Airway hyperresponsiveness reflects corticosteroid-sensitive mast cell involvement across asthma phenotypes

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Background: Airway hyperresponsiveness is a hallmark of asthma across asthma phenotypes. Airway hyperresponsiveness to mannitol specifically relates to mast cell infiltration of the airways, suggesting inhaled corticosteroids to be effective in reducing the response to mannitol, despite low levels of type 2 inflammation.
Objective: We sought to investigate the relationship between airway hyperresponsiveness and infiltrating mast cells, and the response to inhaled corticosteroid treatment.
Methods: In 50 corticosteroid-free patients with airway hyperresponsiveness to mannitol, mucosal cryobiopsies were obtained before and after 6 weeks of daily treatment with 1600 μg of budesonide. Patients were stratified according to baseline fractional exhaled nitric oxide (FENO) with a cutoff of 25 parts per billion.
Results: Airway hyperresponsiveness was comparable at baseline and improved equally with treatment in both patients with FENO-high and FENO-low asthma: doubling dose, 3.98 (95% CI, 2.49-6.38; $P < .001$) and 3.85 (95% CI, 2.51-5.91; $P < .001$), respectively. However, phenotypes and distribution of mast cells differed between the 2 groups. In patients with FENO-high asthma, airway hyperresponsiveness correlated with the density of chymase-high mast cells infiltrating the epithelial layer (ρ, −0.42; $P = .04$), and in those with FENO-low asthma, it correlated with the density in the airway smooth muscle (ρ, −0.51; $P = .02$). The improvement in airway hyperresponsiveness after inhaled corticosteroid treatment correlated with a reduction in mast cells, as well as in airway thymic stromal lymphopoietin and IL-33.
Conclusions: Airway hyperresponsiveness to mannitol is related to mast cell infiltration across asthma phenotypes, correlating with epithelial mast cells in patients with FENO-high asthma and with airway smooth muscle mast cells in patients with FENO-low asthma. Treatment with inhaled corticosteroids was effective in reducing airway hyperresponsiveness in both groups. (J Allergy Clin Immunol 2023;152:107-16.)

Key words: *Asthma, airway hyperresponsiveness, mast cell, inhaled corticosteroids*

Despite increasing awareness of the heterogeneity of underlying disease mechanisms in asthma, guidelines still use a one-size-fits-all approach with inhaled corticosteroids as the main therapy for all patients with asthma.[1] Patients with low markers of type 2 (T2) inflammation (ie, T2-low asthma) comprise a substantial section of patients in whom underlying disease mechanisms are both heterogenic and poorly understood. With less response to inhaled corticosteroids compared with patients with T2-high asthma, these patients are often more difficult to manage.[2] The effect of inhaled corticosteroids in patients with T2-low asthma is however variable, and without any good biomarkers to predict a favorable response, both clinicians and patients are left with a trial-and-error approach.[3-11]

Airway hyperresponsiveness is a key feature of asthma across T2-high and T2-low disease.[12,13] The mannitol test is an indirect bronchial provocation test that assesses airway hyperresponsiveness and has been demonstrated to act through an activation of mast cells.[14-17] Although mast cells infiltrating the airway smooth muscle (ie, airway myositis) have been observed in both patients with T2-high asthma and those with T2-low asthma and have been shown to correlate with the level of airway hyperresponsiveness to direct bronchial challenge tests, mast cells infiltrating the epithelial layer have been shown to correlate with the level of airway hyperresponsiveness to indirect bronchial challenge tests in patients with T2-high asthma.[18-21] Furthermore, we and others have previously shown that there is a correlation between mast cell infiltration and thymic stromal lymphopoietin (TSLP) and IL-33, and indirect airway hyperresponsiveness to mannitol is reduced by blocking TSLP.[21-25]

Inhaled corticosteroids are also effective in suppressing both airway hyperresponsiveness to mannitol and infiltration of mast cells, but the effect has not been studied in patients with T2-low asthma specifically.[26-29]

We hypothesized that airway hyperresponsiveness to mannitol would respond to treatment with inhaled corticosteroids,

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Abbreviations used
ACQ: Asthma Control Questionnaire
BAL: Bronchoalveolar lavage
FENO: Fractional exhaled nitric oxide
HIER: Heat-induced epitope retrieval
MC_T: Tryptase-high mast cell
MC_{TC}: Chymase-high mast cell
PD_{15}: Cumulative provoking dose of mannitol causing a 15% fall in FEV_1
TSLP: Thymic stromal lymphopoietin
T2: Type 2

irrespective of the level of T2 biomarkers, and that this response would be related to a decrease in infiltrating mast cells.

We therefore conducted a prospective intervention study with 50 steroid-free patients with asthma and airway hyperresponsiveness to mannitol, investigating the effect of a 6-week course of high-dose inhaled corticosteroids on the level of airway hyperresponsiveness in patients with T2-high and T2-low asthma and further exploring airway mast cell content, distribution, and phenotype, as well as T2 inflammatory drivers.

METHODS

Study overview

The RECONSTRUCT study was a single-center investigator-initiated prospective intervention study including 50 corticosteroid-free patients with asthma and airway hyperresponsiveness to mannitol. All subjects were assessed with a medical interview, the 6-item Asthma Control Questionnaire (ACQ-6), lung function testing, fractional exhaled nitric oxide (FENO), mannitol provocation test, induced sputum, as well as bronchoscopy by obtaining mucosal cryobiopsies before and after 6 weeks of treatment with a daily dose of 1600 μg of budesonide.

Population and ethics

Patients with asthma aged 18 to 64 years with a positive mannitol provocation test result and no use of inhaled or oral corticosteroids for at least the past 3 months were invited consecutively to participate in the study. The exclusion criteria are provided in Table E1 in this article's Online Repository at www.jacionline.org. A total of 69 steroid-free patients with assumed asthma were screened for inclusion, of whom 12 had a negative mannitol test result, 4 did not consent to participate, 1 did not have asthma, 1 was an active smoker, and 1 was too old and they were thereby excluded.

The study was approved by the Danish National Committee on Health Research Ethics (H-16043663), the Danish Medicines Agency (EUDRACT: 2016-03509-33), and the Danish Data Protection Agency (BFH-2018-018) and monitored by the Danish Good Clinical Practice Unit (Project ID: 2016-898).

Patient stratification

Patients were stratified according to FENO levels at the time of inclusion. FENO was measured at a rate of 0.05 L/s with the Nitric Oxide Analyzer (NIOX; Aerocrine AB, Solna, Sweden) or the Ecomedics CLD88sp (Ecomedics, Duernten, Switzerland). Cutoffs were less than 25 parts per billion for FENO-low asthma and 25 parts per billion or more for FENO-high asthma.[30-34]

Interview and questionnaire

All patients filled in a self-administered Danish version of the ACQ-6 before clinical and physical tests.[35] During the interview with a doctor from the study group, patients were asked about family history of atopy and lung disease, allergy, atopic and respiratory symptoms at any time and during the past 4 weeks, current medication, previous asthma exacerbations, asthma triggers, and smoking history.

