Eur Respir J 2011; 37: 331–341
DOI: 10.1183/09031936.00017810


# Osteopontin expression and relation to disease severity in human asthma

**K. Samitas*,#,¶, E. Zervas*, S. Vittorakis*,#, M. Semitekolou#, T. Alissafi#, A. Bossios¶, H. Gogos+, E. Economidou*, J. Lötvall¶, G. Xanthou#, V. Panoutsakopoulou#,§ and M. Gaga*,§**

**ABSTRACT: Recent studies have associated osteopontin (OPN) with allergic inflammation; however, its role in human asthma remains unclear. The aim of this study was to measure OPN levels in the serum, bronchoalveolar lavage fluid (BALF) and bronchial tissue of healthy controls and asthmatics, identify cellular sources of OPN and examine possible correlations between OPN expression, disease severity and airway remodelling.**

**Serum samples were obtained from 35 mild-to-moderate asthmatics, 19 severe asthmatics and 17 healthy controls in the steady state and in cases of exacerbation. Of these subjects, 29 asthmatics and nine controls underwent bronchoscopy with endobronchial biopsy and BALF collection. OPN expression was determined by ELISA and immunohistochemistry/immunofluorescence. Reticular basement membrane thickness and goblet cell hyperplasia were also determined.**

**Serum and BALF OPN levels were significantly increased in all asthmatics in the steady state, whereas 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 reticular basement membrane thickness and was more prominent in subepithelial inflammatory cells in severe compared to mild-to-moderate asthma.**

**OPN expression is upregulated in human asthma and associated with remodelling changes, and its subepithelial expression correlates with disease severity.**

**KEYWORDS: Airway remodelling, allergic inflammation, asthma, basement membrane, osteopontin, research bronchoscopy**

Asthma is an inflammatory disorder of the conducting airways, typically associated with aberrant type-2 T-helper cell (Th2) 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 remodelling [1]. The prominent role of airway inflammation and remodelling in the pathogenesis and clinical presentation of asthma has led to the current focus on mediators potentially involved in both processes.

Osteopontin (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 that mediates cell adhesion, migration and survival [3], and an intracellular nonsecreted form [4]. OPN is produced by most cells of the immune system, including T-cells, B-cells, macrophages, neutrophils, eosinophils, natural killer cells and mast cells, as well as structural cells, including fibroblasts and 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 Th1-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 Th2-linked inflammation and remodelling. OPN expression is upregulated in nasal tissue samples taken from asthmatic patients with chronic rhinosinusitis [10], and allergic patients undergoing successful long-term venom allergen immunotherapy show increased levels of serum OPN [11]. Moreover, OPN is expressed and functional in peripheral blood eosinophils of atopic human subjects [12],

AFFILIATIONS
*7th Respiratory Medicine Dept and Asthma Centre,
+Pathology Dept, Athens Chest Hospital ''Sotiria'',
#Cellular Immunology Laboratory, Division of Cell Biology, Center for Basic Research, Biomedical Research Foundation of the Academy of Athens, Athens, Greece.
¶Krefting Research Centre, Dept of Internal Medicine, Institute of Medicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden.
§Both authors contributed equally to this study.

CORRESPONDENCE
M. Gaga
7th Respiratory Medicine Dept and Asthma Centre
Mesogeion 152
Athens 11527
Greece
E-mail: minagaga@yahoo.com

Received:
Feb 02 2010
Accepted after revision:
May 28 2010
First published online:
June 18 2010

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

and is also involved in the *in vitro* migration of eosinophils isolated from asthmatics [13]. It has previously been 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 remodelling 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 the previous study [14], preliminary data were reported regarding OPN bronchial tissue expression in a limited number (six) of human asthmatics.

In the present study, it was hypothesised that the upregulation of OPN expression is more pronounced in more severe forms of asthma and that a relationship exists between OPN upregulation and remodelling changes. Furthermore, the aim was to identify the cellular sources of OPN in the bronchial tissue of asthmatics. Therefore, OPN expression was investigated in healthy controls and patients with varying severities of asthma in the steady state and in cases of exacerbation. Levels of OPN in the serum, bronchoalveolar lavage fluid (BALF) and bronchial tissue of mild-to-moderate (MMA) and severe asthma (SA) patients were determined and compared to those of healthy controls. It was also examined whether or not correlations exist between OPN expression, disease severity and control status, as well as tissue remodelling, with respect to reticular basement membrane (RBM) thickening and goblet cell hyperplasia. Finally, cellular sources of OPN were identified in the bronchial tissue of asthmatics using double immunofluorescence staining and confocal microscopy.

## METHODS

### *Study population*

A total of 54 persistently asthmatic patients (35 with MMA and 19 with SA) and 17 non-atopic healthy controls participated in the present study and were followed for $\geqslant$1 yr. From this population, 29 patients with steady-state asthma (12 MMA and 17 SA) and nine healthy controls consented to undergo research fibreoptic bronchoscopy with endobronchial biopsy and BALF collection when possible. Serum samples were collected from all participants in the steady state and the 17 patients that exhibited an exacerbation during the study. In addition, a subpopulation of 17 patients with MMA and SA agreed to receive 2 weeks of placebo-controlled treatment with oral corticosteroids (11 patients received 40 mg prednisolone and six received placebo; 2:1 ratio) in order to evaluate the effect of steroids on serum OPN expression. Informed and written consent was obtained from all participating subjects, and the corresponding protocol was approved by the Hospital Research Ethics Committee of Athens Chest Hospital, as well as the Greek National Organization for Medicines (both Athens, Greece). Subject characteristics are summarised in table 1, and patient groups and procedures are presented in a flow diagram (fig. 1).

Asthma was defined as a clear clinical history with current symptoms plus 15% reversibility in forced expiratory volume in 1 s ($FEV_1$) after two puffs of $\beta_2$-agonist and/or positive methacholine challenge. All patients had a physician-confirmed diagnosis of persistent asthma and were receiving therapy depending on their asthma severity, according to Global Initiative for Asthma 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 $\geqslant$3 days [17]. All asthmatic patients had been attending the asthma outpatient clinic of Athens Chest Hospital for $\geqslant$2 yrs prior to the study. None of the patients were treated with theophylline, antileukotrienes or anti-immunoglobulin (Ig)E during the study. In addition, none of the participants had shown 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 gave normal spirometric results. All subjects participating in the study were nonsmokers. 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, Leiden, the Netherlands).

**TABLE 1** Subject characteristics

| | Control | MMA | SA |
|---|---|---|---|
| **Subjects n** | 17 | 35 | 19 |
| **Age yrs** | 49 (33–70) | 49 (29–68) | 53 (33–71) |
| **Males/females n** | 6/11 | 13/22 | 5/14 |
| **Asthma duration yrs** | | 34 (5–63) | 37 (13–65) |
| **$FEV_1$ % pred** | 92.7±9.8 | 78.7±8.1* | 65.3±17.5$^{+,\S}$ |
| **Atopy n** | | 19 | 7 |
| **LABA n** | | 26 | 19 |
| **ICS μg$^{\#}$** | | 35 (680±420) | 19 (1950±570) |
| **CS mg *p.o.*$^{\P}$** | | | 8 (8.4±2.8) |

Data are presented as mean±SD or mean (range), unless otherwise indicated. MMA: mild-to-moderate asthma; SA: severe asthma; $FEV_1$: forced expiratory volume in 1 s; % pred: % predicted; LABA: long-acting $\beta_2$-agonist; ICS: inhaled corticosteroid; CS: corticosteroid. $^{\#}$: beclometasone or beclometasone equivalent dose; $^{\P}$: prednisolone or prednisolone equivalent dose. *: $p<0.05$; $^{+}$: $p<0.001$ *versus* healthy controls; $^{\S}$: $p<0.05$ *versus* MMA.

