

Exhaled eicosanoids following oral aspirin challenge in asthmatic patients

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Summary

Background Biochemical analysis of expiratory breath condensate is an emerging non-invasive technique for assessment of airway inflammation.

Objective We wondered whether application of expiratory breath condensate could facilitate diagnosis of aspirin-intolerant asthma and reproduce eicosanoids mediators' abnormalities described in this disease.

Methods We measured prostaglandins (PGs) E_2 , $F_{2\alpha}$, $9\alpha11\beta F_2$ and iso- F_2 by gas-chromatography/mass-spectrometry and cysteinyl leukotrienes (cys-LTs) by radioimmunoassay in breath condensates of asthmatic patients undergoing oral aspirin challenge. Fourteen patients with aspirin-induced asthma and 20 aspirin-tolerating asthmatics, most of them on chronic inhaled corticotherapy, were studied and compared with 10 healthy subjects. Additionally, plasma $9\alpha11\beta PGF_2$, the metabolite of PGD_2 and urinary leukotriene (LT) E_4 were measured before and following the challenge.

Results At baseline, PG did not differ between the groups, except for lower $9\alpha11\beta PGF_2$ in aspirin-intolerant asthma. Their concentrations were not changed by the challenge. Breath condensate cys-LTs were similar in the groups studied at base, and after aspirin challenge increased only in aspirin-intolerant patients. Elevated baseline urinary LTE_4 and its further increase following aspirin challenge was highly diagnostic for aspirin-intolerant asthma. The discriminatory value of cys-LTs increase in breath condensates was lower (72.8%) than either basal (99%) or post-challenge increase (94%) of urinary LTE_4 .

Conclusions In asthmatic patients on chronic corticotherapy measurement of urinary LTE_4 excretion rather than cys-LTs in breath condensate is of greater value for diagnosis of aspirin hypersensitivity.

Keywords aspirin intolerance, bronchial asthma, cysteinyl leukotrienes, exhaled breath condensate

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Introduction

Several inflammatory mediators have been identified in the exhaled air [1–3]. Analysis of exhaled breath gave an opportunity to non-invasive monitoring of inflammation and oxidative stress in the respiratory tract. The technique is simple to perform, may be repeated frequently and applied to children. However, the procedure has to be standardized before data from different centres can be compared, and the diagnostic value of mediators measurements have to be confirmed [4–6].

Exhaled breath contains volatile compounds like nitric oxide, carbon monoxide and hydrogen peroxide, which were demonstrated as markers of airway inflammation. A vapour-saturated phase of exhaled air also transports numerous non-volatile molecules that can be condensed by breathing through a cold-trapping system [1, 3]. This fluid, termed exhaled breath condensate (EBC) contains eicosanoids,

arachidonic acid-derived mediators of inflammation. EBC analysis was introduced quite recently and the exact cellular source of eicosanoids remains unknown.

There are few published articles on eicosanoids in EBC [1, 6–11]. Most of these studies concentrated on isoprostanes, the free-radicals-catalysed peroxide derivatives of arachidonic acid. In EBC, leukotrienes (LTs) and prostaglandins (PGs) are usually measured using commercially available ELISA kits; however, these assays have limited sensitivity. These results ought to be validated by more specific analytical techniques, such as gas-chromatography/mass-spectrometry (GC/MS) [3], or by radioimmunoassays (RIAs) offering lower detection threshold. We used these techniques to quantify eicosanoids in EBC of asthma patients. We wondered whether this approach might be of diagnostic value for differentiating aspirin-sensitive from aspirin-tolerant patients with asthma. The former are characterized by alterations in arachidonic acid metabolism at base, which becomes further accentuated following aspirin ingestion [12]. We, therefore, studied the effect of aspirin challenge on EBC eicosanoids in asthma patients as compared with healthy controls.

