Analysis of daily aspirin intake on platelet-associated factors and aggregation in nonsteroidal anti-inflammatory drug exacerbated respiratory disease: A cross-sectional study

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Abstract

Background: Nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (N-ERD) is a clinical syndrome characterized by chronic rhinosinusitis with nasal polyposis (CRSwNP), adult-onset asthma and hypersensitivity to NSAIDs. Long-term aspirin treatment after desensitization (ATAD) is used for clinical improvement in N-ERD patients. However, information on the potential effect of ATAD on the platelet-neutrophil aggregates (PNA) level in N-ERD patients is highly limited.

Objective: This study aimed to explore the impact of PNA on the pathogenesis of N-ERD and the potential effect of ATAD on N-ERD patient profiles from a platelet point-of-view.

Methods: Sixty-one individuals were enrolled, including 16 N-ERD patients with ATAD (ATAD+), 15 N-ERD patients without ATAD (ATAD-), 15 aspirin-tolerant asthma (ATA) patients, and 15 healthy controls (HCs). Lipid mediators classical in N-ERD, including urinary-LTE4 (uLTE4), prostaglandin-D2 (PGD2), and prostaglandin-E2 (PGE2) were assessed by ELISA. Platelet activation was estimated based on expression levels of sP-selectin, CD40L, Platelet Factor-4 (PF4), RANTES, Thromboxane-A2 (TXA2), PAF, 12-HETE in plasma levels by ELISA; and PNA percentage by flow cytometry.

Results: ATAD+; 12-HETE, and PF4 levels were remarkably low, while higher levels were determined in ATAD- and ATA groups. ATAD+; uLTE4 levels were positively correlated with 12-HETE. Another positive correlation was detected between sP-selectin and 12-HETE in ATAD-. Compared to HCs, it was found that among all N-ERD patients, significant increase in PNA.

Conclusions: Plasma levels of PGE2, PF4, and 12-HETE appear to be affected by aspirin treatment. We believe that 12-HETE could play a significant role in the N-ERD pathogenesis by contributing to platelet activation.

Key words: aspirin treatment after desensitization (ATAD), chronic rhinosinusitis with nasal polyposis, lipid mediators, nonsteroidal anti-inflammatory drug exacerbated respiratory disease (N-ERD), platelet-neutrophil aggregates, aspirin

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Introduction

Nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (N-ERD) is characterized by persistent rhinosinusitis, nasal polyposis, and aspirin (acetylsalicylic acid; ASA) or NSAIDs hypersensitivity and a dysregulation of arachidonic acid (AA) metabolism.[1]

After AA is released from membrane phospholipids, it can be metabolized by four different pathways: cyclooxygenases (COX), lipoxygenases (LO), cytochrome oxidases, and anandamide pathways.[2] In the cyclooxygenase pathway, AA is metabolized to prostaglandin (PG)E2, and PGD2 via COX enzymes. In N-ERD patients, even low doses of NSAIDs can permanently inhibit the COX1 enzyme, causing a shift in AA metabolism towards 5-lipoxygenase (5-LO) pathway, resulting in an excessive increase in cysteinyl leukotrienes (CysLTs), especially LTE4, a strong lipid mediator.[3] However, the mechanisms responsible for CysLT overproduction and PGD2/PGE2 imbalance remain to be unknown. In addition to COX and 5-LO pathways, AA can be metabolized via other enzymatic pathways, including the cytochrome P450 (CYP450) and 12-lipoxygenase (12-LO) enzymatic systems producing other hydroxyeicosatetraenoic acids (HETEs). Studies on N-ERD so far mainly focused on 5-LO and COX-1 pathways, and information regarding CYP450 and 12-LO enzyme pathways in N-ERD is highly limited.

Platelets and neutrophils, as well as eosinophils and mast cells, are the main players in N-ERD and crosstalk between these cells contribute to disease pathogenesis. The literature highlights a progressive platelet activity involved in N-ERD pathogenesis.[4] Platelets can form aggregates with leukocytes, including eosinophils, monocytes and neutrophils through cell surface proteins. Among other key inflammatory cells, neutrophils gained attention, since its activation has been associated with the severity of inflammation in N-ERD. Monocytes and eosinophils are able to produce CysLTs themselves, since they have two key enzymes for the production. Neutrophils and platelets, however, cannot produce CysLTs on their own because neutrophils lack one of the enzymes and platelets cannot synthesize other enzyme. However, when these two cells interact, they gain the ability to produce CysLTs.[5] Therefore, platelet-neutrophil aggregation (PNA) may potentially increase the production of CysLTs. The functional contribution of neutrophils in N-ERD seems to be limited.

Among the treatment options for N-ERD, long-term aspirin treatment after desensitization (ATAD) has resulted in considerable improvement in the clinical course and most of the patients have been reported to be able to tolerate aspirin desensitization (AD).[6] Although AD and daily administration of high-dose aspirin appears beneficial for most patients with N-ERD, some unresponsive patients require biologicals, suggesting a need for N-ERD-specific treatment options. Therefore, some studies focus on alternative anti-platelet therapies as an alternative option.[4]

The severity of airway inflammation in N-ERD was reported to be associated with neutrophil activation; however, the precise mechanism is still not completely understood. Furthermore, the effect of ATAD on PNA in N-ERD patients, as well as the relevance of this interaction to disease pathophysiology, remains unknown. This study aimed to find out more about the impact of PNA on the pathogenesis of N-ERD and the potential effect of ATAD on N-ERD patient profile from a platelet point of view. We also assessed plasma samples from patients for platelet-associated factors, which can potentially be associated with N-ERD.

Methods

Study Populations

In the current study, we included 61 individuals divided into four groups: 16 N-ERD patients receiving ATAD (ATAD+), 15 N-ERD patients without ATAD (ATAD-), 15 patients with aspirin-tolerant asthma (ATA), and 15 healthy controls (HCs) recruited at a tertiary care allergy outpatient clinic, which is a reference center experienced in the follow up of N-ERD patients. The diagnosis of chronic rhinosinusitis was made in accordance with the guidelines based on clinical symptoms, nasal endoscopy, and/or computed tomography. NSAID hypersensitivity was confirmed in all N-ERD patients with a single-blind oral aspirin provocation test.[7] Asthma was diagnosed and treated in accordance with the GINA guidelines.[8] ATA patients had physician-diagnosed asthma, could use non-selective COX inhibitors without experiencing allergic reactions or they failed the aspirin challenge test and did not have nasal polyposis. Peripheral venous blood and urine samples were collected from the participants in the morning between 8:00 AM and 10:00 AM. Ethical approval (2020/09-39) for this study was obtained by the Institutional Review Board and Commission at Hacettepe University, and all participants gave written informed consent.

Aspirin treatment after Aspirin Desensitization (ATAD)

ATAD was performed in patients with symptoms of rhinosinusitis who responded inadequately to appropriate medical treatment, with a history of recurrent sinus surgery, or who had severe asthma.[7] AD was initiated with a dose of 25 mg and escalated to doses of 50, 100, 150, and 300 mg each at 60 minutes intervals, and then continued with 300 mg of ASA daily. The details of the ATAD protocol and the interventions taken during the reactions were explained in detail supplementary material.

Plasma Protein Measurements

Blood samples were collected into blood collection tubes with EDTA. Plasma was separated by centrifugation for 10 minutes at room temperature at 1600xg andstored in aliquots at -80°C until analysis. The plasma levels of P-selectin, CD40L, Platelet Factor 4 (PF4), RANTES (R&D Systems, Minneapolis, USA), PAF, PGD2 (Elabscience, Houston, Texas, USA), 12-HETE (Abcam, USA), and PGE2 (Cayman Chemical, Michigan, USA) were detected by ELISA according to manufacturer's instructions.

Urinary Measurements

Spot urine samples were collected on the same day as blood collection. Samples were aliquoted and stored at -80°C. Direct enzyme immunoassay was used to assess uLTE4 in 1:10 diluted urine samples (Cayman Chemical). The measurement was performed in triplicates. Urinary LTE4 levels were reported in picograms per milligram of creatinine (pg/mg Cr).

