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Article type : Letter to the Editor

Correlation of matrix-related airway remodeling and bradykinin B1 receptor expression with fixed airflow obstruction in severe asthma

To the editor:

Severe asthma is associated with marked airway remodeling (AR)^{1,2} leading to airway narrowing, loss of lung function and fixed airflow obstruction (FAO) in some asthmatics³. Bradykinin (BK), via B2 and B1 receptors (B2R/B1R), provokes acute inflammatory effects in the airways and in addition induces fibroblasts to differentiate into α -smooth muscle actin (α -SMA)⁺ myofibroblasts and to release proangiogenic factors, thus contributing to airflow obstruction in chronic asthma⁴. An *in vitro* study showed BK involvement in a cross-talk between airway smooth muscle (ASM) and epithelial cells resulting in AR-signals in asthma⁵.

We investigated the relation between BK and matrix-related remodeling in severe asthma, by assessing the expression of B1R/B2R and remodeling markers in bronchial biopsies of mild-to-severe asthmatics. We evaluated the association between these patterns and lung function parameters.

This observational cross-sectional study enrolled 42 asthmatics (20 mild asthmatics, MA; 22 severe asthmatics, SA) and 9 controls (CTRL) to obtain bronchial biopsies by bronchoscopy. Asthma severity and FAO definition are reported in the *Supporting information. Table S1* and *S2* report the study group characteristics.

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Immunohistochemistry revealed a higher expression of B1R, B2R, α -SMA at 20 and 100 μ m beneath the basal membrane (BM) in the bronchial submucosa of SA than MA or CTRL and a thicker BM in SA than CTRL ($P<.05$, *Figure 1*). Confocal microscopy showed a higher expression of pro-collagen III and tenascin ($P<.01$, *Figure 2A-B*) in SA than MA. Immunofluorescence revealed colocalization of B1R and α -SMA (*Figure 2C*) indicating B1R expression in the α -SMA⁺ mesenchymal cells of the asthmatic bronchial mucosa. Immunohistochemistry revealed B1R⁺ and B2R⁺ cells in the epithelium (*Figure 1*). We found a significant interaction between atopy and asthma severity regarding B2R expression (*Figure S1A*). Linear regression showed a relationship between submucosal B1R and ICS dose, thus suggesting that asthma severity affects B1R expression (*Figure S2*).

α -SMA 20 μ m negatively correlated with pre-FVC(%pred.) and pre-FEV₁(%pred.), PB-FEV₁(%pred.) and PB-FEV₁/FVC ($P<.05$, *Figure 2D-G*); α -SMA 100 μ m correlated negatively with pre-FVC(%pred.), pre-FEV₁(%pred.) and PB-FEV₁(%pred.) and positively with RV(%pred.) ($P<.05$, *Figures 2H-I; S3A-B*). B1R correlated negatively with PB-FEV₁/FVC, PB-FEV₁(%pred.), pre-FEV₁(%pred.) and positively with BM ($P\leq.05$, *Figures 2L-M; S3D-E*). ROC analyses allowed us to discriminate SA from MA: patients with α -SMA 20 μ m >26.28 cells/mm², α -SMA 100 μ m >114.6 cells/mm² or B1R >13.46 cells/mm² had higher probability to be SA (6, 3 and 4-fold, respectively, *Figure 2N and supporting information*). Based on these cut-offs, we found the best thresholds for PB-FEV₁(%pred.), and PB-FEV₁/FVC (*Figure S4*) indicating that the highest probability of having matrix-related AR was associated with the following cut-off combinations: PB-FEV₁(%pred.) <91.96% and α -SMA 20 μ m >26.28 cells/mm² or α -SMA 100 μ m >114.6 cells/mm²; PB-FEV₁/FVC <0.67 and α -SMA 20 μ m >26.28 cells/mm²; PB-FEV₁/FVC <0.69 and α -SMA 100 μ m >114.6 cells/mm²; B1R >13.46 cells/mm² and PB-FEV₁(%pred.) <80.08% or PB-FEV₁/FVC <0.72 (*Figure 2O*).

Here, SA had higher expression of B1R/B2R and the markers of matrix AR in the bronchial submucosa than MA or CTRLS. We detected no correlation between the above-cited parameters and the inflammatory cells. AR is considered to be consequent to inflammation. Evidence demonstrated remodeling properties of inflammatory T2-cytokines and TGF- β , mast cell-induced proliferation of ASM, and profibrotic effects of eosinophils⁶.

However, the association between AR and inflammation is under debate⁷ and has been rebutted by the fact that higher number of allergen-induced myofibroblasts in the MA airways persisted after

the dissipation of inflammatory infiltrate⁷. Moreover, remodeling can occur very early in asthma during infancy without correlation with inflammation⁷.

Here, we highlight AR differences between severe and mild asthma. The myofibroblast/smooth muscle marker α -SMA at 20/100 μ m was higher in SA than in MA and negatively correlated with the lung function parameters. This could indicate an increase in ASM and myofibroblasts⁷, also very close to the epithelium, associated with disease severity⁸. This increment could result either from epithelial-to-mesenchymal transition or epithelial-mediated fibroblast-myofibroblast transition, characteristic events of AR⁷. However, further investigations are needed to clarify this. The higher expression of collagen III and tenascin in SA than MA support the hypothesis of higher degree of AR⁷.

In our study, the expression of B1R/B2R was higher in SA than in MA or CTRL. Furthermore, in all asthmatics α -SMA 20/100 μ m and B1R expressions were associated with impairment of pulmonary function, and we demonstrate here, for the first time, that these markers are associated with FAO. Interestingly, AR may be protective: excessive matrix deposition could lead to progressive FAO which could stiffen the wall, limiting an excessive lumen narrowing³.

Our group previously demonstrated a relation between B2R and vascular-related AR in asthma⁴. In our asthmatics, colocalization of B1R with α -SMA and significant correlation between B1R and BM thickness suggest a role of B1R in matrix-related AR.

Different stimuli induce acute effect of BK and the production of pro-inflammatory mediators which, in turn, upregulate its receptors in the airways^{4,9}. Ricciardolo and colleagues demonstrated that in atopic MA α -SMA and B2R colocalized, and both were more expressed after allergen challenge¹⁰. Similarly, we showed here a greater expression of B2R in atopic SA than atopic MA and non-atopic SA suggesting that both disease severity and atopy influence B2R. To date, no evidence attests that IgE modulates airway B2R expression. Intriguingly, anti-IgE pre-treatment reduced α -SMA expression in the bronchi of a murine model of chronic asthma¹¹.

