Impact of former smoking exposure on airway eosinophilic activation and autoimmunity in patients with severe asthma

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In patients with severe asthma, former smoking exposure is associated with airway eosinophil activation and autoimmunity towards eosinophils and macrophages, as well as an incomplete anti-inflammatory response to systemic corticosteroids. https://bit.ly/3sTBJiF

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Abstract

Introduction Severe eosinophilic asthma is characterised by frequent exacerbations and a relative insensitivity to steroids. Experimentally, smoking may induce eosinophilic airway inflammation, but the impact in patients with severe asthma is not clear.

Objective To investigate the association between smoking exposure in patients with severe asthma, and eosinophilic inflammation and activation, as well as airway autoimmunity and steroid responsiveness.

Methods Patients with severe asthma according to European Respiratory Society/American Thoracic Society criteria were assessed with sputum samples, analysed by cell differential count, and for the presence of free eosinophil granules (FEGs), autoantibodies against eosinophil peroxidase (EPX) and macrophage receptor with collagenous structure (MARCO). A subgroup of patients with eosinophilic airway inflammation was re-assessed after a 2-week course of prednisolone.

Results 132 severe asthmatics were included in the study. 39 (29.5%) patients had ⩾10 pack-years of smoking history: 36 (27.3%) were former smokers and three (2.3%) current smokers; and 93 (70.5%) had <10 pack-years exposure. Eosinophilic airway inflammation was more prevalent among patients with ⩾10 pack-years (66.7%), compared to patients with <10 pack-years (38.7%, p=0.03), as was the level of FEGs (p=0.001) and both anti-EPX and anti-MARCO (p<0.05 and p<0.0001, respectively). Omitting current smokers did not affect these associations. Furthermore, prednisolone reduced, but did not normalise, sputum eosinophils in patients with a ⩾10 pack-year smoking history.

Conclusion In patients with severe asthma, a former smoking history is associated with eosinophilic airway inflammation and activation and relative insensitivity to steroids, as well as airway autoimmunity.

Introduction

Asthma is a clinically and immunologically heterogeneous disease entity, and despite significant advances in our understanding of the role of specific molecular inflammatory pathways, causative factors driving asthma severity are still poorly understood [1]. Although eosinophilic inflammation is a strong predictor of a clinical response to steroids, severe eosinophilic asthma is characterised by a relative refractoriness to steroids, where steroids are effective, but higher doses are required to achieve clinical effect and inflammation control [2]. Increasing evidence points to marked molecular heterogeneity within the type 2 high entity [3], and the need for understanding the mechanisms behind severe eosinophilic asthma, to develop better treatment strategies, has sparked extensive research in "alternative" eosinophilic pathways such as group 2 innate lymphoid cells (ILC2) [4–6]. Emerging evidence has also pointed to a localised immune response within the airways toward cytotoxic and immunogenic eosinophil degranulation products such as eosinophil peroxidase as a mechanism of corticosteroid-resistant eosinophilia [7–9]. Interestingly,

cigarette smoke has been implicated in both ILC2-activation and propensity toward autoimmunity, and cigarette smoke can even modify eosinophil properties, making them more active and prone to degranulation [10], potentially increasing the risk of developing local autoimmune responses. Severe asthma often starts in adulthood, and many patients with severe asthma have a significant smoking history [11]. Asthma patients who smoke have poorer clinical outcomes and worse quality of life across all severities of asthma; immunologically, smoking is associated with oxidative stress in severe asthma, but the impact of smoking in severe eosinophilic asthma has not been described [12–14]. Patients with persistent severe eosinophilic asthma are prone to exacerbations [15, 16], which has recently been linked to signs of eosinophilic activation, with clusters of free eosinophil granules (FEGs) in sputum [17]. FEGs are known to correlate strongly with eosinophil peroxidase (EPX) in the airways [18], suggesting that both are released upon eosinophil degranulation [19, 20]. Peroxidases are proven to be cytotoxic and highly immunogenic when released in an uncontrolled manner to the surroundings, and autoantibodies against EPX (anti-EPX) are found in the airways of severe asthmatics with increased markers of eosinophil degranulation despite maintenance oral corticosteroid therapy [9]. Recently, elevated airway anti-EPX levels were found to be associated with a suboptimal response to anti-interleukin-5 therapy in patients with severe eosinophilic asthma [21]. Additionally, autoimmunity against macrophage scavenger receptors (macrophage receptor with a collagenous structure (MARCO)) has been found to co-exist with anti-EPX [9]; macrophage dysfunction could potentially impair the ability to clear airway inflammation. Additionally, macrophage dysfunction has been strongly implicated in smoking-related pathology [22] and associated with more severe disease in eosinophilic COPD [23].

An association between smoking and autoimmunity was hypothesised >15 years ago. Since then, smoking has been identified as a risk factor for developing several systemic autoimmune conditions [24], most prominently rheumatoid arthritis and systemic lupus erythematosus, possibly through a complex interplay with genetic and epigenetic factors [25, 26].

The potential role of smoking in triggering autoimmune responses in asthma has not been explored previously, but it would appear plausible that activation of eosinophils caused by smoking, with continued release of eosinophilic peroxidases, could lead to a higher risk of airway autoimmunity in patients with eosinophilic asthma, who have a significant smoking history. In the present study, we assessed the impact of smoking on airway levels of eosinophilic inflammation and activation, and autoreactivity to EPX and macrophage scavenger receptors, in patients with severe eosinophilic asthma, as well as the impact on the response to systemic steroids.

Methods

Patient population

Data from two studies of patients with severe asthma according to the 2014 European Respiratory Society (ERS)/American Thoracic Society (ATS) guidelines conducted at the Respiratory Research Unit at Bispebjerg Hospital (Copenhagen, Denmark) were pooled for this analysis: the cross-sectional Severe Eosinophilic Asthma – the Impact of Smoking (SATS) study [27] and a subset of patients (the Systematic Asssessment of Possible Severe Asthma (SEPIA) substudy) from the prospective intervention study SIGNATURE [3, 28]. The SEPIA substudy included only patients with eosinophilic airway inflammation (sputum eosinophil count of ⩾3%) at the time of enrolment, and these patients were re-examined after a 14-day course of prednisolone (37.5 mg daily). Patients were stratified and compared by smoking history, where ⩾10 pack-years of cigarette smoking was defined as a positive smoking history. Smoking history, including smoking status was self-reported.

