



ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Prostaglandin E₂ decrease in induced sputum of hypersensitive asthmatics during oral challenge with aspirin

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Abstract

Background: A special regulatory role for prostaglandin E₂ (PGE₂) has been postulated in nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (NERD).

Objective: To investigate the effect of systemic aspirin (acetylsalicylic acid) administration on airway PGE₂ biosynthesis in induced sputum supernatant (ISS) among subjects with NERD or aspirin-tolerant asthma with chronic rhinosinusitis with nasal polyposis (ATA-CRSwNP), as well as healthy controls (HC).

Methods: Induced sputum (IS) was collected from patients with NERD (n = 26), ATA-CRSwNP (n = 17), and HC (n = 21) at baseline and after aspirin challenge. Sputum differential cell count and IS supernatant (ISS) levels of prostanoids, PGE₂, 8-iso-PGE₂, tetranor-PGE-M, 8-iso-PGF₂α, and leukotriene C₄, D₄, and E₄, were determined using mass spectrometry. Urinary excretion of LTE₄ was measured by ELISA.

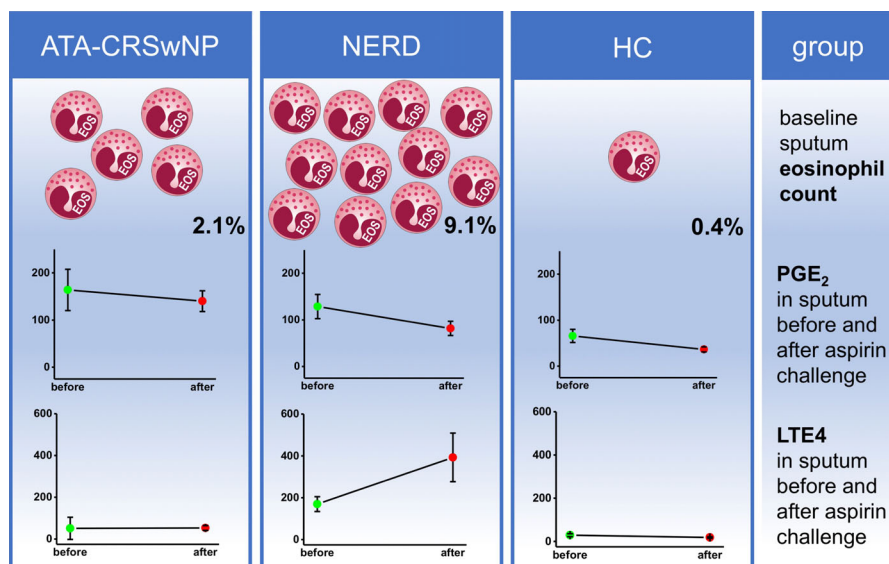
Results: NERD subjects had elevated sputum eosinophilic count as compared to ATA-CRSwNP and HC (median NERD 9.1%, ATA-CRSwNP 2.1%, and HC 0.4%; *P* < 0.01). Baseline ISS levels of PGE₂ were higher in asthmatics as compared to HC at baseline (NERD vs HC *P* = 0.04, ATA-CRSwNP vs HC *P* < 0.05). Post-challenge ISS levels of PGE₂ compared to baseline significantly decreased in NERD and HC (*P* < 0.01 and *P* = 0.01), but not in ATA-CRSwNP. In NERD, a similar decrease in PGE₂ as in HC resulted from 2.8 times lower dose of aspirin.

Conclusion: Aspirin-precipitated bronchoconstriction is associated with a decrease in airway PGE₂ biosynthesis. These results support the mechanism of PGE₂ biosynthesis inhibition as a trigger for bronchoconstriction in NERD.

KEYWORDS

eicosanoids, induced sputum, nonsteroidal anti-inflammatory drug-exacerbated respiratory disease, oral aspirin challenge, prostaglandins

Abbreviations: ATA-CRSwNP, aspirin-tolerant asthma with chronic rhinosinusitis with nasal polyposis; COX, cyclooxygenase; Cys-LTs, cysteinyl leukotrienes; HC, healthy control; LTC₄, leukotriene C₄; LTD₄, leukotriene D₄; LTE₄, leukotriene E₄; NERD, nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease; NSAIDs, nonsteroidal anti-inflammatory drugs; PGE₂, prostaglandin E₂; Tetranor-PGE-M, 9,15-dioxo-11α-hydroxy-2,3,4,5-tetranor-prostan-1,20-dioic acid.



GRAPHICAL ABSTRACT

Prostaglandin E₂ decreases in induced sputum of hypersensitive asthmatics during oral challenge with aspirin. In nonsteroidal anti-inflammatory drug-exacerbated respiratory disease (NERD), sputum eosinophilic count is elevated as compared to aspirin-tolerant asthmatics with chronic rhinosinusitis with nasal polyposis (ATA-CRSwNP) or healthy controls (HC). Prostaglandin E₂ (PGE₂) levels in the induced sputum are higher in asthmatics (NERD and ATA-CRSwNP) reflecting chronic inflammation of bronchi despite control of the disease. During oral aspirin challenge, cyclooxygenases are inhibited. Aspirin-provoked bronchoconstriction in NERD is accompanied by decrease of PGE₂ and rise of leukotriene E₄ in the induced sputum.

1 | INTRODUCTION

Nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (NERD) is a distinct phenotype of asthma characterized by a hypersensitivity to aspirin and other NSAIDs. The clinical presentation of NERD is comprised of chronic rhinosinusitis, nasal polyposis, and an overproduction of cysteinyl leukotrienes (Cys-LTs).^{1,2} Aspirin and other NSAIDs inhibit cyclooxygenase-mediated biosynthesis of prostanoids.³ It was proposed that only cyclooxygenase-1 (COX-1) inhibition is followed by a release of Cys-LTs from mast cells and eosinophils in NERD.⁴⁻⁷ Recently, a transcellular mechanism was suggested of platelet-granulocyte aggregates for Cys-LT biosynthesis in NERD.^{8,9} On the mechanistic level, an outburst of Cys-LT production accompanying an asthmatic attack can result from the inhibition of prostaglandin E₂ (PGE₂) production, whose anti-inflammatory activity controls Cys-LT biosynthetic pathways.¹⁰

