Platelet activation markers overexpressed specifically in patients with aspirin-exacerbated respiratory disease

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Background: Aspirin-exacerbated respiratory disease (AERD) is characterized by respiratory reactions on ingestion of COX-1 inhibitors and cysteinyl leukotriene overproduction. The hypersensitivity reaction is induced by low doses of aspirin that inhibit COX-1 in platelets.

Objective: We sought to explore the role of platelets in the pathogenesis of AERD in patients under stable conditions and during an aspirin challenge test.

Methods: Stable patients with AERD (n = 30), aspirin-tolerant asthma (ATA; n = 21), or idiopathic chronic eosinophilic pneumonia (n = 10) were enrolled. Platelet activation was estimated based on expression levels of P-selectin (CD62P), CD63, CD69, and GPIIb/IIIa (PAC-1) in peripheral platelets; percentages of circulating platelet-adherent leukocytes; and plasma levels of soluble P-selectin (sP-selectin) and soluble CD40 ligand (sCD40L).

Results: In the stable condition, expression of all surface markers on platelets, the percentage of platelet-adherent eosinophils, and the plasma levels of sP-selectin and sCD40L were significantly higher in patients with AERD compared with those in patients with ATA. P-selectin and CD63 expression on platelets and plasma sP-selectin and sCD40L levels were positively correlated with the percentage of platelet-adherent eosinophils. Among these markers, P-selectin expression and plasma sP-selectin levels positively correlated with urinary concentrations of leukotriene E_4. Additionally, plasma sP-selectin and sCD40L levels were negatively correlated with lung function. In contrast, platelet activation markers in patients with AERD did not change during the aspirin challenge test.

Conclusion: Peripheral platelets were activated more in patients with stable AERD compared with those in patients with stable ATA, patients with idiopathic chronic eosinophilic pneumonia, and control subjects. Platelet activation was involved in cysteinyl leukotriene overproduction and persistent airflow limitations in patients with AERD. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: *P-selectin, adhesion, aspirin-exacerbated respiratory disease, asthma, cysteinyl leukotriene, platelet*

Aspirin-exacerbated respiratory disease (AERD) is characterized by persistent eosinophilic inflammation both in the upper and lower airways combined with hypersensitivity to aspirin and other COX-1 inhibitors.[1] Most patients with AERD present with severe asthma,[1-3] and AERD is one of the risk factors for persistent airflow limitations.[4] The pathogenesis of AERD involves an imbalance in eicosanoid metabolism[5,6]; the urinary concentration of leukotriene (LT) E_4, which is excessive even under stable disease conditions,[7,8] further increases after aspirin-induced reactions.[9,10] Leukotriene C_4 synthase (LTC_4S) is markedly overexpressed in eosinophils and mast cells from bronchial biopsy specimens of patients with AERD, and eosinophils in particular have been implicated as the main source of cysteinyl leukotrienes (cysLTs) under stable conditions.[11] However, the mechanisms responsible for eosinophil activation and cysLT overproduction in patients with AERD are not yet completely understood.

Platelets play an important role in asthma, contributing to airway hyperreactivity, bronchoconstriction, airway inflammation, and airway remodeling.[12] Large numbers of pulmonary megakaryocytes have been detected in lung sections from patients who have died from status asthmaticus.[13] Activated platelets bind to leukocytes through P-selectin (CD62P)–P-selectin glycoprotein ligand 1, GPIIb/IIIa-Mac-1, and CD40 ligand (CD40L)–CD40.[14,15] Platelet surface P-selectin is overexpressed and contributes to pulmonary eosinophil recruitment in patients with allergic asthma.[16] In addition, plasma soluble P-selectin (sP-selectin) and soluble CD40L (sCD40L) levels are increased in asthmatic patients after exercise and allergen challenge.[17-19]

Recently, Laidlaw et al[20] have reported that the percentage of platelet-adherent leukocytes is markedly increased in blood and nasal polyps and correlates with cysLT overproduction in patients with AERD. We hypothesized that platelets might contribute to the pathogenesis of AERD in consideration of the facts. The median cumulative dose of aspirin is usually low (median, 60 mg; range, 45-100 mg) in patients with AERD.[21,22] Although low-dose aspirin, which is clinically used for antiplatelet therapy, irreversibly inhibits COX-1 and downregulates thromboxane (TX) A_2 biosynthesis in platelets, it does not inhibit COX-2 activity in endothelial cells and leukocytes.[23,24] Moreover, patients with AERD have a highly characteristic refractory period to aspirin

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Abbreviations used
AERD: Aspirin-exacerbated respiratory disease
ATA: Aspirin-tolerant asthma
CD40L: CD40 ligand
CEP: Idiopathic chronic eosinophilic pneumonia
cysLT: Cysteinyl leukotriene
FEF_{25-75}: Mean forced expiratory flow between 25% and 75% of forced vital capacity
L-ASA: Lysine-aspirin
LT: Leukotriene
LTC_4S: Leukotriene C_4 synthase
sP-selectin: Soluble P-selectin
sCD40L: Soluble CD40L
TX: Thromboxane
$uLTE_4$: Urinary leukotriene E_4

after an aspirin challenge, lasting for several days to 1 week,[25] which is almost equivalent to the platelet lifespan.[26] Furthermore, patients with AERD can be successfully desensitized with as little as 81 mg of aspirin per day.[27,28]

The aim of the present study was to explore the possible role of platelets in the pathogenesis of AERD under stable conditions. Furthermore, we also assessed platelet activation markers in plasma during an aspirin challenge test.

METHODS

For more details, see the Methods section in this article's Online Repository at www.jacionline.org.

Assessment of platelet activation markers at stable condition

Thirty patients with AERD and 21 patients with aspirin-tolerant asthma (ATA), both with stable disease, were recruited at Sagamihara National Hospital. The diagnosis of aspirin intolerance was confirmed by using the lysine-aspirin (L-ASA) intravenous challenge test (n = 24), aspirin oral challenge test (n = 6), or both, as previously described.[10,29] The L-ASA test has not been available in Japan since April 2012, and therefore patients who visited our hospital after April 2012 underwent oral aspirin challenge testing. ATA is defined as asthma with no apparent history of respiratory reaction induced by nonsteroidal anti-inflammatory drugs and a history of using aspirin, nonsteroidal anti-inflammatory drugs, or both at typical doses without any adverse effects within the previous 12 months. The definition of refractory asthma was based on the American Thoracic Society criteria.[30] We also recruited 10 patients with idiopathic chronic eosinophilic pneumonia (CEP) and 14 healthy control subjects, all of whom were aspirin tolerant. The diagnosis of CEP was consistent with the clinical classification proposed by Allen and Davis,[31] and all of these patients had undergone clinical remission.

Inclusion and exclusion criteria for patients and control subjects in the study are listed in the Methods section in this article's Online Repository at www.jacionline.org. Permission to conduct this study was obtained from the ethics committee of our hospital.

Antibodies, ELISA, and flow cytometry

Expression levels of 4 surface markers (P-selectin [CD62P], CD63, CD69 and GPIIb/IIIa [PAC-1]) on platelets and percentages of platelet-adherent leukocytes, including eosinophils, neutrophils, basophils, and T lymphocytes, were analyzed by using flow cytometry. Plasma sP-selectin and sCD40L levels were measured by using ELISAs. Urinary leukotriene E_4 ($uLTE_4$) levels were also measured by using an enzyme immunoassay kit after purification by using high-performance liquid chromatography, as previously described.[32] Methodology pertaining to antibodies, ELISAs, and flow cytometry are described in the Methods section in this article's Online Repository at www.jacionline.org.

Assessment of platelet activation markers during aspirin challenge tests

Twenty-four patients with AERD and 7 patients with ATA underwent an intravenous L-ASA challenge test, which was performed as previously described.[10] Baseline and postchallenge urine and blood samples were collected. Blood samples were collected for measurement of plasma sP-selectin and sCD40L levels at 0 to 1, 1 to 3, 3 to 6, and 9 to 24 hours after ingestion of the dose that produced a positive reaction in patients with AERD or after ingestion of the last dose of L-ASA in patients with ATA. Eight patients with AERD, 4 of them given diagnoses based on an L-ASA challenge test and the others given diagnoses based on an oral aspirin challenge test, were also analyzed for 4 surface markers (P-selectin [CD62P], CD63, CD69 and GPIIb/IIIa [PAC-1]) on platelets at 0 to 1, 1 to 3, and 9 to 24 hours after ingestion of the dose that produced a positive reaction in patients with AERD. Additionally, several patients were also analyzed for the frequency of platelet-adherent leukocytes (eosinophils, n = 4; neutrophils, n = 3; T lymphocytes, n = 3; and basophils, n = 2). The positive reaction was induced approximately 0.5 to 1 hour after the ingestion of the last aspirin dose, and blood at 0 to 1 hour after the last dose was collected within 5 to 20 minutes from the occurrence of the reaction in patients with AERD. Urine samples were also collected for measurements of LTE_4 concentrations during the following periods: 0 to 3, 3 to 6, 6 to 9, and 9 to 24 hours.

