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DOI:
10.1183/13993003.02173-2017

Document Version
Accepted author manuscript

EUROPEAN RESPIRATORY journal
OFFICIAL SCIENTIFIC JOURNAL OF THE ERS

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Journal:	*European Respiratory Journal*
Manuscript ID	ERJ-02173-2017.R1
Manuscript Type:	Original Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Takahashi, Kentaro; Imperial College London; Reaserch Centre For Allergy and Clinical Immunology, Asahi General Hospital Pavlidis, Stelios; Imperial College London, Computing Ng kee kwong, Francois; National Heart and Lung Institute, Airways Hoda, Uruj; Imperial College London Rossios, Christos; Imperial College London Sun, Kai; Imperial College London Loza, matt; Janssen Research and Development Spring House, Immunology Therapeutic Area Baribaud, Frederic; Janseen R&D Ltd, Systems Pharmacology & Biomarkers Chanez, Pascal; University of Marseille, Fowler, Stephen; University of Manchester, Respiratory Research Group; Horváth, Ildikó; Semmelweis University Montuschi, Paolo; Catholic University of the Sacred Heart, Pharmacology Singer, Florian; University Children's Hospital Bern, ; University Children's Hospital Zurich, Musial, Jacek; Jagellonian University Medical College Dahlén, Barbro; Karolinska Institutet, Internal Medicine, Division of Respiratory medicine and Allergy Dahlén, Sven-Erik; Karolinska Institutet, Cfa=Centre for Allergy Research; Krug, Norbert; Fraunhofer Institute for Toxicology and Experimental Medicine, Sandstrom, Thomas; Department of Respiratory Medicine and Allergy, Lutter, Rene; Academic Medical Center, Lab. Exp. Immunology/Dept. Pulmonology Bakke, Per Fleming, Louis; Imperial College London Howarth, Peter; University of Southampton, School of Medicine, Clinical and Experimental Sciences Caruso, Massimo; IUniversity of Catania Sousa, Ana; GlaxoSmithKline Plc, Respiratory therapy Unit Corfield, Julie; Areteva R&D, Shaw, Dominic; University of Nottingham , Respiratory Research Unit Auffray, Charles; EISBM, EISBM Projects Team De Meulder, Bertrand; EISBM,

	Lefaudeux, Diane; EISBM Djukanovic, Ratko; University of Southampton Sterk, Peter; Academic Medical Centre, University of Amsterdam, Respiratory Medicine, F5-259 Guo, Yike; Imperial College, Data Science Institute; Imperial College London Adcock, Ian; Imperial College London, Thoracic Medicine Chung, Kian Fan; Imperial College, National Heart & Lung Institute
Key Words:	asthma, severe asthma, cigarette smoking, differential gene expression, gene set variation analysis

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

Kentaro Takahashi [1,2], Stelios Pavlidis[3], Francois Ng Kee Kwong [1], Uruj Hoda [1], Christos Rossios[1], Kai Sun[3], Matthew Loza[4], Fred Baribaud[4], Pascal Chanez[5], Steve J Fowler[6], Ildiko Horvath[7], Paolo Montuschi[8], Florian Singer[9], Jacek Musial[10], Barbro Dahlen[11], Sven-Eric Dahlen[11], N. Krug[12], Thomas Sandstrom[13], Dominic E. Shaw[14], Rene Lutter [15], Per Bakke[16], Louise J. Fleming[1], Peter H. Howarth[17], Massimo Caruso[18], Ana R Sousa[19], Julie Corfield[20], Charles Auffray[21], Bertrand De Meulder[21], Diane Lefaudeux[21], Ratko Djukanovic[17], Peter J Sterk[16], Yike Guo[3], Ian M. Adcock[1,3], Kian Fan Chung[1,3] *on behalf of the U-BIOPRED study group*[#]

[1]National Heart & Lung Institute, Imperial College London, & Biomedical Research Unit, Biomedical Research Unit, Royal Brompton & Harefield NHS Trust, London, United Kingdom;

[2]Research Centre for Allergy and Clinical Immunology, Asahi General Hospital, Japan;

[3]Department of Computing & Data Science Institute, Imperial College London, United Kingdom;

[4]Janssen Research and Development, High Wycombe, Buckinghamshire, United Kingdom;

[5] Assistance Publique des Hôpitaux de Marseille - Clinique des bronches, allergies et sommeil, Aix Marseille Université, Marseille, France

[6] Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom

[7] Semmelweis University, Budapest, Hungary

[8] Università Cattolica del Sacro Cuore, Milan, Italy;

[9] Bern University Hospital, University of Bern, Bern, Switzerland;

[10] Dept. of Medicine, Jagiellonian University Medical College, Krakow, Poland

[11] Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden

[12] Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany;

[13] Dept of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden;

[14] Respiratory Research Unit, University of Nottingham, UK;

[15] Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;

[16] Department of Clinical Science, University of Bergen, Bergen, Norway;

[17] NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK

[18] Dept. Clinical and Experimental Medicine, University of Catania, Catania, Italy;

[19]Respiratory Therapeutic Unit, GSK, Stockley Park, UK;

[20]AstraZeneca R&D Molndal, Sweden and Areteva R&D, Nottingham, UK;

[21] European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France

[#]Consortium project team members are listed under *Acknowledgements*

Word count: 3506

Figures: 7; Tables: 3

On line Supplementary:

3 supplementary figures

10 Supplementary Tables

One Supplementary file

3 additional xls files

Running title: Distinct pathways in smoking-associated asthma

Corresponding author

Professor K F Chung,

National Heart & Lung Institute,

Imperial College London,

Dovehouse Street,

London SW3 6LY, UK

Email: f.chung@imperial.ac.uk

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 metallopeptidase 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

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

MATERIALS and METHODS

Clinical data

We enrolled totally 374 severe asthma patients in the U-BIOPRED cohort divided into three groups by smoking status; current-smokers with severe asthma (**CSA**), ex-smokers with severe asthma (**ESA**), non-smokers with severe asthma (**NSA**). We narrowed down the NSA to those who had never smoked (0 pack-year), although original non-smokers with severe asthma group in the U-BIOPRED cohort contained the patients whose pack-year was less than 5. Eighty-one non-smoking healthy volunteers (**NH**) whose pack-years were 0 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 obtained (8, 9). Bronchial biopsies, bronchial brushings and sputum were obtained, as previously described (8). Because of the bronchoscopy exclusion criteria (Supplementary File), only 95 bronchial brushings and 69 bronchial biopsies were obtained. The number of sputum samples for proteomic analysis was 88. All subjects whose samples were adequate and underwent omics analyses are shown in **Supplementary Figure S1**. 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™ 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 and 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 CSA subjects were lower than in the other severe asthma groups. Although there were numerical in blood eosinophil counts (CSA 259/μL, ESA 296/μL, NSA 407/μL), 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.

34 DEPs distinguished ESA-NH from NSA-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 ESA-NH and NSA-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 CSA was associated with proteins involved in macrophage recruitment and mucus production and both ESA and NSA 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 ESA than to other groups. However, this was not reflected in a significant difference in sputum neutrophilia in these subjects (Table 1). In addition, the protein expression of CAT, a key antioxidant, was upregulated equally in all severe asthma groups compared with NH (Figure 2G).

Pathway analysis of differentially-expressed proteins

Pathway analysis of sputum DEPs indicated that ESA-NH was associated with phagocytosis, response to chemicals, response to multicellular organisms, chemotaxis, myeloid cell differentiation and innate immunity and inflammation whilst NSA-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 CSA compared to NSA subjects providing bronchial brushings and biopsies for analysis although the proportion of patients who took systemic corticosteroids or the dose of oral corticosteroids was no different between the 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 CSA and NSA

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

The DEGs between CSA and NSA 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 CSA 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 CSA 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 CSA compared to NSA in bronchial brushings and biopsies (Figure 4E).

Pathway analysis using DEGs between CSA and NSA

Pathway analysis indicated that oxidation-reduction, chemical metabolism and endoplasmic reticulum (ER) stress were different between CSA and NSA (Supplementary Table S6, S7). These results suggest that the lung epithelial cells of CSA patients are under more potent chemical, oxidative and ER stresses than those of NSA 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 CSA group. There were no significant differences between ESA and NSA for these pathways. Using the signatures for active smoking obtained from Spira and colleagues (21), we confirmed that bronchial brushings and biopsies from CSA were enriched for the active smoking-related gene and that both CSA and ESA were enriched for the pack-year signature (Supplementary Table 1; Supplementary Figure S3).

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

As we could not detect any DEGs between ESA and NSA at the FDR<0.05 level, we undertook a discovery approach using a less stringent analysis strategy. Genes whose absolute fold-change was ≥2.0 in limma were used to clarify the phenotypic difference between ESA and NSA (Additional File 2). Twenty-seven genes (thirty-five probes) were up-regulated in ESA 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 (22). Innate immunity, including complement system, TLR signalling and neutrophilic inflammation, may be characteristics of ESA.

Six down-regulated DEGs (fold-change ≤ 0.5) distinguished ESA from NSA 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 (23). CST1 is a cysteine proteinase inhibitor that has a protective effect on epithelium (24). MUC2 provides a protective barrier for airways against particles or infectious agents (25). This suggests that ESA has a lesser protective epithelial barrier and reduced mast cell activity compared with NSA.

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

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 (29), which indicates that the CSF2 pathway can mediate smoke-induced inflammation. Future experiments in models of severe asthma linked to smoking or in selected patients may determine whether the elevated CSF2 expression seen here is causal or a marker of other driver mechanisms.

Our data provide evidence for enhanced oxidative and ER stress in airway epithelial cells of CSA 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 (30). Cigarette smoke not only contains high concentrations of reactive oxygen species (ROS) (30), but also activates alveolar macrophages and neutrophils, which also release ROS leading to an increased inflammatory response in a feed-forward process (30, 31). In both asthma and COPD, activated inflammatory cells including neutrophils, macrophages and eosinophils also produce ROS and further generate inflammation and causes injury to the airway epithelium (30). Moreover, impaired upregulation and production of protective antioxidant was reported in smokers, asthma and COPD patients. This oxidant-antioxidant imbalance resulting in oxidative stress is associated with airway hyperresponsiveness and decreased lung function and asthma severity (30, 32).

CSA represented the escalated response to oxidative stress derived from cigarette smoking as CSA bronchial brushings and biopsies alone were enriched for the active smoking-related gene set whereas both CSA and ESA samples showed a similar enrichment of the pack-year signature. Increased antioxidant gene expression and increased enrichment of the gene set showing response to oxidative stress were observed in bronchial brushings, which may suggest that cigarette smoking stimulates airway epithelial cells to respond to oxidative stress in severe asthma. We also showed that ER stress might have a key role in CSA phenotype. ER stress is associated with neutrophilic asthma through NF-κB activation and proinflammatory cytokine production (33). Cigarette smoking

itself may induce ER stress (34) and the activation seen here in severe asthma might relate to active cigarette smoke exposure. However, CSF2 and AGR2 have not been shown to be differentially-expressed in the previously-published proteomic analysis of sputum from healthy current-smokers compared to never-smokers (35). Moreover, endoplasmic stress and lysosome gene sets that we found to be differentially expressed in these 2 groups of severe asthma were not differentially-expressed in healthy current smokers compared to non-smokers (Supplementary Table S9 and S10) (21). These results suggest that DEGs between CSA and NSA were not derived from the influence of cigarette smoking itself.

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

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

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

Acknowledgements:

The U-BIOPRED project is supported through an Innovative Medicines Initiative joint undertaking under grant agreement 115010, resources of which are composed of financial contributions from the European Union's Seventh Framework Programme (FP7/2007-2013) and European Federation of Pharmaceutical Industries and Associations companies' in-kind contributions (www.imi.europa.eu).

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/m2)	27.7±4.7	31.1±6.7	27.5±5.7	25.3±3.2 ǂ	**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 (*/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, ǂp<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, 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 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 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.
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 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.
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±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,

and red rectangles represent samples with high expression for the particular gene. CSA (dark green), ESA (light green) and NSA (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). CSA: circles, ESA: squares, NSA: 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 CSA (red circles), ESA (olive green circles), NSA (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 CSA (red circles), ESA (olive green circles), NSA (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.

