ORIGINAL RESEARCH

A Comprehensive Analysis of the Stability of Blood Eosinophil Levels

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Abstract

Rationale: Blood eosinophil counts are used to inform diagnosis/management of eosinophilic asthma.

Objectives: Examine blood eosinophil variability and identify factors affecting eosinophil levels to inform clinical interpretation.

Methods: *Post hoc* analysis to understand eosinophil variability using data from four randomized controlled asthma trials. We examined *1)* influence of intrinsic/extrinsic factors (comorbidities, medication, and patient history) using baseline data ($n = 2,612$); *2)* monthly variation using placebo-treated patient data ($n = 713$); *3)* stability of eosinophil classification (<150, $150–299$, and ≥ 300 cells/μl) in placebo-treated patients with monthly measurements over a 1-year period ($n = 751$); and *4)* impact of technical factors (laboratory-to-laboratory differences and time from collection to analysis).

Results: Of intrinsic/extrinsic factors examined, nasal polyps increased eosinophil levels by 38%, whereas current smoking decreased levels by 23%. Substantial seasonal differences in eosinophil counts were observed, with differences of ~20% between July and January. Eosinophil levels between 150 and 299 cells/μl were least stable, with 44% of patients remaining in the same classification for seven of 10 measurements versus 59% and 66% of patients in the <150 and ≥ 300 cells/μl subgroups, respectively. Measurements at different laboratories showed high association (Spearman's correlation coefficient, $R = 0.89$); however, eosinophil counts were reduced, with longer time from collection to analysis, and variability increased with increasing eosinophil counts.

Conclusions: Several intrinsic, extrinsic, and technical factors may influence, and should be considered in, clinical interpretation of eosinophil counts. Additionally, a single measurement may not be sufficient when using eosinophil counts for diagnosis/management of eosinophilic asthma.

Keywords: asthma; diagnosis; eosinophil count; patient management

Higher baseline blood eosinophil counts are associated with asthma severity and increased likelihood of future exacerbations in persistent asthma (1). An association has also been shown between blood eosinophil counts and response to corticosteroids (2). More recently, associations of higher blood eosinophil counts with greater response to type 2 biologics directed against interleukin (IL)-5 (mepolizumab, reslizumab) (3, 4), IL-5 receptor α (benralizumab) (5), and IL-4 receptor α (dupilumab) (6) have also been described. These observations, combined with the ease of blood sample collection and eosinophil count testing, have led to the use of blood eosinophil counts for guiding treatment decisions with eosinophilic and type 2 biologics in asthma.

Previous studies have shown that blood eosinophil levels vary substantially over time (7–11). Using a cutoff of 150 cells/μl, eosinophil counts in patients with asthma and healthy control subjects were shown to vary by up to 40% of an individual's baseline value between subsequent counts (8). Similarly, in patients with severe asthma with baseline eosinophil count >150 cells/μl, average eosinophil count over a 1-year timeframe remained >150 cells/μl in 85–90% of patients but did not remain >150 cells/μl in ~10–15% of patients (7, 9). Blood eosinophil levels may also be influenced by intrinsic and extrinsic factors, such as medication, asthma comorbidities, allergic sensitivities, obesity, time of day, and smoking (8, 12–15). Technical factors, such as storage conditions and time from collection to analysis (16, 17), are also thought to contribute to eosinophil count variability, although they have not been thoroughly investigated.

Because fluctuations in eosinophil counts may have practical implications for asthma management, better characterization of blood eosinophil count as a biomarker is needed to understand potential limitations. In this paper, we describe a comprehensive analysis of multiple factors that may impact variability of eosinophil levels and stability of classification. Our analysis focused on intrinsic and extrinsic factors (including comorbidities, asthma medication, demographics, and disease history), monthly/seasonal variation, and eosinophil classification (using cutoffs frequently used in clinical practice) stability. Additionally, we investigated technical variation of measuring eosinophil levels, interlaboratory variation, and time from sampling to measurement.

Methods

Study Population and Design

Data from four randomized, multicenter, double-blind, placebo-controlled lebrikizumab studies (anti–IL-13; LUTE [NCT01545440], VERSE [NCT01545453], LAVOLTA I [NCT01867125], and LAVOLTA II [NCT01868061]) were used for eosinophil variability analysis. Detailed methods, including inclusion and exclusion criteria, for these studies have been published previously (18, 19). Relevant elements are summarized briefly below.

Key patient inclusion criteria for enrollment in LUTE/VERSE and LAVOLTA I/II included age 18–75 years, uncontrolled asthma despite treatment with fluticasone propionate 500–2,000 μg dry powder inhaler or equivalent and a second controller, prebronchodilator forced expiratory volume in 1 second (FEV_1) 40–80% of predicted, and ≥12% FEV_1 reversibility after bronchodilator administration (18, 19). Patients remained on their standard-of-care asthma controller medications for the study duration (18, 19). Patients on maintenance oral corticosteroid therapy, defined as daily or alternate-day oral corticosteroid maintenance therapy, were excluded from the studies (18, 19).

For the intrinsic and extrinsic factor analysis, data on comorbidities and concomitant controller medications were collected during screening in all four studies (n = 2,612 [LAVOLTA I/II, n = 2,149; LUTE/VERSE, n = 463]).

The eosinophil classification (<150, 150–299, or ≥300 cells/μl) stability analysis included placebo-treated patient data from all four studies to prevent influence of treatment on eosinophil levels. Because LUTE/VERSE terminated early, the study period differed between patients, and only patients with at least five monthly eosinophil measures were included (n = 751).

The monthly/seasonal variation analysis only included placebo-treated patient data from LAVOLTA I/II (n = 713), because LUTE/VERSE did not include a full year of data for each patient.

For the technical variability analysis, a standalone study was conducted. To ensure that a range of eosinophil values was obtained, both patients with asthma (n = 30) and healthy control subjects (n = 32) were included.

All study participants provided written informed consent, and study protocols were approved by relevant ethics committees or institutional review boards. All studies were conducted in accordance with the Declaration of Helsinki and Good Clinical Practice Guidelines.

Eosinophil Measurement

Blood samples were collected from patients by venipuncture into ethylenediaminetetraacetic acid tubes. Blood eosinophil levels were measured as part of a total white blood cell count with differential using automated hematology analyzers. In LUTE, VERSE, and LAVOLTA I/II, blood eosinophils were measured in a central laboratory throughout the study as part of safety monitoring. The technical variability analysis was a distinct study to assess if eosinophil measurements themselves would have an impact on eosinophil levels, and it was conducted separately from the lebrikizumab studies. For this analysis, 10 blood samples were collected from each study subject at a single time point and analyzed in one of 10 laboratories. The hematology analyzers used were as follows: Advia 2120i (Siemens): laboratories A (central laboratory), B, and J; Sysmex XE2100 models (Sysmex Corporation): laboratories C, D, and H; LH750, LH780 (Beckman Coulter): laboratory E; DXH800 (Beckman Coulter): laboratory G; and Sysmex 400Xt (Sysmex Corporation): laboratory I. A hematology analyzer was not provided for laboratory F.

