially expressed proteins*

Sputum SomaLogic analysis adjusted for age, sex and systemic corticosteroid use identified 13, 63 and 42 differentially expressed proteins (DEPs) between CSA and NH, ESA and NH, and NSA and NH, respectively (figure 1a–c). The DEPs are shown in figure 1d and online supplementary file 1. Only five proteins distinguished CSA-NH from NSA-NH, including colony stimulating factor (CSF)2, also known as granulocyte-macrophage colony-stimulating factor, CXCL8/interleukin (IL)-8 and anterior gradient protein (AGR)2 (table 2). CXCL8 did not distinguish between the CSA-NH and ESA-NH groups. CSF2 is critical for the proliferation, differentiation and survival of granulocytes, monocytes and macrophages [12], whereas AGR2 is involved in mucin 5AC (MUC5AC) production by asthmatic epithelial cells [13]. Sputum levels of CSF2 and AGR2 and the sputum gene expression of *MUC5AC* were highest in CSA

TABLE 1 Patient characteristics for sputum SomaLogic analyses

| | CSA | Missing values | ESA | Missing values | NSA | Missing values | Healthy nonsmokers | Missing values | p-value |
|---|---|---|---|---|---|---|---|---|---|
| **Subjects** | 11 | | 22 | | 37 | | 18 | | |
| **Female** | 5 (45.5) | | 14 (63.6) | | 22 (59.5) | | 6 (33.3) | | $2.01\times10^{-1\P}$ |
| **Age years** | 50.0±10.6 | | 55.7±9.7 | | 52.6±13.3 | | 39.9±13.8$^{\#\#,\P\P}$ | | $\mathbf{3.95\times10^{-3+}}$ |
| **Onset age of asthma years** | 29.8±19.9 | | 39.5±19.0$^{\P\P}$ | | 25.0±18.1 | | | | $\mathbf{2.45\times10^{-2+}}$ |
| **Age at starting smoking years** | 19.3±4.0 | | 16.2±2.5 | | | | | | $\mathbf{4.93\times10^{-2\S}}$ |
| **Years of smoking cessation** | | | 13.7±10.5 | | | | | | |
| **Smoking pack-years** | 29.0±18.2 | | 20.8±16.1 | | 0±0 | | 0±0 | | $1.17\times10^{-1\S}$ |
| **BMI kg·m$^{-2}$** | 27.7±4.7 | | 31.1±6.7 | | 27.5±5.7 | | 25.3±3.2$^{\#\#}$ | | $\mathbf{2.79\times10^{-2+}}$ |
| **Atopic** | 8 (88.9) | 2 | 10 (62.5) | 6 | 28 (84.8) | 4 | 5 (45.5) | 7 | $\mathbf{3.60\times10^{-2}\P}$ |
| **Blood eosinophils cells·μL$^{-1}$** | 259±173 | | 296±246 | | 407±357 | 2 | 116±71 | | $3.31\times10^{-1+,f}$ |
| **Blood neutrophils ×10$^3$ cells·μL$^{-1}$** | 5.10±1.95 | | 5.84±3.03 | | 4.97±2.16 | 2 | 3.35±1.15 | | $6.03\times10^{-1+,f}$ |
| **Sputum eosinophils %** | 7.2±15.2 | | 14.8±16.8 | | 18.8±24.6 | | 0.36±0.57 | | $2.98\times10^{-1+,f}$ |
| **Sputum neutrophils %** | 53.9±16.1 | | 55.2±20.6 | | 50.8±30.9 | | 41.0±26.5 | | $9.28\times10^{-1+,f}$ |
| **IgE IU·mL$^{-1}$** | 222±201 | 2 | 313±499 | | 305±510 | 3 | 105±178 | | $8.84\times10^{-1+,f}$ |
| **$F_{ENO}$ ppb** | 15.2±16.6$^{\#\#,\P\P}$ | | 40.5±33.9 | 1 | 41.2±36.3 | 3 | 19.4±9.7 | 3 | $\mathbf{7.55\times10^{-4+,f}}$ |
| **Periostin ng·mL$^{-1}$** | 42.8±9.3 | 2 | 53.1±18.9 | 4 | 54.9±20.3 | 9 | 49.7±5.5 | 4 | $2.66\times10^{-1+,f}$ |
| **FEV$_1$ post-bronchodilator$^{\#}$ %** | 73.7±18.2 | | 78.8±21.1 | | 68.6±21.1 | | 105.2±11.5 | | $1.82\times10^{-1+,f}$ |
| **FEV$_1$/FVC post-bronchodilator$^{\#}$ %** | 61.5±10.1 | | 63.4±12.2 | | 60.2±13.9 | | 79.0±5.9 | | $6.27\times10^{-1+,f}$ |
| **Airway reversibility %** | 15.0±9.5 | | 16.7±12.7 | | 17.3±20.4 | 1 | | | $7.45\times10^{-1+}$ |
| **Airflow limitation (missing data n=92 overall)** | 7 (63.6) | | 11 (50.0) | | 28 (75.7) | | | | $1.33\times10^{-1\P}$ |
| **Average ACQ-7** | 2.87±1.31 | 1 | 2.67±0.98 | 3 | 2.68±1.19 | 4 | | | $8.30\times10^{-1+}$ |
| **Average AQLQ** | 4.15±1.57 | 1 | 4.62±1.04 | 5 | 4.35±1.29 | 2 | | | $5.06\times10^{-1+}$ |
| **Exacerbations in previous year** | 2.6±3.3 | | 2.1±1.9 | | 2.4±1.9 | | | | $7.47\times10^{-1+}$ |
| **ER visits due to breathing problems** | 5 (45.5) | | 14 (63.6) | | 25 (62.2) | | | | $4.41\times10^{-1\P}$ |
| **Comorbidities** | | | | | | | | | |
| Allergic rhinitis | 2 (25.0) | 3 | 8 (40.0) | 2 | 16 (55.2) | 8 | | | $2.85\times10^{-1\P}$ |
| Nasal polyps | 2 (20.0) | 1 | 7 (33.3) | 1 | 12 (34.3) | 2 | | | $7.20\times10^{-1\P}$ |
| Sinusitis | 2 (25.0) | 3 | 6 (28.6) | 1 | 9 (28.1) | 5 | | | $1.00^{\P}$ |
| Chronic bronchitis | 1 (11.1) | 1 | 2 (9.1) | | 4 (12.1) | 4 | | | $1.00^{\P}$ |
| Psychiatric disease | 3 (33.3) | 2 | 3 (14.3) | 1 | 5 (13.9) | 1 | | | $3.84\times10^{-1\P}$ |
| GORD | 4 (50.0) | 3 | 15 (71.4)$^{\P\P}$ | 1 | 11 (32.4) | 3 | | | $\mathbf{1.74\times10^{-2}\P}$ |
| **Medications** | | | | | | | | | |
| Inhaled corticosteroids | 11 (100.0) | | 22 (100.0) | | 37 (100.0) | | | | $1.00^{\P}$ |
| Systemic corticosteroids | 3 (30.0) | 1 | 14 (63.6) | | 16 (45.7) | 2 | | | $1.93\times10^{-1\P}$ |
| Oral corticosteroid dose mg·day$^{-1}$ | 2.50±4.71 | 1 | 7.89±8.01 | 3 | 4.18±6.61 | 2 | | | $8.53\times10^{-2+}$ |
| Anti-IgE therapy | 0 (0.0) | 1 | 2 (4.0) | 2 | 0 (0.0) | 2 | | | $1.13\times10^{-1\P}$ |
| Long-acting β-agonists | 11 (100.0) | | 21 (95.5) | | 37 (100.0) | | | | $4.71\times10^{-1\P}$ |
| Leukotriene modifiers | 4 (36.4) | | 11 (52.4) | 1 | 19 (51.4) | | | | $6.80\times10^{-1\P}$ |
| Tiotropium | 3 (30.0) | 1 | 4 (22.2) | 2 | 12 (34.3) | 2 | | | $5.61\times10^{-1\P}$ |
| Macrolide | 2 (18.2) | | 3 (13.6) | | 4 (10.8) | | | | $7.96\times10^{-1\P}$ |

