Osteopontin expression and relation to disease severity in human asthma

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ABSTRACT

Recent studies have associated osteopontin (Opn) with allergic inflammation; however, its role in human asthma remains unclear. We measured Opn levels in serum, bronchoalveolar lavage fluid (BALF) and bronchial tissue of healthy controls and asthmatics, identified cellular sources of Opn and examined possible correlations between Opn expression, disease severity and airway remodeling.

Serum samples were obtained from 35 mild-moderate (MMA), 19 severe asthmatics (SA) and 17 healthy controls in steady state and in case of exacerbation. Of these subjects, 29 asthmatics and 9 controls underwent bronchoscopy with endobronchial biopsy and BALF collection. Opn expression was determined by ELISA and immunohistochemistry/immunofluorescence. Reticular basement membrane (RBM) thickness and goblet cell hyperplasia were also determined.

Serum and BALF Opn levels were significantly increased in all asthmatics in steady state, while serum levels decreased during exacerbations. Opn was upregulated in the bronchial tissue of all patients and expressed by epithelial, airway and vascular smooth muscle cells, myofibroblasts, T-lymphocytes and mast cells. Opn expression correlated with RBM thickness and was more prominent in subepithelial inflammatory cells in severe compared to mild-moderate asthma.

Opn expression is upregulated in human asthma, is associated with remodeling changes and its subepithelial expression correlates to disease severity.

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Keywords: asthma, airway remodeling, allergic inflammation, basement membrane, osteopontin, research bronchoscopy

Abbreviations

Opn : osteopontin
CTRLs : healthy controls
MMA : patients with mild-to-moderate asthma
SA : patients with severe asthma
BALF : bronchoalveolar lavage fluid
RBM : reticular basement membrane thickness
GC : goblet cell
GCH : goblet cell hyperplasia
DAPI : 4’, 6-diamidino-2-phenylindole
α-SMA : alpha-smooth muscle actin
AHR : airway hyper-responsiveness
CS : corticosteroids
ICS : inhaled corticosteroids
INTRODUCTION

Asthma is an inflammatory disorder of the conducting airways, typically associated with an aberrant T Helper-2 (T\textsubscript{H2}) cell activation and response against environmental antigens. Airway inflammation in asthma is a multicellular process that is associated with structural alterations of the airway components, a process known as airway remodeling[1]. The prominent role of airway inflammation and remodeling in the pathogenesis and clinical presentation of asthma has led us to focus on mediators potentially involved in both processes.

Opn is a cytokine originally described as a structural component of the extracellular matrix that has the ability to bind to proteins and most types of collagen[2]. Opn exists in a secreted form (sOpn) that mediates cell adhesion, migration and survival[3], and an intracellular, non-secreted form (iOpn)[4]. Opn is produced by most cells of the immune system, including T cells, B cells, macrophages, neutrophils, eosinophils, NK cells and mast cells, as well as structural cells, including fibroblasts, smooth muscle and epithelial cells[5, 6]. Opn has been typically classified as a type-1 cytokine[7]. In humans, increased Opn expression has been observed in a number of T\textsubscript{H1}-mediated lung diseases, including granulomatous diseases and pulmonary fibrosis[8, 9].

There is now, also, emerging evidence to support an active role for Opn in T\textsubscript{H2}-linked inflammation and remodeling. Opn expression is upregulated in nasal tissue samples taken from asthmatic patients with chronic rhinosinusitis[10], while allergic patients undergoing successful long-term venom allergen immunotherapy have increased levels of serum Opn[11]. Moreover, Opn is expressed and functional in peripheral blood eosinophils of atopic human subjects[12] and is also involved in the migration
in vitro of eosinophils isolated from asthmatics[13]. We have previously demonstrated that Opn plays a crucial role in allergic airway inflammation by regulating distinct dendritic cell subsets[14] and that it is also involved in airway remodeling in well established animal models of allergic airway disease[15]. However, there are no studies investigating the expression pattern and cellular sources of Opn in patients with asthma of varying severity in a clinical setting. In our previous study[14], we reported preliminary data regarding Opn bronchial tissue expression in a limited number (six) of human asthmatics.

In the present study, we hypothesize that the upregulation of Opn expression is more pronounced in more severe forms of the disease and that a relationship exists between Opn upregulation and remodeling changes. Furthermore, we aim to identify the cellular sources of Opn in bronchial tissue of asthmatics. We have, therefore, investigated Opn expression in healthy controls and patients with varying severity of asthma in steady state and in case of exacerbation. We determined the levels of Opn in the serum, bronchoalveolar lavage fluid (BALF) and bronchial tissue of mild-moderate (MMA) and severe asthma (SA) patients and compared it to those of healthy controls. We also examined whether correlations exist between Opn expression, disease severity and control status, as well as tissue remodeling, with respect to reticular basement membrane thickening and goblet cell hyperplasia. Finally, we identified cellular sources of Opn in the bronchial tissue of asthmatics using double immunofluorescence staining and confocal microscopy.
METHODS