Statistical analyses

Data were analyzed by using SPSS for MS windows, version 21 (SPSS, Chicago, Ill). Data are presented as median values with ranges or mean values ± SDs. Categorical variables were assessed by using χ^2 tests. The means (or medians) of continuous variables were compared by using the Student *t* test or Mann-Whitney *U* test. ANOVA or the Kruskal-Wallis test was used for comparing more than 2 groups of patients. The Wilcoxon signed-rank test was used for time-course experiments. Correlations were evaluated by using the Spearman rank correlation test. *P* values of .05 or less were considered statistically significant. The significance threshold was further calculated for the number of group comparisons by using the Bonferroni procedure.

RESULTS

Patients' characteristics under stable conditions

The demographic and clinical characteristics of the study patients are presented in Table I. There were no significant differences among the 4 groups with respect to age, sex, smoking history, and atopic status. Asthma severity was similar in patients with AERD and in patients with ATA. Among patients with CEP, 5 of 10 also had asthma, and the severity did not differ in comparison with that seen in patients with AERD or ATA. Regarding lung function, 25 patients with AERD and 18 patients with ATA showed similar prebronchodilator and postbronchodilator lung function. Patients with CEP had higher prebronchodilator and postbronchodilator lung function compared with patients with AERD (percent predicted prebronchodilator and postbronchodilator FEV_1: $P = .006$ and $P = .027$; percent predicted prebronchodilator and postbronchodilator mean forced expiratory flow between 25% and 75% of forced vital capacity [FEF_{25-75}]: $P = .019$ and $P = .048$, respectively). The $uLTE_4$ level in patients with AERD was higher than that in patients with ATA ($P = .005$) and control subjects ($P < .001$), respectively. Although the $uLTE_4$ level was higher in patients with AERD than in patients

TABLE I. Demographic data and inflammation markers

	Patients with AERD (n = 30)	Patients with ATA (n = 21)	Patients with CEP (n = 10)	Healthy control subjects (n = 14)
Age (y)	52 ± 13	53 ± 17	52 ± 14	41 ± 14*
Age at onset (y)	33 ± 15	40 ± 15	40 ± 13	NA
Duration of asthma (y)	19 ± 11	12 ± 11	10 ± 8*	NA
Male sex (%)	23	14	0	7
BMI (kg/m^2)	22 ± 4	23 ± 3	20 ± 2*	21 ± 2
Smoking history (%)				
Never	57	71	70	100*
Past	33	29	30	0
Current	10	0	0	0
Atopy (%)	60	81	78	100
Comorbidity (%)				
Atopic dermatitis	10	15	10	14
Allergic rhinitis	50	65	20	79*
Rhinosinusitis	90	**38**‡	**10**‡	**18**‡
Family history of asthma (%)	39	50	20	0†
Pediatric asthma (%)	10	19	10	0
Refractory asthma (%)	43	24	44	NA
Prebronchodilator (%)				
FEV_1	89 ± 20	92 ± 19	110 ± 13†	ND
$FEF_{25-75\%}$	47 ± 24	57 ± 25	68 ± 12*	ND
Postbronchodilator (%)				
FEV_1	106 ± 39	106 ± 39	113 ± 20*	ND
$FEF_{25-75\%}$	57 ± 29	85 ± 77	78 ± 19*	ND
Exhaled NO (bpm)	48 ± 29	49 ± 34	61 ± 36	ND
Serum total IgE (kU/L)	369 ± 770	385 ± 506	321 ± 333	ND
Baseline $uLTE_4$ level (pg/mg creatinine)	198 (58-4288)	98 (48-1129)†	147 (54-2361)	**74 (37-101)**‡
ICS dose (μg/d)§	720 (0-3600)	640 (0-1600)	321 (0-1600)	**0 (0-0)**‡
Continuous OCS treatment (mg/d)	0 (0-10)	0 (0-6)	4 (0-20)	0 (0-0)
Patients conducted with L-ASA intravenous challenge test, no. (%)	24/30 (80)	7/21 (33)	NA	NA
Cumulative dose of aspirin (mg)	120 (25-400)	NA	NA	NA

P values surviving Bonferroni correction are shown in boldface ($P < .05/24$). Data are presented as means ± SDs (baseline $uLTE_4$ level, daily ICS dose, continuous OCS treatment, and cumulative dose of aspirin are presented as medians with ranges).
BMI, Body mass index; *ICS*, inhaled corticosteroid; *NO*, nitric oxide; *OCS*, oral corticosteroid; *NA*, not applicable; *ND*, no data.
*$P < .05$.
†$P < .01$.
‡$P < .001$ compared with patients with AERD.
§Dose in budesonide equivalents.

with CEP, the difference was not statistically significant. The median cumulative provocative dose of aspirin in patients with AERD was 120 mg (range, 25-400 mg). Table E1 in this article's Online Repository at www.jacionline.org shows peripheral blood profiles of patients with AERD, ATA, or CEP.

There were no differences in total platelet, eosinophil, and basophil counts among the 3 groups; however, lymphocyte and monocyte counts were significantly higher in patients with AERD compared with those in patients with ATA ($P = .020$ and $P = .008$, respectively) and patients with CEP ($P = .007$ and $P = .020$, respectively). White blood cell, eosinophil, and neutrophil counts were also increased in patients with AERD compared with those in patients with ATA ($P = .002$, $P = .021$, and $P = .014$, respectively).

Platelet activation in patients with AERD under stable conditions

Plasma levels of sP-selectin and sCD40L in patients with AERD were higher than those in patients with ATA ($P = .017$ and $P = .013$, respectively) and control subjects ($P = .015$ and $P = .010$, respectively; Fig 1, *A* and *B*, and Table II). In the comparison of patients with AERD and those with CEP, the only significant difference observed was in the plasma sCD40L level, which was higher in patients with AERD ($P = .028$). There were no significant differences in plasma markers among patients with ATA, patients with CEP, and control subjects.

In the flow cytometric analysis, expression levels of all surface markers (P-selectin, CD63, CD69, and PAC-1) on platelets from patients with AERD were higher than those from patients with ATA ($P = .022$, $P = .001$, $P = .029$, and $P = .014$, respectively; Fig 1, *C-F*, and Table II). In the comparison between patients with AERD and those with CEP, expression levels of CD63 and CD69 in patients with AERD were higher than those in patients with CEP ($P < .001$ and $P = .008$; Fig 1, *D* and *E*, respectively). There were no differences in expression levels of these markers on platelets among patients with ATA, patients with CEP, and control subjects.

