



Clinical Trial Paper

Sputum biomarkers during aspirin desensitization in nonsteroidal anti-inflammatory drugs exacerbated respiratory disease



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ABSTRACT

Background: Aspirin desensitization (AD) is an effective and safe therapeutic option for patients with non-steroidal anti-inflammatory drugs (NSAIDs)-exacerbated respiratory disease (N-ERD). The mechanisms driving its beneficial effects remain poorly understood.

Objective: To investigate the effect of long-term AD on clinical, biochemical and radiological changes in N-ERD patients.

Methods: The study group consisted of twenty-three individuals with N-ERD who underwent AD, followed by ingestion of 325 mg aspirin twice daily. Twenty patients completed the 52 weeks of AD. The following evaluations were conducted at baseline and in the 52nd week of AD: (i) clinical: asthma exacerbations, Asthma Control Test (ACT), Visual Analogue Scale (VAS) for the assessment of nasal symptoms; (ii) blood and induced sputum supernatant (ISS) periostin, (iii) phenotypes based on induced sputum (IS) cells, (iiii) ISS and nasal lavage (NL) concentration of prostaglandin D₂ (PGD₂), prostaglandin E₂ (PGE₂), tetranor-PGD-M, tetranor-PGE-M, 8-iso-PGE₂, leukotriene B₄ (LTB₄), LTC₄, LTD₄ and LTE₄, and urine LTE₄.

Results: A significant improvement was observed in ACT ($P = 0.02$) and VAS score ($P = 0.008$) in the 52nd week of AD. ISS periostin and IS eosinophil count decreased significantly in the 52nd week of AD ($P = 0.04$ and $P = 0.01$, respectively). ISS and NL eicosanoid concentrations did not change following long-term AD.

Conclusion: and Clinical Relevance: AD is associated with a decrease in sputum periostin biosynthesis, which may prevent the recruitment of eosinophils into respiratory tissue and be one of explanation of the clinical benefits of AD. Long-term aspirin administration does not lead to an imbalance between pro- and anti-inflammatory ISS eicosanoids.

1. Introduction

According to the current recommendations nonsteroidal anti-inflammatory drugs (NSAIDs)-exacerbated respiratory disease (abbreviated as N-ERD) is a proper term to describe the distinct phenotype of aspirin (acetyl salicylic acid; ASA) and other NSAIDs hypersensitivity, which involves upper and lower airway mucosa [1,2]. N-ERD is typically characterized by chronic rhinosinusitis with nasal polyposis (CRSwNP) and bronchial asthma, which are exacerbated upon ingestion

of cyclooxygenase (COX)-1 inhibitors such as aspirin. As N-ERD is a heterogenous syndrome, finding new biomarkers and subphenotyping the disease would allow for a more personalized care of N-ERD patients [3]. The hallmarks of the disease are complex alterations of the eicosanoid pathways, which include a reduction in anti-inflammatory prostaglandins and the overproduction of pro-inflammatory cysteinyl leukotrienes (Cys-LTs) due to mast cell and eosinophil activation [4]. The dysregulation of these pro- and anti-inflammatory mediators was observed in induced sputum supernatant (ISS) [5], the sinuses and nasal

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Abbreviations used

ACT	Asthma Control Test
AD	Aspirin desensitization
CRSwNP	Chronic rhinosinusitis with nasal polyposis
Cys-LTs	Cysteinyl leukotrienes
COX	Cyclooxygenase
ELISA	Enzyme-linked immunosorbent assay
FEV ₁	Forced expiratory volume in the first second
GC-MS	Gas chromatography/mass spectrometry
GI	Gastrointestinal
ICS	Inhaled corticosteroids
IL-4	Interleukin 4
IL-13	Interleukin 13

ILC2	Type 2 innate lymphoid cells
IS	Induced sputum
ISS	Induced sputum supernatant
LMS	Lund-Mackay Score
LTB ₄	Leukotriene B ₄
LTC ₄	Leukotriene C ₄
LTD ₄	Leukotriene D ₄
LTE ₄	Leukotriene E ₄
N-ERD	Nonsteroidal anti-inflammatory drugs (NSAIDs)-exacerbated respiratory disease
NL	Nasal lavage
PGD ₂	Prostaglandin D ₂
PGE ₂	Prostaglandin E ₂
VAS	Visual Analogue Scale

polyps of N-ERD patients [6]. Recent studies have also suggested the role of platelet-granulocyte aggregates in respiratory tissue inflammation and increased biosynthesis of Cys-LTs in N-ERD [7]. Antiplatelet agents, such as prasugrel, are currently the subject of interest in terms of the prevention of aspirin-induced reactions in N-ERD individuals [8]. Additionally, there is emerging data on the role of type 2 innate lymphoid cells (ILC2) and the associated cytokine production in the pathogenesis of N-ERD [9,10].

One therapeutic option proposed for patients with N-ERD is aspirin desensitization (AD). AD is indicated in N-ERD individuals who (1) have uncontrolled asthma or CRSwNP, (2) require multiple nasal surgeries due to recurrent polyposis, or (3) demand COX-1 inhibitors for anti-platelet therapy [11]. Several previous studies have shown that AD treatment leads to reduced upper airway congestion, decreased nasal polyp formation and improved asthma control in N-ERD patients [12,13]. However, despite strong evidence supporting clinical efficacy of AD, the mechanism driving its beneficial effects remains poorly understood.

