Targeted eicosanoid lipidomics of exhaled breath condensate provide a distinct pattern in the aspirin-intolerant asthma phenotype

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Background: Eicosanoids, important signaling and inflammatory molecules, are present in exhaled breath condensate (EBC) in very low concentrations, requiring highly sensitive analytic methods for their quantification.

Objective: We sought to assess a vast platform of eicosanoids in different asthma phenotypes, including aspirin-intolerant asthma, by means of a recently developed analytic approach based on mass spectrometry.

Methods: EBC from 115 adult asthmatic subjects (62 with aspirin intolerance) and 38 healthy control subjects were assessed quantitatively for 19 eicosanoids by using complementary HPLC, gas chromatography–mass spectrometry, or both. Palmitic acid concentrations were used as a marker for dilution of condensate samples.

Results: Asthma was characterized by an increase in arachidonate lipoxygenase products and cysteinyl leukotrienes. The COX pathway was also significantly upregulated in asthmatic subjects. Subjects with aspirin-intolerant asthma were distinguished by a sharp increase in the level of prostaglandin D₂ and E₂ metabolites; their 5- and 15-hydroxyeicosatetraenoic acid levels were also higher than in aspirin-tolerant subjects. A classical discriminant analysis permitted us to classify correctly 99% of asthmatic subjects within the study population; the specificity of the analysis was 97%. The eicosanoid profiling allowed for 92% correct classification of aspirin-intolerant subjects.

Conclusions: The highly sensitive eicosanoid profiling in EBC makes it possible to detect alterations in asthma, especially in its distinct phenotype characterized by hypersensitivity to aspirin and other nonsteroidal anti-inflammatory drugs. This permits us to discriminate asthmatic subjects from healthy subjects, as well as to distinguish the 2 asthma phenotypes based on the presence or absence of aspirin hypersensitivity. (*J Allergy Clin Immunol* 2011;127:1141-7.)

Key words: Exhaled breath condensate, asthma, aspirin intolerance, eicosanoids, mass spectrometry

The breath, like the heartbeat, has always been a mark of life, an expression of its mystery. Breath composition has recently started to be analyzed in airway physiology and pathology.¹ This approach appeals to researchers and clinicians. It provides them with a sample of air from the respiratory tract obtained through a noninvasive procedure, which can be repeated several times. The exhaled air contains both volatile and nonvolatile compounds, which might include potential biomarkers. Volatile organic compounds, derived from various metabolic pathways in the lung and elsewhere in the body, can be detected by using an integrative array of microsenors (“electronic nose”). This method might yield distinctive patterns for asthma and chronic obstructive pulmonary disease.² However, it cannot identify individual volatile organic compounds. This requires mass spectrometry,³ which also is a gold standard for detecting nonvolatile compounds. The latter are present in such a low concentration that methods other than tandem mass spectrometry barely reach the threshold of their detection.

Nonvolatile compounds are usually studied in exhaled breath condensate (EBC).⁴ During tidal breathing, lasting 15 to 20 minutes, the air is directed through a 1-way expiratory valve to a cooling trap, resulting in the accumulation of 1 to 2 mL of clear liquid. The vast majority of this liquid is composed of water, but a tiny fraction contains nonvolatile compounds. It is assumed that they are present in the liquid that lines the airway and that they become aerosolized during inflation of the lung and carried up with exhaled airflow.

Of various mediators active in asthma, the derivatives of arachidonic acid, eicosanoids, occupy a special place. Marked alterations in their biosynthesis have been demonstrated in a clear-cut phenotype of asthma (ie, aspirin-induced asthma [AIA])^{5,6} and suggested in other asthma phenotypes.⁷ We hypothesized that EBC analysis might provide new insight into the alteration of these eicosanoids. Therefore we applied our own analytic approach based on mass spectrometry.⁸ This recently developed approach has allowed us to assess a vast platform of eicosanoids in different asthma phenotypes.

METHODS

Subjects studied

The patients were recruited from the outpatient clinic of our department. All were in stable condition, with no signs of recent respiratory tract infection. No changes in current therapy were requested, except withholding of short-acting β -mimetics for 4 hours preceding the EBC collection.

In patients with AIA, the disease developed according to a pattern characterized by a sequence of symptoms.⁹ They had at least 1 asthma attack after an ingestion of aspirin or another nonsteroidal anti-inflammatory drug in the past, and this diagnosis was confirmed by means of either oral or bronchial