ACKNOWLEDGEMENTS

The U-BIOPRED consortium wishes to acknowledge the help and expertise of the following individuals and groups without whom, the study would not have been possible:

U-BIOPRED Supplementary authors	
Name	**Affiliation**
Adcock I M	National Heart and Lung Institute, Imperial College, London, UK;
Ahmed H	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Auffray C	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Bakke P	Department of Clinical Science, University of Bergen, Bergen, Norway;
Banssal A T	Acclarogen Ltd, St. John's Innovation Centre, Cambridge, UK;
Baribaud F	Janssen R&D, USA;
Bates S	Respiratory Therapeutic Unit, GSK, London, UK;
Bel E H	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Bigler J	*Previously Amgen Inc*
Bisgaard H	COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark
Boedigheimer M J	Amgen Inc.; Thousand Oaks, USA
Bønnelykke K	COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark;
Brandsma J	University of Southampton, Southampton, UK
Brinkman P	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Bucchioni E	Chiesi Pharmaceuticals SPA, Parma, Italy
Burg D	Centre for Proteomic Research, Institute for Life Sciences, University of Southampton, Southampton, UK
Bush A	National Heart and Lung Institute, Imperial College, London, UK; Royal Brompton and Harefield NHS trust, UK
Caruso M	Dept. Clinical and Experimental Medicine, University of Catania, Catania, Italy;
Chaiboonchoe A	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Chanez P	Assistance publique des Hôpitaux de Marseille - Clinique des bronches, allergies et sommeil, Aix Marseille Université, Marseille, France
Chung K F	National Heart and Lung Institute, Imperial College, London, UK;
Compton C H	Respiratory Therapeutic Unit, GSK, London, UK
Corfield J	Areteva R&D, Nottingham, UK;
D'Amico A	University of Rome 'Tor Vergata', Rome Italy;
Dahlen S E	Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
De Meulder B	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Djukanovic R	NIHR Southampton Respiratory Biomedical Research Unit and Clinical and Experimental Sciences, Southampton, UK;
Erpenbeck V J	Translational Medicine, Respiratory Profiling, Novartis Institutes for Biomedical Research, Basel, Switzerland;
Erzen D	Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Fichtner K	Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Fitch N	BioSci Consulting, Maasmechelen, Belgium;
Fleming L J	National Heart and Lung Institute, Imperial College, London, UK;

	Royal Brompton and Harefield NHS trust, UK
Formaggio E	*Previously CROMSOURCE, Verona Italy*
Fowler S J	Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom
Frey U	University Children's Hospital, Basel, Switzerland;
Gahlemann M	Boehringer Ingelheim (Schweiz) GmbH,Basel, Switzerland;
Geiser T	Department of Respiratory Medicine, University Hospital Bern, Switzerland;
Guo Y	Data Science Institute, Imperial College, London, UK;
Hashimoto S	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Haughney J	International Primary Care Respiratory Group, Aberdeen, Scotland;
Hedlin G	Dept. Women's and Children's Health & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden;
Hekking P W	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Higenbottam T	Allergy Therapeutics, West Sussex, UK;
Hohlfeld J M	Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany
Holweg C	Respiratory and Allergy Diseases, Genentech, San Francisco, USA
Horváth I	Semmelweis University, Budapest, Hungary
Howarth P	NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK
James A J	Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden;
Knowles R	Arachos Pharma, Stevenge, UK;
Knox A J	Respiratory Research Unit, University of Nottingham, Nottingham, UK;
Krug N	Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany;
Lefaudeux D	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Loza M J	Janssen R&D, USA;
Lutter R	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Manta A	Roche Diagnostics GmbH, Mannheim, Germany
Masefield S	European Lung Foundation, Sheffield, UK;
Matthews J G	Respiratory and Allergy Diseases, Genentech, San Francisco, USA;
Mazein A	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Meiser A	Data Science Institute, Imperial College, London, UK
Middelveld R J M	Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Miralpeix M	Almirall, Barcelona, Spain;
Montuschi P	Università Cattolica del Sacro Cuore, Milan, Italy;
Mores N	Università Cattolica del Sacro Cuore, Milan, Italy;
Murray C S	Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom
Musial J	Dept. of Medicine, Jagiellonian University Medical College, Krakow, Poland
Myles D	Respiratory Therapeutic Unit, GSK, London, UK;
Pahus L	Assistance publique des Hôpitaux de Marseille, Clinique des bronches, allergies et sommeil Espace Éthique Méditerranéen, Aix-Marseille Université, Marseille,

	France;
Pandis I	Data Science Institute, Imperial College, London, UK
Pavlidis S	National Heart and Lung Institute, Imperial College, London, UK
Powel P	European Lung Foundation, Sheffield, UK;
Praticò G	CROMSOURCE, Verona, Italy
Puig Valls M	CROMSOURCE, Barcelona, Spain
Rao N	Janssen R&D, USA;
Riley J	Respiratory Therapeutic Unit, GSK, London, UK;
Roberts A	Asthma UK, London, UK;
Roberts G	NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK;
Rowe A	Janssen R&D, UK;
Sandström T	Dept of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden;
Seibold W	Boehringer Ingelheim Pharma GmbH, Biberach, Germany
Selby A	NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK;
Shaw D E	Respiratory Research Unit, University of Nottingham, UK;
Sigmund R	Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Singer F	University Children's Hospital, Zurich, Switzerland;
Skipp P J	Centre for Proteomic Research, Institute for Life Sciences, University of Southampton, Southampton, UK
Sousa A R	Respiratory Therapeutic Unit, GSK, London, UK;
Sterk P J	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Sun K	Data Science Institute, Imperial College, London, UK
Thornton B	MSD, USA
van Aalderen W M	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
van Geest M	AstraZeneca, Mölndal, Sweden;
Vestbo J	Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom
Vissing N H	COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark;
Wagener A H	Academic Medical Center Amsterdam, Amsterdam, The Netherlands
Wagers S S	BioSci Consulting, Maasmechelen, Belgium
Weiszhart Z	Semmelweis University, Budapest, Hungary;
Wheelock C E	Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden;
Wilson S J	Histochemistry Research Unit, Faculty of Medicine, University of Southampton, Southampton, UK;

Contributors

Aliprantis Antonios, Merck Research Laboratories, Boston, USA;
Allen David, North West Severe Asthma Network, Pennine Acute Hospital NHS Trust, UK
Alving Kjell, Dept Women's & Children's Health, Uppsala University, Uppsala, Sweden
Badorrek P, Fraunhofer ITEM; Hannover, Germany
Balgoma David, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden

Ballereau S, European institute for Systems Biology and Medicine, University of Lyon, France
Barber Clair, NIHR Southampton Respiratory Biomedical Research Unit and Clinical and Experimental Sciences, Southampton, UK;
Batuwitage Manohara Kanangana, Data Science Institute, Imperial College, London, UK
Bautmans An, MSD, Brussels, Belgium
Bedding A, Roche Diagnostics GmbH, Mannheim, Germany
Behndig AF, Umeå University, Umea, Sweden
Beleta Jorge, Almirall S.A., Barcelona, Spain;
Berglind A, MSD, Brussels, Belgium
Berton A, AstraZeneca, Mölndal, Sweden
Bochenek Grazyna, II Department of Internal Medicine, Jagiellonian University Medical College, Krakow, Poland;
Braun Armin, Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany;
Campagna D, Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy;
Carayannopoulos Leon, Previously at: MSD, USA;
Casaulta C, University Children's Hospital of Bern, Switzerland
Chaleckis Romanas, Centre of Allergy Research, Karolinska Institutet, Stockholm, Sweden
Dahlén B, Karolinska University Hospital & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Davison Timothy Janssen R&D, USA;
De Alba Jorge, Almirall S.A., Barcelona, Spain;
De Lepeleire Inge, MSD, Brussels, BE
Dekker Tamara, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Delin Ingrid, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Dennison P, NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences, NIHR-Wellcome Trust Clinical Research Facility, Faculty of Medicine, University of Southampton, Southampton, UK;
Dijkhuis Annemiek, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Dodson Paul, AstraZeneca, Mölndal, Sweden
Draper Aleksandra, BioSci Consulting, Maasmechelen, Belgium;
Dyson K, CROMSOURCE; Stirling, UK
Edwards Jessica, Asthma UK, London, UK;
El Hadjam L, European Institute for Systems Biology and Medicine, University of Lyon
Emma Rosalia, Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy;
Ericsson Magnus, Karolinska University Hospital, Stockholm, Sweden
Faulenbach C, Fraunhofer ITEM; Hannover, Germany
Flood Breda, European Federation of Allergy and Airways Diseases Patient's Associations, Brussels, Belgium

Galffy G, Semmelweis University, Budapest, Hungary;
Gallart Hector, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Garissi D, Global Head Clinical Research Division, CROMSOURCE, Italy
Gent J, Royal Brompton and Harefield NHS Foundation Trust, London, UK;
Gerhardsson de Verdier M, AstraZeneca; Mölndal, Sweden;
Gibeon D, National Heart and Lung Institute, Imperial College, London, UK;
Gomez Cristina, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Gove Kerry, NIHR Southampton Respiratory Biomedical Research Unit and Clinical and Experimental Sciences, Southampton, UK;
Gozzard Neil, UCB, Slough, UK;
Guillmant-Farry E, Royal Brompton Hospital, London, UK
Henriksson E, Karolinska University Hospital & Karolinska Institutet, Stockholm, Sweden
Hewitt Lorraine, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Hoda U, Imperial College, London, UK
Hu Richard, Amgen Inc. Thousand Oaks, USA
Hu Sile, National Heart and Lung Institute, Imperial College, London, UK;
Hu X, Amgen Inc.; Thousand Oaks, USA
Jeyasingham E, UK Clinical Operations, GSK, Stockley Park, UK
Johnson K, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Jullian N, European Institute for Systems Biology and Medicine, University of Lyon
Kamphuis Juliette, Longfonds, Amersfoort, The Netherlands;
Kennington Erika J., Asthma UK, London, UK;
Kerry Dyson, CromSource, Stirling, UK;
Kerry G, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Klüglich M, Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Knobel Hugo, Philips Research Laboratories, Eindhoven, The Netherlands;
Kolmert Johan, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Konradsen J R, Dept. Women's and Children's Health & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Kots Maxim, Chiesi Pharmaceuticals, SPA, Parma, Italy;
Kretsos Kosmas, UCB, Slough, UK
Krueger L, University Children's Hospital Bern, Switzerland
Kuo Scott, National Heart and Lung Institute, Imperial College, London, UK;
Kupczyk Maciej, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Lambrecht Bart, University of Gent, Gent, Belgium;
Lantz A-S, Karolinska University Hospital & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Larminie Christopher, GSK, London, UK
Larsson L X, AstraZeneca, Mölndal, Sweden

Latzin P, University Children's Hospital of Bern, Bern, Switzerland
Lazarinis N, Karolinska University Hospital & Karolinska Institutet, Stockholm, Sweden
Lemonnier N, European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Lone-Latif Saeeda, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Lowe L A, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Manta Alexander, Roche Diagnostics GmbH, Mannheim, Germany
Marouzet Lisa, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Martin Jane, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Mathon Caroline, Centre of Allergy Research, Karolinska Institutet, Stockholm, Sweden
McEvoy L, University Hospital, Department of Pulmonary Medicine, Bern, Switzerland
Meah Sally, National Heart and Lung Institute, Imperial College, London, UK;
Menzies-Gow A, Royal Brompton and Harefield NHS Foundation Trust, London, UK;
Metcalf Leanne, Previously at: Asthma UK, London, UK;
Mikus Maria, Science for Life Laboratory & The Royal Institute of Technology, Stockholm, Sweden;
Monk Philip, Synairgen Research Ltd, Southampton, UK;
Naz Shama, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Nething K, Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Nicholas Ben, University of Southampton, Southampton, UK
Nihlén U, *Previously AstraZeneca; Mölndal, Sweden;*
Nilsson Peter, Science for Life Laboratory & The Royal Institute of Technology, Stockholm, Sweden;
Niven R, North West Severe Asthma Network, University Hospital South Manchester, UK
Nordlund B, Dept. Women's and Children's Health & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Nsubuga S, Royal Brompton Hospital, London, UK
Östling Jörgen, AstraZeneca, Mölndal, Sweden;
Pacino Antonio, Lega Italiano Anti Fumo, Catania, Italy;
Palkonen Susanna, European Federation of Allergy and Airways Diseases Patient's Associations, Brussels, Belgium.
Pellet J, European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Pennazza Giorgio, University of Rome 'Tor Vergata', Rome Italy;
Petrén Anne, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Pink Sandy, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Pison C, European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Postle Anthony, University of Southampton, UK
Rahman-Amin Malayka, Previously at: Asthma UK, London, UK;