### *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].

### *Fibreoptic bronchoscopy*

Bronchoscopy was performed on an outpatient basis at the Athens Chest Hospital according to published recommendations [20]. All bronchoscopic procedures were performed by the same experienced bronchoscopist using a flexible bronchoscope (WM-N60 mobile workstation; Olympus, Tokyo, Japan). After inspection of the bronchial tree, a mini-lavage was performed according to the most recent European Respiratory Society Task Force guidelines [21]. Briefly, 100 mL pre-warmed 0.9% saline were instilled into the right middle lobe in 20-mL aliquots and then gently aspirated. The first aliquot collected was discarded. Only BALF samples with a recovery rate of $\geqslant$60% were kept for further analysis, which was the case in six

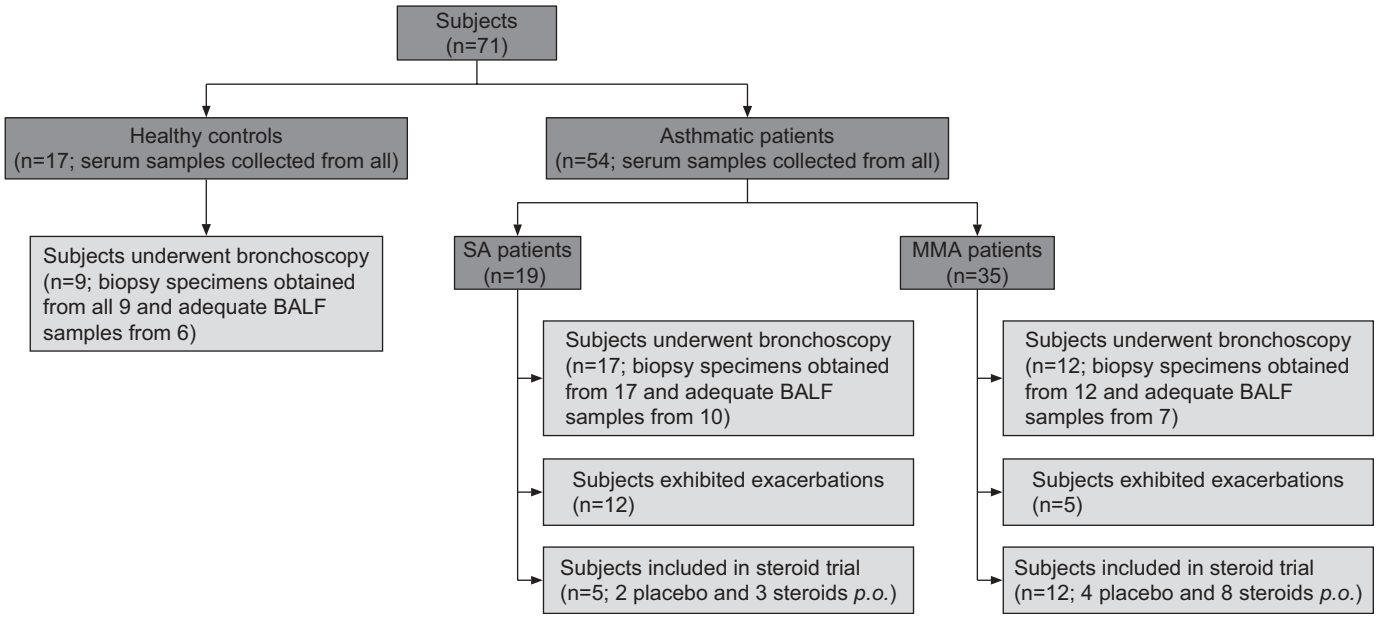


**FIGURE 1.** Flow diagram showing patient groups and procedures included in the present study. MMA: mild-to-moderate asthma; SA: severe asthma; BALF: bronchoalveolar lavage fluid.

healthy controls and 17 patients with asthma. Endobronchial biopsy specimens were obtained following BALF collection, from various sites of the subsegmental carinae of the right lower lobes or right middle lobe. A minimum of three bronchial biopsy specimens were taken from each subject.

### *Sample collection and storage*

Blood samples were centrifuged for 15 min at $1,000 \times g$ at room temperature. BALF samples were centrifuged for 15 min at $300 \times g$ at 4°C. Serum and BALF supernatant samples were aliquoted and stored at -80°C within 2 h after collection. BALF cell pellets were resuspended in 1 mL PBS and used for total and differential cell counts (table 2). Peripheral blood cell counts were also performed (table 3).

**TABLE 2** Subepithelial inflammation grade score and bronchoalveolar lavage fluid (BALF) cell counts

| | Control | MMA | SA |
|---|---|---|---|
| **Subepithelial inflammation score** | 0.33 (0–1) | 1.58 (0–3)* | 2.29 (1–3)*** |
| **BALF cell count** | | | |
| Eosinophils % | 0.5 (0–2) | 1.9 (0.5–5) | 2.6 (0.5–9)* |
| Neutrophils % | 1.5 (0–6) | 2.6 (0–10) | 4.4 (0.5–26) |
| Macrophages % | 88 (81–97) | 81 (45–97) | 78 (40–95) |
| Lymphocytes % | 6.5 (1–12) | 7.7 (0.5–21) | 7.2 (2–35) |
| Total cell count $10^4$ cells·mL$^{-1}$ | 8.1 (6.2–11.0) | 9.5 (7.6–12.8) | 11.7 (8.0–23.2)* |

Data are presented as mean (range) for inflammation score and median (range) for cell counts. Subepithelial inflammation score ranges 0–3. MMA: mild-to-moderate asthma; SA: severe asthma. *: $p<0.05$; ***: $p<0.001$ *versus* healthy controls.

### *ELISA*

OPN concentration in serum and BALF was determined in duplicate using an ELISA kit (DY1433; R&D Systems, Wiesbaden, Germany) 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$^{-1}$). The intra- 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 from 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$^{-1}$, after 4 weeks: 41.61 (25.58–50.61) ng·mL$^{-1}$, after 8 weeks: 40.59 (23.48–52.35) ng·mL$^{-1}$, and after 12 weeks: 38.89 (23.35–49.75)

**TABLE 3** Cell counts in the peripheral blood of healthy controls and asthmatic patients

| | Control | MMA | SA |
|---|---|---|---|
| **WBCs cells·mL$^{-1}$** | 7069±1833 | 7561±2637 | 8340±2802 |
| **Differential cell counts %** | | | |
| Neutrophils | 60.33±6.47 | 57.88±11.67 | 59.10±8.66 |
| Lymphocytes | 30.45±5.85 | 31.41±9.36 | 30.05±6.94 |
| Monocytes | 6.24±2.04 | 5.34±1.95 | 5.01±2.10 |
| Eosinophils | 2.00±0.79 | 4.33±3.24* | 4.71±3.75**,# |
| Basophils | 0.92±0.34 | 0.80±0.49 | 0.89±0.45 |

Data are presented as mean±SD; differential cell counts are presented as a percentage of white blood cells (WBCs). MMA: mild-to-moderate asthma; SA: severe asthma. #: correlates with serum osteopontin levels ($p=0.032$, $r=0.47$). *: $p<0.05$; **: $p<0.01$ *versus* healthy controls.

ng·mL$^{-1}$; p=0.1) and BALF samples (1st week: 1.036 (0.939–1.329) ng·mL$^{-1}$, after 4 weeks: 0.962 (0.785–1.312) ng·mL$^{-1}$, after 8 weeks: 1.037 (0.698–1.357) ng·mL$^{-1}$, and after 12 weeks: 0.9915 (0.755–1.279) ng·mL$^{-1}$; p=0.29).