Analyses

Prespecified threshold. Because determining statistical significance of variability with large sample sizes can result in small differences that may not be clinically meaningful, we defined a prespecified threshold of the acceptable magnitude of eosinophil variability using natural variation within patients. Repeated eosinophil measurements in placebo-treated patients in LUTE/VERSE taken 4 weeks apart were used. Differences between eosinophil levels were calculated, using standard deviation (SD) for the 25th to 75th percentile to determine the threshold for meaningful difference. Subsequently, the 90% prediction interval for difference in eosinophil levels

4 weeks apart was set as the prespecified threshold for meaningful differences. This corresponded to ~20% (~50 cells/µl at the median of 250 cells/µl) and was used to determine whether eosinophil counts were meaningfully different in the intrinsic, extrinsic, and seasonality analyses.

Intrinsic and extrinsic factors. A univariate analysis of variance model using baseline data from LUTE, VERSE, and LAVOLTA I/II was conducted to estimate relative difference in log-transformed eosinophils from a reference category for intrinsic and extrinsic variables. Intrinsic variables included demographics (age, sex, body mass index, race/ethnicity, region), baseline FEV$_1$, history of asthma exacerbations, and comorbidities (chronic sinusitis, nasal and sinus polyps, acute sinusitis, allergic rhinitis, aspirin allergy, atopic dermatitis/eczema, gastroesophageal reflux disease, osteoporosis, and type 2 diabetes). Extrinsic variables included concomitant medications (inhaled corticosteroids [ICSs; ≥1,000 µg], long-acting β-agonists [LABAs], ICSs ≥1,000 µg plus LABAs, leukotriene receptor antagonists, long-acting muscarinic antagonists, and theophylline) and smoking status. Because some variables may be related, a multivariable analysis including variables that showed differences in the univariate analysis was subsequently performed.

Seasonality. A seasonality analysis using longitudinal data from placebo-treated patients in LAVOLTA I/II was conducted for July versus other months. July was chosen as the index month because eosinophil levels may increase before or during exacerbations, and exacerbations are observed less during summer months. Because LAVOLTA I/II were global trials with sites in the northern and southern hemispheres (19), seasonality was adjusted accordingly. LUTE/VERSE were not included in this analysis because many patients did not have a full year of follow-up.

Eosinophil classification stability. The percentage of patients remaining in the most frequently observed eosinophil category (<150, 150–299, and ≥300 cells/µl) was assessed for ≥60%, ≥70%, ≥80%, and ≥90% of the time (six, seven, eight, and nine of 10 measurements, respectively). Percentages were not mutually exclusive. Transition to other eosinophil subgroups between study visits was visualized using a Sankey diagram.

A sensitivity analysis excluding patients with at least one exacerbation during the study period was performed to reduce potential influence from oral corticosteroid use.

Technical variability. Spearman correlation coefficient estimates were determined between all laboratories and between each of the local/regional laboratory (laboratories B–J) eosinophil counts and central laboratory (laboratory A) eosinophil count. Additionally, variability was assessed by plotting eosinophil counts ordered by median values of each subject. A difference plot assessed eosinophil counts by time from collection to analysis for the central laboratory versus each local/regional laboratory. In all instances, data were graphed by laboratory.

Results

Baseline Demographics and Clinical Characteristics
Baseline demographics and clinical characteristics for all analysis populations are shown in Table 1. Randomized controlled trial analysis populations were generally similar.

Intrinsic and Extrinsic Factors
Using combined data from all four randomized controlled trials in a univariate analysis, we assessed which variables were associated with eosinophil variability. We also evaluated LUTE/VERSE and LAVOLTA I/II separately to ensure results were consistent between studies. History of asthma exacerbations (≥3 vs. 0), theophylline use at baseline, sinus polyps, nasal polyps, and chronic sinusitis increased eosinophil levels, whereas current smoking decreased eosinophil levels, beyond the 20% prespecified threshold versus their respective reference groups. Body mass index <25 kg/m^2 versus ≥30 kg/m^2 was associated with reduced eosinophil levels beyond the prespecified 20% threshold in LUTE/VERSE (−20%) but not in LAVOLTA I/II (−10%). However, because eosinophils are known to be impacted by obesity (13), we included body mass index in the multivariable analysis.

The multivariable analysis was performed to adjust for variables that were possibly related. All factors that affected eosinophil variability beyond the 20% threshold in the univariate analysis were

included. Nasal polyps and current smoking only demonstrated changes beyond the prespecified 20% threshold after controlling for other factors. Whereas nasal polyps (39%) were associated with an increase in eosinophil levels, current smoking (−23% vs. never-smokers) was associated with a decrease (Figure 1).

Seasonality
Using July as the reference month, we investigated whether there were seasonal differences in eosinophil levels. Compared with July, other late spring and summer months (May, June, August, and September) showed similar levels. Eosinophil levels increased toward fall and winter, reaching the prespecified threshold in November, December, January, and February. The largest difference was in January (27.9%; Figure 2).

Eosinophil Classification Stability
Because eosinophil levels are used in clinical practice to diagnose or manage eosinophilic asthma using specific thresholds, we investigated eosinophil classification stability by calculating how often a patient remained in the same classification (<150, 150–299, or ≥300 cells/µl) over a 1-year period. The 150–299 cells/µl subgroup showed more variability versus the <150 and ≥300 cells/µl subgroups. The percentage of patients who remained in the same eosinophil subgroup for ≥70%, ≥80%, and ≥90% of the time (seven, eight, or nine of 10 measurements, respectively) was substantially lower in the baseline 150–299 cells/µl subgroup versus other eosinophil subgroups. This is illustrated in the cumulative distribution plot, which demonstrates a larger and more rapid decrease in the percentage of patients remaining on the most frequently observed category when the percentage of time increases (Figure 3A and Table 2).

Approximately 30% of patients transitioned between eosinophil subgroups during consecutive visits. The 150–299 cells/µl subgroup was least stable, with ~40% of patients changing classification between consecutive time points. The ≥300 and <150 cells/µl subgroups showed lower percentages of transitioning patients, with ~20% and 30% transitioning to other eosinophil subgroups at any point in time.

