



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

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

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Take home message

Inflammatory, oxidative/ER stress and epithelial barrier pathways are differentially activated in current smoking and ex-smoking severe asthma patients.

ABSTRACT (199 words)**Background:**

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.

Methods

The U-BIOPRED severe asthma patients containing current-smokers (CSA), ex-smokers (ESA), non-smokers (NSA) and healthy non-smokers (NH) 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.

Results

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 metalloproteinase 12 genes were upregulated in ESA and the epithelial protective genes, mucin 2 and cystatin SN, were downregulated.

Conclusion

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

ABBREVIATIONS

ACOS:	Asthma-COPD Overlap Syndrome
ACQ:	Asthma Control Questionnaire
AQLQ:	Asthma Quality of Life Questionnaire
BMI:	Body Mass Index
COPD:	Chronic Obstructive Pulmonary Disease
CSA:	Current-smokers with Severe Asthma
DEG:	Differentially Expressed Gene
DEP:	Differentially Expressed Protein
ERS:	European Respiratory Society
ESA:	Ex-smokers with Severe Asthma
eTRIKS:	European Translational Information and Knowledge Management Services
ER:	Endoplasmic Reticulum
FDR:	False Discovery Rate
FeNO:	Fraction of exhaled Nitric Oxide
FEV ₁ :	Forced Expiratory Volume in 1 second
FVC:	Forced Vital Capacity
GERD:	Gastroesophageal Reflux Disease
GSVA:	Gene Set Variation Analysis
ICS:	Inhaled Corticosteroids
IgE:	Immunoglobulin E
LABA:	Long-Acting β_2 -Agonist
MANOVA:	Multivariate Analysis Of Variance
NH:	Non-smoking Healthy volunteers
NSA:	Non-smokers with Severe Asthma
SCS:	Systemic Corticosteroids
Th2:	T helper type 2
U-BIOPRED:	Unbiased BIOMarkers for the PREdiction of respiratory diseases outcomes

INTRODUCTION

Severe asthma has been defined as asthma that requires treatment with high dose inhaled corticosteroids (ICS) and long-acting β_2 -agonist (LABA) and often with 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 have been 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 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-COPD overlap syndrome (ACOS) with features of both diseases. In patients who have been recruited as COPD patients in the COPDgene cohort, the patients who have had a history of asthma before the age of 40 and who had a smoking history of at least 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 Unbiased Biomarkers for the PREDiction of respiratory disease outcomes (U-BIOPRED) project recruited patients with severe asthma that included 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

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3 underlie smoking or ex-smoking severe asthma, we examined the differential expression of genes
4 and proteins in various compartments.
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6 **MATERIALS and METHODS**

7 *Clinical data*

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10 We enrolled totally 374 severe asthma patients in the U-BIOPRED cohort divided into three
11 groups by smoking status; current-smokers with severe asthma (**CSA**), ex-smokers with severe
12 asthma (**ESA**), non-smokers with severe asthma (**NSA**). We narrowed down the NSA to those who
13 had never smoked (0 pack-year), although original non-smokers with severe asthma group in the U-
14 BIOPRED cohort contained the patients whose pack-year was less than 5. Eighty-one non-smoking
15 healthy volunteers (**NH**) whose pack-years were 0 were also enrolled. Differential blood and induced
16 sputum cell counts, serum total IgE and skin prick tests, serum periostin and fraction of exhaled
17 nitric oxide (FeNO) and pre- and post- bronchodilator spirometry were obtained (8, 9). Bronchial
18 biopsies, bronchial brushings and sputum were obtained, as previously described (8). Because of the
19 bronchoscopy exclusion criteria (Supplementary File), only 95 bronchial brushings and 69 bronchial
20 biopsies were obtained. The number of sputum samples for proteomic analysis was 88. All subjects
21 whose samples were adequate and underwent omics analyses are shown in **Supplementary Figure**
22 **S1**. The study was approved by the Ethics Committees for each of the 16 clinical recruiting centres.
23 All subjects gave written and signed informed consent.
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44 *Transcriptomic microarray analysis*

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46 Sputum plugs were obtained and separated into cells and supernatants [7]. Cell pellets were
47 used to prepare RNA using the miRNeasy mini kit (Qiagen, CA, USA). Sputum samples with >30%
48 squamous cells were excluded from microarray analysis. Bronchial brushings and biopsy samples
49 were immediately placed in TRIzol reagent (Invitrogen) and preserved at -80°C. Expression profiling
50 of transcriptome was performed using GeneChip[®] Human Genome U133 Plus 2.0 Array (Affymetrix,
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3 Santa Clara, CA) as previously described (8, 9). Pathway analysis, enrichment analysis and
4 functional clustering of differentially-expressed genes were performed as described previously (8, 9)
5 and protein interaction analysis using annotated protein-coding genes was performed by STRING
6 version 10.0 (STRING CONSORTIUM 2016, <http://www.string-db.org>) (10).
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11 12 13 ***SomaLogic proteomic technique***

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15 The SOMAscan proteomic assay of sputum supernatants performed by SomaLogic Inc.,
16 (Boulder, CO) was used (9).
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22 ***Gene Set Variation Analysis (GSVA)***

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24 Gene set variation analysis (GSVA) was performed in R using the Bioconductor GSVA
25 package for estimating variation of gene set enrichment (11). Gene sets were obtained from
26 Molecular Signatures Database v5.2 (MSigDB) (<http://software.broadinstitute.org/gsea/msigdb>) or
27 from published papers (Supplementary Table S1). We used ImmunomapTM graphics (Johnson &
28 Johnson Ltd., NJ, USA) for visualisation.
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37 ***Statistical analysis***

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39 All datasets were quality-controlled and normalized, followed by adjustment of batch effects
40 using ComBat tools and uploaded into transSMART, an open-source knowledge management
41 platform for sharing research data supported by European Translational Information and Knowledge
42 Management Services (eTRIKS) (8, 9). All categorical variables were analysed using Fisher's
43 exact test. Continuous variables were analysed using Kruskal-Wallis test. Gene and protein
44 expression data were analysed using multivariate analysis of variance (MANOVA) with age, gender
45 and systemic corticosteroids (SCS) use were analysed as covariates. A p value <0.05 was considered
46 significant. A linear model for microarray data (Bioconductor limma package for R) with
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3 Benjamini-Hochberg false discovery rate (FDR) correction was used in the analysis of the
4 differentially-expressed genes (DEGs) and for GSVA. Fold change ≥ 1.5 and FDR < 0.05 was
5 considered statistically significant in transcriptomic and proteomic analyses. When using GSVA,
6 FDR < 0.05 was considered statistically significant. Statistical analyses were performed by R version
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11 3.3.1 (R Core Team, 2016).
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RESULTS

Clinical characteristics of subjects with sputum SomaLogic data

Table 1 shows the characteristics of subjects who provided sputum samples for SomaLogic analysis. The levels of fractional exhaled nitric oxide (FeNO) of CSA subjects were lower than in the other severe asthma groups. Although there were numerical in blood eosinophil counts (CSA 259/ μ L, ESA 296/ μ L, NSA 407/ μ L), sputum eosinophils (CSA 7.2%, ESA 14.8%, NSA 18.8%), and the proportion of those on oral corticosteroids (CSA 30.0%, ESA 63.6%, NSA 45.7%), these were not statistically significant. NSA patients had the highest blood and sputum eosinophil counts. No differences were seen among the 3 severe asthma groups in terms of pulmonary function, airway reversibility, clinical (ACQ-7) and AQLQ, and in exacerbations in the previous year.

Comparison of differentially-expressed proteins

Sputum SomaLogic analysis adjusted for age, gender 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 1, A-C). The DEPs are shown in Figure 1D and Additional File 1. Only 5 proteins distinguished CSA-NH from NSA-NH including colony stimulating factor 2 (CSF2, also known as granulocyte-macrophage colony-stimulating factor or GM-CSF), CXCL8/IL-8 and anterior gradient protein 2 (AGR2) (Table 2). CXCL8 did not distinguish between CSA-NH and ESA-NH group. 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 (Figure 2A-B, Supplementary Figure S2). This suggests that CSAPage 20) is associated with macrophage/neutrophil recruitment and mucus production.

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3 34 DEPs distinguished ESA-NH from NSA-NH and included azurocidin 1 (AZU1),
4 neutrophil elastase (ELANE), complement factor properdin (CFP) and CXCL8 (Table 3, Figure 2C-
5 F). AZU1 possesses monocyte chemotactic and antimicrobial activity (14) and CFP positively
6 regulates the alternative complement system (15). 29 proteins overlapped between ESA-NH and
7 NSA-NH and these included C-reactive protein (CRP), colony-stimulating factor 1 receptor (CSF1R),
8 inducible T-cell costimulatory ligand (ICOSLG), FCGR2A and catalase (CAT) (Table 3, Figure 1D).
9 In contrast, there were 13 differentially-expressed proteins including PDIA3, granzyme B (GZMB)
10 and CD5 antigen-like (CD5L) (Table 3, Figure 1D). GZMB is a cytoplasmic granule of cytotoxic T-
11 cells and NK cells, and is involved in apoptosis, chronic inflammation and wound healing (16).
12 CD5L, expressed in lymphoid tissues, lung epithelial cells or tissue macrophages, plays multiple
13 roles in inflammation, such as promoting macrophage phagocytosis (17).
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26 In summary, whilst CSA was associated with proteins involved in macrophage recruitment
27 and mucus production and both ESA and NSA with proteins with inflammatory and immune
28 responses characterized by T-cell-mediated acquired immunity in common, proteins linked to
29 neutrophilic activity were more closely related to ESA than to other groups. However, this was not
30 reflected in a significant difference in sputum neutrophilia in these subjects (Table 1). In addition,
31 the protein expression of CAT, a key antioxidant, was upregulated equally in all severe asthma
32 groups compared with NH (Figure 2G).
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44 **Pathway analysis of differentially-expressed proteins**

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46 Pathway analysis of sputum DEPs indicated that ESA-NH was associated with phagocytosis,
47 response to chemicals, response to multicellular organisms, chemotaxis, myeloid cell differentiation
48 and innate immunity and inflammation whilst NSA-NH was associated with acute-phase
49 inflammation, platelet degranulation, response to wounding and the immune system (Supplementary
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3 [Table S2](#)). Overall, different pathways were activated between CSA and NSA and airway epithelial
4 damage may be associated with ESA.
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9 **Characteristics of patients with transcriptomic analysis in bronchial biopsies and brushings**

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11 We found increased blood neutrophils and lower FeNO levels in CSA compared to NSA
12 subjects providing bronchial brushings and biopsies for analysis although the proportion of patients
13 who took systemic corticosteroids or the dose of oral corticosteroids was no different between the 2
14 severe asthma groups ([Supplementary Tables 3-5](#)). There were no significant differences in blood
15 eosinophil, sputum eosinophil and sputum neutrophil counts, and in pulmonary function, ACQ-7,
16 AQLQ or the number of exacerbations in the previous year among the 3 severe asthma groups. The
17 subjects who provided samples for sputum transcriptomics did not completely overlap with those
18 providing sputum proteomics but the clinical characteristics were similar ([Supplementary Table 5](#)).
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31 **Differentially-expressed genes (DEGs) between CSA and NSA**

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33 We detected 142 significantly differentially-expressed gene (DEG) probes in bronchial
34 brushings, 23 in bronchial biopsies and 15 in sputum samples between CSA and NSA ([Figure 3A-C](#);
35 [Additional File 2](#)). There were no significant DEG probes (FDR>0.05) in any samples between ESA
36 and NSA ([Additional File 3](#)). Hierarchical clustering of the 142 DEG from bronchial brushings
37 indicated that although CSA and NSA were clearly distinct, NSA and ESA did not cluster separately
38 ([Figure 3D](#)).
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46 The DEGs between CSA and NSA are implicated in oxidation-reduction, xenobiotic
47 metabolism and endoplasmic reticulum (ER) stress ([Additional File 2](#)). Cytochrome P450 family 1
48 subfamily B member 1 (CYP1B1) and aldehyde dehydrogenase 3 family member A1 (ALDH3A1),
49 which were over-expressed in bronchial brushings of CSA compared to other groups ([Figure 4A-B](#)),
50 play a role in metabolizing polycyclic aromatic hydrocarbons (PAHs) or aldehydes (18). The
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3 oxidative stress genes, NAD(P)H quinone dehydrogenase 1 (NQO1) and aldo-keto reductase family
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5 1 member C1 (AKR1C1), were also highly expressed in CSA bronchial brushings (Figure 4C-D). ER
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7 plays a central role in the protein biosynthesis, correct protein folding and post-transcriptional
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9 modifications (19). Accumulation of unfolded and misfolded proteins, termed ER stress, leads to the
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11 unfolded protein response (UPR) and inflammation (20). Heat shock protein family A (Hsp70)
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13 member 5 (HSPA5), a key mediator of ER stress, was significantly upregulated in CSA compared to
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15 NSA in bronchial brushings and biopsies (Figure 4E).
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20 **Pathway analysis using DEGs between CSA and NSA**

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22 Pathway analysis indicated that oxidation-reduction, chemical metabolism and endoplasmic
23
24 reticulum (ER) stress were different between CSA and NSA (Supplementary Table S6, S7). These
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26 results suggest that the lung epithelial cells of CSA patients are under more potent chemical,
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28 oxidative and ER stresses than those of NSA patients.
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33 **Gene Set Variation Analysis (GSVA) of bronchial brushings and biopsies**

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35 GSVA confirmed the selective enrichment of xenobiotic metabolism by cytochrome P (CYP)
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37 450, glutathione metabolism, response to oxidative stress, endoplasmic reticulum (ER) stress,
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39 unfolded protein response, lysosome or glycolysis and gluconeogenesis pathways in bronchial
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41 brushings (Figure 5A-G) and biopsies (Figure 6A-G) in the CSA group. There were no significant
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43 differences between CSA and NSA for these pathways. Using the signatures for active smoking
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45 obtained from Spira and colleagues (21), we confirmed that bronchial brushings and biopsies from
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47 CSA were enriched for the active smoking-related gene and that both CSA and NSA were enriched
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49 for the pack-year signature (Supplementary Table 1; Supplementary Figure S3).
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55 **Differentially-expressed genes in sputum, bronchial biopsies and epithelial brushings**

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3 As we could not detect any DEGs between ESA and NSA at the $FDR < 0.05$ level, we
4 undertook a discovery approach using a less stringent analysis strategy. Genes whose absolute fold-
5 change was ≥ 2.0 in limma were used to clarify the phenotypic difference between ESA and NSA
6 ([Additional File 2](#)). Twenty-seven genes (thirty-five probes) were up-regulated in ESA sputum
7 samples included matrix metalloproteinase 12 (MMP12), neuropilin 1 (NRP1), Toll-interleukin 1
8 receptor domain containing adaptor protein (TIRAP), C-X-C motif chemokine ligand 5 (CXCL5)
9 and pro-platelet basic protein (PPBP) ([Supplementary Table S8](#)). MMP12 has been associated with
10 decreased lung function and COPD, TIRAP is involved in the Toll-like receptor (TLR) signalling
11 pathway and both CXCL5/ENA-78 and PPBP/CXCL7 are potent neutrophil chemoattractants and
12 activators ([22](#)). Innate immunity, including complement system, TLR signalling and neutrophilic
13 inflammation, may be characteristics of ESA.

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16 Six down-regulated DEGs (fold-change ≤ 0.5) distinguished ESA from NSA in bronchial
17 brushings, namely carboxypeptidase A3 (CPA3), cystatin SN (CST1), immunoglobulin kappa
18 constant (IGKC), mucin 2, oligomeric mucus/gel-forming (MUC2) and tryptase $\alpha/\beta 1$ (TPSAB1)
19 ([Supplementary Table S8](#)). CPA3 and TPSAB1 are mast cell biomarkers and are found to be
20 elevated in asthma patients ([23](#)). CST1 is a cysteine proteinase inhibitor that has a protective effect
21 on epithelium ([24](#)). MUC2 provides a protective barrier for airways against particles or infectious
22 agents ([25](#)). This suggests that ESA has a lesser protective epithelial barrier and reduced mast cell
23 activity compared with NSA.

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

Protein interaction analysis using combined DEGs from airway samples

Protein interaction analysis by STRING using combined DEGs between CSA and NSA showed direct interactions of oxidation-reduction and pentose phosphate pathway network with the innate immune response via protein production and modification in endoplasmic reticulum (Figure 7). Proteins which play a role in lysosome, mucus production, Golgi homeostasis and tissue structure were also seen in the network.

DISCUSSION

We describe the differences in protein and gene expression between severe asthma patients who actively smoke (CSA), and ex-smokers with a significant history of cigarette smoking (ESA), and those who do not smoke (NSA). 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 whilst 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 ER 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 sensitization and anti-CSF2 antibodies block the allergic response in mouse models of asthma (27). CSF2 is also involved in the lung innate immune response to noxious agents such as LPS and cigarette smoke (28). Acute exposure to

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3 cigarette smoke in mice leads to enhanced CSF2 expression and neutralization using an intranasal
4 anti-CSF2 antibody reduced BALF macrophages and neutrophils and inflammatory analytes (29),
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6 which indicates that the CSF2 pathway can mediate smoke-induced inflammation. Future
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8 experiments in models of severe asthma linked to smoking or in selected patients may determine
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10 whether the elevated CSF2 expression seen here is causal or a marker of other driver mechanisms.
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14 Our data provide evidence for enhanced oxidative and ER stress in airway epithelial cells of
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16 CSA patients. We postulate that the increased activation of the xenobiotic response and oxidative
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18 and ER stress pathways influences innate immunity in these subjects. There is increased oxidative
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20 stress in asthma and COPD patients as well as in healthy smokers (30). Cigarette smoke not only
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22 contains high concentrations of reactive oxygen species (ROS) (30), but also activates alveolar
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24 macrophages and neutrophils, which also release ROS leading to an increased inflammatory
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26 response in a feed-forward process (30, 31). In both asthma and COPD, activated inflammatory cells
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28 including neutrophils, macrophages and eosinophils also produce ROS and further generate
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30 inflammation and causes injury to the airway epithelium (30). Moreover, impaired upregulation and
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32 production of protective antioxidant was reported in smokers, asthma and COPD patients. This
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34 oxidant-antioxidant imbalance resulting in oxidative stress is associated with airway
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36 hyperresponsiveness and decreased lung function and asthma severity (30, 32).
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40 CSA represented the escalated response to oxidative stress derived from cigarette smoking as
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42 CSA bronchial brushings and biopsies alone were enriched for the active smoking-related gene set
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44 whereas both CSA and ESA samples showed a similar enrichment of the pack-year signature.
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46 Increased antioxidant gene expression and increased enrichment of the gene set showing response to
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48 oxidative stress were observed in bronchial brushings, which may suggest that cigarette smoking
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50 stimulates airway epithelial cells to respond to oxidative stress in severe asthma. We also showed
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52 that ER stress might have a key role in CSA phenotype. ER stress is associated with neutrophilic
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54 asthma through NF- κ B activation and proinflammatory cytokine production (33). Cigarette smoking
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3 itself may induce ER stress (34) and the activation seen here in severe asthma might relate to active
4 cigarette smoke exposure. However, CSF2 and AGR2 have not been shown to be differentially-
5 expressed in the previously-published proteomic analysis of sputum from healthy current-smokers
6 compared to never-smokers (35). Moreover, endoplasmic stress and lysosome gene sets that we
7 found to be differentially expressed in these 2 groups of severe asthma were not differentially-
8 expressed in healthy current smokers compared to non-smokers (Supplementary Table S9 and S10)
9 (21). These results suggest that DEGs between CSA and NSA were not derived from the influence of
10 cigarette smoking itself.

