

Airway smooth muscle enlargement is associated with protease-activated receptor 2/ligand overexpression in patients with difficult-to-control severe asthma

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Background: Asthma is a complex disease with heterogeneous features of airway inflammation and remodeling. The increase in airway smooth muscle (ASM) mass is an essential component of airway remodeling in patients with severe asthma, yet the pathobiological mechanisms and clinical outcomes associated with ASM enlargement remain elusive.

Objective: We sought to compare ASM area in control subjects and patients with mild-to-moderate or severe asthma and to identify specific clinical and pathobiological characteristics associated with ASM enlargement.

Methods: Bronchial biopsy specimens from 12 control subjects, 24 patients with mild-to-moderate asthma, and 105 patients with severe asthma were analyzed for ASM area, basement membrane thickness, vessels, eosinophils, neutrophils, T lymphocytes, mast cells, and protease-activated receptor 2 (PAR-2). In parallel, the levels of several ASM mitogenic factors, including the PAR-2 ligands, mast cell tryptase, trypsin, tissue factor, and kallikrein (KLK) 5 and KLK14, were assessed in bronchoalveolar lavage fluid. Data were correlated with asthma severity and control both at inclusion and after 12 to 18 months of optimal management and therapy.

Results: Analyses across ASM quartiles in patients with severe asthma demonstrated that patients with the highest ASM quartile (median value of ASM area, 26.3%) were younger (42.5 vs ≥ 50 years old in the other groups, $P \leq .04$) and had lower asthma control after 1 year of optimal management ($P \leq .006$). ASM enlargement occurred independently of features of airway inflammation and remodeling, whereas it was associated with PAR-2 overexpression and higher alveolar tryptase ($P \leq .02$) and KLK14 ($P \leq .03$) levels.

Conclusion: Increase in ASM mass, possibly involving aberrant expression and activation of PAR-2-mediated pathways, characterizes younger patients with severe asthma with poor asthma control. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Airway remodeling, asthma control, mast cells, tryptase, protease-activated receptor 2, kallikreins

Several clinical phenotypes of severe asthma have been recently recognized according to age of asthma onset, frequency of exacerbations, presence of fixed airflow obstruction, and diminished or no corticosteroid response.¹ Usual therapeutic approaches are focused on high doses of inhaled corticosteroids (ICSs), long-acting β_2 -agonists (LABAs), and omalizumab, the mAb directed against IgE.^{2,3} Identification of eosinophilic, neutrophilic, and mixed cellularity phenotypes of severe asthma⁴ has led to the development of new treatments, such as anti-IL-5 mAb, which showed efficacy in improving asthma outcomes, particularly in steroid-unresponsive hypereosinophilic subjects.^{5,6} However, in some patients symptoms of severe asthma persist despite limited or no inflammatory responses.⁷ In addition, recent studies in asthmatic children demonstrated that certain structural changes of the airway wall, referred to as “remodeling,” occur independently on inflammation.⁸

Increase in airway smooth muscle (ASM) mass is an important component of airway remodeling in asthmatic patients that accounts for airway narrowing and loss of respiratory function.^{9,10} ASM cells from asthmatic patients proliferate faster than cells from nonasthmatic subjects,¹¹ and they sustain airway inflammation and remodeling by producing a large array of proinflammatory and fibrotic mediators.¹² We and others have shown that ASM increased in relation to asthma severity.¹³⁻¹⁵ However, no analyses have been conducted to determine whether clinical and pathobiological features of severe asthma differ depending on the degree of ASM enlargement.

The current study was aimed at comparing the extent of ASM area in bronchial biopsy specimens from patients with severe

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Abbreviations used

ACT:	Asthma Control Test
ASM:	Airway smooth muscle
ATS:	American Thoracic Society
BAL:	Bronchoalveolar lavage
bFGF:	Basic fibroblast growth factor
EGF:	Epidermal growth factor
FVC:	Forced vital capacity
ICS:	Inhaled corticosteroid
KLK:	Kallikrein
LABA:	Long-acting β_2 -agonist
OCS:	Oral corticosteroid
PAR-2:	Protease-activated receptor 2
PCNA:	Proliferating cell nuclear antigen
PDGF:	Platelet-derived growth factor
SBM:	Subepithelial basement membrane
TF:	Tissue factor

asthma in relation to clinical outcomes, in particular asthma control, after 12 to 18 months of maximal therapy. The extent of ASM area was also assessed in healthy volunteers and in patients with mild-to-moderate asthma and compared with that of patients with severe asthma. We also determined whether changes in ASM area were associated with expression of the G protein–coupled receptor protease-receptor 2 (PAR-2)¹⁶ with some of the natural ligands that drive ASM proliferation, including mast cell tryptase, trypsin, tissue factor (TF), and kallikrein (KLK) 5 and KLK14,^{17–21} and with the ASM mitogenic growth factors epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) BB.^{22–24}

METHODS**Subjects and sample collection**

Twelve control subjects, 24 patients with mild-to-moderate asthma, and 105 patients with severe asthma who met the American Thoracic Society (ATS) workshop criteria for severe asthma² were included at the Pneumology A Department of Bichat Hospital in Paris (Table I). Clinical and respiratory function and Asthma Control Test (ACT) values were assessed at inclusion in all patients and both at inclusion and every 6 months for 12 to 18 months in patients with severe asthma (see Table E1 in this article's Online Repository at www.jacionline.org).

Bronchoscopy with a 50-mL bronchoalveolar lavage (BAL) was performed at inclusion after achievement of local anesthesia. One-milliliter aliquots of BAL supernatants were stored at -80°C for further immunoassays, and the proportion of eosinophils and neutrophils was determined in cytopsin preparations after Diff-Quik staining. Two biopsy specimens were taken from the subcarinae of the middle lobe (B4-B5), fixed in 10% formaldehyde, and processed to paraffin wax for immunohistochemical and morphometric studies.¹³

Immunohistochemistry and morphometry

Bronchial biopsy specimens were assessed for determination of subepithelial basement membrane (SBM) thickening and ASM area by using morphometry after hematoxylin and eosin staining and α -actin immunostaining, respectively.¹³ In addition, ASM proliferation was assessed by immunolocalizing proliferating cell nuclear antigen (PCNA) to ASM nuclei, as previously described.²⁵ T lymphocytes (CD3⁺), vessels (CD31⁺), eosinophils and neutrophils (major basic protein and elastase positive, respectively), and mast cells (tryptase and chymase positive) were enumerated (see Table E2 in this article's Online Repository at www.jacionline.org). Finally, the

expression of PAR-2 by the ASM was examined by using immunohistochemistry,²⁶ and its intensity was quantified by means of digital image analysis.²⁷

All the parameters were examined in 2 to 3 noncontiguous sections from the same biopsy specimen at different depths for each biopsy specimen and in sections from 2 different biopsy specimens. Sections were randomly assigned and analyzed blind by 2 observers. The final figure was the mean of all measurements obtained for each patient. The coefficients of variation between biopsy specimens from the same patient and between each parameter of the same biopsy specimen were 8% to 14% and 4% to 9%, respectively.

Mediator assessment in BAL fluid

EGF, bFGF, and PDGF-BB levels were determined in BAL fluid supernatants by using Multiplex multianalyte technology (Merck Millipore, Molsheim, France). In addition, mast cell tryptase was assessed by using the ImmunoCAP assay (Thermo Scientific, Phadia, St Quentin-les-Yvelines, France), whereas trypsin, TF, and KLK-5 and KLK-14 levels were measured by means of ELISA (R&D Systems, Lille, France).

Statistical analysis

Qualitative variables are reported as numbers and percentages and compared by using the Fisher exact test. Normally distributed quantitative variables are reported as means and SDs and compared by using the *t* test and 1-way ANOVA. Nonnormal variables are reported as medians and 25th to 75th percentiles and compared by using the Mann-Whitney *U* test and Kruskal-Wallis analysis. Exacerbation rates were compared between study groups with Poisson regression. We used the paired Student test, Wilcoxon test, and McNemar test to compare the evolution of characteristics measured in the same patients over time (inclusion and follow-up). Potential associations between biological and physiologic variables and ASM area have been assessed by using the nonparametric Spearman rank correlation coefficient (Rho). Benjamini-Hochberg correction with a family-wise error rate of 0.05 has been used to adjust for multiple comparisons. All tests were 2-tailed; *P* values of less than .05 were considered significant. Data were analyzed statistically with R 2.15.2 software (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS
Patients

Twelve control subjects, 24 patients with mild-to-moderate asthma, and 105 patients with severe asthma were examined at inclusion (Table I). The majority of subjects in all groups were female, and the prevalence of atopy did not differ between the asthmatic groups. Blood eosinophilia was increased to a similar extent in patients with mild-to-moderate asthma and those with severe asthma compared with values in control subjects (Table I). Control subjects and patients with mild-to-moderate asthma had normal respiratory function, whereas patients with severe asthma had airflow obstruction, with a prebronchodilator and postbronchodilator FEV₁ of less than 80% and an FEV₁/forced vital capacity (FVC) ratio of less than 70% (Table I). Patients with mild-to-moderate and those with severe asthma were treated with 800 $\mu\text{g}/\text{d}$ or less and 2000 $\mu\text{g}/\text{d}$ or greater of beclomethasone equivalents, respectively. Twenty-one percent of patients with severe asthma also required maintenance with mean daily doses of 20 mg or more of oral prednisone, and 15% of them had also been treated with anti-IgE (omalizumab) since at least 6 months (Table I).

ASM area, airway inflammation, and remodeling

ASM area was significantly increased in patients with severe compared with those with mild-to-moderate asthma (*P* = .013)

TABLE I. Characteristics of control subjects and asthmatic patients at inclusion*

Parameter	Control subjects	Patients with mild-to-moderate asthma	Patients with severe asthma	P value†
No. of patients	12	24	105	
Female sex, no. (%)	8 (66)	20 (83)	65 (67)	.28
Age (y)	48.8 ± 16.6	53.0 ± 15.1	48.5 ± 13.5	.98
Caucasian origin, no. (%)	10 (83)	18 (75)	72 (69)	.57
Former smokers, no. (%)	1 (8)	4 (17)	23 (22)	.63
Packs of cigarettes per year	15	8.3 ± 2.4	9.9 ± 9.2	.66
Body mass index (kg/m ²)	31.8 ± 10.2	29.5 ± 9.6	27.1 ± 6.1	.08
Age of asthma onset (y)	—	26.0 ± 19.6	23.0 ± 15.5	.07
Asthma duration (y)	—	27.0 ± 19.5	27.0 ± 16.2	.83
History of atopy, no. (%)	0	17 (71)	74 (70)	1.00
Blood eosinophils (10 ⁹ /L)	110 (100-240)	180 (120-280)	309 (158-497)	.06
Total serum IgE (IU/mL)	18.0 (11.5-30.3)	192 (83-328)‡	200 (100-452)‡	<.0001
Respiratory function				
Prebronchodilator FEV ₁ (% predicted)	107.3 ± 20.5	94.2 ± 20.3	67.0 ± 18.4‡§	<.0001
Postbronchodilator FEV ₁ (% predicted)	107.9 ± 16.6	101.0 ± 19.6	72.6 ± 18.6‡§	<.0001
Prebronchodilator FEV ₁ /FVC (% predicted)	ND	71.6 ± 10.9	61.4 ± 12.4	.0003
Postbronchodilator FEV ₁ /FVC (% predicted)	ND	74.9 ± 9.6	64.7 ± 12.0	.0002
Reversibility to β_2 -agonists (%)	1.3 ± 2.0	6.8 ± 5.6‡	5.8 ± 5.8‡	.014
Treatments				
LABAs, no. (%)	—	12 (50)	105 (100)	<.0001
Dose of ICS (μ g equivalents of beclomethasone per day)	—	813 ± 247	2667 ± 947	<.0001
On maintenance use of OCS (%)	—	0	22 (21)	.01
Daily dose of OCS (mg)	—	0	21.2 ± 13.9	<.0001
Anti-IgE, no. (%)	—	0	16 (15)	.04

Boldface denotes statistical significance.

