Lipid mediator profiles differ between lung compartments in asthmatic and healthy humans

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Oxylipins are oxidized fatty acids that can exert lipid mediator function in inflammation and several oxylipins derived from arachidonic acid are linked to asthma. This study quantified oxylipin profiles in different regions of the lung to obtain a broad-scale characterization of the allergic asthmatic inflammation in relation to healthy individuals.

Bronchoalveolar lavage (BAL) fluid, bronchial wash (BW), and endobronchial mucosal biopsies were collected from 16 healthy and 16 mild allergic asthmatic individuals. Inflammatory cell counts, immunohistochemical (IHC) staining and oxylipin profiling were performed. Uni- and multivariate statistics were employed to evaluate compartment-dependent and diagnosis-dependent oxylipin profiles in relation to other measured parameters.

Multivariate modelling showed significantly different BW and BAL oxylipin profiles in both groups ($R^2_Y[cum]=0.822$, $Q^2[cum]=0.759$). Total oxylipin concentrations, and five individual oxylipins, primarily from the lipoxygenase (LOX) pathway of arachidonic and linoleic acid, were elevated in BW from asthmatics compared to healthy controls, supported by IHC staining of 15-LOX-1 in the bronchial epithelium. No difference between the groups was found among BAL oxylipins.

In conclusion, BW and BAL fluid contain distinct oxylipin profiles, which may have ramifications for the study of respiratory diseases. Specific protocols for sampling proximal and distal airways separately should be employed.

**Keywords:** Airway inflammation, asthma, bronchoalveolar lavage, bronchial wash, lung compartments, oxylipin profiles
INTRODUCTION

Asthma is a common respiratory disease characterized by chronic airway inflammation, bronchial hyper-responsiveness and airflow limitation that affects ~300 million people worldwide with a substantial disease burden in terms of morbidity, mortality and economic cost [1, 2]. There is a broad range of asthmatic phenotypes with different clinical presentation and pathophysiology [3].

The role of lipid mediators in the pathogenesis of asthma has been extensively studied [4-10]. Lipid mediators are included in a group of compounds broadly called oxylipins; a term that includes eicosanoids derived from arachidonic acid (AA) as well as compounds derived from other related ω-3 and ω-6 fatty acids. These compounds are produced via 3 biosynthetic pathways: lipoxygenase (LOX; e.g., leukotrienes), cyclooxygenase (COX; e.g., prostaglandins) and cytochrome P450 (CYP) [11, 12]. In particular, 15-LOX expression and induction have been shown to be elevated in the lower airways of asthmatics [13, 14]. Both pro- and anti-inflammatory characteristics have been attributed to 15-LOX lung activity, in the form of derivatives such as eoxins (pro-inflammatory) and lipoxins (anti-inflammatory), as well as through the protective ω-3 derived resolvins and protectins [12, 15-17]. The dual properties of 15-LOX activity illustrate how complex biological interactions, involving multiple oxylipin species, affect inflammation initiation, progression, and resolution.

Oxylipin profiles have previously been investigated in large volume bronchoalveolar lavage (BAL) mainly reflecting the peripheral lung [7, 8], but not in small volume bronchial wash (BW) samples, which better reflect the proximal airways. To test the hypothesis that asthmatics, due to their underlying asthmatic inflammation, have altered oxylipin profiles in both distal (BAL) and proximal (BW) regions of the lung compared to healthy individuals, bronchoscopies were performed. A broad selection of compounds was quantified in both BAL and BW through an oxylipin metabolic profiling approach. The resulting profiles were
investigated together with clinical parameters and inflammatory cell counts using uni- and multivariate statistics, highlighting the utility of broad scale metabolic profiling methods to investigate respiratory disease.

MATERIALS AND METHODS

Subjects

Healthy and asthmatic subjects were invited to participate in the present study through advertisement. Subjects fulfilled the following inclusion criteria: age 18-40 years, never smokers, normal lung function (forced expiratory volume in 1 second (FEV₁) and forced vital capacity (FVC) of at least 80% of predicted and a normal FEV₁/FVC ratio) and absence of concomitant diseases, apart from allergy in the asthmatic group. All asthmatics had a positive history of allergy together with at least one positive skin prick test against a standard panel of common aeroallergens. In the asthmatic group, bronchial hyper-responsiveness to methacholine with provocative concentration causing a 20% fall in FEV₁ (PC₂₀) <8 mg/ml was required. All participants were free of respiratory infection for 6 weeks prior to the study. No antioxidant supplementation or anti-inflammatory medication was allowed 2 weeks prior to the study. Asthma severity was classified as intermittent according to GINA guidelines [18], treated only with short-acting inhaled β₂-agonists on demand. Inhaled or nasal corticosteroids were terminated at least three months prior to the study. Thirty-two Caucasian volunteers were included; 16 asthmatics and 16 healthy controls. Subject demographics are shown in Table 1.

Three satisfactorily performed lung function measurements were carried out, according to the recommendations of the American Thoracic Society (ATS) [19], using a Vitalograph spirometer (Vitalograph Ltd. Buckingham, UK). Methacholine challenge was performed using the method described by Juniper et al [20].
Informed consent was obtained from all volunteers after verbal and written information. The study was approved by the local Ethics Review Board at Umeå University, Sweden, and performed according to the declaration of Helsinki.