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Abbreviations used

AIA:	Aspirin-intolerant asthma
ATA:	Aspirin-tolerant asthma
EBC:	Exhaled breath condensate
8-iso-PGF _{2α} :	Isoprostane F _{2α}
11-dehydro-TXB ₂ :	Thromboxane A ₂ metabolite
GC-MS:	Gas chromatography–mass spectrometry
HPLC-MS/MS:	High-performance liquid chromatography–tandem mass spectrometry
HETE:	Hydroxyeicosatetraenoic acid
LO:	Lipoxygenase
LT:	Leukotriene
9α,11β-PGF ₂ :	PGD ₂ semistable metabolite
PG:	Prostaglandin
PGEM:	PGE ₂ stable metabolite
6-keto-PGF _{1α} :	Prostacyclin metabolite

provocation tests with aspirin performed within 1 year before the study. In oral challenge tests ($n = 46$) mean PD₂₀ was 98.3 mg (20–187 mg) of acetylsalicylic acid, and in inhalation tests ($n = 16$) it was 6.4 mg (0.3–26.0 mg) of acetylsalicylic acid. Sixteen patients (10 with aspirin-tolerant asthma [ATA] and 6 with AIA) were receiving leukotriene (LT) receptor antagonists. No patient received an inhibitor of LT biosynthesis or aspirin desensitization treatment.

Asthmatic subjects who tolerated aspirin well within the previous 2 years had occasionally used aspirin and denied any adverse effects when specifically queried. In 16 subjects with ATA selected at random, an aspirin challenge test was performed; results were negative in all cases.

The assessment of asthma severity was based on the National Asthma Education and Prevention Program's Expert Panel Report 3.¹⁰ The current symptoms of asthma, bronchodilator use, limitation of normal activity, current lung function, and frequency of exacerbations in the last year were taken into account. Asthma severity was classified as intermittent, mild persistent, moderate persistent, and severe persistent. Each subject was assigned to the highest level at which any feature occurred.

Asthma control was assessed by using the Asthma Control Test.¹¹ It consisted of 5 items rated on a 1- to 5-point scale, which reflected the level of asthma control in the previous 4 weeks. The maximum score possible was 25. The subject was categorized as having well-controlled asthma if his or her score equaled 25. A score of 20 to 24 categorized the subject as having partly controlled asthma, and a score of less than 20 categorized the subject as having poorly controlled disease.

The control group (Table I) consisted of healthy subjects with no history of asthma or allergy who were recruited from the university hospital staff. All subjects provided informed consent to participate in the study, and the protocol was approved by the university's ethics committee.

EBC collection and sample preparation

Collections of EBC were carried out with the ECO Screen I (Jaeger GmbH, Hoechberg, Germany) equipped with a saliva trap, according to American Thoracic Society/European Respiratory Society recommendations.⁴ After tidal breathing for 15 minutes, 2 mL of clear fluid was collected and immediately transferred on ice to a -70°C freezer. Samples were stored frozen until extraction, which was completed within 3 months. After thawing on ice, the pH of the condensate was adjusted to 3.5 by using acetic acid (2 μL) and added methanol solution of internal deuterated standards containing 5-hydroxyeicosatetraenoic acid (HETE)-d₈, 12-HETE-d₈, 15-HETE-d₈, LTD₄-d₅, LTE₄-d₃, LTB₄-d₄ (0.5 ng each), tetranor-PGE₂ stable metabolite (PGEM)-d₆ (5 ng), prostaglandin (PG) E₂-d₄, PGD₂-d₄, PGD₂ semistable metabolite (9α,11β-PGF₂)-d₄ (0.25 ng each), prostacyclin metabolite (6-keto-PGF_{1α})-d₄, isoprostane F_{2α} (8-iso-PGF_{2α})-d₄, thromboxane A₂ metabolite (11-dehydro-TXB₂)-d₄ (0.5 ng each, all from Cayman Chemical Co, Ann Arbor, Mich), and palmitic acid-d₄ (40 ng; Dr Ehrenstorfer GmbH, Augsburg, Germany). The sample was extracted twice with 1 mL of tert-butyl methyl ether. Organic phases were

combined and evaporated under nitrogen at room temperature. Residue was dissolved in methanol (60 μL) and immediately used for further analyses.

HPLC–tandem mass spectrometry

The EBC extract (10 μL in methanol) was injected onto a reverse phase column (Zorbax Eclipse XDB C-18; Agilent Technologies, Inc, Santa Clara, Calif) stabilized thermally at 37°C . HPLC with an autosampler (Shimadzu SIL-2-AC; Shimadzu Scientific Instruments, Inc, Columbia, Md) was coupled to a tandem mass spectrometer (Qtrap 4000; Applied Biosystems, Foster City, Calif) equipped with an electrospray ion source. Analytes were measured by using the multiple-reaction monitoring mode. Chromatographic solvents, binary gradient and flow conditions, ionization modes, retention times, and diagnostic ions were as previously described.⁸

Gas chromatography–mass spectrometry

EBC extracts were prepared by means of a 3-step derivatization to pentafluorobenzyl ester, trimethylsilyl esters, and methoxyoxime, which modified the carboxyl, hydroxyl, and keto groups of eicosanoids. After purification with thin-layer chromatography, gas chromatography negative-ion chemical ionization mass spectrometry (model Engine 5989B series II; Hewlett-Packard, Palo Alto, Calif; for details see Sanak et al⁸) was used to measure prostanoids that could not be separated with HPLC–tandem mass spectrometry (HPLC-MS/MS; 9α,11β-PGF₂, PGF_{2α}, and 8-iso-PGF₂) and palmitic acid.