ND, Not determined.

*Data are presented as numbers (percentages), means ± SDs, or medians (25th-75th interquartile ranges).

†ANOVA or Fisher exact test (2-tailed).

‡ $P < .05$ compared with control subjects and § $P < .05$ compared with patients with mild-to-moderate asthma (Student *t* test).

and control subjects ($P = .0004$; Fig 1, A-C). In patients with mild-to-moderate and those with severe asthma, ASM area ranged from 0.4% to 18.3% (median, 9.7%; 25th-75th interquartile range, 1.6% to 13.4%) and 1.2% to 42.7% (median, 13.2%; 25th-75th interquartile range, 7.1% to 18.4%), respectively. In large part the increase in ASM area observed in patients with severe asthma was due to cell proliferation because the proportion of PCNA⁺ ASM nuclei over that of total ASM nuclei was significantly greater in patients with severe compared with those with mild-to-moderate asthma ($P = .037$) and control subjects ($P = .029$, see Fig E1 in this article's Online Repository at www.jacionline.org). The proportion of PCNA⁺ nuclei significantly correlated with the extent of ASM area in all asthmatic patients ($r_s = 0.40$, $P = .003$) and patients with severe asthma ($r_s = 0.52$, $P = .001$).

Because of the large variability in the extent of ASM area, we performed analyses after stratification of the 105 patients with severe asthma into quartiles (Fig 1, A and D). Median values of ASM area in the 4 patient groups were as follows: Q1, 4.8% (25th-75th interquartile range, 3.4% to 5.9%); Q2, 10.8% (25th-75th interquartile range, 8.0% to 11.7%); Q3, 15.3% (25th-75th interquartile range, 14.0% to 16.2%); and Q4, 26.4% (25th-75th interquartile range, 21.3% to 29.7%). ASM area from Q3 and Q4, but not from Q1 and Q2, was significantly increased compared with that measured in patients with mild-to-moderate asthma ($P < .0001$ for both comparisons; Fig 1, A).

We then evaluated SBM thickening, density of vessels (CD31⁺ cells), and total numbers of submucosal CD3⁺ T cells, mucosal

and intramuscular tryptase- and chymase-positive mast cells, BAL and mucosal eosinophils, and neutrophils (Table II). When compared with control subjects, patients with mild-to-moderate asthma had significantly higher numbers of T cells, mucosal and BAL fluid eosinophils, and intramuscular mast cells. No changes were found in terms of SBM thickening, blood vessels, and BAL fluid and mucosal neutrophils (Table II). In the 105 patients with severe asthma stratified according to ASM quartiles, SBM thickening and BAL fluid eosinophil counts were higher in patients in ASM Q2, Q3, and Q4 in relation to those in control subjects, patients with mild-to-moderate asthma, or both, whereas the other features of airway inflammation and remodeling were significantly downregulated. These parameters did not differ across ASM quartiles (Table II).

Although the prevalence of patients with severe asthma with detectable intramuscular tryptase- and chymase-positive mast cells was comparable between ASM Q3 and Q4 (60% and 74%, respectively) and patients with mild-to-moderate asthma (71%), values in ASM Q3 and Q4 were higher than those found in ASM Q1 and Q2 (8% and 22%, respectively; see Fig E2 in this article's Online Repository at www.jacionline.org).

Multiple correlation analyses performed in all asthmatic patients and in patients with severe asthma did not provide significant associations between ASM area and the other inflammatory and remodeling parameters examined, both in bronchial biopsy specimens and in BAL fluid (see Table E3 in this article's Online Repository at www.jacionline.org). The unique significant correlation was found between ASM area and the density of

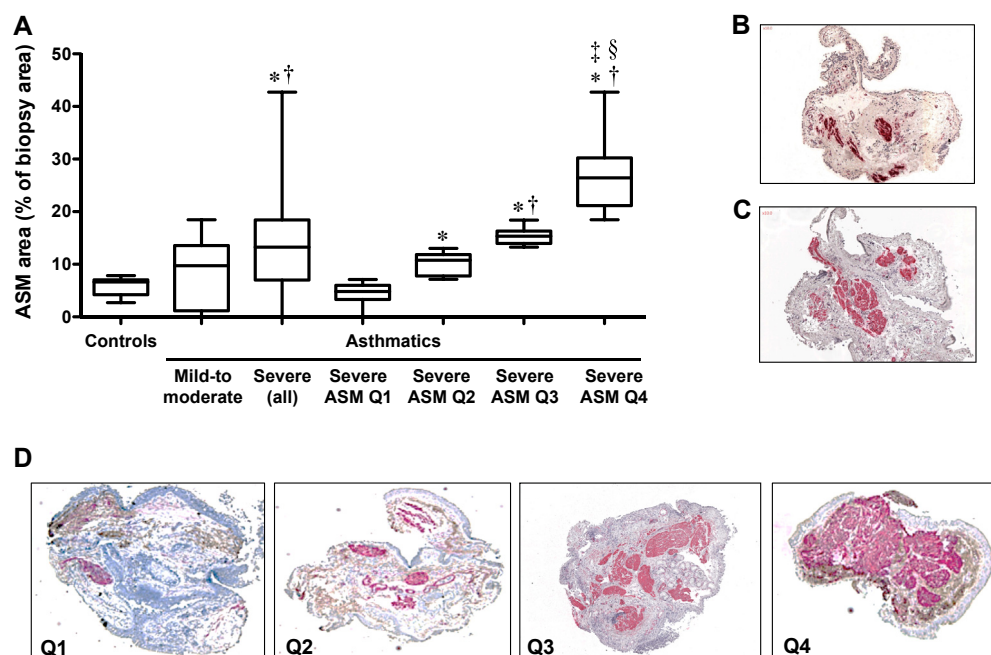


FIG 1. ASM area in control subjects and asthmatic patients. **A**, ASM area (expressed as the proportion of ASM area over the total biopsy area) was determined by using morphometry and image analysis on α -actin-stained bronchial biopsy specimens from 12 healthy volunteers, 24 patients with mild-to-moderate asthma, and 105 patients with severe asthma. This latter patient group was also analyzed across ASM quartiles. Horizontal bars inside boxes represent median values, and vertical bars represent 25% to 75% interquartile ranges. Overall comparison was made with the Kruskal-Wallis test ($P = .0002$). * $P < .05$ compared with control subjects, † $P < .05$ compared with patients with mild-to-moderate asthma, ‡ $P < .05$ compared with patients with severe asthma of ASM Q1, and § $P < .05$ compared with patients with severe asthma of ASM Q2 (Mann-Whitney U test). **B-D**, Representative immunostaining for α -actin (red deposit) in a control subject (Fig 1, B), a patient with mild-to-moderate asthma (Fig 1, C), and 4 patients with severe asthma in ASM Q1, Q2, Q3, and Q4 (Fig 1, D). Original magnification $\times 40$.

intramuscular tryptase- and chymase-expressing mast cells in all asthmatic patients and patients with severe asthma ($P < .0001$ for all comparisons, see Table E3).

BAL levels of ASM mitogenic mediators

To investigate the mechanisms of ASM enlargement in patients with severe asthma, we assessed BAL levels of EGF, bFGF, and PDGF-BB, which are potent mitogenic growth factors for ASM (see Table E4 in this article's Online Repository at www.jacionline.org).²²⁻²⁴ We found a significant increase in BAL levels of these 3 mediators in patients with mild-to-moderate asthma in relation to control subjects. These levels were progressively downregulated in patients with severe asthma, particularly in those in ASM Q3, Q4, or both (see Table E4).

BAL levels of EGF and bFGF negatively correlated with ASM area in asthmatic patients ($r_s = -0.185$, $P = .05$ and $r_s = -0.223$, $P = .02$, respectively), whereas no correlation was found between BAL levels of PDGF-BB and ASM area ($r_s = -0.10$, $P = .30$).

Expression of PAR-2 and PAR-2 ligands

Previous studies have shown that the interaction between PAR-2 and its ligands, including mast cell-derived tryptase, promoted calcium mobilization, contraction, and proliferation of human ASM cells.^{17,18,21} Therefore we examined changes in ASM-associated PAR-2 expression in control subjects and asthmatic patients. Using immunohistochemistry and image

analysis, we found that the proportion of ASM area stained for PAR-2 was greater in patients with severe asthma compared with that in patients with mild-to-moderate asthma ($P < .0001$) and control subjects ($P = .0007$; Fig 2, A). In the 105 patients with severe asthma, this proportion was significantly increased in ASM Q2, Q3, and Q4 compared with control subjects and patients with mild-to-moderate asthma and compared with ASM Q1 (Fig 2, A), although no significant differences were found across ASM Q2, Q3, and Q4.

PAR-2 was localized to defined foci, although ASM areas with widespread and diffuse immunostaining were also observed (Fig 2, F and I). When present, PAR-2 also localized to the bronchial epithelium (Fig 2, E and H) and mucous glands (Fig 2, F).

The proportion of ASM stained for PAR-2 positively correlated with ASM area, both in all asthmatic patients ($r_s = 0.53$, $P < .001$) and those with severe asthma ($r_s = 0.50$, $P = .001$).

By analyzing the ratio of ASM surfaces stained for PAR-2 in bronchial tissue sections from control subjects and asthmatic patients in each of the 3 categories of intensity (1+, 2+, and 3+), we found higher degrees of PAR-2 expression (scores 2+ and 3+) in the ASM of patients with severe compared with those with mild-to-moderate asthma and control subjects ($P \leq .0002$ for all comparisons; Fig 3, A-D). These differences in the intensity of PAR-2 expression in ASM were easily measurable by using image analysis (Fig 3, D, G, and J).

