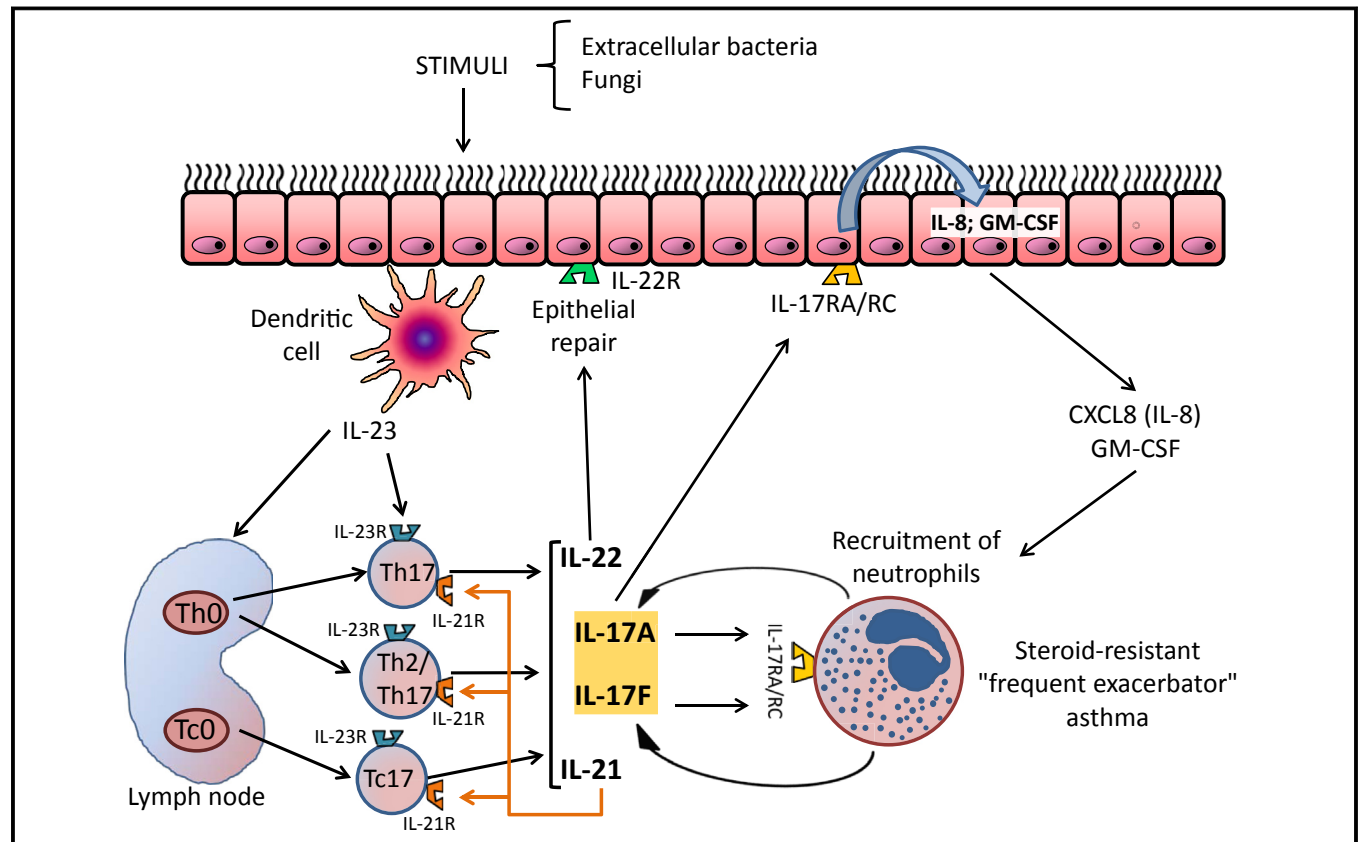


Identification of IL-17F/frequent exacerbator endotype in asthma

Fabio L. M. Ricciardolo, MD, PhD,^a Valentina Sorbello, PhD,^a Anna Folino, PhD,^a Fabio Gallo, MSc,^b Gian Mario Massaglia, MD,^c Gabriella Favatà, MD,^d Salvatore Conticello, MD,^d Davide Vallese, PhD,^e Federica Gani, MD,^c Mario Malerba, MD,^f Gert Folkerts, PhD,^g Giovanni Rolla, MD,^h Mirella Profita, PhD,ⁱ Thais Mauad, PhD,^j Antonino Di Stefano, PhD,^{e*} and Giorgio Ciprandi, MD^{k*} *Torino, Genova, Novara, Brescia, and Palermo, Italy; Utrecht, The Netherlands; and São Paulo, Brazil*

GRAPHICAL ABSTRACT



Background: Severe asthma might be associated with overexpression of T_H17 cytokines, which induce neutrophil recruitment via neutrophil-mobilizing cytokines in airways. **Objective:** To study IL-17-related cytokines in nasal/bronchial biopsies from controls and mild asthmatics (MAs) to severe asthmatics (SAs) in relation to exacerbation rate.

Methods: Inflammatory cells and IL-17A⁺, IL-17F⁺, IL-21⁺, IL-22⁺, and IL-23⁺ cells were examined by immunohistochemistry in cryostat sections of bronchial/nasal biopsies obtained from 33 SAs (21 frequent exacerbators [FEs]), 31 MAs (3 FEs), and 14 controls. IL-17F protein was also measured by ELISA in bronchial/nasal lysates and by

From ^athe Department of Clinical and Biological Sciences, Azienda Ospedaliera Universitaria (AOU) San Luigi Hospital, University of Torino, Torino; ^bthe Health Science Department, University of Genova; ^cthe Division of Respiratory Disease and ^dthe Division of Ear, Nose, and Throat, AOU San Luigi Hospital, Torino; ^ethe Pulmonary Division, Fondazione S. Maugeri, Istituto di Ricovero e Cura a Carattere Scientifico (IRCCS), Novara; ^fthe Department of Internal Medicine, University of Brescia; ^gthe Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, University of Utrecht; ^hthe Allergologia ed Immunologia Clinica, Ospedale Ordine Mauriziano "Umberto I," University of Torino; ⁱthe Institute of Biomedicine and Molecular Immunology, Italian National Research Council, Palermo; ^jthe Department of Pathology, Sao Paulo University Medical School; and ^kthe IRCCS-AOU San Martino, Genova.

*These authors are senior authors.

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Corresponding author: Fabio L.M. Ricciardolo, MD, PhD, Department of Clinical and Biological Sciences, University of Torino, AOU San Luigi Hospital, Regione Gonzole 10, 10043-Orbassano (Torino), Italy. E-mail: fabioluigimassimo.ricciardolo@unito.it. 0091-6749/\$36.00

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immunohistochemistry in bronchial tissue obtained from subjects who died because of fatal asthma. Immunofluorescence/confocal microscopy was used for IL-17F colocalization. **Results:** Higher number ($P < .05$) of neutrophils, IL-17A⁺, IL-17F⁺, and IL-21⁺ cells in bronchial biopsies and higher numbers ($P < .01$) of IL-17F⁺ and IL-21⁺ cells in nasal biopsies were observed in SAs compared with MAs. Bronchial IL-17F⁺ cells correlated with bronchial neutrophils ($r = 0.54$), exacerbation rate ($r = 0.41$), and FEV₁ ($r = -0.46$). Nasal IL-17F⁺ cells correlated with bronchial IL-17F ($r = 0.35$), exacerbation rate ($r = 0.47$), and FEV₁ ($r = -0.61$). FEs showed increased number of bronchial neutrophils/eosinophils/CD4⁺/CD8⁺ cells and bronchial/nasal IL-17F⁺ cells. Receiver operating characteristic curve analysis evidenced predictive cutoff values of bronchial neutrophils and nasal/bronchial IL-17F for discriminating between asthmatics and controls, between MAs and SAs and between FEs and non-FEs. IL-17F protein increased in bronchial/nasal lysates of SAs and FEs and in bronchial tissue of fatal asthma. IL-17F colocalized in CD4⁺/CD8⁺ cells. **Conclusions:** IL-17-related cytokines expression was amplified in bronchial/nasal mucosa of neutrophilic asthma prone to exacerbation, suggesting a pathogenic role of IL-17F in FEs. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Severe asthma, bronchial biopsy, nasal biopsy, IL-17F, neutrophils, frequent exacerbators, phenotype, endotype

Asthma is a heterogeneous disease characterized by chronic airway inflammation, typically sustained by T_H2 cells and eosinophils.¹ Several types of asthma feature neutrophilia such as intrinsic asthma²; asthma in the elderly,³ smokers,⁴ or obese females⁵; and a category of severe asthma (SA).⁶ Neutrophilic SA can be distinguished in 2 subtypes featured by absence or presence of eosinophils.⁶ T_H17 cells seem to play a relevant role in SA related to bronchial neutrophils.^{7,8} IL-17A/F increased expression has been demonstrated in bronchial mucosa of moderate-to-severe asthma.⁸⁻¹⁰ Moreover, IL-8 is prominently expressed in airways secretions of acute SAs,¹¹ and neutrophil airway infiltration is commonly reported in SA,¹² suggesting a key role for T_H17 cells. Although the main function of T_H17 cells is supposed to be induction of protection against potentially harmful fungi and extracellular bacteria, many experiments revealed that T_H17 cells also drive tissue inflammation in patients with several autoimmune diseases and allergy.^{13,14} These cells entail distinct phenotypes of CD4/CD8⁺ cells, characterized by IL-17A, IL-17F, IL-21, and IL-22 production.^{13,14} T_H17 cell differentiation is mainly regulated by IL-23, which is structurally related to IL-12,^{13,14} and whose serum levels are increased and negatively correlated with FEV₁ in asthmatic children.¹⁵ IL-17A/F induce the release of chemokines, such as CXCL1 (GRO α), CXCL8 (IL-8), and GM-CSF from airway epithelial cells and smooth muscle cells: these substances are involved in neutrophilic infiltration.^{16,17} IL-17A/F act on a multiplicity of cells, which respond with upregulation of proinflammatory cytokines, chemokines, and/or metalloproteases.^{18,19} A study demonstrated that IL-17 is able to increase IL-6 and IL-11 synthesis in bronchial fibroblasts derived from asthmatic bronchial biopsies playing an indirect role in asthma remodelling.²⁰ Asthma phenotypes, and more specifically asthma endotypes, are considered essential in clinical practice because of

Abbreviations used

AUC: Area under the curve
FE: Frequent exacerbator
FEV₁: Forced expiratory volume in 1 second
MA: Mild asthma/asthmatic
ROC: Receiver operating characteristic
SA: Severe asthma/asthmatic

their potential help to predict treatment response.²¹ Recently, the frequent exacerbator (FE) phenotype has been described, which is significantly more prone to exacerbations.²² It is known that several comorbidities, including upper airways disorders,²³ and overall exacerbations²⁴ may be risk factors for asthma severity.

Different studies showed a close relationship between upper and lower airways in asthma, indeed rhinitis is considered the main risk factor for asthma onset/worsening.²⁵ Moreover, nasal eosinophils correlate both with bronchial and nasal airflow limitations.²⁵ In addition, T_H17-driven inflammation may affect both upper and lower airways.^{8,26,27}

This study, which is an extension of a previous study,⁸ aimed to evaluate the expression of IL-17-related cytokines in nasal/bronchial biopsies obtained from control subjects and atopic/nonatopic mild asthmatics (MAs) to SAs focusing on exacerbation rate. Finally, IL-17F expression was also examined in fatal asthma.

METHODS

Asthmatic and control subjects

In this observational and cross-sectional study, bronchial biopsies from 78 subjects were analyzed: 33 SAs, 31 MAs, and 14 controls; clinical/demographic characteristics are shown in Table 1. Asthma diagnosis/severity were defined according to Global Initiative for Asthma (GINA) and European Respiratory Society/American Thoracic Society guidelines.^{1,28} Rhinitis/chronic sinusitis were defined according to guidelines.^{29,30} Allergy was assessed by skin prick tests/serum-specific IgE,²⁹ and all subjects performed lung function test and fractional exhaled NO as described in Online Repository at www.jacionline.org. Controls had no respiratory disease history and no airflow limitation. All subjects were in stable condition (see also Online Repository). FEs are defined subjects who have ≥ 2 asthma exacerbations/year that require use of systemic corticosteroids (or an increase from a stable maintenance dose) for at least 3 days or a hospitalization/emergency department visit because of asthma requiring systemic corticosteroids.^{22,31} The local ethics committee (San Luigi Hospital: protocols 1759/2008-14871/2009) approved the study, which conformed to the Declaration of Helsinki; written informed consent was obtained from each subject.

Fatal asthma subjects

Tissues were obtained from 10 patients who died because of asthma attack (fatal asthma) and 10 nonsmoking subjects who died of nonpulmonary causes (deceased controls). All of them underwent autopsy at the Department of Pathology of the São Paulo University between 1996 and 2004. For patients' descriptions and pathological inclusion criteria,³² see Online Repository. This study was approved by the human studies review board of the São Paulo University Medical School.

Measurements of lung function; fiberoptic bronchoscopy/rhinocopy, collection, and processing of bronchial-nasal biopsies/fatal asthma bronchial tissue, immunohistochemistry (see Fig E1 in this article's Online Repository at www.jacionline.org), immunofluorescence, and confocal microscopy; and ELISA test from tissue lysates of bronchial/nasal biopsies are provided in the Online Repository.

TABLE I. Subjects' characteristics

Clinical parameters	Controls (n = 14)	MAAs (n = 31)	SAs (n = 33)	Asthma non-FEs (n = 40)	Asthma FEs (n = 24)
Age (y)	52 ± 13	48.5 ± 9.4	55 ± 10	50 ± 12.8	55 ± 12.4
Sex, M/F	8/6	13/18	20/13	21/19	12/12
Smoke, yes/no	0/14	2/31	4/33	4/10	2/19
Atopy, yes/no	0/14	19/12	18/15	24/16	14/10
Rhinitis, yes/no	0/14	29/2	33/0	39/1	23/1
Severe persistent rhinitis, yes/no		10/21	15/18	12/28	13/11
Sinusitis, yes/no	0/14	15/16	21/12	21/19	15/9
FVC pre GLI, %pred	103 ± 18	100 ± 12	80.5 ± 13*†	95 ± 15	81 ± 14‡
FEV ₁ pre GLI, %pred	102 ± 12	88 ± 18*	55.5 ± 11.5*†	79.7 ± 22	58.3 ± 15‡
FEV ₁ postB2 change (L)	—	+344 ± 115	+289 ± 104	+328 ± 113	+274 ± 106‡
FeNO (ppb)	11 ± 2	32 ± 23*	25 ± 16*	25.5 ± 23	25 ± 14
Asthma duration (y)	—	22 ± 15	28 ± 18	25 ± 15	26 ± 19
Exacerbations, frequency (n/y)	—	0.7 ± 0.9	2.5 ± 2	0.6 ± 0.5	3.4 ± 2
Beclomethasone HFA equivalent daily dose (μg)	—	132 ± 99	629 ± 230†	266 ± 242	592 ± 299‡
Blood eosinophils (cells/mL)	119.3 ± 91	296.3 ± 197§	299.4 ± 215§	294.5 ± 216	286.7 ± 172

Data are expressed as means ± SDs. Seven SAs/FEs were on oral corticosteroid for ≥50% of the previous year (prednisone 5 mg/day at least for 6 months per year).³⁰ Two SAs were on omalizumab.

Five mild asthmatics were not on inhaled corticosteroid therapy.

F, Female; FeNO, fractional exhaled NO; FEV₁ preB2, baseline FEV₁; FEV₁ postB2 change, FEV₁ postB2 change from baseline; FVC, forced vital capacity; GLI, Global Lung Function Initiative 2012; HFA, hydrofluoroalkanes; M, male; ppb, parts per billion.

**P* < .001 significantly different from controls.

†*P* < .001 significantly different from MAAs.

‡*P* < .01 significantly different from non-FEs.

§*P* < .001 significantly different from controls.

Statistical analysis

Data were analyzed using Graph Pad Prism software (version 5.0; GraphPad Software Inc, San Diego, Calif). Clinical/functional variables are expressed as means ± SDs, morphological parameters as medians (interquartile ranges). A normal and nonnormal distribution were assumed, respectively, for clinical/functional data and morphological parameters applying, respectively, ANOVA or the nonparametric Kruskal-Wallis test for multiple comparisons and the Student *t* test or the Mann-Whitney *U* test for comparisons between groups. Spearman rank method was used for correlations. A receiver operating characteristic (ROC) curve was used to assess the accuracy of bronchial/nasal IL-17F in discriminating between asthmatics and controls, MA and SA and between FE and non-FE (see also [Online Repository](#)). The area under the curve (AUC) with the 95% confidence intervals was calculated for each ROC curve, and the best cutoff point was chosen based on the Youden index. *P* values of less than .05 were considered statistically significant, and ROC curves were constructed using R 3.3.1 software environment (R Foundation, Vienna, Austria).³³

RESULTS

Inflammatory cells in bronchial/nasal mucosa

The numbers of inflammatory cells (neutrophils, eosinophils, and CD4⁺ and CD8⁺ cells) were investigated in the bronchial/nasal mucosa (Figs 1 and 2; see [Fig E2](#) in this article's Online Repository at [www.jacionline.org](#)). Bronchial neutrophils were higher in the mucosa of SAs compared with MAAs (*P* < .01) and controls (*P* < .001) and in MAAs compared with controls (*P* < .01), whereas nasal neutrophils were more elevated in SAs (*P* < .01) and MAAs (*P* < .05) compared with controls (Fig 2, A and B).