Eosinophils, neutrophils, basophils, and T lymphocytes were distinguished based on differential light scatter characteristics and relative membrane expression of chemoattractant receptor–homologous molecule expressed on T_H2 lymphocytes, CD16b, CRA1, and CD3, respectively. Because these cell types

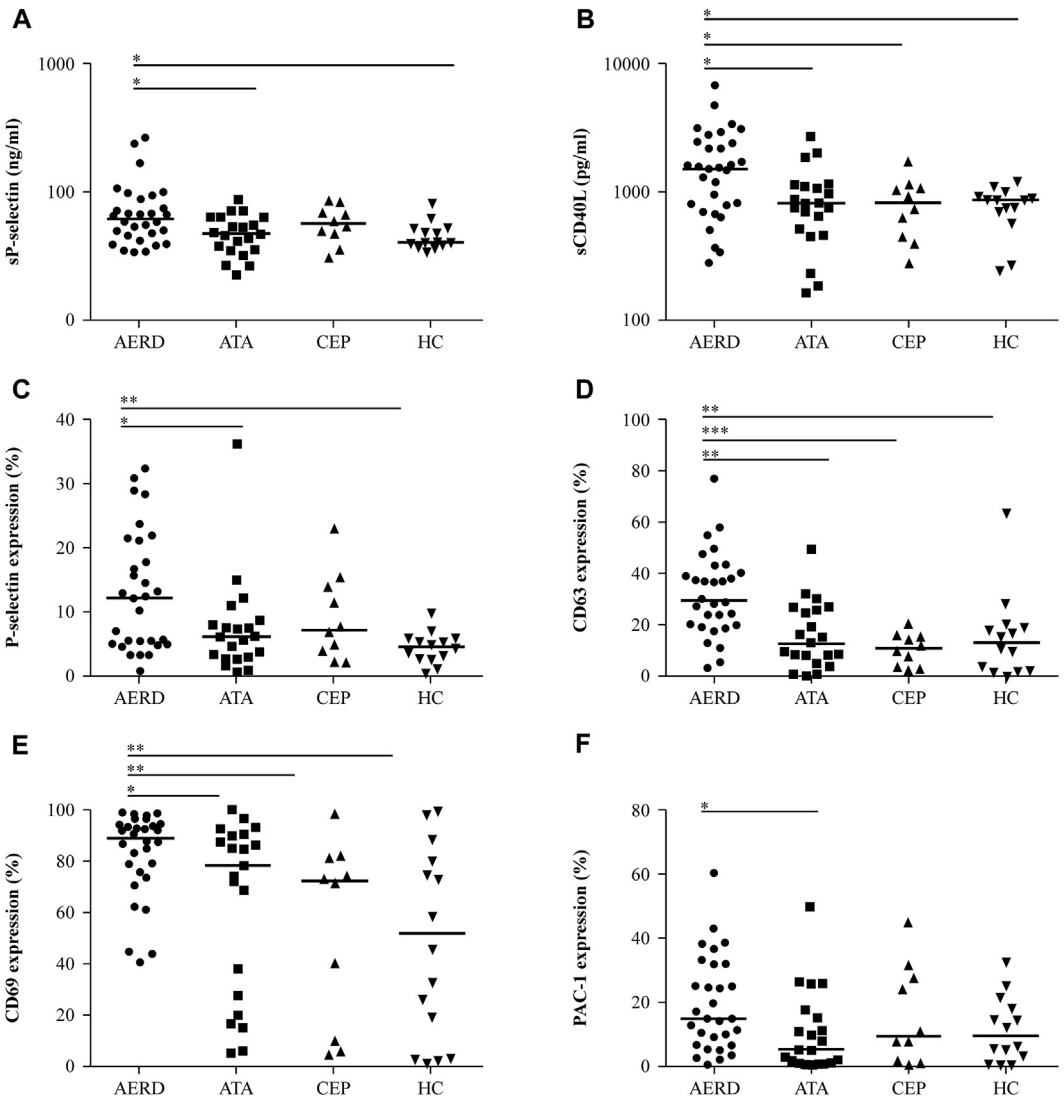

FIG 1. Comparison of plasma levels of sP-selectin **(A)** and sCD40L **(B)** and expression levels of P-selectin **(C)**, CD63 **(D)**, CD69 **(E)**, and PAC-1 **(F)** on platelets among patients with AERD (n = 30), patients with ATA (n = 21), patients with CEP (n = 10), and healthy control subjects (*HC*; n = 14). **P* < .05, ***P* < .01, and ****P* < .001. *Bars* indicate median of data.

also could be distinguished from one another based solely on their light scatter characteristics, we considered that this parameter was sufficient to identify leukocyte cell types. Although platelet-adherent leukocytes were identified in all groups, the percentage of all platelet-adherent leukocyte types, except for lymphocytes, was higher in patients with AERD compared with that seen in the other groups (Fig 2 and Table II). The percentage of platelet-adherent eosinophils was particularly increased in patients with AERD compared with those in patients with ATA and control subjects ($P = .025$ and $P = .016$, respectively), and platelets adhered to 51% of eosinophils in patients with AERD (Fig 2, *A*).

We also analyzed expression levels of surface markers on platelets adherent to leukocytes (Table II). The P-selectin expression level on platelets adherent to basophils was highest in patients with AERD among the 4 groups ($P = .019$) and was positively correlated with the percentage of platelet-adherent basophils in patients with AERD ($r = 0.582$, $P = .004$, data not shown).

Correlation of platelet activation markers with uLTE$_4$ levels and lung function under stable conditions

There were several significant correlations between platelet activation markers; CD63 expression levels correlated positively with P-selectin and CD69 expression levels on platelets (P-selectin: $r = 0.575$, $P = .001$; CD69: $r = 0.604$, $P < .001$; data not shown). There was also a correlation between 2 of the plasma markers: sP-selectin and sCD40L ($r = 0.839$, $P < .001$).

Several platelet activation markers were correlated with the percentage of platelet-adherent eosinophils and with the uLTE$_4$ concentration (see Table E2 in this article's Online Repository at www.jacionline.org); P-selectin and CD63 expression levels on platelets and sP-selectin and sCD40L levels in plasma were positively correlated with the percentage of platelet-adherent eosinophils (P-selectin: $r = 0.308$, $P = .019$; CD63: $r = 0.361$, $P = .005$; sP-selectin: $r = 0.276$, $P = .036$; sCD40L: $r = 0.263$, $P = .046$, respectively; Fig 3, *A*, *B*, *E*, and *F*). P-selectin expression

TABLE II. Platelet activation markers in patients

	Patients with AERD (n = 30)	Patients with ATA (n = 21)	Patients with CEP (n = 10)	Health control subjects (n = 14)
Plasma sP-selectin level (ng/mL)	63 (34-266)	48 (23-89)*	57 (31-86)	41 (35-83)*
Plasma sCD40L level (pg/mL)	1529 (285-6782)	824 (167-2655)*	829 (278-1695)*	867 (245-1204)*
P-selectin expression on platelets (%)	13 ± 9	7 ± 8*	9 ± 7	5 ± 2†
CD63 expression on platelets (%)	32 ± 16	**16 ± 13**†	**11 ± 6**‡	**15 ± 16**†
CD69 expression on platelets (%)	82 ± 17	62 ± 34*	53 ± 36†	48 ± 38†
PAC-1 expression on platelets (%)	19 ± 15	10 ± 13*	15 ± 15	11 ± 10
Platelet adhesion on eosinophils (%)	51 ± 23	36 ± 15*	41 ± 16	34 ± 11*
Platelet adhesion on basophils (%)	70 ± 21	60 ± 18	67 ± 20	62 ± 19
Platelet adhesion on neutrophils (%)	58 ± 25	51 ± 22	52 ± 23	48 ± 18
Platelet adhesion on T lymphocytes (%)	32 ± 10	29 ± 8	33 ± 5	32 ± 6
P-selectin expression on platelet-adherent eosinophils (%)	12 ± 15	11 ± 14	6 ± 6	9 ± 8
CD63 expression on platelet-adherent eosinophils (%)	39 ± 29	35 ± 29	32 ± 21	45 ± 23
CD69 expression on platelet-adherent eosinophils (%)	17 ± 13	17 ± 20	23 ± 10	15 ± 12
P-selectin expression on platelet-adherent basophils (%)	20 ± 18	12 ± 9	9 ± 5	8 ± 6*
CD63 expression on platelet-adherent basophils (%)	60 ± 27	58 ± 26	67 ± 27	42 ± 26*
CD69 expression on platelet-adherent basophils (%)	18 ± 10	15 ± 9	17 ± 13	6 ± 2†
PAC-1 expression on platelet-adherent basophils (%)	7 ± 7	6 ± 9*	7 ± 8	4 ± 2

P values surviving Bonferroni correction are shown in boldface ($P < .05/17$). Data are presented as means ± SDs (plasma levels of sP-selectin and sCD40L are presented as medians with ranges).
*$P < .05$.
†$P < .01$.
‡$P < .001$ compared with patients with AERD.