In subanalyses performed in the 105 patients with severe asthma only, the proportion of patients with no or low PAR-2

TABLE II. Hallmarks of airway inflammation and remodeling in control subjects and asthmatic patients

Parameter	Control subjects	Patients with mild-to-moderate asthma	Patients with severe asthma				P value*
			ASM Q1	ASM Q2	ASM Q3	ASM Q4	
No. of patients	12	24	27	25	27	26	
SBM (μm)	5.0 (4.6-5.8) [†]	5.3 (4.4-5.7)	7.0 (5.9-7.9)	7.3 (6.4-8.6) ^{‡§}	6.8 (5.9-7.8) [§]	7.3 (6.4-7.9) ^{‡§}	.02
CD31 ⁺ cells/mm ² of biopsy area	32.3 (19.7-73.1)	65.7 (16.9-103.8)	33.6 (18.9-56.4)	40.2 (19.4-53.5)	31.7 (25.3-65.8)	33.6 (21.8-49.0)	.69
CD3 ⁺ cells/mm ² of biopsy area	37.0 (12.6-67.9)	118.4 (44.1-197.5) [§]	39.8 (14.0-60.9) [§]	52.1 (25.8-94.6)	41.4 (26.1-82.9) [§]	45.6 (15.2-114.1) [§]	.006
Tryptase-positive cells/mm ² of biopsy area	105.3 (71.5-123.5)	122.3 (78.6-185.1)	40.0 (3.0-62.0) ^{‡§}	62.0 (14.0-94.0) ^{‡§}	53.0 (19.5-96.0) ^{‡§}	60.6 (21.0-106.3) ^{‡§}	<.0001
Tryptase-positive cells/mm ² of ASM	0.0 (0.0-0.0)	43.5 (0.0-101.7) [§]	0.0 (0.0-0.0) [§]	0.0 (0.0-0.0) [§]	12.9 (0.0-28.6) ^{‡§}	38.2 (14.3-54.1) ^{‡§}	<.0001
Chymase-positive cells/mm ² of biopsy area	40.9 (16.8-89.0)	52.5 (34.2-77.1)	11.0 (1.0-27.5) [§]	15.0 (2.0-55.0) [§]	16.0 (5.5-38.0) [§]	17.0 (3.2-42.8) [§]	.0004
Chymase-positive cells/mm ² of ASM	0.0 (0.0-0.0)	22.4 (0.0-40.1) [§]	0.0 (0.0-0.0) [§]	0.0 (0.0-0.0) [§]	0.0 (0.0-13.4) ^{‡§}	7.8 (0.0-24.1) ^{‡§}	<.0001
MBP ⁺ cells/mm ² of biopsy area	0.0 (0.0-0.0)	4.7 (1.8-12.3) [‡]	1.1 (0.0-3.9) ^{‡§}	1.6 (0.0-6.9) [‡]	1.0 (0.0-14.1) [‡]	1.6 (0.0-22.8) [‡]	.01
Elastase-positive cells/mm ² of biopsy area	2.7 (1.9-4.0)	33.4 (5.1-73.9)	15.1 (8.9-35.0)	16.2 (6.2-35.6)	8.1 (2.8-36.4)	14.2 (6.7-27.1)	.40
Eosinophils in BAL fluid (%)	0.0 (0.0-0.7)	2.1 (0.0-5.5) [‡]	1.8 (0.2-12.3) [‡]	2.6 (0.0-10.7) [‡]	5.2 (0.4-14.8) [‡]	3.9 (1.6-9.1) [‡]	.01
Neutrophils in BAL fluid (%)	2.1 (1.1-3.0)	5.3 (1.8-12.3)	6.7 (1.7-14.4) [‡]	11.0 (4.1-35.2) [‡]	5.3 (0.8-11.9)	14.2 (6.7-27.1) [‡]	.05

Boldface indicates statistical significance.

MBP, Major basic protein.

*Kruskal-Wallis test.

†Data are presented as medians (25th-75th interquartile ranges).

‡P < .05 compared with control subjects and §P < .05 compared with patients with mild-to-moderate asthma (Mann-Whitney U test).

expression (ie, scores of 0 and 1+) gradually decreased across the ASM quartiles (Fig 3, E and F), whereas the rate of patients with middle and high PAR-2 expression (scores 2+ and 3+) progressively increased (Fig 3, G and H). In particular, patients with severe asthma in ASM Q4 had the highest proportion of ASM area with increased intensity of PAR-2 expression (score 3+; mean value ± SEM, 27.5% ± 5.7%; Fig 3, H).

We then examined whether PAR-2 overexpression in patients with severe asthma with a large ASM area was accompanied by changes in BAL levels of some of its ligands, such as mast cell tryptase,^{17,18} trypsin,¹⁸ TF,¹⁹ and KLK5 and KLK14.²⁰

A greater prevalence of patients with detectable BAL levels of tryptase was found in patients with severe compared with those with mild-to-moderate asthma (32% vs 17%, respectively; P = .02, Fisher exact test, 2-tailed; see Fig E3 in this article's Online Repository at www.jacionline.org), whereas this protease was undetectable in control subjects (see Fig E2). Analyses across ASM quartiles in patients with severe asthma showed a significantly higher proportion of patients with detectable BAL levels of tryptase in ASM Q3 (37%) and, to a higher extent, in ASM Q4 (62%) compared with the proportion in ASM Q1 and Q2 (8% and 20%, respectively; see Fig E2).

BAL concentrations of tryptase and KLK14 were higher in patients with severe asthma in ASM Q4 compared with control subjects and compared with patients in ASM Q1, Q2, and Q3 (Fig 4, A and D). Given the low proportion of patients with mild-to-

moderate asthma with detectable tryptase in BAL fluid (4 [17%] of 24 patients; Fig 4, A) and the large variability of the values (2500-23600 ng/mL), differences with patients with severe asthma in any of the 4 ASM quartiles did not reach statistical significance.

BAL levels of trypsin tended to increase in patients in ASM Q3 and Q4 compared with those in the other patient groups, yet the high variability of the data prevented the differences from reaching statistical significance (P = .54; Fig 4, B).

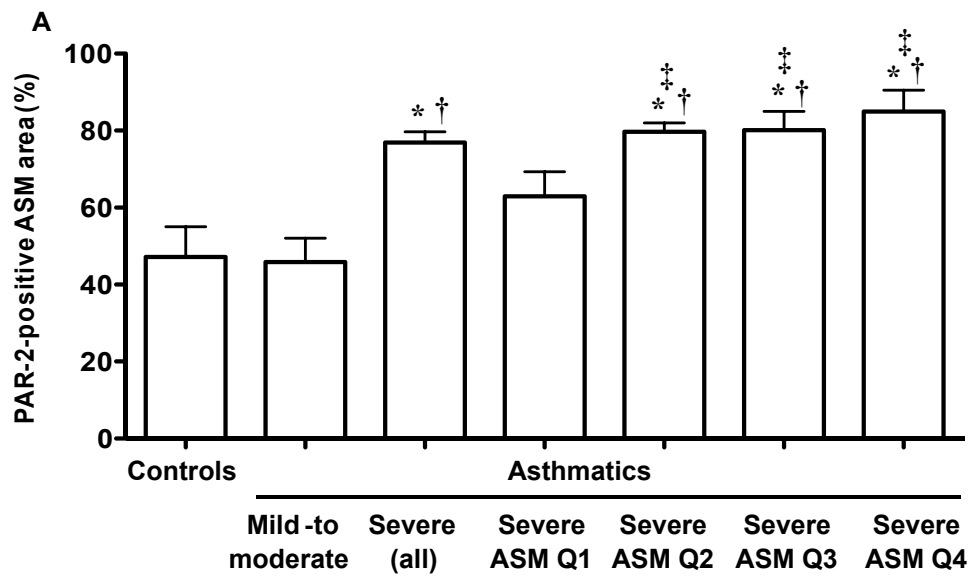
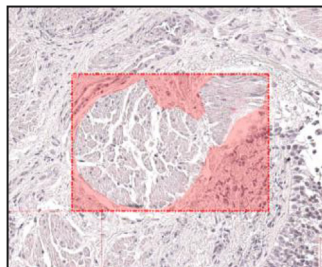
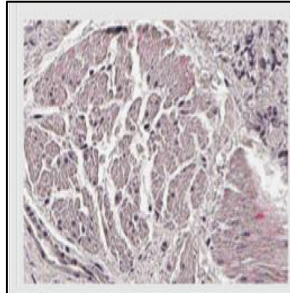
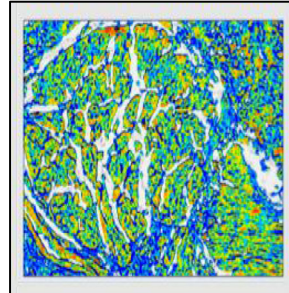
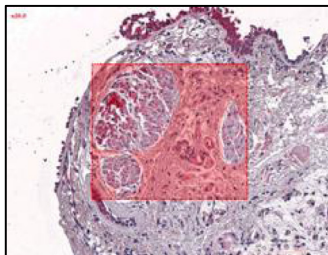
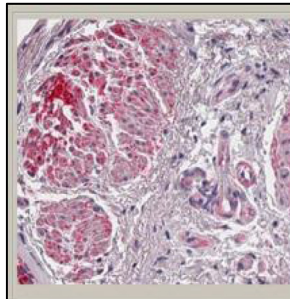
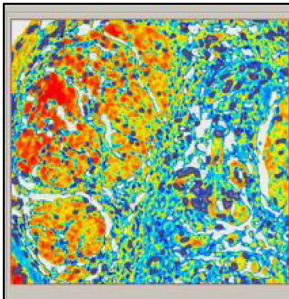
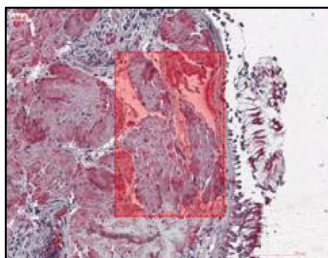
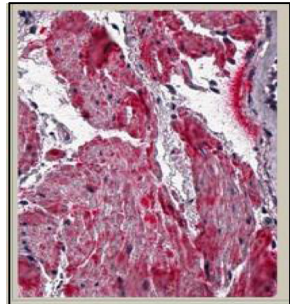
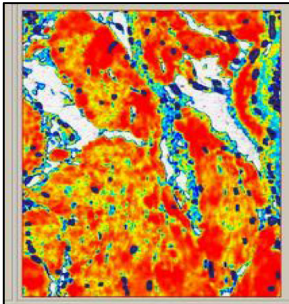
TF was significantly augmented in BAL fluid of patients with severe asthma belonging to all ASM quartiles compared with control subjects, and a significant increase in relation to patients with mild-to-moderate asthma was noted in patients in ASM Q4 (Fig 4, C).

