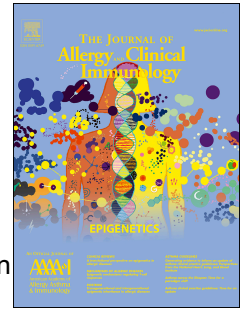


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α IIb-integrin (CD41) associated with blood eosinophils is a potential biomarker for disease activity in eosinophilic esophagitis

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**α IIb-integrin (CD41) associated with blood eosinophils is a potential
biomarker for disease activity in eosinophilic esophagitis**

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in the study design; the data collection, analysis, or interpretation; the writing of the report; or the decision to submit the article for publication.

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Capsule summary: After EoE treatment, association of platelets with blood eosinophils, as reported by CD41, predicted esophageal eosinophil count. Percentage CD41+ circulating eosinophils is a potential non-invasive biomarker for EoE disease activity.

Key words: *Eosinophilic esophagitis, α_{IIb} -integrin, CD41, blood eosinophils, platelets, platelet-eosinophil association, peak eosinophil count, disease activity, biomarker, non-invasive*

To the Editor:

Eosinophilic esophagitis (EoE), a chronic inflammatory disease characterized by symptoms of esophageal dysfunction and eosinophilic infiltration of the esophageal mucosa, has increased in incidence and prevalence the past two decades.^{1, 2} The standard for assessing disease activity is performing endoscopy and pathological examination of esophageal biopsies. There is a critical need for a biomarker to replace such invasive monitoring. A number of potential tests have been evaluated but none have been incorporated into guideline recommendations or routine clinical practice.^{3, 4}

We previously demonstrated by flow cytometry that the activation epitope for monoclonal antibody (mAb) N29 on the β_1 integrin subunit on circulating eosinophils correlates with and predicts decreased pulmonary function in patients with non-severe asthma.⁵ Further, we linked N29 epitope appearance to eosinophil engagement by P-selectin (CD62P) on activated platelets and showed that P-selectin-stimulated eosinophil $\alpha_4\beta_1$ integrin activation causes enhanced adhesion to vascular cell adhesion molecule (VCAM)-1, which is induced on activated endothelium.⁶ We hypothesized that a similar pathway occurs in EoE and undertook the present study to determine if eosinophil surface biomarkers correlate with and predict eosinophil count in esophageal biopsies.

Patients were recruited from the University of Wisconsin (UW) Health Gastroenterology Clinic after failing to respond for two months to proton-pump inhibitor (PPI) therapy and following endoscopy diagnostic of EoE. Informed written consent was obtained at Visit 1 (V1) within two weeks of release of the endoscopy results, according to a protocol approved by the UW-Madison Health Sciences Institutional Review Board (see Online Repository text and Table EI). Patients received standard of care EoE treatment for eight weeks followed by V2 and repeat

endoscopy. N29, CD62P, CD41 (α_{IIb} integrin subunit, a reporter of platelet association), and 13 other surface markers (Table EII) were assayed by flow cytometry of eosinophils gated in whole blood. Peak eosinophil count (PEC) per high power field (HPF) was assessed on esophageal biopsy. Flow cytometry, clinical assessments, and statistical analysis are described in the Online Repository.

Twenty-five patients (Table I) completed V1 and V2. At V1, all patients had active disease, as specified by the enrollment criteria. Changes in PEC and disease scores from V1 to V2 are visualized in Fig E1. PEC (Table I) and scores decreased significantly, from means of 11.0 to 4.6 for EoEHSS (histology, $P = 0.001$), 31.6 to 19.0 for EEsAI (symptoms, $P < 0.001$), and 4.1 to 2.8 for EREFS (endoscopy, $P < 0.001$). At V2, eleven patients (44%) had PEC < 6 /HPF and three others, for a total of 14 (56%) patients, had PEC < 15 /HPF (Fig. E1, A). Correlations among the three scores and PEC at V1 or V2 are shown in Fig E2. The strongest correlation was between EoEHSS and PEC, whereas the other correlations were weaker.

Flow cytometry histogram examples for patients with low or high level activated β_1 integrin (N29 signal) or low or high degree α_{IIb} (CD41) positivity are displayed in Fig E3. N29 intensity and levels of and percentage eosinophils positive for P-selectin (CD62P) and CD41 correlated among each other (Fig E4). CD62P and CD41 positivity correlated with PEC at V2, whereas N29 intensity did not (Fig E5). Adjusting for the RCAT (Rhinitis Control Assessment Test) score, allergy and asthma, or treatment including steroid, did not affect these correlations (Table EIII). In addition, patients on steroids did not have significantly different expression of the markers compared to patients not on steroids (Table EIV). None of the other 13 markers correlated significantly with PEC at V2 (Table III). N29 intensity or CD62P or CD41 positivity did not correlate significantly with the other measures of EoE disease activity (Table EV).

However, CD41 positivity correlated with eosinophil inflammation and lamina propria fibrosis EoEHSS subscores (Table EVI).

We performed receiver operating characteristic (ROC) analysis to investigate the ability of these potential biomarkers to predict PEC below pre-specified cutoffs. CD62P or CD41 significantly predicted PEC < 6/HPF; CD41 was best with an area under curve (AUC) of 0.84, 93% specificity and 82% sensitivity for the statistically optimal criterion < 22.9% CD41-positive cells (Fig 1, A; Table EVII). CD41 also significantly predicted PEC < 15/HPF (Fig E6 legend). Dividing patients according to median CD41 positivity (26.7%), CD41-low patients had median PEC = 0/HPF and CD41-high patients significantly higher median PEC = 31/HPF (Fig 1, B). Alternatively, categorizing the patients according to PEC < or $\geq$ 6 or 15/HPF, PEC-low patients had significantly fewer CD41-positive blood eosinophils than PEC-high patients (Fig E6).