PGE₂, PGD₂, PGI₂ metabolite, and thromboxane A₂ (11-dehydro-TXB₂) values were ascertained by using both methods. A discrepancy observed previously between PGE₂ measurements by means of HPLC-MS/MS and gas chromatography–mass spectrometry (GC-MS)⁸ was resolved ultimately. GC-MS measurements of PGE₂ overestimated its concentration because it was overlaid with isoprostane 8-iso-PGE₂. A corrected GC-MS analysis,¹² which discriminated between the 2 compounds, resulted in a highly significant correlation of PGE₂ measured by using the 2 methods ($R = 0.908$). For other double-measured eicosanoids, correlation coefficients were as follows: $R[6\text{-keto-PGF}_{1\alpha}]$, 0.982; $R[11\text{-dehydro-TXB}_2]$, 0.959; and $R[\text{PGD}_2]$, 0.243. The latter, similar to uncorrected GC-MS measurements of PGE₂, were overestimated by using this method. A possible interference between PGD₂ and isoprostane D₂ or other structurally related compounds could not be resolved in the current study because no appropriate standard compounds were available. It is noteworthy that the GC-MS bias of PGD₂ measurements was highest in the asthmatic subjects, whereas in healthy control subjects the measurements correlated with the HPLC-MS/MS method satisfactorily ($R = 0.529$). Further analysis was restricted to HPLC-MS/MS data, except the eicosanoids were measured only with GC-MS. All solvents were of HPLC grade (Mallin-crodt Baker, Inc, Phillipsburg, NJ), and other chemicals were from Sigma-Aldrich Co (St Louis, Mo).

Concentrations of the measured analytes were calculated by using a stable isotope dilution method. Data are presented as medians with interquartile ranges (25th and 75th percentiles). Both raw data and relative concentrations corrected for EBC dilution with palmitic acid are reported. The rationale for the method of correction for variable EBC dilution factor with palmitic acid has been described previously.⁸

Between-group comparisons were done with a nonparametric Mann-Whitney test. Asthmatic subjects were contrasted with healthy control subjects. Comparisons between subjects with AIA and asthmatic subjects who tolerated aspirin well (ATA) were done with the same method. The statistical significance of differences was verified with the Conover-Inman method for pairwise comparisons. A discriminant analysis was performed on the data corrected for dilution factor by using relative concentrations. For all measured compounds, a quadratic model was used because of a high cross-correlation between eicosanoid concentrations. An alternate forward stepwise discriminant analysis was done with a linear model. The model entry criterion was arbitrarily defined as an increase F statistics by more than 1. Prior probabilities for the discriminant analysis were calculated from the group sizes. All calculations were done with SPSS for Windows version 17.0 (SPSS, Inc, Chicago, Ill).

TABLE I. Clinical characteristics of the study subjects

	Asthmatic subjects (n = 115)	Subjects with aspirin-intolerant asthma (n = 62)	Subjects with ATA (n = 53)	Healthy control subjects (n = 38)
Male/female subjects	41/74	21/41	20/33	12/26
Age ($\pm$ SD)	50.25 $\pm$ 11.2	52.58 $\pm$ 11.3 [‡]	47.57 $\pm$ 10.6	43.03 $\pm$ 13.1*
Current smokers	3	3	0	10
Duration of asthma	18.61 $\pm$ 11.8	19.84 $\pm$ 9.2 [†]	17.11 $\pm$ 14.3	NA
Atopy, no. (%)	56 (48.7)	28 (45.2)	28 (52.8)	NA
Rhinitis, no. (%)	81 (70.4)	44 (71.0)	35 (66.1)	0
Peripheral blood eosinophil count (/mm ³), median (25th-75th percentile)	278 (144-576.5)	305 (197-605)	234 (105-517)	NA
Total serum IgE (IU/mL), median (25th-75th percentile)	101 (41.6-220)	100.2 (44.7-180.0)	116 (24-268.5)	NA
FEV ₁ (%)	80.56 $\pm$ 18.4	77.10 $\pm$ 18.6 [†]	84.87 $\pm$ 17.5	NA
Asthma severity (NAEPP-EPR3)	2.43 $\pm$ 1.1	2.86 $\pm$ 1.0 [§]	1.90 $\pm$ 1.1	NA
Asthma control (ACT)	19.92 $\pm$ 5.0	19.48 $\pm$ 5.2	20.45 $\pm$ 4.7	NA
ICS dose (μ g fluticasone equivalent)	742 $\pm$ 504	688 $\pm$ 486	807 $\pm$ 521	0
OCS dose (mg prednisone equivalent)	3.02 $\pm$ 11.2	2.30 $\pm$ 3.7	3.89 $\pm$ 10.4	0

ACT, Asthma Control Test; NA, not applicable; NAEPP-EPR, National Asthma Education and Prevention Program's Expert Panel Report 3.