Overall, three sets of analyses were performed, comparing subjects with *versus* without a smoking history (⩾10 pack-years *versus* <10 pack-years): 1) a cross-sectional comparison, in the entire combined patient sample; 2) a cross-sectional comparison, only among patients with airway eosinophilia; and 3) a comparison of response to prednisolone among patients with eosinophilic airway inflammation.

Ethics

Written informed consent was obtained from all study subjects prior to inclusion. Study approval was obtained from the local scientific ethics committee (project IDs: H-1-2014-047 (SATS) and H-17004938 (SIGNATURE)).

Sputum phenotyping

The participants' inflammatory phenotype was determined by the sputum cell differential count. Four phenotypic groups were defined: eosinophilic (sputum eosinophil count ⩾3%); neutrophilic (sputum

neutrophil count ⩾61%); mixed granulocytic (sputum neutrophil count ⩾61% and eosinophil count ⩾3%); and paucigranulocytic (sputum neutrophil count <61% and eosinophil count <3%) [29, 30].

Measurements of airway eosinophil activity

As a surrogate marker for eosinophil activation and degranulation, we assessed the sputum for FEGs [18, 19, 31]. A trained lab technician ranked the amount of FEGs in cytospin sputum preparations on a semi-quantitative scale from 0 to 3, representing a range (FEG score) of 0 (none), 1 (few), 2 (moderate) and 3 (extensive). When used as a dichotomous variable, FEG scores of 0–1 are termed "no degranulation" and FEG scores of 2–3 are termed "degranulation".

Measurements of airway autoimmunity

Immunoglobulin reactivity towards anti-EPX and anti-MARCO [32] was assessed in cell-free soluble fractions of processed sputum supernatant. We immunoprecipitated (IP) sputum samples with protein A/G beads to generate IP-immunoglobulins (IP-Igs), using the method described by Mukherjee *et al*. [33]. Anti-EPX and anti-MARCO reactivity was assessed by using an indirect in-house ELISA [9]. Values are expressed as absorbance at 620 nm after background correction. The cut-off for a positive test was defined as the 90th percentile of immunoglobulin reactivity based on 22 healthy controls.

Blood samples

Standard blood parameters, including blood differential count and C-reactive protein, were analysed at the department of clinical biochemistry, Bispebjerg and Frederiksberg Hospital (Copenhagen, Denmark). Blood eosinophilia was defined as values $\geqslant 0.3\times10^9$ cells·L^{-1}.

Statistical analyses

Data was analysed using SPSS Statistics for Windows (version 25.0; IBM, Armonk, NY, USA). Categorical data are presented as numbers and percentages while continuous data are presented as mean±SD for parametric data and median (interquartile range) for nonparametric data.

Results

Patient characteristics

Characteristics of the 132 patients included in the study are summarised in table 1; 39 patients (29.5%) had smoking history of ⩾10 pack-years of smoking. Only three (8%) patients in the group with a ⩾10 pack-years smoking history were active smokers at the time of inclusion, while the remaining 36 (92%) patients were former smokers, who had completed and maintained smoking cessation ⩾1 year prior to inclusion (former smokers).

TABLE 1 Baseline characteristics of the study population

	<10 pack-years	⩾10 pack-years	p-value
Patients	93 (70.5)	39 (29.5)	
Female	54 (58.1)	18 (46.2)	0.33
BMI (kg·m^{-2})	27.4 (24.3–31.5)	28.1 (23.9–31.6)	0.70
Age at inclusion (years)	46.3±13.1	54.0±11.4	0.06
Age at asthma onset	28.9±18.1	33.9±15.2	0.22
Smoking (pack-years)	0 (0–1)	20 (14–33)	0.001
Daily ICS dose (µg)	1600 (1600–2400)	1600 (1600–1600)	0.08
FEV_1 (% predicted)	79.0±20.7	69.5±20.8	0.02
FVC (% predicted)	91.6±18.3	92.4±17.9	0.49
Atopy	60 (64.5)	17 (43.6)	0.03
Total IgE (kU·L^{-1})	115 (33–263)	143 (35–521)	0.08
Blood eosinophils (×10^9 cells·L^{-1})	0.26 (0.11–0.39)	0.35 (0.10–1.47)	0.45
Blood neutrophils (×10^9 cells·L^{-1})	4.3 (3.5–5.2)	4.1 (1.75–15.75)	0.83
F_{eNO} (ppb)	28.0 (18.0–47.0)	42.0 (23.0–59.0)	0.14
Sputum eosinophils (%)	1.75 (0.25–6.75)	6.75 (1.75–15.75)	0.01
Sputum neutrophils (%)	53.75 (33.75–73.00)	60.25 (39.25–77.00)	0.30

Data are presented as n (%), mean±SD or median (interquartile range), unless otherwise stated. BMI: body mass index; ICS: inhaled corticosteroids; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; Ig: immunoglobulin; F_{eNO}: exhaled nitric oxide fraction.

Former smoking exposure is associated with eosinophilic airway inflammation in patients with severe asthma

Out of 132 patients, 62 (47%) had eosinophilic airway inflammation: 44 (33%) had a purely eosinophilic phenotype and 18 (14%) had a mixed granulocytic phenotype (figure 1a). Of the remaining noneosinophilic patients, 37 (28%) were paucigranulocytic and 33 (25%) were neutrophilic. When stratifying patients into subjects with *versus* without a smoking history of ⩾10 pack-years, the prevalence of airway eosinophilia was significantly higher in the group with ⩾10 pack-years compared with the group with <10 pack-years; a total of 26 (66.7%) patients *versus* 36 (38.7%) patients (p=0.003) (figure 1b and c). Furthermore, there was a significant, although moderate, degree of correlation between the total smoking exposure (pack-years) and the level of sputum eosinophils (Spearman's ρ 0.035, p=0.045).

