Blood granulocyte patterns as predictors of asthma phenotypes in adults from the EGEA study

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ABSTRACT To what extent blood granulocyte patterns may predict asthma control remains understudied. Our aim was to study associations between blood neutrophilia and eosinophilia and asthma control outcomes in adults.

Analyses were conducted in 474 asthmatics from the first follow-up of the Epidemiological Study on the Genetics and Environment of Asthma (EGEA2), including 242 asthmatics who were adults a decade earlier (EGEA1). At EGEA2, asthma control was assessed using the Global Initiative for Asthma definition (2015), and asthma exacerbations by use of urgent care or courses of oral corticosteroids in the past year. Blood EOS^{lo}/EOS^{hi} was defined as $</\geqslant 250$ eosinophils·mm^{-3}, respectively, and NEU^{lo}/NEU^{hi} as $</\geqslant 5000$ neutrophils·mm^{-3}, respectively. Estimates were adjusted for age, sex and smoking.

At EGEA2, NEU^{hi} was associated with asthma exacerbations and poor asthma control (OR >2.10). EOS^{hi} was associated with higher bronchial hyperresponsiveness (BHR) (OR (95% CI) 2.21 (1.24–3.97)), poor lung function ($p=0.02$) and higher total IgE level ($p=0.002$). Almost 50% of asthmatics had a persistent pattern between surveys. Persistent NEU^{hi} was associated with poor asthma control at EGEA2 (OR (95% CI) 3.09 (1.18–7.05)). EOS^{hi} at EGEA1 and persistent EOS^{hi} were associated with higher BHR (OR (95% CI) 2.36 (1.10–5.07) and 3.85 (1.11–13.34), respectively), poor lung function ($p<0.06$) and higher immunoglobulin E level ($p<10^{-4}$) at EGEA2.

Granulocyte patterns were differently associated with asthma outcomes, suggesting specific roles for each one, which could be tested as predictive signatures.

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Introduction

Although induced sputum seems to be the gold standard test for phenotyping the inflammation in asthma [1–5], the use of circulating granulocyte counts has been proposed as a more suitable method in large-scale studies [6–8].

Blood neutrophilia and eosinophilia are recognised features of asthma [9–11]. Blood eosinophils are proposed as biological markers to monitor uncontrolled asthma [12], to personalise immunological biotherapies in patients with asthma [11], to target severe asthma with increased blood eosinophils for treatment with anti-interleukin-5 antibodies [13, 14] and to select appropriate patients with chronic obstructive pulmonary disease (COPD) to be treated with inhaled corticosteroids [15]. Regarding neutrophilia, recent studies have shown that neutrophils are closely associated with not only severity, but initiation of allergic inflammation and allergic sensitisation [16]. However, data to support neutrophilic asthma as a specific phenotype are rare, and no consensus exists for the level of blood neutrophilia that should be used to define this phenotype [10].

We were first to describe the use of cut-off points of 250 cells·mm^{-3} and 5000 cells·mm^{-3}, respectively, for blood counts of eosinophils and neutrophils to define granulocyte patterns in adults in the framework of the Epidemiological Study on the Genetics and Environment of Asthma (EGEA) [6, 7]. We showed that the eosinophilic pattern was associated with reports of more asthma attacks and being woken by an attack of shortness of breath in the past 12 months, and that the neutrophilic pattern was associated with fewer positive skin-prick test responses and with reports of more nocturnal symptoms and dyspnoea. A study by Volbeda *et al.* [17] showed that adult patients with uncontrolled asthma had higher numbers of eosinophils in peripheral blood, whereas no association was found with blood neutrophil numbers. High blood eosinophil count (⩾400 cells·mm^{-3}) was also reported as a practical biological marker to identify adult patients with persistent asthma who are at increased risk for future asthma exacerbations [18]. And concomitant systemic (⩾400 cells·mm^{-3}) and bronchial (⩾3%) eosinophilic inflammation has been reported to contribute to poor asthma control [19]. To the best of our knowledge, no studies have reported variations over long periods of time of blood neutrophilia and eosinophilia in adults with asthma, nor investigated the long-term relationship of both the neutrophilic and eosinophilic pattern with asthma control outcomes.

In the EGEA study, using the same blood granulocyte count cut-off points as previously described, we assessed the association of blood eosinophil and neutrophil patterns with asthma control outcomes, both in a cross-sectional and longitudinal way.

Methods

Study design

Data used for the analyses were collected in the framework of the EGEA (https://egeanet.vjf.inserm.fr/), a French cohort study based on an initial group of asthma cases and their first-degree relatives, and controls (first survey: EGEA1). The protocol and descriptive characteristics have been described previously and are included in the online supplementary material [20–22].

The cross-sectional analysis includes asthma cases and their first-degree relatives with asthma who were adults (aged ⩾16 years) at the second survey (EGEA2) with available data on blood eosinophil and neutrophil cell counts (n = 232 + 242 = 474) (figure 1). For the longitudinal analysis, asthma cases and their first-degree relatives already aged ⩾16 years a decade earlier (EGEA1; n=381), and with available data for blood eosinophil and neutrophil cell counts (n=242) were included. Asthmatics included in the longitudinal analyses (n=242) were more often asthma cases and current smokers than those not included in the longitudinal analyses (online supplementary table S1). The two groups did not differ for age, sex, body mass index, asthma, treatment, lung function, allergic sensitisation, immunoglobulin (Ig)E level and blood eosinophil and neutrophil counts.

Ethical approval was obtained from the relevant institutional review board committees (Cochin Port-Royal Hospital and Necker-Enfants Malades Hospital, Paris). Written informed consent was signed by all participants.

Respiratory phenotypes

Inclusion criteria used to define asthma cases were based on self-reported positive responses to four questions from the validated and standardised British Medical Research Council, European Coal and Steel Community, American Thoracic Society (ATS) and European Community Respiratory Health Survey questionnaires: “Have you ever had attacks of breathlessness at rest with wheezing?”, “Have you ever had asthma attacks?”, “Was this diagnosis confirmed by a physician?” and “Have you had an asthma attack in the last 12 months?”, or a positive response to at least two questions and a positive review of the medical records. Asthma in relatives of cases was defined as a positive answer to at least one of the first two questions [23, 24].