### *Immunohistochemistry for OPN*

Immunohistochemical staining for OPN was performed as previously described [14]. Bronchial biopsy specimens were fixed in 10% neutral buffered formalin overnight and embedded in paraffin. Sections 3–4 μm thick were deparaffinised in xylol and rehydrated in a graded alcohol series. Endogenous peroxidase activity was inhibited using 0.3% $H_2O_2$ in methanol for 15 min. Heat-induced epitope retrieval was performed in preheated 10 mM citrate buffer (pH 6.0) using a microwave oven (600 W) for 15 min. Blocking of unspecific binding sites was performed by incubating slides with 10% goat serum for 30 min. Mouse anti-human monoclonal OPN antibody (diluted in PBS containing 1% bovine serum albumin BSA and 0.1% Triton X-100; MAB1433; R&D Systems; 1 μg·mL$^{-1}$) was applied overnight at 4°C, followed by a 45-min incubation at room temperature with a biotinylated secondary goat anti-mouse antibody (BA-9200; Vector Laboratories, Burlingame, CA, USA). Slides were developed with the streptavidin–biotin complex–peroxidase reagent and the peroxidase substrate diaminobenzidine (DAB substrate kit (SK1400); Vector Laboratories) according to the manufacturer's instructions. Negative controls were performed by substitution of the primary antibody with the same concentration of the corresponding isotypic IgG control (R&D Systems). All sections were counterstained with haematoxylin. All stained sections were coded and examined in a blinded manner at the end of the study. All available sections from each patient were evaluated. Biopsy specimens were considered suitable for examination when there was $\geqslant$1.0 mm of basement membrane length with intact epithelium and $\geqslant$0.1 mm$^2$ of subepithelial area. At least three suitable biopsy sections were examined per subject.

Quantitative measurements of OPN-positive cells in the bronchial tissue were performed as previously described [14]. Briefly, OPN-positive and -negative cells were counted in each biopsy specimen separately in the intact epithelium and the submucosa. Bronchial epithelial cells positive for the OPN monoclonal antibody were expressed as a percentage of total epithelial cells, whereas positive subepithelial inflammatory cells were expressed as a 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 <8%.

### *RBM thickness and goblet cell number measurements*

Analysis of RBM thickness and subepithelial infiltration was performed on sections stained with haematoxylin–eosin as previously described [22]. Briefly, basement membrane thickness was measured in micrometers by performing measurements at 20-μm intervals along the whole length of the membrane, taking only the adequately preserved and oriented area into account. A semi-quantitative scoring system, ranging 0–3, 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 (395B-1KT; Sigma-Aldrich, Steinheim, Germany). The reaction of periodic acid selectively oxidises glucose residues and creates aldehydes that react with the Schiff reagent, staining carbohydrates in goblet cells a purple-magenta colour. Goblet cells were quantified as the number of positively staining cells per millimetre of basement membrane, as previously described [23]. Sections were observed using an Axioskop40 microscopy system (Carl Zeiss Jena, Jena, Germany) coupled with an AxioCam MRc digital camera, and basement membrane thickness measurements were performed using AxioVision v4.5.0.0 image analysis software.

### *Double immunofluorescence*

Double immunofluorescence staining was performed in order to identify the cellular source of OPN. Antigen retrieval was performed (S2368 and S2369; Dako, Glostrup, Denmark) and then sections were blocked for 30 min in 10% goat serum, followed by incubation with primary antibodies (diluted in Tris-buffered saline containing 1% BSA and 0.1% Triton X-100) for 1 h at room temperature. The primary antibodies that were used in appropriate combinations were as follows: mouse anti-human OPN (MAB1433; R&D Systems; 2 μg·mL$^{-1}$), rabbit anti-human OPN (O7264; Sigma-Aldrich; 5 μg·mL$^{-1}$), smooth muscle α-actin (α-SMA) (Ab5694; Abcam, Cambridge, UK; 1:100), T-lymphocytes (CD3) (M7254; Dako; 1:5), B-lymphocytes (CD19) (M7296; Dako; 1:5), mast cells (mast cell tryptase) (clone AA1 (M7052); Dako; 1:50), neutrophils (neutrophil elastase) (clone NP57 (M0752); Dako; 1:100) and eosinophils (EG2 (No. 10-9196-01); Kabi Pharmacia Diagnostics, Uppsala, Sweden; 1:50). The secondary antibodies used were the Alexa Fluor (AF) 555-labelled goat F(ab')$_2$ anti-mouse and the AF488-labelled goat F(ab')$_2$ fragment fluorescent anti-rabbit secondary monoclonal antibodies (A11070 and A21425, respectively; Molecular Probes; 5 μg·mL$^{-1}$; incubation for 40 min at room temperature). Antigen retrieval was not performed for the 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 (P36931; Molecular Probes, Eugene, OR, USA), which also contained 4′-6-diamidino-2-phenylindole (DAPI) as a nuclear counterstain, and were examined using an Axioplan2 microscope (Carl Zeiss Jena), with filters set for AF488 and AF555.

### *Confocal laser microscopy*

Specimens that showed double staining on fluorescence microscopy were further examined using 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). DAPI was excited by the 405-nm laser diode and the fluorescence was collected using a long-pass (LP) 420 emission filter; the green (AF488) antibody was excited by the 488-nm argon ion laser line and the fluorescence was collected using a band-pass (BP) 505–530 emission filter, and the red (AF555) antibody was excited by the 543-nm green helium–neon laser line and the fluorescence was collected using an LP560 emission filter. Tissue sections were visualised using the confocal microscope at 1,024×1,024-pixel resolution through a Plan-Neofluar 40×/1.3 oil differential interference contrast objective with eight-times averaging in sequential scanning (multi-track) mode with the pinhole set to obtain an optical section of ~1 μm in all channels.

### Statistical analysis

Data are expressed as median with interquartile range (first and third quartiles) unless specified otherwise. Normality was assessed using Kolmogorov–Smirnov and D'Agostino–Pearson omnibus normality tests. Two-group comparisons were performed using the nonparametric Mann–Whitney U-test, whereas three-group comparisons were performed using the nonparametric Kruskal–Wallis one-way ANOVA accompanied by Dunn's *post hoc* correction. Stability data were evaluated using the nonparametric Friedman test followed by Dunn's *post hoc* analysis. The nonparametric Wilcoxon matched-pair test was applied to evaluate OPN expression in the stable state and during exacerbation. Correlation coefficients were calculated using the nonparametric Spearman's rank method. A statistical software package was used for all data analysis and graph preparation (Prism v5; GraphPad, San Diego, CA, USA). A p-value of $<0.05$ was considered significant.

## RESULTS

### OPN in the serum of patients with asthma

Patients with asthma showed significantly increased serum OPN levels compared with healthy controls (47.92 (34.64–67.32) and 20.25 (22.51–24.92) ng·mL$^{-1}$, respectively; $p<0.0001$). A subgroup analysis revealed that both MMA (49.16 (36.85–69.84) ng·mL$^{-1}$; $p<0.001$) and SA patients (46.77 (33.38–62.03) ng·mL$^{-1}$; $p<0.01$) exhibited significantly higher serum OPN levels than healthy controls, although no significant difference was found between these two patient groups (fig. 2a). Patients with exacerbations demonstrated decreased serum OPN levels compared with those in the steady state (50.33 (35.65–58.87) and 58.97 (45.77–66.96) ng·mL$^{-1}$, respectively; $p=0.0332$) (fig. 2b). No difference was found regarding serum OPN levels in SA patients receiving oral steroids and those not receiving them (table 4). Finally, serum OPN levels did not differ in patients with asthma before and after 2 weeks of treatment with either prednisolone *p.o.* (52.82 (32.32–68.71) and 47.27 (25.69–72.35 serum, respectively; n=11) or placebo (53.08 (44.34–64.97) and 48.32 (37.52–56.78) ng·mL$^{-1}$, respectively; n=6; $p=0.8921$).