Ravanetti Lara, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Ray Emma, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Reinke Stacey, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Reynolds Leanne, Previously at: Asthma UK, London, UK;
Riemann K, Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Robberechts Martine, MSD, Brussels, Belgium
Rocha J P, Royal Brompton and Harefield NHS Foundation Trust
Rossios C, National Heart and Lung Institute, Imperial College, London, UK;
Russell Kirsty, National Heart and Lung Institute, Imperial College, London, UK;
Rutgers Michael, Longfonds, Amersfoort, The Netherlands;
Santini G, Università Cattolica del Sacro Cuore, Milan, Italy;
Santoninco Marco, University of Rome 'Tor Vergata', Rome Italy;
Saqi M, European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Schoelch Corinna, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
Schofield James P. R., Centre for Proteomic Research, Institute for Life Sciences, University of Southampton, Southampton, UK
Scott S, North West Severe Asthma Network, Countess of Chester Hospital, UK
Sehgal N, North West Severe Asthma Network; Pennine Acute Hospital NHS Trust
Sjödin Marcus, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Smids Barbara, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Smith Caroline, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Smith Jessica, Asthma UK, London, UK;
Smith Katherine M., University of Nottingham, UK;
Söderman P, Dept. Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden
Sogbessan A, Royal Brompton and Harefield NHS Foundation Trust, London, UK;
Spycher F, University Hospital Department of Pulmonary Medicine, Bern, Switzerland
Staykova Doroteya, University of Southampton, Southampton, UK
Stephan S, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Stokholm J, University of Copenhagen and Danish Pediatric Asthma Centre Denmark
Strandberg K, Karolinska University Hospital & Karolinska Institutet, Stockholm, Sweden
Sunther M, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Szentkereszty M, Semmelweis University, Budapest, Hungary;
Tamasi L, Semmelweis University, Budapest, Hungary;
Tariq K, NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences, NIHR-Wellcome Trust Clinical Research Facility, Faculty of Medicine, University of Southampton, Southampton, UK;
Thörngren John-Olof, Karolinska University Hospital, Stockholm, Sweden

Thorsen Jonathan, COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark;
Valente S, Università Cattolica del Sacro Cuore, Milan, Italy;
van de Pol Marianne, Academic Medical Centre, University of Amsterdam, Amsterdam ,The Netherlands;
van Drunen C M, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Van Eyll Jonathan, UCB, Slough, UK
Versnel Jenny, Previously at: Asthma UK, London, UK;
Vink Anton, Philips Research Laboratories, Eindhoven, The Netherlands;
von Garnier C, University Hospital Bern, Switzerland;
Vyas A, North west Severe Asthma Network, Lancashire Teaching Hospitals NHS Trust, UK
Wald Frans, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
Walker Samantha, Asthma UK, London, UK;
Ward Jonathan, Histochemistry Research Unit, Faculty of Medicine, University of Southampton, Southampton, UK;
Wetzel Kristiane, Boehringer Ingelheim Pharma GmbH, Biberach, Germany
Wiegman Coen, National Heart and Lung Institute, Imperial College, London, UK;
Williams Siân, International Primary Care Respiratory Group, Aberdeen, Scotland;
Yang Xian, Data Science Institute, Imperial College, London, UK
Yeyasingham Elizabeth, UK Clinical Operations, GSK, Stockley Park, UK;
Yu W, Amgen Inc.; Thousand Oaks, USA
Zetterquist W, Dept. Women's and Children's Health & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Zolkipli Z, NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK;
Zwinderman A H, Academic Medical Centre, University of Amsterdam, The Netherlands;

Partner organisations	
Novartis Pharma AG	University of Southampton, Southampton, UK
Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands	Imperial College London, London, UK
University of Catania, Catania, Italy	University of Rome 'Tor Vergata', Rome, Italy
Hvidore Hospital, Hvidore, Denmark	Jagiellonian Univ. Medi.College, Krakow, Poland
University Hospital, Inselspital, Bern, Switzerland	Semmelweis University, Budapest, Hungary
University of Manchester, Manchester, UK	Université d'Aix-Marseille, Marseille, France
Fraunhofer Institute, Hannover, Germany	University Hospital, Umea, Sweden
Ghent University, Ghent, Belgium	Ctr. Nat. Recherche Scientifique, Lyon, France
Università Cattolica del Sacro Cuore, Rome, Italy	University Hospital, Copenhagen, Denmark
Karolinska Institutet, Stockholm, Sweden	Nottingham University Hospital, Nottingham, UK

University of Bergen, Bergen, Norway	Netherlands Asthma Foundation, Leusden, NL
European Lung Foundation, Sheffield, UK	Asthma UK, London, UK
European. Fed. of Allergy and Airways Diseases Patients' Associations, Brussels, Belgium	Lega Italiano Anti Fumo, Catania, Italy
International Primary Care Respiratory Group, Aberdeen, Scotland	Philips Research Laboratories, Eindhoven, NL
Synairgen Research Ltd, Southampton, UK	Aerocrine AB, Stockholm, Sweden
BioSci Consulting, Maasmechelen, Belgium	Almirall
AstraZeneca	Boehringer Ingelheim
Chiesi	GlaxoSmithKline
Roche	UCB
Janssen Biologics BV	Amgen NV
Merck Sharp & Dome Corp	

MEMBERS OF THE ETHICS BOARD			
Name	**Task**	**Affiliation**	**e-mail**
Jan-Bas Prins	Biomedical research	LUMC/the Netherlands	J.B.Prins@lumc.nl
Martina Gahlemann	Clinical care	BI/Germany	Martina.Gahlemann@boehringer-ingelheim.com
Luigi Visintin	Legal affairs	LIAF/Italy	visintin@inrete.it
Hazel Evans	Paediatric care	Southampton/UK	hazel.evans@uhs.nhs.uk
Martine Puhl	Patient representation (co-chair)	NAF/ the Netherlands	martine@puhl.nl
Lina Buzermaniene	Patient representation	EFA/Lithuania	lina.buzermaniene@pavb.lt
Val Hudson	Patient representation	Asthma UK	hudsonval7@gmail.com
Laura Bond	Patient representation	Asthma UK	lvbond22@googlemail.com
Pim de Boer	Patient representation and pathobiology	IND	deboer.pim@hetnet.nl
Guy Widdershoven	Research ethics	VUMC/the Netherlands	g.widdershoven@vumc.nl
Ralf Sigmund	Research methodology and biostatistics	BI/Germany	ralf.sigmund@boehringer-ingelheim.com

THE PATIENT INPUT PLATFORM	
Name	**Country**
Amanda Roberts	UK
David Supple (chair)	UK
Dominique Hamerlijnck	The Netherlands
Jenny Negus	UK
Juliëtte Kamphuis	The Netherlands
Lehanne Sergison	UK
Luigi Visintin	Italy
Pim de Boer (co-chair)	The Netherlands
Susanne Onstein	The Netherlands

MEMBERS OF THE SAFETY MONITORING BOARD	
Name	**Task**
William MacNee	Clinical care
Renato Bernardini	Clinical pharmacology
Louis Bont	Paediatric care and infectious diseases

Per-Ake Wecksell	Patient representation
Pim de Boer	Patient representation and pathobiology (chair)
Martina Gahlemann	Patient safety advice and clinical care (co-chair)
Ralf Sigmund	Bio-informatician

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

Kentaro Takahashi [1,2], Stelios Pavlidis[3], Francois Ng Kee Kwong [1], Uruj Hoda [1], Christos Rossios[1], Kai Sun[3], Matthew Loza[4], Fred Baribaud[4], Pascal Chanez[5], Steve J Fowler[6], Ildiko Horvath[7], Paolo Montuschi[8], Florian Singer[9], Jacek Musial[10], Barbro Dahlen[11], Sven-Eric Dahlen[11], N. Krug[12], Thomas Sandstrom[13], Dominic E. Shaw[14], Rene Lutter [15], Per Bakke[16], Louise J. Fleming[1], Peter H. Howarth[17], Massimo Caruso[18], Ana R Sousa[19], Julie Corfield[20], Charles Auffray[21], Bertrand De Meulder[21], Diane Lefaudeux[21], Ratko Djukanovic[17], Peter J Sterk[16], Yike Guo[3], Ian M. Adcock[1,3], Kian Fan Chung[1,3] *on behalf of the U-BIOPRED study group*[#]

[1]National Heart & Lung Institute, Imperial College London, & Biomedical Research Unit, Biomedical Research Unit, Royal Brompton & Harefield NHS Trust, London, United Kingdom;

[2]Research Centre for Allergy and Clinical Immunology, Asahi General Hospital, Japan;

[3]Department of Computing & Data Science Institute, Imperial College London, United Kingdom;

[4]Janssen Research and Development, High Wycombe, Buckinghamshire, United Kingdom;

[5] Assistance Publique des Hôpitaux de Marseille - Clinique des bronches, allergies et sommeil, Aix Marseille Université, Marseille, France

[6] Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom

[7] Semmelweis University, Budapest, Hungary

[8] Università Cattolica del Sacro Cuore, Milan, Italy;

[9] Bern University Hospital, University of Bern, Bern, Switzerland;

[10] Dept. of Medicine, Jagiellonian University Medical College, Krakow, Poland

[11] Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden

[12] Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany;

[13] Dept of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden;

[14] Respiratory Research Unit, University of Nottingham, UK;

[15] Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;

[16] Department of Clinical Science, University of Bergen, Bergen, Norway;

[17] NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK

[18] Dept. Clinical and Experimental Medicine, University of Catania, Catania, Italy;

[19]Respiratory Therapeutic Unit, GSK, Stockley Park, UK;

[20]AstraZeneca R&D Molndal, Sweden and Areteva R&D, Nottingham, UK;

[21] European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France

[#]Consortium project team members are listed under *Acknowledgements*

Word count: 3506

Figures: 7; Tables: 3

On line Supplementary:

3 supplementary figures

10 Supplementary Tables

One Supplementary file

3 additional xls files

Running title: Distinct pathways in smoking-associated asthma

Corresponding author

Professor K F Chung,

National Heart & Lung Institute,

Imperial College London,

Dovehouse Street,

London SW3 6LY, UK

Email: f.chung@imperial.ac.uk

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 metallopeptidase 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

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

MATERIALS and METHODS

Clinical data

We enrolled totally 374 severe asthma patients in the U-BIOPRED cohort divided into three groups by smoking status; current-smokers with severe asthma (**CSA**), ex-smokers with severe asthma (**ESA**), non-smokers with severe asthma (**NSA**). We narrowed down the NSA to those who had never smoked (0 pack-year), although original non-smokers with severe asthma group in the U-BIOPRED cohort contained the patients whose pack-year was less than 5. Eighty-one non-smoking healthy volunteers (**NH**) whose pack-years were 0 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 obtained (8, 9). Bronchial biopsies, bronchial brushings and sputum were obtained, as previously described (8). Because of the bronchoscopy exclusion criteria (Supplementary File), only 95 bronchial brushings and 69 bronchial biopsies were obtained. The number of sputum samples for proteomic analysis was 88. All subjects whose samples were adequate and underwent omics analyses are shown in **Supplementary Figure S1**. 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™ 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 and 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 CSA subjects were lower than in the other severe asthma groups. Although there were numerical in blood eosinophil counts (CSA 259/μL, ESA 296/μL, NSA 407/μL), 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.

34 DEPs distinguished ESA-NH from NSA-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 ESA-NH and NSA-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 CSA was associated with proteins involved in macrophage recruitment and mucus production and both ESA and NSA 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 ESA than to other groups. However, this was not reflected in a significant difference in sputum neutrophilia in these subjects (Table 1). In addition, the protein expression of CAT, a key antioxidant, was upregulated equally in all severe asthma groups compared with NH (Figure 2G).

Pathway analysis of differentially-expressed proteins

Pathway analysis of sputum DEPs indicated that ESA-NH was associated with phagocytosis, response to chemicals, response to multicellular organisms, chemotaxis, myeloid cell differentiation and innate immunity and inflammation whilst NSA-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 CSA compared to NSA subjects providing bronchial brushings and biopsies for analysis although the proportion of patients who took systemic corticosteroids or the dose of oral corticosteroids was no different between the 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 CSA and NSA

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

The DEGs between CSA and NSA 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 CSA 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 CSA 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 CSA compared to NSA in bronchial brushings and biopsies (Figure 4E).