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Subjects and methods

Three groups of patients were studied. The first group consisted of 14 patients with aspirin-induced asthma (AIA). Their disease developed according to a pattern characterized by a sequence of symptoms [13]. All had at least one episode of asthma attack after ingestion of aspirin (acetylsalicylic acid (ASA)) or other non-steroidal anti-inflammatory drugs, and the diagnosis was confirmed by oral ASA provocation test, performed within 1 year before the study. Nasal polyposis was diagnosed in 10 of 14 patients. The second group comprised 20 asthmatics who tolerated aspirin (aspirin-tolerant asthma (ATA)). All patients had stable asthma and none had experienced an exacerbation in the 6 weeks preceding the study. The control group consisted of 10 healthy subjects of similar age and sex with no history of asthma or allergy. The patients' clinical characteristics are given in Table 1. The protocol of the study was approved by the University Ethics Committee, and written, informed consent was obtained from each subject.

Aspirin challenge

In asthmatic patients ($n = 34$), oral challenge test with ASA (13) was carried out between 08:00 and 08:30 hours. Short-acting β_2 -agonists were not allowed to use at least 8 h before the challenge, whereas long-acting β_2 -agonists and theophylline were discontinued for at least 48 h. Short-acting antihistamines and cromones were withdrawn 5 days before the procedure. During the challenge, the patients received increasing doses of ASA (27, 44, 117 and 312 mg) p.o. every 1.5 h, until the cumulative ASA dose of 500 mg [14]. Forced

expiratory volume in 1 s (FEV_1) was measured before the first dose of ASA, and subsequently every 30 min. Clinical signs of bronchospasm or dyspnoea, wheezing, rhinorrhoea, nasal congestion, redness of conjunctiva, periorbital swelling or cutaneous erythema were sought. The challenge was discontinued if FEV_1 decreased more than 20% (PD_{20}), or when the cumulative dose of ASA was reached. In healthy volunteers ($n = 10$), after FEV_1 measurement at baseline, a single dose of 500 mg of ASA was given orally, and the spirometric test was repeated 2 h later.

Collection of the samples

In all subjects studied, peripheral venous blood was sampled after EBC collection, before ASA administration and following the drug administration. The patients were asked to collect baseline urine specimen before ASA administration; the second sample was taken 2 h after the last dose of the drug.

EBC was collected using the EcoScreen system for non-invasive collection of non-gaseous substances from exhaled air (Jaeger, Wuerzburg, Germany). Briefly, following initial rinsing of mouth with water, patients were instructed to breathe tidally through a mouthpiece with a saliva trap, connected to a one-way valve separating expiratory air from inspiratory air, with the nose clip applied. Expiratory air flowed through a lamellar condenser placed in a cooling cuff and kept at -30°C . The condensates were collected in disposable tubes within 20–25 min in average volume of 3 mL and were snap-frozen on dry ice and stored not longer than 3 weeks in -70°C until analysed. Paired EBC samples were collected from each patient, before the aspirin challenge and following the test. In AIA patients, the second EBC was obtained after the challenge was stopped because of the positive reaction, while in ATA patients and controls it was gained 2 h after administration of 500 mg of ASA.

Mediators' measurements

In EBC cysteinyl LTs (cys-LTs) were measured directly, by (RIA) method. Duplicate EBC sample (100 μL) was incubated with 2.5 pg of ^3H labelled LTE_4 (DuPont NEN Research Products, Boston, MA, USA) and with 100 μL of monoclonal antibody (1:2000) against cys-LTs (Sigma-Aldrich Co., Milwaukee, WI, USA) in a phosphate-buffered saline containing 0.04% albumin and 0.01% Triton X-100, at 4°C overnight. Unbound tracer was removed by dextran-coated activated charcoal and the supernatant was measured in a liquid scintillation counter (Becton Dickinson, Franklin Lakes, NJ, USA). Cys-LTs content in a sample was calculated from radioactivity (DPM) of bound tracer using a standard curve constructed with a serial dilution of non-labelled LTE_4 in a range from 1 to 400 pg/mL. The coefficient of one-site agonist curve fit for standards was repetitively better than 0.97. The used antibody bound LTC_4 , LTD_4 and LTE_4 with similar reactivity (80–100%), the detection threshold of the assay was 3 pg/mL, the standard error for replicates was less than 5% and the inter-assay error was less than 10%. PGE_2 , $\text{PGF}_{2\alpha}$, $9\alpha 11\text{PGF}_2$ and iso- PGF_2 were measured following addition to the sample of deuterated PGE_2 , iso- PGF_2 and $\text{PGF}_{2\alpha}$ (100 pg/mL, Cayman Chemicals,