Immunoreactivity of IL-17A/F and related cytokines in the bronchial/nasal mucosa

Immunohistochemistry of all bronchial and nasal IL-17-related cytokines is shown in Fig 1 for SA and in the Online Repository for MA (see [Fig E3](#) in this article's Online Repository at

[www.jacionline.org](#)) and controls (see [Fig E4](#) in this article's Online Repository at [www.jacionline.org](#)). IL-17-related cytokines were expressed in inflammatory cells, fibroblasts, and epithelial cells of bronchial/nasal mucosa.

IL-17A/F were significantly increased in asthmatics compared with controls both in bronchial (*P* < .01 and *P* < .01, respectively) and nasal (*P* < .05 and *P* < .001, respectively) mucosa, as well as bronchial IL-21 (*P* < .01), IL-22 (*P* < .001), IL-23 (*P* < .01), and nasal IL-21 (*P* < .01), IL-22 (*P* < .01), and IL-23 (*P* < .0001).

IL-17A/F and IL-21 were more expressed in the bronchial mucosa of SAs compared with both MAAs and controls (*P* ≤ .01) (Fig 2, A). Bronchial IL-17F and IL-21 were higher in MAAs compared with controls (*P* < .05) (Fig 2, A). Bronchial IL-22 and bronchial IL-23 were increased both in SAs (*P* < .05) and MAAs (*P* < .01) compared with controls (Fig 2, A). Nasal IL-17F and IL-21 were significantly higher in SAs compared with MAAs and controls and IL-17F also in MAAs compared with controls (*P* < .01) (Fig 2, B). Nasal IL-17A expression was significantly increased only in SAs compared with controls (*P* < .05) (Fig 2, B).

Finally, nasal IL-22 and nasal IL-23 were higher in SAs (*P* < .001) and MAAs (*P* < .05) compared with controls (Fig 2, B).

Concerning the bronchial and nasal epithelium, IL-17F expression was higher in SAs compared with both MAAs and controls both in bronchial and nasal mucosa (see [Table E1](#) in this article's Online Repository at [www.jacionline.org](#)).

Correlations between IL-17-related cytokines/inflammatory cells and clinical parameters

In all asthmatics, both bronchial and nasal IL-17F expression was negatively related to FEV₁ (*r*_s = −0.46, *P* < .0005 and *r*_s = −0.61, *P* < .0005, respectively) (Fig 3, A and E). Concurrently, both bronchial and nasal IL-17F expression was positively related to exacerbation rate (*r*_s = 0.41, *P* < .005 and *r*_s = 0.47, *P* < .0005, respectively) (Fig 3, B and F). In all

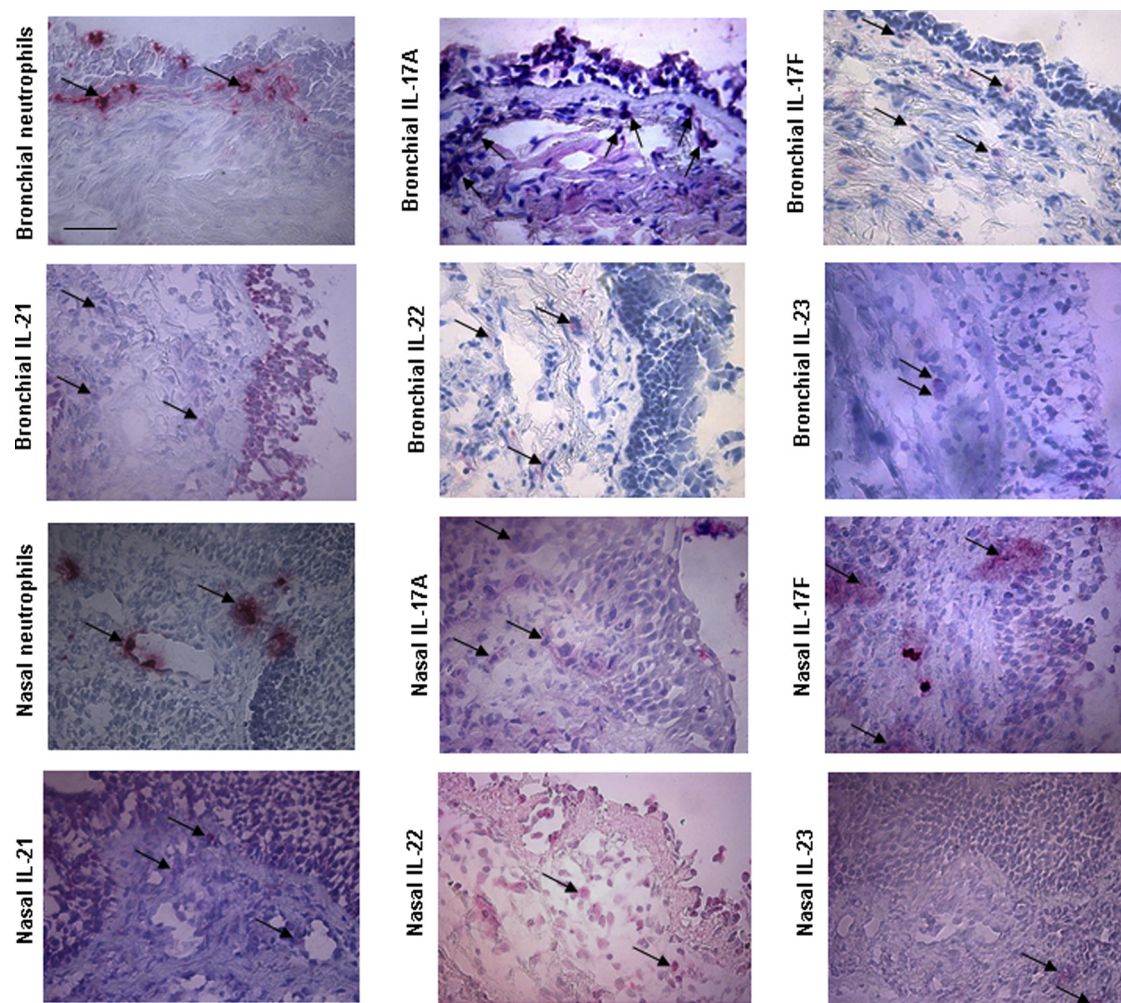


FIG 1. Neutrophils and IL-17-related cytokines expression in bronchial and nasal biopsy specimens obtained from severe asthma. Photomicrographs show the immunostaining for neutrophils, IL-17A, IL-17F, IL-21, IL-22, and IL-23 in the bronchial and nasal mucosa of severe asthma patients. Original magnification 400 $\times$. Bar = 25 μ m. Arrows indicate positive cells.

asthmatics, a negative correlation between FEV₁ and exacerbations number was also observed ($r_s = -0.47$, $P < .0005$) (Fig 3, J). Bronchial IL-17A⁺ ($r_s = -0.38$, $P < .01$) and IL-21⁺ ($r_s = -0.34$, $P < .01$) as well as nasal IL-21⁺ ($r_s = -0.43$, $P < .01$) cells showed a significant negative correlation with FEV₁ in asthmatics. Nasal IL-17A⁺ ($r_s = 0.40$, $P < .01$), IL-21⁺ ($r_s = 0.40$, $P < .01$), and IL-22⁺ ($r_s = 0.50$, $P < .001$) cells showed a significant positive correlation with exacerbation rate in asthmatics. Bronchial neutrophils are related to FEV₁ ($r_s = -0.41$, $P < .005$) (Fig 3, I) and exacerbation rate ($r_s = 0.47$, $P < .0005$) (Fig 3, K). Correlations between forced vital capacity and IL-17-related cytokines are shown in this article's Online Repository (see Fig E5 at www.jacionline.org).

Correlations between inflammatory cells and bronchial/nasal IL-17-related cytokines

In all asthmatics, bronchial IL-17F⁺ cells correlated with bronchial neutrophils ($r_s = 0.54$, $P < .0005$) (Fig 3, C), bronchial IL-17A⁺ ($r_s = 0.67$, $P < .0005$) (Fig 3, D), bronchial IL-21⁺ ($r_s = 0.30$, $P < .05$) (see Fig E6, A in this article's Online

Repository at www.jacionline.org), bronchial IL-22⁺ ($r_s = 0.29$, $P < .05$) (see Fig E6, B in this article's Online Repository at www.jacionline.org), bronchial CD4⁺ ($r_s = 0.36$, $P < .01$) (see Fig E7, C in this article's Online Repository at www.jacionline.org), bronchial CD8⁺ ($r_s = 0.27$, $P < .05$) (see Fig E7, D in this article's Online Repository at www.jacionline.org), and nasal IL-17F⁺ ($r_s = 0.35$, $P < .05$) (Fig 3, G) cells. For the other significant correlations among bronchial/nasal IL-17-related cytokines and inflammatory cells see the Online Repository and Figs E6 and E7 at www.jacionline.org.

Inflammatory cells and IL-17-related cytokines expression in bronchial/nasal mucosa of FEs versus non-FEs

Differences in inflammatory cells and IL-17-related cytokines expression were also investigated in asthmatics stratified in FEs ($n = 24$) and non-FEs ($n = 40$). Subjects' characteristics are reported in Table I. Concerning inflammatory cells expression in the bronchial/nasal mucosa, bronchial neutrophils, eosinophils, and CD4⁺ and CD8⁺ cells were significantly increased in FEs

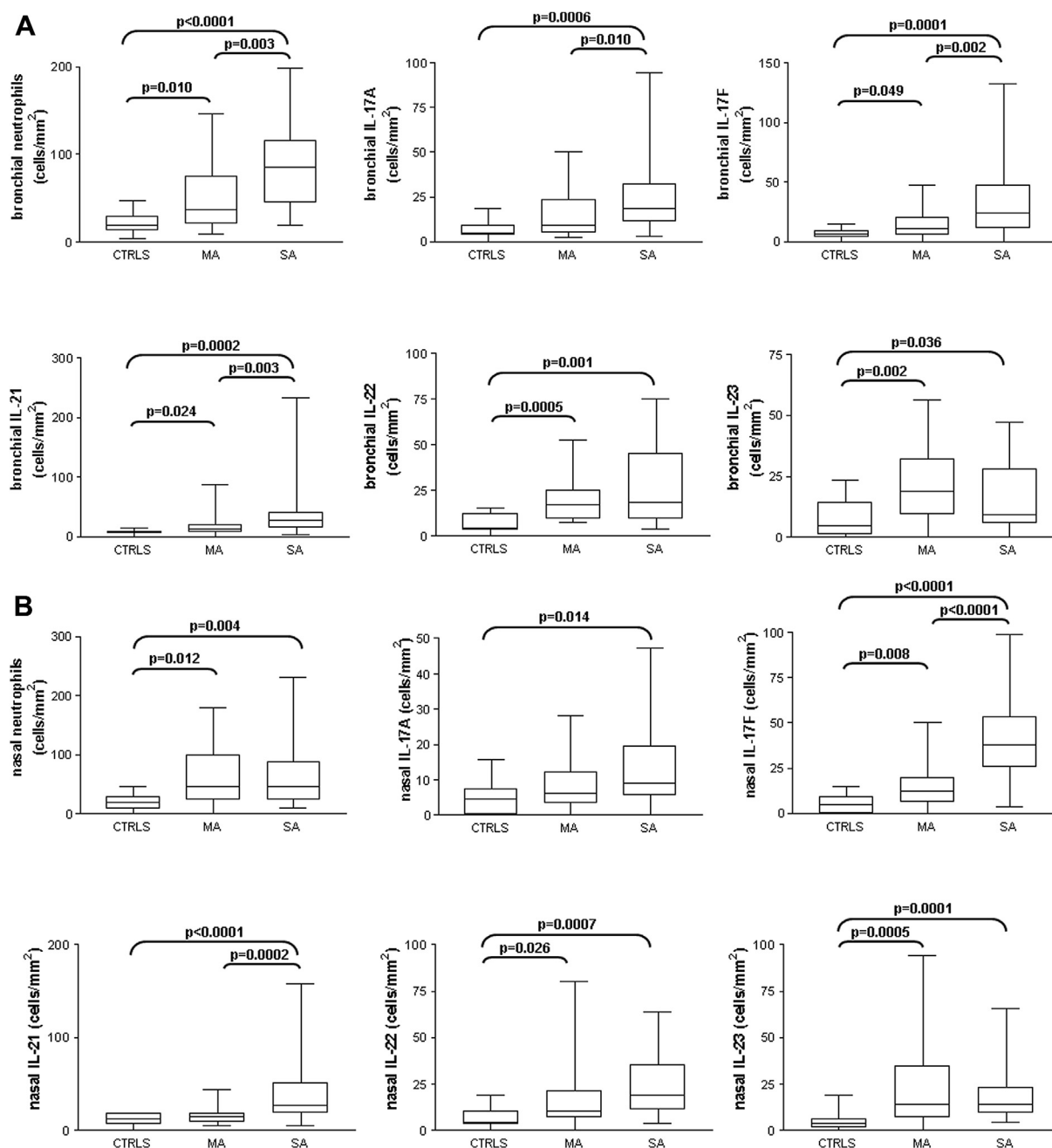


FIG 2. Number of neutrophils, IL-17A⁺, IL-17F⁺, IL-21⁺, IL-22⁺, and IL-23⁺ cells/mm² in the bronchial (A) and nasal (B) mucosa of SAs, MAs, and controls (CTRLS). Data are presented as box plots. Each horizontal line indicates the median value in the respective group; boxes represent the 25th and 75th percentiles and bars the 10th and 90th percentiles.

compared with non-FEs ($P < .05$) (Fig 4). Regarding IL-17-related cytokines, both bronchial ($P < .001$) and nasal ($P < .001$) IL-17F showed significantly higher expressions in FEs compared with non-FEs, as well as bronchial IL-17A, nasal IL-21, and nasal IL-22 ($P < .05$) (Fig 4).

ROC curve analysis

ROC curve analysis of bronchial neutrophils and bronchial eosinophils showed a significant AUC of 0.81 and 0.94, respectively, for discriminating asthmatics from healthy controls

(Fig 5, A). The best cutoff value for bronchial neutrophils was 47.17 cells/mm², whereas for bronchial eosinophils, it was 12.45 cells/mm². For the discrimination of MA from SA, significant AUC values of 0.75 for neutrophils were estimated and the best cutoff value for bronchial neutrophils was 50.31 cells/mm² (Fig 5, B). Finally, ROC curve analysis among all asthmatics for discriminating the FE phenotype (Fig 5, C) showed significant AUC values of 0.84 and 0.77 for neutrophils and eosinophils, respectively. The best cutoff values for neutrophils and eosinophils were 78.62 cells/mm² and 37.74 cells/mm², respectively.

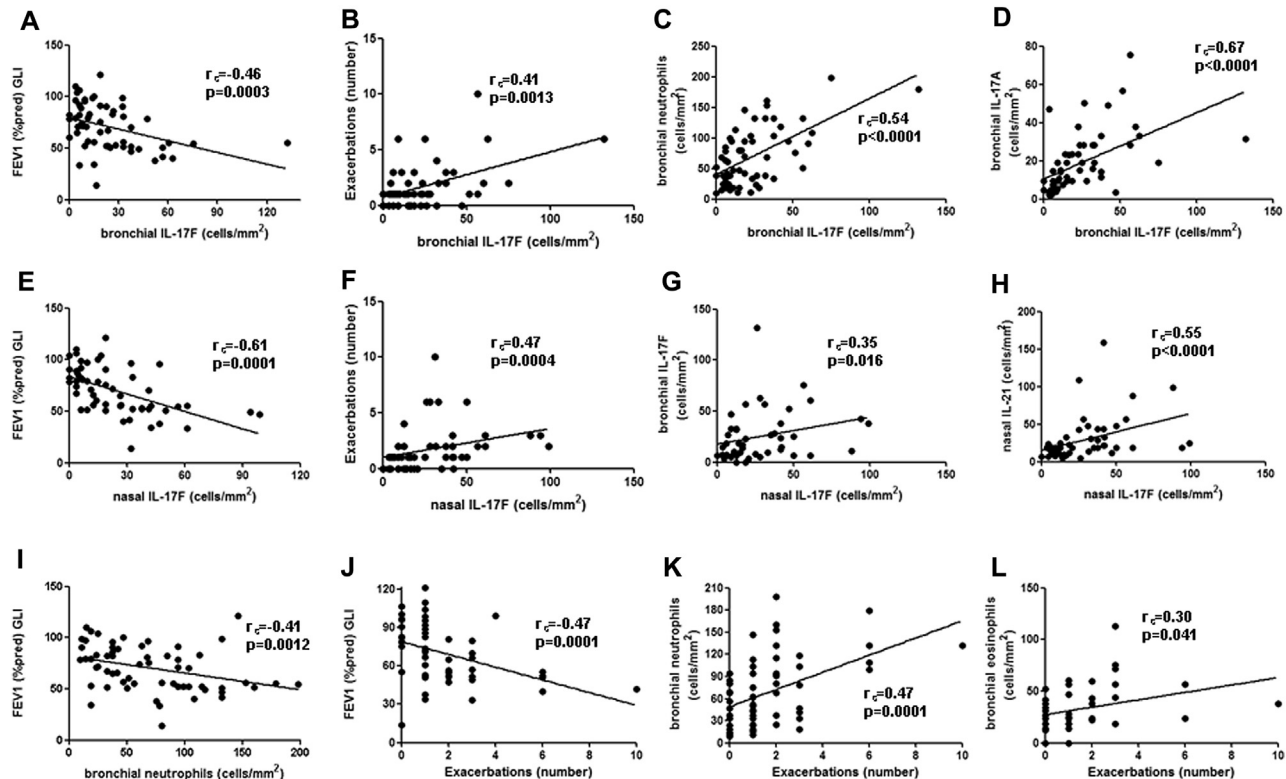


FIG 3. Relationships among the numbers of bronchial IL-17F⁺ cells and FEV₁ (A), exacerbations (B), bronchial neutrophils (C), and bronchial IL-17A⁺ cells (D) in all asthmatics. Relationships among the numbers of nasal IL-17F⁺ cells and FEV₁ (E), exacerbations (F), bronchial IL-17F⁺ cells (G), and nasal IL-21⁺ cells (H) in all asthmatics. Relationships between the numbers of bronchial neutrophils and FEV₁ (I) and among the numbers of exacerbations and FEV₁ (J), bronchial neutrophils (K), and bronchial eosinophils (L). The correlation coefficient was obtained using the Spearman rank method (r_s). GLI, Global Lung Function Initiative 2012.

