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

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ABSTRACT

Background: Severe asthma might be associated with neutrophil recruitment and Th17 cytokines over-expression in bronchial biopsies.

Objective: To study IL-17-related cytokines in nasal/bronchial biopsies from controls and mild (MA)-to-severe (SA) asthmatics 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 (IHC) in cryostat sections of bronchial/nasal biopsies obtained from 33 SA (21 frequent exacerbators (FE)), 31 MA (3 FE) and 14 controls. IL-17F protein was also measured by ELISA in bronchial/nasal lysates and by IHC in bronchial tissue obtained from subjects died for fatal asthma. Immunofluorescence/confocal microscopy was used for IL-17F co-localization.

Results: Higher number ($p<0.05$) of neutrophils, IL-17A$^+$, IL-17F$^+$ and IL-21$^+$ cells in bronchial biopsies and higher number ($p<0.01$) of IL-17F$^+$ and IL-21$^+$ cells in nasal biopsies were observed in SA compared to MA. Bronchial IL-17F$^+$ cells correlated with bronchial neutrophils ($r=0.54$), exacerbation rate ($r=0.41$) and FEV_1 ($r=-0.46$). Nasal IL-17F$^+$ cells correlated with bronchial IL-17F ($r=0.35$), exacerbation rate ($r=0.47$) and FEV_1 ($r=-0.61$). FE showed increased number of bronchial neutrophils/eosinophils/CD4$^+$/CD8$^+$ cells and bronchial/nasal IL-17F$^+$ cells. ROC curve analysis evidenced predictive cut-off values of bronchial neutrophils and nasal/bronchial IL-17F for discriminating between asthmatics and controls, between MA and SA and between FE and non-FE. IL-17F protein increased in bronchial/nasal lysates of SA and FE and in bronchial tissue of fatal asthma. IL-17F co-localized 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 frequent exacerbators.

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Abbreviations used:

FEV_1: forced expiratory volume in one second

FVC : forced vital capacity

GINA: Global Initiative for Asthma

SA: severe asthma

MA: mild asthma

FE: frequent exacerbators

ICS: Inhaled corticosteroid

OCS: oral corticosteroids

ROC: receiver operating characteristic

AUC: area under curve

IHC: immunohistochemistry

ECP: eosinophil cationic protein

ELISA: enzyme-linked immunosorbent assay

CTRLs: control subjects

DCTRLs: deceased controls

Capsule Summary: **35 words**

Overexpression of nasal/bronchial IL-17F is a feature of severe asthma in relation to neutrophils, airway obstruction and exacerbation rate and it is also able to recognize frequent exacerbator phenotype potentially at risk of asthma death.

Key messages:

1) Increased expression of IL-17-related cytokines in nasal and bronchial tissues of severe asthmatics is in relation to neutrophilic inflammation, airway obstruction and exacerbation rate.

2) Recognition of a frequent exacerbator phenotype in mixed neutrophilic/eosinophilic asthma with high values of bronchial/nasal IL-17F mainly produced by CD4/Th17 and CD8/Tc17.

3) Identification of IL-17F/frequent exacerbator endotype might have clinical implications in order to predict treatment response to selective therapeutic options against IL-17F in asthma.

Key words: severe asthma; bronchial biopsy; nasal biopsy; IL-17F; neutrophils; frequent exacerbators; phenotype; endotype.

INTRODUCTION

Asthma is a heterogeneous disease characterized by chronic airway inflammation, typically sustained by T helper 2 (Th2) cells and eosinophils[1]. Several types of asthma are featured by neutrophilia like intrinsic asthma [2], asthma in the elderly[3], in smokers[4], in obese females[5] and a category of severe asthma (SA)[6]. Neutrophilic SA can be distinguished in two subtypes featured by absence or presence of eosinophils[6]. Th17 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 severe asthmatics[11] and neutrophil airway infiltration is commonly reported in SA[12], suggesting a key role for Th17 cells. Although the main function of Th17 cells is supposed to be induction of protection against potentially harmful fungi and extracellular bacteria, many experiments revealed that Th17 cells drive also tissue inflammation in patients with several autoimmune diseases and allergy[13-14]. These cells entail distinct phenotype of CD4/CD8$^+$ cells, characterized by IL-17A, IL-17F, IL-21, and IL-22 production[13-14]. Th17 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 FEV_1 in asthmatic children[15]. IL-17A/F induce chemokines release, like CXCL1 (GROα), CXCL8 (IL-8) and granulocyte-macrophage colony-stimulating factor (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 up-regulation of pro-inflammatory 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 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 exacerbations[24] 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 limitation[25]. In addition, Th-17-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 IL-17-related cytokines expression in nasal/bronchial biopsies obtained from control subjects and atopic/non-atopic mild-to-severe asthmatics focusing on exacerbation rate. Finally, IL-17F expression was also examined in fatal asthma.

MATERIALS AND METHODS

Asthmatic and control subjects

In this observational and cross-sectional study bronchial biopsies from 78 subjects were analysed: 33 SA, 31 mild asthma (MA) and 14 controls; clinical/demographic characteristics are shown in Table 1. Asthma diagnosis/severity were defined according to GINA and ERS/ATS 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 FeNO as described in Online Repository. Controls had no respiratory disease history and no airflow limitation. All subjects were in stable condition (see also Online Repository). Frequent exacerbator is defined the subject who has ≥2 asthma exacerbations/year which requires use of systemic corticosteroids (or an increase from a stable maintenance dose) for at least three 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 for asthma attack (fatal asthma) and 10 non-smoking subjects who died of non-pulmonary causes (deceased controls: DCtrls). All of them underwent autopsy at the Department of Pathology of the São Paulo University between 1996 and 2004. For patients description 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 (CAPPesq-HCFMUSP).

Measurements of lung function; fiberoptic bronchoscopy/rhinoscopy, collection and processing of bronchial-nasal biopsies/fatal asthma bronchial tissue, immunohistochemistry (IHC),

immunofluorescence and confocal microscopy; ELISA test from tissue lysates of bronchial/nasal biopsies are provided in Online Repository.

Statistical analysis

Data were analyzed using Graph Pad Prism software (GraphPad version 5.0 Software Inc., San Diego, CA, USA). Clinical/functional variables are expressed as mean±SD, morphological parameters as median (interquartile ranges). A normal and non-normal distribution were assumed respectively for clinical/functional data and 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 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 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. P-values <0.05 were considered statistically significant and ROC curves were constructed using R 3.3.1 software environment[33].