Table 1. Clinical characteristics of the patients studied

	AIA ($n = 14$) mean $\pm$ SD (range)	ATA ($n = 20$) mean $\pm$ SD (range)
Male : Female	8 : 6	14 : 6
Age (years)	41.4 $\pm$ 13.9 (19–60)	36.5 $\pm$ 12.3 (20–59)
Duration of asthma (years)	10.1 $\pm$ 6.9 (0.75–24)	14.9 $\pm$ 9.7 (2–30)
FEV_1 % predicted	81.5 $\pm$ 12.5 (66.1–114.0)	92.6 $\pm$ 14.9 (77.6–115.3)
Atopy (%)	50	74
Total serum IgE (kU/L)	97 $\pm$ 33 (14.1–286)	110 $\pm$ 149 (17.4–298)
Eosinophilia (per mm^3)	324 $\pm$ 115 (28–846)	112 $\pm$ 108.6 (0–343)
Plasma $9\alpha 11\text{PGF}_2$ at base (ng/mL)	6.2 $\pm$ 2.9	5.8 $\pm$ 2.6
Inhaled steroids (fluticasone)		
No. of users (n)	11	13
Dose ($\mu\text{g/day}$)	515 $\pm$ 333 (200–1000)	493 $\pm$ 510 (125–1500)
Oral methylprednisone		
No. of users (n)	3	4
Dose (mg/day)	7.4 $\pm$ 6.3 (4–12)	6.9 $\pm$ 6.9 (4–12)

ATA, aspirin-tolerant asthma; AIA, aspirin-induced asthma; FEV_1 , forced expiratory volume in 1 s; PG, prostaglandin.

Table 2. Prostanoids and cys-LTs concentration at baseline and following ASA challenge

		Controls baseline	Controls challenge	ATA baseline	ATA challenge	AIA baseline	AIA challenge
EBC	9 α 11 β PGF ₂	5.8 $\pm$ 3.9	6.5 $\pm$ 3.1	5.8 $\pm$ 2.6	5.8 $\pm$ 2.55	4.0 $\pm$ 1.8*	3.76 $\pm$ 1.6*
	PGF _{2α}	11.6 $\pm$ 4.3	13.3 $\pm$ 3.3	13.2 $\pm$ 2.1	13.8 $\pm$ 2.9	12.6 $\pm$ 4.0	14.6 $\pm$ 3.7
	PGE ₂	6.0 $\pm$ 6.8	8.4 $\pm$ 7.0	6.5 $\pm$ 5.1	7.0 $\pm$ 6.0	4.1 $\pm$ 3.7	4.7 $\pm$ 4.3
	8-iso-PGF _{2α}	12.3 $\pm$ 1.5	11.8 $\pm$ 1.0	11.2 $\pm$ 1.8	11.5 $\pm$ 2.0	10.1 $\pm$ 2.4	9.9 $\pm$ 2.1
	Cys-LTs	13.8 $\pm$ 16.2	6.3 $\pm$ 9.5	13.8 $\pm$ 17.1	8.2 $\pm$ 12.4	9.3 $\pm$ 10.3	34.0 $\pm$ 33.5†
Urine	LTE ₄	276 $\pm$ 107	284 $\pm$ 178	262 $\pm$ 133	305 $\pm$ 194	2859 $\pm$ 1719†	9691 $\pm$ 8685†
Plasma	9 α 11 β PGF _{2α}	5.6 $\pm$ 2.4	7.6 $\pm$ 3.6	5.8 $\pm$ 2.6	9.1 $\pm$ 8.6	6.2 $\pm$ 2.9	8.2 $\pm$ 3.0

Concentration in EBC and plasma eicosanoids are expressed in pg/mL. LTE₄ in urine expressed in pg/mg creatinine.