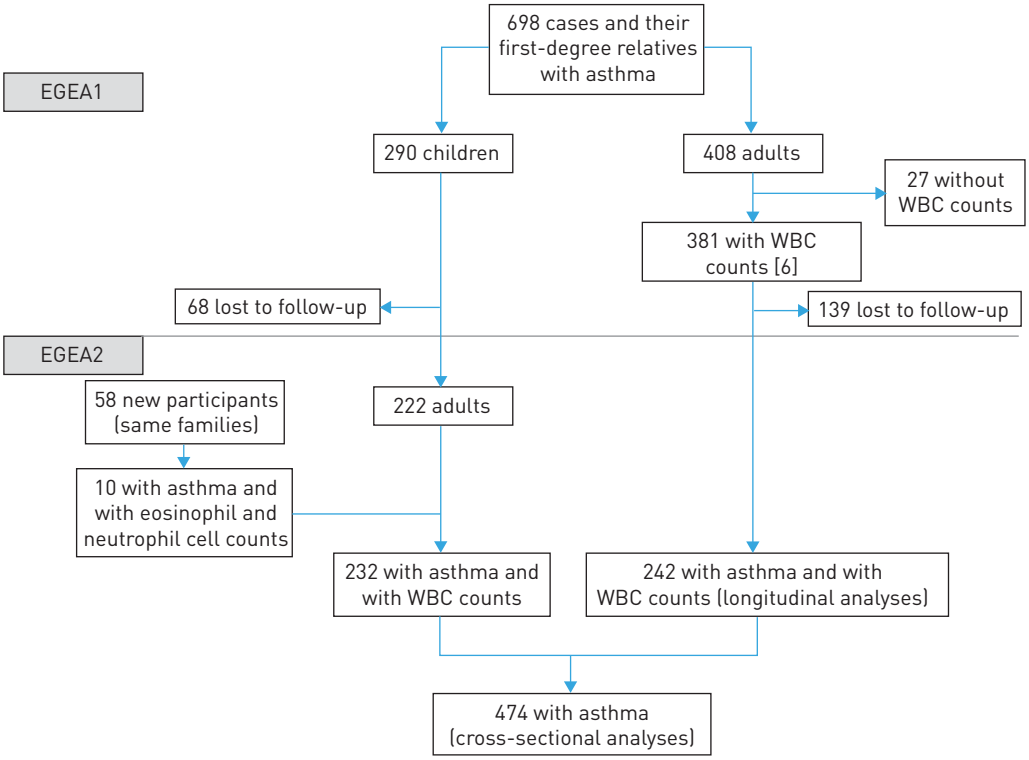

FIGURE 1 Flow chart of the participants included in the cross-sectional and longitudinal analyses. EGEA: Epidemiological Study on the Genetics and Environment of Asthma; WBC: white blood cell.

Symptom control in asthma was assessed in three classes, over a 3-month period, using responses to EGEA2 survey questions to approximate as closely as possible the Global Initiative for Asthma (GINA) 2015 definition, and as previously used [25]. Participants were defined as having controlled, partly controlled and uncontrolled asthma if they had none, 1–2 or 3–4 of the following criteria, respectively: frequent daytime symptoms (defined by at least one asthma attack or one or more instances of trouble breathing per week in the past 3 months), any night-time symptoms (defined by waking because of asthma or by an attack of shortness of breath in the past 3 months), frequent use of reliever medication (defined by on average twice a week in the past 3 months) and any activity limitation (defined by the following answer "totally limited", "extremely limited", "very limited", "moderate limitation" or "some limitation" to the question "Overall, among all the activities that you have done during the last 2 weeks, how limited have you been by your asthma?").

Data on medication for asthma was available for 470 (99.2%) of the participants: 286 (60.3%) participants did not take any medication in the past 12 months. In the past 3 months, 122 (25.7%) participants reported regular use of inhaled corticosteroids (ICS), and 62 (13.1%) reported use of ICS, but not regular use. Furthermore, main daily ICS dose over the past 12 months is available for the 122 participants with a median (minimum–maximum) of 250 (50–500) µg of beclometasone equivalent.

Asthma exacerbation was defined at EGEA2 by hospital or emergency admissions because of respiratory problems or use of oral steroids for breathing difficulties in the past year.

A lung function test with spirometry and methacholine challenge was performed using a standardised protocol with similar equipment across centres according to the ATS/European Respiratory Society guidelines [26]. Change in forced expiratory volume in 1 s (FEV_1) between first and second spirometry was assessed as annual lung function change (mL per year) with a negative value representing a decline.

Blood granulocyte patterns

At both surveys, four granulocyte patterns were defined from white blood cell counts according to the eosinophil (EOS) and neutrophil (NEU) count cut-off points previously described [6]. Briefly, samples with $\geqslant$250 EOS·mm^{-3} were classified as EOS^{hi}, and those with $\geqslant$5000 NEU·mm^{-3} as NEU^{hi}. The cut-off point for eosinophils is the one commonly used in epidemiology, and corresponded to the 75th percentile in the 1356 adults at the EGEA1 study. Only 20 patients with asthma had a neutrophil count equal to or higher than the upper limit adult references [27], and a cut-off point of 5000 NEU·mm^{-3} was chosen,

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which corresponded to the 75th percentile of the distribution. Other cut-off points were also studied: 300 and 400 EOS·mm^{-3}, reported in the literature, 6040 NEU·mm^{-3}, corresponding to the 90th percentile in the 1356 adults from the EGEA1 study and optimal cut-off points calculated using the receiver operating characteristic (ROC) curve (see the online supplementary material for more details).

Statistical methods

Standard statistical tests were performed. Due to the familial aggregation of the data, multivariate analyses (except polytomous logistic regression) were conducted using generalised estimated equations to take into account dependence between observations. Controlled asthma was the reference group. Due to similarity in results in partly controlled and uncontrolled asthma, and due to the small number of asthmatics in the uncontrolled group, asthma control was also expressed as a dichotomous variable: partly controlled plus uncontrolled *versus* controlled. A sensitivity analysis was performed to test associations with different cut-off points for eosinophils and neutrophils.