Study population

A total of 54 asthmatic patients [35 patients with mild to moderate persistent asthma (MMA), 19 patients with severe persistent asthma (SA)] and 17 non-atopic healthy controls participated in the study and were followed for at least one year. From this population, 29 patients with steady-state asthma (12 MMA and 17 SA) and 9 healthy controls consented to undergo research fiberoptic bronchoscopy with endobronchial biopsy and bronchoalveolar lavage fluid (BALF) collection when possible. Serum samples were collected from all participants in steady state and in 17 patients that exhibited an exacerbation during the study. In addition, a subpopulation of 17 patients with moderate and severe asthma agreed to receive two weeks of placebo-controlled treatment with oral corticosteroids (11 patients received 40 mg of prednisolone and 6 patients received placebo, 2:1 ratio), in order to evaluate the effect of steroids in Opn serum expression. Informed and written consent was obtained from all participating subjects and the corresponding protocol was approved by the Hospital Research Ethics Committee as well as the Greek National Organization for Medicines (EOF). Subject characteristics are summarized in table 1, while patient groups and procedures are presented in a flow diagram.

Asthma was defined as a clear clinical history with current symptoms plus 15% reversibility in FEV1 after two puffs of β2 agonist and/or positive metacholine challenge. All patients had physician confirmed diagnosis of persistent asthma and were receiving therapy depending on their asthma severity, according to GINA guidelines[16]. Asthma exacerbations were defined as events of severe deterioration of symptoms and rescue medication use that required the use of systemic
corticosteroids, or an increase from a stable maintenance dose, for at least 3 days[17]. All asthmatic patients had been attending our Asthma outpatient clinic for at least 2 years prior to the study. None of the patients were treated with theophylline, anti-leukotrienes or anti-IgE during the study. In addition, none of the participants had upper respiratory tract infection for 2 months prior to bronchoscopy or serum sample collection. Healthy individuals had no history of asthma or any other chronic disease, were not receiving any medication and had normal spirometry. All subjects participating in the study were non-smokers. Atopy was defined according to the recently revised nomenclature of the World Allergy Organization[18] and was assessed by means of skin prick tests for 18 common aeroallergens (HAL Allergy, Benelux).

**Pulmonary function testing**

Pulmonary function tests were performed on the same day prior to bronchoscopy or on the day that serum samples were collected, as previously described[19].

**Fiberoptic bronchoscopy**

Bronchoscopy was performed on an outpatient basis at the Athens Chest Hospital according to published recommendations[20]. All bronchoscopies were performed by the same experienced bronchoscopist using a flexible bronchoscope (WM-N60 Mobile Workstation). After inspection of the bronchial tree, a mini lavage was performed according to most recent ERS Taskforce guidelines[21]. Briefly, 100mL of prewarmed 0.9% saline were instilled into the right middle lobe in 20mL aliquots and then gently aspirated. The first aliquot collected was discarded. Only BAL fluid samples with a recovery rate equal or higher than 60% were kept for further analysis, which was the case in 6 healthy controls and 17 patients with asthma. Endobronchial
biopsies were obtained after BALF collection from various sites of the subsegmental carinae of the right lower lobes or right middle lobe. A minimum of three bronchial biopsies were taken from each subject.

**Sample collection and storage**

Blood samples were centrifuged at 1000g for 15 min at room temperature. BALF samples were centrifuged at 300g for 15 min at 4°C. Serum and BALF supernatant samples were aliquoted and stored in -80°C within two hours from collection. BALF cell pellets were resuspended in 1mL PBS and used for total and differential cell counts (table 2). Peripheral blood cell counts were also performed (table 3).

**ELISA**

Opn concentration in serum and BALF was determined in duplicates using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, DY1433), according to the manufacturer’s guidelines. Serum samples were diluted 50-fold before measurement. Opn levels in all samples were higher than the calculated detection limit of the assay (0.045 ng/mL). The intra-assay and inter-assay variability were determined to be 7.8% and 9.8%, respectively. The stability of the frozen serum and BALF samples was tested in 16 randomly selected samples for both patients (n=10) and controls (n=6), as previously described[19]. The evaluation of the stability of Opn did not show significant differences among the four measurements performed in the serum [1st week: 39.93 (24.99 – 54.72) ng/mL, after 4 weeks: 41.61 (25.58 – 50.61) ng/mL, after 8 weeks: 40.59 (23.48 – 52.35) ng/mL and after 12 weeks: 38.89 (23.35 – 49.75) ng/mL, p = 0.1] and BALF samples [1st week: 1.036 (0.939 – 1.329) ng/mL, after 4 weeks: 0.962 (0.785 – 1.312) ng/mL, after 8 weeks: 1.037 (0.698 – 1.357) ng/mL and after 12 weeks: 0.9915 (0.755 – 1.279) ng/mL, p = 0.29].
**Immunohistochemistry for Opn**