ROC analyses provided cut-offs potentially useful to identify severe to mild asthmatics, based on α -SMA and B1R expression: α -SMA 100 μ m and B1R appear to be moderate markers, whereas α -SMA 20 μ m provided a better characterization of SA. Further analyses demonstrated a significant interaction between B1R expression, AR and FAO and for the first time, we obtained significant combination of cut-offs allowing to discriminate SA with matrix-related AR and FAO. Here, we used bronchial biopsies. We know that bronchoscopy is limited to specialized medical centres as it

is invasive and not easy to perform in daily clinical practice. However, bronchial biopsies remain the gold standard to describe airway inflammation and AR in asthma.

In conclusion, we provide new evidence regarding the involvement of B1R in matrix-related AR in SA, and the importance of FAO in identifying SA with a greater degree of matrix-related AR.

Word count: 1000/1000

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Author contributions

Experiments and data analysis: FB, VC, MB, AES, ID, VS; conception and design: FLMR; drafting of the manuscript: FB, VC; review and final acceptance of the manuscript: FB, VC, AES, MB, ID, VS, FG, ADS, FLMR.

Conflict of Interest

All Authors declare no relevant conflicts of interest.

Abbreviations

α -SMA: α -smooth muscle actin; ASM: smooth muscle cells; AUC: area under the curve; B1R: bradykinin receptor 1; B2R: bradykinin receptor 2; BK: bradykinin; BM: basal membrane thickness; CTRL: control; ECP: eosinophil cationic protein; FAO: fixed airflow obstruction; FeNO: fractional exhaled nitric oxide; FEV₁: forced expiratory volume in 1 second; FVC: forced vital capacity; ICS: inhaled corticosteroids; IgE: immunoglobulin E; MA: mild asthmatics; NE: neutrophilic elastase; NPV: negative predictive value; OCS: oral corticosteroid; OR: odds ratio; PB: post-bronchodilation; PPV: positive predictive value; ROC: receiver operating characteristic; RV: residual volume; SA: severe asthmatics.

Keywords

Asthma remodeling, bradykinin receptors, alpha-SMA, basal membrane thickness, fixed airflow obstruction.

Figure legend

Figure 1. Representative photomicrographs showing cells staining positively for the B1R, B2R, α -SMA 20 μ m, α -SMA 100 μ m, and basal membrane thickness (BM) studied in bronchial biopsies of control (left), mild asthma (MA, middle), and severe asthma (SA, right) subjects (40 $\times$ magnification). Arrows indicate positively stained cells.

Panels A-E: Data regarding the expression B1R, B2R, α -SMA 20 μ m, α -SMA 100 μ m, and basal membrane thickness in control (CTRL), all asthmatics (ALL), mild asthmatics (MA) and severe asthmatics (SA). *: $P < .05$; **: $P < .01$, ***: $P < .001$. Data are presented as box plots and the horizontal line indicates the mean value in the respective group. The differences between the groups are assessed by Unpaired T-test or Mann-Whitney test.

Figure 2.

Panels A-B: Confocal images of immunofluorescence expression in the lamina propria of bronchial biopsies for pro-collagen III and tenascin in mild (MA) and severe asthmatics (SA) and the quantification of the fluorescent colour intensity data (mean grey value, MGV). **: $P < .01$; ***: $P < .001$. Data are presented as box plots and the horizontal line indicates the mean value in the respective group. The differences between the groups are assessed by Mann-Whitney test.

Panel C: Confocal images of immunofluorescence showing expression in the bronchial biopsies for B1R (red), α -SMA (green) and their colocalization (yellow and white arrows).

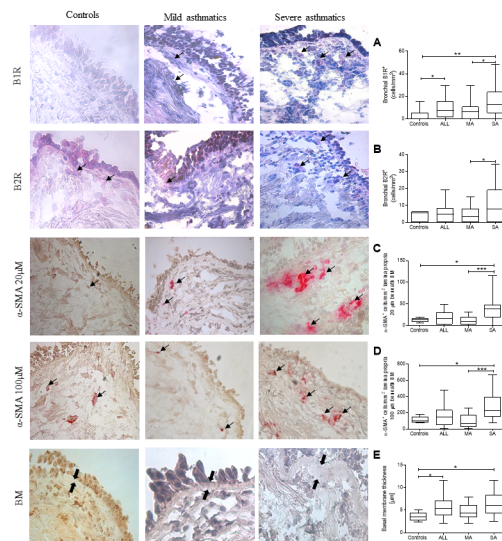
Panels D-M: Correlations between B1R, matrix-related airway remodeling, and lung function parameters in all asthmatics. The box groups all the correlations pertaining to α -SMA 20 μ m (D-G).

Panels H-I: Correlations between α -SMA 100 μ m and pre-FVC or pre-FEV₁(%pred.) respectively.