For the cross-sectional analyses at EGEA2, a sensitivity analysis was performed to test associations between granulocyte patterns and asthma control in participants without respiratory infections in the past 4 weeks. To investigate the modifying effect of current smoking status (smokers *versus* non- or ex-smokers) and ICS treatment in the past year on associations between granulocyte patterns and asthma control, analyses were conducted in the dataset stratified by each of these factors. Sensitivity analyses were also performed with adjustment for ICS use in the past 3 months expressed as three classes (no use, irregular use and regular use), with adjustment for daily dose of ICS and by excluding participants with chronic bronchitis. Moreover, to take into account the complex interplay between eosinophilia and allergic sensitisation, associations between eosinophil patterns and asthma control were further adjusted for allergic sensitisation (yes/no). For the longitudinal analysis, the association between blood granulocyte patterns at EGEA1 with the subsequent risk of poor asthma control (partly controlled or uncontrolled asthma) at EGEA2 was investigated. Persistence of the granulocyte patterns (NEU^{hi} at EGEA1 and 2 and EOS^{hi} at EGEA1 and 2) and changes between EGEA1 and EGEA2 were also considered.

All multiple regression models considered age (continuous), sex, smoking status (never-, ex- or current smokers) and ICS treatment in the past year (yes/no) as potential confounding factors. These factors were measured at EGEA2 for the cross-sectional analyses and at EGEA1 for the longitudinal analyses (except for current smoking, for which the status over the follow-up time was also considered). All statistical analyses were performed using SAS software (version 9.4; SAS Institute, Inc., Cary, NC, USA).

Results

The overall characteristics of the participants are shown in table 1: at EGEA2, 48.9%, 10.6%, 31.6% and 8.9% of them belonged to the paucigranulocytic (EOS^{lo}/NEU^{lo}), neutrophilic (EOS^{lo}/NEU^{hi}), eosinophilic (EOS^{hi}/NEU^{lo}) and mixed (EOS^{hi}/NEU^{hi}) pattern groups, respectively. These frequencies were similar among the sub-population involved in the longitudinal analyses. Characteristics of the participants according to these four blood granulocyte patterns are shown in online supplementary table S2.

Neutrophil counts were significantly higher in females, in participants with respiratory infections in the past 4 weeks and increased with age (all p<0.01). Among all participants, neutrophil counts increased with the number of smoking pack-years (p=0.07). Among ex- and current smokers, neutrophil counts increased with the number of cigarettes smoked per day (mean (95% CI) 4054 (3527–4235) cells·mm^{-3}, 3901 (3511–4290) cells·mm^{-3}, 4380 (3878–4882) cells·mm^{-3} and 4576 (3724–5427) cells·mm^{-3} for 0 cigarettes per day, 1–10 cigarettes per day, 11–20 cigarettes per day and >20 cigarettes per day, respectively; p-value for trend 0.02). Eosinophil and neutrophil counts were significantly higher in ICS users than in nonusers (adjusted mean (95% CI), 288 (259–317) cells·mm^{-3} *versus* 231 (206–256) cells·mm^{-3} (p=0.003) and 4286 (4078–4493) cells·mm^{-3} *versus* 3905 (3724–4087) cells·mm^{-3} (p=0.006), respectively). No other significant associations were found.

Granulocyte patterns and phenotypic characteristics in cross-sectional analyses (n=474)

Participants with the NEU^{hi} pattern reported more nocturnal cough in the past year, more exacerbations, more chronic cough and had poorer asthma control than those with the NEU^{lo} pattern (table 2). They also had a nonsignificant tendency for lower FEV1 (mean (95% CI) 92.4 (88.7–96.0)% predicted *versus* 95.6 (93.5–97.7)% pred, p=0.10) than those with the NEU^{lo} pattern. Participants with the EOS^{hi} pattern had higher bronchial hyperresponsiveness (BHR: lower provocative dose causing a 20% fall in FEV1) (table 2), higher total IgE level (mean (95% CI) 213 (175–261) IU·mL^{-1} *versus* 144 (122–171) IU·mL^{-1}, p=0.002) and lower FEV1 (mean (95% CI) 93.2 (90.4–96.0)% pred *versus* 97.3 (95.0–99.6)% pred, p=0.02) than those with the EOS^{lo} pattern. No significant association was found between EOS^{hi} pattern and allergic sensitisation (OR (95% CI) 1.30 (0.67–1.90)).

TABLE 1 Characteristics of adults with asthma included in the analyses of the second survey of the Epidemiological Study on the Genetics and Environment of Asthma

	Cross-sectional analyses	Longitudinal analyses
Subjects n	474	242
Age years	38.2±16.1	48.2±13.1
Female	47.9	53.3
Body mass index		
<20 kg·m^{-2}	12.2	8.3
20–25 kg·m^{-2}	53.3	47.5
25–30 kg·m^{-2}	24.4	30.8
⩾30 kg·m^{-2}	10.1	13.4
Smoking habits		
Smokers	24.0	16.5
Ex-smokers	24.3	34.3
Nonsmokers	51.7	49.2
Age of asthma onset		
⩽4 years	31.6	19.4
4–16 years	36.3	33.5
>16 years	32.1	47.1
Total immunoglobulin E IU·mL^{-1}	155 (68.7–377)	123 (48.6–304)
Current asthma (past 12 months)	89.2	91.3
Skin-prick test positivity[#]	82.1	77.2
White blood cell counts		
Eosinophils cells·mm^{-3}	259±198	255±189
Neutrophils cells·mm^{-3}	4047±1442	4225±1536
FEV_1 % pred	96.2±18.2	93.1±21.4
FEV_1 <80% pred	14.4	21.7
Methacholine challenge[¶] **n**	282	126
PD_{20} ⩽4 mg	71.6	65.9
Asthma attacks in past 12 months	41.8	40.9
Nocturnal symptoms in past 12 months		
Cough	37.0	38.0
Chest tightness	22.8	27.3
Shortness of breath	48.9	51.6
Asthma control		
Uncontrolled	10.6	13.2
Partly controlled	32.7	33.5
Controlled	56.7	53.3
Chronic cough	12.5	14.5
Chronic phlegm	10.8	12.9
Cough or phlegm all days during 3 months	13.6	17.0
Dyspnoea grade 3	16.2	23.4
Inhaled corticosteroids in past 12 months	44.5	55.6
Respiratory infection in past 4 weeks	15.0	15.8

Data are presented as mean±SD, % or geometric mean (interquartile range), unless otherwise stated. FEV_1: forced expiratory volume in 1 s; PD_{20}: provocative dose causing a 20% fall in FEV_1. [#]: defined by a mean weal diameter ⩾3 mm greater than the negative control for at least one of 12 aeroallergens; [¶]: not performed if baseline FEV_1 <80% pred.