Data are presented as n, n (%) or mean±SD, unless otherwise stated. Bold type represents statistical significance (p<0.05). CSA: severe asthma current smokers; ESA: severe asthma ex-smokers; NSA: severe asthma nonsmokers; BMI: body mass index; Ig: immunoglobulin; $F_{ENO}$: fractional exhaled nitric oxide; FEV$_1$: forced expiratory volume in 1 s; FVC: forced vital capacity; ACQ: asthma control questionnaire; AQLQ: asthma quality of life questionnaire; ER: emergency room; GORD: gastro-oesophageal reflux disease. $^{\#}$: spirometry data without bronchodilator were used for healthy subjects; statistical analysis was performed using $^{\P}$: Fisher's exact test, $^{+}$: Kruskal–Wallis test; or $^{\S}$: Mann–Whitney U-test; $^{f}$: healthy subjects were excluded from statistical analyses of several items; $^{\#\#}$: p<0.05 *versus* ESA; $^{\P\P}$: p<0.052 *versus* NSA.

patients (figure 2a and b and online supplementary figure S2). This suggests that CSA is associated with macrophage/neutrophil recruitment and mucus production.

34 DEPs distinguished ESA-NH from NSA-NH and included azurocidin (AZU)1, neutrophil elastase (ELANE), complement factor properdin (CFP) and C-X-C motif chemokine ligand (CXCL)8 (table 3, figure 2c–f). AZU1 possesses monocyte chemotactic and antimicrobial activity [14] and CFP positively regulates the alternative complement system [15]. 29 proteins overlapped between ESA-NH and NSA-NH, including C-reactive protein, CSF1 receptor, inducible T-cell costimulatory ligand, FCGR2A and catalase (CAT) (table 3, figure 1d). In contrast, there were 13 DEPs, including protein disulfide isomerase family A

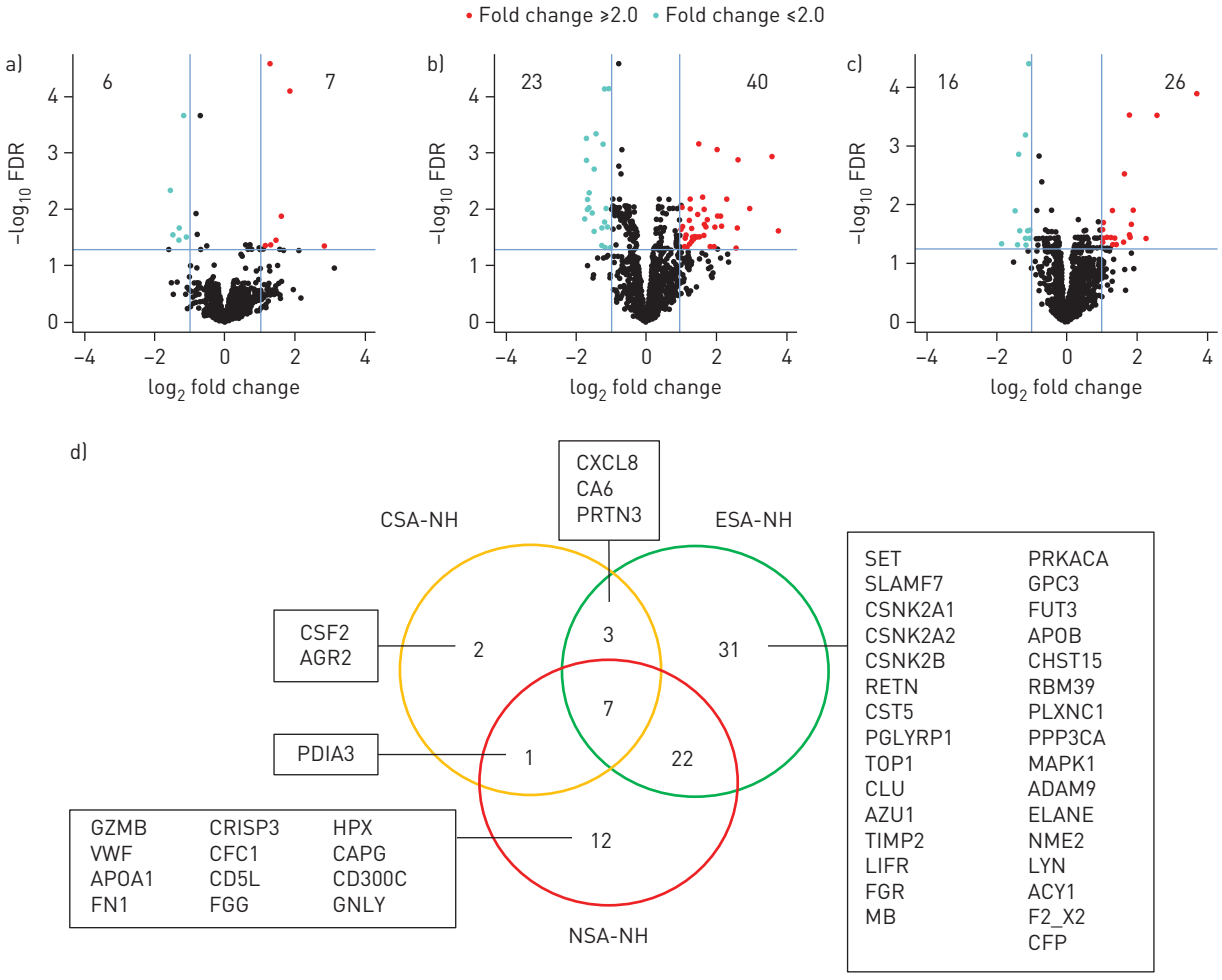


FIGURE 1 Phenotypic differences among severe asthma current smokers (CSA), severe asthma ex-smokers (ESA) and severe asthma nonsmokers (NSA) were unveiled using the SomaLogic linear model for microarray (limma) analysis of sputum. a–c) Volcano plots showing differentially expressed proteins (DEPs) in limma of sputum SomaLogic in the following comparisons: a) CSA and healthy nonsmokers (NH); b) ESA and NH; and c) NSA and NH. The proteins whose absolute fold change ⩾2.0 at false discovery rate (FDR) <0.05 were regarded as DEPs. The number of DEPs of each comparison is shown in the left and right upper areas of each plot. d) Venn diagram showing the numbers and names of DEPs in each comparison.

member 3, granzyme B (GZMB) and CD5 antigen-like (CD5L) (table 3, figure 1d). GZMB is a cytoplasmic granule of cytotoxic T-cells and natural killer cells, and is involved in apoptosis, chronic inflammation and wound healing [16]. CD5L, expressed in lymphoid tissues, lung epithelial cells or tissue macrophages, plays multiple roles in inflammation, such as promoting macrophage phagocytosis [17].

In summary, while CSA was associated with proteins involved in macrophage recruitment and mucus production and both ESA and NSA were associated with proteins with inflammatory and immune responses characterised by T-cell-mediated acquired immunity in common, proteins linked to neutrophilic activity were more closely related to ESA than to other groups. However, this was not reflected in a significant difference in sputum neutrophilia in these subjects (table 1). In addition, the protein expression of CAT, a key antioxidant, was upregulated equally in all severe asthma groups compared with NH (figure 2g).