### OPN in the BALF of patients with asthma

OPN levels were significantly increased in the BALF of asthmatic patients (n=6) compared with healthy controls (n=17) (1.108 (0.918–1.464) and 0.746 (0.672–1.009) ng·mL$^{-1}$, respectively; $p=0.0129$) (fig. 2c). Although a subgroup analysis revealed elevated BALF OPN levels for both MMA (1.108 (0.958–1.824) ng·mL$^{-1}$; n=7) and SA patients (1.165 (0.889–1.419) ng·mL$^{-1}$; n=10), only MMA patients showed significantly higher levels of OPN than healthy controls ($p<0.05$). When BALF OPN levels were compared between SA patients receiving oral steroids and those not receiving them (fig. 2c), 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 significance ($p=0.058$, $r=0.4$).

### OPN in 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 (fig. 3b and d), whereas healthy controls either did not express OPN or demonstrated very low OPN expression by some bronchial epithelial cells (fig. 3a). 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 (fig. 4e–l), myofibroblasts (fig. 4m–p), T-lymphocytes (fig. 4q–t) and mast cells (fig. 4u–x) 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)%; $p<0.0001$) than healthy controls (5 (3.5–9.5) and 2 (0.5–2)%, respectively). Regarding epithelial expression of OPN, 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 (fig. 5a).

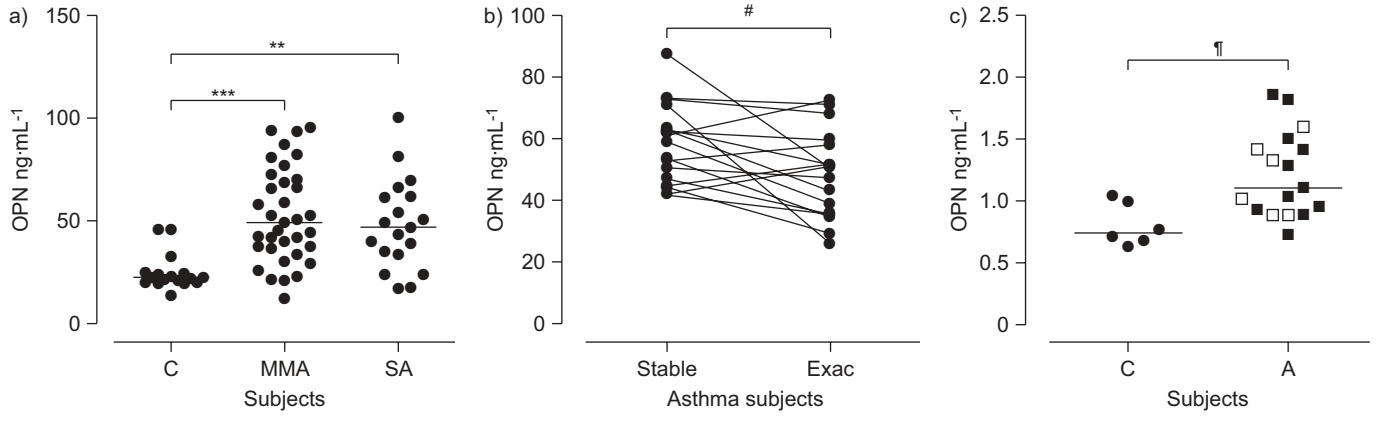


**FIGURE 2.** Osteopontin (OPN) levels in a, b) serum and c) bronchoalveolar lavage fluid (BALF) of asthmatic (A) subjects. Data are presented as medians. a) Serum OPN is increased in both mild-to-moderate asthma (MMA; n=35) and severe asthma (SA) patients (n=19) compared with healthy controls (C; n=17), with no difference found between the two patient groups. b) Asthmatic patients exhibiting exacerbation (exac) show decreased levels of serum OPN compared to those in the steady state. c) OPN in the BALF of asthmatic patients (n=17; □: asthmatic patients receiving steroids *p.o.*; ■: patients not receiving steroids *p.o.*) is increased compared with healthy controls (n=6). **: $p<0.01$; ***: $p<0.001$; #: $p=0.032$; ¶: $p=0.0129$.

**TABLE 4** Osteopontin (OPN) expression in the tissue, serum and bronchoalveolar lavage fluid (BALF) of severe asthmatics by corticosteroids use

| | Group A | Group B | p-value |
|---|---|---|---|
| **Patients (total/bronchoscopy) n** | 8/8 | 11/9 | |
| **OPN ng·mL$^{-1}$** | | | |
| Serum | 46.93 (27.9–65.71) | 46.77 (33.38–62.03) | 0.77 |
| BALF | 1.188 (0.895–1.466) | 1.087 (0.771–1.387) | 0.39 |
| **OPN positive cells % total** | | | |
| Epithelial | 47.0 (36.0–55.75) | 40.0 (28.5–51.5) | 0.41 |
| Subepithelial | 30.0 (20.5–36.95) | 29.0 (15.5–34.0) | 0.5 |

Data are presented as median (interquartile range), unless otherwise indicated. Patients in group A received corticosteroids *p.o.*; patients in group B did not.

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 than MMA ($p<0.05$) (fig. 5b). A significant correlation was found between epithelial and subepithelial OPN expression in all asthmatics ($r=0.83$, $p<0.0001$), whereas OPN expression in bronchial epithelial cells correlated weakly with BALF OPN levels ($r=0.46$, $p<0.0267$) (fig. 5c and 4d). Furthermore, OPN expression in the bronchial epithelium, and especially the subepithelium, correlated inversely with FEV1 ($p=0.0184$, $r=-0.4142$ and $p=0.0001$, $r=-0.6227$, respectively). Finally, the comparison between eight SA patients who were receiving steroids *p.o.* and nine who were not revealed no significant difference in OPN expression in either epithelial or subepithelial infiltrating cells (table 4).

### *RBM thickness and goblet cells in patients with asthma and RBM thickness correlation with OPN tissue expression*

RBM thickness was increased in patients with asthma compared to healthy controls (6.3 (5.35–7.9) and 4.8 (4.25–5.4) μm respectively; $p=0.0051$) (fig. 6a and b). Performing subgroup analysis demonstrated that the RBM was thicker in SA (7.5 (5.9–8.65) μm) than in MMA patients (5.35 (4.4–6.475) μm; $p<0.05$) and controls ($p<0.001$), whereas no significant difference was found between MMA and controls (fig. 6e). Furthermore, RBM thickness correlated with OPN expression in both the epithelium ($r=0.58$, $p=0.0001$) and subepithelium ($r=0.62$, $p<0.0001$) (fig. 6f and g). Finally, a weak but statistically significant inverse correlation was observed between RBM thickness and FEV1 ($p=0.0075$, $r=-0.46$).

Goblet cell numbers, although highly variable, were significantly increased in asthmatic patients (90 (72.5–117) cells·mm$^{-1}$) compared to healthy controls (68 (45–88) cells·mm$^{-1}$; $p=0.0234$) (fig. 6c and d). However, subgroup analysis revealed only a near-significant difference between the control, MMA (87 (68.25–116) cells·mm$^{-1}$) and SA (102 (78–117) cells·mm$^{-1}$) groups ($p=0.0506$). No correlation was found between the number of goblet cells and epithelial and/or subepithelial tissue OPN expression ($p=0.22$ and $p=0.1$, respectively).

