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Title page:

Severe persistent allergic rhinitis: inflammation but no histologic features of structural upper airway remodeling.

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At a Glance Commentary:

Scientific Knowledge on the Subject:

Rhinitis and asthma are common clinical presentations of allergic airway disease and manifest Th2-mediated inflammation. Whereas airway remodeling is considered to be an important feature of asthma, little is known about the extent of tissue remodeling in nasal mucosa in allergic rhinitis.

What This Study Adds to the Field

Despite the presence of allergic inflammation in persistent rhinitis, there is no upper airway structural remodeling compared to healthy subjects.

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Abstract

Rationale: Increases in airway-smooth-muscle, extracellular-matrix, and vascularity are prominent features of airway-remodeling in asthma whereas the extent of such remodeling in persistent allergic rhinitics (PAR) patients is unknown. We aimed to test the hypothesis that upper-airway remodeling is a feature of PAR.

Methods: Total nasal symptom scores, nasal biopsies and Th1 and Th2 cytokines from nasal lavage were assessed in subjects with severe PAR (n=46) and healthy controls (n=19). Angio-lymphangiogenesis was examined using immunohistochemistry staining against CD31 (Vascular endothelial cells), VEGF-A and D2-40 (Lymphatic endothelial cells). Collagen and extracellular matrix proteins such as HSP-47 (markers of collagen synthesis), MMP7-9 and TIMP1, and α-smooth–muscle-actin (myofibroblasts) were evaluated as markers of activation of upper airway remodeling using image analysis, together with reticular basement membrane (RBM) thickness, mucus gland area, collagen area and sub-mucosal effector inflammatory cells.

Results: Total nasal symptoms scores, visual analogue scale and total quality of life were significantly higher in PAR compared to healthy controls ($p<0.0001$). Nasal lavage cytokine levels of IL-4 ($p<0.01$), IL-5, and IL-13 ($p<0.001$, respectively) were significantly higher in PAR compared to healthy controls. In addition there was an increase in sub-mucosal eosinophils ($p=0.06$). No statistical difference in terms of angiogenesis, lymphangiogenesis, deposition of extracellular matrix, collagen markers, RBM thickness or glandular percentage area was observed between PAR and healthy controls.

Conclusion: Our data suggest that tissue remodeling is not a feature of PAR and argues that in contrast to asthma, targeting remodeling in allergic rhinitis may not be appropriate as a therapeutic approach.

Key words: Collagen, allergen, MMP, lymphangiogenesis, angiogenesis, fibroblasts, allergic rhinitis.

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Introduction

Tissue remodeling is by definition the reorganization or renovation of existing tissues. This is a process which is required to maintain normal tissue homeostasis and repair damaged tissue. Remodeling occurs in all inflammatory responses although the mechanism and severity vary depending on the disease (1,2).

Allergic asthma is a chronic inflammatory disease involving ongoing inflammation and repair leading to structural airway remodeling. Although multiple mediators and growth factors such as Th2 cytokines, TGF-beta, MMP-9, ADAM-33 and VEGF have been considered to influence the airway remodeling process, currently there are no reliable clinical characteristics or surrogate markers that could identify lower airways remodeling (3). Structural changes that are implicated in lower airways remodeling such as epithelial shedding, thickening of basement membrane, angiogenesis, smooth muscle hypertrophy and deposition of collagens with subepithelial fibrosis are the features which are considered to contribute to airway obstruction and bronchial hyperreactivity (4-6).

In allergic rhinitis, remodeling has received scarce interest and is not well understood. There are very few studies that investigate upper-airway structural remodeling in the nasal mucosa of allergic rhinitis and data is conflicting. It was reported that perennial allergic rhinitics had increased vascularity in nasal mucosa compared to healthy controls (7), while another study examined lower and upper airways of perennial allergic rhinitis patients with and without asthma and compared them with healthy controls (8). In this study, it was demonstrated that even though similar inflammatory changes were present in both lower and upper airways of allergic rhinitis with and without asthma, no evidence of structural changes such as reticular basement thickness, epithelial desquamation and vascularity; was found in upper airways in allergic rhinitis as compared to healthy controls.

We aimed to test the hypothesis that airway remodeling is a feature of allergic rhinitis and that it's being driven by Th2 inflammatory responses in nasal mucosa. Nasal lavage and nasal mucosal biopsies were taken from severe persistent allergic rhinitis patients (PAR) and healthy controls. Local tissue inflammation, remodeling markers and tissue structure were evaluated. These studies have been previously been reported in part as an abstract (9).