**Study design**

The study was performed outside pollen season. Prior to the study visit, subjects fasted from midnight and were asked to refrain from alcohol-containing beverages for 24 h. Measurements of exhaled nitric oxide (FENO) and bronchoscopy were performed in the morning. Healthy and asthmatic participants were examined in a randomised order.

**Fraction of exhaled nitric oxide (FENO)**

Before measurements, the participants rinsed their mouths with water. The participants wore a nose-clip while first inhaling deeply and then slowly exhaling against a resistance according to ATS/ERS recommendations [21]. FENO was measured three times at each flow rate (10, 50 and 100 ml/s) using a chemiluminescence analyser (NiOX; Aerocrine AB, Stockholm, Sweden).

**Bronchoscopy**

Bronchoscopy was performed as previously described [22], using a flexible video bronchoscope (Olympus BF-1T160, Tokyo, Japan). A detailed method description is provided in the Supporting Information. Endobronchial mucosal biopsies were taken from proximal criptae. Bronchial wash (BW, 2x20 ml) and bronchoalveolar lavage (BAL, 3x60 ml) were carried out with saline solution on the contralateral side. The aspirates recovered from the instillations of BW and BAL were processed and analysed as previously described [23].
Immunohistochemistry

Endobronchial mucosal biopsies were processed and embedded into glycol methacrylate resin (Polyscience; Northampton, UK), according to the method developed by Britten et al [24]. Two-micron thick sections were cut and stained immunohistochemically. The monoclonal antibodies used were directed against mast cells, eosinophils, neutrophils and 15-LOX-1. The immunostaining procedure has been described previously [23]. Stained inflammatory cells were counted in the epithelium and in the submucosa excluding glands, blood vessels and muscle. The cell counts were expressed as cells/mm in the epithelium and cells/mm² in the submucosa and counted using a light microscope. 15-LOX-1 staining was quantified and expressed as % of stained epithelial area. Length and areas of the epithelium and submucosa and positive epithelial staining areas were determined using the program LeicaQWin V3 (Leica Microsystems, Wetzlar, Germany).

Oxylipin extraction and analysis

Oxylipin analysis was performed according to previously published protocols [25, 26]. Standards (native and deuterated) and N-cyclohexyl-N'-dodecanoic acid urea (CUDA) used for recovery calculations were obtained from Cayman Chemical (Ann Arbor, MI, USA), Larodan Fine Chemicals AB (Malmö, Sweden) and Biomol International (Plymouth Meeting, PA, USA). The list of analysed oxylipins is given in Supporting Information (Table S1). Solid phase extraction (SPE) was performed using Waters Oasis HBL 60 mg cartridge columns (Milford, MA, USA). Oxylipins were analysed using an Aqutiy Ultra Performance Liquid Chromatography (UPLC) separation module, equipped with a 2.1 × 150 mm BEH C18 column with a 1.7 µm particle size (Waters) coupled to a Xevo TQ triple quadrupole tandem mass spectrometer run in negative ionization mode. Quantification of oxylipins above the limit of quantification (LOQ), defined as peaks with signal to noise ratio (S/N) >10, was
performed using the stable isotope dilution method. Oxylipins below the LOQ, but above the limit of detection (LOD), defined as peaks with S/N >3, were assigned the method LOD value in the subsequent statistical analyses.

**Statistical analysis**

Univariate statistical analysis using Mann-Whitney U test was performed with PASW Statistics 18.0 (SPSS; Chicago, IL, USA) or the R software (R Foundation for Statistical Computing, Vienna, Austria). A p-value <0.05 was considered significant. Multivariate data analysis (MVA) using principal component analysis (PCA) and orthogonal projections to latent structures with discriminant analysis (OPLS-DA) was performed with SIMCA software v.13 (Umetrics AB, Umeå, Sweden) [27]. Model validity was assessed using the cumulative amount of systematic variation among the variables summarized by the model (R^2_X[cum] and R^2_Y[cum]), the predictive ability of the model (Q^2[cum]), and p-values calculated by ANOVA based on the cross-validated score vectors (CV-ANOVA) [28]. All data were scaled to unit variance and mean-centred prior to modelling.

A description of these multivariate methods is provided in the Supplementary Material, of which a summary is provided here. MVA can be described as tools for reducing the dimensionality of large datasets to render the visualization and interpretation more manageable. The relationship between the clinical samples (observations) and collected metabolite data (variables) is described. PCA forms the basis in MVA, representing a multivariate data table as a low-dimensional plane, which provides an overview of the data. This overview may reveal groupings, trends, and outliers. The first principal component (PC1) is the line in the metabolic space that best approximates all the data. Each sample is thereafter projected onto this PC-line, which becomes its co-ordinate value along the line. This co-ordinate value for each sample is called a *score*. One PC line is usually insufficient to
model the systematic variation of an entire metabolite dataset. Accordingly, a second PC (PC2) is calculated, which is orthogonal (perpendicular) to PC1. When two PCs have been derived, they together define a plane, or a “window” into the high-dimensional metabolite space that can be plotted as a scatter plot (PC1 vs. PC2) — termed a scores plot. There is a corresponding plot for the metabolites called the loadings plot. This plot reveals how the metabolites contribute to the structure of the scores plot, and can be used to link information between individual metabolites and clinical samples.