Spirometry, bronchial challenge test, and induced sputum

Spirometry was performed according to the European Respiratory Society/American Thoracic Society guidelines using Jaeger spirometers (Intramedic, Gentofte, Denmark).[36]

The mannitol test (Aridol; Pharmaxis, French Forest, NSW, Australia) was performed in accordance with the instructions provided by the manufacturer. Following inhalation of an empty (0 mg) capsule, participants inhaled increasing doses of mannitol from 5 mg to 160 mg up to the cumulated dose causing a 15% reduction in FEV_1, with a maximum cumulated dose of 635 mg. In between each dose, FEV_1 was measured. Airway hyperresponsiveness was reported as the cumulative provoking dose causing a 15% fall in FEV_1 (PD_{15}). A test result with a PD_{15} less than 635 mg was considered positive. If the mannitol test result was negative at follow-up, a PD_{15} of 635 mg was assigned.[14,37]

Directly following the bronchial challenge with mannitol, patients were given inhalation of 0.4 mg of salbutamol and asked to cough up sputum.[38,39] If the bronchial challenge was not adequate to induce sputum, induction with inhalation of hypertonic saline in increasing concentrations (3%, 4%, and 5%) for 3 time periods, each of 6 minutes, was performed. Sputum plugs were selected, standard cytospins were prepared, and a differential cell count was performed.[40-43]

Bronchoscopy and tissue sampling

Bronchoscopies were performed within 1 week of the baseline and follow-up visit, respectively. These were performed as standard outpatient procedures with a flexible bronchoscope (Olympus BF-1TQ180/BF-1TH190; Olympus, Hamburg, Germany).[44] To obtain larger and more intact airway biopsies for the evaluation of distribution of mast cells, 2 mucosal biopsies were obtained with cryotechnique (Erbecryo 2, Ø 1.9 mm; Erbe, Tübingen, Germany). The method of obtaining mucosal cryobiopsies was developed in our research unit and has previously been described.[45,46] In short, biopsies were obtained from longitudinal stretches at subsegmental levels at the discretion of the operator using a freeze cycle of 2 to 3 seconds. Biopsies were retracted and thawed in a saline bath. After thawing, the biopsies were immediately subjected to overnight fixation in 4% buffered formaldehyde before dehydration and paraffin embedment. Bronchoalveolar lavage (BAL) was performed by introducing 2 × 60 mL saline, resulting in a return of approximately 20 mL.

Study medication and adherence

All patients with asthma were treated for 6 weeks with 1600 μg of budesonide daily as 2 times 2 inhalations of Spirocort Turbohaler 400 μg (AstraZeneca, Copenhagen, Denmark). During the 6-week treatment period, patients were contacted once weekly by phone and asked for how many days during the last week they have been taking their inhaler (Foster score).[47] Patients could use as needed short-acting β_2-agonists except for an 8-hour period before tests. No long-acting β_2-agonists, muscarinic antagonists, leukotriene receptor antagonists, or biologics were used during the study period.

Histology

The immunohistochemical staining of tryptase-high mast cell (MC_T) and chymase-high mast cell (MC_{TC}) subtypes was performed in an automated slide staining robot (Autostainer Plus; Agilent/Dako, Glostrup, Denmark) after heat-induced epitope retrieval (HIER) at pH 6 and with a peaking temperature at 98°C in a PT-link HIER machine (Agilent Technologies, Glostrup, Denmark). A modified double-staining protocol was used for simultaneous visualization of MC_{TC} and MC_T. Formalin-fixed and paraffin-embedded sections (4 μm) were dewaxed and subjected to rehydration HIER and dual endogenous enzyme-blocking agents that quench endogenous alkaline phosphatase and peroxidase. Chymase-containing mast cells were first labeled with an antichymase antibody (HPA052634; Atlas Antibodies, Bromma, Sweden) diluted 1:3000 and incubated

for 1 hour at room temperature. After a washing step, the primary antibody was detected by a secondary antibody conjugated to horseradish peroxidase polymers (K5007; Agilent/Dako). Next, the chymase immunoreactivity was visualized by horseradish peroxidase substrate DAB (3,3′-Diaminobenzidine) to yield a brown inert chromogen precipitate. Next, a blocking step (DNS001L; Biocare, Pacheco, Calif) was applied, making the first antibody inert to further staining by chemically destroying the antigenicity. The remaining MC_T subclass was then visualized by the same immunohistochemical procedure but now with an antitryptase antibody (MAB1222A; Merck Millipore, Burlington, Mass) diluted 1:10,000 and incubated for 1 hour at room temperature. Tryptase immunoreactivity was visualized with the chromogen Vina Green (BRR807A; Biocare). Finally, sections were counterstained with Mayer hematoxylin, dipped in xylene, and mounted with Pertex (Histolab, Gothenburg, Sweden). The resulting staining thus detects chymase-containing MC_{TC} as brown cells and the chymase-negative but tryptase-positive MC_T as green cells.

All primary antibodies and the associated antigen retrieval and immunohistochemistry protocols to identify MC_T and MC_{TC} have been validated extensively for use on paraffin sections.[48,49] Negative controls were produced by replacing the primary antibody with isotype controls.

High-resolution digital images of the entire tissue areas and chromogens were generated using a slide-scanning robot (ScanScope Slide Scanner; Aperio Technologies, Vista, Calif).[50] Epithelial and airway smooth muscle compartments were identified visually in each image and outlined by manual cursor tracing. Marker-positive staining was quantified using computerized image analysis (Visiomorph^{DP}; Visiopharm, Hørsholm, Denmark) after automatic identification of marker-/chromogen-positive pixels by color segmentation with "locked" RGB threshold values corresponding to the specific chromogens. Next, the area (pixel numbers) of each chromogen was normalized against the total analyzed area and presented as the fraction (%) of the total analyzed tissue area that contained marker-positive staining. As a rough correlation to mast cell numbers, 1% marker positivity reflects roughly 100 to 200 mast cells/mm^2 tissue.

Cytokine measurements in BAL

Cytokines involved in the activation of mast cells and T2 inflammation were measured in BAL using the Human Th9/Th17/Th22 Magnetic Luminex Performance Assay 17-plex (LKTM009; R&D Systems, Abingdon, UK), following manufacturer's instructions. Furthermore, TSLP protein analyses were performed by means of Human TSLP DuoSet ELISA (DY1398; R&D Systems), following manufacturer's instructions. Optical densities at 450 nm with a wavelength correction at 540 nm were recorded in a Multiskan GO (Thermo Fisher Scientific, Uppsala, Sweden) spectrophotometer. Protein concentration was calculated from a standard curve.