Finally, KLK5 was detectable in 24% of BAL samples from all patient groups, and no difference in its levels was found between patients with mild-to-moderate and those with severe asthma (data not shown).

Spearman rank-order analysis showed positive correlations between BAL levels of tryptase and KLK14 (but neither trypsin nor TF) and ASM area in all asthmatic patients and patients with severe asthma (see Table E5 in this article's Online Repository at www.jacionline.org).

Clinical analyses

Analyses of categorical and continuous variables in asthmatic patients demonstrated that patients with severe asthma in ASM

**B****C****D****E****F****G****H****I****J**

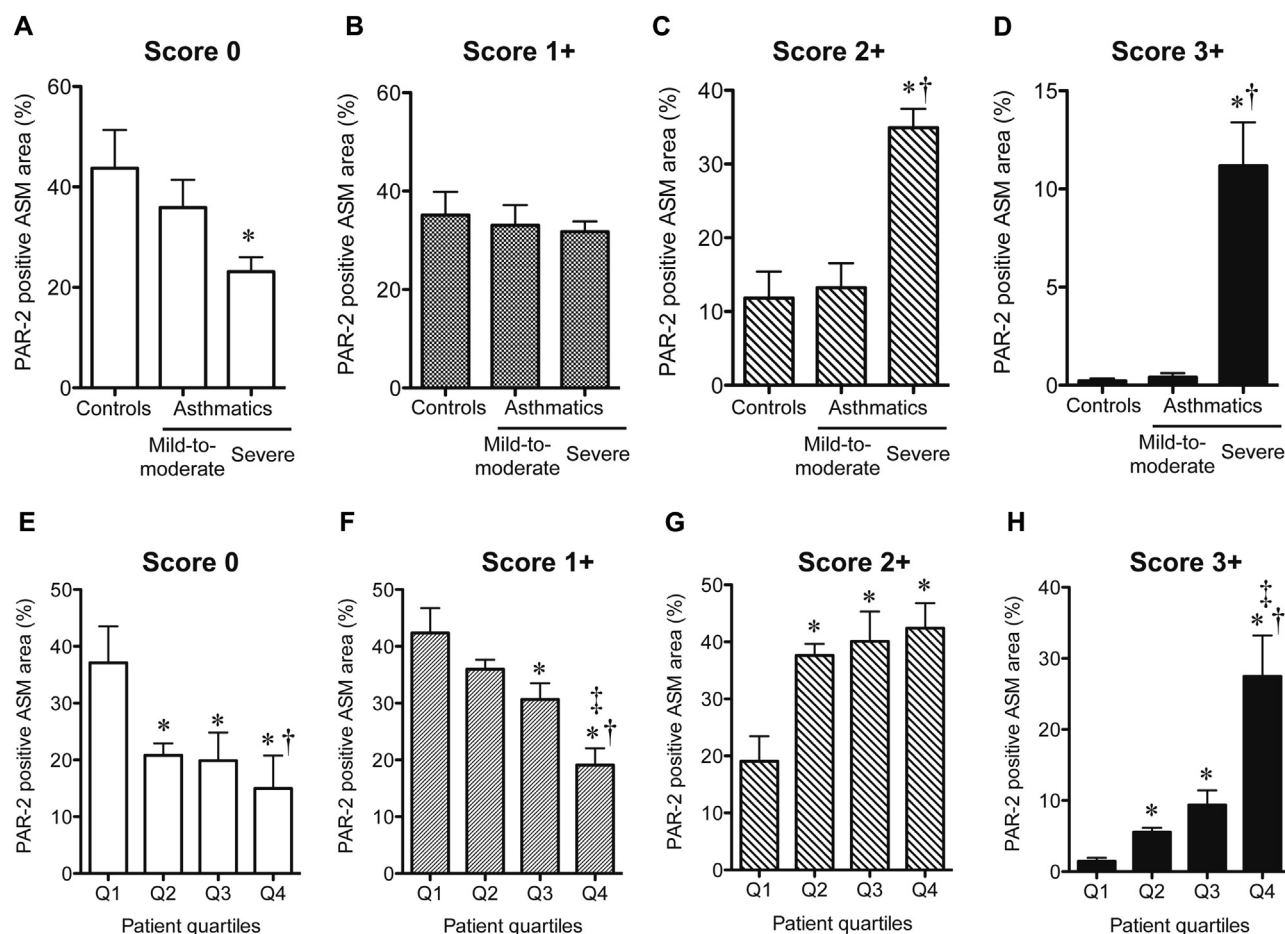


FIG 3. ASM-associated PAR-2 expression in control subjects and asthmatic patients. Comparison of PAR-2 expression in the ASM of control subjects, patients with mild-to-moderate asthma, and patients with severe asthma (A-D) and of patients with severe asthma, either considered as a whole population or classified according to ASM quartiles (E-H). The intensity of PAR-2 immunostaining was quantified by using an immunosurface algorithm that defined 4 categories of magnitude of expression (ie, absence = score 0 [Fig 3, A and E], low = score 1+ [Fig 3, B and F], moderate = score 2+ [Fig 3, C and G], and high = score 3+ [Fig 3, D and H]). Patients with severe asthma had a lower proportion of ASM area negative for PAR-2 immunostaining than patients with mild-to-moderate asthma and control subjects (Fig 3, A). In addition, the proportion of patients with severe asthma with no or low (score 1+) PAR-2 expression gradually decreased across the ASM quartiles (Fig 3, E and F), whereas the rate of patients with middle and high PAR-2 expression (scores 2+ and 3+) progressively increased (Fig 3, G and H). Patients with severe asthma in ASM Q4 had the highest proportion of ASM area with increased intensity of PAR-2 expression (Fig 3, H). Data are presented as means $\pm$ SEMs, and overall comparisons were made by using the Kruskal-Wallis test ($P = .027$ for Fig 3, A; $P = .342$ for Fig 3, B; $P < .0001$ for Fig 3, C; $P < .0001$ for Fig 3, D; $P = .01$ for Fig 3, E; $P = .001$ for Fig 3, F; $P = .0083$ for Fig 3, G; and $P < .0001$ for Fig 3, H). * $P < .05$ compared with control subjects, † $P < .05$ compared with patients with mild-to-moderate asthma (Fig 3, A-D). * $P < .05$ compared with Q1, † $P < .05$ compared with Q2, and ‡ $P < .05$ compared with Q3 in Fig 3, E-H (Mann-Whitney U tests).

FIG 2. Changes in ASM-associated PAR-2 expression in control subjects and asthmatic patients. **A**, Proportion of ASM area stained for PAR-2 in control subjects ($n = 12$), patients with mild-to-moderate asthma ($n = 24$), and patients with severe asthma ($n = 105$) and in the 105 patients with severe asthma analyzed across ASM quartiles. Data are presented as means $\pm$ SEMs, and overall comparisons were made by using the Kruskal-Wallis test ($P < .0001$). * $P < .05$ compared with control subjects, † $P < .05$ compared with patients with mild-to-moderate asthma, and ‡ $P < .05$ and § $P < .05$ compared with ASM Q1 (Mann-Whitney U test). **B-J**, Exemplary PAR-2 immunostaining in sections from bronchial biopsy specimens of a patient with mild-to-moderate asthma (Fig 2, B-D) and patients with severe asthma in ASM Q3 with a score of 1+ (Fig 2, E-G) and in ASM Q4 with a score of 3+ (Fig 2, H-J). Fig 2, B, E, and H, indicate PAR-2 immunostaining (red deposit), with high-power inserts in Fig 2, C, F, and I. Fig 2, D, G, and J show the corresponding immunosurface algorithm results (blue = negative, yellow = low intensity, orange = moderate intensity, and red = high intensity). Original magnification $\times 40$ (Fig 2, B, E, and H) and $\times 400$ (Fig 2, C, D, E-G, I, and J).

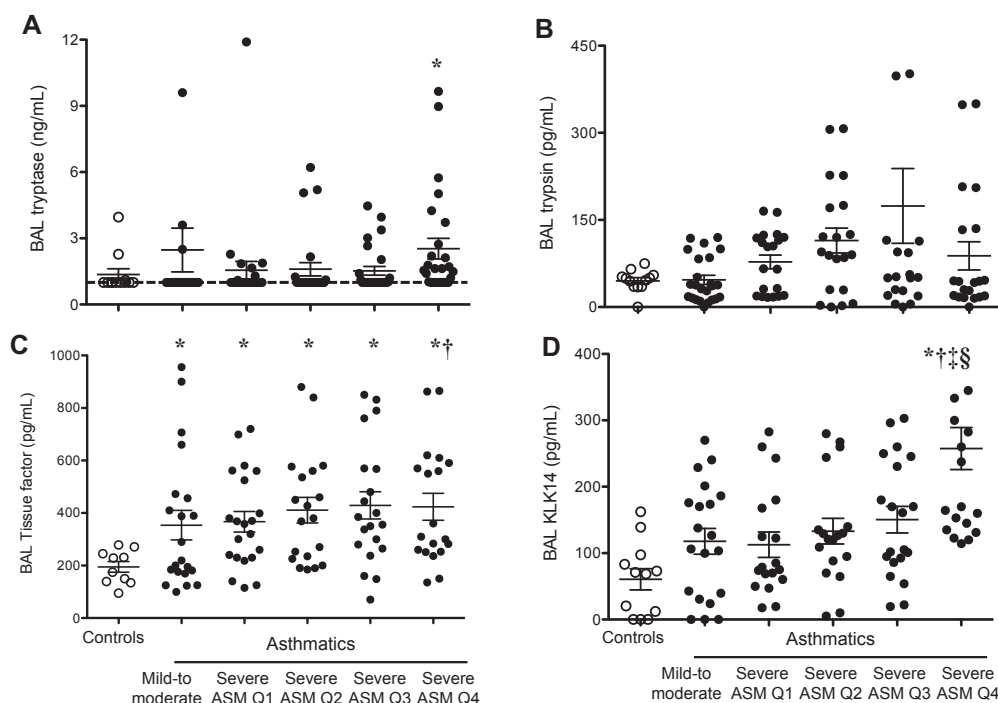


FIG 4. BAL fluid levels of PAR-2 ligands in control subjects and asthmatic patients. Scatter plot analyses showing individual levels of tryptase (**A**), trypsin (**B**), TF (**C**), and KLK14 (**D**) in BAL fluid of control subjects, patients with mild-to-moderate asthma, and patients with severe asthma classified according to ASM quartiles are shown. Horizontal bars indicate median values, and vertical bars denote 25% to 75% interquartile ranges. Overall P values were .0003 in Fig 4, **A**; .54 in Fig 4, **B**; .05 in Fig 4, **C**; and .04 in Fig 4, **D** (Kruskal-Wallis test). * $P < .05$ compared with control subjects, † $P < .05$ compared with patients with mild-to-moderate asthma, ‡ $P < .05$ compared with patients with severe asthma in ASM Q1, and § $P < .05$ compared with those in ASM Q2 (Mann-Whitney U test).