Flow Cytometry Analysis

PNAs were determined by flow cytometry. Whole peripheral blood was collected into EDTA-anticoagulant tubes, stored at room temperature, and assayed within 1 hour of collection. We incubated 50 μl whole blood with directly conjugated antibodies specific for CD61, CD62P, CD11b, CD15, and CD16 antibodies (**Table S1**). PNAs were evaluated by counting double-positive cells for the platelet-specific markers (CD62P-CD61) on a dot plot obtained from the neutrophil gate (CD11b-CD15-CD16). Methodology relevant to flow cytometry and gating strategy were presented in the Supporting Information file (**Figure S1**). Antibody details were given in **Table S1**.

Statistical Analysis

The statistical analysis was performed using the SPSS, version 21 (SPSS, Chicago, Ill) and GraphPad Prism 9.0. Categorical variables were determined by using chi-square test. One-way ANOVA and Dunnette's post hoc or Kruskal-Wallis test were used to compare the groups and data are presented as median (interquartile range). Spearman's rank correlation test was applied for correlation analysis. A p-value below 0.05 was considered statistically significant. Flow cytometry results were analyzed with KALUZA V2.1.2 software.

Results

The clinical characteristics of participants

The clinical characteristics of the study population were shown in **Table 1**. Asthma severity was similar in N-ERD groups. The number of patients having nasal polyps (CRSwNP) and undergoing polypectomy was substantially lower in ATA patients than both NERD groups. ICS + LABA and LTRA use was lower in ATA group than ATAD+ patients ($p = 0.008$ and $p = 0.044$, respectively). Furthermore, all N-ERD patients had substantially higher eosinophil level than HCs. Serum total IgE levels were also significantly higher in N-ERD groups than HCs. ATA group also had higher total IgE levels compared to HCs, however this difference was not statistically significant ($p = 0.093$). The Short Form-36 (SF-36) quality of life scale's "general health" and "physical function" parameters were significantly higher in HCs than in N-ERD patients ($p = 0.001$ and $p = 0.002$, respectively). Sino-Nasal Outcome Test-22 (SNOT-22) scores were not different between groups.

Table 1. Clinical characteristics of the participants.

Characteristic	HC (n = 15)	ATAD + (n = 16)	ATAD - (n = 15)	ATA (n = 15)	p-value
Age (years)	40 (32-44)	48.5 (33-58.25)	40 (36-55)	49 (39-57)	0.414
Females, n (%)	10 (66.7)	13 (81.3)	10 (66.7)	12 (80)	0.672
Atopy, n (positive, %)	na	5 (31.3)	5 (33.3)	5 (33.3)	0.990
Asthma Duration (years)	na	15 (7.2-27.0)	10 (5-22)	9 (5-12)	0.205
ACT Score	na	24 (13.2-25)	22 (15-25)	23 (15-25)	0.947
Severe Asthma, n (%)	na	5 (31.1)	5 (33.3)	5 (33.3)	0.876
Family history of asthma, n (%)	na	10 (62.5)	7 (46.7)	4 (26.7)	0.134
CRSwNP, n (%)	na	16 (100.0)	14 (93.3)	**2 (13.3)**[††††/####]	**< 0.0001**
Number of polypectomies in the past	na	2 (2-3)	2 (1-4)	**0 (0-0)**[††††/####]	**< 0.0001**
Polyp Duration (years)	na	11.5 (6.7-19.5)	13 (1-20)	0 (0-0)	0.361
NSAIDs Hypersensitivity (years)	na	8.5 (3.5-17)	10 (2-10)	na	0.416
ICS use, n (%)	na	2 (12.5)	5 (33.3)	6 (40.0)	0.205
ICS + LABA use, n(%)	na	13 (81.3)	7 (46.7)	**4 (26.7)**[††]	**0.009**
LTRA use, n (%)	na	14 (87.5)	11 (73.3)	**7 (46.7)**[†]	**0.044**
Nasal Steroid use, n (%)	na	12 (75.0)	10 (66.7)	6 (40.0)	0.117

Table 1. (Continued)

Characteristic	HC (n = 15)	ATAD + (n = 16)	ATAD - (n = 15)	ATA (n = 15)	p-value
Hemoglobin (gr/dL)	13.3 (12.3-15.2)	13.10 (12.58-14.45)	14 (13.3-15.0)	14 (13.0-14.5)	0.553
Neutrophil (× 10³/uL)	4.1 (2.9-5.1)	4.4 (3.22-5.57)	5.0 (3.8-7.0)	4.4 (3.7-5.1)	0.265
Eosinophil (× 10³/uL)	0.1 (0.1-0.2)	**0.3 (0.1-0.87)***	**0.4 (0.2-0.5)***	0.1 (0.1-0.3)	**0.006**
Neutrophil (%)	58.8 (55.8-60.8)	57.6 (52.05-61.87)	60.4 (58.7-63.3)	59.6 (56.5-65.2)	0.333
Eosinophil (%)	1.40 (1.0-1.9)	**4.45 (2.05-10.5)***	**4.10 (3.2-5.8)***	1.70 (0.8-3.9)	**0.004**
Platelet (× 10³/uL)	277.0 (245.0-331.0)	249.5 (220.2-335.7)	267.0 (240.0-324.0)	257.0 (236.0-318.0)	0.703
Serum Total IgE (UI/mL)	25.8 (9.9-57.0)	**99.1 (58.4-168.5)****	**95.5 (35.1-197.0)***	73.3 (33.6-180.0)	**0.002**
Urine creatinine (mg/dL)	106.9 (60.1-204.9)	105.8 (44.9-128.7)	104.3 (42.7-183.0)	104.5 (85.5-144.7)	0.817
ATAD duration (weeks)	na	241.5 (39.75-618.50)	na	na	-
SF-36 Score					
Physical function	95.00 (90.00-100.00)	**65.00 (46.25-85.00)****	**75.00 (40.00-90.00)***	75.00 (55.00-90.00)	**0.002**
Role-physical	100.00 (75.00-100.00)	100.00 (31.25-100.00)	**50.00 (0.00-75.00)***	100.00 (50.00-100.00)	**0.050**
Role-emotional	66.67 (66.67-100.00)	66.67 (33.33-66.67)	33.33 (33.33-100.00)	66.67 (33.33-66.67)	0.059
Vitality	62.50 (50.00-75.00)	56.25 (37.50-62.50)	50.00 (37.50-75.00)	62.50 (37.50-75.00)	0.411
Mental health	68.75 (62.50-81.25)	59.45 (43.75-79.75)	62.50 (43.75-75.00)	56.25 (43.75-75.00)	0.313
Social functioning	100.00 (75.00-100.00)	75.00 (50.00-100.00)	75.00 (50.00-100.00)	50.00 (25.00-100.00)	0.203
General health	70.00 (65.00-85.00)	**40.00 (30.00-60.00)****	**45.00 (20.00-50.00)****	**50.00 (35.00-55.00)***	**0.001**
Bodily pain	80.00 (67.50-100.00)	77.50 (45.00-90.00)	57.50 (45.00-100.00)	70.00 (35.00-100.00)	0.315
SNOT-22 Score	na	44.00 (20.50-66.25)	57.00 (23.00-66.00)	37.00 (24.00-59.00)	0.651

N-ERD, Nonsteroidal anti-inflammatory drug exacerbated respiratory disease; NSAID, Nonsteroidal anti-inflammatory drug; ATAD, aspirin treatment after aspirin desensitization; ATAD+, N-ERD patients recieving ATAD; ATAD-, N-ERD patients without ATAD; ATA, aspirin tolerant asthma; ACQ, asthma control test; CRS, chronic rhinosinusitis; CRSwNP, CRS with nasal polyposis; ICS, inhaled corticosteroid; LABA, long-acting β2 agonist; LTRA, Leukotriene receptor antagonist; na, nonapplicable, SF-36, Short Form-36; SNOT-22, Sino-Nasal Outcome Test-22. Data are presented as medians (interquartile)

*(p < 0.05), **(p < 0.01) Significantly different compared with healthy controls
†(p < 0.05), ††(p < 0.01), ††††(p < 0.0001) Significantly different compared with ATAD+
‡‡‡‡(p < 0.0001) Significantly different compared with ATAD-

N-ERD-associated lipid mediators

Although there is an increasing trend in urinary LTE4 (uLTE4) levels in ATAD+ patients, there were no significant differences between groups (**Figure 1A**). We observed a slight increase in the plasma PGD2 level in ATAD- group compared to HCs (p = 0.158). The plasma PGD2 level, on the other hand, was significantly higher in ATAD+ and ATA group compared to HC (p = 0.007 and p < 0.0001, respectively) but there were no differences between ATAD+ and ATAD- groups (p = 0.144) (**Figure 1B** and **Table 2**). Also, PGE2 levels were markedly lower in the ATAD+ group than HC (p < 0.0001) and ATAD- group (p = 0.0001) (**Figure 1C**). Similar to PGD2 levels, PGE2 plasma levels were also significantly higher in ATA group (p < 0.0001). There was no correlation between ATAD duration and lipid mediators in ATAD+ patients (**Table S2**).