RESULTS

Inflammatory cells in bronchial/nasal mucosa

Inflammatory cells (neutrophils, eosinophils, $CD4^+$ and $CD8^+$ cells) numbers were investigated in the bronchial/nasal mucosa (see Online Repository). Bronchial neutrophils were higher in the mucosa of SA compared to MA ($p<0.01$) and controls ($p<0.001$) and in MA compared to controls ($p<0.01$), while nasal neutrophils were more elevated in SA ($p<0.01$) and MA ($p<0.05$) compared to controls (Figure 2A/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 Figure 1 for SA and in Online Repository for MA (Figure E2) and controls (Figure E3). 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 to controls both in bronchial ($p<0.01$ and $p<0.01$ respectively) and nasal ($p<0.05$ and $p<0.001$ respectively) mucosa, as well as bronchial IL-21 ($p<0.01$), IL-22 ($p<0.001$), IL-23 ($p<0.01$) and nasal IL-21 ($p<0.01$), IL-22 ($p<0.01$) and IL-23 ($p<0.0001$).

IL-17A/F and IL-21 were more expressed in the bronchial mucosa of SA compared to both MA and controls ($p\leq0.01$; Figure 2A). Bronchial IL-17F and IL-21 were higher in MA compared to controls ($p<0.05$; Figure 2A). Bronchial IL-22 and bronchial IL-23 were increased both in SA ($p<0.05$) and MA ($p<0.01$) compared to controls (Figure 2A). Nasal IL-17F and IL-21 were significantly higher in SA compared to MA and controls and IL-17F also in MA compared to controls ($p<0.01$; Figure 2B). Nasal IL-17A expression was significantly increased only in SA compared to controls ($p<0.05$; Figure 2B).

Finally, nasal IL-22 and nasal IL-23 were higher in SA ($p<0.001$) and MA ($p<0.05$) compared to controls (Figure 2B).

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

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 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). Correlations between FVC and IL-17 related cytokines are shown in Online Repository (Figure E7).

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 E5A), bronchial IL-22^+ (r_s=0.29, p<0.05; Figure E5B), bronchial $CD4^+$ (r_s=0.36, p<0.01; Figure E6C), bronchial $CD8^+$ (r_s=0.27, p<0.05; Figure E6D) and nasal IL-$17F^+$ (r_s=0.35, p<0.05; Figure 3G) cells. For the other significant correlations among bronchial/nasal IL-17 related cytokines and inflammatory cells see Online Repository and Figures E5-E6.

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/mm^2, while for bronchial eosinophils was 12.45 cells/mm^2. 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/mm^2 (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/mm^2 and 37.74 cells/mm^2 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/mm^2 for bronchial IL-17F was estimated, while for nasal IL-17F was 11.32 cells/mm^2.

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/mm^2, while for nasal IL-17F was 18.87 cells/mm^2. 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/mm^2 and for nasal IL-17F a

significant AUC of 0.78 and a related best cut-off value of 26.41 cells/mm^2. 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 E1.

IL-17F in bronchial/nasal lysates

Bronchial IL-17F lysates concentrations in SA (n=10; 64±14 pg/ml) were significantly higher compared to MA (n=10; 43±15 pg/ml; $p<0.01$) and CTRL (n=8; 29±5 pg/ml; $p<0.001$) groups (Figure 6). In addition, nasal IL-17F lysates concentrations in SA (n=10; 72±22 pg/ml) were significantly higher compared to MA (n=10; 47±12 pg/ml; $p<0.01$) and CTRL (n=8; 35±9 pg/ml; $p<0.001$) groups (Figure 6). Bronchial and nasal IL-17F lysates concentrations in FE (n=10 and n=9 respectively) group were significantly increased compared to non-FE (n=10 and n=11 respectively) 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 SA (n=6) 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 MA (n=3) 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 FE (n=4), 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 SA and MA and between FE and non-FE (n=5) were not statistically significant both in bronchial and nasal mucosa.
In bronchial mucosa of FE (n=3) 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 ($p<0.05$; Figure 8), but no significant difference was observed for neutrophils between the two groups ($p=0.68$; Figure E9).

DISCUSSION

This study showed higher expression of IL-17 related cytokines, especially of IL-17F, in the nasal/bronchial mucosa of atopic/non atopic SA compared to MA and controls. IL-17F was significantly related to bronchial neutrophilia, FEV_1 and exacerbation rate in all asthmatics. In particular, phenotyping the population of asthmatics in FE and non-FE both nasal and bronchial IL-17F expression in conjunction with bronchial neutrophils (and to a lesser extent with eosinophils, $CD4^+$ and $CD8^+$ cells) was considerably increased in asthma FE. Furthermore, IL-17F protein increased in bronchial/nasal lysates of SA and FE in line with IHC results. ROC curve analysis allowed to identify an optimal predictive cut-off value of bronchial neutrophils and nasal/bronchial IL-17F for discriminating asthmatics, SA and FE. Finally, bronchial IL-17F expression was higher in fatal asthma compared to controls.

Bronchial mucosa in SA was characterized by a more intense neutrophilic infiltration compared to MA and controls, suggesting that SA is typified by a distinct neutrophilic phenotype with increased expression of Th17-related cytokines (IL-17F, IL-17A and IL-21) at bronchial level. Among asthmatics neutrophils and nasal/bronchial IL-17F resulted significantly increased in FE compared to non-FE as well as bronchial IL-17F/A correlated with bronchial neutrophils in all asthmatics strengthening the link between Th17 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 compared to deceased controls unveils the involvement of this bio-molecular pathway in the risk of death in asthma.