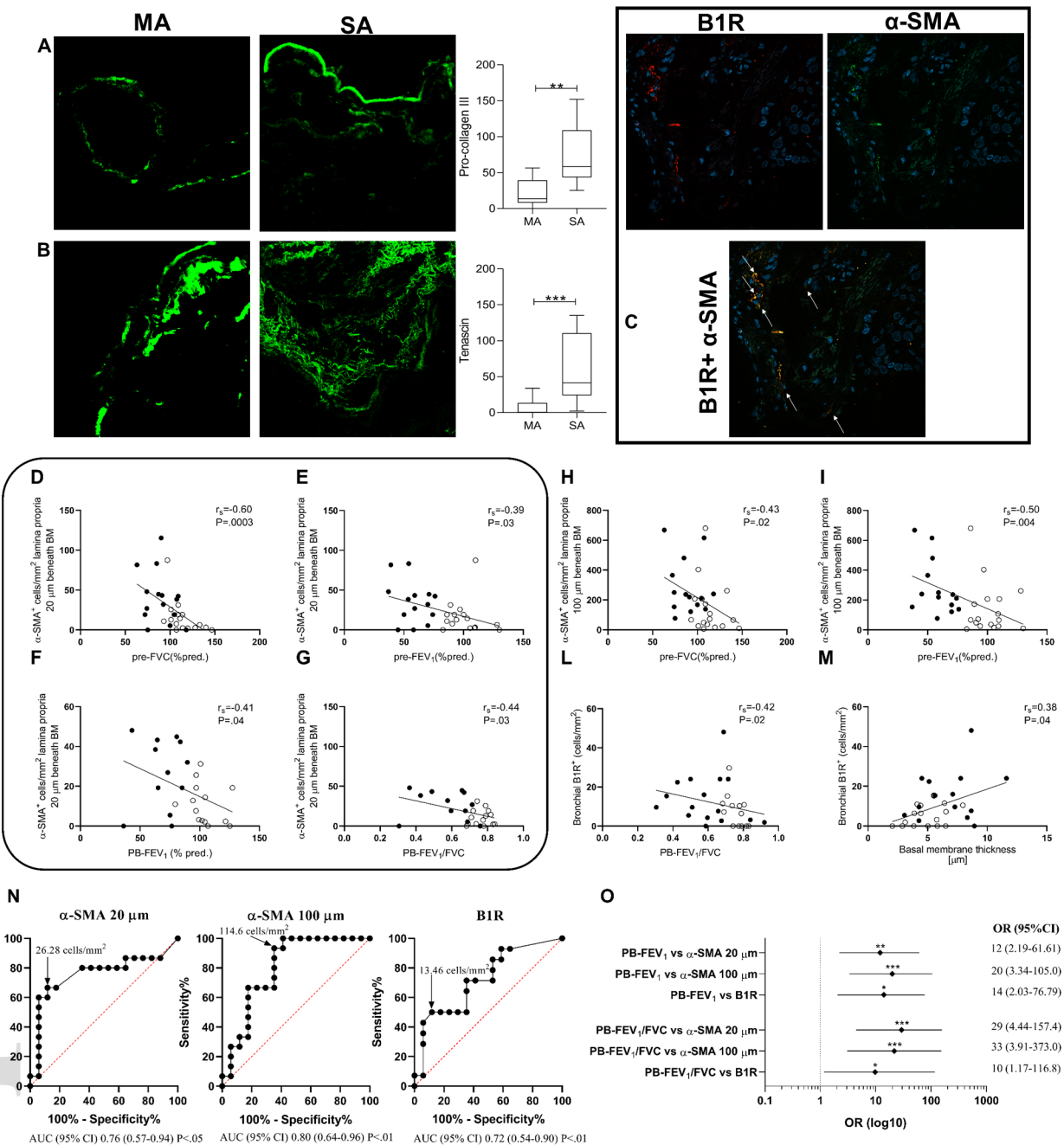
Panels L-M: Correlations between B1R and PB-FEV₁/FVC or basal membrane thickness. The straight line represents the best-fitting line. r_s = Spearman correlation coefficient; PB= Post-bronchodilation.

Panel N: ROC curves for optimal cut-off points of α -SMA 20 μ m, α -SMA 100 μ m and B1R.

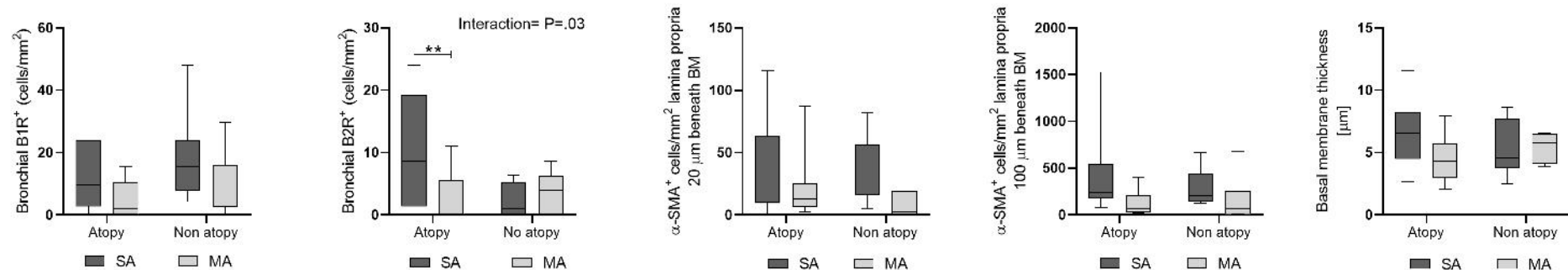
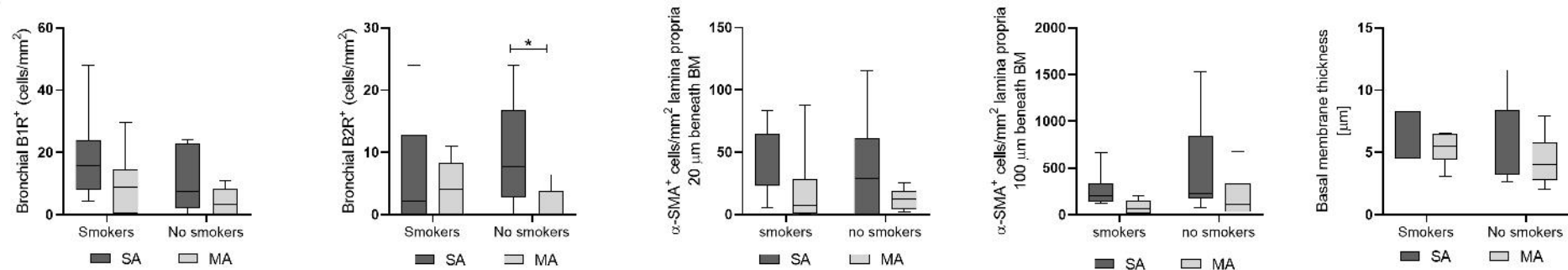
Panel O: Forest plot indicating predictors of airway remodeling in asthmatics. The points represent the confidence interval (95%). OR: odds ratio *: $P < .05$; **: $P < .01$; ***: $P < .001$.