### *Pathway analysis of DEPs*

Pathway analysis of sputum DEPs indicated that ESA-NH was associated with phagocytosis, response to chemicals, response to multicellular organisms, chemotaxis, myeloid cell differentiation and innate immunity and inflammation, while NSA-NH was associated with acute-phase inflammation, platelet degranulation, response to wounding and the immune system (online supplementary table S2). Overall, different pathways were activated between CSA and NSA and airway epithelial damage may be associated with ESA.

TABLE 2 Differentially expressed proteins between severe asthma current smokers (CSA) and healthy nonsmokers (NH) and severe asthma nonsmokers (NSA) and NH by sputum assay

| Probe ID | Protein target | Gene symbol | Gene name | Function |
|---|---|---|---|---|
| **CSA-NH** | | | | |
| SL001726 | CSF2 | CSF2 (or GM-CSF) | Colony-stimulating factor 2 | Granulocyte, monocyte, macrophage expansion |
| SL004925 | AGR2 | AGR2 | Anterior gradient protein 2 | Mucin (MUC5AC and MUC5B) overproduction in asthma<br>Localised in endoplasmic reticulum of bronchial epithelial cells |
| SL000039 | IL-8 | CXCL8 (=IL8) | C-X-C motif chemokine ligand 8 | Acts as one of the major mediators of the inflammatory response by recruiting neutrophils |
| **NSA-NH** | | | | |
| SL000342 | Catalase | CAT | Catalase | A key antioxidant enzyme in the body's defence against oxidative stress |
| SL000051 | CRP | CRP | C-reactive protein | Host defence based on its ability to recognise foreign pathogens and damaged cells of the host and to initiate their elimination by interacting with humoral and cellular effector systems in the blood |
| SL004153 | M-CSF R | CSF1R | Colony-stimulating factor 1 receptor | The receptor for CSF-1, a cytokine which controls the production, differentiation and function of macrophages |
| SL004853 | B7-H2 | ICOSLG | Inducible T-cell costimulatory ligand | This protein acts as a costimulatory signal for T-cell proliferation and cytokine secretion and induces B-cell proliferation and differentiation into plasma cells |
| SL006108 | CD5L | CD5L | CD5 molecule like | This secreted protein is mainly expressed by macrophages in lymphoid and inflamed tissues and regulates mechanisms in inflammatory responses. Regulation of intracellular lipids mediated by this protein has a direct effect on transcription regulation mediated by nuclear receptors ROR-γ (RORC) |
| SL004068 | GZMB | GZMB | Granzyme B | Targets cell lysis in cell-mediated immune responses. Processes cytokines and degrades extracellular matrix proteins; these roles are implicated in chronic inflammation and wound healing |
| **CSA-NH and NSA-NH** | | | | |
| SL017613 | FCG2A/B | FCGR2A | Fc fragment of immunoglobulin-γ receptor IIa | The protein is a cell surface receptor found on phagocytic cells such as macrophages and neutrophils, and is involved in the process of phagocytosis and clearing of immune complexes |
| SL003524 | Protein disulfide isomerase A3 | PDIA3 | Protein disulfide isomerase family A member 3 | Formation of the final antigen conformation, export from the endoplasmic reticulum to the cell surface and adaptation to oxidative damage |

Assay performed by SomaLogic (Boulder, CO, USA). GM-CSF: granulocyte-macrophage colony-stimulating factor; IL: interleukin.

### *Characteristics of patients with transcriptomic analysis in bronchial biopsies and brushings*

We found increased blood neutrophils and lower $F_{eNO}$ levels in CSA compared to NSA subjects providing bronchial brushings and biopsies for analysis, although the proportion of patients who took systemic corticosteroids or the dose of oral corticosteroids was no different between the two severe asthma groups (online supplementary tables S3–S5). There were no significant differences in blood eosinophil, sputum eosinophil and sputum neutrophil counts, and in pulmonary function, ACQ-7, AQLQ or the number of exacerbations in the previous year among the three severe asthma groups. The subjects who provided samples for sputum transcriptomics did not completely overlap with those providing sputum proteomics, but the clinical characteristics were similar (online supplementary table S5).

### *DEGs between CSA and NSA*

We detected 142 significant DEG probes in bronchial brushings, 23 in bronchial biopsies and 15 in sputum samples between CSA and NSA (figure 3a–c and online supplementary file 2). There were no significant DEG probes (FDR >0.05) in any samples between ESA and NSA (online supplementary file 3). Hierarchical clustering of the 142 DEGs from bronchial brushings indicated that although CSA and NSA were clearly distinct, NSA and ESA did not cluster separately (figure 3d).