Q4 were younger than those with mild-to-moderate asthma and severe asthma in ASM Q1, Q2, and Q3 (overall $P = .05$, Table III). These patients tended to be predominantly male (50% in ASM Q4 compared with 17% to 37% in patients with mild to-moderate and those with severe asthma in ASM Q1, Q2, and Q3; $P = .07$, Table III).

No changes were found between patients with mild-to-moderate and those with severe asthma in all ASM quartiles in terms of ethnic origin, smoking history, atopy, age of asthma onset and duration, numbers of peripheral blood eosinophils, and levels of total IgE (Table III). In contrast, patients with severe asthma in ASM Q3 and Q4 had lower prebronchodilator and postbronchodilator FEV₁ compared with those in ASM Q1, ASM Q2, or both (Table III). Irrespective of the ASM quartiles, more than 75% of patients with severe asthma had poor asthma control at inclusion, and they experienced at least 5 severe exacerbations requiring treatment with oral corticosteroids (OCSs) in the previous year (Table III).

The same analyses were performed in the 105 patients with severe asthma after at least 1 year of optimal treatment. We found that the proportion of patients requiring maintenance use of OCSs tended to be higher in ASM Q4 than in ASM Q1, Q2, and Q3 (31% vs 15%, 8%, and 17%, respectively), although the differences were not statistically significant ($P = .24$). In addition, the prevalence of patients with severe asthma with poor asthma control was greater in ASM Q4 (65% in relation to 28%, 41% and to 47% in ASM Q1, Q2, and Q3; $P \leq .001$, Table III), with a significantly lower ACT score than that observed

in patients in ASM Q1 and Q2 ($P = .02$ and $P = .006$, respectively; Table III).

Multiple regression analyses showed that ASM area negatively correlated with the age of the patients and prebronchodilator FEV₁ both in the whole population of asthmatic patients and in those with severe asthma only (see Table E5). In all asthmatic patients ASM was also negatively associated with postbronchodilator FEV₁ and prebronchodilator FEV₁/FVC ratio (see Table E6 in this article's Online Repository at www.jacionline.org). Finally, ASM area negatively correlated with ACT scores at follow-up ($P = .04$, see Table E6).

In a separate analysis we confirmed previous studies performed in other cohorts,^{28,29} showing a significant correlation between peripheral blood eosinophil counts and the number of severe exacerbations occurring in the previous year ($r_s = 0.232$, $P = .018$). This contrasted with BAL and tissue eosinophil counts, which were not associated with the number of exacerbations ($r_s = 0.09$, $P = .37$ and $r_s = 0.178$, $P = .07$, respectively).

DISCUSSION

Using a cohort of 129 patients with mild-to-moderate or severe asthma and performing analyses across ASM quartiles in patients with severe asthma, we found that patients with larger ASM area (ASM Q4; median value of ASM area, 26.4%) were younger than patients with mild-to-moderate or severe asthma with low ASM areas. In addition, within the group of patients with severe asthma, the prevalence of patients whose symptoms remained

TABLE III. Clinical parameters in asthmatic patients according to ASM area: Data at inclusion and follow-up

Parameter	Patients with mild-to-moderate asthma	Patients with severe asthma				P value*
		ASM Q1	ASM Q2	ASM Q3	ASM Q4	
No. of patients	24	27	25	27	26	
Male sex, no. (%)	4 (17) [†]	10 (37)	5 (20)	7 (26)	13 (50)	.07
Age (y)	53.0 ± 15.1	51.7 ± 12.9	50.2 ± 14.7	49.6 ± 12.6	42.5 ± 12.5 ^{‡§ ¶}	.05
Caucasian origin, no. (%)	18 (75)	19 (70)	17 (68)	17 (63)	19 (73)	.91
Former smokers, no. (%)	2 (16)	8 (30)	7 (28)	5 (19)	5 (19)	.24
Body mass index (kg/m ²)	29.5 ± 9.6	26.3 ± 4.6	26.3 ± 6.2	29.9 ± 7.5	25.6 ± 3.9	.08
Age of asthma onset (y)	26.0 ± 19.6	21.1 ± 18.4	25.7 ± 16.0	20.5 ± 16.5	22.7 ± 10.1	.65
Asthma duration (y)	27.0 ± 19.5	29.8 ± 18.9	24.9 ± 16.1	28.5 ± 13.4	20.1 ± 14.9	.25
History of atopy, no. (%)	17 (71)	19 (70)	17 (68)	19 (70)	20 (77)	.97
Blood eosinophils (10 ⁹ /L)	180 (120-280)	210 (129-505)	240 (166-480)	345 (232-570)	335 (200-490)	.26
Total serum IgE (IU/mL)	192 (83-328)	151 (72-451)	244 (98-622)	201 (152-443)	211 (123-353)	.59
Respiratory function at inclusion						
Prebronchodilator FEV ₁ (% predicted)	94.2 ± 20.3 [‡]	71.2 ± 21.7 [‡]	71.0 ± 19.3 [‡]	63.5 ± 17.0 [‡]	62.3 ± 13.9 [‡]	<.0001
Postbronchodilator FEV ₁ (% predicted)	101.0 ± 19.6	74.5 ± 22.0 [‡]	78.3 ± 20.2 [‡]	70.0 ± 17.7 [‡]	68.0 ± 12.3 [‡]	<.0001
Prebronchodilator FEV ₁ /FVC (% predicted)	71.6 ± 10.9	63.2 ± 14.9	62.7 ± 10.3 [‡]	60.3 ± 13.6 [‡]	59.4 ± 10.0 [‡]	.0005
Postbronchodilator FEV ₁ /FVC (% predicted)	74.9 ± 9.6	66.6 ± 13.8	65.1 ± 10.2	64.3 ± 13.7 [‡]	62.6 ± 10.0 [‡]	<.0001
Reversibility to β_2 -agonists (%)	6.8 ± 5.6	3.6 ± 3.8	7.5 ± 6.6	6.6 ± 5.8	5.9 ± 6.2	.13
Respiratory function at follow-up						
Prebronchodilator FEV ₁ (% predicted)	ND	70.2 ± 22.3	73.0 ± 19.0	74.5 ± 17.6	68.2 ± 17.6	.46
Postbronchodilator FEV ₁ (% predicted)	ND	74.2 ± 20.4	77.9 ± 19.1	79.5 ± 18.4	74.5 ± 17.1	.32
Prebronchodilator FEV ₁ /FVC (% predicted)	ND	60.8 ± 15.6	60.2 ± 9.6	65.6 ± 12.7	63.2 ± 10.7	.52
Postbronchodilator FEV ₁ /FVC (% predicted)	ND	62.1 ± 15.7	63.4 ± 10.4	67.7 ± 11.8	65.5 ± 10.4	.14
Reversibility to β_2 -agonists (%)	ND	6.5 ± 5.1	4.9 ± 4.9	5.3 ± 4.6	6.3 ± 5.1	.20
Treatments at inclusion						
LABAs, no. (%)	12 (50)	27 (100)	25 (100)	27 (100)	26 (100)	
Dose of ICS (μ g/d beclomethasone equivalents)	813 ± 274	2815 ± 1001 [‡]	2720 ± 980 [‡]	2667 ± 961 [‡]	2461 ± 859 [‡]	<.0001
Maintenance use of OCS (%)	0	6 (22)	4 (16)	7 (26)	5 (19)	.68
Daily dose of OCS (mg)	0	16.8 ± 14.5	32.5 ± 22.2	28.6 ± 16.8	17.6 ± 14.0	.39
Anti-IgE, no. (%)	0	6 (22)	6 (23)	1 (4)	3 (11)	.16
Treatments at follow-up						
LABAs, no. (%)	ND	27 (100)	25 (100)	27 (100)	26 (100)	—
Dose of ICS (μ g/d beclomethasone equivalents)	ND	2311 ± 1108	2520 ± 963	2519 ± 1051	2392 ± 989	.79
Maintenance use of OCS (%)	ND	4 (15)	2 (8)	4 (17)	8 (31)	.24
Daily dose of OCS (mg)	ND	27.0 ± 21.7	16 ± 8.5	21.3 ± 6.3	16.8 ± 12.6	.74
Anti-IgE, no. (%)	ND	8 (31)	11 (42)	4 (15)	8 (31)	.18
Asthma control at inclusion						
With uncontrolled asthma, no. (%)	0	22 (81)	19 (76)	21 (78)	23 (88)	.70
ACT score	ND	11.6 ± 5.8	14.5 ± 4.7	11.2 ± 4.3	11.4 ± 5.2	.89
No. of severe exacerbations in previous year	0	6.0 ± 3.4	5.7 ± 3.9	5.2 ± 3.6	5.5 ± 5.2	.75
Asthma control at follow-up						
With uncontrolled asthma, no. (%)	0	10 (37)	7 (28)	11 (41)	17 (65)	.05
ACT score	0	18.5 ± 6.1	19.0 ± 3.7	17.6 ± 4.8	14.3 ± 6.2 [‡]	.04
No. of severe exacerbations in previous year	0	4.1 ± 3.6	2.3 ± 1.6	2.4 ± 2.9	2.0 ± 2.9	.38

Boldface indicates statistical significance.

ND, Not determined.

*ANOVA or χ^2 test.

[†]Values are presented as numbers (percentages), means $\pm$ SDs, or medians (25th-75th interquartile ranges). [‡] $P < .05$ related to patients with mild-to-moderate asthma, [§] $P < .05$ related to patients with severe asthma in ASM Q1, ^{||} $P < .05$ related to patients with severe asthma in ASM Q2, and [¶] $P < .05$ related to patients with severe asthma in ASM Q3 (Student t test).

uncontrolled despite 1 year of optimal management was greater in ASM Q4, with a lower ACT score than measured in patients with ASM Q1 to Q3. In contrast, the number of severe exacerbations did not change in relation to ASM enlargement, a finding supporting previous data showing that exacerbations are closely related to eosinophilic inflammation,^{28,29} whereas ASM is mainly involved in airway hyperresponsiveness and in wheezing and breathlessness.¹⁰ We confirmed these observations in our patient cohort by showing that peripheral blood eosinophil counts significantly correlated with the number of severe exacerbations,

whereas ASM area was highly associated with airflow obstruction, both in all asthmatic patients and in those with severe asthma. In this setting BAL fluid and tissue eosinophil counts did not correlate with exacerbations, a finding in agreement with most, but not all, studies.³⁰⁻³²

ASM expansion arises early in childhood,^{8,33} and a recent study demonstrated that increased ASM area, without changes in SBM thickening and tissue eosinophil numbers, predicted asthma onset at school age.³⁴ Whether ASM enlargement observed in these children is maintained over life and accounts for the younger

age of adults with severe asthma with increased ASM area presently investigated remain to be established.

