

# Airway Platelet Activation Is Associated With Airway Eosinophilic Inflammation in Asthma

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**Background:** Allergic asthma is characterized by airway inflammation associated with recruitment and activation of eosinophils. In mice, allergen exposure induces platelet migration to the airways that is necessary for eosinophil recruitment and activation. We therefore hypothesized that in the airways of human subjects with asthma, platelet activation would be positively associated with eosinophil activation and platelet and eosinophil activation would both be associated with clinical asthma characteristics.

**Methods:** Nasal wash levels of P-selectin (a measure of platelet activation) and eosinophil cationic protein (ECP) (a measure of eosinophil activation) were compared with each other and with clinical asthma characteristics in a cross-sectional study of urban children and adolescents (age range, 6–20 years) with asthma.

**Results:** Regression analysis revealed a significantly positive association between  $\log_{10}$  P-selectin levels and  $\log_{10}$  ECP levels ( $\beta = 0.50$  ng/mL [95% confidence interval, 0.05–0.94 ng/mL];  $P = 0.029$ ). Additionally, ECP was significantly and negatively associated with 2 asthma-related quality of life measurements, and P-selectin was associated with one of these.

**Conclusions:** Our study shows the first significant association between platelet and eosinophil activation in airways of human subjects with asthma. These data provide a first step toward delineating what seems to be an important role for platelets in airway eosinophilia.

**Key Words:** asthma, eosinophils, platelets

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The airway inflammation seen in allergic asthma is associated with recruitment and activation of inflammatory cells (eg, eosinophils, lymphocytes, mast cells).<sup>1</sup> Recent evidence has shown that activated platelets play a critical role in the development of inflammation in humans with allergic asthma as well as a variety of other disease states (ie, rhinitis, chronic obstructive pulmonary disease, rheumatoid arthritis, inflammatory bowel syndrome, atherosclerosis).<sup>2</sup>

In a mouse model of allergic asthma, allergen exposure induces platelet activation and migration to the airways<sup>3–5</sup> where they in turn attach to and activate leukocytes. Activated leukocytes show an increase in expression of CD11b and Very Late Antigen-4 (VLA-4), adhesion molecules that are necessary for inflammatory cell attachment to the airway vascular endothelium.<sup>3</sup> The platelet-leukocyte complexes also have been shown in mice to be associated with eosinophil and lymphocyte activation and migration to the lungs where they subsequently adhere to the endothelial walls and cause inflammation.<sup>6</sup> Additionally, it also has been shown that platelets play a critical role in airway remodeling as a result of chronic allergen exposure.<sup>7</sup>

Therefore, we hypothesized that there would be a positive association between platelet activation and eosinophil activation in the airways of children and adolescents with asthma. Furthermore, we hypothesized that increased platelet and eosinophil activation would be associated with more severe clinical asthma characteristics.

## METHODS

### Study Cohort

The subjects studied were enrolled as part of the ongoing Asthma Severity Modifying Polymorphisms (AsthMaP) Project, a cross-sectional study at Children's National Medical Center in Washington, DC. It is made up of 6- to 20-year-old urban children and adolescents living in the District of Columbia metropolitan area with physician-diagnosed asthma present for at least 1 year. Details of the AsthMaP Project have been previously described.<sup>8,9</sup> Briefly, subjects were recruited in the emergency department at Children's National Medical Center and then returned to the Clinical Research Center for 1 study visit at least 4 weeks after completion of their most recent oral steroid dose. Study visits were conducted at approximately the same time of day. Informed consent and assent were obtained from participants and/or their guardians as appropriate. The study was approved by our institutional review board.

### Clinical Characteristics

Multiple historical and physiological characteristics were assessed for each subject in the asthma cohort. Of note, nasal washes were performed by instilling isotonic sterile saline into each nare, holding for 10 seconds, and then collecting the fluid in a specimen container. Eosinophils and neutrophils from these nasal washes were manually counted on slides stained with Wright stain. Additionally, serum immunoglobulin E (IgE) was measured using chemiluminescence with an Immulite 2000 system (Siemens Healthcare Diagnostics, Deerfield, IL). Parental interviews were conducted using the Integrated Therapeutics Group's (ITG) Child Asthma Short Form,<sup>10</sup> National Institutes of Health, National Asthma Education and Prevention Program (NAEPP) 2007 criteria,<sup>11,12</sup> and, depending on age, either the Asthma Control Test (ACT) or Childhood Asthma Control Test (cACT) (Quality Metric Incorporated, Lincoln, RI).