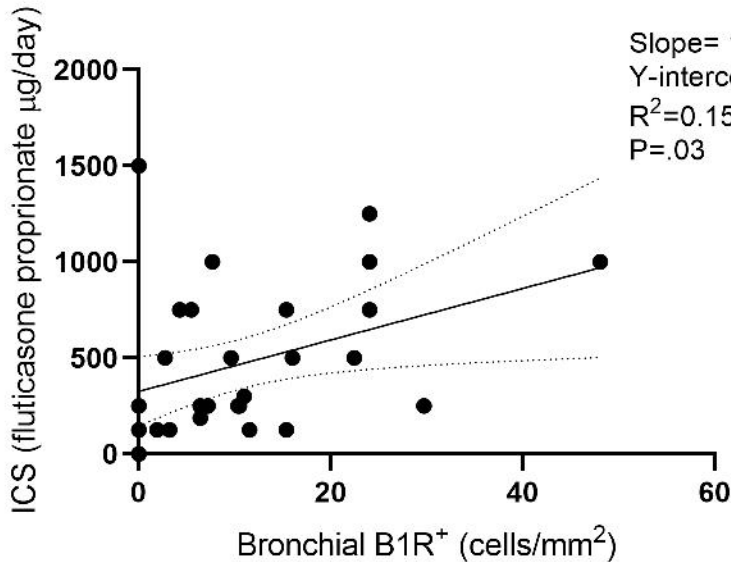


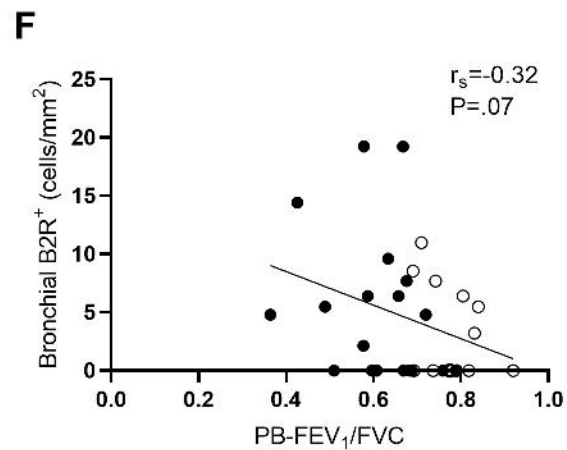
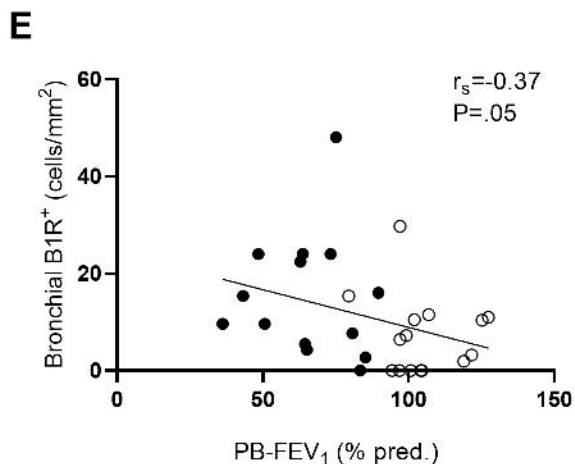
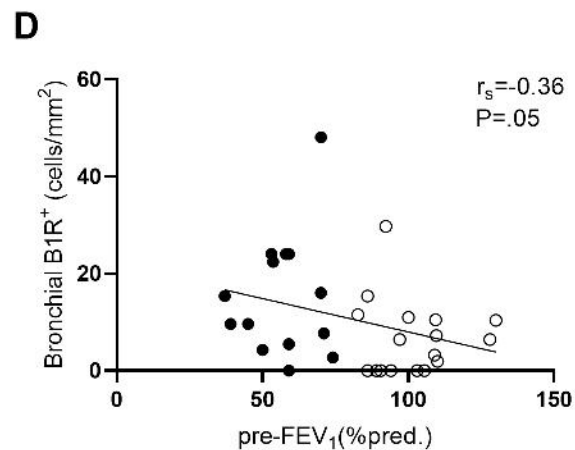
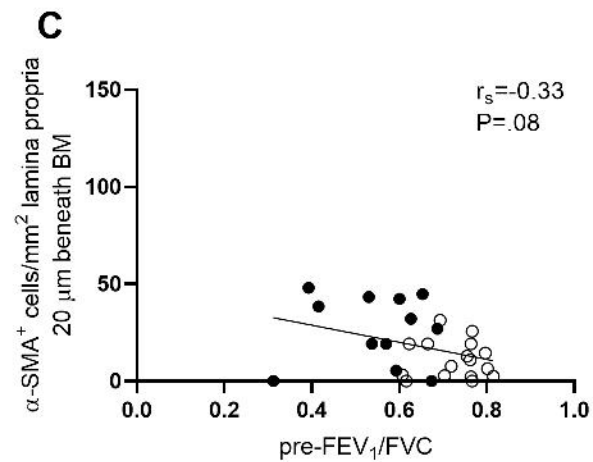
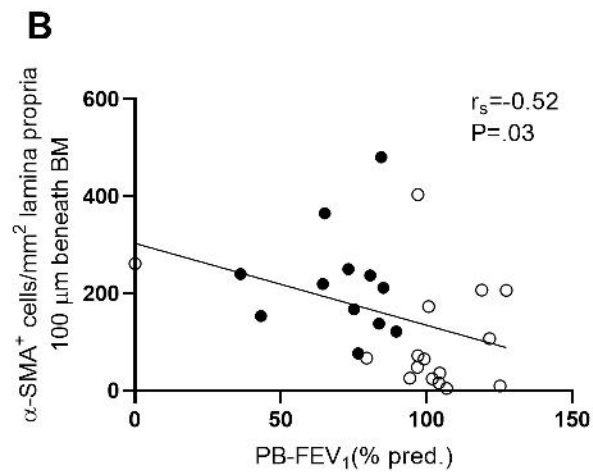
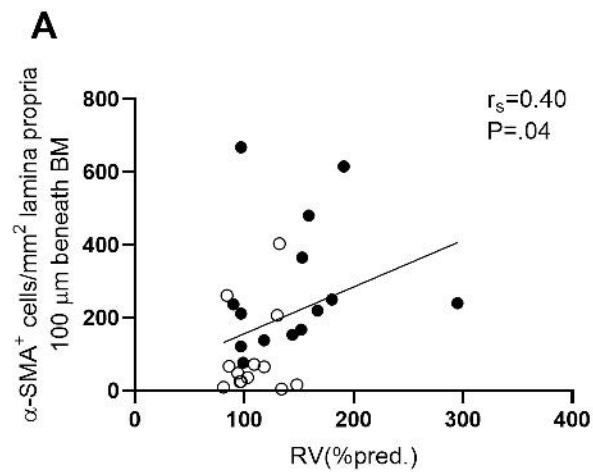
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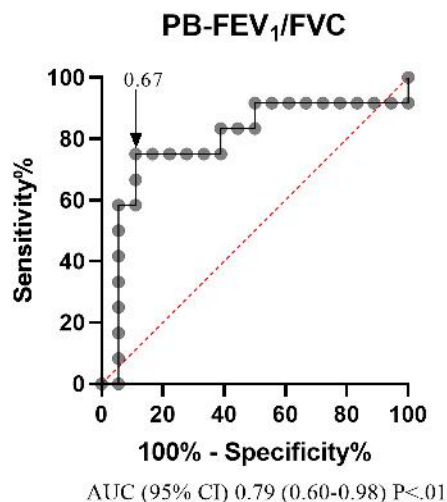
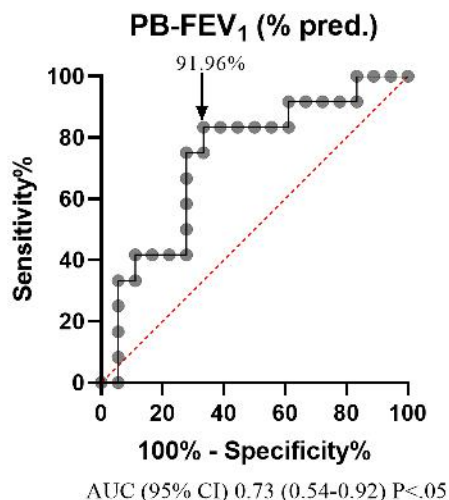
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A**B**