ROC curve analysis of bronchial/nasal IL-17F showed a significant AUC of 0.84 and 0.85, respectively, for discriminating asthmatics from healthy controls (Fig 5, D). A best cutoff value of 10.29 cells/mm² for bronchial IL-17F was estimated, whereas for nasal IL-17F, it was 11.32 cells/mm².

To differentiate between MA and SA, a significant AUC of 0.73 and 0.83 was estimated for bronchial and nasal IL-17F, respectively (Fig 5, E). The best cutoff value for bronchial IL-17F was 23.58 cells/mm², whereas for nasal IL-17F, it was 18.87 cells/mm². Finally ROC curve analysis among all asthmatics for discriminating the FE phenotype (Fig 5, F) showed for bronchial IL-17F a significant AUC of 0.79 and a related best cutoff value of 23.58 cells/mm² and for nasal IL-17F, a significant AUC of 0.78 and a related best cutoff value of 26.41 cells/mm². The percentages of sensitivity, specificity, positive predictive value and negative predictive value for all variables in all subgroups are reported in Table E2 in the Online Repository.

IL-17F in bronchial/nasal lysates

Bronchial IL-17F lysates concentrations in SAs ($n = 10$; 64 ± 14 pg/mL) were significantly higher compared with MAs ($n = 10$; 43 ± 15 pg/mL; $P < .01$) and controls ($n = 8$; 29 ± 5 pg/mL; $P < .001$) (Fig 6). In addition, nasal IL-17F lysates concentrations in SAs ($n = 10$; 72 ± 22 pg/mL) were significantly

higher compared with MAs ($n = 10$; 47 ± 12 pg/mL; $P < .01$) and controls ($n = 8$; 35 ± 9 pg/mL; $P < .001$) (Fig 6). Bronchial and nasal IL-17F lysates concentrations in FEs ($n = 10$ and $n = 9$, respectively) were significantly increased compared with non-FEs ($n = 10$ and $n = 11$, respectively; $P \leq .001$).

Double staining/confocal microscopy for CD4⁺ and CD8⁺ cells with IL-17F in the bronchial/nasal mucosa of asthmatics

In bronchial/nasal mucosa of asthmatics merging the green (IL-17F) and red (CD4⁺ or CD8⁺ cells) pixels revealed that IL-17F positivity coexpressed CD4⁺ or CD8⁺ cells (yellow pixels) (Fig 7).

Colocalization by quantitative analysis in SAs ($n = 6$) showed that among all IL-17F⁺ cells $26.5 \pm 5\%$ and $22.1 \pm 8.1\%$ were CD8⁺ and $23.3 \pm 6.54\%$ and $21 \pm 3.5\%$ were CD4⁺ lymphocytes in the bronchial and nasal mucosa, respectively. Colocalization by quantitative analysis in MAs ($n = 3$) revealed that among all IL-17F⁺ cells $23.2 \pm 6.6\%$ and $22.6 \pm 8.3\%$ were CD8⁺ and $23.3 \pm 7.5\%$ and $20.3 \pm 5.7\%$ were CD4⁺ lymphocytes in the bronchial and nasal mucosa, respectively. In FEs ($n = 4$), among all IL-17F⁺ cells, $23.4 \pm 1.3\%$ and $25 \pm 8.1\%$ were CD8⁺ and $24.3 \pm 3.1\%$ and $23.5 \pm 3.5\%$ were CD4⁺ lymphocytes in the bronchial and nasal mucosa, respectively. The differences between SAs and MAs and between

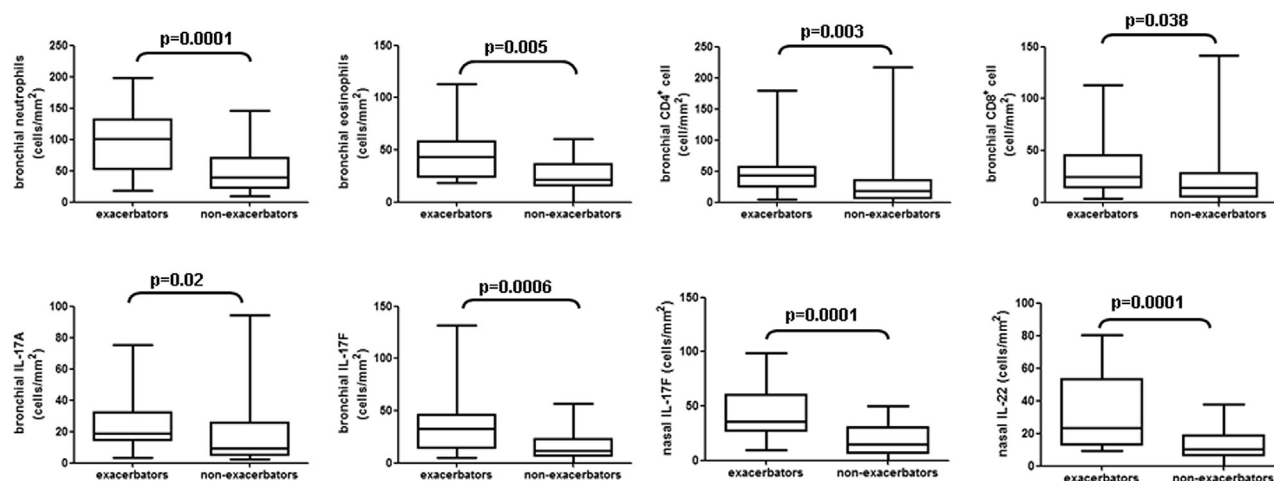


FIG 4. Number of bronchial neutrophils, eosinophils, CD4⁺ cells, CD8⁺ cells, IL-17A⁺, IL-17F⁺, and nasal IL-17F⁺ and IL-22⁺ cells in the bronchial/nasal mucosa of FEs versus non-FEs. Data are presented as box plots. Each horizontal line indicates the median value in the respective group; boxes represent the 25th and 75th percentiles and bars the 10th and 90th percentiles.

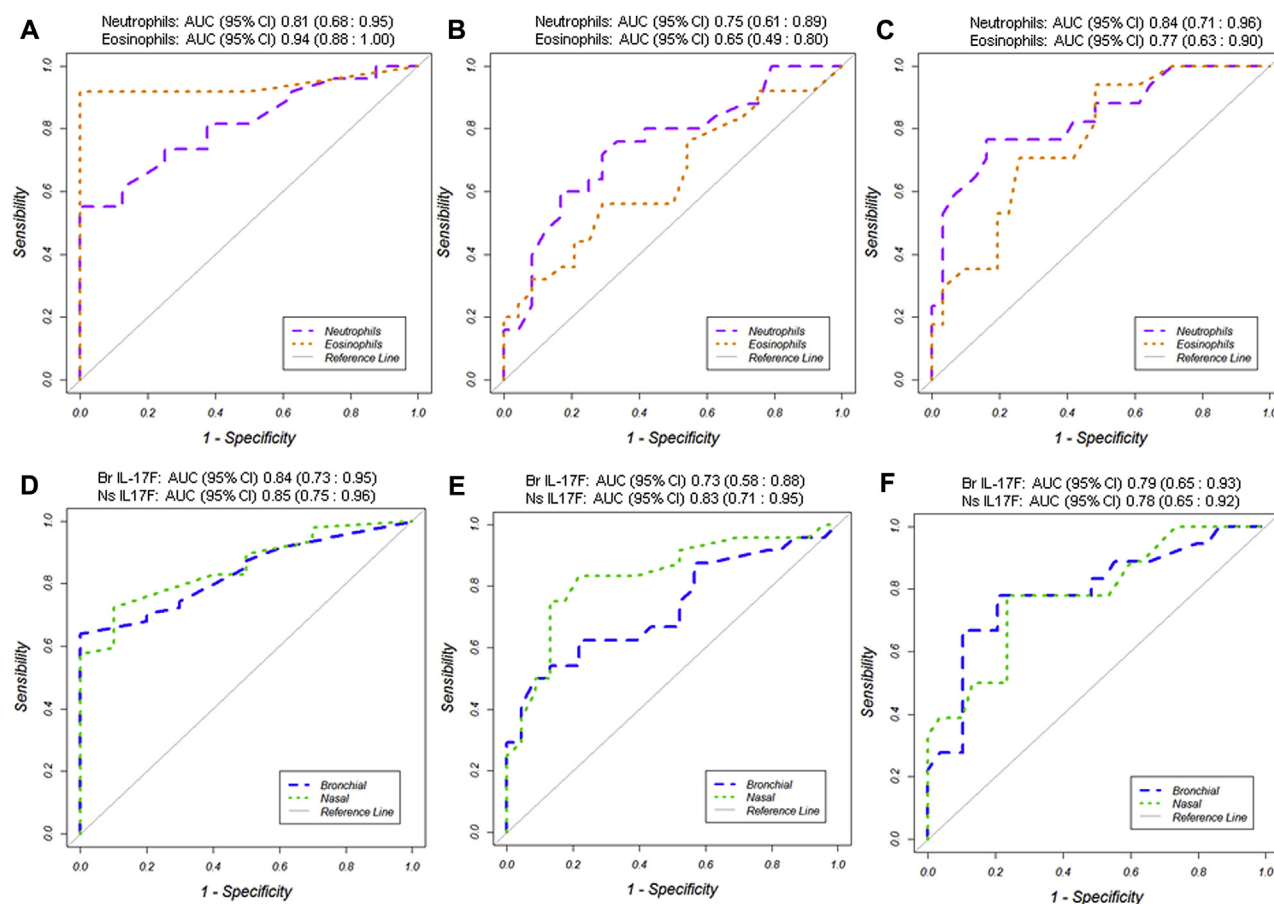


FIG 5. ROC curves for optimal cutoff points at which bronchial (Br) neutrophils and bronchial eosinophils discriminate asthmatic patients from healthy controls (A), SAs from MAs (B) and asthma FE from asthma non-FEs (C). ROC curves for optimal cutoff points at which bronchial and nasal (Ns) IL-17F discriminate asthmatic patients from healthy controls (D), SAs from MAs (E), and asthma FE from asthma non-FEs (F).

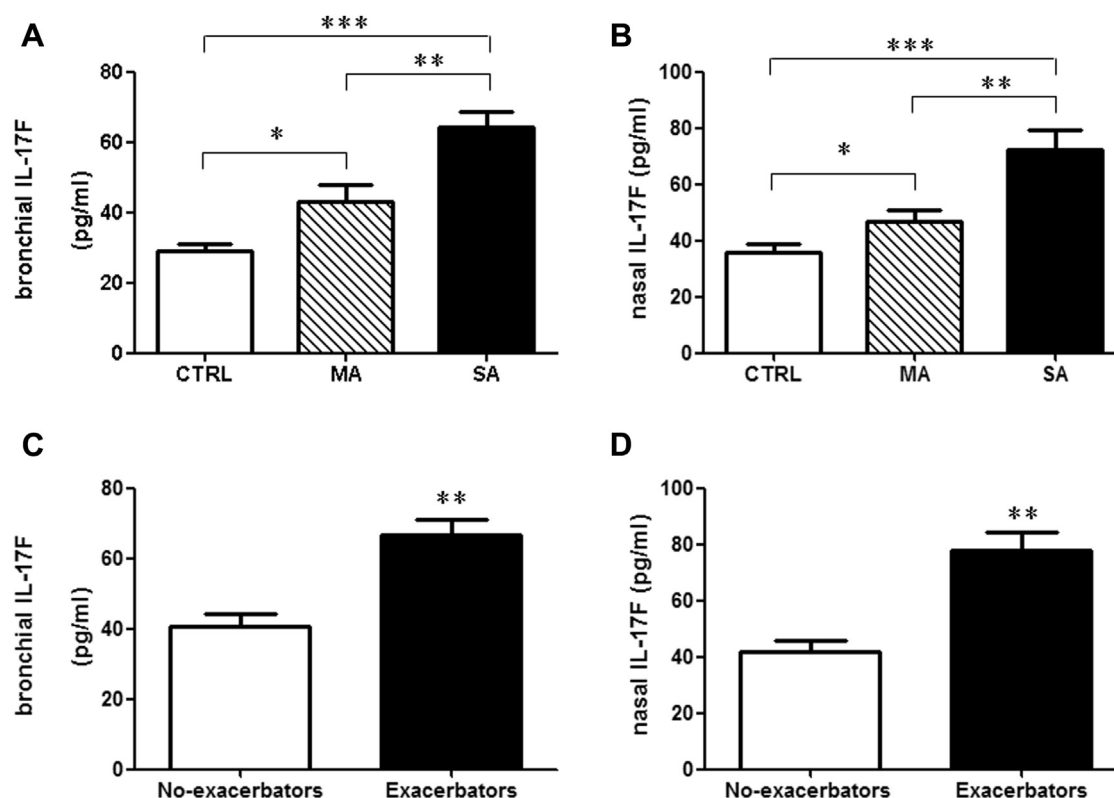


FIG 6. IL-17F concentration (pg/mL) in bronchial (A and C) and nasal biopsies (B and D) from CTRLs, MAs and SAs and from FEs and non-FEs. Data are presented as columns. Each column represents mean $\pm$ SD. * $P \leq .05$, ** $P \leq .001$, and *** $P \leq .0001$.

FEs and non-FEs ($n = 5$) were not statistically significant both in bronchial and nasal mucosa.

In bronchial mucosa of FEs ($n = 3$) the colocalization of IL-17F⁺/neutrophil elastase⁺ cells was $7.3 \pm 4\%$, but it was almost absent for IL-17F⁺/CD31⁺ cells (see Fig E8 in this article's Online Repository at www.jacionline.org).

IL-17F expression in fatal asthma

IL-17F and neutrophils expression was evaluated in a small group of subjects ($n = 10$) who died because of a fatal asthma attack and of controls ($n = 10$). IL-17F was significantly higher in fatal asthma patients than in controls ($P < .05$) (Fig 8), but no significant difference was observed for neutrophils between the 2 groups ($P = .68$) (see Fig E9 in this article's Online Repository at www.jacionline.org).

DISCUSSION

This study showed higher expression of IL-17-related cytokines, especially of IL-17F, in the nasal/bronchial mucosa of atopic/nonatopic SAs compared with MAs and controls. IL-17F was significantly related to bronchial neutrophilia, FEV₁, and exacerbation rate in all asthmatics. In particular, phenotyping the population of asthmatics in FEs and non-FEs, both nasal and bronchial IL-17F expression in conjunction with bronchial neutrophils (and to a lesser extent with eosinophils and CD4⁺ and CD8⁺ cells) was considerably increased in asthmatic FEs. Furthermore, IL-17F protein increased in bronchial/nasal lysates

of SAs and FEs in line with immunohistochemistry results. ROC curve analysis allowed us to identify an optimal predictive cutoff value of bronchial neutrophils and nasal/bronchial IL-17F for discriminating asthmatics, SAs, and FEs. Finally, bronchial IL-17F expression was higher in fatal asthma patients than in controls.

Bronchial mucosa in SAs was characterized by a more intense neutrophilic infiltration compared with MAs and controls, suggesting that SA is typified by a distinct neutrophilic phenotype with increased expression of T_H17-related cytokines (IL-17F, IL-17A, and IL-21) at bronchial level. Among asthmatics, neutrophils and nasal/bronchial IL-17F were significantly increased in FEs compared to non-FEs as well as bronchial IL-17F/A correlated with bronchial neutrophils in all asthmatics, strengthening the link between T_H17 cytokines and neutrophilia in relation to disease severity and exacerbation rate. This pivotal role of nasal/bronchial IL-17F was confirmed by ROC curve analysis. The tendency of higher IL-17F expression in bronchial mucosa of fatal asthma patients versus deceased controls unveils the involvement of this biomolecular pathway in the risk of death due to asthma.