In its simplest form, OPLS-DA is used instead of PCA when additional sample knowledge exists (e.g., healthy vs. asthmatics). OPLS-DA divides the variation in the data into two parts, one part that models the class-separating variance (or predictive variation) and another part that models the within-class separating variance (or orthogonal variation). OPLS can also be used to predict the class identity of unknown samples. For PCA and OPLS-DA models, the amount of modeled variation is defined as the goodness of fit (R²). An R² value of one (1) indicates that all variation in the data is modeled; a value of zero (0) means that no variation in the data is modeled. The goodness of prediction (Q²) is based on cross validation. Q² values of one (1) reflect perfect predictive precision, while values equal to or below zero (0) indicate that a random guess is more accurate. These metrics are used to evaluate the quality of PCA and OPLS-DA models.

RESULTS

Airway inflammation

FE_{NO} was measured as a non-invasive marker of airway inflammation. Asthmatics showed apparent higher FE_{NO}-values in all flow rates, compared to healthy controls. However, significance was only reached at the 10 ml/s flow rate (Table 2). Airway inflammation was further assessed using bronchoscopy to sample inflammatory cells from
different regions of the lung. Median BAL recoveries (reflecting peripheral lung compartments) were 70% for asthmatics and 75% for healthy controls with median BW recoveries (reflecting more proximal compartments) 39% and 45%, respectively. There was no significant difference between recovered volumes between the two groups.

Eosinophils and mast cells were elevated in BW from asthmatics compared to healthy individuals (Table 3), with no differences between cell counts in BAL. Endobronchial mucosal biopsies revealed higher eosinophil and mast cell numbers in the asthmatic epithelium, while submucosal neutrophil numbers were higher in the healthy individuals (Table 3).

**Oxylipin profiles**

Of the 88 screened oxylipins using LC-MS/MS, 17 were above the LOQ and used in subsequent uni- and multivariate statistical analyses. PCA analysis of the oxylipin data revealed a gross asthmatic outlier, which was removed. A simplified pathway map of oxylipins included in the statistical analyses together with relative amounts in BW and BAL from asthmatics and healthy controls is shown in Figure 1. The complete list of the 88 screened oxylipins and acronym definitions along with values above LOD and LOQ, is provided in Supporting Information (Table S1). Notably, a common isoprostane (8-iso-PGE₂) was not detected in any of the samples.

The medians of the oxylipin concentrations in BW and BAL in both asthmatics and healthy individuals ranged over 3 orders of magnitude from ~1 pM to 1 nM (Table 4). Five BW oxylipins (15-HETE, 9-HODE, 13-HODE, 13-KODE, and 15-HETrE), predominantly originating from the 15-LOX pathway and auto-oxidation, as well as total oxylipin levels in BW, were significantly elevated in asthmatics vs. healthy individuals (Table 4). No BAL oxylipin levels differed significantly between asthmatics and healthy individuals. Comparison
of the oxylipin levels in BW vs. BAL evidenced several differences in both asthmatic and healthy subjects (Table 4). Generally, 15-LOX-derived lipids were greater in BW and CYP-derived products greater in BAL, independent of disease. Levels of the 15-LOX products 12-HETE, 15-HETE, 15-KETE, 15-HETrE, and 13-HOTE were higher in BW in both groups, while 13-HODE levels were higher in only the asthmatic subjects. Levels of the CYP-derived 9(10)-EpOME and 12(13)-EpOME were higher in BAL of both groups, while 9,10-DiHOME was only higher in asthmatics. The synthetic origin of the linoleic-derived 9,10,13-TriHOME and 9,12,13-TriHOME is unclear, but they are potentially products of a mixture of 15-LOX and CYP activity and/or autooxidation. However, TriHOME response mirrored that of the CYP-derived compounds, with 9,10,13-TriHOME higher in BAL of both groups and 9,12,13-TriHOME higher in BAL of healthy individuals.

**15-lipoxygenase**

Bronchial biopsies were stained for 15-LOX-1, with staining observed only in the epithelium. The epithelial expression of 15-LOX-1 was higher in the asthmatics compared to the healthy controls; however, levels did not reach statistical significance (p=0.08, Figure 2). OPLS modelling evidenced a correlation between 15-LOX-1 expression and oxylipin profile (R²Y=0.61, Q²[cum]=0.471, p=0.002) using data for 15-HETE, 9-HODE, 13-HODE, 13-KODE, and 15-HETrE (significantly elevated in BW from asthmatics compared to healthy controls) to predict 15-LOX-1 expression.