Serum levels of platelet-associated factors

The level of PF4 was higher in the ATAD- and ATA groups than ATAD+ group (p = 0.026 and p = 0.004, respectively; **Figure 2A**). sP-selectin levels were not significantly different between both N-ERD groups in our study, but there was a significant increase in ATA group compared to ATAD+ patients (p = 0.006; **Figure 2B**). RANTES levels in ATAD+ group showed a decreasing trend compared to ATAD- group, but it was not statistically significant (**Figure 2C** and **Table 2**). Furthermore, there were a profound increase in RANTES levels in ATA group compared to ATAD+ patients (p < 0.001; **Figure 2C**). We observed a marginal difference in 12-HETE level of ATAD- group compared ATAD+ patients (p = 0.049; **Figure 2G**). ATA group also had higher plasma 12-HETE levels than ATAD+ participants (p = 0.002). There were no significant differences in plasma levels of sCD40L, PAF, and TXA2 molecules between the groups (**Figure 2E-F-G**).
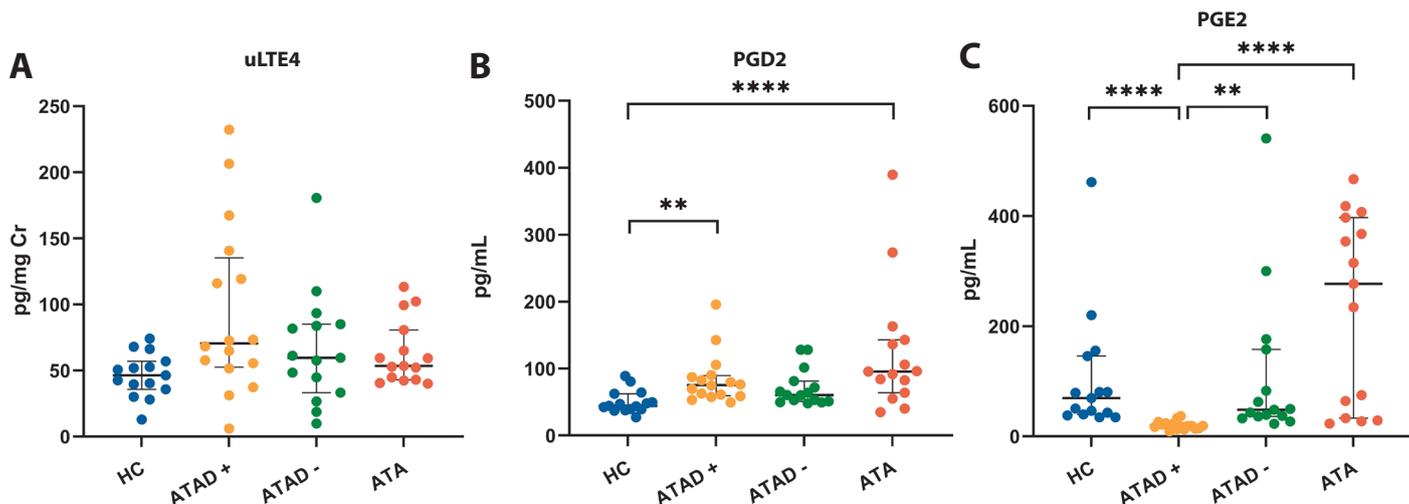
Figure 1. Comparison of urinary leukotriene E4 (uLTE4) (A), plasma levels of prostaglandin (PG)D2 (B), and PGE2 levels (C) in N-ERD patients receiving ATAD (ATAD+; n = 16), N-ERD patients without ATAD (ATAD-; n = 15), patients with aspirin-tolerant asthma (ATA; n = 15) and healthy control (HC; n = 15). N-ERD: nonsteroidal anti-inflammatory drug exacerbated respiratory disease, ATAD: aspirin therapy after aspirin desensitization. Data are presented as median (interquartile range). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

Table 2. Comparisons of the levels of platelet-associated factors between groups.

ELISA	HC n = 15	ATAD+ n = 16	ATAD- n = 15	ATA n = 15	p
sP-Selectin (ng/mL)	40.21 (33.72-58.91)	34.39 (25.98-43.38)	40.73 (32.99-106.70)	86.06 (54.03-105.26)[††]	0.012
CD40L (pg/mL)	310.3 (92.71-1146.61)	184 (30.98-995.6)	297 (77.11-535.1)	280.4 (115.8-1213)	0.492
PF4 (ng/mL)	670.02 (260.43-1271.08)	426.06 (135.91-785.83)	1690.09 (463.73-5353.10)[†]	3025.74 (1286.07-3340.75)[**,††††]	0.002
PAF (pg/mL)	1429 (366.7-1853)	1022 (328.4-1984)	571.8 (161.1-1481)	1249 (437.4-2099)	0.597
TXA2 (pg/mL)	487.4 (399.7-552)	379.3 (360.6-483.8)	389.3 (369.8-446.3)	494.9 (416.2-520.7)	0.063
RANTES (ng/mL)	13.32 (7.42-21.93)	8.09 (4.75-11.18)	18.65 (4.84-23.66)	24.33 (15.75-27.16)[††††]	0.001
PGD2 (pg/mL)	44.32 (39.17-62.46)	75.46 (59.43-89.49)[**]	60.57 (51.06-81.37)	95.58 (63.89-142.80)[****]	< 0.0001
PGE2 (pg/mL)	69.47 (39.81-145.90)	19.03 (14.40-23.95)[****]	48.18 (36.34-158)[††]	276.90 (33.05-397)[††††]	< 0.0001
12-HETE (ng/mL)	4.212 (2.442-8.606)	2.445 (1.073-4.568)	4.130 (2.803-10.205)[†]	25.884 (3.457-36.598)[††]	0.003
uLTE4 (pg/mg Cr)	46.39 (35.76-56.86)	70.48 (52.52-135.3)	59.55 (33.27-85.06)	53.43 (42.98-80.60)	0.059

Definition of abbreviations: N-ERD, nonsteroidal anti-inflammatory drug exacerbated respiratory disease; ATA, aspirin-tolerant asthmatic; ATAD, aspirin treatment after aspirin desensitization; ATAD+, N-ERD patients receiving ATAD; ATAD-, N-ERD patients without ATAD; uLTE4, urinary leukotriene E4; sP-selectin, soluble P-selectin; sCD40L, soluble CD40 ligand; TXA2, thromboxane A2; 12-HETE, 12-hydroxyeicosatetraenoic acid; RANTES, regulated on activation, normal T cell expressed and secreted; PGD2, prostaglandin D2; PGE2, prostaglandin E2. Data are presented as medians (interquartile)