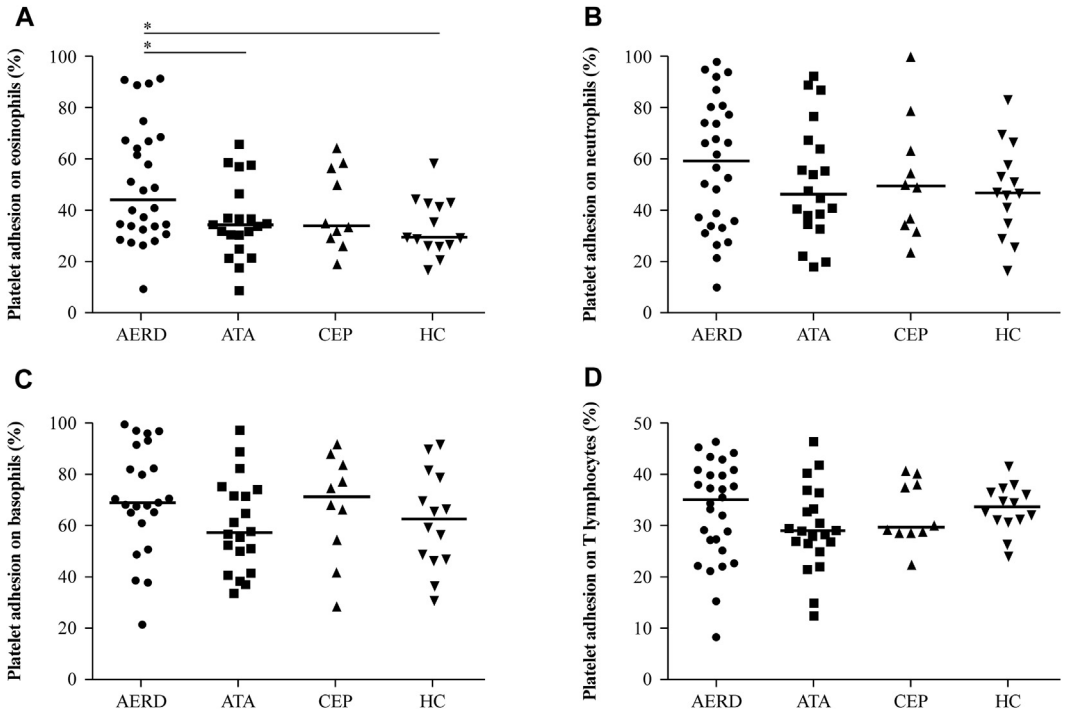

FIG 2. Comparison of platelet adhesion on leukocytes analyzed by using flow cytometry. Platelet adhesion on eosinophils **(A)**, neutrophils **(B)**, basophils **(C)**, and T lymphocytes **(D)** from patients with AERD (n = 30), patients with ATA (n = 21), patients with CEP (n = 10), and healthy control subjects (*HC*; n = 14). *$P < .05$. *Bars* indicate median of data.

levels on platelets ($r = 0.310$, $P = .015$) and plasma levels of sP-selectin ($r = 0.300$, $P = .019$) were positively correlated with $uLTE_4$ concentrations (Fig 4, *A* and *E*). CD63 expression levels showed a weak correlation with $uLTE_4$ concentrations ($r = 0.240$, $P = .063$; Fig 4, *B*).

Some platelet activation markers were correlated with prebronchodilator and postbronchodilator lung function in all patients (see Table E3 in this article's Online Repository at www.jacionline.org). Plasma sP-selectin levels were negatively correlated with pulmonary function, including percent

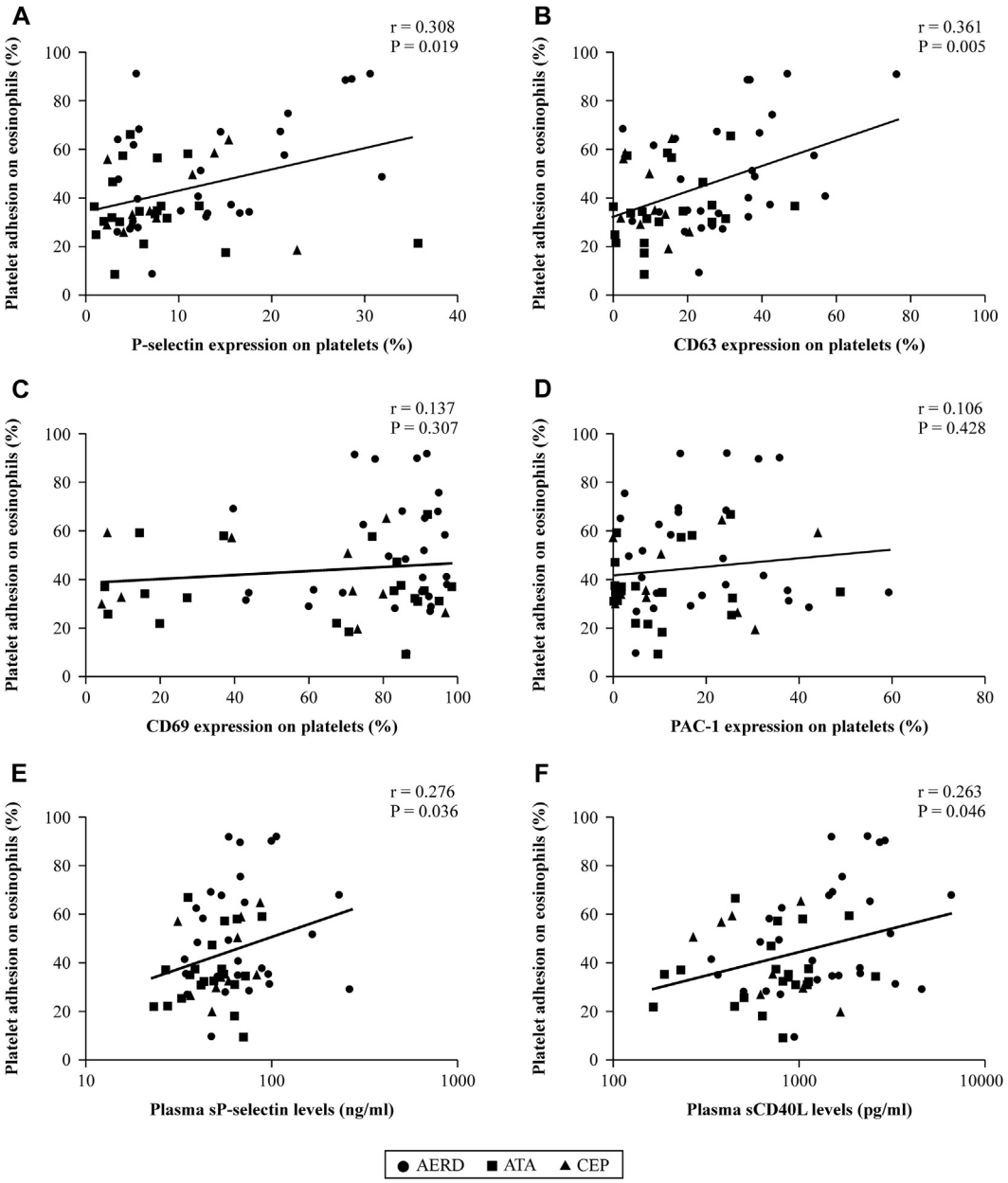

FIG 3. Correlation of platelet activation markers with the percentage of platelet adhesion on eosinophils were assessed in all patients. Expression levels of P-selectin **(A)**, CD63 **(B)**, CD69 **(C)**, and PAC-1 **(D)** on platelets and plasma levels of sP-selectin **(E)** and sCD40L **(F)** are shown. *P* values surviving after Bonferroni correction are shown in boldface.

predicted prebronchodilator and postbronchodilator FEV_1 ($r = -0.324$, $P = .019$ and $r = -0.370$, $P = .007$, respectively; Fig 5, *A* and *B*) and percent predicted prebronchodilator and postbronchodilator FEF_{25-75} ($r = -0.381$, $P = .005$ and $r = -0.472$, $P < .001$, respectively; Fig 5, *C* and *D*). Plasma sCD40L levels were negatively correlated only with percent predicted postbronchodilator FEF_{25-75} ($r = -0.336$, $P = .015$ and $r = -0.367$, $P = .007$; Fig 5, *G* and *H*). Among surface markers measured, expression levels of CD63 and CD69 were also associated with percent predicted prebronchodilator and postbronchodilator FEF_{25-75}, although the correlation between the CD63 expression level and percent predicted prebronchodilator FEF_{25-75} was marginally nonsignificant (see Table E3, and Figs E1 and E2, in this article's Online Repository at www.jacionline.org).