11
12 We found decreased production of protective agents in ESA airways. Cigarette smoke injures
13 the airway epithelium in several ways, including decreased protective protein expression (36),
14 disruption of tight junctions (37), and through innate immune and inflammatory response (31).
15 Cigarette smoke-activated alveolar macrophages produce pro-inflammatory molecules, reactive
16 oxygen species (ROS), tissue proteases and chemokines for recruitment and survival of neutrophils
17 in the lung tissue (31), and activated neutrophils secrete proteases and breakdown collagen into
18 fragments, which can activate neutrophils in a positive feedback manner (38). We showed decreased
19 expression of MUC2 and CST1 in ESA, which both play a protective role for airway epithelium (39,
20 40). Conversely, the expression of MMP12, CXCL8 and PPBP, which can enhance lung damage,
21 were upregulated in ESA.

22
23 Sputum microbiota, which is associated with neutrophilic airway inflammation in adult
24 severe asthma has been reported to be different from that of healthy controls or non-severe
25 asthmatics (41, 42). Our results imply that a reduction in airway protective agents might change the
26 airway microbiome, affecting neutrophilic airway inflammation, especially in ESA; on the other
27 hand, the heightened mucin production might have had a beneficial effect in keeping the airway
28 epithelium free from bacterial colonization in CSA.

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3 There are important limitations in our study. First, the numbers of smoking and ex-smokers in
4
5 our groups were relatively small particularly when analysing data from sputum and biopsy and
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7 brushing samples, and the results should be considered as exploratory and will need confirmation in
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9 a larger cohort. Secondly, the lack of a control group of age-matched non-asthmatic active smokers
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11 does not allow us to determine the exact contribution of cigarette smoking to the changes observed.
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13 Thirdly, we did not observe differences in blood or sputum neutrophil counts although neutrophil
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15 chemoattractants were more upregulated in airways of ESA and CSA patients compared to controls.
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17

18 In conclusion, we found that current-smokers with severe asthma were characterized by
19
20 increased sputum CFS2 and AGR2 protein expression indicating enhanced macrophage recruitment
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22 and mucus production in addition to airway tissue genes associated with increased xenobiotic
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24 metabolism and responses to oxidative stress and ER stress. In contrast, ex-smokers with severe
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26 asthma were characterized by pathways involved in the recruitment and activity of neutrophils and
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28 with decreased airway protective factors. Airway gene expression analysis showed little difference
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30 between severe asthmatics who were ex-smokers and those who were never smokers.
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Author Contributions:

KT, CR, ML, SH, KS and SP performed the analysis; KT, KFC, ML, FD, IMA and YG designed the analytical approaches taken and analyzed the results; UH, PSB, PC, SJF, IH, NK, TS, DES, LJF, PHH, MC, LF, BD participated in the clinical characterization of the patients; KS, ARS, JC, YK were part of the data curation team; IMA, RD, PJS, and KFC conceived of and designed the study; and KT, I.M.A., and KFC coordinated the data and drafted the manuscript. All authors read the final version of the manuscript.

The transcriptomic data have been deposited in the Gene Expression Omnibus database, <http://www.ncbi.nlm.nih.gov/geo> (accession no. GSE76225 for gene expression data of bronchial biopsies).

Table 1. Patient characteristics for sputum SomaLogic analyses

	Severe asthma			Healthy	P value
	Current-smoker (CSA) (n=11)	Ex-smoker (ESA) (n=22)	Non-smoker (NSA) (n=37)	Non-smoker (NH) (n=18)	
Female	5 (45.5)	14 (63.6)	22 (59.5)	6 (33.3)	2.01E-01*
Age (y)	50.0±10.6	55.7±9.7	52.6±13.3	39.9±13.8 [¶]	3.95E-03 [§]
Onset age of asthma (y)	29.8±19.9	39.5±19.0 [¶]	25.0±18.1	N.R.	2.45E-02 [§]
Age at starting smoking (y)	19.3±4.0	16.2±2.5	N.R.	N.R.	4.93E-02 [†]
Years of smoking cessation (y)	N.R.	13.7±10.5	N.R.	N.R.	
Smoking pack-year	29.0±18.2	20.8±16.1	0.0±0.0	0.0±0.0	1.17E-01 [†]
BMI (kg/m ²)	27.7±4.7	31.1±6.7	27.5±5.7	25.3±3.2 [‡]	2.79E-02 [§]
Atopic (%)	8 (88.9) [2]	10 (62.5) [6]	28 (84.8) [4]	5 (45.5) [7]	3.60E-02 *
Blood eosinophil (*10 ³ /μL)	259±173	296±246	407±357 [2]	(116±71)	3.31E-01 ^{§#}
Blood neutrophil (*10 ³ /μL)	5.10±1.95	5.84±3.03	4.97±2.16 [2]	(3.35±1.15)	6.03E-01 ^{§#}
Sputum eosinophil (%)	7.2±15.2	14.8±16.8	18.8±24.6	(0.36±0.57)	2.98E-01 ^{§#}
Sputum neutrophil (%)	53.9±16.1	55.2±20.6	50.8±30.9	(41.0±26.5)	9.28E-01 ^{§#}
IgE (IU/mL)	222±201 [2]	313±499	305±510 [3]	(105±178)	8.84E-01 ^{§#}
FeNO (ppb)	15.2±16.6 [¶]	40.5±33.9 [1]	41.2±36.3 [3]	(19.4±9.7) [3]	7.55E-04 ^{§#}
Periostin (ng/mL)	42.8±9.3 [2]	53.1±18.9 [4]	54.9±20.3 [9]	(49.7±5.5) [4]	2.66E-01 ^{§#}
%FEV1 post-bronchodilator (%) ^{##}	73.7±18.2	78.8±21.1	68.6±21.1	(105.2±11.5)	1.82E-01 ^{§#}
FEV1/FVC post-bronchodilator (%) ^{##}	61.5±10.1	63.4±12.2	60.2±13.9	(79.0±5.9)	6.27E-01 ^{§#}
Airway reversibility (%)	15.0±9.5	16.7±12.7	17.3±20.4 [1]	N.R.	7.45E-01 [§]
Airflow limitation [92]	7 (63.6)	11 (50.0)	28 (75.7)	N.R.	1.33E-01*
Average ACQ-7	2.87±1.31 [1]	2.67±0.98 [3]	2.68±1.19 [4]	N.R.	8.30E-01 [§]
Average AQLQ	4.15±1.57 [1]	4.62±1.04 [5]	4.35±1.29 [2]	N.R.	5.06E-01 [§]
Exacerbation in previous year (n/y)	2.6±3.3	2.1±1.9	2.4±1.9	N.R.	7.47E-01 [§]
ER visit due to breathing problems	5 (45.5)	14 (63.6)	25 (62.2)	N.R.	4.41E-01*
Co-morbidities					
Allergic rhinitis (%)	2 (25.0) [3]	8 (40.0) [2]	16 (55.2) [8]	N.R.	2.85E-01*
Nasal polyp (%)	2 (20.0) [1]	7 (33.3) [1]	12 (34.3) [2]	N.R.	7.20E-01*
Sinusitis (%)	2 (25.0) [3]	6 (28.6) [1]	9 (28.1) [5]	N.R.	1.00E+00*
Chronic bronchitis (%)	1 (11.1) [1]	2 (9.1)	4 (12.1) [4]	N.R.	1.00E+00*
Psychiatric disease (%)	3 (33.3) [2]	3 (14.3) [1]	5 (13.9) [1]	N.R.	3.84E-01*
GERD (%)	4 (50.0) [3]	15 (71.4) [1] [¶]	11 (32.4) [3]	N.R.	1.74E-02 *
Medications					
Inhaled corticosteroids (%)	11 (100.0)	22 (100.0)	37 (100.0)	N.R.	1.00E+00*
Systemic corticosteroids (%)	3 (30.0) [1]	14 (63.6)	16 (45.7) [2]	N.R.	1.93E-01*
Oral corticosteroid dose (mg/day)	2.50±4.71 [1]	7.89±8.01 [3]	4.18±6.61 [2]	N.R.	8.53E-02 [§]
Anti-IgE therapy (%)	0 (0.0) [1]	2 (4.0) [2]	0 (0.0) [2]	N.R.	1.13E-01*
Long-acting beta agonist (%)	11 (100.0)	21 (95.5)	37 (100.0)	N.R.	4.71E-01*
Leukotriene modifiers (%)	4 (36.4)	11 (52.4) [1]	19 (51.4)	N.R.	6.80E-01*
Tiotropium (%)	3 (30.0) [1]	4 (22.2) [2]	12 (34.3) [2]	N.R.	5.61E-01*
Macrolide (%)	2 (18.2)	3 (13.6)	4 (10.8)	N.R.	7.96E-01*

Data are presented as mean±SD or n (%). The numbers of missing values are shown in square brackets. ^{##}Spirometry data without bronchodilator were used for healthy subjects. Statistical analysis was performed by Fisher's exact test*, Kruskal-Wallis test[§] or Mann-Whitney U test[†]. [#]Healthy subjects were excluded from statistical analyses of several items. Variables with P values in bold were significantly different (<0.05). [‡]p<5.00E-02 vs CSA, [‡]p<5.00E-02 vs ESA, [¶]p< 5.00E-02 vs NSA. ACQ: Asthma Control Questionnaire, AQLQ: Asthma Quality of Life Questionnaire, BMI: body mass index, ER: emergency room, ERS: European Respiratory Society, FeNO: fractional exhaled nitric oxide, FEV₁: forced expiratory volume in one second, FVC: forced vital capacity, GERD: gastroesophageal reflux disease, IgE: immunoglobulin E, N.R.: not relevant.

Table 2. Differentially expressed proteins between CSA-NH and NSA-NH by sputum somaLogic.

Probe ID	Protein target	Gene symbol	Gene name	Function
CSA-NH				
SL001726	CSF2	CSF2 (= GM-CSF)	colony stimulating factor 2	Granulocyte, monocyte, macrophage expansion
SL004925	AGR2	AGR2	anterior gradient protein 2	Mucin (MUC5AC and MUC5B) overproduction in asthma Localized in endoplasmic reticulum of bronchial epithelial cells
SL000039	IL-8	CXCL8 (=IL8)	C-X-C motif chemokine ligand 8	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 defence against oxidative stress.
SL000051	CRP	CRP	C-reactive protein	Host defence based on its ability to recognize 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 colony stimulating factor 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-gamma (RORC).
SL004068	GZMB	GZMB	granzyme B	Target 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.
CSA-NH and NSA-NH				
SL017613	FCG2A/B	FCGR2A	Fc fragment of immunoglobulin gamma 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.

Table 3. Differentially expressed key proteins in sputum somaLogic in comparison between ESA-NH and NSA-NH.

Probe ID	Protein target	Gene symbol	Gene name	Function
ESA-NH				
SL004589	AZU1	AZU1	azurocidin 1	A preproprotein of a mature azurophil granule antibiotic protein with monocyte chemotactic and antimicrobial activity.
SL000401	ELANE	ELANE	neutrophil elastase	This protease hydrolyzes proteins within specialized neutrophil lysosomes, called azurophil granules, as well as proteins of the extracellular matrix.
SL003192	CFP	CFP	complement factor properdin	A positive regulator of the alternate pathway of complement system.
SL000039	IL-8	CXCL8 (=IL8)	C-X-C motif chemokine ligand 8	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-gamma (RORC).
SL004068	GZMB	GZMB	granzyme B	Target 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 gamma receptor IIa	This 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.
SL000342	catalase	CAT	catalase	A key antioxidant enzyme in the body defence against oxidative stress.
SL000051	CRP	CRP	C-reactive protein	Host defence based on its ability to recognize 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 colony stimulating factor 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.

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FIGURE LEGENDS**Figure 1. Phenotypic differences among CSA, ESA and NSA were unveiled by limma of sputum SomaLogic.**

(A-C) Volcano plots showing differentially-expressed proteins (DEPs) in linear model for microarray (limma) of sputum SomaLogic in following comparisons; (A) CSA and NH, (B) ESA and NH, (C) NSA and NH. The proteins whose absolute fold change (FC) ≥ 2.0 at false discovery rate (FDR) < 0.05 were regarded as DEPs shown as coloured dots (red: FC ≥ 2.0 , turquoise: FC ≤ -2.0). The number of DEPs of each comparison is shown in the left box and right upper areas of each plot. (D) Venn diagram showing the numbers and names of DEPs in each comparison.

Figure 2. Differentially-expressed proteins (DEPs) in severe asthma sputum according to smoking status.

Dot plots with mean \pm SD showing signal intensity levels of protein expression of CSF2 (A), AGR2 (B), AZU1 (C), CXCL8 (D), ELANE (E), CFP (F) and CAT (G) in sputum by SomaLogic analysis in CSA (circles), ESA (squares), NSA (triangles) and NH (inverted triangles). RFU: Relative Fluorescence Units, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3. Differentially-expressed genes (DEGs) in current smokers (CSA) and non-smokers (NSA) with severe asthma.

Volcano plots showing differentially expressed genes (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 (red: FC ≥ 1.5 , turquoise: FC ≤ -1.5). 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. Blue rectangles represent samples with low expression for the particular gene,

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3 and red rectangles represent samples with high expression for the particular gene. CSA (dark
4 green), ESA (light green) and NSA (cyan).

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9 **Figure 4. Differentially-expressed genes (DEGs) associated with metabolism of xenobiotics,**
10 **oxidative stress and ER stress in bronchial brushings.**

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13 Dot plots showing DEGs in bronchial brushings associated with xenobiotic metabolism CYP1B1
14 (A), ALDH3A1 (B), NQO1 (C), AKR1C1 (D) and HSPA5 (E). CSA: circles, ESA: squares,
15 NSA: triangles, NH: inverted triangles. RFU: Relative Fluorescence Units, * $p < 0.05$; ** $p < 0.01$;
16 *** $p < 0.001$.

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24 **Figure 5. Gene Set Variation Analysis of selected stress-related pathways in bronchial**
25 **brushings according to smoking status.**

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28 Box-and-whisker plots showing pathway enrichment of (A) xenobiotic metabolism by CYP450,
29 (B) glutathione metabolism, (C) response to oxidative stress, (D) endoplasmic reticulum (ER)
30 stress, (E) unfolded protein response, (F) lysosome and (G) glycolysis and gluconeogenesis in
31 bronchial brushings of CSA (red circles), ESA (olive green circles), NSA (cyan circles) and NH
32 (purple circles) subjects. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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42 **Figure 6. Gene Set Variation Analysis of selected stress-related pathways in bronchial**
43 **biopsies according to smoking status.**

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46 Box-and-whisker plots showing pathway enrichment of (A) xenobiotic metabolism by CYP450,
47 (B) glutathione metabolism, (C) response to oxidative stress, (D) endoplasmic reticulum (ER)
48 stress, (E) unfolded protein response, (F) lysosome and (G) glycolysis and gluconeogenesis in
49 bronchial biopsies of CSA (red circles), ESA (olive green circles), NSA (cyan circles) and NH
50 (purple circles) subjects. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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5 **Figure 7. Protein interaction analysis by STRING using combined DEGs.**
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7 Combined differentially expressed genes (DEGs) in limma from bronchial brushings, biopsies
8 and sputa were used for protein interaction analysis by STRING. The large pink-coloured area is
9 filled with proteins related to xenobiotic metabolism and oxidation-reduction which contains
10 pentose-phosphate pathway (orange-coloured area). These proteins function with those in charge
11 of redox (small pink-coloured area) and connect with protein production or modification (yellow).
12 Some proteins are associated with innate immunity (blue). The other proteins function as
13 lysosomal (sky blue), membranous (coral red), mucus productive (apple green), Golgi
14 homeostatic (purple) or structural proteins (olive green). Overall, this reveals the relationship
15 between oxidative stress, ER stress, metabolism of xenobiotics and innate immunity.
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**Sputum proteomics and airway cell transcripts of current and ex-smokers with severe asthma
in U-BIOPRED: an exploratory analysis**

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Take home message

Inflammatory, oxidative/ER stress and epithelial barrier pathways are differentially activated in current smoking and ex-smoking severe asthma patients.

ABSTRACT (199 words)**Background:**

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.

Methods

The U-BIOPRED severe asthma patients containing current-smokers (CSA), ex-smokers (ESA), non-smokers (NSA) and healthy non-smokers (NH) 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.

Results

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 metalloproteinase 12 genes were upregulated in ESA and the epithelial protective genes, mucin 2 and cystatin SN, were downregulated.

Conclusion

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

ABBREVIATIONS

ACOS:	Asthma-COPD Overlap Syndrome
ACQ:	Asthma Control Questionnaire
AQLQ:	Asthma Quality of Life Questionnaire
BMI:	Body Mass Index
COPD:	Chronic Obstructive Pulmonary Disease
CSA:	Current-smokers with Severe Asthma
DEG:	Differentially Expressed Gene
DEP:	Differentially Expressed Protein
ERS:	European Respiratory Society
ESA:	Ex-smokers with Severe Asthma
eTRIKS:	European Translational Information and Knowledge Management Services
ER:	Endoplasmic Reticulum
FDR:	False Discovery Rate
FeNO:	Fraction of exhaled Nitric Oxide
FEV ₁ :	Forced Expiratory Volume in 1 second
FVC:	Forced Vital Capacity
GERD:	Gastroesophageal Reflux Disease
GSVA:	Gene Set Variation Analysis
ICS:	Inhaled Corticosteroids
IgE:	Immunoglobulin E
LABA:	Long-Acting β_2 -Agonist
MANOVA:	Multivariate Analysis Of Variance
NH:	Non-smoking Healthy volunteers
NSA:	Non-smokers with Severe Asthma
SCS:	Systemic Corticosteroids
Th2:	T helper type 2
U-BIOPRED:	Unbiased BIOMarkers for the PREdiction of respiratory diseases outcomes

INTRODUCTION

Severe asthma has been defined as asthma that requires treatment with high dose inhaled corticosteroids (ICS) and long-acting β_2 -agonist (LABA) and often with 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 have been 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 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-COPD overlap syndrome (ACOS) with features of both diseases. In patients who have been recruited as COPD patients in the COPDgene cohort, the patients who have had a history of asthma before the age of 40 and who had a smoking history of at least 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 Unbiased Biomarkers for the PREDiction of respiratory disease outcomes (U-BIOPRED) project recruited patients with severe asthma that included 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

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3 underlie smoking or ex-smoking severe asthma, we examined the differential expression of genes
4 and proteins in various compartments.

7 **MATERIALS and METHODS**

9 *Clinical data*

11 We enrolled totally 374 severe asthma patients in the U-BIOPRED cohort divided into three
12 groups by smoking status; current-smokers with severe asthma (CSA), ex-smokers with severe
13 asthma (ESA), non-smokers with severe asthma (NSA). We narrowed down the NSA to those who
14 had never smoked (0 pack-year), although original non-smokers with severe asthma group in the U-
15 BIOPRED cohort contained the patients whose pack-year was less than 5. Eighty-one non-smoking
16 healthy volunteers (NH) whose pack-years were 0 were also enrolled. Differential blood and induced
17 sputum cell counts, serum total IgE and skin prick tests, serum periostin and fraction of exhaled
18 nitric oxide (FeNO) and pre- and post- bronchodilator spirometry were obtained (8, 9). Bronchial
19 biopsies, bronchial brushings and sputum were obtained, as previously described (8). Because of the
20 bronchoscopy exclusion criteria (Supplementary File), only 95 bronchial brushings and 69 bronchial
21 biopsies were obtained. The number of sputum samples for proteomic analysis was 88. All subjects
22 whose samples were adequate and underwent omics analyses are shown in **Supplementary Figure**
23 **S1**. The study was approved by the Ethics Committees for each of the 16 clinical recruiting centres.
24 All subjects gave written and signed informed consent.

