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Stratification of asthma by lipidomic profiling of induced sputum supernatant

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PII: S0091-6749(23)00293-2

DOI: https://doi.org/10.1016/j.jaci.2023.02.032

Reference: YMAI 15904

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 9 August 2022

Revised Date: 7 February 2023

Accepted Date: 14 February 2023

Please cite this article as: Brandsma J, Schofield JPR, Yang X, Strazzeri F, Barber C, Goss VM, Koster G, Bakke PS, Caruso M, Chanez P, Dahlén S-E, Fowler SJ, Horváth I, Krug N, Montuschi P, Sanak M, Sandström T, Shaw DE, Chung KF, Singer F, Fleming LJ, Adcock IM, Pandis I, Bansal AT, Corfield J, Sousa AR, Sterk PJ, Sánchez-García RJ, Skipp PJ, Postle AD, Djukanović R, on behalf of the U-BIOPRED Study Group, Stratification of asthma by lipidomic profiling of induced sputum supernatant, *Journal of Allergy and Clinical Immunology* (2023), doi: https://doi.org/10.1016/j.jaci.2023.02.032.

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source: https://doi.org/10.48350/180098 | downloaded: 12.10.2024

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Stratification of asthma by lipidomic profiling of induced sputum supernatant

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Funding: The U-BIOPRED consortium receives funding from the European Union and from the European Federation of Pharmaceutical Industries and Associations as an IMI JU funded project (no. 115010). Additional funding for the analytical equipment was obtained from a Wellcome Trust equipment grant (no. 093500/Z/10/Z).

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U-BIOPRED Ethics Board and Study Group: The study was overseen and approved by the U-BIOPRED Ethics Board which was comprised of Pim de Boer (chair), Jan-Bas Prins, Martina Gahlemann, Luigi Visintin, Hazel Evans, Martine Puhl, Lina Buzermaniene, Val Hudson, Laura Bond, Guy Widdershoven and Ralf Sigmund (http://www.europeanlung.org/en/projects-and-research/projects/u-biopred/home). A comprehensive list of members of the U-BIOPRED Study Group has been provided. All members are acknowledged for their help and expertise, without which the study would not have been possible.

Conflict of Interest: Pascal Chanez reports receiving grants and personal fees from Almirall, Boehringer Ingelheim, ALK, GSK, AstraZeneca, Novartis, Teva, and Chiesi; Kian Fan Chung has received honoraria for participating in Advisory Board meetings of GSK, AstraZeneca, Roche, Novartis, Merck, Nocion, Shionogi, and Rickett-Beckinson regarding treatments for asthma, COPD and cough, and has also been renumerated for speaking engagements; Sven-Erik Dahlén reports receiving personal fees from AstraZeneca, Cayman Chemical, GSK, Novartis, Regeneron, Sanofi, and Teva; Ratko Djukanović reports receiving grants from the Innovative Medicines Initiative, UK Medical Research Council, and UK National Institute for Health and Care Research, a grant and personal fees from Novartis, personal fees from TEVA, Sanofi, Boehringer Ingelheim, Synairgen, and Kymab, as well as holding stocks of Synairgen; Louise Fleming reports receiving grants from the Innovative Medicines Initiative during the conduct of the study, as well as personal fees from Novartis, AstraZeneca, and Sanofi; Graham Roberts reports receiving grants from the Innovative Medicines Initiative during the conduct of the study; Florian Singer reports receiving personal fees from Novartis and Vertex Pharmaceuticals, and non-financial support from Chiesi;

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Paul Skipp is a director and shareholder of TopMD Precision Medicine Ltd; Peter Sterk is a scientific advisor to, and has an officially non-substantial interest, in SME Breathomix BV; Fabio Strazzeri is a director and shareholder of TopMD Precision Medicine Ltd. None of the disclosed conflicts of interest relate to any of the work presented in this manuscript. The remaining authors declare no Conflicts of Interest.

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Abstract

Background: Asthma is a chronic respiratory disease with significant heterogeneity in its clinical presentation and pathobiology. There is need for improved understanding of respiratory lipid metabolism in asthma patients and its relation to observable clinical features.

Objective: To perform a comprehensive, prospective, cross-sectional analysis of the lipid composition of induced sputum supernatant obtained from asthma patients with a range of disease severities, as well as healthy controls.

Methods: Induced sputum supernatant was collected from 211 asthmatic adults and 41 healthy individuals enrolled in the U-BIOPRED study. Sputum lipidomes were characterised by semi-quantitative shotgun mass spectrometry, and clustered using topological data analysis to identify lipid phenotypes.

Results: Shotgun lipidomics of induced sputum supernatant revealed a spectrum of nine molecular phenotypes, highlighting not just significant differences between the sputum lipidomes of asthmatics and healthy controls, but within the asthmatic population as well. Matching clinical, pathobiological, proteomic and transcriptomic data informed on the underlying disease processes. Sputum lipid phenotypes with higher levels of non-endogenous, cell-derived lipids were associated with significantly worse asthma severity, worse lung function, and elevated granulocyte counts.

Conclusion: We propose a novel mechanism of increased lipid loading in the epithelial lining fluid of asthmatics, resulting from the secretion of extracellular vesicles by granulocytic inflammatory cells, which could reduce the ability of pulmonary surfactant to lower surface tension in asthmatic small airways, as well as compromise its role as an immune regulator.

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Clinical Implication: Immunomodulation of extracellular vesicle secretion in the lungs may provide a novel therapeutic target for severe asthma.

Capsule Summary: We used lipid phenotyping of induced sputum to stratify a heterogeneous asthma cohort, and propose a novel mechanism of pulmonary surfactant dysregulation by extracellular vesicles secreted in asthmatic airways.