The aim of this prospective study was to assess the clinical, biochemical and radiological changes in N-ERD patients subjected to long-term (52-week) AD. Well-defined efficacy outcomes allowed us to evaluate the clinical effectiveness of 52-week AD with daily aspirin dose of 650 mg. We evaluated changes in blood and ISS levels of periostin, as it is known that this biomarker plays a role in asthma pathogenesis and may represent a promising therapeutic target [14]. However, its influence on AD has so far not been investigated. Additionally, we sought to determine whether long-term AD leads to any specific alterations in local eicosanoid biosynthesis measured in ISS and nasal lavage (NL) of N-ERD individuals.

2. Materials and methods

2.1. Study group

The study group consisted of 23 patients with asthma and CRSwNP, whose aspirin hypersensitivity had been confirmed by oral provocation test during the period of three years preceding the study [15]. All study participants were at clinically stable conditions and their baseline forced expiratory volume in the first second (FEV₁) was at least 70% 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. None had been treated with systemic corticosteroids or leukotriene modifiers 6 weeks before proper study. All study participants were treated with the same dose of inhaled corticosteroids (ICS)- 1000 mg/day (fluticasone eq.), inhaled long-acting beta₂-agonists- 100 µg/day (salmeterol eq.) and nasal corticosteroids - 110 µg/day (fluticasone eq.) for 6 preceding weeks and throughout the study. All patients had CRSwNP evidenced by endoscopic and CT findings, and seventeen patients (74%) had a history of at least one

Table 1

Characteristics of patients at baseline.

Variable	Patients (n = 23)
Age (years)	49 (29–67)
Sex (female/male ratio)	16/7
Asthma duration (years)	8 (3–32)
Age at asthma onset > 12 years (yes/no)	23/0
ACT score	21 (12–25)
Baseline FEV ₁ (% predicted)	94.2 (70.1–126.6)
ICS (yes/no)	23/0
Dose of ICS (µg/day) fluticasone eq.	1000 (1000–1000)
CRSwNP	23/0
History of sinonasal surgery (yes/no)	17/6
Time to initiation of AD after sinonasal surgery (months) (12 months/ > 12 months)	12 (3–60) 9/8
Overall nasal symptoms VAS score	6 (0–10)
Sense of smell VAS score	5 (0–10)
Lund-Mackay Score	18 (0–24)
Nasal corticosteroids (yes/no)	23/0
Dose of nasal corticosteroids (µg/day) fluticasone eq.	110 (110–110)
Eosinophils (mm ³)	350 (20–930)
Skin tests (positive/negative)	7/16
IgE total (IU/mL)	126 (17.1–1730.0)

Values are expressed as median (min-max value).

ACT- Asthma Control Test; FEV₁- Forced expiratory volume in the first second; ICS- inhaled corticosteroids; CRSwNP- Chronic rhinosinusitis with nasal polyposis; AD-aspirin desensitization.

sinonasal surgery within the median time to the AD initiation of 12 (3–60) months (Table 1). The study was approved by the Jagiellonian University Bioethics Committee (approval number: 122.6120.277.2016) and written informed consent was obtained from all participants.

2.2. Study design

Study participants were recruited from the N-ERD patient cohort (n = 121) of the 2nd Department of Internal Medicine, Jagiellonian University Medical College. Run-in period lasted for 52 weeks, when patients remained under the ambulatory care of our clinic (Fig. 1A and B). In the course of the proper study patients had two main hospitalizations-the first in order to perform oral aspirin challenge, which is required to initiate an acute AD, and the second in the 52nd week of AD. Additionally, all study participants were controlled in the 8th and the 26th week of the study. During first hospitalization, on the day of aspirin administration, acute AD was initiated. At baseline (one day before aspirin challenge) and in the 52nd week of AD the following evaluations were conducted: (i) clinical (asthma exacerbations; asthma control; nasal symptoms; spirometry), (ii) biochemical (blood eosinophil count, blood and ISS periostin; cell phenotypes based on induced sputum (IS); ISS, NL and urine concentrations of eicosanoids); and (iii)