In summary, our results indicate that a pathway of platelet activation and platelet-eosinophil association, likely also P-selectin-triggered $\alpha_4\beta_1$ integrin activation and $\alpha_4\beta_1$ -mediated eosinophil arrest on VCAM-1 on activated endothelium in the esophagus, occurs in EoE, as suggested in the lung in asthma.^{5,6} Such a scenario is consistent with evidence for platelet activation and platelet-eosinophil interactions, as visualized by immunofluorescence microscopy *in vitro* of α_{IIb} /CD41-positive eosinophils,⁷ which we have also observed *in vivo* in a patient with EoE (Fig. E7), leading to eosinophil recruitment in allergic diseases.⁸ It is also consistent with upregulated VCAM-1 in the esophagus in EoE and association between response to treatment and decreased VCAM-1.⁹

PEC was our primary measure of disease status. PEC correlated strongly with the histology score, whereas the other correlations among scores were more modest. This is consistent with the struggles to identify an ideal metric for EoE disease activity, as there is a

loose relationship among symptoms, pathology, and visualization of the esophagus. To date, PEC remains the primary metric for EoE diagnosis and monitoring. Limitations of the present study include the relatively small sample size, exclusion of children, and that subjects were recruited by EoE guidelines at the time of study design, i.e., subjects with PPI-responsive esophageal eosinophilia were excluded. To validate our findings and address these and other limitations, we plan to examine a larger cohort including patients with PPI-responsive esophageal eosinophilia and pediatric patients.

In conclusion, we demonstrate that α_{Ib} integrin (CD41) associated with circulating eosinophils is a potential non-invasive biomarker for residual esophageal eosinophilic inflammation, after a period of recommended standard of care treatment.

(Word count 1000)

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Author contributions:

Conception and design MWJ, LDL, SMC, EAG, SKM; Data acquisition and validation MWJ, EMM, PSF, JLB, SMC; Data analysis MWJ, ELA, KEL, MDE, SMC; Data interpretation:

MWJ, KEL, MDE, SMC, SKM: Manuscript draft MWJ; Manuscript editing and revision MWJ, KEL, DFM, SMC, EAG, SKM; All authors reviewed the manuscript critically for intellectual content and approved the final manuscript.

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TABLE**TABLE I.** Patient characteristics

Variable	Value
<i>n</i>	25
Age (years) (mean $\pm$ SD)	38 $\pm$ 14
Sex (<i>n</i> [percentage] male)	19 (76%)
Race/ethnicity (white non-Hispanic) (<i>n</i> [percentage])	24 (96%)*
Time since symptom onset (years) (median [25 th , 75 th percentiles])	5 (2, 10)
V1 blood eosinophils (per μ l) (median [25 th , 75 th percentiles])	194 (70, 299)
V2 blood eosinophils (per μ l) (median [25 th , 75 th percentiles])	158 (114, 273)
Allergic rhinitis diagnosis or symptoms (<i>n</i> [percentage])	20 (80%)
V1 RCAT score (mean $\pm$ SD) (<i>n</i> = 21)	24.1 $\pm$ 4.3
V2 RCAT score (mean $\pm$ SD) (<i>n</i> = 22)	23.3 $\pm$ 4.4
Asthma diagnosis (<i>n</i> [percentage])	2 (8%)
Treatment from V1 to V2 (<i>n</i>)	12 topical steroid
	7 food elimination
	5 both
	1 other
Reported use of NSAID(s) (including aspirin) (<i>n</i>)	13
EoE severity:	
Required dilations (<i>n</i> [percentage])	12 (48%)

Required ED visits for EoE (<i>n</i> [percentage])	13 (52%)
Allergic sensitivities:	
Trees (<i>n</i> [percentage])	17 (68%)
Grass (<i>n</i> [percentage])	12 (48%)
Ragweed/late fall pollen (<i>n</i> [percentage])	10 (40%)
Mold (<i>n</i> [percentage])	5 (20%)
Dust mite (<i>n</i> [percentage])	10 (40%)
Cat or dog (<i>n</i> [percentage])	9 (36%)
Food sensitivities:	
Egg (<i>n</i> [percentage])	9 (36%)
Milk (<i>n</i> [percentage])	2 (8%)
Peanut (<i>n</i> [percentage])	6 (24%)
Shrimp (<i>n</i> [percentage])	5 (20%)
Soy (<i>n</i> [percentage])	1 (4%)
Wheat (<i>n</i> [percentage])	3 (12%)
Any above food (<i>n</i> [percentage])	13 (52%)
V1 PEC (per HPF) (median [25 th , 75 th percentiles])	42 (28, 68)
(percentage $\geq$ 6/HPF or $\geq$ 15/HPF)	100% $\geq$ 6, 96% $\geq$ 15
V2 PEC (per HPF) (median [25 th , 75 th percentiles])	12 (0, 44)†
(percentage $\geq$ 6/HPF or $\geq$ 15/HPF)	56% $\geq$ 6, 44% $\geq$ 15

ED, emergency department; *EoE*, eosinophilic esophagitis; *HPF*, high power field; *n*, number of subjects; *NSAID*, non-steroidal anti-inflammatory drug; *PEC*, peak eosinophil count; *RCAT*, rhinitis control assessment test; *V*, visit.

Data are presented as mean $\pm$ SD (if data are normally distributed) or median (25th, 75th percentiles) (if data are not normally distributed).

*One patient was white and American Indian/Alaska Native.

†*P* = 0.02 versus V1.

