Distinct pathways underlying neutrophilic inflammation in smoking-associated severe asthma in U-BIOPRED

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Take home message

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

ABSTRACT (198 words)

Background:

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

Methods

The U-BIOPRED severe asthma patients containing current-smokers (CSSA), ex-smokers (ESSA), non-smokers (NSSA) and healthy non-smokers (NH) was examined. Blood and sputum cell counts, fractional exhaled nitric oxide and spirometry were obtained. 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 CSSA sputum supernatants with azurocidin 1, neutrophil elastase and CXCL8 upregulated in ESSA. Phagocytosis and innate immune pathways were associated with neutrophilic inflammation in ESSA. Gene Set Variation Analysis of bronchial epithelial cell transcriptome from CSSA showed enrichment of xenobiotic metabolism, oxidative stress and endoplasmic reticulum stress compared to other groups. CXCL5 and matrix metallopeptidase 12 genes were upregulated in ESSA and the epithelial protective genes, mucin 2 and cystatin SN, were downregulated.

Conclusion

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

ABBREVIATIONS

ACQ:	Asthma Control Questionnaire
AQLQ:	Asthma Quality of Life Questionnaire
BMI:	Body Mass Index
CSSA:	Current-smokers with Severe Asthma
ESSA:	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
FEV1:	Forced Expiratory Volume in 1 second
FVC:	Forced Vital Capacity
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
NSSA:	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 severe 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 underlie

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

MATERIALS and METHODS

Clinical data

U-BIOPRED is a cross-sectional cohort consisting of 420 severe asthma patients divided into three groups by smoking status: current-smokers with severe asthma (**CSSA**), ex-smokers with severe asthma (**ESSA**), non-smokers with severe asthma (**NSSA**) (7). Eighty-eight non-smoking healthy volunteers (**NH**) were also enrolled. Differential blood and induced sputum cell counts, serum total IgE and skin prick tests, serum periostin and fraction of exhaled nitric oxide (FeNO) and pre- and post-bronchodilator spirometry were measured (8, 9). Bronchial biopsies, bronchial brushings and sputum were obtained as previously described (8). The study was approved by the Ethics Committees for each of the 16 clinical recruiting centres. All subjects gave written and signed informed consent.

Transcriptomic microarray analysis

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

SomaLogic proteomic technique

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

Gene Set Variation Analysis (GSVA)

Gene set variation analysis (GSVA) was performed in R using the Bioconductor GSVA package for estimating variation of gene set enrichment (11). Gene sets were obtained from Molecular Signatures Database v5.2 (MSigDB) (http://software.broadinstitute.org/gsea/msigdb) or from published papers (Supplementary Table S1). We used Immunomap^{TM} graphics (Johnson & Johnson Ltd., NJ, USA) for visualisation.

Statistical analysis

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

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 CSSA subjects were lower than in the other severe asthma groups, but there were no differences in either blood or sputum eosinophil and neutrophil counts among the 3 severe asthma groups. 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 CSSA and NH, ESSA and NH, and NSSA and NH, respectively (Figure 1, A-C). The DEPs are shown in Figure 1D and Additional File 1. Only 5 proteins distinguished CSSA-NH from NSSA-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 CSSA-NH and ESSA-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 CSSA (Figure 2A-B, Supplementary Figure S1). This suggests that CSSA is associated with macrophage/neutrophil recruitment and mucus production.

34 DEPs distinguished ESSA-NH from NSSA-NH and included azurocidin 1 (AZU1), neutrophil elastase (ELANE), complement factor properdin (CFP) and CXCL8 (Table 3, Figure 2C-F). AZU1 possesses monocyte chemotactic and antimicrobial activity (14) and CFP positively regulates the alternative complement system (15). 29 proteins overlapped between ESSA-NH and

NSSA-NH and these included C-reactive protein (CRP), colony-stimulating factor 1 receptor (CSF1R), inducible T-cell costimulatory ligand (ICOSLG), FCGR2A and catalase (CAT) (Table 3, Figure 1D). In contrast, there were 13 differentially-expressed proteins including PDIA3, granzyme B (GZMB) and CD5 antigen-like (CD5L) (Table 3, Figure 1D). GZMB is a cytoplasmic granule of cytotoxic T-cells and NK cells, and is involved in apoptosis, chronic inflammation and wound healing (16). CD5L, expressed in lymphoid tissues, lung epithelial cells or tissue macrophages, plays multiple roles in inflammation, such as promoting macrophage phagocytosis (17).