Prostaglandins are produced in airways by numerous cells, and their pro- or anti-inflammatory properties depend on their local concentration and cellular receptors for these autacoids. Airway production of pro-inflammatory prostanoids such as PGD₂ and PGF₂α by mast cells or Cys-LTs by eosinophils is increased,^{11,12} whereas structural cells of airways, including epithelial, smooth muscle, and endothelial ones, maintain biosynthesis of anti-inflammatory prostaglandin E₂ and prostacyclin.¹³ PGE₂ can mediate both pro- and anti-inflammatory effects, depending on the stimulation of four receptors subtypes (EP1-EP4).^{14,15} EP2 activation can block

eosinophil or mast cell degranulation. Deficiency in anti-inflammatory activity of PGE₂ seems relevant to NSAID-precipitated symptoms of NERD.^{16,17} Indeed, a spontaneous production of PGE₂ is decreased in epithelial cells from nasal polyps¹⁷ and nasal explants collected from NERD donors, and explained by a lower expression of cyclooxygenase-2 (COX-2) in nasal mucosa¹⁸ or nasal polyps.¹⁹ However, COX-2 expression in the bronchial mucosa obtained by biopsy does not show any difference between NERD and aspirin-tolerant asthma with chronic rhinosinusitis with nasal polyposis (ATA-CRSwNP) subjects.²⁰ Moreover, arachidonic acid-induced PGE₂ release from peripheral blood cells after aspirin challenge is not altered in NERD.¹⁶ Data on local airway PGE₂ concentration in response to aspirin challenge are scarce and contradictory.²¹⁻²³

In our previous study on NERD, we observed an unexpected rise in the main urinary metabolites of PGE₂ following oral administration of aspirin.²⁴ Moreover, no change of PGE₂ was noticeable in exhaled breath condensate following inhaled aspirin challenge,²⁵ although a decline in PGE₂ concentration in the induced sputum supernatant (ISS) was present.²¹ Thus, the rationale for this study was to investigate bronchial biosynthesis of eicosanoids in ISS after oral aspirin challenge. No study had evaluated these mediators in induced sputum of NERD following systemic administration of aspirin. We expected to validate the hypothesis on the inhibition of PGE₂ biosynthesis; thus, the main endpoint of this study was a change in ISS PGE₂ concentrations and its dependence on the cumulative

dose of aspirin in NERD, ATA-CRSwNP, and healthy controls (HC). This study was controlled for corticosteroids and other asthma therapies.

2 | METHODS

2.1 | Study groups

The study groups comprised of 26 asthmatics with previously diagnosed NERD, 17 ATA-CRSwNP who tolerated aspirin well and 21 HC. Diagnosis of asthma and the disease control were ascertained using GINA 2014 update. The diagnosis of aspirin hypersensitivity was confirmed by oral aspirin challenge, as previously described.²⁶ ATA-CRSwNP and HC reported aspirin or other NSAID use without any adverse reactions. All study participants were at clinically stable conditions, and their baseline FEV₁ exceeded 70% of the predicted value on the challenge days. None of the asthmatics experienced any exacerbation or respiratory tract infection within a 6-week period preceding the study. Inhaled corticosteroids were allowed at a daily dose up to 2000 µg budesonide equivalent. All other asthma medications were withheld for the duration of their pharmacological action prior to aspirin challenge according to the guidelines.²⁶ None of the asthmatics were treated with systemic corticosteroids or leukotriene-modifying drugs. Healthy controls had no history of asthma or intolerance to NSAIDs, and none of them used any medication within the preceding 2 months. The use of aspirin or NSAIDs within the previous 2 months was among exclusion criteria.

Sputum induction (IS) for cytospin slides, prostaglandins (PGE₂, 8-iso-PGE₂, 8-iso-PGF_{2α}, and tetranor-PGE-M), and leukotriene C₄ (LTC₄), D₄ (LTD₄), and E₄ (LTE₄), as well as urine collection for LTE₄, was carried out in all study participants. Induced sputum was collected twice, at baseline and after the challenge. Urine was collected at baseline, and next at the time of symptoms appearance (0 h) and at 2, 4, 6, and 24 h. If no symptoms occurred, urine was collected 1.5 h after the last aspirin dose (0 h) and next in intervals of 2, 4, 6, and 24 h.

All study participants gave written informed consent to participate in the study, and the protocol was approved by the Jagiellonian University Ethics Committee.

2.2 | Study design

We performed a single-blind, placebo-controlled oral challenge test with aspirin over two consecutive days. The study participants were examined for their stable clinical condition as described.²⁶ Gelatin capsules with lactose monohydrate were prepared by the hospital pharmacy and administered as placebo. The same formulation was used for aspirin, and the capsules were indistinguishable. Oral placebo challenge was performed firstly, and then oral aspirin challenge was performed the next day.

Placebo challenge started 1.5 h after induced sputum collection. At least a 70% of baseline FEV₁ was required to start the procedure.²⁶ Three capsules of placebo were administered at 1.5-h intervals. FEV₁ was measured at baseline and every 30 min, and any

patient with a greater FEV₁ variation than 15% was excluded from the study due to an unstable clinical condition.

The next day, aspirin challenge was commenced by FEV₁ measurements and 70% of the predicted value was required during three consecutive attempts to continue with increasing doses of aspirin (27, 44, 117, and 312 mg) administered every 1.5 h until the cumulative dose of 500 mg was reached. FEV₁ was measured before each dose of aspirin and subsequently every 30 min, that is, after 30, 60, and 90 min. Any presence of extrabronchial symptoms was monitored. The challenge was positive and next interrupted if baseline FEV₁ decreased by 20% or more. If the cumulative dose of aspirin was reached without a fall in FEV₁, the challenge was negative. A provocative dose causing a 20% fall in baseline FEV₁ (PD₂₀) was the total of the aspirin administered.²⁶

2.3 | Induced sputum collection

Induced sputum was collected according to ERS recommendations²⁷ by inhalation of hypertonic saline solution at concentrations from 3% to 5% delivered using ultrasonic nebulizer (Ultraneb 2000; DeVilbiss, Somerset, Philadelphia, PA). Sputum expectorated on a Petri dish kept on ice was immediately transferred to the laboratory. Sputum plugs were manually separated from saliva under magnifying glass and solubilized with SPUTOLYSIN Reagent (Calbiochem EMD Millipore, Billerica, MA) at 4:1 volume ratio, next used for cytospin preparation or supernatant stored in -70°C.

A short-acting beta₂-agonist was administered 4.5 h before IS collection in all study participants at least 6 h before placebo administration. Following aspirin challenge, IS was collected in patients with positive reaction immediately after bronchial symptoms were reversed with inhaled bronchodilator. In ATA-CRSwNP and HC, IS was collected 1.5 h after the last aspirin dose. Patients were hospitalized and monitored for at least 24 h after aspirin challenge and sputum induction. Hypersensitivity symptoms were treated with beta₂-agonists and antihistamines.