Our current findings demonstrated that ASM area was accompanied by neither changes in the number of mucosal T cells, mast cells, and granulocytes nor increased vascularity. These findings suggest that ASM enlargement occurs independently of other structural alterations and airway inflammation. In contrast, SBM thickening was greater in patients with severe asthma in ASM Q2, Q3, and Q4 compared with patients with mild-to-moderate asthma and control subjects, although these parameters did not mutually correlate.

Both ASM hypertrophy and hyperplasia contribute to the increase in ASM mass in asthmatic patients,^{13,15,25,35} and we confirmed previous findings showing an increased proliferation rate in ASM cells from patients with severe asthma compared with those with mild-and-moderate asthma and control subjects.²⁵ ASM proliferation can be stimulated by a number of mediators, including EGF, PDGF, and bFGF,²²⁻²⁴ which are produced by inflammatory and airway structural cells and by products of mast cells infiltrating the ASM bundles, such as histamine, tryptase, and cysteine leukotrienes.^{36,37} Our results showing higher BAL levels of EGF, PDGF, and bFGF in patients with mild-to-moderate asthma compared with those in control subjects and their decrease in patients with severe asthma suggest that production of these factors might not participate in ASM enlargement in patients with severe asthma.

Similarly, ASM infiltration by mast cells was markedly reduced in patients with severe asthma, particularly those in ASM Q1 to Q3.³⁸ In contrast, patients in the highest ASM quartile maintained numbers of ASM-infiltrating mast cells comparable with those observed in patients with mild-to-moderate asthma, despite a similar treatment regimen than that of patients in ASM Q1, Q2, and Q3. This finding suggests that mast cell homing to ASM bundles in a subpopulation of patients with severe asthma is a steroid-insensitive component of airway inflammation that might participate in the excessive ASM enlargement.

Among mast cell-derived mediators, tryptase was shown to promote ASM cell proliferation and mediator release,^{17,18} partly through its interaction with PAR-2.^{18,39,40} PAR-2 overexpression has been demonstrated in the bronchial epithelium⁴¹ and in *ex vivo*-cultured ASM cells from patients with mild-to-moderate asthma compared with healthy subjects.⁴⁰ In large part, PAR-2 immunolocalization in bronchial biopsy specimens was heterogeneous and difficult to appreciate precisely.⁴¹ Therefore, using an immunosurface algorithm allowing the simultaneous determination of the rate of ASM area staining for PAR-2 and the intensity of this expression,²⁷ we found that the proportion of ASM area with detectable PAR-2 was increased to a similar extent in patients with severe asthma in ASM Q2 to Q4 compared with those in ASM Q1 and patients with mild-to-moderate asthma. However, the intensity of PAR-2 expression was drastically increased exclusively in patients from the highest ASM quartile. Notably, these patients also showed a greater prevalence of having detectable BAL levels of mast cell tryptase and KLK14, a serine proteinase that signals to cells on cleavage and activation of PAR-2.²⁰ Both tryptase and KLK14 levels significantly correlated with ASM area, suggesting that they support persistent PAR-2 activation in the ASM of patients with severe asthma.

It is well established that mechanical stretch⁴² and a range of naturally occurring substances that are upregulated in the airways

of patients with asthma, such as IL-1 β , IL-6, TNF- α , and TGF- β ,^{43,44} are potent PAR-2 expression-promoting agents in various cell types, including ASM cells. Whether these factors also operate by inducing and/or activating PAR-2 in the ASM of patients with severe asthma remains to be established.

PAR-1 is another member of the PAR G protein-coupled receptor family that is expressed by the ASM, although to a weaker extent than PAR-2.⁴¹ Recent findings demonstrated that the activated plasma circulating coagulant factor FXa promotes proliferation and cytokine release from human ASM cells, partly through PAR-1 activation.⁴⁵ Whether PAR-1 activation also participates in ASM enlargement in patients with severe asthma remains a matter for further studies.

Airway remodeling is considered one of the most intractable problems in patients with severe asthma.^{9,10,46} Patients with severe asthma with increased ASM area did not show increased incidence of atopy and increased blood and tissue eosinophilia and neutrophilia. These data predict a poor response of this patient phenotype to current and future biotherapeutics targeting T_H2- or T_H17-type pathways and underline the need to identify new treatments directed specifically against ASM abnormalities.

The current study demonstrated that symptoms in patients with severe asthma with increased ASM area are less controlled by maximal therapy than those with lower ASM area. ASM enlargement was associated with increased expression of PAR-2 and high BAL levels of their ligands, particularly mast cell tryptase and KLK14. Although larger studies are required to identify other biomarkers specifically characterizing these patients, we postulate that aberrant PAR-2 expression and activation represent an important physiopathologic mechanism involved in the excessive and irreversible ASM expansion in patients with severe asthma.

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Clinical implications: ASM enlargement, possibly engaging an aberrant PAR-2 ligand interaction, characterizes a novel phenotype of patients with severe uncontrolled asthma.

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METHODS

Subjects

Between March 2008 and July 2013, we selected 12 healthy volunteers and 129 asthmatic patients at the Pulmonary A Department of the Bichat Hospital (Paris, France; [Table 1](#)). These patients belong to the longitudinal multicenter French Cohort Of Bronchial Obstruction and Asthma. This cohort is aimed at identifying relevant outcomes and biomarkers in patients with severe asthma based on thorough initial investigation.

The group of 12 healthy subjects had normal pulmonary function, no bronchodilator response, no chronic illness, no history of allergy or asthma, and negative skin prick test responses. They received no medication, 11 of them were never smokers, and 1 was a former smoker (<10 cigarette packs per year with smoking intake arrest since at least 1 year).

We used the recommendations of the Global Initiative for Asthma^{E1} to classify the 129 asthmatic patients into those with mild-to-moderate persistent ($n = 24$) and severe persistent ($n = 105$) asthma by considering the frequency and intensity of daytime and nighttime asthma symptoms, the need and dose of medications, and FEV₁ and FVC values. Four and 23 patients in the mild-to-moderate and severe groups, respectively, were former smokers, and none of them was a current smoker.

A validated standardized recording folder was used for data collection at every visit. During the first visit, personal and family history, demographic data, age at asthma onset, clinical examination, atopic manifestation and testing, and, for patients with severe asthma, intensive care unit admissions, hospitalization requirement in the past, exacerbation rates in the last 12 months, number of the exacerbations (defined as a worsening of asthma symptoms requiring treatment with systemic corticosteroids), and OCS burst were recorded.

Patients with mild-to-moderate persistent asthma had normal lung function ([Table 1](#)) and daytime and nighttime symptoms, and they were treated with short-acting β_2 -agonists alone ($n = 12$) or in association with LABAs ($n = 12$), ICSs (800 μ g/d budesonide or 500-1000 μ g/d inhaled beclomethasone equivalents), or both.

Patients with severe asthma were recruited if they had a known diagnosis of severe asthma, according to international European Respiratory Society/ATS guidelines^{E2} for at least 1 year and underwent all clinical and paraclinical examinations, as described below. Severity at inclusion was confirmed on the basis of inadequate symptom control and/or 2 or more exacerbations requiring OCSs in the 12 previous months before inclusion, despite at least 1000 μ g of fluticasone equivalents combined with LABAs. Patients declaring nonadherence to their asthma treatments were not considered for the study.

Asthma control was assessed at inclusion and every 6 months for 12 to 18 months by using the ACT.^{E3} ACT scores are based on a response scale from 1 to 25, where a score of greater than 21 over the last 4 weeks indicates complete control and a score of between 16 and 20 indicates somewhat controlled, a score of less than 15 indicates poorly controlled, and a score of less than 7.5 indicates not controlled asthma. In the current study patients with severe uncontrolled asthma were defined as having an ACT score of less than 15.

Every 6 months, patients with severe asthma underwent assessment of flow volume, FEV₁, and FVC before and after inhalation of 400 μ g of salbutamol. Clinical and biological data used in the current study were obtained at inclusion and 12 or 18 months thereafter. At inclusion, these patients showed airflow obstruction, with mean prebronchodilator and postbronchodilator FEV₁ of less than 80% of predicted value and prebronchodilator and postbronchodilator FEV₁/FVC ratios of less than 70%. Eighty-seven (83%) of the 105 patients also had uncontrolled symptoms, with a mean ACT value of 15 or less, 6 or more severe exacerbations, and 1 or more hospitalization for asthma or intensive care unit stay in the previous year. All patients with severe asthma had normal lung diffusion capacity, with values of transfer factor of the lung for carbon monoxide of 90% or greater and no signs of emphysema, as assessed by using computed tomographic scans.

Treatments throughout the study were recorded, detailing the OCS daily dose when required, annual OCS dose and burst frequency, ICS daily dose, LABA daily dose, and use of other controller medication (leukotriene receptor

antagonists, other bronchodilators, and IgE monoclonal therapy). Total IgE levels and blood eosinophilia were recorded at each visit and expressed as the absolute number of cells per cubic milliliter.

Patients were managed according to the best standard of care in our tertiary teaching hospital's outpatient clinic. In a subanalysis we also compared clinical and physiopathologic characteristics in the 105 patients with severe asthma at entry and after 12 to 18 months of follow-up with optimal management and maximal medication ([Table E1](#)). At this time, patients with severe asthma were treated with doses of ICSs and OCSs similar to those administered at baseline. In contrast, 30% of these patients received omalizumab instead of 15% at inclusion ($P = .017$). Under these conditions, a significant improvement in prebronchodilator and postbronchodilator FEV₁ ($P = .001$) and lower numbers of severe exacerbations and hospitalizations were noted, whereas ACT scores were significantly augmented ($P < .0001$). Overall, 44% of patients' symptoms remained uncontrolled at follow-up compared with 83% observed at inclusion ($P < .0001$).

The protocol was approved by the CPP Ile-de-France I Ethics Committee (no. 09-11962), and all subjects provided written informed consent before being enrolled in the study.