In the <150 and ≥300 cells/µl subgroups, transitioning occurred most often between adjacent eosinophil subgroups. In

Table 1. Baseline demographics and clinical characteristics

Characteristic	EOS Stability over Time Analysis: LUTE, VERSE, LAVOLTA I/II* (N = 751)	Intrinsic/Extrinsic Analysis: LUTE, VERSE, LAVOLTA I/II (N = 2,612)	Seasonality Analysis: LAVOLTA I/II* (N = 713)	Technical Variability Analysis (N = 62)
Age, mean (SD), yr	50.3 (12.9)	50.3 (12.8)	50.4 (12.9)	33.7 (18, 70)[†]
Sex, female, n (%)	473 (63.0)	1,646 (63.0)	448 (62.8)	36 (58.1)
Race, n (%)				
White	575 (76.6)	2,029 (77.7)	554 (77.7)	37 (59.7)
Black	69 (9.2)	215 (8.2)	51 (7.2)	8 (12.9)
Other/multiple	107 (14.2)	368 (14.1)	108 (15.1)	17 (27.4)
BMI, median (IQR), kg/m^2	28.2 (7.3)	28.2 (8.0)	27.8 (7.1)	—
Prebronchodilator FEV$_1$, mean (SD), % predicted	61.4 (10.4)	61.1 (10.5)	61.1 (10.6)	—
FE$_{NO}$, median (IQR), ppb	25.0 (28.0)	25.0 (28.0)	25.0 (32.0)	—
Blood EOS, median (IQR), cells/μl	250.0 (240.0)	240.0 (250.0)	240.0 (240.0)	—
Total IgE, median (IQR), pg/ml	177.0 (397.5)	156.0 (351.0)	172.0 (371.0)	—
Patients with asthma exacerbation in year before enrollment, n (%)	464 (61.8)	1,592 (61.0)	452 (63.4)	—

Definition of abbreviations: BMI = body mass index; EOS = eosinophils; FE$_{NO}$ = fractional exhaled nitric oxide; FEV$_1$ = forced expiratory volume in 1 second; IgE = immunoglobulin E; IQR = interquartile range; SD = standard deviation.
*Indicates analysis included placebo-treated patient data only.
[†]Mean (minimum, maximum).

patients with eosinophil counts <150 cells/μl, ~30% of patients transitioned to the 150–299 cells/μl subgroup, and ~20% of patients with eosinophil counts ≥300 cells/μl transitioned to the 150–299 cells/μl subgroup. However, transitions to nonadjacent subgroups also occurred from the <150 to the ≥300 cells/μl subgroup and

from the ≥300 to the <150 cells/μl subgroup. Transitions to nonadjacent subgroups were less common, with only 6% and 4% of patients in the <150 and ≥300 cells/μl subgroups transitioning to the ≥300 and <150 cells/μl subgroups, respectively. Patients in the 150–299 cells/μl subgroup had an almost equal likelihood

(~20%) of transitioning to the <150 and ≥300 cells/μl subgroups (Figure 3B).

In a sensitivity analysis excluding patients treated with oral corticosteroids for an exacerbation during the study, the proportion of patients remaining in the <150, 150–299, and ≥300 cells/μl subgroups for ≥60%, ≥70%, ≥80%, and

Parameter	Unadjusted	Adjusted
Sinus Polyps	56%	14%
Nasal Polyps	51%	39%
Chronic Sinusitis	26%	9%
Past Exacerbations: ≥3 (vs. 0)	26%	18%
Past Exacerbations: 1–2 (vs. 0)	4%	2%
Theophylline	23%	16%
BMI: ≥30 (vs. <25)	−12%	−7%
BMI: 25 to <30 (vs. <25)	−2%	0%
Smoker: Current (vs. Never)	−25%	−23%
Smoker: Previous (vs. Never)	−4%	−4%
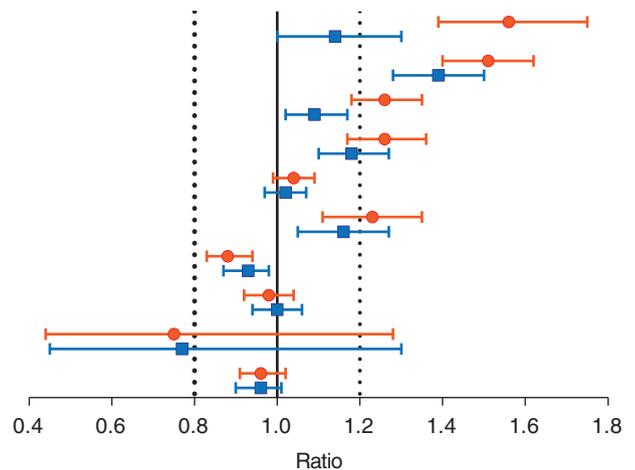
Figure 1. Factors identified as having an influence on eosinophil levels beyond the prespecified threshold of 20% in the univariate (unadjusted) analyses and included in the multivariable (adjusted) analyses of LUTE/VERSE and LAVOLTA I/II. Percentages are relative difference from reference categories. Data are presented as a ratio with the relative reference category. Dotted lines represent nonmeaningful differences (20% plotted as a ratio on a log scale [i.e., 0.8–1.25]). BMI = body mass index.

(vs. July)

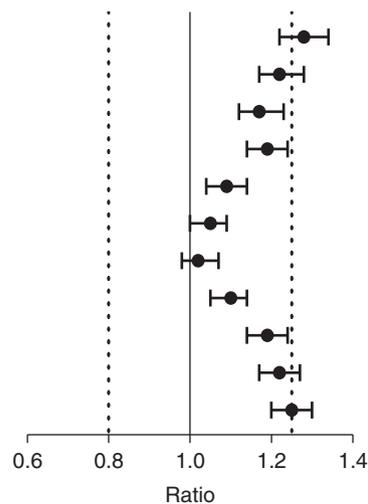| Month		
January	27.9%	
February	22.4%	
March	17.4%	
April	18.8%	
May	9.0%	
June	4.5%	
August	2.0%	
September	9.6%	
October	19.0%	
November	21.8%	
December	25.0%	

Figure 2. Monthly influence on blood eosinophil levels beyond the prespecified threshold of 20% by month in LAVOLTA I/II. Percentages are relative difference from July. Data in the graph are presented as a ratio with July. Dotted lines represent nonmeaningful differences (20% plotted as a ratio on a log scale [i.e., 0.8–1.25]).

≥90% of the time was similar to the analysis of the total population. Furthermore, the 150–299 cells/μl subgroup showed a similar degree of instability versus the <150 and ≥300 cells/μl subgroups (Table 2).

Technical Variability

Although blood eosinophils are used in asthma management, the assays to measure eosinophils were not developed for this purpose. Because technical variation may have an impact on reliability of eosinophil counts, we studied differences between laboratories and associated variables. Eosinophil counts from 62 subjects measured at 10 different laboratories were compared. There was good association between eosinophil counts measured at laboratory A versus all other laboratories (Spearman's correlation coefficient: minimum, $R = 0.83$; maximum, $R = 0.95$). Eosinophil counts were generally higher in local/regional laboratories versus the central laboratory, laboratory A (150 [90] cells/μl). Mean (SD) eosinophil counts in local/regional laboratories were 180 (120) cells/μl at laboratory C, 180 (130) cells/μl at laboratory D, 170 (120) cells/μl at laboratory E, 170 (110) cells/μl at laboratory F, 190 (140) cells/μl at laboratory G, 170 (130) cells/μl at laboratory H, and 160 (120) cells/μl at laboratory I. Mean (SD) eosinophil counts were lower than laboratory A at laboratories B (120 [90] cells/μl) and J (140 [90] cells/μl).