2.4 | Lung function

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

2.5 | Biochemical assays

The concentration of eicosanoids in ISS was measured by gas chromatography/tandem mass spectrometry (GC-MS/MS) for PGE₂, 8-iso-PGE₂, and 8-iso-PGF_{2α} or by high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) for tetranor-PGE-M, LTC₄, LTD₄, and LTE₄. ISS samples (1 mL) were acidified to pH 3.5 with acetic acid and extracted with organic phase in the presence of chemically identical deuterated internal standards added (PGE₂, tetranor-PGE-M, 8-iso-PGE₂, 8-iso-PGF_{2α}, LTC₄, LTD₄, and LTE₄, all from Cayman Chemical Co, Ann Arbor, MI). Concentrations of eicosanoids were calculated using a stable isotope dilution method from the area under the peak and expressed in picograms per milliliter. Analytical details were described in the Appendix S1 or elsewhere.^{21,28}

TABLE 1 Clinical characteristics of the study groups

	NERD (n = 26)	ATA-CRSwNP (n = 18)	HC (n = 21)	P-value
Age (years)				
Mean $\pm$ SD	48.8 (11.3)	46.3 (8.7)	46.4 (16.5)	0.77
Median (25%-75%)	49 (40-57)	45 (41-50)	53 (29-59)	
Sex				
(F/M)	16/10	12/6	16/5	0.56
Age at asthma onset >12 year (Yes/No)	26/0	17/1	-	0.42
BMI >30 kg/m ² (Yes/No)	5/21	4/14	3/18	0.84
Present level of asthma control (Well controlled/Partially controlled/Uncontrolled)	12/12/2	8/7/3	-	0.75
Nasal polyps, whenever (Yes/No)	25/1	17/1	0/21	<0.01 ^{d,f}
FESS, whenever (Yes/No)	21/5	14/4	0/21	<0.01 ^{d,f}
Baseline FEV1 (% predicted) Median (min-max)	88.7% (70%-127%)	102.5% (72%-119%)	94.1% (75%-115%)	0.07
Positive skin prick test responses, No. (%)	7 (28%)	7 (38.9%)	-	0.67
IgE total (IU/mL)				
Median (min-max)	107 (16.5-959)	179.3 (16.5-1700)	27.5 (16.5-308)	0.07
>100 IU/mL, No. (%)	12 (48%)	7 (38.9%)	5 (23.8%)	
Blood eosinophil count per mm ²				
Median (min-max)	300 (100-1710)	400 (200-900)	140 (40-350)	<0.01 ^{d,f}
>400/mm ² , No. (%)	4 (16%)	9 (50%)	0 (0%)	
Treatment of asthma				
No. (%)	6 (24%)	5 (27.8%)	-	1
Without ICS, no. (%)	19 (76%)	13 (72.2%)		
ICS dose (mg of fluticasone eq./day), Median (min-max)	400 (0-1000)	400 (0-1000)	-	0.86
Asthma severity, no. (%)				
Intermittent	1 (4%)	4 (22.2%)	-	0.74
Mild persistent	6 (24%)	1 (5.6%)		
Moderate persistent	16 (64%)	12(66.7%)		
Severe persistent	2 (8%)	1 (5.6%)		
Dose of nasal steroids (mg of fluticasone eq./day)	110	110	-	1
Median (min-max)	(110-110)	(110-110)		

P-value for ANOVA or Freeman-Halton extension of the Fisher exact test when appropriate. Post hoc tests: d— $P < 0.01$ for NERD vs HC; f— $P < 0.01$ for ATA-CRSwNP vs HC. ATA-CRSwNP, aspirin-tolerant asthma with chronic rhinosinusitis with nasal polyposis; BMI, body mass index; CS, corticosteroids; FESS, functional endoscopic sinus surgery; HC, healthy control; ICS, inhaled corticosteroids; NERD, nonsteroidal anti-inflammatory drug-exacerbated respiratory disease.

2.6 | Urinary LTE₄ excretion

Urinary LTE₄ was measured in diluted (1:10-1:30) crude urine samples by direct enzyme immunoassay (Cayman Chemical Co). Results were recalculated in picograms per mg of urinary creatinine.

2.7 | Statistical analysis

All calculations were performed with Statistica 12 data analysis software (StatSoft, Inc. Tulsa, Oklahoma, United States). Categorical variables were presented as numbers and percentages and analyzed using Fisher exact test and Freeman-Halton extension of the Fisher exact test for two rows by three columns contingency table for small samples. Continuous variables were expressed as mean and standard

deviation (SD) or median and lower and upper quartiles. ANOVA models with post hoc Tukey's procedure were applied for multiple group comparisons. The variance stabilizing Box-Cox transformation was used when needed. Type I statistical error P value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Clinical characteristics of the study participants and oral challenge results

No demographic differences were present between the study groups (Table 1). Elevated peripheral blood eosinophilia, nasal polyps, and a history of functional endoscopic sinus surgery (FESS) were present in

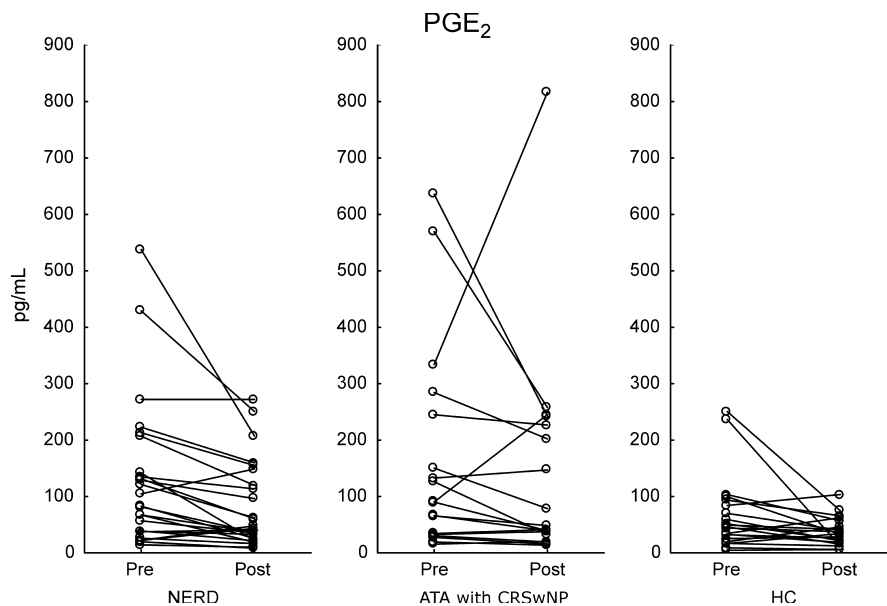


FIGURE 1 Induced sputum supernatant concentrations of PGE₂ before and after oral aspirin challenge in nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (NERD), aspirin-tolerant asthma with chronic rhinosinusitis with nasal polyposis (ATA-CRSwNP), and healthy control (HC).

