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Induced sputum supernatant bioactive lipid mediators can identify subtypes of asthma

Lipid mediators identify subtypes of asthma

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#### Abstract

#### Background:

Induced sputum (IS) allows to measure mediators of asthmatic inflammation in bronchial secretions. The specific role of induced sputum supernatant (ISS) endogenous bioactive lipid mediators in subtypes of asthma is not well understood.

Objective: In order to investigate interactions between airway inflammation and clinical phenotypes of asthma, we integrated induced sputum supernatant (ISS) eicosanoids and quantitative assessment of infiltrating cells into new subtypes with the means of latent class analysis (LCA).

Methods: 139 asthmatics with and without aspirin hypersensitivity underwent sputum induction. High-performance liquid chromatography or gas chromatography coupled with mass spectrometry were used to profile eicosanoids. 19 variables covering clinical characteristics, IS inflammatory cells and eicosanoids were considered in the LCA.

#### Results:

Four phenotypic asthma classes were distinguished. Class 1 with mild to moderate asthma, chronic

rhinosinusitis (CRS), high  $\text{PGA}_2$  in ISS and almost equal distribution of inflammation cell patterns.

Class 3 subjects also had mild to moderate asthma but without upper airway symptoms. Induced sputum was often paucigranulocytic with low levels of lipid mediators. Class 2 and 4 represented severe asthma with CRS and impaired lung function despite high doses of steroids. High blood and sputum eosinophilia was in line with high cysteinyl leukotrienes and  $\text{PGD}_2$  in ISS only in class 2. Class 4 subjects tended to have increased sputum neutrophilia and  $\text{PGE}_2$  in ISS. Aspirin hypersensitivity was most frequent among class 2 subjects.

**Conclusions & Clinical Relevance:** The LCA revealed four distinct asthma classes differing in eicosanoid pathways.

**Key words:** Asthma phenotype; induced sputum; eicosanoids; latent class analysis.

**Abbreviations used:**

AERD: aspirin exacerbated respiratory disease

ATA: aspirin-tolerant asthma

LCA: latent class analysis

$\text{PGE}_2$ : prostaglandin  $\text{E}_2$

$\text{PGD}_2$ : prostaglandin  $\text{D}_2$

$\text{PGA}_2$ : prostaglandin  $\text{A}_2$

Cys-LTs: cysteinyl leukotrienes

$\text{LTC}_4$ : leukotriene  $\text{C}_4$

$\text{LTD}_4$ : leukotriene  $\text{D}_4$

$\text{LTE}_4$ : leukotriene  $\text{E}_4$

5-,12-,15-HETE: 5-,12-,15-hydroxyeicosatetraenoic acid

COX: cyclooxygenase

NSAIDs: nonsteroidal anti-inflammatory drugs

IS: induced sputum

ISS: induced sputum supernatant

## Introduction

Adult phenotypes of asthma were first divided into three categories in 2006: 1) clinical or physiological phenotypes (i.e. severe asthma), 2) phenotypes related to triggers (allergens, occupational allergens, aspirin, exercise, etc.), and 3) inflammatory phenotypes; eosinophilic phenotype defined as induced sputum (IS) eosinophilia >2%, neutrophilic defined as IS neutrophilia >50%, and paucigranulocytic – that is no predominant inflammation pattern present<sup>1</sup>. Also, phenotypes of asthma were determined as Th2 - high or Th2 - low types of asthma<sup>2</sup>. Most of the phenotype concepts were based on statistical methods, such as cluster analysis<sup>3 4</sup> and latent class analysis<sup>5 6</sup>. This pathobiological approach led to the concept of distinct disease entities with a specific mechanism determining its characteristics, called “asthma endotypes”. Using these criteria, Lotvall et al. were able to identify several asthma endotypes (i.e. allergic asthma, etc.)<sup>7,8</sup>. The importance of phenotypic/endotypic definition has been increasing as personalized treatment of asthma on the basis of understanding underlying genetic bases, molecular pathomechanisms, treatment response and prognosis has also been evolving.

Quantitative assessment of infiltrating cells present in sputum provide a reliable and relatively noninvasive method to assess chronic airway inflammation in the asthmatic lung<sup>9</sup>. Phenotypes based on sputum cells have identified cellular heterogeneity in asthmatic patients in routine clinical practice<sup>1</sup>. So far, no studies have attempted to identify adult asthma “subtypes” using a more comprehensive approach integrating sputum cell count and induced sputum supernatant (ISS) bioactive lipid mediators.

Transcellular eicosanoids metabolism in the airway occurs during multicellular interaction, which leads to the generation of bioactive lipid mediators able to regulate inflammation and functional responses. The imbalance between proinflammatory and proresolving mediators in the pathogenesis of asthma has been addressed in the last few decades of research. A lower biosynthetic capacity for anti-inflammatory mediators known as lipoxins<sup>10 11</sup>, prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)<sup>12 13</sup>, and higher biosynthetic capacity for potentially pro-inflammatory mediators such as cysteinyl-leukotrienes (cys-LTs) and PGD<sub>2</sub><sup>14</sup> have been linked to both severe asthma and aspirin-exacerbated respiratory disease (AERD)<sup>15</sup>. AERD is recognized as a distinct asthma phenotype. It usually has a severe course accompanied by chronic sinusitis with nasal polyps, blood eosinophilia, and increased concentrations of urinary leukotriene E<sub>4</sub> (LTE<sub>4</sub>). A more insightful analysis of individual patients has shown this group to be nonhomogeneous<sup>6</sup>.

We hypothesized that the integration of pathobiologic measures such as inflammatory cells and eicosanoids in induced sputum with clinical characteristics in a single multivariate analysis would lead to identification of subject groups that not only share the same phenotypic variant but also molecular mechanisms possibly determining these features. Previous studies analyzing lipid mediators in bronchoscopic samples have limited applicability<sup>16</sup>, so we considered whether methods using sputum induction would be more broadly applicable<sup>17</sup>. We assumed that the inflammatory mediators measured in induced sputum supernatant would reflect the inflammation process in the bronchial tree *in vivo*.