We also observed that higher absolute eosinophil counts were associated with higher interlaboratory variability (Figure 4A). Because eosinophil cutoffs are commonly used in clinical practice, this may lead to a potential misclassification if counts are used for diagnosis of eosinophilic asthma. Indeed, of 16 subjects who had one or more measurements of ≥300 cells/μl, only three subjects had eosinophil counts of ≥300 cells/μl at all laboratories. One subject had a missing value and one had nine of 10 measurements of ≥300 cells/μl. For 11 subjects, measurements were inconsistent across laboratories (Figure 4A). Using a cutoff of 150 cells/μl, 36 subjects had one or more measurements of ≥150 cells/μl, of which only 15 (42%) subjects had a consistent reading >150 cells/μl.

Time from collection to analysis also showed some impact on eosinophil counts. Mean (SD) time from collection to analysis ranged from 7.6 (3.4) hours at laboratory F to 31.2 (3.7) hours at laboratory B and was 25.1 (3.9) hours at the central laboratory. A longer relative time from sample collection to analysis was associated with a small relative reduction in absolute eosinophil counts (Figure 4B).

An additional observation was that some laboratories reported eosinophil counts to one decimal place (laboratories C, D, G, and J), whereas others reported two decimal places. Rounding of values in laboratories that used one decimal place led to

consistently lower correlation with the central laboratory (Spearman's correlation coefficient: minimum, $R = 0.83$; maximum, $R = 0.91$) than those reporting to two decimal places (Spearman's correlation coefficient: minimum, $R = 0.93$; maximum, $R = 0.95$).

Discussion

We performed a comprehensive analysis on factors that can influence eosinophil counts, including intrinsic and extrinsic factors, seasonality, variation over time, and technical variation. Comorbid nasal polyps were associated with substantially higher eosinophil levels, whereas smoking was associated with decreased levels. Additionally, there was a strong seasonal effect, with higher levels in winter than in summer months. We also observed that eosinophil classifications based on commonly used cut points were not stable, with eosinophil levels between 150 and 299 cells/μl being least stable. Lastly, there are several technical factors that can influence reported eosinophil counts, which could affect diagnosis or management of eosinophilic asthma.

Blood eosinophil thresholds of ≥150 and ≥300 cells/μl are commonly applied to identification of patients with eosinophilic asthma and treatment decisions regarding initiation of type 2 biologics because of greater efficacy demonstrated in patients with higher eosinophil counts (3–6). However, there is no consensus regarding number of eosinophil count measurements before initiating biologics (7–10). Analyses of patients from mepolizumab trials suggested that single measurements were sufficient to guide treatment because most patients' average eosinophil counts remained in the same subgroup during the study (7, 9). However, our findings corroborate findings by Mathur and colleagues (8) demonstrating substantial variability in eosinophil counts over time. Indeed, if eosinophilic asthma is defined by ≥300 cells/μl, only 6.6 of 10 patients showed an eosinophil count of ≥300 cells/μl in seven of 10 measurements. The proportion of patients demonstrating measurements above this threshold decreased incrementally when the requirement increased from eight of 10 to nine of 10 measurements. These data suggest that one eosinophil count may not be sufficient, and multiple eosinophil measurements over time may better reflect a
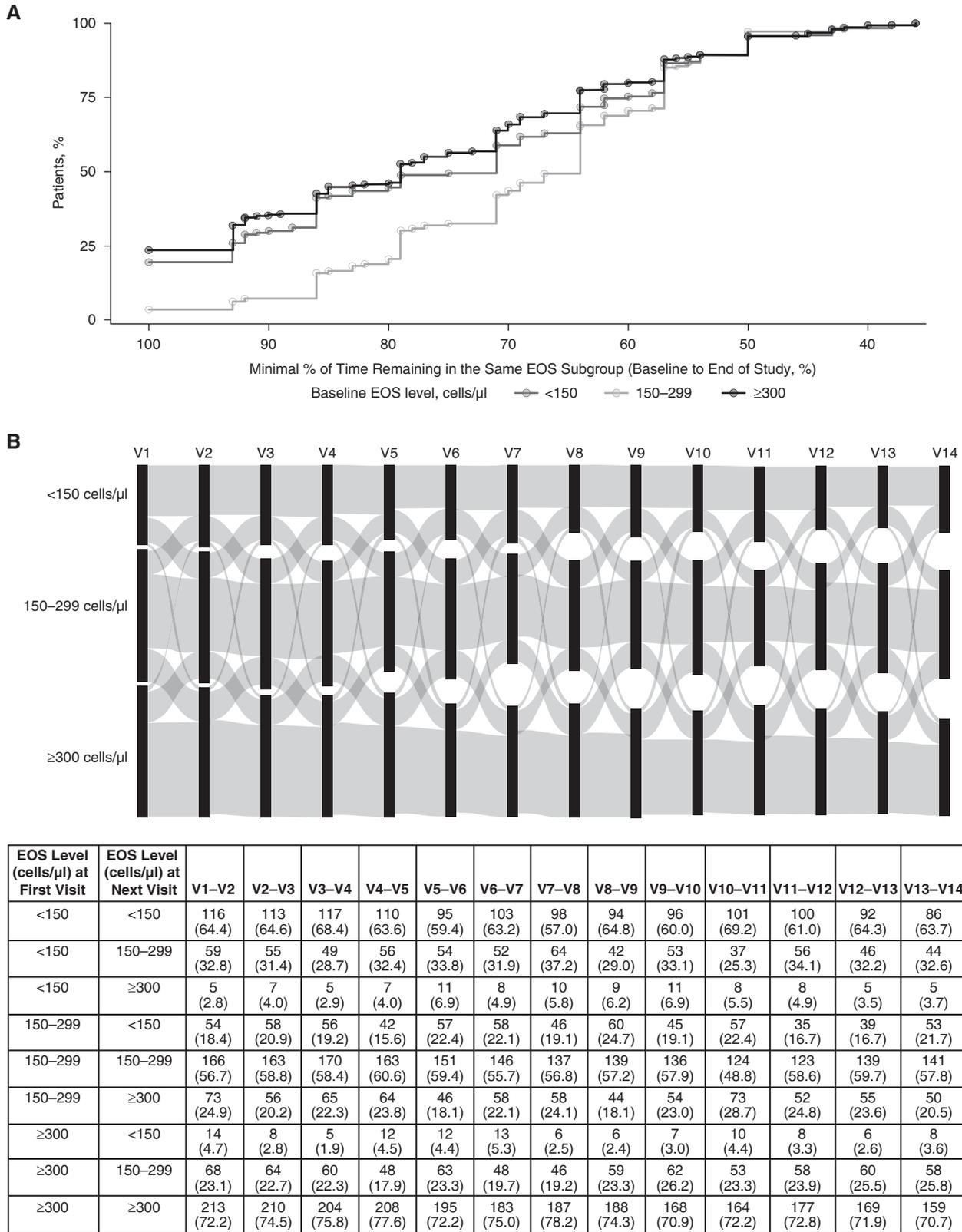
Figure 3. Blood eosinophil (EOS) stability by EOS subgroup. (*A*) Cumulative distribution plot demonstrating minimal percentage of time remaining in the most commonly observed EOS subgroup. (*B*) Sankey diagram demonstrating blood EOS subgroup changes by study visit over 1 year. Each of the three black bars aligned vertically represents patients in the three EOS subgroups at a specific visit; the three bars are ordered as <150 cells/μl (top), 150–299 cells/μl (middle), and ≥300 cells/μl (bottom) for every visit (1 month apart). Gray bars show how many