Initially, Sun et al³⁴ showed IL-17A protein increase in induced sputum from SAs compared with from controls, which was accompanied by a rise in sputum neutrophils. The investigators also examined IL-17A protein in all disease stages and found that IL-17A increase in sputum clearly paralleled disease severity in conjunction with the concentrations of both the neutrophil-recruiting chemokine IL-8 and the activity marker myeloperoxidase, thus illustrating IL-17A association with

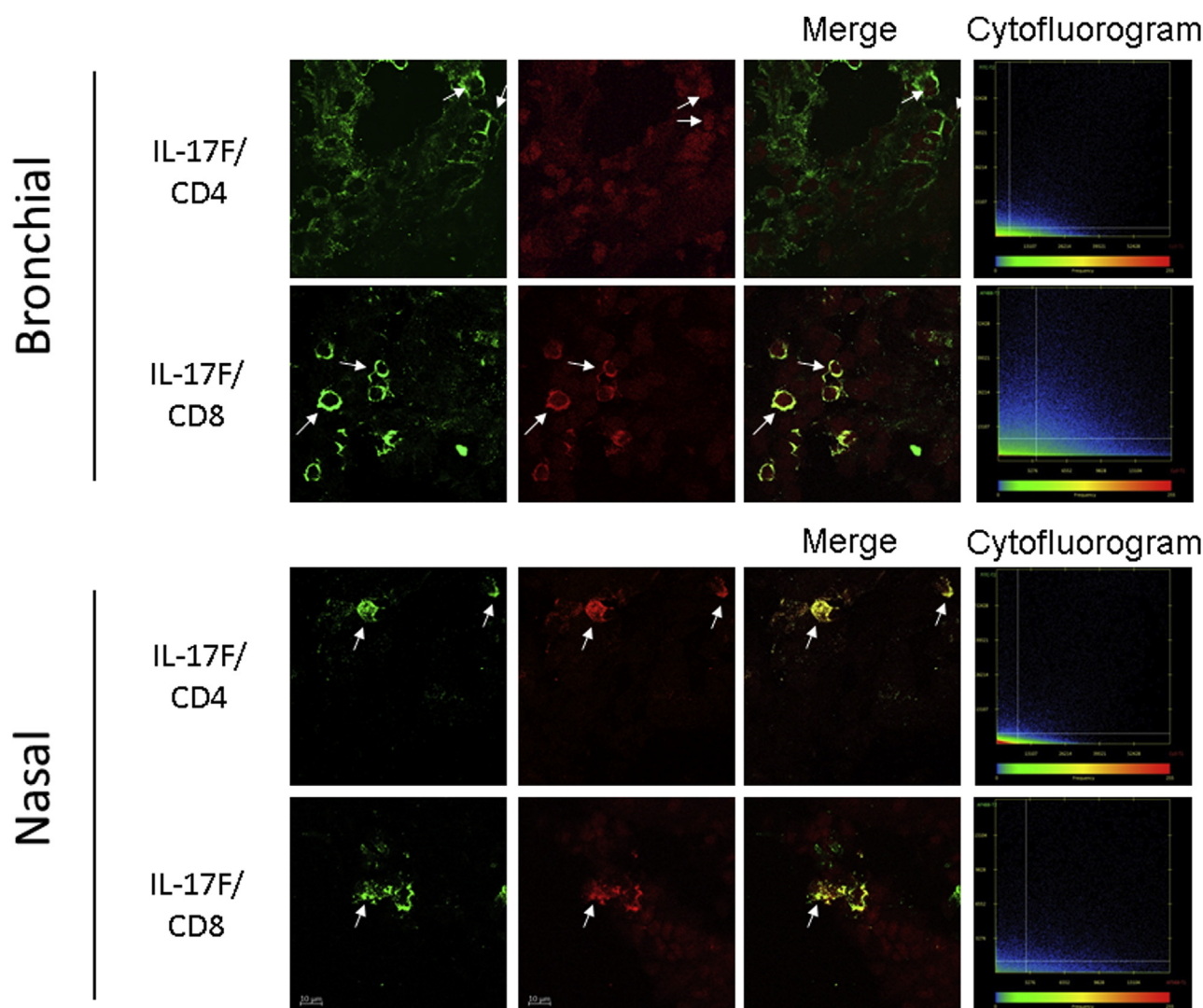


FIG 7. Confocal images of double immunostaining for the identification of IL-17F⁺/CD4⁺ and IL-17F⁺/CD8⁺ cells in the bronchial and nasal mucosa of asthma FEs. IL-17F positivity is shown in green (AF488) and positivity for CD4 or CD8 is shown in red (AF568), colocalization is shown in the merge panel as yellow pixels. In the cytofluorogram, colocalization is represented the as blue pixels. Original magnification 1000 $\times$.

neutrophil mobilization in asthma.³⁴ IL-17A was described in bronchial tissue from asthmatics with a more marked expression in MA to SA.³⁵ This increase was complement to the corresponding TGF- β and type I collagen increase, showing the involvement of IL-17A-producing cells in remodelling.³⁵ Al-Ramli et al⁹ demonstrated that the magnitude of the increase in bronchial IL-17A matches with disease severity and that this increase is robust in SAs, supporting the idea that IL-17A is involved in SA pathogenesis.³⁶ Similarly, the relevant role of IL-17F in asthma was demonstrated,³⁷ evidencing that also this cytokine stimulates the production and release of neutrophil-mobilizing cytokines (eg, GM-CSF and IL-8) in human bronchial epithelial cells³⁷ and bronchial fibroblasts.^{14,38} Some studies referred an increase of IL-17F expression in bronchial lamina propria related to disease severity^{9,10} and, interestingly, Al-Ramli et al⁹ found IL-17F protein in the epithelial layer and even within epithelial cells in the very same patients that expressed IL-17A in asthmatics' submucosa, suggesting that both cytokines may be expressed

simultaneously. There is now a growing evidence that, just like neutrophils *per se*, IL-17A/F are critically involved in mammalian host defense against bacteria, fungi and, possibly, viruses; thus, these 2 cytokines as well as the IL-17RA/RC receptor complex they share emerge as promising therapeutic targets.⁷

Concerning IL-21, we observed a significant increase in SAs compared with MAs and controls either in nasal or bronchial mucosa. IL-21 acts in an autocrine manner to promote IL-17A production and further inhibits IFN- γ production from T_H1 cells.^{39,40} The current study also showed an increased expression of IL-22 and IL-23 in the nasal/bronchial mucosa of both MAs and SAs compared with controls and, additionally, both IL-21 and IL-22 were higher in the nasal mucosa of FEs than non-FEs, indicating a role for IL-21 and IL-22 in modulating IL-17 cascade in asthma, particularly in the nose of subjects prone to exacerbations. IL-22 is expressed by T_H17 cells and promotes innate immunity against infection, which connect the immune

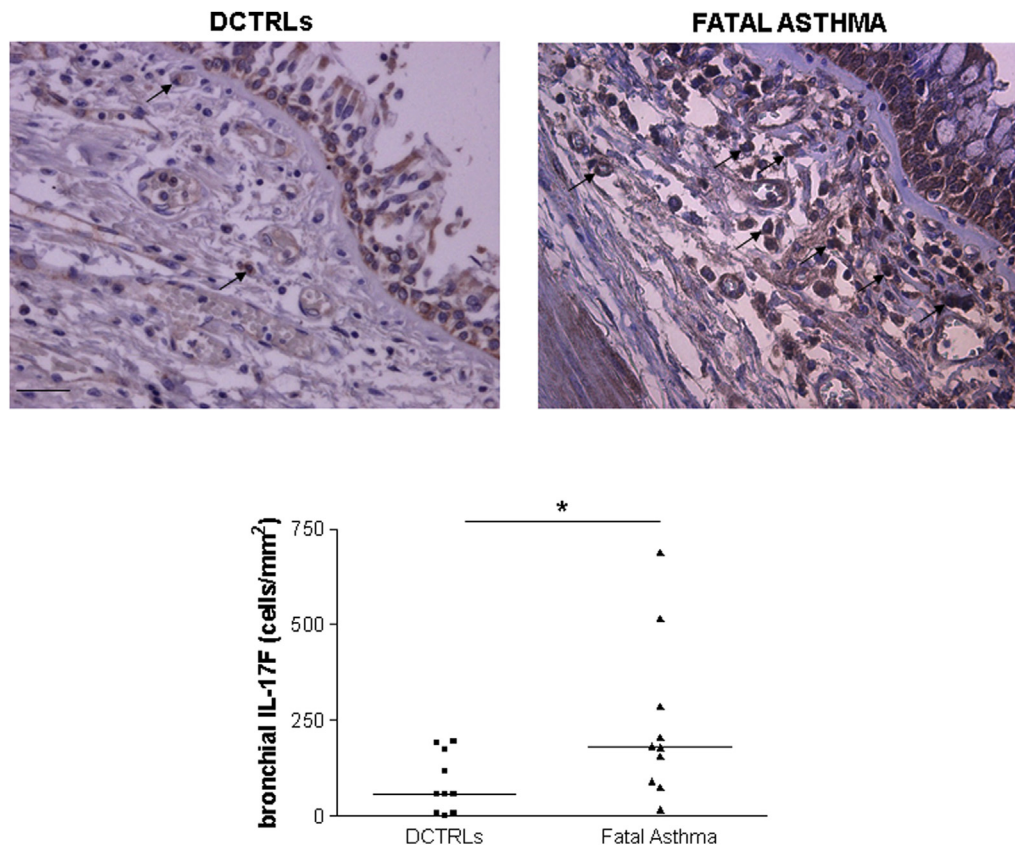


FIG 8. IL-17F expression in bronchial tissue obtained from fatal asthma. Photomicrographs show the immunostaining for IL-17F in the bronchial mucosa of fatal asthma patients and deceased controls (DCTRLs). Original magnification 400 $\times$. Bar = 25 μ m. Arrows indicate positive cells. Results are expressed as number of bronchial IL-17F⁺ cells/mm² in the bronchial mucosa. Each horizontal line indicates the median value in the respective group. * $P < .05$.

response to tissue inflammation.⁴¹ Both IL-17 and IL-22 play roles in immune defense to extracellular bacteria: IL-17 contributing to antibacterial immunity and IL-22 promoting epithelial proliferation and repair following injury.⁴² Furthermore, IL-23 is the most important differentiating cytokine, with proinflammatory properties, which drives the immune response toward T_H17 cells. In asthma, IL-23 promotes T_H17 cells' proliferation to maintain IL-17A/F production and neutrophils recruitment, indicating that the IL-23–T_H17 axis might become a novel therapeutic target especially for the severe corticosteroid-dependent asthma.⁴³ A recent study⁴⁴ suggested that IL-17/IL-23 cytokines hamper both anti-inflammatory and immunosuppressant actions of glucocorticoids in peripheral lymphocytes via increased glucocorticoid receptor- β –glucocorticoid receptor- α ratios.

The nasal mucosa of SAs showed a significantly more abundant infiltrate of CD4⁺ and CD8⁺ cells compared with those of MAs and controls, together with an increased nasal expression of IL-17F and IL-21, whereas nasal IL-17A, IL-22, and IL-23 were enhanced in all asthmatics compared with controls. A significant positive correlation between the number of IL-17A⁺ cells in sinonasal tissues and the sinusitis radiologic severity was observed in chronic rhinosinusitis with nasal polyps by Makiyama et al,²⁷ who also found a significant negative correlation between the number of IL-17A⁺ cells and

FEV₁–forced vital capacity ratio. Furthermore, it has been shown that T_H17 cells may promote both eosinophilic and neutrophilic inflammation in allergic rhinitis and chronic rhinosinusitis.⁴⁵ Thus, SA is characterized by a more intense T_H17-polarized nasal inflammation than MA confirming the close pathogenic association between nose and bronchi independently of atopic status. Nasal inflammation appears to play a relevant role in worsening lower airways function as previously demonstrated.²⁵ The role of T_H17 cytokines in an asthmatic's nose is further emphasized by the significant correlation between nasal IL-17F⁺ and bronchial IL-17F⁺ cells, FEV₁ (negative) or exacerbations number (positive), suggesting a similar pattern and degree of inflammation in upper and lower airways in asthma related to severity. This study strengthens the concept of united airways disease,⁴⁶ as nasal inflammation severity is constantly associated with asthma severity: the nose should be ever assessed in asthmatics and envisaged as the window of bronchi.⁴⁷

In all asthmatics a positive correlation between IL-17F and neutrophils in the bronchial mucosa and a negative correlation between both nasal and bronchial IL-17F and FEV₁ or forced vital capacity were observed, confirming consistently with our previous study⁸ the direct role for IL-17F in nasal/bronchial neutrophils recruitment and in lung function decline in asthma.⁹ A positive correlation between nasal and bronchial IL-17F⁺ cells and exacerbations number was also evidenced, indicating that

IL-17F can be associated to exacerbation frequency, which is a strong risk factor for pulmonary function decline^{24,48} and near-fatal events in SAs.²³

The current study also demonstrated higher numbers of bronchial CD4⁺ and CD8⁺ cells in FEs, both in relationship with bronchial IL-17F in all asthmatics and the colocalization of IL-17F in CD8⁺ (25%) and CD4⁺ lymphocytes (25%) in the bronchial/nasal mucosa of asthmatics, indicating an immune modulator role depending on IL-17 cascade for both T-cell subsets. Thereupon, Annunziato et al¹⁴ recently reported that the innate and adaptive immune response converge into 3 major kinds of cell-mediated effector immunity categorized as type 1, type 2, and type 3 that respond to distinct species of microbes. In particular, type 3 immunity is mediated by retinoic acid-related orphan receptor- γ ⁺ innate lymphoid cell 3s, T_H17 cells, and T_H17 cells producing IL-17, IL-22, or both, which promote neutrophils recruitment in tissues and induce antimicrobial peptide production by epithelial cells, thus protecting against extracellular bacteria and fungi.¹⁴

The use of high inhaled corticosteroid doses and oral corticosteroids maintenance therapy in SAs and FEs compared with low inhaled corticosteroid dose in MAs and non-FEs could be a limitation of this study. Previous reports showed that neutrophil survival is increased by corticosteroids,⁴⁹ suggesting that daily inhaled corticosteroid high doses could influence neutrophil counts but, on the other hand, oral corticosteroids treatment reduced sputum neutrophils in SAs.⁵⁰ In addition, it is fundamental to point out that IL-17 provokes steroid insensitivity in PBMCs by glucocorticoid receptor-beta upregulation⁴⁴ and steroid-resistant neutrophil activity, suggesting that IL-17 directly induces insensitivity of neutrophils to corticosteroids.⁵¹

In conclusion, the current study confirms previous preliminary data⁸ on nasal/bronchial IL-17F, extending to a larger population of both atopic and nonatopic asthmatics, in conjunction with a role for IL-17A and IL-21 in neutrophilic SA. Moreover, this study highlights the importance of nasal and bronchial IL-17F expression as a potential biomarker to identify the FE endotype among asthmatics. These factors might allow us to obtain an early prognosis that is clinically relevant to predicting treatment response to specific therapeutic options against IL-17F in asthma and to improving the clinical management of the respiratory disability in FE as well as considering the risk of asthma death.

Key messages

- Increased expression of IL-17-related cytokines in nasal and bronchial tissues of severe asthmatics is related to neutrophilic inflammation, airway obstruction, and exacerbation rate.
- Recognition of a FE phenotype in mixed neutrophilic/eosinophilic asthma with high values of bronchial/nasal IL-17F is mainly produced by CD4/T_H17 and CD8/T_H17.
- Identification of IL-17F/FE endotype might have clinical implications for predicting treatment response to selective therapeutic options against IL-17F in asthma.