Fiberoptic bronchoscopy

The 129 asthmatic patients underwent bronchoscopy by the same operator, according to the guidelines of the ATS,^{E4} as previously described.^{E5} The subjects were premedicated with 200 μ g of inhaled albuterol, and the nose and larynx were anesthetized with 2% and 0.5% xylocain spray, respectively. BAL fluid was collected by injecting and recovering 50 mL of 0.9% NaCl. After centrifugation (400g for 5 minutes at 4°C), aliquots of cell-free supernatants were stored at -80°C until mediator analysis (see below). The proportion of BAL eosinophils and neutrophils was determined on cytopsin preparations after Diff-Quick staining by counting at least 200 cells in randomly selected fields. Two biopsy specimens were then taken from the subcarinae of the right mild lobe (RML, B4-B5) by using standard forceps and a bronchoscope (Olympus Optical, Tokyo, Japan). Biopsy specimens were immediately fixed in 10% formaldehyde and paraffin wax. Serial biopsy sections (5 μ m) were collected on glass slides previously coated with β -methacryloxypropyltrimethoxysilane (Sigma-Aldrich, St Louis, Mo).^{E5}

Immunohistochemistry and morphometry

Bronchial tissue sections were deparaffinized in xylene and dehydrated in ethanol, and antigens were retrieved under citrate buffer solution at pH 6.0 in a steam-heating device at 97°C for 40 minutes. Approximately 40 serial sections of 5 μ m were obtained for each biopsy specimen, and morphology was established every 10 sections after staining with hematoxylin and eosin. Sections were incubated with primary antibodies and reagents that are listed in [Table E2](#), followed by horse anti-mouse biotinylated IgG (1:300 dilution; Vector Laboratories, Eurobio/Abcys, Les Ulis, France). Antigen detection was assessed by use of the Avidin Biotin Complex from the Vectastain ABC-Alkaline Phosphatase kit (Vector Laboratories), and immunostaining was visualized with Fast red (Dako, Glostrup, Denmark), followed by light nuclear Mayer hematoxylin counterstaining. The specificity of immunostaining was assessed by using the corresponding isotypes (mouse IgG₁ or IgG_{2a}, Sigma-Aldrich) instead of primary antibodies ([Table E2](#)).

Biopsy area was determined by means of morphometry at $\times 60$ magnification and computer-assisted image analysis (AnalySIS soft Imaging System 3.2, Olympus, France).^{E5} Eosinophils (major basic protein-positive cells), neutrophils (elastase-positive cells), mast cells (tryptase- and chymase-positive cells), and T lymphocytes (CD3⁺ cells) were visualized at $\times 400$ magnification. Results were expressed as the number of positive cells per square millimeter of bronchial tissue.^{E5} Mast cells were also enumerated within the ASM bundles, and values were expressed as numbers per square millimeter of ASM area.^{E6}

ASM area, SBM thickening, and number of vascular sections were assessed by using morphometry and computer-assisted image analysis. The area of the bronchial submucosa occupied by the ASM was determined

on α -actin-stained tissue sections, and final results were expressed as the percentage ASM area to total biopsy area.^{E5} The maximum area of ASM measured by using morphometry was 352 mm². This procedure was initially developed by our group and further validated extensively.^{E7-E12} SBM thickness (in micrometers) was determined in tissue sections stained with Mayer hematoxylin from the base of the bronchial epithelium to the outer limit of the reticular lamina of the basement membrane. Measurements (20-50) were made at regular intervals of 50 μ m along the length of the SBM at $\times 400$ magnification.^{E5} The total number of vessels in each tissue section was determined after CD31 immunostaining, which recognizes platelet endothelial cell adhesion molecule expressed by endothelial cells.^{E13} ASM area and number of vessels were assessed at $\times 60$ magnification. Proliferation of ASM cells was detected by means of immunolocalization of PCNA, as previously described,^{E14} with a specific mouse mAb (clone PC10, dilution 1:100; Chemicon-Millipore, Temecula, Calif). Tissue slides were examined at $\times 20$ magnification by using a slide scanner coupled to an image analyzer (Calopix; TRIBVN, Chatillon, France). Binding was visualized with biotin-conjugated anti-mouse antibody and avidin-biotinylated peroxidase complex from the Vectastain kit (Vector, Burlingame, Calif), followed by incubation with 3,3'-diaminobenzidine (Dako Cytomation) and light Mayer hematoxylin counterstaining. The proportion of PCNA⁺ ASM nuclei over total ASM nuclei was calculated.^{E14}

Finally, PAR-2 immunostaining was performed, as previously described,^{E15} and quantified by using image analysis.^{E16} Briefly, digital images of PAR-2 immunohistochemically stained tissue slides were examined at $\times 20$ magnification with an image analyzer (Calopix). The proportion of PAR-2⁺ ASM area was assessed by using an interval scale (0%, 1% to 20%, 21% to 40%, 41% to 60%, 61% to 80%, and 81% to 100%). In addition, the intensity of PAR-2 immunostaining in the ASM was expressed by using an immunosurface algorithm that defined 4 categories of magnitude, as follows: absence, score of 0 (blue); low, score of 1 (yellow); moderate, score of 2 (orange); and high, score of 3 (red).

In this assay PAR-2 immunostaining was carried out simultaneously in all slides according to the same experimental procedure to avoid interexperiment variability. A positive control consisting of a lung tissue section from a patient with idiopathic pulmonary fibrosis was included.^{E15} The global intensity of PAR-2 expression likely reflects changes in receptor levels.

For all immunohistochemical analyses, negative controls included mouse IgG₁ or IgG_{2a} (Sigma-Aldrich) instead of the primary antibody.

All the parameters were examined in 2 to 3 noncontiguous sections from the same biopsy specimen at different depths for each biopsy specimen and in sections from 2 different biopsy specimens. Sections were randomly assigned and analyzed by 2 observers (FH and JB) who were unaware of the clinical group assignment. The final figure was the mean of all the measurements obtained for each patient. The coefficients of variation between biopsy specimens from the same patient and between each parameter of the same biopsy specimen were 8% to 14% and 4% to 9%, respectively.

Assessment of ASM mitogenic factors and PAR-2 ligands in BAL fluid

EGF, bFGF, and PDGF-BB levels were determined in BAL supernatants by using Multiplex multianalyte technology (Merck Millipore). Threshold sensitivities were 0.2 pg/mL for EGF and bFGF and 0.8 pg/mL for PDGF-BB. In addition, using an ELISA (R&D Systems), we measured amounts of the PAR-2 activators trypsin,^{E17} TF,^{E18} and KLK5 and KLK14.^{E19,E20} In parallel, BAL mast cell tryptase levels^{E21-E24} were assessed by using the ImmunoCAP assay (Thermo Scientific, Phadia, St. Quentin-les-Yvelines, France), with a threshold of sensitivity of 1000 ng/mL.

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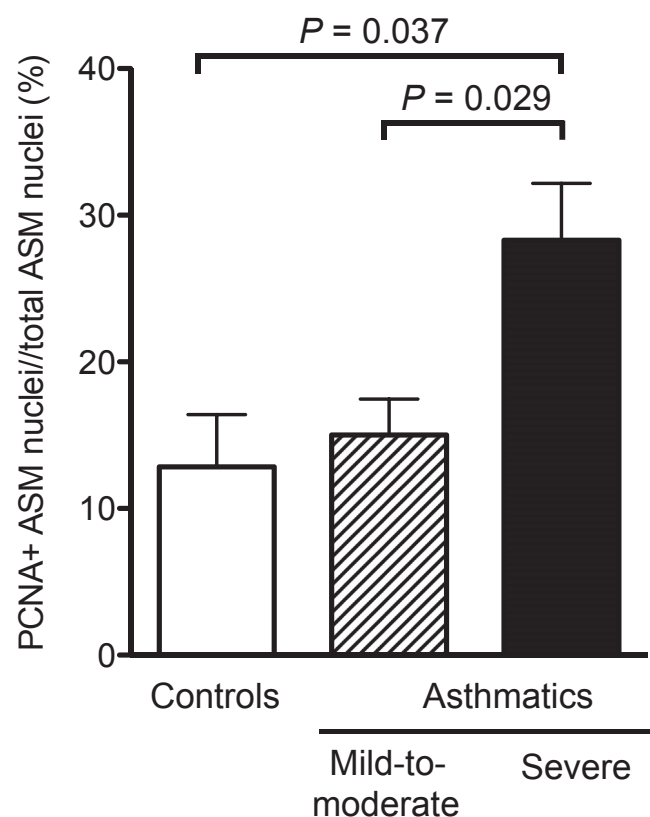


FIG E1. Assessment of ASM proliferation in bronchial biopsy specimens. Sections from control subjects, patients with mild-to-moderate asthma, and patients with severe asthma were stained with a mouse anti-human PCNA mAb, and the proportion of PCNA⁺ nuclei over the total number of ASM nuclei was determined by using image analysis. Data are expressed as means $\pm$ SEMs. Overall comparison was made with the Kruskal-Wallis test ($P = .024$), and P values were calculated with the Mann-Whitney U test.

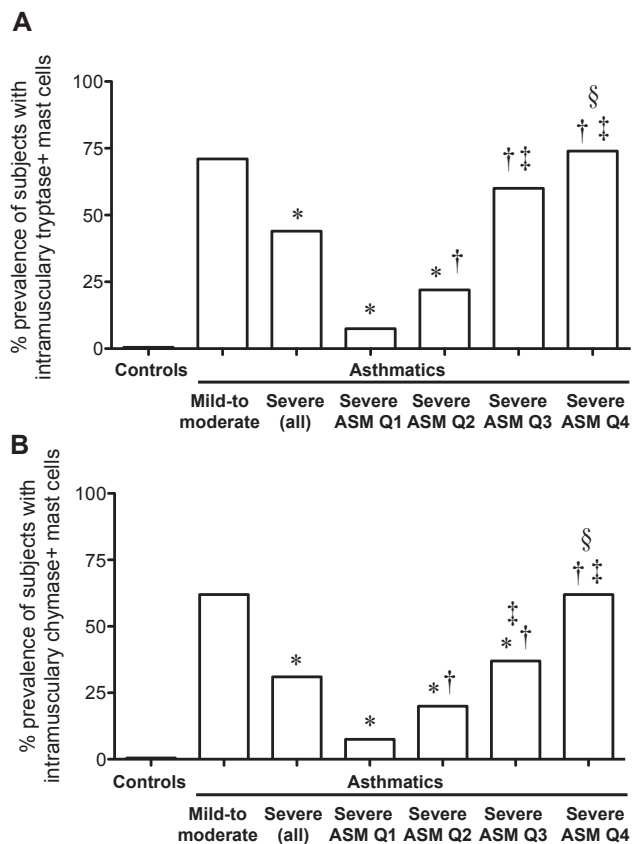


FIG E2. Prevalence of control subjects and asthmatic patients with intramuscular mast cells. Frequency distribution of tryptase-positive (**A**) and chymase-positive (**B**) mast cells within the ASM bundles of control subjects, patients with mild-to-moderate asthma, and patients with severe asthma. Patients with severe asthma were also distributed into ASM quartiles. *P* values were calculated by using the Fisher exact test (2-tailed). No intramuscular mast cells were found in control subjects. Data are presented as means $\pm$ SEMs. **P* < .05 in relation to patients with mild-to-moderate asthma, †*P* < .05 in relation to patients with severe asthma in ASM Q1, ‡*P* < .05 in relation to patients with severe asthma in ASM Q2, and §*P* < .05 in relation to patients with severe asthma in ASM Q3.