Changes in platelet activation marker levels during an aspirin challenge test

Plasma levels of sP-selectin and sCD40L and $uLTE_4$ concentrations were measured in 24 patients with AERD and 7 patients with ATA during an L-ASA challenge test (see Tables E4 and E5 in this article's Online Repository at www.jacionline.org). Plasma sP-selectin and sCD40L levels did not change relative to baseline during the time course after a positive reaction in either patients with AERD or those with ATA (Fig 6, *A* and *B*). As reported

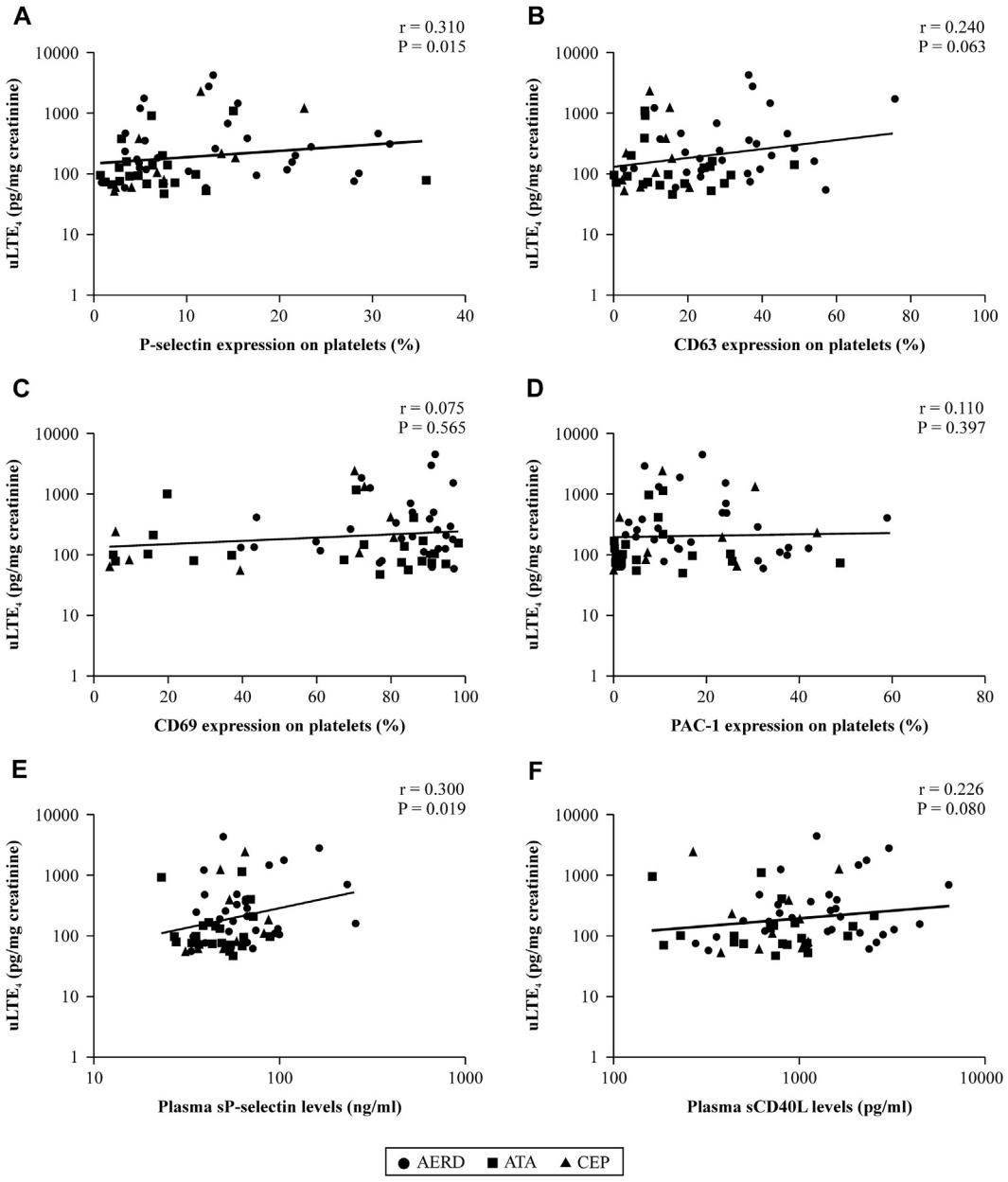

FIG 4. Correlation of platelet activation markers with $uLTE_4$ concentrations were assessed in all patients. Expression levels of P-selectin **(A)**, CD63 **(B)**, CD69 **(C)**, and PAC-1 **(D)** on platelet and plasma levels of sP-selectin **(E)** and sCD40L **(F)** are shown.

previously, $uLTE_4$ concentrations in patients with AERD at 0 to 3, 3 to 6, 6 to 9, and 9 to 24 hours increased significantly from the baseline concentration (Fig 6, *C*). There was no change in the $uLTE_4$ concentration during the L-ASA challenge test in patients with ATA (Fig 6, *C*).

Fluorescence-activated cell sorting analysis was also conducted in 8 patients with AERD; 4 of these patients underwent an L-ASA challenge test, and 4 received an oral aspirin challenge test (see Tables E6 and E7 in this article's Online Repository at www.jacionline.org). Expression levels of all 4 surface markers (ie, P-selectin, CD63, CD69, and PAC-1) on platelets from the 8 patients with AERD showed no significant changes during the aspirin challenge test (Fig 6, *D-G*). In addition, platelet-adherent leukocyte numbers also did not increase during the aspirin challenge test (see Table E7).

DISCUSSION

This study demonstrated platelet activation in patients with AERD compared with those with ATA or CEP and identified adhesion molecules on platelets that contribute to the pathogenesis of AERD. Some platelet activation markers were correlated with the $uLTE_4$ concentration and the decrease in lung function. Additionally, this is the first study that analyzed changes in platelet activation markers during an aspirin challenge test in patients with AERD.