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## P-selectin as a Measurement of Platelet Activation

Soluble P-selectin (ie, CD62P) is an  $\alpha$ -granule membrane protein that migrates to the cell surface of platelets upon release of  $\alpha$ -granules and subsequent platelet activation.<sup>13</sup> It is a well-established marker for platelet activation.<sup>14,15</sup> Levels of soluble p-selection were measured in nasal wash samples by flow cytometry on a FACSCalibur System (BD Biosciences, San Jose, CA) using a FlowCytomix Simplex Kit (Bender MedSystems, Burlingame, CA). Results were analyzed with FlowCytomix Pro 2.3 software (Bender MedSystems, Burlingame, CA).

## Eosinophil Cationic Protein as a Measurement of Eosinophil Activation

Levels of eosinophil cationic protein (ECP), a cytotoxic protein released by activated eosinophils, were measured in nasal wash samples using a standard enzyme-linked immunosorbent assay kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) with a minimal detectable level of 0.125 ng/mL. Eosinophil cationic protein is a cytotoxic protein released by eosinophils after activation by an immune stimulus<sup>16</sup> and was thus chosen as the marker for eosinophil activation.

## Statistical Analysis

Linear regression analysis was used to identify significant associations. Data were  $\log_{10}$  transformed when not normally distributed. All beta coefficients and *P* values were corrected for age and sex. All statistical tests were performed with SPSS Statistics 17.0 (SPSS Inc., Chicago, IL).

## RESULTS

At the time of this study, 109 children and adolescents had been enrolled in AsthmaP. Of those, 61% were male subjects, and the mean age was 11.4 (SE, 0.4) years. Of the 109 subjects, 100 (92%) were self-identified African Americans (AAs), and 89 (82%) had persistent asthma as defined by NAEPP 2007 criteria.<sup>11</sup> The median blood eosinophil level was 3.6% (interquartile range [IQR], 1.8–6.8). A detailed description of select asthma characteristics can be found in Table 1.

Nasal wash samples were available from a subgroup of subjects (*n* = 75). There were no significant differences between these 75 subjects and the remaining 34 with respect to age, sex, and NAEPP classification. From those samples, P-selectin and ECP levels were measured to assess platelet and eosinophil activation, respectively. The median P-selectin level in the nasal washes was 0.83 ng/mL (IQR, 0.45–2.41), and the median ECP level in the nasal washes was 11.16 ng/mL (IQR, 1.27–46.77). Linear regression analysis showed that for every unit increase in  $\log_{10}$  p-selection, there was a corresponding significant 0.5 unit increase in  $\log_{10}$  ECP ( $\beta$  = 0.50 [95% CI, 0.05–0.94]; age and sex adjusted *P* = 0.029).

In addition, using regression analysis, we tested for associations between ECP and subjects' clinical asthma characteristics. We chose to analyze 8 asthma-related quality of life and allergic markers that were relevant to allergic asthma and have previously been shown to be associated with eosinophil activation.<sup>17,18</sup> (Table 2)  $\log_{10}$  ECP was found to be significantly and negatively associated with 2 asthma-related quality of life characteristics: (1)  $\log_{10}$  ITG–nighttime score (–0.06 [–0.12 to 0.00]; adjusted *P* = 0.015) and (2)  $\log_{10}$  ITG–composite score (–0.04 [–0.08 to –0.01]; adjusted *P* = 0.018). To assess whether similar associations were present with P-selectin, regression analyses were carried out between  $\log_{10}$  P-selectin and the same 8 clinical asthma characteristics tested with ECP. P-selectin was found to be significantly and negatively associated with  $\log_{10}$  ITG–nighttime score (–0.12 [–0.22 to –0.01]; adjusted *P* = 0.035).

TABLE 1. Clinical Characteristics of Subjects

Variable	Cases (n = 109)
Sex, % male	60.6
Age, yr (SE)	11.4 (0.4)
Body mass index percentile (SE)	68.2 (2.5)
Age of asthma onset, yr (SE)	2.9 (0.3)
Prebronchodilator FEV1, % predicted (SE)	88.1 (1.8)
Post-bronchodilator FEV1, % predicted (SE)	93.1 (1.6)
ACT/cACT <sup>†</sup> (SE)	20.6 (0.3)
NAEPP* severity classification, %	
1 - Intermittent	11.0
2 - Mild persistent	24.8
3 - Moderate persistent	33.9
4 - Severe persistent	30.3
ITG‡ - composite score (SE)	75.3 (2.0)
Nasal wash eosinophils, % (IQR)	52 (2, 90)
Nasal wash neutrophils, % (IQR)	30 (3, 71)
Eosinophil cationic protein from nasal washes, ng/mL (IQR)	11.2 (1.3, 46.8)
Neutrophil elastase from nasal washes, ng/mL (IQR)	1.4 (0.6, 2.3)
P-selection from nasal washes, ng/mL (IQR)	0.8 (0.5, 2.4)
Blood eosinophils, % (IQR)	3.6 (1.8, 6.8)
Total serum IgE, IU/mL (IQR)	205.5 (72.3, 525.5)
One or more positive allergen skin prick tests, %	36.4

\*National Asthma Education and Prevention Program.