EOS Level (cells/μl) at First Visit	EOS Level (cells/μl) at Next Visit	V1–V2	V2–V3	V3–V4	V4–V5	V5–V6	V6–V7	V7–V8	V8–V9	V9–V10	V10–V11	V11–V12	V12–V13	V13–V14
<150	<150	116 (64.4)	113 (64.6)	117 (68.4)	110 (63.6)	95 (59.4)	103 (63.2)	98 (57.0)	94 (64.8)	96 (60.0)	101 (69.2)	100 (61.0)	92 (64.3)	86 (63.7)
<150	150–299	59 (32.8)	55 (31.4)	49 (28.7)	56 (32.4)	54 (33.8)	52 (31.9)	64 (37.2)	42 (29.0)	53 (33.1)	37 (25.3)	56 (34.1)	46 (32.2)	44 (32.6)
<150	≥300	5 (2.8)	7 (4.0)	5 (2.9)	7 (4.0)	11 (6.9)	8 (4.9)	10 (5.8)	9 (6.2)	11 (6.9)	8 (5.5)	8 (4.9)	5 (3.5)	5 (3.7)
150–299	<150	54 (18.4)	58 (20.9)	56 (19.2)	42 (15.6)	57 (22.4)	58 (22.1)	46 (19.1)	60 (24.7)	45 (19.1)	57 (22.4)	35 (16.7)	39 (16.7)	53 (21.7)
150–299	150–299	166 (56.7)	163 (58.8)	170 (58.4)	163 (60.6)	151 (59.4)	146 (55.7)	137 (56.8)	139 (57.2)	136 (57.9)	124 (48.8)	123 (58.6)	139 (59.7)	141 (57.8)
150–299	≥300	73 (24.9)	56 (20.2)	65 (22.3)	64 (23.8)	46 (18.1)	58 (22.1)	58 (24.1)	44 (18.1)	54 (23.0)	73 (28.7)	52 (24.8)	55 (23.6)	50 (20.5)
≥300	<150	14 (4.7)	8 (2.8)	5 (1.9)	12 (4.5)	12 (4.4)	13 (5.3)	6 (2.5)	6 (2.4)	7 (3.0)	10 (4.4)	8 (3.3)	6 (2.6)	8 (3.6)
≥300	150–299	68 (23.1)	64 (22.7)	60 (22.3)	48 (17.9)	63 (23.3)	48 (19.7)	46 (19.2)	59 (23.3)	62 (26.2)	53 (23.3)	58 (23.9)	60 (25.5)	58 (25.8)
≥300	≥300	213 (72.2)	210 (74.5)	204 (75.8)	208 (77.6)	195 (72.2)	183 (75.0)	187 (78.2)	188 (74.3)	168 (70.9)	164 (72.2)	177 (72.8)	169 (71.9)	159 (70.7)

Table 2. Patients remaining in the most commonly identified eosinophil subgroup for ≥60%, ≥70%, ≥80%, and ≥90% of the time, for all patients (top) and a sensitivity analysis excluding patients who experienced one or more exacerbations during the study period (bottom)

	All Patients		
	Eosinophil Subgroup		
Patients Remaining in the Most Commonly Observed Eosinophil Subgroup	**<150 Cells/μl** (n = 170)	**150–299 Cells/μl** (n = 292)	**≥300 Cells/μl** (n = 289)
≥60% of the time	128 (75.3)	206 (70.5)	231 (79.9)
≥70% of the time	100 (58.8)	127 (43.5)	191 (66.1)
≥80% of the time	76 (44.7)	60 (20.5)	134 (46.4)
≥90% of the time	51 (30.0)	21 (7.2)	102 (35.3)
	Sensitivity Analysis of Patients without Systemic Corticosteroid Use		
	Eosinophil Subgroup		
Patients Remaining in the Most Commonly Observed Eosinophil Subgroup	**<150 Cells/μl** (n = 118)	**150–299 Cells/μl** (n = 209)	**≥300 Cells/μl** (n = 162)
≥60% of the time	94 (79.7)	148 (70.8)	127 (78.4)
≥70% of the time	74 (62.7)	91 (43.5)	109 (67.3)
≥80% of the time	56 (47.5)	45 (21.5)	76 (46.9)
≥90% of the time	37 (31.4)	17 (8.1)	59 (36.4)

Data are shown as n (%).
All patients had five or more eosinophil values available for evaluation.

patient's eosinophilic status to guide treatment decisions with asthma biologics.

Although changes over time reported in this study occurred mostly between adjacent eosinophil subgroups, a small percentage of patients (4–6%) experienced larger changes between nonadjacent eosinophil subgroups. Particularly large changes in eosinophil counts may reflect active infections, antimicrobial treatment, and exacerbations (20, 21). Patients in this study were on stable background medications; however, corticosteroids were permitted for exacerbations. Because corticosteroids reduce eosinophil counts proportionally to dose (22), we completed a sensitivity analysis excluding patients who experienced an exacerbation requiring corticosteroid use. Eosinophil stability was similar, indicating that our findings were not driven by systemic corticosteroid use.

Results from the univariate/multivariable analysis showed an association between nasal polyps and increased eosinophil counts and smoking and reduced eosinophil counts. The association between nasal polyps and increased eosinophil counts is not unexpected; in addition to showing marked tissue eosinophilia, nasal polyps are frequently associated with increased blood eosinophil levels (23), which decrease substantially following endoscopic sinus surgery (24). Sinus polyps showed a larger effect than nasal polyps in the unadjusted model, which was reduced to below the prespecified threshold in the adjusted model, likely reflecting that sinus and nasal polyps coexist. Moreover, these findings reflect the frequent coassociation between asthma, sinus inflammation with nasal polyps, and sensitivity to aspirin, which defines Samter's Triad (25). The identification that aspirin allergy and sinusitis were not associated with increases in eosinophil counts suggests that nasal polyps may drive the higher eosinophil count observed.