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Methods:

Detailed methods and study design are described in the online supplement.

Study Subjects

Subjects aged 18-55 years with a clinical history of moderate-severe persistent allergic rhinitis according to ARIA (10) with a history of seasonal allergic rhinoconjuctivitis (May-July) and/or perennial rhinitis to perennial allergens such as house dust mite with duration of at least two years or more requiring treatment were included. The study was approved by the ethics committee of The Royal Brompton and Harefield Hospitals NHS Trust and was performed with the subjects' written informed consent (*See supplement*).

Nasal Lavage procedure and measurement of nasal lavage cytokine levels

Details of nasal lavage procedure and nasal lavage cytokine processing are in the online supplement. Briefly, for the electrochemiluminescence studies, the levels of IFN-γ, IL-4, IL-5, IL-10, and IL-13 in the nasal lavage samples were analyzed in duplicates using the Human Th1/Th2 10-plex Ultra-Sensitive kit from Meso Scale Discovery (MSD, Gaithersburg, MD). Assays were conducted according to standard manufacturer's protocols. MSD plates were analyzed on the Sector Imager 6000 using MSD Discovery workbench software (v 3.0.17.3).

Nasal Biopsy, immunohistochemistry staining and quantification

Details of nasal biopsy processing are in the online supplement. Briefly, nasal biopsy specimens (2.5 mm) were taken from the under surface of the inferior turbinate using Gerritsma forceps. Immunohistochemistry was performed using the avidin biotin complex-alkaline phosphatase (ABC-AP) method as described previously (11) (*see supplement*).

Biopsy sections were stained using monoclonal antibodies against eosinophils (MBP), mast cells (AAI) and basophils (2D7). Activation of upper airway remodeling was assessed by

staining vascular (CD31) and lymphatic endothelial (D2-40) cells, and vascular endothelial growth factor-A (VEGF-A). Furthermore, extracellular matrix activation was evaluated by quantification of fibroblasts, alpha-SMA, MMP-7, MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1). Epithelial integrity and RBM were assessed using haematoxylin stained slides. Epithelial goblet cells and the glandular mucus area were evaluated using PAS staining. The collagen content was assessed by picrosirius red staining (12). Biopsies were coded and evaluated blindly by three investigators without the knowledge of clinical characteristics.

Nasal mucosal inflammatory cells, were quantified using a BH-2 Olympus microscope. Epithelial integrity was scored on 0-4 scale, with 4 being an intact epithelium. Vascular and lymphatic staining was assessed using computer assisted image analysis (Image Pro-Plus 7.0 system, Media Cybernetics, Inc). The whole specimen was captured at x200 magnification, using Nikon 80*i* light microscope coupled to colour camera (QImaging, Media Cybernetics, Inc) that transmits the image to a computer. Epithelial and sub-mucosal mucus glands, total sub-mucosal and vascular area were measured separately using computer image analysis. Reticular basement membrane thickness was measured as proposed by Sullivan et al (13). Epithelial and sub-mucosa cell counts were expressed as the number of positively stained nucleated cells per mm^2.

The total numbers of $CD31^+$ and $D2\text{-}40^+$cells in sub-mucosa were counted to a depth of 200 μm below the epithelium. For each sample the percentage vascular area, the mean vessel size (mm^2), and the vessel number (per mm^2) within the total area of assessed sub-mucosa were evaluated. Percentage vascular area was calculated as [(total vascular area/sub-mucosal area) x 100]; number of blood vessels/mm^2 (vessels density) as (total number of blood vessels/ total sub-mucosal area); and mean vessel size/mm^2 (total vascular area/total number of blood

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vessels). Furthermore, the relative lymphatic vessel density was studied by calculating the ratio of D2-40/CD31 positive vessels/mm^2 (*see supplement*).

Picrosirius stained collagens were quantified as previously described (12). Briefly, color images of two sections from each biopsy were acquired at x200 magnification (with a polarizing lens for picrosirius red–stained sections; Leica QWin version 3). Subsequently, images were converted to grayscale, and the area of positive staining that includes reticular basement membrane and whole of sub-mucosa was determined at a preset pixel intensity by using the Leica QWin software. The mean area of collagen and total sub-mucosal area was used to calculate the percentage area of collagen in each nasal biopsy.