†Asthma Control Test/Childhood Asthma Control Test.

‡Integrated Therapeutics Group's Child Asthma Short Form.

We have previously identified, using cluster analysis, 3 distinct phenotypic clusters within the AsthmaP cohort.<sup>8</sup> One of the clusters was characterized by an allergic asthma phenotype with increased nasal wash eosinophils and worse asthma control. Nasal washes were available for 25 participants within this cluster. As in the overall cohort, we used regression analysis to test for associations within this allergic phenotypic cluster between  $\log_{10}$  ECP and the 8 relevant asthma characteristics. The 2 significant associations previously identified in the overall cohort also were present within this cluster:  $\log_{10}$  ITG–nighttime score (–0.14 [–0.26 to –0.02]; adjusted *P* = 0.021) and  $\log_{10}$  ITG–composite score (–0.09 [–0.17 to –0.02]; adjusted *P* = 0.020). Additionally, within the cluster, both  $\log_{10}$  ITG–daytime score (–0.28 [–0.53 to –0.04]; adjusted *P* = 0.025) and  $\log_{10}$  ITG–nighttime score (–0.36 [–0.68 to –0.04]; adjusted *P* = 0.029) were found to be significantly associated with  $\log_{10}$  P-selectin. Table 2 highlights these significant associations found both in the overall cohort and within the allergic phenotypic cluster.

## DISCUSSION

We found a significant positive association between markers of platelet (ie, P-selectin) and eosinophil (ie, ECP) activation in the airways of children and adolescents with asthma. Additionally, P-selectin was significantly associated with 1 ECP-associated asthma-related quality of life measurement in the overall cohort. When looking within a previously identified allergic phenotypic cluster of the cohort, the same significant association was found along with an additional significant association between P-selectin and another asthma-related quality of life measurement.

**TABLE 2.** Associations Between Asthma Characteristics and Either Eosinophil Activation or Platelet Activation Overall and Within an Allergic Phenotypic Subgroup

Variables	All (n = 75)		Allergic Subgroup (n = 25)		
	$\beta$ Coefficient* $\dagger$ (95% CI)	P $\dagger$	$\beta$ Coefficient* $\dagger$ (95% CI)	P $\dagger$	
ECP	ACT/cACT	-0.41 (-1.41 to 0.58)	0.414	-2.24 (-4.49 to 0.02)	0.052
	ITG-daytime score $\ddagger$	-0.03 (-0.07 to 0.02)	0.195	-0.09 (-0.20 to 0.00)	0.052
	ITG-nighttime score $\ddagger$	-0.06 (-0.12 to 0.00)	<b>0.015</b>	-0.14 (-0.26 to -0.02)	<b>0.021</b>
	ITG-function score $\ddagger$	-0.04 (-0.07 to 0.00)	0.076	-0.08 (-0.17 to 0.01)	0.094
	ITG-composite score $\ddagger$	-0.04 (-0.08 to -0.01)	<b>0.018</b>	-0.09 (-0.17 to -0.02)	<b>0.020</b>
	Total serum IgE	0.05 (-0.12 to 0.22)	0.542	0.03 (-0.26 to 0.31)	0.851
	Blood eosinophils, %	-0.03 (-0.14 to 0.07)	0.546	-0.13 (-0.31 to 0.05)	0.156
P-selectin	Nasal wash eosinophils, %	-0.02 (-0.18 to 0.13)	0.774	0.00 (-0.13 to 0.12)	0.973
	ACT/cACT	0.10 (-2.13 to 2.32)	0.932	-2.61 (-8.99 to 3.76)	0.406
	ITG-daytime score $\ddagger$	0.08 (-0.02 to 1.18)	0.115	-0.28 (-0.53 to -0.04)	<b>0.025</b>
	ITG-nighttime score $\ddagger$	-0.12 (-0.22 to -0.01)	<b>0.035</b>	-0.36 (-0.68 to -0.04)	<b>0.029</b>
	ITG-function score $\ddagger$	0.02 (-0.07 to 0.10)	0.718	0.03 (-0.23 to 0.29)	0.800
	ITG-composite $\ddagger$	0.00 (-0.08 to 0.08)	0.979	-0.17 (-0.39 to 0.04)	0.106
	Total serum IgE	0.04 (-0.33 to 0.42)	0.829	0.05 (-0.72 to 0.81)	0.903
	Blood eosinophils, %	0.05 (-0.19 to 0.28)	0.692	-0.35 (-0.84 to 0.13)	0.147
	Nasal wash eosinophils, %	0.23 (-0.12 to 0.58)	0.201	0.13 (-0.20 to 0.46)	0.428

\* $\beta$  coefficient is for each  $\log_{10}$  increase in either ECP or P-selectin.