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

Pathway analysis of differentially-expressed proteins

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

Characteristics of patients with transcriptomic analysis in bronchial biopsies and brushings

We found increased blood neutrophils and lower FeNO levels in CSSA compared to NSSA

subjects providing bronchial brushings and biopsies for analysis although the proportion of patients who took systemic corticosteroids or the dose of oral corticosteroids was no different between the 2 severe asthma groups (Supplementary Tables 3-5). There were no significant differences in blood eosinophil, sputum eosinophil and sputum neutrophil counts, and in pulmonary function, ACQ-7, AQLQ or the number of exacerbations in the previous year among the 3 severe asthma groups. The subjects who provided samples for sputum transcriptomics did not completely overlap with those providing sputum proteomics but the clinical characteristics were similar (Supplementary Table 5).

Differentially-expressed genes (DEGs) between CSSA and NSSA

We detected 142 significantly differentially-expressed gene (DEG) probes in bronchial brushings, 23 in bronchial biopsies and 15 in sputum samples between CSSA and NSSA (Figure 3A-C; Additional File 2). There were no significant DEG probes (FDR>0.05) in any samples between ESSA and NSSA (Additional File 3). Hierarchical clustering of the 142 DEG from bronchial brushings indicated that although CSSA and NSSA were clearly distinct, NSSA and ESSA did not cluster separately (Figure 3D).

The DEGs between CSSA and NSSA are implicated in oxidation-reduction, xenobiotic metabolism and endoplasmic reticulum (ER) stress (Additional File 2). Cytochrome P450 family 1 subfamily B member 1 (CYP1B1) and aldehyde dehydrogenase 3 family member A1 (ALDH3A1), which were over-expressed in bronchial brushings of CSSA compared to other groups (Figure 4A-B), play a role in metabolizing polycyclic aromatic hydrocarbons (PAHs) or aldehydes (18). The oxidative stress genes, NAD(P)H quinone dehydrogenase 1 (NQO1) and aldo-keto reductase family 1 member C1 (AKR1C1), were also highly expressed in CSSA bronchial brushings (Figure 4C-D). ER plays a central role in the protein biosynthesis, correct protein folding and post-transcriptional modifications (19). Accumulation of unfolded and misfolded proteins, termed ER stress, leads to the unfolded protein response (UPR) and inflammation (20). Heat shock protein family A (Hsp70) member 5 (HSPA5), a

key mediator of ER stress, was significantly upregulated in CSSA compared to NSSA in bronchial brushings and biopsies (Figure 4E).

Pathway analysis using DEGs between CSSA and NSSA

Pathway analysis indicated that oxidation-reduction, chemical metabolism and endoplasmic reticulum (ER) stress were different between CSSA and NSSA (Supplementary Table S6, S7). These results suggest that the lung epithelial cells of CSSA patients are under more potent chemical, oxidative and ER stresses than those of NSSA patients.

Gene Set Variation Analysis (GSVA) of bronchial brushings and biopsies

GSVA confirmed the selective enrichment of xenobiotic metabolism by cytochrome P (CYP) 450, glutathione metabolism, response to oxidative stress, endoplasmic reticulum (ER) stress, unfolded protein response, lysosome or glycolysis and gluconeogenesis pathways in bronchial brushings (Figure 5A-G) and biopsies (Figure 6A-G) in the CSSA group. There were no significant differences between ESSA and NSSA for these pathways. Using signatures for active smoking obtained from Spira and colleagues (20), we confirmed that bronchial brushings and biopsies from CSSA were enriched for the active smoking-related gene and that both CSSA and ESSA were enriched for the pack-year signature (Supplementary Table 1; Supplementary Figure S2).

Differentially-expressed genes in sputum, bronchial biopsies and epithelial brushings

As we could not detect any DEGs between ESSA and NSSA at the FDR<0.05 level, we undertook a discovery approach using a less stringent analysis strategy. Genes whose absolute fold-change was ≥2.0 in limma were used to clarify the phenotypic difference between ESSA and NSSA (Additional File 2). Twenty-seven genes (thirty-five probes) were up-regulated in ESSA sputum samples included matrix metallopeptidase 12 (MMP12), neuropilin 1 (NRP1), Toll-interleukin 1

receptor domain containing adaptor protein (TIRAP), C-X-C motif chemokine ligand 5 (CXCL5) and pro-platelet basic protein (PPBP) (Supplementary Table S8). MMP12 has been associated with decreased lung function and COPD, TIRAP is involved in the Toll-like receptor (TLR) signalling pathway and both CXCL5/ENA-78 and PPBP/CXCL7 are potent neutrophil chemoattractants and activators (21). Innate immunity, including complement system, TLR signalling and neutrophilic inflammation, may be characteristics of ESSA.