The prevalence of blood eosinophilia groups was comparable between the two groups (p=0.17) (figure 1d). The prevalence of neutrophilic inflammation was furthermore comparable between the two groups: 23 (25%) *versus* 10 (26%) in the group with ⩾10 pack-years (Chi-squared test p=0.91).

Eosinophilic activation is more pronounced in patients with former smoking exposure. As described in supplementary table S1, patients with eosinophilic airway inflammation (sputum eosinophils ⩾3%, n=58), who had a smoking history ⩾10 pack-years, also had lower lung function, higher use of oral corticosteroids (OCS), and a higher exacerbation rate, compared to patients with <10 pack-years (supplementary table S1).

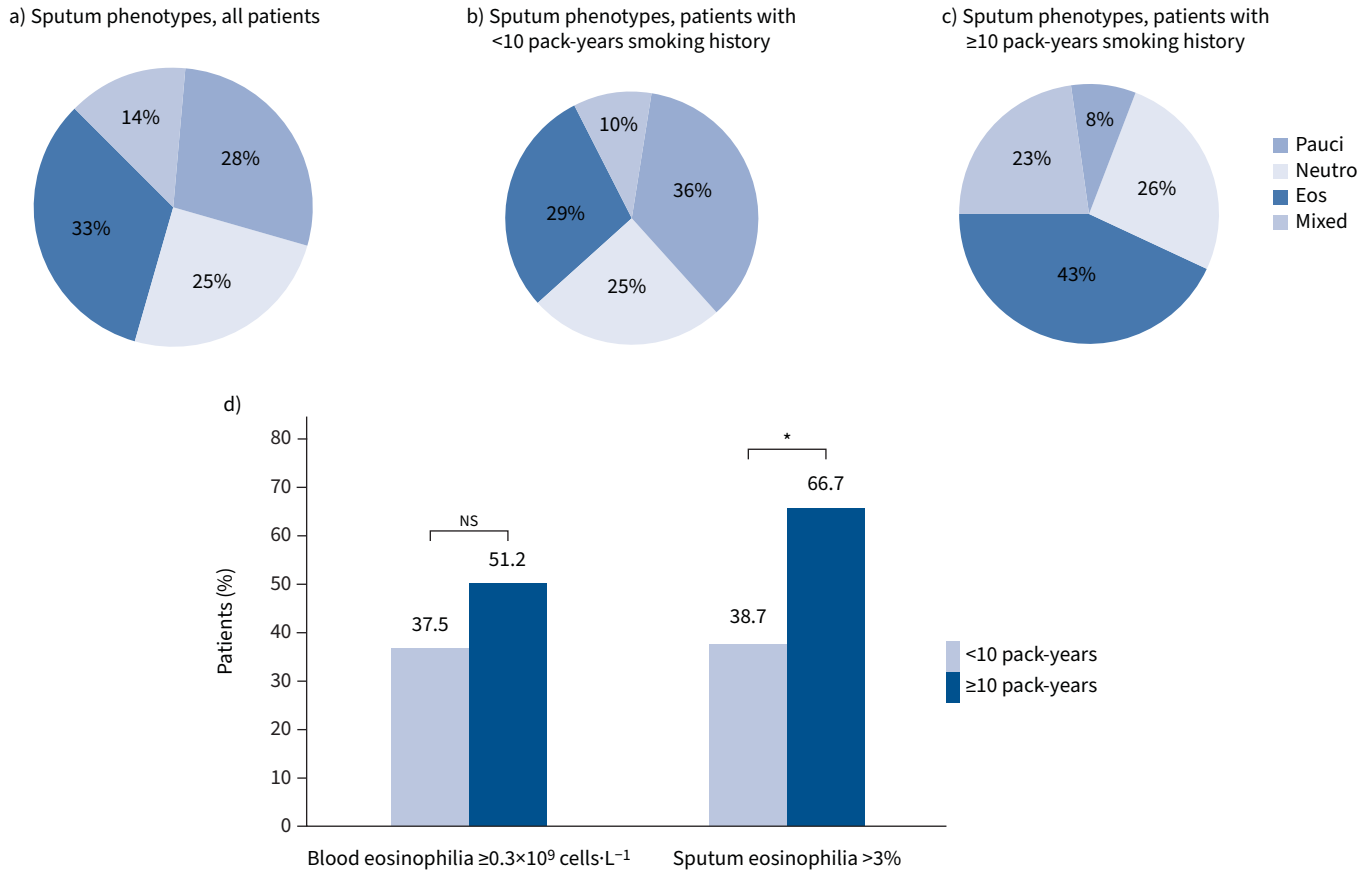

FIGURE 1 Eosinophilic airway inflammation is more prevalent in patients with severe asthma, with ⩾10 pack-years of predominantly former smoking exposure. Inflammatory phenotypes in patients with severe asthma assessed by a–c) sputum differential count and d) blood and sputum differential count, expressed as percentages. a) All patients (n=132); b) patients with <10 pack-years (n=93); and c) patients with ⩾10 pack-years (n=39). d) Proportion of patients with blood and sputum eosinophilia, in patients with <10 pack-years, *versus* patients with ⩾10 pack-years smoking history (n=132). Pauci: paucigranulocytic inflammatory phenotype; neutro: neutrophilic inflammatory phenotype; eos: eosinophilic inflammatory phenotype; mixed: mixed inflammatory phenotype; NS: nonsignificant. Chi-squared test. *: p<0.05.

We found the FEG score to be significantly higher in patients with ⩾10 pack-years compared to patients without a former smoking history (p=0.003), and, in addition, found the FEG score significantly correlated with the absolute number of pack-years in the cohort as a whole (Pearson correlation r=0.29, p<0.01) (figure 2a and b). Furthermore, the FEG score was significantly correlated with exacerbation rate (p=0.036) (figure 2c).