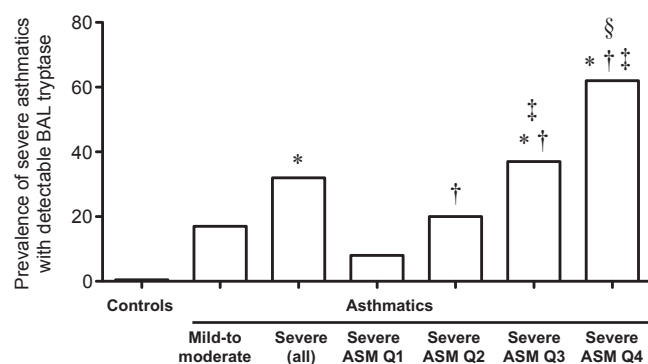


FIG E3. Prevalence of control subjects and asthmatic patients with detectable BAL fluid tryptase levels. Frequency distribution of control subjects, patients with mild-to-moderate asthma, and patients with severe asthma and asthmatic patients with BAL fluid levels of tryptase of greater than the lower limit of detection (eg, 1000 ng/mL). Patients with severe asthma were also distributed into ASM quartiles. *P* values were calculated by using the Fisher exact test (2-tailed). BAL fluid tryptase was undetectable in control subjects. Data are presented as means $\pm$ SEMs. **P* < .05 in relation to patients with mild-to-moderate asthma, †*P* < .05 in relation to patients with severe asthma in ASM Q1, ‡*P* < .05 in relation to patients with severe asthma in ASM Q2, and §*P* < .05 in relation to patients with severe asthma in ASM Q3.

TABLE E1. Clinical characteristics of the 105 patients with severe asthma at inclusion and after 12 to 18 months of follow-up

Parameter	Patients at inclusion*	Patients at follow-up	P value†
Respiratory function			
Prebronchodilator FEV ₁ (% predicted)	67.0 ± 18.4	71.5 ± 19.1	.001
Postbronchodilator FEV ₁ (% predicted)	72.6 ± 18.6	76.6 ± 18.6	.001
Prebronchodilator FEV ₁ /FVC (% predicted)	61.4 ± 12.4	62.5 ± 12.3	.159
Postbronchodilator FEV ₁ /FVC (% predicted)	64.7 ± 12.0	64.0 ± 13.7	.501
Reversibility to β ₂ -agonists (mL)	189.0 ± 232.1	192.5 ± 259.6	.910
Treatments			
LABAs, no. (%)	102 (97)	105 (100)	
Dose of ICS (μg/d beclomethasone equivalents)	2667 ± 947	2434 ± 1020	.059
Maintenance use of OCSs (%)	22 (21)	19 (18)	.721
Daily dose of OCSs (mg)	21.2 ± 13.9	24.6 ± 16.5	.451
Anti-IgE, no. (%)	16 (15)	31 (30)	.017
Asthma control			
With uncontrolled asthma, no. (%)	87 (83)	46 (44)	<.0001
ACT score	12.1 ± 5.1	16.6 ± 5.5	<.0001
With severe exacerbations in previous year, no. (%)	95 (90)	60 (57)	<.0001
No. of severe exacerbations in previous year	6.0 ± 3.9	3.4 ± 2.8	<.0001
Hospitalized for asthma in previous year, no. (%)	46 (44)	4 (4)	<.0001
No. of hospitalization for asthma in previous year	1.3 ± 2.7	0.1 ± 0.3	<.0001
Hospitalized in intensive care units in previous year, no. (%)	12 (11)	0	<.0001
No. of hospitalization in intensive care units in previous year	1.2 ± 0.4	0	<.0001

Boldface denotes statistical significance.

*Data are presented numbers (percentages), means ± SDs, or medians (interquartile ranges).

†Student *t* test for paired values of exact Fisher test (2-tailed).

TABLE E2. Primary mAbs used for immunohistochemistry

Primary antibody	Isotype	Clone	Dilution, incubation	Manufacturer
Anti- α -smooth muscle actin	Mouse IgG _{2a}	1A4	1:200, 1 h	Sigma-Aldrich, St Louis, Mo
Anti-PCNA	Mouse IgG _{2a}	PC10	1:100, overnight	Chemicon-Millipore, Temecula, Calif
Anti-eosinophil MBP	Mouse IgG ₁	BMK13	1:50, 1 h	AbD Serotec, Oxford, United Kingdom
Anti-neutrophil elastase	Mouse IgG _{1k}	NP57	1:100, 1 h	Dako, Glostrup, Denmark
Anti-CD3	Mouse IgG _{1k}	F 7.2.38	1:50, 1 h	Dako
Anti-mast cell tryptase	Mouse IgG _{1k}	AA1	1:50, 1 h	Dako
Anti-mast cell chymase	Mouse IgG _{1k}	B7	1:80, 1 h	Chemicon-Millipore
Anti-CD31	Mouse IgG _{1k}	JC70A	1:20, 1 h	Dako
Anti-PAR-2	Mouse IgG _{2a}	SAM11	1:200, overnight	Santa Cruz Biotechnology, Santa Cruz, Calif

MBP, Major basic protein.

TABLE E3. Correlation analysis between ASM area and hallmarks of airway inflammation and remodeling in asthmatic patients

Parameter	All asthmatic patients (n = 129)		Patients with severe asthma (n = 105)	
	<i>r_s</i>	<i>P</i> value*	<i>r_s</i>	<i>P</i> value*
SBM (μm)	0.161	.07	0.056	.57
CD31 ⁺ cells/mm ² of biopsy area	−0.048	.59	−0.059	.54
CD3 ⁺ cells/mm ² of biopsy area	0.001	.99	0.099	.31
Tryptase-positive cells/mm ² of biopsy area	0.034	.70	0.163	.10
Chymase-positive cells/mm ² of biopsy area	−0.006	.94	0.096	.33
Tryptase-positive cells/mm ² of ASM area	0.500	<.0001	0.600	<.0001
Chymase-positive cells/mm ² of ASM area	0.364	<.0001	0.410	<.0001
MBP ⁺ cells/mm ² of biopsy area	0.049	.58	0.120	.22
Elastase-positive cells/mm ² of biopsy area	−0.053	.55	−0.027	.78
Eosinophils in BAL fluid (%)	−0.071	.43	0.061	.53
Neutrophils in BAL fluid (%)	0.001	.99	−0.086	.38

Boldface denotes statistical significance.

MBP, Major basic protein.

*Spearman rank-order method and Benjamini-Hochberg correction.

TABLE E4. ASM mitogenic growth factors in BAL fluid from control subjects and asthmatic patients

Mediator in BAL fluid (pg/mL)*	Threshold (pg/mL)	Control subjects (n = 12)	Patients with mild-to-moderate asthma (n = 24)	Patients with severe asthma				P value†
				ASM Q1 (n = 27)	ASM Q2 (n = 25)	ASM Q3 (n = 27)	ASM Q4 (n = 26)	
EGF	0.2	0.22 (0.22-0.22)‡	9.8 (6.2-13.2)§	7.3 (0.2-65.3)§	16.1 (0.2-35.2)§	7.7 (0.5-30.5)§	0.22 (0.22-14.2)	.001
bFGF	0.2	0.4 (0.2-2.8)	12.6 (10.4-14.8)§	11.0 (5.1-14.0)§	8.4 (5.2-12.6)§	7.4 (3.9-10.2)§	6.3 (3.2-11.8)§	<.0001
PDGF-BB	0.8	5.1 (3.0-9.0)	14.5 (9.2-22.1)§	6.2 (3.2-14.4)	5.8 (3.4-8.0)	5.8 (4.5-9.9)	6.1 (5.1-9.5)	.04

Boldface denotes statistical significance.

ND, Not detectable.

*Mediators were assessed by using Luminex.

†Kruskal-Wallis test.

‡Data are medians (25th-75th interquartile range).

§P < .05 related to control subjects and ||P < .05 related to patients with mild-to-moderate asthma (Mann-Whitney U test).

TABLE E5. Correlation analysis between ASM area and BAL levels of PAR-2 ligands in asthmatic patients

PAR-2 ligand (pg/mL BAL fluid)	All asthmatic patients (n = 129)		Patients with severe asthma (n = 105)	
	<i>r_s</i>	<i>P</i> value*	<i>r_s</i>	<i>P</i> value
Tryptase	0.39	.02	0.48	.002
Trypsin	0.09	.51	0.08	.62
TF	0.14	.30	0.12	.46
KLK14	0.29	.03	0.42	.008

Boldface denotes statistical significance.

*Spearman rank-order method.

TABLE E6. Correlation analysis between ASM area and clinical parameters in all asthmatic patients and in patients with severe asthma

Parameter	All asthmatic patient (n = 129)		Patients with severe asthma (n = 105)	
	<i>r_s</i>	<i>P</i> value*	<i>r_s</i>	<i>P</i> value*
Age (y)	−0.269	.002	−0.262	.007
Body mass index (kg/m ²)	−0.034	.70	−0.003	.98
Age of asthma onset (y)	−0.020	.82	0.004	.97
Asthma duration (y)	−0.159	.07	−0.157	.11
Blood eosinophils (10 ⁹ /L)	0.097	.28	0.103	.30
Total serum IgE (IU/mL)	0.136	.12	0.105	.29
Values at patient inclusion				
Prebronchodilator FEV ₁ (% predicted)	−0.253	.004	−0.208	.033
Postbronchodilator FEV ₁ (% predicted)	−0.228	.009	−0.164	.09
Prebronchodilator FEV ₁ /FVC (% predicted)	−0.170	.05	−0.145	.14
Postbronchodilator FEV ₁ /FVC (% predicted)	−0.142	.11	−0.107	.27
Reversibility to β ₂ -agonists (%)	0.114	.20	0.148	.13
Daily dose of ICS (μg beclomethasone equivalents)	0.084	.35	−0.153	.12
Daily dose of OCS (mg)	—	—	0.017	.87
No. of severe exacerbations in previous year	—	—	−0.084	.39
ACT score	—	—	−0.088	.37
Values at patient follow-up				
Prebronchodilator FEV ₁ (% predicted)	—	—	−0.031	.76
Postbronchodilator FEV ₁ (% predicted)	—	—	−0.011	.91
Prebronchodilator FEV ₁ /FVC (% predicted)	—	—	0.073	.47
Postbronchodilator FEV ₁ /FVC (% predicted)	—	—	0.034	.74
Reversibility to β ₂ -agonists (%)	—	—	0.029	.78
Daily dose of ICS (μg beclomethasone equivalents)	—	—	0.026	.79
Daily dose of OCS (mg)	—	—	0.101	.30
No. of severe exacerbations in previous year	—	—	0.101	.30
ACT score	—	—	−0.201	.04

Boldface denotes statistical significance.

*Spearman rank-order method and Benjamini-Hochberg correction.