Statistics

Values are presented as median [IQ 25-75 range], unless otherwise specified. Comparisons for quantitative variables were performed by non-parametrical analysis, Mann–Whitney *U* test for non-related samples. Differences between three groups were assessed using Kruskal-Wallis test (nonnormal distribution) followed by Dunn's multiple comparisons test. Within-group comparisons were performed using the Wilcoxon matched-pairs signed-rank test. Interobserver reproducibility was measured using Bland-Altman test, acceptable results were within 2 standard deviation. All tests were two-tailed. Significance was set at $P<0.05$. GraphPad Prism 5 Project software was used for analysis (*see supplements statistics*).

Results

Subjects

The demographic characteristics of the participants are summarised in Table 1 (*see supplement*). Among 46 persistent allergic rhinitics (PAR) subjects evaluated during the grass pollen season, 23 had a history of severe seasonal allergic rhinitis symptoms sensitised to Timothy grass and the other 23 had moderate-severe perennial symptoms and were sensitized to house dust mite *(see supplement Table 2*). Nineteen subjects were non-atopic healthy controls. Thirty one percent of PAR had current eczema or asthma (Table 1).

Nasal symptoms, Visual analogue and Total Quality of Life scores

Total nasal symptoms scores were statistically significantly higher in PAR groups compared to healthy controls (median [IQR]; 6 [4-9] vs 0 [0-1] respectively, $p<0.0001$). Total visual analogue score was significantly higher in PAR compared to controls (45 [23-67] vs 1 [0-7], $p<0.0001$) and the total quality of life scores were higher in PAR compared to controls (2 [1-3.4] vs 0 [0-0.2], $p<0.0001$) (Table 1). Furthermore, on comparing subgroup analysis based on sensitization, both seasonal and perennial AR had significantly higher total nasal symptoms, visual analogue and total quality of life scores compared to healthy controls (*see supplement Table 2*).

Nasal lavage cytokines in PAR and healthy controls

Nasal lavage levels of IL-4, IL-5 and IL-13 were significantly higher in PAR patients compared to healthy controls (IL-4: 0.4 [0.2-0.6] vs 0.2 [0.1-0.3] $p=0.01$; IL5: 7 [5-12] vs 3.3 [2.4-4.5] $p<0.001$; and IL-13: 7 [5-10] vs 5 [3-7] $p<0.001$, respectively) (Fig 1). Subgroup analysis showed IL-4, IL-5 and IL-13 were significantly higher in seasonal allergic rhinitis compared to healthy controls ($p=0.02$, $p<0.001$, and $p<0.0001$, respectively), while perennial

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allergic rhinitis had higher nasal lavage IL-4 and IL-5 levels compared to healthy controls ($p=0.04$ and $p<0.01$, respectively). However, there was no difference in the levels of IL-10 and IFN-γ between PAR and healthy controls or on subgroup analysis (data not shown).

Nasal mucosal inflammatory cells

The numbers of MBP^+ eosinophil were increased in the PAR group compared to controls, $p=0.06$, Fig 1 (for seasonal allergic rhinitis increases in eosinophils were significant, $p=0.04$). No changes in either mast cell or basophil numbers were observed in PAR group compared to healthy controls or when comparing subgroups and healthy controls (data not shown).

Analysis of Epithelium, Reticular basement membrane and Glands

The epithelial integrity and RBM thickness showed no difference between PAR and healthy controls (Table 2). Furthermore, total epithelial mucus and sub-mucosal glandular percentage area were similar in both groups (Fig 2 A & B, Table 2). On subgroup analysis according to sensitization, no differences were observed in terms of epithelial integrity, RBM thickness or glandular percentage area in the epithelium or sub-mucosa between seasonal and perennial allergic rhinitis and healthy controls. Additionally, no changes where observed when compared between in season and out of season (*supplement table 3*).

Changes in Vascularity in Nasal sub-mucosa (blood and lymphatic vessels)

There was no significant difference in the numbers of blood vascular endothelial cells ($CD31^+$ cells), lymphatic endothelial cells ($D2\text{-}40^+$ cells) or VEGF-A stained cells between PAR and healthy controls. Total numbers of blood and lymphatic vessels, percentage vascular area and mean vascular size in the nasal sub-mucosa showed no difference between the two groups (Fig 2 C-F, Table 2). There was no significant difference when analysing the numbers of VEGF-A positive blood vessels, $VEGF\text{-}A^+$ vascular area or mean $VEGF\text{-}A^+$

vascular size between the groups. The relative lymphatic vessel density was analysed by determining the ratio of D2-40 / CD31 positive vessels/mm^2, which showed no statistical difference between the two groups (Table 2).