43 *Transcriptomic microarray analysis*

45 Sputum plugs were obtained and separated into cells and supernatants [7]. Cell pellets were
46 used to prepare RNA using the miRNeasy mini kit (Qiagen, CA, USA). Sputum samples with >30%
47 squamous cells were excluded from microarray analysis. Bronchial brushings and biopsy samples
48 were immediately placed in TRIzol reagent (Invitrogen) and preserved at -80°C. Expression profiling
49 of transcriptome was performed using GeneChip[®] Human Genome U133 Plus 2.0 Array (Affymetrix,
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3 Santa Clara, CA) as previously described (8, 9). Pathway analysis, enrichment analysis and
4 functional clustering of differentially-expressed genes were performed as described previously (8, 9)
5 and protein interaction analysis using annotated protein-coding genes was performed by STRING
6 version 10.0 (STRING CONSORTIUM 2016, <http://www.string-db.org>) (10).
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11 12 13 ***SomaLogic proteomic technique***

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15 The SOMAscan proteomic assay of sputum supernatants performed by SomaLogic Inc.,
16 (Boulder, CO) was used (9).
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22 ***Gene Set Variation Analysis (GSVA)***

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24 Gene set variation analysis (GSVA) was performed in R using the Bioconductor GSVA
25 package for estimating variation of gene set enrichment (11). Gene sets were obtained from
26 Molecular Signatures Database v5.2 (MSigDB) (<http://software.broadinstitute.org/gsea/msigdb>) or
27 from published papers ([Supplementary Table S1](#)). We used ImmunomapTM graphics (Johnson &
28 Johnson Ltd., NJ, USA) for visualisation.
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37 ***Statistical analysis***

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39 All datasets were quality-controlled and normalized, followed by adjustment of batch effects
40 using ComBat tools and uploaded into transSMART, an open-source knowledge management
41 platform for sharing research data supported by European Translational Information and Knowledge
42 Management Services (eTRIKS) (8, 9). All categorical variables were analysed using Fisher's
43 exact test. Continuous variables were analysed using Kruskal-Wallis test. Gene and protein
44 expression data were analysed using multivariate analysis of variance (MANOVA) with age, gender
45 and systemic corticosteroids (SCS) use were analysed as covariates. A p value <0.05 was considered
46 significant. A linear model for microarray data (Bioconductor limma package for R) with
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3 Benjamini-Hochberg false discovery rate (FDR) correction was used in the analysis of the
4 differentially-expressed genes (DEGs) and for GSVA. Fold change ≥ 1.5 and FDR < 0.05 was
5 considered statistically significant in transcriptomic and proteomic analyses. When using GSVA,
6 FDR < 0.05 was considered statistically significant. Statistical analyses were performed by R version
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11 3.3.1 (R Core Team, 2016).
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RESULTS

Clinical characteristics of subjects with sputum SomaLogic data

Table 1 shows the characteristics of subjects who provided sputum samples for SomaLogic analysis. The levels of fractional exhaled nitric oxide (FeNO) of CSA subjects were lower than in the other severe asthma groups. Although there were numerical in blood eosinophil counts (CSA 259/ μ L, ESA 296/ μ L, NSA 407/ μ L), sputum eosinophils (CSA 7.2%, ESA 14.8%, NSA 18.8%), and the proportion of those on oral corticosteroids (CSA 30.0%, ESA 63.6%, NSA 45.7%), these were not statistically significant. NSA patients had the highest blood and sputum eosinophil counts. No differences were seen among the 3 severe asthma groups in terms of pulmonary function, airway reversibility, clinical (ACQ-7) and AQLQ, and in exacerbations in the previous year.

Comparison of differentially-expressed proteins

Sputum SomaLogic analysis adjusted for age, gender 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 1, A-C). The DEPs are shown in Figure 1D and Additional File 1. Only 5 proteins distinguished CSA-NH from NSA-NH including colony stimulating factor 2 (CSF2, also known as granulocyte-macrophage colony-stimulating factor or GM-CSF), CXCL8/IL-8 and anterior gradient protein 2 (AGR2) (Table 2). CXCL8 did not distinguish between CSA-NH and ESA-NH group. 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 (Figure 2A-B, Supplementary Figure S2). This suggests that CSAPage 20) is associated with macrophage/neutrophil recruitment and mucus production.

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3 34 DEPs distinguished ESA-NH from NSA-NH and included azurocidin 1 (AZU1),
4 neutrophil elastase (ELANE), complement factor properdin (CFP) and CXCL8 (Table 3, Figure 2C-
5 F). AZU1 possesses monocyte chemotactic and antimicrobial activity (14) and CFP positively
6 regulates the alternative complement system (15). 29 proteins overlapped between ESA-NH and
7 NSA-NH and these included C-reactive protein (CRP), colony-stimulating factor 1 receptor (CSF1R),
8 inducible T-cell costimulatory ligand (ICOSLG), FCGR2A and catalase (CAT) (Table 3, Figure 1D).
9 In contrast, there were 13 differentially-expressed proteins including PDIA3, granzyme B (GZMB)
10 and CD5 antigen-like (CD5L) (Table 3, Figure 1D). GZMB is a cytoplasmic granule of cytotoxic T-
11 cells and NK cells, and is involved in apoptosis, chronic inflammation and wound healing (16).
12 CD5L, expressed in lymphoid tissues, lung epithelial cells or tissue macrophages, plays multiple
13 roles in inflammation, such as promoting macrophage phagocytosis (17).
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26 In summary, whilst CSA was associated with proteins involved in macrophage recruitment
27 and mucus production and both ESA and NSA with proteins with inflammatory and immune
28 responses characterized by T-cell-mediated acquired immunity in common, proteins linked to
29 neutrophilic activity were more closely related to ESA than to other groups. However, this was not
30 reflected in a significant difference in sputum neutrophilia in these subjects (Table 1). In addition,
31 the protein expression of CAT, a key antioxidant, was upregulated equally in all severe asthma
32 groups compared with NH (Figure 2G).
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44 **Pathway analysis of differentially-expressed proteins**

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46 Pathway analysis of sputum DEPs indicated that ESA-NH was associated with phagocytosis,
47 response to chemicals, response to multicellular organisms, chemotaxis, myeloid cell differentiation
48 and innate immunity and inflammation whilst NSA-NH was associated with acute-phase
49 inflammation, platelet degranulation, response to wounding and the immune system (Supplementary
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3 [Table S2](#)). Overall, different pathways were activated between CSA and NSA and airway epithelial
4 damage may be associated with ESA.
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9 **Characteristics of patients with transcriptomic analysis in bronchial biopsies and brushings**

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11 We found increased blood neutrophils and lower FeNO levels in CSA compared to NSA
12 subjects providing bronchial brushings and biopsies for analysis although the proportion of patients
13 who took systemic corticosteroids or the dose of oral corticosteroids was no different between the 2
14 severe asthma groups ([Supplementary Tables 3-5](#)). There were no significant differences in blood
15 eosinophil, sputum eosinophil and sputum neutrophil counts, and in pulmonary function, ACQ-7,
16 AQLQ or the number of exacerbations in the previous year among the 3 severe asthma groups. The
17 subjects who provided samples for sputum transcriptomics did not completely overlap with those
18 providing sputum proteomics but the clinical characteristics were similar ([Supplementary Table 5](#)).
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31 **Differentially-expressed genes (DEGs) between CSA and NSA**

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33 We detected 142 significantly differentially-expressed gene (DEG) probes in bronchial
34 brushings, 23 in bronchial biopsies and 15 in sputum samples between CSA and NSA ([Figure 3A-C](#);
35 [Additional File 2](#)). There were no significant DEG probes (FDR>0.05) in any samples between ESA
36 and NSA ([Additional File 3](#)). Hierarchical clustering of the 142 DEG from bronchial brushings
37 indicated that although CSA and NSA were clearly distinct, NSA and ESA did not cluster separately
38 ([Figure 3D](#)).
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46 The DEGs between CSA and NSA are implicated in oxidation-reduction, xenobiotic
47 metabolism and endoplasmic reticulum (ER) stress ([Additional File 2](#)). Cytochrome P450 family 1
48 subfamily B member 1 (CYP1B1) and aldehyde dehydrogenase 3 family member A1 (ALDH3A1),
49 which were over-expressed in bronchial brushings of CSA compared to other groups ([Figure 4A-B](#)),
50 play a role in metabolizing polycyclic aromatic hydrocarbons (PAHs) or aldehydes (18). The
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3 oxidative stress genes, NAD(P)H quinone dehydrogenase 1 (NQO1) and aldo-keto reductase family
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5 1 member C1 (AKR1C1), were also highly expressed in CSA bronchial brushings (Figure 4C-D). ER
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7 plays a central role in the protein biosynthesis, correct protein folding and post-transcriptional
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9 modifications (19). Accumulation of unfolded and misfolded proteins, termed ER stress, leads to the
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11 unfolded protein response (UPR) and inflammation (20). Heat shock protein family A (Hsp70)
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13 member 5 (HSPA5), a key mediator of ER stress, was significantly upregulated in CSA compared to
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15 NSA in bronchial brushings and biopsies (Figure 4E).
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20 **Pathway analysis using DEGs between CSA and NSA**

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22 Pathway analysis indicated that oxidation-reduction, chemical metabolism and endoplasmic
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24 reticulum (ER) stress were different between CSA and NSA (Supplementary Table S6, S7). These
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26 results suggest that the lung epithelial cells of CSA patients are under more potent chemical,
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28 oxidative and ER stresses than those of NSA patients.
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33 **Gene Set Variation Analysis (GSVA) of bronchial brushings and biopsies**

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35 GSVA confirmed the selective enrichment of xenobiotic metabolism by cytochrome P (CYP)
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37 450, glutathione metabolism, response to oxidative stress, endoplasmic reticulum (ER) stress,
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39 unfolded protein response, lysosome or glycolysis and gluconeogenesis pathways in bronchial
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41 brushings (Figure 5A-G) and biopsies (Figure 6A-G) in the CSA group. There were no significant
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43 differences between CSA and NSA for these pathways. Using the signatures for active smoking
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45 obtained from Spira and colleagues (21), we confirmed that bronchial brushings and biopsies from
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47 CSA were enriched for the active smoking-related gene and that both CSA and NSA were enriched
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49 for the pack-year signature (Supplementary Table 1; Supplementary Figure S3).
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55 **Differentially-expressed genes in sputum, bronchial biopsies and epithelial brushings**

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3 As we could not detect any DEGs between ESA and NSA at the $FDR < 0.05$ level, we
4 undertook a discovery approach using a less stringent analysis strategy. Genes whose absolute fold-
5 change was ≥ 2.0 in limma were used to clarify the phenotypic difference between ESA and NSA
6 (Additional File 2). Twenty-seven genes (thirty-five probes) were up-regulated in ESA sputum
7 samples included matrix metalloproteinase 12 (MMP12), neuropilin 1 (NRP1), Toll-interleukin 1
8 receptor domain containing adaptor protein (TIRAP), C-X-C motif chemokine ligand 5 (CXCL5)
9 and pro-platelet basic protein (PPBP) (Supplementary Table S8). MMP12 has been associated with
10 decreased lung function and COPD, TIRAP is involved in the Toll-like receptor (TLR) signalling
11 pathway and both CXCL5/ENA-78 and PPBP/CXCL7 are potent neutrophil chemoattractants and
12 activators (22). Innate immunity, including complement system, TLR signalling and neutrophilic
13 inflammation, may be characteristics of ESA.
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18 Six down-regulated DEGs (fold-change ≤ 0.5) distinguished ESA from NSA in bronchial
19 brushings, namely carboxypeptidase A3 (CPA3), cystatin SN (CST1), immunoglobulin kappa
20 constant (IGKC), mucin 2, oligomeric mucus/gel-forming (MUC2) and tryptase $\alpha/\beta 1$ (TPSAB1)
21 (Supplementary Table S8). CPA3 and TPSAB1 are mast cell biomarkers and are found to be
22 elevated in asthma patients (23). CST1 is a cysteine proteinase inhibitor that has a protective effect
23 on epithelium (24). MUC2 provides a protective barrier for airways against particles or infectious
24 agents (25). This suggests that ESA has a lesser protective epithelial barrier and reduced mast cell
25 activity compared with NSA.
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30 In the biopsies, 16 DEGs (fold-change ≥ 2.0) were detected and these included follicular
31 dendritic cell secreted protein (FDCSP), periostin (POSTN), PPBP, immunoglobulin λ constant 1
32 (IGLC1) and immunoglobulin λ variable cluster (IGLV). FDCSP and PPBP were upregulated in
33 ESA whilst POSTN, IGLC1 and IGLV were downregulated. Overall, the data suggests that
34 neutrophilic innate immunity is more characteristic of ESA than IL-4/13 signalling and humoral
35 immunity.
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Protein interaction analysis using combined DEGs from airway samples

Protein interaction analysis by STRING using combined DEGs between CSA and NSA showed direct interactions of oxidation-reduction and pentose phosphate pathway network with the innate immune response via protein production and modification in endoplasmic reticulum ([Figure 7](#)). Proteins which play a role in lysosome, mucus production, Golgi homeostasis and tissue structure were also seen in the network.

DISCUSSION

We describe the differences in protein and gene expression between severe asthma patients who actively smoke (CSA), and ex-smokers with a significant history of cigarette smoking (ESA), and those who do not smoke (NSA). 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 whilst 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 ER 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 sensitization and anti-CSF2 antibodies block the allergic response in mouse models of asthma (27). CSF2 is also involved in the lung innate immune response to noxious agents such as LPS and cigarette smoke (28). Acute exposure to

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3 cigarette smoke in mice leads to enhanced CSF2 expression and neutralization using an intranasal
4 anti-CSF2 antibody reduced BALF macrophages and neutrophils and inflammatory analytes (29),
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6 which indicates that the CSF2 pathway can mediate smoke-induced inflammation. Future
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8 experiments in models of severe asthma linked to smoking or in selected patients may determine
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10 whether the elevated CSF2 expression seen here is causal or a marker of other driver mechanisms.
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14 Our data provide evidence for enhanced oxidative and ER stress in airway epithelial cells of
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16 CSA patients. We postulate that the increased activation of the xenobiotic response and oxidative
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18 and ER stress pathways influences innate immunity in these subjects. There is increased oxidative
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20 stress in asthma and COPD patients as well as in healthy smokers (30). Cigarette smoke not only
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22 contains high concentrations of reactive oxygen species (ROS) (30), but also activates alveolar
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24 macrophages and neutrophils, which also release ROS leading to an increased inflammatory
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26 response in a feed-forward process (30, 31). In both asthma and COPD, activated inflammatory cells
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28 including neutrophils, macrophages and eosinophils also produce ROS and further generate
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30 inflammation and causes injury to the airway epithelium (30). Moreover, impaired upregulation and
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32 production of protective antioxidant was reported in smokers, asthma and COPD patients. This
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34 oxidant-antioxidant imbalance resulting in oxidative stress is associated with airway
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36 hyperresponsiveness and decreased lung function and asthma severity (30, 32).
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40 CSA represented the escalated response to oxidative stress derived from cigarette smoking as
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42 CSA bronchial brushings and biopsies alone were enriched for the active smoking-related gene set
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44 whereas both CSA and ESA samples showed a similar enrichment of the pack-year signature.
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46 Increased antioxidant gene expression and increased enrichment of the gene set showing response to
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48 oxidative stress were observed in bronchial brushings, which may suggest that cigarette smoking
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50 stimulates airway epithelial cells to respond to oxidative stress in severe asthma. We also showed
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52 that ER stress might have a key role in CSA phenotype. ER stress is associated with neutrophilic
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54 asthma through NF- κ B activation and proinflammatory cytokine production (33). Cigarette smoking
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3 itself **may** induce ER stress (34) and the activation seen here in severe asthma **might** relate to active
4 cigarette smoke exposure. **However, CSF2 and AGR2 have not been shown to be differentially-**
5 **expressed in the previously-published proteomic analysis of sputum from healthy current-smokers**
6 **compared to never-smokers (35). Moreover, endoplasmic stress and lysosome gene sets that we**
7 **found to be differentially expressed in these 2 groups of severe asthma were not differentially-**
8 **expressed in healthy current smokers compared to non-smokers (Supplementary Table S9 and S10)**
9 **(21). These results suggest that DEGs between CSA and NSA were not derived from the influence of**
10 **cigarette smoking itself.**

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20 We found decreased production of protective agents in ESA airways. Cigarette smoke injures
21 the airway epithelium in several ways, including decreased protective protein expression (36),
22 disruption of tight junctions (37), and through innate immune and inflammatory response (31).
23 Cigarette smoke-activated alveolar macrophages produce pro-inflammatory molecules, reactive
24 oxygen species (ROS), tissue proteases and chemokines for recruitment and survival of neutrophils
25 in the lung tissue (31), and activated neutrophils secrete proteases and breakdown collagen into
26 fragments, which can activate neutrophils in a positive feedback manner (38). We showed decreased
27 expression of MUC2 and CST1 in ESA, which both play a protective role for airway epithelium (39,
28 40). Conversely, the expression of MMP12, CXCL8 and PPBP, which can enhance lung damage,
29 were upregulated in ESA.
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42 Sputum microbiota, which is associated with neutrophilic airway inflammation in adult
43 severe asthma has been reported to be different from that of healthy controls or non-severe
44 asthmatics (41, 42). Our results imply that a reduction in airway protective agents might change the
45 airway microbiome, affecting neutrophilic airway inflammation, especially in ESA; on the other
46 hand, the heightened mucin production might have had a beneficial effect in keeping the airway
47 epithelium free from bacterial colonization in CSA.
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3 There are important limitations in our study. First, the numbers of smoking and ex-smokers in
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5 our groups were relatively small particularly when analysing data from sputum and biopsy and
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7 brushing samples, and the results should be considered as exploratory and will need confirmation in
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9 a larger cohort. Secondly, the lack of a control group of age-matched non-asthmatic active smokers
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11 does not allow us to determine the exact contribution of cigarette smoking to the changes observed.
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13 Thirdly, we did not observe differences in blood or sputum neutrophil counts although neutrophil
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15 chemoattractants were more upregulated in airways of ESA and CSA patients compared to controls.
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18 In conclusion, we found that current-smokers with severe asthma were characterized by
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20 increased sputum CFS2 and AGR2 protein expression indicating enhanced macrophage recruitment
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22 and mucus production in addition to airway tissue genes associated with increased xenobiotic
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24 metabolism and responses to oxidative stress and ER stress. In contrast, ex-smokers with severe
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26 asthma were characterized by pathways involved in the recruitment and activity of neutrophils and
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28 with decreased airway protective factors. Airway gene expression analysis showed little difference
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30 between severe asthmatics who were ex-smokers and those who were never smokers.
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Author Contributions:

KT, CR, ML, SH, KS and SP performed the analysis; KT, KFC, ML, FD, IMA and YG designed the analytical approaches taken and analyzed the results; UH, PSB, PC, SJF, IH, NK, TS, DES, LJF, PHH, MC, LF, BD participated in the clinical characterization of the patients; KS, ARS, JC, YK were part of the data curation team; IMA, RD, PJS, and KFC conceived of and designed the study; and KT, I.M.A., and KFC coordinated the data and drafted the manuscript. All authors read the final version of the manuscript.

The transcriptomic data have been deposited in the Gene Expression Omnibus database, <http://www.ncbi.nlm.nih.gov/geo> (accession no. GSE76225 for gene expression data of bronchial biopsies).