The association between high neutrophil inflammation and asthma control remained significant after further adjustment on eosinophil count (OR (95% CI) 2.95 (1.74–5.00) and 3.08 (1.42–6.69) for partly controlled and uncontrolled asthma, respectively). Associations beetween neutrophil inflammation and poor asthma control (partly controlled or uncontrolled *versus* controlled) were consistently observed in participants without respiratory infections in the past 4 weeks, or after stratification according to current smoking status or ICS treatment (figure 2) (p-values for Breslow–Day interaction test <0.5). The associations remained also significant after adjustment on both current smoking status and ICS treatment in the past 12 months (figure 2), but also after adjustment for ICS use in the past 3 months (OR (95% CI) 2.82 (1.73–4.62)), after adjustment for the daily dose of ICS (OR (95% CI) 3.24 (1.56–6.76)), or when excluding the 64 participants with chronic bronchitis (OR (95 %CI) 2.21 (1.31–3.72)). Furthermore, associations between neutrophil inflammation and poor asthma control remained significant with

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TABLE 2 Adjusted cross-sectional associations between neutrophilic or eosinophilic granulocyte patterns and asthma or asthma-related phenotypes in the second survey of the Epidemiological Study on the Genetics and Environment of Asthma

	NEU^{hi} *versus* NEU^{lo}	EOS^{hi} *versus* EOS^{lo}
Subjects n/n	92/382	192/282
Skin-prick test positivity[#]	0.91 (0.49–1.70)	1.13 (0.67–1.90)
Methacholine challenge[¶] **PD_{20} ⩽4 mg**	1.60 (0.75–3.40)	**2.21 (1.24–3.97)**[+]
At least one asthma attack in past 12 months	1.55 (0.98–2.46)	**1.48 (0.99–2.21)**[§]
Nocturnal symptoms in past 12 months		
Cough	**1.74 (1.10–2.74)**[+]	1.16 (0.78–1.72)
Chest tightness	1.34 (0.78–2.29)	1.33 (0.82–2.17)
Shortness of breath	1.38 (0.87–2.17)	1.22 (0.83–1.80)
Asthma control		
Controlled	1	1
Partly controlled	**2.88 (1.70–4.87)**[+]	1.32 (0.87–2.02)[f]
Uncontrolled	**2.99 (1.38–6.46)**[+]	1.62 (0.83–3.16)[f]
Asthma exacerbations in past 12 months	**2.19 (1.07–4.50)**[+]	0.81 (0.48–1.38)
Chronic cough	**2.20 (1.15–4.21)**	1.22 (0.68–2.17)
Chronic phlegm	1.73 (0.84–3.59)	1.40 (0.78–2.54)
Dyspnoea grade 3	1.71 (0.91–3.22)	1.26 (0.73–2.18)

Data are presented as adjusted odds ratio (95% confidence interval), unless otherwise stated. OR (95% CI) were adjusted on age, sex, current smoking status and use of inhaled corticosteroids in the past year, taking into account familial dependence of the participants. Bold type represents statistical significance. n=474. NEU^{lo}: <5000 neutrophils·mm^{-3}; NEU^{hi}: ⩾5000 neutrophils·mm^{-3}; EOS^{lo}: <250 eosinophils·mm^{-3}; EOS^{hi}: ⩾250 eosinophils·mm^{-3}; PD_{20}: provocative dose causing a 20% fall in forced expiratory volume in 1 s (FEV_1). [#]: defined by a mean weal diameter ⩾3 mm greater than the negative control for at least one of 12 aeroallergens; [¶]: not performed if baseline FEV_1 <80% pred; [+]: remained significant in participants without respiratory infections in the plast 4 weeks (n=404); [§]: became significant in participants without respiratory infections in the past 4 weeks (OR (95% CI) 1.65 (1.06–2.55), n=404); [f]: OR (95% CI) 1.52 (1.01–2.28) and 2.03 (1.08–3.80) for partly controlled and uncontrolled asthma, respectively, in a model with adjustment for age and sex.

6040 cells·mm^{-3} as the cut-off point for neutrophils (OR (95% CI) 2.55 (1.26–5.18)), or with optimal cut-off points calculated using the ROC curve (online supplementary figure S1).

A high level of eosinophil inflammation was positively but inconsistently associated with poor asthma control (figure 2). The associations were significant after adjustment for age and sex, in participants without respiratory infections in the past 4 weeks, and in non-/ex-smokers. These associations did not remain significant after further adjustment for current smoking status and ICS treatment (p-values for Breslow–Day interaction test <0.3). Analyses performed with 300 cells·mm^{-3} and 400 cells·mm^{-3} as the cut-off points for eosinophils gave similar inconsistent findings (OR (95% CI) 1.50 (1.01–2.24) and 1.51 (0.91–2.50) for 300 cells·mm^{-3} and 400 cells·mm^{-3}, respectively), due to reduced power because of the unbalanced distribution of participants between classes. Further adjustment for allergic sensitisation did not change the results.

Furthermore, participants with the mixed pattern (both high eosinophil and high neutrophil counts) had significantly higher risks of poor asthma control, asthma attack and asthma exacerbation compared to participants having only high eosinophil counts (table 3). Such findings were not found when comparisons were performed with participants having only high neutrophil counts.