Initially, Sun and colleagues[34] showed IL-17A protein increase in induced sputum from SA compared to controls which was accompanied by a rise in sputum neutrophils. The authors 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

neutrophil mobilisation in asthma[34]. IL-17A was described in bronchial tissue from asthmatics with a more marked expression in moderate-to-severe asthma[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 al.[9] demonstrated that the magnitude of the increase in bronchial IL-17A matches with disease severity and that this increase is robust in SA supporting the idea that IL-17A is involved in severe asthma 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-mobilising cytokines (like G-CSF and IL-8) in human bronchial epithelial cells[37] and bronchial fibroblasts[38-39]. Some studies referred an increase of IL-17F expression in bronchial lamina propria related to disease severity[9-10] and, interestingly, Al-Ramli and co-workers 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[9]. There is now a growing evidence that, just like neutrophils *per se*, IL-17A/F are critically involved in mammalian host defence against bacteria, fungi and, possibly, viruses; thus, these two 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 SA compared to MA 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 Th1 cells[40-41]. The current study also showed an increased expression of IL-22 and IL-23 in the nasal/bronchial mucosa of both MA and SA compared to controls and, additionally, both IL-21 and IL-22 were higher in the nasal mucosa of FE compared to non-FE 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 Th17 cells and by innate lymphocytes, which connect the immune response to tissue inflammation[42]. Both IL-17 and IL-22 play roles in immune defense to extracellular bacteria, IL-17 contributing with antibacterial immunity and IL-22 promoting epithelial proliferation and repair following injury[43]. Furthermore,

IL-23 is the most important differentiating cytokine, with pro-inflammatory properties, which drives the immune response toward Th17 cells. In asthma IL-23 promotes Th17 cells' proliferation to maintain IL-17A/F production and neutrophils recruitment, indicating that the IL-23/Th17 axis might become a novel therapeutic target especially for the severe corticosteroid-dependent asthma[44]. A recent study suggested that IL-17/IL-23 cytokines hamper both anti-inflammatory and immunosuppressant actions of glucocorticoids in peripheral lymphocytes via increased GR-β/GR-α ratios[45].

The nasal mucosa of SA showed a significantly more abundant infiltrate of $CD4^+$ and $CD8^+$ cells compared to MA and controls, together with an increased nasal expression of IL-17F and IL-21, while nasal IL-17A, IL-22 and IL-23 were enhanced in all asthmatics compared to 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 Makihara *et al*., who also found a significant negative correlation between the number of IL-$17A^+$ cells and FEV_1/FVC ratio[27]. Furthermore, it has been shown that Th17 cells may promote both eosinophilic and neutrophilic inflammation in allergic rhinitis and chronic rhinosinusitis[46]. Thus, SA is characterized by a more intense Th17-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]. Th17 cytokines role in asthmatics nose is further emphasized by the significant correlation between nasal IL-$17F^+$ and bronchial IL-$17F^+$ cells, FEV_1 (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[47] 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[48].

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 FEV_1 or FVC were observed,

confirming, consistently with our previous study[8], 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[24,49] and near-fatal event in SA[23].

The current study also demonstrated higher number of bronchial CD4$^+$ and CD8$^+$ cells in FE, both in relationship with bronchial IL-17F in all asthmatics and the co-localization of IL-17F in CD8$^+$ (25%) and CD4$^+$ (25%) lymphocytes 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 al*. 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[38]. In particular, Type 3 immunity is mediated by retinoic acid–related orphan receptor (ROR)γt innate lymphoid cell (ILC)3s, Tc17 cells, and Th17 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[38].

The use of high ICS doses and OCS maintenance therapy in SA and FE compared to low ICS dose in MA and non-FE can be a limitation of this study. Previous reports showed that neutrophil survival is increased by CS[50] suggesting that daily ICS high dose could influence neutrophil counts but, on the other hand, OCS treatment reduced sputum neutrophils in SA[51]. In addition, it is fundamental to point out that IL-17 provokes steroid insensitivity in PBMCs by glucocorticoid receptor-beta upregulation[45] and steroid-resistant neutrophil activity suggesting that IL-17 directly induces insensitivity of neutrophils to CS[52].

In conclusion, the current study confirms previous preliminary data[8] on nasal/bronchial IL-17F, extending to a larger population of both atopic and non-atopic 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 potential biomarker able to identify among asthmatics the FE endotype. These factors might allow to obtain an early prognosis clinically relevant to predict treatment response to specific therapeutic options against IL-17F in asthma and to improve the clinical management of the respiratory disability in FE also considering the risk of asthma death.

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FIGURE LEGENDS

Figure 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 400x. Internal scale: 25 μm. Arrows indicate positive cells.

Figure 2. Number of neutrophils, IL-17A$^+$, IL-17F$^+$, IL-21$^+$, IL-22$^+$ and IL-23$^+$ cells/mm^2 in the bronchial (panel A) and nasal (panel B) mucosa of severe asthma (SA), mild asthma (MA) 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, bars the 10th and 90th percentiles.

Figure 3. A, B, C, D: Relationship between the number of bronchial IL-17F$^+$ cells and FEV_1 (A), number of exacerbations (B), bronchial neutrophils (C) and bronchial IL-17A$^+$ cells (D) in all asthmatics. E, F, G, H: Relationship between the number of nasal IL-17F$^+$ cells and FEV_1 (E), number of exacerbations (F), number of bronchial IL-17F$^+$ cells (G) and nasal IL-21$^+$ cells (H) in all asthmatics. I, J, K, L: Relationship between the number of bronchial neutrophils and FEV_1 (I) and between the number of exacerbations and FEV_1 (J), bronchial neutrophils (K) and bronchial eosinophils (L). The correlation coefficient was obtained using the Spearman rank method (r_s).

Figure 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 frequent *versus* non-frequent exacerbators. 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 5. Panels A-B-C: ROC curves for optimal cut-off points at which bronchial neutrophils and bronchial eosinophils discriminate asthmatic patients from healthy controls (A), severe asthmatics from mild asthmatics (B) and asthma frequent from asthma non-frequent exacerbators (C). Panels

D-E-F: ROC curves for optimal cut-off points at which bronchial and nasal IL-17F discriminate asthmatic patients from healthy controls (D), severe asthmatics from mild asthmatics (E) and asthma frequent from asthma non-frequent exacerbators (F).

Figure 6. IL-17F concentration (pg/ml) in bronchial (A-C) and nasal biopsies (B-D) from CTRL, mild and severe asthma and from frequent and non-frequent exacerbators. Data are presented as columns. Each columns represent mean±SD.*$p \leq 0.05$, **$p \leq 0.001$, ***$p \leq 0.0001$.

Figure 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 frequent exacerbators. IL-17F positivity is showed in green (AF488) and positivity for CD4 or CD8 is showed in red (AF568), co-localization is showed in the merge panel as yellow pixels. In the cytofluorogram is represented the co-localization as blue pixels. Original magnification 1000x.

Figure 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 400x. Internal scale: 25 μm. Arrows indicate positive cells. Results are expressed as number of bronchial IL-17F$^+$ cells/mm^2 in the bronchial mucosa. Each horizontal line indicates the median value in the respective group. *$p<0.05$

Table 1. Subjects' characteristics.