asthmatics only. No difference in blood eosinophilic count between NERD and ATA-CRSwNP was observed. There was a common feature of nasal polyps (96% and 94%) and the history of FESS (80% and 78%) in NERD and ATA-CRSwNP, respectively. All study participants had baseline FEV₁ value at least 70% on challenge days. No FEV₁ variation >15% or any symptoms were noted during placebo administration. Aspirin challenge caused bronchial reaction after 44 mg in four, after 117 mg in 14 and after 312 mg in eight subjects from NERD group. Thus, the mean provocation dose of aspirin was 176.7 mg and all NERD subjects reacted with bronchoconstriction. None of the subjects from ATA-CRSwNP or HC group had any clinical symptoms following aspirin.

3.2 | Prostanoids in ISS

Baseline ISS levels of PGE₂ differed significantly between all study groups ($P < 0.05$ ANOVA). The concentration of PGE₂ was the lowest in HC (45.90 [25.24-84.01]) and significantly lower in comparison with NERD (82.71 [38.02-141.42], $P = 0.04$) and ATA-CRSwNP (90.13 [32.81-244.94], $P < 0.05$). No differences were present between NERD and ATA-CRSwNP ($P = 0.75$) (Figure 1, Table 2a).

Following oral aspirin challenge, ISS PGE₂ decreased in NERD (by 34.6% of the baseline 95% CI [19.3%-49.9%]; $P < 0.01$) and in HC (by 29.5% of the baseline 95% CI [4%-35%]; $P = 0.01$). No significant change of ISS PGE₂ was observed in ATA-CRSwNP group (by 19% of the baseline, 95% CI [-5%-43%]; $P = 0.10$) (Figure 1; Table 2b). The cumulative dose of aspirin had no effect on PGE₂ decrease following the challenge in NERD subjects ($r = -0.09$, $P = 0.71$). Inhaled corticosteroids did not affect baseline and post-challenge level of PGE₂ in asthmatics.

Baseline levels of the stable metabolite of PGE₂ differed significantly between all study groups ($P < 0.01$, ANOVA). The highest tetranor-PGE-M concentration was found in ATA-CRSwNP (40.95 [31.00-57.23]), whereas concentrations in NERD (26.65 [19.61-41.02])

or HC (24.57 [16.90-36.12]) were significantly lower ($P = 0.01$ and $P < 0.01$, respectively) (Table 2a). Aspirin oral challenge had no impact on tetranor-PGE-M concentration in ISS (Table 2b).

Baseline 8-iso-PGE₂ levels differed significantly between study groups ($P < 0.05$, ANOVA). This isoprostane was significantly higher in ATA-CRSwNP (15.64 [7.55-32.29]) as compared with NERD (6.83 [3.84-16.80]; $P = 0.04$) or HC (7.07 [3.89-18.16]; $P = 0.04$) (Table 2a). A significant decrease in 8-iso-PGE₂ concentration occurred only in ATA-CRSwNP group ($P = 0.03$) following oral aspirin challenge (Table 2b).

No differences between the study groups were observed in ISS 8-iso-PGF_{2α} concentration. No effect of aspirin oral challenge was noted on this isoprostane (Table 2a,b).

3.3 | Cys-LTs in ISS

ISS concentration of Cys-LTs (LTC₄, LTD₄, and LTE₄) before the challenge was different between the study groups ($P < 0.01$, ANOVA). Baseline ISS concentration of Cys-LTs was significantly lower in HC (25.6 [18.9-77.3]) in comparison with NERD (165.4 [54.0-395.0], $P < 0.01$) and ATA-CRSwNP (101.6 [47.6-140.0], $P = 0.03$) (Table 2a). Following oral aspirin challenge, Cys-LT concentration had a tendency to increase in NERD subjects only (by 27%, to 210.7 [44.9-783.1], $P = 0.05$). The concentration of Cys-LTs in HC decreased significantly (by 14%, to 22.5 [15.4-32.7], $P = 0.02$), whereas no change was observed in ATA-CRSwNP group (92.4 [56.0-172.7], $P = 0.81$) (Table 2b).

Baseline ISS concentration of LTC₄ did not differ markedly between the study groups ($P = 0.11$, ANOVA). LTD₄ ISS level at baseline was different between study groups ($P < 0.01$, ANOVA). Baseline level of LTD₄ did not differ between NERD (59.33 [17.15-81.69]) and ATA-CRSwNP (61.88 [26.06-94.19]) group, whereas it was significantly lower in HC (9.19 [4.02-44.39]) in comparison with both NERD ($P = 0.01$) and ATA-CRSwNP ($P < 0.01$). ISS

TABLE 2 (a) Baseline concentrations of eicosanoids in the studied groups. (b) Post-challenge concentrations of eicosanoids in the studied groups