To address this goal we analyzed a group of 139 asthmatics representing all asthma spectrum severity, taking into consideration phenotypic features such as aspirin hypersensitivity, atopic status and chronic rhinosinusitis. All subjects underwent sputum induction to determine inflammation cellular pattern and eicosanoid profile. Latent class analysis identified four asthma subphenotypes differing in clinical characteristics, cell influx and bioactive lipid mediators.

## Methods

### Subjects

The study groups consisted of 75 asthmatic patients, and 64 asthmatics who tolerated aspirin well. Aspirin hypersensitivity was diagnosed by bronchial provocation test<sup>18</sup>. Aspirin-tolerant asthmatics (ATA) occasionally used non-steroidal anti-inflammatory drugs (NSAIDs) without any adverse reactions and their aspirin challenge test result was negative. They were asked to avoid NSAIDs for 4 weeks before participating in the study. None of AERD subjects underwent aspirin desensitisation. The asthma diagnosis and control was ascertained according to GINA 2014 update. The definition of severe asthma complied with ATS/ERS 2013 recommendations<sup>19</sup>. Patients who had an asthma exacerbation or a lower respiratory tract infection within the previous 6 weeks were excluded. Baseline spirometry was performed in asthmatics after bronchodilator withhold if their asthma symptoms permitted (4 hours for short-acting  $\beta$ -agonists and 12 hours for long-acting  $\beta$ -agonists). No other modification in asthma treatment was requested. Additional phenotypic characterization of subjects included: skin prick tests, sinus computer tomography to confirm the diagnosis of chronic rhinosinusitis, blood eosinophil count and total serum IgE levels. The clinical characteristic of the study patients is presented in Table 1. The patients gave informed consent to participate in the study and official approval from the Jagiellonian University Ethics Committee was obtained (KBET/7/B/2010).

### Study design

All subjects underwent sputum induction. Induced sputum samples were investigated for sputum differential cell count and sputum supernatant concentrations of selected eicosanoids. Latent class analysis considering 19 variables covering clinical characteristics such as: sex, age at asthma onset, BMI, asthma control and severity, use of inhaled and/or oral steroids, presence of

chronic rhinosinusitis, history of sinus surgery, FEV<sub>1</sub>, blood eosinophil count, total serum IgE, skin prick tests, induced sputum inflammatory cells and selected eicosanoids - PGD<sub>2</sub>, PGA<sub>2</sub>, PGE<sub>2</sub>, LTE<sub>4</sub>, 5-HETE; was applied on collected dataset. PGF<sub>2α</sub>, TXA<sub>2</sub>, and PGI<sub>2</sub> were not assessed in the lipid profile in ISS (none of these mediators appeared to be different in any of the groups).

#### Induced sputum

Induced sputum was collected and processed according to ERS recommendations<sup>17</sup>. Subjects with FEV<sub>1</sub> less than 50% of predicted value after salbutamol administration did not undergo sputum induction, but a spontaneous sputum sample was collected if possible. In brief, sputum induction was performed by inhalation of hypertonic saline solution at increasing concentrations, from 3% to 5%, using an ultrasonic nebulizer (Ultraneb 2000; De Vilbiss, Somerset Philadelphia USA). Samples were processed to obtain cytopsin slides for differential cell count and supernatant for measurements of selected eicosanoids as previously described<sup>21</sup>. Slides with greater than 80% squamous cells were deemed inadequate for further analysis. Differential count of sputum induced cells, has allowed for classification into inflammatory patterns: eosinophilic- defined as induced sputum eosinophilia above 2%, neutrophilic - that is induced sputum neutrophilia above 50%, mixed with sputum eosinophilia above 2% and neutrophilia above 50%, and paucigranulocytic – when no predominant inflammation pattern was present<sup>17,20</sup>. Concentrations of selected anti-inflammatory eicosanoids (PGE<sub>2</sub>, and tetranor-PGE-M), pro-inflammatory eicosanoids (LTD<sub>4</sub>, LTE<sub>4</sub>, LTB<sub>4</sub>, PGD<sub>2</sub>, tetranor-PGD-M, PGF<sub>2α</sub>, TXA<sub>2</sub>, PGI<sub>2</sub> and PGA<sub>2</sub>), and pro-inflammatory precursors of lipoxygenation 5-, 12- and 15-hydroxyeicosatetraenoic acid (5-HETE, 12-HETE, 15-HETE) were measured in induced sputum supernatant.

## Biochemical assays

The concentration of eicosanoids in ISS was measured by gas chromatography/mass spectrometry (GC-MS) for PGE<sub>2</sub>, PGD<sub>2</sub>, by high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) for 5-HETE, 12-HETE, 15-HETE, leukotrienes B<sub>4</sub>, D<sub>4</sub>, E<sub>4</sub>, PGA<sub>2</sub>, tetranor-PGE-M and tetranor-PGD-M. Before organic phase extraction with ethyl acetate, samples were acidified to pH 3.5 using acetic acid and chemically identical deuterated internal standards were added (Supporting information). Concentrations of eicosanoids were calculated using a stable isotope dilution method from the area under the peak of an analyte and the deuterated standard. All eicosanoid concentrations were expressed in picograms per milliliter of processed sputum samples. These analytical method details were presented elsewhere<sup>21</sup> and are given in supporting information. Limits of quantification for measured eicosanoids are presented in Table 2, together with the results.

## Lung function

Pulmonary function tests were performed on a flow-integrating computerized pneumotachograph (Pneumoscreen, E. Jaeger, Germany).

## Analytic strategy and selection of variables

The R package polCA<sup>22</sup> was used for the estimation of latent class models and latent class regression models for the analysis of multivariate categorical data. The latent class regression model generalizes the basic latent class model by permitting the inclusion of covariates to predict individuals' latent class membership<sup>23</sup>. This is a so-called "one – step" technique for estimating the effects of covariates, because the coefficients on the covariates are estimated simultaneously as a part of the latent class model. As was demonstrated by Bolck, Croon and Hagenaars<sup>24</sup>, it is

preferable to estimate the latent class regression model all at once instead of using an alternate estimation procedure called the “three-step” model due to biased coefficient estimates produced by a “three-step” procedure. Covariates are included in the latent class regression model through their effects on the priors probabilities of latent class membership. In the basic latent class model, it is assumed that every individual has the same prior probabilities of latent class membership. The latent class regression model, in contrast, allows individuals’ priors to vary depending upon their observed covariates. Identification of the optimal model was done using AIC (Akaike Information Criterion) in a stepwise manner by analysis of models with the number of classes starting from 1 to 5. AIC is founded on information theory: it offers a relative estimate of the information lost when a given model is used to represent the process that generates the data. In doing so, it deals with the trade-off between the goodness of fit of the model and the complexity of the model. More information about AIC one can find in [http:// en.wikipedia.org/wiki/Akaike\\_information\\_criterion](http://en.wikipedia.org/wiki/Akaike_information_criterion). When the best model was determined, each subject was then allocated to the single latent class according to the highest posterior probability of membership rule. We estimate, that for the number of items we used for LCA (n=19) and assuming benchmark value of Cohen’s w (measure of effect size used for chi square tests)=0.5, the statistical power of LCA was between 0.7 and 0.8 at type I error = 0.05.