REFERENCES

1. Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma (GINA). NHLBI/WHO workshop report. Bethesda, Md: National Heart, Lung and Blood Institute; 2016. Available at: www.ginasthma.org/. Accessed September 2016.
2. Amin K, Lúdvíksdóttir D, Janson C, Nettelbladt O, Björnsson E, Roomans GM, et al. Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. Am J Respir Crit Care Med 2000;162:2295-301.
3. Ricciardolo FL, Sabatini F, Sorbello V, Benedetto S, Defilippi I, Petecchia L, et al. Expression of vascular remodelling markers in relation to bradykinin receptors in asthma and COPD. Thorax 2013;68:803-11.
4. Chalmers GW, MacLeod KJ, Thomson L, Little SA, McSharry C, Thomson NC. Smoking and airway inflammation in patients with mild asthma. Chest 2001;120:1917-22.
5. Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, et al. Cluster analysis and clinical asthma phenotypes. Am J Respir Crit Care Med 2008;178:218-24.
6. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, et al. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. Am J Respir Crit Care Med 1999;160:1001-8.
7. Lindén A, Dahlén B. Interleukin-17 cytokine signalling in patients with asthma. Eur Respir J 2014;44:1319-31.
8. Sorbello V, Ciprandi G, Di Stefano A, Massaglia GM, Favatà G, Conticello S, et al. Nasal IL-17F is related to bronchial IL-17F/neutrophilia and exacerbations in stable atopic severe asthma. Allergy 2015;70:236-40.
9. Al-Ramli W, Préfontaine D, Chouiali F, Martin JG, Olivenstein R, Lemièrre C, et al. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. J Allergy Clin Immunol 2009;123:1185-7.
10. Doe C, Bafadhel M, Siddiqui S, Desai D, Mistry V, Rugman P, et al. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. Chest 2010;138:1140-7.
11. Ordonez CL, Shaughnessy TE, Matthay MA, Fahy JV. Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: clinical and biologic significance. Am J Respir Crit Care Med 2000;161:1185-90.
12. Wenzel S. Severe asthma in adults. Am J Respir Crit Care Med 2005;172:149-60.
13. Nembrini C, Marsland BJ, Kopf M. IL-17-producing T cells in lung immunity and inflammation. J Allergy Clin Immunol 2009;123:986-94; quiz 995-6.
14. Annunziato F, Romagnani C, Romagnani S. The 3 major types of innate and adaptive cell-mediated effector immunity. J Allergy Clin Immunol 2015;135:626-35.
15. Ciprandi G, Cuppari C, Salpietro AM, Tosca MA, Rigoli L, Grasso L, et al. Serum IL-23 strongly and inversely correlates with FEV1 in asthmatic children. Int Arch Allergy Immunol 2012;159:183-6.
16. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgamma directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121-33.
17. Rahman MS, Yamasaki A, Yang J, Shan L, Halayko AJ, Gounni AS. IL-17A induces eotaxin-1/CC chemokine ligand 11 expression in human airway smooth muscle cells: role of MAPK (Erk1/2, JNK, and p38) pathways. J Immunol 2006;177:4064-71.
18. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005;6:1133-41.
19. Hizawa N, Kawaguchi M, Huang SK, Nishimura M. Role of interleukin-17F in chronic inflammatory and allergic lung disease. Clin Exp Allergy 2006;36:1109-14.
20. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Pagé N, et al. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol 2001;108:430-8.
21. Agache I, Akdis C, Jutel M, Virchow JC. Untangling asthma phenotypes and endotypes. Allergy 2012;67:835-46.
22. Kupczyk M, ten Brinke A, Sterk PJ, Bel EH, Papi A, Chanez P, et al. BIOAIR Investigators. Frequent exacerbators—a distinct phenotype of severe asthma. Clin Exp Allergy 2014;44:212-21.
23. Moore WC, Bleeker ER, Curran-Everett D, Erzurum SC, Ameredes BT, Bacharier L, et al. National Heart, Lung, and Blood Institute's Severe Asthma Research Program. Characterization of the severe asthma phenotype by the National Heart, Lung, and Blood Institute's Severe Asthma Research Program. J Allergy Clin Immunol 2007;119:405-13.
24. Bai TR, Vonk JM, Postma DS, Boezen HM. Severe exacerbations predict excess lung function decline in asthma. Eur Respir J 2007;30:452-6.
25. Ciprandi G, Cirillo I. The lower airway pathology of rhinitis. J Allergy Clin Immunol 2006;118:1105-9.

26. Ciprandi G, De Amici M, Murdaca G, Fenoglio D, Ricciardolo F, Marseglia G, et al. Serum interleukin-17 levels are related to clinical severity in allergic rhinitis. *Allergy* 2009;64:1375-8.
27. Makihara S, Okano M, Fujiwara T, Kariya S, Noda Y, Higaki T, et al. Regulation and characterization of IL-17A expression in patients with chronic rhinosinusitis and its relationship with eosinophilic inflammation. *J Allergy Clin Immunol* 2010;126:397-400, 400.e1-11.
28. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014;43:343-73.
29. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy* 2008; 63(suppl 86):8-160.
30. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012: a summary for otorhinolaryngologists. *Rhinology* 2012;50:1-12.
31. Fuhrbrigge A, Peden D, Apter AJ, Boushey HA, Camargo CA Jr, Gern J, et al. Asthma outcomes: exacerbations. *J Allergy Clin Immunol* 2012;129(3 suppl): S34-48.
32. Mauad T, Ferreira DS, Costa MB, Araujo BB, Silva LF, Martins MA, et al. Characterization of autopsy-proven fatal asthma patients in São Paulo, Brazil. *Rev Panam Salud Publica* 2008;23:418-23.
33. R Core Team. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2016. Available at: <http://www.R-project.org/>. Accessed September 2016.
34. Sun YC, Zhou QT, Yao WZ. Sputum interleukin-17A is increased and associated with airway neutrophilia in patients with severe asthma. *Chin Med J (Engl)* 2005; 118:953-6.
35. Chakir J, Shannon J, Molet S, Fukakusa M, Elias J, Laviolette M, et al. Airway remodeling-associated mediators in moderate to severe asthma: effect of steroids on TGF-beta, IL-11, IL-17A, and type I and type III collagen expression. *J Allergy Clin Immunol* 2003;111:1293-8.
36. Agache I, Ciobanu C, Agache C, Anghel M. Increased serum IL-17 is an independent risk factor for severe asthma. *Respir Med* 2010;104: 1131-7.
37. Kawaguchi M, Onuchic LF, Li XD, Essayan DM, Schroeder J, Xiao HQ, et al. Identification of a novel cytokine, ML-1, and its expression in subjects with asthma. *J Immunol* 2001;167:4430-5.
38. Newcomb DC, Peebles RS Jr. Th17-mediated inflammation in asthma. *Curr Opin Immunol* 2013;25:755-60.
39. Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 2007;8:967-74.
40. Korn T, Bettelli E, Gao W, Awasthi A, Jäger A, Strom TB, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 2007;448:484-7.
41. Chung Y, Yang X, Chang SH, Ma L, Tian Q, Dong C. Expression and regulation of IL-22 in the IL-17-producing CD4⁺ T lymphocytes. *Cell Res* 2006;16:902-7.
42. McAleer JP, Kolls JK. Directing traffic: IL-17 and IL-22 coordinate pulmonary immune defense. *Immunol Rev* 2014;260:129-44.
43. Li Y, Hua S. Mechanisms of pathogenesis in allergic asthma: role of interleukin-23. *Respirology* 2014;19:663-9.
44. Vazquez-Tello A, Halwani R, Hamid Q, Al-Muhsen S. Glucocorticoid receptor-beta up-regulation and steroid resistance induction by IL-17 and IL-23 cytokine stimulation in peripheral mononuclear cells. *J Clin Immunol* 2013;33:466-78.
45. Liu Y, Zeng M, Liu Z. Th17 response and its regulation in inflammatory upper airway diseases. *Clin Exp Allergy* 2015;45:602-12.
46. Rimmer J, Ruhno JW. Rhinitis and asthma: united airway disease. *Med J Aust* 2006;185:565-71.
47. Amorim MM, Araruna A, Caetano LB, Cruz AC, Santoro LL, Fernandes AL. Nasal eosinophilia: an indicator of eosinophilic inflammation in asthma. *Clin Exp Allergy* 2010;40:867-74.
48. McDonald VM, Gibson PG. Exacerbations of severe asthma. *Clin Exp Allergy* 2012;42:670-7.
49. Saffar AS, Ashdown H, Gounni AS. The molecular mechanisms of glucocorticoids-mediated neutrophil survival. *Curr Drug Targets* 2011;12:556-62.
50. Louis R, Lau LC, Bron AO, Roldaan AC, Radermecker M, Djukanovic R. The relationship between airways inflammation and asthma severity. *Am J Respir Crit Care Med* 2000;161:9-16.
51. Murcia RY, Vargas A, Lavoie JP. The interleukin-17 induced activation and increased survival of equine neutrophils is insensitive to glucocorticoids. *PLoS One* 2016;11:e0154755.

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Identification of IL-17F/frequent exacerbator endotype in asthma

Fabio L.M. Ricciardolo, MD, PhD^{1*}, Valentina Sorbello, PhD¹, Anna Folino, PhD¹, Fabio Gallo, MSc², Gian Mario Massaglia, MD³, Gabriella Favatà⁴, MD, Salvatore Conticello⁴, MD, Davide Vallese, PhD⁵, Federica Gani³, MD, Mario Malerba⁶, MD, Gert Folkerts⁷, PhD, Giovanni Rolla⁸, MD, Mirella Profita⁹, PhD, Thais Mauad¹⁰, PhD, Antonino Di Stefano^{5§}, PhD, Giorgio Ciprandi^{2§}, MD

¹Department of Clinical and Biological Sciences, University of Torino, AOU San Luigi Hospital, Orbassano (Torino), Italy; ²Health Science Department, University of Genova, Italy; ³Division of Respiratory Disease and ⁴Division of Ear, Nose and Throat, AOU San Luigi Hospital, Orbassano (Torino), Italy; ⁵Pulmonary Division, Fondazione S. Maugeri, IRCCS, Veruno (Novara), Italy; ⁶Department of Internal Medicine, University of Brescia, Italy; ⁷Department of Pharmacology and Pathophysiology, Utrecht Institute for Pharmaceutical Sciences, University of Utrecht, The Netherlands; ⁸Allergologia ed Immunologia Clinica, Ospedale Ordine Mauriziano “Umberto I”, University of Torino, Italy; ⁹Institute of Biomedicine and Molecular Immunology (IBIM), Italian National Research Council (CNR), Palermo, Italy; ¹⁰Department of Pathology, Sao Paulo University Medical School, São Paulo, Brazil; ¹¹IRCCS-AOU San Martino, Genova, Italy.

[§]Both authors are senior authors.

MATERIALS AND METHODS

Asthmatic and control subjects

In this observational and cross-sectional study bronchial biopsies from 78 subjects were analysed by immunohistochemistry: 33 severe asthma, 31 mild asthma and 14 healthy controls; clinical and demographic characteristics are shown in Table 1. Asthma diagnosis and severity were defined according to GINA and ERS/ATS guidelines^{E1,E2}.

Rhinitis was diagnosed according to Allergic Rhinitis and its Impact on Asthma (ARIA) guidelines^{E3}. Chronic Sinusitis was diagnosed according to European position paper on rhinosinusitis and nasal polyps (EPOS) guidelines^{E4}. Allergy was diagnosed when sensitization, documented by positive skin prick test and serum allergen-specific IgE assay, was consistent with symptoms occurrence after the exposure to the sensitizing allergen^{E3}.

All subjects performed lung function test and FeNO as below described. All control subjects had no history of respiratory disease and no airflow limitation. All subjects were in stable condition and none of them was treated with theophylline, antibiotics, antioxidants, mucolytics, and/or systemic glucocorticoids in the 6 weeks prior to bronchial biopsy.

Frequent exacerbator is defined the subject who has two or more severe asthma exacerbations per year which requires use of systemic corticosteroids (or an increase from a stable maintenance dose) for at least three days or a hospitalization or emergency department visit because of asthma requiring systemic corticosteroids^{E5}. The local Ethics Committee (San Luigi Hospital: protocols 1759/2008-14871/2009) approved the study which conformed to the Declaration of Helsinki; written informed consent was obtained from each subject.

Fatal asthma subjects

Tissues were obtained from the lungs of 10 patients (3/2 non-atopic/atopic males-3/2 non-atopic/atopic females; age 40±15 years; all non-smokers; 1/10 under regular use of corticosteroids)

who died due to an asthma attack (fatal asthma) and underwent autopsy at the Department of Pathology of the São Paulo University between 1996 and 2004. All patients had their deaths ascribed to asthma by the pathologist and were part of a previous study^{E6}. Clinical data were obtained from an interview with the next of kin^{E6}. Pathological inclusion criteria were lung hyperinflation and mucus hypersecretion and the following histological changes related to asthma: epithelial shedding, basement membrane thickening, airway smooth muscle hypertrophy and cellular inflammatory infiltrate with or without eosinophils. Ten non-smoking subjects (5 males/5 females; age 50±8 years) who died of non-pulmonary causes, had no previous history of asthma, wheeze or other lung diseases and no gross or microscopic lung alterations were included as deceased controls (DCTRLs). A mean of 14 h elapsed between death and autopsy/tissue collection for both asthmatics and controls. This study was approved by the human studies review board of the São Paulo University Medical School.

Pulmonary Function Tests

Spirometric values were obtained using a pneumotachograph with volume integrator (Quark Spiro, Cosmed; Roma, Italy). The pulmonary function tests (PFTs) and bronchodilator response (reversibility test) were performed following ATS/ERS criteria^{E7,E8}. All values were expressed as percentage of predicted normal which were derived using prediction equations from the Global Lung Function Initiative (GLI-2012 <http://www.lungfunction.org/>)^{E9}.

Exhaled nitric oxide (FeNO)

FeNO was measured with a chemiluminescence analyser (Eco Medics CLD88 sp, Duernten, Switzerland) as previously described^{E10}.

Fibreoptic bronchoscopy and rhinoscopy, collection and processing of bronchial/nasal biopsies and fatal asthma bronchial tissue

Bronchoscopy and rhinoscopy (1 week later) were performed in all subjects using a flexible fibreoptic bronchoscope (Pentax FB-18P; Asahi Optical Co. LTD, Tokyo, Japan) and a flexible fibreoptic nasal endoscope (ENF Type P4, Olympus; Tokyo, Japan) respectively, after the appropriate premedication [for bronchoscopy: atropine (0.5 mg) and diazepam (10 mg), both given by intramuscular injection followed by a local anaesthesia with lidocaine (4%) to the upper airways and larynx and by lidocaine (2%) sprayed into the lower airways; for nasal endoscopy: 0.05% oxymetazoline moistened nasal cottonoids with Xylocaine and 1:100.000 adrenaline solution for 10 minutes before procedure]. Bronchial (segmental/lobar airways) and nasal (inferior turbinate) biopsies were gently extracted and processed for light microscopy as previously described^{E11,E12}. Samples were embedded in Tissue Tek II OCT (Miles Scientific, Naperville, IL), frozen within 15 min in isopentane pre-cooled in liquid nitrogen, and stored at -80°C . For all samples 6 μm thick cryostat sections were cut for immunohistochemistry.

Fatal asthma and deceased control subjects had random samples from central areas of the lung collected during autopsy. Tissue was fixed in 4% buffered formalin, routinely processed and embedded in paraffin.

Immunohistochemistry

Sections, obtained by selection of the best oriented biopsy for each patient after preliminary analysis, were stained with immunohistochemical methods^{E13}. One immunostained section for each antigen was used for quantitative purposes. Cracks of tissue or artificial mechanical areas were excluded from the quantification process and quantification was performed in a blinded fashion^{E14}. Primary antibodies directed against the following markers were used: lymphocytes (mouse anti CD4, 1:100, code M716; mouse anti CD8, 1:200, code M7103; Dakocytomation, Milan, Italy), neutrophils (mouse anti-elastase, 1:100, code M752; Dakocytomation, Milan, Italy), eosinophils (rabbit anti ECP, 1:200, code bs8615R; Bioss Inc, Woburn, USA), Th-17 cytokines (goat-anti-IL-17A, 1:50, code AF317NA; goat-anti-IL-17F, 1:50, code AF1335NA; goat-anti-IL-22, 1:50, code

AF782 [R&D Systems, Minneapolis, USA]; goat-anti-IL-21, 1:50, code SC17649; and goat-anti-IL23, 1:50, code sc21079; [Santa Cruz Biotechnology Inc. Texas]). Irrelevant primary antibodies from Sigma (Milan, Italy; irrelevant rabbit, 1:200, code I8140; irrelevant mouse, 1:100-1:200, code I8765; irrelevant goat, 1:50, code I9140) were applied at the same concentration of the related specific primary antibodies for immunostaining of negative control slides (Figure E1). In addition, as a further negative control, secondary antibody alone was used (Figure E1).

After blocking non-specific binding sites using serum derived from the same animal species as the secondary antibody, primary antibodies were applied at optimal dilutions in TRIS-buffered saline (0.15 M saline containing 0.05 M TRIS-hydrochloric acid at pH 7.6) and incubated (1 h) at room temperature in a humidified chamber. Antibody binding was demonstrated with the use of secondary antibodies anti mouse (Vector, 1:200, code BA 2000) or anti goat (Vector, 1:200, code BA 5000) or anti rabbit (Vector, 1:200, code BA1000) followed by Strept AB Complex/AP (Dako, K0391) and fast-red substrate (pink colour).

To evaluate IL-17F in fatal asthma, 5 µm thick paraffin-embedded sections obtained from bronchial tissue were analysed using mouse anti-human IL-17F (diluted 1:50, Thermo Scientific, Waltham, MA, USA) by the BenchMark Ultra instrument (Ventana Medical Systems, Roche, Basel, Switzerland) according to the manufacturer's instructions.

Morphometric measurements were performed with a light microscope (Leitz Biomed, Leica Cambridge, UK) connected to a video recorder linked to a computerised image system (Quantimet 500 Image Processing and Analysis System, Software Qwin V0200B, Leica). Light-microscopic analysis was performed at a magnification of 400x. IL-17A, IL-17F, IL-21, IL-22 and IL-23 immunostaining was scored in the complete bronchial epithelium (constituted by both columnar and basal epithelial cells); range: 0 (absence of immunostaining) to 3 (extensive immunostaining). Immunostained cells were quantified in the area 100 µm beneath the epithelial 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 each biopsy. We quantified the immunostained cells with at least a portion of the nucleus seen close to immunopositivity^{E14}.

IL-17F ELISA

IL-17F concentration in bronchial and nasal tissues was detected by ELISA (Life Span Biosciences, Inc, Seattle, Washington). Briefly, slices of 250µm were taken from biopsies and suspended in 200µl of PBS. Tissues were sonicated and Bradford's quantitation method was used to uniform quantity of protein among samples. The detection procedure was carried out in 96-well plate following the manufacturer's instruction.