## DISCUSSION

In the present study, we demonstrate that OPN is upregulated in bronchial tissue in asthma and show, for the first time, that subepithelial inflammatory cells in SA patients express significantly more OPN than in MMA patients, suggesting that OPN expression correlates with disease severity. Furthermore, we show that OPN expression correlates with RBM thickness, suggesting a role for OPN in asthma remodelling. We also demonstrate that OPN levels are increased in the serum of patients with asthma and that they decrease during exacerbations, and confirm that BALF OPN levels are elevated in asthma [12]. Finally, our findings reveal, for the first time, that cellular sources of OPN in the bronchial tissue in asthma include not only epithelial cells and macrophages but also OPN localisation 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 remodelling, remain 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. It has previously been shown that OPN has a protective role during secondary pulmonary antigenic challenge, and that therapeutic administration of recombinant OPN during secondary antigenic challenge 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 has previously been demonstrated [14, 15] and further shown in the present study is that OPN overexpression in chronic inflammation may also result in tissue remodelling.

RBM thickness is a widely used marker of airway remodelling [1], and, in the present study, the RBM was significantly thicker in SA patients than in either MMA patients or healthy controls, in accordance with previous results [22]. Interestingly, significant correlations were found between RBM thickness and bronchial tissue OPN expression, between FEV1 and RBM thickness, and between FEV1 and bronchial tissue OPN expression, suggesting that OPN is involved in remodelling and the resulting airway obstruction. Studies performed by our group have implicated OPN in fibrosis and remodelling in murine models of asthma [15]. Moreover, OPN expression has

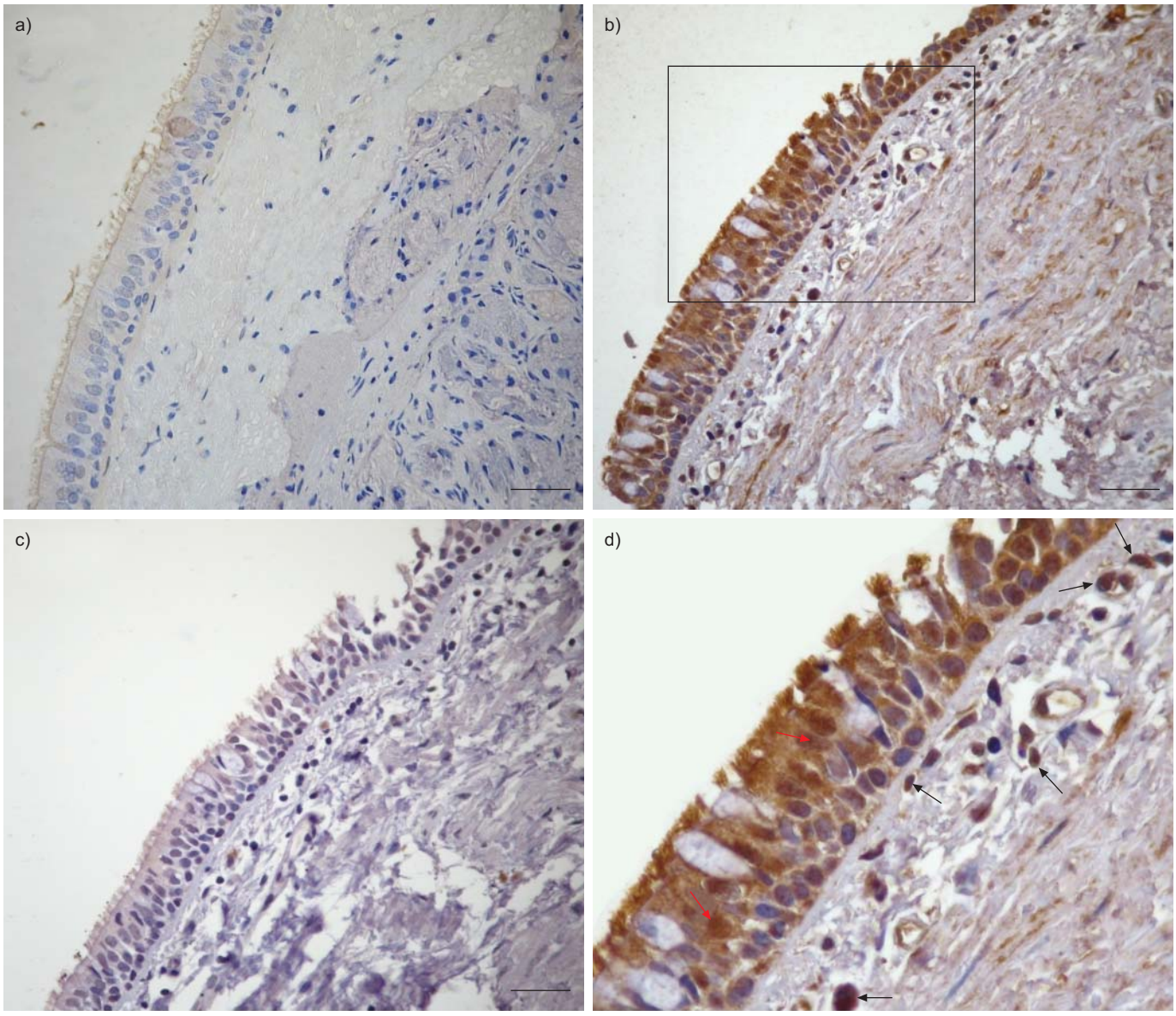


**FIGURE 3.** Expression of osteopontin (OPN) in the bronchial tissue of patients with asthma and healthy controls. Bronchial tissue obtained from healthy and asthmatic subjects who underwent fibreoptic bronchoscopy was processed immunohistochemically to show tissue OPN expression. Specific staining for OPN is brown, whereas nuclei are stained blue. Representative photomicrographs are presented from a) a healthy individual and b) a patient with severe asthma, and c) the corresponding isotypic control, performed on a consecutive section. d) The region selected in b) is presented at higher magnification. Red arrows indicate positively stained epithelial cells and black arrows point to positively stained subepithelial cells. Scale bars=20 μm.

recently been shown to be essential for the migration and differentiation of myofibroblasts [27], which are important contributors to RBM thickening. This is in accordance with the present 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 pathological characteristics of asthma and airway remodelling [28]. Using a murine model, it has previously been demonstrated that OPN is associated with increased mucus production [15]; however, in the present study, no significant correlation was found. 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 the steady state or during exacerbation have not previously been documented. Here, it is reported that serum OPN levels are increased in patients with asthma, possibly reflecting overspill from local infiltration and upregulation of OPN-producing cells in the bronchial tissue. However, although increased bronchial tissue OPN expression was found in SA, no differences were detected in the levels of OPN in either serum or BALF between MMA and SA patients. A hypothesis explaining this lack of difference could be that the higher