**Integrated multivariate modelling**

Multivariate statistical analysis was used to investigate patterns and relationships in the data. The component summarizing the majority of the systematic variability was used in Figure 3 to highlight the diagnosis-dependent and compartment-dependent variability of the
profiles, respectively. The position of each subject along the y-axis was determined by its predicted OPLS-DA class definition value, based on cross-validation calculations using each subject’s profile of variables in relation to the rest of the subjects’ profiles. Thereby, subjects with similar profiles (in the same class) clustered together either below or above 0.5, equivalent to the class definition threshold. See Table S2 for validation parameters.

An OPLS-DA model (R²Y[ cum] = 0.419, Q² = 0.241) for all collected data (demographics, cell numbers, oxylipins, FENO, and 15-LOX staining) separated asthmatics and healthy subjects along the y-axis, except for six asthmatics above 0.5, and one healthy subject below 0.5 (Figure 3A). The information was summarized by a model component capturing ~20% of the data variability related to diagnosis. The healthy individuals were more homogenously clustered along the y-axis than the asthmatic group, indicating that the asthmatics display a larger variation in the outcomes. There was no clustering with respect to gender.

To interpret the influence of each parameter on the different profiles displayed by asthmatics and healthy subjects, loading values, i.e., the individual contribution from each parameter to the class separation, were calculated (Table S3). Variables with large absolute loading values and small confidence intervals were the main contributors to the diagnosis-dependent profiles, while variables close to zero displayed insignificant influence. Variables with large negative values were typical for the asthmatic group and variables with large positive values were typical for the healthy group. In accordance with the univariate analysis, the major asthma drivers were FE(NO) and eosinophils in the epithelium together with the oxylipins 13-HODE, 13-KODE and EKODE in BW. Age and BMI were among the variables with little or no influence on the diagnosis.

An OPLS-DA model of the oxylipin profiles displayed clustering with respect to lung compartment (Figure 3B). Approximately 20% of the oxylipin data were related to lung
compartment. The inter-subject variation was similar in both BW and BAL, as well as among the asthmatic and healthy subjects, indicating that the baseline difference between BW and BAL oxylipin profiles exists in both asthmatic and healthy subjects. Consequently, there was a relationship between separate OPLS-DA oxylipin models for asthmatics and healthy subjects, displayed in a shared and unique structure (SUS) plot (R^2=0.83, Figure 4). See Lundström et al [8] for an in-depth discussion on interpreting SUS plots. Oxylipin profiles in asthmatics (x-axis) vs. healthy individuals (y-axis) revealed the same characteristic oxylipins for BW (e.g., 13-HOTE and 5(6)-EpETrE in the lower left corner), as well as for BAL (e.g., 9(10)-EpOME and 12(13)-EpOME in the upper right corner) (Figure 4). Hence, the difference in oxylipin profiles between BW and BAL was largely independent of diagnosis. The class prediction and loading plots for the two different models are shown in Figure S1 (asthmatics) and Figure S2 (healthy).

DISCUSSION

Oxylipin baseline profiles differed between proximal and distal airways in both asthmatics and healthy individuals, indicating lung region-specific lipid mediator production. These regional differences in oxylipin levels were further supported by allergic airway inflammation in terms of higher eosinophil and mast cell counts in BW and in the bronchial epithelium of asthmatics, compared to healthy controls, with no significant alterations in BAL. The asthmatics also expressed higher FENO levels than the healthy controls at 10 ml/s. These data suggest that enhanced lipid mediator production is associated with allergic asthmatic airway inflammation, predominately in the proximal airways.

While auto-oxidation and CYP products were more common in BAL, the majority of the significantly elevated oxylipins in BW from asthmatics were derived from the 15-LOX pathway of arachidonic and linoleic acid. These findings were supported by: 1)
immunohistochemical data on stained endobronchial mucosal biopsies, and 2) multivariate
correlations between 15-LOX data predicted by the five oxylipins elevated in BW and 15-
LOX-1 levels. Leukotoxin and iso-leukotoxin, (9[10]-EpOME and 12[13]-EpOME), were
among the most prominent species in the BAL oxylipin profile. These compounds, and their
corresponding diols (9,10-DiHOME and 12,13-DiHOME; leukotoxin-diol and iso-
leukotoxin-diol, respectively), are produced in leukocytes, and previous studies have shown
that BAL levels of leukotoxin are elevated in respiratory distress syndrome [29].
Significantly elevated levels of 12,13-DiHOME have also been found in exhaled breath
condensate from an asthmatic individual after allergen exposure [30]. Accordingly, these
CYP products and their downstream soluble epoxide hydrolase (sEH) metabolites are
potentially of interest in the pathology of respiratory disease and merit further study.

Asthma-specific shifts in lipid mediators were only observed in the proximal lung
regions, sampled through collection of BW, in contrast to previous findings showing baseline
asthma-related oxylipin alterations in BAL fluid [8]. These seemingly contradictory findings
most likely demonstrate the impact of employing different lavage protocols for airway
sampling. The aim of the present study was to address oxylipin levels in different lung
compartments. Towards that end, a separate BW collection step was performed, which
contrasted to previous studies [8], in which the lavage procedure was not designed to study
the difference between proximal and distal airways. In the current study, BW (2x20 ml) and
BAL (3x60 ml) were carried out sequentially with saline solution, while in the previous study
BAL (5x50 ml) was carried out with phosphate buffered saline solution. Accordingly, the
lack of observed differences in BAL fluid between asthmatics and healthy individuals in the
current study is attributed to the respective oxylipins being removed in the initial BW,
whereas previous studies contained these oxylipin species in the BAL fluid. These results
emphasize the importance of well-defined lavage sampling protocols and the need for
rigorous descriptions of the sampling methods. In addition, the findings demonstrate the added benefit in distinguishing different lung compartments provided by a sequential lavage procedure in which BW and BAL fluids are collected separately.