As smokers had a more predominant airway eosinophilia, we also examined the association between FEGs in sputum and eosinophilia in blood *versus* sputum, and found marked differences in degranulation across the different groups (figure 2d and e, n=132), with the highest prevalence of degranulation in patients with concomitant blood and sputum eosinophilia (p<0.01).

Former smoking exposure is associated with airway autoimmunity towards eosinophils and macrophages

In the airway eosinophilic subgroup cohort (n=58), we found that anti-EPX and anti-MARCO IgG titres in sputum were significantly higher in patients with ⩾10 pack-years than in both patients with <10 pack-years (p<0.05 and p<0.0001, respectively) and healthy controls (p<0.05 for both comparisons) (figure 3a–d). In

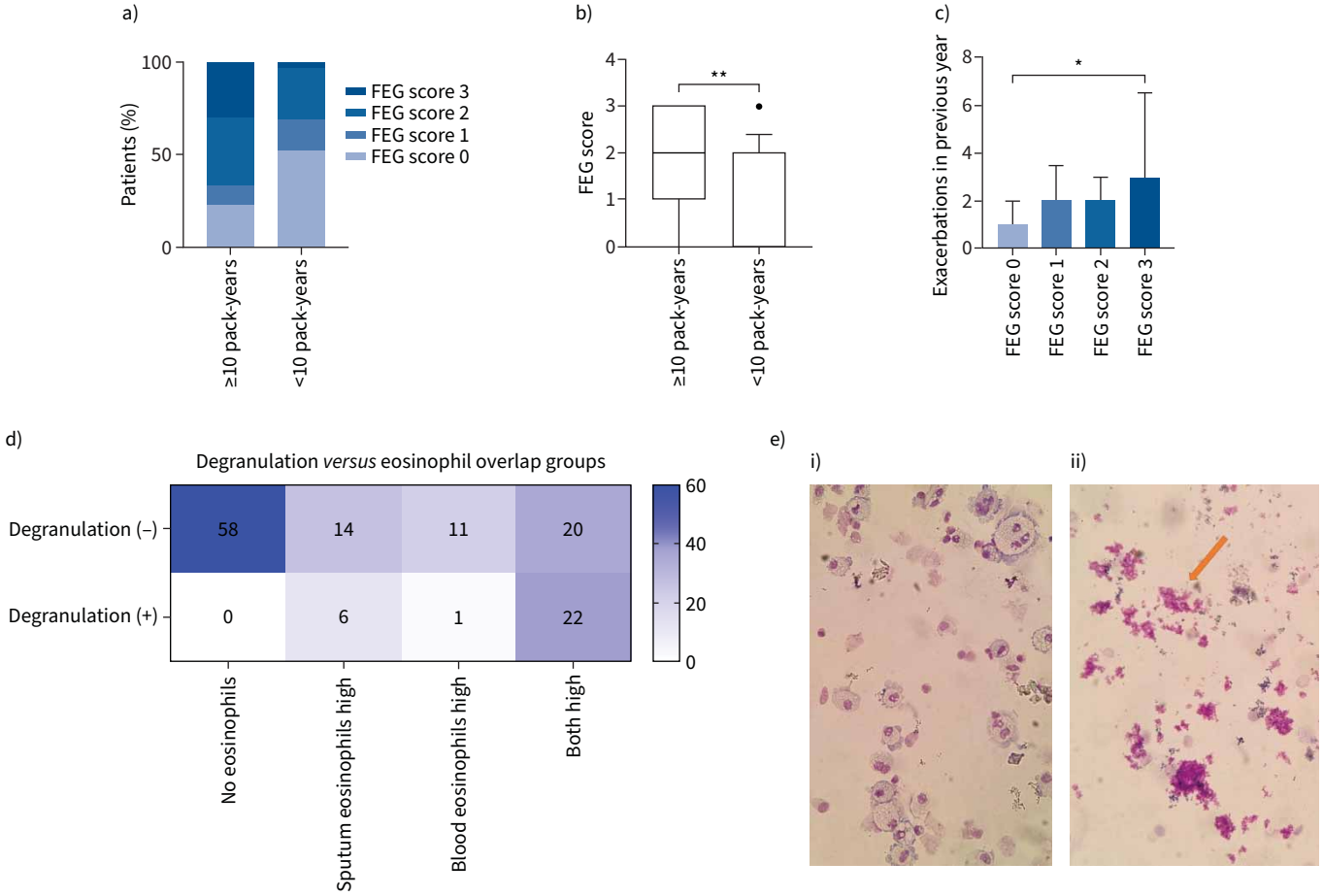

FIGURE 2 Eosinophilic inflammation is associated with eosinophil activation and is more pronounced in patients with severe asthma, with ⩾10 pack-years of predominantly former smoking exposure. Eosinophilic activation assessed by free eosinophil granules (FEGs) in sputum from patients with a–c) severe eosinophilic asthma (n=58) and d) severe asthma (n=132). a) FEG scores 0–3 in patients with ⩾10 pack-years *versus* patients with <10 pack-years. b) Median (interquartile range) FEG scores in patients with ⩾10 pack-years *versus* patients with <10 pack-years. c) Mean±SD exacerbation rate in severe asthma patients with FEG scores 0, 1, 2 and 3. d) Prevalence of degranulation (FEG score 2–3) *versus* no degranulation (FEG score 0–1) across different eosinophilic compartment groups in severe asthma: no eosinophils in sputum or blood (none out of 58, 0%), elevated sputum eosinophils (six out of 20, 30%), elevated blood eosinophils (one out of 12, 8%) or both (22 out of 44, 52%). e) Examples of induced sputum from i) a patient without FEGs, and ii) a patient with a high FEG score (3). Eosinophilic granules are indicated by the arrow. *: p<0.05, **: p<0.01.