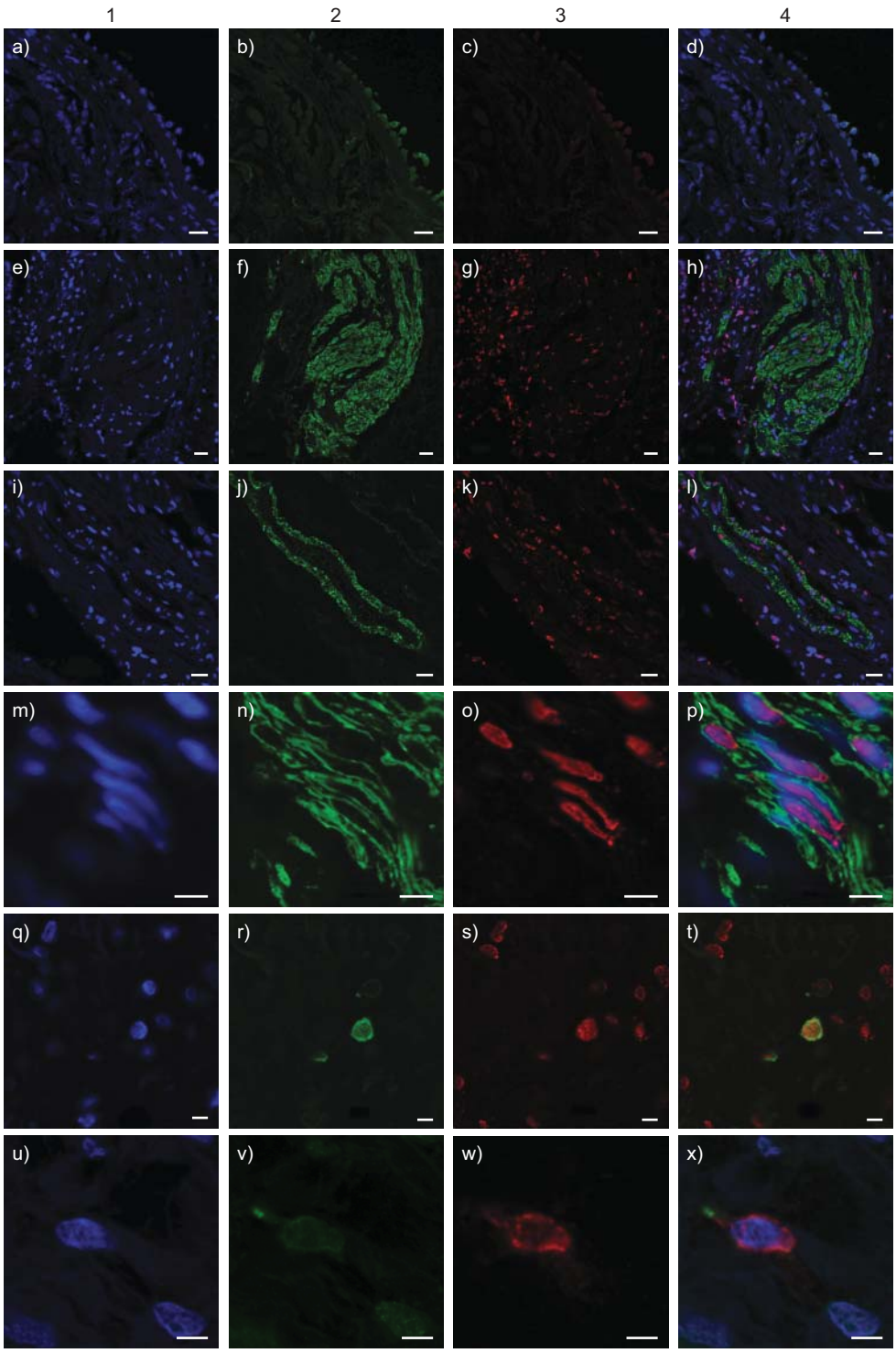


**FIGURE 4.** Identification of cellular sources of osteopontin (OPN) in the bronchial tissue of asthmatic patients using confocal laser microscopy. Double immunofluorescence staining was performed on formalin-fixed paraffin-embedded 3–4-μm tissue sections, obtained from patients with asthma, to identify the cellular source of OPN. Counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI) to visualise nuclear DNA. Column 1 shows DAPI nuclear counterstaining and columns 2 and 3 positive staining for primary antibodies; column 4 is the result of merging images from columns 1–3. Either a mouse monoclonal antibody with an Alexa Fluor (AF) 488-conjugated goat anti-mouse secondary antibody (g, k and o) or a rabbit polyclonal antibody with an AF555-conjugated goat anti-rabbit secondary antibody (s) were used to specifically stain intracellular or secreted OPN. Isotypic controls using mouse and rabbit anti-human immunoglolin G primary antibodies followed by AF488/AF555-conjugated goat anti-mouse and anti-rabbit secondary antibodies, respectively, showed no specific staining (a–d). Airway smooth muscle cells (green) stained with a rabbit monoclonal antibody directed against human smooth muscle α-actin (α-SMA) demonstrated positive staining for OPN (red) (e–h). Vascular smooth muscle cells, as well as some endothelial cells, express OPN (i–l). α-SMA-positive myofibroblasts (green) were also found to produce OPN (red) (m–p). Furthermore, T-lymphocytes positively stained with anti-CD3 monoclonal antibody (green) displayed positive staining for OPN (red) (q–t). Finally, mast cells positively stained for mast cell tryptase (red) demonstrated positive staining for OPN (green) (u–x). Scale bars: a–l) 20 μm; m–x) 5 μm.

bronchial OPN expression in SA occurs due to upregulation of the intracellular form of OPN. It was also found that serum OPN levels decrease during exacerbation; however, the reported significance is borderline and the number of cases is relatively small.

There is limited information regarding the cellular origin of OPN production in the bronchial airways of asthmatic patients. It has previously been reported that bronchial tissue macrophages from asthmatic patients can produce OPN [14], and, in the present study, it has been shown for the first time that OPN also localises 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 previously been demonstrated to induce proliferation of human bronchial smooth muscle *in vitro* [15].

There is currently no information regarding 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, no differences were found in serum, BALF and bronchial tissue OPN expression between SA patients on long-term oral steroids and those on inhaled corticosteroids alone. Furthermore, no differences were found 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 BALF OPN levels and lung tissue mRNA expression [34]. However, the amount of steroids used in this study (3 $mg \cdot kg^{-1}$ dexamethasone or 20 $mg \cdot kg^{-1}$ prednisolone) is ~200 times greater than the dose of oral prednisolone used in the present 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