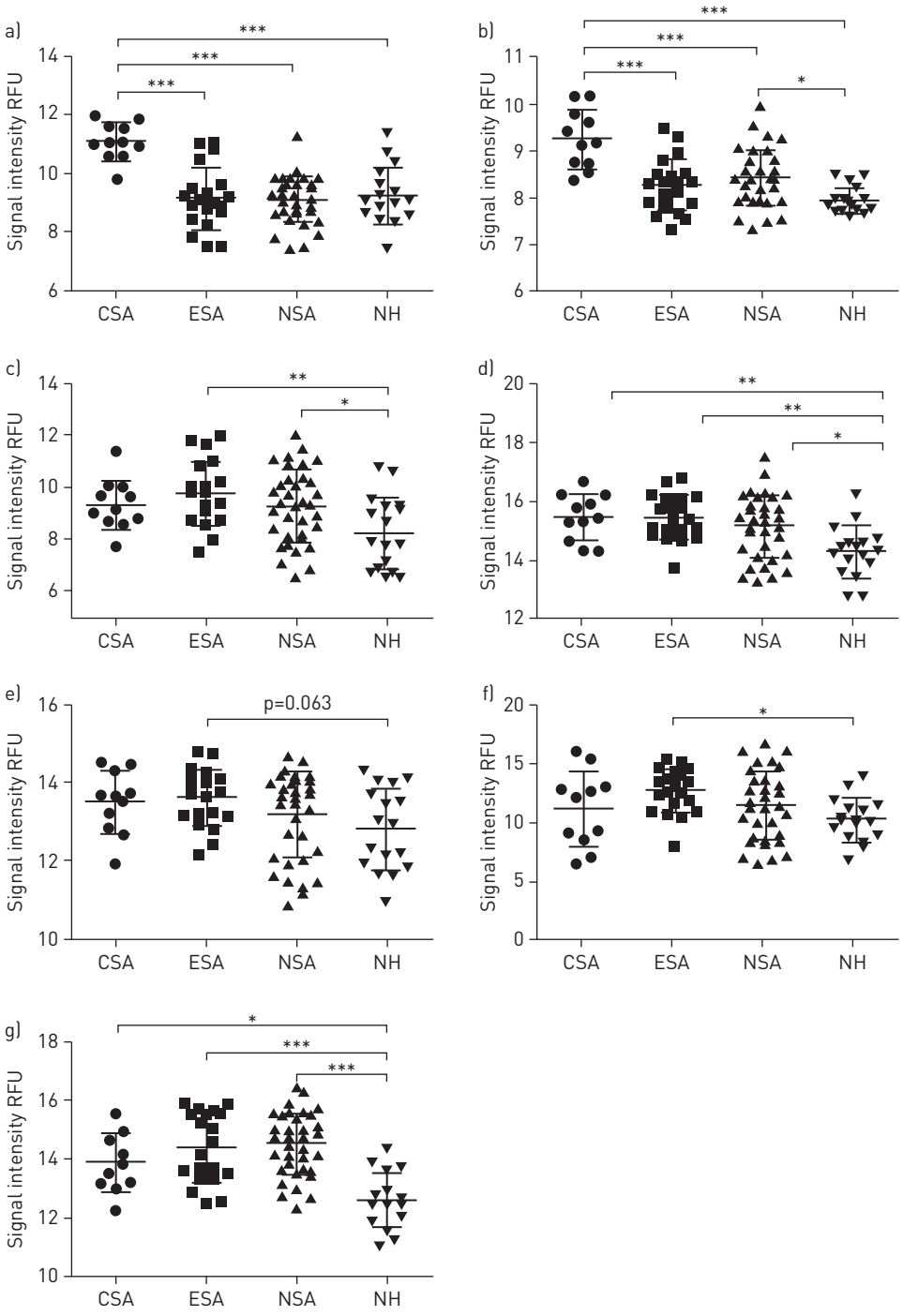


FIGURE 2 Differentially expressed proteins in severe asthma sputum according to smoking status. Dot plots with mean±SD showing signal intensity levels of protein expression of a) colony-stimulating factor (CSF)-2, b) anterior gradient protein (AGR)2, c) azurocidin (AZU)1, d) C-X-C motif chemokine ligand (CXCL)8, e) neutrophil elastase (ELANE), f) complement factor properdin (CFP) and g) catalase (CAT) in sputum by SomaLogic (Boulder, CO, USA) analysis in severe asthma current smokers (CSA), severe asthma ex-smokers (ESA), severe asthma nonsmokers (NSA) and healthy nonsmokers (NH). RFU: relative fluorescence units, *: p<0.05; **: p<0.01; ***: p<0.001.

The DEGs between CSA and NSA are implicated in oxidation–reduction, xenobiotic metabolism and endoplasmic reticulum stress (online supplementary file 2). Cytochrome P450 family 1 subfamily B member 1 (CYP1B1) and aldehyde dehydrogenase 3 family member A1 (ALDH3A1), which were overexpressed in bronchial brushings of CSA compared to other groups (figure 4a and b) play a role in

TABLE 3 Differentially expressed key proteins in sputum SomaLogic analysis comparing severe asthma ex-smokers (ESA) and healthy nonsmokers (NH) with severe asthma nonsmokers (NSA) and NH

| Probe ID | Protein target | Gene symbol | Gene name | Function |
|---|---|---|---|---|
| **ESA-NH** | | | | |
| SL004589 | AZU1 | AZU1 | Azurocidin 1 | A pre-proprotein of a mature azurophil granule antibiotic protein with monocyte chemotactic and antimicrobial activity |
| SL000401 | ELANE | ELANE | Neutrophil elastase | This protease hydrolyses proteins within specialised neutrophil lysosomes, called azurophil granules, as well as proteins of the extracellular matrix |
| SL003192 | CFP | CFP | Complement factor properdin | A positive regulator of the alternative pathway of complement system |
| SL000039 | IL-8 | CXCL8 (=IL-8) | C-X-C motif chemokine ligand 8 | Acts as one of the major mediators of the inflammatory response by recruiting neutrophils |
| **NSA-NH** | | | | |
| SL003524 | Protein disulfide isomerase A3 | PDIA3 | Protein disulfide isomerase family A member 3 | Formation of the final antigen conformation, export from the endoplasmic reticulum to the cell surface and adaptation to oxidative damage |
| SL006108 | CD5L | CD5L | CD5 molecule like | This secreted protein is mainly expressed by macrophages in lymphoid and inflamed tissues and regulates mechanisms in inflammatory responses. Regulation of intracellular lipids mediated by this protein has a direct effect on transcription regulation mediated by nuclear receptors ROR-γ (RORC) |
| SL004068 | GZMB | GZMB | Granzyme B | Targets cell lysis in cell-mediated immune responses. This protein also processes cytokines and degrades extracellular matrix proteins, and these roles are implicated in chronic inflammation and wound healing |
| **ESA-NH and NSA-NH** | | | | |
| SL017613 | FCG2A/B | FCGR2A | Fc fragment of immunoglobulin-γ receptor IIa | Cell surface receptor found on phagocytic cells such as macrophages and neutrophils, is involved in the process of phagocytosis and clearing of immune complexes |
| SL000342 | Catalase | CAT | Catalase | A key antioxidant enzyme in the body's defence against oxidative stress |
| SL000051 | CRP | CRP | C-reactive protein | Host defence based on its ability to recognise foreign pathogens and damaged cells of the host and to initiate their elimination by interacting with humoral and cellular effector systems in the blood |
| SL004153 | M-CSF R | CSF1R | Colony-stimulating factor 1 receptor | The receptor for CSF1, a cytokine which controls the production, differentiation and function of macrophages |
| SL004853 | B7-H2 | ICOSLG | Inducible T-cell costimulatory ligand | This protein acts as a costimulatory signal for T-cell proliferation and cytokine secretion and induces B-cell proliferation and differentiation into plasma cells |

Assay performed by SomaLogic (Boulder, CO, USA). IL: interleukin.

metabolising polycyclic aromatic hydrocarbons or aldehydes [18]. The oxidative stress genes, NAD(P)H quinone dehydrogenase 1 (NQO1) and aldo-keto reductase family 1 member C1 (AKR1C1), were also highly expressed in CSA bronchial brushings (figure 4c and d). The endoplasmic reticulum plays a central role in the protein biosynthesis, correct protein folding and post-transcriptional modifications [19]. Accumulation of unfolded and misfolded proteins, termed endoplasmic reticulum stress, leads to the unfolded protein response and inflammation [20]. Heat shock protein family A (Hsp70) member 5 (HSPA5), a key mediator of endoplasmic reticulum stress, was significantly upregulated in CSA compared to NSA in bronchial brushings and biopsies (figure 4e).

### *Pathway analysis using DEGs between CSA and NSA*

Pathway analysis indicated that oxidation–reduction, chemical metabolism and endoplasmic reticulum stress were different between CSA and NSA (online supplementary tables S6 and S7). These results suggest that the lung epithelial cells of CSA patients are under more potent chemical, oxidative and endoplasmic reticulum stresses than those of NSA patients.