Pathway analysis using DEGs between CSA and NSA

Pathway analysis indicated that oxidation-reduction, chemical metabolism and endoplasmic reticulum (ER) stress were different between CSA and NSA (Supplementary Table S6, S7). These results suggest that the lung epithelial cells of CSA patients are under more potent chemical, oxidative and ER stresses than those of NSA 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 CSA group. There were no significant differences between ESA and NSA for these pathways. Using the signatures for active smoking obtained from Spira and colleagues (21), we confirmed that bronchial brushings and biopsies from CSA were enriched for the active smoking-related gene and that both CSA and ESA were enriched for the pack-year signature (Supplementary Table 1; Supplementary Figure S3).

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

As we could not detect any DEGs between ESA and NSA at the FDR<0.05 level, we undertook a discovery approach using a less stringent analysis strategy. Genes whose absolute fold-change was ≥2.0 in limma were used to clarify the phenotypic difference between ESA and NSA (Additional File 2). Twenty-seven genes (thirty-five probes) were up-regulated in ESA 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 (22). Innate immunity, including complement system, TLR signalling and neutrophilic inflammation, may be characteristics of ESA.

Six down-regulated DEGs (fold-change ≤ 0.5) distinguished ESA from NSA 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 (23). CST1 is a cysteine proteinase inhibitor that has a protective effect on epithelium (24). MUC2 provides a protective barrier for airways against particles or infectious agents (25). This suggests that ESA has a lesser protective epithelial barrier and reduced mast cell activity compared with NSA.

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

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 (29), which indicates that the CSF2 pathway can mediate smoke-induced inflammation. Future experiments in models of severe asthma linked to smoking or in selected patients may determine whether the elevated CSF2 expression seen here is causal or a marker of other driver mechanisms.

Our data provide evidence for enhanced oxidative and ER stress in airway epithelial cells of CSA 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 (30). Cigarette smoke not only contains high concentrations of reactive oxygen species (ROS) (30), but also activates alveolar macrophages and neutrophils, which also release ROS leading to an increased inflammatory response in a feed-forward process (30, 31). In both asthma and COPD, activated inflammatory cells including neutrophils, macrophages and eosinophils also produce ROS and further generate inflammation and causes injury to the airway epithelium (30). Moreover, impaired upregulation and production of protective antioxidant was reported in smokers, asthma and COPD patients. This oxidant-antioxidant imbalance resulting in oxidative stress is associated with airway hyperresponsiveness and decreased lung function and asthma severity (30, 32).

CSA represented the escalated response to oxidative stress derived from cigarette smoking as CSA bronchial brushings and biopsies alone were enriched for the active smoking-related gene set whereas both CSA and ESA samples showed a similar enrichment of the pack-year signature. Increased antioxidant gene expression and increased enrichment of the gene set showing response to oxidative stress were observed in bronchial brushings, which may suggest that cigarette smoking stimulates airway epithelial cells to respond to oxidative stress in severe asthma. We also showed that ER stress might have a key role in CSA phenotype. ER stress is associated with neutrophilic asthma through NF-κB activation and proinflammatory cytokine production (33). Cigarette smoking

itself may induce ER stress (34) and the activation seen here in severe asthma might relate to active cigarette smoke exposure. However, CSF2 and AGR2 have not been shown to be differentially-expressed in the previously-published proteomic analysis of sputum from healthy current-smokers compared to never-smokers (35). Moreover, endoplasmic stress and lysosome gene sets that we found to be differentially expressed in these 2 groups of severe asthma were not differentially-expressed in healthy current smokers compared to non-smokers (Supplementary Table S9 and S10) (21). These results suggest that DEGs between CSA and NSA were not derived from the influence of cigarette smoking itself.

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

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

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

Acknowledgements:

The U-BIOPRED project is supported through an Innovative Medicines Initiative joint undertaking under grant agreement 115010, resources of which are composed of financial contributions from the European Union's Seventh Framework Programme (FP7/2007-2013) and European Federation of Pharmaceutical Industries and Associations companies' in-kind contributions (www.imi.europa.eu).

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/m2)	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 (*/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, 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 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 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.
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 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.
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±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,

and red rectangles represent samples with high expression for the particular gene. CSA (dark green), ESA (light green) and NSA (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). CSA: circles, ESA: squares, NSA: 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 CSA (red circles), ESA (olive green circles), NSA (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 CSA (red circles), ESA (olive green circles), NSA (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.

ACKNOWLEDGEMENTS

The U-BIOPRED consortium wishes to acknowledge the help and expertise of the following individuals and groups without whom, the study would not have been possible:

U-BIOPRED Supplementary authors	
Name	**Affiliation**
Adcock I M	National Heart and Lung Institute, Imperial College, London, UK;
Ahmed H	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Auffray C	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Bakke P	Department of Clinical Science, University of Bergen, Bergen, Norway;
Banssal A T	Acclarogen Ltd, St. John's Innovation Centre, Cambridge, UK;
Baribaud F	Janssen R&D, USA;
Bates S	Respiratory Therapeutic Unit, GSK, London, UK;
Bel E H	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Bigler J	*Previously Amgen Inc*
Bisgaard H	COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark
Boedigheimer M J	Amgen Inc.; Thousand Oaks, USA
Bønnelykke K	COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark;
Brandsma J	University of Southampton, Southampton, UK
Brinkman P	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Bucchioni E	Chiesi Pharmaceuticals SPA, Parma, Italy
Burg D	Centre for Proteomic Research, Institute for Life Sciences, University of Southampton, Southampton, UK
Bush A	National Heart and Lung Institute, Imperial College, London, UK; Royal Brompton and Harefield NHS trust, UK
Caruso M	Dept. Clinical and Experimental Medicine, University of Catania, Catania, Italy;
Chaiboonchoe A	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Chanez P	Assistance publique des Hôpitaux de Marseille - Clinique des bronches, allergies et sommeil, Aix Marseille Université, Marseille, France
Chung K F	National Heart and Lung Institute, Imperial College, London, UK;
Compton C H	Respiratory Therapeutic Unit, GSK, London, UK
Corfield J	Areteva R&D, Nottingham, UK;
D'Amico A	University of Rome 'Tor Vergata', Rome Italy;
Dahlen S E	Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
De Meulder B	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Djukanovic R	NIHR Southampton Respiratory Biomedical Research Unit and Clinical and Experimental Sciences, Southampton, UK;
Erpenbeck V J	Translational Medicine, Respiratory Profiling, Novartis Institutes for Biomedical Research, Basel, Switzerland;
Erzen D	Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Fichtner K	Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Fitch N	BioSci Consulting, Maasmechelen, Belgium;
Fleming L J	National Heart and Lung Institute, Imperial College, London, UK;

	Royal Brompton and Harefield NHS trust, UK
Formaggio E	*Previously CROMSOURCE, Verona Italy*
Fowler S J	Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom
Frey U	University Children's Hospital, Basel, Switzerland;
Gahlemann M	Boehringer Ingelheim (Schweiz) GmbH,Basel, Switzerland;
Geiser T	Department of Respiratory Medicine, University Hospital Bern, Switzerland;
Guo Y	Data Science Institute, Imperial College, London, UK;
Hashimoto S	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Haughney J	International Primary Care Respiratory Group, Aberdeen, Scotland;
Hedlin G	Dept. Women's and Children's Health & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden;
Hekking P W	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Higenbottam T	Allergy Therapeutics, West Sussex, UK;
Hohlfeld J M	Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany
Holweg C	Respiratory and Allergy Diseases, Genentech, San Francisco, USA
Horváth I	Semmelweis University, Budapest, Hungary
Howarth P	NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK
James A J	Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden;
Knowles R	Arachos Pharma, Stevenge, UK;
Knox A J	Respiratory Research Unit, University of Nottingham, Nottingham, UK;
Krug N	Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany;
Lefaudeux D	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France;
Loza M J	Janssen R&D, USA;
Lutter R	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Manta A	Roche Diagnostics GmbH, Mannheim, Germany
Masefield S	European Lung Foundation, Sheffield, UK;
Matthews J G	Respiratory and Allergy Diseases, Genentech, San Francisco, USA;
Mazein A	European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Meiser A	Data Science Institute, Imperial College, London, UK
Middelveld R J M	Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Miralpeix M	Almirall, Barcelona, Spain;
Montuschi P	Università Cattolica del Sacro Cuore, Milan, Italy;
Mores N	Università Cattolica del Sacro Cuore, Milan, Italy;
Murray C S	Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom
Musial J	Dept. of Medicine, Jagiellonian University Medical College, Krakow, Poland
Myles D	Respiratory Therapeutic Unit, GSK, London, UK;
Pahus L	Assistance publique des Hôpitaux de Marseille, Clinique des bronches, allergies et sommeil Espace Éthique Méditerranéen, Aix-Marseille Université, Marseille,

	France;
Pandis I	Data Science Institute, Imperial College, London, UK
Pavlidis S	National Heart and Lung Institute, Imperial College, London, UK
Powel P	European Lung Foundation, Sheffield, UK;
Praticò G	CROMSOURCE, Verona, Italy
Puig Valls M	CROMSOURCE, Barcelona, Spain
Rao N	Janssen R&D, USA;
Riley J	Respiratory Therapeutic Unit, GSK, London, UK;
Roberts A	Asthma UK, London, UK;
Roberts G	NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK;
Rowe A	Janssen R&D, UK;
Sandström T	Dept of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden;
Seibold W	Boehringer Ingelheim Pharma GmbH, Biberach, Germany
Selby A	NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK;
Shaw D E	Respiratory Research Unit, University of Nottingham, UK;
Sigmund R	Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Singer F	University Children's Hospital, Zurich, Switzerland;
Skipp P J	Centre for Proteomic Research, Institute for Life Sciences, University of Southampton, Southampton, UK
Sousa A R	Respiratory Therapeutic Unit, GSK, London, UK;
Sterk P J	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Sun K	Data Science Institute, Imperial College, London, UK
Thornton B	MSD, USA
van Aalderen W M	Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
van Geest M	AstraZeneca, Mölndal, Sweden;
Vestbo J	Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom
Vissing N H	COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark;
Wagener A H	Academic Medical Center Amsterdam, Amsterdam, The Netherlands
Wagers S S	BioSci Consulting, Maasmechelen, Belgium
Weiszhart Z	Semmelweis University, Budapest, Hungary;
Wheelock C E	Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden;
Wilson S J	Histochemistry Research Unit, Faculty of Medicine, University of Southampton, Southampton, UK;

Contributors

Aliprantis Antonios, Merck Research Laboratories, Boston, USA;
Allen David, North West Severe Asthma Network, Pennine Acute Hospital NHS Trust, UK
Alving Kjell, Dept Women's & Children's Health, Uppsala University, Uppsala, Sweden
Badorrek P, Fraunhofer ITEM; Hannover, Germany
Balgoma David, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden

Ballereau S, European institute for Systems Biology and Medicine, University of Lyon, France
Barber Clair, NIHR Southampton Respiratory Biomedical Research Unit and Clinical and Experimental Sciences, Southampton, UK;
Batuwitage Manohara Kanangana, Data Science Institute, Imperial College, London, UK
Bautmans An, MSD, Brussels, Belgium
Bedding A, Roche Diagnostics GmbH, Mannheim, Germany
Behndig AF, Umeå University, Umea, Sweden
Beleta Jorge, Almirall S.A., Barcelona, Spain;
Berglind A, MSD, Brussels, Belgium
Berton A, AstraZeneca, Mölndal, Sweden
Bochenek Grazyna, II Department of Internal Medicine, Jagiellonian University Medical College, Krakow, Poland;
Braun Armin, Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany;
Campagna D, Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy;
Carayannopoulos Leon, Previously at: MSD, USA;
Casaulta C, University Children's Hospital of Bern, Switzerland
Chaleckis Romanas, Centre of Allergy Research, Karolinska Institutet, Stockholm, Sweden
Dahlén B, Karolinska University Hospital & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Davison Timothy Janssen R&D, USA;
De Alba Jorge, Almirall S.A., Barcelona, Spain;
De Lepeleire Inge, MSD, Brussels, BE
Dekker Tamara, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Delin Ingrid, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Dennison P, NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences, NIHR-Wellcome Trust Clinical Research Facility, Faculty of Medicine, University of Southampton, Southampton, UK;
Dijkhuis Annemiek, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Dodson Paul, AstraZeneca, Mölndal, Sweden
Draper Aleksandra, BioSci Consulting, Maasmechelen, Belgium;
Dyson K, CROMSOURCE; Stirling, UK
Edwards Jessica, Asthma UK, London, UK;
El Hadjam L, European Institute for Systems Biology and Medicine, University of Lyon
Emma Rosalia, Department of Clinical and Experimental Medicine, University of Catania, Catania, Italy;
Ericsson Magnus, Karolinska University Hospital, Stockholm, Sweden
Faulenbach C, Fraunhofer ITEM; Hannover, Germany
Flood Breda, European Federation of Allergy and Airways Diseases Patient's Associations, Brussels, Belgium