EBC, exhaled breath condensate; ATA, aspirin-tolerant asthma; AIA, aspirin-induced asthma; PG, prostaglandin; cys-LTs, cysteinyl leukotrienes; ASA, acetylsalicylic acid.

* $P < 0.05$; † $P < 0.001$.

Ann Arbor, MI, USA) as internal standards to calculate compounds' recovery. EBCs were prepared by a thin-layer chromatography and derivatization to pentafluorobenzyl ester. A gas-chromatography negative-ion chemical ionization mass-spectrometry (GC-NICI-MS) was used to measure the prostanoids following formation of trimethylsilyl ether derivatives (model 5896 series II Hewlett Packard, Palo Alto, CA, USA). Diagnostic ions were 524 Mz PGE₂ (528 Mz for d₄PGE₂), and 569 Mz for PGF_{2 α} , 9 α 11 β PGF₂ and iso-PGF₂ (573 Mz for d₄iso-PGF₂ and d₄PGF_{2 α}). Blood plasma samples were additionally pre-purified using solid-phase extraction stage on C18 Sep-Pack cartridge (Waters Corp., Milford, MA, USA). Cys-LTs in urine were measured by ELISA (Cayman Chemicals) from unpurified samples and standardized for creatinine concentration [15].

Statistical methods

Results were expressed as means $\pm$ SD. Groups were compared using the following statistical tests: normally distributed data of PG levels were compared between the groups by ANOVA and between time-points of the challenge by paired *t*-test, skewed data of cys-LTs in EBC and in urine were compared between the groups by Mann-Whitney *U*-test and between time-points by Wilcoxon's matched pair test using STATISTICA package (StatSoft, Inc., Tulsa, OK, USA). The discrimination power of measured eicosanoids for diagnosis of AIA was calculated using receiver operator characteristics curves (ROC) (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA). The area under ROC examines the performance of a laboratory test not taking into account the level arbitrarily set as a cut-off, instead determining sensitivity vs. specificity across its full range of values.

Results

In 14 asthmatic (AIA) patients, aspirin challenge caused dyspnoea and fall in FEV₁ $\geq 20\%$. Additionally, nasal discharge developed in nine subjects and nasal blockage in eight, periorbital swelling and itching of the eyes in seven, facial flush in four. The average cumulative dose of aspirin was 180 mg, ranging from 27 to 500 mg. In two other groups,

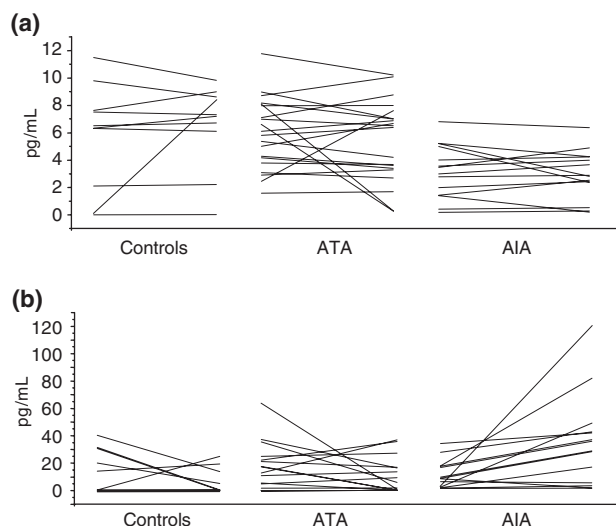


Fig. 1. Eicosanoids' levels in exhaled breath condensate before and after aspirin challenge test. Panel a, 9 α 11 β PGF₂; panel b, cys-LTs. PG, prostaglandin; cys-LTs, cysteinyl leukotrienes; ATA, aspirin-tolerant asthma; AIA, aspirin-induced asthma.

consisting of ATA and controls, aspirin challenge produced neither clinical symptoms nor fall in FEV₁.