* $P < .001$ for the contrast between asthmatic and healthy control subjects.

[†] $P < .05$, [‡] $P < 0.01$, or [§] $P < .001$ for the contrast between subjects with aspirin-intolerant asthma and subjects with ATA.

RESULTS

Characteristics of asthmatic subjects

One hundred fifteen asthmatic subjects were recruited, 62 (41 female subjects) of whom had AIA. Asthmatic subjects who tolerated aspirin well (ie, those with ATA; $n = 53$; 33 female subjects) were younger, on average, by 5 years ($P < .01$) than subjects with AIA. The duration of asthma was longer by 2.7 years in subjects with AIA ($P < .05$). Atopy status, defined as a documented positive skin prick test reaction for common allergens, the presence of allergen-specific IgE at a concentration exceeding 35 IU/mL, or both, did not differ between the groups. Peripheral blood eosinophil counts tended to be higher in subjects with AIA ($P = .08$), whereas total serum IgE levels were not different. Subjects with ATA had lower forced expiratory flow ($P < .05$) and asthma severity scores ($P < .001$). Roughly 50% of asthmatic subjects had at least partly controlled and the rest poorly controlled disease, without any differences ($P = .43$) between the groups. The groups did not differ in terms of dosage of topical or systemic corticosteroids. The characteristics of the study subjects are summarized in Table I.

Palmitic acid and eicosanoids in EBC: Correlation with pulmonary function in asthmatic subjects

Palmitic acid did not show any correlation ($R = 0.151$) with FEV₁ in the entire asthmatic group. However, subjects with ATA analyzed alone demonstrated a significant negative correlation between the concentration of palmitic acid and FEV₁ ($R = -0.384$, $P = .005$), whereas such a correlation was absent in the AIA group ($R = 0.019$). Of other eicosanoids quantified, 6-keto-PGF_{1 α} correlated positively and significantly ($R = 0.378$, $P = .003$) with FEV₁ in subjects with ATA ($R = 0.024$) but not in the AIA group. In the AIA group the only significant predictor of decreased FEV₁ was increased 9 α ,11 β -PGF₂, the metabolite of PGD₂ ($R = -0.374$, $P = .015$), which did not correlate with FEV₁ in subjects with ATA ($R = 0.121$).

Sex differences

Nonparametric comparison of EBC eicosanoids and palmitic acid between men and women did not reveal any significant differences. Within the whole asthmatic group, LTD₄ levels tended to be higher in men (2.2 vs 1.9 ppm, $P = .048$), and PGEM levels were higher in women (323 vs 138 ppm, $P = .023$). Further stratification of asthmatic subjects into aspirin-intolerant and aspirin-tolerant phenotypes showed higher eoxin D₄ levels in female subjects in cases of aspirin tolerance (2.4 vs 1.4 ppm, $P = .032$).

Eicosanoid EBC concentrations in asthmatic versus healthy control subjects

Subjects with asthma had levels of all 3 lipoxygenase (LO) products increased. A significant increase in 5-HETE, 12-HETE, and 15-HETE levels was accompanied by an increase in LTC₄, LTD₄, and LTE₄, but not LTB₄, levels. Eoxins C₄, D₄, and E₄ and lipoxin A₄ levels did not differ from those in the control group. Metabolites of PGD₂, PGE₂, and prostacyclin (PGI₂), as well as PGF_{2 α} , 8-iso-PGF₂, and 11-dehydro-TXB₂, were all increased in asthmatic subjects. The results were similar when expressed as dilution-unadjusted concentrations, although statistical significance was less frequently encountered (Table II and see Table E1 in this article's Online Repository at www.jacionline.org).

Differences between subjects with aspirin-intolerant asthma and those with ATA

The most striking difference between aspirin-intolerant asthma and ATA was a vast increase of metabolites of PGE₂ and PGD₂ in the former group. In subjects with AIA, PGE₂ showed a tendency toward decreased values, which did not reach statistical significance. These patients had also increased levels of 5-HETE and 15-HETE. Cysteinyl LT and eoxin levels, however, were lower in subjects with AIA. Results obtained by means of evaluation