Table 1. Patient characteristics for sputum SomaLogic analyses

	Severe asthma			Healthy	P value
	Current-smoker (CSA) (n=11)	Ex-smoker (ESA) (n=22)	Non-smoker (NSA) (n=37)	Non-smoker (NH) (n=18)	
Female	5 (45.5)	14 (63.6)	22 (59.5)	6 (33.3)	2.01E-01*
Age (y)	50.0±10.6	55.7±9.7	52.6±13.3	39.9±13.8 [¶]	3.95E-03§
Onset age of asthma (y)	29.8±19.9	39.5±19.0 [¶]	25.0±18.1	N.R.	2.45E-02§
Age at starting smoking (y)	19.3±4.0	16.2±2.5	N.R.	N.R.	4.93E-02†
Years of smoking cessation (y)	N.R.	13.7±10.5	N.R.	N.R.	
Smoking pack-year	29.0±18.2	20.8±16.1	0.0±0.0	0.0±0.0	1.17E-01†
BMI (kg/m ²)	27.7±4.7	31.1±6.7	27.5±5.7	25.3±3.2 [‡]	2.79E-02§
Atopic (%)	8 (88.9) [2]	10 (62.5) [6]	28 (84.8) [4]	5 (45.5) [7]	3.60E-02*
Blood eosinophil (* μ L)	259±173	296±246	407±357 [2]	(116±71)	3.31E-01§#
Blood neutrophil (* $10^3/\mu$ L)	5.10±1.95	5.84±3.03	4.97±2.16 [2]	(3.35±1.15)	6.03E-01§#
Sputum eosinophil (%)	7.2±15.2	14.8±16.8	18.8±24.6	(0.36±0.57)	2.98E-01§#
Sputum neutrophil (%)	53.9±16.1	55.2±20.6	50.8±30.9	(41.0±26.5)	9.28E-01§#
IgE (IU/mL)	222±201 [2]	313±499	305±510 [3]	(105±178)	8.84E-01§#
FeNO (ppb)	15.2±16.6 [¶]	40.5±33.9 [1]	41.2±36.3 [3]	(19.4±9.7) [3]	7.55E-04§#
Periostin (ng/mL)	42.8±9.3 [2]	53.1±18.9 [4]	54.9±20.3 [9]	(49.7±5.5) [4]	2.66E-01§#
%FEV1 post-bronchodilator (%) ^{##}	73.7±18.2	78.8±21.1	68.6±21.1	(105.2±11.5)	1.82E-01§#
FEV1/FVC post-bronchodilator (%) ^{##}	61.5±10.1	63.4±12.2	60.2±13.9	(79.0±5.9)	6.27E-01§#
Airway reversibility (%)	15.0±9.5	16.7±12.7	17.3±20.4 [1]	N.R.	7.45E-01§
Airflow limitation [92]	7 (63.6)	11 (50.0)	28 (75.7)	N.R.	1.33E-01*
Average ACQ-7	2.87±1.31 [1]	2.67±0.98 [3]	2.68±1.19 [4]	N.R.	8.30E-01§
Average AQLQ	4.15±1.57 [1]	4.62±1.04 [5]	4.35±1.29 [2]	N.R.	5.06E-01§
Exacerbation in previous year (n/y)	2.6±3.3	2.1±1.9	2.4±1.9	N.R.	7.47E-01§
ER visit due to breathing problems	5 (45.5)	14 (63.6)	25 (62.2)	N.R.	4.41E-01*
Co-morbidities					
Allergic rhinitis (%)	2 (25.0) [3]	8 (40.0) [2]	16 (55.2) [8]	N.R.	2.85E-01*
Nasal polyp (%)	2 (20.0) [1]	7 (33.3) [1]	12 (34.3) [2]	N.R.	7.20E-01*
Sinusitis (%)	2 (25.0) [3]	6 (28.6) [1]	9 (28.1) [5]	N.R.	1.00E+00*
Chronic bronchitis (%)	1 (11.1) [1]	2 (9.1)	4 (12.1) [4]	N.R.	1.00E+00*
Psychiatric disease (%)	3 (33.3) [2]	3 (14.3) [1]	5 (13.9) [1]	N.R.	3.84E-01*
GERD (%)	4 (50.0) [3]	15 (71.4) [1] [¶]	11 (32.4) [3]	N.R.	1.74E-02*
Medications					
Inhaled corticosteroids (%)	11 (100.0)	22 (100.0)	37 (100.0)	N.R.	1.00E+00*
Systemic corticosteroids (%)	3 (30.0) [1]	14 (63.6)	16 (45.7) [2]	N.R.	1.93E-01*
Oral corticosteroid dose (mg/day)	2.50±4.71 [1]	7.89±8.01 [3]	4.18±6.61 [2]	N.R.	8.53E-02§
Anti-IgE therapy (%)	0 (0.0) [1]	2 (4.0) [2]	0 (0.0) [2]	N.R.	1.13E-01*
Long-acting beta agonist (%)	11 (100.0)	21 (95.5)	37 (100.0)	N.R.	4.71E-01*
Leukotriene modifiers (%)	4 (36.4)	11 (52.4) [1]	19 (51.4)	N.R.	6.80E-01*
Tiotropium (%)	3 (30.0) [1]	4 (22.2) [2]	12 (34.3) [2]	N.R.	5.61E-01*
Macrolide (%)	2 (18.2)	3 (13.6)	4 (10.8)	N.R.	7.96E-01*

Data are presented as mean±SD or n (%). The numbers of missing values are shown in square brackets. ^{##}Spirometry data without bronchodilator were used for healthy subjects. Statistical analysis was performed by Fisher's exact test*, Kruskal-Wallis test[§] or Mann-Whitney U test[†]. [#]Healthy subjects were excluded from statistical analyses of several items. Variables with P values in bold were significantly different (<0.05). [‡]p<5.00E-02 vs CSA, [‡]p<5.00E-02 vs ESA, [¶]p< 5.00E-02 vs NSA. ACQ: Asthma Control Questionnaire, AQLQ: Asthma Quality of Life Questionnaire, BMI: body mass index, ER: emergency room, ERS: European Respiratory Society, FeNO: fractional exhaled nitric oxide, FEV₁: forced expiratory volume in one second, FVC: forced vital capacity, GERD: gastroesophageal reflux disease, IgE: immunoglobulin E, N.R.: not relevant.

Table 2. Differentially expressed proteins between CSA-NH and NSA-NH by sputum somaLogic.

Probe ID	Protein target	Gene symbol	Gene name	Function
CSA-NH				
SL001726	CSF2	CSF2 (= GM-CSF)	colony stimulating factor 2	Granulocyte, monocyte, macrophage expansion
SL004925	AGR2	AGR2	anterior gradient protein 2	Mucin (MUC5AC and MUC5B) overproduction in asthma Localized in endoplasmic reticulum of bronchial epithelial cells
SL000039	IL-8	CXCL8 (=IL8)	C-X-C motif chemokine ligand 8	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 defence against oxidative stress.
SL000051	CRP	CRP	C-reactive protein	Host defence based on its ability to recognize 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 colony stimulating factor 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-gamma (RORC).
SL004068	GZMB	GZMB	granzyme B	Target 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.
CSA-NH and NSA-NH				
SL017613	FCG2A/B	FCGR2A	Fc fragment of immunoglobulin gamma 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.

Table 3. Differentially expressed key proteins in sputum somaLogic in comparison between ESA-NH and NSA-NH.

Probe ID	Protein target	Gene symbol	Gene name	Function
ESA-NH				
SL004589	AZU1	AZU1	azurocidin 1	A preproprotein of a mature azurophil granule antibiotic protein with monocyte chemotactic and antimicrobial activity.
SL000401	ELANE	ELANE	neutrophil elastase	This protease hydrolyzes proteins within specialized neutrophil lysosomes, called azurophil granules, as well as proteins of the extracellular matrix.
SL003192	CFP	CFP	complement factor properdin	A positive regulator of the alternate pathway of complement system.
SL000039	IL-8	CXCL8 (=IL8)	C-X-C motif chemokine ligand 8	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-gamma (RORC).
SL004068	GZMB	GZMB	granzyme B	Target 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 gamma receptor IIa	This 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.
SL000342	catalase	CAT	catalase	A key antioxidant enzyme in the body defence against oxidative stress.
SL000051	CRP	CRP	C-reactive protein	Host defence based on its ability to recognize 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 colony stimulating factor 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.

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FIGURE LEGENDS**Figure 1. Phenotypic differences among CSA, ESA and NSA were unveiled by limma of sputum SomaLogic.**

(A-C) Volcano plots showing differentially-expressed proteins (DEPs) in linear model for microarray (limma) of sputum SomaLogic in following comparisons; (A) CSA and NH, (B) ESA and NH, (C) NSA and NH. The proteins whose absolute fold change (FC) ≥ 2.0 at false discovery rate (FDR) < 0.05 were regarded as DEPs shown as coloured dots (red: FC ≥ 2.0 , turquoise: FC ≤ -2.0). The number of DEPs of each comparison is shown in the left box and right upper areas of each plot. (D) Venn diagram showing the numbers and names of DEPs in each comparison.

Figure 2. Differentially-expressed proteins (DEPs) in severe asthma sputum according to smoking status.

Dot plots with mean \pm SD showing signal intensity levels of protein expression of CSF2 (A), AGR2 (B), AZU1 (C), CXCL8 (D), ELANE (E), CFP (F) and CAT (G) in sputum by SomaLogic analysis in CSA (circles), ESA (squares), NSA (triangles) and NH (inverted triangles). RFU: Relative Fluorescence Units, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure 3. Differentially-expressed genes (DEGs) in current smokers (CSA) and non-smokers (NSA) with severe asthma.

Volcano plots showing differentially expressed genes (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 (red: FC ≥ 1.5 , turquoise: FC ≤ -1.5). 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. Blue rectangles represent samples with low expression for the particular gene,

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3 and red rectangles represent samples with high expression for the particular gene. CSA (dark
4 green), ESA (light green) and NSA (cyan).
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9 **Figure 4. Differentially-expressed genes (DEGs) associated with metabolism of xenobiotics,**
10 **oxidative stress and ER stress in bronchial brushings.**
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13 Dot plots showing DEGs in bronchial brushings associated with xenobiotic metabolism CYP1B1
14 (A), ALDH3A1 (B), NQO1 (C), AKR1C1 (D) and HSPA5 (E). CSA: circles, ESA: squares,
15 NSA: triangles, NH: inverted triangles. RFU: Relative Fluorescence Units, * $p < 0.05$; ** $p < 0.01$;
16 *** $p < 0.001$.
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24 **Figure 5. Gene Set Variation Analysis of selected stress-related pathways in bronchial**
25 **brushings according to smoking status.**
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28 Box-and-whisker plots showing pathway enrichment of (A) xenobiotic metabolism by CYP450,
29 (B) glutathione metabolism, (C) response to oxidative stress, (D) endoplasmic reticulum (ER)
30 stress, (E) unfolded protein response, (F) lysosome and (G) glycolysis and gluconeogenesis in
31 bronchial brushings of CSA (red circles), ESA (olive green circles), NSA (cyan circles) and NH
32 (purple circles) subjects. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
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41 **Figure 6. Gene Set Variation Analysis of selected stress-related pathways in bronchial**
42 **biopsies according to smoking status.**
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45 Box-and-whisker plots showing pathway enrichment of (A) xenobiotic metabolism by CYP450,
46 (B) glutathione metabolism, (C) response to oxidative stress, (D) endoplasmic reticulum (ER)
47 stress, (E) unfolded protein response, (F) lysosome and (G) glycolysis and gluconeogenesis in
48 bronchial biopsies of CSA (red circles), ESA (olive green circles), NSA (cyan circles) and NH
49 (purple circles) subjects. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
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Figure 7. Protein interaction analysis by STRING using combined DEGs.

Combined differentially expressed genes (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 pentose-phosphate pathway (orange-coloured area). These proteins function with those in charge of redox (small pink-coloured area) and connect with protein production or modification (yellow). Some proteins are associated with innate immunity (blue). The other proteins function as lysosomal (sky blue), membranous (coral red), mucus productive (apple green), Golgi homeostatic (purple) or structural proteins (olive green). Overall, this reveals the relationship between oxidative stress, ER stress, metabolism of xenobiotics and innate immunity.

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

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Supplementary Tables S1- S10

Supplementary Table S1. Gene Sets for GSA.

Gene signatures	Cell types	Gene symbols
Xenobiotic metabolism by CYP450 [#]	Human cells	ADH1A ADH1B ADH1C ADH4 ADH5 ADH6 ADH7 AKR1C1 AKR1C2 AKR1C3 AKR1C4 ALDH1A3 ALDH3A1 ALDH3B1 ALDH3B2 CYP1A1 CYP1A2 CYP1B1 CYP2B6 CYP2C18 CYP2C19 CYP2C8 CYP2C9 CYP2E1 CYP2F1 CYP2S1 CYP3A4 CYP3A43 CYP3A5 CYP3A7 DHDH EPHX1 GSTA1 GSTA2 GSTA3 GSTA4 GSTA5 GSTK1 GSTM1 GSTM2 GSTM3 GSTM4 GSTM5 GSTO1 GSTO2 GSTP1 GSTT1 GSTT2 GSTZ1 MGST1 MGST2 MGST3 UGT1A1 UGT1A10 UGT1A3 UGT1A4 UGT1A5 UGT1A6 UGT1A7 UGT1A8 UGT1A9 UGT2A1 UGT2A3 UGT2B10 UGT2B11 UGT2B15 UGT2B17 UGT2B28 UGT2B4 UGT2B7
Glutathione metabolism [#]	Human cells	ANPEP G6PD GCLC GCLM GGCT GGT1 GGT5 GGT6 GGT7 GPX1 GPX2 GPX3 GPX4 GPX5 GPX6 GPX7 GSR GSS GSTA1 GSTA2 GSTA3 GSTA4 GSTA5 GSTK1 GSTM1 GSTM2 GSTM3 GSTM4 GSTM5 GSTO1 GSTO2 GSTP1 GSTT1 GSTT2 GSTZ1 IDH1 IDH2 LAP3 MGST1 MGST2 MGST3 ODC1 OPLAH PGD RRM1 RRM2 RRM2B SMS SRM TXNDC12
Response to oxidative stress*	Human cells	ABCC2 ABL1 ADA ADAM9 ADIPOQ ADNP2 ADPRHL2 AGER AIF1 AIFM1 AKR1B1 AKR1C3 AKT1 ALAD ALDH3A2 ALDH3B1 ALS2 ANGPTL7 ANKRD2 ANXA1 APEX1 APOA4 APOD APOE APP APTX AQP1 AREG ARG1 ARL6IP5 ARNT ARNTL ATF4 ATOX1 ATP13A2 ATP2A2 ATP7A ATRN BAD BAK1 BCL2 BMP4 BNIP3 C20orf111 CA3 CASP3 CAT CBX8 CCL19 CCL5 CCNA2 CCR7 CCS CD36 CD38 CDK1 CDK2 CHD6 CHRNA4 CHUK CLN8 COL1A1 CPEB2 CRYAB CRYGD CYBA CYBB CYCS CYGB CYP1B1 CYP2E1 DDIT3 DGKK DHCR24 DHR52 DIABLO DNM2 DPEP1 DUOX1 DUOX2 DUSP1 ECT2 EDN1 EEF2 EGLN1 ENDOG EP300 EPAS1 EPX ERCC1 ERCC2 ERCC3 ERCC6 ERCC8 ERO1L ETFDH ETS1 ETV5 EZH2 F3 FABP1 FAS FER FGF8 FKBP1B FN1 FOS FOSL1 FOXO1 FOXO3 FXN G6PD GAB1 GCLC GCLM GJA3 GJB2 GLRX2 GNAO1 GPX1 GPX2 GPX3 GPX4 GPX5 GPX6 GPX7 GPX8 GSK3B GSR GSS GSTP1 GUCY1B3 HAO1 HBA1 HBA2 HBB HDAC2 HDAC6 HIF1A HMOX1 HNRNPD HP HSPA1A HSPA1B HTRA2 HYAL1 HYAL2 IDH1 IL18BP IL18RAP IL1B IL1R1 IL6 IMPACT IPCEF1 JAK2 JUN KAT2B KCNA5 KCNC2 KDM6B KLF2 KLF4 KLF6 KPNA4 KRT1 LDHA LIAS LONP1 LPO LRRK2 MAP3K5 MAPK7 MAPT MB MBL2 MDM2 MELK MGMT MGST1 MICB MMP14 MMP3 MPO MPV17 MSRA MSRB2 MSRB3 MST4 MT3 MTF1 MTR MUTYH NAPRT1 NDUFA12 NDUFA6 NDUFB4 NDUFS2 NDUFS8 NEFH NEIL1 NET1 NFE2L1 NFE2L2 NFKB1 NGFR NOS1 NOS3 NOX4 NOX5 NQO1 NR4A2 NR4A3 NUDT1 NUDT2 OGG1 OLR1 OXR1 OXSR1 P4HB PARK2 PARK7 PARP1 PAX2 PCGF2 PDCD10 PDGFD PDGFRA PDGFRB PDK1 PDK2 PDLIM1 PENK PINK1 PKD2 PLA2G4A PLA2R1 PLEKHA1 PLK3 PML PNKP PNPT1 PON2 PPARGC1A PPARGC1B PPIF PPP1R15B PPP2CB PPP5C PRDX1 PRDX2 PRDX3 PRDX5 PRDX6 PRKAA1 PRKCD PRKD1 PRKRA PRNP PRODH PSEN1 PSIP1 PSMB5 PTGS1 PTGS2 PTK2B PTPRK PTPRN PXDN PXDNL PXN PYCR1 PYCR2 RAD52 RBM11 RELA RGS14 RHOB ROMO1 RPS3 RRM2B S100A7 SCARA3 SCGB1A1 SDC1 SELK SELS SEPNI SEPP1 SEPX1 SETX SGK2 SHC1 SIN3A SIRT1 SIRT2 SLC11A2 SLC23A2 SLC25A24 SLC7A11 SLC8A1 SNCA SOD1 SOD2 SOD3 SPHK1 SRC SRXN1 STAR STAT6 STAU1 STC2 STK24 STK25 STX2 STX4 TACR1 TAT TMEM161A TNFAIP3 TOR1A TP53 TP53INP1 TPM1 TPO TRAF2 TRPA1 TRPC6 TRPM2 TXN TXN2 TXNDC2 TXNDC3 TXNDC8 TXNIP TXNL1 TXNRD1 TXNRD2 UCN UCP2 UCP3 VKORC1L1 VNN1 VRK2 WNT16 WRN XBP1 XPA ZC3H12A ZNF277 ZNF580 ZNF622
Endoplasmic reticulum stress [1]	Mouse embryo fibroblasts	WFS1 ERO1A CLCN3 ACAT2 CHKA DDIT3 ETS2 RBM38 TCEA1 IFI16 PPP1R15A POLR1A PPAN CA6 MTM1 CDCA7 PTX3 STBD1 ADH7 RAD1 LONP1 AMPD3 IFRD2 PARD6A KITLG PTRH2
Unfolded protein response ^s	Human cells	ACADVL ADD1 ARFGAP1 ASNS ATF3 ATF4 ATF4P3 ATF6 ATP6V0D1 C19orf10 CALR CCL2 CTDSP2 CUL7 CXXC1 DCP2 DCTN1 DDIT3 DDX11 DDX12P DIS3 DNAJB11 DNAJB9 DNAJC3 EDEM1 EIF2AK3 EIF2S1 ERN1 EXOSC1 EXOSC2 EXOSC3 EXOSC4 EXOSC5 EXOSC6 EXOSC7 EXOSC8 EXOSC9 EXTL3 FKBP14 GOSR2 GSK3A HDGF HERPUD1 HSP90B1 HSPA5 HYOU1 IGFBP1 IL8 KDELR3 KHSRP KLHDC3 LMNA LOC730136 MBTPS1 MBTPS2 NFYA NFYB PARN PDIA5 PDIA6 PPP2R5B PREB SEC31A SERP1 SHC1 SRPR SRPRB SSR1 SULT1A3 SULT1A4 SYVN1 TATDN2 TLN1 TPP1 TSPYL2 WFS1 WIP1 XBP1 YIF1A ZBTB17
Lysosome [#]	Human cells	ABCA2 ABCB9 ACP2 ACP5 AGA AP1B1 AP1G1 AP1M1 AP1M2 AP1S1 AP1S2 AP1S3 AP3B1 AP3B2 AP3D1 AP3M1 AP3M2 AP3S1 AP3S2 AP4B1