Six down-regulated DEGs (fold-change ≤ 0.5) distinguished ESSA from NSSA in bronchial brushings, namely carboxypeptidase A3 (CPA3), cystatin SN (CST1), immunoglobulin kappa constant (IGKC), mucin 2, oligomeric mucus/gel-forming (MUC2) and tryptase α/β1 (TPSAB1) (Supplementary Table S8). CPA3 and TPSAB1 are mast cell biomarkers and are found to be elevated in asthma patients (22). CST1 is a cysteine proteinase inhibitor that has a protective effect on epithelium (23). MUC2 provides a protective barrier for airways against particles or infectious agents (24). This suggests that ESSA has a lesser protective epithelial barrier and reduced mast cell activity compared with NSSA.

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

Protein interaction analysis using combined DEGs from airway samples

Protein interaction analysis by STRING using combined DEGs between CSSA and NSSA 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 (CSSA), and ex-smokers with a significant history of cigarette smoking (ESSA), and those who do not smoke (NSSA). There was a difference in the sputum proteome between NSSA and CSSA (CSF2, AGR2 and CXCL8) and ESSA (AZU1, ELANE, CFP and CXCL8) subjects with CXCL8 not discriminating between ESSA and CSSA. Distinct pathways were activated in CSSA and NSSA sputum whilst the sputum protein data also suggested that ESSA was associated with airway epithelial cell damage. In addition, gene expression profiles between bronchial epithelial cells from CSSA and NSSA were significantly different as determined by pathway analysis, GSVA and protein-protein interaction analysis. There were no significant DEGs (FDR<0.05) between ESSA and NSSA. Hierarchical clustering indicated that although CSSA and NSSA were clearly distinct, NSSA and ESSA did not cluster separately. Airway epithelial cells in CSSA patients show an enrichment of oxidative and ER stress and innate immune pathways compared to ESSA or NSSA patients and there were no significant differences between ESSA and NSSA for these pathways. Using a less stringent analysis ESSA subjects showed upregulated expression of neutrophil chemotactic genes and downregulated expression of genes related to mast cells, humoral immunity and epithelial protection compared to NSSA. Overall, proteomics and transcriptomics were able to differentiate CSSA from NSSA but ESSA and NSSA could only be discriminated using sputum proteomics as airway transcriptomics clustered ESSA and CSSA 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 (25). CSF2 transgenic mice have an enhanced Th2 response to ovalbumin sensitization and anti-CSF2 antibodies block the allergic response in mouse models of asthma (26). CSF2 is also involved in the lung innate immune response to noxious agents such as LPS and cigarette smoke (27). Acute exposure to cigarette

smoke in mice leads to enhanced CSF2 expression and neutralization using an intranasal anti-CSF2 antibody reduced BALF macrophages and neutrophils and inflammatory analytes (28), which indicates that the CSF2 pathway can mediate smoke-induced inflammation. Future experiments in models of severe asthma linked to smoking or in selected patients may determine whether the elevated CSF2 expression seen here is causal or a marker of other driver mechanisms.

Our data provide evidence for enhanced oxidative and ER stress in airway epithelial cells of CSSA patients. We postulate that the increased activation of the xenobiotic response and oxidative and ER stress pathways influences innate immunity in these subjects. There is increased oxidative stress in asthma and COPD patients as well as in healthy smokers (29). Cigarette smoke not only contains high concentrations of reactive oxygen species (ROS) (29), but also activates alveolar macrophages and neutrophils, which also release ROS leading to an increased inflammatory response in a feed-forward process (29, 30). In both asthma and COPD, activated inflammatory cells including neutrophils, macrophages and eosinophils also produce ROS and further generate inflammation and causes injury to the airway epithelium (29). Moreover, impaired upregulation and production of protective antioxidant was reported in smokers, asthma and COPD patients. This oxidant-antioxidant imbalance resulting in oxidative stress is associated with airway hyperresponsiveness and decreased lung function and asthma severity (29, 31).

