

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

Chihiro Mitsui, MD,^{a,c} Keiichi Kajiwar, BSc,^a Hiroaki Hayashi, MD,^a Jun Ito, MD, PhD,^a Haruhisa Mita, PhD,^a Emiko Ono, MD, PhD,^a Noritaka Higashi, MD, PhD,^{a,b} Yuma Fukutomi, MD, PhD,^a Kiyoshi Sekiya, MD,^a Takahiro Tsuburai, MD, PhD,^a Kazuo Akiyama, MD,^a Kazuhiko Yamamoto, MD, PhD,^c and Masami Taniguchi, MD, PhD^a
 Kanagawa, Kumamoto, and Tokyo, Japan

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 E₄. 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) E₄, which is excessive even under stable disease conditions,^{7,8} further increases after aspirin-induced reactions.^{9,10} Leukotriene C₄ synthase (LTC₄S) 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 al²⁰ 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) A₂ 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

From ^athe Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagami National Hospital, Kanagawa; ^bthe Japanese Red Cross Kumamoto Health Care Center, Kumamoto; and ^cthe Department of Allergy and Rheumatology, Graduate School of Medicine, University of Tokyo.

Supported by a Health, Labor, and Sciences research grant on allergic disease and immunology from the Ministry of Health, Labour and Welfare of Japan (grant no. H26-ITAKU (MEN)–IPPAN-002).

Disclosure of potential conflict of interest: The authors have been supported by a Health, Labor, and Sciences research grant on allergic disease and immunology from the Ministry of Health, Labour and Welfare of Japan (grant no. H26-ITAKU (MEN)–IPPAN-002).

Received for publication January 7, 2015; revised May 3, 2015; accepted for publication May 20, 2015.

Corresponding author: Masami Taniguchi, MD, PhD, Clinical Research Center for Allergy and Rheumatology, National Hospital Organization, Sagami National Hospital, Sakuradai 18-1, Minami-ku, Sagami, Kanagawa, Japan. E-mail: m-taniguchi@sagami-hosp.gr.jp.

0091-6749/\$36.00

© 2015 American Academy of Allergy, Asthma & Immunology

<http://dx.doi.org/10.1016/j.jaci.2015.05.041>

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 ₄ S:	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 Sagami 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 E₄ (uLTE₄) 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 LTE₄ 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 $\pm$ 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 FEV₁: $P = .006$ and $P = .027$; percent predicted prebronchodilator and postbronchodilator mean forced expiratory flow between 25% and 75% of forced vital capacity [FEF₂₅₋₇₅]: $P = .019$ and $P = .048$, respectively). The uLTE₄ level in patients with AERD was higher than that in patients with ATA ($P = .005$) and control subjects ($P < .001$), respectively. Although the uLTE₄ 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 uLTE₄ 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

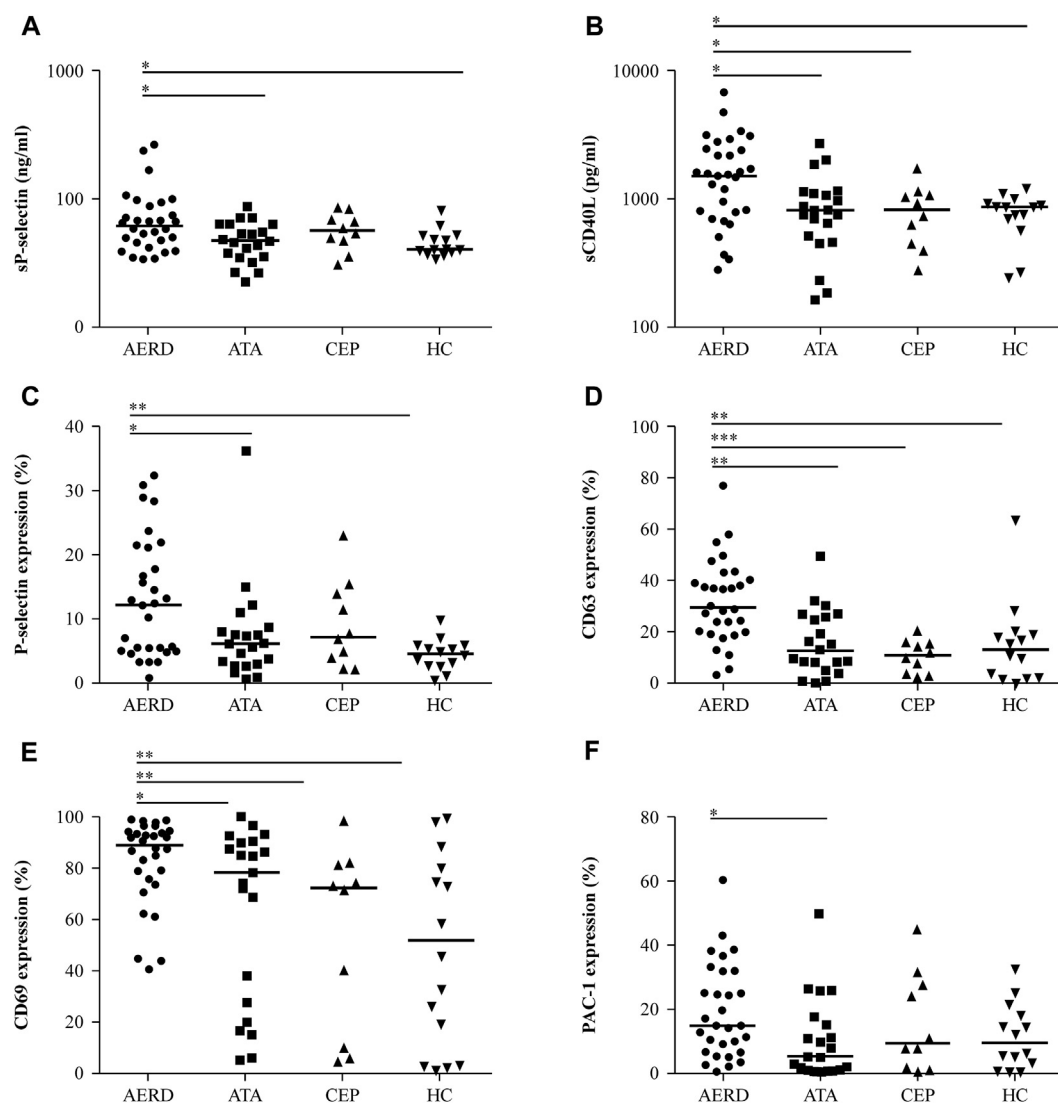


FIG 1. Comparison of plasma levels of sP-selectin (A) and sCD40L (B) and expression levels of P-selectin (C), CD63 (D), CD69 (E), and PAC-1 (F) on platelets among patients with AERD (n = 30), patients with ATA (n = 21), patients with CEP (n = 10), and healthy control subjects (HC; n = 14). * $P < .05$, ** $P < .01$, 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 uLTE₄ 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 uLTE₄ 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.