CLINICAL PARAMENTERS	CONTROLS (N=14)	MILD ASTHMA (N=31)	SEVERE ASTHMA (N=33)	Asthma non-frequent exacerbators (N=40)	Asthma frequent exacerbators (N=24)
Age (years)	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 postβ2 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 (years)	-	22±15	28±18	25±15	26±19
Exacerbations Frequency (N/year)	-	0.7±0.9	2.5±2	0.6±0.5	3.4±2
Beclomethasone HFA daily dose (mcg)	-	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 mean±SD. FEV_1 preβ2= baseline FEV_1; FEV_1 postβ2 change= FEV_1 postβ2 change from baseline. N=number.

Seven severe asthmatics/frequent exacerbators were on oral corticosteroid for ≥ 50% of the previous year (prednisone 5 mg/day at least for six months per year) [30]. Two severe asthmatics were on omalizumab.

Five mild asthmatics were not on inhaled corticosteroid therapy. GLI stands for Global Lung Function Initiative 2012.

°$p<0.001$ significantly different from controls

§$p<0.001$ significantly different from controls

*$p<0.001$ significantly different from mild asthma

#$p<0.01$ significantly different from non-frequent exacerbators.

Table 1. Subjects' characteristics.

CLINICAL PARAMENTERS	**CONTROLS** (N=14)	**MILD ASTHMA** (N=31)	**SEVERE ASTHMA** (N=33)	**Asthma non-frequent exacerbators** (N=40)	**Asthma frequent exacerbators** (N=24)
Age (years)	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 postβ2 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 (years)	-	22±15	28±18	25±15	26±19
Exacerbations Frequency (N/year)	-	0.7±0.9	2.5±2	0.6±0.5	3.4±2
Beclomethasone HFA daily dose (mcg)	-	132±99	629±230*	266±242	592±299#
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Seven severe asthmatics/frequent exacerbators were on oral corticosteroid for ≥ 50% of the previous year (prednisone 5 mg/day at least for six months per year) [30]. Two severe asthmatics were on omalizumab.

Five mild asthmatics were not on inhaled corticosteroid therapy. GLI stands for Global Lung Function Initiative 2012.

°$p<0.001$ significantly different from controls

§$p<0.001$ significantly different from controls

*$p<0.001$ significantly different from mild asthma

#$p<0.01$ significantly different from non-frequent exacerbators.

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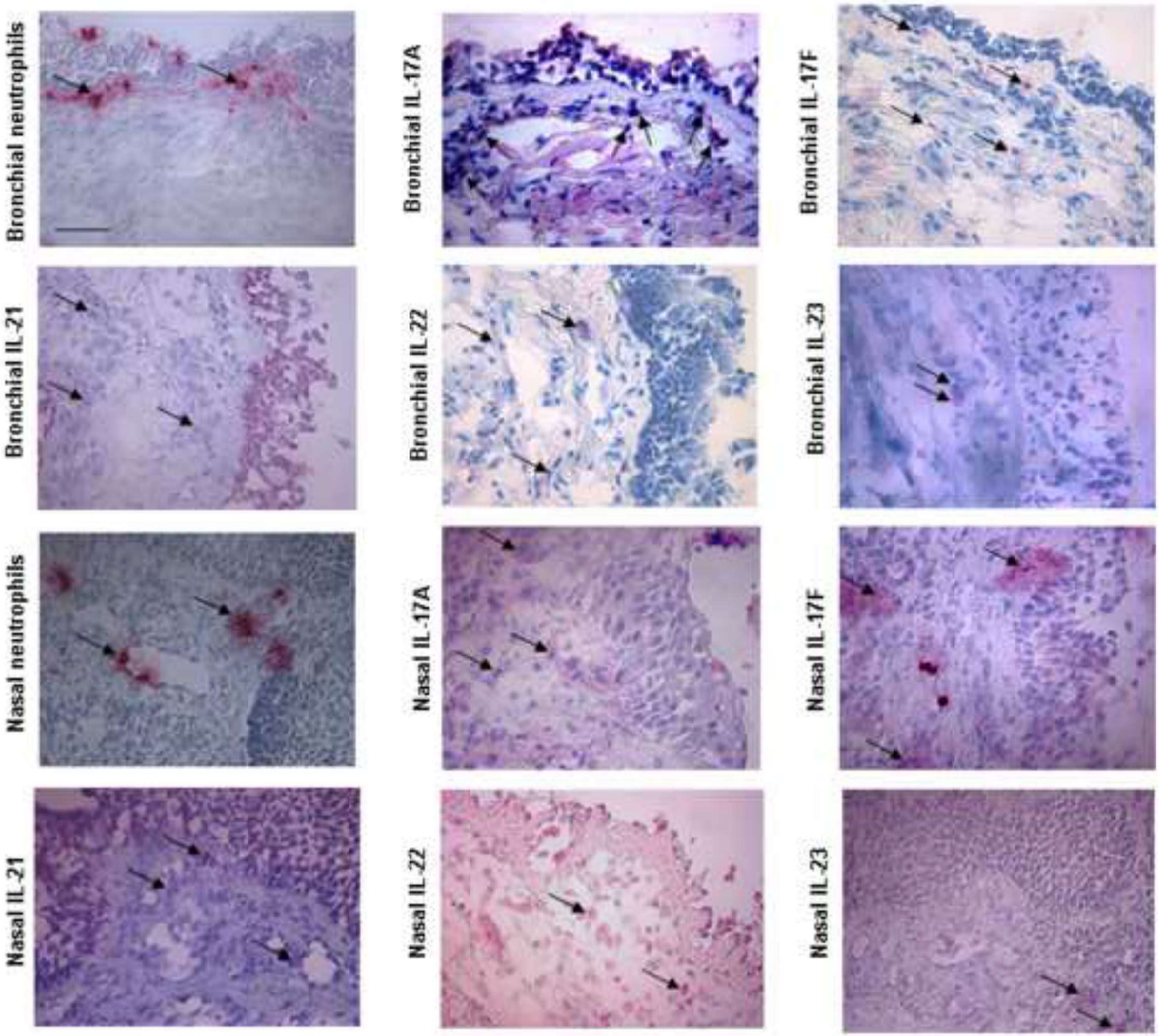
Figure 1