Galffy G, Semmelweis University, Budapest, Hungary;
Gallart Hector, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Garissi D, Global Head Clinical Research Division, CROMSOURCE, Italy
Gent J, Royal Brompton and Harefield NHS Foundation Trust, London, UK;
Gerhardsson de Verdier M, AstraZeneca; Mölndal, Sweden;
Gibeon D, National Heart and Lung Institute, Imperial College, London, UK;
Gomez Cristina, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Gove Kerry, NIHR Southampton Respiratory Biomedical Research Unit and Clinical and Experimental Sciences, Southampton, UK;
Gozzard Neil, UCB, Slough, UK;
Guillmant-Farry E, Royal Brompton Hospital, London, UK
Henriksson E, Karolinska University Hospital & Karolinska Institutet, Stockholm, Sweden
Hewitt Lorraine, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Hoda U, Imperial College, London, UK
Hu Richard, Amgen Inc. Thousand Oaks, USA
Hu Sile, National Heart and Lung Institute, Imperial College, London, UK;
Hu X, Amgen Inc.; Thousand Oaks, USA
Jeyasingham E, UK Clinical Operations, GSK, Stockley Park, UK
Johnson K, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Jullian N, European Institute for Systems Biology and Medicine, University of Lyon
Kamphuis Juliette, Longfonds, Amersfoort, The Netherlands;
Kennington Erika J., Asthma UK, London, UK;
Kerry Dyson, CromSource, Stirling, UK;
Kerry G, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Klüglich M, Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Knobel Hugo, Philips Research Laboratories, Eindhoven, The Netherlands;
Kolmert Johan, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Konradsen J R, Dept. Women's and Children's Health & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Kots Maxim, Chiesi Pharmaceuticals, SPA, Parma, Italy;
Kretsos Kosmas, UCB, Slough, UK
Krueger L, University Children's Hospital Bern, Switzerland
Kuo Scott, National Heart and Lung Institute, Imperial College, London, UK;
Kupczyk Maciej, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Lambrecht Bart, University of Gent, Gent, Belgium;
Lantz A-S, Karolinska University Hospital & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Larminie Christopher, GSK, London, UK
Larsson L X, AstraZeneca, Mölndal, Sweden

Latzin P, University Children's Hospital of Bern, Bern, Switzerland
Lazarinis N, Karolinska University Hospital & Karolinska Institutet, Stockholm, Sweden
Lemonnier N, European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Lone-Latif Saeeda, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Lowe L A, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Manta Alexander, Roche Diagnostics GmbH, Mannheim, Germany
Marouzet Lisa, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Martin Jane, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Mathon Caroline, Centre of Allergy Research, Karolinska Institutet, Stockholm, Sweden
McEvoy L, University Hospital, Department of Pulmonary Medicine, Bern, Switzerland
Meah Sally, National Heart and Lung Institute, Imperial College, London, UK;
Menzies-Gow A, Royal Brompton and Harefield NHS Foundation Trust, London, UK;
Metcalf Leanne, Previously at: Asthma UK, London, UK;
Mikus Maria, Science for Life Laboratory & The Royal Institute of Technology, Stockholm, Sweden;
Monk Philip, Synairgen Research Ltd, Southampton, UK;
Naz Shama, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Nething K, Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Nicholas Ben, University of Southampton, Southampton, UK
Nihlén U, *Previously AstraZeneca; Mölndal, Sweden;*
Nilsson Peter, Science for Life Laboratory & The Royal Institute of Technology, Stockholm, Sweden;
Niven R, North West Severe Asthma Network, University Hospital South Manchester, UK
Nordlund B, Dept. Women's and Children's Health & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Nsubuga S, Royal Brompton Hospital, London, UK
Östling Jörgen, AstraZeneca, Mölndal, Sweden;
Pacino Antonio, Lega Italiano Anti Fumo, Catania, Italy;
Palkonen Susanna, European Federation of Allergy and Airways Diseases Patient's Associations, Brussels, Belgium.
Pellet J, European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Pennazza Giorgio, University of Rome 'Tor Vergata', Rome Italy;
Petrén Anne, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Pink Sandy, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Pison C, European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Postle Anthony, University of Southampton, UK
Rahman-Amin Malayka, Previously at: Asthma UK, London, UK;

Ravanetti Lara, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Ray Emma, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Reinke Stacey, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Reynolds Leanne, Previously at: Asthma UK, London, UK;
Riemann K, Boehringer Ingelheim Pharma GmbH & Co. KG; Biberach, Germany
Robberechts Martine, MSD, Brussels, Belgium
Rocha J P, Royal Brompton and Harefield NHS Foundation Trust
Rossios C, National Heart and Lung Institute, Imperial College, London, UK;
Russell Kirsty, National Heart and Lung Institute, Imperial College, London, UK;
Rutgers Michael, Longfonds, Amersfoort, The Netherlands;
Santini G, Università Cattolica del Sacro Cuore, Milan, Italy;
Santoninco Marco, University of Rome 'Tor Vergata', Rome Italy;
Saqi M, European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
Schoelch Corinna, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
Schofield James P. R., Centre for Proteomic Research, Institute for Life Sciences, University of Southampton, Southampton, UK
Scott S, North West Severe Asthma Network, Countess of Chester Hospital, UK
Sehgal N, North West Severe Asthma Network; Pennine Acute Hospital NHS Trust
Sjödin Marcus, Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Smids Barbara, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Smith Caroline, NIHR Southampton Respiratory Biomedical Research Unit, Southampton, UK
Smith Jessica, Asthma UK, London, UK;
Smith Katherine M., University of Nottingham, UK;
Söderman P, Dept. Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden
Sogbessan A, Royal Brompton and Harefield NHS Foundation Trust, London, UK;
Spycher F, University Hospital Department of Pulmonary Medicine, Bern, Switzerland
Staykova Doroteya, University of Southampton, Southampton, UK
Stephan S, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Stokholm J, University of Copenhagen and Danish Pediatric Asthma Centre Denmark
Strandberg K, Karolinska University Hospital & Karolinska Institutet, Stockholm, Sweden
Sunther M, Centre for respiratory medicine and allergy, Institute of Inflammation and repair, University Hospital of South Manchester, NHS Foundation Trust, Manchester, UK
Szentkereszty M, Semmelweis University, Budapest, Hungary;
Tamasi L, Semmelweis University, Budapest, Hungary;
Tariq K, NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences, NIHR-Wellcome Trust Clinical Research Facility, Faculty of Medicine, University of Southampton, Southampton, UK;
Thörngren John-Olof, Karolinska University Hospital, Stockholm, Sweden

Thorsen Jonathan, COPSAC, Copenhagen Prospective Studies on Asthma in Childhood, Herlev and Gentofte Hospital, University of Copenhagen, Copenhagen, Denmark;
Valente S, Università Cattolica del Sacro Cuore, Milan, Italy;
van de Pol Marianne, Academic Medical Centre, University of Amsterdam, Amsterdam ,The Netherlands;
van Drunen C M, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
Van Eyll Jonathan, UCB, Slough, UK
Versnel Jenny, Previously at: Asthma UK, London, UK;
Vink Anton, Philips Research Laboratories, Eindhoven, The Netherlands;
von Garnier C, University Hospital Bern, Switzerland;
Vyas A, North west Severe Asthma Network, Lancashire Teaching Hospitals NHS Trust, UK
Wald Frans, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany
Walker Samantha, Asthma UK, London, UK;
Ward Jonathan, Histochemistry Research Unit, Faculty of Medicine, University of Southampton, Southampton, UK;
Wetzel Kristiane, Boehringer Ingelheim Pharma GmbH, Biberach, Germany
Wiegman Coen, National Heart and Lung Institute, Imperial College, London, UK;
Williams Siân, International Primary Care Respiratory Group, Aberdeen, Scotland;
Yang Xian, Data Science Institute, Imperial College, London, UK
Yeyasingham Elizabeth, UK Clinical Operations, GSK, Stockley Park, UK;
Yu W, Amgen Inc.; Thousand Oaks, USA
Zetterquist W, Dept. Women's and Children's Health & Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
Zolkipli Z, NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK;
Zwinderman A H, Academic Medical Centre, University of Amsterdam, The Netherlands;

Partner organisations	
Novartis Pharma AG	University of Southampton, Southampton, UK
Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands	Imperial College London, London, UK
University of Catania, Catania, Italy	University of Rome 'Tor Vergata', Rome, Italy
Hvidore Hospital, Hvidore, Denmark	Jagiellonian Univ. Medi.College, Krakow, Poland
University Hospital, Inselspital, Bern, Switzerland	Semmelweis University, Budapest, Hungary
University of Manchester, Manchester, UK	Université d'Aix-Marseille, Marseille, France
Fraunhofer Institute, Hannover, Germany	University Hospital, Umea, Sweden
Ghent University, Ghent, Belgium	Ctr. Nat. Recherche Scientifique, Lyon, France
Università Cattolica del Sacro Cuore, Rome, Italy	University Hospital, Copenhagen, Denmark
Karolinska Institutet, Stockholm, Sweden	Nottingham University Hospital, Nottingham, UK

University of Bergen, Bergen, Norway	Netherlands Asthma Foundation, Leusden, NL
European Lung Foundation, Sheffield, UK	Asthma UK, London, UK
European. Fed. of Allergy and Airways Diseases Patients' Associations, Brussels, Belgium	Lega Italiano Anti Fumo, Catania, Italy
International Primary Care Respiratory Group, Aberdeen, Scotland	Philips Research Laboratories, Eindhoven, NL
Synairgen Research Ltd, Southampton, UK	Aerocrine AB, Stockholm, Sweden
BioSci Consulting, Maasmechelen, Belgium	Almirall
AstraZeneca	Boehringer Ingelheim
Chiesi	GlaxoSmithKline
Roche	UCB
Janssen Biologics BV	Amgen NV
Merck Sharp & Dome Corp	

MEMBERS OF THE ETHICS BOARD			
Name	**Task**	**Affiliation**	**e-mail**
Jan-Bas Prins	Biomedical research	LUMC/the Netherlands	J.B.Prins@lumc.nl
Martina Gahlemann	Clinical care	BI/Germany	Martina.Gahlemann@boehringer-ingelheim.com
Luigi Visintin	Legal affairs	LIAF/Italy	visintin@inrete.it
Hazel Evans	Paediatric care	Southampton/UK	hazel.evans@uhs.nhs.uk
Martine Puhl	Patient representation (co-chair)	NAF/ the Netherlands	martine@puhl.nl
Lina Buzermaniene	Patient representation	EFA/Lithuania	lina.buzermaniene@pavb.lt
Val Hudson	Patient representation	Asthma UK	hudsonval7@gmail.com
Laura Bond	Patient representation	Asthma UK	lvbond22@googlemail.com
Pim de Boer	Patient representation and pathobiology	IND	deboer.pim@hetnet.nl
Guy Widdershoven	Research ethics	VUMC/the Netherlands	g.widdershoven@vumc.nl
Ralf Sigmund	Research methodology and biostatistics	BI/Germany	ralf.sigmund@boehringer-ingelheim.com

THE PATIENT INPUT PLATFORM	
Name	**Country**
Amanda Roberts	UK
David Supple (chair)	UK
Dominique Hamerlijnck	The Netherlands
Jenny Negus	UK
Juliëtte Kamphuis	The Netherlands
Lehanne Sergison	UK
Luigi Visintin	Italy
Pim de Boer (co-chair)	The Netherlands
Susanne Onstein	The Netherlands

MEMBERS OF THE SAFETY MONITORING BOARD	
Name	**Task**
William MacNee	Clinical care
Renato Bernardini	Clinical pharmacology
Louis Bont	Paediatric care and infectious diseases