Keywords: Asthma, induced sputum, epithelial lining fluid, pulmonary surfactant, lipidomics, molecular phenotyping, extracellular vesicles, granulocytic inflammation

Abbreviations:

ACQ	Asthma Control Questionnaire
ATII	Alveolar type II
Chol	Cholesterol
CE	Cholesterol ester
Cer	Ceramide
DG	Diglyceride
DPPC	Dipalmitoyl-phosphatidylcholine
ELF	Epithelial lining fluid
EV	Extracellular vesicle
HC	Healthy control

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HexCer	Hexosyl-ceramide
IgE	Immunoglobulin E
IPA	Ingenuity Pathway Analysis
JT-test	Jonckheere-Terpstra test
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LPC	Lyso-phosphatidylcholine
MDS	Multi-dimensional scaling
MMA	Mild-to-moderate asthmatic
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
SM	Sphingomyelin
QC	Quality control
SAc/ex	Current or ex-smoking severe asthmatic
SAn	Non-smoking severe asthmatic
TDA	Topological data analysis
TG	Triglyceride

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U-BIOPRED	Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes

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Introduction

Asthma is a chronic respiratory disease characterised by recurrent attacks of breathlessness and wheezing, variable airflow limitation and loss of lung function, with airways inflammation and remodelling as the underlying pathobiological processes. The most significant challenge in asthma treatment is its heterogeneity in clinical presentation and underlying pathobiology. A variety of asthma phenotypes have been described to date based on demographic, clinical or pathophysiological characteristics. Amongst these, blood and sputum eosinophilia have been of greatest value for understanding the risk of exacerbations and response to treatments with inhaled corticosteroids and biologics.[1] However, there is still a large unmet need for understanding the underlying disease mechanisms and for finding correlations with specific pathobiological processes or treatment responses in order to provide a clearer delineation of the various disease phenotypes and endotypes.[2-4] Improved stratification along mechanistic lines will open up new directions for targeted drug development and more personalised disease management strategies.

The U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes) study[5] has employed an 'unbiased' multi-omics systems biology approach to stratify patients with asthma, elucidate biochemical pathways, and define new sets of diagnostic molecular biomarkers.[6-11] The aims of the current study were: to provide a comprehensive analysis of the lipid composition of induced sputum supernatant across the entire disease spectrum, from health to severe asthma; to stratify the heterogeneous U-BIOPRED cohort according to its sputum lipid molecular phenotypes; and to infer mechanisms of lipid biology that are either affected by, or contribute to, the observed phenotypes and their clinical features.

Sputum supernatant comprises a mixture of pulmonary surfactant and soluble material secreted by immune cells and the respiratory epithelium within the lungs, with small quantities of saliva. In healthy adult volunteers the sputum lipidome is dominated by a comparatively restricted number of molecular species, in particular di-saturated phosphatidylcholines, and small amounts of other glycerophospholipids, sphingolipids, glycerolipids and sterols.[12-14] Thus, its lipid composition matches its primary source: pulmonary surfactant secreted by ATII cells in the alveolar epithelium.[15-17] The tight and rapid regulation of lipids, from the cellular to systemic level, combined with their large molecular diversity and involvement in a wide range of intra and inter-cellular signalling pathways, makes them a rich source of molecular biomarkers of disease and a valuable component of systems-based disease phenotyping studies.[18,19] However, despite significant interest in the role of lipids in respiratory diseases, studies of the sputum lipidome remain scarce.[12-14,20-22] We have previously reported on the lipid composition of sputum supernatant in a cohort of 41 healthy adults[14] and now extend our analysis to an additional 211 U-BIOPRED study participants from across the asthma severity spectrum. Semi-quantitative shotgun lipidomic measurements were clustered using topological data analysis (TDA) and complemented with matched clinical, immunoassay and transcriptomic data to inform on the underlying disease processes. This multi-dimensional approach to patient characterisation stratified healthy and asthmatic participants into nine different groups based on their sputum lipid phenotypes, suggesting distinct biological mechanisms that could provide novel targets for asthma therapeutics.

Methods

U-BIOPRED study design and tranSMART data repository

All samples were obtained from the U-BIOPRED cohort recruited in 14 European clinical centres.[5] Processed biological samples from clinical sites were fully blinded and stored in a central biobank (CIGMR Biobank, University of Manchester). All clinical, laboratory and 'omics data collected for U-BIOPRED are hosted on the tranSMART knowledge management platform, and these were only released to study group members upon completion of all 'omics analyses.

Lipid analysis and data processing

Shotgun lipidomics of induced sputum samples (n=252) followed the methodology previously published by us,[14] and a comprehensive description is given in this article's Online Repository at www.jacionline.org. Briefly, lipids were extracted using a modified Bligh-Dyer extraction protocol[23] and characterised by flow injection analysis on a Dionex 3000 ultra-high performance liquid chromatography system (Thermo Scientific Dionex, Sunnyvale, CA, USA), coupled to a MaXis 3G quadrupole time-of-flight mass spectrometer, equipped with an electrospray ionisation source (Bruker Daltonics, Billerica, MA, USA). Measurements were performed in full scan mode for both positive and negative ionisation at *m/z* 300-1000. Fragmentation analyses for lipid identification were performed on pooled QC samples, using the same instrumental setup, but in LC-MS/MS mode with a Waters Acquity C8 column (Waters, Milford, MA, USA). Data-independent product ion scans were acquired over the entire LC run via broadband collision induced dissociation. Precursor and fragment ions were matched by their chromatographic retention time and using well-established fragmentation rules for lipids[24] for identity confirmation.

After removal of ions with <60% detection rate, data were corrected for potential batch effects using the *R* script "SVA ComBat".[25] All ion counts were normalised using synthetic internal standards and the original sample volume to obtain semi-quantitative

results. Because sputum is subject to variable dilution of analytes during sampling and processing,[26,27] data were also normalised to the amount of total lipid.