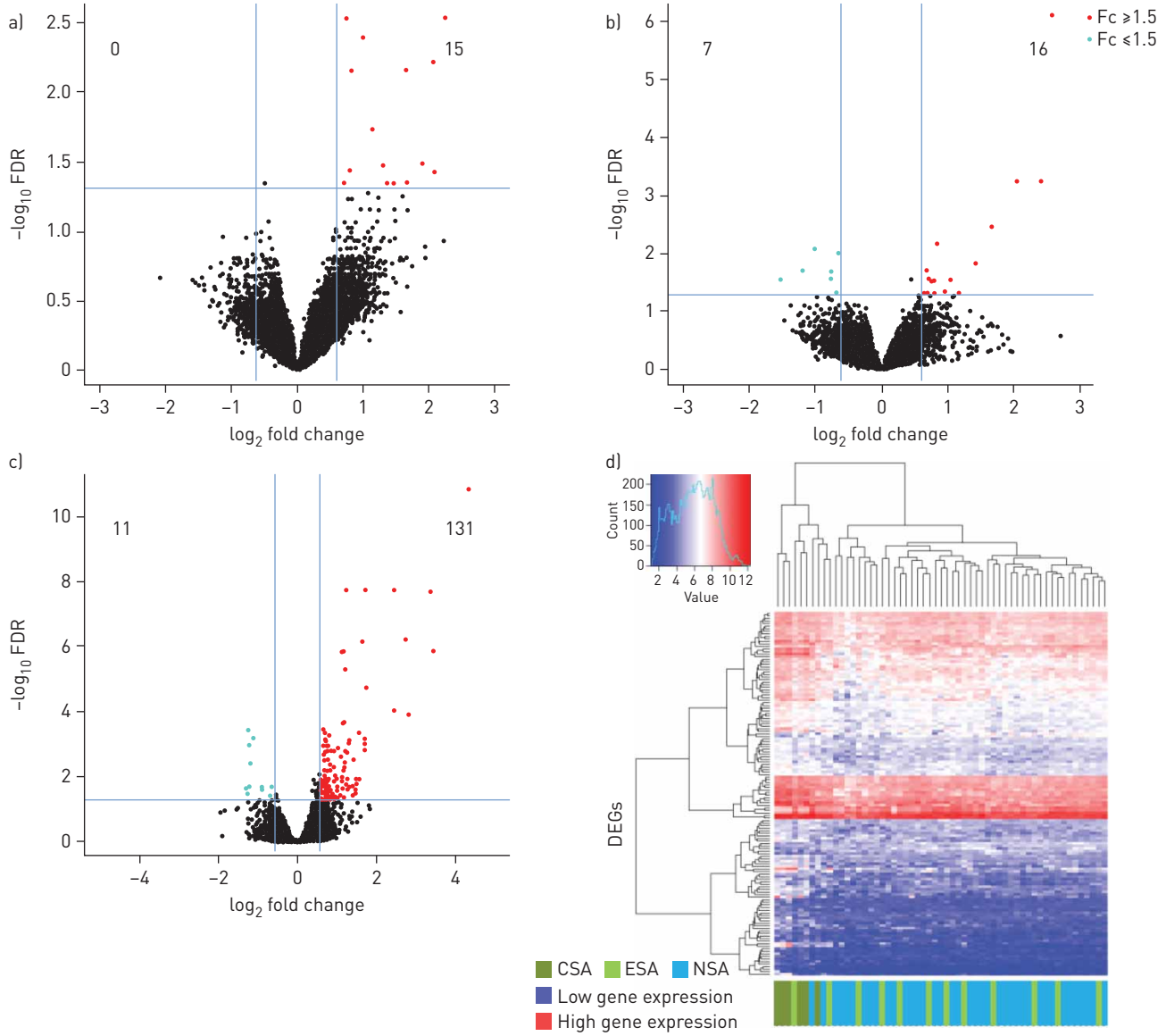


FIGURE 3 Differentially expressed genes (DEGs) in current smokers (CSA) and nonsmokers (NSA) with severe asthma. Volcano plots showing DEGs between CSA and NSA in a) sputa, b) bronchial biopsies and c) bronchial brushings. The genes whose absolute fold change (FC) ⩾1.5 at a false discovery rate (FDR) <0.05 are shown as coloured dots. The number of DEGs in each sample is shown in the left and right upper areas of each plot. d) Hierarchical clustering for DEGs from bronchial brushings in severe asthma patients. ESA: severe asthma ex-smokers.

### *GSVA of bronchial brushings and biopsies*

GSVA confirmed the selective enrichment of xenobiotic metabolism by cytochrome P450, glutathione metabolism, response to oxidative stress, endoplasmic reticulum stress, unfolded protein response, lysosome or glycolysis and gluconeogenesis pathways in bronchial brushings (figure 5a–g) and biopsies (figure 6a–g) in the CSA group. There were no significant differences between ESA and NSA for these pathways. Using the signatures for active smoking obtained from Spira *et al.* [21], we confirmed that bronchial brushings and biopsies from CSA were enriched for the active smoking-related gene and that both CSA and ESA were enriched for the pack-year signature (online supplementary table S1 and figure S3).

### *DEGs in sputum, bronchial biopsies and epithelial brushings*

As we could not detect any DEGs between ESA and NSA at the FDR <0.05 level, we undertook a discovery approach using a less stringent analysis strategy. Genes whose absolute fold change was ⩾2.0 in limma were used to clarify the phenotypic difference between ESA and NSA (online supplementary file 2). 27 genes (35 probes) were upregulated in ESA sputum samples including matrix metallopeptidase (MMP)

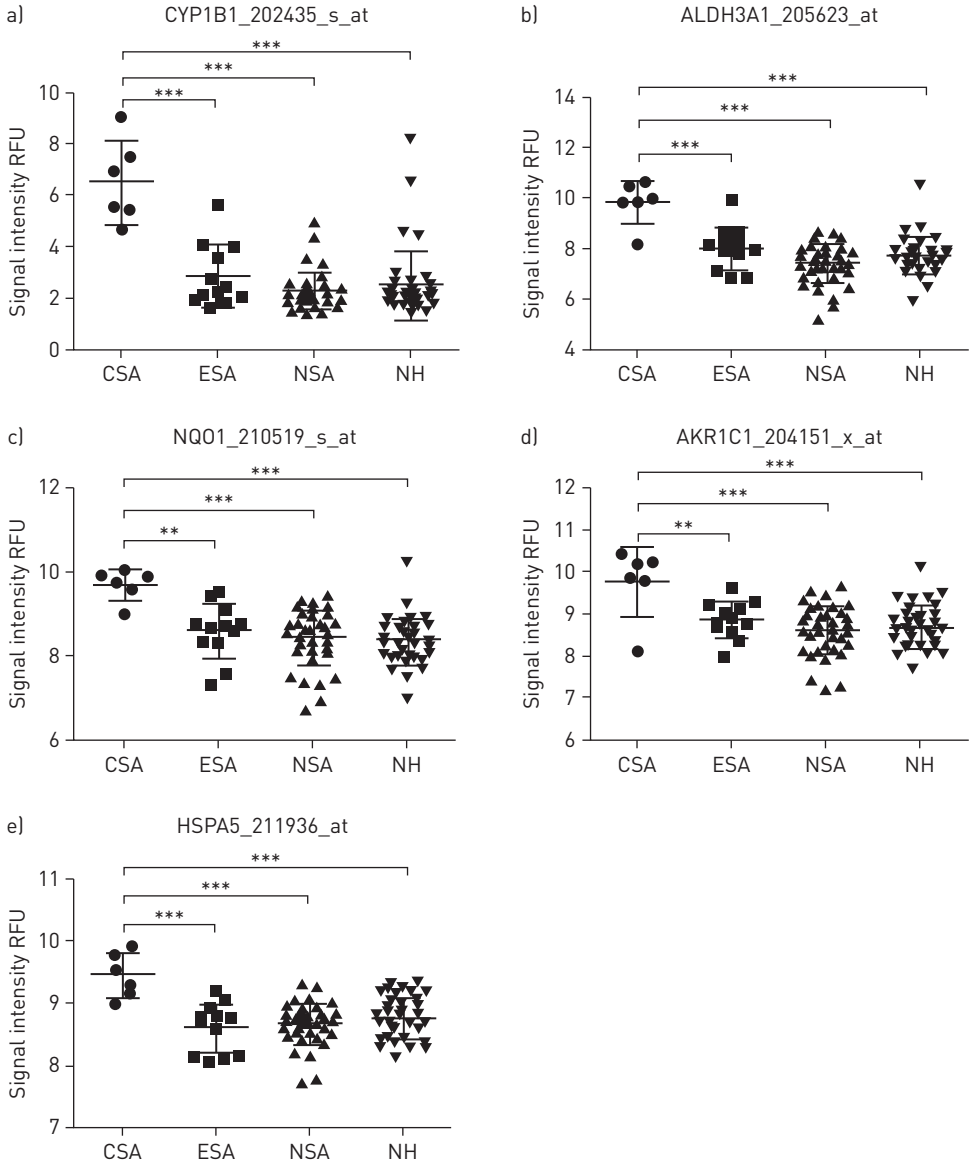


FIGURE 4 Differentially expressed genes (DEGs) associated with metabolism of xenobiotics, oxidative stress and endoplasmic reticulum stress in bronchial brushings. Dot plots showing DEGs in bronchial brushings associated with a) xenobiotic metabolism by cytochrome P450 family 1 subfamily B member 1 (CYP1B1), b) aldehyde dehydrogenase 3 family member A1 (ALDH3A1), c) NAD(P)H quinone dehydrogenase 1 (NQO1), d) aldo-keto reductase family 1 member C1 (AKR1C1) and e) heat shock protein family A (Hsp70) member 5 (HSPA5). RFU: relative fluorescence units; CSA: severe asthma current smokers; ESA: severe asthma ex-smokers; NSA: severe asthma nonsmokers; NH: healthy nonsmokers. **: p<0.01; ***: p<0.001.