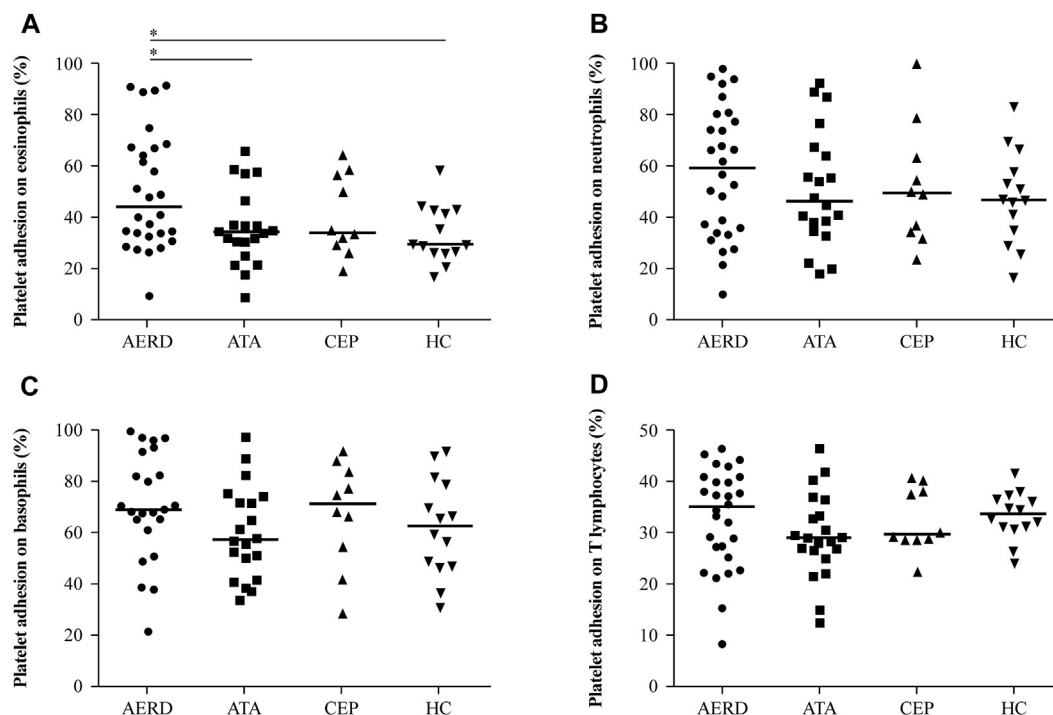


FIG 2. Comparison of platelet adhesion on leukocytes analyzed by using flow cytometry. Platelet adhesion on eosinophils (A), neutrophils (B), basophils (C), and T lymphocytes (D) from patients with AERD (n = 30), patients with ATA (n = 21), patients with CEP (n = 10), and healthy control subjects (HC; 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 uLTE₄ concentrations (Fig 4, A and E). CD63 expression levels showed a weak correlation with uLTE₄ 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

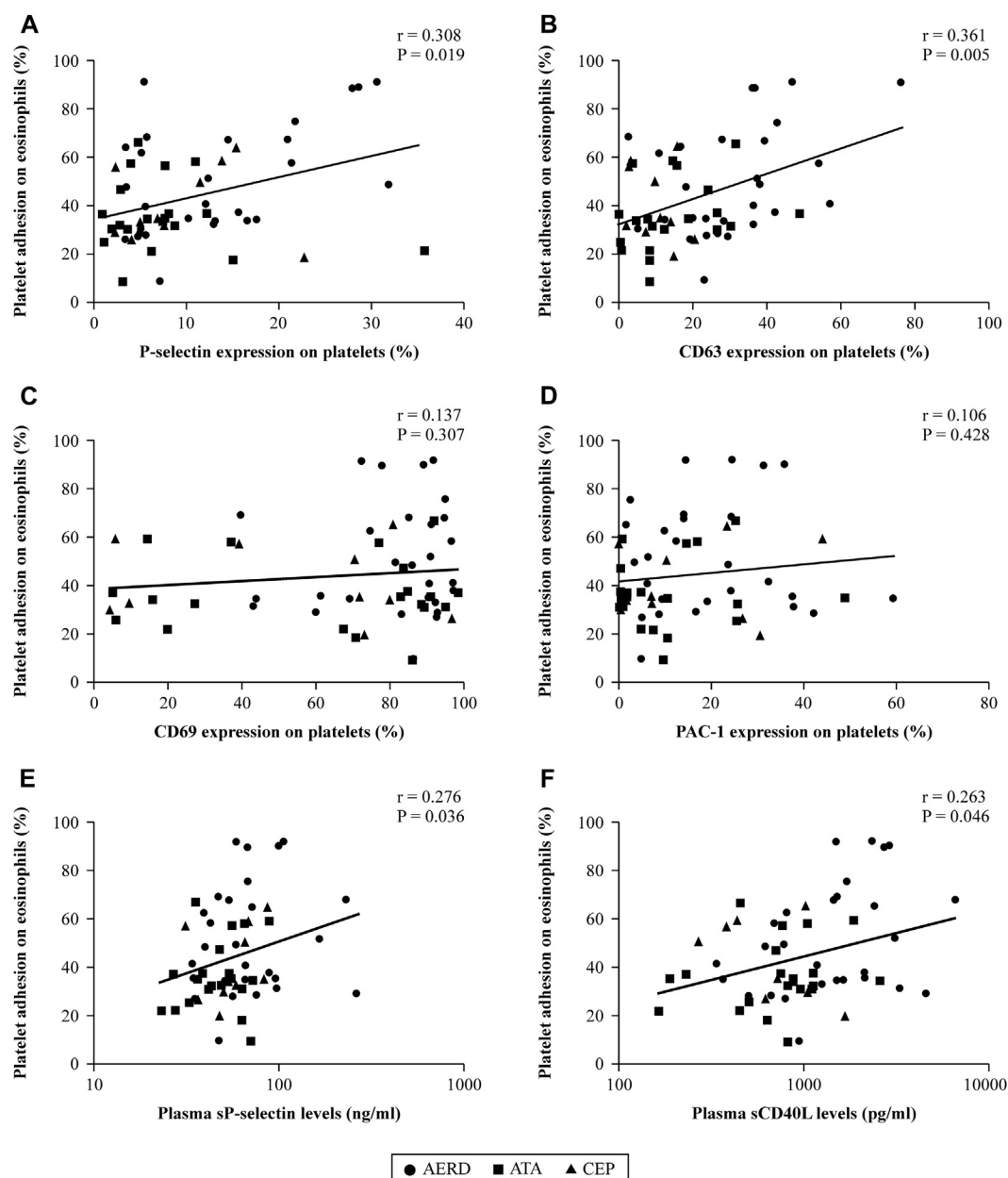


FIG 3. Correlation of platelet activation markers with the percentage of platelet adhesion on eosinophils were assessed in all patients. Expression levels of P-selectin (**A**), CD63 (**B**), CD69 (**C**), and PAC-1 (**D**) on platelets and plasma levels of sP-selectin (**E**) and sCD40L (**F**) are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

predicted prebronchodilator and postbronchodilator FEV₁ ($r = -0.324$, $P = .019$ and $r = -0.370$, $P = .007$, respectively; Fig 5, A and B) and percent predicted prebronchodilator and postbronchodilator FEF₂₅₋₇₅ ($r = -0.381$, $P = .005$ and $r = -0.472$, $P < .001$, respectively; Fig 5, C and D). Plasma sCD40L levels were negatively correlated only with percent predicted postbronchodilator FEF₂₅₋₇₅ ($r = -0.336$, $P = .015$ 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 FEF₂₅₋₇₅, although the correlation between the CD63 expression level and percent predicted prebronchodilator FEF₂₅₋₇₅ 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 uLTE₄ 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