Data analysis and statistics

In order to identify lipid phenotypes, topological data analysis (TDA)[9,28,29] was used to group participants with comparable sputum lipid profiles. TDA was performed on the SymphonyAI machine intelligence platform (SymphonyAI, Palo Alto, CA, USA), using a normalised correlation metric combined with MDS lenses. Groups of participants with similar sputum lipid profiles (*i.e.*, sputum lipid phenotypes) were defined within the TDA structure using density mode clustering.[30,31] Statistical significance of trends across the TDA structure was assessed in SPSS Statistics 24 (IBM, Armonk, NY, USA): Jonckheere-Terpstra tests for ordered alternatives were performed for sputum lipids, as well as a range of demographic, clinical and pathobiological measurements, selected blood protein concentrations, and sputum cell pellet gene expression (see below). Trends were considered significant if $p<0.05$, and highly significant if $p<0.001$ (after Bonferroni correction).

Blood protein data

Concentrations of 32 protein markers of inflammation and tissue function were downloaded from the U-BIOPRED database hosted on the tranSMART knowledge management platform. Protein measurements were performed on plasma samples, using Mesoscale Discovery (MSD) electrochemiluminescence (11), or on serum samples, using either of the following immunoassay platforms: Luminex (16), Impact (2), Singulex (1), Elecsys (1) or Immulite (1).

Pathway analysis of sputum cell pellet transcriptomics data

Transcriptomic data from RNA extracts of 97 matching sputum cell pellets were downloaded from the U-BIOPRED dataset[32], and subjected to Ingenuity Pathway Analysis (QIAGEN Bioinformatics, Redwood City, CA, USA) to identify potential upstream regulators of differential gene expression in each of the lipid phenotypes. IPA core analysis was performed on the top 4000 differentially expressed genes, using the ordered TDA groups as described above. The results were subjected to a comparison analysis to identify trends of IPA-predicted upstream regulator activation/inhibition across the sputum lipidomics TDA structure.

Results

Study cohort

Of the 610 adult individuals recruited in U-BIOPRED, 252 successfully provided sputum samples that passed QC based on cell viability, resuspension volume and a squamous epithelial cell cut-off of ≤40% of total sputum inflammatory cells.[5] The study group thus comprised 137 females and 115 males of predominately white origin, clinically categorised as either non-smoking severe asthmatics (SAn; n=117), current or ex-smoking severe asthmatics (SAc/ex; n=51), mild-to-moderate asthmatics (MMA; n=43), or healthy controls (HC; n=41) (Table 1).

Topological data analysis of sputum lipid phenotypes

A total of 291 lipid molecular ions were quantified in the sputum samples (for methodology, QC and selection procedures see this article's Online Repository at www.jacionline.org). Of these, 92 lipid species were identified using LC-MS/MS of pooled QC samples. The remaining ions were classified as 'unknown lipids', but together these comprised only 5% of the total lipid signal.

Initial TDA of all samples produced a network comprising a tight, highly interconnected "core" group (~60% of participants), connected to a more diffuse "flare" (~40% of participants) via a small number of edges (Fig. 1A). This indicates that sputum lipid profiles were similar amongst members of the core group, but were markedly different in the flare group. To gain deeper insight, the "core" (C) and "flare" (F) sets were split and re-analysed in individual TDAs. This analysis yielded a ring-like network for the core set, consisting of four connected groups, which were labelled C1 to C4 (Fig. 1B). The flare set comprised a V-shaped string of five distinct groups, which were labelled F1 to F5 (Fig. 1B). Edges connecting the flare set to the core set in the original TDA network were restricted to C3, C4, F1 and F2 in the central part of the structure. The nine identified groups constitute a continuous spectrum of partly overlapping sputum lipid phenotypes, starting with the 'basal' phenotype of group C1, via 'intermediate' groups C2-C4, then F1-F2, to the 'terminal' groups F3-F5 (Fig. 1B).

To examine which components of the sputum lipidome were driving this structure, a trend analysis for ranked data was performed (JT-test). C1 and F5 were selected as the first and last group of the series, respectively, with the remaining intermediate groups ranked according to their distance from either end of the TDA structure (where groups were equidistant the order was assigned arbitrarily, *e.g.*, C2 and C3). The results showed highly significant trends from C1 to F5 for 85% of the measured lipids (Fig. 2 and Fig. E1 in this article's Online Repository at www.jacionline.org). Relative amounts of DPPC and other palmitic acid-containing PC species progressively decreased from C1 onwards, being lowest in group F5. There was a reciprocal increase in the relative quantities of other lipids, including long-chain polyunsaturated fatty acid-containing PCs, mixed alkyl-acyl PCs, other glycerophospholipids such as PE and PS, sphingolipids, sterols (Chol and its esters), and triacylglycerols (TG).

Importantly, trends in relative abundance were not always matched by the actual lipid concentration data. For example, actual concentrations of the surfactant-specific lipid DPPC were comparable across all TDA groups (JT-test, *p*-value: 0.823, *z*-score: 0.223). Moreover, a number of differences between individual phenotypes did not conform to the general trend described. For example, relative to the other core groups, C2 was enriched in PC[16:0/18:0] and PC[16:0/18:1], whereas F3 was highly enriched in cholesterol and CE species, but not as enriched in PS[36:1] as the other flare groups (Fig. E1 in this article's Online Repository at www.jacionline.org).