Immunofluorescence double staining and confocal microscopy

For confocal microscopy, sections were fixed with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS) and incubated for 1 h with PBS containing 5% bovine serum albumin (BSA). After blocking, the preparations were incubated overnight with the primary antibodies diluted in PBS containing 5% BSA. The following antibodies were used: goat anti-human IL17-F (1:50, R&D System, Minneapolis, USA), mouse anti-human CD4 and CD8 (1:200; Dakocytomation, Milan, Italy), mouse anti-human CD31 (1:50; Dakocytomation) and mouse anti-human elastase (1:100; Dakocytomation, Milan, Italy). After washing with PBS, the preparations were incubated for a further 1 hour with the appropriate secondary Alexa Fluor 488 or Alexa Fluor 648 (Molecular Probes, Invitrogen S.R.L., Milan, Italy) conjugated antibodies diluted 1:200 in PBS. Alexa Fluor 488-tagged anti-IL-17F was showed in green and Alexa Fluor 648-tagged anti-CD4, -CD8, -CD31 or -elastase were visualized in red. The slides were mounted using a specific mounting medium containing DAPI (Fluka 10979, Sigma) in order to identify nuclei.

Images were captured with confocal microscope Zeiss LSM 800 (Carl Zeiss, Germany). Final results, expressed as the percentage of co-localization, were calculated as the average of the cellular counts performed in each section. Co-localized pixels representing co-expression of IL-17F and

CD4, CD8, CD31 or neutrophil elastase were displayed in yellow pixels in the merged images and in blue pixels (upper right area) in the co-localization scatterplot and the relative results were reported in graphs^{E15}.

Statistical analysis

Data were analyzed using Graph Pad Prism software (GraphPad version 5.0 Software Inc., San Diego, CA, USA). Clinical and functional variables are expressed as mean $\pm$ SD, morphological parameters as median (interquartile ranges). A normal distribution was assumed for clinical/functional data and a non-normal distribution for morphological parameters applying respectively the analysis of variance (ANOVA) or the non-parametric Kruskal-Wallis test for multiple comparisons and the Student's t test or the Mann-Whitney U test for comparisons between groups. Spearman's rank method was used for correlations. A receiver operating characteristic (ROC) curve, a graphical plot of the sensitivity (true positive rate; y axis) versus 1-specificity (false positive rate; x axis), was used to assess the accuracy of bronchial/nasal IL-17F in discriminating between asthmatics and controls, MA and SA and between FE and non-FE. The Area Under Curve (AUC) with the 95% confidence intervals was calculated for each ROC curve, and the best cut-off point was chosen based on Youden's Index. Differences, with a p-value less than 0.05, were selected as significant and the ROC curves were constructed using R 3.3.1 software environment^{E16}.

RESULTS

Inflammatory cells in bronchial/nasal mucosa

The expression of inflammatory cells (neutrophils, eosinophils, CD4 and CD8) was investigated in the bronchial and nasal mucosa (Figure 1, Figure 2 and Figure E2). Neutrophils and eosinophils

were significantly increased in the group of all asthmatics compared to controls both in the bronchial ($p=0.0002$ and $p=0.011$ respectively) and the nasal mucosa ($p=0.003$ and $p=0.0008$ respectively), whereas $CD4^+$ and $CD8^+$ cells were significantly increased in the nasal ($p=0.005$ and $p=0.032$ respectively) but not in the bronchial mucosa ($p=0.37$ and $p=1.0$ respectively). In particular, bronchial neutrophils were significantly higher in the mucosa of severe asthma compared to mild asthma ($p=0.003$) and to controls ($p<0.0001$) and in mild asthma compared to controls ($p=0.010$), while nasal neutrophils were significantly more elevated in severe ($p=0.004$) and mild ($p=0.012$) asthma compared to controls (Figure 2A/B). Both bronchial and nasal eosinophils were increased in severe ($p=0.0001$ and $p=0.0002$ respectively) and mild ($p=0.0001$ and $p=0.004$ respectively) asthma compared to controls (Figure E2). Nasal $CD4^+$ cells were more elevated in the mucosa of severe asthma compared to mild asthma ($p=0.0008$) and to controls ($p=0.0004$) and in mild asthma compared to controls ($p=0.013$), whereas nasal $CD8^+$ cells were increased in severe asthma compared to mild asthma ($p=0.015$) and to controls ($p=0.0002$, Figure E2).

Immunoreactivity of IL-17A/F and related cytokines in the bronchial/nasal mucosa

Immunohistochemistry of all bronchial and nasal IL-17 related cytokines is shown in Figure 1 for severe asthma and in supplementary material for mild asthma (Figure E3) and controls (Figure E4). IL-17 related cytokines were expressed in inflammatory cells, fibroblasts and epithelial cells of bronchial/nasal mucosa.

IL-17A/F were significantly increased in all asthmatics compared to controls both in the bronchial ($p=0.007$ and $p=0.001$ respectively) and nasal ($p=0.046$ and $p=0.0001$ respectively) mucosa, as well as bronchial IL-21 ($p=0.001$), IL-22 ($p=0.0003$), IL-23 ($p=0.006$) and nasal IL-21 ($p=0.006$), IL-22 ($p=0.002$) and IL-23 ($p<0.0001$).

IL-17A/F and IL-21 were more expressed in the bronchial mucosa of severe asthma compared to both mild asthma and controls ($p\leq 0.01$; Figure 2A). Bronchial IL-17F and IL-21 were higher in mild asthma compared to controls ($p<0.05$; Figure 2A). Bronchial IL-22 and bronchial IL-23 were

increased both in severe ($p<0.05$) and mild ($p<0.01$) asthma compared to controls (Figure 2A). Nasal IL-17F and IL-21 were significantly higher in severe compared to mild asthma and controls and IL-17F also in mild asthma compared to controls ($p<0.01$; Figure 2B). Nasal IL-17A expression was significantly increased only in severe asthma compared to controls ($p=0.014$; Figure 2B). Finally, nasal IL-22 and nasal IL-23 were higher in severe asthma ($p<0.001$) and mild asthma ($p<0.05$) compared to controls (Figure 2B).

Concerning the bronchial and nasal epithelium, IL-17F expression was higher in severe asthma compared to both mild asthma and controls both in bronchial and nasal mucosa (Table E1).

Correlations between IL-17 related cytokines and clinical parameters

In all asthmatics both bronchial and nasal IL-17F expression was negatively related to FEV_1 ($r_s=-0.46$, $p<0.0005$ and $r_s=-0.61$, $p<0.0005$ respectively; Figure 3A,E). Concurrently, both bronchial and nasal IL-17F expression was positively related to exacerbation rate ($r_s=0.41$, $p<0.005$ and $r_s=0.47$, $p<0.0005$ respectively; Figure 3B,F). In all asthmatics a negative correlation between FEV_1 and exacerbations number was also observed ($r_s=-0.47$, $p<0.0005$; Figure 3J). Bronchial IL-17A⁺ ($r_s=-0.38$, $p<0.01$) and IL-21⁺ ($r_s=-0.34$, $p<0.01$) as well as nasal IL-21⁺ ($r_s=-0.43$, $p<0.01$) cells showed a significant negative correlation with FEV_1 in asthmatics. Nasal IL-17A⁺ ($r_s=0.40$, $p<0.01$), IL-21⁺ ($r_s=0.40$, $p<0.01$) and IL-22⁺ ($r_s=0.50$, $p<0.001$) cells showed a significant positive correlation with exacerbation rate in asthmatics. Bronchial neutrophils are related to FEV_1 ($r_s=-0.41$, $p<0.005$; Figure 3I) and exacerbation rate ($r_s=0.47$, $p<0.0005$; Figure 3K).

In all asthmatics both bronchial and nasal IL-17F expression was negatively related to FVC ($r_s=-0.32$, $p=0.015$; Figure E5; and $r_s=-0.52$, $p<0.0001$ respectively). In all asthmatics a negative correlation between FVC and exacerbations number was also observed ($r_s=-0.38$, $p<0.0021$; Figure E5). Bronchial neutrophils ($r_s=-0.28$, $p=0.026$), CD8⁺ ($r_s=-0.31$, $p=0.014$), IL-17A⁺ ($r_s=-0.29$, $p=0.026$) and IL-21⁺ ($r_s=-0.35$, $p=0.007$) cells (Figure E5) as well as nasal CD4⁺ ($r_s=-0.33$,

$p=0.015$), $CD8^+$ ($r_s=-0.11$, $p=0.002$), $IL-21^+$ ($r_s=-0.34$, $p=0.013$) and $IL-22^+$ ($r_s=-0.32$, $p=0.024$) cells showed a significant negative correlation with FVC in asthmatics.

Correlations between inflammatory cells and bronchial/nasal IL-17 related cytokines

In all asthmatics bronchial $IL-17F^+$ cells correlated with bronchial neutrophils ($r_s=0.54$, $p<0.0005$; Figure 3C), bronchial $IL-17A^+$ ($r_s=0.67$, $p<0.0005$; Figure 3D), bronchial $IL-21^+$ ($r_s=0.30$, $p<0.05$; Figure E6A), bronchial $IL-22^+$ ($r_s=0.29$, $p<0.05$; Figure E6B), bronchial $CD4^+$ ($r_s=0.36$, $p<0.01$; Figure E7C), bronchial $CD8^+$ ($r_s=0.27$, $p<0.05$; Figure E7D) and nasal $IL-17F^+$ ($r_s=0.35$, $p<0.05$; Figure 3G) cells.

In addition, bronchial $IL-17A^+$ cells correlated with bronchial $IL-22^+$ cells ($r_s=0.43$, $p\leq 0.001$; Figure E6C). Nasal $IL-17F^+$ cells were significantly related to nasal $IL-21^+$ cells ($r_s=0.55$, $p<0.0001$; Figure 3H) while nasal $IL-17A^+$ cells were related to nasal $IL-21^+$ ($r_s=0.29$, $p<0.05$; Figure E6D) and nasal $IL-22^+$ ($r_s=0.38$, $p<0.01$; Figure E6E) cells in all asthmatics.

Concerning inflammatory cells, bronchial neutrophils significantly correlated also with bronchial $IL-17A^+$ ($r_s=0.34$, $p<0.01$; Figure E7A) and bronchial $IL-22^+$ cells ($r_s=0.36$, $p<0.01$; Figure E7B); nasal neutrophils significantly correlated with nasal $IL-22^+$ cells ($r_s=0.34$, $p<0.05$; Figure E7E). In all asthmatics bronchial $CD8^+$ and bronchial $CD4^+$ cells showed a significant correlation with bronchial $IL-17F^+$ cells ($r_s=0.27$, $p<0.05$ and $r_s=0.36$, $p<0.01$, respectively; Figure E7C,D), and nasal $CD4^+$ cells significantly correlated with nasal $IL-17F^+$ cells ($r_s=0.43$, $p\leq 0.001$; Figure E7F). Finally, bronchial $IL-17F^+$ cells significantly correlated with nasal $IL-22^+$ cells ($r_s=0.42$, $p<0.005$; Figure E7G), while nasal $IL-17F^+$ cells significantly correlated with bronchial $IL-17A^+$ cells ($r_s=0.43$, $p<0.005$; Figure E7H).

Inflammatory cells and IL-17 related cytokines expression in bronchial/nasal mucosa of frequent versus non-frequent exacerbators

Differences in inflammatory cells and IL-17 related cytokines expression were also investigated in asthmatics stratified in FE (n=24) and non-FE (n=40). Subjects' characteristics are reported in Table 1. Concerning inflammatory cells expression in the bronchial/nasal mucosa, bronchial neutrophils, eosinophils, CD4⁺ and CD8⁺ cells were significantly increased in FE compared to non-FE ($p<0.05$; Figure 4). Regarding IL-17-related cytokines, both bronchial ($p<0.001$) and nasal ($p<0.001$) IL-17F showed a significant higher expression in FE compared to non-FE, as well as bronchial IL-17A, nasal IL-21 and nasal IL-22 ($p<0.05$; Figure 4).

ROC curve analysis

ROC curve analysis of bronchial neutrophils and bronchial eosinophils showed a significant AUC of 0.81 and 0.94 respectively for discriminating asthmatics from healthy controls (Figure 5A). The best cut-off value for bronchial neutrophils was 47.17 cells/mm², while for bronchial eosinophils was 12.45 cells/mm². For the discrimination of MA from SA, significant AUC values of 0.75 for neutrophils was estimated and the best cut-off value for bronchial neutrophils was 50.31 cells/mm² (Figure 5B). Finally, ROC curve analysis among all asthmatics for discriminating the FE phenotype (Figure 5C) showed significant AUC values of 0.84 and 0.77 for neutrophils and eosinophils respectively. The best cut-off values for neutrophils and eosinophils were 78.62 cells/mm² and 37.74 cells/mm² respectively.

ROC curve analysis of bronchial/nasal IL-17F showed a significant AUC of 0.84 and 0.85 respectively for discriminating asthmatics from healthy controls (Figure 5D). A best cut-off value of 10.29 cells/mm² for bronchial IL-17F was estimated, while for nasal IL-17F was 11.32 cells/mm². To differentiate between MA and SA, a significant AUC of 0.73 and 0.83 was estimated for bronchial and nasal IL-17F, respectively (Figure 5E). The best cut-off value for bronchial IL-17F was 23.58 cells/mm², while for nasal IL-17F was 18.87 cells/mm². Finally ROC curve analysis among all asthmatics for discriminating the FE phenotype (Figure 5F) showed for bronchial IL-17F a significant AUC of 0.79 and a related best cut-off value of 23.58 cells/mm² and for nasal IL-17F a

significant AUC of 0.78 and a related best cut-off value of 26.41 cells/mm². The percentages of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for all variables in all sub-groups are reported in Table E2.

IL-17F in bronchial/nasal lysates

Bronchial IL-17F lysates concentrations in SA (64±14 pg/ml) were significantly higher compared to MA (43±15 pg/ml; $p<0.01$) and CTRL (29±5 pg/ml; $p<0.001$) groups (Figure 6). In addition, nasal IL-17F lysates concentrations in SA (72±22 pg/ml) were significantly higher compared to MA (47±12 pg/ml; $p<0.01$) and CTRL (35±9 pg/ml; $p<0.001$) groups (Figure 6). Bronchial and nasal IL-17F lysates concentrations in FE group were significantly increased compared to non-FE group ($p\leq0.001$).

Double staining/confocal microscopy for CD4⁺ and CD8⁺ cells with IL-17F in the bronchial/nasal mucosa of asthmatics

In bronchial/nasal mucosa of asthmatics merging the green (IL-17F) and red (CD4⁺ or CD8⁺ cells) pixels revealed that IL-17F positivity co-expressed CD4⁺ or CD8⁺ cells (yellow pixels) (Figure 7). Co-localization by quantitative analysis in severe asthmatics showed that among all IL-17F⁺ cells 26.5±5% and 22.1±8.1% were CD8⁺ and 23.3±6.54% and 21±3.5% were CD4⁺ lymphocytes in the bronchial and nasal mucosa respectively. Co-localization by quantitative analysis in mild asthmatics revealed that among all IL-17F⁺ cells 23.2±6.6% and 22.6±8.3% were CD8⁺ and 23.3±7.5% and 20.3±5.7% were CD4⁺ lymphocytes in the bronchial and nasal mucosa respectively. In frequent exacerbators, among all IL-17F⁺ cells, 23.4±1.3% and 25±8.1% were CD8⁺ and 24.3±3.1% and 23.5±3.5% were CD4⁺ lymphocytes in the bronchial and nasal mucosa respectively. The differences

between severe and mild asthmatics and between frequent and non-frequent exacerbators were not statistically significant both in bronchial and nasal mucosa.

In bronchial mucosa of FE the co-localization of IL-17F⁺/neutrophil elastase⁺ cells was 7,3±4%, but it was almost absent for IL-17F⁺/CD31⁺ cells (Figure E8).

IL-17F expression in fatal asthma

IL-17F and neutrophils expression was evaluated in a small group of subjects (n=10) dead for a fatal asthma attack and of controls (n=10). IL-17F was significantly higher in fatal asthma patients compared to controls (p=0.035; Figure 8), while no significant difference was observed for neutrophils between the two groups (p=0.684; Figure E9).

REFERENCES

- E1. Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma (GINA). NHLBI/WHO workshop report. Bethesda, National Heart, Lung and Blood Institute. Available from www.ginasthma.org/. Updated 2012.
- E2. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J*. 2014 Feb;43(2):343-73. doi:10.1183/09031936.00202013.
- E3. Bousquet J, Khaltaev N, Cruz AA, Denburg J, Fokkens WJ, Togias A, et al. Allergic Rhinitis and its Impact on Asthma (ARIA) 2008 update (in collaboration with the World Health Organization, GA(2)LEN and AllerGen). *Allergy*. 2008;63 Suppl 86:8-160.
- E4. Fokkens WJ, Lund VJ, Mullol J, Bachert C, Alobid I, Baroody F, et al. EPOS 2012: European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology*. 2012 Mar;50(1):1-12. doi:10.4193/Rhino50E2.
- E5. Fuhlbrigge A, Peden D, Apter AJ, Boushey HA, Camargo CA Jr, Gern J, Heymann PW, Martinez FD, Mauger D, Teague WG, Blaisdell C. Asthma outcomes: exacerbations. *J Allergy Clin Immunol* 2012;129(3 Suppl):S34-S48.
- E6. Mauad T, Ferreira DS, Costa MB, Araujo BB, Silva LF, Martins MA, Wenzel SE, Dolhnikoff M. Characterization of autopsy-proven fatal asthma patients in São Paulo, Brazil. *Rev Panam Salud Publica* 2008;23(6):418-423.
- E7. Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, et al. Standardisation of spirometry. *Eur Respir J* 2005; 26:319-338.
- E8. Pellegrino R, Viegi G, Brusasco V, Crapo RO, Burgos F, Casaburi R, et al. Interpretative strategies for lung function tests. *Eur Respir J* 2005; 26:948-968.