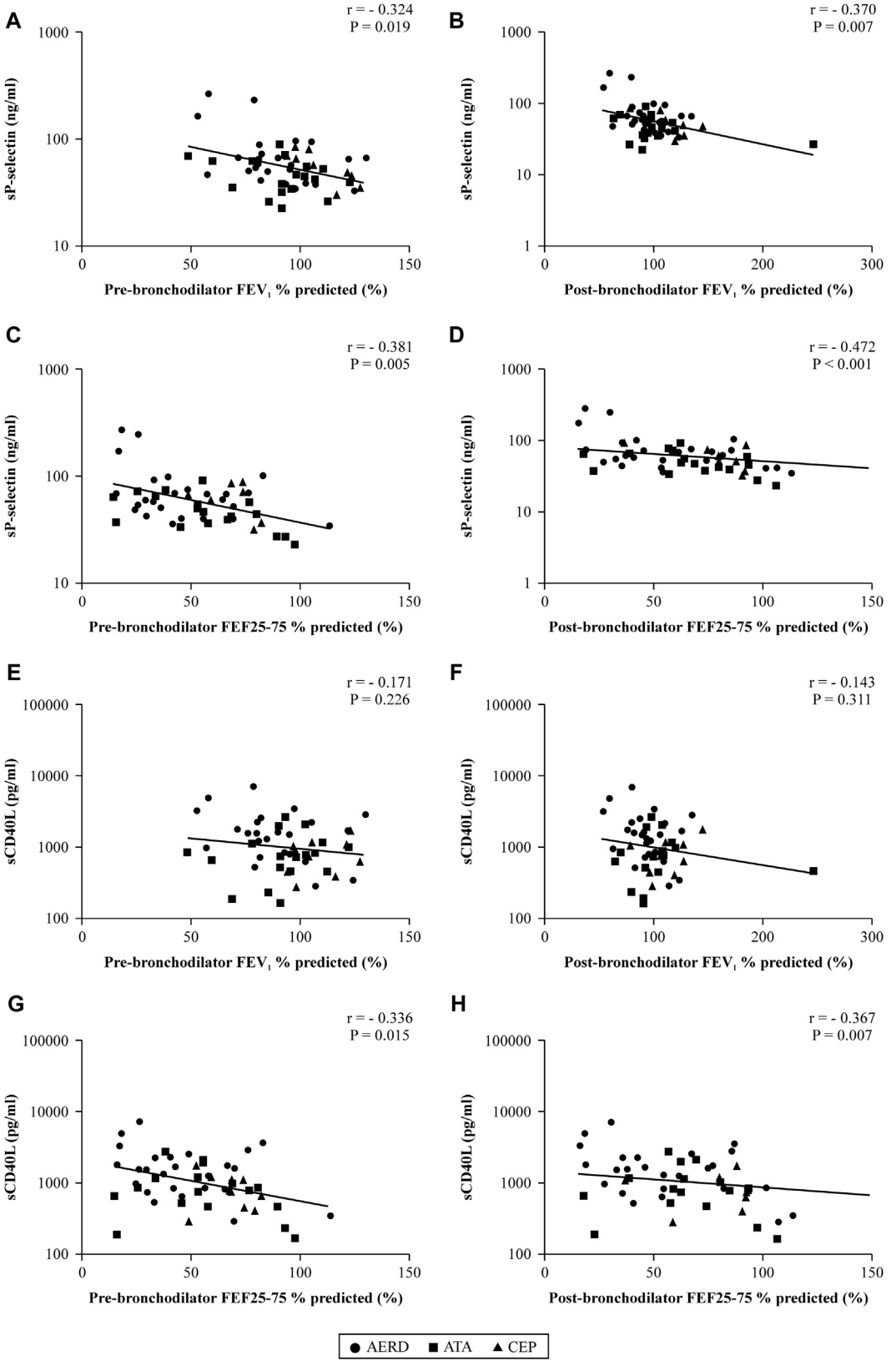

FIG 5. Correlation of platelet activation markers in plasma with lung function was assessed in all patients. The correlation of plasma sP-selectin **(A-D)** and sCD40L **(E-H)** levels with percent predicted FEV_1 and percent predicted $FEF_{25-75\%}$ in prebronchodilator and postbronchodilator spirometry.

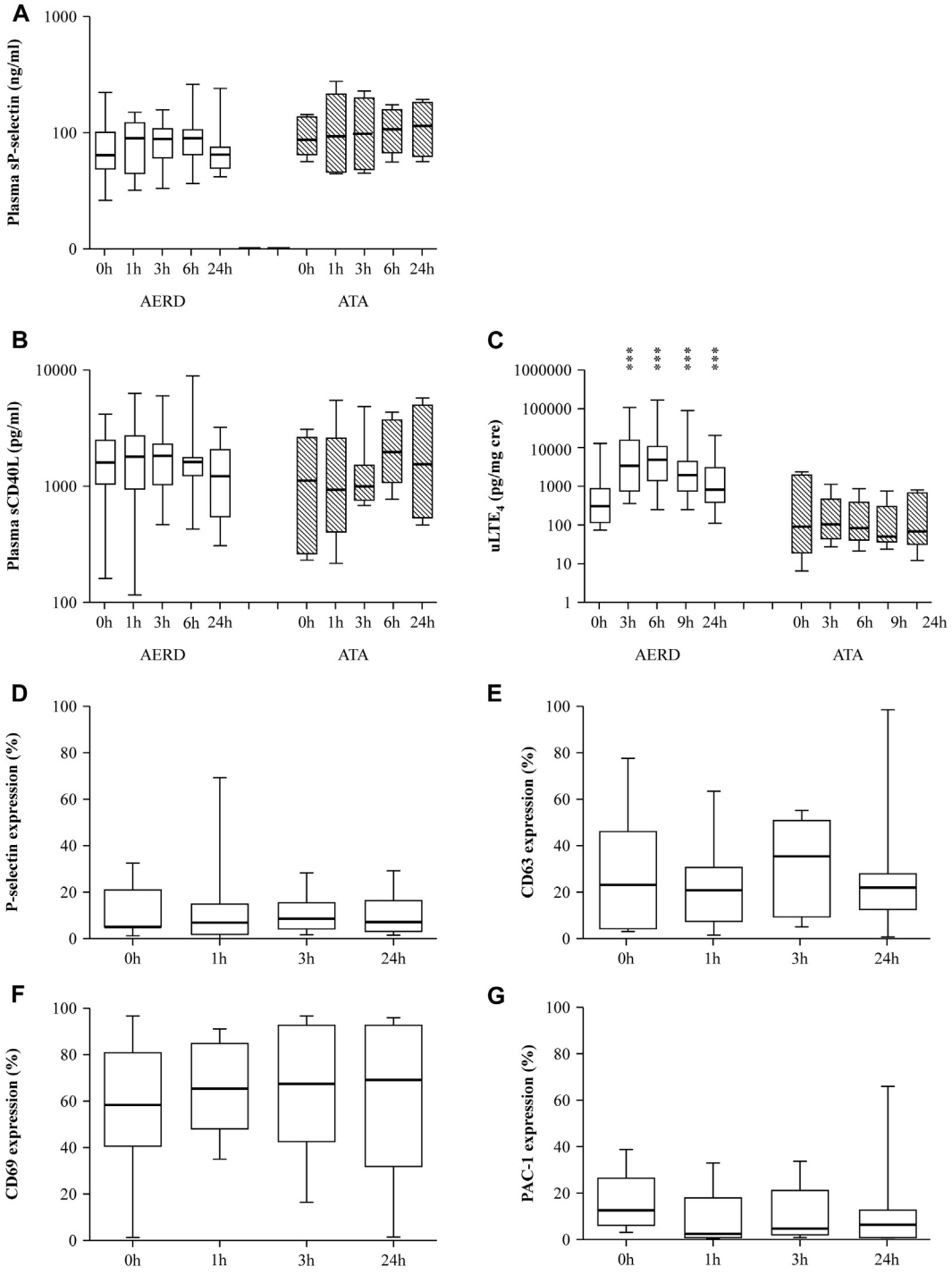

FIG 6. Changes in plasma sP-selectin **(A)** and sCD40L levels **(B)** and $uLTE_4$ levels **(C)** during the L-ASA challenge test were evaluated in patients with AERD (n = 24) and patients with ATA (n = 7). P-selectin **(D)**, CD63 **(E)**, CD69 **(F)**, and PAC-1 **(G)** expression levels on platelets were also assessed in patients with AERD (n = 8). ***$P < .001$.

We found that 2 plasma markers, all 4 surface markers on platelets, and the percentage of platelet-adherent eosinophils were significantly higher in patients with AERD compared with those in patients with ATA under stable conditions or control subjects (Figs 1 and 2, *A*, and Table II). Furthermore, there were no significant differences in any of these markers between patients with ATA and control subjects. These findings indicate that platelet activation occurred very specifically in patients with AERD. Because activated platelets rapidly shed surface P-selectin after platelet activation,[33] it seems that ongoing platelet activation takes place specifically in patients with AERD. However, the cause of platelet activation in patients with AERD has not been clarified.

Platelets express numerous adhesion molecules and ligands that facilitate interactions among platelets, leukocytes, and

endothelium. P-selectin on activated platelets adheres to P-selectin glycoprotein ligand 1 on leukocytes and subsequently regulates integrin expression and accelerates firm adhesion to the endothelium.[12] Platelets lack 5-lipoxygenase; however, adherent platelets contribute to transcellular metabolism of LTs from leukocyte-derived LTA_4 through LTC_4S in platelets.[34,35] Therefore an increase in platelet-adherent leukocyte counts is involved in cysLT overproduction and infiltration of inflammatory cells into the airway.

Platelets are activated by distinct mechanisms in different settings and express P-selectin on their surfaces. Cummings et al[36] revealed that LTC_4, but not LTD_4 and LTE_4, binds to cysLT receptor 2 and subsequently causes an autocrine ADP-mediated response through P_2Y_{12} receptors, which induce surface expression of P-selectin on murine platelets, although cysLTs, including LTE_4, do not directly amplify human platelet adhesion on leukocytes or their recruitment into the airways.[37] A functional impairment of the protein kinase A system through prostaglandin E receptors on granulocytes might cause release of mediators from leukocytes, which results in platelet activation.[38] CD40L, which is a GPIIb/IIIa ligand, is also associated with P-selectin expression on platelets. The trimeric form of sCD40L also enhances P-selectin expression on platelets and platelet adhesion to neutrophils, which is caused by a pathway distinct from signaling through GPIIb/IIIa.[39]

In our study, platelet surface expression of P-selectin and CD63 and plasma levels of sP-selectin and sCD40L were positively correlated with the percentage of platelet-adherent eosinophils (Fig 3, *A*, *B*, *E*, and *F*, and see Table E2). Platelet surface P-selectin expression and plasma sP-selectin levels were positively correlated with $uLTE_4$ concentrations (Fig 4, *A* and *E*, and see Table E2). Our results fit with those of Laidlaw et al,[20] indicating that the percentage of platelet-adherent leukocytes correlates with $uLTE_4$ levels. These findings suggest that platelets adhere subsequently to eosinophils through P-selectin and CD40L and contribute to cysLT overproduction through synthesis of LTC_4 from leukocyte-derived LTA_4 (see Fig E3).