Immunohistochemical staining for Opn was performed as previously described[14]. Bronchial biopsies were fixated in 10% neutral buffered formalin overnight and embedded in paraffin. Sections 3-4 μm thick were deparaffinized in xylol and rehydrated in graded alcohol series. Endogenous peroxidase activity was inhibited using 0.3% H₂O₂ in methanol for 15 min. Heat induced epitope retrieval (HIER) was performed in preheated 10mM citrate buffer (pH 6) using a microwave oven (600W) for 15 min. Blocking of unspecific binding sites was performed by incubating slides with 10% goat serum for 30 min. Mouse antihuman monoclonal Opn antibody (diluted in PBS, 1% BSA, 0.1% TritonX) was applied overnight (1 μg/mL, R&D Systems, MAB1433) at 4°C, followed by a 45 min incubation at room temperature (RT) with a biotinylated secondary goat anti-mouse antibody (Vector, BA-9200). Slides were developed with the Strep-ABC-Peroxidase reagent and the peroxidase substrate diaminobenzidine (DAB Substrate Kit, Vector Laboratories, SK4100) according to the manufacturer’s instructions. Negative controls were performed by substitution of the primary antibody with the same concentration of the corresponding isotype IgG control (R&D Systems). All sections were counterstained with hematoxylin. All stained sections were coded and examined in a blinded manner at the end of the study. All available sections per patient were evaluated. Biopsies were considered suitable for examination when there was at least 1.0 mm of basement membrane length with intact epithelium and 0.1 mm² of subepithelial area. At least 3 suitable biopsy sections were examined per subject.

Quantitative measurements for Opn positive cells in the bronchial tissue were performed as previously described[14]. Briefly, Opn positive and negative cells were counted in each biopsy separately in the intact epithelium and the submucosa.
Bronchial epithelial cells positive for the Opn monoclonal antibody were expressed as percentage of total epithelial cells, while positive subepithelial inflammatory cells were expressed as percentage of total cells. The intra-observer error was assessed by performing three separate counts of the same section on different occasions and was calculated to be less than 8%.

**Reticular basement membrane thickness and goblet cell number measurements**

Analysis of reticular basement membrane (RBM) thickness and subepithelial infiltration was performed on sections stained with hematoxylin–eosin as previously described[22]. Briefly, basement membrane thickness was measured in micrometers (μm) by performing measurements at 20μm intervals along the whole length of the membrane, while taking only the adequately preserved and oriented area into account. A semi-quantitative scoring system, ranging from 0 to 3, was used to grade the number of inflammatory cells infiltrating the subepithelial layer (table 2). Goblet cells were examined on lung sections stained with Periodic-Acid-Schiff (PAS, Sigma-Aldrich, 395B-1KT). The reaction of periodic acid selectively oxidizes glucose residues and creates aldehydes that react with the Schiff reagent, staining carbohydrates in goblet cells with a purple-magenta color. GCs were quantified as number of positive cells per mm of basement membrane as previously described[23]. Sections were observed using an Axioskop40 microscopy system (Carl Zeiss, Jena GmbH) coupled with an AxioCam MRc digital camera, while basement membrane thickness measurements were done using the AxioVision v4.5.0.0 image analysis software.

**Double immunofluorescence**
Double immunofluorescence staining was performed to identify the cellular source of osteopontin. Antigen retrieval was performed (Dako S2368, S2369) and then sections were blocked for 30 minutes in 10% goat serum, followed by incubation with primary antibodies (diluted in TBS, 1% BSA, 0.1% TritonX) for 1 hour at RT. The primary antibodies that were used in appropriate combinations were the following: mouse anti-human Opn (2 μg/mL, R&D Systems, MAB1433), rabbit anti-human Opn antibody (5 μg/mL, Sigma Aldrich, O7264), alpha-smooth muscle actin (a-SMA, 1:100, Abcam, Ab5694), T-lymphocytes (CD3, 1:5, Dako, M7254), B-lymphocytes (CD19, 1:5, Dako, M7296), mast cells (mast cell tryptase, 1:50, clone AA1, Dako, M7052), neutrophils (neutrophil elastase, 1:100, clone NP57, Dako, M0752) and eosinophils (EG2, 1:50, Kabi Pharmacia Diagnostics AB, #10-9196-01). The secondary antibodies used were the AF555 goat F(ab')2 anti-mouse and the AF488 goat F(ab')2 anti-rabbit fluorescent secondary monoclonal antibodies (5 μg/mL, Molecular Probes, A11070 and A21425 respectively, 40 min incubation at RT). Antigen retrieval was not performed for Opn/neutrophil elastase and Opn/EG2 combinations, as it was found destructive of the epitopes. Finally, tissue sections were mounted with ProLong Gold Antifade Reagent (Molecular Probes, P36931), that also contained 4’-6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain, and were examined using an Axioplan2 microscope (Carl Zeiss, Jena GmbH) with filters set for AF488 and AF555.