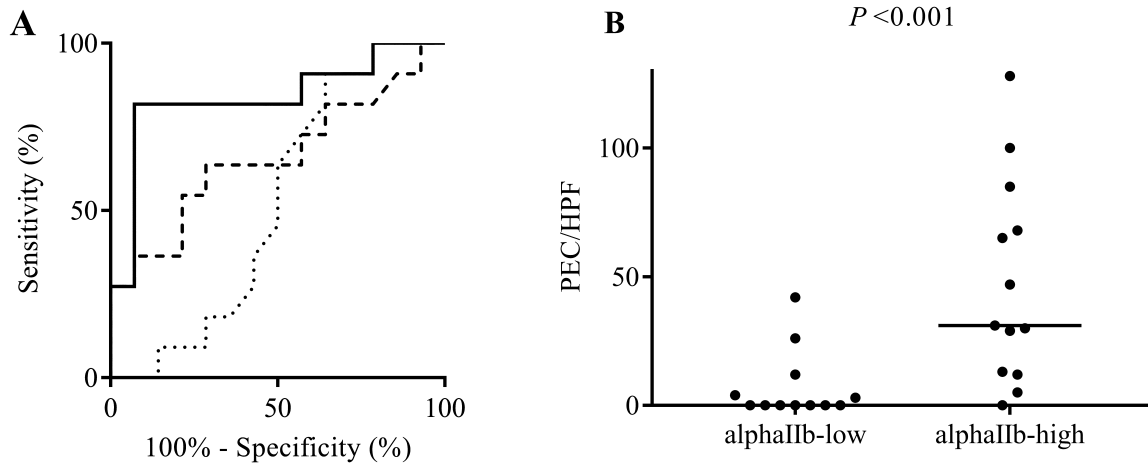
For blood eosinophils, *P* = 0.96 between V2 and V1.

Allergic and food sensitivities are based on skin test.

Note: Information on NSAID use was captured as part of the information on medication but not captured in relation to study visits.

FIGURE LEGEND

FIG 1. ROC curve for the ability of blood eosinophil activated β_1 integrin (mAb N29), P-selectin (CD62P)- or α_{IIb} integrin (CD41)-positive blood eosinophils to predict PEC at V2, and PEC at V2 in α_{IIb} -low or -high patients. **A**, Dotted line, N29; dashed line, P-selectin (CD62P); solid line, α_{IIb} (CD41). To predict PEC < 6/HPF, area under curve (AUC) = 0.52 for N29, 0.66 for P-selectin, and 0.84 for α_{IIb} ($P < 0.001$); for criterion < 22.9% α_{IIb} -positive cells, specificity = 93% and sensitivity = 82%. **B**, Patients were divided according to median α_{IIb} -positive cells (26.7%) at V2; Bar, median PEC in each group (median in the α_{IIb} -low group = 0).



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Online Repository

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METHODS

Patients

In a protocol approved by the University of Wisconsin (UW)-Madison Health Sciences Institutional Review Board, adult patients (> 18 years of age) with established eosinophilic esophagitis (EoE) were recruited from the UW Health Gastroenterology Clinic following a diagnostic endoscopy for esophageal dysfunction. Patients with esophageal biopsy showing ≥ 15 eosinophils/high power field (HPF), with one patient enrolled with a peak eosinophil count (PEC) of 11/HPF who remained symptomatic, despite use of high dose proton pump inhibitor (PPI, equivalent of omeprazole ≥ 40 mg daily) for at least two months (with exception for one patient who had contraindication to PPI use), were invited to participate and enroll within two weeks of receiving the endoscopy results. Histology staining was used to identify eosinophils. The PEC was derived from either the proximal or distal esophagus, as the greatest value. A total of 28 patients were recruited for a 9-week prospective observational study between 2016 and 2018. A summary of the visits is shown in Table EI. Informed written consent was obtained from each subject prior to participation in the study at visit (V) 1. Exclusion criteria included major health problem such as autoimmune disease, heart disease, type I or II diabetes, uncontrolled hypertension, or lung disease other than asthma; or pregnancy, lactating, or a planned pregnancy during the course of the study. Twenty-five patients completed V1 and V2 and are included in the analyses.

Study visits and assessments

A complete medical history including other diagnoses, medications, and scoring of symptoms was performed at V1. Symptom scoring used the eosinophilic esophagitis activity

index (EESAI), a validated seven-day recall tool of seven questions developed to quantify EoE symptoms.^{E1} Patients with concomitant asthma had spirometry performed and completed the Asthma Control Questionnaire-7.^{E2} Patients with concomitant allergic rhinitis completed the Rhinitis Control Assessment Test (RCAT).^{E3} Updates to the medical history, medication changes, and symptom scores for EoE, asthma, and allergic rhinitis were recorded at V2. Allergy skin prick testing for aeroallergens and foods was performed at V1. A 55 ml blood draw was performed at V1 and V2. Ten ml was aliquoted into vacuum tubes containing CTAD (citrate, theophylline, adenosine, and dipyridamole) anticoagulant solution (BD Vacutainer Systems, Franklin Lakes, NJ), to minimize artifactual platelet activation,^{E4-6} and were used for flow cytometry (see below).

Standard of care EoE therapy, specifically recommendations of either swallowed steroid or food elimination were offered. For the food elimination option, in patients with no positive food tests, the recommendation was a six-food elimination diet (milk, egg, peanut, wheat, soy, and shellfish). Patients with positive food tests were offered the option of six-food elimination or restricted elimination of foods to which they tested positive. Patients then received this standard of care treatment, appropriate to each patient, for eight weeks, followed by V2 (Table EI).

Endoscopies were performed at the UW Digestive Health Center on an outpatient basis and not during acute food impaction nor during acute respiratory infection or sinusitis. The validated Endoscopic Reference Score (EREFS) for endoscopic assessment of EoE was used to uniformly score the endoscopic signs of EoE for the endoscopy report.^{E7} Biopsies of the esophageal epithelium were obtained using standard protocol: A 3 mm cup biopsy forceps was used to obtain four to six biopsies of the distal esophagus and a second sampling of four to six biopsies were performed of the proximal esophagus. Esophageal mucosal samples were assessed

by the UW Pathology Department to determine PEC/HPF and provide assessment of the EoE Histological Scoring System (EoEHSS).^{E8}

Antibodies and flow cytometry

Primary mAbs used in this study included clones used previously for flow cytometry on eosinophils in whole blood from patients with asthma (by us) or EoE (by others).^{E4, 5, 9-14} The primary mAbs and isotype controls are listed in Table EII. Notably, for β_1 integrin activation, P-selectin (CD62P), and α_{IIb} integrin (CD41), the following clones were used: N29 (Millipore Sigma, Burlington, MA), AC1.2 (BD Biosciences, San Jose, CA), and HIP8 (BD). Phycoerythrin (PE)-conjugated anti-mouse and anti-rat immunoglobulin (Ig) G secondary antibodies, and fluorescein isothiocyanate (FITC)-conjugated anti-CD14 mAb clone M5E2 and anti-CD16 3G8^{E6} were from BD.