		AP4E1 AP4M1 AP4S1 ARSA ARSB ARSG ASAHI ATP6AP1 ATP6V0A1 ATP6V0A2 ATP6V0A4 ATP6V0B ATP6V0C ATP6V0D1 ATP6V0D2 ATP6V1H CD164 CD63 CD68 CLN3 CLN5 CLTA CLTB CLTC CLTCL1 CTNS CTSA CTSB CTSC CTSD CTSE CTSF CTSG CTSH CTSK CTSL1 CTSL2 CTSO CTSS CTSW CTSZ DNASE2 DNASE2B ENTPD4 FUCA1 GAA GALC GALNS GBA GGA1 GGA2 GGA3 GLA GLB1 GM2A GNPTAB GNPTG GNS GUSB HEXA HEXB HGSNAT HYAL1 IDS IDUA IGF2R LAMP1 LAMP2 LAMP3 LAPTM4A LAPTM4B LAPTM5 LGMN LIPA M6PR MAN2B1 MANBA MCOLN1 MFSD8 NAGA NAGLU NAGPA NAPSA NEU1 NPC1 NPC2 PLA2G15 PPT1 PPT2 PSAP PSAPL1 SCARB2 SGSH SLC11A1 SLC11A2 SLC17A5 SMPD1 SORT1 SUMF1 TCIRG1 TPP1
Glycolysis and gluconeogenesis [#]	Human cells	ACSS1 ACSS2 ADH1A ADH1B ADH1C ADH4 ADH5 ADH6 ADH7 AKR1A1 ALDH1A3 ALDH1B1 ALDH2 ALDH3A1 ALDH3A2 ALDH3B1 ALDH3B2 ALDH7A1 ALDH9A1 ALDOA ALDOB ALDOC BPGM DLAT DLD ENO1 ENO2 ENO3 FBP1 FBP2 G6PC G6PC2 GALM GAPDH GCK GPI HK1 HK2 HK3 LDHA LDHAL6A LDHAL6B LDHB LDHC PCK1 PCK2 PDHA1 PDHA2 PDHB PFKL PFKM PFKP PGAM1 PGAM2 PGAM4 PGK1 PGK2 PGM1 PGM2 PKLR PKM2 TPI1
Current-smoking [2]	Human bronchial epithelial cells	GMDS ZNF323 GALNT12 AP2B1 HN1 GMDS ABCC1 RAB11A MSMB MAFG ABHD2 ANXA3 VND2 FTH1 UGT1A3 TSPAN-1 CTGF PGD HTATIP2 CYP4F11 GCLM ADH7 GCLC UPK1B PLEKHB2 TCN1 TRIM16 UGT1A9 UGT1A1 UGT1A6 HDO1 TXNRD1 PRDX1 ME1 PIR TALDO1 GPX2 HDO1 HDO1 AKR1C3 AKR1C1 AKR1C-pseudo AKR1C2 ALDH3A1 CLDN10 TXN TKT CYP1B1 AKR1C1 CBR1 AKR1B1 KLF4 NET-6 NUDT4 GALNT3 GALNT7 CEACAM6 AP1G1 CA12 FLJ20151 BCL2L13 SRPUL FLJ13052 GALNT6 OASIS MUC5B MUC5B S100P NUDT4 ME1 SDR1 PLA2G10 DPYSL3
Smoking index-related [2]	Human bronchial epithelial cells	CST6 EIF2C3 BRD2 HBP17 PCDHGC3 MGC13053 C22orf3 SFN TCF20 C9orf7 EPS8L1 BDKRB2 FAXDC1 ARIH2 EPS8L1 FLJ10404 PRG1 RPN2 GMPPA TCIRG1 BAIAP2 S100A8 DKFZp564B0769 TRAP95 SRRM2 MUC5AC MYL6 PURA BAIAP3 ARFGAP1 FLJ10849 PCDHGC3 LMNA MGC14376 LYPLA2 KIAA0992 C11orf13 CPT1B EPHA2 MUC5AC QSCN6 RPA1 PPAP2B KIAA0971 ZNF258

Curated gene sets from [#]KEGG, ^{*}GO or [§]Reactome in MSigDB.

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Supplementary Table S2. GO enrichment analysis for biological process using differentially expressed proteins in ESA or NSA.

GO biological process	Fold enrichment	P value
<i>ESA-NH</i>		
Regulation of phagocytosis	21.11	3.71E-02
Response to hydrogen peroxide	15.45	2.33E-02
Response to monosaccharide	13.38	8.55E-03
Regulation of chemotaxis	12.8	1.93E-03
Protein autophosphorylation	10.8	3.47E-02
Myeloid cell differentiation	10.75	3.59E-02
Leukocyte migration	8.87	2.90E-02
Positive regulation of cell migration	8.15	8.49E-04
Response to bacterium	7.26	1.98E-04
Leukocyte activation	7.02	1.31E-02
Inflammatory response	6.94	4.18E-03
Response to hormone	6.83	1.73E-07
Activation of immune response	6.71	1.95E-02
Response to wounding	6.27	3.50E-03
Positive regulation of kinase activity	6.07	4.71E-02
Immune effector process	6.07	4.71E-02
Positive regulation of cell proliferation	5.93	2.03E-05
Tube development	5.78	2.41E-02
Regulation of cytokine production	5.72	2.66E-02
Regulation of proteolysis	5.55	4.29E-03
Regulation of protein kinase activity	5.38	6.02E-03
Innate immune response	5.38	4.83E-02
Response to cytokine	5.26	7.77E-03
Positive regulation of transport	4.78	3.30E-03
Positive regulation of protein phosphorylation	4.72	1.01E-02
Negative regulation of catalytic activity	4.66	2.94E-02
Positive regulation of intracellular signal transduction	4.63	1.28E-02
Positive regulation of cell differentiation	4.52	4.08E-02
Negative regulation of protein metabolic process	4.38	1.01E-02
Negative regulation of multicellular organismal process	4.17	4.25E-02
Positive regulation of multicellular organismal process	3.75	6.50E-03
Cellular response to organic substance	3.74	5.00E-05
Cell adhesion	3.74	3.22E-02
Cell surface receptor signaling pathway	3.62	1.85E-05
Regulation of multicellular organismal development	3.5	4.11E-03
Negative regulation of cellular process	2.73	1.51E-06
Regulation of biological quality	2.56	1.92E-03
<i>NSA-NH</i>		
Acute-phase response	36.41	4.09E-02
Platelet degranulation	20.61	3.91E-02
Response to carbohydrate	13.94	3.83E-02
Response to wounding	7.51	5.59E-03
Cell adhesion	4.7	6.65E-03
Immune system process	4.46	8.67E-06
Positive regulation of response to stimulus	3.52	3.99E-02
Regulation of biological quality	2.82	5.93E-03

GO: Gene Ontology.

Supplementary Table S3. Patient characteristics in transcriptomic analyses (bronchial brushings)

	Severe asthma			Healthy	P value
	Current-smoker (CSA) (n=6)	Ex-smoker (ESA) (n=12)	Non-smoker (NSA) (n=40)	Non-smoker (NH) (n=37)	
Female	4 (66.7)	3 (25.0)	22 (55.0)	13 (35.1)	1.05E-01*
Age (y)	49.7±7.6	53.0±8.8	48.6±13.9	36.2±13.9 [¶]	2.95E-04 [§]
Onset age of asthma (y)	27.3±20.0	29.8±17.1	22.1±19.9	N.R.	5.38E-01 [§]
Age at starting smoking (y)	16.5±3.7	16.2±3.7	N.R.	N.R.	9.61E-01 [†]
Years of smoking cessation (y)	N.R.	17.7±9.5	N.R.	N.R.	
Smoking pack-year	22.4±8.6	29.9±23.1	0.0±0.0	0.0±0.0	8.51E-01 [†]
BMI (kg/m ²)	22.4±2.4 [¶]	32.5±4.4	31.5±6.3	24.5±3.0 [¶]	9.99E-09
Atopic (%)	2 (66.7) [3]	10 (83.3)	28 (71.8) [1]	12 (40.0) [7] [¶]	1.44E-02 *
Blood eosinophil (*μL)	178±68	308±321	298±246	(158±153)	5.18E-01 ^{§#}
Blood neutrophil (*10 ³ /μL)	8.14±4.02 [¶]	6.19±2.46	4.45±1.61	(3.34±2.13)	8.24E-03 ^{§#}
Sputum eosinophil (%)	0.87±1.03 [3]	17.0±20.2 [8]	12.6±17.2 [24]	(0.33±0.50) [16]	4.44E-01 ^{§#}
Sputum neutrophil (%)	61.8±26.0 [3]	39.6±18.1 [8]	51.4±17.9 [24]	(35.0±26.7) [16]	3.39E-01 ^{§#}
IgE (IU/mL)	184±198 [1]	807±1851	300±456	(60.4±99.7) [2]	7.49E-01 ^{§#}
FeNO (ppb)	14.1±13.5 [¶]	35.1±27.4 [1]	41.0±30.1 [4]	(21.9±15.5) [2]	1.12E-02 ^{§#}
Periostin (ng/mL)	44.1±6.7 [3]	47.9±14.8 [1]	51.4±13.9 [5]	(51.9±10.0) [5]	5.18E-01 ^{§#}
%FEV1 post-bronchodilator (%) ^{##}	76.8±7.3	70.7±19.1	84.6±21.4	(100.8±12.7)	9.33E-02 ^{§#}
FEV1/FVC post-bronchodilator (%) ^{##}	61.4±6.5	62.2±12.7	69.8±12.1	(79.4±6.1)	5.94E-02 ^{§#}
Airway reversibility (%)	16.8±7.1	11.4±11.4	12.5±16.2	N.R.	8.07E-02 [§]
Airflow limitation [92]	5 (83.3)	8 (66.7)	15 (37.5)	N.R.	5.30E-02 [§]
Average ACQ-7	3.12±0.79 [1]	2.27±1.29 [4]	2.19±1.16 [9]	N.R.	2.06E-01 [§]
Average AQLQ	4.25±1.19	5.01±1.47 [5]	4.98±0.98 [6]	N.R.	2.97E-01 [§]
Exacerbation in previous year (n/y)	2.6±3.3	2.1±1.9	2.4±1.9	N.R.	3.80E-01 [§]
ER visit due to breathing problems	2 (33.3)	7 (58.3)	25 (62.5)	N.R.	3.80E-01*
Comorbidities					
Allergic rhinitis (%)	1 (16.7)	5 (41.7)	21 (60.0) [5]	N.R.	1.14E-01*
Nasal polyp (%)	1 (16.7)	6 (54.5) [1]	12 (34.3) [5]	N.R.	3.05E-01*
Sinusitis (%)	3 (50.0)	2 (16.7)	11 (30.6) [4]	N.R.	3.44E-01*
Chronic bronchitis (%)	0 (0.0) [1]	4 (33.3)	6 (15.0)	N.R.	2.54E-01*
Psychiatric disease (%)	3 (50.0)	3 (25.0)	6 (16.2) [3]	N.R.	1.52E-01*
GERD (%)	4 (80.0) [1]	7 (58.3)	21 (56.8) [3]	N.R.	7.51E-01*
Medications					
Inhaled corticosteroids (%)	6 (100.0)	12 (100.0)	40 (100.0) [5]	N.R.	1.00E+00*
Systemic corticosteroids (%)	1 (16.7)	8 (72.7) [1]	13 (35.1) [3]	N.R.	4.41E-02 *
Oral corticosteroid dose (mg/day)	1.67±4.08	14.0±17.6 [2]	3.81±7.00 [4]	N.R.	4.17E-02 [§]
Anti-IgE therapy (%)	0 (0.0)	1 (12.5) [4]	2 (5.7) [5]	N.R.	6.45E-01*
Long-acting beta agonist (%)	6 (100.0)	12 (100.0)	40 (100.0)	N.R.	1.00E+00*
Leukotriene modifiers (%)	2 (33.3)	6 (60.0) [2]	22 (59.5) [3]	N.R.	5.67E-01*
Tiotropium (%)	2 (33.3)	3 (42.9) [5]	4 (11.4) [5]	N.R.	5.72E-02*
Macrolide (%)	1 (16.7)	1 (8.3)	5 (12.5)	N.R.	1.00E+00*

Data are presented as mean±SD or n (%). The numbers of missing values are shown in square brackets. ^{##}Spirometry data without bronchodilator were used for healthy subjects. Statistical analysis was performed by Fisher's exact test*, Kruskal-Wallis test[§] or Mann-Whitney U test[†]. [#]Healthy subjects were excluded from statistical analyses of several items. Variables with P values in bold were significantly different (<0.05). [¶]p<5.00E-02 vs CSA, [‡]p<5.00E-02 vs ESA, [§]p<5.00E-02 vs NSA. BMI: body mass index, IgE: immunoglobulin E, FeNO: fractional exhaled nitric oxide, FEV: forced expiratory volume, FVC: forced vital capacity, ERS: European Respiratory Society, ACQ: Asthma Control Questionnaire, AQLQ: Asthma Quality of Life Questionnaire, ER: emergency room, GERD: gastroesophageal reflux disease, N.R.: not relevant.

Supplementary Table S4. Patient characteristics in transcriptomic analyses (bronchial biopsies)

	Severe asthma			Healthy	P value
	Current-smoker (CSA) (n=7)	Ex-smoker (ESA) (n=6)	Non-smoker (NSA) (n=34)	Non-smoker (NH) (n=22)	
Female	5 (71.4)	0 (0.0) ^{¶¶}	21 (61.8)	9 (40.9)	1.55E-02*
Age (y)	50.0±7.0	58.5±7.4	49.8±13.1	39.6±15.9	2.11E-02
Onset age of asthma (y)	29.9±19.5	28.5±21.6 [1]	21.8±20.6	N.R.	6.56E-01 [§]
Age at starting smoking (y)	17.0±3.7	18.0±4.1	N.R.	N.R.	7.72E-01 [†]
Years of smoking cessation (y)	N.R.	21.3±11.4	N.R.	N.R.	
Smoking pack-year	22.6±7.9	29.8±18.6	0.0±0.0	0.0±0.0	4.74E-01 [†]
BMI (kg/m ²)	23.6±3.7	33.2±4.8	30.9±5.8	24.5±2.9	3.14E-06 [§]
Atopic (%)	2 (50.0) [3]	5 (83.3)	24 (72.7) [1]	9 (42.9) [1]	9.45E-02 *
Blood eosinophil (*μL)	210±104	457±404	278±229	(157±184)	5.06E-01 ^{§#}
Blood neutrophil (*10 ³ /μL)	7.92±3.71 [¶]	5.37±2.45	4.65±1.56	(3.70±2.57)	4.80E-02 ^{§#}
Sputum eosinophil (%)	1.99±2.40 [3]	N.D. [6]	11.7±16.3 [16]	(0.43±0.53) [9]	2.49E-01 ^{†#}
Sputum neutrophil (%)	46.7±36.9 [3]	N.D. [6]	51.3±16.1 [16]	(32.8±28.0) [9]	9.32E-01 ^{†#}
IgE (IU/mL)	153±192 [1]	1418±2567	288±385	(61.8±88.8)	4.99E-01 ^{§#}
FeNO (ppb)	16.5±13.9 [¶]	32.5±29.8	38.8±29.5 [5]	(27.2±18.5) [3]	3.22E-02 ^{§#}
Periostin (ng/mL)	42.1±6.7 [3]	51.4±16.6 [1]	49.9±12.9 [4]	(55.0±11.6) [5]	4.05E-01 ^{§#}
%FEV1 post-bronchodilator (%) ^{##}	76.7±6.6	73.9±22.6	82.4±21.1	(98.6±13.8)	4.77E-01 ^{§#}
FEV1/FVC post-bronchodilator (%) ^{##}	61.4±6.0	61.8±15.5	67.4±12.7	(80.0±6.2)	3.02E-01 ^{§#}
Airway reversibility (%)	21.9±15.0	7.0±14.8	14.2±16.7	N.R.	8.32E-02 [§]
Airflow limitation [92]	6 (85.7)	4 (66.7)	15 (44.1)	N.R.	1.03E-01 *
Average ACQ-7	3.12±0.79 [2]	2.37±1.54 [1]	2.17±1.21 [5]	N.R.	2.28E-01 [§]
Average AQLQ	4.25±1.19 [1]	4.48±1.36 [1]	5.02±1.06 [3]	N.R.	2.32E-01 [§]
Exacerbation in previous year (n/y)	4.1±3.1	2.0±1.7	2.3±2.2 [1]	N.R.	2.26E-01 [§]
ER visit due to breathing problems	3 (42.9)	3 (50.0)	20 (58.8)	N.R.	8.04E-01 *
Comorbidities					
Allergic rhinitis (%)	2 (28.6)	2 (33.3)	19 (61.3) [3]	N.R.	2.29E-01 *
Nasal polyp (%)	1 (14.3)	4 (66.7)	10 (31.3) [2]	N.R.	1.51E-01 *
Sinusitis (%)	4 (57.1)	0 (0.0)	9 (28.1) [2]	N.R.	6.12E-02 *
Chronic bronchitis (%)	0 (0.0) [1]	2 (33.3)	5 (14.7)	N.R.	4.11E-01 *
Psychiatric disease (%)	3 (42.9)	1 (16.7)	6 (18.2) [1]	N.R.	3.92E-01 *
GERD (%)	4 (66.7) [1]	4 (66.7)	16 (48.5) [1]	N.R.	7.05E-01 *
Medications					
Inhaled corticosteroids (%)	7 (100.0)	6 (100.0)	34 (100.0)	N.R.	1.00E+00 *
Systemic corticosteroids (%)	2 (28.6)	3 (50.0)	14 (43.8) [2]	N.R.	7.99E-01 *
Oral corticosteroid dose (mg/day)	2.86±4.88	3.00±4.47 [1]	4.98±7.82 [3]	N.R.	8.47E-01 [§]
Anti-IgE therapy (%)	0 (0.0) [1]	0 (0.0) [1]	2 (6.5) [3]	N.R.	1.00E+00 *
Long-acting beta agonist (%)	7 (100.0)	6 (100.0)	34 (100.0)	N.R.	1.00E+00 *
Leukotriene modifiers (%)	3 (42.9)	4 (66.7)	18 (56.3) [2]	N.R.	8.02E-01 *
Tiotropium (%)	2 (33.3) [1]	2 (40.0) [1]	7 (22.6) [3]	N.R.	6.10E-01 *
Macrolide (%)	1 (14.3)	0 (0.0)	5 (14.7)	N.R.	1.00E+00 *

Data are presented as mean±SD or n (%). The numbers of missing values are shown in square brackets. ^{##}Spirometry data without bronchodilator were used for healthy subjects. Statistical analysis was performed by Fisher's exact test*, Kruskal-Wallis test[§] or Mann-Whitney U test[†]. [#]Healthy subjects were excluded from statistical analyses of several items. Variables with P values in bold were significantly different (<0.05). [¶]p<5.00E-02 vs CSA, [‡]p<5.00E-02 vs ESA, [§]p<5.00E-02 vs NSA. BMI: body mass index, IgE: immunoglobulin E, FeNO: fractional exhaled nitric oxide, FEV: forced expiratory volume, FVC: forced vital capacity, ERS: European Respiratory Society, ACQ: Asthma Control Questionnaire, AQLQ: Asthma Quality of Life Questionnaire, ER: emergency room, GERD: gastroesophageal reflux disease, **N.R.:** not relevant.