Confocal laser microscopy

Specimens that showed double staining in fluorescent microscopy were further examined in a confocal laser microscope. Fluorescence images of the tissue sections were acquired, using an Axiovert 200/LSM 510 META confocal microscopy system (Carl Zeiss, Jena GmbH). DAPI was excited by the diode 405 nm laser and the
fluorescence was collected using a LP420 emission filter; the green (AF488) antibody was excited by the 488 nm Argon ion laser line and the fluorescence was collected using a BP505-530 emission filter, while the red (AF555) antibody was excited by the 543 nm line of the Green Helium-Neon laser and the fluorescence was collected using a LP560 emission filter. Tissue sections were visualized using the confocal microscope at 1024x1024 pixel resolution through a Plan-Neofluar 40x/1.3 Oil DIC objective with 8 times averaging in sequential scanning (multi track) mode with the pinhole set to obtain an optical section of about 1 μm in all channels.

**Statistical analysis**

Data are expressed as median with interquartile range (1\textsuperscript{st} and 3\textsuperscript{rd} quartiles) unless specified otherwise. Normality was assessed using Kolmogorov – Smirnov and D’Agostino – Pearson omnibus normality tests. Two-group comparisons were performed using the non-parametric Mann-Whitney U test, while three-group comparisons were performed using the non parametric Kruskal-Wallis one way analysis of variance accompanied by Dunn’s post hoc correction. Stability data were evaluated using the non-parametric Friedman test followed by Dunn’s post hoc analysis. The non-parametric Wilcoxon matched-pair test was applied to evaluate Opn expression in stable state and exacerbation. Correlation coefficients were calculated using the non parametric Spearman’s rank method. A statistical software package was used for all data analysis and graph preparations (GraphPad Prism v5, San Diego, CA). A \( p \) value of less than 0.05 was considered significant.
RESULTS

Opn is increased in the serum of patients with asthma

Patients with asthma had significantly increased serum Opn levels compared to healthy controls [47.92 (34.64 – 67.32) and 20.25 (22.51 – 24.92) ng/mL respectively, \( p < 0.0001 \)]. A subgroup analysis revealed that both mild-moderate (MMA) [49.16 (36.85 – 69.84) ng/mL, \( p < 0.001 \)] and severe asthmatics (SA) [46.77 (33.38 – 62.03) ng/mL, \( p < 0.01 \)] had significantly higher serum Opn levels compared to healthy controls, although no significant difference was found between these two patient groups (Figure 1A). Patients with exacerbations demonstrated decreased serum Opn levels compared to steady state [50.33 (35.65 – 58.87) and 58.97 (45.77 – 66.96) ng/mL respectively, \( p = 0.0332 \)] (Figure 1B). No difference was found regarding Opn serum levels in SA patients receiving oral steroids and those that did not (table 4). Finally, serum Opn levels did not differ in patients with asthma before and after two weeks of treatment with either prednisolone p.o. [\( n=11 \), mean values: 52.82 (32.32 – 68.71) and 47.27 (25.69 – 72.35) ng/mL respectively] or placebo [\( n=6 \), mean values: 53.08 (44.34 – 64.97) and 48.32 (37.52 – 56.78) ng/mL respectively, \( p = 0.8921 \)].

Opn is increased in the BAL fluid of patients with asthma

Opn levels were significantly increased in the BALF of asthmatic patients compared to healthy controls [1.108 (0.918 – 1.464), \( n = 6 \), and 0.746 (0.672 – 1.009) ng/mL, \( n =17 \), respectively, \( p = 0.0129 \)] (Figure 1C). Although a subgroup analysis revealed elevated BALF Opn levels for both MMA [1.108 (0.958 – 1.824) ng/mL, \( n = 7 \)] and SA [1.165 (0.889 – 1.419) ng/mL, \( n = 10 \)], only MMA had significantly higher levels of Opn compared to healthy controls (\( p < 0.05 \)). When Opn BAL fluid levels were
compared between SA patients receiving oral steroids (designated by “S” in figure 1) and those that did not, no significant difference was found (table 4). Finally, there was a trend for a positive correlation between BALF Opn levels and BALF eosinophils, which did not reach statistical significance ($p = 0.058$, $r = 0.4$).

**Opn is highly upregulated by bronchial epithelial and subepithelial inflammatory cells in patients with asthma**

Immunohistochemical staining revealed that Opn was highly expressed by both bronchial epithelial and subepithelial infiltrating inflammatory cells in asthmatic patients (Figure 2B and 2D), while healthy controls either didn’t express Opn or demonstrated very low Opn expression by some bronchial epithelial cells (Figure 2A). Detailed examination of the cellular sources of Opn in the lung revealed that Opn was expressed by cells positive for either α-SMA, CD3 or mast cell tryptase, suggesting that airway and vascular smooth muscle cells (Figure 3B and C), myofibroblasts (Figure 3D), T-lymphocytes (Figure 3E) and mast cells (Figure 3F), respectively, are sources of Opn production in the lung.