Whole unfractionated CTAD blood from V1 and V2 was directly processed (100 μ l per flow cytometry tube) for flow cytometry, and data were acquired at the UW Comprehensive Cancer Center Flow Cytometry Facility as previously described.^{E4, 5, 10, 11} Data were collected, analyzed, and expressed as specific geometric channel fluorescence (gMCF).^{E5, 6, 11} P-selectin (CD62P) and α_{IIb} integrin (CD41), which have been observed to have heterogeneous distributions, were also expressed as percentage positive cells.^{E11} This is in contrast to other markers, such as N29, which have homogeneous distributions and for which expression level is a more representative way to present data, as previously discussed.^{E10} FITC-labeled anti-CD14 and anti-CD16 were used to gate eosinophils based on scatter and lack of FITC staining, as done previously.^{E4, 5, 10, 11} Mid-range one-peak rainbow fluorescent beads (Spherotech, Lake Forest, IL) were run at setup to set the sensitivity of the detectors in a standardized manner, thus

maximizing reproducibility and optimizing data comparisons among subjects and visits.

Compensation was performed with unlabeled, FITC-labeled, and PE-labeled Calibrite (BD) beads. Data analysis was performed using FlowJo software (Ashland, OR).

Data in previous asthma studies, and the initial phase and larger part of this study were acquired using a FACSCalibur cytometer (BD Biosciences, San Jose, CA) as before.^{E4-6, 9-11}

During the course of the current study, the Flow Cytometry Facility discontinued the Calibur cytometers and replaced them with Attune cytometers (ThermoFisher, Waltham, MA). In order to transition the study from the Calibur to an Attune cytometer, transform new Attune data into Calibur equivalents, and compare the Attune data to data generated with the Calibur, the following measures were taken, after consultation with Flow Facility staff. From a whole blood sample, 100 μ l blood was analyzed in each tube using the primary mAb or isotype controls listed in Table EII, or buffer only, giving a total of 22 tubes (16 primary mAbs, 4 isotype controls, 2 tubes with buffer plus anti-mouse or anti-rat secondary antibody). These tubes were processed according to the standard flow cytometry protocol (see above). At the end of the protocol, cells in each tube were resuspended in 500 μ l FACS buffer. This volume was divided in 2 x 250 μ l aliquots. Data from one set of aliquots, i.e., 22 aliquots, were acquired in the Calibur as before. The other set of aliquots was diluted with 250 μ l FACS buffer to a total volume of 500 μ l. Data from these aliquots, i.e., also 22 aliquots, were acquired in the Attune. The same type of rainbow beads, to set the sensitivity of the detectors in a standardized manner, and the same type of Calibrite beads for compensation (see above) were used with the two instruments. With the Calibur data, eosinophils were gated as before (see above), i.e., first by a region within a side scatter-height (SSC-H) versus forward scatter (FSC)-H plot, then by gating this region in a SSC-H versus FITC-H (CD14/CD16) plot. With the Attune data, eosinophils were similarly first gated

by SSC-H versus FSC-H, then for singlets by FSC-H versus FSC-area (A)^{E6} and SSC-H versus SSC-A, and then, similarly as for the Calibur data, by SSC-H versus the BL1 (FITC)-H channel. After analysis of the data sets from the two instruments as obtained after rainbow-bead-guided detector settings, linear regression was performed to compare the Attune-generated to the Calibur-generated data. This yielded the equation $y = 0.07404x + 18.34$ with $r^2 = 0.9998$ ($n = 22$), where y = Calibur geometric mean and x = Attune geometric mean fluorescent intensities. As the goodness of fit was excellent, this equation was used from then on to transform new Attune data to Calibur equivalents. Thus, the data presented in the manuscript are based on fluorescence intensities obtained on the Calibur cytometer or as would have been obtained on the Calibur cytometer.

Immunohistochemistry

Slides with formalin-fixed, paraffin-embedded esophageal tissue biopsy sections from an EoE patient were stained with hematoxylin and eosin as well as immunohistochemistry for platelets using mouse mAb clone 2f2 against β_3 integrin (CD61) (Cell Marque, Rocklin, CA), the β subunit that heterodimerizes with the α_{IIb} integrin subunit (CD41) to form the platelet $\alpha_{IIb}\beta_3$ integrin (CD41/CD61), and the UltraView Universal 3,3'-diaminobenzidine (DAB) Detection Kit containing a horseradish peroxidase-conjugated anti-mouse secondary antibody, according to the manufacturer's instructions (Ventana Medical Systems, Tucson, AZ).

Statistical analysis

Study data were collected and managed using REDCap (Research Electronic Data Capture) tools, hosted at the UW-Madison Department of Medicine, for data downloads to common statistical packages.^{E15} Mann-Whitney U test or Kruskal-Wallis rank sum comparison

test was used to compare data between two groups of subjects. Wilcoxon matched-pairs signed-rank test was used to compare data between two visits by the same subjects. Spearman rank test was used to analyze correlations. A level of probability ($P \leq 0.05$) was considered significant. Group data are reported as mean $\pm$ standard deviation (SD) if the variable was normally distributed or median with 25th and 75th percentiles if the variable was not normally distributed. Receiver operating characteristic (ROC) curve analysis was done to evaluate the ability of blood eosinophil-surface protein expression data to predict PEC. Analyses were performed using Prism (GraphPad, San Diego, CA), SAS (Cary, NC), or R software (Vienna, Austria).

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TABLES**TABLE EI.** Timeline and procedure chart

	Pre-recruitment	V1	V2
Time	- $\leq$ 2 wks	0	8 wks
Informed consent		x	
Procedures:			
Medical history/symptoms		x	x
Food allergy testing		x	
Blood draw		x	x
Endoscopy with biopsy	x		x

V, visit; wk, week.