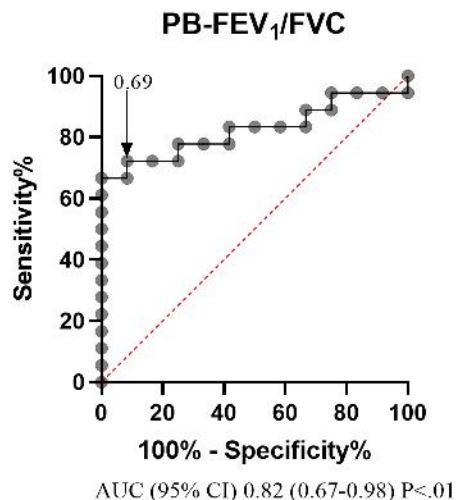
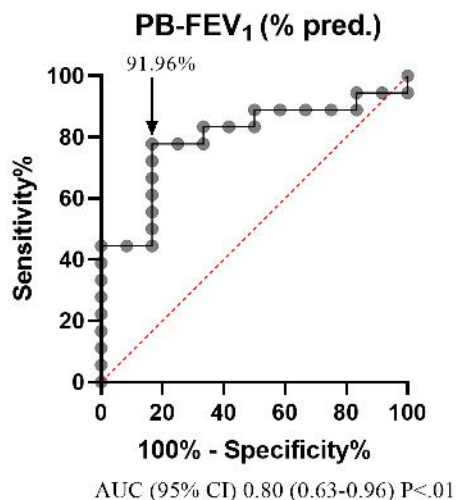




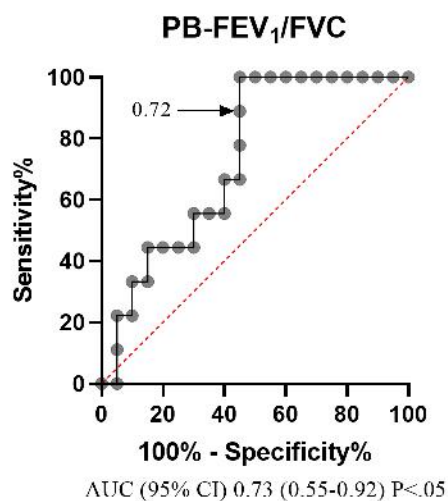
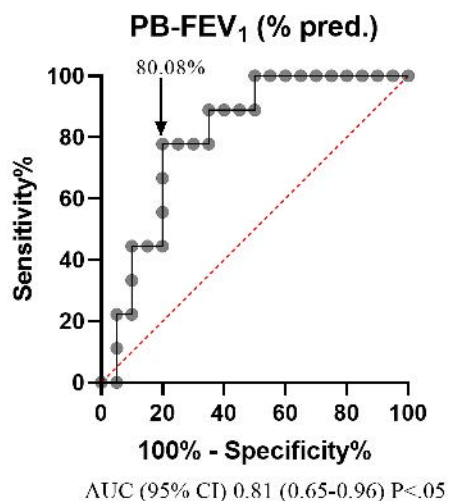
α -SMA 20 μ m



α -SMA 100 μ m



B1R



SUPPORTING INFORMATION

Supporting methods

Patients

This observational cross-sectional study was conducted on 42 asthmatics and 9 controls enrolled at the San Luigi Gonzaga University Hospital (Orbassano, Torino, Italy). We evaluated bronchial biopsies obtained by bronchoscopy¹. Tissue sections of 51 subjects were studied (20 mild asthmatics, MA; 22 moderate-to-severe asthmatics, SA; and 9 controls, CTRL). Asthma was defined according to GINA² and ERS/ATS Guidelines³. Clinical data and patient history were obtained at recruitment when spirometry, post-bronchodilation reversibility tests and hematological exams were performed. Post-bronchodilation (PB) FEV₁ (%pred.) <80% and FEV₁/FVC ratio ≤0.70 were used as thresholds to define the presence of FAO⁴. We ruled out patients with asthma-COPD overlap (ACO) based on the GINA 2017 definition². We considered as controls subjects without asthma and/or COPD diagnosis who underwent bronchoscopy for other clinical reasons (e.g. unknown origin of hemoptysis). Written informed consent was obtained from each participant. The study conformed to the Declaration of Helsinki and was approved by the local Ethics Committees (San Luigi Gonzaga University Hospital: protocols 1759/2008 and 27164/2010). At the time of the study, all asthmatics were in stable condition and none had been treated with theophylline, antibiotics, antioxidants, mucolytics, and/or systemic glucocorticoids in the 6 weeks before the bronchial biopsy.

Evaluation of clinical, functional and blood parameters

Pulmonary function testing was performed assessing spirometry and lung volumes before and 15 min after the administration of albuterol (400 µg) using body plethysmograph (Vmax Encore 62, Carefusion, Germany). White blood cell (WBC) count and WBC differential count were based on optical and impedance characteristics using a Cell-Dyn Sapphire (Abbott, Rome, Italy) automated hematology analyzer (data were expressed as absolute values). Fractional exhaled nitric oxide (FeNO) was measured according to the previous study¹. Smoking history was defined as ≥10 pack-years, based on previous data. Patients who quit smoking in the previous 12 months were classified as former smokers. Skin prick tests and specific serum total IgE levels were performed to identify allergic patients according to validated criteria⁵. Patients sensitized to 2 or more allergens were considered as poly-sensitized⁶.