Quantification of lung Opn expression revealed that patients with asthma demonstrated significantly higher Opn expression by both bronchial epithelial [40 (28 – 48.5) %, $p < 0.0001$] and subepithelial cells [21 (11 – 33) %, $p < 0.0001$] compared to healthy controls [5 (3.5 – 9.5) % and 2 (0.5 – 2) %, respectively]. Regarding epithelial expression of Opn, a subgroup analysis revealed that this was true for both MMA [32.5 (18 – 42.75) %, $p < 0.01$] and SA [46 (32 – 52) %, $p < 0.001$], although no statistically significant difference was found between the two patient groups (Figure 4A). In addition, Opn expression by subepithelial infiltrating cells was also higher in both MMA and SA [12 (3.75 -20) %, $p < 0.05$ and 29 (18 – 36.5) %, $p <$
0.001, respectively] compared to healthy controls, with SA exhibiting significantly higher Opn levels compared to MMA \((p < 0.05)\) (Figure 4B). A significant correlation was found between epithelial and subepithelial Opn expression in all asthmatics \((r = 0.83, p < 0.0001)\), while Opn expression in bronchial epithelial cells weakly correlated with Opn BALF levels \((r = 0.46, p < 0.0267)\) (Figure 4C and 4D). Furthermore, Opn expression in the bronchial epithelium and especially the subepithelium inversely correlated with FEV\(_1\) \((p = 0.0184, r = -0.4142\) and \(p = 0.0001, r = -0.6227\), respectively). Finally, the comparison between eight severe asthma patients that were receiving steroids p.o. (designated by “S” in figure 4) and nine that were not, revealed no significant difference in Opn expression by both epithelial and subepithelial infiltrating cells (table 3).

**RBM thickness and goblet cells are increased in patients with asthma and RBM thickness correlates with Opn tissue expression**

RBM thickness was increased in patients with asthma compared to healthy controls \([6.3 (5.35 – 7.9) \mu m and 4.8 (4.25 – 5.4) \mu m respectively, p = 0.0051]\) (Figure 5A). Performing a subgroup analysis demonstrated that RBM was thicker in SA \([7.5 (5.9 – 8.65) \mu m]\) compared to MMA \([5.35 (4.4 – 6.475) \mu m, p < 0.05]\) and CTRLs \((p < 0.001)\), while no significant difference was found between MMA and CTRLs (Figure 5B). Furthermore, RBM thickness correlated with Opn expression both in the epithelium \((r = 0.58, p = 0.0001)\) as well as the subepithelium \((r = 0.62, p < 0.0001)\) (Figure 5C and 5D). Finally, a weak but statistically significant inverse correlation was observed between RBM thickness and FEV\(_1\) \((p = 0.0075, r = -0.46)\).

Goblet cell numbers, although highly variable, were significantly increased in asthmatic patients \([90 (72.5 – 117) cells/mm]\) compared to healthy controls \([68 (45 –\])
88) cells/mm, \( p = 0.0234 \) (Figure 5E). However, a subgroup analysis revealed only a near-significant difference between controls, MMA [87 (68.25 – 116) cells/mm] and SA [102 (78 – 117) cells/mm] groups (\( p = 0.0506 \)). No correlation was found between the number of goblet cells and Opn epithelial and/or subepithelial tissue expression (\( p = 0.22 \) and \( p = 0.1 \), respectively).
DISCUSSION

In this study we demonstrate that Opn is upregulated in the bronchial tissue in asthma and show for the first time that subepithelial inflammatory cells in SA patients express significantly more Opn compared to MMA patients, suggesting that Opn expression correlates with disease severity. Furthermore, we show that Opn expression correlates to RBM thickness, suggesting a role for Opn in asthma remodeling. We also demonstrate that Opn levels are increased in the serum of patients with asthma and that they decrease during exacerbations and we confirm that BALF Opn levels are elevated in asthma[12]. Finally, our findings reveal for the first time that cellular sources for Opn in the bronchial tissue in asthma include not only epithelial cells and macrophages but also that Opn localizes in airway and vascular smooth muscle cells, mast cells, lymphocytes and myofibroblasts.

The reasons behind the upregulation of Opn expression in asthma, as well as its role in the development of allergic inflammation and airway remodeling are still largely unknown. Opn is generally classified as a pro-inflammatory cytokine[5, 13, 24, 25]. However, emerging evidence supports an anti-inflammatory role of Opn in allergic disease. We have previously shown that Opn has a protective role during secondary pulmonary antigenic challenges and that therapeutic administration of rOpn during secondary antigenic challenges protects from allergic disease[14]. Furthermore, Opn appears to be associated with successful long-term venom allergen immunotherapy in allergic patients[11], it is involved in the prevention of viral infections[7] and may have a protective role in immune cell responses in the epithelium[26]. Although it is possible that Opn upregulation in the bronchial tissue in asthma may initially serve as a protective mechanism, what we have previously demonstrated[14, 15] and further
show in this study is that Opn over-expression in chronic inflammation may also result in tissue remodeling.