We based our hypothesis on the assumption that airway lavages reflecting different regions of the lung would reveal asthma-related oxylipin-specific attributes due to the underlying asthmatic inflammation in connection to oxidative stress. While this was true for BW, it was not the case for BAL in this particular study group of mild allergic asthmatics. Previous studies using differentiated lavage volumes have given support to the notion that the asthmatic inflammation is more localised in the proximal airways and better sampled by small volume BW, than the limited events occurring in the alveolar region, which is mostly sampled by a large volume BAL [31].

The oxylipin composition of BW and BAL differ, which has potential physiological implications for the study of asthma. The majority of oxylipin metabolic profiling work performed to date has focused on BAL fluid; however, our results demonstrate that BW provides a distinct oxylipin signature, which highlights the physiological implications of compartment-dependent sampling. Given that asthma is a disease primarily of the proximal airways, the sampling of BW may provide increased insight into disease. In addition, asthma pathophysiology is linked to oxidative stress and lipid peroxidation, with isoprostanes often employed as oxidative markers [9]. While 8-iso-PGE$_2$ (a common isoprostane) was not detected, we found elevated levels of five 15-LOX/auto-oxidation derived oxylipins in BW from asthmatics compared to healthy subjects. It is purported that ROS are generated as by-products during conversion of the intermediate 15-LOX peroxide metabolites by glutathione reductase (e.g., formation of 15-HETE or 13-HODE) [32], which could subsequently affect the oxidative environment. It would therefore be of interest to explore whether differences in LOX metabolism affect the oxidative environment and associate with disease – on a lung
compartment-specific basis. Taken together, our findings illustrate the value of metabolic profiling of lipid mediators in respiratory disease and suggest that the effects of lung compartment-specific sampling should be considered in experimental design.

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Table 1. Characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Asthmatics</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 16</td>
<td>n = 16</td>
</tr>
<tr>
<td>Male/female</td>
<td>6/10</td>
<td>11/5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>26 (6)</td>
<td>25 (2)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24 (3)</td>
<td>23 (2)</td>
</tr>
<tr>
<td>FEV₁ (% of predicted)</td>
<td>98 (10)</td>
<td>105 (8)</td>
</tr>
<tr>
<td>Methacholine PC₂₀ (mg/ml)</td>
<td>1.1 (0.7-4.4)</td>
<td>-</td>
</tr>
<tr>
<td>Skin Prick Test (SPT)</td>
<td>Positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Data are given as mean (standard deviation) except for Methacholine PC₂₀, which is given as median (interquartile range).
BMI=body mass index; FEV₁=forced expiratory volume in one second; PC₂₀=provocative concentration causing a 20% fall in FEV₁.

Table 2. Fraction of exhaled nitric oxide (FE_{NO}) in asthmatic and healthy individuals

<table>
<thead>
<tr>
<th>FE_{NO} flow rate (ppb)</th>
<th>Asthmatics</th>
<th>Healthy</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 16</td>
<td>n = 16</td>
<td></td>
</tr>
<tr>
<td>FE_{NO} 100 ml/s</td>
<td>12.8 (7.4-26.0)</td>
<td>8.3 (6.5-10.3)</td>
<td>0.050</td>
</tr>
<tr>
<td>FE_{NO} 50 ml/s</td>
<td>20.3 (11.2-48.3)</td>
<td>14.1 (9.9-17.1)</td>
<td>0.053</td>
</tr>
<tr>
<td>FE_{NO} 10 ml/s</td>
<td>60.1 (43.8-118)</td>
<td>42.0 (32.0-57.4)</td>
<td><strong>0.027</strong></td>
</tr>
</tbody>
</table>

Data are given as medians (interquartile range).
Table 3. Inflammatory cell counts in bronchial wash (BW), bronchoalveolar lavage (BAL), submucosa (SM) and epithelium (EP) in asthmatic and healthy individuals