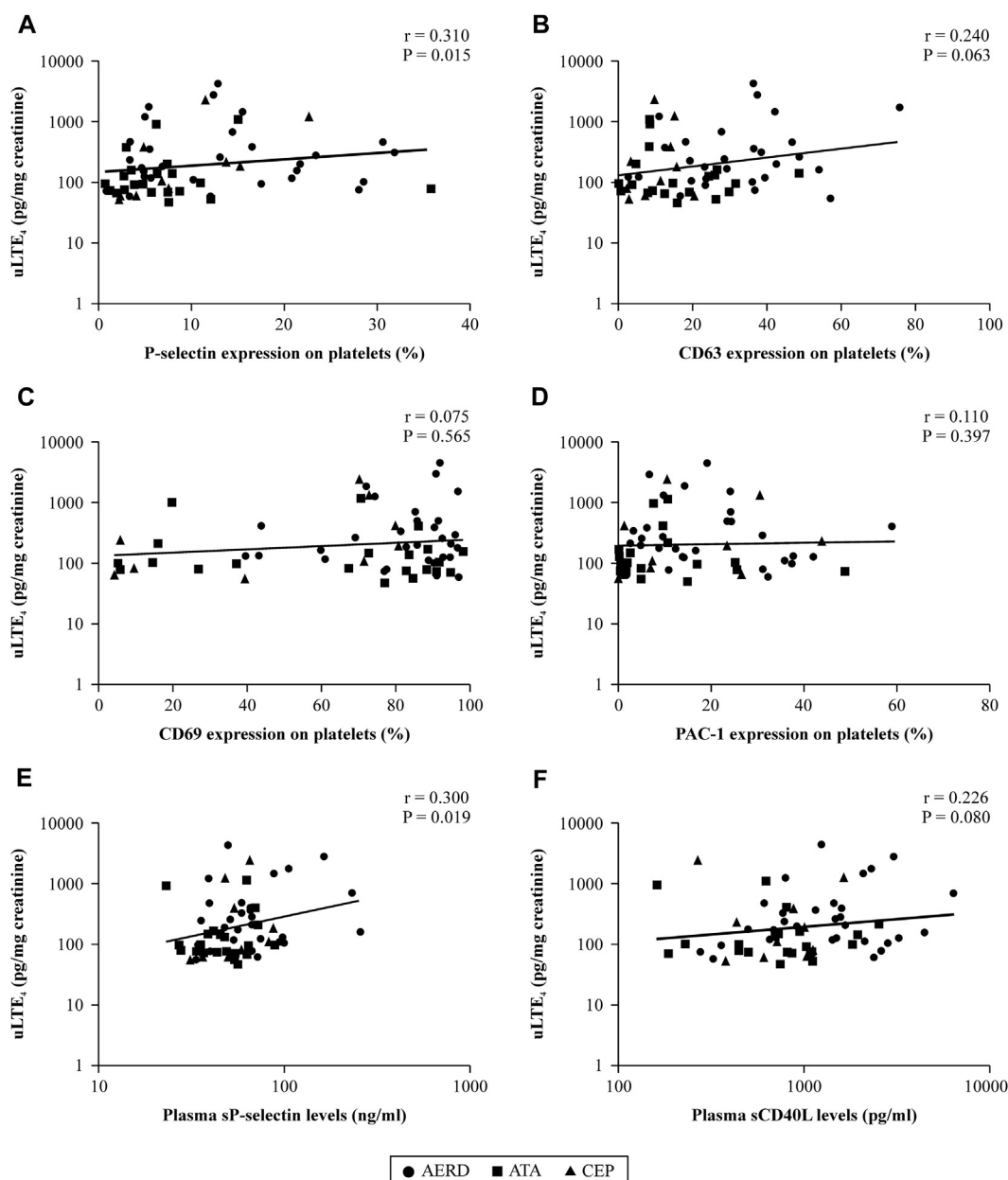


FIG 4. Correlation of platelet activation markers with uLTE₄ concentrations were assessed in all patients. Expression levels of P-selectin (A), CD63 (B), CD69 (C), and PAC-1 (D) on platelet and plasma levels of sP-selectin (E) and sCD40L (F) are shown.

previously, uLTE₄ 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 uLTE₄ 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 uLTE₄ 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.

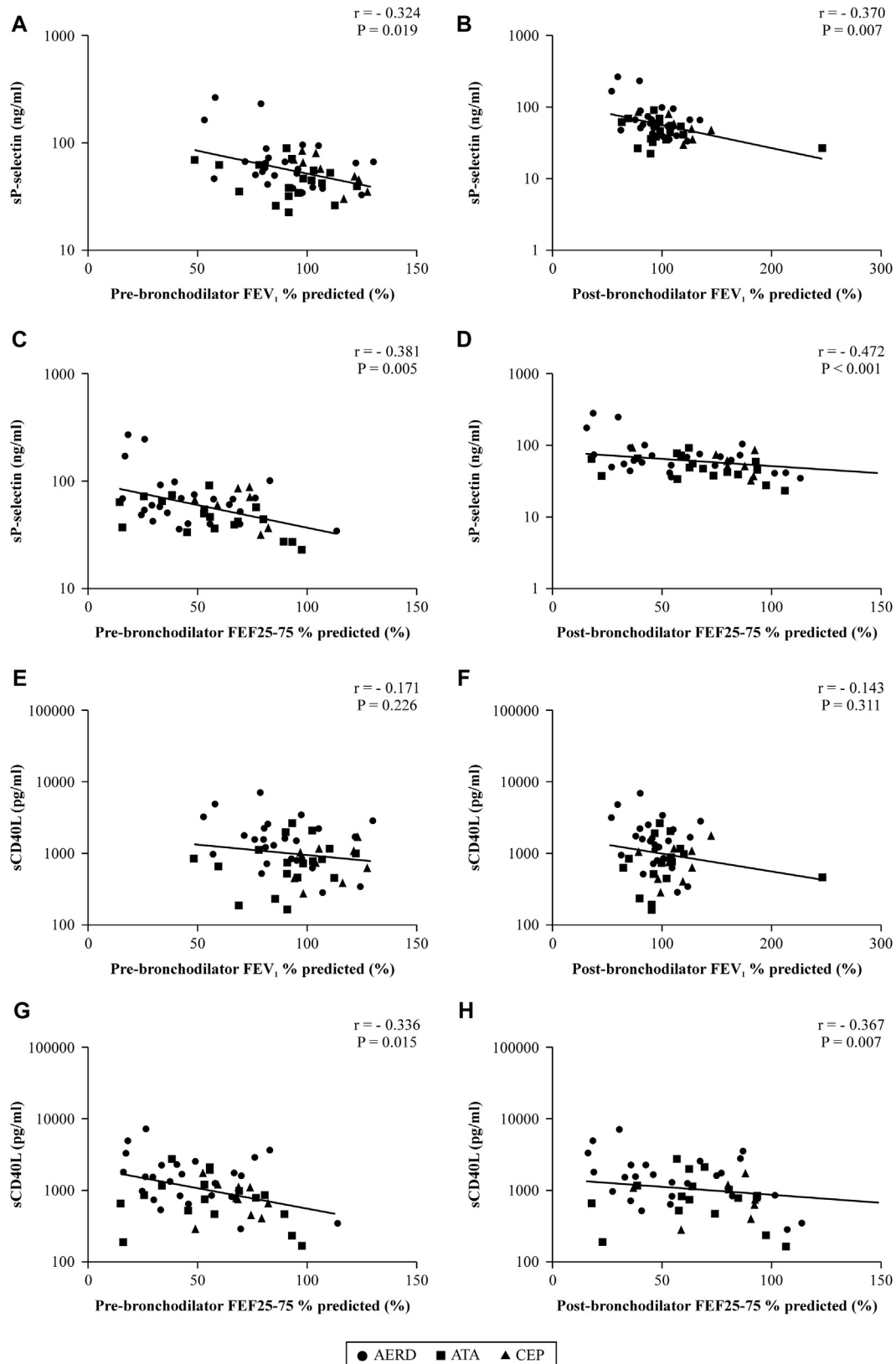


FIG 5. Correlation of platelet activation markers in plasma with lung function was assessed in all patients. The correlation of plasma sP-selectin (A-D) and sCD40L (E-H) levels with percent predicted FEV₁ and percent predicted FEF₂₅₋₇₅ in prebronchodilator and postbronchodilator spirometry.