	NERD (n = 26)	ATA-CRSwNP (n = 18)	HC (n = 21)	ANOVA (P-value)
(a)				
LTC ₄ (pg/mL)				
Mean ± SD	2.61 ± 1.90	2.19 ± 1.06	3.04 ± 7.13	0.11
Median (25%-75%)	1.95 (1.03-3.81)	2.33 (1.10-2.81)	1.25 (0.80-2.06)	
LTD ₄ (pg/mL)				
Mean ± SD	80.15 ± 87.25	72.62 ± 63.86	43.15 ± 81.81	<0.01 ^{c,f}
Median (25%-75%)	59.33 (17.15-81.69)	61.88 (26.06-94.19)	9.19 (4.02-44.39)	
LTE ₄ (pg/mL)				
Mean ± SD	169.68 ± 176.99	51.38 ± 93.24	29.24 ± 30.56	<0.01 ^{b,d}
Median (25%-75%)	89.74 (42.65-239.66)	29.13 (15.42-34.92)	17.33 (9.32-34.48)	
Cys-LTs (pg/mL)				
Mean ± SD	252.4 ± 231.6	126.2 ± 144.9	75.4 ± 107.0	<0.01 ^{d,e}
Median (25%-75%)	165.4 (54.0-395.0)	101.6 (47.6-140.0)	25.6 (18.9-77.3)	
PGE ₂ (pg/mL)				
Mean ± SD	128.69 ± 129.25	163.88 ± 185.23	65.63 ± 66.00	<0.05 ^{c,e}
Median (25%-75%)	82.71 (38.02-141.42)	90.13 (32.81-244.94)	45.90 (25.24-84.01)	
Tetranor-PGE ₂ -M (pg/mL)				
Mean ± SD	31.65 ± 15.12	48.26 ± 25.30	30.23 ± 17.86	<0.01 ^{a,f}
Median (25%-75%)	26.65 (19.61-41.02)	40.95 (31.00-57.23)	24.57 (16.90-36.12)	
8-iso-PGE ₂ (pg/mL)				
Mean ± SD	15.31 ± 20.09	21.95 ± 18.65	13.50 ± 15.74	<0.05 ^{a,e}
Median (25%-75%)	6.83 (3.84-16.80)	15.64 (7.55-32.29)	7.07 (3.89-18.16)	
8-iso-PGF _{2α} (pg/mL)				
Mean ± SD	5.38 ± 4.22	5.07 ± 4.55	4.41 ± 3.04	0.82
Median (25%-75%)	4.05 (3.03-6.60)	3.76 (2.14-6.54)	3.19 (2.46-6.91)	
(b)				
LTC ₄ (pg/mL)				
Mean ± SD	3.49 ± 3.10	3.29 ± 2.56	5.36 ± 16.35	0.21
Median (25%-75%)	2.79 (1.09-4.64)	2.39 (1.72-4.05)	1.53 (0.97-2.39)	
LTD ₄ (pg/mL)				
Mean ± SD	208.70 ± 338.1	63.97 ± 52.04	14.63 ± 28.99	<0.01 ^{d,f}
Median (25%-75%)	44.80 (17.2-163.2)	54.55 (24.01-97.05)	7.57 (5.41-11.86)	
LTE ₄ (pg/mL)				
Mean ± SD	392.80 ± 581.15	53.03 ± 49.03	18.34 ± 15.99 ^g	<0.01 ^{a,d}
Median (25%-75%)	127.40 (33.20-348.9)	34.26 (15.77-72.23)	12.41 (9.91-18.91)	
Cys-LTs (pg/mL)				
Mean ± SD	604.9 ± 870.3 ^g	120.28 ± 93.20	38.33 ± 47.56 ^g	<0.01 ^{d,f}
Median (25%-75%)	210.67 (44.90-783.10)	92.43 (56.0-172.70)	22.50 (15.43-32.65)	
PGE ₂ (pg/mL)				
Mean ± SD	81.67 ± 76.92 ^h	140.15 ± 192.33	36.31 ± 24.95 ^g	0.02 ^e
Median (25%-75%)	43.49 (25.5-119.6)	44.82 (32.6-226.7)	32.77 (18.49-45.90)	
Tetranor-PGE ₂ -M (pg/mL)				
Mean ± SD	28.98 ± 12.73	39.91 ± 16.45	26.75 ± 9.40	0.01 ^{a,f}
Median (25%-75%)	25.11 (20.70-32.13)	41.18 (26.93-47.44)	25.55 (20.34-31.00)	

(Continues)

TABLE 2 (Continued)

	NERD (n = 26)	ATA-CRSwNP (n = 18)	HC (n = 21)	ANOVA (P-value)
8-iso-PGE ₂ (pg/mL)				
Mean ± SD	14.08 ± 19.55	15.99 ± 13.20 ^g	12.40 ± 20.39	0.27
Median (25%-75%)	4.52 (3.01-18.49)	15.20 (5.73-21.27)	5.01 (3.57-11.46)	
8-iso-PGF _{2α} (pg/mL)				
Mean ± SD	4.49 ± 2.85	5.40 ± 7.05	5.14 ± 6.57	0.94
Median (25%-75%)	4.69 (1.67-7.27)	2.89 (1.30-5.42)	2.39 (1.69-5.55)	

Post hoc tests: a— $P < 0.05$ or b— $P < 0.01$ for NERD vs ATA-CRSwNP; c— $P < 0.05$ or d— $P < 0.01$ for NERD vs HC; e— $P < 0.05$ or f— $P < 0.01$ for ATA-CRSwNP vs HC. Within the groups, pre- vs post-challenge difference g— $P < 0.05$ and h— $P < 0.01$. ATA-CRSwNP, aspirin-tolerant asthma with chronic rhinosinusitis with nasal polyposis; HC, healthy controls; NERD, nonsteroidal anti-inflammatory drug-exacerbated respiratory disease.