Statistical analyses

Continuous data are presented as median with interquartile range for nonnormally distributed data and mean with 95% CI for normally distributed data. Comparisons were done using the Mann-Whitney *U* test and *t* test. Categorical data are presented as numbers and percentages, and chi-square tests were used for comparisons. Data on hyperresponsiveness (PD_{15}), FENO, and sputum differential counts were logarithmically transformed using the logarithm to base 2 scale, and independent *t* tests were used for comparison between them. For sputum counts of 0, the minimal detectable lower limit of 1 cell, corresponding to 0.25%, was assigned to allow for logarithmical transformation. For ACQ-6 scores and tissue cell counts, the Wilcoxon signed-rank test was used. For correlations between measures, the Pearson correlation was used. One-way ANOVA was used to test for difference in between groups adjusted for age, time since asthma diagnosis, and FEV_1/forced vital capacity.

All data were analyzed using IBM SPSS statistics version 25 (IBM Software, Chicago, Ill).

RESULTS

Fifty patients were included in the study. Four patients withdrew consent: 2 before the first bronchoscopy because of personal reasons and 2 after the first bronchoscopy because of procedure-related discomfort. A total of 46 patients (92%), 25 with FENO-high and 21 with FENO-low asthma, completed the study. The baseline characteristics of the patients are provided in Table I.

Airway hyperresponsiveness

The degree of airway hyperresponsiveness to mannitol at baseline was comparable among patients with FENO-high and FENO-low asthma: geometric mean PD_{15}, 126 mg (95% CI, 73-215) and 134 mg (95% CI, 77-232), respectively ($P = .87$). There was an improvement in both groups after treatment with inhaled corticosteroids, corresponding to a doubling dose of 3.98 (95% CI, 2.49-6.38; $P < .001$) and 3.85 (95% CI, 2.51-5.91; $P < .001$), respectively (*P* for difference in between groups, .92). Among patients with FENO-high asthma, 17 (68%) had a negative mannitol test result at follow-up, whereas this was 18 (86%) in the group of patients with FENO-low asthma ($P = .16$) (Fig 1).

Infiltrating mast cells

Mast cells infiltrating the airway wall were visualized in mucosal cryobiopsies, as exemplified in Fig 2. As presented in Table II, mast cell content differed among the 2 patient groups with a higher density of total mast cell markers. The difference was primarily driven by a higher density of chymase-positive mast cells in the epithelium in patients with FENO-high asthma ($P = .001$) (Fig 3). Furthermore, the density of chymase-high mast cells infiltrating the epithelium in patients with FENO-high asthma correlated with the degree of airway hyperresponsiveness (ρ, −0.42; $P = .04$), whereas in patients with FENO-low asthma, it correlated with chymase-high mast cells infiltrating the airway smooth muscle (ρ, −0.51; $P = .02$) (Fig 3; see also Table E2 in this article's Online Repository at www.jacionline.org).

After treatment with inhaled corticosteroids, there was a reduction in chymase-high mast cells infiltrating the airway smooth muscle in patients with FENO-low asthma (change factor, 0.61; 95% CI, 0.43-0.89; $P = .01$). In patients with FENO-high asthma, there was a reduction in epithelial chymase-high mast cells (change factor, 0.74; 95% CI, 0.58-0.95; $P = .02$) (Fig 4). The density of tryptase-high mast cells decreased in both the airway smooth muscle and the epithelium in patients with FENO-low asthma, and in the airway epithelium in those with FENO-high asthma (Fig 5).

Cytokines in BAL fluid

Levels of IL-33 were the highest in patients with FENO-high asthma and correlated with airway hyperresponsiveness ($\rho = -0.40$; $P = .05$). Levels were reduced in both patients with FENO-high and FENO-low asthma after treatment with inhaled corticosteroids. In patients with FENO-high asthma, this reduction correlated with the reduction in epithelial chymase-high mast cells ($\rho = 0.43$; $P = .04$). TSLP concentrations were reduced in patients with FENO-high asthma after treatment with inhaled corticosteroids, which correlated with the reduction in chymase-high mast cells infiltrating both the epithelium ($\rho = 0.53$; $P = .02$) and the airway smooth muscle ($\rho = 0.83$; $P < .001$) (Fig 6).

Levels of all measured cytokines are provided in Table E3 in this article's Online Repository at www.jacionline.org. The T2

TABLE I. Baseline characteristics of patients with T2-high asthma (FENO ≥ 25 ppb) and T2-low asthma (FENO < 25 ppb) and adherence

Characteristic	FENO-high asthma	FENO-low asthma	*P*
No. of patients	25	21	—
FENO (ppb)	56.9 (71.5)	14.2 (8.75)	—
Age (y)	26 (6)	23 (4)	.02
Female participants, n	11 (44)	12 (57)	.38
FEV_1 (L)	3.91 (1.61)	3.71 (0.97)	.70
FEV_1% predicted	95 (16)	102 (19)	.13
FEV_1/FVC	0.73 (0.11)	0.78 (0.07)	.03
BMI	23.5 (5.4)	23.2 (4.4)	.83
Former smokers, n	4 (16)	3 (14)	.51
Age at asthma onset (y)	8 (24)	16 (14)	.27
Asthma duration (y)	15 (11)	9 (9)	.04
ACQ score	1.50 (1.42)	1.17 (1.00)	.35
Atopy, n	22 (88)	6 (29)	<.001
Blood total IgE (U/mL)	145 (364)	33 (93)	<.001
Blood eosinophils (μL^{-1})	200 (260)	100 (160)	.001
Sputum eosinophils (%)	3.81 (95% CI, 2.05-7.10)	1.25 (95% CI, 0.68-2.32)	.01
Sputum neutrophils (%)	18.2 (95% CI, 9.7-34.3)	21.7 (95% CI, 12.3-38.2)	.68
Adherence, Foster score	6.83 (0.25)	6.83 (0.33)	.85

Data are presented as median with interquartile range or geometric mean with 95% CI and numbers with percentages. Comparisons of T2-high and T2-low asthma were made with the Mann-Whitney *U* test or the *t* test for continuous data and the chi-square test for categorical data.
BMI, Body mass index; *FVC*, forced vital capacity; *ppb*, parts per billion.