There were no differences between controls and asthmatic patients in EBC PGE₂, PGF_{2 α} and iso-PGF₂ content; levels of these prostanoids did not change after ASA administration (Table 2). AIA patients were distinguished by a lower EBC concentration of 9 α 11 β PGF₂ (before and after challenge, $P < 0.05$) but the concentration of this PGD₂ metabolite in exhaled air remained unaffected by ASA administration (Fig. 1). Plasma levels of 9 α 11 β PGF₂ increased after ASA challenge by 30.1% ($P < 0.01$) in all studied individuals; however, no differences between controls and asthmatics were observed in the magnitude of the response.

EBC cys-LTs levels did not differ at baseline between controls and asthmatics. Following ASA challenge, the average cys-LTs concentration in EBC increased 2.3 ± 1.7 times ($P < 0.001$) only in AIA patients (Fig. 1). However, in two cases (14%) cys-LTs after the challenge remained below RIA detection level; in one of them the level was lower than before ASA administration.

AIA patients had distinctly elevated urine concentration of LTE₄ before the challenge ($P < 0.001$, Fig. 2). ASA

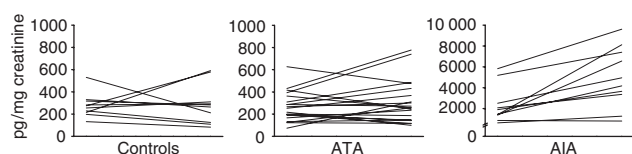


Fig. 2. Urinary leukotriene (LT) E_4 before and after aspirin challenge test.

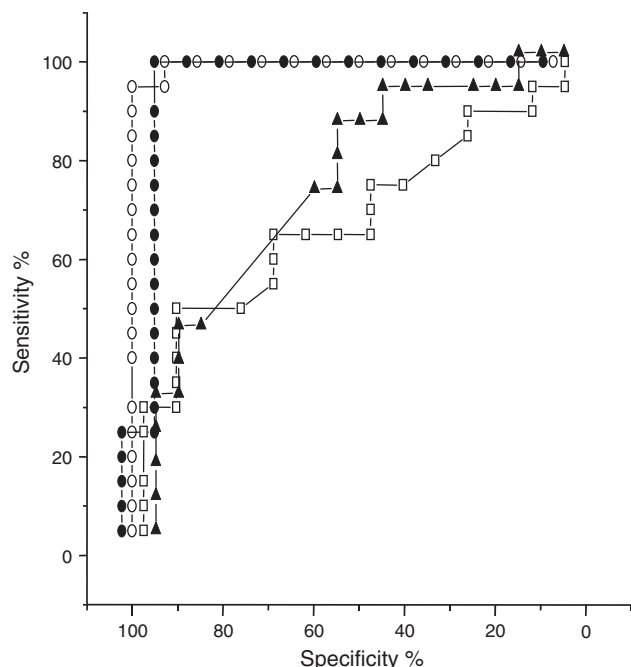


Fig. 3. Receiver Operator Characteristic Curve plots of sensitivity vs. specificity of eicosanoids levels in aspirin-induced asthma: ○, leukotriene (LT) E_4 in urine at base; ●, LTE $_4$ increase in urine following aspirin challenge; □, 9 α 11 β prostaglandin F $_2$ in exhaled breathe condensate (EBC) at base; ▲, cysteinyl leukotrienes (cys-LTs) increase in EBC following aspirin challenge.

administration led to further increase of LTE $_4$ excretion 3.3 ± 2.0 times the baseline ($P < 0.001$).