12, neuropilin (NRP)1, Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), CXCL5 and pro-platelet basic protein (PPBP) (online supplementary table S8). MMP12 has been associated with decreased lung function and COPD, TIRAP is involved in the Toll-like receptor (TLR) signalling pathway and both CXCL5/ENA-78 and PPBP/CXCL7 are potent neutrophil chemoattractants and activators [22]. Innate immunity, including the complement system, TLR signalling and neutrophilic inflammation may be characteristics of ESA.

Six downregulated DEGs (fold change ⩽0.5) distinguished ESA from NSA in bronchial brushings, namely carboxypeptidase (CP)A3, cystatin SN (CST1), immunoglobulin-κ constant (IGKC), mucin 2, oligomeric mucus/gel-forming (MUC2) and tryptase α/β1 (TPSAB1) (online supplementary table S8). CPA3 and TPSAB1 are mast cell biomarkers and are found to be elevated in asthma patients [23]. CST1 is a cysteine proteinase inhibitor that has a protective effect on epithelium [24]. MUC2 provides a protective barrier for airways against particles or infectious agents [25]. This suggests that ESA subjects have a lesser protective epithelial barrier and reduced mast cell activity compared with NSA.

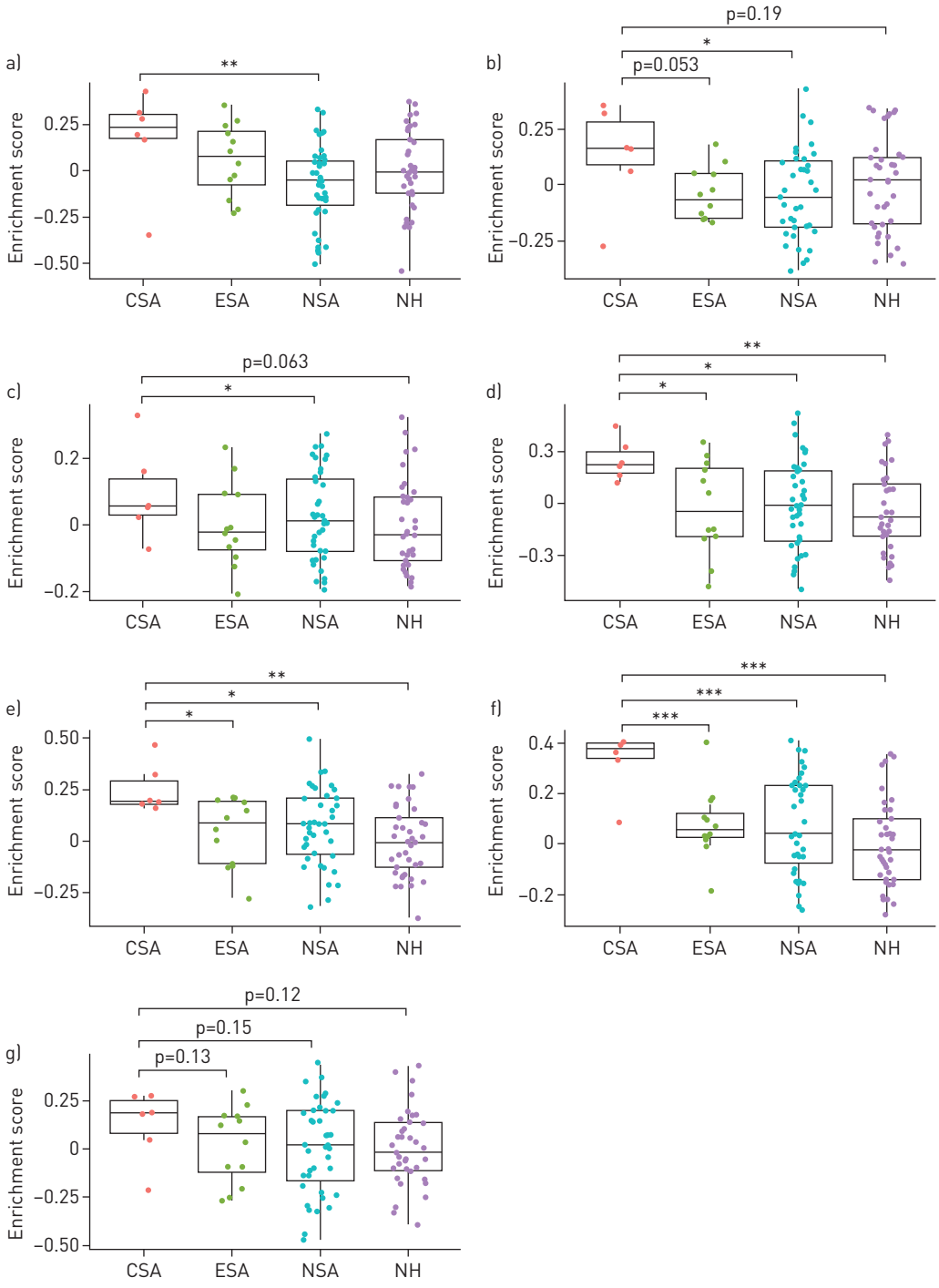


FIGURE 5 Gene set variation analysis of selected stress-related pathways in bronchial brushings according to smoking status. Box-and-whisker plots showing pathway enrichment of a) xenobiotic metabolism by CYP450, b) glutathione metabolism, c) response to oxidative stress, d) endoplasmic reticulum stress, e) unfolded protein response, f) lysosome and g) glycolysis and gluconeogenesis in bronchial brushings of severe asthma current smokers (CSA), severe asthma ex-smokers (ESA), severe asthma nonsmokers (NSA) and healthy nonsmokers (NH). *: p<0.05; **: p<0.01; ***: p<0.001.

In the biopsies, 16 DEGs (fold change ⩾2.0) were detected and these included follicular dendritic cell secreted protein (FDCSP), periostin (POSTN), PPBP, immunoglobulin λ constant 1 (IGLC1) and immunoglobulin λ variable cluster (IGLV). FDCSP and PPBP were upregulated in ESA subjects, while POSTN, IGLC1 and IGLV were downregulated. Overall, the data suggest that neutrophilic innate immunity is more characteristic of ESA than IL-4/13 signalling and humoral immunity.