TABLE II. Eicosanoid concentration in EBC as parts per million of palmitic acid

	Asthmatic subjects (n = 116)	Subjects with aspirin-intolerant asthma (n = 62)	Subjects with ATA (n = 54)	Healthy control subjects (n = 38)
5-HETE	7.51 (4.18-13.48)	8.92 (4.26-17.56)§	6.23 (4.15-9.49)	4.29 (3.15-7.55)†
12-HETE	9.57 (5.69-14.91)	9.98 (5.55-28.46)	8.74 (6.11-15.82)	5.20 (3.73-7.73)‡
15-HETE	10.53 (5.74-22.72)	15.09 (5.85-28.46)§	8.79 (4.40-14.96)	5.19 (2.87-7.44)‡
PGE ₂	1.74 (0.79-3.06)	1.13 (0.49-3.11)	1.82 (1.12-2.83)	1.64 (0.90-3.10)
PGEM	292 (128-450)	387 (222-515)¶	144 (77.4-293)	76.1 (36.5-220)‡
PGD ₂	1.79 (1.15-3.71)	1.85 (1.13-3.88)	1.65 (1.16-3.05)	1.74 (0.82-3.36)
9 α ,11 β PGF ₂	1.31 (0.5-2.16)	1.72 (0.98-2.61)¶	0.62 (0.29-1.19)	0.55 (0.35-0.86)†
LTB ₄	12.92 (7.15-19.38)	16.18 (7.68-23.04)	10.02 (7.00-16.11)	16.19 (11.08-25.20)*
LTC ₄	4.28 (2.68-8.13)	3.69 (2.39-7.03)	5.59 (3.01-9.56)	2.28 (0.81-5.43)†
LTD ₄	2.69 (1.32-5.42)	1.93 (0.96-4.09)	3.77 (2.09-6.94)	1.80 (0.80-4.09)*
LTE ₄	3.40 (1.99-5.49)	2.54 (1.37-4.66)¶	4.52 (2.96-6.87)	2.03 (1.39-4.05)*
Eoxin C ₄	1.07 (0.60-1.99)	0.77 (0.34-1.52)¶	1.42 (0.80-2.81)	1.06 (0.65-1.92)
Eoxin D ₄	1.30 (0.65-2.60)	0.70 (0.40-1.30)¶	2.02 (1.21-3.08)	1.47 (1.95-3.73)
Eoxin E ₄	4.23 (2.19-9.09)	2.82 (1.82-6.75)	4.81 (2.49-11.40)	6.14 (3.05-14.80)
11-Dehydro-TXB ₂	13.41 (8.35-19.24)	10.41 (6.82-17.57)§	15.36 (12.21-19.50)	10.05 (7.98-12.91)*
6-Keto-PGF _{1α}	34.62 (19.56-49.97)	35.65 (23.29-57.32)	28.46 (18.26-46.81)	15.72 (10.39-19.56)‡
PGF _{2α}	1.74 (0.92-3.02)	1.48 (0.91-2.65)	2.12 (1.04-3.33)	0.66 (0.42-1.39)‡
8-Iso-PGF ₂	0.77 (0.45-1.26)	0.79 (0.36-1.52)	0.76 (0.54-1.08)	0.46 (0.34-0.87)*
Lipoxin A ₄	0.69 (0.40-1.11)	0.63 (0.39-1.09)	0.85 (0.48-1.17)	0.94 (0.44-1.90)

Values are presented as medians (25th-75th percentiles).

* $P < .05$, † $P < .01$, or ‡ $P < .001$ for the contrast between asthmatic and healthy control subjects.

§ $P < .05$, || $P < .01$, or ¶ $P < .001$ for the contrast between subjects with aspirin-intolerant asthma and subjects with ATA.

of dilution-unadjusted concentrations were very similar (Table II and see Table E1).

Effect of glucocorticosteroid therapy on palmitic acid and eicosanoids in EBC

The palmitic acid concentration in EBC was significantly lower in asthmatic subjects treated with glucocorticosteroids (459 ± 197 vs 543 ± 257 ng/mL, $P = .03$). This difference, however, disappeared when FEV₁ was entered as a covariate into ANOVA ($P = .4$). Asthmatic subjects receiving glucocorticosteroids (oral corticosteroid treatment and/or inhaled corticosteroid dose exceeding 250 μ g of fluticasone equivalent daily) had noticeably lower 8-isoPGF_{2 α} levels than untreated subjects ($n = 91$, 1.0 vs 1.64 ppm, $P = .034$). Only subjects with AIA had higher eoxin E₄ levels if treated with glucocorticosteroids ($n = 45$, 5.5 vs 1.9 ppm, $P = .03$). Neither of the measured eicosanoids correlated with glucocorticosteroid treatment in the whole group of asthmatic subjects or in asthmatic subjects stratified by tolerance to aspirin.

Discriminant analysis of eicosanoids as predictors of asthmatic phenotype

By using a classical stepwise binary discriminant analysis, in which the presence of asthma was predicted on adjusted concentrations of all measured eicosanoids, only 4 markers fulfilled the criteria of probabilities to enter to the model. Canonical discriminant function, which predicted the presence of asthma, had the following standardized coefficients: 5-HETE, 0.41; LTD₄, 0.30; PGE₂, -0.72; and 6-keto-PGF_{1 α} , 1.01. The mean function value for control subjects was significantly lower than for asthmatic subjects (-1.11 vs 0.32 , $P < .001$). By using this function, 95% of asthmatic subjects were correctly stratified, but 53% of control subjects were misclassified as having asthma.