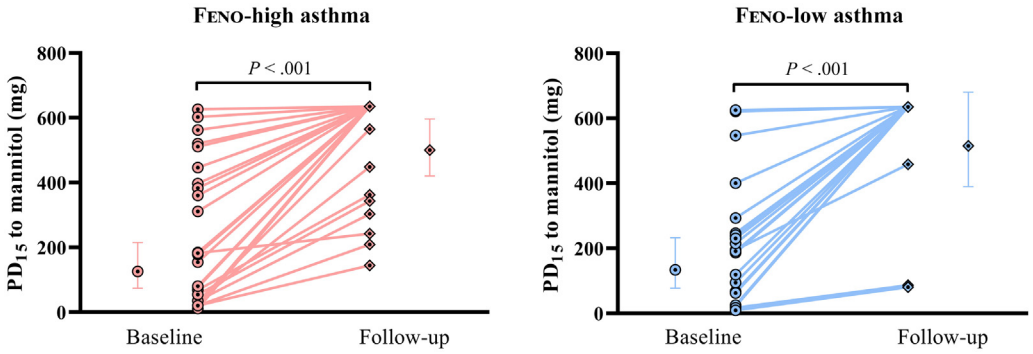

FIG 1. Airway hyperresponsiveness to mannitol before and after treatment with inhaled corticosteroids in patients with T2-high and T2-low asthma. Absolute measures and geometric mean with 95% CI. If the test result was negative at follow-up, a PD_{15} of 635 mg (maximum dose) was assigned.

inflammatory cytokines IL-4, IL-5, and IL-13 were generally below detection rate.

Asthma symptoms and lung function parameters

Improvements in the ACQ-6 scores after treatment with inhaled corticosteroids were seen in both patients with FENO-high and FENO-low asthma, but were most noticeable in patients with FENO-high asthma, among whom 17 patients (68%) improved more than 0.5 point versus 7 (33%) among patients with FENO-low asthma ($P = .02$). There were no significant improvements in lung function measures.

Sputum cell counts

Sputum induction yield at baseline was 89%. The cell counts are provided in Table I. After 6 weeks of treatment with inhaled corticosteroids, sputum eosinophils were reduced in patients with FENO-high asthma by a factor of 0.35 (95% CI, 0.17-0.73; $P = .007$) but not in patients with FENO-low asthma.

DISCUSSION

In corticosteroid-free patients with asthma and airway hyperresponsiveness to mannitol, the level of airway hyperresponsiveness correlated with the infiltration of chymase-high mast cells in both patients with high and low FENO, but with different distributions: in the airway epithelium of patients with FENO-high asthma and in the airway smooth muscle of those with FENO-low asthma. Treatment with high-dose inhaled corticosteroids for 6 weeks resulted in significant improvements in airway hyperresponsiveness concomitantly, with a decrease in mast cells in both groups. Furthermore, the reduction in mast cells correlated with a decrease in the epithelial alarmins TSLP and IL-33 in patients with FENO-high asthma but not in those with FENO-low asthma. The results suggest that infiltrating mast cells have different modes of activation and distribution in the airways but may be potential treatment targets in both patients with FENO-high and FENO-low asthma.

The present study demonstrates a robust effect of inhaled corticosteroids on airway hyperresponsiveness in both patients with FENO-high and FENO-low asthma, confirming what previous