Supporting the present findings, patients with asthma who smoke are more likely to have reductions in eosinophil counts than nonasthmatic smokers (26). Also consistent with previous studies (27), former smokers in the present study had a blood eosinophil count closer to that of nonsmokers. It should be noted that the number of smokers in the analysis was low, and thus, error bars are wide, because LUTE/VERSE and LAVOLTA I/II excluded smokers, making it difficult to draw definitive conclusions (18, 19).

Surprisingly, we did not find any evidence of background asthma medication influencing eosinophil levels beyond the prespecified threshold. Although patients who used theophylline at baseline showed evidence of higher eosinophil levels in the univariate analysis, this could have been theoretically due to its antiinflammatory effects—including the induction of eosinophil apoptosis, reduction of degranulation, adhesion, and migration of eosinophils—leading to reduced eosinophil influx to the airways and accumulation of systemic eosinophils (28, 29). However, this finding was not significant in the multivariate analysis. Although corticosteroids impact eosinophil counts, patients on maintenance oral corticosteroids were excluded from the clinical trials used in

Figure 3. (*Continued*). patients flow from one EOS subgroup to another or stay stable from visit to visit, because the width of the gray bars is proportional to the number of patients flowing from one EOS subgroup to another. The table complements the figure by showing the number of patients changing EOS categories from visit to visit. V = visit.
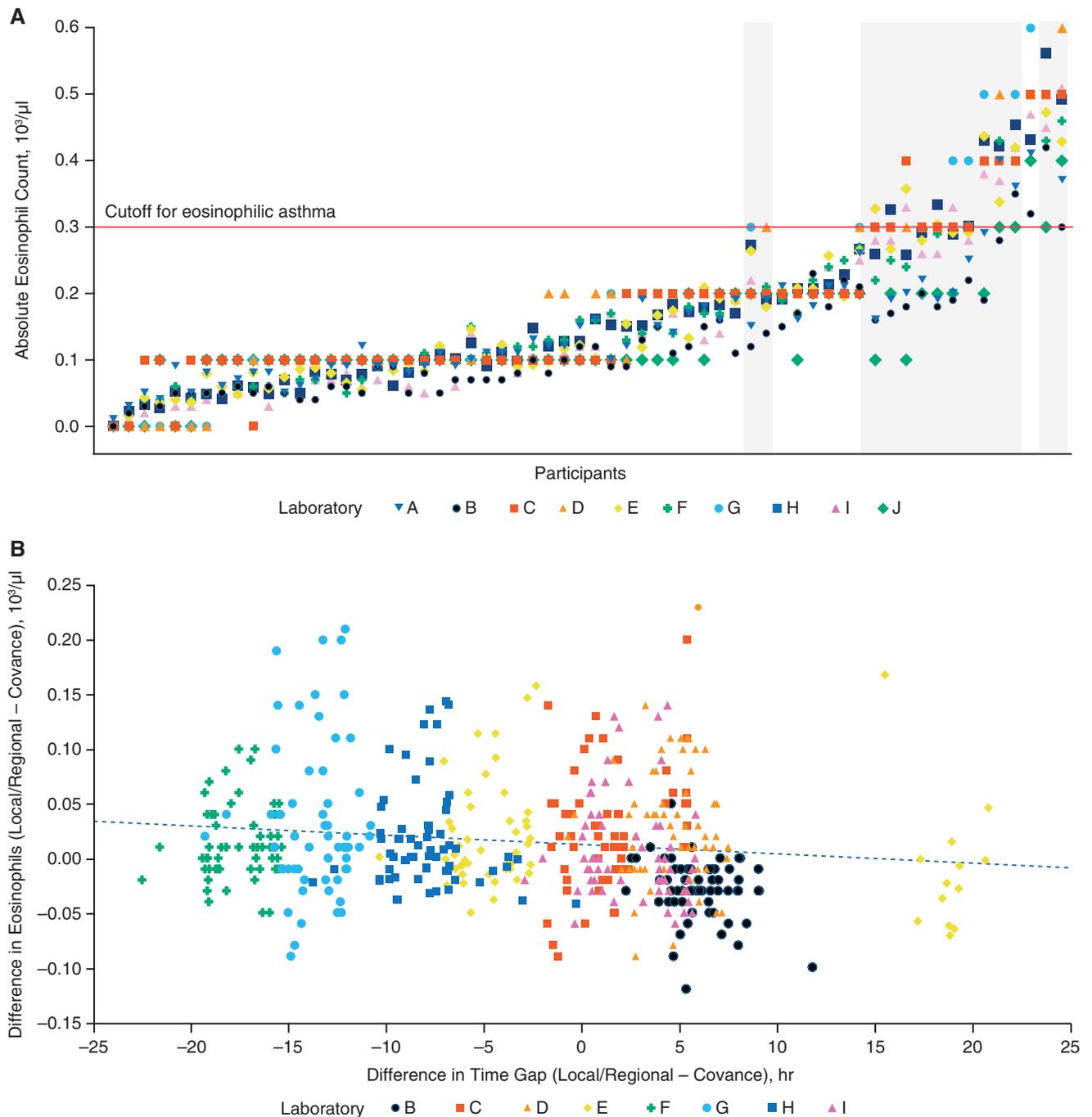
Figure 4. (*A*) Absolute eosinophil counts ordered by median values of each patient. Gray shaded area in *A* indicates patients whose diagnosis of eosinophilic asthma (based on a 300 cells/μl cutoff) is dependent upon the laboratory. (*B*) Relationship between differences in time from collection to analysis differences and differences in eosinophil counts for the local/regional – central laboratories.

this analysis and ICSs may not reach the systemic levels needed to show a sufficiently large effect on eosinophil levels. Indeed, a study investigating potential reductions in eosinophils after ICS use in patients with chronic obstructive pulmonary disease only found decreases of ∼30 cells/μl, which were

below our 20% (∼50 cells/μl) prespecified threshold (30). LABA use (formoterol) has also been shown to reduce eosinophils in pediatric and adolescent patients (31); however, that study withdrew LABA therapy for 4 weeks followed by its reintroduction, whereas our studies required that patients

were on stable doses with no permitted changes to medication.

Previous studies looking at seasonal effects on eosinophil counts have been scarce, but tend to show a peak during peak pollen seasons in the United States (March to August). Similarly, we observed higher levels

in early spring and late fall versus the reference month (July) and summer months. However, the highest eosinophil levels were in winter months. Differences in findings are unlikely related to atopy because seasonal differences have been shown in both nonatopic (32) and atopic individuals (33), although patients with allergen sensitization may be exposed to their respective allergens more during winter months owing to a greater percentage of time being spent indoors (34–36). However, another possibility, and the reason that July was chosen as the reference month, is that eosinophil count changes are related to exacerbations, often being caused by viral infections (37, 38), which also peak in winter in adults, and are lowest in the summer (39, 40). Viral infections may increase eosinophil levels (41).