Classification of the study subjects into healthy control or asthma groups, irrespective of their tolerance to aspirin, was almost perfect by using all the measurements of eicosanoids (total of 99% correctly classified). Misclassification was encountered in the control group, in which 3% of subjects were categorized as being asthmatics.

A similar but ternary model built on a stepwise entry of eicosanoid measurements had slightly limited specificity for classification of the study subjects into 3 groups: subjects with AIA, subjects with ATA, and healthy control subjects. It required 12 of 19 measured analytes. Misclassification affected subjects with ATA (total of 7%, 2% categorized as aspirin intolerant and 5% as healthy control subjects; Fig 1). By using this limited set of measurements, the worst specificity was in healthy control subjects (total of 73% correctly categorized, 5% misclassified as having AIA and 19% as having ATA). Nevertheless, overall performance showed a 92% correct diagnosis. The ternary model of a discriminant analysis using all the measurements correctly categorized 99% of the study subjects (Fig 2). The only misclassification was 1 subject with AIA categorized in the ATA group. The eicosanoids entered into discrimination function are listed in Table E2 (available in this article's Online Repository at www.jacionline.org) for both ternary models. In these models of discriminant analysis, healthy control subjects were characterized by a lower value of canonical factor 2 than seen in asthmatic subjects. The phenotype of AIA versus ATA was discriminated by a difference in the load of canonical factor 1, being smaller for subjects with ATA in the all measurements model.

DISCUSSION

In the present study we used a validated analytic platform^{13,14} to analyze eicosanoids in EBC of asthmatic subjects. The study subjects were contrasted for the triggering phenotype (ie, AIA [aspirin-

FIG 1. Discriminant analysis based on stepwise selection of 12 eicosanoids in EBC. The overall performance of the ternary model built on a stepwise entry of eicosanoid measurements was a 92% correct diagnosis. Color codes for subjects are as follows: *red*, subjects with aspirin-intolerant asthma; *blue*, subjects with ATA; and *green*, healthy control subjects.

FIG 2. Discriminant analysis using all the measurements of eicosanoids in EBC. The ternary model of a discriminant analysis using all the measurements correctly categorized 99% of the study subjects. Color codes for subjects are as follows: *red*, subjects with aspirin-intolerant asthma; *blue*, subjects with ATA; and *green*, healthy control subjects.

exacerbated respiratory disease] vs ATA). Patterns of eicosanoid mediators in subjects with AIA have previously been reported in several biological matrices: blood plasma,¹⁵ bronchoalveolar lavage fluid,¹⁶ nasal lavage fluid,^{17,18} sputum,^{19,20} and urine^{21,22} and, to a much lesser extent, EBC.^{23,24} After the oral aspirin challenge test, the starker differences in eicosanoid production were found in urine,^{21,22} reflecting increased systemic biosynthesis of cysteinyl LTs, the main eicosanoid culprit for asthmatic attack. In aspirin-intolerant subjects, even those in stable clinical condition, increased LTE₄ urinary excretion is a hallmark of the disease.

The aim of this study was to compare patterns of eicosanoids released to the epithelial surface of the asthmatic lung in subjects with well-defined aspirin hypersensitivity. Because of the lack of an *in vitro* test to diagnose aspirin hypersensitivity, the rationale of our study was to investigate the discriminant properties of EBC eicosanoids. To determine the patterns of eicosanoids produced within the lung during an asthmatic inflammation, we introduced some novel concepts for the standardization of EBC material measurements. A highly sensitive method of mass spectrometry was used; it still requires some improvements to avoid a bias caused by the similarity between PGs and isoprostanes.¹⁴ The concurrent measurement of palmitic acid content in EBC seems a convenient surrogate for compensating the dilution factor,⁸ although alternative methods are available.²⁵ A dilution of nonvolatile compounds with water has recently been demonstrated to vary between the subjects by more than 1 order of magnitude and to depend on ventilation mechanics.^{26,27} It is of interest that in our data forced expiratory flow was higher in subjects with increased levels of 6-keto-PGF_{1α} metabolite, possibly because prostacyclin in some,²⁸ but not all,²⁹ phenotypes of asthma was demonstrated to exert a protective effect. This effect was absent in subjects with AIA, a phenotype in which prostacyclin was proved to have no bronchoprotective effect.³⁰ A different mechanism of bronchial obstruction³¹ might operate in subjects with AIA, in whom enhanced biosynthesis of PGD₂ correlated with the trapping of epithelial line fluid aerosol. Comparing 2 phenotypes of asthma defined by hypersensitivity for a triggering factor (ie, aspirin), we demonstrated a distinct inflammatory endophenotype in EBC of subjects with AIA.