Granulocyte patterns at baseline and follow-up and phenotypic characteristics 12 years later in longitudinal analyses (n=242)

In our population, 46% of participants with the NEU^{hi} pattern at EGEA1, and 52% of those with the EOS^{hi} pattern at EGEA1 continued to have the same pattern at EGEA2 (see online supplementary table S3 for the other patterns).

The NEU^{hi} pattern at EGEA1 was unrelated to the asthma phenotypes assessed at EGEA2 (table 4). However, participants with persistent NEU^{hi} pattern (NEU^{hi} at EGEA1 and EGEA2) and those moving from NEU^{lo} to NEU^{hi} between the two surveys had poorer asthma control compared to those with the NEU^{lo} pattern at both surveys (table 4 and figure 3). Similar findings were obtained when analyses were performed with 6040 cells·mm^{-3} as the cut-off point for neutrophils (OR (95% CI) 3.21 (0.73–14.04)), or

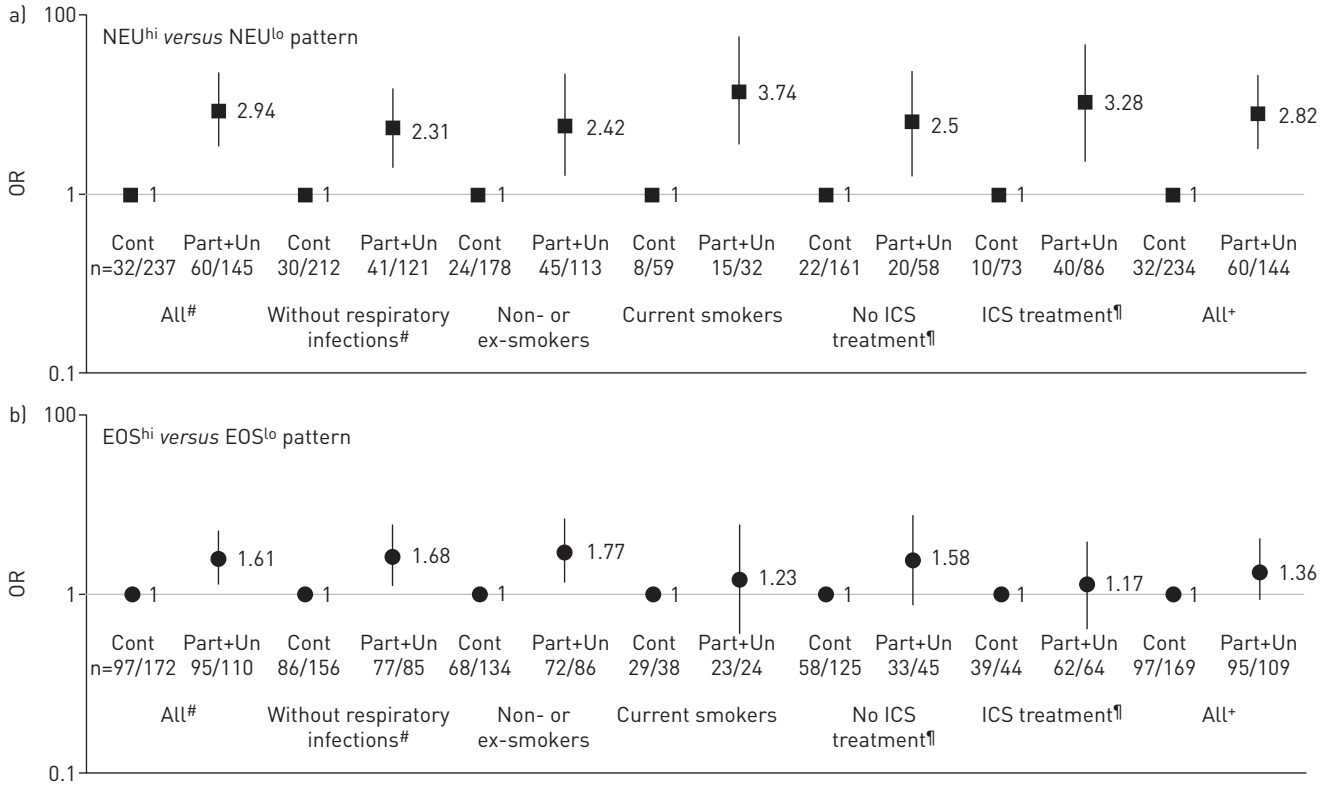

FIGURE 2 Cross-sectional association between a) neutrophilic or b) eosinophilic pattern and asthma control according to smoking status or inhaled corticosteroid (ICS) treatment in the past 12 months. ICS use was not available for four participants (n=470). NEU^{hi}: ⩾5000 neutrophils·mm^{-3}; NEU^{lo}: <5000 neutrophils·mm^{-3}; EOS^{hi}: ⩾250 eosinophils·mm^{-3}; EOS^{lo}: <250 eosinophils·mm^{-3}; cont: controlled; part: partly controlled; un: uncontrolled. #: odds ratios adjusted for age and sex; ¶: odds ratios adjusted for age, sex and current smoking status; +: odds ratios adjusted for age, sex, current smoking status and ICS treatment. Data are presented as n/n NEU^{hi} /NEU^{lo} and EOS^{hi} /EOS^{lo} in a) and b), respectively.

with optimal cut-off points calculated using the ROC curve (online supplementary figure S1). No associations were found with the decline in lung function (table 5).

Participants with an EOS^{hi} pattern at baseline had higher BHR at follow-up (table 4) than those with the EOS^{lo} pattern. They also had higher total IgE level (geometric mean (95% CI) 199 (153–259) IU·mL^{-1} *versus* 101 (80–128) IU·mL^{-1}, p<10^{-4}), and lower FEV_1 at follow-up (table 5). Participants with persistent EOS^{hi} patterns had higher BHR, higher total IgE level (270 (188–387) IU·mL^{-1} *versus* 115 (89–149) IU·mL^{-1}, p<10^{-4}) and lower FEV_1 (table 5) than those with persistent EOS^{lo} patterns. Changes in eosinophilic patterns between EGEA1 and EGEA2 were unrelated to asthma control (figure 3). No associations were found with decline in lung function (table 5).