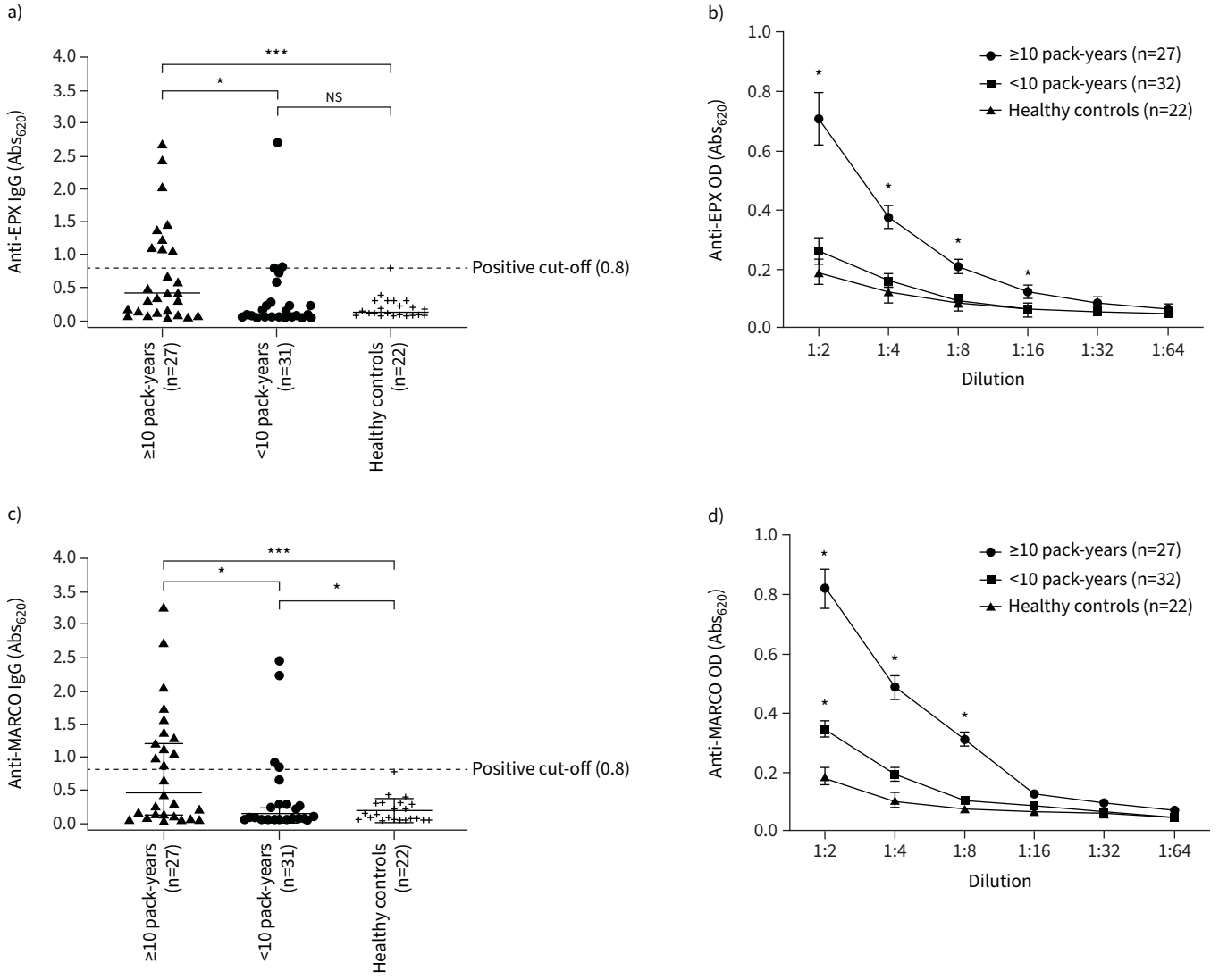

FIGURE 3 Airway autoimmunity towards eosinophils and macrophages in patients with severe eosinophilic asthma is more prevalent in patients with severe asthma, with ⩾10 pack-years of predominantly former smoking exposure. Presence of autoantibodies in induced sputum, against eosinophil peroxidase (EPX) and macrophage receptor with collagenous structure (MARCO). Comparison of patients with >10 pack-years *versus* patients with <10 pack-years, *versus* healthy controls: a) anti-EPX immunoglobulin (Ig)G levels; b) anti-EPX titres; c) anti-MARCO IgG levels; and d) anti-MARCO titres. *: p<0.05; ***: p<0.001. OD: optical density; Abs_{620}: absorbance at 620 nm; NS: nonsignificant.

addition, patients with a smoking history <10 pack-years had significantly higher titres of anti-MARCO compared with healthy controls (p<0.05), while we found no difference in anti-EPX titres between these two groups.

Overall, 17 (29.3%) out of 58 patients were autoimmune-positive. In patients with ⩾10 pack-years, nine (33%) out of 27 were positive for anti-EPX, while only three (9.7%) out of 31 patients with <10 pack-years were positive (p=0.04) (figure 3a and b). For anti-MARCO, 12 (44.4%) out of 27 patients with ⩾10 pack-years, compared to five (16.1%) out of 31 patients with <10 pack-years were anti-MARCO positive (p=0.02) (figure 3c and d). All 17 autoimmune-positive patients were positive for anti-MARCO, while 12 (20.7%) patients were positive for both anti-EPX and anti-MARCO.

In the overall population, three subjects were current smokers; excluding these from the analysis did not impact on the results significantly. The low number of current smokers prohibited a subgroup analysis on the impact of current smoking. The three current smokers were not part of the OCS intervention substudy.

To assess whether autoantibodies decline over time, after smoking cessation, we assessed the correlation between the duration of smoking cessation, and the current level of anti-EPX and anti-MARCO, but did not find any associations.