Per-Ake Wecksell	Patient representation
Pim de Boer	Patient representation and pathobiology (chair)
Martina Gahlemann	Patient safety advice and clinical care (co-chair)
Ralf Sigmund	Bio-informatician

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

an exploratory analysis

Kentaro Takahashi 1,2, Stelios Pavlidis3, Francois Ng Kee Kwong 1, Uruj Hoda 1, Christos Rossios1, Kai Sun3, Matthew Loza4, Fred Baribaud4, Pascal Chanez5, Steve J Fowler6, Ildiko Horvath7, Paolo Montuschi8, Florian Singer9, Jacek Musial10, Barbro Dahlen11, Sven-Eric Dahlen11, N. Krug12, Thomas Sandstrom13, Dominic E. Shaw14, Rene Lutter 15, Per Bakke16, Louise J. Fleming1, Peter H. Howarth17, Massimo Caruso18, Ana R Sousa19, Julie Corfield20, Charles Auffray21, Bertrand De Meulder21, Diane Lefaudeux21, Ratko Djukanovic17, Peter J Sterk16, Yike Guo3, Ian M. Adcock1,3, Kian Fan Chung1,3 on behalf of the U-BIOPRED study group#

1National Heart & Lung Institute, Imperial College London, & Biomedical Research Unit, Biomedical Research Unit, Royal Brompton & Harefield NHS Trust, London, United Kingdom;
2Research Centre for Allergy and Clinical Immunology, Asahi General Hospital, Japan;
3Department of Computing & Data Science Institute, Imperial College London, United Kingdom;
4Janssen Research and Development, High Wycombe, Buckinghamshire, United Kingdom;
5 Assistance Publique des Hôpitaux de Marseille - Clinique des bronches, allergies et sommeil, Aix Marseille Université, Marseille, France
6 Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom
7 Semmelweis University, Budapest, Hungary
8 Università Cattolica del Sacro Cuore, Milan, Italy;
9 Bern University Hospital, University of Bern, Bern, Switzerland;
10 Dept. of Medicine, Jagiellonian University Medical College, Krakow, Poland
11 Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden
12 Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany;
13 Dept of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden;
14 Respiratory Research Unit, University of Nottingham, UK;
15 Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
16 Department of Clinical Science, University of Bergen, Bergen, Norway;
17 NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK
18 Dept. Clinical and Experimental Medicine, University of Catania, Catania, Italy;
19Respiratory Therapeutic Unit, GSK, Stockley Park, UK;
20AstraZeneca R&D Molndal, Sweden and Areteva R&D, Nottingham, UK;
21 European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
#Consortium project team members are listed under Acknowledgements

Supplementary Tables S1- S10

Supplementary Table S1. Gene Sets for GSVA.

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 DHRS2 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 SEPN1 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[§]	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 WIPI1 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 ASAH1 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.

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

GO biological process	Fold enrichment	P value
ESA-NH		
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
NSA-NH		
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	
	Current-smoker (CSA) (n=6)	**Ex-smoker (ESA)** (n=12)	**Non-smoker (NSA)** (n=40)	**Non-smoker (NH)** (n=37)	**P value**
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/m2)	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	
	Current-smoker (CSA) (n=7)	**Ex-smoker (ESA)** (n=6)	**Non-smoker (NSA)** (n=34)	**Non-smoker (NH)** (n=22)	**P value**
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/m2)	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	
	Current-smoker (CSA) (n=8)	**Ex-smoker (ESA)** (n=15)	**Non-smoker (NSA)** (n=47)	**Non-smoker (NH)** (n=15)	**P value**
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/m2)	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 (*/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 metallopeptidase 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-kB, 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-κB: 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

Supplementary file

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

Kentaro Takahashi 1,2, Stelios Pavlidis3, Francois Ng Kee Kwong 1, Uruj Hoda 1, Christos Rossios1, Kai Sun3, Matthew Loza4, Fred Baribaud4, Pascal Chanez5, Steve J Fowler6, Ildiko Horvath7, Paolo Montuschi8, Florian Singer9, Jacek Musial10, Barbro Dahlen11, Sven-Eric Dahlen11, N. Krug12, Thomas Sandstrom13, Dominic E. Shaw14, Rene Lutter 15, Per Bakke16, Louise J. Fleming1, Peter H. Howarth17, Massimo Caruso18, Ana R Sousa19, Julie Corfield20, Charles Auffray21, Bertrand De Meulder21, Diane Lefaudeux21, Ratko Djukanovic17, Peter J Sterk16, Yike Guo3, Ian M. Adcock1,3,

Kian Fan Chung1,3 on behalf of the U-BIOPRED study group#

1National Heart & Lung Institute, Imperial College London, & Biomedical Research Unit, Biomedical Research Unit, Royal Brompton & Harefield NHS Trust, London, United Kingdom;

2Research Centre for Allergy and Clinical Immunology, Asahi General Hospital, Japan;

3Department of Computing & Data Science Institute, Imperial College London, United Kingdom;

4Janssen Research and Development, High Wycombe, Buckinghamshire, United Kingdom;

5 Assistance Publique des Hôpitaux de Marseille - Clinique des bronches, allergies et sommeil, Aix Marseille Université, Marseille, France

6 Centre for Respiratory Medicine and Allergy, Institute of Inflammation and Repair, University of Manchester and University Hospital of South Manchester, Manchester Academic Health Sciences Centre, Manchester, United Kingdom

7 Semmelweis University, Budapest, Hungary

8 Università Cattolica del Sacro Cuore, Milan, Italy;

9 Bern University Hospital, University of Bern, Bern, Switzerland;

10 Dept. of Medicine, Jagiellonian University Medical College, Krakow, Poland

11 Centre for Allergy Research, Karolinska Institutet, Stockholm, Sweden

12 Fraunhofer Institute for Toxicology and Experimental Medicine, Hannover, Germany;

13 Dept of Public Health and Clinical Medicine, Umeå University, Umeå, Sweden;

14 Respiratory Research Unit, University of Nottingham, UK;

15 Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands;
16 Department of Clinical Science, University of Bergen, Bergen, Norway;
17 NIHR Southampton Respiratory Biomedical Research Unit, Clinical and Experimental Sciences and Human Development and Health, Southampton, UK
18 Dept. Clinical and Experimental Medicine, University of Catania, Catania, Italy;
19Respiratory Therapeutic Unit, GSK, Stockley Park, UK;
20AstraZeneca R&D Molndal, Sweden and Areteva R&D, Nottingham, UK;
21 European Institute for Systems Biology and Medicine, CNRS-ENS-UCBL-INSERM, Lyon, France
#Consortium project team members are listed under Acknowledgements

1. Inclusion criteria of severe asthma patients:

Participants with asthma had either airflow reversibility (increase in forced expiratory volume in 1s (FEV_1) >12% predicted and 200 mL following inhalation of 400 μg salbutamol), airway hyperresponsiveness (methacholine provocative concentration causing a 20% fall in FEV_1 <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 (FEV_1) 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 FEV_1<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.

02-Jan-2018
ERJ-02173-2017

Dear Prof. Chung:

Your manuscript entitled "Distinct pathways underlying neutrophilic inflammation in smoking-associated severe asthma in U-BIOPRED" has been evaluated by anonymous reviewers and the editors.

Based on the reviewers' recommendations, our own views, and the editorial standards used by the editorial board, we have to inform you that your manuscript cannot be accepted in its present form. However, we will reconsider this decision if you are prepared to submit an adequately revised manuscript.

The main points of criticism can be summarised as follows:

Main issues summarised by the associate and chief editor:

Comments to Author:
There are some critical issues that need to be fixed and satisfied entirely to get this paper acceptable. See detailed reviews below.

RESPONSE:

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

The full comments of the reviewers were the following:

Reviewer: 1

Comments to the Author

General comments

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

Response:

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

Major specific comments

1. Healthy smoker controls

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

RESPONSE:

We appreciate the interest of the Reviewer in our manuscript and for pointing out the important findings in our study. We appreciate also the comment regarding the major limitation of our study regarding the lack of the healthy non-asthmatic smokers. However, we used previously-published reports that have looked at healthy smokers compared to healthy non-smokers. In the previous human proteome publications analysing sputum in healthy current smokers and never smokers, CSF2 and AGR2 were not shown to be differentially expressed proteins [1]. We therefore believe that severe asthma current smokers have different molecular signatures compared to non-asthmatic healthy smokers. Various publications describing sputum or bronchial epithelial cell transcriptomic profile in cigarette smokers showed upregulated expression of xenobiotic and oxidative stress [1, 2]. Pathway analyses using GO and DAVID that were performed by us, as shown in Supplementary Tables, S9 and S10, showed that the significantly upregulated gene sets of endoplasmic stress and lysosome were seen in our severe asthma smoking subjects compared to the equivalent non-smoking subject using bronchial epithelial cells. This was not seen within Avi Spira's 97 differentially-expressed genes from bronchial epithelial cells between current-smokers and never-smokers [2]. These observations indicate that differently-expressed genes between CSA and NSA are not due to the influence of cigarette smoking itself. We have added supplementary Tables S8 and S9 and comments in the DISCUSSION to that effect as follows:

"CSF2 and AGR2 have not been shown to be differentially-expressed in the previously-published proteomic analysis of sputum from healthy current-smokers compared to never-smokers (35). Moreover, endoplasmic stress and lysosome gene sets

that we found to be differentially-expressed in these 2 groups of severe asthma were not differentially-expressed in healthy current smokers compared to non-smokers (Supplementary Table S9 and S10) (21). These results suggest that DEGs between CSA and NSA were not derived from the effect of cigarette smoking itself."

2. Small sample size

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

RESPONSE:

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

3. Title

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

RESPONSE:

We agree with this comment and have changed the Title accordingly to: "Sputum proteomics and airway cell transcripts of current and ex-smokers with severe asthma in U-BIOPRED: an exploratory analysis". We have also added "exploratory" to the Abstract.

4. Bacterial colonization

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

RESPONSE:

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

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

Minor comments

1. Abstract: Rationale

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

Response:

We have removed the term "eosinophilia" and replaced it with 'reported neutrophilia'.

2. Introduction Page 7, line 12: If reference #2 is used, then revise the sentence 'significant number of patients with severe asthma are current smokers or have been

ex-smokers (2)' by omitting the word 'severe'. This was a population-based study and did not specifically investigate patients with severe asthma.

RESPONSE:

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

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

RESPONSE:

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

4. Figure 1 (Sputum supernatant proteomics):

a) Is CA6 the symbol for Carbonic anhydrase VI and is CA6 present because of salivary contamination?

RESPONSE:

We did our best to exclude salivary contamination by rinsing the mouth with water prior to sputum induction and we excluded sputum samples whose squamous cells were more than 30% for transcriptomic assay to lessen salivary contamination, as described in MATERIALS and METHODS. However, this salivary contamination cannot be entirely excluded but can be reduced.

b) Use consistent abbreviations: CSSA, ESSA and NSSA used in legend and CSA, ESA and NSA used in figure. Also applies to other sections of the manuscript 5. Discussion Page 19, line 10: the study did not show 'increased neutrophilia in ESSA patients', at least in terms of sputum neutrophilia compared to the other severe asthma groups. Please rephrase

Response:

We have changed CSSA, ESSA and NSSA to CSA, ESA and NSA, respectively, in all sections. We have excluded the term 'increased neutrophilia in ESA patients'.

Reviewer: 2

Comments to the Author

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

Major Points

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

RESPONSE:

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

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

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/μL, ESA 296/μL, NSA 407/μL), % 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

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

RESPONSE:

We apologise for this mistake. We modified the numbers from16.7% to 28.6%, 72.7% to 50.0% and 35.1% to 43.8%.

Minor Points

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

RESPONSE:

We changed "eosinophilia" to "reported neutrophilia".

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

RESPONSE:

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

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

RESPONSE:

We have added a new file "Supplementary File" where we describe the following inclusion criteria:

"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. "

4. Materials and methods: please make it clear that you are using a subset of patients for this study. It reads currently as though all 420 patients had sputum and bronchial biopsies etc.

RESPONSE:

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

We enrolled totally 374 severe asthma patients in the U-BIOPRED cohort divided into three groups by smoking status; current-smokers with severe asthma (CSA), ex-smokers with severe asthma (ESA), non-smokers with severe asthma (NSA). We narrowed down the NSA to those who had never smoked (0 pack-year), although original non-smokers with severe asthma group in the U-BIOPRED cohort contained the patients whose pack-year was less than 5. Eighty-one non-smoking healthy volunteers (NH) whose pack-years were 0 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 obtained (8, 9). Bronchial biopsies, bronchial brushings and sputum were obtained, as previously described (8). Because of the bronchoscopy exclusion criteria (Supplementary File), only 95 bronchial brushings and 69 bronchial biopsies were obtained. The number of sputum samples for proteomic analysis was 88. All subjects whose samples were adequate and underwent omics analyses are shown in Supplementary Figure S1.