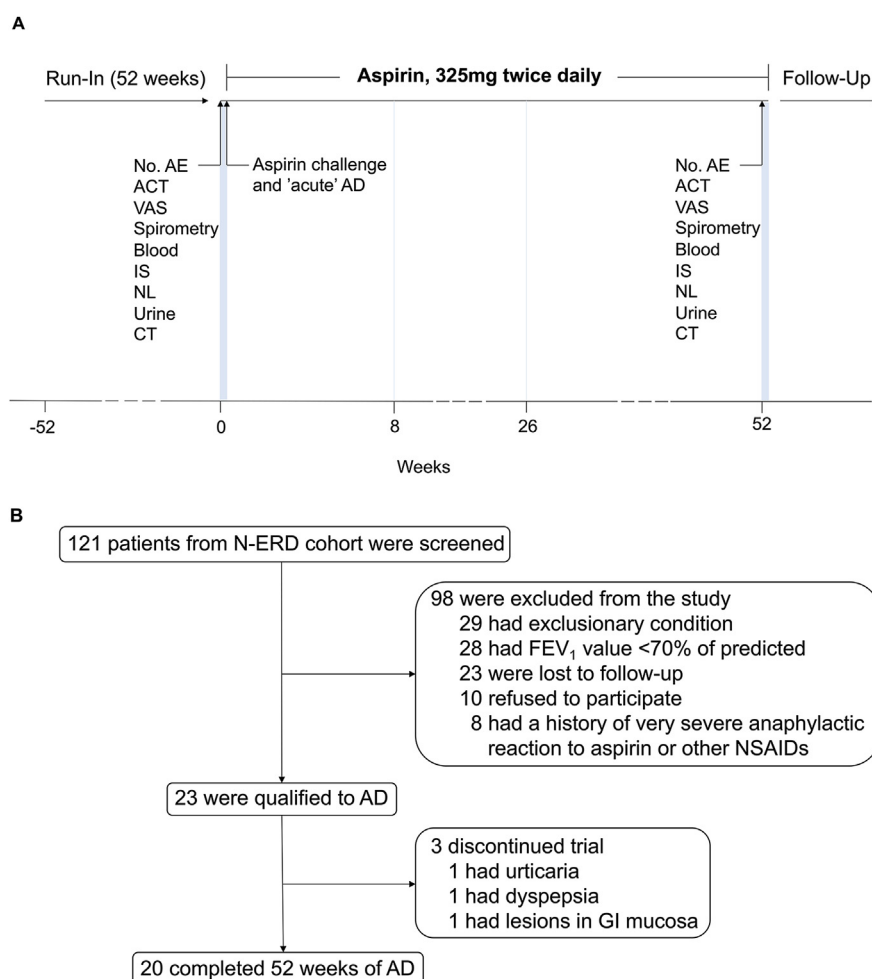


Fig. 1. Patients and study design. (A) An overview of the trial design and procedures. Blue vertical lines denote study visits. AD = aspirin desensitization; No. AE = number of asthma exacerbations; ACT = Asthma Control Test; VAS = Visual Analogue Scale; IS = induced sputum; NL = nasal lavage; CT = computed tomography. (B) The summary of the numbers of patients involved in screening, qualification, and trial completion. GI = gastrointestinal.

radiological (CT scan images of the sinuses). Asthma control, nasal symptoms and spirometry were additionally evaluated by an allergologist in the 8th and 26th weeks of the study.

The number of clinically relevant asthma exacerbations in the year prior to the study and during 52 weeks of AD was precisely assessed. Exacerbations were defined as asthma symptoms requiring hospitalization for the treatment of asthma or systemic glucocorticoid therapy. Asthma control was evaluated based on Asthma Control Test (ACT). Visual Analogue Scale (VAS) was used for the assessment of overall nasal symptoms and separately for the assessment of the sense of smell.

2.3. Aspirin challenge and desensitization

Single-blind, placebo controlled oral aspirin challenges were performed on two consecutive days according to the EAACI/GA2LEN guidelines [15]. AD was performed based on our own modification of the Scripps Clinic protocol [16]. 'Acute AD' was initiated on the same day after aspirin challenge completed, and the patient was clinically stabilized. The patient received a provocative dose of aspirin established during aspirin challenge. If the patient remained asymptomatic, the dose was gradually increased every 1.5 h until the target dose of 650 mg of aspirin was reached. If a patient presented any clinical symptoms, the same dose, which provoked symptoms, was repeated when the reaction resolved. On the second day of AD procedure the patient was able to take the overall daily dose of 650 mg. Aspirin was given immediately after meals. All patients received stomach protectors

during the 52-week AD, the daily dose was 40 mg taken once.

2.4. Induced sputum

IS collection was performed according to European Respiratory Society (ERS) recommendations [17]. Four cell phenotypes based on IS were distinguished-eosinophilic ($\geq 3\%$ eosinophils and $< 60\%$ neutrophils), neutrophilic ($\geq 60\%$ neutrophils and $< 3\%$ eosinophils), mixed ($\geq 3\%$, eosinophils and $\geq 60\%$ neutrophils) and paucigranulocytic ($< 3\%$ eosinophils and $< 60\%$ neutrophils) [18].

2.5. Nasal lavage

NL was performed before sputum induction to avoid contact of hypertonic saline with nasal epithelium as previously described [19,20].

2.6. Blood and ISS levels of periostin

Blood and ISS periostin levels were assessed using ELISA: Periostin/OSF-2 human, BioVision Inc, Milpitas, CA. According to manufacturer recommendation Periostin (human) ELISA kit is to be used in cell culture supernatants, serum and plasma. Assay range: 78 pg/mL–5000 pg/mL. Detection limit: 15 pg/mL. As per the manufacturer's protocol blood was diluted 1/60 since the expected human periostin levels in plasma and serum range from 10 to > 100 ng/mL. ISS samples were not diluted

as the results were expected to fall within the assay range. Blood and ISS periostin concentrations were presented in ng/mL.

2.7. Eicosanoid evaluation in ISS and NL

The concentration of eicosanoids was measured by gas chromatography/tandem mass spectrometry (GC-MS/MS) for PGD₂, PGE₂, 8-iso-PGE₂ or by high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS/MS) for tetranor-PGE-M, tetranor-PGD-M, LTB₄, LTC₄, LTD₄ and LTE₄. 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 (PGD₂, PGE₂, 8-iso-PGE₂, tetranor-PGD-M, tetranor-PGE-M, LTB₄, LTC₄, LTD₄ and LTE₄, all from Cayman Chemical Co, Ann Arbor, MI).

2.7.1. High performance liquid chromatography – tandem mass spectrometry

Methanol dissolved extract (10 µL) was injected onto a reverse phase column (Zorbax Eclipse XDB C-18 - Agilent Technologies, Santa Clara, CA, USA), stabilized thermally at 37 °C and eluted with a gradient consisting of two mobile phases: A - acetonitrile/water/acetic acid (20/80/0.0001) and B - acetonitrile/iso-propanol/acetic acid (55/45/0.0001). The flow rate was 0.11 mL/min (Shimadzu Sil-2-AC -

Shimadzu Scientific Instruments, Kyoto, Japan). All solvents were HPLC grade and purchased from Avantor (formerly Mallinckrodt Baker, Phillipsburg, NJ), other chemicals were from Sigma-Aldrich Co. (St. Louis, MO). The mobile phase binary linear gradient was 1 min 8% B, 9.5 min 8–95% B, 0.5 min 95% B, 0.5 min 95–100% B, 2 min 100% B. All eicosanoids were measured using multiple reaction monitoring mode (MRM) tandem mass spectrometry (Qtrap 4000, Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ion source negative ionization mode, using a batch profile for ion pairs detection.