($p < 0.01$), **($p < 0.0001$) Significantly different compared with healthy controls
[†]($p < 0.05$), [††]($p < 0.01$), [††††]($p < 0.0001$) Significantly different compared with ATAD+
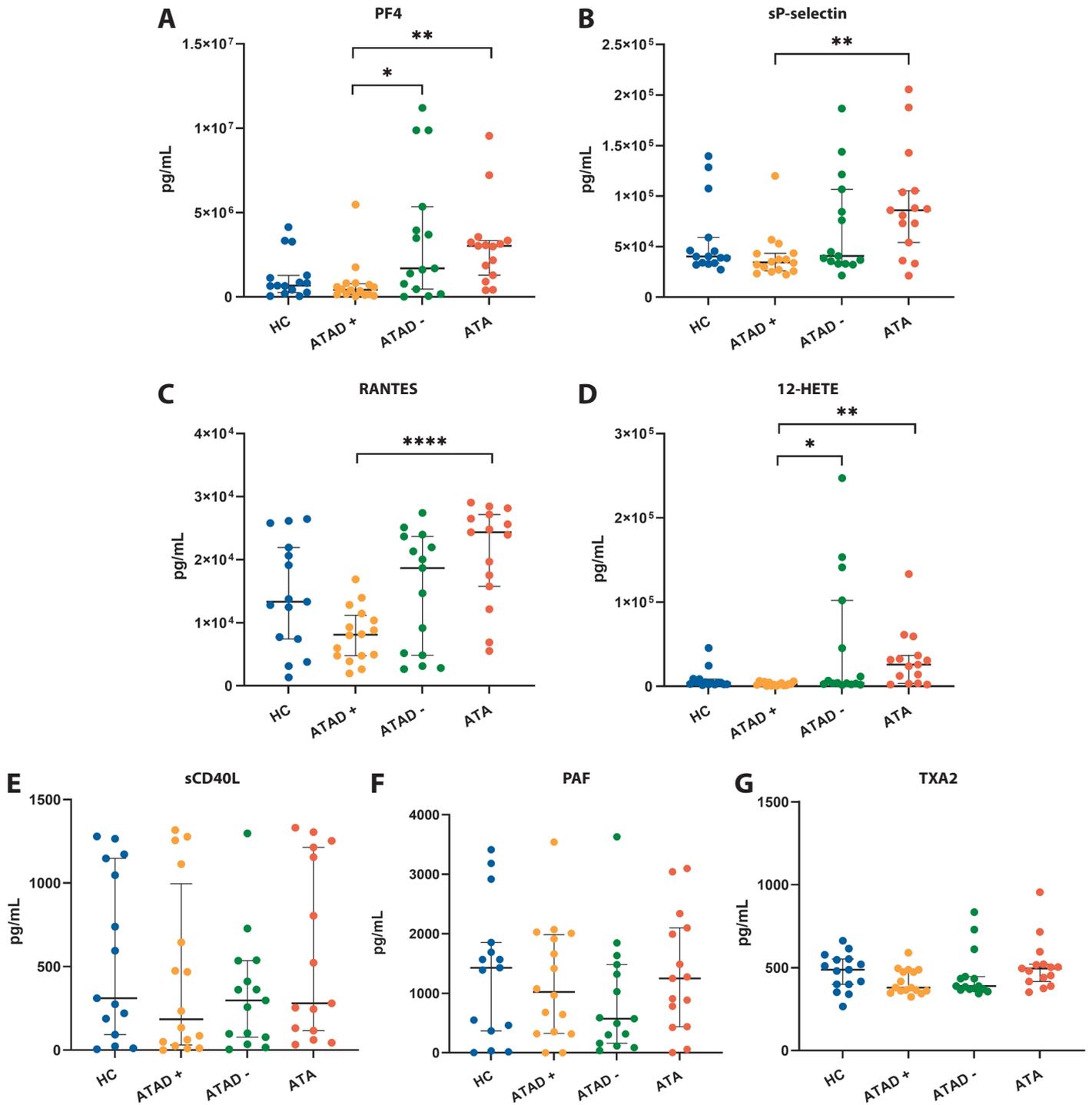
Figure 2. Comparison of plasma levels of Platelet-factor 4 (PF4) (A), sP-selectin (B), RANTES (C), 12-hydroxyeicosatetraenoic acid (12-HETE) (D), CD40L (E), Platelet activating factor (PAF) (F), and Thromboxane A2 (TXA2) (G) in N-ERD patients receiving ATAD (ATAD+) (n = 16) and N-ERD patients without ATAD (ATAD-) (n = 15), patients with aspirin-tolerant asthma (ATA; n = 15) and healthy control (HC; n = 15). N-ERD: nonsteroidal anti-inflammatory drug exacerbated respiratory disease, ATAD: aspirin therapy after aspirin desensitization. Data are presented as median (interquartile range). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

In ATAD+ group, a positive correlation was detected between uLTE4 and 12-HETE as well as between RANTES and PF4, but no significant difference was found between platelet-associated factors and ATAD duration (**Table S2**). sP-selectin level was positively correlated with 12-HETE, PGE2, and RANTES, while a negative correlation was observed with PGD2 (**Table S3**) in ATAD- group.

Flow cytometry results of platelet-neutrophil aggregates (PNA)