Reticular basement membrane thickness is a widely used marker of airway remodeling[1] and in our study, RBM was significantly thicker in SA patients compared to both MMA patients and healthy controls, in accordance with previous results[22]. Interestingly, we did find significant correlations between RBM thickness and bronchial tissue Opn expression, between FEV$_1$ and RBM thickness and between FEV1 and bronchial tissue Opn expression, suggesting that Opn is involved in remodeling and the resulting airway obstruction. Studies performed by our group have implicated Opn in fibrosis and remodeling in murine models of asthma[15]. Moreover, Opn expression has recently been shown to be essential for the migration and differentiation of myofibroblasts[27], which are important contributors of RBM thickening. This is in accordance with our findings, showing that myofibroblasts in the bronchial tissue of asthmatics express Opn in vivo. The relationship between Opn expression and subepithelial changes deserves further investigation, as it may suggest a target for therapeutic intervention.

Goblet cell hyperplasia and hypertrophy are also established pathologic characteristics of asthma and airway remodeling[28]. Using a murine model, we have previously demonstrated that Opn is associated with increased mucous production[15], however, in the present study, we found no significant correlation. This could possibly be attributed to treatment, since steroids have been reported to ameliorate goblet cell hyperplasia and mucus production[29].

Opn levels in the serum of asthmatic patients in steady state or during exacerbation have not been previously documented. Here we report that serum Opn is increased in
patients with asthma, possibly reflecting overspill from local infiltration and upregulation of Opn-producing cells in the bronchial tissue. However, although we find increased bronchial tissue Opn expression in SA, we did not detect differences in the levels of Opn in either serum or BALF between MMA and SA. A hypothesis explaining this lack of differences could be that the higher bronchial Opn expression in SA is due to the upregulation of the intracellular form of Opn. We also found that Opn serum levels decrease during exacerbation, however, the reported significance is borderline and the number of cases is relatively small.

There is limited information on the cellular origin of Opn production in the bronchial airways of asthmatic patients. We have previously reported that bronchial tissue macrophages from asthmatic patients can produce Opn[14] and in this study, we showed for the first time that Opn also localizes in airway and vascular smooth muscle cells, mast cells, lymphocytes and myofibroblasts. This is in agreement with a previous report showing that Opn is expressed in circulating T cells of subjects with atopic asthma[30] and that it is produced by mast cells and is involved in mast cell degranulation and migration[25]. Furthermore, bronchial myofibroblasts from patients with usual interstitial pneumonia express Opn in vivo and in vitro[31] and Opn expression is required for myofibroblast differentiation and activity[27]. Lastly, Opn has been previously demonstrated to induce proliferation of human bronchial smooth muscle in vitro[15].

There is currently no information about the effect of steroids on Opn expression in human lung. Moreover, the information available from animal models and human tissues other than lung is very limited and rather controversial[32, 33]. In the present study, we found no differences in the serum, BALF and bronchial tissue Opn expression between SA patients on long-term oral steroids to those on ICS only.
Furthermore, we found no differences in serum Opn levels before and after 2 weeks of oral steroid treatment. In a recent study using a murine model of asthma, dexamethasone was found to decrease Opn BALF levels and lung tissue mRNA expression[34]. However, the amount of steroids used in this study (3 mg/kg dexamethasone or 20 mg/kg prednisolone) is approximately 200 times larger than the dose of oral prednisolone used in our study, and 40 times higher than what current guidelines suggest for the management of severe exacerbations of asthma[16]. It is, therefore, questionable whether the effects of dexamethasone on OPN expression reported in this experimental study have clinical relevance.

Our study presents certain limitations. In an observational cross-sectional study it is difficult to determine specific mechanisms of action. In addition, the number of patients that exhibited an exacerbation was relatively small and the observed results could be subject to statistical error. This might also apply to BALF data; although 38 subjects underwent research bronchoscopy, BALF samples were processed from only 23 subjects in order to adhere to current guidelines regarding the recovery rate (>60%). In addition, it was not possible to differentiate between secreted and intracellular forms of OPN in the bronchial tissue. Finally, although we performed a subgroup analysis and found no differences in Opn expression with respect to steroid treatment, we cannot exclude a possible bias introduced by asthma medication.

Interest regarding Opn has grown remarkably over the last decade, during which Opn has evolved from a structural bone matrix component to an increasingly complex and multifaceted cytokine that has a pivotal role in the regulation of immune responses. In our study we demonstrate an upregulation of Opn expression in human asthma that is associated with remodeling changes and correlates to disease severity. Further
research on Opn function and regulation in human asthma could lead to novel therapeutic approaches targeting the regulation of Opn expression and function \textit{in situ}. 
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COMPETING INTERESTS

The authors declare that they have no competing interests.
Table 1: Subject characteristics

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<td>92.7±9.8</td>
<td>78.7±8.1 ‡</td>
<td>65.3±17.5 †</td>
</tr>
<tr>
<td>Atopy</td>
<td>-</td>
<td>19</td>
<td>7</td>
</tr>
<tr>
<td>LABA</td>
<td>-</td>
<td>26</td>
<td>19</td>
</tr>
<tr>
<td>ICS§</td>
<td>-</td>
<td>35 (680±420)</td>
<td>19 (1950±570)</td>
</tr>
<tr>
<td>CS p.o. ‡</td>
<td>-</td>
<td>-</td>
<td>8 (8.4±2.8)</td>
</tr>
</tbody>
</table>

± denotes standard deviation (SD)

† p < 0.05 compared to MMA and p < 0.001 compared to healthy controls

‡ p < 0.05 compared to healthy controls

§ Dose of beclomethasone; all other ICS doses were converted to their equivalent doses of beclomethasone

‖ Dose of prednisolone; all other CS p.o. doses were converted to their equivalent doses of prednisolone
Flow Diagram. Patient groups and procedures included in the study.