Oral corticosteroid treatment fails to normalise airway eosinophilic inflammation in patients with a former smoking history ⩾10 pack-years

To assess the impact of a former smoking history on the effect of anti-inflammatory treatment, 23 patients with sputum eosinophils ⩾3% at baseline (n=12 with a smoking history of ⩾10 pack-years and n=11 with a smoking history <10 pack-years) were included in the OCS treatment substudy. Baseline characteristics were comparable between groups, including Asthma Control Questionnaire (ACQ) scores, lung function and blood and sputum eosinophils, apart from a significantly higher FEG score in patients with a smoking history ⩾10 pack-years (supplementary table S2).

OCS treatment lowered blood eosinophils in both groups (p=0.003 for both), with no significant differences between the two groups (figure 4a and b), as well as a significant reduction of sputum eosinophils (p=0.002 and p=0.026) (figure 4c and d). However, the degree of normalisation was more pronounced in patients without a significant smoking history; only three (25%) out of 12 still had elevated sputum eosinophils post-OCS treatment, compared to eight (73%) out of 11 in the group of patients with ⩾10 pack-years (figure 4c and d). Furthermore, whereas OCS treatment lowered exhaled nitric oxide fraction levels in both groups, improvements in forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC) were only observed in patients with a significant smoking history, and ACQ and FEG scores were unchanged (table 2). In contrast, OCS treatment did not significantly reduce the levels of airway autoantibodies towards EPX or MARCO in either group (figure 4e–h), although there was a trend for anti-MARCO levels in patients with <10 pack-years (figure 4g; p=0.091).

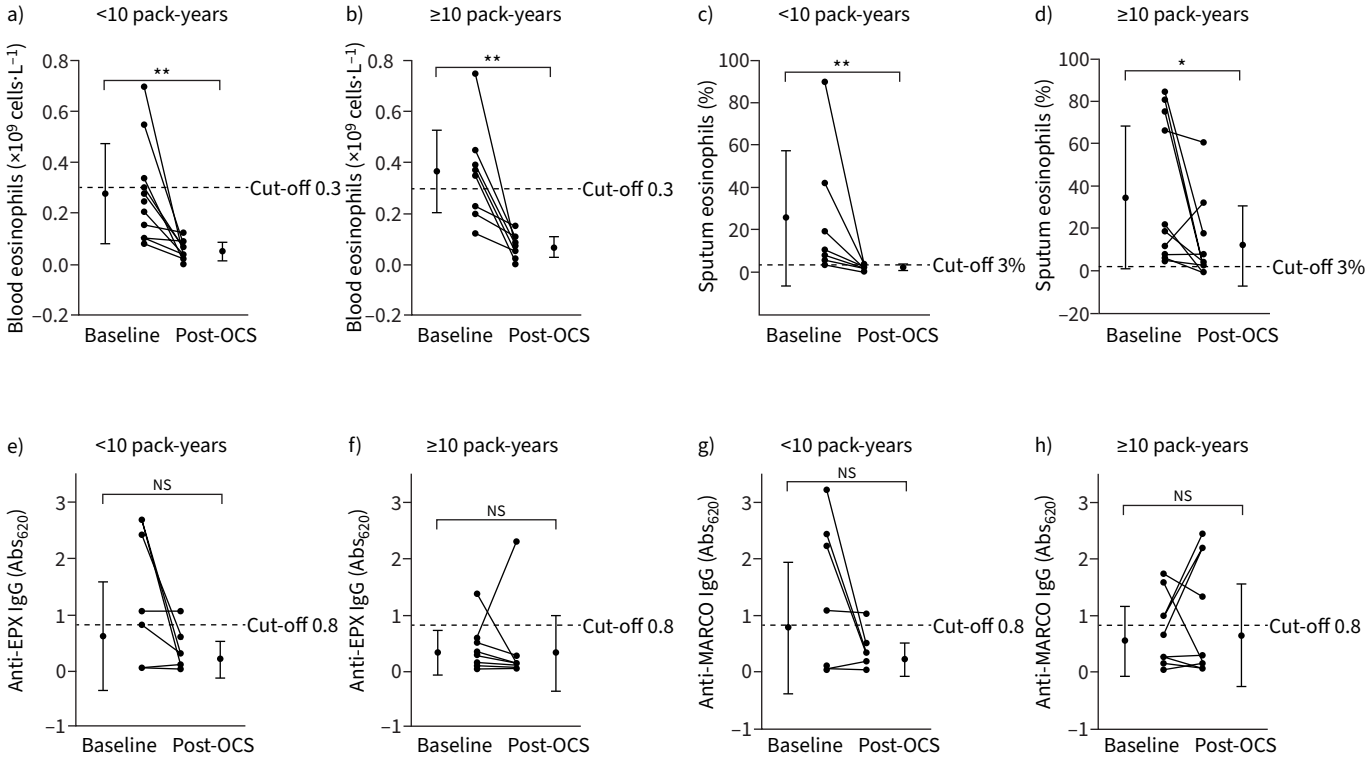

FIGURE 4 2 weeks of oral corticosteroid (OCS) treatment reduces systemic and airway eosinophils regardless of smoking history, but does not normalise airway eosinophils in patients with ⩾10 pack-years of smoking history. Effect of 2 weeks of prednisone treatment (37.5 mg daily) in patients with <10 pack-years and patients with ⩾10 pack-years of smoking history: a and b) blood eosinophils (×10^9 cells·L^{-1}), c and d) sputum eosinophils (%), e and f) anti-eosinophil peroxidase (EPX) IgG and g and h) anti-macrophage receptor with collagenous structure (MARCO) immunoglobulin (Ig)G levels. Abs_{620}: absorbance at 620 nm.