Analyses performed with 300 and 400 cells·mm^{-3} as the cut-off points for eosinophils gave similar findings for the association with asthma control (OR (95% CI) 2.10 (1.01–4.35) and 1.97 (0.83–4.65) for

TABLE 3 Associations between asthma control outcomes and exacerbations and mixed patterns (cross-sectional analyses)

	EOS^{lo}/NEU^{hi}	Mixed: EOS^{hi}/NEU^{hi}	EOS^{hi}/NEU^{lo}	Mixed: EOS^{hi}/NEU^{hi}
Subjects	50	42	150	42
Methacholine challenge# PD_{20} ⩽4 mg	Ref.	NA¶	Ref.	2.89 (0.77–10.8)
Asthma attack in past 12 months %	Ref.	2.23 (0.87–5.71)	Ref.	**2.12 (1.02–4.40)**
Asthma control: partly controlled/uncontrolled %	Ref.	1.84 (0.64–5.31)	Ref.	**3.46 (1.54–7.76)**
Exacerbations in past 12 months %	Ref.	0.79 (0.29–2.18)	Ref.	**2.56 (1.02–6.46)**

Data are presented as n or OR (95% CI) and were adjusted for age, sex, current smoking status and inhaled corticosteroid use in the past year, and taking into account familial dependence of the participants. n=474. Bold type represents statistical significance. EOS^{lo}: <250 eosinophils·mm^{-3}; NEU^{hi}: ⩾5000 neutrophils·mm^{-3}; EOS^{hi}: ⩾250 eosinophils·mm^{-3}; NEU^{lo}: <5000 neutrophils·mm^{-3}; PD_{20}: provocative dose causing a 20% fall in forced expiratory volume in 1 s (FEV_1); NA: not available. #: not performed if baseline FEV_1 <80% predicted; ¶: due to smallest sample sizes.

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TABLE 4 Adjusted longitudinal associations between neutrophilic or eosinophilic granulocyte patterns at baseline (Epidemiological Study on the Genetics and Environment of Asthma (EGEA)1), or similar patterns at baseline and follow-up (EGEA1 and EGEA2) and asthma or asthma-related phenotypes at follow-up (EGEA2)

	NEU^{hi} *versus* NEU^{lo} at baseline	**NEU^{hi} *versus* NEU^{lo} at baseline and follow-up**	**EOS^{hi} *versus* EOS^{lo} at baseline**	**EOS^{hi} *versus* EOS^{lo} at baseline and follow-up**
Subjects n/N	52/190	24/159	106/136	55/99
Methacholine challenge[#] PD_{20} ⩽4 mg	0.67 (0.27–1.69)	NA[¶]	**2.36 (1.10–5.07)**	**3.85 (1.11–13.3)**
Asthma attack in past 12 months	0.90 (0.48–1.69)	1.22 (0.52–2.87)	1.02 (0.61–1.71)	1.30 (0.67–2.54)
Nocturnal symptoms in past 12 months				
Cough	0.80 (0.42–1.50)	0.93 (0.37–2.30)	0.63 (0.36–1.09)	0.65 (0.31–1.39)
Chest tightness	0.77 (0.39–1.53)	1.10 (0.43–2.81)	1.11 (0.63–1.95)	1.21 (0.58–2.51)
Shortness of breath	0.77 (0.42–1.42)	1.08 (0.46–2.52)	1.01 (0.60–1.68)	1.07 (0.53–2.16)
Asthma control				
Controlled	1	1	1	1
Partly controlled/uncontrolled	1.52 (0.79–2.89)	**3.09 (1.18–8.09)**	1.30 (0.77–2.21)	1.68 (0.84–3.34)
Exacerbations in past 12 months	0.85 (0.37–1.95)	1.97 (0.70–5.53)	1.26 (0.65–2.43)	0.85 (0.33–2.23)
Chronic cough	0.96 (0.39–2.36)	1.89 (0.59–6.07)	0.81 (0.38–1.75)	1.31 (0.47–3.63)
Chronic phlegm	0.90 (0.34–2.41)	2.11 (0.27–5.08)	1.32 (0.61–2.84)	1.87 (0.67–5.20)
Dyspnoea grade 3	1.30 (0.65–2.62)	1.66 (0.59–4.68)	1.24 (0.68–2.26)	1.33 (0.57–3.07)

Data are presented as n/N or adjusted OR (95% CI). OR (95% CI) were adjusted for age, sex and smoking at baseline, and taking into account familial dependence of the participants. n=242. Bold type represents statistical significance. NEU^{hi}: ⩾5000 neutrophils·mm^{-3}; NEU^{lo}: <5000 neutrophils·mm^{-3}; EOS^{hi}: ⩾250 eosinophils·mm^{-3}; EOS^{lo}: <250 eosinophils·mm^{-3}; PD_{20}: provocative dose causing a 20% fall in forced expiratory volume in 1 s (FEV_1); NA: not available. [#]: not performed if baseline FEV_1 <80% predicted; [¶]: due to smallest sample sizes.

300 and 400 cells·mm^{-3}, respectively, in participants with a persistent EOS^{hi} pattern), although these findings were less significant due to reduced power.

Discussion

In the present study, we showed that blood neutrophil and eosinophil counts are relatively stable over 10 years, and that they are differently associated with clinical features of asthma: namely, the NEU^{hi} pattern was associated with exacerbations, poor asthma control and nocturnal symptoms, and the EOS^{hi} pattern was associated with higher IgE level, higher bronchial hyperresponsiveness and lower lung function.