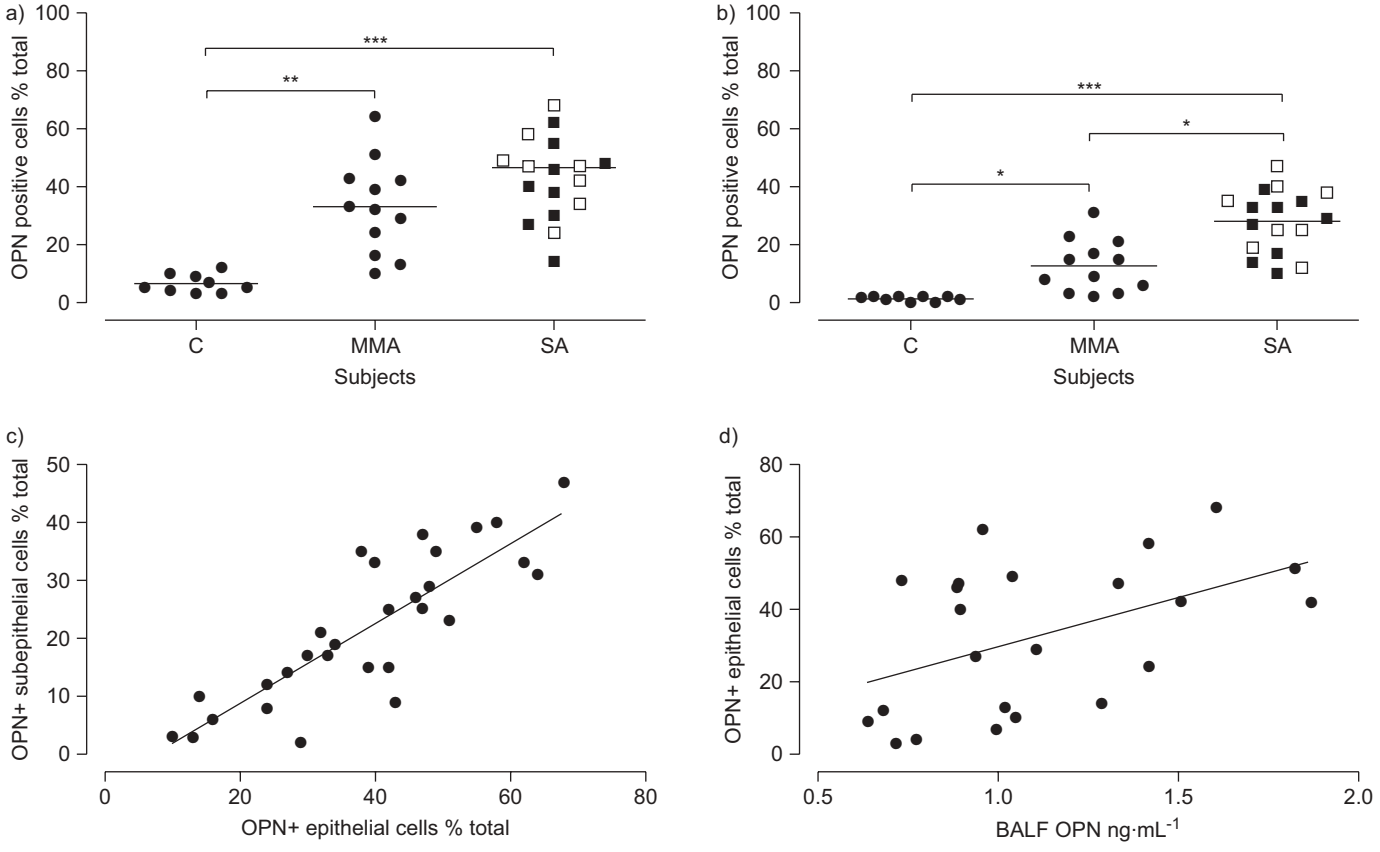


**FIGURE 5.** Osteopontin (OPN) is upregulated in the bronchial tissue of patients with asthma. Bronchial biopy specimens specifically stained for OPN in healthy controls (C, n=9) and patients with asthma (n=29) were further evaluated in order to quantify OPN tissue expression. OPN positive and negative cells were counted separately in the epithelium (expressed as a percentage of total epithelial cells) and submucosa (expressed as a percentage of total cells). Data are presented as medians. Both mild-to-moderate asthma (MMA) patients (n=12) and patients with severe asthma (SA) (n=17; □: asthmatic patients receiving steroids *p.o.*; ■: patients not receiving steroids *p.o.*) 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 than in MMA (b). A significant correlation was found between epithelial and subepithelial OPN expression in all asthmatics (c) ($r=0.83$; $p<0.0001$), whereas OPN expression in bronchial epithelial cells weakly correlated with OPN in bronchoalveolar lavage fluid (BALF) (d) ($r=0.46$, $p<0.0267$). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

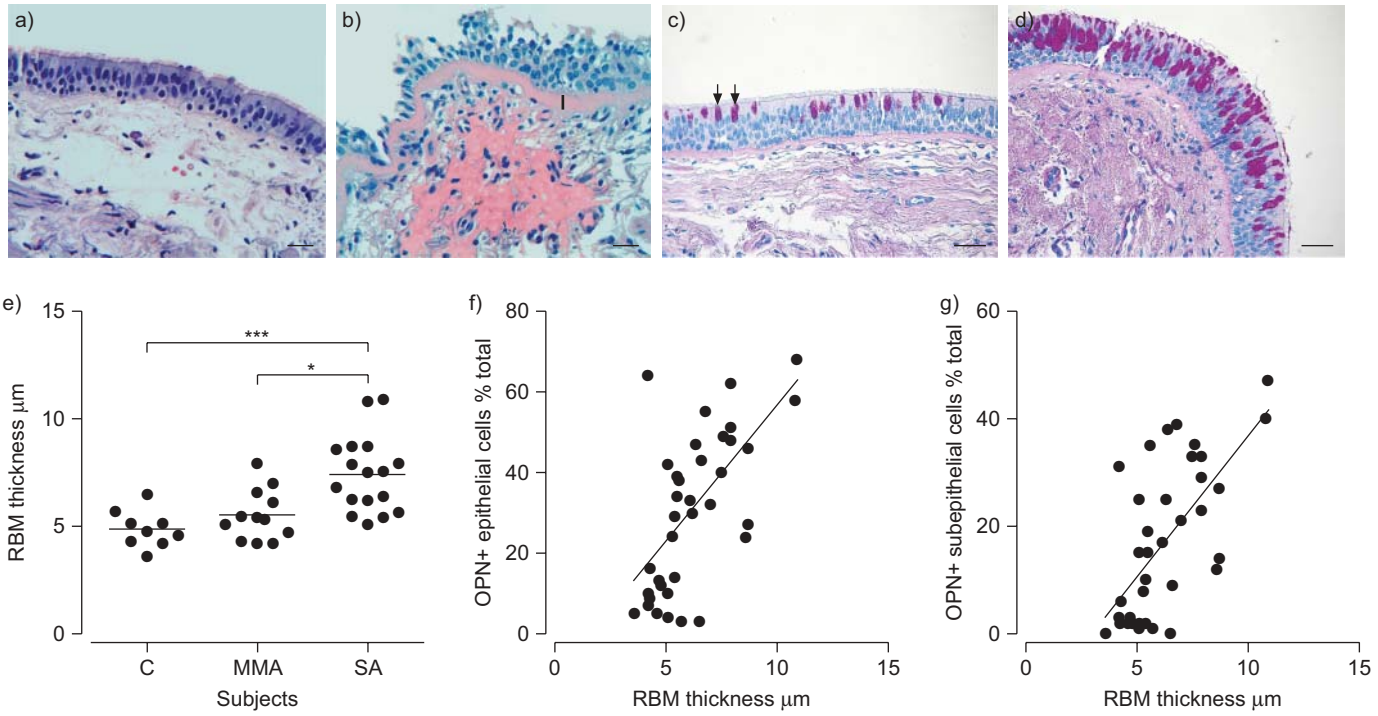


**FIGURE 6.** Reticular basement membrane (RBM) thickness and goblet cell number are increased in patients with asthma, and RBM thickness correlates with tissue osteopontin (OPN) expression. Bronchial tissue sections obtained from subjects who had undergone fibreoptic bronchoscopy were stained with haematoxylin–eosin. Data are presented as medians. Representative photomicrographs are depicted from a) a healthy individual and b) a patient with severe asthma (SA), showing a marked increase in membrane thickness. e) Patients with SA (n=17) showed increased RBM thickness compared to both mild-to moderate asthma (MMA) patients (n=12) and healthy controls (C; n=9). RBM thickness correlated with OPN expression in both the epithelium (f) (r=0.58, p<0.0001) and the subepithelium (g) (r=0.62, p<0.0001) of all subjects who had undergone bronchoscopy. Bronchial tissue sections from c) healthy controls and d) patients with asthma were also stained with periodic acid–Schiff to identify goblet cells (arrows) in the bronchial epithelium. Sections were counterstained with haematoxylin. Scale bars=20 μm. b) Vertical scale bar=12 μm. *: p<0.05; ***: p<0.001.

expression reported in this experimental study are of clinical relevance.

The current 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 low, 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 a subgroup analysis was performed and no differences found in OPN expression with respect to steroid treatment, possible bias introduced by asthma medication cannot be excluded.

Interest regarding OPN has grown remarkably since the late 1990s, during which time OPN has progressed 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 the present study, we demonstrate upregulation of OPN expression in human asthma that is associated with remodelling changes and correlates with 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 *in situ*.

## SUPPORT STATEMENT

K. Samitas was funded by a European Academy of Allergy and Clinical Immunology (Zurich, Switzerland) fellowship. G. Xanthou and V. Panoutsakopoulou are supported by the Hellenic Ministries of Health (Athens, Greece) and Education (Marousi, Greece), and V. Panoutsakopoulou by a grant award (03ED750) from the General Secretariat for Research and Technology of the Hellenic Ministry of Development (Athens, Greece). A. Bossios and J. Lötvall are funded by the VBG GROUP Centre for Asthma and Allergy Research (Vänersborg, Sweden) and the Swedish Heart and Lung Foundation (Stockholm, Sweden; project No. 20070575).