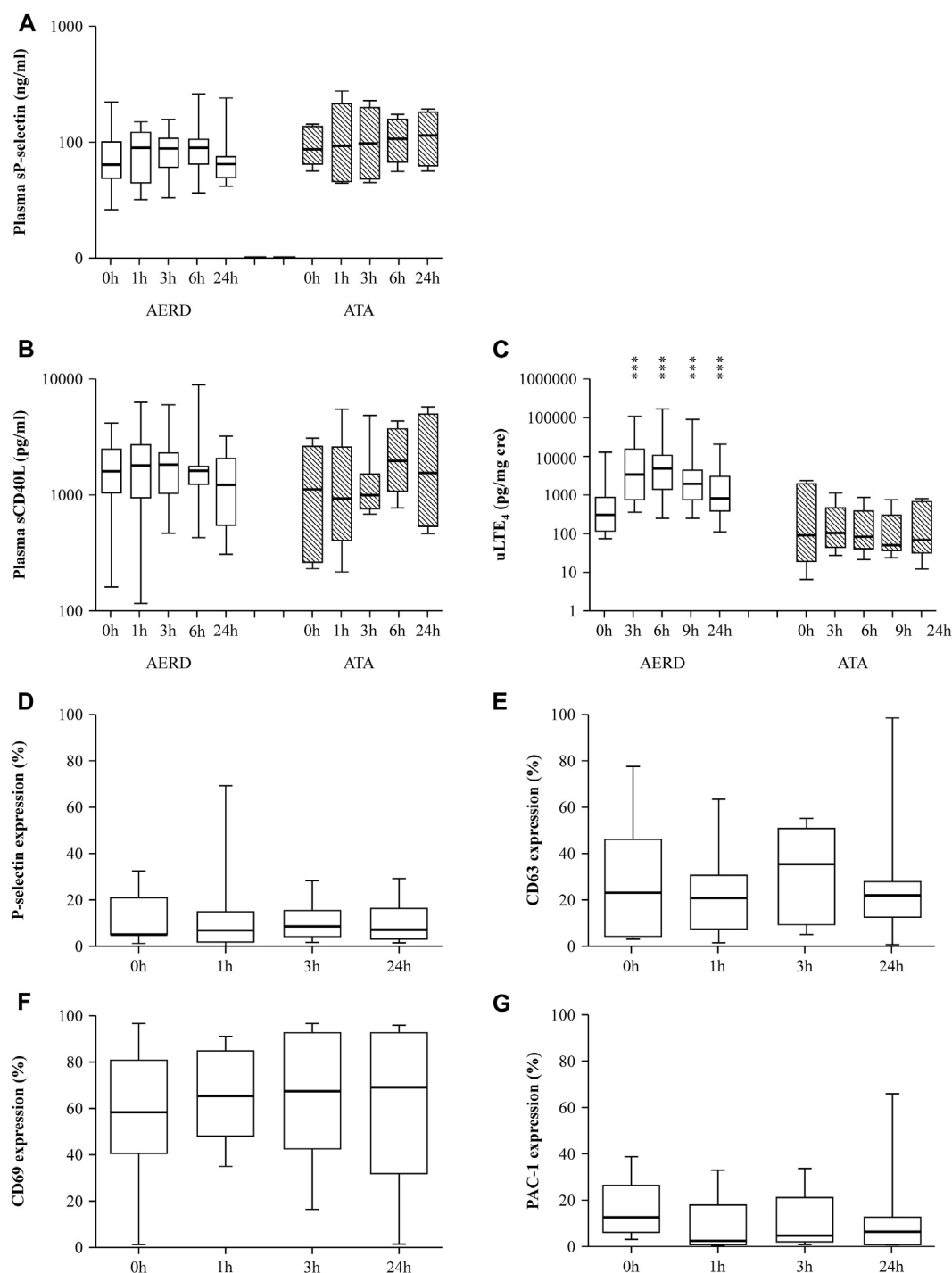


FIG 6. Changes in plasma sP-selectin (A) and sCD40L levels (B) and uLTE₄ levels (C) during the L-ASA challenge test were evaluated in patients with AERD (n = 24) and patients with ATA (n = 7). P-selectin (D), CD63 (E), CD69 (F), and PAC-1 (G) expression levels on platelets were also assessed in patients 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 LTA₄ through LTC₄S 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 al³⁶ revealed that LTC₄, but not LTD₄ and LTE₄, binds to cysLT receptor 2 and subsequently causes an autocrine ADP-mediated response through P₂Y₁₂ receptors, which induce surface expression of P-selectin on murine platelets, although cysLTs, including LTE₄, 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 uLTE₄ 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 uLTE₄ levels. These findings suggest that platelets adhere subsequently to eosinophils through P-selectin and CD40L and contribute to cysLT overproduction through synthesis of LTC₄ from leukocyte-derived LTA₄ (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 uLTE₄ 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 al¹⁸ 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 TXA₂, 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 E₂ synthesis and uncouples the downregulation of LTC₄S activity through inhibition of TXA₂ 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 P₂Y₁ antagonist, but not a P₂Y₁₂ or P₂Y₁ 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 uLTE₄ 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.

We thank Ms Itsuko Ito for excellent technical assistance.

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

REFERENCES

- Stevenson DD, Szczeklik A. Clinical and pathologic perspectives on aspirin sensitivity and asthma. *J Allergy Clin Immunol* 2006;118:773-86.
- 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.
- 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.
- 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.
- 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.
- Laidlaw TM, Boyce JA. Pathogenesis of aspirin-exacerbated respiratory disease and reactions. *Immunol Allergy Clin North Am* 2013;33:195-210.
- Kumlin M, Dahlen B, Björck 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.
- 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.
- 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.
- Mita H, Endoh S, Kudoh M, Kawagishi 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.
- 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.
- Page C, Pitchford S. Platelets and allergic inflammation. *Clin Exp Allergy* 2014;44:901-13.
- Slater D, Martin J, Trowbridge A. The platelet in asthma. *Lancet* 1985;1:110.
- Jawien J, Lomnicka M, Korb 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.
- Li N. Platelet-lymphocyte cross-talk. *J Leukoc Biol* 2008;83:1069-78.
- 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.
- Zietkowski Z, Skiepkio 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.
- Kowal K, Pampuch A, Kowal-Bielecka O, DuBuske LM, Bodzenta-Lukaszyk A. Platelet activation in allergic asthma patients during allergen challenge with *Der-matophagoides pteronyssinus*. *Clin Exp Allergy* 2006;36:426-32.
- 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.
- 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.
- Kowalski ML, Grzelewska-Rzymowska I, Rozniecki J, Szmidt M. Aspirin tolerance induced in aspirin-sensitive asthmatics. *Allergy* 1984;39:171-8.
- 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.
- 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.
- Capone ML, Tacconelli S, Sciuilli 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.
- 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.
- 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.
- 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.
- Fruth K, Pogorzelski B, Schmidtman 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.
- Stevenson DD. Diagnosis, prevention, and treatment of adverse reactions to aspirin and nonsteroidal anti-inflammatory drugs. *J Allergy Clin Immunol* 1984;74:617-22.
- 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.
- Allen JN, Davis WB. Eosinophilic lung diseases. *Am J Respir Crit Care Med* 1994;150:1423-38.
- 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.
- 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.
- Maclof 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.
- Patrignani P, Dovizio M. Inside platelet-leukocyte cross-talk. *Blood* 2012;119:3649-50.
- 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.
- Paruchuri S, Tashiro 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.
- 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.
- 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.
- 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.
- 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.
- Pitchford SC, Yano H, Lever R, Riffio-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.
- 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.
- 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.
- Tornhamre S, Ehnage 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.
- 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.