Fiberoptic bronchoscopy and immunohistochemistry

Bronchoscopy was performed using a flexible fiberoptic bronchoscope (Pentax FB-35 18P; Asahi Optical Co. LTD, Tokyo, Japan) and bronchial biopsies were obtained as previously described^{1,7}. Four bronchial mucosal biopsy specimens were taken from segmental and subsegmental airways of the right lower and upper lobes using size 19 cupped forceps for all subjects included in this study. Immunostaining was performed on 6 µm-thick frozen sections using specific antibodies against eosinophil cationic protein (ECP, 1:100, rabbit polyclonal IgG, Bioss, Woburn, MA, USA); neutrophil elastase (1:100, mouse monoclonal IgG, Agilent Dako, Santa Clara, CA, USA); CD4 for T helper lymphocytes (1:100 mouse monoclonal, Dako, Denmark); tryptase for mast cells (1:500 mouse monoclonal, EMD Millipore Corporation, CA, USA); CD68 for macrophages (1:100 mouse monoclonal, Thermo Fisher Scientific, UK); rabbit anti-human bradykinin B1R (1:50 Santa Cruz Biotechnology, Wembley, UK); mouse anti-human B2R (1:100 BD Biosciences Inc., Milan, Italy); and mouse anti-human α-SMA (1:100 DAKO cytometry, Milan, Italy). Appropriated biotinylated secondary antibodies (Vector Laboratories, Peterborough, UK) were then applied (1:200); antibody binding was revealed by the use of Bloxall Blocking Solution (SP-6000, Vector Laboratories) and colour development was achieved by treatment with DAB substrate (Sigma Aldrich S.r.l., Milan, Italy). Cell counts were performed on immune-histostained tissues at 40x magnification, by the same operator, who was blinded to the subject ID and diagnosis. Immunostained cells were quantified in the area 100 µm (or 20 µm only for α-SMA) beneath the basement membrane (lamina propria) in

several non-overlapping high power fields until all the available area was covered. The final result expressed as the number of positive cells/mm², was calculated as the average of all the cellular counts performed in all fields. We quantified the immunostained cells with at least a portion of the nucleus seen close to immunopositivity. Basal membrane (BM) thickness was measured in Hematoxylin-Eosin stained cryostat slices of bronchial biopsies using the method described by Sullivan et al.⁸.

Immunofluorescence

We randomly selected and evaluated 20 bronchial biopsies (10 MA and 10 SA). These sections were processed according to the previous study⁹. The following primary antibodies were used: mouse anti-human tenascin (1:100, Tebu-bio, Magenta, Italy) and mouse anti-human pro-collagen III (1:100 Tebu-bio, Magenta, Italy). After washing with PBS, the preparations were incubated for 1 hour with secondary Alexa Fluor 488 antibodies (Invitrogen S.R.L., Milan, Italy) shown in green. The expression of pro-collagen III and tenascin in the bronchial mucosa of asthmatics were expressed in mean grey value (MGV) (derived from the quantification of the fluorescent colour intensity)¹⁰. We assessed colocalization of B1R and α -SMA in 3 bronchial biopsies. The following primary antibodies were used: mouse anti-human α -SMA (1:50 DAKO cytometry, Milan, Italy) and rabbit anti-human bradykinin B1R (1:50 Santa Cruz Biotechnology, Wembley, UK). After washing with PBS, the preparations were incubated for 1 hour with secondary Alexa Fluor 488 and Alexa Fluor 568 antibodies (Invitrogen S.R.L., Milan, Italy) shown in green and red, respectively. Images were analyzed by using a laser-scanning confocal microscope ZEISS LSM 800 at a final magnification of 63 $\times$. The Zeiss ZEN2 software package was used for acquisition, storage, and analysis.

Statistical analysis

Statistical evaluations were performed with GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Data distributions were assessed using the D'Agostino-Pearson test. Unpaired T-test (Welch-corrected, if a different standard deviation was detected) or Mann-Whitney test were used to compare differences between groups. Chi-squared (χ^2) tests were used to compare frequencies. Outliers were identified using ROUT methods and excluded from analysis. Correlations were determined using the Pearson correlation coefficient interpreted as follows: >0.8 = very strong; 0.6–0.79 = strong; 0.4–0.59 = moderate; 0.2–0.39 = weak; and <0.2 = very weak¹¹. The effects of atopy or smoke and disease severity were studied with two-way ANOVA and Šídák's multiple comparison post-tests. P-values $\leq .05$ were considered significant. Optimal cut-off values of airway remodeling predictor (α -SMA 20 and 100 μ m and B1R) were determined by receiver operating characteristic (ROC) curves; the area under the ROC curve (AUC) was used to measure the accuracy of each score. The different odds ratios (OR) obtained were used to estimate the probability of airway remodeling occurring. General linear model with univariate analysis was performed to assess the possible association between clinical data and B1R and B2R expression levels. The simple linear regression model was used to confirm the univariate result.

Supporting results

Evaluation of clinical, functional and biological characteristics

Characteristics of the study group are reported in *Table S1*. The 42 asthmatics included 20 MA and 22 SA. The groups of CTRL, MA, and SA were comparable for age, sex, body mass index (BMI) and smoking history. Compared to CTRL, all asthmatics (MA and SA combined) displayed a lower percentage of current smokers ($P<.05$) and a higher prevalence of former smokers ($P<.05$). All asthmatics showed higher FeNO values compared to CTRL ($P<.05$). There were more patients with atopy amongst all asthmatics than in CTRL ($P<.05$), while SA and MA did not differ in terms of their serum IgE levels and allergen sensitization pattern. Significantly lower values of FEV₁, FVC and FEV₁/FVC pre and PB(%pred.) as well as higher values of RV and FRC(%pred.) were observed in SA compared to CTRL and MA. All asthmatics showed lower values of pre FEV₁ (%pred.) and pre

FEV₁/FVC compared to CTRL (P<.05). Moreover, SA had a higher prevalence of FAO (68.2% PB-FEV₁% pred. <80%, and 86.4% PB-FEV₁/FVC ≤0.70) than MA (P<.01 and P<.05, respectively). In SA, ICS dose (equivalent to fluticasone propionate hydrofluoroalkane [HFA]) was 8 times higher (P<.0001) than in MA, with 6/22 SA patients receiving OCS for ≥6 months/year (P<.05) and ≥2 exacerbations occurring in 14/22 SA patients in the previous year (P<.01). Finally, blood eosinophil counts were significantly higher in MA, SA and all asthmatics compared to CTRL (P<.01); on the other hand, blood neutrophil and total WBC counts differed only between SA and MA (P<.05).

Inflammatory cell counts in the bronchial submucosa

Table S2 shows the comparisons between inflammatory cell counts in the bronchial mucosa of the study groups. In SA we found higher numbers of bronchial eosinophils (P<.01), neutrophils (P<.001), CD4⁺ cells (P<.001), CD68⁺ cells (P<.01) and mast cells (P<.05) than in CTRL. Moreover, we observed significantly increased numbers of bronchial eosinophils (p<0.01), neutrophils (P<.05), and CD68⁺ (P<.05) cells in MA than in CTRL. In addition, SA had more CD4⁺ cells (P<.01) but less mast cells (P<.05) than MA. Finally, we observed higher numbers of bronchial eosinophils (P<.001), neutrophils, and CD4⁺ and CD68⁺ cells (P<.01) in all asthmatics compared to CTRL.