Although these findings support previous findings demonstrating high correlation in eosinophil counts obtained at different laboratories (42, 43), technical factors associated with eosinophil measurements contributed to differences in eosinophil counts. For instance, eosinophil counts decreased slightly as time from collection to analysis increased, likely because of cell apoptosis (44, 45). Rounding to one decimal place was also associated with loss of data that resulted in generally higher overall average eosinophil counts and reduced association with counts obtained at the central laboratory. The important observation is that these technical factors may influence diagnosis and management of eosinophilic asthma. We showed that in subjects with eosinophil counts near the $\geq$300 cells/μl cutoff, subjects may be classified as eosinophilic or noneosinophilic, depending on which laboratory the sample was sent to and/or time to sample analysis.

Strengths of our analyses include the large patient population on stable medication, as mandated by trial inclusion criteria. In addition, for baseline analysis, blood samples were obtained before the investigational drug was administered, and for longitudinal analysis, only patients on placebo were included, limiting the effect of changes to medication use. Furthermore, the large number of patients in combination with a large number of variables made it possible to do a comprehensive analysis to characterize blood eosinophil level as a biomarker and identify factors that should be taken into account when interpreting levels in the context of eosinophilic asthma. However, some parameters had small n values, making observations less certain. The prespecified threshold was fairly large ($\pm$50 cells/μl), which may have resulted in having missed variables that may also be important. The retrospective nature of these analyses also potentially makes these data more prone to confounding and bias. Furthermore, only a few aspects of technical variability were included, and it is unknown whether other factors, such as ethylenediaminetetraacetic acid tube filling and mixing, transportation, and sample preparation, may have contributed to the differences observed (16, 46).

Conclusions

This study adds to a body of evidence suggesting that eosinophil counts are variable and may be influenced by multiple factors, including nasal polyps, smoking, seasonality, natural variation over time, and analytical factors. If eosinophil counts are used for diagnosis or management of eosinophilic asthma, a single blood eosinophil measurement may not be sufficient and should be interpreted in the context of each patient's medical history. ■

References

1 Zeiger RS, Schatz M, Li Q, Chen W, Khatry DB, Gossage D, *et al.* High blood eosinophil count is a risk factor for future asthma exacerbations in adult persistent asthma. *J Allergy Clin Immunol Pract* 2014;2: 741–750.e4.

2 Nair P, O'Byrne PM. Measuring eosinophils to make treatment decisions in asthma. *Chest* 2016;150:485–487.

3 Ortega HG, Yancey SW, Mayer B, Gunsoy NB, Keene ON, Bleecker ER, *et al.* Severe eosinophilic asthma treated with mepolizumab stratified by baseline eosinophil thresholds: a secondary analysis of the DREAM and MENSA studies. *Lancet Respir Med* 2016;4:549–556.

4 Castro M, Zangrilli J, Wechsler ME, Bateman ED, Brusselle GG, Bardin P, *et al.* Reslizumab for inadequately controlled asthma with elevated blood eosinophil counts: results from two multicentre, parallel, double-blind, randomised, placebo-controlled, phase 3 trials. *Lancet Respir Med* 2015;3:355–366.

5 FitzGerald JM, Bleecker ER, Nair P, Korn S, Ohta K, Lommatzsch M, *et al.*; CALIMA study investigators. Benralizumab, an anti-interleukin-5 receptor α monoclonal antibody, as add-on treatment for patients with severe, uncontrolled, eosinophilic asthma (CALIMA): a randomised, double-blind, placebo-controlled phase 3 trial. *Lancet* 2016;388:2128–2141.

6 Castro M, Corren J, Pavord ID, Maspero J, Wenzel S, Rabe KF, *et al.* Dupilumab efficacy and safety in moderate-to-severe uncontrolled asthma. *N Engl J Med* 2018;378:2486–2496.

7 Katz LE, Gleich GJ, Hartley BF, Yancey SW, Ortega HG. Blood eosinophil count is a useful biomarker to identify patients with severe eosinophilic asthma. *Ann Am Thorac Soc* 2014;11:531–536.

8 Mathur SK, Fichtinger PS, Evans MD, Schwantes EA, Jarjour NN. Variability of blood eosinophil count as an asthma biomarker. *Ann Allergy Asthma Immunol* 2016;117:551–553.

9 Ortega H, Gleich G, Mayer B, Yancey S. Reproducibility of a single blood eosinophil measurement as a biomarker in severe eosinophilic asthma. *Ann Am Thorac Soc* 2015;12:1896–1897.

10 Spector SL, Tan RA. Is a single blood eosinophil count a reliable marker for "eosinophilic asthma?". *J Asthma* 2012;49:807–810.

11 Rakowski E, Zhao S, Liu M, Ahuja S, Durmus N, Grunig G, *et al.* Variability of blood eosinophils in patients in a clinic for severe asthma. *Clin Exp Allergy* 2019;49:163–170.

12 Hilderink JM, Klinkenberg LJJ, Aakre KM, de Wit NCJ, Henskens YMC, van der Linden N, *et al.* Within-day biological variation and hour-to-hour reference change values for hematological parameters. *Clin Chem Lab Med* 2017;55:1013–1024.

13 Sunadome H, Matsumoto H, Izuhara Y, Nagasaki T, Kanemitsu Y, Ishiyama Y, *et al.* Correlation between eosinophil count, its genetic background and body mass index: the Nagahama study. *Allergol Int* 2020;69:46–52.

14 Wenzel S, Ford L, Pearlman D, Spector S, Sher L, Skobieranda F, *et al.* Dupilumab in persistent asthma with elevated eosinophil levels. *N Engl J Med* 2013;368:2455–2466.

15 Pavord ID, Korn S, Howarth P, Bleecker ER, Buhl R, Keene ON, *et al.* Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. *Lancet* 2012;380:651–659.

16 Schapkaitz E, Pillay D. Prolonged storage-induced changes in haematology parameters referred for testing. *Afr J Lab Med* 2015;4:a208.

17 Turhan T, Sezer S, Yücel C, Koca Y. Effects of storage conditions on complete blood cell count parameters. *Turk J Biochem* 2011;36:165–174.

18 Hanania NA, Noonan M, Corren J, Korenblat P, Zheng Y, Fischer SK, *et al.* Lebrikizumab in moderate-to-severe asthma: pooled data from two randomised placebo-controlled studies. *Thorax* 2015;70:748–756.

19 Hanania NA, Korenblat P, Chapman KR, Bateman ED, Kopecky P, Paggiaro P, *et al.* Efficacy and safety of lebrikizumab in patients with uncontrolled asthma (LAVOLTA I and LAVOLTA II): replicate, phase 3, randomised, double-blind, placebo-controlled trials. *Lancet Respir Med* 2016;4:781–796.