Online Data Supplement

Platelet activation markers overexpressed specifically in aspirin-exacerbated
respiratory disease

Chihiro Mitsui, Keiichi Kajiwarra, Hiroaki Hayashi, Jun Ito, Haruhisa Mita,
Emiko Ono, Noritaka Higashi, Yuma Fukutomi, Kiyoshi Sekiya, Takahiro
Tsuburai, Kazuo Akiyama, Kazuhiko Yamamoto, Masami Taniguchi

Methods

Subjects

Prior to enrollment, all subjects were asked about their regular medications and medical history. Subjects with autoimmune disease, ischemic cardiovascular disease, cerebrovascular disease, or undertreatment of hypertension, hyperlipidemia, or diabetes mellitus were excluded from the study. Undertreatment of hypertension was defined by casual blood pressure $\geq 140/90$ mmHg. Undertreatment of hyperlipidemia was defined by a fasting blood triglyceride level ≥ 150 mg/dl or low density lipoprotein level ≥ 140 mg/dl. Undertreatment of diabetes mellitus was defined by a fasting blood glucose level ≥ 126 mg/dl. None of the subjects were regular users of aspirin, nonsteroidal anti-inflammatory drugs or cyclooxygenase-2 selective inhibitors. Subjects who had asthma exacerbation or lower respiratory tract infection within the previous 6 weeks were also excluded from the study. Idiopathic chronic eosinophilic pneumonia (CEP) patients had been diagnosed using the following criteria (based on the classification proposed by Allen et al.^{E1}: 1) a suggestive radiological profile; 2) blood and/or alveolar eosinophilia; 3) general and respiratory symptoms for > 2 weeks; 4) a prompt

response to corticosteroids; and 5) uncertain cause, and were treated to induce remission prior to the study.

Assessments

All the patients were evaluated by chest radiograph, spirometry analyses, and exhaled nitric oxide (FeNO). Pulmonary function tests (according to the American Thoracic Society (ATS) guidelines^{E2}) were performed before and after administration of 400 µg salbutamol.^{E3} Data that were evaluated included forced expiratory volume in 1 second (FEV₁) and mean forced expiratory flow between 25% and 75% of forced vital capacity (FVC) as a percentage of predicted values. FeNO was collected and measured by offline methods at a flow rate of 50 mL/s (Sievers Instruments, Boulder, CO).^{E4} Total white blood cell, differential, and platelet counts were also measured. Total serum immunoglobulin E (IgE) levels were measured with the Uni-CAP system (Phadia, Uppsala, Sweden). Atopy was defined as showing a positive response to at least one aeroallergen in a skin test or IgE CAP RAST. Urine and blood samples were collected between 08:00 AM and 12:00 noon for measurement of urinary leukotriene E₄ (uLTE₄)

concentrations and platelet activation markers in patients with stable disease. Blood was carefully obtained using a 21G needle by several physicians experienced with this procedure.

Aspirin challenge test

All of the patients were in clinically stable condition at the time of the challenge test, and their FEV₁/FVC ratio was higher than 70%. All medications except for daily oral corticosteroids were stopped for at least 24 h prior to the aspirin challenge test. At the beginning of the study, we collected blood samples and urine samples from stored urine between 08:00 AM and 10:00 AM.

Twenty-four AERD patients, who visited our hospital between 2002 and 2012 March, underwent an intravenous lysine-aspirin (L-ASA) challenge test, as reported previously.^{E5} After intravenous injection of 1 ml saline, if FEV₁ did not change by more than 5% from the pre-challenge baseline, doubling doses of L-ASA (25-200 mg equivalent to aspirin) were intravenously administered. As L-ASA became unavailable in Japan after 2012, 6 AERD patients, who visited our hospital after 2012 April, underwent

an oral aspirin challenge test.^{E6} The dosage of ASA was started at 30 mg, and the dose was doubled at 2-hour intervals up to 480 mg until a positive reaction occurred. FEV₁ was measured by spirometry 3 times at each time point, and the highest value was used. A decrease in FEV₁ by 20% or greater as compared with the baseline or a decrease in FEV₁ by 10% to 20% with exacerbating clinical symptoms, such as nasal obstruction, were defined as a positive reaction.

Blood samples were collected for measurements of plasma soluble P-selectin (sP-selectin) and soluble CD40 ligand (sCD40L) levels at 0-1, 1-3, 3-6, and 9-24 hours after the ingestion of the dose producing a positive reaction in 24 AERD patients, or after the last dose (cumulative dose of 375 mg) of L-ASA in 7 ATA controls. Additionally, we analyzed expression levels of 4 surface markers on platelets [P-selectin (CD62P), CD63, CD69 and GPIIb/IIIa (PAC-1)] at 0-1, 1-3, and 9-24 hours after the ingestion of the dose producing a positive reaction in 8 AERD patients. L-ASA was administered to 4 of them and oral aspirin was administered to the remaining patients. Some of the patients were also assessed to determine the percentage of platelet-adherent leukocytes, including eosinophils (n = 4), neutrophils (n =

3), T lymphocytes (n = 3), and basophils (n = 2). Urine samples were also collected for measurement of LTE₄ levels during the following periods: 0-3, 3-6, 6-9, and 9-24 h after the dose producing a positive reaction in AERD patients, or after the last dose in ATA patients.

Antibodies

For fluorescence-activated cell sorter (FACS) analysis studies, the following antibodies were used; phycoerythrin-cyanine 7 (PC7)-conjugated anti-CD61 (GPIIIa); phycoerythrin (PE)-conjugated anti-CD63 and anti-CD69; fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and anti-CD16b (FcγRIIIb) were purchased from Beckman Coulter (Fullerton, CA). PE-conjugated anti-CD62P (P-selectin) and FITC-conjugated PAC-1 (activated form of GPIIb/IIIa) were from BD Biosciences (San Jose, CA). FITC-conjugated anti-chemoattractant receptor-homologous molecule expressed by Th2 (CRTH-2) was from Santa Cruz Biotechnology (Santa Cruz, CA). FITC-conjugated anti-FcεRIα chain (CRA-1) was from eBiosciences (San Diego, CA). The corresponding isotypes (Beckman Coulter) were used as controls.

109

110 **Flow cytometric analysis of platelets**

111 Samples were analyzed using a FACSCalibur flow cytometer with
112 CellQuest software (Becton Dickinson, San Jose, CA). Whole peripheral
113 blood was drawn using a 21G needle into 3.2% sodium citrate tube, and
114 were stained within 10 min after collection. Unstimulated whole blood (10 μ l)
115 was incubated with directly-conjugated specific antibodies to CD61 and
116 P-selectin (CD62P), CD63, CD69, or GPIIbIIIa (PAC-1) for 15 minutes, and
117 then fixed by the addition of 1% paraformaldehyde. Platelets were detected
118 on the basis of forward/side scatter characteristics and expression of CD61.
119 At least 20000 CD61⁺ platelet events were collected in each determination.
120 The percentage of cells positive for each surface marker and the mean
121 fluorescence intensity (MFI) in the platelet gate (determined by
122 CD61-staining) were quantified as a measure of platelet activation.