Furthermore, on subgroup analysis no differences were observed when comparing seasonal, perennial and healthy controls in terms of blood and lymphatic endothelial positive cells, total vascular numbers, vascular area or mean vascular size for both blood and lymphatic vessels. There was also no difference in lymphatic vessel density on subgroup analysis (*supplement table 3*).

Sub-mucosal Extracellular and Collagen Markers

Expression of sub-mucosal MMP-7, MMP-9 and TIMP-1 were not significantly different between PAR and healthy controls (Fig 3 A-F, Table 2). The ratio of MMP9/TIMP-1 was not significantly different between the two groups. No difference was observed when comparing between seasonal, perennial and healthy controls for all analysed extracellular matrix protein markers. On comparing seasonal allergic rhinitis subjects during and out of season, a significant increase in TIMP-1 was observed in out of season biopsies compared to during pollen season, while no changes were observed for MMP-7 and MMP-9 when seasonal allergic subjects were compared in season and out of season (*supplement table 3*).

The expression of nasal mucosal fibroblasts (Fig 4 A,B) and α-SMA cell numbers (Fig 4 C,D) were not significantly different between PAR and healthy controls (Table 2). No difference was observed when comparing seasonal, perennial and healthy controls for all analysed fibrotic markers. Furthermore, no changes were observed when seasonal allergic subjects were compared in season and out of season (*supplement table 3*). When analysing collagen content in nasal mucosa using polarised light, both larger collagen fibers in bright

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orange and thinner fibers in green were present in nasal sub-mucosa of all subjects. There was no significant difference in collagen area between PAR and healthy controls (Fig 4 E,F).

Discussion

The current study demonstrated that persistent allergic rhinitis is characterised by increased levels of local nasal secretion of IL-4, IL-5 and IL-13 Th2 cytokines and local tissue eosinophilia but no structural changes nor evidence of remodelling. Epithelial integrity, reticular basement membrane thickness and the nasal mucosal glandular area were not different between PAR and controls. Furthermore, there was no difference in blood and lymphatic vascularity, fibrosis or collagen deposition nor degradation of extracellular matrix markers observed between the groups. In contrast to asthma, remodeling is not a feature of allergic rhinitis. This questions the current dogma on the relation between the upper and lower airways and argues that in contrast to asthma, targeting remodeling in allergic rhinitis may not be appropriate as a therapeutic approach.

To our knowledge, this is the first study to compile and evaluate the expression of remodeling-related proteins in the upper-airways of allergic rhinitis patients looking both in nasal lavage and nasal biopsies in one setting. The current study included adequate sample size and used strict inclusion criteria to ensure that a well characterised group of subjects with moderate to severe persistent allergic rhinitis according to Aria guidelines could be observed alongside a group of normal healthy controls.

Lower airway remodeling is considered to occur due to chronic inflammation and eosinophils are thought to be the cells that orchestrate the production of cytokines, growth factors and mediators leading to proliferation and differentiation of fibrotic cells that leads to collagen deposition and tissue fibrosis (14-16). The current study found an increase in Th2 cytokines within nasal secretions and sub-mucosal eosinophils in persistent allergic rhinitis confirming a local intense inflammation. This corresponds to our previous finding that both seasonal (17, 18) and perennial allergic rhinitis (19) are associated with an increase in tissue eosinophils and with an increase in nasal mucosal cells expressing mRNA for the Th2-type

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cytokines compared to healthy controls. This is in line with previous studies that compared nasal and bronchial tissue with eosinophilia in persistent allergic rhinitis and found that the upper airway has similar intense inflammation compared to lower airways in the same individuals (8, 20) but that the chronic inflammation didn't alter upper airway mucosal structural changes (21). Hence, the present study confirms that although there is allergic inflammation in the upper airways of PAR subjects, remodeling changes such as epithelial integrity loss, RBM thickness, nasal tissue glandular hypertrophy, vascularity and collagen or extracellular matrix deposition are indistinguishable between PAR and healthy controls.