Expression of BK receptors (B1R and B2R), Alpha-SMA and basal membrane thickness in bronchial submucosa of mild and severe asthmatics and controls

In the bronchial submucosa, the positive immunostaining for B1R and B2R was observed in the epithelial cells, fibroblasts and endothelial cells in the lamina propria.

In the bronchial submucosa, both B1R and B2R were significantly more expressed in SA than MA (P<.05), but only the B1R⁺ cell count was higher in SA and all asthmatics than in CTRL (P<.01, Figure 1A-B). Cell counts in the lamina propria of bronchial sections showed a higher expression of α-SMA at 20 and 100 μm beneath the basement membrane in SA than in MA (P<.001) and in CTRL (P<.05; Figure 1C-D). Further, SA showed a significantly increased BM thickness compared to CTRL (P<.01, Figure 1E), but no significant difference was observed between SA and MA. Comparing all asthmatics to CTRL, we detected a greater BM thickness (P<.05). Moreover, we observed a significantly higher expression of B2R in severe non-smoker asthmatics than in mild non-smokers (Figure S1B; P<.05), but no interaction was detected between smoking status and asthma severity. No significant interaction between allergy or smoking status and disease severity on the expression of B1R, α-SMA 20 μm, α-SMA 100 μm and BM was observed (Figure S1A-B). Assessing the clinical parameter influence on B1R expression, we performed univariate analysis suggesting a significant relationship between ICS/day (μg fluticasone propionate HFA eq.) and B1R (P=.03). Based on this result we performed simple linear regression test confirming univariate result regarding B1R and ICS/day (slope=13.4 (1.4-25.4); Y-intercept=325.9 (144.9-506.9); R²=0.15 and P=.03) (Figure S2).

Correlations between B1R/B2R or matrix-related airway remodeling markers and pulmonary function parameters

Analyzing the relationship between lung function and matrix-related airway remodeling markers in all asthmatics, we also detected, negative correlation between B1R with pre-FEV₁(%pred.) and PB-FEV₁ (%pred.) (P=.05 for both; Figure S3 D, E), and only as a trend, negative correlations between α-SMA 20μm with pre-FEV₁/FVC (P=.08; Figure S3 C), and between B2R and PB-FEV₁/FVC (P=.07; Figure S2 F).

Correlations between B1R/B2R, markers of airway remodeling and inflammatory cell counts

No significant correlation was found between the inflammatory cells analyzed and the markers of matrix-related airway remodeling or B1R and B2R in all asthmatics.

ROC curve analyses

ROC curve analyses provided the best cut-off values to discriminate SA and MA based on B1R and matrix-related airway remodeling markers (*Figure 2N*). Patients with values of α -SMA $20\mu\text{m} > 26.28$ cells/ mm^2 were 6 times more likely to be severe asthmatics (AUC 0.76; diagnostic OR=5.7); sensitivity was 66.7%, specificity 88.2%, PPV 66.7%, and NPV 88.2%. Patients with values of α -SMA $100\mu\text{m} > 114.6$ cells/ mm^2 had a 3-fold higher probability of being severe asthmatics (AUC 0.80; diagnostic OR=2.7); sensitivity was 93.3%, specificity 64.7%, PPV 95.4%, and NPV 55.0%. Furthermore, patients with values of B1R > 13.46 cells/ mm^2 had a 4-fold higher probability of being a severe asthmatic (AUC 0.72; diagnostic OR=4.2); sensitivity was 50.0%, specificity 88.2%, PPV 88.2%, and NPV 50.0%. Based on the airway remodeling cut-offs, we searched for the best cut-off values for the PB-FEV₁(%pred.), PB-FVC (%pred.), and PB-FEV₁/FVC (*Figure S4*). Unfortunately, the ROC curve for PB-FVC (%pred.) was not statistically significant, so we excluded this parameter from subsequent analyses. We then assessed the predictive validity of the cut-offs and found that patients with PB-FEV₁(%pred.) $< 91.96\%$ and α -SMA $20\mu\text{m} > 26.28$ cells/ mm^2 had a significantly higher probability of having matrix-related airway remodeling than those with PB-FEV₁ (%pred.) $\geq 91.96\%$ and α -SMA $20\mu\text{m} \leq 26.28$ cells/ mm^2 (OR=12, $P < .01$). Instead, patients with PB-FEV₁/FVC < 0.67 and α -SMA $20\mu\text{m} > 26.28$ cells/ mm^2 had a significantly greater probability of having matrix-related airway remodeling compared to those with ≥ 0.67 and ≤ 26.28 cells/ mm^2 (OR=29, $P < .001$). Patients with α -SMA $100\mu\text{m} > 114.6$ cells/ mm^2 and PB-FEV₁ (%pred.) $< 91.96\%$, or PB-FEV₁/FVC < 0.69 had a significantly higher probability (OR=20, $P < .001$; OR=33, $P < .001$) of having matrix remodeling. Finally, patients with B1R > 13.46 cells/ mm^2 and PB-FEV₁ (%pred.) $< 80.08\%$ or PB-FEV₁/FVC < 0.72 had a higher probability of having matrix remodeling processes (OR=14, $P < .05$; OR=10, $P < .05$) (*Figure 2O*).