Supplementary Table S5. Patient characteristics in transcriptomic analyses (sputa)

	Severe asthma			Healthy	P value
	Current-smoker (CSA) (n=8)	Ex-smoker (ESA) (n=15)	Non-smoker (NSA) (n=47)	Non-smoker (NH) (n=15)	
Female	4 (50.0)	8 (53.3)	28 (59.6)	3 (20.0) [¶]	6.15E-02*
Age (y)	48.6±11.9	54.8±10.5	52.3±12.7	37.5±13.6	2.56E-03 [§]
Onset age of asthma (y)	25.6±20.7	40.7±18.7 [¶]	21.9±18.6	N.R.	8.97E-03 [§]
Age at starting smoking (y)	18.8±4.4	15.9±3.1	N.R.	N.R.	2.29E-01
Years of smoking cessation (y)	N.R.	13.6±8.8	N.R.	N.R.	
Smoking pack-year	19.6±9.6	23.1±18.8	0.0±0.0	0.0±0.0	9.49E-01
BMI (kg/m ²)	26.7±4.0	30.8±4.9	27.6±5.5	25.8±2.9	4.82E-02 [§]
Atopic (%)	5 (83.3) [2]	8 (72.7) [4]	36 (85.7) [5]	4 (44.4) [6]	4.96E-02 *
Blood eosinophil (*10 ³ /μL)	294±157	303±226	393±339 [2]	(113±74)	6.17E-01 ^{§#}
Blood neutrophil (*10 ³ /μL)	6.31±2.66	5.92±2.38	5.10±2.31 [2]	(3.66±1.40)	2.28E-01 ^{§#}
Sputum eosinophil (%)	9.4±17.5	14.6±15.0	14.4±22.3	(0.13±0.26)	2.70E-01 ^{§#}
Sputum neutrophil (%)	53.8±19.3	57.1±20.4	60.3±28.5	(45.0±25.6)	6.19E-01 ^{§#}
IgE (IU/mL)	238±202 [2]	324±547	266±471 [3]	(119±178)	5.47E-01 ^{§#}
FeNO (ppb)	18.3±18.9 [¶]	42.9±31.2 [1]	40.8±35.1 [2]	(20.1±10.2) [2]	1.41E-02 ^{§#}
Periostin (ng/mL)	45.4±10.7 [1]	54.6±20.4 [4]	53.2±19.5 [8]	(47.4±5.6) [2]	6.85E-01 ^{§#}
%FEV1 post-bronchodilator (%) ^{##}	81.6±12.6	75.0±22.8	66.7±22.2	(106.4±10.4)	1.08E-01 ^{§#}
FEV1/FVC post-bronchodilator (%) ^{##}	63.2±7.9	59.4±11.5	58.1±14.3	(78.5±6.0)	6.57E-01 ^{§#}
Airway reversibility (%)	16.6±9.8	17.0±14.2 [1]	17.2±18.5	N.R.	8.46E-01 [§]
Airflow limitation [92]	5 (62.5)	10 (66.7)	36 (76.6)	N.R.	5.46E-01*
Average ACQ-7	2.53±1.42 [1]	2.36±0.92 [2]	2.68±1.40 [3]	N.R.	7.24E-01 [§]
Average AQLQ	4.51±1.65 [1]	5.05±1.07 [3]	4.56±1.23 [2]	N.R.	4.31E-01 [§]
Exacerbation in previous year (n/y)	2.9±3.4	2.1±2.1	2.2±1.9	N.R.	8.99E-01 [§]
ER visit due to breathing problems	3 (37.5)	9 (60.0)	30 (63.8)	N.R.	3.95E-01*
Comorbidities					
Allergic rhinitis (%)	2 (28.6) [1]	5 (35.7) [1]	19 (48.7) [8]	N.R.	5.36E-01*
Nasal polyp (%)	2 (28.6) [1]	6 (40.0)	16 (35.6) [2]	N.R.	9.29E-01*
Sinusitis (%)	2 (33.3) [2]	5 (33.3)	11 (25.0) [3]	N.R.	6.81E-01*
Chronic bronchitis (%)	0 (0.0) [2]	2 (13.3)	8 (18.2) [3]	N.R.	1.94E-01*
Psychiatric disease (%)	2 (28.6) [1]	2 (13.3)	6 (13.0) [1]	N.R.	4.90E-01*
GERD (%)	4 (80.0) [2]	9 (60.0)	17 (37.8) [2]	N.R.	2.03E-01*
Medications					
Inhaled corticosteroids (%)	8 (100.0)	15 (100.0)	47 (100.0)	N.R.	1.00E+00*
Systemic corticosteroids (%)	3 (42.9) [1]	10 (71.4) [1]	20 (43.5) [1]	N.R.	2.17E-01*
Oral corticosteroid dose (mg/day)	3.57±5.37 [1]	8.75±7.83 [1]	4.06±6.25 [2]	N.R.	6.29E-02 [§]
Anti-IgE therapy (%)	0 (0.0) [1]	1 (7.7) [2]	1 (2.2) [2]	N.R.	5.24E-01*
Long-acting beta agonist (%)	8 (100.0)	14 (93.3)	47 (100.0)	N.R.	3.29E-01*
Leukotriene modifiers (%)	4 (50.0)	7 (50.0) [1]	23 (48.9)	N.R.	1.00E+00*
Tiotropium (%)	3 (42.9) [1]	2 (15.4) [2]	14 (31.1) [2]	N.R.	3.89E-01*
Macrolide (%)	2 (25.0)	2 (13.3)	6 (12.8)	N.R.	5.90E-01*

Data are presented as mean±SD or n (%). The numbers of missing values are shown in square brackets. ^{##}Spirometry data without bronchodilator were used for healthy subjects. Statistical analysis was performed by Fisher's exact test*, Kruskal-Wallis test[§] or Mann-Whitney U test[†]. [#]Healthy subjects were excluded from statistical analyses of several items. Variables with P values in bold were significantly different (<0.05). [¶]p<5.00E-02 vs CSA, [‡]p<5.00E-02 vs ESA, [§]p<5.00E-02 vs NSA. BMI: body mass index, IgE: immunoglobulin E, FeNO: fractional exhaled nitric oxide, FEV: forced expiratory volume, FVC: forced vital capacity, ERS: European Respiratory Society, ACQ: Asthma Control Questionnaire, AQLQ: Asthma Quality of Life Questionnaire, ER: emergency room, GERD: gastroesophageal reflux disease, N.R.: not relevant.

Supplementary Table S6. GO enrichment analysis for biological process using differentially expressed genes from bronchial brushings in comparison between CSA and NSA.

GO biological process	Fold enrichment	P value
Pentose biosynthetic process	> 100	8.37E-03
Cellular response to jasmonic acid stimulus	> 100	8.37E-03
Doxorubicin metabolic process	> 100	4.19E-06
Daunorubicin metabolic process	> 100	4.19E-06
Primary alcohol catabolic process	92.66	4.44E-02
Cellular aldehyde metabolic process	32.03	1.89E-06
Retinoid metabolic process	19.43	9.02E-05
Cellular hormone metabolic process	14.55	5.32E-03
Response to endoplasmic reticulum stress	8.07	1.67E-02
Oxidation-reduction process	4.74	1.99E-05

GO: Gene Ontology.

Supplementary Table 7. Functional clustering of annotated probes by DAVID using differentially expressed gene probes from bronchial brushings in comparison between CSA and NSA.

DAVID Function cluster	Enrichment score
Endoplasmic reticulum	5.71
Cytoplasmic membrane-bounded vesicle	4.37
Oxidation reduction	4.18
Signal peptide	3.51
Redox	2.56
Xenobiotic metabolic process	2.55
Terpenoid metabolic process	2.34
Hormone metabolic process	2.16
NADP and nicotinamide metabolic process	1.96
Glycosylation	1.84
Lysosome	1.37

DAVID: Database for Annotation, Visualization and Integrated Discovery. NADP: nicotinamide adenine dinucleotide phosphate. Only significant clusters (enrichment score > 1.3) are shown.

Supplementary Table S8. Critical genes whose absolute fold change was more than or equal to 2.0 in limma in comparison between ESA and NSA.

Probe ID	Gene symbol	Gene name	Function
Sputum			
215101_s_at	CXCL5	C-X-C motif chemokine ligand 5	The encoded protein is proposed to bind the G-protein coupled receptor chemokine (C-X-C motif) receptor 2 to recruit neutrophils, to promote angiogenesis and to remodel connective tissues.
204580_at	MMP12	matrix metalloproteinase 12	The encoded protease degrades soluble and insoluble elastin. The mutations in this gene are associated with lung function and COPD.
212298_at	NRP1	neuropilin 1	Neuropilins affect cell survival, migration, and attraction. Some of the ligands and co-receptors bound by neuropilins are VEGF and semaphorin family members. This encoded protein plays versatile roles in angiogenesis, axon guidance, cell survival, migration, and invasion.
214146_s_at	PPBP	pro-platelet basic protein	This platelet-derived growth factor is a potent chemoattractant and activator of neutrophils.
1554091_a_at	TIRAP	Toll-interleukin 1 receptor domain containing adaptor protein	The protein encoded by this gene is a Toll-interleukin 1 receptor adaptor protein involved in the TLR4 signaling pathway of the immune system. It activates NF- κ B, MAPK1, MAPK3 and JNK, which then results in cytokine secretion and the inflammatory response.
Bronchial Brushings			
205624_at	CPA3	carboxypeptidase A3	The encoded preproprotein is proteolytically processed to generate a mature protease that is released by mast cells and may be involved in the degradation of endogenous proteins and the inactivation of venom-associated peptides.
206224_at	CST1	cystatin SN	This protein is a member of type 2 cystatin proteins that are a class of cysteine proteinase inhibitors found in a variety of human fluids and secretions, where they appear to provide protective functions. This gene is located in the cystatin locus and encodes a cysteine proteinase inhibitor found in saliva, tears, urine, and seminal fluid.
224795_x_at 221651_x_at	IGKC	immunoglobulin kappa constant	This is a human gene that encodes the constant domain of kappa-type light chains for antibodies. It is associated with humoral immunity to a variety of self and non-self antigens, and it is also a risk factor for several immune-mediated diseases, including some cancers.
204673_at	MUC2	mucin-2	This protein coats the epithelia of the intestines, airways, and other mucus membrane-containing organs. It is thought to provide a protective, lubricating barrier against particles and infectious agents at mucosal surfaces.
216474_x_at	TPSAB1	tryptase alpha/beta 1	This gene encodes tryptase alpha-1 and tryptase beta-1, that have been implicated as mediators in the pathogenesis of asthma and other allergic and inflammatory disorders. Beta tryptases appear to be the main isoenzymes expressed in mast cells; whereas in basophils, alpha tryptases predominate.
Biopsies			
229152_at	FDCSP	follicular dendritic cell secreted protein	This protein secreted from follicular dendritic cells specifically binds to activated B cells, and functions as a regulator of antibody responses.
215214_at	IGLC1	immunoglobulin lambda constant 1	This encoded protein is the constant domain of lambda-type light chains for antibodies. It is associated with humoral immunity to a variety of self and non-self antigens.
234764_x_at	IGLV@	immunoglobulin lambda variable cluster	This gene encodes the variable region protein of lambda-type light chains for antibodies. It is associated with humoral immunity to a variety of self and non-self antigens.
210809_s_at 1555778_a_at	POSTN	periostin	The encoded protein is a secreted extracellular matrix protein which functions in tissue development and regeneration through IL-4/13 signalling.
214146_s_at	PPBP	pro-platelet basic protein	This platelet-derived growth factor is a potent chemoattractant and activator of neutrophils.

COPD: chronic obstructive pulmonary disease, VEGF: vascular endothelial growth factor, TLR: Toll-like receptor, NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells, MAPK: mitogen-activated protein kinase, JNK: Jun N-terminal kinase.

Supplementary Table S9. GO enrichment analysis for biological process using current-smoking signature gene set [2].

GO biological process	Fold enrichment	P value
Pentose biosynthetic process	> 100	7.34E-04
Cellular response to thyroxine stimulus	> 100	1.34E-02
Cellular response to jasmonic acid stimulus	> 100	7.18E-04
Extracellular matrix constituent secretion	> 100	1.91E-02
NADP biosynthetic process	> 100	2.58E-02
Doxorubicin metabolic process	> 100	1.08E-04
Daunorubicin metabolic process	> 100	1.02E-04
Xenobiotic glucuronidation	> 100	1.10E-04
Flavonoid glucuronidation	> 100	1.05E-04
Response to prostaglandin D	99.02	3.32E-02
Pentose-phosphate shunt, non-oxidative branch	82.52	4.05E-02
Vitamin K metabolic process	82.52	4.00E-02
Cobalamin transport	70.73	4.88E-02
Cellular response to follicle-stimulating hormone stimulus	61.89	5.50E-03
Retinal metabolic process	57.13	6.42E-03
Progesterone metabolic process	53.05	7.24E-03
Regulation of cell migration involved in sprouting angiogenesis	32.29	1.87E-02
Prostaglandin metabolic process	25.61	2.99E-02
Arachidonic acid metabolic process	22.50	1.13E-03
O-glycan processing	20.98	1.51E-03
Cell redox homeostasis	13.75	2.67E-02
Positive regulation of JAK-STAT cascade	13.03	3.10E-02
Cellular response to oxidative stress	10.95	5.84E-05
Response to corticosteroid	9.46	8.40E-03
Oxidation-reduction process	4.70	5.69E-05
Response to growth factor	4.48	2.23E-02
Response to wounding	4.00	4.06E-02
Chemical homeostasis	3.07	4.75E-02
Regulation of apoptotic process	2.81	1.36E-02

GO: Gene Ontology. JAK: Janus kinase. STAT: Signal Transducers and Activator of Transcription.

Supplementary Table S10. Functional clustering of annotated probes by DAVID using current-smoking signature gene set [2].

DAVID Function cluster	Enrichment score
Oxidation reduction	4.86
Metallothionein	3.93
Metabolism of xenobiotics by cytochrome P450	3.89
CTCK domain	2.74
Glycosyltransferase	2.54
Response to reactive oxygen species	1.92
Calcium ion binding	1.69
Pentose-phosphate shunt	1.57
Extracellular space	1.44

DAVID: Database for Annotation, Visualization and Integrated Discovery. CTCK: C-terminal cystine knot-like. Only significant clusters (enrichment score > 1.3) are shown.

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

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16 Lyon, France

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18 #Consortium project team members are listed under Acknowledgements
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1. Inclusion criteria of severe asthma patients:

Participants with asthma had either airflow reversibility (increase in forced expiratory volume in 1s (FEV₁) >12% predicted and 200 mL following inhalation of 400 µg salbutamol), airway hyperresponsiveness (methacholine provocative concentration causing a 20% fall in FEV₁ <8 mg/mL, or diurnal peak expiratory flow amplitude >8% of mean), or a decrease in FEV₁ of 12% predicted or 200 mL within 4 weeks after tapering maintenance treatment. Severe asthma was defined as asthma with uncontrolled symptoms according to Global Initiative for Asthma (GINA) guidelines and/or frequent exacerbations (more than two per year) despite high-dose inhaled corticosteroids (≥1000 µg fluticasone propionate per day or equivalent dose) plus additional controller therapy.

2. Bronchoscopy exclusion criteria

Bronchoscopy exclusion criteria included the subjects who had a severe exacerbation of asthma requiring the new administration of oral corticosteroids or a doubling of their usual oral corticosteroid dose within the last three months, a ≥10% decrease in forced expiratory volume in 1s (FEV₁) after inhalation of 0.9% saline during sputum induction, increase use of rescue short acting β-agonist by six or more puffs/day over and above usual use during the last three days, severe bronchoconstriction or other adverse reactions at previous attempts to perform bronchoscopy, a post-bronchodilator FEV₁<40% predicted, a transcutaneous oxygen saturation by pulse-oximetry of <90%, a heart rate >130 beats/minutes to pre-medication, known serious ventricular heart rhythm disturbances or treatment with warfarin or other anticoagulants.

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5 02-Jan-2018

6 ERJ-02173-2017
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9 Dear Prof. Chung:
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12 Your manuscript entitled "Distinct pathways underlying neutrophilic inflammation in
13 smoking-associated severe asthma in U-BIOPRED" has been evaluated by anonymous
14 reviewers and the editors.
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17 Based on the reviewers' recommendations, our own views, and the editorial standards
18 used by the editorial board, we have to inform you that your manuscript cannot be
19 accepted in its present form. However, we will reconsider this decision if you are
20 prepared to submit an adequately revised manuscript.
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24 The main points of criticism can be summarised as follows:
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27 Main issues summarised by the associate and chief editor:
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30 Comments to Author:
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32 There are some critical issues that need to be fixed and satisfied entirely to get this
33 paper acceptable. See detailed reviews below.
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35

36 **RESPONSE:**

37 **We thank the Editors and the Reviewers for their comments. We are grateful to them**
38 **for allowing us to submit a response and a revised manuscript. We are aware of some**
39 **mistakes in the Tables which we have corrected. We are also aware of the limitations of**
40 **our conclusions given the number of subjects who were actively smoking that were**
41 **recruited into U-BIOPRED. We believe that we have responded to all the reviewers'**
42 **comments as attached to the best of our ability, and have resubmitted a revised**
43 **manuscript based on all the comments raised by the Reviewers.**
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5 The full comments of the reviewers were the following:

6 Reviewer: 1

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9 Comments to the Author

10 General comments

11
12 Little is known about the molecular pathways involved in the pathogenesis of airway
13 disease in patients with severe asthma who either are current smokers or former
14 smokers. The authors investigated the molecular phenotypes of severe asthma in
15 relationship to smoking status, using data on sputum proteomics and sputum, bronchial
16 brush and bronchial biopsy transcripts obtained in the U-BIOPRED study of severe
17 asthma (current-smokers, former smokers and non-smokers) plus from healthy
18 non-smokers. In the current smokers with severe asthma, the main findings were: 1)
19 increased sputum supernatant proteomic colony stimulating factor 2 (CSF2) and
20 anterior gradient protein 2 (AGR2) expression levels compared to non-smokers or
21 former smokers with severe asthma and 2) increased markers of xenobiotic (chemical)
22 metabolism, oxidative stress and endoplasmic reticulum stress pathways in airway
23 epithelial cells compared to non-smokers with severe asthma. In former smokers with
24 severe asthma, the main findings were 1) increased sputum supernatant proteomic
25 azurocidin 1 (AZU1), neutrophil elastase and CXCL8 expression levels compared to
26 non-smokers with severe asthma and 2) no difference in differentially-expressed genes
27 probes (FDR>0.05) compared with non-smokers with severe asthma, although using a
28 less stringent analysis cut-off found upregulation in some gene expression e.g. MMP-12
29 and downregulation on other genes (mast cells, humoral immunity, IL4/IL13 signaling
30 and epithelial protection).The authors conclude that although clinical characteristics of
31 the participants with severe asthma recruited to U-BIOPRED are similar, the current
32 smokers with severe asthma could be distinguished from former smokers with severe
33 asthma at the sputum proteomic level and formers smokers showed loss of epithelial
34 barrier processes compared with non-smokers with severe asthma. The measurement
35 techniques appear to be appropriate. The manuscript includes new and interesting data,
36 although I found the description of the results at time hard to follow. There are some
37 specific criticisms of the study.
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50 Response:

51 We thank the Reviewer for these comments and for taking time to assimilate the large
52 amount of data that was presented. We hope to have addressed all the comments and
53 criticisms he/she has raised.
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7 Major specific comments

8 1. Healthy smoker controls

9 The lack of a healthy smoker control group is a major limitation to the interpretation of
10 the results. It is not clear whether the changes in markers of oxidative stress are due
11 to cigarette smoking alone, as has been reported previously in healthy smokers, or due
12 to an interaction between cigarette smoking and severe asthma. I think it would be
13 helpful for the readership of the Journal, if in the discussion, the authors could briefly
14 speculate on whether they think the changes in current smokers with severe asthma
15 are likely to differ from healthy smokers, perhaps by making indirect comparisons with
16 relevant published data in healthy smokers.
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22 **RESPONSE:**

23 We appreciate the interest of the Reviewer in our manuscript and for pointing out the
24 important findings in our study. We appreciate also the comment regarding the major
25 limitation of our study regarding the lack of the healthy non-asthmatic smokers.
26 However, we used previously-published reports that have looked at healthy smokers
27 compared to healthy non-smokers. In the previous human proteome publications
28 analysing sputum in healthy current smokers and never smokers, CSF2 and AGR2
29 were not shown to be differentially expressed proteins [1]. We therefore believe that
30 severe asthma current smokers have different molecular signatures compared to
31 non-asthmatic healthy smokers. Various publications describing sputum or bronchial
32 epithelial cell transcriptomic profile in cigarette smokers showed upregulated
33 expression of xenobiotic and oxidative stress [1, 2]. Pathway analyses using GO and
34 DAVID that were performed by us, as shown in Supplementary Tables, S9 and S10,
35 showed that the significantly upregulated gene sets of endoplasmic stress and lysosome
36 were seen in our severe asthma smoking subjects compared to the equivalent
37 non-smoking subject using bronchial epithelial cells. This was not seen within Avi
38 Spira's 97 differentially-expressed genes from bronchial epithelial cells between
39 current-smokers and never-smokers [2]. These observations indicate that
40 differently-expressed genes between CSA and NSA are not due to the influence of
41 cigarette smoking itself. We have added supplementary Tables S8 and S9 and
42 comments in the DISCUSSION to that effect as follows:
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51 "CSF2 and AGR2 have not been shown to be differentially-expressed in the
52 previously-published proteomic analysis of sputum from healthy current-smokers
53 compared to never-smokers (35). Moreover, endoplasmic stress and lysosome gene sets
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5 **that we found to be differentially-expressed in these 2 groups of severe asthma were not**
6 **differentially-expressed in healthy current smokers compared to non-smokers**
7 **(Supplementary Table S9 and S10) (21). These results suggest that DEGs between CSA**
8 **and NSA were not derived from the effect of cigarette smoking itself.”**
9