- **71 Subjects**
  - **17 Healthy Controls** (serum samples collected from all)
    - 9 healthy controls underwent bronchoscopy (biopsies were obtained from all 9 subjects, and adequate BALF samples from 6 subjects)
  - **54 Asthmatic patients** (serum samples collected from all)
    - **35 MMA**
      - 12 patients underwent bronchoscopy (biopsies obtained from 12 patients and adequate BALF samples from 7 patients)
      - 5 patients exhibited exacerbation during the study
      - 12 patients included in steroid trial (4 placebo and 8 steroids p.o.)
    - **19 SA**
      - 17 patients underwent bronchoscopy (with 17 biopsies and 10 adequate BALF samples)
      - 12 patients exhibited exacerbation during the study
      - 5 patients included in steroid trial (2 placebo and 3 steroids p.o.)
**Table 2:** Subepithelial inflammation grade score (grade 0 to 3, mean ± range) and BAL fluid cell counts (median ± range).

<table>
<thead>
<tr>
<th></th>
<th>CTRL</th>
<th>MMA</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subepithelial inflammation</td>
<td>0.33 (0-1)</td>
<td>1.58 (0-3)*</td>
<td>2.29 (1-3)†</td>
</tr>
<tr>
<td>BAL fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.5 (0-2)</td>
<td>1.9 (0.5-5)</td>
<td>2.6 (0.5-9)*</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>1.5 (0-6)</td>
<td>2.6 (0-10)</td>
<td>4.4 (0.5-26)</td>
</tr>
<tr>
<td>Macrophages (%)</td>
<td>88 (81-97)</td>
<td>81 (45-97)</td>
<td>78 (40-95)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>6.5 (1-12)</td>
<td>7.7 (0.5-21)</td>
<td>7.2 (2-35)</td>
</tr>
<tr>
<td>Total cell count (x10⁴)</td>
<td>8.1 (6.2-11)</td>
<td>9.5 (7.6-12.8)</td>
<td>11.7 (8-23.2)*</td>
</tr>
</tbody>
</table>

* *p* < 0.05 compared to healthy controls
† *p* < 0.001 compared to healthy controls
Table 3. Cell counts in the peripheral blood of healthy controls and asthmatic patients. White blood cells (WBC) are presented as mean absolute number of cells ± SD, while differential counts are presented as mean percentage of WBC ± SD.

<table>
<thead>
<tr>
<th></th>
<th>CTRL</th>
<th>MMA</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC</td>
<td>7069 ± 1833</td>
<td>7561 ± 2637</td>
<td>8340 ± 2802</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>60.33 ± 6.47</td>
<td>57.88 ± 11.67</td>
<td>59.1 ± 8.66</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>30.45 ± 5.85</td>
<td>31.41 ± 9.36</td>
<td>30.05 ± 6.94</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>6.24 ± 2.04</td>
<td>5.34 ± 1.95</td>
<td>5.01 ± 2.1</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>2 ± 0.79</td>
<td>4.33 ± 3.24*</td>
<td>4.71 ± 3.75†‡</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.92 ± 0.34</td>
<td>0.8 ± 0.49</td>
<td>0.89 ± 0.45</td>
</tr>
</tbody>
</table>

* $p < 0.05$ compared to healthy controls  
† $p < 0.01$ compared to healthy controls  
‡ Blood eosinophils count correlates with Opn serum levels ($p = 0.032$, $r = 0.47$)
Table 4: Opn expression levels in the tissue, serum and BALF of severe asthmatics receiving corticosteroids p.o. (group A) compared to those that didn’t (group B). All values are presented as median ± interquartile range.

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>8/8</td>
<td>11/9</td>
<td></td>
</tr>
<tr>
<td>(total/undergone bronchoscopy)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Opn (ng/mL)</td>
<td>46.93 (27.9 – 65.71)</td>
<td>46.77 (33.38 – 62.03)</td>
<td>0.77</td>
</tr>
<tr>
<td>BALF Opn (ng/mL)</td>
<td>1.188 (0.895 – 1.466)</td>
<td>1.087 (0.771 – 1.387)</td>
<td>0.39</td>
</tr>
<tr>
<td>Opn⁺ epithelial cells</td>
<td>47 (36 – 55.75)</td>
<td>40 (28.5 – 51.5)</td>
<td>0.41</td>
</tr>
<tr>
<td>(% of total)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Opn⁺ subepithelial cells</td>
<td>30 (20.5 – 36.95)</td>
<td>29 (15.5 - 34)</td>
<td>0.5</td>
</tr>
<tr>
<td>(% of total)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIGURE LEGENDS

Figure 1. Osteopontin (Opn) levels are increased in the serum and BAL fluid of asthmatic subjects. Serum Opn is increased in both mild-moderate asthmatics (MMA) [n = 35] and severe asthmatic subjects (SA) [n = 19] compared to healthy controls [n = 17], with no difference found between the two patient groups (A). Asthmatic patients exhibiting exacerbation have decreased levels of serum Opn compared to steady state (B). Opn in the BAL fluid of asthmatic patients [n = 17] is increased compared to healthy controls [n = 6] (C). “S” denotes asthmatic patients receiving steroids p.o. Results are expressed as median.