123

124 **Flow cytometric analysis of platelet-leukocyte aggregates**

125 Whole peripheral blood was drawn using a 21G needle into tubes
126 containing EDTA anticoagulant solution and assayed within 30 minutes of

collection. Whole blood (100 μ l) was incubated with directly-labeled antibodies against CD61 and CRTH-2, Fc ϵ RI α (CRA-1), CD16b, or CD3 for 20 minutes. Erythrocytes were lysed in 1 ml of 1X red blood cell lysis solution (BD Biosciences). Cell samples were washed with 1 ml PBS and then fixed in 0.1% paraformaldehyde in PBS. At least 10000 of each leukocyte-specific markers-positive cell type was measured. Eosinophils, neutrophils, basophils, and T lymphocytes were distinguished based on their typical appearance in the forward/side scatter and their relative membrane expression of CRTH-2, CD16b, CRA1 and CD3, respectively. Each leukocyte was gated into CD61-negative and -positive populations, and assessed for the presence of adherent platelets by relative expression of CD61.

Enzyme-linked immunosorbent assay (ELISAs)

Plasma was prepared from blood drawn into tubes containing EDTA anticoagulant solution (1.5mg/ml) and centrifuged for 10 min at room temperature at 1600 $\times$ g. The final supernatant was stored in aliquots at -35°C for up to 4 weeks until measurement. The concentrations of sP-selectin and sCD40L were determined by ELISA kit (R&D Systems, Minneapolis,

MN) both in patients with stable disease and during an aspirin challenge test. Samples were diluted with ELISA buffer to a final dilution of 1:20 for sP-selectin analysis, and 1:2 for sCD40L analysis. The absorbance of the colored product was measured at 450nm in a microplate reader, (model 3550-UV, Bio-Rad Laboratories, Hercules, CA). Each dilution was run in duplicate. The detection limits of the assays were 0.5 ng/ml for sP-selectin and 10.1 pg/ml for sCD40L, respectively.

Quantification of uLTE₄

Urine samples were collected in polypropylene bottles containing 4-hydroxy-TEMPO (Sigma-Aldrich, St Louis, MO) at a final concentration of 1mmol/L, and stored at -35°C until measurement. LTE₄ in urine (2 ml) was purified using an Empore C18 disk cartridge and high-performance liquid chromatography system (Shimadzu LC 6A) equipped with a NOVA-PACK C18 column (Waters, Milford, MA), and quantified using a commercial enzyme immunoassay kit (Cayman, Ann Arbor, MI).^{E7} The concentrations of uLTE₄ was normalized to the urinary creatinine concentration and expressed as pg per mg of creatinine.

References

E1. Allen JN, Davis WB. Eosinophilic lung diseases. *Am J Respir Crit Care Med* 1994;150:1423-1438.

E2. Standardization of Spirometry, 1994 Update. American Thoracic Society. *Am J Respir Crit Care Med* 1995;152:1107-1136.

E3. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R et al. Interpretative strategies for lung function tests. *Eur Respir J* 2005;26:948-968.

E4. American Thoracic Society, European Respiratory Society. ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. *Am J Respir Crit Care Med* 2005;171:912-930.

E5. Mita H, Endoh S, Kudoh M, Kawagishi Y, Kobayashi M, Taniguchi M et al. Possible involvement of mast-cell activation in aspirin provocation of aspirin-induced asthma. *Allergy* 2001;56:1061-1067.

E6. Stevenson DD. Diagnosis, prevention, and treatment of adverse reactions to aspirin and nonsteroidal anti-inflammatory drugs. *J Allergy Clin Immunol* 1984;74:617-622.

181 E7. Mita H, Oosaki R, Mizushima Y, Kobayashi M, Akiyama K. Efficient
182 method for the quantitation of urinary leukotriene E4: extraction using an
183 Empore C18 disk cartridge. J Chromatogr B Biomed Sci Appl.
184 1997;692:461-466.

185

186 TableE1. Peripheral blood profiles of patients

	AERD N=30	ATA N=21	CEP N=10
Total WBC, / μ l	7715 $\pm$ 2753	5629 $\pm$ 1531**	6896 $\pm$ 1556
Peripheral platelet count, $\times 10^4$ / μ l	25 $\pm$ 6	22 $\pm$ 3	22 $\pm$ 2
Peripheral eosinophil count, / μ l	465 $\pm$ 402	303 $\pm$ 374*	557 $\pm$ 618
Peripheral neutrophil count, / μ l	4885 $\pm$ 2476	3497 $\pm$ 1208*	4663 $\pm$ 1556
Peripheral basophil count, / μ l	40 $\pm$ 24	37 $\pm$ 26	39 $\pm$ 20
Peripheral lymphocyte count, / μ l	1936 $\pm$ 586	1510 $\pm$ 464*	1340 $\pm$ 369**
Peripheral monocyte count, / μ l	389 $\pm$ 142	282 $\pm$ 120**	267 $\pm$ 173*

187 P values surviving Bonferroni correction are shown in boldface (P < .05/7).

188 Definition of abbreviations: AERD, aspirin-exacerbated respiratory disease; ATA,
189 aspirin-tolerant asthma; CEP, chronic eosinophilic pneumonia; WBC, white blood count;

190 Data are presented as mean $\pm$ SD.

191 *P<0.05, **P<0.01 compared with AERD patients.

192

193 Table E2. Correlation of platelet activation markers with platelet-adherent eosinophils and
194 uLTE₄ levels among AERD patients or all patients

	Platelet-adherent eosinophils				uLTE ₄			
	AERD patients		All patients		AERD patients		All patients	
	r	p-value	r	p-value	r	p-value	r	p-value
P-selectin expression levels, %	0.461	0.014	0.308	0.019	0.085	0.656	0.310	0.015
CD63 expression levels, %	0.395	0.037	0.361	0.005	0.199	0.291	0.240	0.063
CD69 expression levels, %	0.063	0.749	0.137	0.307	0.020	0.917	0.075	0.565
PAC-1 expression levels, %	0.033	0.866	0.106	0.428	-0.066	0.730	0.110	0.397
sP-selectin levels, ng/ml	0.187	0.340	0.276	0.036	0.181	0.338	0.300	0.019
sCD40L levels, pg/ml	0.322	0.095	0.263	0.046	0.172	0.362	0.226	0.080

195 P values surviving Bonferroni correction are shown in boldface (P < .05/6).
196 Definition of abbreviations: AERD, aspirin-exacerbated respiratory disease; uLTE₄, urinary
197 leukotriene E₄; sP-selectin, soluble P-selectin; sCD40L, soluble CD40 ligand

198
199
200
201
202

203 Table E3. Correlation of pre- and post-bronchodilator pulmonary function with platelet
204 activation markers among AERD patients or all patients