PNA percentage was higher in both ATAD- ($p = 0.028$; **Figure 3**) and ATAD+ ($p = 0.015$) groups compared to HCs. However, the difference in PNA between the ATAD+ and ATAD- patients was not statistically significant, although a slight decrease was observed.
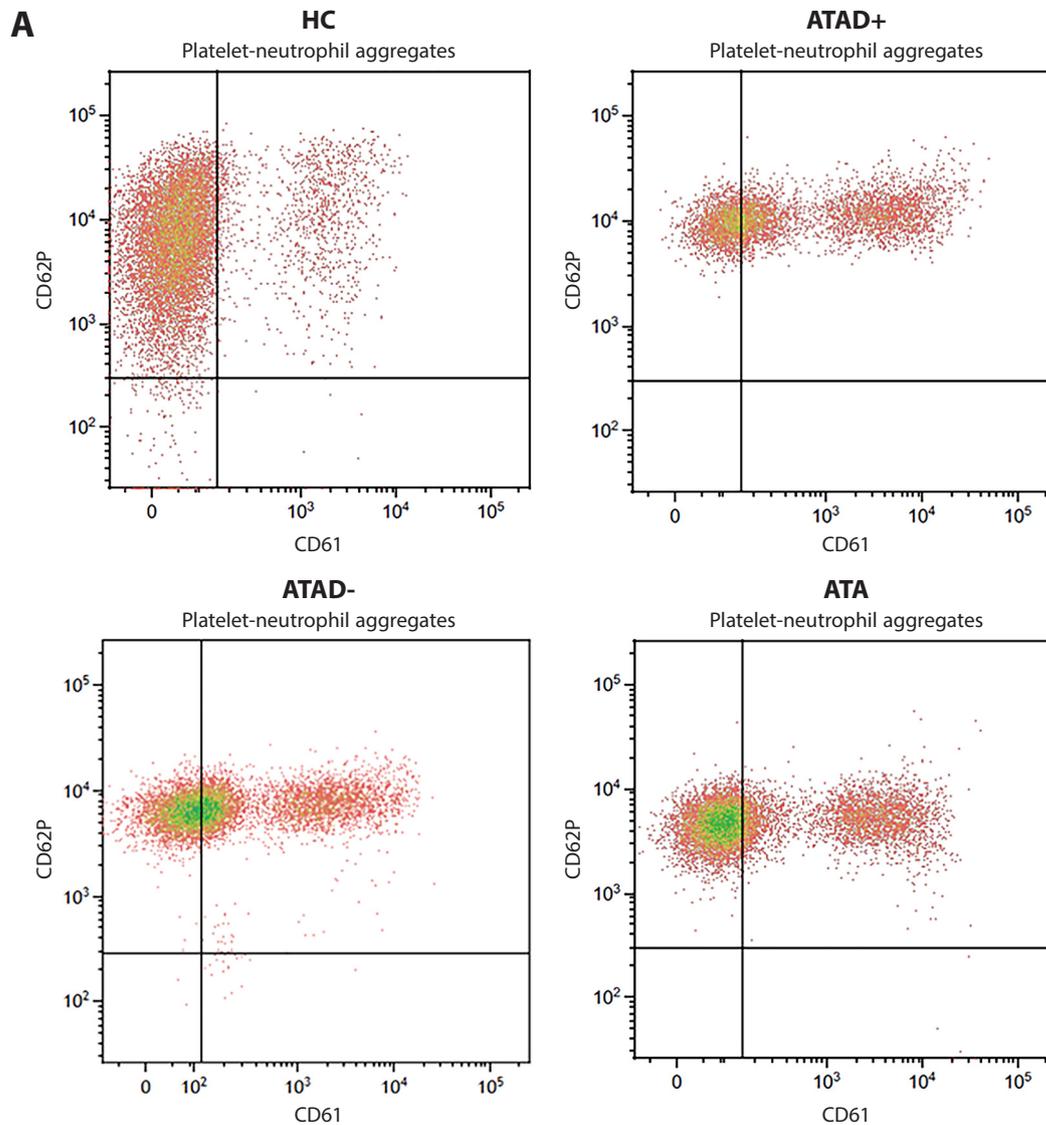
Figure 3. Comparison of whole blood platelet-neutrophil aggregates (PNA) (A) Representative histogram results for the groups (B) Percentage of PNAs from all participants (C) N-ERD (ATAD+) (n = 16) and N-ERD (ATAD-) (n = 15), patients with ATA; (n = 15) and HC; (n = 15). N-ERD: nonsteroidal anti-inflammatory drug exacerbated respiratory disease, ATAD: aspirin therapy after aspirin desensitization, CD62P (also known as P-selectin): platelet activation marker, CD61: platelet marker. Data are presented as median (interquartile range). *$p < 0.05$, **$p < 0.01$.
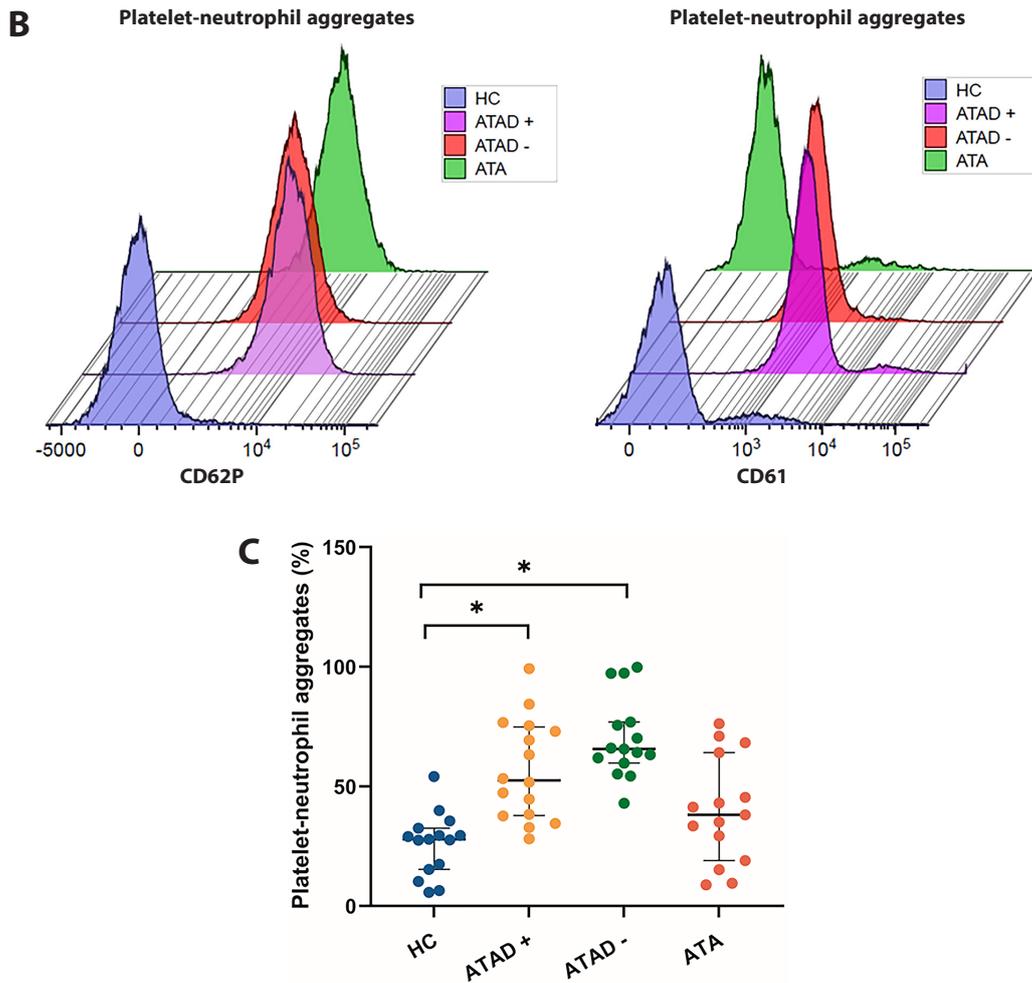
Figure 3. (Continued)

uLTE4 levels were positively correlated with the percentage of PNA in all participants (**Table S4**). Also, PNA percentage was positively correlated with plasma sP-selectin (CD62P) levels and PF4 in ATAD+ patients (**Table S4**). No significant difference was found when comparing the duration of aspirin treatment and PNA levels (**Table S4**).

Discussion

The current study evaluates the key lipid mediators and platelet-related molecules involved in the pathogenesis of N-ERD, particularly focusing on the differences with and without ATAD. We observed lower plasma levels of PGE2, PF4, and 12-HETE levels in ATAD+ group, suggesting a potential involvement of these molecules in N-ERD pathophysiology. Additionally, the correlations identified between lipid mediators, including 12-HETE and platelet activation may provide insight into the mechanism behind platelet aggregation and neutrophil recruitment in this disease, although it requires confirmation with further investigations. Our findings may indicate that aspirin desensitization therapy (ATAD) promote clinical improvement by potentially modulating inflammatory markers like RANTES and PF4.

Lipid mediators, including uLTE4, PGD2, and PGE2, which are the major players in AA metabolism were evaluated. We observed an increasing trend in uLTE4 levels in N-ERD patients, although it was not statistically significant. The current literature suggests that elevated uLTE4 levels are common in N-ERD patients but also indicates high variability among studies. Higashi et al. demonstrated that uLTE4 levels can vary depending on disease severity, showing only a trend in intermittent and mild asthma cases, which was not statistically significant.[9] Similarly, Cahill et al. reported no significant difference in basal uLTE4 levels between NERD patients who can tolerate aspirin desensitization protocol and ATA groups.[10] Another study reported that although higher uLTE4 levels were found in N-ERD patients than ATA participants, uLTE4 measurement has limited specifity to correctly identify all aspirin-intolerant individuals.[11] Researchers also stated that uLTE4 might not be a powerful biomarker as only some subtypes had greater levels, but it could be utilized to identify N-ERD subtypes for better disease management.[12] These findings support the idea that although uLTE4 is a potential biomarker, its utility may be limited due to variability among N-ERD phenotypes.

Prostaglandin D2 (PGD2) is another critical mediator in N-ERD pathophysiology. We found that both N-ERD groups, but particularly aspirin-tolerant asthmatics, had higher PGD2 levels. The literature on PGD2 levels following AD presents contradictory findings. A study showed that despite clinical improvement, there was an increasing trend in uLTE4 and PGD metabolite levels during aspirin treatment, aligning with our findings.[13] However, Lee et al. found that baseline plasma PGD2 level was not different between NERD and ATA patients.[14] Mastalerz et al. also showed that PGD2 level in exhaled breath condensate were not different between aspirin intolerant asthmastics and aspirin tolerant asthmatics both at baseline and during aspirin challenge.[15] This may suggest that PGD2 levels may not serve as a robust biomarker for aspirin desensitization. These findings could be an indicator of the complexity of lipid mediator dynamics in N-ERD and the variability coming from different phenotypes may limits the generalizability of specific biomarkers for disease characterization.

Another AA metabolite PGE2 is suggested to have a protective function in the pathogenesis of N-ERD.[16] We observed that PGE2 level was lower in ATAD+ group. Basal PGE2 levels have been reported to be diminished in N-ERD patients compared to HCs, however others indicated that, as in uLTE4 and PGD2, basal PGE2 levels also varied among N-ERD patients from different subtypes.[11] Aspirin has a complex effect on PGE2 levels. While there are conflicting reports in the literature about how aspirin treatment impacts PGE2 levels, the overall inhibitory effects of NSAIDs on AA metabolism—especially their ability to lower PGE2 levels in various biological contexts—are well-documented[17,18] and consistent with our findings. Researchers demonstrated a significant decline in PGE2 levels following both long time low and high dose aspirin treatment.[19] Additionally, Boutaud et al reported a drastic decrease in PGE2 production during aspirin challenge.[20] Moreover, Kodela et al. established that all positional isomers of aspirin can effectively decrease PGE2 production by nearly 80%.[21] The robust reduction in PGE2 production observed with ATAD further emphasizes its pivotal role in regulating inflammatory responses in N-ERD. This dual nature—being both a protective mediator and a key molecule in aspirin's mechanism of action—highlights the complex dynamics of PGE2 within the AA pathway and brings the idea of a potential biomarker for guiding personalized treatment approaches in N-ERD.