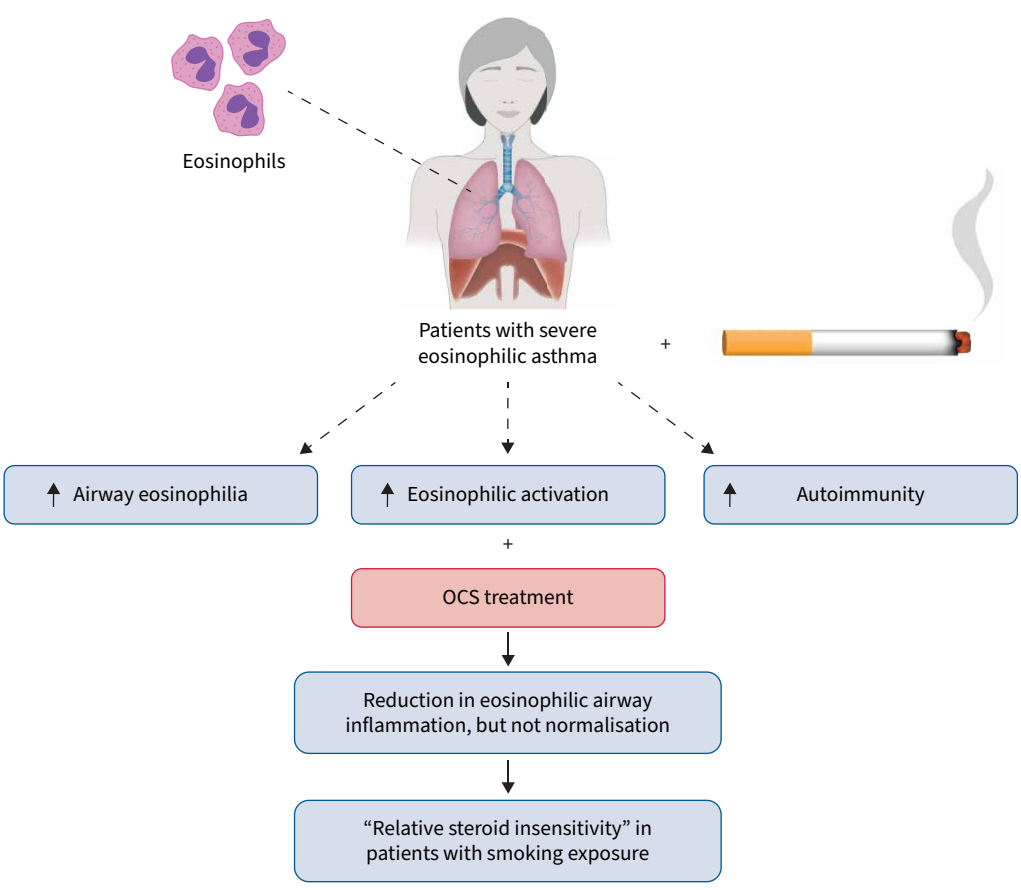

FIGURE 5 Former smoking exposure of ⩾10 pack-years is associated with airway eosinophilia, eosinophilic activation and airway autoimmunity, as well as a reduced effect of systemic steroids in patients with severe asthma.

Overall, these findings suggest that smoking exposure can induce airway eosinophilia as well as eosinophilic activation and autoimmunity towards eosinophils and macrophages, possibly causing a reduced sensitivity to steroid treatment (figure 5).

Discussion

We found a former smoking history of ⩾10 pack-years to be associated with airway eosinophilia in patients with severe asthma. Furthermore, based on the presence of FEGs in sputum, former smoking-exposed patients exhibited significantly more eosinophil activation and degranulation than in patients without a former smoking exposure of ⩾10 pack-years, implying a more volatile and immunoreactive environment conducive for local autoimmune responses. Indeed, we observed that a former smoking history was

TABLE 2 Clinical parameters pre- and post-oral corticosteroid (OCS) treatment

	<10 pack-years (n=12)				⩾10 pack-years (n=11)				Δ p-value
	Pre-OCS	Post-OCS	Δ (95% CI)	p-value	Pre-OCS	Post-OCS	Δ (95% CI)	p-value	
ACQ-5 score	2.2 (1.7–3.3)	2.4 (0.8–3.6)	−0.25 (−0.9–1.5)	0.53	2 (1–2.6)	1.4 (1–2)	−0.5 (−0.4–1.2)	0.25	0.38
FEV_1 (%)	77 (66–101)	91 (60–105)	4.3 (−1.0–12.0)	0.14	72 (60–92)	86 (69–108)	9.5 (3.5–17.0)	0.021	0.12
FVC (%)	94 (86–118)	104 (93–114)	3.3 (−4.0–12.5)	0.47	89 (77–126)	97 (85–138)	7.8 (4.0–13.5)	0.020	0.17
F_{eNO} (ppb)	26 (16–30)	16 (12–26)	−6.5 (1.5–11.0)	0.013	39 (24–50)	20 (16–30)	−15.5 (−26.5– −2.5)	0.021	0.19
FEG score	0 (0–1.8)	0 (0–1)	0 (−0.5–0)	0.16	1 (1–3)	1 (1–3)	0 (0–0)	0.32	0.35

Data are presented as median (interquartile range), unless otherwise stated. ACQ: Asthma Control Questionnaire; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; F_{eNO}: exhaled nitric oxide fraction; FEG: free eosinophil granule.

associated with airway autoimmunity towards both eosinophil- and macrophage-derived targets. Airway autoreactivity was correlated to the level of eosinophilic inflammation and activation, as well as to higher exacerbation rates. Finally, we found that airway eosinophils decreased after a course of systemic steroids in patients with a former smoking history, but in most patients, did not normalise. These findings suggest that in patients with severe eosinophilic asthma, former smoking exposure may induce activation and degranulation of eosinophils that is relatively insensitive to steroid treatment, potentially resulting in persistent eosinophilic activation, in turn inducing airway autoimmunity and increased disease activity. As there were very few current smokers in the present study, the results cannot be extrapolated to the impact of ongoing smoking exposure, and studies on current smokers with severe asthma are needed.