#### Statistical Analysis

Summary statistics were presented as mean, standard deviation, median, 25th and 75th percentiles and number in each category with percentage of total. We transformed mediator concentrations to their logarithm or used Box-Cox transformation to normalize the distributions before analysis. Normality was checked by using the Shapiro-Wilk W test. A general linear model (GLM) and post-hoc Tukey test analysis was applied to analyze differences in mediator levels between latent classes. Categorical data was analyzed by using the  $\chi^2$  and Fisher exact tests. A p-

value  $\leq 0.05$  was considered statistically significant. All other statistical analysis was performed with the use of Statistica software (v.10, StatSoft Tulsa OK) and Origin Pro (v.9.1 Origin Lab Corp.).

## Results

### Latent class analysis

Using clinical and laboratory criteria, a model with four latent classes was selected as the best model fitting the analyzed data. The mean highest posterior probability was - 98.0% (median: 99.0 %; 25th and 75th percentile:98.7%-100% ), indicating that participants were assigned to classes with a fairly high probability. Characteristics of the particular classes and probability of having a patient allocated to one of the 4 classes are given in Table 3 and 4 (see supporting information).

Class 1 was comprised of 54 asthmatic subjects, 67% of whom were AERD, 57% female and 43% male, with a mean age of subjects  $46.4 \pm 12.1$  y. All subjects had chronic rhinosinusitis (CRS). Slightly above half of the group was atopic (56%). This class was characterized by an almost equal prevalence of phenotypes based on induced sputum cell counts - that is 24% had eosinophilic, 30% neutrophilic, 28% paucigranulocytic and 18% mixed eosinophilic and neutrophilic asthma (Fig. 1). Asthma tended to be mild to moderate (59%), or intermittent (22%), without lung function impairment ( $FEV_1$   $98\% \pm 12.1\%$ ). None of the subjects had uncontrolled asthma (mean ACT score  $23.8 \pm 1.8$ ), furthermore, asthmatic subjects required lower doses of inhaled corticosteroids (ICS) to achieve disease control (median 250  $\mu$ g of fluticasone daily). This class was characterized by a significantly higher level of  $PGA_2$  in ISS compared to class 3 ( $P < 0.001$ ) (Fig. 2).

Class 2 comprised of 35 asthmatic subjects - predominantly AERD (86 %) and women (77%), with mean age of  $47.3 \pm 14.6$  y. All subjects had CRS. They were less likely to be atopic (26%). None of the subjects had childhood asthma, with mean disease duration of  $16.1 \pm 9.8$  y. The induced sputum specimens were mainly eosinophilic (74%) and mixed eosinophilic and neutrophilic (23%) (Fig. 1). This class was characterized by high sputum (median 14.1%) and high blood eosinophilia (median 641 count/ $\mu$ l). A high level of eosinophilia was accompanied by a more severe course of asthma with lower FEV<sub>1</sub> values ( $80.8\% \pm 18.8$ ). 71% of patients met the criteria for severe asthma, 29% mild and moderate, no subjects had intermittent asthma. Asthma was uncontrolled in 40% of patients, despite all of them having corticosteroid treatment, mean ACT result was  $19.1 \pm 4.2$ . High doses of ICS were administered (median 1000  $\mu$ g of fluticasone daily), 14% of subjects required oral corticosteroid (OCS) treatment. LTE<sub>4</sub> and LTD<sub>4</sub> in ISS were significantly higher in this class in contrast to classes 1, 3 and 4 ( $P < 0.001$  for LTE<sub>4</sub>, LTD<sub>4</sub> respectively). Similarly, PGD<sub>2</sub> in ISS was higher in comparison to classes 1 and 3 ( $P < 0.001$ ) (Fig. 2).

Class 3 comprised of 33 asthmatic subjects tolerant to aspirin, 58% women and 42% male with mean age of  $46.3 \pm 16.6$  y. Only 3% of subjects suffered from CRS. They were predominantly atopic (73%). 15% had childhood asthma with a disease duration of  $13.9 \pm 10.6$  y. The induced sputum specimens were mainly paucigranulocytic (49%) and neutrophilic (33%) (Fig. 1). This class was characterized by low sputum (median 0.4%) and blood (median 165 count/ $\mu$ l) eosinophilia. Low concentrations of ISS LTE<sub>4</sub>, LTD<sub>4</sub> and PGD<sub>2</sub> in comparison to class 2 ( $P < 0.001$  for LTE<sub>4</sub>, LTD<sub>4</sub> and PGD<sub>2</sub> respectively), PGA<sub>2</sub> in comparison to class 1 ( $P < 0.001$  respectively) and lower PGE<sub>2</sub> with higher tetranor-PGE<sub>2</sub>-M in comparison to class 4 ( $P = 0.02$  and  $P = 0.04$  respectively) were observed (Fig. 2). This class can be defined as having non severe (21% intermittent, 55% mild and moderate), well (70%) and partially (30%) controlled asthma (mean ACT score of  $23.1 \pm 2.3$ ) requiring lower doses of ICS (median 250  $\mu$ g of fluticasone daily).