2.7.2. Gas chromatography – mass spectrometry

Organic phase extracts (2 µL) were prepared by a 3-step derivatization to pentafluorobenzyl and trimethylsilyl esters and methoxyoxime which modified carboxyl and hydroxyl groups of the compounds, with subsequent purification by a thin-layer chromatography (TLC) and methanol elution from the TLC silica. A tandem quadrupole mass spectrometer (GCMS-TQ8030 Shimadzu, Columbia, MD, USA) in gas chromatography negative-ion methane chemical ionization mode (GC-NICI-MS) with a 30 m capillary column was used for quantification.

2.7.3. Data acquisition

Ion pairs (deuterated standard and analyte Mass/Charge ratio) for

Table 2

Concentrations of eicosanoids [pg/mL] in induced sputum supernatant (ISS) and nasal lavage (NL) in patients with aspirin exacerbated respiratory disease (N-ERD) at baseline and in the 52nd week of aspirin desensitization (AD). Cys-LTs = total of LTC₄, LTD₄ and LTE₄. LLOQ = the lowest limit of quantification (signal to noise ratio > 10).

Eicosanoid (LLOQ)	Induced sputum supernatant			Nasal lavage		
	Baseline [pg/mL]	52nd week [pg/mL]	P value	Baseline [pg/mL]	52nd week [pg/mL]	P value
PGD ₂ (4.19)						
mean ± SD	109.57 ± 89.52	70.29 ± 88.49	0.17	103 ± 178.64	86.27 ± 105.06	0.71
Median	90.09	33.5		37.84	49.86	
(25%–75%)	(35.29–149.85)	(13.05–86.06)		(26.55–116.20)	(31.42–81.42)	
PGE ₂ (1.62)						
mean ± SD	93.08 ± 71.3	96.66 ± 168.45	0.65	305.77 ± 283.87	307.24 ± 445.98	0.85
Median	65.79	31.39		163.08	221.29	
(25%–75%)	(28.72–147.57)	(11.62–110.37)		(102.91–500.91)	(107.3–289.62)	
Tetranor-PGD-M (1.55)						
mean ± SD	6.55 ± 2.56	5.37 ± 1.37	0.12	7.49 ± 2.75	8.13 ± 3.15	0.37
Median	6.33	5.39		7.27	7.64	
(25%–75%)	(4.52–7.90)	(4.15–6.56)		(5.51–9.06)	(5.96–10.03)	
Tetranor-PGE-M (1.45)						
mean ± SD	9.01 ± 5.68	6.50 ± 1.94	0.19	8.96 ± 2.54	8.98 ± 4.17	0.85
Median	6.64	6.42		9.40	8.89	
(25%–75%)	(5.25–10.02)	(5.04–7.98)		(6.94–10.75)	(5.24–11.34)	
8-iso-PGE ₂ (1.65)						
mean ± SD	17.97 ± 20.04	18.98 ± 17.71	0.46	61.78 ± 90.42	53.99 ± 76.61	0.74
Median	12.40	12.35		29.21	26.34	
(25%–75%)	(7.17–19.99)	(6.59–21.65)		(17.80–56.86)	(11.72–62.78)	
LTB ₄ (2.96)						
mean ± SD	1054.21 ± 1929.9	1598.13 ± 2928.94	0.83	217.60 ± 389.23	275.64 ± 358.94	0.47
Median	310.72	244.92		85.03	132.81	
(25%–75%)	(55.92–724.28)	(118.25–1820.27)		(38.23–155.88)	(53.00–341.79)	
LTC ₄ (2.42)						
mean ± SD	3.16 ± 4.01	5.80 ± 9.95	0.51	5.93 ± 4.74	10.84 ± 24.27	0.50
Median	1.36	1.79		4.39	3.78	
(25%–75%)	(0.47–4.57)	(1.00–4.01)		(3.46–5.65)	(2.19–8.86)	
LTD ₄ (2.85)						
mean ± SD	66.35 ± 110.33	80.96 ± 132.32	0.73	36.08 ± 47.96	45.97 ± 71.57	0.95
Median	17.04	20.54		21.09	19.34	
(25%–75%)	(5.75–79.19)	(5.91–130.96)		(12.48–32.37)	(9.53–42.18)	
LTE ₄ (3.22)						
mean ± SD	95.48 ± 142.37	146.88 ± 324.46	0.83	207.00 ± 374.91	206.59 ± 285.24	0.59
Median	29.22	52.19		56.07	48.03	
(25%–75%)	(5.4–160.42)	(9.7–120.18)		(22.50–185.60)	(18.32–292.78)	
Cys-LTs						
mean ± SD	164.99 ± 204.77	233.65 ± 450.31	0.93	249.02 ± 420.19	263.40 ± 347.23	0.95
Median	58.15	79.95		77.27	85.00	
(25%–75%)	(12.56–310.79)	(17.76–283.69)		(48.48–221.51)	(37.00–352.80)	

HPLC-MS² measurements were as follows:

- tetranor-PGE-M-d₆ 333–315 and tetranor-PGE-M 327–309*
- tetranor-PGD-M-d₆ 333–315 and tetranor-PGD-M 327–309.
- LTB₄-d₄ 339–130 and LTB₄ 335–129.
- LTC₄-d₅ 631–194 and LTC₄ 626–189.
- LTD₄-d₅ 502–194 and LTE₄ 497–189.
- LTE₄-d₃ 441–336 and LTE₄ 438–333.

