SHORT COMMUNICATION

Comparison of Normal and Metaplastic Epithelium in Patients with Stable versus Persistently Symptomatic Severe Asthma Using Laser-Capture Microdissection and Data-Independent Acquisition—Mass Spectrometry

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Accepted for publication
August 22, 2019.

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A proportion of patients with severe asthma (SA) show poor responses to traditional asthma medications; however, it remains unknown why some patients remain persistently symptomatic. Our objective was to explore the use of laser-capture microdissection of specific epithelial structures combined with quantitative data-independent acquisition mass spectrometry to elucidate differences in protein composition in patients with SA with varying symptom control. Unbiased label-free quantitative proteome analyses were performed on laser-capture—microdissected areas of specific epithelial structures from patients with SA with varying degrees of symptom control. A total of 1993 stable SA and 1652 symptomatic SA proteins in normal epithelium and 1458 stable SA and 1647 symptomatic SA proteins in metaplastic epithelium were quantified. When comparing proteome profiles based on symptom control, 33 proteins in patients with stable SA ($\geq$ twofold change; $P \leq 0.05$) and 13 proteins in patients with persistently symptomatic SA ($\geq$ twofold change; $P \leq 0.05$) were enriched significantly. When comparing proteome profiles based on epithelial status, 21 proteins in normal epithelium ($\geq$ twofold change; $P \leq 0.05$) and 6 proteins in metaplastic epithelium ($\geq$ twofold change; $P \leq 0.05$) were enriched significantly. New treatment strategies are needed for patients with severe asthma and exploratory studies of unbiased nature such as this may help when searching for new mechanisms and potential targets involved in the disease pathology. (*Am J Pathol* 2019, 189: 2358–2365; <https://doi.org/10.1016/j.ajpath.2019.08.003>)

Asthma is a chronic inflammatory airway disorder characterized by variable airflow obstruction with an increasing prevalence worldwide.¹ Severe asthma (SA) is defined by the requirement of treatment with high-dose inhaled and/or systemic corticosteroids in combination with a second long-term controller medication, and represents approximately 5% to 10% of all asthma patients.² In addition, a proportion of patients with SA show poor responses to traditional

Supported by Heart-Lung Foundation grant SBE 13-0130 (G.W.-T.), Swedish Medical Research Council grant 2016-01190 (G.W.-T.), Swedish Asthma and Allergy Associations Research Foundation grant F2017-0034 (M.W.), Evy and Gunnar Sandberg Foundation grant 2015 (G.W.-T.), Greta and John Kock Foundation grant 20171027GWT (G.W.-T.), Alfred Österlunds Foundation grant GWT2015 (G.W.-T.), Royal Physiographical Society grant 20181114 Weitoft (M.W.), and the ALF Agreement between the Government and the County Councils (Sweden).

Disclosures: None declared.

asthma medications; the reason why some patients remain persistently symptomatic is unknown.

The airway epithelium serves as a first line of defense against all inhaled substances, including allergens and infectious agents, and, in addition to forming a physical barrier, epithelial cells coordinate and communicate with cells of the innate and adaptive immune system.^{3,4} The loss of pseudostratified epithelium and an increase in metaplastic epithelium, as seen in patients with SA,⁵ can be caused by a number of external assaults such as viral infections or cigarette smoke, which can disturb the innate immune functions of the epithelium.^{6,7} A better understanding of the nature of epithelial pathology in SA is needed and may provide insights into the persistence of symptoms in certain individuals with SA. The aim of this study was to explore the use of laser-capture microdissection (LCM) of specific epithelial structures combined with quantitative data-independent acquisition mass spectrometry (DIA-MS) to elucidate spatial differences in protein composition in SA patients with varying symptom control. The proteome differences in patients with stable SA compared with patients with symptomatic SA were found to be more pronounced than the differences between normal and metaplastic epithelium; these findings should be explored in larger cohorts.

Materials and Methods

Patient Characteristics

The present study involved 12 patients with SA treated with oral corticosteroids according to the American Thoracic Society criteria,⁸ recruited at Aarhus University Hospital (Aarhus, Denmark). The ethics committee in Aarhus approved the study. All subjects provided informed consent to participate in the study.