In AA metabolism, 12-HETE, produced by 12-lipoxygenase (12-LO), is another important metabolite, which has been shown to modulate platelet activation and neutrophil chemotaxis.[22] We found that plasma 12-HETE level was extremely low in ATAD+ group, while higher levels were determined in other disease groups. However, the potential role of 12-HETE in N-ERD is scarce in the current literature and studies assessing its involvement in asthma often focus on general asthma phenotypes rather than N-ERD specifically. Reports showed that aspirin/ASA can reduce the production of 12-HETE.[23] Scott et al. showed that 12-HETE level was lower in the mucus of N-ERD and CRSwNP patients than in HCs and no correlation was detected

between 12-HETE and asthma severity.[22] One potential reason for the disparity between our results and those of Scott et al. could be attributed to the origin of the 12-HETE. In ATAD+ patients, uLTE4 levels were positively correlated with 12-HETE, raising the possibility that AA metabolism in N-ERD pathogenesis may also shift towards 12-LO pathway. Also, another positive correlation was detected between sP-selectin and 12-HETE in ATAD- group, which is compatible with the fact that 12-HETE is involved in pro-inflammatory processes and molecules produced by platelets. Mastalerz et al. showed that 12-HETE level was halved in aspirin intolerant asthmastics after aspirin challenge.[15] Others reported that aspirin challenge significantly reduced platelet-derived 12-HETE production in healthy individuals.[24] The same study also indicated that lower 12-HETE levels were correlated with a higher reduction in aspirin-mediated platelet aggregation, which may suggest a role for 12-HETE in mediating platelet aggregation. Such role for 12-HETE was also attributed by others.[25] Although the decrease in 12-HETE level in ATAD+ group in our study was not directly reflected to platelet-neutrophil aggregation, it may be associated with clinical improvement. Thus, we believe that 12-HETE could be an important player in the pathogenesis of N-ERD through its contribution to platelet activation.

PF4 has been implicated in bronchoconstriction, as well as in the activation of eosinophil and neutrophils, which are central to the inflammatory processes in N-ERD.[26] We found that plasma PF4 levels were higher in ATAD- and ATA patients than ATAD+ group, which suggesting that aspirin therapy may potentially mitigate platelet activation in individuals with N-ERD. Although Laidlaw et al. previously presumed a role for PF4 in N-ERD,[26] there is no study investigating the effect of ATAD on PF4 or its involvement in the clinical improvement in this disease. PF4 has been shown to promote IgG and IgE receptor expression as well as the activation of basophil, eosinophil, and other inflammatory cells, exacerbating allergic response.[27] Although we could not observe a direct correlation between plasma PF4 and IgE levels in our study, we detected a positive correlation between PF4 and inflammatory cytokine RANTES, as well as platelet-neutrophil aggregation in ATAD+ group, which may suggest that decrease in PF4 may be associated with clinical improvement in N-ERD patients who took long term aspirin therapy.

Platelets can interact with inflammatory cells through sP-selectin, which can promote subsequent inflammatory responses.[28] Our findings showed that N-ERD patients not receiving ATAD had slightly increased sP-selectin levels compared to HCs, which may be an indicator of an ongoing inflammation. This finding is consistent with existing literature, which has reported higher basal plasma sP-selectin levels in N-ERD patients compared to HCs and no significant change in neither N-ERD group nor ATA patients after aspirin challenge compared to baseline levels.[28] We observed no significant change in sP-selectin levels following ATAD, further suggesting that aspirin therapy may not directly influence this marker of platelet activation.

Shifting focus to another important molecule mediating platelet-leukocyte interactions, we also measured soluble CD40 ligand (sCD40L) levels. Previous studies have demonstrated an increase in sCD40L levels following exercise and allergen challenge in asthmatic patients.[28] However, the role of sCD40L in N-ERD patients and its response to aspirin desensitization remains to be clarified. Mitsui et al. reported higher basal sCD40L levels in N-ERD patients compared to both HCs and ATA patients, with no subsequent changes observed after aspirin challenge.[28] Our study showed no significant difference in sCD40L levels between the groups. The disparity between these findings may be partly due to methodological differences as Mitsui et al. assessed the response after aspirin challenge but not long-term aspirin therapy. Our finding suggests that sCD40L may not serve as an essential mediator in N-ERD pathophysiology.

RANTES is a well-established potent chemoattractant which can be stored in granules of platelets and mediates leukocyte migration and inflammation.[29] While RANTES has been widely investigated in various inflammatory conditions, its involvement in N-ERD pathogenesis remains largely unknown and the potential contribution of RANTES in clinical improvement following ATAD has not yet been investigated. Pods et al. previously reported elevated RANTES production in nasal secretion of N-ERD patients.[30] Our findings also revealed RANTES level was slightly higher in ATAD- group although N-ERD patients who received ATAD exhibited lower RANTES levels. This finding may suggest that ATAD may be at least partially responsible for the reduction in this inflammatory mediator, thus contributing to the clinical improvement observed in these patients.

Although platelet-leukocyte aggregates are also found in healthy individuals, their level has been shown to be elevated in various diseases. In our study, we found a significant increase in platelet-neutrophil aggregates (PNA) among all N-ERD patients compared to healthy controls. Previous studies by Mitsui et al. and Laidlaw et al. also reported higher PNA levels in N-ERD patients compared to healthy controls and ATA patients.[28,31] These findings suggest that platelet-neutrophil aggregates may be actively involved in N-ERD pathogenesis, as indicated by the elevated PNA levels. The substantial variation in PNA levels among N-ERD patients further suggests that the inflammatory status may also vary among patients, probably due to the different phenotypes of the disease. Interestingly, N-ERD patients receiving ATAD exhibited slightly lower PNA levels compared to those without ATAD, although this difference did not reach statistical significance. This finding may suggest that while ATAD treatment improves general clinical status in N-ERD, this improvement may not be strictly reflected to PNA level in N-ERD patients.

There are several limitations to this study. The major limitation of this study is that we could not evaluate the same participants before and after desensitization, which would eliminate the undesired effects of confounding factors. However, in our cross-sectional study design, we tried to match all the confounding factors as much as possible,

and in this context, the clinical characteristics of both N-ERD groups were highly similar. Additionally, the sample size of the groups remained slightly smaller due to an insufficient number of patients. In our previous studies, we demonstrated the clinical benefits of using 300 mg of aspirin for comparable efficacy with better safety profile.[32-34] Since effective dose for ATAD may range from 300 to 1300 mg,[7] the difference in dosage may affect the levels of mediators we evaluated, thereby influencing the outcomes. However, the significant differences between different groups support the presence of some molecules that take part in the neutrophil and platelet aggregation may play a role in the pathogenesis of N-ERD patients. Further research is required to understand the nature of platelet activation in N-ERD, since plasma markers may not always accurately reflect platelet activity in the airways.

Conclusion

Taken together, plasma levels of PGE2, PF4, and 12-HETE seem to be affected by daily aspirin intake. PNA percentage also showed a decreasing tendency, although the change was not statistically significant. Our study results suggest that suppression of platelet activation in patients with N-ERD may be a potential therapeutic target for disease pathogenesis. However, since N-ERD shows high heterogeneity, developing a standardized process to identify different N-ERD subtypes or classes is critical to discover more accurate biomarkers.

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Supporting Information

Study population and management of aspirin intolerance

Sixty-one participants in four groups, including 16 N-ERD patients with ATAD (ATAD+), 15 N-ERD patients without ATAD (ATAD-), 15 with aspirin tolerant asthma (ATA) who were followed up at Hacettepe University Faculty of Medicine, Department of Allergy and Asthma, and 15 healthy controls (HCs) were recruited in the study.