TABLE EII. Primary mAbs used for flow cytometry

Antigen	Clone	Isotype	Company
β_1 integrin (CD29)	MAR4	mouse IgG ₁	BD
Activated β_1 integrin	N29	mouse IgG ₁	Millipore Sigma
β_2 integrin (CD18)	L130	mouse IgG ₁	BD
α_L integrin (CD11a)	HI111	mouse IgG ₁	BD
α_M integrin (CD11b)	LT11	mouse IgG ₁	MyBioSource
α_X integrin (CD11c)	B-ly6	mouse IgG ₁	BD
ICAM1 (CD54)	HCD54	mouse IgG ₁	BioLegend
Fc ϵ RII (CD23)	M-L233	mouse IgG ₁	BD
CD40	5C3	mouse IgG ₁	BD
CD44	BJ18	mouse IgG ₁	BioLegend
PSGL-1 (CD162)	KPL-1	mouse IgG ₁	BioLegend
α_{IIb} integrin (CD41)	HIP8	mouse IgG ₁	BD
P-selectin (CD62P)	AC1.2	mouse IgG ₁	BD
CCR3 (CD193)	5E8	mouse IgG _{2b}	BioLegend
CEACAM8 (CD66b)	G10FS	mouse IgM	BD
CRTH2 (CD294)	BM16	rat IgG _{2a}	BD
Isotype control	MOPC-21	mouse IgG ₁	BD
Isotype control	27-35	mouse IgG _{2b}	BD
Isotype control	G155-228	mouse IgM	BD

Isotype control	R35-95	rat IgG _{2a}	BD
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CD, cluster of differentiation; *CCR*, C-C type chemokine receptor; *CEACAM*, carcinoembryonic antigen-related cell adhesion molecule; *CRTH2*, chemoattractant receptor-homologous molecule expressed on Th2 cells (or prostaglandin D₂ receptor 2); *Fc*, fragment, crystallizable (of immunoglobulin); *ICAM*, intercellular adhesion molecule; *Ig*, immunoglobulin; *mAb*, monoclonal antibody; *PSGL*, P-selectin glycoprotein ligand; *R*, receptor; *Th2*, T helper (cell) type 2.

For antigens, conventional names and CD numbers are given.

Companies: BD Biosciences, San Jose, CA; BioLegend, San Diego, CA; Millipore Sigma, Burlington, MA; MyBioSource, San Diego, CA.

TABLE EIII. Correlations between potential blood eosinophil surface biomarkers and PEC at V2.

	r_s	P	r_s	P	r_s	P	r_s	P
Correlation adjusted for:	Not adjusted		RCAT		Allergy		Treatment group	
Antigen					and asthma		and steroid	
β_1 integrin (CD29)	-0.32	0.12	-0.27	0.24	-0.38	0.07	-0.31	0.15
Activated β_1 integrin/N29	-0.09	0.66	-0.09	0.69	-0.10	0.65	-0.13	0.55
β_2 integrin (CD18)	-0.21	0.31	-0.20	0.38	-0.15	0.50	-0.24	0.26
α_L integrin (CD11a)	-0.05	0.80	0.09	0.71	0.02	0.91	-0.10	0.64
α_M integrin (CD11b)	-0.08	0.69	-0.05	0.83	-0.04	0.86	-0.11	0.63
α_X integrin (CD11c)	0.02	0.91	0.03	0.91	-0.06	0.79	-0.08	0.70
ICAM1 (CD54)	0.08	0.72	0.06	0.78	0.02	0.95	0.08	0.71
FcεRII (CD23)	0.34	0.10	0.28	0.22	0.28	0.19	0.28	0.19
CD40	-0.31	0.14	-0.38	0.08	-0.28	0.19	-0.32	0.14
CD44	-0.34	0.10	-0.22	0.34	-0.30	0.16	-0.32	0.13
PSGL-1 (CD162)	-0.24	0.24	-0.21	0.36	-0.26	0.23	-0.29	0.18
α_{IIb} integrin (CD41): level	0.39	0.05	0.42	0.06	0.47	0.02	0.35	0.10
% positive cells	0.60	0.002	0.56	0.009	0.55	0.006	0.61	0.002
P-selectin (CD62P): level	0.25	0.24	0.33	0.14	0.32	0.14	0.24	0.27
% positive cells	0.47	0.02	0.52	0.02	0.55	0.007	0.51	0.01
CCR3 (CD193)	-0.29	0.15	-0.25	0.27	-0.31	0.15	-0.28	0.20

CEACAM8 (CD66b)	-0.08	0.71	-0.04	0.86	-0.11	0.63	-0.13	0.55
CRTH2 (CD294)	0.01	0.98	0.06	0.79	0.04	0.85	-0.06	0.77

P, probability; *RCAT*, Rhinitis Control Assessment Test; *r_s*, Spearman rank correlation coefficient; *V*, visit.

For abbreviations of antigens, see Table EII legend.

Data for all antigens are based on expression level, except for α_{IIb} integrin (CD41) and P-selectin (CD62P) based on expression level or percentage positive cells.

TABLE EIV. Expression of the potential blood eosinophil surface biomarkers and PEC at V2 in patients on or off steroid

Antigen or PEC	On steroid (n = 17)	Off steroid (n = 8)	P
	Median (quartiles)	Median (quartiles)	
PEC (per HPF)	4 (0, 47)	28 (12, 30)	0.42
β_1 integrin (CD29)	320 (180, 400)	290 (240, 390)	0.93
Activated β_1 integrin/N29	140 (80, 180)	130 (70, 190)	0.95
β_2 integrin (CD18)	1020 (690, 1060)	960 (770, 1140)	1.00
α_L integrin (CD11a)	810 (730, 1010)	770 (650, 1030)	0.67
α_M integrin (CD11b)	1130 (900, 1230)	1120 (830, 1170)	0.59
α_X integrin (CD11c)	210 (120, 300)	320 (250, 380)	0.11
ICAM1 (CD54)	30 (10, 40)	30 (10, 60)	0.77
Fc ϵ RII (CD23)	0 (0, 10)	5 (0, 40)	0.47
CD40	0 (0, 20)	6 (0, 30)	0.45
CD44	350 (240, 480)	330 (190, 430)	0.67
PSGL-1 (CD162)	1440 (1060, 1560)	1370 (1330, 1470)	0.84
α_{IIb} integrin (CD41): level	230 (170, 420)	460 (150, 670)	0.32
% positive cells	24.2 (15.7, 33.9)	38.8 (23.6, 56.7)	0.12
P-selectin (CD62P): level	80 (20, 90)	70 (50, 130)	0.55
% positive cells	3.5 (2.5, 5.5)	2.8 (2.0, 5.7)	0.73
CCR3 (CD193)	1560 (1370, 1770)	1600 (1410, 1680)	0.59