- E9. Quanjer PH, Stanojevic S, Cole TJ, Baur X, Hall GL, Culver BH, et al. Multi-ethnic reference values for spirometry for the 3-95-yr age range: the global lung function 2012 equations. *Eur Respir J*. 2012 Dec;40(6):1324-43. doi:10.1183/09031936.00080312.
- E10. Ricciardolo FL, Sorbello V, Bellezza Fontana R, Schiavetti I, Ciprandi G. Exhaled nitric oxide in relation to asthma control: A real-life survey. *Allergol Immunopathol (Madr)*. 2015 Nov 14. pii: S0301-0546(15)00123-8. doi: 10.1016/j.aller.2015.05.012.
- E11. Ricciardolo FL, Sabatini F, Sorbello V, Benedetto S, Defilippi I, Petecchia L, et al. Expression of vascular remodelling markers in relation to bradykinin receptors in asthma and COPD. *Thorax*. 2013 Sep;68(9):803-11. doi:10.1136/thoraxjnl-2012-202741.
- E12. Sorbello V, Ciprandi G, Di Stefano A, Massaglia GM, Favatà G, Conticello S, et al. Nasal IL-17F is related to bronchial IL-17F/neutrophilia and exacerbations in stable atopic severe asthma. *Allergy*. 2015 Feb;70(2):236-40. doi: 10.1111/all.12547.
- E13. Di Stefano A, Caramori G, Gnemmi I, Contoli M, Bristot L, Capelli A, et al. Association of increased CCL5 and CXCL7 chemokine expression with neutrophil activation in severe stable COPD. *Thorax*. 2009 Nov;64(11):968-75. doi: 10.1136/thx.2009.113647.
- E14. Jeffery P, Holgate S and Wenzel S. Methods for the assessment of endobronchial biopsies in clinical research. *Am J Respir Crit Care Med* 2003;168:S1-S17.
- E15. Ricciardolo FL, Petecchia L, Sorbello V, Di Stefano A, Usai C, Massaglia GM, et al. Bradykinin B2 receptor expression in the bronchial mucosa of allergic asthmatics: the role of NF-kB. *Clin Exp Allergy*. 2016 Mar;46(3):428-38. doi: 10.1111/cea.12676.
- E16. R Core Team (2015) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna <http://www.R-project.org/>.

FIGURE LEGENDS

Figure E1.

Photomicrographs showing staining by IHC of nasal polyps with normal goat, mouse and rabbit isotype irrelevant non-specific immunoglobulins (negative controls; panel A, C and E respectively) and with anti-goat, -mouse and -rabbit secondary antibody only (panel B, D and F respectively). Original magnification 400x.

Figure E2. Eosinophils (ECP positivity) in bronchial biopsy from CTRL, mild and severe asthma. Arrows indicate positive cells. Original magnification 400x. Internal scale: 25 μm . Number of bronchial eosinophils and nasal eosinophils, CD8^+ and CD4^+ cells/ mm^2 in the bronchial and nasal mucosa obtained from biopsy specimens of severe asthma (SA) and mild asthma (MA) patients and of controls (CTRLS). Data are presented as box plots. Each horizontal line indicates the median value in the respective group; boxes represent the 25th and 75th percentiles, bars the 10th and 90th percentiles.

Figure E3. IL-17 related cytokines expression in bronchial and nasal biopsy specimens obtained from mild asthma. Photomicrographs show the immunostaining for IL-17A, IL-17F, IL-21, IL-22 and IL-23 in the bronchial and nasal mucosa of mild asthma patients. Original magnification 400x. Internal scale: 25 μm . Arrows indicate positive cells.

Figure E4. IL-17 related cytokines expression in bronchial and nasal biopsy specimens obtained from healthy controls. Photomicrographs show the immunostaining for IL-17A, IL-17F, IL-21, IL-22 and IL-23 in the bronchial and nasal mucosa of healthy controls. Original magnification 400x. Internal scale: 25 μm . Arrows indicate positive cells.

Figure E5. Relationship between the FVC (% pred) and exacerbation rate (panel A) and bronchial neutrophils (panel B), CD8⁺ cells (panel C), IL-17A (panel D), IL-17F (panel E) and IL-21 (panel F) in all asthmatics. The correlation coefficient was obtained using the Spearman rank method (r_s).

Figure E6. A, B: Relationship between the number of bronchial IL-17F⁺ cells and the number of bronchial IL-21⁺ cells (A) and bronchial IL-22⁺ cells (B) in all asthmatics. C: Relationship between the number of bronchial IL-17A⁺ cells and bronchial IL-22⁺ cells in all asthmatics. D,E: Relationship between the number of nasal IL-17A⁺ cells and the number of nasal IL-21⁺ cells (D) and nasal IL-22⁺ cells (E) in all asthmatics. The correlation coefficient was obtained using the Spearman rank method (r_s).

Figure E7. A, B: Relationship between the number of bronchial neutrophils and the number of bronchial IL-17A⁺ cells (A) and bronchial IL-22⁺ cells (B) in all asthmatics. C, D: Relationship between the number of bronchial CD4⁺ (C) or CD8⁺ cells (D) and the number of bronchial IL-17F⁺ cells in all asthmatics. E, F: Relationship between the number of nasal neutrophils and the number of nasal IL-22⁺ cells (E) and between the number of nasal CD4⁺ cells and the number of nasal IL-17F⁺ cells (F) in all asthmatics. G: Relationship between the number of bronchial IL-17F⁺ cells and the number of nasal IL-22⁺ cells in all asthmatics. H: Relationship between the number of nasal IL-17F⁺ cells and the number of bronchial IL-17A⁺ cells in all asthmatics. The correlation coefficient was obtained using the Spearman rank method (r_s).

Figure E8. Double immunostaining for the identification of IL-17F⁺/CD31⁺ cells and IL-17F⁺/neutrophil elastase⁺ cells in the bronchial mucosa of frequent exacerbators. IL-17F positivity is showed in green and positivity for CD31 or neutrophil elastase is showed in red, co-localization is showed in the merge panel as yellow pixels. In the cytofluorogram is represented the co-localization as blue pixels in the graph. Original magnification 1000x

Figure E9. Neutrophils expression in bronchial tissue obtained from fatal asthma. Photomicrographs show the immunostaining for neutrophils in the bronchial mucosa of fatal asthma patients and deceased controls (DCTRLs). Original magnification 400x. Internal scale: 25 μm . Arrows indicate positive cells. Results are expressed as number of bronchial neutrophils/ mm^2 in the bronchial mucosa. Each horizontal line indicates the median value in the respective group.

Table E1. Epithelial score of bronchial and nasal epithelium in mild to severe asthmatics and controls.

Cytokine	Controls	Mild asthma	Severe asthma	Asthma non-frequent exacerbators	Asthma frequent exacerbators
Bronchial IL-17A	0.5 (0.0-0.5)	0.5 (0.3-1.5)	0.75 (0.5-1.8)#	0.5 (0.5-1.0)	0.5 (0.5-1.1)
Bronchial IL-17F	0.0 (0.0-1.0)	1.0 (0.0-1.5)	1.5 (0.8-2.0)#*	0.5 (0.5-1.0)	0.5 (0.5-1.0)
Bronchial IL-21	0.5 (0.5-2.0)	2.0 (1.5-2.5)#	2.5 (2.0-3.0)#	0.5 (0.5-1.0)	1.0 (0.8-1.8)°
Bronchial IL-22	0.0 (0.0-1.0)	1.5 (0.8-2.3)#	1.0 (0.5-1.8)	1.0 (0.5-1.5)	1.5 (0.8-1.5)
Bronchial IL-23	0.0 (0.0-0.3)	0.5 (0.5-1.0)#	0.5 (0.0-1.3)	0.5 (0.5-1.0)	1.0 (0.9-1.0)
Nasal IL-17A	0.0 (0.0-0.3)	0.25 (0.0-1.3)	0.5 (0.5-1.3)#	0.0 (0.0-0.5)	0.25 (0.0-0.5)
Nasal IL-17F	0.0 (0.0-0.5)	0.5 (0.0-0.8)	1.5 (0.5-1.5)#*	0.5 (0.5-1.5)	2.0 (1.0-2.5)°
Nasal IL-21	1.0 (0.8-1.5)	1.5 (0.8-2.0)	2.0 (1.5-2.8)#	2.0 (1.5-2.5)	2.5 (1.9-2.5)
Nasal IL-22	0.5 (0.3-0.8)	1.5 (0.5-1.8)#	1.75 (1.0-2.0)#	2.0 (1.5-2.5)	1.5 (1.5-2.5)
Nasal IL-23	0.0 (0.0-0.5)	1.5 (0.5-2.3)#	0.5 (0.5-0.8)#	1.5(0.5-1.9)	0.5 (0.5-1.1)

Immunostaining was scored in the complete bronchial epithelium (constituted by both columnar and basal epithelial cells); range: 0 (absence of immunostaining) to 3 (extensive immunostaining).

Data are expressed as median (lower quartile-upper quartile).

#p<0.05 significantly different from controls.

*p<0.05 significantly different from mild asthma.

°p<0.05 significantly different from non-frequent exacerbators

Median (Range) = median with range; AUC (95%CI) = area under the ROC curve with 95% confidence interval; p-value = p-values refer to the Mann-Whitney U test; Cut-off = best cut-off values; SE % = sensitivity; SP %, =specificity; PPV% = Positive Predictive Value; NPV% = Negative Predictive Value.

		Descriptive Statistics Median (Range)	ROC Analysis						
Variable	Subgroups		AUC (95%CI)	p-value	Cut-off	SP%	SE%	PPV%	NPV%
Neutrophils	Controls	20.76 (3.77 : 47.16)	0.81 (0.68 : 0.95)	0.0024	47.17	100	55	100	27
	All Asthmatics	50.31 (9.43 : 198.11)							
Eosinophils	Controls	1.18 (0.00 : 4.72)	0.94 (0.88 : 1.00)	<0.0001	12.45	100	92	100	67
	All Asthmatics	26.42 (0.00 : 113.21)							
Neutrophils	Mild Asthma	37.73 (9.43 : 146.23)	0.75 (0.61 : 0.89)	0.0015	50.31	71	72	72	71
	Severe Asthma	80.19 (18.87 : 198.11)							
Eosinophils	Mild Asthma	24.05 (0.00 : 59.62)	0.65 (0.49 : 0.80)	0.0506	37.74	71	56	67	61
	Severe Asthma	37.74 (0.00 : 113.21)							
Neutrophils	Asthma non frequent exacerbators	37.73 (9.43 : 146.23)	0.84 (0.71 : 0.96)	<0.0001	78.62	84	77	72	87
	Asthma frequent exacerbators	108.49 (25.16 : 198.11)							
Eosinophils	Asthma non frequent exacerbators	18.87 (0.00 : 60.38)	0.77 (0.63 : 0.90)	0.0013	37.74	74	71	60	82
	Asthma frequent exacerbators	43.39 (18.86 : 113.21)							
Bronchial IL-17F	Controls	5.50 (0.00 : 9.43)	0.84 (0.73 : 0.95)	0.0005	10.29	100	64	100	37
	All Asthmatics	16.77 (0.00 : 132.07)							
Nasal IL-17F	Controls	6.4 (0.00 : 15.09)	0.85 (0.75 : 0.96)	0.0002	11.32	90	72	97	41
	All Asthmatics	18.87 (0.00 : 99.06)							
Bronchial IL-17F	Mild Asthma	12.58 (0.00 : 47.17)	0.73 (0.58 : 0.88)	0.0035	23.58	78	63	75	67
	Severe Asthma	30.66 (0.00 : 132.07)							
Nasal IL-17F	Mild Asthma	12.58 (0.00 : 50.31)	0.83 (0.71 : 0.95)	<0.0001	18.87	78	83	80	82
	Severe Asthma	35.38 (3.77 : 99.06)							
Bronchial IL-17F	Asthma non frequent exacerbators	10.29 (0.00 : 56.60)	0.79 (0.65 : 0.93)	0.0004	23.58	79	78	70	85
	Asthma frequent exacerbators	33.02 (4.72 : 132.07)							
Nasal IL-17F	Asthma non frequent exacerbators	14.62 (0.00 : 50.31)	0.78 (0.65 : 0.92)	0.0006	26.41	77	78	67	85
	Asthma frequent exacerbators	37.27 (9.43 : 99.06)							

MILD ASTHMA

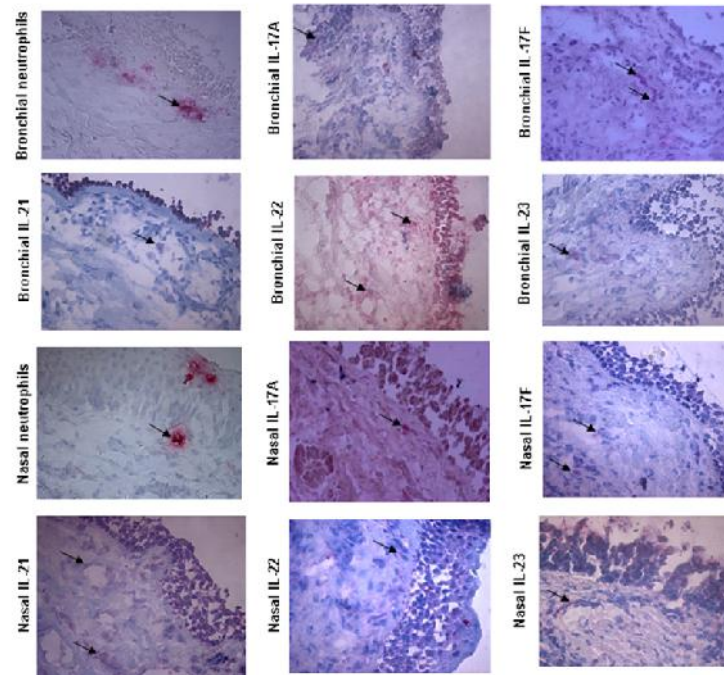


Fig E3.

Fabio L.M. Ricciardolo, Valentina Sorbello, Anna Folino, Fabio Gallo, Gian Mario Massaglia, Gabriella Favatà, Salvatore Conticello, Davide Vallese, Federica Gani, Mario Malerba, Gert Folkerts, Giovanni Rolla, Mirella Profita, Thais Mauad, Antonino Di Stefano, Giorgio Ciprandi
Identification of IL-17F/frequent exacerbator endotype in asthma

Journal of Allergy and Clinical Immunology, 2016, Available online 5 December 2016

<http://dx.doi.org/10.1016/j.jaci.2016.10.034>

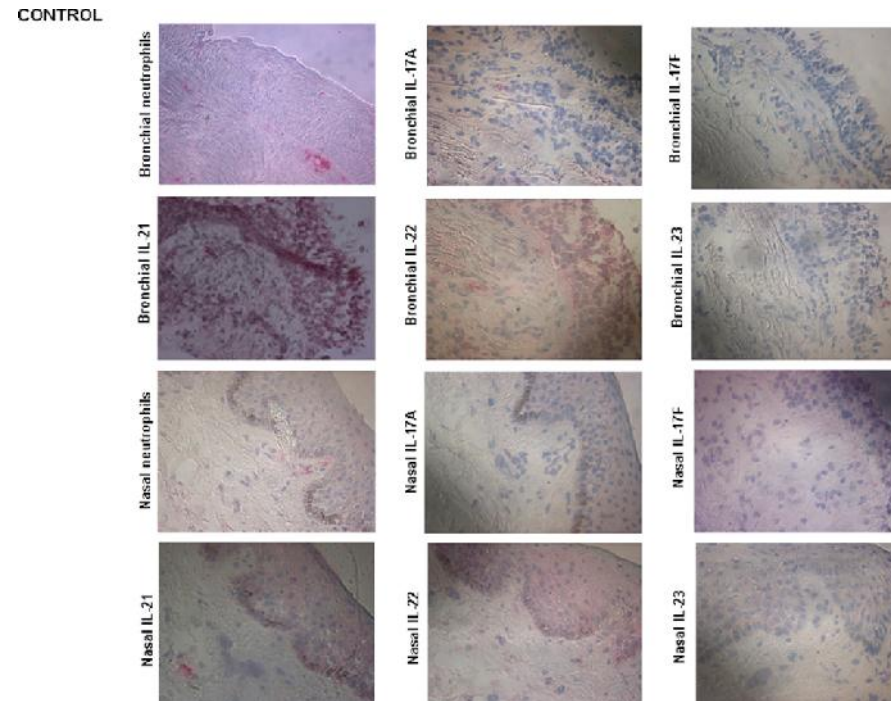


Fig E4.