SEVERE ASTHMA



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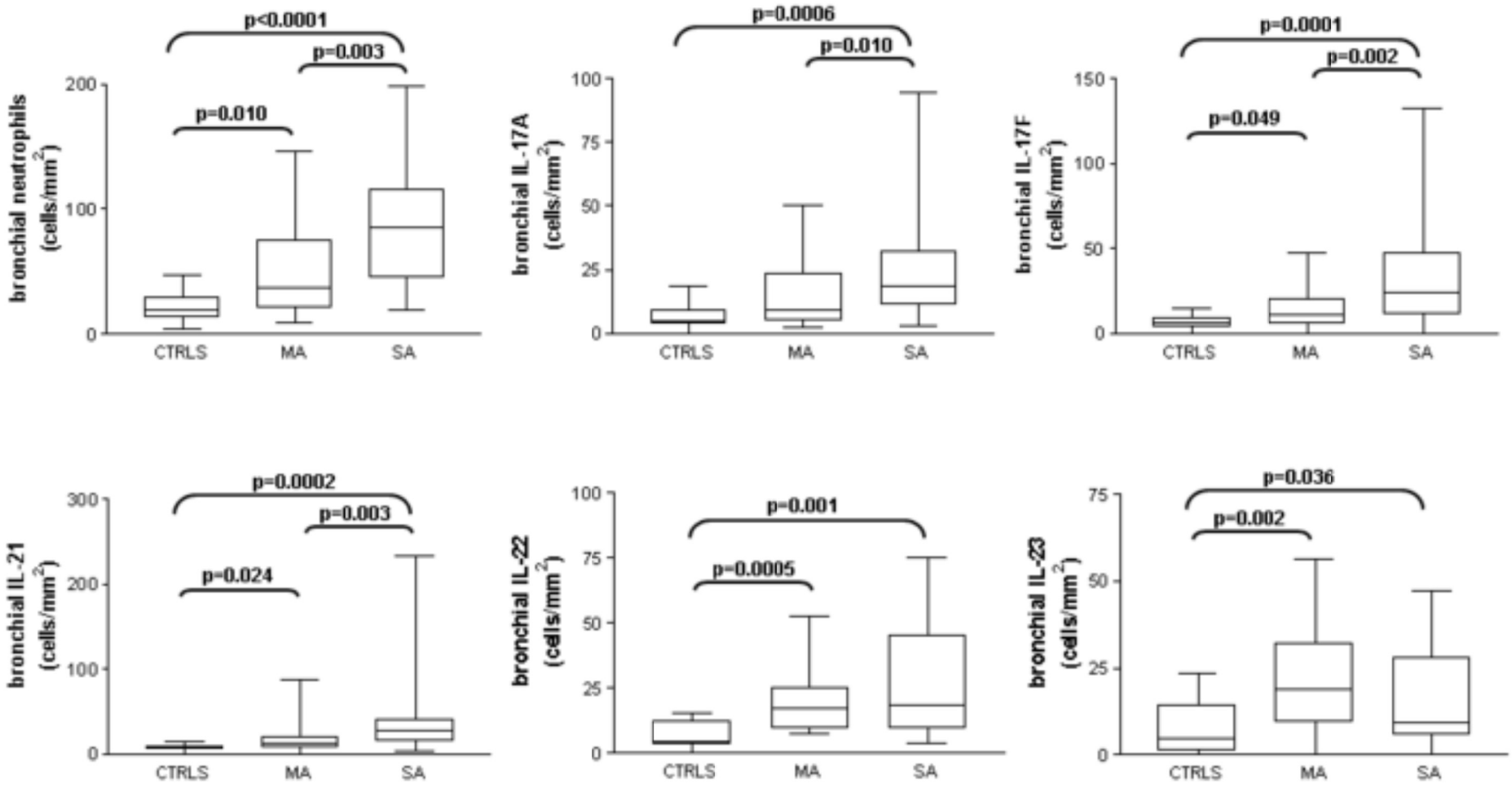

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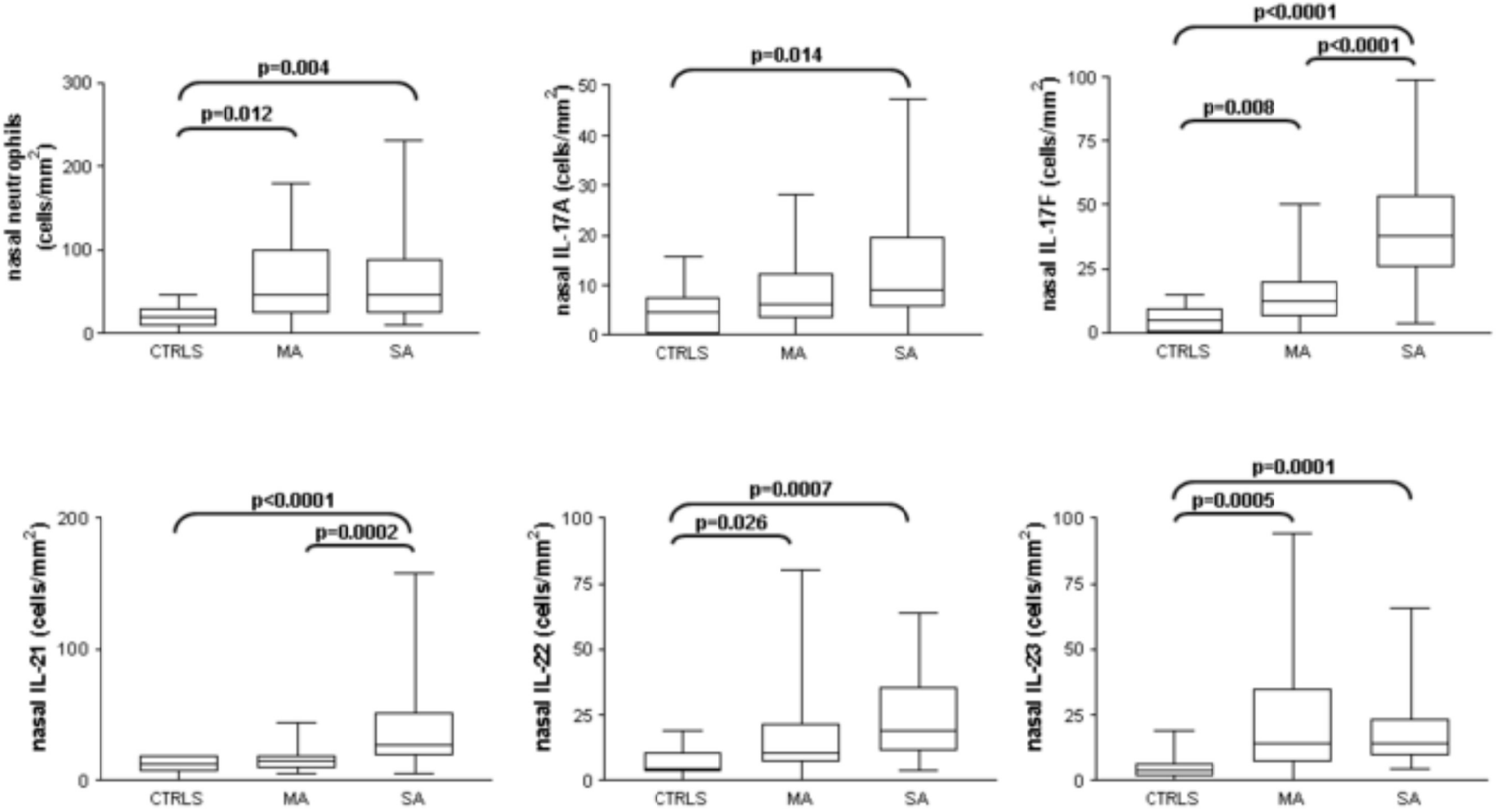