Class 4 was comprised of 17 asthmatics of whom 12 (71%) were female with a mean age of  $48.9 \pm 13.6$  y. 53% of subjects were hypersensitive to aspirin. This class was defined as having poorly controlled, severe "non eosinophilic" asthma. 35% of subjects had childhood asthma, 65% were atopic, 77% had CRS. Almost all patients had uncontrolled asthma (82%). Despite the highest doses of ICS administered (median 1000  $\mu$ g of fluticasone daily) impaired lung function ( $FEV_1$   $78.9\% \pm 14.8\%$ ) was observed in this class. Additionally, 47% of subjects required OCS. Many of the subjects (47%) were obese ( $BMI \geq 30$   $kg/m^2$ ). The dominant phenotypes were neutrophilic (41%) and paucigranulocytic (35%) (Fig. 1). Sputum (0.3%) and blood eosinophilia (197 count/ $\mu$ l) were lower, as well as concentrations of ISS  $LTD_4$  and  $LTE_4$  compared to class 2.  $PGE_2$  was significantly higher compared to classes 3 and 1.  $PGE_2$  metabolite (tetranor- $PGE$ -M) was higher only compared to class 3 (Fig. 2).

## Discussion

This study has shown that the direct *in vivo* measurements of induced sputum supernatant bioactive lipid mediators provides some new useful information regarding the underlying pathogenesis of airway inflammation in the asthmatic lung.

The application of the latent class analysis based on clinical data, induced sputum cell count and lipids mediators revealed four clearly defined classes. Class 1 with a high  $PGA_2$  level in ISS comprised of both aspirin hypersensitive and tolerant subjects, all with chronic rhinosinusitis, usually with well-controlled mild to moderate asthma, without a clear indication for a dominant inflammation pattern in the bronchial airway. Whereas subjects in class 2 with high levels of pro-inflammatory mediators: cys-LTs and  $PGD_2$  in the ISS had dominantly severe, poorly controlled eosinophilic aspirin hypersensitivity and less frequently aspirin tolerant asthma. Class 3 was characterized by low levels

of proinflammatory mediators in ISS. The asthmatics tended to be atopic, rarely had chronic rhinosinusitis, the disease was well-controlled and mild, frequently induced sputum was paucigranulocytic. Class 4, with lower levels of proinflammatory mediators (cysLTs, PGD<sub>2</sub>) and high levels of PGE<sub>2</sub>, showed a fairly frequent involvement of the nasal mucosa and paranasal sinuses. The disease was typically severe and poorly controlled. The majority of subjects were overweight and obese women, with long lasting, and frequently, childhood onset atopic asthma.

AERD subjects tend to be located in class 2, but also in classes 1 and 4. The persistence of eosinophilia in severe asthma is often associated in adult onset disease with aspirin hypersensitivity<sup>25</sup>. Cysteinyl leukotrienes: LTD<sub>4</sub> and LTE<sub>4</sub> that were overproduced in class 2 are strong mediators of allergic inflammation mainly released by eosinophils and mast cells<sup>26</sup>. In numerous studies, the key role of cys-LTs overproduction in AERD was well documented on the systemic level and increased urinary excretion of LTE<sub>4</sub> as a hallmark of this disease<sup>27,28, 29,30</sup>. Increased local and systemic biosynthesis of proinflammatory PGD<sub>2</sub><sup>14,31</sup> and its metabolites was also observed in AERD patients. In accordance with the studies above, our observations point out that the majority of AERD subjects belong to severe eosinophilic asthma class with high cysLTs and PGD<sub>2</sub> in ISS.

The increasing frequency of eosinophilic inflammation in class 2 together with high levels of eosinophil derived mediators, despite high doses of corticosteroids suggests a more complex mechanism influencing asthma severity. Increased sputum eosinophil count was described in asthma from intermittent to severe and is associated with decreased asthma control and more frequent exacerbations<sup>32</sup>. Treatment with ICS is known to decrease sputum eosinophilia and helps to achieve disease control<sup>33,34</sup>. Asthmatics with severe asthma and persistence of eosinophilic inflammation despite chronic treatment with high doses of ISC and in some cases OCS might represent a specific CS recalcitrant subpopulation. Yet the influence of steroid treatment on eosinophilic inflammation

and in consequence class the asthmatic locates is not obvious. Brusselle et al.<sup>35</sup> distinguished two different pathways leading to eosinophilic inflammation in allergic and non-allergic asthma. In allergic asthma, responding well to CS therapy, dendritic cells induce Th2 cells producing IL-4, IL-5 and IL-13, and promote IgE switch. Since non-allergic eosinophilic inflammation in asthma is CS resistant, it was suggested that innate lymphoid cells type-2 (ILC2) might be alternate source for high amounts of IL-5 and IL-13. A limitation of this study is that no lymphocytes population or markers were included. Sputum eosinophil percentages significantly decreased during prednisone therapy<sup>36</sup>. On the contrary it was shown that there is no correlation between eicosanoids measured *in vivo* in EBC<sup>15</sup> and BAL<sup>36</sup> with glucocorticosteroid treatment in asthmatic subjects. In class 1, representing rather mild to moderate asthma, the distribution of inflammation patterns was almost equal. Neutrophilic phenotype is associated with severe as well as milder asthma<sup>37 38</sup>. Neutrophilic phenotype in contrast to eosinophilic inflammation is associated with a worse response to steroid therapy<sup>39</sup>. In a study by Moore<sup>4</sup> (SARP study), eosinophilic phenotype was also quite frequent among asthmatics with a good control of the disease and low administered ICS doses, similarly to subjects located in class 1 in this study. Severe asthma was in 83% characterized by sputum neutrophilia either alone or with concurrent eosinophila. Probably because the higher incidence of AERD in our cohort more eosinophilic inflammation was observed among severe asthma in class 2. This observation supports the role of eosinophils in mild to moderate asthma. Asthma possibly responds well to corticosteroid therapy in this class, what is reflected by low proinflammatory cysLTs and PGD<sub>2</sub>. The findings of this study stress that a single biomarker is unlikely to classify asthmatics.

Neutrophil predominant and mixed patterns in induced sputum were most frequently observed in this study in class 4 representing severe, poorly controlled asthma with low FEV<sub>1</sub> despite high corticosteroid regimens. The presence of atopy and childhood asthma was relatively high in granulocytic inflammation class in line with a study by Moore et al.<sup>4</sup>. This suggests that neutrophilic phenotype does not exclude atopy underlying asthmatic inflammation in such cases. The highest

doses of corticosteroids were administered in this class. It was previously suggested that sputum neutrophils might be a specific biological side effect of exposure to high doses of corticosteroids<sup>40</sup>.