Asthmatic inflammation was characterized by a significant increase in major arachidonate lipoxygenation products (ie, 5-HETE, 12-HETE, and 15-HETE). These findings corroborate with well-studied overexpression of LOs in the asthmatic lung. 5-LO and 15-LO are expressed not only in eosinophils and activated macrophages³² but also in lymphocytes and mast cells.^{33,34} The cellular origin of 12-LO is less clear; blood platelets are regarded as its main source. However, 12-LO was originally cloned from respiratory epithelia, where 15-LO activity was also found.³⁵ In our study an increased concentration of arachidonate lipoxygenation products in the whole asthma group was accompanied by an increase in more active downstream mediators: the cysteinyl LTs LTC₄, LTD₄, and LTE₄. As noticed previously,²⁴ cysteinyl LT levels were not increased in EBC of subjects with AIA despite enhanced capacity for their biosynthesis, possibly because a vast majority of our patients were not steroid naive²³ but received a chronic steroid therapy. Glucocorticosteroid therapy affects only limited cell types *in vivo*, with major inhibiting effects limited to alveolar macrophages.³⁶ In contrast to childhood asthma,³⁷ 15-LO analogues of cysteinyl LTs (ie, eoxins C₄, D₄, and E₄) showed no increase. This might be perhaps due to a less frequent IgE-dependent mechanism operating in adults with asthma. However, atopy status did not correlate with eoxin levels in our data (data not shown).

The COX pathway was significantly upregulated in asthmatic subjects, as manifested by an excess of the most abundant prostanoid metabolites (ie, PGI₂ and thromboxane A₂). Levels of PGF_{2α} and its nonenzymatic isomer, 8-iso-PGF₂, were also increased. In subjects with AIA, PGE₂ showed a tendency toward decreased values, which failed to reach significance; PGD₂ values were unaltered. Anomalies in the synthesis of PGE₂ along with the expression of its receptor, PGE₂ receptor isoform 2, have been associated with aspirin sensitivity (for reference see, Szczeklik et al³⁸). Decreased PGE₂ production was reported in epithelial cells of nasal polyps, peripheral blood cells, and bronchial fibroblasts compared with that seen in subjects with ATA, healthy subjects, or both. In a large genomic study³⁹ PGE₂ receptor isoform 2 gene polymorphisms located in the regulatory region have been associated with aspirin hypersensitivity in asthmatic patients. We now demonstrate an excessive concentration of the PGE₂ end metabolite PGEM in the lung of subjects with AIA,

suggesting an enhanced turnover of PGE₂. Upregulation of the PGE₂ catabolic pathway could explain previously reported decreases in PGE₂ levels in subjects with AIA but had to be accompanied by at least normal capacity for its synthesis. Indeed, at the systemic level, subjects with AIA had increased PGEM urinary excretion after aspirin challenge.²² More information derived from the mass spectrometric assays on other PGE₂ metabolites, including EBC levels of the dinor-PGE₂ intermediate, might shed more light on the site and nature of the anomalous PGE₂ kinetics in subjects with AIA. In contrast to the constant finding of increased urinary LTE₄ levels in subjects with AIA,⁴⁰ the subjects with controlled disease did not have increased cysteinyl LTs in EBC. The content of eicosanoids in the epithelial lining fluid is affected not only by biosynthesis but also by a polarized transepithelial release of prostanoids,⁴¹ and thus a possible interpretation of this phenomenon would suggest an inflammatory mechanism distinct from the asthmatic paradigm but more similar to a persistent viral infection. The current results are in line with our previous observation²¹ of increased urinary excretion of PGE₂ stable metabolites in subjects with aspirin-intolerant asthma after aspirin challenge. In subjects with AIA, we also detected an increase in levels of the stable PGD₂ metabolite, namely 9α,11βPGF₂. The increased values of this metabolite have been reported in the blood of subjects with AIA.¹⁵ In addition, after aspirin challenge, the blood and urinary levels of this metabolite increase.¹⁵ Mast cells are likely the source of this overproduction.

In summary, the highly sensitive eicosanoid profiling in EBC points to alterations in asthma, especially in its distinct phenotype characterized by aspirin and other nonsteroidal anti-inflammatory drug hypersensitivity. In our opinion the assessment of EBC does not yet seem to be practically applicable in routine clinical workup because of the limited availability of expensive equipment. Instead, the monitoring of anti-inflammatory intervention, as well as the progress of asthma, seems to fit this noninvasive procedure best.

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Clinical implications: Eicosanoid profiling in EBC points to alterations in asthma, especially in its phenotype characterized by aspirin hypersensitivity. The method seems suitable for monitoring the disease.