Trends in matched clinical and pathobiological data

Trend tests were also performed for a variety of metadata available from the U-BIOPRED tranSMART repository, including demographic and clinical measurements, blood proteins and sputum cell pellet gene expression. There was a highly significant trend in asthma severity (JT-test, *p*-value <0.001, *z*-score: 6.336), as judged by the proportion of participants from each of the four clinically characterised U-BIOPRED categories (Table 1). The proportion of healthy controls was highest in basal group C1 (42%) and decreased progressively through the intermediate groups to 12% in C4, being only 6-7% in F1, F2 and F4. The two terminal flare groups (F3 and F5) contained only mild-to-moderate and severe asthmatics, but no healthy participants. Among the clinical and pathobiological variables, highly significant negative trends were observed for lung function measurements (spirometry and reversibility), whereas subject age, ACQ scores, serum IgE levels, blood inflammatory cells and blood platelets all increased significantly from C1 to F5 (Fig. E2 in this article's Online Repository at www.jacionline.org). The sputum differential cell counts showed reciprocal increases in eosinophils and neutrophils and significant decreases in macrophages and lymphocytes. Although median sputum eosinophil levels reached 3% of total

inflammatory cell counts in four of the TDA groups, a threshold viewed as clinically relevant,[33] they were significantly higher in group F3 (26.8%) than in any other group. Similarly, neutrophil levels gradually increased from 40% in C1 to 60% in groups F1-F3, and peaked in groups F4 and F5 (medians 83% and 91%, respectively).

Of the 32 blood protein biomarkers of inflammation and tissue function available for this analysis, more than half increased significantly from C1 to F4/F5 (Fig. E3 in this article's Online Repository at www.jacionline.org). Some of the between-group differences appeared to be independent of the overall trend. For example, high levels of serum Eotaxin-3 and IL-13 were associated with the high-eosinophil group F3, whereas levels of CCL17 and Galectin-3 in that group did not differ from the basal group C1. Finally, a number of upstream transcriptional regulators of inflammation, predicted by pathway analysis of the sputum cell pellet transcriptome, also showed consistent, significant trends across the TDA structure (Fig. E4 in this article's Online Repository at www.jacionline.org). These included increasing expressions (from C1 to F4/F5) of *RAB1B*, *PLA2R1*, *SYVN1*, *CD24*, *HSP90B1* and *DNMT3B*, and decreasing expressions of *miR-10*, *miR-122*, *WT1*, *SMARCA4*, *NANOG*, *KDM5B*, *ETS1* and *SMAD3*. However, for most of the regulators any differences in expression relative to C1 appeared to be specific to discrete, smaller parts of the structure.

Discussion

In this comprehensive cross-sectional assessment of sputum lipid biomarkers, we show the existence of a continuous spectrum of molecular phenotypes from health to severe neutrophilic and eosinophilic asthma (Figs. 1 and 3). TDA of the sputum lipidome showed a progressive reduction in relative quantities of DPPC and other di-

saturated PC species from the basal group C1 to the flare groups F1-F5 (Fig. 2). This trend was matched by a progressive increase in both absolute and relative abundances of alkyl-acyl PCs, longer-chain/polyunsaturated fatty acid-containing PCs, various PE and PS species, sphingolipids, and neutral lipids (Chol, CE, DAG and TG species), in particular in the eosinophilic and neutrophilic severely asthmatic flare groups. In contrast, absolute concentrations of surface-active di-saturated PC species, the main lipid component of pulmonary surfactant,[15,34,35] did not vary significantly between TDA groups. Lipid metabolism is highly dynamic, responding to developmental, nutritional and environmental challenges by up- or down-regulating lipid synthetic and catabolic pathways that maintain homeostasis. Due to its unique role in reducing surface tension at the air–liquid interface in the alveoli, and thereby preventing collapse of these structures at end expiration, the lipid composition of pulmonary surfactant is tightly regulated by the ATII cells.[35] The constant levels of surface-active di-saturated PC species observed in this study strongly suggest that surfactant production and secretion are not significantly altered in asthma. Rather, the progressive increase in lipids that are not secreted by ATII cells as part of the pulmonary surfactant points to the presence of another source for this material, particularly in the TDA flare groups. Given the nature of this sample type, the number of potential sources is limited. Saliva was ruled out as a major source since the numbers of squamous epithelial cells derived from the upper airways, an indicator of salivary contamination,[36,37] were on average only 10% and did not vary significantly between TDA groups. Moreover, concentrations of neutral lipids, which predominate in saliva,[38] were low. Plasma infiltration in the upper airways has been shown to disrupt the respiratory lipidome, in particular during asthma attacks and allergen challenges, leading to significantly increased levels in the ELF of typical plasma lipids, such as

linoleic acid-containing PCs.[39,40] This pattern did not match any of the phenotypes described here, and lipid species such as PC[34:1] and PC[34:2] either followed similar trends to DPPC, or did not show strong trends across the TDA network at all. This indicates that plasma infiltration is not a major factor in driving the sputum lipid phenotypes observed in this study.

In contrast, highly significant associations were seen between the lipidomic trends and inflammatory cell numbers. The flare groups all contained high numbers of granulocytic inflammatory cells, predominately eosinophils in F3, neutrophils in F4 and F5, and a combination of both in F1 and F2 (Fig. 3). We speculate, therefore, that the ELF lipidome in these phenotypes is enriched by material derived from airway granulocytes. The sampling protocol required removal by centrifugation of whole cells, and samples rich in dead or damaged cells were excluded from analysis as part of the QC. Thus, we postulate that the lipid material could be derived either from small membrane fragments or secreted extracellular vesicles (EVs). The latter were recently identified in both bronchoalveolar lavage fluid and induced sputum samples from mild allergic asthma patients.[41-43]

Knowledge of the lipid pathobiology of neutrophils and eosinophils is unfortunately limited. Neutrophils are rich in PC, PE, PS, PI, SM and cholesterol, with high levels of mixed alkyl-acyl.[44-47] In eosinophils, research has mainly focused on activation-induced formation of intracellular lipid droplets and their metabolism of arachidonic acid as the precursor for pro-inflammatory lipid mediators,[48-50] but there has been no systematic profiling of the eosinophil lipidome. Both neutrophils and eosinophils release EVs in response to inflammatory stimuli, either as endosome-derived exosomes or outer membrane-derived micro-vesicles.[51,52] Such EVs were shown to be rich in sphingolipids, PS and neutral lipids, and may also reflect the lipid

compositions of their progenitor cells.[53-55] The TDA flare groups were all significantly enriched in lipids that fit this inflammatory cell profile. For example, levels of arachidonic acid-containing lipids such as PC[16:0/20:4] and PC[18:0/20:4], as well as cholesterol and its esters, were highest in these groups (especially in the eosinophil-rich group F3), as were levels of SM species and other sphingolipids (especially in F4 and F5). Levels of mixed alkyl-acyl species such as PC[O-16:0/18:1], common in neutrophils, were only enriched in the two neutrophil-rich groups.