LCM

From each patient, formalin-fixed, paraffin-embedded tissue samples were sectioned at 4 μ m using a microtome. Sections were placed onto polyethylene naphthalate membrane slides (membrane slide 1.0 polyethylene, 415101-4401-000; Carl Zeiss MicroImaging, Jena, Germany) to allow RoboLCM-mode. Before use, polyethylene naphthalate slides were activated by 30 minutes of UV irradiation (254 nm) using a Spectrolinker/Crosslinker XL-1000 UV (Techtum, Umeå, Sweden). Sections were air-dried, deparaffinized (2 $\times$ xylene for 5 minutes, 2 $\times$ absolute ethanol for 2 minutes, 95% ethanol 1 minute, 70% ethanol 1 minute, and two changes of distilled H₂O), and stained with Mayers hematoxylin (Histolab, Askim, Sweden) for 30 seconds to visualize nuclei. After three changes of H₂O, sections were dehydrated (2 $\times$ 95% ethanol for 1 minute, absolute ethanol for 2 minutes), air dried, and stored at -20°C in sealed containers. Before LCM, sections were allowed to equilibrate to room temperature, avoiding condensation. LCM was performed on a PALM MicroBeam system

(Carl Zeiss MicroImaging) using the PALM Robo 4.6 Pro Software (Carl Zeiss MicroImaging), with a 10 $\times$ objective in the RoboLPC mode (defined structures are cut out and catapulted into the lid of the adhesive tube). Images of the slides were acquired using an AxioCamICc1 (Carl Zeiss MicroImaging), and the CapCheck function was used to control successful collection of dissected tissue into the lid (Figure 1).

MS Sample Preparation

Sample preparation was performed according to Braakman et al,⁹ with modifications. LCM material in the lids of the tubes was dissolved in 20 μ L per lid with 0.1% RapiGest w/v (186001861; Waters Corporation, Milford, MA) in 50 mmol/L ammonium bicarbonate and 8 mol/L urea and incubated at room temperature for 30 minutes. Subsequently, the lysate was centrifuged to the bottom of the tube (30 s, 6082 $\times$ g; Biofuge pico, Heraeus; Thermo Fisher Scientific, Waltham, MA). Tissue slices were sonicated in Bioruptor Plus (Diagenode SA, Seraing, Belgium) at 4°C for 20 cycles for 15 seconds on/off. Proteins were denatured at 99°C at 3 $\times$ g for 5 minutes, reduced by incubation with 5 mmol/L dithiothreitol at 60°C for 30 minutes, and alkylated with 15 mmol/L iodoacetamide for 30 minutes. Samples were diluted with 50 mmol/L ammonium bicarbonate to 1.6 mol/L urea concentration and digested with 1 μ g Lys-C/Trypsin mix (Promega, Madison WI) overnight at 37°C at 300 rpm. The digestion was stopped by addition of 10% trifluoroacetic acid to a final concentration of 0.5% (v/v) and RapiGest was degraded by incubation for 60 minutes at 37°C after acidifying the sample. The peptide samples were desalted using SP3 beads (Thermo Scientific, Fremont, CA) as described previously.¹⁰ Because the starting material was peptides, the peptide SP3 clean-up procedure was followed. Briefly, acidified peptide sample were supplemented with 4 μ L SP3 beads and acetonitrile was added to a final concentration of >95%. After incubation for 8 minutes, samples were washed with 100% acetonitrile. Peptides were eluted by incubation for 5 minutes in aqueous buffer with 2% dimethyl sulfoxide, followed by sonication. Peptide samples were mixed 1:1 (v/v) with 2 $\times$ buffer A [4% acetonitrile, 0.4% formic acid, iRT peptides (1:10 v/v; Biognosys AG, Schlieren, Switzerland)], providing a final concentration of 2% acetonitrile, 0.2% formic acid, 1% dimethyl sulfoxide, and iRT peptides 1:20. To compensate for differences between the collected tissue areas, the injection volumes were adjusted to the sample with the smallest area collected.