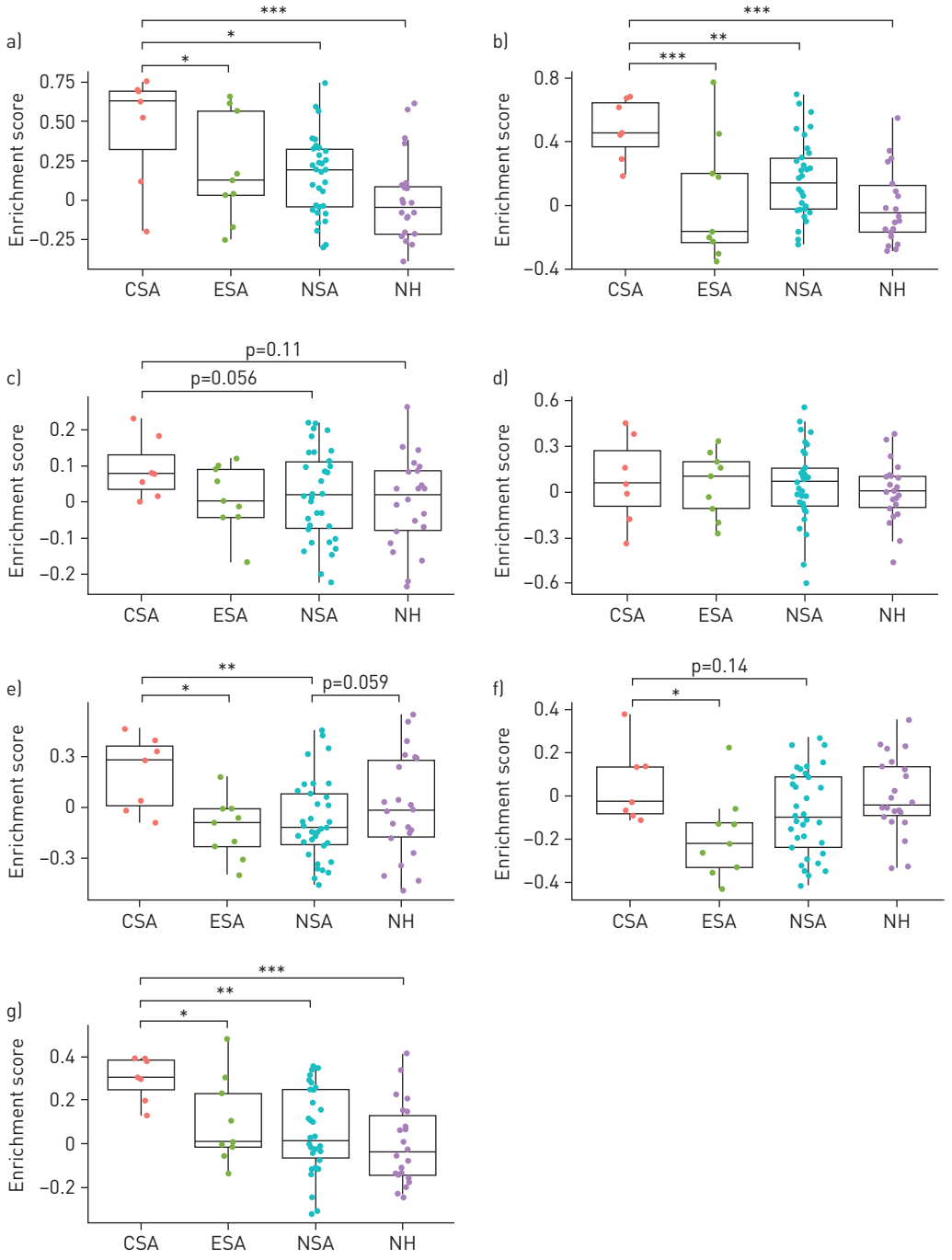


FIGURE 6 Gene set variation analysis of selected stress-related pathways in bronchial biopsies according to smoking status. Box-and-whisker plots showing pathway enrichment of a) xenobiotic metabolism by CYP450, b) glutathione metabolism, c) response to oxidative stress, d) endoplasmic reticulum stress, e) unfolded protein response, f) lysosome and g) glycolysis and gluconeogenesis in bronchial biopsies of severe asthma current smokers (CSA), severe asthma ex-smokers (ESA), severe asthma nonsmokers (NSA) and healthy nonsmokers (NH). *: p<0.05; **: p<0.01; ***: p<0.001.

### *Protein interaction analysis using combined DEGs from airway samples*

Protein interaction analysis in STRING using combined DEGs between CSA and NSA showed direct interactions of oxidation–reduction and the pentose phosphate pathway network with the innate immune response *via* protein production and modification in endoplasmic reticulum (figure 7). In addition, proteins that play a role in lysosome, mucus production, Golgi homeostasis and tissue structure were seen in the network.

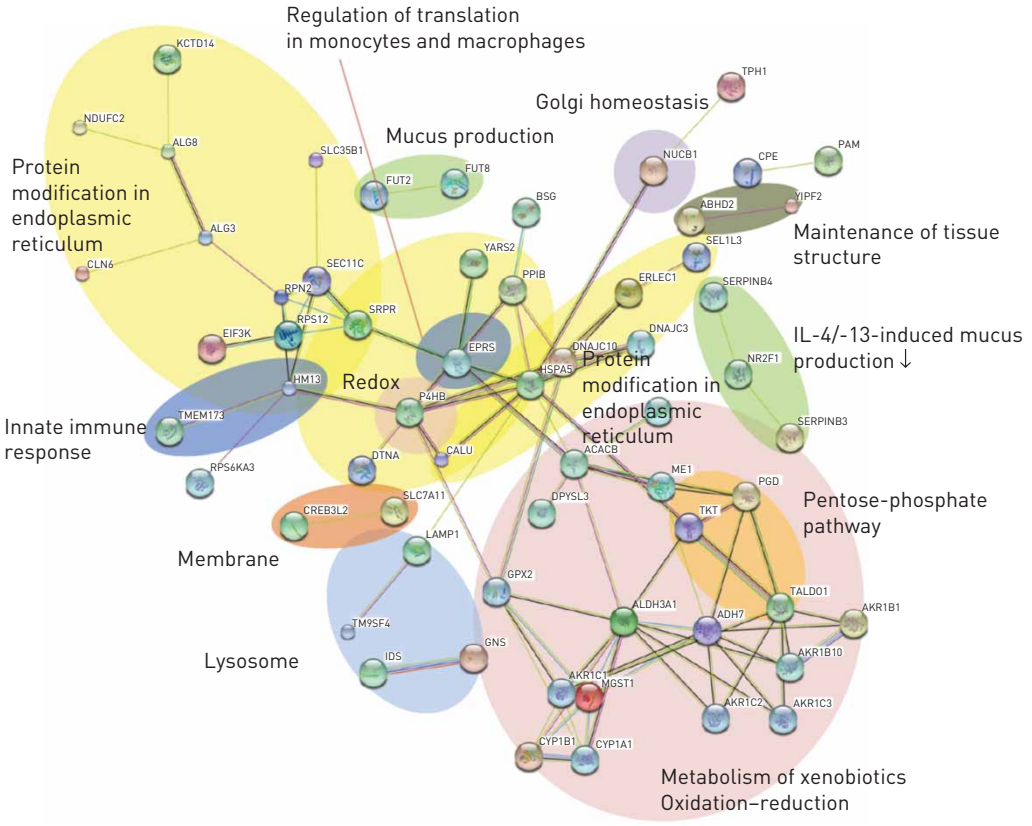


FIGURE 7 Protein interaction analysis in STRING (version 10.0; STRING Consortium 2016, www.string-db.org) using combined differentially expressed genes (DEGs). Combined DEGs in limma from bronchial brushings, biopsies and sputa were used for protein interaction analysis by STRING. The large pink-coloured area is filled with proteins related to xenobiotic metabolism and oxidation–reduction which contains the pentose-phosphate pathway. These proteins function with those in charge of redox and connect with protein production or modification. Some proteins are associated with innate immunity. The other proteins function as lysosomal, membranous, mucus productive, Golgi homeostatic or structural proteins. Overall, this reveals the relationship between oxidative stress, endoplasmic reticulum stress, metabolism of xenobiotics and innate immunity.