## STATEMENT OF INTEREST

Statements of interest for M. Gaga, K. Samitas and E. Zervas and the study itself can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

## ACKNOWLEDGEMENTS

The authors are grateful to all of the patients and healthy volunteers for their cooperation in the present study. The authors would also like to thank C. Malmhäll (Krefting Research Centre, Gothenburg, Sweden) for comments and advice on experiments and K. Papadopoulou (Pathology Dept, Athens Chest Hospital, Athens, Greece) for valuable

technical assistance with the immunohistochemical procedures performed in this study. Finally, the authors also acknowledge the Centre for Cellular Imaging at the Sahlgrenska Academy (University of Gothenburg, Gothenburg, Sweden), and J. Fernandez-Rodriguez (Centre for Cellular Imaging) personally for comments and expert technical assistance with the confocal imaging equipment.

## REFERENCES

1 James AL, Wenzel S. Clinical relevance of airway remodelling in airway diseases. *Eur Respir J* 2007; 30: 134–155.
2 Butler WT. Structural and functional domains of osteopontin. *Ann N Y Acad Sci* 1995; 760: 6–11.
3 Weber GF, Ashkar S, Glimcher MJ, *et al.* Receptor–ligand interaction between CD44 and osteopontin (Eta-1). *Science* 1996; 271: 509–512.
4 Shinohara ML, Kim HJ, Kim JH, *et al.* Alternative translation of osteopontin generates intracellular and secreted isoforms that mediate distinct biological activities in dendritic cells. *Proc Natl Acad Sci USA* 2008; 105: 7235–7239.
5 Koh A, da Silva AP, Bansal AK, *et al.* Role of osteopontin in neutrophil function. *Immunology* 2007; 122: 466–475.
6 Patarca R, Freeman GJ, Singh RP, *et al.* Structural and functional studies of the early T lymphocyte activation 1 (Eta-1) gene. Definition of a novel T cell-dependent response associated with genetic resistance to bacterial infection. *J Exp Med* 1989; 170: 145–161.
7 Ashkar S, Weber GF, Panoutsakopoulou V, *et al.* Eta-1 (osteopontin): an early component of type-1 (cell-mediated) immunity. *Science* 2000; 287: 860–864.
8 O'Regan A. The role of osteopontin in lung disease. *Cytokine Growth Factor Rev* 2003; 14: 479–488.
9 Pardo A, Gibson K, Cisneros J, *et al.* Up-regulation and profibrotic role of osteopontin in human idiopathic pulmonary fibrosis. *PLoS Med* 2005; 2: e251.
10 Lu X, Zhang XH, Wang H, *et al.* Expression of osteopontin in chronic rhinosinusitis with and without nasal polyps. *Allergy* 2009; 64: 104–111.
11 Konno S, Golden DB, Schroeder J, *et al.* Increased expression of osteopontin is associated with long-term bee venom immunotherapy. *J Allergy Clin Immunol* 2005; 115: 1063–1067.
12 Puxeddu I, Berkman N, Ribatti D, *et al.* Osteopontin is expressed and functional in human eosinophils. *Allergy* 2010; 65: 168–174.
13 Takahashi A, Kurokawa M, Konno S, *et al.* Osteopontin is involved in migration of eosinophils in asthma. *Clin Exp Allergy* 2009; 39: 1152–1159.
14 Xanthou G, Alissafi T, Semitekolou M, *et al.* Osteopontin has a crucial role in allergic airway disease through regulation of dendritic cell subsets. *Nat Med* 2007; 13: 570–578.
15 Simoes DC, Xanthou G, Petrochilou K, *et al.* Osteopontin deficiency protects against airway remodeling and hyperresponsiveness in chronic asthma. *Am J Respir Crit Care Med* 2009; 179: 894–902.
16 Global Initiative for Asthma (GINA). Global Strategy for Asthma Management and Prevention. NHLBI/WHO workshop Report. Updated 2009. New York, Medical Communications Resources, Inc., 2010.
17 Reddel HK, Taylor DR, Bateman ED, *et al.* An official American Thoracic Society/European Respiratory Society statement: asthma control and exacerbations: standardizing endpoints for clinical asthma trials and clinical practice. *Am J Respir Crit Care Med* 2009; 180: 59–99.
18 Johansson SG, Bieber T, Dahl R, *et al.* Revised nomenclature for allergy for global use: report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol* 2004; 113: 832–836.
19 Samitas K, Chorianopoulos D, Vittorakis S, *et al.* Exhaled cysteinyl-leukotrienes and 8-isoprostane in patients with asthma and their relation to clinical severity. *Respir Med* 2009; 103: 750–756.
20 Jeffery P, Holgate S, Wenzel S. Methods for the assessment of endobronchial biopsies in clinical research: application to studies of pathogenesis and the effects of treatment. *Am J Respir Crit Care Med* 2003; 168: S1–S17.
21 Haslam PL, Baughman RP. Report of ERS Task Force: guidelines for measurement of acellular components and standardization of BAL. *Eur Respir J* 1999; 14: 245–248.
22 Bourdin A, Neveu D, Vachier I, *et al.* Specificity of basement membrane thickening in severe asthma. *J Allergy Clin Immunol* 2007; 119: 1367–1374.
23 Saetta M, Turato G, Baraldo S, *et al.* Goblet cell hyperplasia and epithelial inflammation in peripheral airways of smokers with both symptoms of chronic bronchitis and chronic airflow limitation. *Am J Respir Crit Care Med* 2000; 161: 1016–1021.
24 Andre E, Stoeger T, Takenaka S, *et al.* Inhalation of ultrafine carbon particles triggers biphasic pro-inflammatory response in the mouse lung. *Eur Respir J* 2006; 28: 275–285.
25 Nagasaka A, Matsue H, Matsushima H, *et al.* Osteopontin is produced by mast cells and affects IgE-mediated degranulation and migration of mast cells. *Eur J Immunol* 2008; 38: 489–499.
26 Sodek J, Batista Da Silva AP, Zohar R. Osteopontin and mucosal protection. *J Dent Res* 2006; 85: 404–415.
27 Lenga Y, Koh A, Perera AS, *et al.* Osteopontin expression is required for myofibroblast differentiation. *Circ Res* 2008; 102: 319–327.
28 Fahy JV. Remodeling of the airway epithelium in asthma. *Am J Respir Crit Care Med* 2001; 164: S46–S51.
29 Chen Y, Nickola TJ, DiFronzo NL, *et al.* Dexamethasone-mediated repression of MUC5AC gene expression in human lung epithelial cells. *Am J Respir Cell Mol Biol* 2006; 34: 338–347.
30 Hansel NN, Cheadle C, Diette GB, *et al.* Analysis of CD4+ T-cell gene expression in allergic subjects using two different microarray platforms. *Allergy* 2008; 63: 366–369.
31 Kaarteenaho-Wiik R, Paakko P, Sormunen R. Ultrastructural features of lung fibroblast differentiation into myofibroblasts. *Ultrastruct Pathol* 2009; 33: 6–15.
32 Chen J, Thomas HF, Sodek J. Regulation of bone sialoprotein and osteopontin mRNA expression by dexamethasone and 1,25-dihydroxyvitamin $D_3$ in rat bone organ cultures. *Connect Tissue Res* 1996; 34: 41–51.
33 Kirton JP, Wilkinson FL, Canfield AE, *et al.* Dexamethasone downregulates calcification-inhibitor molecules and accelerates osteogenic differentiation of vascular pericytes: implications for vascular calcification. *Circ Res* 2006; 98: 1264–1272.
34 Kurokawa M, Konno S, Matsukura S, *et al.* Effects of corticosteroids on osteopontin expression in a murine model of allergic asthma. *Int Arch Allergy Immunol* 2009; 149: Suppl. 1, 7–13.