Eotaxin production is elevated in the airways of asthmatic patients, and this chemokine can both recruit eosinophils[56] and stimulate the formation of lipid droplets in these cells, which are enriched in arachidonic acid and can act as sites of eicosanoid formation.[57,58] Such lipid droplets may selectively contribute lipid material for EVs, in particular exosomes, the formation and secretion of which is also induced by eotaxin and other inflammatory stimuli.[51] A similar mechanism was recently proposed for neutrophil-derived exosomes, which were shown to contribute to airway smooth muscle remodelling.[52] In the current study, circulating levels of a range of chemokine and cytokine markers of inflammation and tissue function were significantly higher in the serum of participants in the TDA flare groups. In addition, the upstream transcriptional regulator with the strongest positive trend across the TDA structure was *RAB1B*. Members of the Rab GTPases protein family are key regulators of intracellular membrane trafficking and are present on the membranes of lipid droplets.[59,60] Combined with the sputum lipidomics results, this suggests that pro-inflammatory mediators have additional, and potentially damaging, biological effects beyond cell recruitment. We also note a significant upregulation of the protein-coding gene *PLA2R1* in the granulocytic flare groups (F3-F5). This receptor is produced to counteract the biological effects of secreted phospholipase A2 enzymes,[61] and

PLA2R1 was previously shown to be overexpressed in the bronchial epithelium of children with atopic asthma.[62] We did not observe a direct effect of potentially increased phospholipase A2 activity on the sputum lipidome of groups F3-F5 (*e.g.*, an increase in lysophospholipid levels[63,64]). Nevertheless, phospholipase activity is likely an important driver in the pathobiology of eosinophilic and neutrophilic asthma, and additional insight is required into their localisation, substrate specificity and kinetics.

The lipid phenotypic differences observed within the TDA core group were more subtle than in the flare groups. Group C1 contained a mixed population of healthy and mildly asthmatic participants with a sputum lipid profile that matches that of healthy adults.[12-14] The remaining core groups (C2-C4) contained smaller numbers of healthy participants, and mostly comprised a mixture of mild-to-moderate and non-inflammatory severe asthmatics. Phenotype C4 was paucigranulocytic, with mean eosinophil and neutrophil counts of 2% and 43% respectively. Its lipid composition was intermediate between the healthy and severe granulocytic flare phenotypes, containing relatively less DPPC, elevated levels of the other lipid classes mentioned above, and a specific enrichment in PI species. The lipid phenotype of group C3 was intermediate between C1 and C4, with notably low levels of PIs. The main pathobiological features distinguishing these two groups of asthmatics were their atopy status and serum IgE levels, both of which were high in C4 and low in C3, suggesting the presence of a distinct sputum lipid phenotype for 'non-atopic' asthmatics.[65] Finally, group C2 had a lipid phenotype similar to that of the 'healthy' group, but with relatively less DPPC and concomitantly increased levels of C18 fatty acid-containing PC species. This group was characterised by a higher body mass index and waist circumference, suggesting that the differences were related to body weight status, rather than a particular type of asthma. Several studies have

demonstrated dysregulation of lung lipid metabolism in obese animal models,[66-68] and we have previously reported on a distinct ELF lipid phenotype in overweight, but otherwise healthy, human adults.[14]

Finally, we examined associations between the lipidome and upstream regulators of inflammation identified by IPA of sputum cell pellets. In addition to upregulated *RAB1B* and *PLA2R1* in the TDA flare groups, significant associations were observed between asthma severity and expression of *SYVN1, CD24, HSP90B1* and *DNMT3B* (upregulation), and *miR-10*, *miR-122*, *WT1*, *SMARCA4*, *NANOG*, *KDM5B*, *ETS1* and *SMAD3* (downregulation). Many of these have previously been indicated in asthma and other inflammatory diseases. The upregulation of *CD24*, known to be expressed on granulocytes and lymphocytes, matched the high-neutrophil TDA groups F2, F4 and F5.[69] In contrast, *ETS1* is a negative regulator of Th17 cells, proposed to drive specific phenotypes of asthma.[70-73] Overexpression of *DNMT3B* promotes macrophage polarization into a 'classically-activated' M1 phenotype and enhances macrophage inflammation,[74] and this gene was upregulated in all of the asthmatic groups apart from C2 and C4. Finally, Wilms Tumour 1 (*WT1*) is known to regulate the expression of Matrix metalloproteinase-9 (MMP-9), an enzyme responsible for extracellular matrix degradation and airway remodelling in asthma.[75] Our results suggest that this mechanism may be activated in the most severe neutrophilic asthmatics (F4, F5), with *WT1* downregulation leading to more MMP-9 mediated tissue.[76]

In summary, we have shown that lipidomic profiling of induced sputum stratifies asthma into a spectrum of distinct molecular phenotypes, and that the abundance and proportion of lipids that are non-endogenous to the pulmonary surfactant increases significantly with asthma severity. Based on matching trends in the clinical,

immunoassay and transcriptomic data, we propose a hypothesis for a novel mechanism of surfactant dysregulation in severe asthma, wherein granulocytes recruited into the airways are activated to produce both intracellular lipid droplets and EVs (exosomes and/or microvesicles) (Fig. 4). Upon release, these lipid and protein-rich EVs could disrupt the tightly regulated structure of the pulmonary surfactant component of the ELF,[34] in a similar way as has been proposed for granulocyte-derived proteins in asthma.[77] In turn, this would reduce the surfactant's ability to lower surface tension in the small airways, leading to collapsibility, and potentially also compromise its immunological function.[34,42]

We wish to highlight that our findings require external validation, and that the variable efficacy of widely used asthma medication such as inhaled corticosteroids may play an important role in refining the respiratory lipid phenotype model presented here. Further *in vivo* or *in vitro* studies are needed to verify the proposed mechanism, including detailed analyses of the lipidomes of granulocytes and their EVs. Nonetheless, immunomodulation of EV secretion by granulocytes in the lungs could provide a new and attractive therapeutic target for severe asthma. If the proposed mechanism is substantiated, then efforts should be directed at identifying drugs that could modulate the observed sputum lipid phenotypes, thereby further exploring the relevance of respiratory lipid metabolic changes in asthma and the potential for additional therapeutics.