![Graph A - Serum Opn levels](image1)

![Graph B - Serum Opn levels](image2)

![Graph C - BAL Opn levels](image3)

Figure 2. Expression of osteopontin (Opn) levels in the bronchial tissue of patients with asthma and healthy controls. Bronchial tissue obtained from healthy and asthmatic subjects that underwent fiberoptic bronchoscopy was processed immunohistochemically to show tissue Opn expression. Specific staining for Opn is depicted with brown color, whereas nuclei are stained blue. Representative microphotographs (x400) are presented from a healthy individual (A) and an asthmatic (SA) patient (B), followed by the corresponding isotype control (C) performed on a consecutive section (bars = 20μm). In (D), the region within the black square in (B) is presented in higher magnification. Red arrows indicate positively stained epithelial cells and black arrows point to positively stained subepithelial cells.

![Microphotograph A](image4)

![Microphotograph B](image5)

![Microphotograph C](image6)

![Microphotograph D](image7)
Figure 3. Identification of cellular sources of Opn in the bronchial tissue of asthmatic patients using confocal laser microscopy. Double immunofluorescent staining was performed on formalin-fixed paraffin-embedded 3-4μm tissue sections, obtained from patients with asthma, to identify the cellular source of Opn. Counterstaining was performed with 4’, 6-diamidino-2-phenylindole (DAPI) to visualize nuclear DNA. Column 1 shows DAPI nuclear counterstain, columns 2 and 3 represent positive staining for primary antibodies and column 4 is the result of merging images from columns 1 to 3. Either a mouse monoclonal with an AF488-conjugated goat anti-mouse secondary (B3, C3 and D3) or a rabbit polyclonal antibody with an AF555-conjugated goat anti-rabbit secondary (E3) were used to specifically stain intracellular or secreted Opn. Isotype controls using mouse and rabbit anti-human IgG primary antibodies followed by AF488/AF555-conjugated goat anti-mouse and anti-rabbit secondary antibodies, respectively, showed no specific staining (A). Airway smooth muscle cells (green) stained with a rabbit monoclonal against human alpha-smooth muscle actin (α-SMA) demonstrated positive staining for Opn (red) (B). Vascular smooth muscle cells (green arrows), as well as some endothelial cells (white arrow), express Opn in (C). α-SMA positive myofibroblasts (green) were also found to produce Opn (red) (D). Furthermore, T-lymphocytes stained positive with anti-CD3 monoclonal antibody (green) displayed positive staining for Opn (red) (E). Finally, mast cells positively stained for mast-cell tryptase (red) demonstrated positive staining for Opn (green) (F).
Figure 4. Opn is upregulated in the bronchial tissue of patients with asthma. Bronchial biopsies stained specifically for Opn in healthy controls \([n = 9]\) and patients with asthma \([n = 29]\) were further evaluated to quantify Opn tissue expression. Opn positive and negative cells were counted separately in the epithelium (expressed as percentage of total epithelial cells) and the submucosa (expressed as percentage of total cells). Both mild-moderate asthmatics (MMA) \([n = 12]\) and patients with severe asthma (SA) \([n = 17]\) exhibit increased epithelial (A) and subepithelial (B) Opn expression compared to healthy controls \([n = 9]\), although Opn is expressed by more subepithelial infiltrating cells in SA compared to MMA (B). “S” represents patients with severe asthma receiving steroids p.o. A significant correlation was found between epithelial and subepithelial Opn expression in all asthmatics (C), while Opn expression in bronchial epithelial cells weakly correlated with Opn in BAL fluid (D). Values are expressed as median.
Figure 5. Reticular basement membrane thickness (RBMt) and goblet cells are increased in patients with asthma and RBMt correlates with Opn tissue expression. Bronchial tissue sections obtained from subjects that underwent fiberoptic bronchoscopy were stained with hematoxylin – eosin (HE). Representative microphotographs are depicted from a healthy individual and a patient with severe asthma (A), showing a marked increase in membrane thickness. Patients with severe asthma \([n = 17]\) had increased RBM thickness compared to both MMA \([n = 12]\) and healthy controls \([n = 9]\). RBMt correlated with Opn expression both in the epithelium (C) as well as the subepithelium (D) of all subjects that underwent bronchoscopy. Bronchial tissue sections from healthy controls and patients with asthma were also stained with Periodic-Acid-Schiff (PAS) to identify goblet cells (GCs) in the bronchial epithelium (E). Sections were counterstained with hematoxylin. Magnification in (A) and (E) is x400. Values are expressed as median.
Reference List