	Pre-bronchodilator				Post-bronchodilator			
	AERD patients		All patients		AERD patients		All patients	
	r	p-value	r	p-value	r	p-value	r	p-value
FEV₁, %								
P-selectin expression levels, %	-0.052	0.807	-0.017	0.905	-0.073	0.727	-0.048	0.737
CD63 expression levels, %	-0.208	0.319	-0.158	0.264	-0.228	0.274	-0.147	0.297
CD69 expression levels, %	-0.140	0.504	-0.214	0.127	-0.190	0.363	-0.145	0.306
PAC-1 expression levels, %	0.345	0.091	0.068	0.632	0.303	0.141	0.042	0.769
sP-selectin levels, ng/ml	-0.349	0.087	-0.324	0.019	-0.447	0.025	-0.370	0.007
sCD40L levels, pg/ml	-0.297	0.149	-0.171	0.226	-0.378	0.063	-0.143	0.311
FEF_{25-75%}, %								
P-selectin expression levels, %	-0.140	0.504	-0.130	0.357	-0.182	0.385	-0.190	0.178
CD63 expression levels, %	-0.286	0.166	-0.248	0.076	-0.318	0.121	-0.285	0.040
CD69 expression levels, %	-0.330	0.107	-0.325	0.019	-0.301	0.143	-0.309	0.026
PAC-1 expression levels, %	0.315	0.125	0.023	0.870	-0.244	0.241	-0.032	0.822
sP-selectin levels, ng/ml	-0.302	0.142	-0.381	0.005	-0.426	0.034	-0.472	< 0.001
sCD40L levels, pg/ml	-0.251	0.226	-0.336	0.015	-0.363	0.074	-0.367	0.007

205 P values surviving Bonferroni correction are shown in boldface (P < .05/6).

206 Definition of abbreviations: AERD, aspirin-exacerbated respiratory disease; FEV₁, forced
207 expiratory volume in 1 second; FEF_{25-75%}, mean forced expiratory flow between 25% and 75%
208 of forced vital capacity; sP-selectin, soluble P-selectin; sCD40L, soluble CD40 ligand

209

210 Table E4. Demographic data of patients assessed for platelet activation during the L-ASA
211 challenge test

	AERD	ATA
	N=24	N=7
Age, year	52 ± 12	55 ± 19
Male, %	21	14
BMI, kg/m ²	23 ± 4	25 ± 3
Smoking History,%		
Never	67	71
Past	21	29
Current	13	0
Refractory asthma, %	38	43
Atopy, %	63	86
Continuous OCS treatment, %	25	14
Cumulative dose of aspirin, mg	125 (25-420)	N.A.
Peripheral eosinophil count, /μl	350 (193-2070)	390 (160-1000)
FEV ₁ , %	85 ± 14	79 ± 25
FEF _{25-75%} , %	45 ± 24	49 ± 26

212 P values surviving Bonferroni correction are shown in boldface (P < .05/9).

213 Abbreviations: L-ASA, lysine-aspirin; AERD, aspirin-exacerbated respiratory disease; ATA,
214 aspirin-tolerant asthma; BMI, body mass index; OCS, oral corticosteroid; FEV₁, forced
215 expiratory volume in 1 second; FEF_{25-75%}, mean forced expiratory flow between 25% and 75%
216 of forced vital capacity; N.A., not applicable;

217 Data are presented mean ± SD. (Cumulative dose of aspirin and peripheral eosinophil count
218 are presented as median with range.)

219

220

221 Table E5. Changes in platelet activation marker levels in plasma and uLTE₄ concentrations
 222 during the L-ASA challenge test

Plasma marker	Before	0-1 h Onset of reaction	1-3 h	3-6 h	12-24 h
AERD / ATA, number	24 / 7	19 / 7	24 / 7	13 / 4	10 / 4
sP-selectin, ng/ml					
AERD	65 (25-219)	90 (32-153)	88 (33-159)	88 (36 -260)	64 (41-241)
ATA	88 (56-140)	93 (44 -274)	97 (45-228)	107 (56-175)	116 (56-191)
sCD40L, ng/ml					
AERD	1.6 (0-4.1)	1.8 (0.1-6.1)	1.8 (0.5-5.8)	1.6 (0.4-8.7)	1.2 (0.3-3.2)
ATA	1.1 (0.2-3.0)	0.7 (0.2-5.5)	1.0 (0.7-4.8)	2.0 (0.8-4.2)	1.5 (0.5-5.7)
uLTE ₄	Before	0-3h	3-6h	6-9h	9-24h
AERD / ATA, number	24 / 7	24 / 7	24 / 7	24 / 7	24 / 7
uLTE ₄ , pg/mg creatinine					
AERD	315 (71-13257)	3393*** (346-111202)	5063*** (254-163334)	2017*** (246-90943)	823*** (107-18925)
ATA	89 (7-2171)	97 (0-1149)	55 (0-952)	51 (23-703)	70 (12-744)

223 P values surviving Bonferroni correction are shown in boldface (P < .05/10).

224 Abbreviations: uLTE₄, urinary leukotriene E₄, L-ASA; lysine-aspirin; AERD,

225 aspirin-exacerbated respiratory disease; ATA, aspirin-tolerant asthma; sP-selectin, soluble

226 P-selectin; sCD40L, soluble CD40 ligand

227 Data are presented as median with range.

228 ***P <0.001 compared to baseline.

229

230

Table E6. Demographic data of patients assessed for platelet activation using flow cytometry during the aspirin challenge test

	AERD
	N=8
Age, year	38± 11
Male, n (%)	1 (12)
BMI, kg/m ²	23± 4
Smoking History,%	
Never	63
Past	25
Current	12
Refractory asthma, %	12
Atopy, %	75
Continuous OCS treatment, %	0
L-ASA challenge test, n (%)	4 (50)
Cumulative dose of aspirin, mg	120 (45-350)
Peripheral eosinophil count, /μl	410 (193-1040)
Basal uLTE ₄ level, pg /mg creatinine	267 (75-3239)
FEV ₁ , %	95± 9
FEF _{25-75%} , %	66± 19

Abbreviations: AERD, aspirin-exacerbated respiratory disease; BMI, body mass index; OCS, oral corticosteroid; L-ASA, lysine-aspirin; uLTE₄, urinary leukotriene E₄; FEV₁, forced expiratory volume in 1 second; FEF_{25-75%}, mean forced expiratory flow between 25% and 75% of forced vital capacity

Data are presented mean ± SD. (Cumulative dose of aspirin, peripheral eosinophil count, and basal uLTE₄ level are presented as median with range.)

240 Table E7. Changes in expression levels of platelet activation marker on platelets and the
241 percentage of adherent platelets on leukocytes during the challenge test in AERD patients

	Before	0-1 h	1-3 h	9-24 h
P-selectin expression on platelets, %	11.4 ± 10.9 n = 8	14.5 ± 23.0 n = 8	10.5 ± 8.7 n = 8	10.9 ± 9.8 n = 7
CD63 expression on platelets, %	28.2 ± 26.2 n = 8	23.7 ± 19.5 n = 8	31.6 ± 21.7 n = 8	29.3 ± 31.7 n = 7
CD69 expression on platelets, %	57.1 ± 30.6 n = 8	64.6 ± 19.9 n = 8	64.5 ± 29.9 n = 8	64.1 ± 35.5 n = 7
PAC-1 expression on platelets, %	16.0 ± 12.5 n = 8	8.2 ± 12.5 n = 8	10.1 ± 12.7 n = 8	13.8 ± 23.5 n = 7
Platelet adhesion on eosinophils, %	37.7 ± 8.4 n = 4	37.2 ± 24.8 n = 4	38.1 ± 9.9 n = 4	48.3 ± 23.3 n = 4
Platelet adhesion on neutrophils, %	37.7 ± 5.3 n = 3	36.9 ± 15.4 n = 3	40.3 ± 14.5 n = 3	39.3 ± 6.8 n = 3
Platelet adhesion on lymphocytes, %	33.4 ± 10.1 n = 3	33.9 ± 11.8 n = 3	34.5 ± 2.3 n = 3	32.7 ± 7.6 n = 3
Platelet adhesion on basophils, %	47.3 ± 1.6 n = 2	75.3 ± 28.3 n = 2	54.1 ± 5.8 n = 2	57.7 ± 15.4 n = 2

242 Abbreviations: AERD, aspirin-exacerbated respiratory disease

243 Data are presented as mean±SD.

244

245

246

Figure legends

FIG E1. Correlations of surface markers on platelets with percentage predicted FEV₁ in all patients. **A, B**, P-selectin, **C, D**, CD63, **E, F**, CD69, and **G, H**, PAC-1 expression levels on platelets had no relation to the percentage predicted FEV₁ in pre- and post-bronchodilator spirometry.