Acknowledgements

The authors thank John Langley, Julie Herniman and Jon Paul Townsend for their analytical support, and Dominic Burg, Ben Nicholas, Kamran Tariq and Jeanne-Marie

Perotin-Collard for additional discussion. This study was performed on behalf of The U-BIOPRED Study Group with input from the U-BIOPRED Patient Input Platform and patient representatives from the Ethics Board and Safety Management Board. A list of the U-BIOPRED Study Group Supplementary authors and their affiliations has been provided in this article's Online Repository at www.jacionline.org).

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738 **Table 1**

		Severe asthmatic Active or ex-smoker n=51	Severe asthmatic Non-smoker n=117	Mild/moderate asthmatic Non-smoker n=43	Healthy Non-smoker n=41
Age	mean [range]	55 [29-74]	53 [21-78]	42 [18-72]	37 [18-65]
Sex (m/f)	ratio	19/32	43/74	24/19	29/12
Race (Caucasian/non-Caucasian)	ratio	49/2	109/8	40/3	37/4
Age at first diagnosis	mean [range]	35 [1-67]	24 [0-68]	20 [1-63]	NA
BMI	mean [range]	30.1 [20.6-48.4]	29.1 [17.8-51.1]	25.5 [17.9-36.6]	25.5 [18.9-32.0]
Serum IgE (mL^{-1})	mean [range]	274 [5-2690]	398 [0-6811]	328 [7-3520]	89 [0-574]
FEV1 (% predicted)	mean [range]	66.1 [24.3-113.0]	65.4 [18.4-120.6]	90.4 [40.9-132.3]	102.1 [66.9-123.6]
FVC (% predicted)	mean [range]	90.6 [54.6-129.1]	87.8 [40.2-131.3]	107.4 [68.6-151.6]	108.5 [72.2-136.4]
FEV1/FVC ratio	mean [range]	62.6 [35.2-90.0]	62.1 [31.0-92.2]	75.0 [52.4-93.9]	NA
Exacerbations (past 12 months)	mean [range]	2.5 [0-10]	2.1 [0-8]	0.4 [0-4]	NA
Smoking pack-years	mean [range]	24.2 [5-70]	0.4 [0-5]	0.7 [0-5]	0.3 [0-5]
Intubation (ever)	count [pct]	1 [2%]	13 [11%]	0 [0%]	NA
ICU admission (ever)	count [pct]	7 [14%]	28 [24%]	0 [0%]	NA
Positive atopy test	count [pct]	27 [53%]	73 [62%]	36 [84%]	14 [34%]
ACQ1-5 score	mean [range]	2.2 [0.2-4.4]	2.2 [0-5.0]	0.8 [0-2.4]	NA
ACQ7 score	mean [range]	4.2 [0-7.0]	3.9 [0-7.0]	1.8 [0-7.0]	NA
AQLQ score	mean [range]	4.4 [2.3-6.8]	4.6 [1.9-6.8]	5.9 [3.2-6.9]	NA
Oral corticosteroid use (current)	count [pct]	25 [49%]	49 [42%]	0 [0%]	NA

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Inhaled corticosteroid use (current)	count [pct]	50 [98%]	113 [97%]	43 [100%]	NA
Injectable corticosteroid use (current)	count [pct]	0 [0%]	8 [7%]	0 [0%]	NA
Long-acting ß-agonist use (current)	count [pct]	48 [94%]	112 [96%]	1 [2%]	NA
Short-acting ß-agonist use (current)	count [pct]	37 [73%]	91 [78%]	32 [74%]	NA
Corticosteroid dose (mg day^{-1})	mean [range]	13.7 [2.5-40.0]	12.4 [5.0-37.5]	NA	NA

Demographics of study participants according to the U-BIOPRED cohorts (see main text for definitions). Ex-smokers with a pack-year smoking history of ≤5 were considered to have a 'negative' smoking status, whereas ex-smokers with pack-year ≥5 were only included in the study if also diagnosed with severe asthma. Abbreviations: BMI = body mass index; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity; IgE = Immunoglobulin E; ICU = intensive care unit; ACQ = Asthma Control Questionnaire; AQLQ = Asthma Quality of Life Questionnaire; NA = not applicable/not assessed. Systemic dosage of corticosteroids for severe asthmatic participants is expressed in prednisolone-equivalent doses.

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Figure legends

Fig. 1 TDA structures of (A) the complete study cohort (n=252) and (B) the 'core' and 'flare' subgroups side by side (n=164 and n=107 respectively), coloured by FEV_1 (forced expiratory volume in 1 second) from 50% (red) to 100% (green; see histogram in inset). TDA was performed on 291 sputum lipid ions, using a normalised correlation metric and two MDS lenses. The TDA groups, as delineated by density mode clustering, along with the proportion of participants present in each group are shown in B. The original figures were obtained with the SymphonyAI machine intelligence platform (https://www.symphonyai.com/).

Fig. 2 Box plots of representative lipid species demonstrating the trends across the TDA structure. Relative abundances are given as a percentage of the total lipid, and boxplots were coloured from blue (low) to red (high) to highlight trends. The original plots were created in SPSS Statistics 24 which defines outliers as 'near' (open circles: more than 1.5 times the interquartile range) and 'far' (stars: more than 3 times the interquartile range). See main text for abbreviations of the lipid classes.