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TABLE E1. Eicosanoid concentration in EBC: unadjusted concentration

	Asthmatic subjects (n = 115)	Subjects with aspirin-intolerant asthma (n = 62)	Subjects with ATA (n = 54)	Healthy control subjects (n = 38)
5-HETE	2.82 (1.88-5.41)	3.90 (2.25-5.80)	2.26 (1.43-3.32)	2.75 (1.80-4.89)
12-HETE	3.50 (2.52-6.42)	3.63 (2.76-6.70)	3.36 (2.22-6.14)	3.50 (2.39-4.51)
15-HETE	4.81 (3.16-7.02)	6.37 (3.74-9.30)	3.67 (2.30-5.74)	2.60 (2.15-3.93)‡
PGE ₂	0.64 (0.39-1.07)	0.55 (0.33-0.98)	0.66 (0.49-1.12)	0.78 (0.39-1.56)
PGEM	99.2 (48.7-191)	169 (91-300)¶	55.0 (27.6-103)	108 (24.6-184)
PGD ₂	0.79 (0.50-1.26)	0.91 (0.66-1.25)	0.68 (0.46-1.27)	0.70 (0.43-1.68)
9α,11βPGF ₂	0.49 (0.20-0.89)	0.73 (0.47-1.18)¶	0.22 (0.12-0.48)	0.38 (0.22-0.63)
LTB ₄	5.10 (2.83-7.63)	5.83 (3.41-8.87)	3.68 (2.58-6.09)	8.07 (4.65-16.41)‡
LTC ₄	1.98 (1.21-3.06)	1.95 (1.09-2.68)	1.98 (1.31-3.24)	1.63 (0.75-3.10)
LTD ₄	1.03 (0.59-2.18)	0.72 (0.48-1.76)	1.18 (0.77-2.66)	1.14 (0.49-2.09)
LTE ₄	1.37 (0.77-1.91)	1.09 (0.64-1.69)¶	1.71 (1.22-2.29)	1.16 (0.75-2.00)
Eoxin C ₄	0.43 (0.30-0.93)	0.37 (0.26-0.86)	0.51 (0.33-0.95)	0.69 (0.37-0.94)
Eoxin D ₄	0.57 (0.37-0.93)	0.47 (0.34-0.61)	0.67 (0.43-1.03)	0.87 (0.57-1.62)*
Eoxin E ₄	1.80 (0.99-4.23)	2.07 (1.02-4.64)	1.70 (0.98-4.19)	3.70 (2.18-8.72)*
11-Dehydro-TXB ₂	5.16 (4.10-6.45)	4.77 (4.03-5.93)	6.25 (4.40-6.83)	5.37 (4.19-6.67)
6-Keto-PGF _{1α}	14.08 (10.47-17.16)	15.84 (13.21-17.50)¶	11.90 (7.64-14.54)	7.93 (6.92-14.15)‡
PGF _{2α}	0.73 (0.37-1.16)	0.75 (0.40-1.07)	0.69 (0.28-1.28)	0.41 (0.23-0.80)†
8-Iso-PGF _{2α}	0.32 (0.20-0.47)	0.35 (0.25-0.49)	0.29 (0.18-0.37)	0.26 (0.15-0.9)
Lipoxin A ₄	0.29 (0.18-0.45)	0.26 (0.19-0.41)	0.31 (0.17-0.55)	0.36 (0.22-1.02)*
PA	391 (308-495)	404 (323-666)§	372 (300-472)	617 (431-731)‡

Eicosanoids are shown in picograms per milliliter, and palmitic acid is shown in nanograms per milliliter. Values are presented as medians (25th-75th percentile).

PA, Palmitic acid.

* $P < .05$, † $P < .01$, or ‡ $P < .001$ for the contrast between asthmatic and healthy control subjects.

§ $P < .05$, || $P < 0.01$, or ¶ $P < .001$ for the contrast between subjects with aspirin-intolerant asthma and subjects with ATA.

TABLE E2. Canonical discrimination functions standardized by within-group variances for the full model and a stepwise selection

Eicosanoid	Full model		Stepwise model	
	Factor (1)	Factor (2)	Factor (1)	Factor (2)
5-HETE	0.168	0.238		
12-HETE	0.260	−0.564		
15-HETE	0.003	0.248	0.108	0.311
PGE ₂	−0.452	−0.867	−0.411	0.508
PGEM	0.710	0.301		
PGD ₂	0.442	0.425	0.650	0.024
9 α ,11 β PGF ₂	1.034	−0.568	0.652	0.783
LTB ₄	0.103	−0.272		
LTC ₄	0.089	0.084	−0.822	0.301
LTD ₄	−0.107	0.329		
LTE ₄	−0.601	0.396	−0.182	−0.239
Eoxin C ₄	−0.206	0.192		
Eoxin D ₄	−0.141	−0.114	0.188	0.236
Eoxin E ₄	−0.260	−0.441		
11-Dehydro-TXB ₂	−0.558	0.041	−0.298	0.601
6-Keto-PGF _{1α}	0.179	0.464	0.596	0.314
PGF _{2α}	−0.745	0.562	0.373	−0.422
8-Iso-PGF _{2α}	−0.277	0.183	−0.950	−0.723
Lipoxin A ₄	0.050	−0.115	0.411	−0.976