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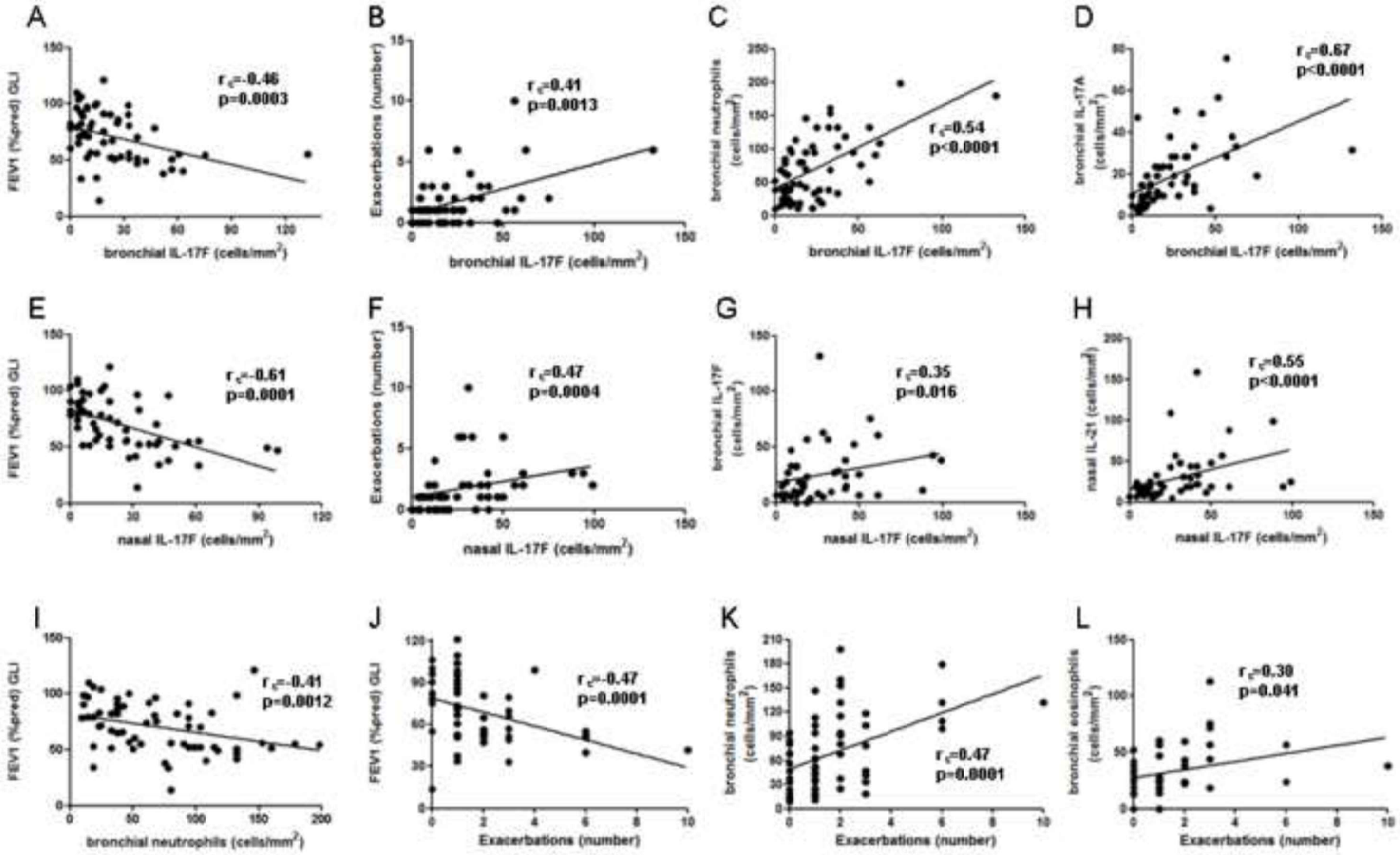

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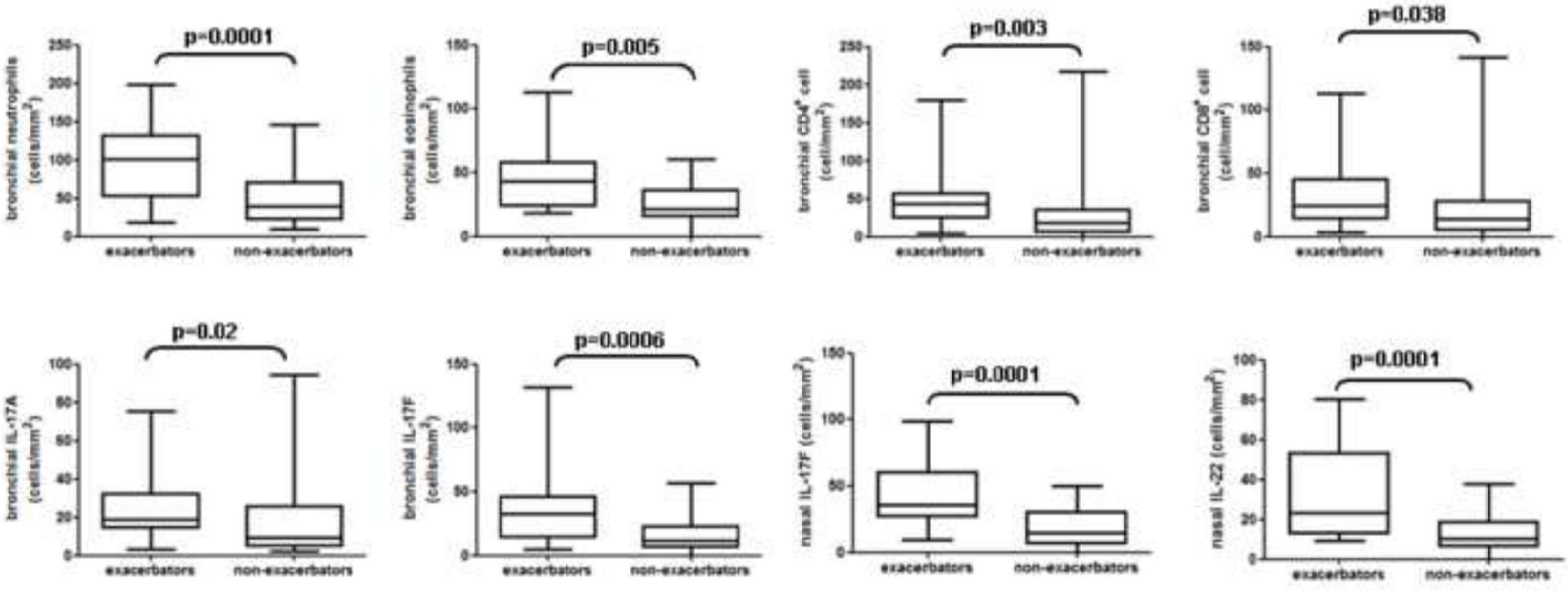