Supporting table

Table S1. Clinical, functional, and biologic parameters of the study population

CLINICAL PARAMETERS	All asthmatics (n=42)	Mild asthma (n=20)	Severe asthma (n=22)	Control (n=9)
Age, years (range)	53.7 $\pm$ 10.6 (37-72)	50.9 $\pm$ 7.9 (39-66)	55.6 $\pm$ 11.6 (37-72)	57.2 $\pm$ 13.9 (31-74)
Sex, M/F	22/20	10/10	12/10	5/4
BMI, Kg/m ²	26.7 $\pm$ 4.9	27.5 $\pm$ 5.2	26.0 $\pm$ 4.8	23.71 $\pm$ 3.3
Asthma onset, mean $\pm$ SD	29.1 $\pm$ 17.3	30.2 $\pm$ 17.0	28.1 $\pm$ 18.0	/
Asthma onset, years (< 18 years)	12/42 (28.6%)	5/20 (25.0%)	7/22 (31.8%)	/
Asthma duration, years	24.3 $\pm$ 15.9	20.8 $\pm$ 16.6	27.5 $\pm$ 14.9	/
Current smokers [§]	2/42 (4.7%) [°]	1/20 (5%)	1/22 (4.5%)	3/9 (33.3%)
Former smokers [§]	13/42 (30.9%) [°]	4/20 (20.0%)	9/22 (40.9%)	1/9 (11.1%)
Atopy	25/42 (59.5%) ^{°°}	13/20 (65.0%) [°]	12/22 (54.4%) [°]	0/9 (0.0%)
Polysensitized	21/42 (50.0%)	12/13 (92.3%)	9/12 (75.0%)	/
Perennial allergen sensitization	17/42 (40.5%)	9/13 (69.2%)	8/12 (66.7%)	/
IgE, KUI/L	222.0 $\pm$ 367.7	230.4 $\pm$ 424.6	214.4 $\pm$ 317.3	/
FeNO, ppb [¥]	29.8 $\pm$ 23.3 ^{°°}	35.9 $\pm$ 28.7 [°]	24.2 $\pm$ 15.7 [°]	12.6 $\pm$ 1.0
WBC, cells/ μ L	7403 $\pm$ 2312	6673 $\pm$ 1242	8067 $\pm$ 2841*	7343 $\pm$ 3882
Blood eosinophils, cells/ μ L	290.7 $\pm$ 204.0 ^{°°°}	281.0 $\pm$ 158.3 ^{°°}	299.5 $\pm$ 241.7 ^{°°}	81.6 $\pm$ 50.0
Blood neutrophils, cells/ μ L	3904 $\pm$ 1778	3190 $\pm$ 720	4553 $\pm$ 2185*	4978 $\pm$ 3221
pre-FVC(% pred.)	100.8 $\pm$ 20.1	114.4 $\pm$ 15.9	88.5 $\pm$ 15.2****\ ^{°°}	112.1 $\pm$ 15.5
PB-FVC(% pred.)	103.8 $\pm$ 16.0	110.8 $\pm$ 13.5	98.0 $\pm$ 15.8**	/
pre-FEV ₁ (% pred.)	78.6 $\pm$ 23.2 ^{°°}	101.3 $\pm$ 14.5	58.0 $\pm$ 10.8****\ ^{°°°°}	107.8 $\pm$ 12.9
PB-FEV ₁ (% pred.)	80.9 $\pm$ 28.5	93.8 $\pm$ 34.3	69.1 $\pm$ 14.7****	/
PB-FEV ₁ (% pred.) ($< 80\%$) [#]	18/42 (42.8%)	3/20 (15.0%)	15/22 (68.2%)**	/
pre-FEV ₁ /FVC	0.63 $\pm$ 0.13 [°]	0.73 $\pm$ 0.08	0.55 $\pm$ 0.10****\ ^{°°°}	0.76 $\pm$ 0.06

PB-FEV ₁ /FVC	0.67±0.14	0.78±0.07	0.58±0.12****	/
PB-FEV ₁ /FVC (≤0.70) [#]	22/42 (52.4%)	3/20 (15.0%)	19/22 (86.4%)*	/
RV(% pred.)	129.6±41.3	108.7±20.2	143.8±46.1** [°]	100.5±13.03
TLC(%pred.)	114.5±20.2	110.0±4.8	118.3±27.5	101.4±11.7
FRC(%pred.)	112.0±30.8	100.8±16.8	121.1±36.6*	109.2±12.9
ICS/day, µg fluticasone propionate HFA eq.	522.9±393.7	179.4±78.4	835.2±287.1****	/
OCS (≥6 months/year)	6/42 (14.3%)	0/20 (0.0%)	6/22 (27.3%)*	/
Omalizumab	3/42 (7.1%)	0/20 (0.0%)	3/22 (13.6%)	/
Frequent exacerbation (≥2/year)	15/42 (35.7%)	1/20 (5%)	14/22 (63.6%)**	/

Continuous variables are presented as mean ± SD. For age, the min to max range is also provided. Incidences are reported as occurrence/exposed cases and as percentage. Exacerbation means severe exacerbation with the use of OCS burst.

§ indicates the inclusion of patients with smoking history of ≥10 pack-years.

¥ indicates that we excluded current smokers FeNO values.

indicates the number of subjects with fixed airway obstruction.

*: P<.05; **: P<.01; ***: P<.001; ****: P<.0001 compared to MA.

°: P<.05; °°: P<.01; °°°: P<.001; °°°°: P<.0001 compared to CTRL.

Table S2. Inflammatory positive cells in the bronchial mucosa of patients

Cell counts mm ² lamina propria	Controls (n=9)	All subjects (n=42)	Mild asthmatics (n=20)	Severe asthmatics (n=22)
ECP ⁺	5.0±3.0	33.4±20.1***	34.0±22.3**	43.7±32.6**
NE ⁺	9.7±3.7	71.6±50.2**	57.4±42.1*	84.6±54.3****
CD4 ⁺	4.9±3.4	30.2±20.7**	19.9±15.5	39.0±20.9*** ^{°°}
CD68 ⁺	96.4±39.1	240.9±146.1**	267.0±154.6*	217.4±137.4**
Mast cells ⁺	26.2±19.6	68.2±55.4	88.9±62.1	48.6±40.7* [°]

Continuous variables are presented as mean ± SD.

*: P<.05; **: P<.01; ***: P<.001 compared to CTRL.

°: P<.05; °°: P<.01 compared to MA.

Supporting figure legends

Figure S1.

Panel A shows the effect of atopy and asthma severity on B1R⁺, B2R⁺, α-SMA⁺ 20µm, α-SMA⁺ 100µm cell counts, and basal membrane thickness in the lamina propria of bronchial biopsies.

Panel B shows the effect of smoking and asthma severity on B1R⁺, B2R⁺, α-SMA⁺ 20µm, α-SMA⁺ 100µm cell counts, and basal membrane thickness in the lamina propria of bronchial biopsies.

Two-way ANOVA showed significant interaction only for atopy for B2R⁺ (P=.03) and no significant interaction for B1R⁺, B2R⁺, α-SMA⁺ 20µm, α-SMA⁺ 100µm cell counts, and basal membrane thickness for allergy or smoking status. Šídák's multiple comparison post-tests.

*: P<.05; **: P<.01.

Figure S2.

Simple linear regression between B1R and ICS/day (µg fluticasone propionate HFA). The solid line represents the regression line and the dotted lines stand for the regression lines' 95% confidence intervals.

1 **Figure S3.**

2 Correlations among lung function parameters and B1R/B2R and matrix-related airway remodeling
3 markers in all asthmatic patients. Straight-line represents the best-fitting line. r_s = Spearman
4 correlation coefficient.

5 **Figure S4.**

6 ROC curves for optimal cut-off points of PB-FEV₁(% pred.) and PB-FEV₁/FVC based on the cut-off
7 of α -SMA 20 μ m (26.28 cells/mm²), α -SMA 100 μ m (114.6 cells/mm²), and B1R (13.46 cells/mm²).
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