LC-MS/MS Analysis

Peptides were separated on an EASY-nLC 1200 HPLC system (Thermo Fisher Scientific) using a 50-cm EASY-spray PepMapRSLC C18 column (Thermo Fisher Scientific). For peptide separation the following segmented gradient of solvent B (0.1% formic acid in 80% acetonitrile) over solvent A (0.1% formic acid) was used; 3% to 10% solvent B for 11 minutes, 10% to 30% solvent B for 87 minutes, 30% to 45% solvent B for 22

Figure 1 Laser-capture microdissection (LCM) of normal and metaplastic epithelium. Normal pseudostratified epithelium is indicated by the presence of cilia and goblet cells, whereas metaplastic epithelium is disorganized and lacks clear cilia and goblet cells. During LCM, a region of interest is delineated (green lines), dissected by the laser, and can be visualized in the tube (cap check). Numbers in yellow rectangles are annotations of each area that is marked for dissection; numbers in white rectangles are area in μm^2 of dissected area shown. Arrow indicates ciliated epithelium; asterisk, goblet cells; blue dots, where the laser lifts the tissue from the glass slide. LCM samples were subsequently analyzed using label-free quantitative data-independent acquisition mass spectrometry (DIA-MS). Scale bars = 150 μm . PEN, polyethylene naphthalate.

Proteome analysis using DIA-MS

minutes, 45% to 80% solvent B for 4 minutes, followed by 100% solvent B for 10 minutes, using a flow of 350 nL/minute. Data acquisitions were performed on a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific). For DIA, full MS scans were acquired at a resolution of 120,000 at 200 m/z , with automatic gain control target $3e6$, and a maximum injection time of 100 ms at a mass range of 350 to 1650 m/z . Each full scan was followed by 26 MS/MS fragmentation scans using variable isolation windows with 30,000 resolution at 200 m/z ,

automatic gain control target $3e6$, and a maximum injection time of 50 ms. The 26 variable isolation windows had the following consecutive m/z : 33, 26, 22, 20, 20, 18, 20, 19, 19, 19, 20, 21, 21, 23, 23, 24, 26, 31, 32, 37, 40, 53, 66, 99, and 574.

MS Data Analysis

All data analyses were performed in openBIS¹¹ using the peptide assay library described previously¹² for DIA

Figure 2 Label-free quantitative characterization of normal and metaplastic epithelium proteome profiles from patients with stable and persistently symptomatic severe asthma (SA) using data-independent acquisition mass spectrometry. **A:** Venn diagram of the total number of proteins identified in each of the four sample groups (symptomatic SA normal epithelium, symptomatic SA metaplastic epithelium, stable SA normal epithelium, and stable SA metaplastic epithelium). **B:** Spearman correlation matrix of laser-capture microdissection (LCM) samples. **C** and **D:** Volcano plots for stable SA versus symptomatic SA (**C**) and normal versus metaplastic epithelium (**D**). Red dots indicate proteins that are significantly differentially expressed and have a fold change of ≥ 2 or ≤ -2 and a P value ≤ 0.05 .

quantifications. DIA files were analyzed using open-SWATH¹³ where workflows included FDR calculations of 1% for both peptide and protein, followed by feature alignment with TRIC (transfer of identification confidence).¹⁴

Statistical Analysis

Data were analyzed statistically using RStudio version 1.1.442 (RStudio, Boston, MA). The t -test was used to calculate P values, where P values of <0.05 were considered statistically significant.

Results

Demographic and Clinical Characteristics

Lung biopsy specimens were taken at Aarhus University Hospital under anesthesia at the lobar carina level using a stiff bronchoscope. Biopsy specimens with well-preserved morphology were serially sectioned and analyzed at Lund University. The 12 patients with SA included in the study were divided based on their symptom profile according to patient journals, which included patient self-assessment data. The two groups, stable SA ($n = 6$) and symptomatic SA ($n = 6$), were matched in terms of sex, age (median,

Figure 3 Clustering of significantly differentially expressed proteins isolated from normal and metaplastic epithelium of patients with stable or persistently symptomatic severe asthma (SA). Heat map of differentially expressed proteins from patients with stable SA and symptomatic SA (**A**) and normal and metaplastic epithelium (**B**), with a fold change of ≥ 2 or ≤ -2 and a P value ≤ 0.05 . Data are presented as a z-score, in which red color codes for higher abundance and blue color codes for lower abundance.