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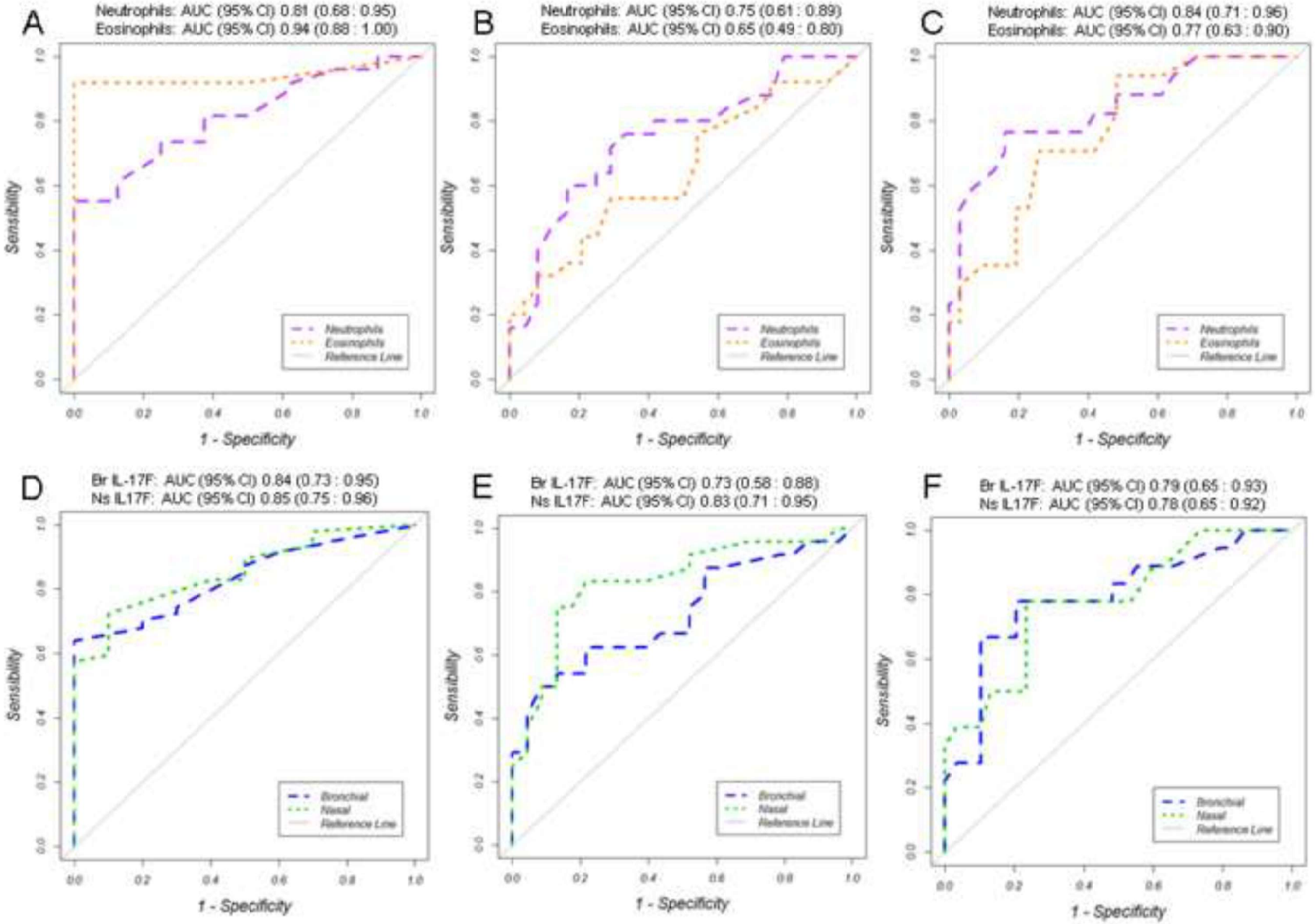