$\dagger$ Adjusted for age and sex.  $P < 0.05$  are listed in **bold**.

$\ddagger$ Variables were log transformed to approximate a normal distribution.

It is well established that allergic asthma is associated with inflammation and airway epithelial damage.<sup>1</sup> Specifically, after an allergic stimulus, inflammatory cell (eg, eosinophils, lymphocytes, mast cells) activation and migration to asthmatic airways is followed by subsequent obstruction and inflammation.<sup>17,18</sup> Emerging literature has begun to explore the role of platelets in this inflammatory response.<sup>19</sup> For example, activated platelets have been shown to be increased in asthmatic airways.<sup>20</sup> Additionally, it has been demonstrated in vitro that, in the presence of platelets derived from patients with asthma, eosinophil attachment to airway endothelium is enhanced.<sup>21,22</sup> It also has been shown that mitogens and enzymes released by activated platelets play a direct role in the chronic inflammatory events that lead to airway remodeling in asthma.<sup>23</sup>

Pitchford et al.<sup>6</sup> showed in a mouse model of allergic asthma that P-selectin expression on activated platelets is necessary for contact-dependent leukocyte activation. Furthermore, they found an association between P-selectin expression on the surfaces of activated platelets and eosinophil and lymphocyte activation. These findings suggest that platelets play an indirect, albeit crucial, role on inflammatory cell activation and recruitment through the binding and activation of leukocytes. Our data extend these findings into human asthma by providing initial evidence for a positive association between platelet activation and eosinophil activation in human airways. Additionally, we show that eosinophil and platelet activation are both associated with asthma-related quality of life characteristics in a pediatric asthmatic cohort. Although we only uncovered a few significant associations because of the small sample size, the associations that we did find support the current literature showing that inflammation leads to increased symptoms and worse disease control.<sup>17,18</sup>

We had previously used cluster analysis to reduce the heterogeneity of the AsthMaP cohort and to identify distinct phenotypic clusters.<sup>8</sup> In this current study, we now show that the significant associations found in the overall cohort were stronger

when analyzed within the allergic phenotypic cluster, despite its small sample size. Furthermore, one additional association between platelet activation and ITG-daytime score that was not present in the overall cohort was found to be significant within the cluster. Thus, looking within this allergic phenotypic cluster to identify new associations and strengthen previous associations supports the concept that distinct phenotypic subgroups will display different responses to stimuli.<sup>18</sup>

This study has several limitations. First, this study had a relatively small sample size. However, despite the low power to detect significant associations, the relationships detected are important first steps to understanding the role of platelets in inflammation in human airways. Second, the use of ECP as a measure of eosinophil activity presents several concerns. The sticky nature of this protein, along with the fact that it is highly charged, makes recoverability difficult.<sup>24</sup> Furthermore, eosinophils have been shown to release less intracellular ECP compared with other granule proteins.<sup>25</sup> However, despite these inherent difficulties in measuring ECP, the significant association that we found between ECP and P-selectin provides initial evidence for the role of platelet activation in airway eosinophil activation and recruitment. Third, AsthMaP is composed largely of AA children and adolescents. Therefore, it is difficult to extend our findings into other populations. However, it is important to note that our study provides insight into AA children and adolescents with asthma, which is one of the highest-risk asthma populations. Finally, a fourth limitation is our use of nasal washes in lieu of the gold standard bronchoalveolar lavage. Because it has been shown in cystic fibrosis and respiratory syncytial virus that inflammatory cell proportions in nasal samples accurately reflect what is observed in the lower airways,<sup>26,27</sup> we argue that these samples are valuable as a less invasive means to measure markers of airway inflammation.

In summary, this study extends to humans the current literature showing platelet involvement in airway inflammation

in mice. Using a cohort of urban children and adolescents with asthma, we were able to identify a positive association between platelet activation and eosinophil activation in nasal secretions that had previously been demonstrated in the lower airways of a mouse model of allergic asthma. This finding provides a first step to better understanding the relationship between platelets and inflammation and should be further explored to better delineate the role of platelets in asthma.

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