CSSA represented the escalated response to oxidative stress derived from cigarette smoking as CSSA bronchial brushings and biopsies alone were enriched for the active smoking-related gene set whereas both CSSA and ESSA samples showed a similar enrichment of the pack-year signature. Increased antioxidant gene expression and increased enrichment of the gene set showing response to oxidative stress were observed in bronchial brushings, which may suggest that cigarette smoking stimulates airway epithelial cells to respond to oxidative stress in severe asthma. We also showed that ER stress might have a key role in CSSA phenotype. ER stress is associated with neutrophilic asthma through NF-κB activation and proinflammatory cytokine production (32). However, cigarette

smoking induces ER stress (33) and the activation seen here in severe asthma probably relates to active cigarette smoke exposure.

In addition to increased neutrophilia in ESSA patients, we found decreased production of protective agents in ESSA airways. Cigarette smoke injures the airway epithelium in several ways, including decreased protective protein expression (34), disruption of tight junctions (35), and through innate immune and inflammatory response (30). Cigarette smoke-activated alveolar macrophages produce pro-inflammatory molecules, reactive oxygen species (ROS), tissue proteases and chemokines for recruitment and survival of neutrophils in the lung tissue (30), and activated neutrophils secrete proteases and breakdown collagen into fragments, which can activate neutrophils in a positive feedback manner (36). We showed decreased expression of MUC2 and CST1 in ESSA, which both play a protective role for airway epithelium (37, 38). Conversely, the expression of MMP12, CXCL8 and PPBP, which can enhance lung damage, were upregulated in ESSA.

Neutrophilic airway inflammation has been linked to bacterial infection or colonization (39, 40). Sputum microbiota in adult severe asthma is different from that of healthy controls or non-severe asthmatics (41, 42). Moreover, changes in the lung microbiome might modulate host inflammatory and immune responses (42). Similar results are seen in COPD with decreased microbiota diversity that has been associated with exacerbations (43). The direct effects of smoking on the microbiome are not well understood. The bronchoalveolar lavage microbiome was similar between healthy non-smokers and smokers (44) whilst the abundance of *Veillonella spp.* and *Megasphaera spp.* was elevated in the sputum of current smokers (45). Our results imply that the airway microbiome might affect neutrophilic airway inflammation in ESSA. In CSSA, the heightened mucin production might have a beneficial effect to keep the airway epithelium from bacterial colonization.

There are important limitations in our study. First, the numbers of smoking and ex-smokers in our groups were relatively small particularly when analysing data from sputum and biopsy and brushing samples, and the results should be considered as exploratory and will need confirmation in a

larger cohort. Secondly, the lack of a control group of age-matched non-asthmatic active smokers does not allow us to determine the exact contribution of cigarette smoking to the changes observed. Thirdly, we did not observe differences in blood or sputum neutrophil counts although neutrophil chemoattractants were more upregulated in airways of ESSA and CSSA patients compared to controls.