43 years; $P = 0.23$), body mass index (median, 28 kg/m²; $P = 0.13$), dose of oral corticosteroids (median, 15 mg/day; $P = 0.63$), dose of inhaled corticosteroids (median, 2000 µg; $P = 0.63$), duration of disease (median, 18 years; $P = 0.69$), and forced expiratory volume in one second/forced vital capacity (FEV₁/FVC) (median, 0.69; $P = 0.94$). One of six patients with stable SA and two of six patients with symptomatic SA were ex-smokers, all patients in both groups used long-acting β_2 -agonists and five of six patients with stable SA and all six patients with symptomatic SA used leukotriene-receptor antagonists.

Extraction of Normal and Metaplastic Epithelium by LCM

Normal epithelium was identified by being pseudostratified with clearly visible cilia, goblet cells, and basal cells.

Metaplastic epithelium was identified by the disturbed pseudostratified organization with general absence of goblet cells, basal cells, and cilia (**Figure 1**). Dissected material was collected in the lids of adhesive cap tubes (opaque 500 µL; Zeiss Adhesive Cap tubes), in four pools, as follows: i) stable SA/normal epithelium, total area collected: 989,760 µm²; ii) stable SA/metaplastic epithelium, total area collected: 5,420,655 µm²; iii) symptomatic SA/normal epithelium, total area collected: 1,360,367 µm²; and iv) symptomatic SA/metaplastic epithelium, total area collected: 432,930 µm².

Protein Identifications in Microdissected Regions

LCM regions of normal and metaplastic epithelium from patients with stable SA and symptomatic SA were analyzed using label-free quantitative DIA-MS (**Figures 1** and **2A**). In

Figure 4 Pathway enrichment analysis of biological processes connected to proteins of significantly increased abundance in patients with stable severe asthma (SA) compared with symptomatic SA using the String database.¹⁵ Processes with a false discovery rate $\leq 4\%$ containing at least two proteins are presented.

normal epithelium, 1993 proteins were quantified in stable patients with SA and 1652 in symptomatic patients with SA. In metaplastic epithelium, 1458 proteins were quantified in stable patients with SA and 1647 in symptomatic patients with SA. Data are summarized in a Spearman correlation matrix in [Figure 2B](#). Among all identified proteins, 47% were detected in all four groups, that is, in normal and metaplastic epithelium from patients with stable and persistently symptomatic SA. A list of identified proteins and calculations from sample group comparisons can be found in [Supplemental Table S1](#).

Differences in Proteome Profiles Based On Symptom Control/Clinical Parameters

When comparing proteome profiles based on symptom control, 33 proteins were found to be enriched significantly in patients with stable SA ($\geq$ twofold change; $P \leq 0.05$) and 13 proteins were found to be enriched significantly in patients with persistently symptomatic SA ($\geq$ twofold change; $P \leq 0.05$) ([Figure 2C](#)).

Differences in Normal versus Metaplastic Epithelium Proteome Profiles

When comparing proteome profiles based on epithelial status, 21 proteins were found to be enriched significantly in normal epithelium ($\geq$ twofold change; $P \leq 0.05$) and six proteins were found to be enriched in metaplastic epithelium ($\geq$ twofold change; $P \leq 0.05$) ([Figure 2D](#)).

Clustering of Differentially Expressed Proteins

Unsupervised hierarchical clustering of Z-score values of the significantly differentially enriched proteins showed two distinct protein clusters when comparing stable with symptomatic SA ([Figure 3A](#)) and normal with metaplastic epithelium ([Figure 3B](#)). Thirteen proteins were increased in abundance and 33 proteins were decreased in patients with persistently symptomatic SA compared with patients with stable SA. Twenty-one proteins were increased in abundance and six were decreased in normal epithelium compared with metaplastic epithelium.