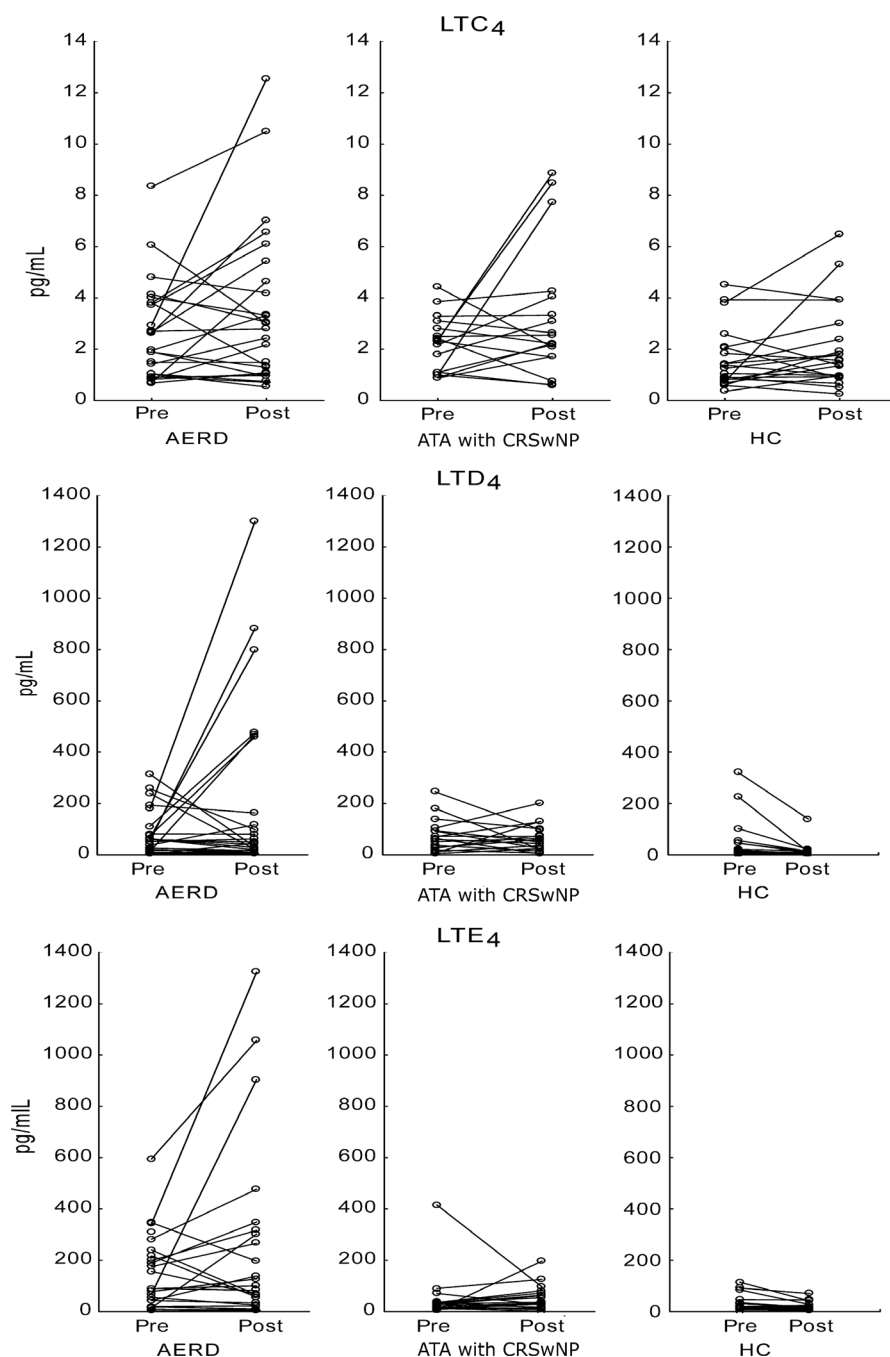
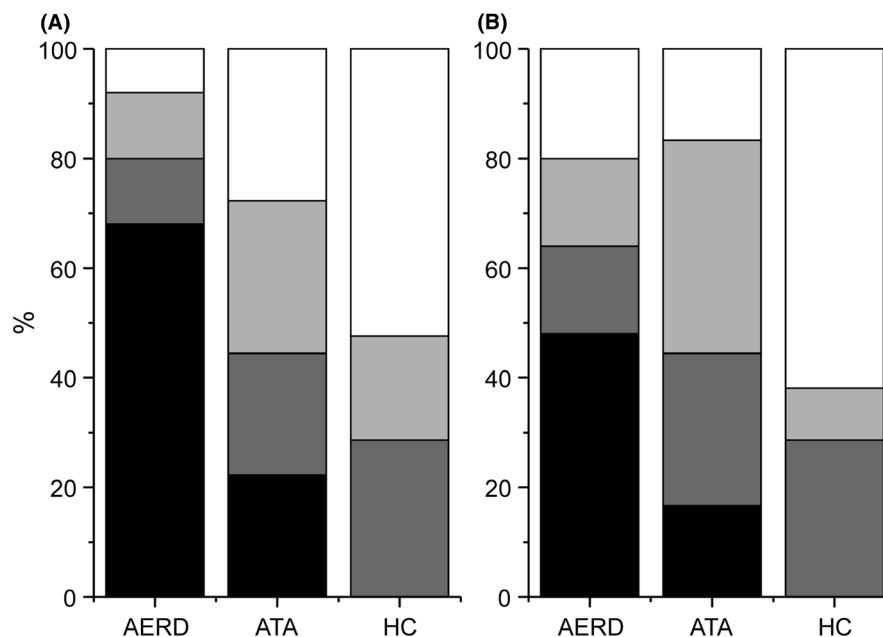


FIGURE 2 Induced sputum supernatant level of leukotrienes before and after oral aspirin challenge in nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (NERD), aspirin-tolerant asthma with chronic rhinosinusitis with nasal polyposis (ATA-CRSwNP), and healthy control (HC)

FIGURE 3 Phenotypes of asthma based on the sputum cytology before (A) and after (B) oral aspirin challenge. The percentages of eosinophilic (black), neutrophilic, paucigranulocytic, and mixed phenotypes (white) in nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (NERD), aspirin-tolerant asthma with chronic rhinosinusitis with nasal polyposis (ATA-CRSwNP) and healthy control (HC) groups



concentration of LTE_4 before the challenge was different between the study groups ($P < 0.01$, ANOVA). The highest level of LTE_4 was in NERD patients (89.74 [42.65-239.66]) as compared with ATA-CRSwNP (29.13 [15.42-34.92], $P < 0.01$) and HC (17.33 [9.32-34.48], $P < 0.01$) (Figure 2; Table 2a).

Following oral aspirin challenge, no significant change of LTC_4 and LTD_4 concentration was observed in study groups (Table 2). LTE_4 concentration had a tendency to increase in NERD subjects only (by 29.5%, $P = 0.06$). In HC, the concentration of LTE_4 decreased significantly (by 29.4%, $P = 0.02$), whereas no significant change was observed in ATA-CRSwNP group (increase by 17.6%, $P = 0.22$) (Figure 2). No effect of the cumulative dose of aspirin or inhaled corticosteroids was present in NERD group as tested by comparison with pre- or post-challenge LTC_4 , LTD_4 , LTE_4 or total Cys-LTs.

3.4 | Cellular phenotypes of asthma based on the sputum examination

Differential cell count in IS significantly varied between the study groups ($P < 0.01$, χ^2 test) before the oral aspirin challenge (Figure 3A). Higher eosinophilic count was in asthmatics (NERD vs HC $P < 0.01$, ATA-CRSwNP vs HC $P = 0.04$), especially in NERD group (NERD vs ATA-CRSwNP $P = 0.01$). Eosinophilic phenotype had the highest frequency in NERD (68.0%), whereas both mixed (27.8%) and paucigranulocytic (27.8%) were common in ATA-CRSwNP. Paucigranulocytic phenotype was the most common (52.4%) in HC. Cellular phenotypes did not change significantly following oral aspirin challenge (Figure 3B); thus, inter-group differences were preserved ($P < 0.01$, χ^2 test).