Four EP receptors 1-4 for PGE<sub>2</sub> in human lungs act individually or in various combinations to maintain homeostasis. While EP2 and EP4 receptor are involved in bronchodilation, antifibrotic and anti-inflammatory effect, EP1 and EP3 stimulation mediates pulmonary artery constriction in the lungs and plays a potential role in type I allergies<sup>41</sup>. The imbalance between the EP receptor types in this asthma class could explain the tendency to impaired lung function and severity of symptoms regardless higher PGE<sub>2</sub> levels measured in ISS. A tendency towards lower levels of eosinophil derived mediators like cys-LTs and PGD<sub>2</sub> suggests that different mediators are crucial for triggering and sustaining asthmatic inflammation.

Subjects with the lowest lung function and poor control of the disease were characterized by a dominance of eosinophilic, neutrophil or mixed inflammatory pattern of induced sputum, despite having received the highest doses of corticosteroids. These results point out that a subject with decreased baseline FEV<sub>1</sub> despite high corticosteroid regimens and second controller medication might be considered for guiding therapy based on induced sputum.

Chronic rhinosinusitis is well known to strongly aggravate the course of asthma. AERD studies show that the incidence of inflammation of upper airways is associated with higher levels of cysLTs. Subjects with nasal polyps show the highest levels of urinary LTE4 (uLTE4), followed by those with hyperplastic rhinitis<sup>42</sup>. CysLTs were also high in severe ATA patients especially accompanied by nasal polyps or chronic eosinophilic rhinitis. A significant reduction of uLTE4 was observed after sinus surgery in both AERD and ATA<sup>43</sup>. It has been suggested that chronic nasal mucosa inflammation with increased polymorphonuclear cells (PMNs) infiltrating the upper airways determines the overproduction of cysLTs rather than that it is a unique characteristic of AERD. In this study chronic

rhinosinusitis is associated with significantly higher levels of cysLTs and PGD<sub>2</sub> in ISS in class 2. CRS was frequent in severe asthma classes 2 and 4 but also mild asthma class 1. Bronchial mucosa in asthmatics is prone to undergo a similar influx of mediator releasing cells in the upper respiratory tract but upper airway inflammation does not always combine with severe eosinophilic asthma. Higher levels of PGA<sub>2</sub> in ISS were measured in class 1. PGA<sub>2</sub> is a PGE<sub>2</sub> spontaneously occurring dehydration product which stimulates mucus glycoproteins release in human airways<sup>44</sup>. This eicosanoid belongs to cyclopentenone prostanoids of potential anti-inflammatory capacities and inhibitory activity to inflammasome complexes<sup>45</sup>. Moreover, a nuclear orphan receptor NR4A3 induced in macrophages by inflammation, can respond to PGA<sub>2</sub><sup>46</sup> which may explain PGA<sub>2</sub> as a marker of this mild class of asthma. It seems that special attention should be paid to chronic rhinosinusitis as an important comorbidity influencing the course of asthma not only among AERD subjects.

Our study points out the heterogeneity of AERD population. AERD subjects tend to be located in class 2, but also classes class 1 and 4. The LCA was previously applied to identify possible AERD subphenotypes in a study by Bochenek et al.<sup>6</sup>. Four classes with disease severity ranging from mild to severe with frequent exacerbations, and high health care utilisation were established. In this study the highest levels of uLTE<sub>4</sub> among AERD were observed among subjects in classes with moderate course, intensive upper airway symptoms and high blood eosinophil levels. Our results reveal that severe, poorly controlled asthma with eosinophilic inflammation of the airways is associated with highest levels of cysLTs in ISS. Higashi et al.<sup>43</sup> has suggested that overproduction of cysLTs is strongly associated with chronic hyperplastic rhinosinusitis, as a significant decrease in uLTE<sub>4</sub> was observed after the surgical treatment of CRS. This supports our observations that a subphenotype of AERD with intense upper and lower airway symptoms is distinguished.

In conclusion, a latent class analysis on a cohort of both aspirin tolerant and hypersensitive asthmatics, using both clinical data, inflammatory cells and lipid mediators together identified 4 distinct classes. They represent a severity spectrum from mild and intermittent allergic asthma with low sputum eosinophilia to severe eosinophil and mixed predominant often aspirin hypersensitive asthma or severe neutrophil and mixed predominant, atopic, early onset asthma. Eosinophils and their mediators - cysLTs, PGD<sub>2</sub> were identified as having special importance in the pathobiology of a severe asthma phenotypes. Severe hypereosinophilic asthma with chronic rhinosinusitis should always be considered to be a potentially aspirin-exacerbated respiratory disease. Our study implies that both eosinophilic and neutrophilic counts in induced sputum without analysis of mediators do not give enough information to profile a phenotype. Determination of airway inflammatory molecular pattern should be more helpful in understanding mechanisms of severe asthma.

#### Clinical implications

A better understanding of asthma phenotypic characteristics should help identify patients at increased risk of uncontrolled asthma and asthma with aspirin hypersensitivity.

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Fig. 1 Distribution of phenotypes based on induced sputum cell count in four latent classes. \*

indicates significant differences ( $P < 0.05$ ) in inflammatory patterns between classes.

Fig. 2 Induced sputum supernatant concentrations of selected eicosanoids [pg/ml] in four latent classes. \* indicates significant differences ( $P < 0.05$ ) in ISS eicosanoid levels between classes.