<table>
<thead>
<tr>
<th>Cell type and location</th>
<th>Unit</th>
<th>Asthmatics n = 16</th>
<th>Healthy n = 16</th>
<th>p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages BW</td>
<td>Cells x 10⁴/ml</td>
<td>5.3 (4.6-9.2)</td>
<td>7.2 (5.2-12.3)</td>
<td>0.070</td>
</tr>
<tr>
<td>Neutrophils BW</td>
<td>Cells x 10⁴/ml</td>
<td>0.2 (0.1-0.6)</td>
<td>0.7 (0.3-1.0)</td>
<td>0.059</td>
</tr>
<tr>
<td>Lymphocytes BW</td>
<td>Cells x 10⁴/ml</td>
<td>0.6 (0.3-0.9)</td>
<td>0.3 (0.2-0.7)</td>
<td>0.346</td>
</tr>
<tr>
<td>Eosinophils BW</td>
<td>Cells x 10⁴/ml</td>
<td>0.1 (0.0-0.3)</td>
<td>0.0 (0.0-0.1)</td>
<td>0.012</td>
</tr>
<tr>
<td>Mast cells BW</td>
<td>Cells x 10⁴/ml</td>
<td>0.03 (0.01-0.04)</td>
<td>0.01 (0.00-0.02)</td>
<td>0.013</td>
</tr>
<tr>
<td>Macrophages BAL</td>
<td>Cells x 10⁴/ml</td>
<td>12.0 (10.6-16.1)</td>
<td>13.9 (8.9-17.6)</td>
<td>0.598</td>
</tr>
<tr>
<td>Neutrophils BAL</td>
<td>Cells x 10⁴/ml</td>
<td>0.1 (0.0-0.2)</td>
<td>0.1 (0.0-0.2)</td>
<td>0.663</td>
</tr>
<tr>
<td>Lymphocytes BAL</td>
<td>Cells x 10⁴/ml</td>
<td>1.1 (0.6-1.4)</td>
<td>0.7 (0.5-1.0)</td>
<td>0.200</td>
</tr>
<tr>
<td>Eosinophils BAL</td>
<td>Cells x 10⁴/ml</td>
<td>0.1 (0.0-0.2)</td>
<td>0.0 (0.0-0.1)</td>
<td>0.180</td>
</tr>
<tr>
<td>Mast cells BAL</td>
<td>Cells x 10⁴/ml</td>
<td>0.02 (0.01-0.04)</td>
<td>0.01 (0.00-0.03)</td>
<td>0.127</td>
</tr>
<tr>
<td>CD3+ lymphocytes SM</td>
<td>Cells/mm²</td>
<td>20.9 (4.6-51.2)</td>
<td>20.4 (4.2-135.0)</td>
<td>0.777</td>
</tr>
<tr>
<td>CD4+ lymphocytes SM</td>
<td>Cells/mm²</td>
<td>15.5 (0.8-45.2)</td>
<td>5.7 (0.0-23.5)</td>
<td>0.251</td>
</tr>
<tr>
<td>CD8+ lymphocytes SM</td>
<td>Cells/mm²</td>
<td>18.7 (2.0-33.4)</td>
<td>5.1 (0.0-39.6)</td>
<td>0.515</td>
</tr>
<tr>
<td>Mast cells SM</td>
<td>Cells/mm²</td>
<td>29.0 (18.0-36.2)</td>
<td>31.1 (16.1-52.0)</td>
<td>0.474</td>
</tr>
<tr>
<td>Neutrophils SM</td>
<td>Cells/mm²</td>
<td>38.8 (23.1-93.9)</td>
<td>104.4 (55.9-167.3)</td>
<td>0.029</td>
</tr>
<tr>
<td>Eosinophils SM</td>
<td>Cells/mm²</td>
<td>1.1 (0.0-9.3)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.083</td>
</tr>
<tr>
<td>CD3+ lymphocytes EP</td>
<td>Cells/mm</td>
<td>3.7 (0.0-6.0)</td>
<td>1.3 (0.0-6.6)</td>
<td>0.538</td>
</tr>
<tr>
<td>CD4+ lymphocytes EP</td>
<td>Cells/mm</td>
<td>0.0 (0.0-0.8)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.572</td>
</tr>
<tr>
<td>CD8+ lymphocytes EP</td>
<td>Cells/mm</td>
<td>5.4 (0.0-6.9)</td>
<td>3.5 (0.0-8.4)</td>
<td>0.599</td>
</tr>
<tr>
<td>Mast cells EP</td>
<td>Cells/mm</td>
<td>0.0 (0.0-1.3)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.006</td>
</tr>
<tr>
<td>Neutrophils EP</td>
<td>Cells/mm</td>
<td>0.5 (0.0-1.7)</td>
<td>0.0 (0.0-2.3)</td>
<td>0.441</td>
</tr>
<tr>
<td>Eosinophils EP</td>
<td>Cells/mm</td>
<td>0.0 (0.0-1.3)</td>
<td>0.0 (0.0-0.0)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

aData are given as medians (interquartile range). Data are not corrected for multiple hypothesis testing. Significance is indicated at the $\alpha=0.05$ level, giving 1.2 potential false positives for the 22 measured values. The Bonferroni corrected p-value for these data is $p=0.002$. 