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

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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	
	Current-smoker (CSSA) (n=11)	**Ex-smoker (ESSA)** (n=22)	**Non-smoker (NSSA)** (n=37)	**Non-smoker (NH)** (n=18)	**P value**
Female	5 (45.5)	14 (63.6)	22 (59.5)	6 (33.3)	0.201*
Age (y)	50.0±10.6	55.7±9.7	52.6±13.3	39.9±13.8 ¦¶	**0.00395**§
Onset age of asthma (y)	29.8±19.9	39.5±19.0 ¶	25.0±18.1	N.R.	**0.0245**§
Age at starting smoking (y)	19.3±4.0	16.2±2.5	N.R.	N.R.	**0.0493**†
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	N.R.	N.R.	0.117†
BMI (kg/m2)	27.7±4.7	31.1±6.7	27.5±5.7	25.3±3.2 ¦	**0.0279**§
Atopic (%)	8 (88.9) [2]	10 (62.5) [6]	28 (84.8) [4]	5 (45.5) [7]	**0.0360***
Blood eosinophil (*/µL)	259±173	296±246	407±357 [2]	(116±71)	0.331§#
Blood neutrophil (*10^3/µL)	5.10±1.95	5.84±3.03	4.97±2.16 [2]	(3.35±1.15)	0.603§#
Sputum eosinophil (%)	7.2±15.2	14.8±16.8	18.8±24.6	(0.36±0.57)	0.298§#
Sputum neutrophil (%)	53.9±16.1	55.2±20.6	50.8±30.9	(41.0±26.5)	0.928§#
IgE (IU/mL)	222±201 [2]	313±499	305±510 [3]	(105±178)	0.884§#
FeNO (ppb)	15.2±16.6 ¦¶	40.5±33.9 [1]	41.2±36.3 [3]	(19.4±9.7) [3]	**0.000755**§#
Periostin (ng/mL)	42.8±9.3 [2]	53.1±18.9 [4]	54.9±20.3 [9]	(49.7±5.5) [4]	0.266§#
%FEV1 post-bronchodilator (%)##	73.7±18.2	78.8±21.1	68.6±21.1	(105.2±11.5)	0.182§#
FEV1/FVC post-bronchodilator (%)##	61.5±10.1	63.4±12.2	60.2±13.9	(79.0±5.9)	0.627§#
Airway reversibility (%)	15.0±9.5	16.7±12.7	17.3±20.4 [1]	N.R.	0.745§
Airflow limitation [92]	7 (63.6)	11 (50.0)	28 (75.7)	N.R.	0.133*
Average ACQ-7	2.87±1.31 [1]	2.67±0.98 [3]	2.68±1.19 [4]	N.R.	0.830§
Average AQLQ	4.15±1.57 [1]	4.62±1.04 [5]	4.35±1.29 [2]	N.R.	0.506§
Exacerbation in previous year (n/y)	2.6±3.3	2.1±1.9	2.4±1.9	N.R.	0.747§
ER visit due to breathing problems	5 (45.5)	14 (63.6)	25 (62.2)	N.R.	0.441*
Commorbidities					
Allergic rhinitis (%)	2 (25.0) [3]	8 (40.0) [2]	16 (55.2) [8]	N.R.	0.285*
Nasal polyp (%)	2 (20.0) [1]	7 (33.3) [1]	12 (34.3) [2]	N.R.	0.720*
Sinusitis (%)	2 (25.0) [3]	6 (28.6) [1]	9 (28.1) [5]	N.R.	1.00*
Chronic bronchitis (%)	1 (11.1) [1]	2 (9.1)	4 (12.1) [4]	N.R.	1.00*
Psychiatric disease (%)	3 (33.3) [2]	3 (14.3) [1]	5 (13.9) [1]	N.R.	4.63E-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.	1.00*
Systemic corticosteroids (%)	3 (30.0) [1]	14 (63.6)	16 (45.7) [2]	N.R.	0.193*
Oral corticosteroid dose (mg/day)	2.50±4.71 [1]	7.89±8.01 [3]	4.18±6.61 [2]	N.R.	0.0853§
Anti-IgE therapy (%)	0 (0.0) [1]	2 (4.0) [2]	0 (0.0) [2]	N.R.	0.113*
Long-acting beta agonist (%)	11 (100.0)	21 (95.5)	37 (100.0)	N.R.	0.471*
Leukotriene modifiers (%)	4 (36.4)	11 (52.4) [1]	19 (51.4)	N.R.	0.680*
Tiotropium (%)	3 (30.0) [1]	4 (22.2) [2]	12 (34.3) [2]	N.R.	0.926*
Macrolide (%)	2 (18.2)	3 (13.6)	4 (10.8)	N.R.	1.00*

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 CSSA, ¦p<5.00E-02 vs ESSA, ¶p< 5.00E-02 vs NSSA. 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, FEV_1: 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 CSSA-NH and NSSA-NH by sputum somaLogic.

Probe ID	Protein target	Gene symbol	Gene name	Function
CSSA-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.
NSSA-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 inflammed 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.
CSSA-NH and NSSA-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 ESSA-NH and NSSA-NH.

Probe ID	Protein target	Gene symbol	Gene name	Function
ESSA-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.
NSSA-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 inflammed 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.
ESSA-NH and NSSA-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 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.

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.

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).

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). CSSA: circles, ESSA: squares, NSSA: triangles, NH: inverted triangles. RFU: Relative Fluorescence Units, *p<0.05; **p<0.01; ***p<0.001.

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 CSSA (red circles), ESSA (olive green circles), NSSA (cyan circles) and NH (purple circles) subjects. *p<0.05; **p<0.01; ***p<0.001.

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 CSSA (red circles), ESSA (olive green circles), NSSA (cyan circles) and NH (purple circles) subjects. *p<0.05; **p<0.01; ***p<0.001.

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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