3.5 | LTE_4 in urine

Study groups differed in pre-challenge urinary LTE_4 (ANOVA, $P < 0.01$). NERD group had the highest concentration, whereas the

lowest one was observed in HC group ($P < 0.01$). The level of urinary LTE_4 in ATA-CRSwNP was intermediate not differing significantly from the concentration in NERD ($P = 0.131$) or HC ($P = 0.356$). Oral aspirin challenge resulted in a significant rise of the urinary LTE_4 concentration exclusively in NERD group (ANOVA, $P < 0.01$). Urinary LTE_4 raised at 0 h ($P = 0.02$), 2, 4, and 6 h ($P < 0.01$) when compared with the pre-test value. A return to the baseline level of urinary LTE_4 occurred within 24 h after the challenge ($P = 0.07$).

4 | DISCUSSION

No previous studies compared eicosanoid concentrations of IS before and after oral aspirin challenge in NERD and their most similar disease control ATA with CRSwNP. Both groups did not differ in the frequency of rhinosinusitis and nasal polyposis.

IS collection is a non-invasive method of bronchial secretions sampling. The method allows to measure a variety of bioactive compounds.²⁹ Lipid mediators are especially interesting in NERD because cyclooxygenases are the pharmacological targets for NSAIDs. Elevated systemic biosynthesis of LTE_4 is a common feature of NERD.³⁰ The main aim of the study was to measure the concentration of anti-inflammatory PGE_2 ¹³ and its stable degradation product, tetranor-PGE-M, before and after a clinically relevant oral dose of aspirin. We also quantified two bronchoconstrictive isoprostanes: 8-iso-PGE₂ and 8-iso-PGF₂ α .^{11,25} We expected to observe the pharmacological effect of aspirin on the biosynthesis of prostaglandins. Systemic administration of aspirin decreased bronchial PGE_2 production in NERD. Decrease in PGE_2 production in HC was expected due to cyclooxygenases inhibition, but there was a sharp difference of the drug dose delivered. PGE_2 inhibition by 30%-35% required in average a 2.8 times lower dose of aspirin in NERD than in HC. This was in contrast with ATA-CRSwNP.

Like in NERD, biosynthesis of PGE_2 was elevated at baseline, but only tended to decrease following 500 mg of aspirin. Our results fully support the pivotal role of PGE_2 triggering asthmatic attacks in NERD. PGE_2 inhalation can protect against bronchospasm following aspirin challenge.³¹ PGE_2 can decrease platelet interaction with granulocytes; thus, a transcellular mechanism has recently received some solid evidence.^{8,9} In the light of our findings, NERD asthmatics fail to maintain adequate bronchial production of PGE_2 when exposed to NSAIDs. Hence, inhibition of PGE_2 biosynthesis by NSAIDs reveals the phenotype of aspirin hypersensitivity leading to bronchoconstriction. Following positive oral aspirin challenge in NERD, a decrease in PGE_2 ISS concentration is accompanied by a surge in Cys-LTs, also present in our study.²¹ Peripheral blood leukocytes from asthmatics with NERD release less PGE_2 than from HC.³² Similarly, bronchial fibroblasts' PGE_2 production is lower in NERD as compared to aspirin-tolerant subjects.³³

Our results support that bronchial cells in asthmatics produce more PGE_2 by inducible COX-2, significantly above the level present in HC. COX-2 activity rises in response to numerous inflammatory stimuli and is overexpressed in the bronchi of asthmatics. A cellular phenotype of IS can influence PGE_2 biosynthesis, which was also confirmed by our study. We observed the highest baseline concentration of PGE_2 in subjects with mixed phenotype. PGE_2 is produced by all cell types of the body. Epithelia, fibroblasts, and infiltrating inflammatory cells represent the major sources during inflammatory response.³⁴ The percentage of COX-2-positive eosinophils and mast cells is increased in patients with NERD.³⁵ Therefore, elevated PGE_2 production in asthma is attributed to COX-2 expression.³⁶ Aspirin is a several-fold more potent inhibitor of COX-1 than COX-2 expressed in respiratory mucosa.^{35,37} COX-2 expression can vary over time in asthma patients.³⁸ Following pro-inflammatory stimulation, COX-2 mRNA is produced within 30 min and reaches a plateau within a few hours.³⁹ Defective post-challenge induction of COX-2 in the airways of NERD could explain post-challenge decrease in PGE_2 . Increased sensitivity of NERD patients to deficiency in bronchial PGE_2 can be aggravated by a lower expression of EP2 receptor.⁴⁰ High levels of PGE_2 before the challenge were not related to IS cellular phenotype, suggesting PGE_2 production from residential cells.¹³ Our previous results showed increased urinary PGE_2 metabolites (13,14-dihydro,15-keto- PGE_2 and tetranor- PGE_2 -M) in NERD patients,²⁴ but bronchial PGE_2 end-metabolite tetranor- PGE_2 -M was higher in ATA-CRSwNP than NERD patients, suggesting accelerated PGE_2 metabolism.

NERD asthmatics overproduce Cys-LTs,³⁰ particularly LTE_4 . This biomarker has variable concentration in spot urine samples. Recently, benefits of 24-h urine collection were presented.³⁰ In our study, Cys-LTs were released following the ingestion of aspirin and correlated with bronchial symptoms similarly to previously reported.^{6,21} Interestingly, a positive correlation of ISS or urinary LTE_4 and eosinophilic phenotype (Appendix S1) suggests eosinophils as the main source of LTE_4 . Platelet-leukocyte aggregates can produce Cys-LTs by a transcellular biosynthesis,⁸ and this mechanism can complement LTC_4 synthase activity in eosinophils from NERD subjects.¹²

8-iso- PGE_2 is a bronchoconstrictory isoprostane formed in vivo by a free radical peroxidation of arachidonate.^{41,42} 8-iso- PGE_2 decreased in ISS following aspirin challenge only in ATA-CRSwNP. We previously described an elevation of this isoprostane in exhaled breath condensate following bronchial aspirin challenge in NERD.²⁵ To explain this discrepancy, caused by a different route of aspirin administration, further studies are needed.

This study evaluated eicosanoid concentrations of induced sputum before and after aspirin challenge. Induced sputum is considered to reflect the inflammatory processes in the respiratory tract well, as it originates from both large and small airways. This is also a minimally invasive and well-validated sample collection method, whose methodology has been standardized. We have enrolled ATA-CRSwNP patients whom only a few studies have compared with NERD. Patients with NERD have more severe upper and lower respiratory tract disease, intensified sinonasal inflammation, require repeated sinus surgeries, and are more likely to have oral corticosteroid-dependent disease than ATA-CRSwNP.⁴³ There were no differences in blood eosinophil count between the two asthmatic populations in our study. In most prospective studies, NERD was characterized by higher blood eosinophilia than ATA-CRSwNP.^{24,44} It is probable that rhinosinusitis in ATA-CRSwNP had eosinophilic etiology because increased blood eosinophilia was described before.⁴⁵

4.1 | Limitations of the study

Limitations of the study were mostly imposed by its design. We enrolled subjects with a known health status who gave informed consent to participate in a time-consuming two-day challenge procedure. Besides the risk for enrollment bias, a successful sampling of the induced sputum required reversal of the airway obstruction in asthmatics in response to hypertonic saline or following a positive challenge. To minimize confounding effect of a single dose of short-acting beta-agonist, this procedure of beta-mimetic inhalation was administered to all the study subjects. For the same reason, time interval of sputum sampling after short-acting beta-agonist was different on placebo and aspirin challenge day.