Most of the participants with asthma included in the analysis were recruited in chest clinics as asthma cases, with a careful procedure set up to include true asthmatics using standardised and validated

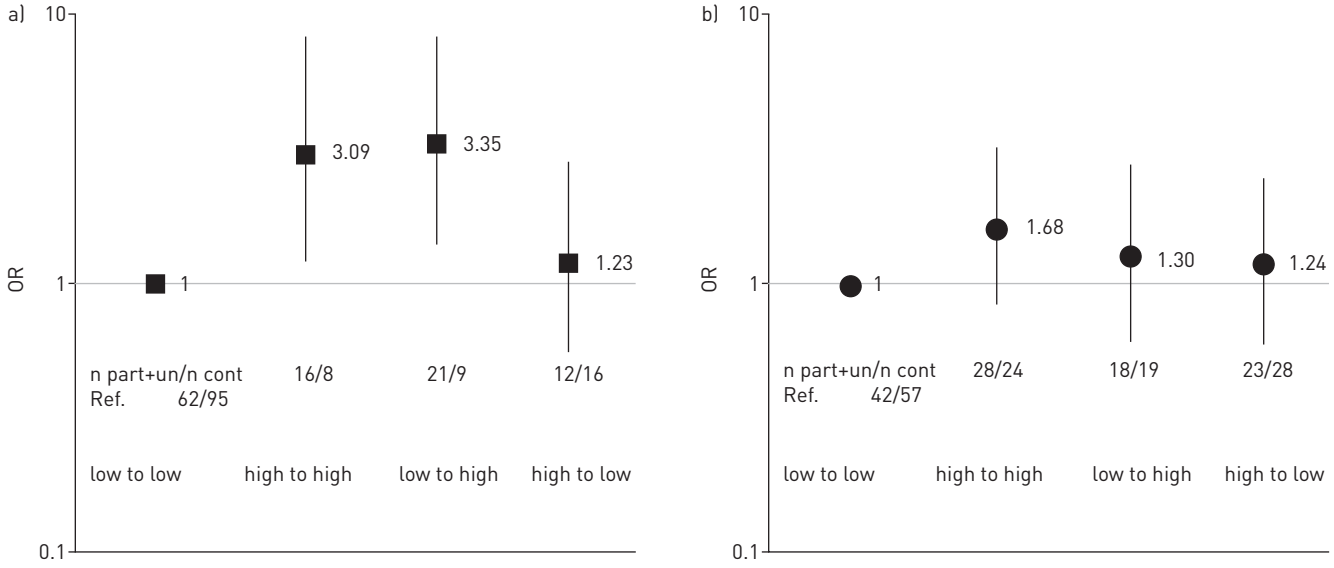

FIGURE 3 Longitudinal association between changes in a) neutrophilic or b) eosinophilic pattern between Epidemiological Study on the Genetics and Environment of Asthma (EGEA)1 and 2 and asthma control. Odds ratios (OR) were adjusted for age, sex and current smoking status at baseline. part: partly controlled; un: uncontrolled; cont: controlled.

TABLE 5 Adjusted longitudinal associations between neutrophilic or eosinophilic granulocyte patterns at baseline (Epidemiological Study on the Genetics and Environment of Asthma (EGEA)1), or stable patterns between baseline and follow-up (EGEA1 and EGEA2) and lung function or lung function decline (EGEA2, n=242)

	NEU^{lo} at baseline	NEU^{hi} at baseline	p-value	NEU^{lo} at baseline and follow-up	NEU^{hi} at baseline and follow-up	p-value	EOS^{lo} at baseline	EOS^{hi} at baseline	p-value[#]	EOS^{lo} at baseline and follow-up	EOS^{hi} at baseline and follow-up	p-value[#]
Subjects n	190	52		159	24		136	106		99	55	
FEV_1 % predicted[#]	93.7 (90.2–97.1)	91.5 (90.2–97.1)	0.5	94.6 (90.8–98.4)	89.9 (81.4–98.5)	0.3	95.3 (91.4–99.1)	90.5 (86.2–94.7)	**0.06**	96.0 (91.3–100.7)	88.6 (82.3–94.9)	**0.04**
Change in FEV_1 slope mL·year^{-1}[¶]	−27.9 (−35.0–−20.8)	−27.6 (−37.8–−13.7)	0.7	−27.8 (−35.4–−20.1)	−33.6 (−51.1–−16.1)	0.5	−30.7 (−38.6–−22.7)	−23.3 (−32.0–−14.6)	0.2	−28.6 (−38.2–−19.0)	−31.9 (−44.5–−19.4)	0.6

Data are presented as n or mean (95% CI). Means (95% CI) were adjusted for age, sex and smoking at baseline, and taking into account familial dependence of the participants. NEU^{hi}: ⩾5000 neutrophils·mm^{-3}; NEU^{lo}: <5000 neutrophils·mm^{-3}; EOS^{hi}: ⩾250 eosinophils·mm^{-3}; EOS^{lo}: <250 eosinophils·mm^{-3}; FEV_1: forced expiratory volume in 1 s. [#]: at follow-up (EGEA2); [¶]: calculated as annual lung function change (mL·year^{-1}) with a negative value representing a decline.

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questionnaires. Others were recruited as first-degree relatives of asthmatic cases, based on anwers to questions on asthma diagnosis. This leads to a group of asthmatics with a wide range of severity and response to methacholine. No follow-up bias related to the asthma status and asthma-related phenotypes was shown in the EGEA study, and the adult asthmatics included in the present study are representative of the original study populations of asthmatic cases and their first-degree relatives with asthma. Furthermore, the association between high neutrophil inflammation and asthma control remained significant excluding participants with chronic bronchitis. The detailed respiratory questionnaire used in our study allowed to retrospectively assess asthma symptoms control following the principles of the GINA 2015 classification, although over a longer time frame (3 months rather than 1 month). The GINA 2015 definition takes into account daytime and night-time symptoms, use of reliever medication and activity limitation, but not lung function. Granulocyte patterns were defined according to eosinophil and neutrophil counts in blood, an easy approach in participants of all ages using standardised collection procedures. The approach of using blood markers to approximate airway inflammation has biological plausability since the infiltrating granulocytes in the airways derive from the bone marrow and access the airways *via* the circulation. The eosinophil count cut-off point used (250 cells·mm^{-3}) was most commonly used in epidemiology, close to the threshold value of 220 cells·mm^{-3} found as the best compromise for predicting sputum eosinophil count $\geqslant$2% in 995 asthmatic adults [4], or for predicting uncontrolled airway eosinophilic inflammation in a population of 508 asthmatic adults [19]. The neutrophil cut-off point was the corresponding 75th percentile of the distribution in the EGEA adult population, and was very similar to the optimal cut-off points obtained from a ROC curve to assess asthma control.