FIG E2. Correlations of surface markers on platelets with percentage predicted FEF_{25-75%} in all patients. **A, B**, P-selectin, **C, D**, CD63, **E, F**, CD69, and **G, H**, PAC-1 expression levels on platelets were assessed the association with percentage predicted FEF_{25-75%} in pre- and post-bronchodilator spirometry.

FIG E3. Platelet-adherent eosinophils may be involved in cysLT overproduction and eosinophil recruitment into the airway in AERD patients. Activated platelets adhere to leukocytes mainly via P-selectin/PSGL-1 and contribute to transcellular metabolism of leukotrienes from leukocyte-derived LTA₄ via LTC₄S in platelets.

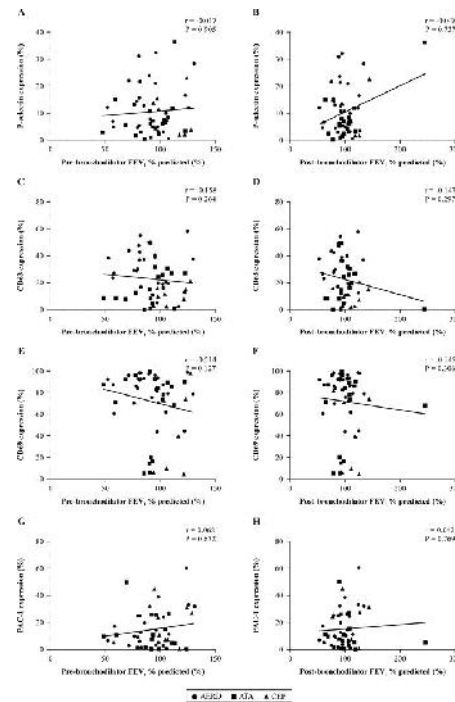


Fig E1.

Chihiro Mitsui, Keiichi Kajiura, Hiroaki Hayashi, Jun Ito, Haruhisa Mita, Emiko Ono, Noritaka Higashi, Yuma Fukutomi, Kiyoshi Sekiya, Takahiro Tsuburai, Kazuo Akiyama, Kazuhiko Yamamoto, Masami Taniguchi

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

Journal of Allergy and Clinical Immunology, 2015, Available online 17 July 2015

<http://dx.doi.org/10.1016/j.jaci.2015.05.041>

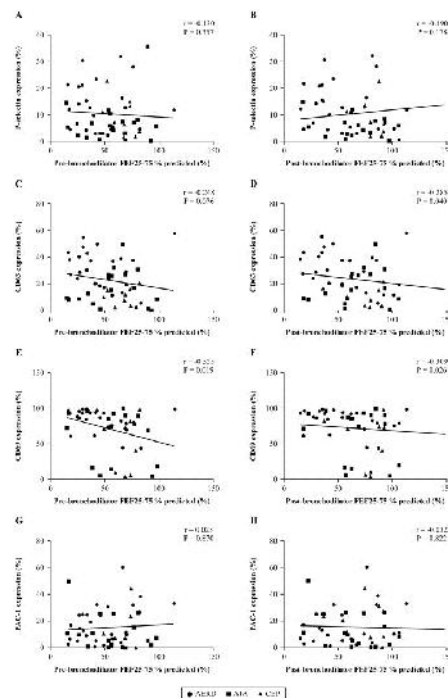


Fig E2.

Chihiro Mitsui, Keiichi Kajiura, Hiroaki Hayashi, Jun Ito, Haruhisa Mita, Emiko Ono, Noritaka Higashi, Yuma Fukutomi, Kiyoshi Sekiya, Takahiro Tsuburai, Kazuo Akiyama, Kazuhiko Yamamoto, Masami Taniguchi

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

Journal of Allergy and Clinical Immunology, 2015, Available online 17 July 2015

<http://dx.doi.org/10.1016/j.jaci.2015.05.041>

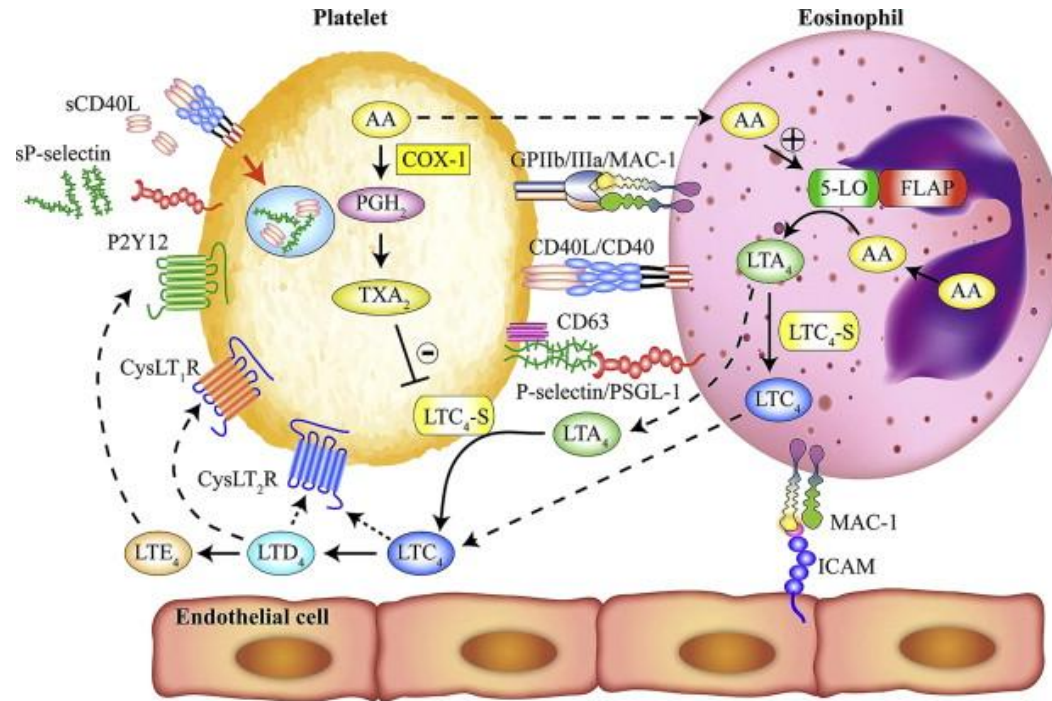


Fig E3.

Chihiro Mitsui, Keiichi Kajiwara, Hiroaki Hayashi, Jun Ito, Haruhisa Mita, Emiko Ono, Noritaka Higashi, Yuma Fukutomi, Kiyoshi Sekiya, Takahiro Tsuburai, Kazuo Akiyama, Kazuhiko Yamamoto, Masami Taniguchi

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

Journal of Allergy and Clinical Immunology, 2015, Available online 17 July 2015

<http://dx.doi.org/10.1016/j.jaci.2015.05.041>