Previous studies on the impact of smoking on airway inflammation in asthma patients have provided variable results, but overall, current smoking appears to be associated with higher levels of airway neutrophils, and lower levels of eosinophils, compared to never-smokers, whereas ever-smokers have comparable airway neutrophils, but lower eosinophils [14, 34–36]. Differences in the total exposure to smoking may explain some of the discrepancies between studies, but could also reflect differences in the selection of asthma patients: in patients with early-onset asthma, smoking exposure could potentially have a different effect on airway inflammation, compared to late-onset eosinophilic asthma in patients with a precedent smoking history. In the present study, the majority of patients had late-onset asthma, with a mean age of onset ~30 years of age. Whether onset of eosinophilic airway inflammation in an airway environment exposed to smoke leads to more activation of eosinophils is unclear. In a study by Granger *et al.* [37], circulating extracellular eosinophilic traps were not related to the severity of asthma, but were higher in ex-smokers, suggesting increased eosinophilic activation in this group of patients. Furthermore, eosinophil activation has been shown to be associated with airway remodelling and fixed airflow obstruction in asthma in general [20]. Similar to our observation of higher exacerbation rates in patients with increased eosinophilic granules, eosinophil activation has previously been linked to frequent asthma exacerbations [17].

The presence of FEGs in the airways could expose the epithelium to EPX, ultimately leading to formation of IgG autoantibodies against EPX [8]. We found airway autoimmune responses in approximately one-third of the ever-smoking patients, most of whom were not on maintenance OCS. This prevalence is comparable with the severe eosinophilic asthma population on maintenance OCS described by Mukherjee *et al.* [9]. Levels of autoantibodies were generally lower than previously reported by Mukherjee and co-workers [9, 21]; this may reflect that our study population had comparatively less-severe asthma, reflected by fewer subjects requiring regular oral steroids.

The observed effect of steroids on reducing, but not normalising, eosinophilic airway inflammation in ever-smokers is in line with a study from Telenga *et al.* [34], who found levels of sputum eosinophils to improve less in ex-smokers and current smokers compared to never-smokers. However, in that study, patients were not severe eosinophilic asthmatics, and the ability of steroids to achieve control of eosinophilic airway inflammation has not previously been assessed specifically in patients with severe eosinophilic asthma.

The present study has some limitations: when allowing ever-smoking asthma patients in a study, COPD must be considered as a potential bias. While we cannot rule out a component of COPD in this patient population, all patients fulfilled the clinical criteria for the diagnosis of asthma. Ever-smokers had a mean baseline post-bronchodilator FEV_1 of 75%, which is greater than what we would typically associate with symptomatic COPD. Additionally, it seems unlikely that COPD as a confounder in the cohort would skew the results in a direction supporting an increased prevalence of airway eosinophilia. A further limitation is that only very few current smokers participated in the study, and we can therefore only conclude on the effect of a former smoking exposure; further studies looking specifically at current smokers are required to understand the role of current *versus* previous smoking. While we did not see any association between the duration of smoking cessation and the level of airway autoantibodies, we cannot rule out that there would be a decline over time after smoking cessation. To answer this question, studies examining patients either also at the time of smoking cessation, or repeatedly over time, would be required.

The primary aim of the substudy on the effect of OCS was to examine the impact of smoking exposure on the effect of OCS on eosinophilic airway inflammation. Few of the included subjects had evidence of autoimmunity, which prohibits firm conclusions on the impact of autoimmunity on the effect of OCS, as well as the effect of OCS on autoantibody levels, and further studies are clearly needed to address this question.

When assessing the autoimmune status in this cohort, we found a complete overlap between anti-MARCO and anti-EPX IgG. This could be caused by either unspecific binding or a shared pathogenesis (possibly the same polyclonal autoimmune response). When looking at the titres of both anti-MARCO and anti-EPX, we saw saturation points over the dilution ranges, making unspecific binding less likely. Regarding the hypothesis of a shared pathogenesis, no longitudinal data exists on the progression of localised autoimmune responses in asthma. Furthermore, only IgG autoantibodies were measured in this study, even though it is known that memory cells are able to produce both IgA and IgM autoantibodies. We chose this approach since the anti-EPX IgG autoantibodies are reported to be the primary drivers of eosinophil pathogenesis; including formation of eosinophil extracellular traps and immune-complex mediated corticosteroid subsensitivity [38]. As for other markers of autoimmunity, we did not assess antinuclear antibodies (ANAs) in this study, but chose to focus on macrophage targets, since macrophage dysfunction has been implicated in the pathogenesis of airway disease in a smoke model [30–32], but largely unexplored in real-life studies, while the correlation between ANAs and anti-EPX was already well established [8, 9].

In a clinical context, the present study indicates that a former smoking history in patients with severe eosinophilic asthma is associated with a more airway-dominant eosinophilic inflammation with signs of eosinophilic degranulation. Importantly, this inflammatory pattern would not be recognised based on eosinophil cell counts alone, nor would the lack of complete suppression of airway eosinophilia after a standard course of systemic steroids be detected in the absence of an induced sputum sample, highlighting the importance of airway sampling combined with more advanced diagnostics, such as quantification of FEGs, in severe asthma patients with a smoking history, who are poorly controlled.

In the substudy with patients on 2 weeks of OCS, none of the patients were current smokers, suggesting that smoking exposure may have lasting detrimental effects in patients with eosinophilic asthma. Overall, our findings suggest that smoking exposure over time could elicit nonspecific immune responses in the airways driving persistent eosinophilic inflammation, possibly causing the development of an "autoimmune" endotype of asthma secondary to eosinophil degranulation.

In conclusion, former smoking exposure was associated with activated airway eosinophils and autoreactivity towards eosinophils and macrophages, as well as an incomplete anti-inflammatory response to systemic corticosteroids. Overall, our findings suggest the existence of a more active and exacerbation-prone phenotype of severe eosinophilic asthma among predominantly former smokers that is potentially more treatment refractory. Our observations indicate an effect of former smoking exposure: The present findings need to be validated in larger populations, and importantly, studies examining current smokers are warranted, to describe whether a similar impact is seen with an ongoing smoking exposure. Finally, further studies are warranted to clarify the best treatment strategies in patients with severe asthma with a smoking history: to determine if biological therapies are equally effective as compared to never-smokers, and if more intensive, longer-term therapy is required to achieve adequate inflammation and symptom control in ever-smoking patients with severe asthma.

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