In asthmatic patients platelets migrate into the lung tissue and contribute to remodeling of the airway by releasing mitogens and enzymes, which induce a synthetic response in airway structural cells.[12] In our study only plasma sP-selectin was associated with large- and small-airway obstructions (Fig 5 and see Table E3). These results suggest that plasma sP-selectin levels are the most useful marker for the assessment of remodeling caused by platelet activation.

CEP and AERD resemble each other in that they both show airway eosinophilia and cysLT overproduction.[40] However, the present study revealed a difference in the profiles of platelet activation markers between patients with CEP and those with AERD; namely, the expression level of CD63 on platelets was significantly higher in patients with AERD than in patients with CEP. It has been reported that CD63 is an essential cofactor for P-selectin and that together they cooperate to promote migration of platelet-adherent leukocytes.[41] In our study, platelet surface P-selectin and CD63 expression levels were correlated with each other and positively correlated with the percentage of platelet-adherent eosinophils and $uLTE_4$ levels (Figs 3, *A* and *B*, and 4, *A* and *B*, and see Table E2). These 2 markers might cooperate to produce severe eosinophil infiltration into the airway and cysLT overproduction in patients with AERD.

This is the first study in which platelet activation was estimated during an aspirin challenge test in patients with AERD. Kowal et al[18] reported that platelet activation markers were increased 30 minutes after mite challenge, and the activation persisted until the late phase in some patients. Another study also revealed a significant increase in platelet-adherent leukocytes at 8 hours after allergen challenge.[42] Conversely, platelet activation markers in plasma, expression levels of surface markers on platelets, and frequencies of platelet-adherent leukocytes did not change after the aspirin-induced reaction in our study (see Tables E5 and E7). A previous study showed that prostacyclin, which has an antagonist action to TXA_2, does not prevent asthmatic attacks induced by aspirin.[43] In addition, aspirin does not affect P-selectin expression on platelets from patients with aspirin-intolerant chronic urticaria.[44] These studies are in good agreement with the results of our study. Plasma markers do not necessarily reflect platelet activation in the airways, and further study is needed to investigate local platelet activation. In the aspirin challenge test aspirin further suppresses prostaglandin E_2 synthesis and uncouples the downregulation of LTC_4S activity through inhibition of TXA_2 formation.[45] Additionally, arachidonic acid derived from adherent platelets activates 5-lipoxygenase in granulocytes and results in cysLT overproduction in patients with AERD (see Fig E3).[35]

The results of our study suggest that antiplatelet therapy, especially with P-selectin antagonist, could be therapeutically beneficial in patients with AERD. Among antiplatelet agents, P2Y12 antagonist and P-selectin antagonist, but not GPIIb/IIIa antagonist, reduce the formation of the platelet-leukocyte complex in human subjects.[46,47] P-selectin antagonist was shown to significantly reduce allergen-induced airway hyperreactivity and peribronchial eosinophilic inflammation in a murine model of asthma.[48] However, a recent study in an allergic model mouse showed that a P_2Y_1 antagonist, but not a P_2Y_{12} or P_2X_1 antagonist, inhibited both platelet adhesion on leukocytes through P-selectin and P-selectin–dependent leukocyte migration.[49] The effects of purinergic receptor antagonists on patients with AERD remains controversial, and further studies are needed.

There are several limitations to this study. First, the patients were limited to the Japanese population, and interpopulational differences might exist. Additionally, because of the small sample size, some *P* values in multiple statistical comparisons did not survive after Bonferroni correction.

Second, platelets are easily activated by blood drawing. However, the effect of blood drawing might be minimal in this study because we carefully drew blood with a 21-gauge needle into a container with 3.2% sodium citrate and stained the platelets within 10 minutes.

Third, platelet activation in the airways was not estimated during the aspirin challenge test. Furthermore, the results could have been affected by epinephrine and corticosteroid use after the aspirin-induced reaction because these drugs can induce pharmacologic aggregation.[50]

In summary, platelet activation markers were significantly increased in patients with AERD compared with those in patients with ATA or CEP. More importantly, we also demonstrated that P-selectin is the key molecule that correlates with $uLTE_4$ levels and persistent airflow limitations in patients with AERD with stable disease. Suppression of platelet activation is a potential therapeutic target for severe eosinophilic inflammation in patients with AERD.

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Clinical implications: This study indicated that activated platelets are involved in the pathogenic processes underlying AERD, particularly cysLT overproduction and persistent airflow limitation.