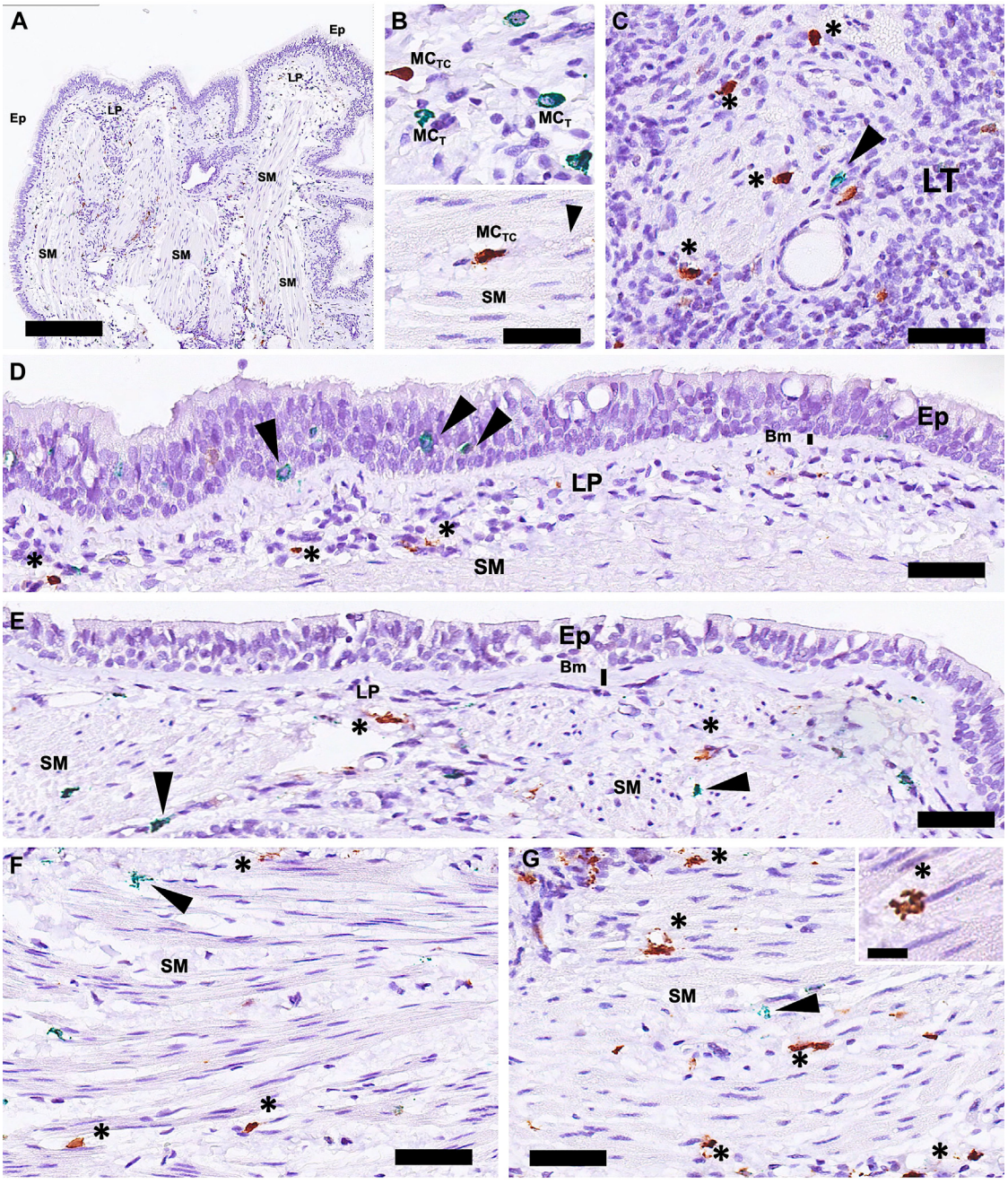

FIG 2. Bright field micrographs exemplifying the immunohistochemical identification of tryptase-high mast cells (MC_T) and chymase-high mast cells (MC_{TC}) in mucosal cryobiopsies. **A,** Low-power image providing an overview of common histological structures. **B,** Zoomed-in areas illustrating individual MC_T (*green*) and MC_{TC} (*brown*) in the subepithelial lamina propria tissue (*upper panel*) and the airway smooth muscle (*lower*). **C,** Example of mast cells within LT. **D** and **E,**) Mast cells in the epithelial region before (Fig 2, *D*) and after (Fig 2, *E*) treatment with inhaled corticosteroids. **F** and **G,** Examples of mast cells in the airway smooth muscle region before (Fig 2, *F*) and after (Fig 2, *G*) treatment with inhaled corticosteroids. In panels *C* to *G*, arrowheads denote Vina Green chromogen MC_T, whereas brown DAB-stained MC_{TC} are marked with asterisks. *DAB*, 3,3′-Diaminobenzidine; *Ep*, airway epithelium; *Bm*, lamina reticularis layer of the basement membrane; *LP,* lamina propria; *LT*, lymphoid tissue; *SM*, airway smooth muscle. Scale bars: A = 350 μm; B = 40 μm; C = 50 μm; D-G = 60 μm (inset in *G* = 15 μm).

studies have shown in general populations of patients with asthma and real-life observations in patients with T2-high or T2-low asthma.[12,26,29] The absence of T2 inflammation in patients with asthma is often considered a predictor of a poor response to inhaled corticosteroids, but the effect is most likely variable in this heterogeneous group of patients.[4-6,51] In this study, the effect of inhaled corticosteroids on airway hyperresponsiveness in patients with low FENO was both significant and uniform, with 86% of patients having a negative mannitol test result at follow-up. Furthermore, all patients had a clinically relevant response,

TABLE II. Density (%) of mast cells in mucosal cryobiopsies obtained before and after 6-wk treatment with high-dose inhaled corticosteroids in patients with FENO-high or FENO-low asthma

	FENO-high asthma		FENO-low asthma		
Mast cells	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**	***P***
MC_{total}	0.54 (0.46-0.64)	0.39 (0.31-0.50)*	0.40 (0.32-0.51)†	0.25 (0.20-0.33)*	.37
MC_T	0.079 (0.052-0.12)	0.043 (0.025-0.072)*	0.14 (0.076-0.24)	0.051 (0.032-0.072)*	.07
MC_{TC}	0.41 (0.33-0.51)	0.31 (0.24-0.40)*	0.19 (0.14-0.27)†	0.17 (0.12-0.24)	.74
Epithelium MC_T	0.13 (0.08-0.17)	0.09 (0.04-0.13)*	0.20 (0.13-0.27)	0.10 (0.05-0.10)*	.11
Epithelium MC_{TC}	0.52 (0.38-0.65)	0.41 (0.25-0.57)*	0.23 (0.17-0.28)†	0.22 (0.14-0.30)	.30
Airway smooth muscle MC_T	0.13 (0.07-0.19)	0.11 (0.05-0.16)	0.23 (0.007-0.44)	0.07 (0.03-0.11)*	.02
Airway smooth muscle MC_{TC}	0.20 (0.10-0.30)	0.24 (0.12-0.36)	0.33 (0.17-0.48)	0.21 (0.08-0.35)*	<.001

*Significant change from baseline to follow-up. *P* for difference in change in mast cell density in between groups.
†Significant difference between FENO-high and FENO-low asthma.

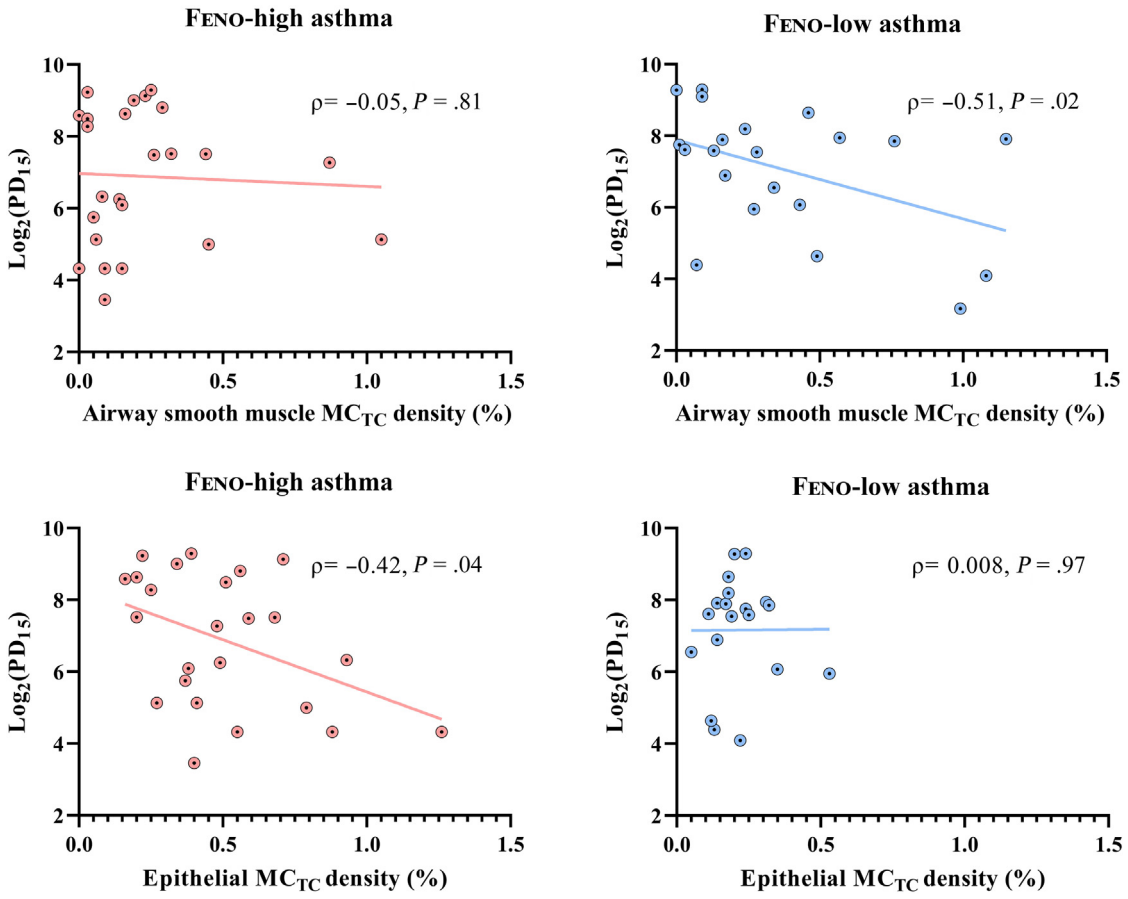

FIG 3. Correlations between airway hyperresponsiveness and the density of infiltrating chymase-high mast cells (MC_{TC}) in the epithelium and airway smooth muscle in patients with FENO-high and FENO-low asthma at baseline.

with improvement in airway hyperresponsiveness corresponding to at least 2 doubling doses of mannitol.[52] We therefore suggest that airway hyperresponsiveness to mannitol may serve as a marker of response to inhaled corticosteroids in patients with an otherwise T2-low profile; however, whether these results from a population of patients with mild asthma can be applied to patients with more severe disease needs to be further validated.