We have added bronchoscopy exclusion criteria in Supplementary File.

"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."

5. Results: please state the numbers of patients in each of your groups in the main text. Please make it clear how many of your patients were different in your sputum analysis and your bronchial biopsy analysis (the baseline demographics data make them look quite distinct). Please state the number of sites from which patients in each subset were recruited.

RESPONSE:

We added the numbers of patients in all comparisons. We have made a new figure showing subjects whose samples were applied for proteomic and transcriptomic analyses (Supplementary Figure S1), referred to in MATERIALS and METHODS.

6. You sometimes use CSSA/ESSA/NSSA and sometimes CSA/ESA/NSA. Please be consistent.

RESPONSE:

We have changed CSSA, ESSA and NSSA to CSA, ESA and NSA, respectively, in all sections.

7. In all data tables "comorbidities" is spelled incorrectly

RESPONSE: We modified the spelling from "commorbidities" to "comorbidities".

8. Across data tables there is inconsistency in the p-value presentation.

RESPONSE: All p-values in data tables now are in same format.

9. Figure 1: I think you might have confused your labelling? You state red dots were those proteins with an absolute fold change of ≥ 2.0 and turquoise was used for those ≤ 2.0. Do you mean a fold change of ≤ -1.0 for red and ≥ 1.0 for turquoise? Also you need a colon rather than semi-colon after "in following comparisons".

RESPONSE:

The labelling of transverse line is log2 (fold change). Absolute fold change 2.0 means log2 (fold change) >1.0 or <-1.0. I modified the legend by adding "-". The proteins whose

absolute fold change (FC) ≥2.0 at false discovery rate (FDR) <0.05 were DEPs shown as coloured dots (red: FC ≥2.0, turquoise: FC ≤-2.0).

10. In your discussion, please consider changing "There was a difference in the sputum proteome between NSSA and CSSA (CSF2, AGR2 and CXCL8) and ESSA (AZU1, ELANE, CFP and CXCL8)," which might be interpreted as a difference between NSSA and CSSA on the one hand with ESSA on the other, to "There was a difference in the sputum proteome between NSSA and CSSA (CSF2, AGR2 and CXCL8) and between NSSA and ESSA (AZU1, ELANE, CFP and CXCL8)."

RESPONSE: We have modified accordingly.

Questions

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

RESPONSE:

Yes, we have performed these analyses. CSF2 and AGR2 were recognized as DEPs in the comparisons between CSA and ESA and between CSA and NSA but we could not find any DEPs in the comparison between ESA and NSA.

2. Why have you adjusted for certain demographic variables in your sputum DEP analyses but not in your bronchial biopsy DEG analyses? Age is adjusted for in the sputum DEP but not biopsy DEG despite being significantly different between groups in both cohorts; oral corticosteroid use is adjusted for in the sputum DEP but not biopsy DEG despite apparently not being significantly different between groups in either cohort; BMI is very significantly different between groups in both cohorts but was not adjusted for in any analysis.

RESPONSE:

We have performed all analyses of DEPs and DEGs by adjusting demographic variables, such as age, gender and systemic corticosteroids use because these factors seemed to affect the expression of mRNAs or proteins in airways. BMI seemed not to affect gene

expression in airways. I modified the sentence in "*Statistical analysis*" as follows: **Gene and protein expression data were analysed using multivariate analysis of variance (MANOVA) with age, gender and systemic corticosteroids (SCS) use analysed as covariates.**

References:

1. Titz B, Sewer A, Schneider T, Elamin A, Martin F, Dijon S, Luettich K, Guedj E, Vuillaume G, Ivanov NV, Peck MJ, Chaudhary NI, Hoeng J, Peitsch MC. Alterations in the sputum proteome and transcriptome in smokers and early-stage COPD subjects. J Proteomics. 2015; 128: 306-20.
2. Spira A, Beane J, Shah V, Liu G, Schembri F, Yang X, Palma J, Brody JS. Effects of cigarette smoke on the human airway epithelial cell transcriptome. Proc Natl Acad Sci U S A 2004; 101: 10143-8.

Fig. 1

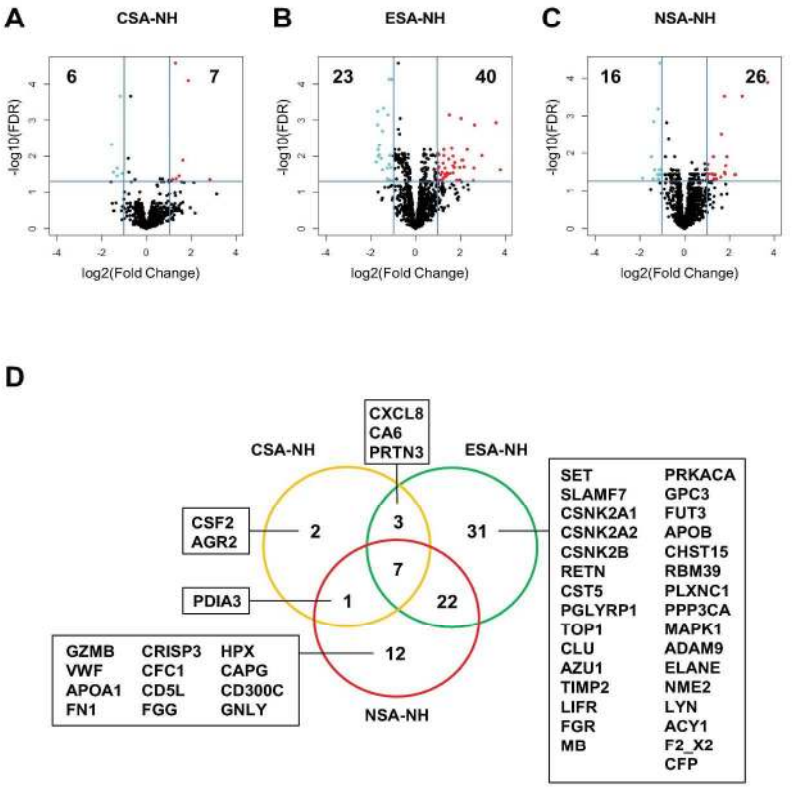

Figure 1. Phenotypic differences among CSSA, ESSA and NSSA were unveiled by limma of sputum SomaLogic.
(A-C) Volcano plots showing differentially-expressed proteins (DEPs) in linear model for microarray (limma) of sputum SomaLogic in following comparisons; (A) CSSA and NH, (B) ESSA and NH, (C) NSSA and 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

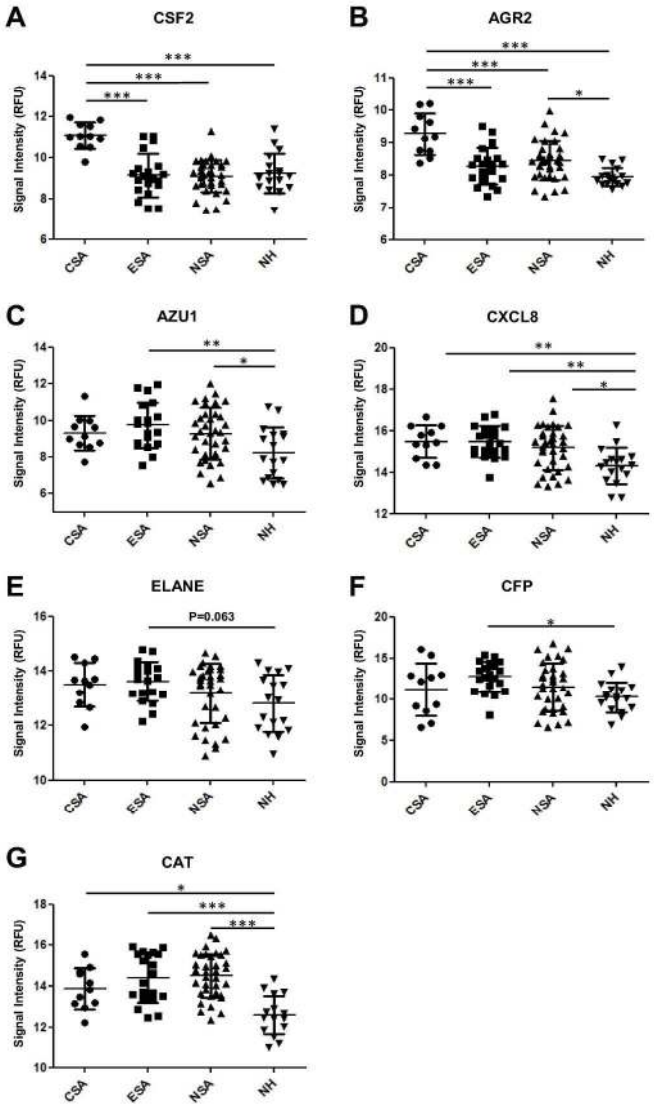

Figure 2. Differentially-expressed proteins (DEPs) in severe asthma sputum according to smoking status. Dot plots with mean±SD showing signal intensity levels of protein expression of CSF2 (A), AGR2 (B), AZU1 (C), CXCL8 (D), ELANE (E), CFP (F) and CAT (G) in sputum by SomaLogic analysis in CSSA (circles),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

188x266mm (300 x 300 DPI)

Fig. 3

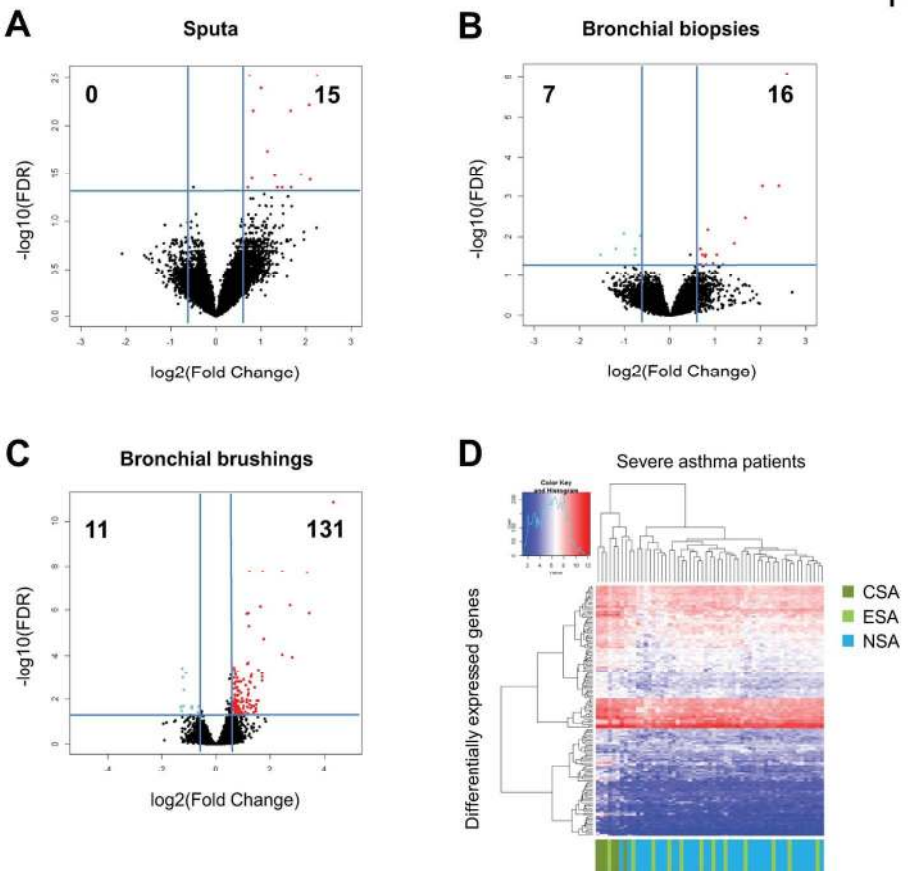

Figure 3. Differentially-expressed genes (DEGs) in current smokers (CSSA) and non-smokers (NSSA) with severe asthma.
Volcano plots showing differentially expressed genes (DEGs) between CSSA and NSSA in (A) sputa, (B) bronchial biopsies and (C) bronchial brushings. The genes whose absolute fold change (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