4.2 | Clinical implications

Following the oral aspirin challenge, NERD phenotype is characterized by a specific decrease in PGE_2 concentration in ISS. Elevated sensitivity to aspirin results in inhibition of bronchial PGE_2 biosynthesis and triggers bronchoconstriction. Measurements of induced sputum supernatant eicosanoids concentration without drug provocation are not useful for the diagnosis of NERD.

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CONFLICTS OF INTEREST

None of the authors have a conflict of interest in relation to this work.

AUTHOR CONTRIBUTIONS

LM and MS designed the study. KET, MI, MB, and AK recruited study subjects and performed challenges and samples collections. LM, EK, KO, FM, and MS analyzed data. AĆ and FM did statistical analysis. LM, KET, and MS wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Online Supplementary material

Validation of eicosanoids measurement in the induced sputum supernatant

Mass spectrometry method was validated using pure chemical compounds spiked into a pooled sample. The lowest levels of quantification (LLOQ) in ISS were: PGE₂ – 3.02 pg/mL, tetranor-PGE₂-M – 1.45 pg/mL, 8-iso-PGE₂ – 2.40 pg/mL, 8-iso-PGF₂α – 1.82 pg/mL, LTC₄ – 4.76 pg/mL, LTD₄ – 3.62 pg/mL, LTE₄ – 3.22 pg/mL. Measured concentrations were not corrected for the sputum plugs dilution (1:4) required for their solubilisation with Sputolysin Reagent (Methods, Induced Sputum Collection).

Correlation between PGE₂ and tetranor-PGE₂-M in ISS

There was a positive correlation between PGE₂ and tetranor-PGE₂-M concentration in ISS at baseline in NERD group ($r=0.41$; $P=0.04$). No correlation was present in ATA with CRSwNP or HC subjects ($r=0.22$, $P=0.38$ and $r=0.27$, $P=0.24$, respectively). Similarly, a positive correlation was observed between PGE₂ and tetranor-PGE₂-M following aspirin challenge in NERD group ($r=0.41$, $P=0.04$). No correlation was found in ATA with CRSwNP and HC subjects ($r=0.06$, $P=0.81$ and $r=0.07$, $P=0.75$, respectively).

Analysis of correlation between ISS PGE₂ and ISS Cys-LTs levels at baseline and following aspirin challenge

At baseline there was a statistically significant positive correlation between ISS level of PGE₂ and Cys-LTs only in ATA with CRSwNP group ($r=0.64$, $P<0.01$). No associations between ISS PGE₂ and Cys-LTs were observed in NERD ($r=-0.16$, $P=0.45$) and HC ($r=0.35$, $P=0.12$). Analyzing baseline levels of each Cys-LTs separately, the only statistically significant correlation was found between ISS PGE₂ and LTD₄ concentration in ATA with CRSwNP group exclusively ($r=0.64$, $P<0.01$). Additionally, there was a weak positive

correlation between ISS PGE₂ and LTE₄ concentration only in ATA with CRSwNP group ($r=0.42$, $P=0.08$).

There was no correlation in post-challenge concentrations of ISS PGE₂ and ISS Cys-LTs in NERD ($r=-0.23$, $P=0.26$) and HC ($r=-0.04$, $P=0.86$). A weak positive correlation of ISS PGE₂ and ISS Cys-LTs was observed in ATA with CRSwNP ($r=0.47$, $P=0.05$). No correlations were found between post-challenge concentrations of ISS PGE₂ and ISS LTC₄, LTD₄ or LTE₄ in NERD and HC. A weak positive correlation of ISS PGE₂ and ISS LTE₄ following aspirin challenge was observed in ATA with CRSwNP ($r=0.43$, $P=0.07$).

Analysis of correlation between blood eosinophil count [$/\text{mm}^3$] and eosinophils in sputum cytology [%]

In all study subjects, a positive correlation was found between blood eosinophil count and percentage of eosinophils in ISS before ($r=0.54$; $P<0.01$) and after aspirin challenge ($r=0.65$; $P<0.01$).

There was a positive correlation between blood eosinophil levels and the percentage of eosinophils in sputum in ATA with CRSwNP (Spearman $r=0.63$; $P=0.01$) and HC subjects at baseline (Spearman $r=0.57$; $P=0.01$). At baseline, a poor positive correlation was found between blood eosinophil levels and the percentage of eosinophils in sputum in NERD NERD (Spearman $r=0.45$; $P=0.05$).

Similarly, after the aspirin challenge we observed a positive correlation only in ATA with CRSwNP (Spearman $r=0.53$; $P=0.03$) and HC subjects (Spearman $r=0.71$; $P<0.01$) and no significant correlation in NERD NERD subject (Spearman $r=0.35$; $P=0.14$).

Analysis of correlation between baseline and post-challenge level of LTE₄ in urine and ISS

After placebo challenge, a weak positive correlation between levels of LTE₄ in urine and ISS was observed in an analysis of all study groups altogether ($r=0.48$, $P<0.01$). No associations were found in an analysis in each study group separately.

After aspirin challenge, a stronger positive correlation between level of LTE₄ in urine and ISS was found in all study groups altogether (Spearman, $r=0.52$, $P<0.01$) as well as in NERD group at 4h ($r=0.58$, $P<0.01$), 6h ($r=0.45$, $P=0.03$) and 24h ($r=0.56$, $P<0.01$).

Analysis of correlation between baseline and post-challenge level of urine LTE₄ and blood eosinophil count

There was a mild correlation between blood eosinophil count and urine LTE₄ at baseline ($r=0.45$, $P<0.01$) and at 0h ($r=0.54$, $P<0.01$) in all study participants and a stronger correlation at baseline ($r=0.51$, $P=0.03$) and at 0h ($r=0.65$, $P<0.01$) in ATA with CRSwNP. There were no correlations between blood eosinophil count and urine LTE₄ following aspirin challenge in any of the study groups.