*PGE₂ and PGD₂ metabolites have the same molecular mass but have different retention time during HPLC.

Pseudomolecular ions (deuterated standard and analyte) for GC-MS measurements were as follows:

- PGE₂-d₄ - 694.
- PGD₂-d₄ - 694.
- PGE₂, 8-iso-PGE₂, PGD₂ - 690*

* PGE₂, 8-iso-PGE₂, PGD₂ - 690 derivatives have the same molecular mass but have different migration distance on thin layer chromatography.

The area under the peak (AUP) for the eicosanoid analyte and corresponding deuterated internal standard (IS) were integrated. Concentration was calculated from the formula: [analyte] = [IS] x (AUP_{analyte}/AUP_{IS}).

Limits of quantification for eicosanoid measurements are presented in Table 2.

2.8. Urine LTE₄

Urine samples were collected for the assessment of LTE₄ excretion with ELISA method (Cayman Chemical Co., Ann Arbor, MI). The results were recalculated in picograms per mg of creatinine.

2.9. CT scans of the sinuses

The results of the CT scans were assessed independently by two experienced radiologists according to the Lund-Mackay Score (LMS) [21].

2.10. Statistical analysis

Statistical analysis was performed with Statistica 13.1 (TIBCO Software Inc., Palo Alto, CA). Summary statistics were expressed as mean (M) and standard deviation (SD) as well as median (Me) and 25% and 75% percentiles. For the two-group comparison we used t-Student test for independent samples and Mann-Whitney *U* test to compare two independent groups and t-Student for dependent samples and Wilcoxon Signed Rank Test for matched pair studies. The relationships between variables were studied with Spearman's correlation coefficient. The level of significance was set at $P \leq 0.05$.

3. Results

3.1. Clinical evaluation

Twenty of the twenty-three enrolled patients completed the 52 weeks of AD. One patient dropped out of the study in the 2nd week due to urticaria, one in the 13th week due to dyspepsia and one in the 26th week because of lesions in gastrointestinal mucosa confirmed by endoscopic findings.

The rate of protocol-defined exacerbations in the AD year decreased significantly in reference to the year prior to AD (0.86 ± 1.35 vs 0.00 ± 0.00 , $P < 0.001$). Eight patients had at least one exacerbation in the year prior to AD. During 52 weeks of AD none of the study participants had any exacerbations.

A significant improvement was also observed in ACT score in 52nd week of AD in comparison to baseline (19.95 ± 4.33 vs 21.35 ± 3.88 , $P = 0.02$) (Fig. 2A). Changes in ACT in the 8th week were insignificant (19.95 ± 4.33 vs 20.1 ± 4.67 , $P = 0.59$), while in the 26th week they were on the verge of statistical significance (19.95 ± 4.33 vs 21.00 ± 3.88 , $P = 0.05$). VAS score for overall nasal symptoms already improved in the 26th week (4.70 ± 2.52 vs 3.20 ± 2.57 , $P = 0.03$) and then in the 52nd week of AD (4.70 ± 2.52 vs 2.8 ± 2.33 , $P = 0.008$) (Fig. 2B). The improvement of VAS score for