The eosinophilic feature is recognised as a pivotal trait of asthma [28]. In the present study, we confirmed the well-documented associations of high blood eosinophil counts with high IgE level, increased BHR, lower FEV1 and more asthma attacks, as previously reported in general or occupational populations [29, 30], and in the EGEA study at baseline [6]. Furthermore, we found associations between EOS^{hi} and subsequent risk of increased BHR. However, we found inconsistent associations between EOS^{hi} and asthma control, and no clear association with subsequent risk of poor asthma control, or subsequent risk of asthma attacks or exacerbations, whatever the threshold. We found that high blood eosinophil counts were associated with exacerbations defined as asthma attack (considered as mild exacerbation), but not with exacerbations defined as admissions to hospital or to emergency or use of oral steroids (considered as severe exacerbation). Previously, higher blood eosinophil counts (300–500 cells·mm^{-3}) have been associated with asthma attacks in 9223 adults from the National Health and Nutrition Examination Survey study [31] and in 3162 adults with asthma [32]. Regarding severe exacerbations, high blood eosinophil counts defined by a threshold of 400 or 300 cells·mm^{-3} were associated with increased risk for future asthma exacerbations in a cohort of 2392 adults with persistent asthma [18], and in a very large cohort, 130 248 patients with asthma with blood eosinophil counts >300 cells·mm^{-3} defined as nine ascending eosinophil count categories (*versus* $\leqslant$200 cells·mm^{-3}) had a greater rate of asthma exacerbations over the subsequent year [33]. Recently, the threshold of 400 cells·mm^{-3} was reported as a risk factor for multiple exacerbations in the same cohort [34]. Asthma attack and asthma exacerbation may identify exacerbations of different severity, which might partly explain the differences in the associations found with blood eosinophils. The cut-off point for blood eosinophilia (250 cells·mm^{-3}) applied in our study was in the lower range of the published cut-offs. Further, neither adjustment on blood neutrophilic inflammation, nor changes in granulocyte pattern over time were taken into account in these studies, and might explain part of the between-study discrepancies.

Regarding asthma control, previous studies have also shown quite different results to ours: lack of control of asthma was significantly associated with higher blood eosinophils in 111 patients with asthma [17] and blood eosinophilic inflammation ($\geqslant$400 cells·mm^{-3}) contributed to poor asthma control in 508 patients with asthma [19]. In a very large cohort of 130 248 patients with asthma, blood eosinophil counts >400 cells·mm^{-3} were associated with lower risk of achieving asthma control over the subsequent year [33]. Adjustment on blood neutrophilic inflammation was performed in one study [32], and changes in granulocyte pattern over time were not taken into account in these studies. In our study, the definition of asthma control differed from the definition used in previous studies that included either airflow variability or FEV1 level, and such phenotypic differences might explain part of the between-study discrepancies.

Variations over time of inflammatory markers in the blood or in the sputum are an important issue when trying to predict asthma evolution. Short-term and long-term stability of granulocyte patterns in sputum has been reported previously in adults [9, 35], mostly based on two sequential sputum samples. However, stable granulocyte patterns were found in only one-third of adults with moderate and severe asthma undergoing frequent (up to monthly) sputum induction over a 1-year period [36]. In our study, we reported variations over time of blood granulocyte patterns, and found that $\geqslant$50% of the participants retained the same pattern 12 years apart. In adults with severe asthma from the Dose Ranging Efficacy and

Safety with Mepolizumab in Severe Asthma study, 85% of those who had a screening blood eosinophil count of $\geqslant$150 cells·μL^{-1} retained the same pattern in the following year [37]. Recently, variations over time of blood eosinophilia in COPD patients have been studied and a good stability of the data 1 year apart was reported [15]. Overall, available data suggest that blood granulocyte patterns seem at least as stable as sputum patterns in adults with asthma.

To the best of our knowledge, only one study has investigated the role of blood neutrophils in asthma control in a very small sample of adults [38], reporting no differences between neutrophils from patients with well-controlled (n=11) *versus* suboptimally controlled asthma (n=7). In the present study, we found that the NEU^{hi} pattern was associated with poor asthma control according to the GINA 2015 classification, and that persistent NEU^{hi} pattern was associated with subsequent risk of poor asthma symptom control. Interestingly, as for eosinophilia, the associations between high blood neutrophil counts and exacerbations varied according to the phenotype we used. Neutrophilia was increased in ICS users in our study; this may be partly due to the inhibitory effect of corticosteroids on neutrophil apoptosis that may, in some settings, contribute to neutrophil activation, suggesting that corticosteroid treatment itself is likely to have some role in the development of neutrophilia [39]. The association that we observed between NEU^{hi} pattern and poor asthma control persisted after accounting for respiratory infections in the past 4 weeks, current smoking status and ICS treatment in the past year. To our knowledge, our study is the first to report that persistent NEU^{hi} pattern and change from NEU^{lo} to NEU^{hi} pattern were associated with poor asthma control (partly controlled or uncontrolled asthma) compared to other patterns. In the EGEA study, using unsupervised methods, we showed that blood neutrophil counts were the highest in the phenotype labelled "active treated adult-onset asthma" [40]. The latter phenotype showed similar characteristics as clusters that exhibited the highest sputum and blood neutrophil counts found in studies by Moore and colleagues [41, 42]. All these studies highlight the interest of using blood neutrophil counts when classifying adults with asthma. Overall, these results suggest that blood eosinophilia and neutrophilia may be associated with two particular and specific endotypes of asthma in adults, which may be related to different characteristics (symptoms, lung function and activity) of the disease. This is in line with new orientations for the clinical management of asthma, based on precise phenotyping and endotyping. New therapies targeting eosinophilic asthma are already available; however, due to the clinical importance of neutrophilic asthma, more research is needed to understand the basic mechanisms of neutrophilic asthma and offer opportunities of translational research for a more personalised and efficient treatment approach.

In conclusion, the present longitudinal study identified for the first time that blood granulocyte patterns are differently associated with subsequent asthma control outcomes in adults with asthma. More generally, this study adds evidence for the interest of blood eosinophils and neutrophils to help identifying adults with subsequent risk of asthma burden that could be targeted for specific therapies.

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