N-ERD patients had a history of physician-diagnosed asthma and chronic rhinosinusitis with nasal polyposis (CRSwNP), as well as clinical hypersensitivity responses to oral aspirin with upper and lower airway involvement verified by aspirin challenge test. Nasal polyps were diagnosed based on the clinical examination performed by ENT clinicians and, if necessary, by paranasal CT, and confirmed by pathological results in patients who underwent endoscopic sinus surgery. Patients with physician-diagnosed asthma who could use any non-selective COX inhibitor without an allergic reaction, or who did not have a reaction in the aspirin challenge test, and who did not have nasal polyposis were included in the ATA group. The diagnosis of asthma was made by a pulmonologist or allergist according to the Global Initiative for Asthma (GINA) standards. All asthma patients met the criteria for asthma. No asthma exacerbations or respiratory infections were observed in asthmatic patients during the six weeks of the study.

Single-blind and placebo-controlled aspirin challenge test was performed in the allergy clinic under medical supervision. All patients underwent pre-test clinical examination and FEV1 measurement by spirometry. Only patients with controlled asthma and FEV1 > 70% predicted were included in the test. Aspirin was administered in increasing doses, 100-250-500 mg, at a maximum dose of 850 mg, to the patients who had no reaction to the placebo on the first day. The test was considered positive if there was a 20% decrease in Forced expiratory volume in 1s (FEV1) from baseline, nasoocular/bronchial symptoms, and/or severe extra-bronchial symptoms of aspirin hypersensitivity during the provocation test.

The N-ERD patient group receiving AD treatment was selected from patients whose aspirin sensitivity was proven by an aspirin challenge test and who had been using regular aspirin treatment for at least eight weeks after AD. Patients receiving aspirin treatment after desensitization (ATAD) consisted of patients desensitized with 300 mg aspirin and using aspirin daily. ATAD was performed in patients with symptoms of rhinosinusitis who responded inadequately to pharmacological treatment, with a history of recurrent sinus surgery, and who had symptoms of difficult-to-treat asthma. Before desensitization, all patients were carefully examined and FEV1 values were noted, and those who had no clinical signs of asthma exacerbation and had an estimated FEV1 above 70% were included in the desensitization protocol.

Since acetylsalicylic acid medications are presented as 100-300 and 500 mg tablets in Turkey, smaller doses were obtained by dividing the tablets. Aspirin desensitization was performed starting from a dose of 25 mg and increasing the doses of 50, 100, 150, and 300 mg, respectively, at 60 minutes intervals, and then continued with 300 mg of ASA daily. The procedure was performed in the allergy clinic and supervised by experienced allergists. Blood pressure, pulse rate, and peak expiratory flow were measured at baseline and every 30 minutes during the procedure. If any clinical symptoms occurred during desensitization, desensitization was stopped and necessary treatment was administered. If the reaction resolved within 2 hours, the same reaction dose was repeated on the same day, otherwise the next day, and the procedure was continued as planned. On the second day of the AD protocol, the patients were observed for 3 hours after 300 mg of aspirin and if no reaction was observed, 300 mg of aspirin per day was continued.

In addition, no intervention was made in the treatments of the four researched groups. All participants were between the ages of 18 and 70, and they were not pregnant, non-breastfeeding and were not current smokers. Patients were excluded if they currently used an anticoagulant or any antiplatelet drugs. Furthermore, participants with autoimmune disease, cardiovascular disease, or undertreatment of hypertension and diabetes mellitus were excluded from the study. None of the patients were users of biological agents. The patient reported nasal symptom severity was recorded using the Sinonasal Outcome Test-22 (SNOT-22). Also, Short Form-36 (SF-36) quality-of-life questionnaires was administered.

SNOT-22 is a widely used, validated, patient-reported outcome measure for chronic rhinosinusitis (CRS). It consists of 22 items rated from 0 to 5, with 0 indicating the best 5 worst clinical symptoms. Overall, SNOT-22 scores range from 0 to 110, with higher SNOT-22 total scores indicating more severe symptoms. Although there are no specific thresholds for staging SNOT-22, a statistically validated definition of the total score has been proposed as 8-20 mild, > 20-50 moderate, and > 50 severe.

SF-36 is a validated scale used to assess the quality of life in both patients and the healthy population. It evaluates the last 4 weeks and is divided into two main groups as physical and mental health. Items are summed between 0-100 points. Lower outcomes represent a lower quality of life.

Flow cytometric analysis of platelet-neutrophil aggregates

Whole blood (50 µl) was incubated with directly labeled antibodies for 20 minutes. 1.5 mL of 1X red blood cell lysis solution (BioLegend, France) was used to lyse erythrocytes. Cell samples were washed with 1 ml PBS 2 times and assessed immediately using a BD FACSCelesta™ flow cytometer. Cytometer settings were initially adjusted for the sample analysis and not changed during the experiment.

Phycoerythrin (PE) conjugated anti-CD61, brilliant violet (BV)785 conjugated anti-CD62P (P-selectin) and BV421 conjugated anti-CD15 were purchased from BioLegend. Fluorescein isothiocyanate (FITC)-conjugated anti-CD11b was from Elabscience. Alexa Fluor® 700 anti-human CD16 was from BD Biosciences. The corresponding isotypes (BioLegend, Elabscience) were utilized as controls. The antibody list containing clone information, catalogue numbers, and brands were given in **Table S1**. The preparation of blood samples and gating strategy for flow cytometric analysis were presented in **Figure S1**.

Correlation analysis

Spearman's rank correlation test was used for correlation analysis. A p-value below 0.05 was considered statistically significant. Correlation results of N-ERD-related factors among N-ERD patients were presented in **Table S2** for ATAD+ group and in **Table S3** for ATAD- patients. Correlation between platelet-adherent neutrophil with the levels of other N-ERD-associated factors among all participants were given in **Table S4**.

Table S1. List of antibodies used for flow cytometer analysis.

Antibody	Cell Type	Clone	Isotype	Company	Cat #	Vol/Test
Brilliant Violet 421 Anti-Human CD15	Neutrophil	W6D3 MOPC-21	Mouse IgG1, κ	BioLegend	323040	2.5 μL
Brilliant Violet 421 Isotype Control				BioLegend	400158	2.5 μL
Alexa Fluor 700 Anti-Human CD16	Neutrophil	3G8 MOPC-21	Mouse IgG1, κ	BD	560713	2.5 μL
Alexa Fluor 700 Isotype Control				BioLegend	400144	2.5 μL
FITC Anti-Human CD11b	Neutrophil	ICRF4 MOPC-21	Mouse IgG1, κ	Elabscience	E-AB-F1146C	2.5 μL
FITC Isotype Control				Elabscience	E-AB-F09792C	2.5 μL
PE Anti-Human CD61	Platelet	VI-PL2 MOPC-21	Mouse IgG1, κ	BioLegend	336406	2.5 μL
PE Isotype Control				BioLegend	400114	2.5 μL
Brilliant Violet 785 Anti-Human CD62P	Platelet	AK4 MOPC-21	Mouse IgG1, κ	BioLegend	304942	2.5 μL
Brilliant Violet 785 Isotype Control				BioLegend	400170	2.5 μL

Table S2. Correlation of N-ERD-related factors in ATAD+ patients.