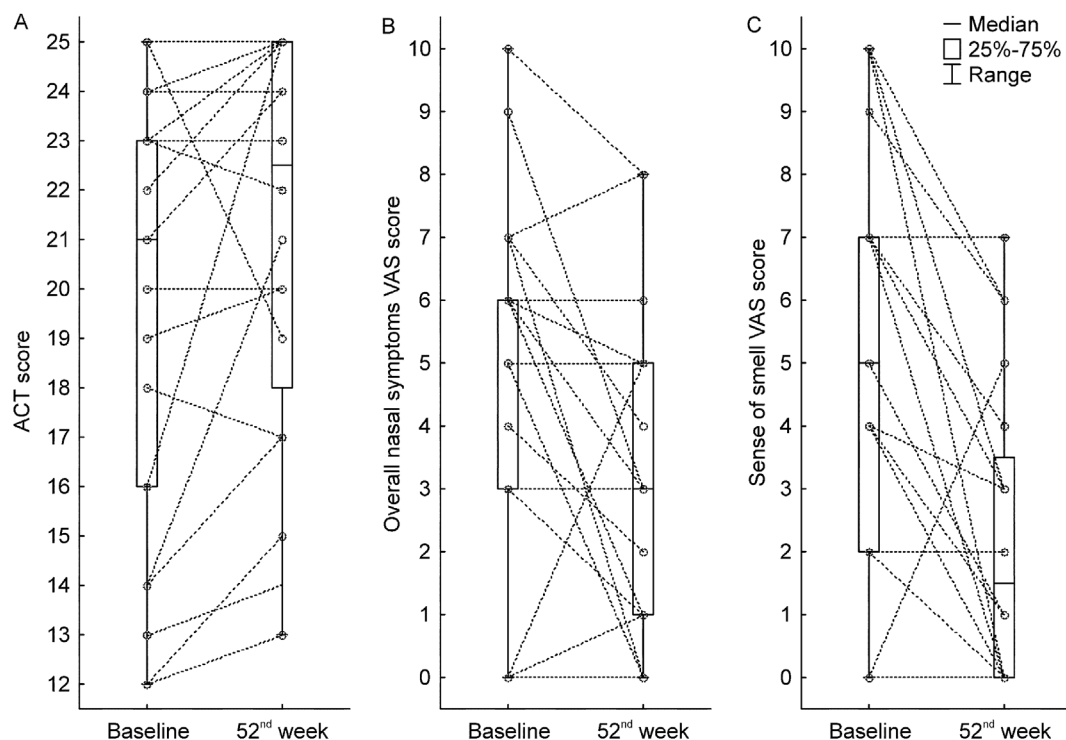


Fig. 2. Clinical assessment at baseline and in the 52nd week of aspirin desensitization (AD). (A) Asthma Control Test (ACT) score, $P = 0.02$; (B) Visual Analogue Scale (VAS) score for overall nasal symptoms, 0–10 cm scale: 0 = not troublesome, 10 = worst possible troublesome, $P = 0.008$; (C) Visual Analogue Scale (VAS) score for the sense of smell, $P = 0.008$.

overall nasal symptoms in the 8th week was insignificant (4.70 ± 2.52 vs 3.45 ± 3.85 , $P = 0.20$). VAS score for the sense of smell improved in 52nd week of AD (5.05 ± 3.04 vs 2.20 ± 2.30 , $P = 0.001$) (Fig. 2C). Changes in VAS score for the sense of smell in the 8th and 26th weeks were insignificant (5.05 ± 3.04 vs 3.45 ± 3.19 , $P = 0.11$ and 5.05 ± 3.04 vs 4.45 ± 3.36 , $P = 0.75$, respectively). 52 weeks of AD had no significant effect on baseline FEV_1 (2675 ± 638 vs 2683 ± 533 , $P = 0.94$).

3.2. Periostin in blood

There was no change in blood periostin level in the 52nd week of AD (63.92 ± 41.24 vs 70.51 ± 56.94 ng/mL, $P = 0.27$). There was a significant positive correlation between blood periostin level and ACT score at baseline ($r = 0.55$, $P = 0.01$), while no correlation was observed in the 52nd week of AD ($r = 0.22$, $P = 0.36$). No other significant correlations were found.

3.3. Eosinophils in blood

There was a statistically significant increase in both the absolute count and the percentage of blood eosinophils in the 52nd week of AD (353.33 ± 241.32 vs 612.63 ± 487.58 , $P = 0.03$ and 5.46 ± 3.22 vs 8.43 ± 6.31 , $P = 0.01$, respectively).

3.4. Cell phenotypes based on sputum

There was a significant decrease in both the absolute count and the percentage of IS eosinophils in the 52nd week of AD ($P = 0.01$ and $P = 0.03$, respectively) (Fig. 3). No significant changes were observed in the absolute count and the percentage of IS neutrophils in the 52nd week of AD ($P = 0.29$ and $P = 0.14$, respectively). There was a positive correlation between baseline absolute eosinophil count in blood and the percentage of eosinophils in IS ($r = 0.51$, $P = 0.03$).

3.5. Sputum phenotypes

Eleven patients at baseline had eosinophilic ($n = 11$; 55%), one patient had neutrophilic ($n = 1$; 5%), one patient had mixed ($n = 1$; 5%) and eight patients had paucigranulocytic ($n = 7$; 35%) sputum phenotypes. In the 52nd week of AD five patients had eosinophilic ($n = 5$; 25%), five patients had neutrophilic ($n = 5$; 25%), one patient had mixed ($n = 1$; 5%) and nine patients had paucigranulocytic ($n = 9$; 45%) sputum phenotypes. Due to the small number of cases representing particular phenotypes, any statistical comparisons would be invalid.

3.6. Periostin in ISS

A significant decrease in ISS periostin concentration was observed in the 52nd week of AD (0.29 [0.14–0.78] vs 0.22 [0.09–0.57] ng/mL, $P = 0.04$) (Fig. 4). Periostin levels were significantly lower in ISS than in blood both at baseline as well as in 52nd week of AD ($P < 0.001$ and $P < 0.001$, respectively). There was no correlation between ISS and blood periostin levels at baseline ($r = 0.34$; $P = 0.17$) nor in 52nd week of AD ($r = 0.07$; $P = 0.79$). No correlation between ISS periostin level and ACT score, FEV_1 , blood and IS eosinophil count and blood IgE was observed neither at baseline nor in the 52nd week of AD.

3.7. Eicosanoids in ISS, NL and urine

No significant changes were observed in the 52nd week of AD in concentrations of ISS and NL prostanoids, their metabolites and leukotrienes (Table 2).

There was no change in the concentration of urinary LTE_4 in the 52nd week of AD (1996 ± 914 at baseline vs 2697 ± 1249 in 52nd

week of AD, $P = 0.07$).

3.8. Blood periostin and eicosanoid concentrations

There was a statistically significant positive correlation at baseline between blood periostin level and LTB_4 in ISS ($r = 0.58$; $P = 0.03$). No correlations were observed between blood periostin concentration and levels of any other eicosanoids measured in ISS or in NL at baseline and in the 52nd week of AD.

The concentration of blood periostin did not correlate with LTE_4 in urine at baseline ($r = -0.09$; $P = 0.71$), but there was a positive correlation between blood periostin level and LTE_4 in urine in the 52nd week of AD ($r = 0.52$; $P = 0.03$).