Table 4. Oxylipin concentrations (pM) in bronchoalveolar lavage (BAL) and bronchial wash (BW) from healthy and asthmatic individuals\(^a\)

<table>
<thead>
<tr>
<th>Compound (pM)</th>
<th>Asthmatics</th>
<th>Healthy</th>
<th>p-values</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BW n=15</td>
<td>BAL n=15</td>
<td>BW n=16</td>
<td>BAL n=16</td>
<td>A vs H</td>
<td>A vs H</td>
<td>Asthmatics BW vs BAL</td>
</tr>
<tr>
<td>12-HETE</td>
<td>100 (16.7-183)</td>
<td>0.0 (0.0-17.3)</td>
<td>56.8 (12.5-87.1)</td>
<td>0.0 (0.0-5.6)</td>
<td>0.19</td>
<td>0.38</td>
<td><strong>0.002</strong></td>
</tr>
<tr>
<td>15-HETE</td>
<td>362 (221-1168)</td>
<td>13.2 (2.8-61.0)</td>
<td>180 (139-234)</td>
<td>5.6 (0.0-21.8)</td>
<td><strong>0.03</strong></td>
<td>0.39</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td>15-KETE</td>
<td>57.0 (15.0-116)</td>
<td>6.6 (2.5-13.6)</td>
<td>19.0 (10.7-29.0)</td>
<td>5.1 (0.8-7.2)</td>
<td>0.11</td>
<td>0.37</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>5(6)-EpET(_E)</td>
<td>58.4 (41.6-85.2)</td>
<td>5.6 (0.0-5.6)</td>
<td>41.9 (12.5-63.0)</td>
<td>5.6 (0.0-5.6)</td>
<td>0.11</td>
<td>0.19</td>
<td><strong>0.0003</strong></td>
</tr>
<tr>
<td>9-HODE</td>
<td>197 (128-284)</td>
<td>107 (8.3-370)</td>
<td>122 (25.0-170)</td>
<td>150 (21.4-370)</td>
<td><strong>0.04</strong></td>
<td>0.62</td>
<td>0.14</td>
</tr>
<tr>
<td>13-HODE</td>
<td>1277 (425-2444)</td>
<td>327 (37.4-789)</td>
<td>343 (121-433)</td>
<td>178 (34.7-498)</td>
<td><strong>0.01</strong></td>
<td>0.70</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>9-KODE</td>
<td>53.9 (8.3-141)</td>
<td>96.4 (32.3-262)</td>
<td>8.3 (8.3-50.8)</td>
<td>132 (33.6-412)</td>
<td>0.09</td>
<td>0.86</td>
<td>0.20</td>
</tr>
<tr>
<td>13-KODE</td>
<td>332 (97.6-849)</td>
<td>156 (19.4-332)</td>
<td>97.2 (58.3-184)</td>
<td>138 (19.4-428)</td>
<td><strong>0.03</strong></td>
<td>0.94</td>
<td>0.17</td>
</tr>
<tr>
<td>9,10,13-TriHOME</td>
<td>58.3 (58.3-58.3)</td>
<td>223 (107-511)</td>
<td>85.3 (58.3-58.3)</td>
<td>368 (156-1162)</td>
<td>0.07</td>
<td>0.55</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>9,12,13-TriHOME</td>
<td>58.3 (58.3-58.3)</td>
<td>138 (35.0-244)</td>
<td>58.3 (58.3-58.3)</td>
<td>262 (91.5-849)</td>
<td>0.24</td>
<td>0.42</td>
<td>0.44</td>
</tr>
<tr>
<td>EKODE</td>
<td>248 (189-642)</td>
<td>537 (248-897)</td>
<td>203 (169-243)</td>
<td>774 (172-2648)</td>
<td>0.08</td>
<td>0.68</td>
<td>0.15</td>
</tr>
<tr>
<td>9(10)-EpOME</td>
<td>324 (305-439)</td>
<td>654 (466-839)</td>
<td>301 (216-355)</td>
<td>627 (500-938)</td>
<td>0.34</td>
<td>1.00</td>
<td><strong>0.0003</strong></td>
</tr>
<tr>
<td>12(13)-EpOME</td>
<td>412 (283-529)</td>
<td>624 (473-791)</td>
<td>340 (286-382)</td>
<td>657 (569-932)</td>
<td>0.32</td>
<td>0.60</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>9,10-DiHOME</td>
<td>21.2 (8.3-48.6)</td>
<td>61.8 (41.3-72.6)</td>
<td>44.7 (10.9-67.3)</td>
<td>63.0 (49.9-84.4)</td>
<td>0.31</td>
<td>0.55</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>12,13-DiHOME</td>
<td>106 (80.5-152)</td>
<td>57.3 (38.4-87.1)</td>
<td>137 (108-179)</td>
<td>67.7 (51.1-100)</td>
<td>0.13</td>
<td>0.34</td>
<td><strong>0.003</strong></td>
</tr>
<tr>
<td>15-HET(_E)</td>
<td>102 (42.5-128)</td>
<td>0.8 (0.0-3.4)</td>
<td>16.9 (2.5-26.7)</td>
<td>0.8 (0.0-2.0)</td>
<td><strong>0.001</strong></td>
<td>0.67</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td>13-HOTE</td>
<td>151 (87.8-270)</td>
<td>9.2 (0.0-34.1)</td>
<td>98.5 (0.0-163)</td>
<td>9.2 (0.0-9.2)</td>
<td>0.17</td>
<td>0.21</td>
<td><strong>0.003</strong></td>
</tr>
<tr>
<td>TOTAL</td>
<td>4446 (2505-8993)</td>
<td>2782 (222-6002)</td>
<td>2128 (1781-2609)</td>
<td>3797 (2401-7819)</td>
<td><strong>0.006</strong></td>
<td>0.71</td>
<td>0.39</td>
</tr>
</tbody>
</table>