	ATAD+													
	uLTE4		sP-selectin		PF4		12-HETE		PGD2		PGE2		RANTES	
	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value
uLTE4	-	-	0.082	0.762	0.294	0.269	**0.544**	**0.029***	-0.188	0.485	-0.053	0.846	0.332	0.208
sP-selectin	0.082	0.762	-	-	0.326	0.217	0.126	0.641	0.138	0.610	0.415	0.110	0.385	0.141
PF4	0.294	0.269	0.326	0.217	-	-	0.109	0.688	-0.062	0.820	-0.271	0.311	**0.703**	**0.002****
12-HETE	**0.544**	**0.029***	0.126	0.641	0.109	0.688	-	-	0.241	0.368	0.124	0.649	0.335	0.204
PGD2	-0.188	0.485	0.138	0.610	-0.062	0.820	0.241	0.368	-	-	-0.032	0.905	0.203	0.451
PGE2	-0.053	0.846	0.415	0.110	-0.271	0.311	0.124	0.649	-0.032	0.905	-	-	0.003	0.991
RANTES	0.332	0.208	0.385	0.141	**0.703**	**0.002****	0.335	0.204	0.203	0.451	0.003	0.991	-	-
ATAD Duration	0.277	0.300	0.113	0.676	0.353	0.180	0.359	0.172	-0.182	0.499	0.104	0.700	0.362	0.168

Definition of abbreviations: N-ERD, nonsteroidal anti-inflammatory drug exacerbated respiratory disease; ATAD, aspirin treatment after aspirin desensitization; ATAD+, N-ERD patients receiving ATAD; uLTE4, urinary leukotriene E4; sP-selectin, soluble P-selectin; sCD40L, soluble CD40 ligand; 12-HETE, 12-hydroxyeicosatetraenoic acid; RANTES, regulated on activation, normal T cell expressed and secreted; PGD2, prostaglandin D2; PGE2, prostaglandin E2

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

Table S3. Correlation of N-ERD-related factors in ATAD- patients.

| | ATAD- | | | | | | | | | | | | | |
| | uLTE4 | | sP-selectin | | PF4 | | 12-HETE | | PGD2 | | PGE2 | | RANTES | |
	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value
uLTE4	-	-	0.264	0.341	-0.279	0.315	0.268	0.334	-0.311	0.260	0.225	0.420	0.364	0.182
sP-selectin	0.264	0.341	-	-	0.189	0.499	**0.954**	**< 0.0001******	**-0.518**	**0.048***	**0.679**	**0.005****	**0.739**	**0.002****
PF4	-0.279	0.315	0.189	0.499	-	-	0.271	0.328	-0.304	0.271	0.182	0.516	0.029	0.919
12-HETE	0.268	0.334	**0.954**	**< 0.0001******	0.271	0.328	-	-	**-0.546**	**0.035***	**0.586**	**0.022***	**0.693**	**0.004****
PGD2	-0.311	0.260	**-0.518**	**0.048***	-0.304	0.271	**-0.546**	**0.035***	-	-	-0.457	0.087	-0.496	0.060
PGE2	0.225	0.420	0.679	0.005	0.182	0.516	**0.586**	**0.022***	-0.457	0.087	-	-	**0.664**	**0.007****
RANTES	0.364	0.182	**0.739**	**0.002****	0.029	0.919	**0.693**	**0.004****	-0.496	0.060	**0.664**	**0.007****	-	-

Definition of abbreviations: N-ERD, nonsteroidal anti-inflammatory drug exacerbated respiratory disease; ATAD, aspirin treatment after aspirin desensitization; ATAD-, N-ERD patients without ATAD; uLTE4, urinary leukotriene E4; sP-selectin, soluble P-selectin; sCD40L, soluble CD40 ligand; 12-HETE, 12-hydroxyeicosatetraenoic acid; RANTES, regulated on activation, normal T cell expressed and secreted; PGD2, prostaglandin D2; PGE2, prostaglandin E2

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
****Correlation is significant at the 0.0001 level (2-tailed).

Table S4. Correlation of platelet-adherent neutrophils with the levels of other N-ERD-associated factors among all participants.

| | Platelet-neutrophil aggregates | | | | | | | | | |
| | ATAD+ | | ATAD- | | ATA | | HC | | All participants | |
	r	*p* value	r	*p* value	r	*p* value	r	*p* value	r	*p* value
uLTE4	0.147	0.588	0.196	0.483	0.314	0.254	**0.720**	**0.002****	**0.290**	**0.023***
sP-selectin	**0.540**	**0.031***	0.193	0.491	-0.329	0.232	0.289	0.296	0.030	0.818
PF4	**0.533**	**0.034***	-0.100	0.723	-0.304	0.271	-0.132	0.639	0.028	0.828
12-HETE	-0.276	0.301	0.282	0.308	-0.250	0.369	0.286	0.302	0.121	0.352
RANTES	0.024	0.930	0.275	0.321	-0.171	0.541	0.136	0.630	-0.055	0.674
PGD2	-0.114	0.674	-0.239	0.390	0.407	0.132	0.100	0.723	0.045	0.730
PGE2	0.005	0.985	0.189	0.499	-0.250	0.369	0.400	0.140	-0.055	0.674
ATAD Treatment	-0.243	0.365	-	-	-	-	-	-	-	-

Definition of abbreviations: N-ERD, nonsteroidal anti-inflammatory drug exacerbated respiratory disease; ATA, aspirin-tolerant asthmatic; HC, healthy control; ATAD, aspirin treatment after aspirin desensitization; ATAD+, N-ERD patients receiving ATAD; ATAD-, N-ERD patients without ATAD; uLTE4, urinary leukotriene E4; sP-selectin, soluble P-selectin; sCD40L, soluble CD40 ligand; 12-HETE, 12-hydroxyeicosatetraenoic acid; RANTES, regulated on activation, normal T cell expressed and secreted; PGD2, prostaglandin D2; PGE2, prostaglandin E2

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).
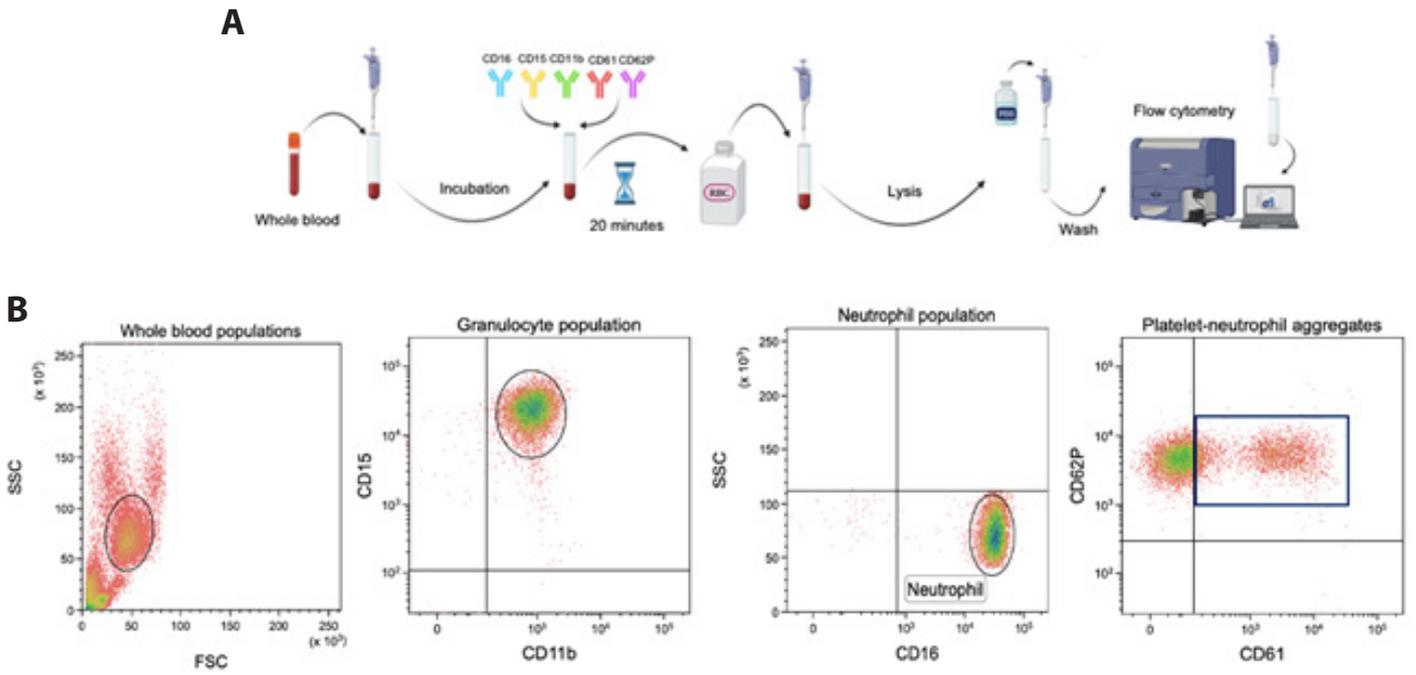
Figure S1. Preparation of blood samples (A) and gating strategy for flow cytometric analysis (B)