To explore underlying mechanisms of the response observed, we performed histological analysis of mucosal cryobiopsies, which showed a differentiated distribution of mast cells among patients with high and low FENO and a differentiated correlation with airway hyperresponsiveness. A correlation between airway hyperresponsiveness and mast cells infiltrating the airways has been shown previously. Infiltration of the airway smooth muscle has been shown to correlate with airway hyperresponsiveness to direct challenges in both patients with T2-high and T2-low asthma,[18-20,53-55] whereas epithelial mast cell infiltration has been shown to correlate with airway hyperresponsiveness to indirect challenges in patients with T2-high asthma.[25,56-60] This study confirms a role of epithelial mast cell infiltration in airway hyperresponsiveness in patients with high FENO, which also correlated with the concentration of the epithelial alarmin IL-33 in BAL.

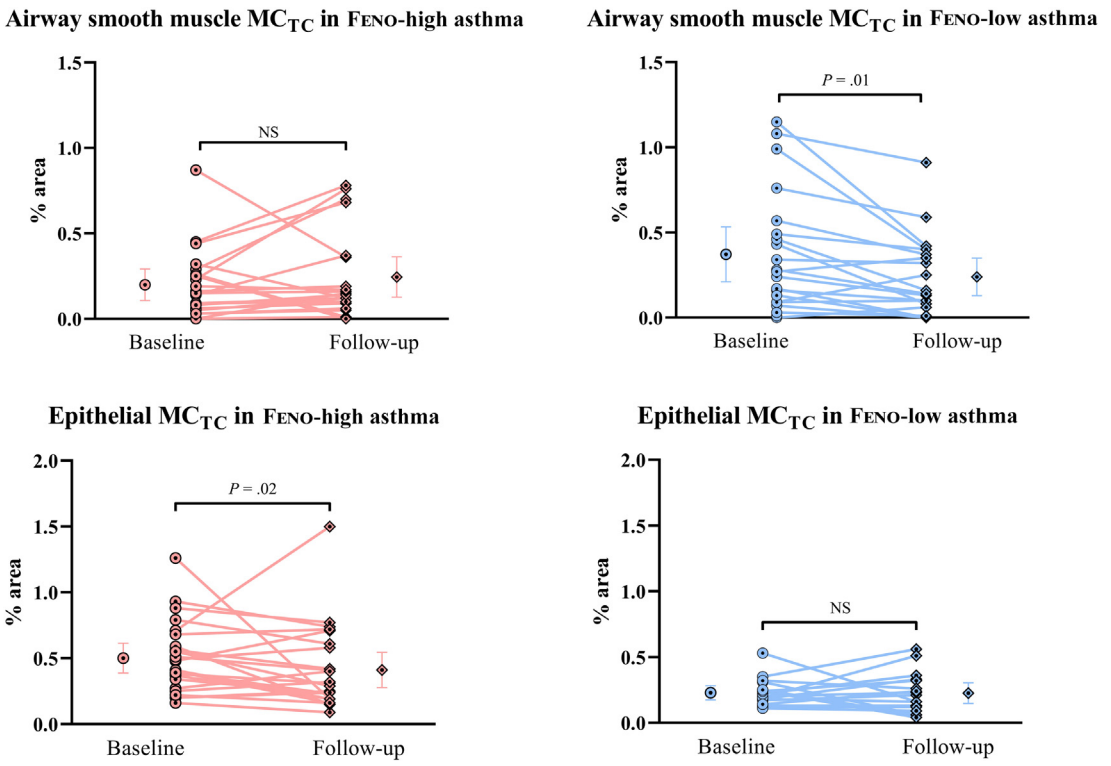

FIG 4. Density of chymase-high mast cells (MC_{TC}) infiltrating the airway smooth muscle and the epithelium in patients with FENO-high and FENO-low asthma before and after treatment with inhaled corticosteroids. Absolute measures and geometric mean with 95% CI. *NS*, Not significant.

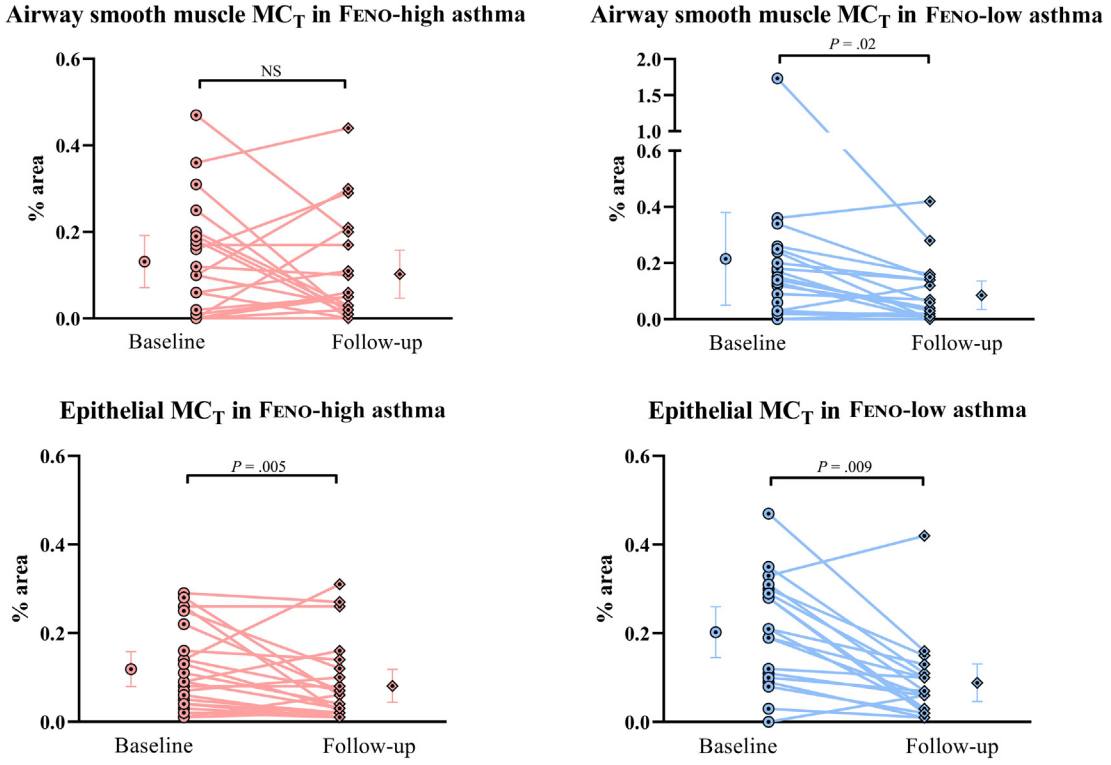

FIG 5. Density of tryptase-high mast cells (MC_T) infiltrating the airway smooth muscle and the epithelium in patients with FENO-high and FENO-low asthma before and after treatment with inhaled corticosteroids. Absolute measures and geometric mean with 95% CI. *NS*, Not significant.