20 Schulte C, Krebs B, Jelinek T, Nothdurft HD, von Sonnenburg F, Löscher T. Diagnostic significance of blood eosinophilia in returning travelers. *Clin Infect Dis* 2002;34:407–411.

21 Davido B, Makhloufi S, Matt M, Calin R, Senard O, Perronne C, *et al.* Changes in eosinophil count during bacterial infection: revisiting an old marker to assess the efficacy of antimicrobial therapy. *Int J Infect Dis* 2017;61:62–66.

22 Prazma CM, Bel EH, Price RG, Bradford ES, Albers FC, Yancey SW. Oral corticosteroid dose changes and impact on peripheral blood eosinophil counts in patients with severe eosinophilic asthma: a post hoc analysis. *Respir Res* 2019;20:83.

23 Wang K, Deng J, Yang M, Chen Y, Chen F, Gao WX, *et al.* Concordant systemic and local eosinophilia relates to poorer disease control in patients with nasal polyps. *World Allergy Organ J* 2019;12:100052.

24 Brescia G, Parrino D, Zanotti C, Tealdo G, Barion U, Sfriso P, *et al.* Blood eosinophil and basophil values before and after surgery for eosinophilic-type sinonasal polyps. *Am J Rhinol Allergy* 2018;32:194–201.

25 Kim S-D, Cho K-S. Samter's triad: state of the art. *Clin Exp Otorhinolaryngol* 2018;11:71–80.

26 Sunyer J, Springer G, Jamieson B, Conover C, Detels R, Rinaldo C, *et al.* Effects of asthma on cell components in peripheral blood among smokers and non-smokers. *Clin Exp Allergy* 2003;33:1500–1505.

27 Higuchi T, Omata F, Tsuchihashi K, Higashioka K, Koyamada R, Okada S. Current cigarette smoking is a reversible cause of elevated white blood cell count: cross-sectional and longitudinal studies. *Prev Med Rep* 2016;4:417–422.

28 Choo JH, Nagata M, Sutani A, Kikuchi I, Sakamoto Y. Theophylline attenuates the adhesion of eosinophils to endothelial cells. *Int Arch Allergy Immunol* 2003;131:40–45.

29 Tsukadaira A, Okubo Y, Horie S, Koyama S. Theophylline inhibits TNF-alpha-induced CD4 expression on human eosinophils and CD4+ eosinophil migration. *Int Arch Allergy Immunol* 2001;125:335–343.

30 Kreindler JL, Watkins ML, Lettis S, Tal-Singer R, Locantore N. Effect of inhaled corticosteroids on blood eosinophil count in steroid-naïve patients with COPD. *BMJ Open Respir Res* 2016;3:e000151.

31 Stelmach I, Gorski P, Jerzynska J, Stelmach W, Majak P, Kuna P. A randomized, double-blind trial of the effect of treatment with formoterol on clinical and inflammatory parameters of asthma in children. *Ann Allergy Asthma Immunol* 2002;89:67–73.

32 Alam J, Qureshi F, Suliman MI, Qureshi Z. Seasonal variation in eosinophil count in normal healthy adult females. *Gomal J Med Sci* 2008;6:69–71.

33 Kämpe M, Stålenheim G, Janson C, Stolt I, Carlson M. Systemic and local eosinophil inflammation during the birch pollen season in allergic patients with predominant rhinitis or asthma. *Clin Mol Allergy* 2007;5:4.

34 Matz CJ, Stieb DM, Davis K, Egyed M, Rose A, Chou B, *et al.* Effects of age, season, gender and urban-rural status on time-activity: CanadianHuman Activity Pattern Survey 2 (CHAPS 2). *Int J Environ Res Public Health* 2014;11:2108–2124.

35 Jie Y, Ismail NH, Jie X, Isa ZM. Do indoor environments influence asthma and asthma-related symptoms among adults in homes?: a review of the literature. *J Formos Med Assoc* 2011;110:555–563.

36 Stemeseder T, Schweidler B, Doppler P, Klinglmayr E, Moser S, Lueftenegger L, *et al.* Exposure to indoor allergens in different residential settings and its influence on IgE sensitization in a geographically confined Austrian cohort. *PLoS One* 2017;12:e0168686.

37 Price DB, Bosnic-Anticevich S, Pavord ID, Roche N, Halpin DMG, Bjermer L, *et al.* Association of elevated fractional exhaled nitric oxide concentration and blood eosinophil count with severe asthma exacerbations. *Clin Transl Allergy* 2019;9:41.

38 Deschildre A, Pichavant M, Engelmann I, Langlois C, Drumez E, Pouessel G, *et al.* Virus-triggered exacerbation in allergic asthmatic children: neutrophilic airway inflammation and alteration of virus sensors characterize a subgroup of patients. *Respir Res* 2017;18:191.

39 Szefler SJ, Raphiou I, Zeiger RS, Stempel D, Kral K, Pascoe S. Seasonal variation in asthma exacerbations in the AUSTRI and VESTRI studies. *ERJ Open Res* 2019;5:00153-2018.

40 Staton TL, Arron JR, Olsson J, Holweg CTJ, Matthews JG, Choy DF. Seasonal variability of severe asthma exacerbations and clinical benefit from lebrikizumab. *J Allergy Clin Immunol* 2017;139:1682–1684.e3.

41 Ehlenfield DR, Cameron K, Welliver RC. Eosinophilia at the time of respiratory syncytial virus bronchiolitis predicts childhood reactive airway disease. *Pediatrics* 2000;105:79–83.

42 Kidd BA, Hoffman G, Zimmerman N, Li L, Morgan JW, Glowe PK, *et al.* Evaluation of direct-to-consumer low-volume lab tests in healthy adults. *J Clin Invest* 2016;126:1734–1744.

43 Meintker L, Ringwald J, Rauh M, Krause SW. Comparison of automated differential blood cell counts from Abbott Sapphire, Siemens Advia 120, Beckman Coulter DxH 800, and Sysmex XE-2100 in normal and pathological samples. *Am J Clin Pathol* 2013;139:641–650.

44 Belloni P, Meschini R, Palitti F. Effects of storage conditions of human whole blood on the viability of lymphocytes. *Int J Radiat Biol* 2008;84:613–619.

45 Bergman M, Bessler H, Salman H, Djaldetti M. Relationship between temperature and apoptosis of human peripheral blood mononuclear cells. *Int J Hematol* 2003;77:351–353.

46 Olson WC, Smolkin ME, Farris EM, Fink RJ, Czarkowski AR, Fink JH, *et al.* Shipping blood to a central laboratory in multicenter clinical trials: effect of ambient temperature on specimen temperature, and effects of temperature on mononuclear cell yield, viability and immunologic function. *J Transl Med* 2011;9:26.