CEACAM8 (CD66b)	680 (450, 750)	710 (630, 800)	0.38
CRTH2 (CD294)	860 (730, 1040)	820 (710, 940)	0.55

HPF, high power field; *P*, probability; *PEC*, peak eosinophil count; *V*, visit.

For abbreviations of antigens, see Table EII legend.

Data for all antigens are based on expression level as specific geometric mean channel fluorescence, except for α_{IIb} integrin (CD41) and P-selectin (CD62P) based on expression level or percentage positive cells.

TABLE EV. Correlations between blood eosinophil β_1 integrin activation (mAb N29), P-selectin (CD62P)- or α_{IIb} integrin (CD41)-positive blood eosinophils and disease activity scores at V2.

Antigen	Score					
	EoEHSS		EEsAI		EREFS	
	r_s	P	r_s	P	r_s	P
Activated β_1 integrin/N29:						
expression level	-0.21	0.36	0.12	0.60	-0.36	0.10
α_{IIb} integrin (CD41):						
% positive cells	0.23	0.31	0.20	0.36	0.09	0.70
P-selectin (CD62P):						
% positive cells	0.23	0.31	-0.35	0.11	-0.17	0.46

EEsAI, Eosinophilic Esophagitis Activity Index; *EoEHSS*, EoE Histological Scoring System; *EREFS*, Endoscopic Reference Score; *P*, probability; r_s , Spearman rank correlation coefficient; V, visit.

TABLE EVI. Correlations between blood eosinophil α_{Ib} integrin (CD41)-positive blood eosinophils and EoEHSS subscores at V2.

Subscore	r_s	P
EI (eosinophil inflammation)	0.43	0.03
BZH (basal zone hyperplasia)	0.26	0.20
EA (eosinophil abscess)	0.26	0.20
SL (eosinophil surface layering)	0.18	0.40
DIS (dilated intracellular space)	0.16	0.46
SEA (surface epithelial alteration)	0.14	0.49
DEC (dyskeratotic epithelial cells)	0.03	0.87
LPF (lamina propria fibrosis)	0.40	0.05 (0.046)

EoEHSS, EoE Histological Scoring System; P , probability; r_s , Spearman rank correlation coefficient; V , visit.

TABLE EVII. Area under curve (AUC) values for the potential blood eosinophil surface biomarkers to predict $PEC \leq 6$ or $15/HPF$ at V2.

Antigen	AUC	AUC
	$PEC \leq 6/HPF$	$PEC \leq 15/HPF$
β_1 integrin (CD29)	0.60	0.62
Activated β_1 integrin/N29	0.52	0.54
β_2 integrin (CD18)	0.47	0.59
α_L integrin (CD11a)	0.53	0.52
α_M integrin (CD11b)	0.53	0.47
α_X integrin (CD11c)	0.60	0.53
ICAM1 (CD54)	0.53	0.49
Fc ϵ RII (CD23)	0.70	0.62
CD40	0.55	0.64
CD44	0.66	0.62
PSGL-1 (CD162)	0.56	0.59
α_{IIb} integrin (CD41): level	0.68	0.73
% positive cells	0.84	0.79
P-selectin (CD62P): level	0.60	0.62
% positive cells	0.66	0.72
CCR3 (CD193)	0.60	0.61
CEACAM8 (CD66b)	0.50	0.51

CRTH2 (CD294)	0.58	0.57
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AUC, area under curve (by receiver operating characteristic curve analysis); *HPF*, high power field; *PEC*, peak eosinophil count; *V*, visit.

For abbreviations of antigens, see Table EII legend.

Data for all antigens are based on expression level, except for α_{IIb} integrin (CD41) and P-selectin (CD62P) based on expression level or percentage positive cells.

FIGURE LEGENDS

FIG E1. Changes in PEC and scores from V1 to V2. Changes in PEC/HPF (**A**), histological score (**B**), symptom score (**C**), and endoscopic score (**D**). *EEsAI*, Eosinophilic Esophagitis Activity Index; *EoEHSS*, EoE Histological Scoring System; *EREFS*, Endoscopic Reference Score; *HPF*, high power field; *PEC*, peak eosinophil count. Dashed and dotted lines in **A** represent $PEC = 6$ and $15/HPF$, respectively.

FIG E2. Correlations among PEC and scores at V1 or V2. Plots with visualization of correlation between each pair of variables, i.e, a total of six correlations at each visit. Stronger correlations are represented by darker color. Numbers represent r_s (Spearman rank correlation coefficient) values. For example, the correlation between *EoEHSS* and *PEC* at V1 has $r_s = 0.81$ and is represented by a dark blue box. *EEsAI*, Eosinophilic Esophagitis Activity Index; *EoEHSS*, EoE Histological Scoring System; *EREFS*, Endoscopic Reference Score; *PEC*, peak eosinophil count.