Table 1. Clinical characteristics of the study cohort.

|                                                                    | <b>Asthma<br/>(n=139)</b> | <b>AERD<br/>(n=75)</b>  | <b>ATA<br/>(n=64)</b>     |
|--------------------------------------------------------------------|---------------------------|-------------------------|---------------------------|
| <b>Male/female subjects</b>                                        | 50/89                     | 19/56*                  | 31/33*                    |
| <b>Age y (<math>\pm</math>SD)</b>                                  | 46.9 $\pm$ 14.0           | 46.2 $\pm$ 12.7         | 47.8 $\pm$ 15.4           |
| <b>Smokers yes/no</b>                                              | 16/123                    | 7/68                    | 9/55                      |
| <b>Duration of asthma, median<br/>(min – max)</b>                  | 12(2-55)                  | 11(2-36)                | 12(2-55)                  |
| <b>ICS yes/no</b>                                                  | 120/19                    | 69/6*                   | 51/13*                    |
| <b>OCS yes/no</b>                                                  | 15/124                    | 8/65                    | 7/65                      |
| <b>ICS (<math>\mu</math>g/d) fluticasone, median<br/>(min-max)</b> | 500(0-2500)               | 500(0-2500)             | 500(0-2000)               |
| <b>Severe/mild or<br/>moderate/intermittent no(%)</b>              | 56 (40)/63<br>(46)/19(14) | 35(47)/34<br>(45)/6(8)* | 21(33)/30(47)/13<br>(20)* |
| <b>ACT median (min-max)</b>                                        | 23(7-25)                  | 22(7-25)                | 23(8-25)                  |
| <b>Controlled/partially/not<br/>controlled asthma<br/>no(%)</b>    | 67(48)/43(31)/29(21)      | 30(40)*/26(35)/19(25)   | 37(58)*/17(27)/10(15)     |
| <b>FEV1 baseline (%predicted) <math>\pm</math>SD</b>               | 91.0 $\pm$ 6.8            | 88.3 $\pm$ 16.8§        | 94.0 $\pm$ 16.2           |

|                                                                                                        |              |               |               |
|--------------------------------------------------------------------------------------------------------|--------------|---------------|---------------|
| <b>Positive skin prick test, yes/no</b>                                                                | 75/64        | 29/46*        | 45/19*        |
| <b>CRS yes/no</b>                                                                                      | 102/37       | 75/0*         | 27/37*        |
| <b>Peripheral blood eosinophil count<br/>count/<math>\mu</math>l median<br/>(25th-75th percentile)</b> | 324(165-612) | 405(254-664)* | 240(111-461)* |
| <b>Total serum IgE<br/>(IU/ml), median<br/>(25th-75th percentile)</b>                                  | 102 (35-256) | 96(32-216)    | 105(37.5-298) |
| <b>IS cellular phenotype<br/>eos/neu/mixed/pauci</b>                                                   | 44/35/23/37  | 32*/14/14/15  | 12*/21/9/22   |

ICS - inhaled corticosteroids, OCS- oral corticosteroids, ACT - asthma control test, CRS - chronic rhinosinusitis, IS - induced sputum, eos - eosinophil predominant, neu - neutrophil predominant, mixed - both eosinophil and neutrophil predominant, pauci - paucigranulocytic; \* AERD vs ATA P<0.05.

Table 2. Concentrations of selected eicosanoids in induced sputum supernatant in particular classes.

| <b>(pg/ml), median<br/>(25th-75th<br/>percentile)</b><br><br><b>Metabolite,<br/>method,<br/>LLOQ</b> | <b>Class 1<br/>(n=54)</b>             | <b>Class 2<br/>(n=35)</b>                 | <b>Class 3<br/>(n=33)</b>            | <b>Class 4<br/>(n=17)</b>         | <b>P</b> |
|------------------------------------------------------------------------------------------------------|---------------------------------------|-------------------------------------------|--------------------------------------|-----------------------------------|----------|
| <b>LTD<sub>4</sub></b><br><br><b>HPLC-MS/MS</b><br><br><b>2.85</b>                                   | 19.3[9.1-<br>59.2] <sup>CL:2,3</sup>  | 99.4[42.4-<br>164.2] <sup>CL:1,3,4</sup>  | 19.4[8.9-<br>27.8] <sup>CL:1,2</sup> | 20.7[7.1-<br>33.8] <sup>CL2</sup> | <0.0001  |
| <b>LTE<sub>4</sub></b><br><br><b>HPLC-MS/MS</b>                                                      | 26.8[10.5-<br>62.2] <sup>CL:2,3</sup> | 124.0[63.4-<br>262.4] <sup>CL:1,3,4</sup> | 11.0[6.5-<br>23.8] <sup>CL:1,2</sup> | 14.9[3.8-<br>36.0] <sup>CL2</sup> | <0.0001  |

|                                                            |                                |                                  |                                |                                |         |
|------------------------------------------------------------|--------------------------------|----------------------------------|--------------------------------|--------------------------------|---------|
| <b>3.22</b>                                                |                                |                                  |                                |                                |         |
| <b>PGE<sub>2</sub></b><br><b>GC-MS</b><br><b>1.62</b>      | 50.1[42.5-68.1]                | 59.6[45.6-76.1]                  | 48.2[33.9-55.0] <sup>CL4</sup> | 71.2[55.9-97.0] <sup>CL3</sup> | 0.02    |
| <b>LTB<sub>4</sub></b><br><b>HPLC-MS/MS</b><br><b>2.96</b> | 523.5[191.8-1479.4]            | 309.2[172,2-1218.6]              | 384.2[199.8-1415.5]            | 463.5[215.6-872.0]             | NS      |
| <b>Tetranor PGE-M</b><br><b>HPLC/MS</b><br><b>1.45</b>     | 33.0[25.5-39.1]                | 33.9[25.8-50.2]                  | 35.3[30.8-46.4] <sup>CL4</sup> | 28.0[16.3-32.8] <sup>CL3</sup> | 0.002   |
| <b>PGA<sub>2</sub></b><br><b>HPLC-MS/MS</b><br><b>12</b>   | 21.5[13.5-31.0] <sup>CL3</sup> | 15.8[12.2-28.1]                  | 13.4[11.5-16.1] <sup>CL1</sup> | 13.3[10.7-24.3]                | 0.003   |
| <b>PGD<sub>2</sub></b><br><b>GC-MS</b><br><b>2.09</b>      | 22.4[13.8-35.2] <sup>CL2</sup> | 47.1[26.3-82.7] <sup>CL1,3</sup> | 16.2[10.3-23.9] <sup>CL2</sup> | 21.4[8.1-101.5]                | <0.0001 |
| <b>Tetranor PGD-M</b><br><b>HPLC-MS/MS</b><br><b>1.55</b>  | 9.2[4.5-17.8]                  | 9.3[4.9-14.4]                    | 5.5[4.8-11.0]                  | 4.9[4.2-15.9]                  | NS      |
| <b>5-HETE</b><br><b>HPLC-MS/MS</b><br><b>0.82</b>          | 664.0[416.7-1835.1]            | 535.8[273.6-1289.9]              | 935.3[525.6-1840.9]            | 427.0[287.0-778.5]             | NS      |
| <b>12-HETE</b><br><b>HPLC-MS/MS</b><br><b>0.63</b>         | 1386.4[701.0-2851.9]           | 979.7[473.4-1537.3]              | 1908.3[993.7-3110.7]           | 1524.3[846.1-2055.8]           | NS      |
| <b>15-HETE</b><br><b>HPLC-MS/MS</b>                        | 674.1[283.3-1632.1]            | 1101.1[544.9-2749.2]             | 831.5[301.8-1655.7]            | 387.8[190.5-600.4]             | NS      |