3.9. ISS periostin and eicosanoid concentrations

There were no correlations at baseline between ISS periostin level and concentrations of any ISS eicosanoids measured. ISS periostin level correlated positively in the 52nd week of AD with LTE_4 and Cys-LTs (LTC_4 , LTD_4 and LTE_4) in ISS ($r = 0.55$; $P = 0.03$ and $r = 0.53$; $P = 0.04$, respectively). There were no correlations between ISS periostin level and the concentration of any other eicosanoids measured in ISS in the 52nd week of AD.

There were no correlations between the concentration of periostin in ISS and LTE_4 in urine before ($r = -0.10$; $P = 0.67$) and in the 52nd week of AD ($r = 0.05$; $P = 0.86$).

3.10. CT scans of the sinuses

There was no statistically significant change in total LMS in the 52nd week of AD (9.16 ± 9.74 vs 9.95 ± 9.92 , $P = 0.25$).

4. Discussion

Since mechanisms of long-term AD are poorly understood [12,13,22], we have investigated changes in a variety of biomarkers before and after 52 weeks of AD in N-ERD individuals. We focused primarily on local (bronchial and nasal) response to long-term aspirin

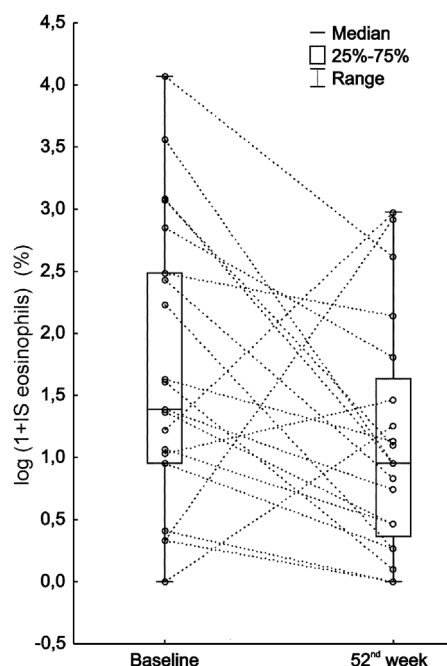


Fig. 3. Induced sputum (IS) eosinophils at baseline and in the 52nd week of aspirin desensitization (AD), $P = 0.03$.

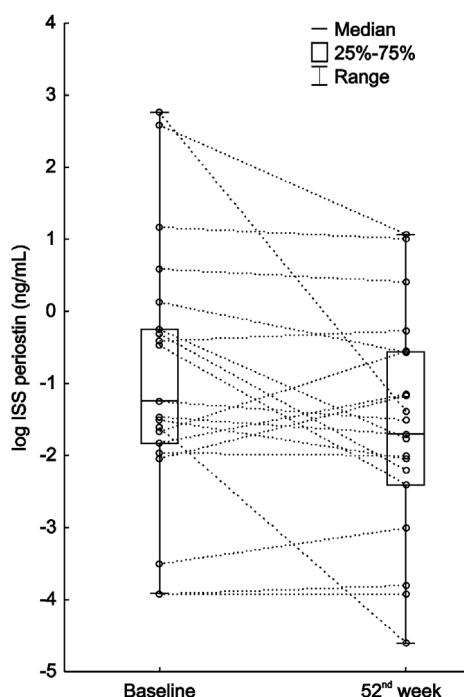


Fig. 4. Induced sputum supernatant (ISS) level of periostin at baseline and in the 52nd week of aspirin desensitization (AD), $P = 0.04$.

intake by measuring a number of mediators in ISS as well as in NL, as we hypothesized this may be relevant to the clinical response to AD.

AD is a provocative procedure but if performed in a properly equipped clinical setting it is a safe option that should be made available to all patients with N-ERD [12,23]. All participants of our study initially tolerated the ASA maintenance dose of 650 mg. The discontinuation of subsequent AD was necessary in three patients—one presented with urticaria and the other two developed a GI reaction. None of our patients experienced any exacerbation in the course of 52-week AD. Likewise, our study showed some discrepancies between subjective and objective measurements of sinonasal outcomes. VAS is commonly used as a subjective method to evaluate nasal symptoms [24]. A significant improvement in nasal outcomes is the most frequently described positive effect of AD [13,22] and was also the most pronounced advantage of the therapy reported by our patients. Most of them emphasized a long-lasting return of their sense of smell, which is consistent with other studies evaluating nasal symptoms in the course of AD [12,22,25]. These observations were, however, not reflected in CT scan images, as no improvement was observed in the total LMS. The discrepancies between clinical and radiological findings were also observed in other reports [22,25]. Such divergence is difficult to explain and some authors suggested that it might be associated with the lack of sinonasal surgery shortly before AD [26–28].

Different cells and mediators are probably involved in AD mechanisms. Periostin is a matricellular protein known to be produced by fibroblasts, epithelial and endothelial cells stimulated by IL-4 and IL-13 [29,30]. Activated cells secrete large quantities of periostin into the underlying matrix, which among others contributes to the mechanisms of airway remodeling in asthma [30]. Kanemitsu et al. [14] showed that deposition of periostin in bronchial subepithelium of asthma patients is inversely correlated with FEV₁ decline. We have observed a significant decrease in ISS periostin level after 52 weeks of AD. On the contrary, we have not found any changes in blood periostin level as well as no correlation between its blood and ISS concentrations. This may indicate that a long-term AD does not influence the blood biosynthesis of this biomarker, but somehow selectively affects its epithelial synthesis, at least in ISS. Katial et al. [31] observed that six-month AD in N-ERD