FIG E3. Examples of flow cytometry histograms for blood eosinophil activated β_1 integrin (mAb N29) and surface α_{IIB} integrin (CD41) at V2. Low (**A**) or high (**B**) N29 reactivity patient (subject No. 1 and 10, respectively); and α_{IIB} integrin-low (**C**), with 0.1% positive blood eosinophils and $PEC = 0/HPF$, or high (**D**) patient, with 69.4% positive cells and $PEC = 85/HPF$ (subject No. 8 and 13). Red = specific mAb, blue = isotype control. Note in **B** that the entire N29 distribution is to the right of the isotype and is monophasic or homogeneous. Thus, this distribution compared to the control is best described with a value based on fluorescent intensity. Note in **D** that the CD41 distribution is biphasic or heterogeneous, with one peak or

subpopulation overlapping with the isotype and the second one to the right. Thus, this distribution is best described as percentage CD41-positive cells.

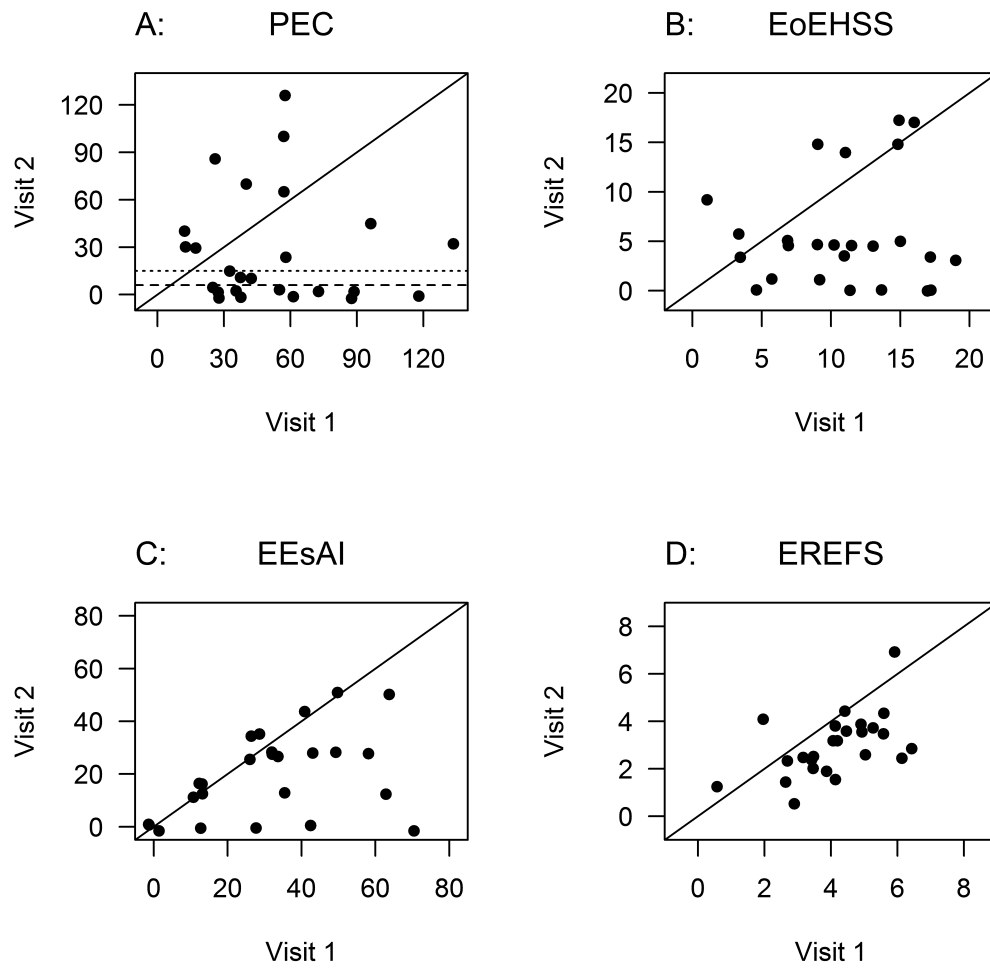
FIG E4. Correlations among blood eosinophil activated β_1 integrin (mAb N29), blood eosinophil surface P-selectin (CD62P) and α_{IIb} integrin (CD41) at V1 or V2. Correlation plots as in Fig E2. *MFI*, based on specific geometric mean fluorescence intensity as geometric mean channel fluorescence; *pp*, as percentage positive cells.

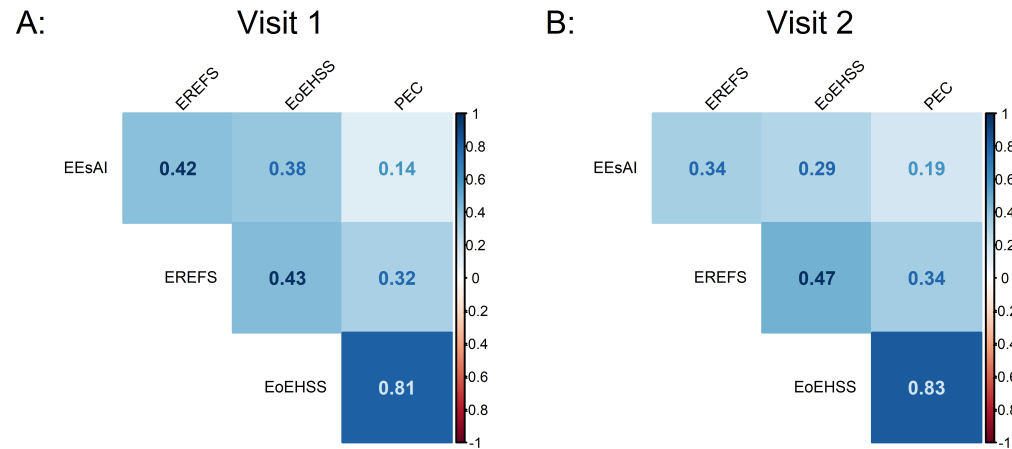
FIG E5. Relationships between blood eosinophil activated β_1 integrin (mAb N29), P-selectin (CD62P)- or α_{IIb} integrin (CD41)-positive blood eosinophils and PEC at V2. N29 reactivity (**A**); eosinophil-surface P-selectin (CD62P), as percentage positive cells (**B**); or eosinophil-surface α_{IIb} integrin (CD41), as percentage positive cells (**C**), versus PEC/HPF. *gMCF*, Geometric mean channel fluorescence; *P*, probability; *r_s*, Spearman rank correlation coefficient; line, linear regression.