|                                                                       |                 |                 |                 |                 |    |
|-----------------------------------------------------------------------|-----------------|-----------------|-----------------|-----------------|----|
| <b>2.18</b>                                                           |                 |                 |                 |                 |    |
| <b>6-keto-PGF<sub>1α</sub></b><br><b>GC-MS</b><br><b>2.41</b>         | 21.8[16.8-27.4] | 19.6[14.6-27.5] | 20.4[14.8-23.7] | 21.6[17.2-26.4] | NS |
| <b>11-dehydro-TXB<sub>2</sub></b><br><b>HPLC-MS/MS</b><br><b>1.84</b> | 17.8[15.6-24.0] | 20.3[17.5-30.8] | 19.8[17.5-23.4] | 17.9[16.4-23.5] | NS |
| <b>8-iso-PGE<sub>2</sub></b><br><b>GC-MS</b><br><b>1.65</b>           | 8.9[5.5-12.2]   | 7.7[5.4-9.8]    | 7.3[4.6-11.1]   | 8.8[5.7-13.6]   | NS |
| <b>8-iso-PGF<sub>2α</sub></b><br><b>GC-MS</b><br><b>0.73</b>          | 5.7[3.0-10.2]   | 4.4[2.6-8.8]    | 6.3[2.6-9.1]    | 6.8[2.7-11.8]   | NS |

GC-MS - gas chromatography/mass spectrometry, HPLC-MS/MS - high-performance liquid chromatography/tandem mass spectrometry, LLOQ - lowest limit of quantification. Significant differences (P<0.05) in eicosanoid level between the classes are marked as superscripts, where the number (CL:1-4) refers to the class.

**Table 3.** Clinical characteristics of the particular classes.

|                                             | Class 1                     | Class 2                      | Class 3                     | Class 4                     | P      |
|---------------------------------------------|-----------------------------|------------------------------|-----------------------------|-----------------------------|--------|
| No. of subjects                             | 54                          | 35                           | 33                          | 17                          |        |
| AERD/ ATA, no.                              | 36/18 <sup>CL:2,3</sup>     | 30/5 <sup>CL:1,3,4</sup>     | 0/33 <sup>CL:1,2,4</sup>    | 9/8 <sup>CL:2,3</sup>       | <0.05  |
| Female/male, no.                            | 31/23                       | 27/8                         | 19/14                       | 12/5                        | NS     |
| Age (y), mean ± SD                          | 46.4±12.1                   | 47.3±17.6                    | 46.3±16.6                   | 48.9±13.6                   | NS     |
| Age at asthma onset ≥12y<br>yes/no          | 51/3 <sup>CL:4</sup>        | 35/0 <sup>CL:4</sup>         | 28/5                        | 11/6 <sup>CL:1,2</sup>      | <0.05  |
| Duration of asthma (y), median<br>(min-max) | 8(2-34) <sup>CL:4</sup>     | 16(3-26)                     | 14(2-46)                    | 20(6-55) <sup>CL:1</sup>    | 0.01   |
| BMI kg/m <sup>2</sup> , mean ± SD           | 26.7±4.1                    | 27.1±5.5                     | 25.3±3.4 <sup>CL:4</sup>    | 30.2±7.3 <sup>CL:3</sup>    | 0.049  |
| ACT mean ± SD                               | 23.8±1.8 <sup>CL:2,4</sup>  | 19.1±4.2 <sup>CL:1,3,4</sup> | 23.1±2.3 <sup>CL:2,4</sup>  | 14.1±4 <sup>CL:1,2,3</sup>  | <0.001 |
| FEV <sub>1</sub> % predicted mean ± SD      | 97.5±12.1 <sup>CL:2,4</sup> | 80.8±18.8 <sup>CL:1,3</sup>  | 97.2±13.4 <sup>CL:2,4</sup> | 78.9±14.8 <sup>CL:1,3</sup> | <0.001 |
| Present level of asthma control,<br>no. (%) |                             |                              |                             |                             |        |
| 1. well controlled                          | 42(78) <sup>CL:2,4</sup>    | 2(6) <sup>CL:1</sup>         | 23(70) <sup>CL:4</sup>      | 0 <sup>CL:1,3</sup>         | <0.05  |
| 2. partially controlled                     | 12(22) <sup>CL:2</sup>      | 19(54) <sup>CL:1</sup>       | 10(30)                      | 3(18)                       | <0.05  |
| 3. not controlled                           | 0 <sup>CL:2,4</sup>         | 14(40) <sup>CL:1,3,4</sup>   | 0 <sup>CL:2,4</sup>         | 14(82) <sup>CL:1,2,3</sup>  | <0.05  |
| Asthma severity, no. (%)                    |                             |                              |                             |                             |        |
| 1. severe                                   | 10(19) <sup>CL:2,4</sup>    | 25(71) <sup>CL:1,3</sup>     | 8(24) <sup>CL:2,4</sup>     | 15(88) <sup>CL:1,3</sup>    | <0.05  |
| 2. mild to moderate                         | 32(59) <sup>CL:2,4</sup>    | 10(29) <sup>CL:1,3</sup>     | 18(55) <sup>CL:2,4</sup>    | 2(12) <sup>CL:1,3</sup>     | <0.05  |
| 3. intermittent                             | 12(22) <sup>CL:2,4</sup>    | 0 <sup>CL:1</sup>            | 7(21) <sup>CL:2,4</sup>     | 0 <sup>CL:1,3</sup>         | <0.05  |
| Oral steroids yes/no                        | 1/53 <sup>CL:2,4</sup>      | 5/30 <sup>CL:1,4</sup>       | 1/32 <sup>CL:4</sup>        | 8/9 <sup>CL:1,2,3</sup>     | <0.05  |