patients leads to the significant suppression of ISS IL-4, which is one of the inducers of periostin production. This suggests that cells capable of producing IL-4 and IL-13 (e.g. Th2 and ILC2) [10] may also be affected by long-term AD. ILC2 was shown to migrate to the nasal mucosa after challenge with COX-1 inhibitor and to correlate positively with symptom scores from upper and lower airways in N-ERD individuals. These changes were additionally accompanied by an increase in the concentration of the stable PGD₂ metabolite 11-PGF₂ and LTE₄ in the urine [32], both were known to induce ILC₂ to cytokine production [10]. On the other hand, the overproduction of PGD₂ and Cys-LTs following aspirin challenge was not observed during long-term AD in our study. We speculated that long-term AD might modulate epithelial cell activation by decreasing the biosynthesis of periostin and alarming-like cytokines such as TSLP, IL-25 and IL-33, which stimulate ILC2 to pro-inflammatory cytokine production. IL-33 has been described to be overexpressed in nasal polyp tissue in N-ERD individuals [33], which might contribute to the typical N-ERD symptoms.

In light of the presented findings, we suppose that long-term aspirin intake (i) might lead to the decrease in IL-4 [31] production from Th2 and ILC2, and, subsequently, to the lower periostin biosynthesis; (ii) might similarly influence bronchial epithelium leading to the decreased production of periostin and alarming-like cytokines; (iii) unlike aspirin challenge, long-term AD might decrease the influx of lymphocytes, most likely Th2 and ILC2, to respiratory tissue.

Periostin has also been described to be associated with sputum eosinophilia in asthma [34] and to be overexpressed in eosinophilic nasal polyps in N-ERD [35,36]. Besides a decrease in ISS periostin concentration, we have also observed a decrease in IS eosinophils. We hypothesize that AD leads to a decrease in a production of periostin in the respiratory tract with accompanying lower eosinophilic activity and, as a result, a reduced inflammatory state and a resolution of asthma symptoms. Unlike after the aspirin challenge, when numbers of blood eosinophils markedly decline [9], possibly due to their migration to the airways, long-term AD only weakly affects tissue distribution of these cells, even resulting in the increase of blood eosinophil count. A raised peripheral blood eosinophil count in N-ERD individuals subjected to high-dose aspirin therapy was also observed by other authors [37].

The hallmark of N-ERD is the dysregulation of pro- and anti-inflammatory products of arachidonic acid metabolism. PGE₂ exerts its anti-inflammatory effect through the control of Cys-LTs production, whose exact mechanism is not fully understood [38]. A single dose of aspirin blocks the biosynthesis of PGE₂ and leads to the abnormal biosynthesis of Cys-LTs [39]. The imbalance between pro- and anti-inflammatory eicosanoids after aspirin challenge was confirmed in previous studies by our research group [5,40]. The present study, however, showed that such changes do not occur during long-term aspirin intake since there were no alterations in any of the eicosanoids measured in ISS or NL after 52 weeks of AD in N-ERD patients. In course of a long-term AD, when no symptoms of bronchospasm as well as no clinically significant decline in FEV₁ value occur, there is also no decrease in PGE₂ concentration. Similarly, the levels of LTB₄ and Cys-LTs also remained unaltered in our study participants after 52 weeks of AD. On the contrary, Bobolea et al. [41] showed that AD resulted in the decreased ISS PGD₂ concentration on a constant LTC₄/PGE₂ ratio in the 1st and 6th months of aspirin administration. Interestingly, the PGE₂ concentration itself increased after the 6th month of AD. However, the eicosanoid evaluation methods, dose of aspirin and the time point of sputum collection during AD differed from our study, therefore the results cannot be directly compared. In light of our findings, it can be concluded that long-term aspirin administration does not lead to an imbalance between prostaglandin and leukotriene levels but somehow stabilizes cyclooxygenase and lipoxygenase pathways. It is plausible that there are some defects in COX enzyme activity in N-ERD, which results in its different activation state after single and during long-term aspirin intake. Sousa et al. [42] have shown a significant reduction of

Cys-LT₁ receptors (Cys-LT₁R) in nasal mucosa after AD. This indicates that beneficial effects of AD on respiratory mucosa might be mediated by the lower activity of Cys-LTs due to Cys-LT₁R downregulation, while the overall production of Cys-LTs remains unaltered.

The generation of Cys-LTs is also modulated by platelet-adherent inflammatory leukocytes, whose role has already been proposed in N-ERD [7]. As aspirin irreversibly blocks COX-1 and the formation of thromboxane A₂ in platelets, leading to an inhibition of platelet aggregation [43], its long-term intake probably results in a lower number of platelet-leucocyte aggregates.

The results of this study should be interpreted within the context of its limitations. The possible confounding factor in the evaluation of local periostin concentration could be ICS treatment, which is known to substantially reduce concentrations of IL-13, to downregulate *POSTN* gene expression [44] and, as a result, to decrease periostin level in the affected tissue. All participants were treated with the same dose of ICS either before or throughout AD in our study, which should minimize their effect on the overall results. However, inter-individual sensitivity to ICS and its influence on gene expression cannot be excluded.

5. Conclusions

The present study shows for the first time that 52 weeks of AD leads to a decrease in ISS periostin biosynthesis and IS eosinophil count. We have also demonstrated that AD stabilizes local prostanoid and leukotriene concentrations in ISS and NL. As the mechanism of AD remains the least understood aspect of N-ERD, we believe that our study has provided some missing links to previous observations and paves a new way to a better understanding of the clinical benefits of AD and optimal patient care.

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Conflicts of interest

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

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