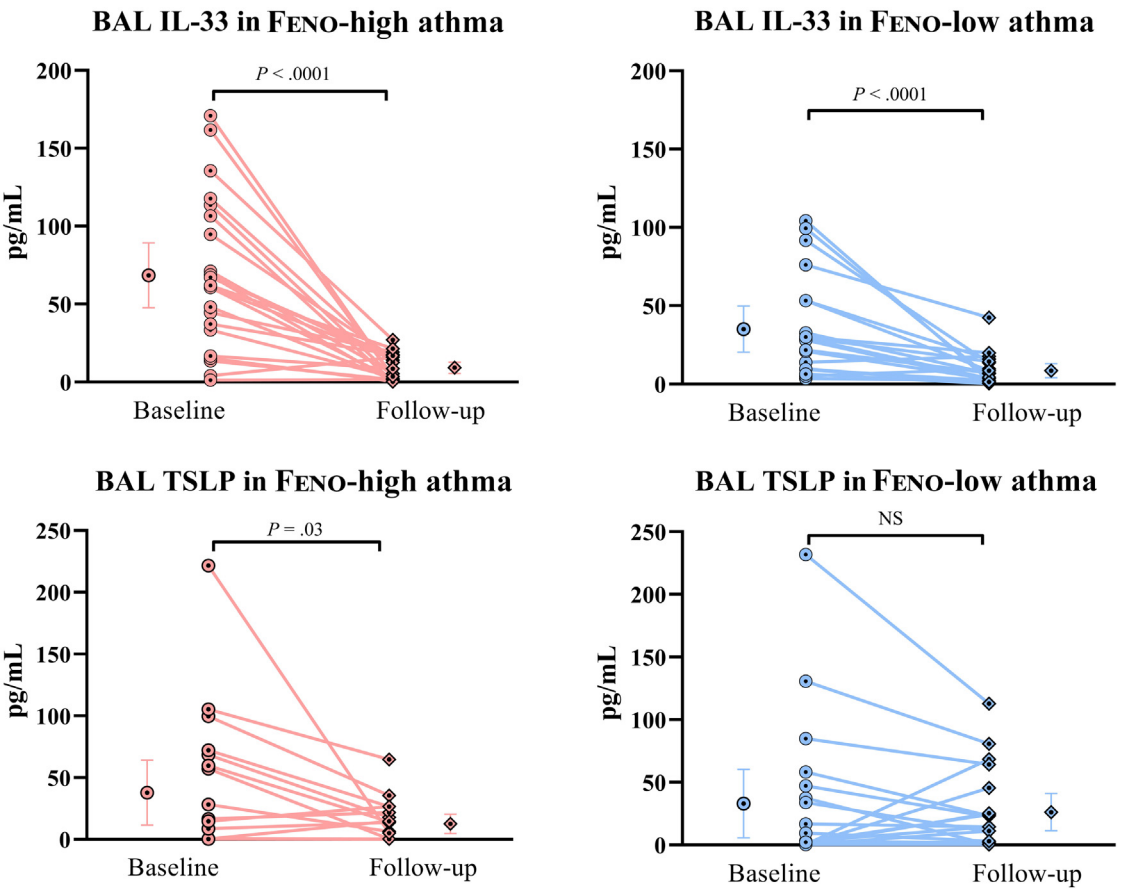

FIG 6. IL-33 and TSLP concentrations in BAL fluid from patients with FENO-high and FENO-low asthma before and after treatment with inhaled corticosteroids. Absolute measures and geometric mean with 95% CI. *NS,* Not significant.

Furthermore, this study demonstrates a correlation between mast cells infiltrating the airway smooth muscle and airway hyperresponsiveness to indirect challenges in patients with low FENO.

Chymase-high mast cells predominated in the airways, which is in contrast with other studies of patients with mild to moderate asthma. In this study, all patients had airway hyperresponsiveness to mannitol at inclusion, which we have previously shown to be related to chymase-positive mast cell infiltration, and may therefore explain this discrepancy.[24,60]

Treatment with high-dose inhaled corticosteroids showed a significant reduction in chymase-high mast cells, with a concomitant improvement in airway hyperresponsiveness in both patient groups. These findings are supported by other studies showing reduction in mast cell numbers after treatment with inhaled corticosteroids.[27] In patients with severe asthma, persistent mast cells infiltrating the airway smooth muscle have however also been linked to corticosteroid insensitivity, which may emphasize the need of treatment options specifically targeting mast cells.[61] Following treatment, we also observed a reduction in BAL IL-33 in both patients with FENO-high and FENO-low asthma and TSLP in patients with FENO-high asthma, and we speculate that mast cell activation through secretion of IL-33 and TSLP from the epithelium or other sites may be of importance, but because this is in contrast with earlier studies, this needs to be further investigated.[22,23,62]

Although a major strength of the present study is the high-quality airway specimens obtained from corticosteroid-free patients with asthma before and after treatment, the study holds some inherent limitations. Because no placebo arm was incorporated in the study, the effects of inhaled corticosteroids observed could be attributed to random effects or regression toward the mean. Although no placebo-controlled studies have cemented the effect of inhaled corticosteroids, several observational studies on general asthma populations have shown similar effects of inhaled corticosteroids on airway hyperresponsiveness[12,26,29,63] as have placebo-controlled studies investigating the additional effect of tezepelumab in patients already treated with inhaled corticosteroids.[22,23]

We used nitric oxide concentrations in exhaled breath as a marker of T2 inflammation to stratify patients into 2 different clinically recognizable phenotypes. FENO is not a perfect marker of T2 inflammation; however, in this cohort of corticosteroid-free nonsmoking patients with asthma, we considered it to be fairly good especially when using a low cutoff of 25 parts per billion.[64,65] This is strengthened by the sputum eosinophilic counts, but we were unfortunately not able to detect T2 cytokine concentrations in BAL fluid, which could have been used further to characterize the findings. Plots of FENO levels against MC_{TC} in filtrating the airway smooth muscle and the epithelium, respectively, are shown in Fig E1 in this article's Online Repository at www.jacionline.org.