10 2. Small sample size

11 From the total number of participants with severe asthma recruited to U-BIOPRED
12 (n=420), only a small number were included in this analysis (n=70), of whom only a
13 small number were current smokers with severe asthma [sputum proteomic analysis,
14 n=11; transcriptomic analyses, bronchial brushings, n=6; bronchial biopsies, n=7; sputa,
15 n=8] and the number of samples differ. The authors acknowledge this limitation. I
16 suggest adding the term ‘exploratory analysis’ to the title and including this term in the
17 abstract.
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23 **RESPONSE:**

24 **We are fully cognisant of the low number of current smokers that we recruited in**
25 **U-BIOPRED. In general, we found it more difficult to recruit these patients across**
26 **Europe compared to the recruitment of non-smokers or ex-smokers and could not**
27 **pinpoint the underlying reason(s) for this. We believe that active smokers do not wish to**
28 **come forward with perhaps the fear of being told to give up smoking. Despite the small**
29 **numbers, the data from current smokers was quite clear-cut compared to the**
30 **ex-smokers and non-smokers. However, we do acknowledge the limitation (as**
31 **mentioned in the Discussion) and agree with the Reviewer to add ‘exploratory analysis’**
32 **in the Title.**
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39 3. Title

40 By including the term ‘neutrophilic inflammation’ in the title does not give an accurate
41 summary of the contents of the paper. Perhaps a more descript title could be considered
42 by the authors, such as ‘Sputum proteomics and airway cell transcripts of current and
43 ex-smokers with severe asthma in U-BIOPRED: an exploratory analysis’.
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47 **RESPONSE:**

48 **We agree with this comment and have changed the Title accordingly to: “Sputum**
49 **proteomics and airway cell transcripts of current and ex-smokers with severe asthma in**
50 **U-BIOPRED: an exploratory analysis”. We have also added “exploratory” to the**
51 **Abstract.**
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5 4. Bacterial colonization

6 Page 19, para 3: In the absence of data on bacterial colonization this section should be
7 considerably reduced in length
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11 **RESPONSE:**

12 **Although we do not have data on microbiome, decreased expression of protective agents,**
13 **such as mucin, and pathway analysis results support our hypothesis that bacterial**
14 **colonization might be associated with ESA. However, we do not have direct proof as**
15 **pointed out. We have therefore reduced the length of the paragraph as requested to:**

16
17 **“Sputum microbiota, which is associated with neutrophilic airway inflammation in**
18 **adult severe asthma has been reported to be different from that of healthy controls or**
19 **non-severe asthmatics. Our results imply that a reduction in airway protective agents**
20 **might change the airway microbiome, affecting neutrophilic airway inflammation,**
21 **especially in ESA; on the other hand, the heightened mucin production might have had**
22 **a beneficial effect in keeping the airway epithelium free from bacterial colonization in**
23 **CSA.”**
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31 Minor comments

32 1. Abstract: Rationale

33 Page 5, the authors state that ‘Severe asthma patients with a significant smoking
34 history represent a distinct group of asthma, associated with airflow obstruction and
35 eosinophilia’. This statement is based on a cluster analysis of the U-BIOPRED cohort
36 (ref 4) that combined a small number of current smokers and ex-smokers with severe
37 asthma. Many other published studies have reported predominately non-eosinophilic
38 inflammation in current smokers with asthma and similar levels of eosinophilic
39 inflammation in former smokers with asthma and non-smokers with asthma.
40 Furthermore, sputum and blood eosinophil counts of the current smokers and former
41 smoker with severe asthma did not differ in the participants to the study (Table 1 &
42 supplementary tables S3, S4, S5)
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49 **Response:**

50 **We have removed the term “eosinophilia” and replaced it with ‘reported neutrophilia’.**
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53 2. Introduction Page 7, line 12: If reference #2 is used, then revise the sentence
54 ‘significant number of patients with severe asthma are current smokers or have been
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5 ex-smokers (2)' by omitting the word 'severe'. This was a population-based study and did
6 not specifically investigate patients with severe asthma.
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10 **RESPONSE:**

11 **We have omitted the word "severe" from this sentence.**

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14 3. Page 9 Methods: Entry criteria for the non-smokers with severe asthma included < 5
15 pack-year smoking history and healthy non-smoker included < 5 pack-year history
16 (Shaw, ERJ 2015, Ref 7). Provide pack-year history for these groups in Table 1.
17

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19 **RESPONSE:**

20 **The pack year history was zero in both groups of non-smokers. This is added in the**
21 **Table 1.**
22

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25 4. Figure 1 (Sputum supernatant proteomics):

26 a) Is CA6 the symbol for Carbonic anhydrase VI and is CA6 present because of salivary
27 contamination?
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30 **RESPONSE:**

31 **We did our best to exclude salivary contamination by rinsing the mouth with water**
32 **prior to sputum induction and we excluded sputum samples whose squamous cells were**
33 **more than 30% for transcriptomic assay to lessen salivary contamination, as described**
34 **in MATERIALS and METHODS. However, this salivary contamination cannot be**
35 **entirely excluded but can be reduced.**
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40 b) Use consistent abbreviations: CSSA, ESSA and NSSA used in legend and CSA, ESA
41 and NSA used in figure. Also applies to other sections of the manuscript 5. Discussion
42 Page 19, line 10: the study did not show 'increased neutrophilia in ESSA patients', at
43 least in terms of sputum neutrophilia compared to the other severe asthma groups.
44 Please rephrase
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49 **Response:**

50 **We have changed CSSA, ESSA and NSSA to CSA, ESA and NSA, respectively, in all**
51 **sections. We have excluded the term 'increased neutrophilia in ESA patients'.**
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5 Reviewer: 2
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8 Comments to the Author

9 Many thanks for the chance to review your abstract. Unfortunately, some of the data
10 you present seems to be incorrect and I would ask that you address this in the first
11 instance to ensure the validity of your results and conclusions.
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13

14
15 Major Points

- 16 1. There appear to be significant errors in your data tables, which if to be
17 believed suggest that around half of the severe asthmatics in one of your
18 analyses do not meet the criteria for severe asthma. Please see in detail as
19 listed below.
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23 **RESPONSE:**

24 **We apologise for these errors in The Tables which are now corrected.**
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26

27
28 2. I wonder about the validity of many of your statements that there is no
29 difference in baseline demographics between various groups. For instance in your
30 bronchial transcriptomic analyses (Table S4) you state there is no significant difference
31 in sputum eosinophil counts. However, in current smokers, mean sputum eosinophils
32 was 0.87% and in ex-smokers 17%. You do not have sputum results for half the current
33 smokers, and two thirds of the ex-smokers. This leaves very few numbers for analysis
34 and I suspect that this is why there is no statistically significant difference. (In contrast
35 you had DEP/DEG data for all patients, by definition, so the chance of finding a
36 significant result here was much higher.) This is similar across your data: in Table S4
37 the proportion of patients on oral corticosteroid varies from 16.7% to 72.7%; in Table 1,
38 non-smokers had 7.2% sputum eosinophils compared to 18.8% in current-smokers;
39 blood eosinophil differences between these groups would typically classify one group as
40 eosinophilic (407/uL) and one as non-eosinophilic (259u/L); the number on systemic
41 corticosteroids varied from 30% to 60%; macrolide use almost doubles one group to
42 another; average age of onset varied from 25 to 39.5 years old; in Table S3 there is
43 double the MCID in ACQ-7 between non- and current-smokers. (This is not a complete
44 list.) It risks appearing disingenuous if you state in your text there is no difference
45 between the groups without mentioning what I think is a significant caveat to this
46 statement.
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RESPONSE:

We appreciate this major limitation in our study as pointed out with the small number of smokers and non-smokers who underwent bronchoscopy. We have modified our manuscript to “Although there were numerical differences in blood eosinophil counts (CSA 259/ μ L, ESA 296/ μ L, NSA 407/ μ L), % sputum eosinophils (CSA 7.2%, ESA 14.8%, NSA 18.8%), and the proportion of those on oral corticosteroids (CSA 30.0%, ESA 63.6%, NSA 45.7%), these were not statistically significant. NSA patients had the highest blood and sputum eosinophil counts.”

We also agree that the differences in mean value of ACQ7 between the group would appear substantial, but because of the low numbers, this did not achieve statistical significance. The issue of limited numbers is addressed in the shortcomings paragraph (Page 20).

Table S3

· Only 1 of your 6 current smokers was on inhaled corticosteroids (41.7% of ex-smokers and 60% of non-smokers). By definition this is not a severe asthma cohort. · You also find a p-value of 1.00 for the difference between these groups, which seems unlikely when the values range from 16.7% to 60% across the three groups.

· You have very high macrolide use in your non-smokers, up at 60%. Incidentally the numbers on macrolides are identical in all groups to the number on inhaled corticosteroids.

RESPONSE:

We apologise for the various mistakes in that Table. The corrected Table S3 is now included.

· The mean dose of the 1 current smoker on systemic corticosteroids was 1.67mg, and you present a standard deviation for this one patient.

RESPONSE:

Each actual OCS dose was 10, 0, 0, 0, 0 and 0 mg/day. Therefore, the mean dose is 1.67, SD is 4.08, as shown in the Table.

Table S4

· These figures seem more believable. However there still seem to be errors in your calculations: you state that 2 of 7 patients being on systemic corticosteroids is

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5 16.7% rather than 28.6%; 3 out of 6 is 72.7%; and 14 out of 32 is 35.1%.

6 **RESPONSE:**

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8 **We apologise for this mistake. We modified the numbers from 16.7% to 28.6%, 72.7% to**
9 **50.0% and 35.1% to 43.8%.**

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11
12 **Minor Points**

- 13
14 1. Abstract: you state “severe asthmatics with a significant smoking history have
15 airflow obstruction and eosinophilia.” You don’t provide evidence for this
16 statement, and actually appear to contradict it in your introduction where you
17 state that smoking related asthma “has been considered a non T helper type 2
18 neutrophilic asthma.”

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22 **RESPONSE:**

23 **We changed “eosinophilia” to “reported neutrophilia”.**

- 24
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26 2. Abbreviations: some are missed out in your list, eg DEG and DEP

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29 **RESPONSE:**

30 **We added several words such as DEG and DEP in ABBREVIATIONS.**

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33 3. Materials and methods: please state how asthma was diagnosed. For sputum
34 (Table 1) the mean age of asthma onset in your non-smokers was 25 and in your
35 ex-smokers was almost 39.5 – you are claiming these are asthmatic so we need
36 evidence that we are not dealing with a COPD cohort in your ex-smokers.

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40 **RESPONSE:**

41 **We have added a new file “Supplementary File” where we describe the following**
42 **inclusion criteria:**

43 **“Severe asthma patients”**

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45 **“Participants with asthma had either airflow reversibility (increase in forced**
46 **expiratory volume in 1s (FEV1) >12% predicted and 200 mL following inhalation of**
47 **400 µg salbutamol), airway hyperresponsiveness (methacholine provocative**
48 **concentration causing a 20% fall in FEV1 <8 mg/mL, or diurnal peak expiratory flow**
49 **amplitude >8% of mean), or a decrease in FEV1 of 12% predicted or 200 mL within 4**
50 **weeks after tapering maintenance treatment. Severe asthma was defined as asthma**
51 **with uncontrolled symptoms according to Global Initiative for Asthma (GINA)**
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5 **guidelines and/or frequent exacerbations (more than two per year) despite high-dose**
6 **inhaled corticosteroids (≥ 1000 μg fluticasone propionate per day or equivalent dose)**
7 **plus additional controller therapy. “**
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12 4. Materials and methods: please make it clear that you are using a subset of
13 patients for this study. It reads currently as though all 420 patients had
14 sputum and bronchial biopsies etc.
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18 **RESPONSE:**

19 **We modified the part of *Clinical data* as follows.**

20
21 **We enrolled totally 374 severe asthma patients in the U-BIOPRED cohort divided**
22 **into three groups by smoking status; current-smokers with severe asthma (CSA),**
23 **ex-smokers with severe asthma (ESA), non-smokers with severe asthma (NSA). We**
24 **narrowed down the NSA to those who had never smoked (0 pack-year), although**
25 **original non-smokers with severe asthma group in the U-BIOPRED cohort**
26 **contained the patients whose pack-year was less than 5. Eighty-one non-smoking**
27 **healthy volunteers (NH) whose pack-years were 0 were also enrolled. Differential**
28 **blood and induced sputum cell counts, serum total IgE and skin prick tests, serum**
29 **perioxin and fraction of exhaled nitric oxide (FeNO) and pre- and post-**
30 **bronchodilator spirometry were obtained (8, 9). Bronchial biopsies, bronchial**
31 **brushings and sputum were obtained, as previously described (8). Because of the**
32 **bronchoscopy exclusion criteria (Supplementary File), only 95 bronchial brushings**
33 **and 69 bronchial biopsies were obtained. The number of sputum samples for**
34 **proteomic analysis was 88. All subjects whose samples were adequate and**
35 **underwent omics analyses are shown in Supplementary Figure S1.**
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43 **We have added bronchoscopy exclusion criteria in Supplementary File.**

44 **“Bronchoscopy exclusion criteria”**

45
46 **“Bronchoscopy exclusion criteria included the subjects who had a severe**
47 **exacerbation of asthma requiring the new administration of oral corticosteroids or a**
48 **doubling of their usual oral corticosteroid dose within the last three months, a $\geq 10\%$**
49 **decrease in forced expiratory volume in 1s (FEV1) after inhalation of 0.9% saline**
50 **during sputum induction, increase use of rescue short acting β -agonist by six or**
51 **more puffs/day over and above usual use during the last three days, severe**
52 **bronchoconstriction or other adverse reactions at previous attempts to perform**
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5 **bronchoscopy, a post-bronchodilator FEV1<40% predicted, a transcutaneous oxygen**
6 **saturation by pulse-oximetry of <90%, a heart rate >130 beats/minutes to**
7 **pre-medication, known serious ventricular heart rhythm disturbances or treatment**
8 **with warfarin or other anticoagulants.”**
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12 5. Results: please state the numbers of patients in each of your groups in the main
13 text. Please make it clear how many of your patients were different in your
14 sputum analysis and your bronchial biopsy analysis (the baseline demographics
15 data make them look quite distinct). Please state the number of sites from
16 which patients in each subset were recruited.
17
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21 **RESPONSE:**

22 **We added the numbers of patients in all comparisons. We have made a new figure**
23 **showing subjects whose samples were applied for proteomic and transcriptomic**
24 **analyses (Supplementary Figure S1), referred to in MATERIALS and METHODS.**
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28 6. You sometimes use CSSA/ESSA/NSSA and sometimes CSA/ESA/NSA. Please
29 be consistent.
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32 **RESPONSE:**

33 **We have changed CSSA, ESSA and NSSA to CSA, ESA and NSA, respectively, in all**
34 **sections.**
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38 7. In all data tables “comorbidities” is spelled incorrectly

39 **RESPONSE: We modified the spelling from “commorbidities” to “comorbidities”.**
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42 8. Across data tables there is inconsistency in the p-value presentation.

43 **RESPONSE: All p-values in data tables now are in same format.**
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46 9. Figure 1: I think you might have confused your labelling? You state red dots
47 were those proteins with an absolute fold change of ≥ 2.0 and turquoise was used for
48 those ≤ 2.0 . Do you mean a fold change of ≤ -1.0 for red and ≥ 1.0 for turquoise? Also you
49 need a colon rather than semi-colon after “in following comparisons”.
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51 **RESPONSE:**

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53 **The labelling of transverse line is log2 (fold change). Absolute fold change 2.0 means**
54 **log2 (fold change) >1.0 or <-1.0. I modified the legend by adding “-“. The proteins whose**
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5 **absolute fold change (FC) ≥ 2.0 at false discovery rate (FDR) < 0.05 were DEPs shown as**
6 **coloured dots (red: FC ≥ 2.0 , turquoise: FC ≤ 2.0).**
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10 10. In your discussion, please consider changing “There was a difference in the
11 sputum proteome between NSSA and CSSA (CSF2, AGR2 and CXCL8) and ESSA
12 (AZU1, ELANE, CFP and CXCL8),” which might be interpreted as a difference between
13 NSSA and CSSA on the one hand with ESSA on the other, to “There was a difference in
14 the sputum proteome between NSSA and CSSA (CSF2, AGR2 and CXCL8) and between
15 NSSA and ESSA (AZU1, ELANE, CFP and CXCL8).”
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19 **RESPONSE: We have modified accordingly.**
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22 Questions

- 23
24 1. For the sputum DEPS it seems you have performed comparisons between each
25 group and healthy controls and drawn conclusions as to what underlies each
26 group based on this. Have you performed similar analyses comparing the
27 current-, ex- and non-smoker groups with each other directly?
28
29

30 **RESPONSE:**

31 **Yes, we have performed these analyses. CSF2 and AGR2 were recognized as DEPs in**
32 **the comparisons between CSA and ESA and between CSA and NSA but we could not**
33 **find any DEPs in the comparison between ESA and NSA.**
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- 39 2. Why have you adjusted for certain demographic variables in your sputum DEP
40 analyses but not in your bronchial biopsy DEG analyses? Age is adjusted for in
41 the sputum DEP but not biopsy DEG despite being significantly different
42 between groups in both cohorts; oral corticosteroid use is adjusted for in the
43 sputum DEP but not biopsy DEG despite apparently not being significantly
44 different between groups in either cohort; BMI is very significantly different
45 between groups in both cohorts but was not adjusted for in any analysis.
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50 **RESPONSE:**

51 **We have performed all analyses of DEPs and DEGs by adjusting demographic variables,**
52 **such as age, gender and systemic corticosteroids use because these factors seemed to**
53 **affect the expression of mRNAs or proteins in airways. BMI seemed not to affect gene**
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5 expression in airways. I modified the sentence in “*Statistical analysis*” as follows: Gene
6 **and** protein expression data were analysed using multivariate analysis of variance
7 (MANOVA) with age, gender and systemic corticosteroids (SCS) use analysed as
8 **covariates.**
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12 References:

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Fig. 1

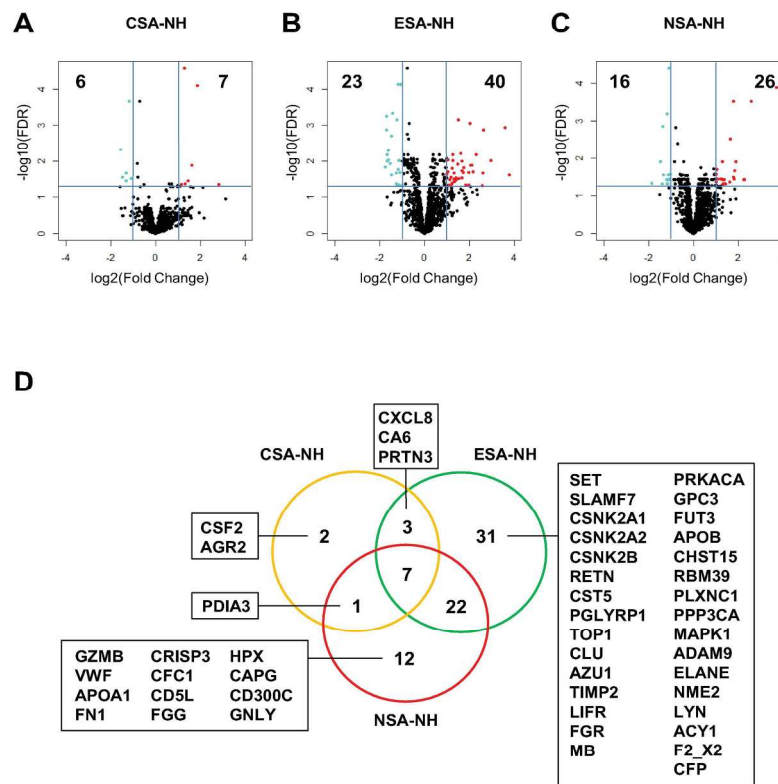


Figure 1. Phenotypic differences among CSSA, ESSA and NSSA were unveiled by limma of sputum SomaLogic.