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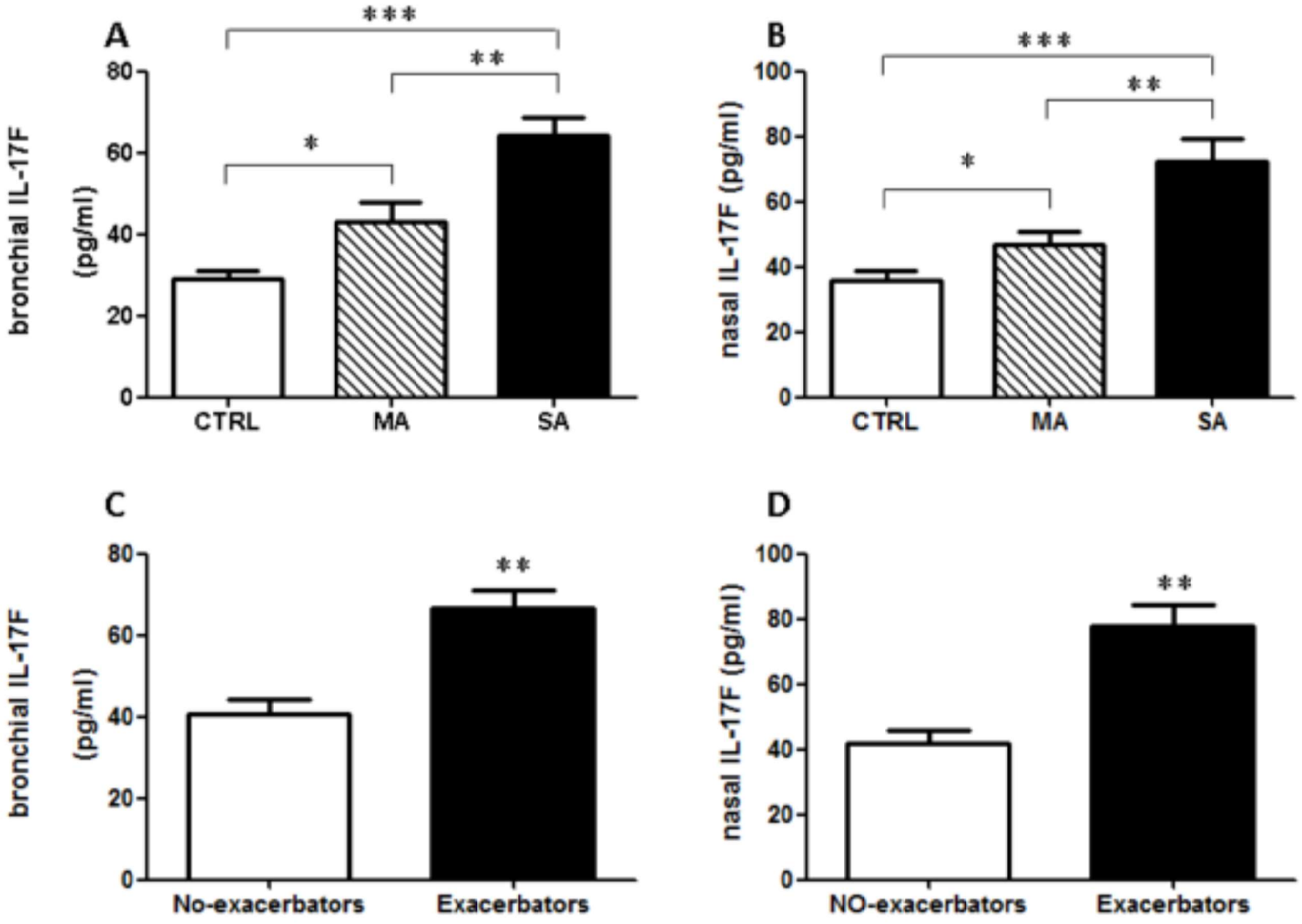

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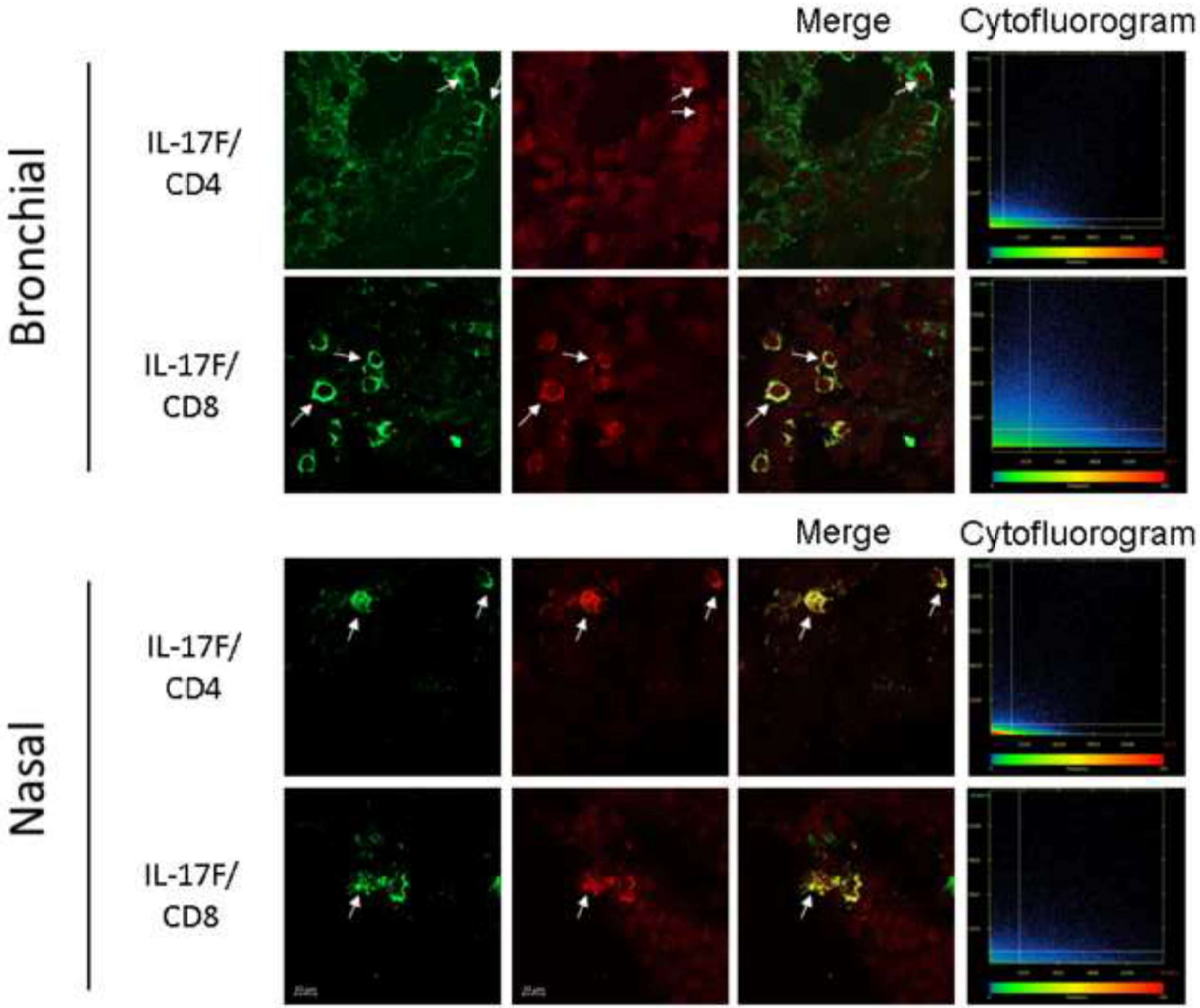

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