Fabio L.M. Ricciardolo, Valentina Sorbello, Anna Folino, Fabio Gallo, Gian Mario Massaglia, Gabriella Favatà, Salvatore Conticello, Davide Vallese, Federica Gani, Mario Malerba, Gert Folkerts, Giovanni Rolla, Mirella Profita, Thais Mauad, Antonino Di Stefano, Giorgio Ciprandi
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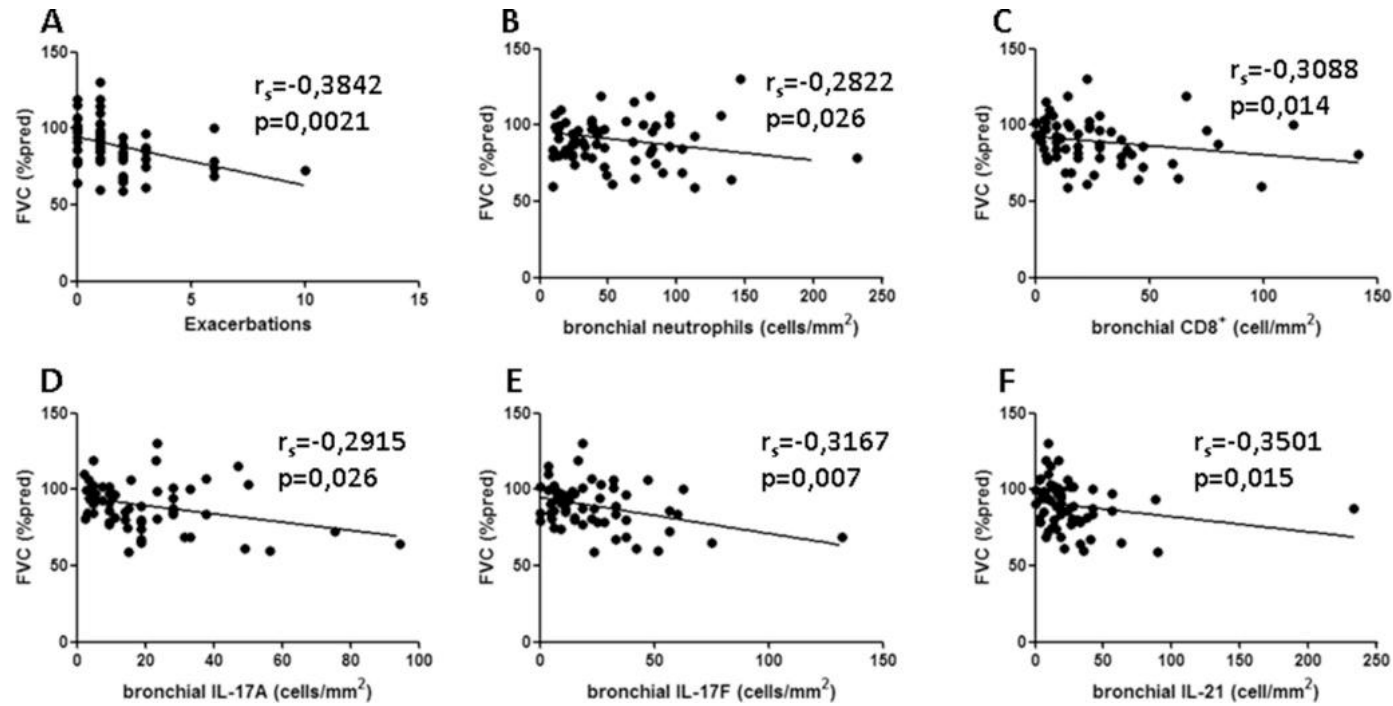


Fig E5.

Fabio L.M. Ricciardolo, Valentina Sorbello, Anna Folino, Fabio Gallo, Gian Mario Massaglia, Gabriella Favatà, Salvatore Conticello, Davide Vallese, Federica Gani, Mario Malerba, Gert Folkerts, Giovanni Rolla, Mirella Profita, Thais Mauad, Antonino Di Stefano, Giorgio Ciprandi
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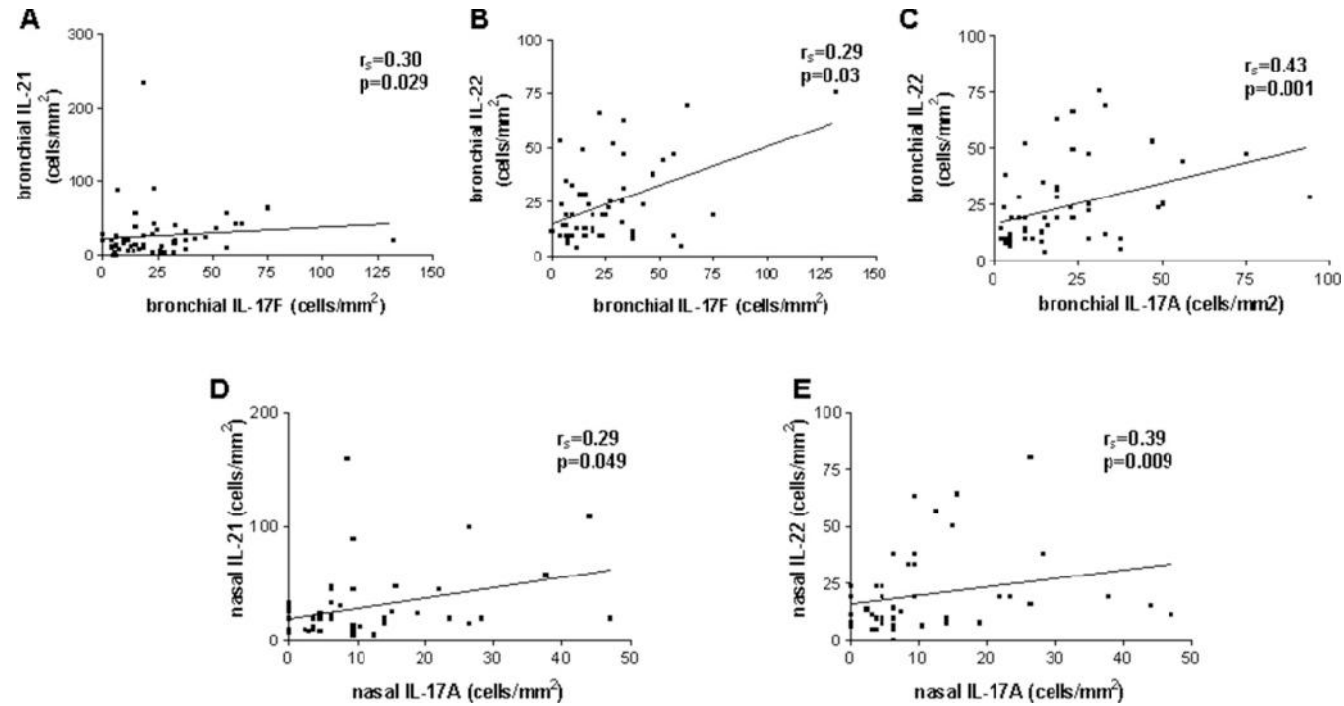


Fig E6.

Fabio L.M. Ricciardolo, Valentina Sorbello, Anna Folino, Fabio Gallo, Gian Mario Massaglia, Gabriella Favatà, Salvatore Conticello, Davide Vallese, Federica Gani, Mario Malerba, Gert Folkerts, Giovanni Rolla, Mirella Profita, Thais Mauad, Antonino Di Stefano, Giorgio Ciprandi
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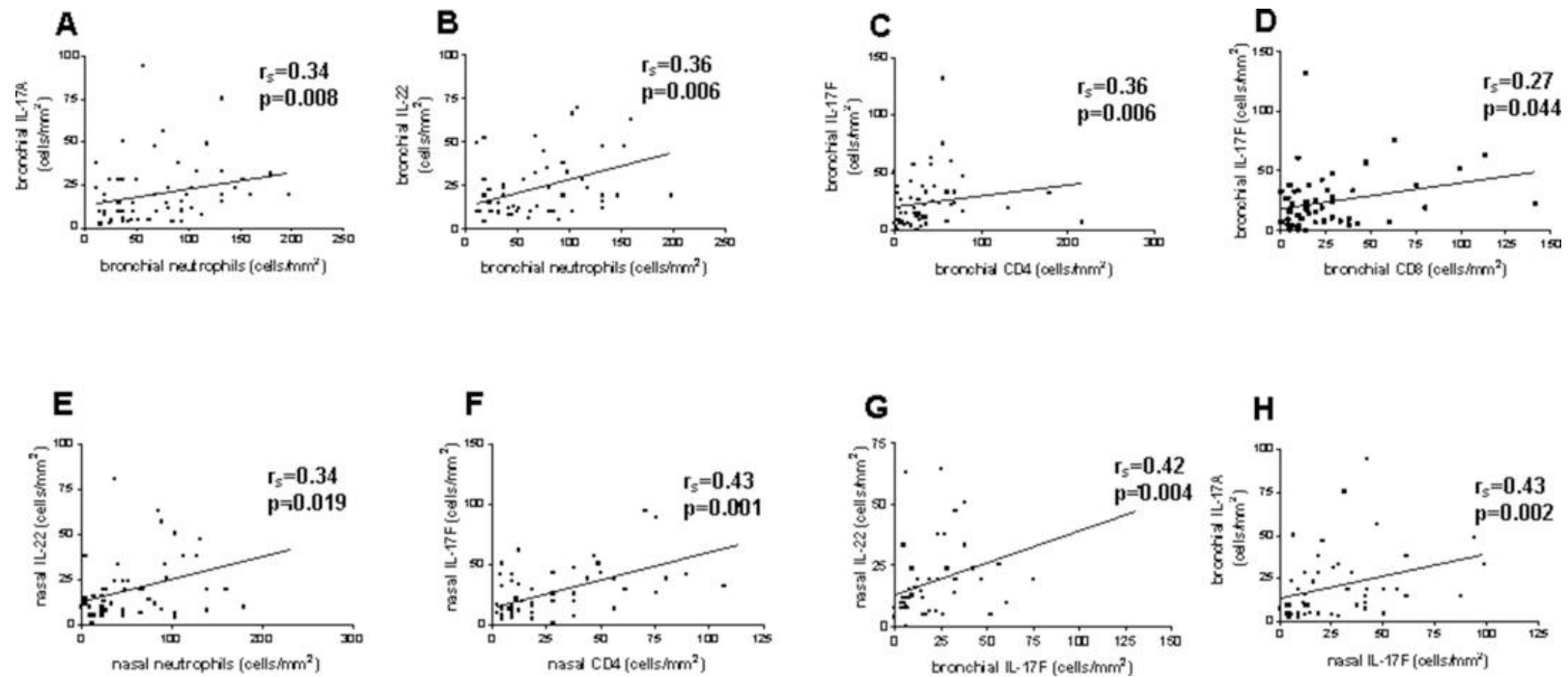


Fig E7.

Fabio L.M. Ricciardolo, Valentina Sorbello, Anna Folino, Fabio Gallo, Gian Mario Massaglia, Gabriella Favatà, Salvatore Conticello, Davide Vallese, Federica Gani, Mario Malerba, Gert Folkerts, Giovanni Rolla, Mirella Profita, Thais Mauad, Antonino Di Stefano, Giorgio Ciprandi
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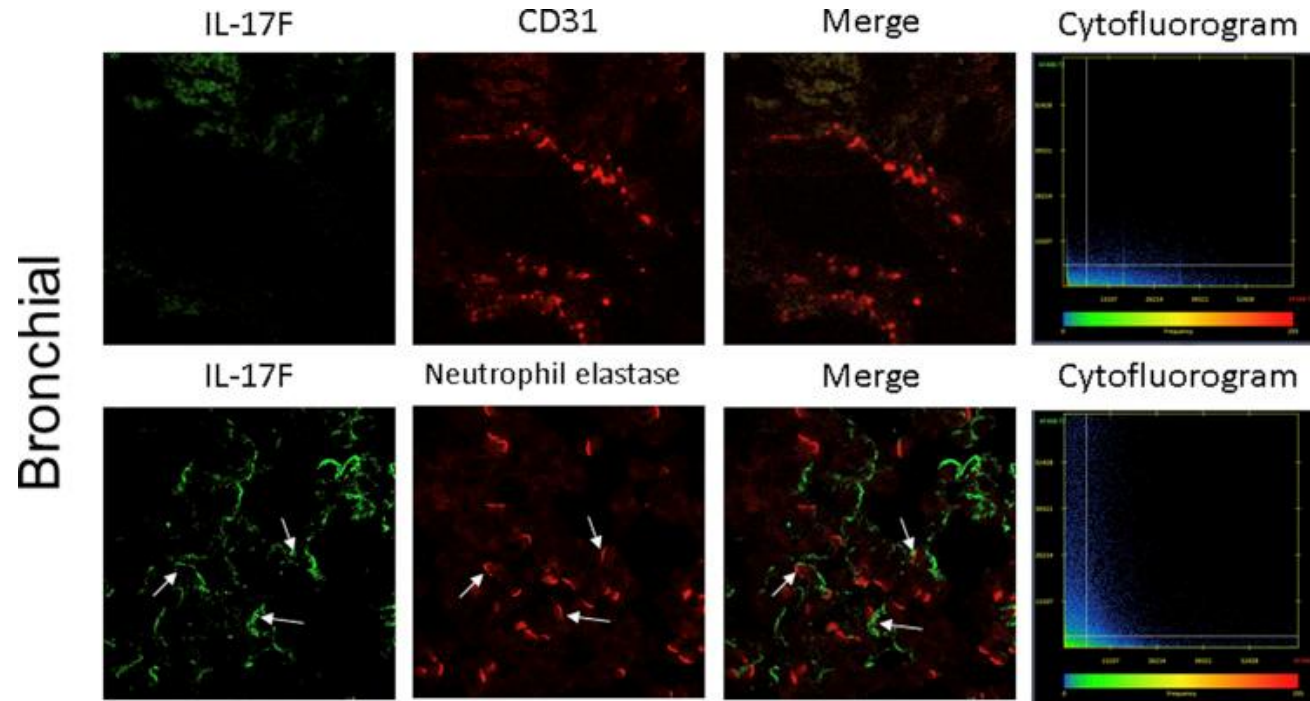


Fig E8.

Fabio L.M. Ricciardolo, Valentina Sorbello, Anna Folino, Fabio Gallo, Gian Mario Massaglia, Gabriella Favatà, Salvatore Conticello, Davide Vallese, Federica Gani, Mario Malerba, Gert Folkerts, Giovanni Rolla, Mirella Profita, Thais Mauad, Antonino Di Stefano, Giorgio Ciprandi
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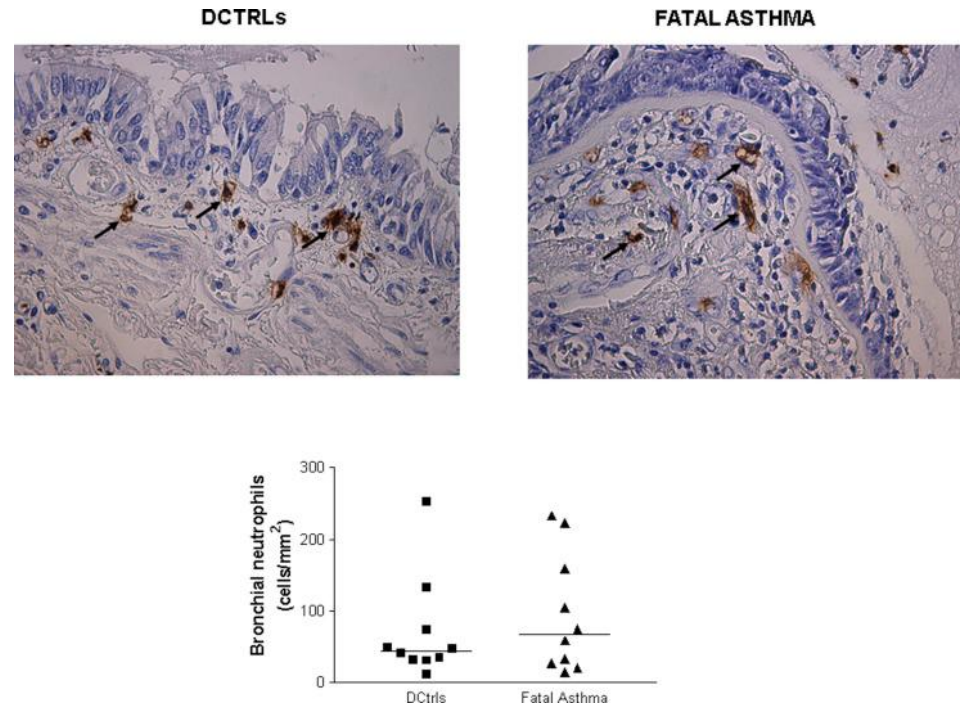


Fig E9.

Fabio L.M. Ricciardolo, Valentina Sorbello, Anna Folino, Fabio Gallo, Gian Mario Massaglia, Gabriella Favatà, Salvatore Conticello, Davide Vallese, Federica Gani, Mario Malerba, Gert Folkerts, Giovanni Rolla, Mirella Profita, Thais Mauad, Antonino Di Stefano, Giorgio Ciprandi
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