|                                                                                                 |                                  |                                     |                                 |                                      |        |
|-------------------------------------------------------------------------------------------------|----------------------------------|-------------------------------------|---------------------------------|--------------------------------------|--------|
| <b>Inhaled steroids (<math>\mu\text{g}/\text{d}</math>)<br/>fluticasone, median (25pc-75pc)</b> | 250 (100-500) <sup>CL:2,4</sup>  | 1000 (500-1000) <sup>CL:1,2,3</sup> | 250 (100-500) <sup>CL:2,4</sup> | 1000 (1000-1500) <sup>CL:1,2,3</sup> | <0.001 |
| <b>positive skin prick test<br/>responses, yes/no</b>                                           | 30/24 <sup>CL:2</sup>            | 9/26 <sup>CL:1,3,4</sup>            | 24/9 <sup>CL:2</sup>            | 11/6 <sup>CL:2</sup>                 | <0.05  |
| <b>CRS yes/no</b>                                                                               | 54/0 <sup>CL:3,4</sup>           | 35/0 <sup>CL:3,4</sup>              | 1/32 <sup>CL:1,2,4</sup>        | 13/4 <sup>CL:1,2,3</sup>             | <0.05  |
| <b>CRS surgery yes/no</b>                                                                       | 32/22 <sup>CL:3</sup>            | 23/12 <sup>CL:3</sup>               | 1/32 <sup>CL:1,2,4</sup>        | 9/8 <sup>CL:3</sup>                  | <0.05  |
| <b>Peripheral blood eosinophil<br/>count</b>                                                    | 398(267-573) <sup>CL:2,3,4</sup> | 641(462-836) <sup>CL:1,3,4</sup>    | 165 (108-324) <sup>CL:1,2</sup> | 197(59-250) <sup>CL:1,2</sup>        | <0.001 |
| <b>count/<math>\mu\text{l}</math> median (25th-75th<br/>percentile)</b>                         |                                  |                                     |                                 |                                      |        |
| <b>Total serum IgE (IU/ml), median<br/>(25th-75th percentile)</b>                               | 95.5(33.5-159)                   | 104(37-318)                         | 96.4(31.9-254)                  | 124(39.8-214)                        | NS     |

ACT - asthma control test, BMI - body mass index, IS - induced sputum, ICS - inhaled corticosteroids, OCS - oral corticosteroids, CRS - chronic rhinosinusitis

Significant differences ( $P < 0.05$ ) in eicosanoid level between the classes are marked in the upper case numbers, where the number (1-4) refers to the class.

Table 4. Characteristics of the particular classes and probability of having a patient allocated to one of the 4 classes based on LCA.

|                                                         | <b>Class 1</b> | <b>Class 2</b> | <b>Class 3</b> | <b>Class 4</b> |
|---------------------------------------------------------|----------------|----------------|----------------|----------------|
| <b>Female subjects</b>                                  | 0.573          | 0.765          | 0.575          | 0.716          |
| <b>Male subjects</b>                                    | 0.427          | 0.235          | 0.425          | 0.284          |
| <b>Age at asthma onset <math>\geq 12\text{y}</math></b> | 0.944          | 1.000          | 0.848          | 0.642          |
| <b>BMI <math>\geq 30 \text{ kg}/\text{m}^2</math></b>   | 0.169          | 0.334          | 0.091          | 0.477          |
| <b>Present level of asthma control (ACT)</b>            |                |                |                |                |
| <b>1. well controlled</b>                               | 0.773          | 0.075          | 0.699          | 0.000          |
| <b>2. partially controlled</b>                          | 0.277          | 0.527          | 0.301          | 0.181          |
| <b>3. not controlled</b>                                | 0.000          | 0.398          | 0.000          | 0.819          |

|                                                                                   |       |       |       |       |
|-----------------------------------------------------------------------------------|-------|-------|-------|-------|
| <b>Asthma severity</b>                                                            |       |       |       |       |
| <b>1. severe</b>                                                                  | 0.187 | 0.700 | 0.211 | 0.892 |
| <b>2. mild to moderate</b>                                                        | 0.588 | 0.300 | 0.577 | 0.108 |
| <b>3. intermittent</b>                                                            | 0.224 | 0.000 | 0.213 | 0.000 |
| <b>ICS yes</b>                                                                    | 0.776 | 0.972 | 0.757 | 1.000 |
| <b>OCS yes</b>                                                                    | 0.019 | 0.140 | 0.030 | 0.476 |
| <b>CRS yes</b>                                                                    | 1.000 | 1.000 | 0.000 | 0.758 |
| <b>CRS surgery yes</b>                                                            | 1.000 | 1.000 | 0.000 | 0.758 |
| <b>FEV1 &lt;80% (% predicted)</b>                                                 | 0.041 | 0.572 | 0.120 | 0.534 |
| <b>positive skin prick test responses</b>                                         | 0.555 | 0.266 | 0.727 | 0.645 |
| <b>IgE total ≥100 IU/ml</b>                                                       | 0.501 | 0.510 | 0.484 | 0.594 |
| <b>Blood eosinophil count ≥400/μL</b>                                             | 0.480 | 0.763 | 0.212 | 0.062 |
| <b>IS cell count phenotypes</b>                                                   |       |       |       |       |
| <b>eosinophilic (≥2% eosinophils)</b>                                             | 0.245 | 0.723 | 0.121 | 0.063 |
| <b>neutrophilic (≥50% neutrophils)</b>                                            | 0.291 | 0.040 | 0.334 | 0.418 |
| <b>paucigranulocytic</b>                                                          | 0.282 | 0.000 | 0.485 | 0.355 |
| <b>mixed eosinophilic and neutrophilic (≥2% eosinophils and ≥50% neutrophils)</b> | 0.183 | 0.237 | 0.060 | 0.164 |

ACT - asthma control test, BMI - body mass index, IS - induced sputum, ICS - inhaled corticosteroids, OCS - oral corticosteroids, CRS - chronic rhinosinusitis