## Discussion

We describe the differences in protein and gene expression between severe asthma patients who actively smoke, ex-smokers with a significant history of cigarette smoking and those who do not smoke. There was a difference in the sputum proteome between NSA and CSA (CSF2, AGR2 and CXCL8) and between NSA and ESA (AZU1, ELANE, CFP and CXCL8) subjects, with CXCL8 not discriminating between ESA and CSA. Distinct pathways were activated in CSA and NSA sputum, while the sputum protein data also suggested that ESA was associated with airway epithelial cell damage. In addition, gene expression profiles between bronchial epithelial cells from CSA and NSA were significantly different as determined by pathway analysis, GSVA and protein–protein interaction analysis. There were no significant DEGs (FDR <0.05) between ESA and NSA. Hierarchical clustering indicated that although CSA and NSA were clearly distinct, NSA and ESA did not cluster separately. Airway epithelial cells in CSA patients show an enrichment of oxidative and endoplasmic reticulum stress and innate immune pathways compared to ESA or NSA patients and there were no significant differences between ESA and NSA for these pathways. Using a less stringent analysis, ESA subjects showed upregulated expression of neutrophil chemotactic genes and downregulated expression of genes related to mast cells, humoral immunity and epithelial protection compared to NSA. Overall, proteomics and transcriptomics were able to differentiate CSA from NSA, but ESA and NSA could only be discriminated using sputum proteomics, as airway transcriptomics clustered ESA and CSA together.

The role of the increased sputum expression of CSF2 is unknown. CSF2 is secreted by macrophages, epithelial cells and T-cells in response to inflammatory and noxious stimuli and its expression is enhanced in asthmatic airway epithelial cells *in situ* and after culture [26]. CSF2 transgenic mice have an enhanced Th2 response to ovalbumin sensitisation and anti-CSF2 antibodies block the allergic response in mouse

models of asthma [27]. In addition, CSF2 is involved in the lung innate immune response to noxious agents such as lipopolysaccharide and cigarette smoke [28]. Acute exposure to cigarette smoke in mice leads to enhanced CSF2 expression, and neutralisation using an intranasal anti-CSF2 antibody reduced bronchoalveolar lavage fluid macrophages and neutrophils and inflammatory analytes [29], which indicates that the CSF2 pathway can mediate smoke-induced inflammation. Future experiments in models of severe asthma linked to smoking or in selected patients may determine whether the elevated CSF2 expression seen here is causal or a marker of other driver mechanisms.

Our data provide evidence for enhanced oxidative and endoplasmic reticulum stress in airway epithelial cells of CSA patients. We postulate that the increased activation of the xenobiotic response and oxidative and endoplasmic reticulum stress pathways influences innate immunity in these subjects. There is increased oxidative stress in asthma and COPD patients as well as in healthy smokers [30]. Cigarette smoke not only contains high concentrations of reactive oxygen species (ROS) [30], but also activates alveolar macrophages and neutrophils, which also release ROS, leading to an increased inflammatory response in a feed-forward process [30, 31]. In both asthma and COPD, activated inflammatory cells including neutrophils, macrophages and eosinophils produce ROS and further generate inflammation and cause injury to the airway epithelium [30]. Moreover, impaired upregulation and production of protective antioxidant was reported in smokers, asthma and COPD patients. This oxidant–antioxidant imbalance resulting in oxidative stress is associated with airway hyperresponsiveness and decreased lung function and asthma severity [30, 32].

CSA represented the escalated response to oxidative stress derived from cigarette smoking as CSA bronchial brushings and biopsies alone were enriched for the active smoking-related gene set, whereas both CSA and ESA samples showed a similar enrichment of the pack-year signature. Increased antioxidant gene expression and increased enrichment of the gene set showing response to oxidative stress were observed in bronchial brushings, which may suggest that cigarette smoking stimulates airway epithelial cells to respond to oxidative stress in severe asthma. In addition, we showed that endoplasmic reticulum stress might have a key role in CSA phenotype. Endoplasmic reticulum stress is associated with neutrophilic asthma through nuclear factor-κB activation and pro-inflammatory cytokine production [33]. Cigarette smoking itself may induce endoplasmic reticulum stress [34] and the activation seen here in severe asthma might relate to active cigarette smoke exposure. However, CSF2 and AGR2 have not been shown to be differentially expressed in the previously published proteomic analysis of sputum from healthy current smokers compared to never-smokers [35]. Moreover, endoplasmic stress and lysosome gene sets that we found to be differentially expressed in these two groups of severe asthma were not differentially expressed in healthy current smokers compared to nonsmokers (online supplementary tables S9 and S10) [21]. These results suggest that DEGs between CSA and NSA were not derived from the influence of cigarette smoking itself.

We found decreased production of protective agents in ESA airways. Cigarette smoke injures the airway epithelium in several ways, including decreased protective protein expression [36], disruption of tight junctions [37] and through innate immune and inflammatory response [31]. Cigarette smoke-activated alveolar macrophages produce pro-inflammatory molecules, ROS, tissue proteases and chemokines for recruitment and survival of neutrophils in the lung tissue [31] and activated neutrophils secrete proteases and breakdown collagen into fragments, which can activate neutrophils in a positive-feedback manner [38]. We showed decreased expression of MUC2 and CST1 in ESA, which both play a protective role for airway epithelium [39, 40]. Conversely, the expression of MMP12, CXCL8 and PPBP, which can enhance lung damage, were upregulated in ESA.

Sputum microbiota, which is associated with neutrophilic airway inflammation in adult severe asthma has been reported to be different from that of healthy controls or nonsevere asthmatics [41, 42]. Our results imply that a reduction in airway protective agents might change the airway microbiome, affecting neutrophilic airway inflammation, especially in ESA; conversely, the heightened mucin production might have had a beneficial effect in keeping the airway epithelium free from bacterial colonisation in CSA.

There are important limitations in our study. First, the numbers of smoking and ex-smokers in our groups were relatively small, particularly when analysing data from sputum and biopsy and brushing samples, and the results should be considered as exploratory and will need confirmation in a larger cohort. Second, the lack of a control group of age-matched nonasthmatic active smokers does not allow us to determine the exact contribution of cigarette smoking to the changes observed. Third, we did not observe differences in blood or sputum neutrophil counts, although neutrophil chemoattractants were more upregulated in airways of ESA and CSA patients compared to controls.

In conclusion, we found that current smokers with severe asthma were characterised by increased sputum CFS2 and AGR2 protein expression, indicating enhanced macrophage recruitment and mucus production

in addition to airway tissue genes associated with increased xenobiotic metabolism and responses to oxidative stress and endoplasmic reticulum stress. In contrast, ex-smokers with severe asthma were characterised by pathways involved in the recruitment and activity of neutrophils and with decreased airway protective factors. Airway gene expression analysis showed little difference between severe asthmatics who were ex-smokers and those who were never-smokers.

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Conflict of interest: K. Takahashi received personal fees from Asahi General Hospital, during the conduct of the study. M. Loza is employed by and owns stock in Johnson & Johnson, the parent company of Janssen R&D. F. Baribaud is an employee and shareholder of Janssen R&D. P. Chanez has provided consultancy services for Boehringer Ingelheim, Johnson & Johnson, GlaxoSmithKline, Merck Sharp & Dohme, AstraZeneca, Novartis, Teva, Chiesi, Sanofi and SNCF; has served on advisory boards for Almirall, Boehringer Ingelheim, Johnson & Johnson, GlaxoSmithKline, AstraZeneca, Novartis, Teva, Chiesi and Sanofi; has received lecture fees from Boehringer Ingelheim, Centocor, GlaxoSmithKline, AstraZeneca, Novartis, Teva, Chiesi, Boston Scientific and ALK; and has received industry-sponsored grants from Roche, Boston Scientific, Boehringer Ingelheim, Centocor, GlaxoSmithKline, AstraZeneca, ALK, Novartis, Teva and Chiesi. I. 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