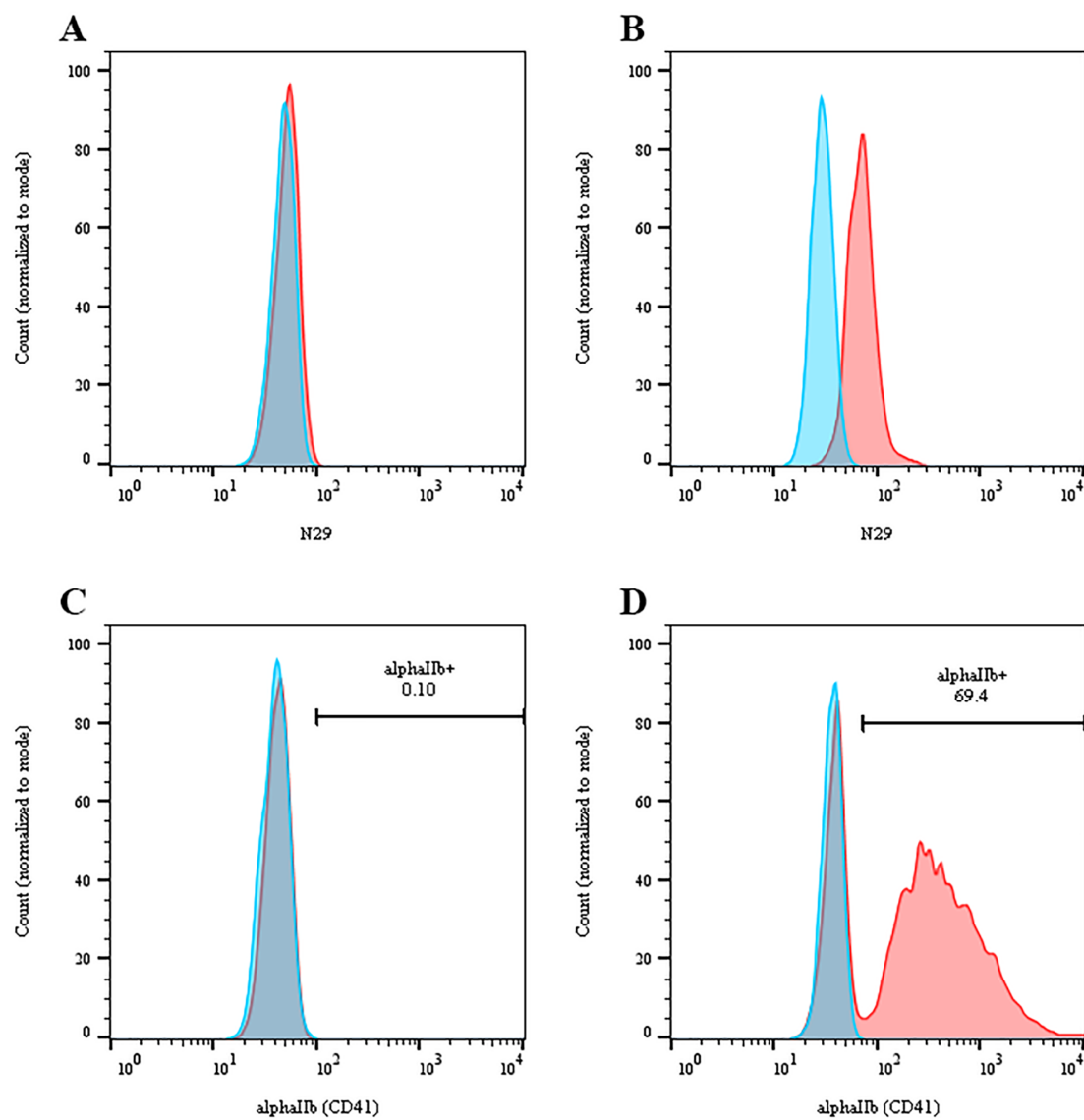
FIG E6. α_{IIb} integrin (CD41) in patients with PEC < or > 6 or 15/HPF. Patients were divided according to PEC < or > 6 (**A**) or 15 (**B**) /HPF. Bar, median in each group. Dotted line indicates the cutoff of < 22.9% (**A**) or 26.7% (**B**) α_{IIb} integrin-positive blood eosinophils, respectively, to predict PEC < 6/HPF (**A**, see Fig 1) or < 15/HPF (**B**), the latter with AUC (area under curve) = 0.79 (*P* < 0.01), specificity = 82%, and sensitivity = 71%.

FIG E7. EoE esophageal tissue biopsy stained with hematoxylin and eosin (**A**) and immunohistochemistry staining for platelets using anti- β_3 integrin (CD61) (**B**). Please note group

of intravascular, vessel-, or vessel wall-associated eosinophils (arrow in **A**) and group of platelets in the same area (arrow in **B**), which appear to interact or be associated with the eosinophils. Also note that biopsies from normal subjects usually have no or only an occasional vascular eosinophil (not shown). Arrowhead in **A**, eosinophil in tissue apparently not associated with platelets. These observations are compatible with a scenario in which, in EoE, eosinophils with associated platelets accumulate in vessels in esophageal tissue, extravasate, and at some point during or after extravasation the platelets dissociate from the eosinophils. Esophageal lumen at bottom. Bar, 20 μ m. This patient had PEC = 25/HPF.

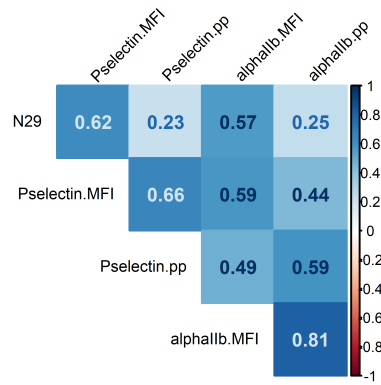






A:

Visit 1



B:

Visit 2