Analysis of the discrimination power of the eicosanoid measurements revealed that based on 9 α 11 β PGF $_{2\alpha}$ baseline levels in EBC, 70.5% of patients could be correctly assigned aspirin intolerance status (area under ROC, $P < 0.05$; 95% confidence interval (CI) 53.2–87.9%, Fig. 3). The sensitivity was 92.9%, specificity 50%, positive predictive value (PPV) 56.5% and negative predictive value (NPV) 90.9% based on EBC 9 α 11 β PGF $_{2\alpha}$ lower than 5.85 pg/mL. ASA challenge did not improve the power of this test. In contrast, only increase of EBC cys-LTs following ASA challenge demonstrated some diagnostic value, discriminating 72.8% cases correctly (area under ROC, $P < 0.03$; 95% CI 55.6–90.1%). In this case, the cut-off point of increase cys-LTs in EBC exceeding 1.0 pg/mL gave 78.6% sensitivity, 75% specificity, 68.8% PPV and 83.3% NPV of the test. LTE $_4$ urinary levels before aspirin challenge and LTE $_4$ increase in urine following the challenge, both showed the best discrimination (99.6% and 94.6% area under ROC, $P < 0.001$) for diagnosis of AIA. Basal urinary LTE $_4$ greater than 626 pg/mg creatinine discriminated AIA patients from ATA with 92.9% sensitivity, 95% specificity, 92.9% PPV and 95% NPV. A threshold value for post-challenge increase of urinary LTE $_4$ exceeding 493 pg/mg

creatinine corresponded to 92.9% sensitivity, 100% specificity, 100% PPV and 95.2% NPV of the test.

Basal urinary LTE $_4$ and its increase following the challenge were closely correlated in AIA patients (Kendal's $\tau = 0.702$, $P < 0.001$). A negative correlation was observed between urinary increase of LTE $_4$ and EBC increase of cys-LTs following ASA challenge in AIA patients (Spearman's $\rho = -0.697$, $P < 0.01$).

No other eicosanoid measurements demonstrated any relation specific to positive ASA challenge outcome.

Discussion

This study demonstrates that GC/MS can be applied for measurement of a spectrum of prostanoids in EBC. The higher specificity of GC/MS over the immunoassay methods explains why the prostanoids concentration recorded by us were of lower magnitude than in the reports based on immunoassays. Similarly, fivefold better sensitivity of RIA used by us for cys-LTs measurements permitted determination of these mediators if present below the ELISA detection threshold.

In EBC, the baseline prostanoids levels were similar to those reported in bronchoalveolar lavage fluid (BALF) [16]. Furthermore, in EBC likewise in BALF, there was no difference in baseline levels between aspirin-tolerant and aspirin-sensitive asthmatics, except for PGD $_2$ metabolite. In EBC, PGE $_2$ and 8-isoprostane were reported not to differ [7] between steroid-treated AIA patients as compared with ATA. It came as a surprise to us that aspirin at a dose that precipitated bronchial obstruction had no effect on any prostanoids in either of the asthmatic groups studied or in healthy controls. Previous study [16] showed that instillation of 10 mg lysine-aspirin in segmental bronchus led to rapid depression of PGE $_2$ and thromboxane B $_2$ levels in BALF of asthmatics. The plausible explanation for this discrepancy is that aspirin applied locally reaches higher concentration, inhibiting topical prostanoid biosynthesis in the lung segment.

Our AIA patients had significantly lower levels of EBC 9 α 11 β PGF $_{2\alpha}$, a PGD $_2$ metabolite. Biosynthesis of this PG is coupled to cyclooxygenase (COX)-1 or COX-2 activity in a limited number of cells. Restricted capacity for biosynthesis of PG in lung fibroblasts from AIA subjects has been demonstrated recently [17] and other studies [18–19] documented down-regulation of cyclooxygenation pathway in the airway of AIA patients. Nevertheless, PGD $_2$ is considered a marker for mast cells activation; a rapid increase in plasma 9 α 11 β PGF $_{2\alpha}$ was observed in asthmatic patients following ASA challenge [20]. In the present study, plasma 9 α 11 β PGF $_{2\alpha}$ following ASA challenge increased in all groups of subjects. We measured plasma level of 9 α 11 β PGF $_{2\alpha}$ following ASA challenge only once, i.e. 5 min after bronchoconstriction in AIA or 2 h after ASA in ATA or controls. Thus, we did not study the difference in time-response between the two groups [20]. Increase of plasma 9 α 11 β PGF $_{2\alpha}$ following aspirin also corroborates with our observation [21] that aspirin can non-specifically activate basophil/mast cell differentiated cell line due to inhibition of PGE $_2$ biosynthesis. Depressed

9 α 11 β PGF₂ levels in EBC of AIA are difficult to explain and require further studies.