Fig. 3 Summary of the sputum lipid phenotypes found in this study and their assignments based on associations with the demographic, clinical and pathobiological data (predominately sputum differential cell counts). As shown in Fig. 1 and discussed in the main text, the nine phenotypes represent a spectrum of asthma severity from C1 (low) to F3 and F5 (high). Key characteristics of the main 'core' and 'flare' groups

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are listed on the left and can be found in Figs. E1-E4 in this article's Online Repository (www.jacionline.org).

Fig. 4 Conceptual representation of the potential role of granulocytic inflammation in producing EVs (exosomes and/or micro-vesicles) that may alter the lipid composition of the ELF (shown as light blue layer) in asthma. Pro-inflammatory chemokines and cytokines (red arrows) recruit eosinophils and neutrophils into the airways and stimulate intracellular lipid droplet formation and the secretion of EVs. The latter are rich in cellular lipids and proteins, which could impair the function of the pulmonary surfactant component of ELF, thereby reducing its ability to lower surface tension in the small airways and potentially compromising its role as an immunological barrier.

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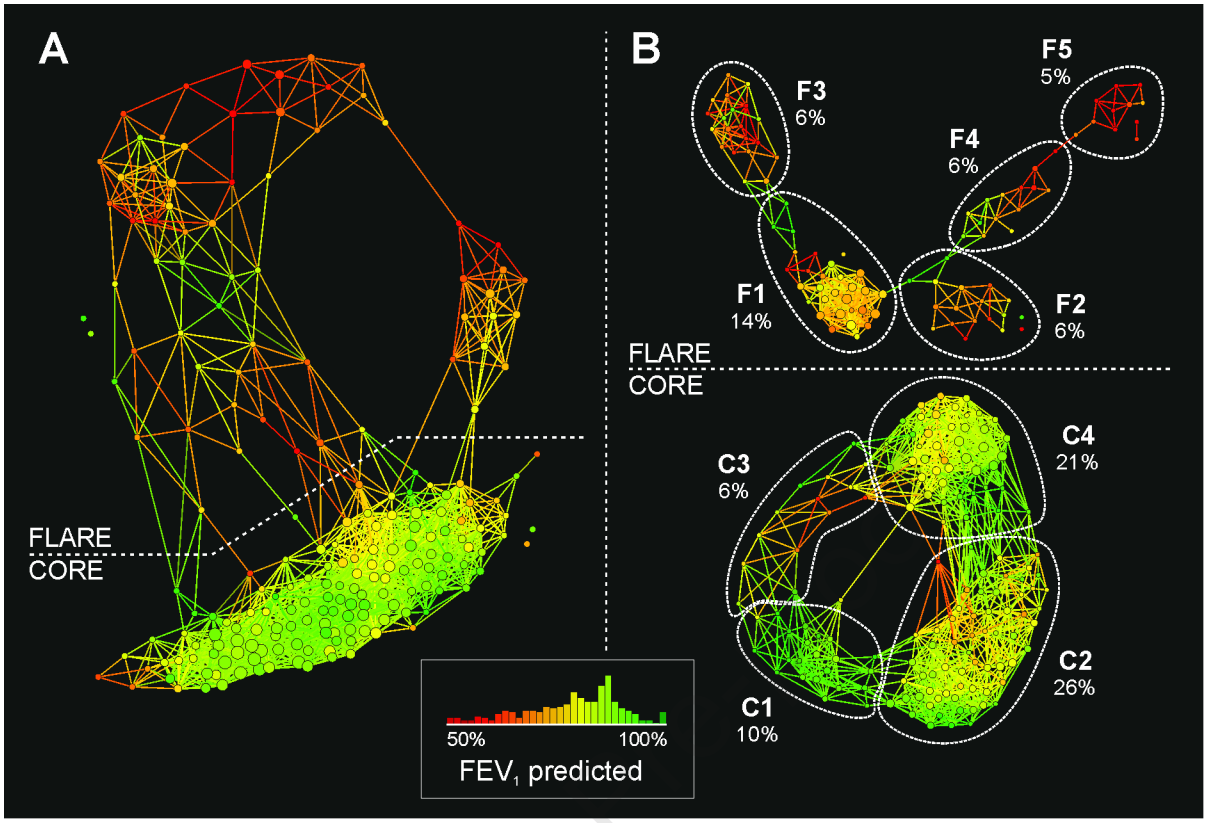