In the characterization of infiltrating mast cells, we used a validated protocol for differentiation between mast cells containing *only* tryptase (MC_T) and mast cells containing both tryptase and

chymase (MC_{TC}).[48,49] Although this MC_T and MC_{TC} classification is commonly used to classify mast cells, other mast cell proteases or mediators may also potentially contribute to an airway hyperresponsiveness–associated mast cell phenotype. Indeed, knowledge of the significance of protease content in mast cells and the effects in asthma is limited and makes clinical interpretation difficult.[57,58] However, the suggested relationship between airway hyperresponsiveness and chymase-positive mast cells seems to be further strengthened by the present data.[24,60]

The present histology-based assessment of tissue mast cells is based on the calculation of the fraction of tissue area positive for mast cell protease markers. As such, and in contrast with robust enumeration of mast cell numbers by, for example, stereology approaches,[21] differences in marker tissue density could in theory be influenced by the degranulation status of the mast cells. However, the protease-based cell objects had similar granular appearance across the study material, suggesting that the present correlation of epithelial and smooth muscle mast cell marker with airway hyperresponsiveness was a result mainly caused by differences in mast cell numbers rather than altered degranulation.

The clinical effect of inhaled corticosteroids that was demonstrated in patients with low FENO and airway hyperresponsiveness to mannitol emphasizes a rationale of inhaled corticosteroids in at least some patients without signs of ongoing T2 inflammation. Currently, there are no available biomarkers to predict response in these patients, but we believe the mannitol test may hold the ability to identify a subgroup that may benefit not only from treatment with inhaled corticosteroids but perhaps also from new drugs specifically targeting mast cell activity; however, this needs to be further validated.[66,67]

Conclusions

This study proposes a relationship between airway hyperresponsiveness and mast cells infiltrating the epithelium in patients with asthma and a high FENO level and mast cells infiltrating the airway smooth muscle in patients with asthma and a low FENO level. Airway hyperresponsiveness and infiltrating mast cell numbers were both reduced following a 6-week course of high-dose inhaled corticosteroid treatment irrespective of FENO levels, leading to the conclusion that airway hyperresponsiveness is related to a differentiated distribution of mast cells in the airways among patients with high and low FENO levels, which in both patient groups are corticosteroid-sensitive and potential treatment targets.

Key messages

- **Airway hyperresponsiveness is related to mast cells infiltrating the airway epithelium in FENO-high asthma phenotypes and the airway smooth muscle in FENO-low asthma phenotypes.**
- **Treatment with inhaled corticosteroids improves airway hyperresponsiveness and decreases infiltrating mast cells across asthma phenotypes.**

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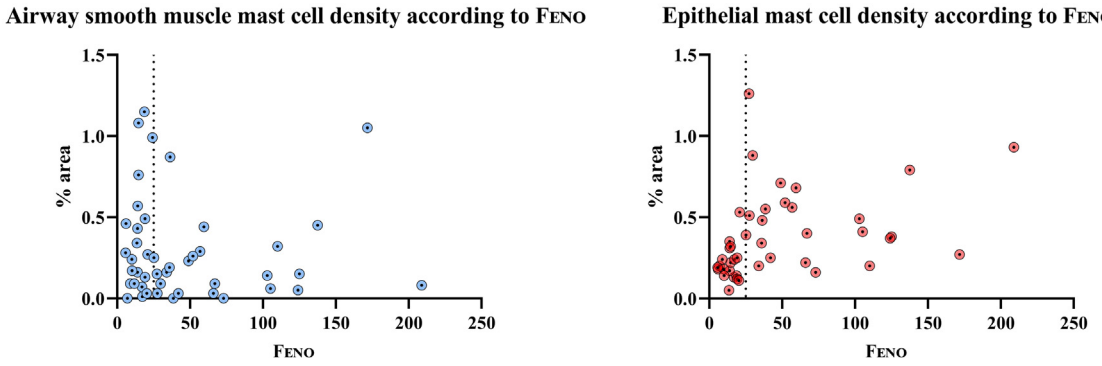

FIG E1. FENO levels at baseline against chymase-high mast cells infiltrating the epithelium and the airway smooth muscle. FENO cutoff of 25 ppb marked with dashed line. *ppb*, Parts per billion.

TABLE E1. Study exclusion criteria

Any corticosteroid-containing medication for past 3 mo
FEV_1 < 70% of predicted
Active smoking or a history of smoking with more than 10 pack years
Competing respiratory diseases including lower respiratory tract infections within the past 4 wk
Significant comorbidity (ASA < 2)
Pregnancy or breast-feeding
Hypersensitivity to study medication
Uncontrolled hypertension
Acute myocardial infarction within past 6 mo
Aorta or cerebral aneurisms
Recent abdominal operation
Failure to comply with the study protocol in general

ASA, American Society of Anesthesiologists.

TABLE E2. Pearson correlations between infiltrating mast cell phenotypes in airway smooth muscle and epithelium and airway hyperresponsiveness in patients with FENO-high and FENO-low asthma at baseline

Mast cell phenotype and location	FENO-high asthma		FENO-low asthma	
	ρ	*P*	ρ	*P*
Total biopsy				
Total MC	−0.317	.13	**−0.501**	**.024**
MC_T	−0.033	.88	0.101	.67
MC_{TC}	−0.329	.12	**−0.500**	**.025**
Epithelium				
Total MC	**−0.418**	**.042**	**−0.508**	**.022**
MC_T	0.116	.59	0.115	.63
MC_{TC}	**−0.418**	**.042**	0.008	.97
Airway smooth muscle				
Total MC	−0.058	.07	−0.425	.055
MC_T	−0.024	.91	−0.128	.58
MC_{TC}	−0.051	.81	**−0.447**	**.042**

Statistical significant correlations are highlighted in boldface.

TABLE E3. Proinflammatory mediator levels in BAL fluid in patients with T2-high and T2-low asthma in response to inhaled corticosteroid treatment

	FENO-high asthma		FENO-low asthma		
Cytokine (pg/mL)	Baseline	Follow-up	Baseline	Follow-up	*P*
IL-1β	5.68 (8.35)	2.56 (7.75)	6.05 (7.68)	1.03 (4.51)	.82
IL-2	4.43 (7.15)	2.18 (3.69)	3.19 (5.30)	2.00 (3.37)*	.48
IL-6	9.98 (15.16)	5.37 (14.92)	10.75 (19.13)	5.18 (25.21)	.91
IFN-γ	2.48 (4.79)	2.07 (4.79)	2.48 (4.61)	0.49 (1.42)*	.70
CCL-20	29.89 (41.32)	16.92 (52.41)	63.49 (118.89)	22.96 (46.51)*	.70

Data are presented as median with interquartile range. Wilcoxon signed-rank test was used for difference. All other measured cytokines (CD40 ligand/TNF-SF5, GM-CSF, IL-4, IL-5, IL-10, IL-12, p70, IL-13, IL-15, IL-17/IL-17A, IL-17E/IL-25, and TNF) were under detection level.
*Significant change from baseline to follow-up. *P* for difference in change in between groups.