At baseline, EBC cys-LTs were similar in the two groups of asthmatics studied. Since a vast majority of our patients were on chronic steroid therapy, this finding agrees with the recent report of Antczak et al. [7] and points to the diagnostic weakness of the test. EBC might still be of use in diagnosis of steroid-naïve AIA patients. In children, as recently reported [11], EBC cys-LTs were elevated in comparison with levels of adults. Asthmatic children had further increased cys-LTs in EBC. Such patients and steroid-naïve adult asthmatics were reported [7] to have higher EBC cys-LTs than steroid-treated AIA patients. Unfortunately, over 2/3 of AIA patients both in Europe [13] and in the USA [22] are on chronic corticotherapy, which limits the diagnostic usefulness of cys-LTs measurements in EBC. The response of EBC cys-LTs to ASA was different from that of prostanoids. In AIA patients, the mean cys-LTs rose significantly at the time of clinical reaction. Such a response was specific for AIA patients and did not occur in asthmatic patients tolerating aspirin well.

Our study design was based on a simplified, 1-day oral aspirin challenge test for AIA patients and the test surrogate for ATA patients and healthy controls. The study end-point had to be different for AIA, where aspirin administration was stopped due to bronchoconstriction and samples were taken at this manifestation. Because of lack of symptoms, in ATA and controls second samples were taken 2 h after a single oral dose of aspirin estimating peak blood level of the drug. The observed increase of cys-LTs corroborates suggestion that cys-LTs are important mediators in AIA. Indeed, a key enzyme, LTC₄ synthase, is overexpressed in bronchial mucosa of patients with AIA [23], and the patients' circulating blood eosinophils carry more mRNA transcripts for this enzyme [24]. However, it should be pointed out that in two of 14 AIA patients no increase in EBC cys-LTs occurred at the time of the reaction and in one patient both EBC samples had cys-LTs below 3 pg/mL. It is interesting to note that following lysine aspirin bronchial instillation cys-LTs concentration rose in 11 of 14 AIA patients, but remained unaltered in the remaining three subjects [16]. These observations point to the non-homogeneity of the AIA syndrome at the biochemical level. An inverse correlation between EBC and urinary cys-LTs is an interesting phenomenon. In a recent study, Csoma et al. [11] observed a similar relationship between EBC LTB₄ and cys-LTs. Perhaps variation in transcellular biosynthesis of cys-LTs in some patients promotes systemic metabolism of LTs followed by urinary excretion, while in others biosynthesis remains confined to the lung.

AIA remains a common but underdiagnosed clinical condition [25]. This is mainly due to lack of a predictive laboratory test that could substitute the ASA challenge procedure. In the present study, both resting and post-aspirin mean urinary LTE₄ excretion were consistently higher in AIA than in ATA patients or controls. This has been previously reported [26–27], but because of some overlap between the groups it was felt [28] that measurement of LTE₄ in a single sample of urine could not accurately diagnose aspirin sensitivity. In our study, basal urinary concentration of LTE₄ showed a similar power of discrimination for aspirin-sensitivity as the post-challenge urinary LTE₄ increase. This

result was obtained despite a simple method of cys-LTs immunoassay in diluted raw material. A significant individual variation in cys-LTs urinary content requires a serial dilution of the material for immunoassay studies, especially if the radioisotope method was planned for use. We found that ELISA on non-purified samples of urine [14], although characterized by higher levels of cys-LTs because of an unknown assay interference, is suitable and highly predictive for AIA. Unmetabolized LTE₄ constituted 7–8% of the intravenously infused radiolabelled compound in urine of healthy volunteers [29]. A highly sensitive method based on mass-spectrometry measurement of LTE₄ excreted in urine, proof against substances cross-reacting in immunoassay, seems an adequate diagnostic option for AIA screening.

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