Pathway Enrichment Analysis of Proteins with Increased Abundance

String database (<https://string-db.org>, last accessed May 23, 2019) and Gene Ontology were used to examine protein–protein interactions and to perform an enrichment analysis of the significantly differentially expressed proteins in the four different groups based on symptom control and epithelial status. An over-representation tool of biological processes determines a probability score for which pathways are significantly up-regulated, with assessment of the false-discovery rate using the Benjamini-Hochberg method.¹⁵ There were 36 pathways associated with the significantly enriched proteins in patients with stable SA compared with symptomatic SA, irrespective of epithelial status. Enriched pathways include the oxidation-reduction process, the regulation of biological quality, and the immune-effector process. The most significantly enriched pathways with a false-discovery rate of $\leq 4\%$ are summarized in [Figure 4](#).

Discussion

Here, unbiased label-free quantitative proteome analyses were performed on laser-capture microdissected areas of specific epithelial structures from patients with SA with varying degrees of symptom control. Asthma is a global health problem and approximately 10% of all patients with asthma have a severe form of the disease, some persistently symptomatic, with great consequences on general quality of life.

The airway epithelium is a complex structure functioning both as a physical barrier that prevents provocants such as allergens, irritants, and oxidative radicals from entering the airway tissue, and as an immunomodulator it communicates with cells of adaptive and innate immunity and is believed to play an important role in asthma pathogenesis.^{3,4,16–18}

The approach with LCM combined with DIA-MS allowed quantifying 1993 proteins from small amounts of formalin-fixed, paraffin-embedded tissue on histologic slides ($\leq 0.43 \text{ mm}^2$). Interestingly, a larger number of significantly differentially abundant proteins were found based on symptom control (46 proteins) rather than epithelial status (27 proteins), suggesting that the regulation of symptom control has a greater impact on the tissue proteome than the epithelial status. Although significant, the results should be interpreted with some caution because of the low number of patients, and future studies should confirm the results in larger subject groups. For further stratification of degree of symptom control and its relation to normal and abnormal (metaplastic) epithelium, pathway enrichment analyses of the quantified proteome profiles were performed. Enriched processes, irrespective of epithelial status, relating to immune effector processes, regulation of exocytosis, and oxidation-reduction processes were found among the pathways enriched in patients with stable SA.

Among the proteins with significantly increased abundance in patients with stable SA as compared with symptomatic SA were peroxiredoxin-1, fibronectin, major vault protein, and complement component C3. These proteins all have been found previously to be involved in inflammation and have potential roles in asthma pathology.^{19–31}

N-myc downstream-regulated gene 1 protein (NDGR1), which is important for maintaining airway epithelial integrity, showed increased abundance in patients with symptomatic SA compared with stable SA, which could indicate ongoing repair mechanisms.³² In addition, NDGR1 also has been found to act as a metastasis suppressor, interrupting epithelial–mesenchymal transition and migration and invasion.^{33,34} Leucine-rich α -2-glycoprotein also was increased in patients with symptomatic SA. Inflamed tissues can produce leucine-rich α -2-glycoprotein, and sputum leucine-rich α -2-glycoprotein has been proposed as a biomarker for local airway inflammation in asthma.³⁵

Proteomic analysis of sputum have shown a protein profile that can differentiate symptomatic from stable asthma, primarily by neutrophil markers and extracellular matrix degradation proteins.³⁶

Olfactomedin-like 3 and apoptosis inhibitor-5 were among the differentially expressed proteins with increased abundance in normal compared with metaplastic epithelium irrespective of symptom control status. Apoptosis inhibitor-5 is a protein with potent anti-apoptotic signaling in tumor cells and olfactomedin-like 3 has been found to have a proangiogenic effect within the tumor microenvironment.^{37,38}

LCM combined with DIA-MS is a good approach to better understand pathologic mechanisms and their impact on tissue integrity and spatial proteome profiles, which can be used as a starting point to look further into underlying disease mechanisms, even in limited patient materials. In conclusion, new treatment strategies are needed for patients with SA and exploratory studies of an unbiased nature may help when searching for new mechanisms and potential targets involved in the disease pathology.

Acknowledgments

We thank Karin Jansner for skillful technical assistance; Ingrid Hedenfalk and Anna Ebbesson (Lund University) for providing the LCM infrastructure and support; and Sven-Ingvær Weitoft for proofreading the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.08.003>.

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