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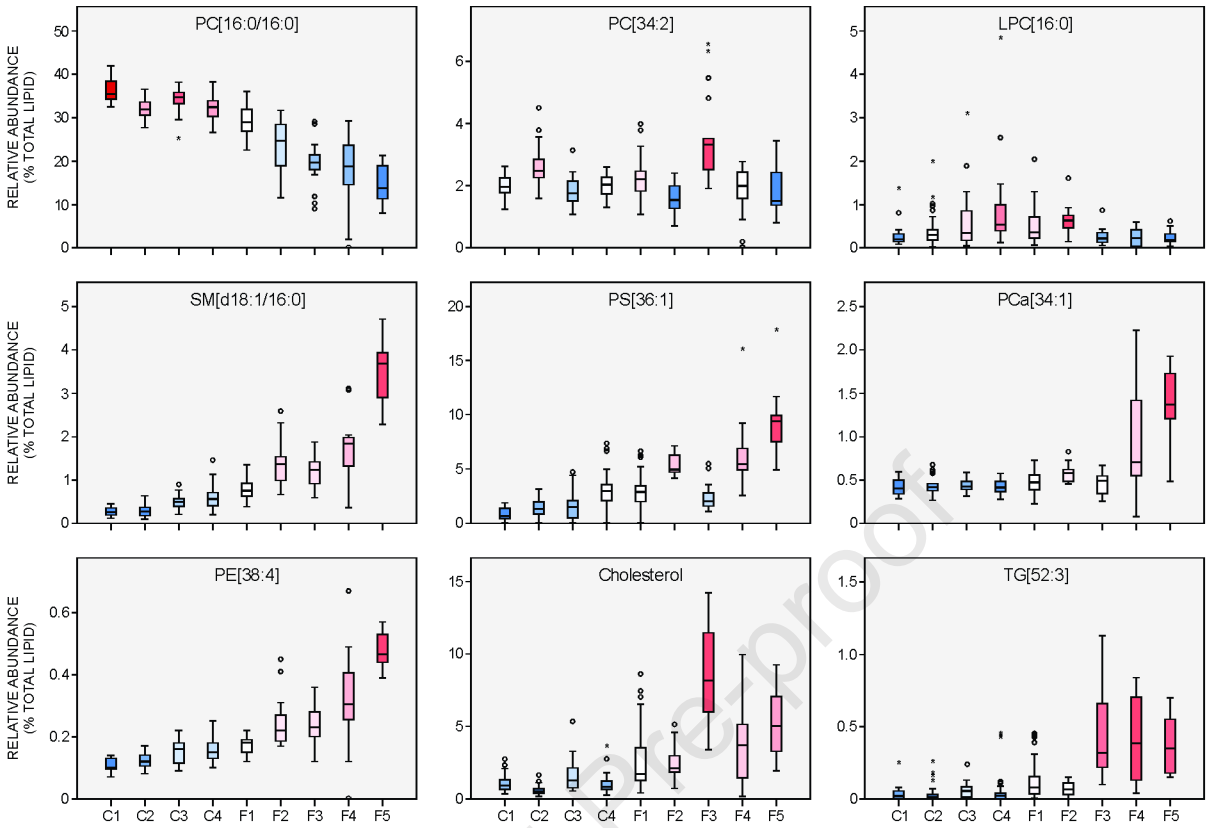

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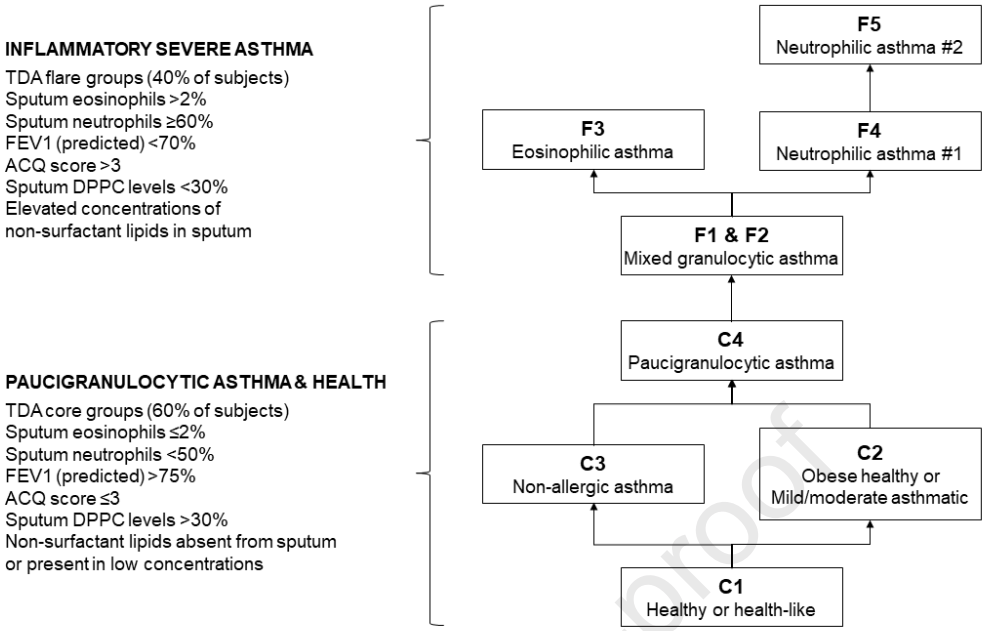

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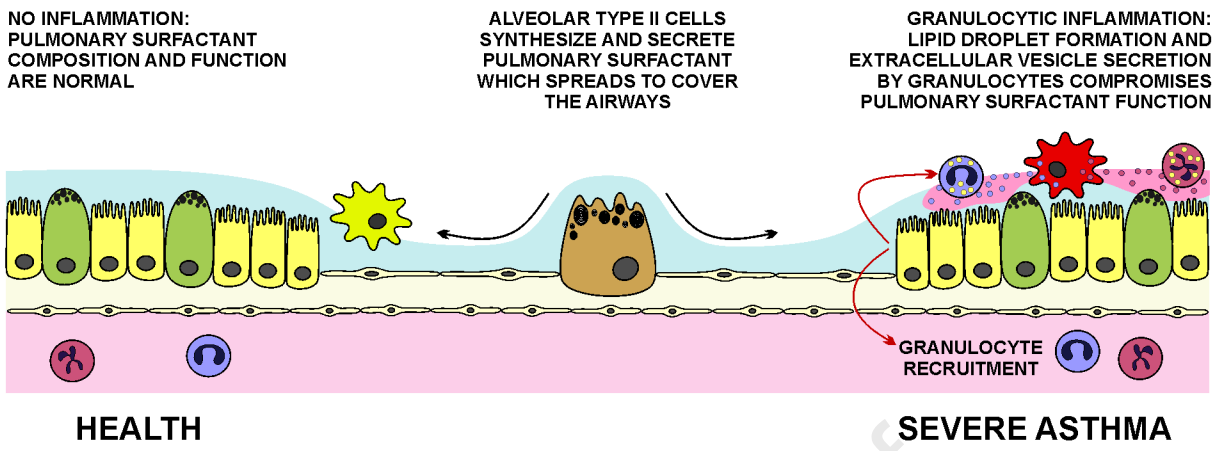

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