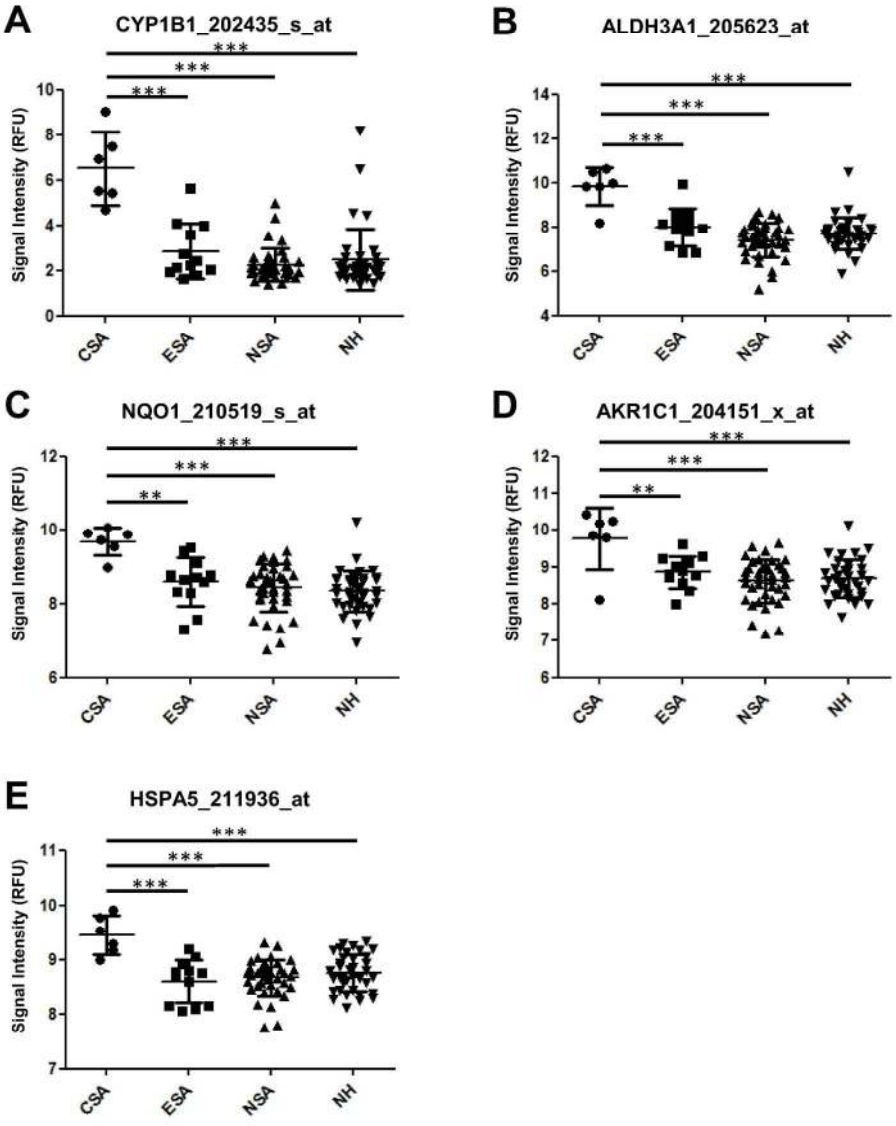

Figure 4. Differentially-expressed genes (DEGs) associated with metabolism of xenobiotics, oxidative stress and ER stress in bronchial brushings.
Dot plots showing DEGs in bronchial brushings associated with xenobiotic metabolism CYP1B1 (A), ALDH3A1 (B), NQO1 (C), AKR1C1 (D) and HSPA5 (E). CSSA: circles, ESSA: squares, NSSA: triangles, NH: inverted triangles. RFU: Relative Fluorescence Units, *p<0.05; **p<0.01; ***p<0.001.

201x240mm (300 x 300 DPI)

Fig. 5

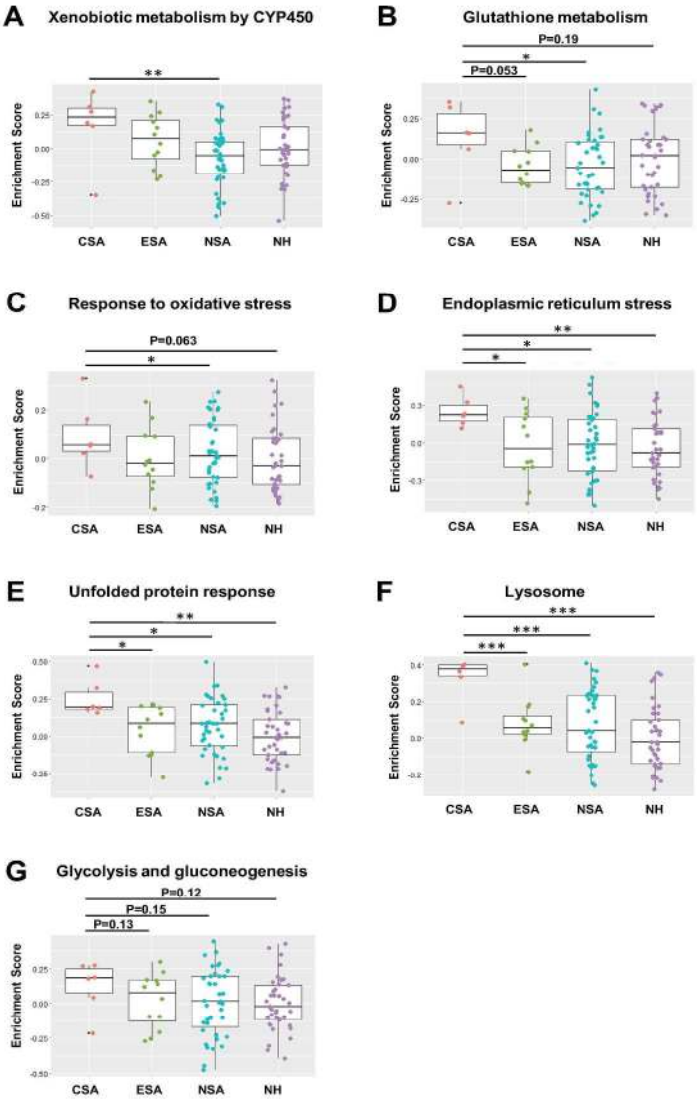

Figure 5. Gene Set Variation Analysis of selected stress-related pathways in bronchial brushings according to smoking status.

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

225x308mm (300 x 300 DPI)

Fig. 6

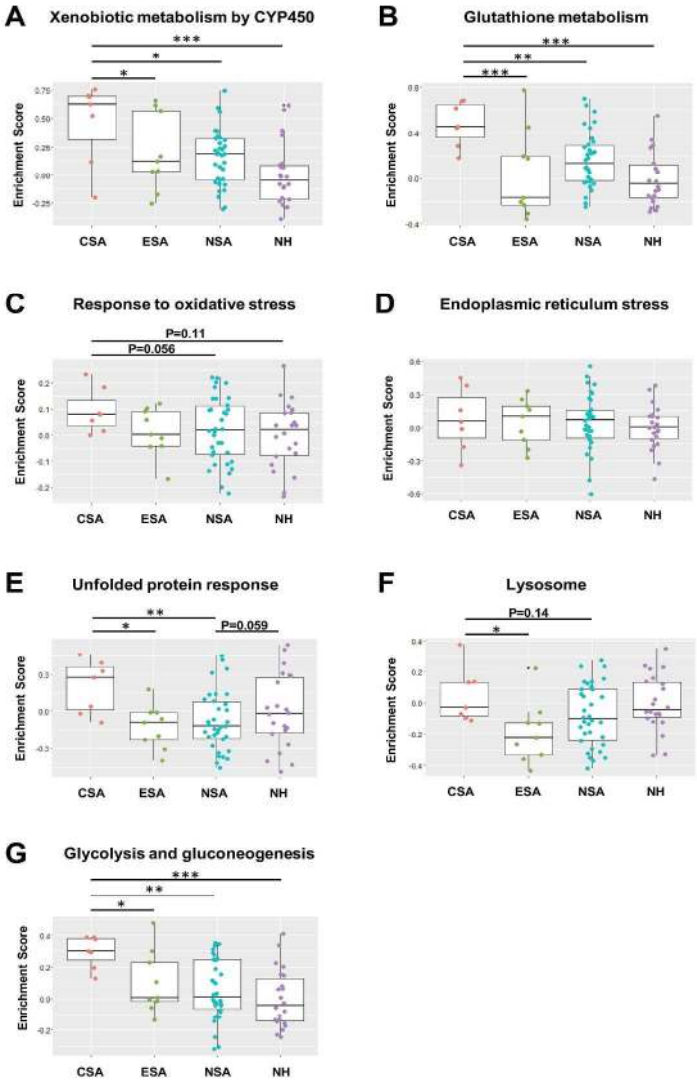

Figure 6. Gene Set Variation Analysis of selected stress-related pathways in bronchial biopsies according to smoking status.
Box-and-whisker plots showing pathway enrichment of (A) xenobiotic metabolism by CYP450, (B) glutathione metabolism, (C) response to oxidative stress, (D) endoplasmic reticulum (ER) stress, (E) unfolded protein response, (F) lysosome and (G) glycolysis and gluconeogenesis in bronchial biopsies of CSSA (red circles), ESSA (olive green circles), NSSA (cyan circles) and NH (purple circles) subjects. *p<0.05; **p<0.01; ***p<0.001.

217x298mm (300 x 300 DPI)

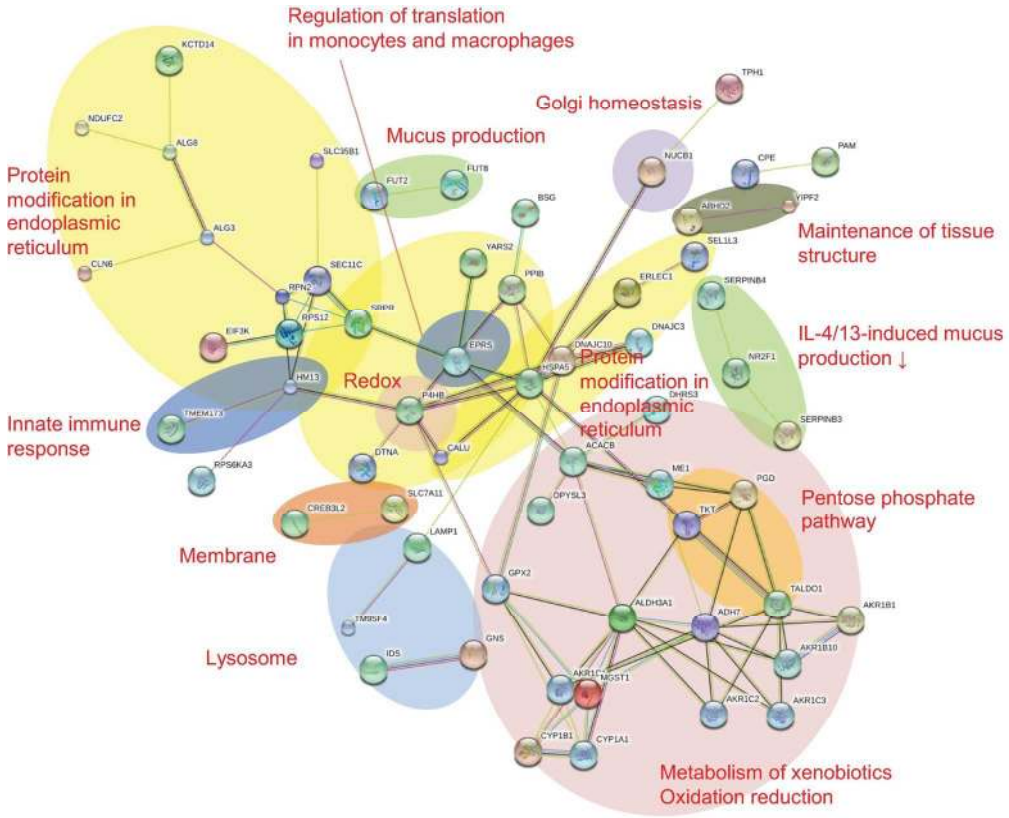

Fig 7

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.

177x180mm (300 x 300 DPI)

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