(A-C) Volcano plots showing differentially-expressed proteins (DEPs) in linear model for microarray (limma) of sputum SomaLogic in following comparisons; (A) CSSA and NH, (B) ESSA and NH, (C) NSSA and NH. The proteins whose absolute fold change (FC) ≥ 2.0 at false discovery rate (FDR) < 0.05 were regarded as DEPs shown as coloured dots (red: FC ≥ 2.0 , turquoise: FC ≤ 2.0). The number of DEPs of each comparison is shown in the left box and right upper areas of each plot. (D) Venn diagram showing the numbers and names of DEPs in each comparison.

196x275mm (300 x 300 DPI)

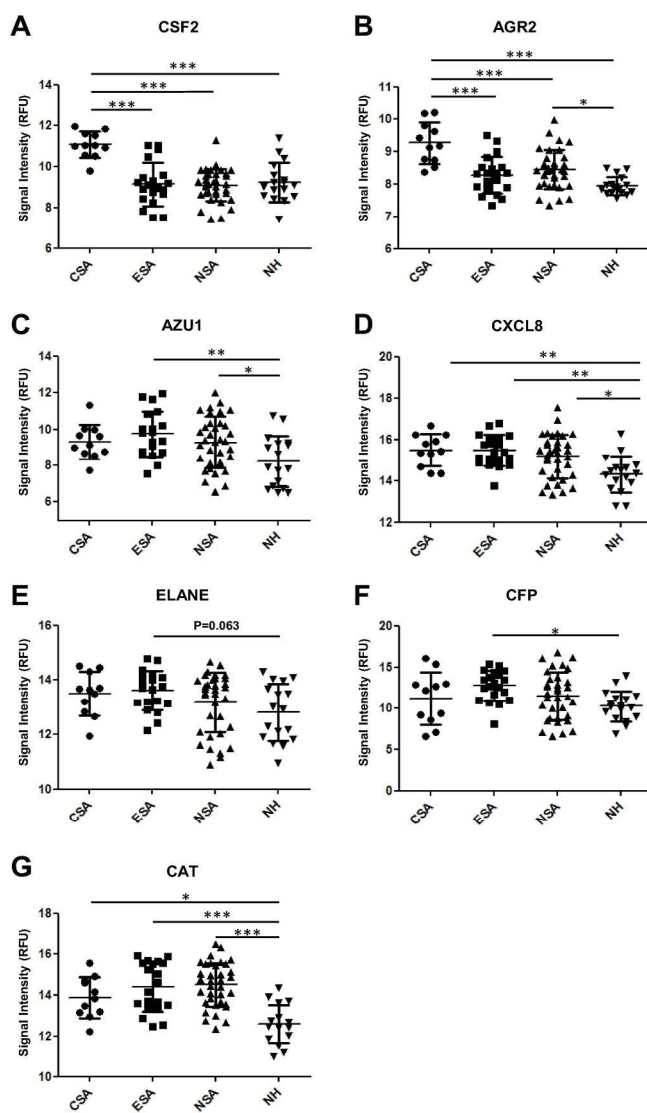


Fig. 2

Figure 2. Differentially-expressed proteins (DEPs) in severe asthma sputum according to smoking status. Dot plots with mean±SD showing signal intensity levels of protein expression of CSF2 (A), AGR2 (B), AZU1 (C), CXCL8 (D), ELANE (E), CFP (F) and CAT (G) in sputum by SomaLogic analysis in CSSA (circles), ESSA (squares), NSSA (triangles) and NH (inverted triangles). RFU: Relative Fluorescence Units, *p<0.05; **p<0.01; ***p<0.001.

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Fig. 3

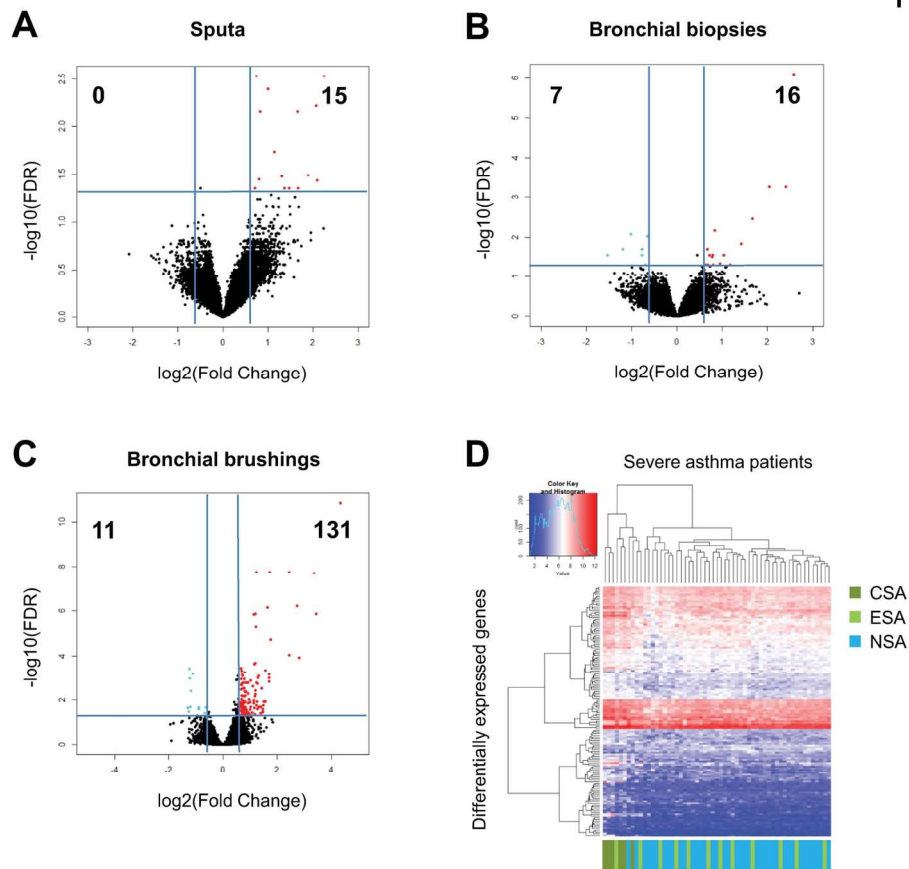


Figure 3. Differentially-expressed genes (DEGs) in current smokers (CSSA) and non-smokers (NSSA) with severe asthma.

Volcano plots showing differentially expressed genes (DEGs) between CSSA and NSSA 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 (red: FC ≥ 1.5 , turquoise: FC ≤ 1.5). 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. Blue rectangles represent samples with low expression for the particular gene, and red rectangles represent samples with high expression for the particular gene. CSSA (dark green), ESSA (light green) and NSSA (cyan).

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Fig. 4

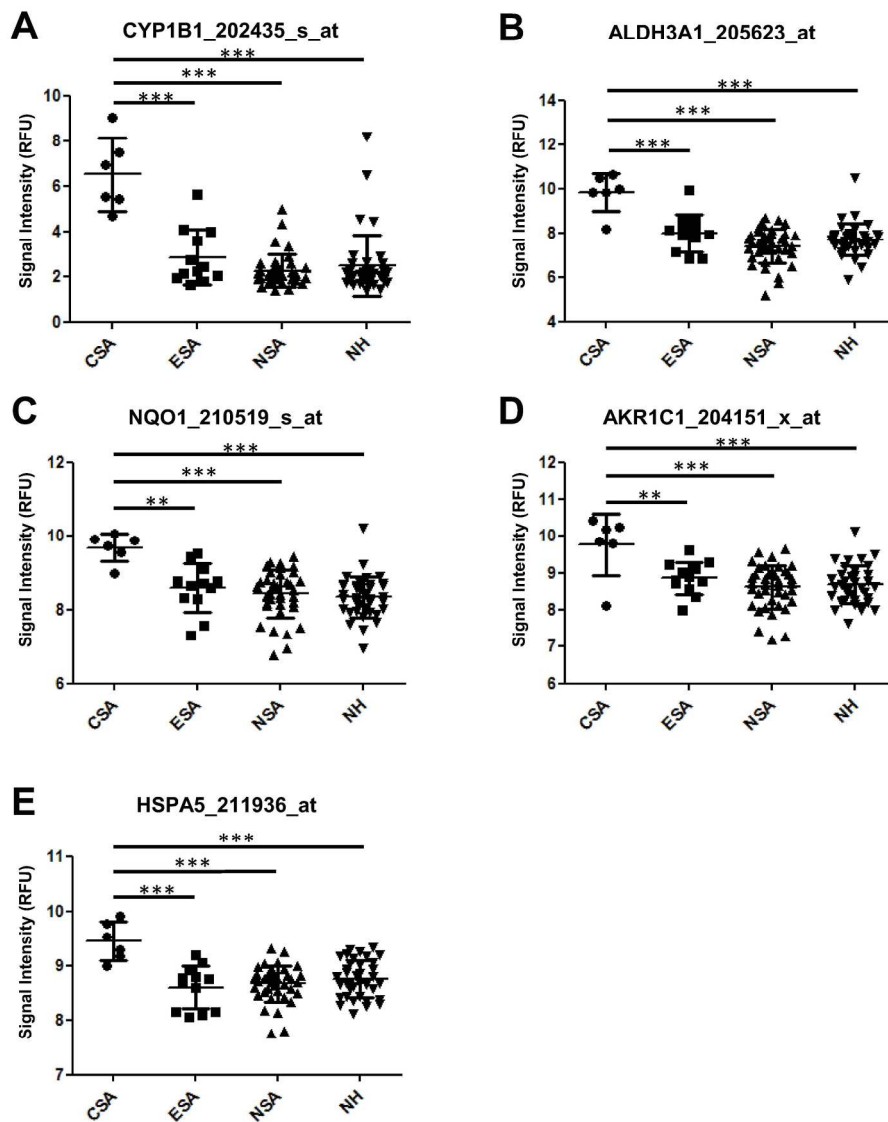


Figure 4. Differentially-expressed genes (DEGs) associated with metabolism of xenobiotics, oxidative stress and ER stress in bronchial brushings.

Dot plots showing DEGs in bronchial brushings associated with xenobiotic metabolism CYP1B1 (A), ALDH3A1 (B), NQO1 (C), AKR1C1 (D) and HSPA5 (E). CSA: circles, ESA: squares, NSA: triangles, NH: inverted triangles. RFU: Relative Fluorescence Units, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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Fig. 5

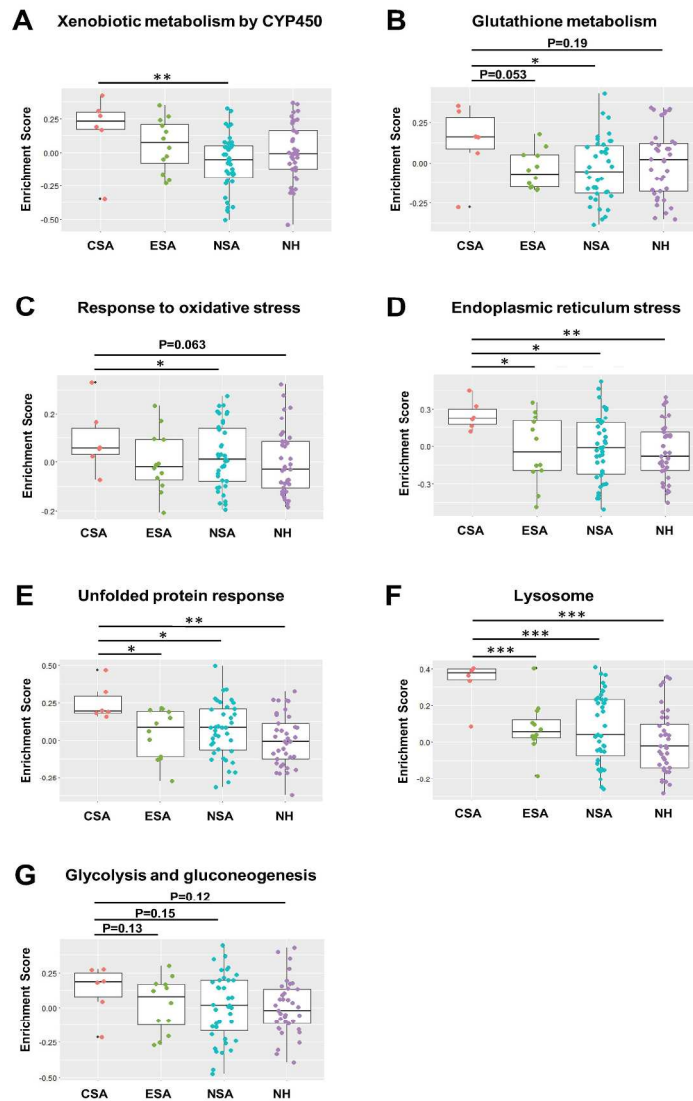


Figure 5. Gene Set Variation Analysis of selected stress-related pathways in bronchial brushings according to smoking status.

Box-and-whisker plots showing pathway enrichment of (A) xenobiotic metabolism by CYP450, (B) glutathione metabolism, (C) response to oxidative stress, (D) endoplasmic reticulum (ER) stress, (E) unfolded protein response, (F) lysosome and (G) glycolysis and gluconeogenesis in bronchial brushings of CSA (red circles), ESSA (olive green circles), NSA (cyan circles) and NH (purple circles) subjects.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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Fig. 6

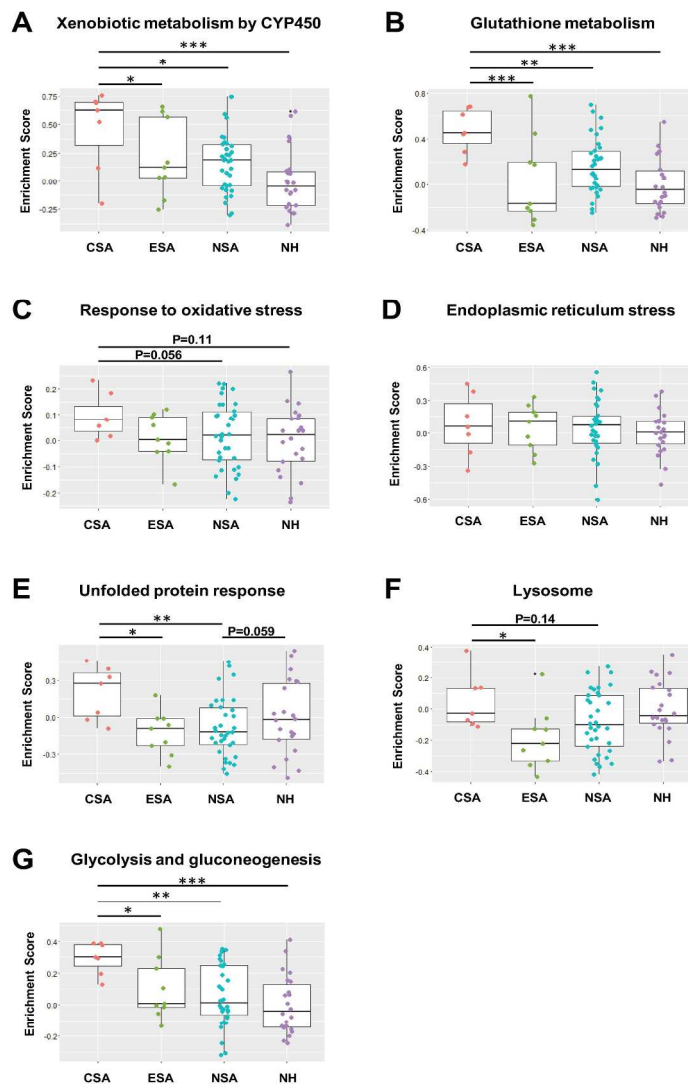


Figure 6. Gene Set Variation Analysis of selected stress-related pathways in bronchial biopsies according to smoking status.

Box-and-whisker plots showing pathway enrichment of (A) xenobiotic metabolism by CYP450, (B) glutathione metabolism, (C) response to oxidative stress, (D) endoplasmic reticulum (ER) stress, (E) unfolded protein response, (F) lysosome and (G) glycolysis and gluconeogenesis in bronchial biopsies of CSA (red circles), ESSA (olive green circles), NSSA (cyan circles) and NH (purple circles) subjects.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

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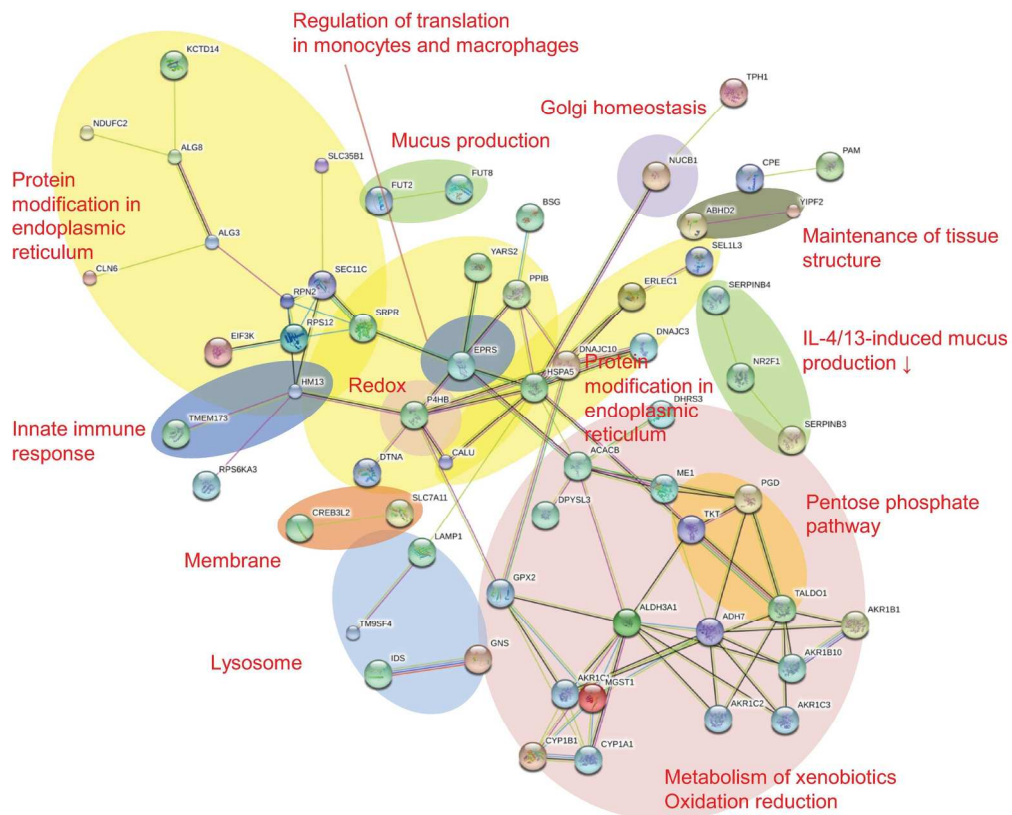


Fig 7

Figure 7. Protein interaction analysis by STRING using combined DEGs. Combined differentially expressed genes (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 pentose-phosphate pathway (orange-coloured area). These proteins function with those in charge of redox (small pink-coloured area) and connect with protein production or modification (yellow). Some proteins are associated with innate immunity (blue). The other proteins function as lysosomal (sky blue), membranous (coral red), mucus productive (apple green), Golgi homeostatic (purple) or structural proteins (olive green). Overall, this reveals the relationship between oxidative stress, ER stress, metabolism of xenobiotics and innate immunity.

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