REFERENCES

1. Stevenson DD, Szczeklik A. Clinical and pathologic perspectives on aspirin sensitivity and asthma. J Allergy Clin Immunol 2006;118:773-86.
2. Szczeklik A, Nizankowska E, Duplaga M. Natural history of aspirin-induced asthma. AIANE Investigators. European Network on Aspirin-Induced Asthma. Eur Respir J 2000;16:432-6.
3. Fukutomi Y, Taniguchi M, Tsuburai T, Tanimoto H, Oshikata C, Ono E, et al. Obesity and aspirin intolerance are risk factors for difficult-to-treat asthma in Japanese non-atopic women. Clin Exp Allergy 2012;42:738-46.
4. Mascia K, Haselkorn T, Deniz YM, Miller DP, Bleecker ER, Borish L. Aspirin sensitivity and severity of asthma: evidence for irreversible airway obstruction in patients with severe or difficult-to-treat asthma. J Allergy Clin Immunol 2005;116:970-5.
5. Yamaguchi H, Higashi N, Mita H, Ono E, Komase Y, Nakagawa T, et al. Urinary concentrations of 15-epimer of lipoxin A(4) are lower in patients with aspirin-intolerant compared with aspirin-tolerant asthma. Clin Exp Allergy 2011;41:1711-8.
6. Laidlaw TM, Boyce JA. Pathogenesis of aspirin-exacerbated respiratory disease and reactions. Immunol Allergy Clin North Am 2013;33:195-210.
7. Kumlin M, Dahlen B, Bjorck T, Zetterstrom O, Granstrom E, Dahlen SE. Urinary excretion of leukotriene E4 and 11-dehydro-thromboxane B2 in response to bronchial provocations with allergen, aspirin, leukotriene D4, and histamine in asthmatics. Am Rev Respir Dis 1992;146:96-103.
8. Higashi N, Taniguchi M, Mita H, Kawagishi Y, Ishii T, Higashi A, et al. Clinical features of asthmatic patients with increased urinary leukotriene E4 excretion (hyperleukotrienuria): Involvement of chronic hyperplastic rhinosinusitis with nasal polyposis. J Allergy Clin Immunol 2004;113:277-83.
9. Christie PE, Tagari P, Ford-Hutchinson AW, Black C, Markendorf A, Schmitz-Schumann M, et al. Urinary leukotriene E4 after lysine-aspirin inhalation in asthmatic subjects. Am Rev Respir Dis 1992;146:1531-4.
10. Mita H, Endoh S, Kudoh M, Kawagashi Y, Kobayashi M, Taniguchi M, et al. Possible involvement of mast-cell activation in aspirin provocation of aspirin-induced asthma. Allergy 2001;56:1061-7.
11. Cowburn AS, Sladek K, Soja J, Adamek L, Nizankowska E, Szczeklik A, et al. Overexpression of leukotriene C4 synthase in bronchial biopsies from patients with aspirin-intolerant asthma. J Clin Invest 1998;101:834-46.
12. Page C, Pitchford S. Platelets and allergic inflammation. Clin Exp Allergy 2014;44:901-13.
13. Slater D, Martin J, Trowbridge A. The platelet in asthma. Lancet 1985;1:110.
14. Jawien J, Lomnicka M, Korbut R, Chlopicki S. The involvement of adhesion molecules and lipid mediators in the adhesion of human platelets to eosinophils. J Physiol Pharmacol 2005;56:637-48.
15. Li N. Platelet-lymphocyte cross-talk. J Leukoc Biol 2008;83:1069-78.
16. Ulfman LH, Joosten DP, van Aalst CW, Lammers JW, van de Graaf EA, Koenderman L, et al. Platelets promote eosinophil adhesion of patients with asthma to endothelium under flow conditions. Am J Respir Cell Mol Biol 2003;28:512-9.
17. Zietkowski Z, Skiepko R, Tomasiak MM, Bodzenta-Lukaszyk A. Soluble CD40 ligand and soluble P-selectin in allergic asthma patients during exercise-induced bronchoconstriction. J Investig Allergol Clin Immunol 2008;18:272-8.
18. Kowal K, Pampuch A, Kowal-Bielecka O, DuBuske LM, Bodzenta-Lukaszyk A. Platelet activation in allergic asthma patients during allergen challenge with *Dermatophagoides pteronyssinus*. Clin Exp Allergy 2006;36:426-32.
19. Kowal K, Pampuch A, Kowal-Bielecka O, Iacoviello L, Bodzenta-Lukaszyk A. Soluble CD40 ligand in asthma patients during allergen challenge. J Thromb Haemost 2006;4:2718-20.
20. Laidlaw TM, Kidder MS, Bhattacharyya N, Xing W, Shen S, Milne GL, et al. Cysteinyl leukotriene overproduction in aspirin-exacerbated respiratory disease is driven by platelet-adherent leukocytes. Blood 2012;119:3790-8.
21. Kowalski ML, Grzelewska-Rzymowska I, Rozniecki J, Szmidt M. Aspirin tolerance induced in aspirin-sensitive asthmatics. Allergy 1984;39:171-8.
22. Hope AP, Woessner KA, Simon RA, Stevenson DD. Rational approach to aspirin dosing during oral challenges and desensitization of patients with aspirin-exacerbated respiratory disease. J Allergy Clin Immunol 2009;123:406-10.
23. Bertele V, Falanga A, Tomasiak M, Dejana E, Cerletti C, de Gaetano G. Platelet thromboxane synthetase inhibitors with low doses of aspirin: possible resolution of the "aspirin dilemma". Science 1983;220:517-9.
24. Capone ML, Tacconelli S, Sciulli MG, Grana M, Ricciotti E, Minuz P, et al. Clinical pharmacology of platelet, monocyte, and vascular cyclooxygenase inhibition by naproxen and low-dose aspirin in healthy subjects. Circulation 2004;109:1468-71.
25. Pleskow WW, Stevenson DD, Mathison DA, Simon RA, Schatz M, Zeiger RS. Aspirin desensitization in aspirin-sensitive asthmatic patients: clinical manifestations and characterization of the refractory period. J Allergy Clin Immunol 1982;69:11-9.
26. Taytard A, Guenard H, Vuillemin L, Bouvot JL, Vergeret J, Ducassou D, et al. Platelet kinetics in stable atopic asthmatic patients. Am Rev Respir Dis 1986;134:983-5.
27. Stevenson DD, Pleskow WW, Simon RA, Mathison DA, Lumry WR, Schatz M, et al. Aspirin-sensitive rhinosinusitis asthma: a double-blind crossover study of treatment with aspirin. J Allergy Clin Immunol 1984;73:500-7.
28. Fruth K, Pogorzelski B, Schmidtmann I, Springer J, Fennan N, Fraessdorf N, et al. Low-dose aspirin desensitization in individuals with aspirin-exacerbated respiratory disease. Allergy 2013;68:659-65.
29. Stevenson DD. Diagnosis, prevention, and treatment of adverse reactions to aspirin and nonsteroidal anti-inflammatory drugs. J Allergy Clin Immunol 1984;74:617-22.
30. Proceedings of the ATS workshop on refractory asthma: current understanding, recommendations, and unanswered questions. American Thoracic Society. Am J Respir Crit Care Med 2000;162:2341-51.
31. Allen JN, Davis WB. Eosinophilic lung diseases. Am J Respir Crit Care Med 1994;150:1423-38.
32. Mita H, Oosaki R, Mizushima Y, Kobayashi M, Akiyama K. Efficient method for the quantitation of urinary leukotriene E4: extraction using an Empore C18 disk cartridge. J Chromatogr B Biomed Sci Appl 1997;692:461-6.
33. Michelson AD, Barnard MR, Hechtman HB, MacGregor H, Connolly RJ, Loscalzo J, et al. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. Proc Natl Acad Sci U S A 1996;93:11877-82.
34. Maclouf JA, Murphy RC. Transcellular metabolism of neutrophil-derived leukotriene A4 by human platelets. A potential cellular source of leukotriene C4. J Biol Chem 1988;263:174-81.
35. Patrignani P, Dovizio M. Inside platelet-leukocyte cross-talk. Blood 2012;119:3649-50.
36. Cummings HE, Liu T, Feng C, Laidlaw TM, Conley PB, Kanaoka Y, et al. Cutting edge: leukotriene C4 activates mouse platelets in plasma exclusively through the type 2 cysteinyl leukotriene receptor. J Immunol 2013;191:5807-10.
37. Paruchuri S, Tashimo H, Feng C, Maekawa A, Xing W, Jiang Y, et al. Leukotriene E4-induced pulmonary inflammation is mediated by the P2Y12 receptor. J Exp Med 2009;206:2543-55.
38. Laidlaw TM, Cutler AJ, Kidder MS, Liu T, Cardet JC, Chhay H, et al. Prostaglandin E2 resistance in granulocytes from patients with aspirin-exacerbated respiratory disease. J Allergy Clin Immunol 2014;133:1692-701.
39. Inwald DP, McDowall A, Peters MJ, Callard RE, Klein NJ. CD40 is constitutively expressed on platelets and provides a novel mechanism for platelet activation. Circ Res 2003;92:1041-8.
40. Ono E, Taniguchi M, Mita H, Higashi N, Fukutomi Y, Tanimoto H, et al. Increased urinary leukotriene E4 concentration in patients with eosinophilic pneumonia. Eur Respir J 2008;32:437-42.
41. Doyle EL, Ridger V, Ferraro F, Turmaine M, Saftig P, Cutler DF. CD63 is an essential cofactor to leukocyte recruitment by endothelial P-selectin. Blood 2011;118:4265-73.
42. Pitchford SC, Yano H, Lever R, Riffo-Vasquez Y, Ciferri S, Rose MJ, et al. Platelets are essential for leukocyte recruitment in allergic inflammation. J Allergy Clin Immunol 2003;112:109-18.
43. Nizankowska E, Czerniawska-Mysik G, Szczeklik A. Lack of effect of i.v. prostacyclin on aspirin-induced asthma. Eur J Respir Dis 1986;69:363-8.
44. Palikhe S, Palikhe NS, Kim SH, Yoo HS, Shin YS, Park HS. Elevated platelet activation in patients with chronic urticaria: a comparison between aspirin-intolerant and aspirin-tolerant groups. Ann Allergy Asthma Immunol 2014;113:276-81.
45. Tornhamre S, Ehnhage A, Kolbeck KG, Edenius C, Lindgren JA. Uncoupled regulation of leukotriene C4 synthase in platelets from aspirin-intolerant asthmatics and healthy volunteers after aspirin treatment. Clin Exp Allergy 2002;32:1566-73.
46. Klinkhardt U, Graff J, Harder S. Clopidogrel, but not abciximab, reduces platelet leukocyte conjugates and P-selectin expression in a human ex vivo in vitro model. Clin Pharmacol Ther 2002;71:176-85.

47. Caron A, Theoret JF, Mousa SA, Merhi Y. Anti-platelet effects of GPIIb/IIIa and P-selectin antagonism, platelet activation, and binding to neutrophils. J Cardiovasc Pharmacol 2002;40:296-306.
48. John AE, Lukacs NW, Berlin AA, Palecanda A, Bargatze RF, Stoolman LM, et al. Discovery of a potent nanoparticle P-selectin antagonist with anti-inflammatory effects in allergic airway disease. FASEB J 2003;17:2296-8.
49. Amison RT, Momi S, Morris A, Manni G, Keir S, Gresele P, et al. Rho A signaling through platelet P2Y1 receptor controls leukocyte recruitment in allergic mice. J Allergy Clin Immunol 2015;135:528-38.
50. McDonald JR, Tan EM, Stevenson DD, Vaughan JH. Platelet aggregation in asthmatic and normal subjects. J Allergy Clin Immunol 1974;54:200-8.