\(^a\)Data are given as medians (interquartile range). Data are not corrected for multiple hypothesis testing. Significance is indicated at the \(\alpha=0.05\) level, giving 0.85 potential false positives for the 17 measured values. The Bonferroni corrected p-value for these data is \(p=0.003\).
Figure 1. Schematic overview of oxylipins produced from fatty acid precursors via auto-oxidation, the lipoxygenase (LOX) and cytochrome P450 (CYP) enzymatic pathways, and their median relative contribution to the bronchial wash (BW) and bronchoalveolar (BAL) fluid profiles. The fatty acids linoleic acid, arachidonic acid, $\alpha$-linolenic acid, and dihomo-$\gamma$-linolenic acid are precursors for hydroxyeicosatetraenoic acids (HETEs), hydroxyoctadienoic acids (HODEs), trihydroxyoctamonoenoic acids (TriHOMEs), hydroxyeicosatrienoic acids (HETrEs), hydroxyoctadecatrienoic acids (HOTEs), oxo-eicosatetraenoic acids (KETEs), oxo-octadecadienoic acids (KODEs), epoxyketoctadecenoic acid (EKODE), epoxyeicosatrienoic acids (EpETrEs), epoxyoctamonoenoic acids (EpOMEs), as well as the downstream soluble epoxide hydrolase (sEH) metabolites dihydroxyoctamonoenoic acids (DiHOMEs). Some of the oxylipins can be produced through alternative pathways, for instance 9-HODE, which also might be derived through the LOX pathway.
Figure 2. Representative pictures of endobronchial biopsies with 15-lipoxygenase-1 (15-LOX-1) staining (a) compared with tris-buffered saline (TBS) control (b) and fraction of epithelial area stained with 15-lipoxygenase-1 in asthmatics vs. healthy controls (c). The staining was localised to the epithelium.
Figure 2.

a) 15-LOX

b) TBS

c) 15-LOX

% staining of epithelium

Asthmatic  Healthy

p=0.08
Figure 3. Diagnosis-dependent (a) and compartment-dependent (b) cross-validated OPLS-DA class definition values. In the diagnosis-dependent model (a), asthmatics (green circles) and healthy controls (blue squares) evidenced different profiles based on demographics, cell numbers, FE\textsubscript{NO}, 15-LOX staining, and oxylipins, resulting in different class definition values (below 0.5 for asthmatics and above 0.5 for healthy subjects), except for six asthmatic subjects with similar profiles as the healthy group (above 0.5), and one healthy subject with similar profile as the asthmatic group (below 0.5). Inter-subject variation was larger in the asthmatic than in the healthy group, while male (M) and female (F) subjects showed similar inter-subject variation in both groups. In the compartment-dependent model (b), bronchoalveolar lavage (BAL, green circles) and bronchial wash (BW, blue squares) evidenced different oxylipin profiles, resulting in different class definition values (below 0.5 for BW and above 0.5 for BAL). Inter-subject variation was similar in both BW and BAL, as well as within the asthmatic (A) and healthy (H) group.
Figure 3.

(a)

(b)
Figure 4. Shared and unique structure plot of two separate OPLS-DA models for oxylipin profiles in asthmatic and healthy subjects, respectively. Oxylipins were correlated to BW (grey circles) and BAL (closed circles) to the same extent in both models (R²=0.83). Only 13-KODE and 9-HODE (open circles) were not significantly different between BW and BAL in either of the two groups (p = 0.05).
Supporting Information

Supplemental Methods Section
Detailed description of bronchoscopy and processing of samples

Table S1. List of standards used for quantification and recovery calculations (native and deuterated) including nomenclature and information about fraction among analyzed samples with oxylipin values above limit of detection (LOD) and limit of quantification (LOQ) in bronchial wash (BW) and bronchoalveolar lavage (BAL). Oxylipins used for uni- and multivariate calculations are indicated in bold.

Table S2. Validation parameters of OPLS-DA models

Table S3. Individual contribution of each parameter to diagnosis-dependent separation (loading values and confidence intervals)

Figure S1. Compartment-dependent cross-validated OPLS-DA (A) class prediction values, and (B) loadings (p[1]cv[1]) for the asthmatic subjects. Bronchoalveolar lavage (BAL, green circles) and bronchial wash (BW, blue squares) among the asthmatic (A) subjects evidenced different oxylipin profiles resulting in class prediction values below 0.5 for BW and above 0.5 for BAL. Inter-subject variation was similar in both BW and BAL.

Figure S2. Compartment-dependent cross-validated OPLS-DA (A) class prediction values, and (B) loadings (p[1]cv[1]) for the healthy subjects. Bronchoalveolar lavage
(BAL, green circles) and bronchial wash (BW, blue squares) among the healthy (H) subjects evidenced different oxylipin profiles resulting in class prediction values below 0.5 for BW and above 0.5 for BAL. Inter-subject variation was similar in both BW and BAL.
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