It has been well documented that angiogenesis is a characteristic feature of remodeling in asthma (22,23). Although increased vascular permeability is observed in allergic rhinitis (24), evidence showing angiogenesis or gross structural vascular changes are lacking. Our data confirms previously published reports (8,25) showing no difference in the total number of $CD31^+$ cells, blood vessels, vascular density nor mean vascular size in the nasal mucosa of allergic rhinitics compared to controls. Furthermore, on evaluating angiogenic growth factors, there were no changes in the upregulation of VEGF-A expressing cells or their stained vessels in the nasal mucosa observed between the groups. In addition, using D2-40 staining which is considered a sensitive and specific marker for lymphatic endothelium (26), no difference in the numbers, density or mean volume of lymphatic vessels was observed between the two groups. Taken all together, our study does not indicate that angiogenesis or lymphangiogenesis is the feature of upper airway remodeling in allergic rhinitics.

Matrix metalloproteinases are major proteolytic enzymes that are involved in extracellular matrix turnover. We examined MMP-7 and MMP-9 because they are believed to cleave major collagen components of extracellular matrix and basement membrane. The extracellular activity of MMPs is regulated by TIMPs, which are produced by the same cell types that produce MMPs, and TIMP-1 has been implicated as a natural inhibitor of both

MMP-7 and 9 (27-28). It has been shown that MMP7 and 9 is upregulated in chronic rhino-sinusitis with or without polyps (29) and the ratio of MMP-9/TIMP-1 is increased in asthmatics (30-31). Recently it has been demonstrated that only MMP-9 but not MMP-2 or TIMP-1 mRNA expression is increased in nasal mucosa of severe perennial allergic rhinitis compared to mild rhinitics and healthy controls (32). We had anticipated that patients with PAR would have increased expression of MMPs and an imbalance of TIMP. However, in the present study we did not find any difference in MMP-7, MMP-9 or TIMP-1 between PAR and healthy controls. This is in line with previous reports that found no significant difference in both protein and mRNA transcript levels of MMP-9 or TIMP-1 in the nasal mucosa of perennial allergic rhinitis compared to healthy controls (33).

Subepithelial fibrosis is an important feature of airway remodeling in asthma which is attributable to an increased deposition of extracellular matrix (34). Fibroblasts and myo-fibroblasts are the main sources of extracellular matrix proteins in the airways subepithelial fibrosis with TGF-beta being the main player that promotes differentiation of fibroblasts to myo-fibroblasts phenotypes (those expressing α -SMA) triggering their proliferation (31, 35, 36). We found a similar degree of expression of HSP-47^{+} fibroblasts cells and α -SMA^{+} cells in both PAR and healthy controls. In addition, total collagen area was the same in both PAR and healthy control nasal mucosa. Thickening of RBM due to deposition of collagens is a common feature of asthmatic airway remodeling, however the present study demonstrated no difference in changes in nasal mucosal subepithelial basement membrane between healthy and PAR patients confirming that there is no upper-airway structural difference between allergic rhinitics compared to healthy individuals. Recently, it has been shown in an *in-vitro* study that TGF-beta enhances expression of both α-SMA and collagen production in lung fibroblasts but not in nasal polyp fibroblasts highlighting the heterogeneous response of fibroblasts in the airway to TGF-beta between the upper and lower airway tissues (37).

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Recent studies have suggested that structural airway changes in asthmatics can occur after resolution of or in the absence of inflammation due to the induction of epithelial stress by airway hyperreactivity which initiates bronchial airway remodeling (38,39). Hence supporting the emerging concept that the epithelial-mesenchymal trophic unite (EMTU), the embryologic unit driving airway development, is being reactivated in airway remodeling through genetic and environmental interactions. This concept suggests that activated airway epithelial cells promote mesenchymal signalling inducing myofibroblast transformation which initiates wound healing in asthmatics (40).

A recent study (41) examined sinonasal epithelial markers and lineage differentiation in participants with chronic rhinosinusitis (CRS) with and without polyps, allergic rhinitis and healthy controls. In CRS (with and without polyps) but not in allergic rhinitis, a significant decrease was observed in the expression of proteins associated with adherens and tight junctions, and an increase in intracellular vimentin filaments that correlated with subepithelial fibrosis and disease severity. These findings imply that alterations in the EMTU occur in CRS but not in allergic rhinitis or healthy controls. Interestingly, CRS with and without polyps differ in immunopathological mechanisms, hence indicating that EMTU and remodeling might occur independently of inflammatory or immune changes. This study adds weight to our findings that Th2 inflammation as occurs in allergic rhinitis might not contribute to tissue remodeling or activation of the EMTU.

Furthermore, a recent report that analysed gene expression profiles of upper and lower airway epithelial cells in the same individuals comparing those with rhinitis and asthma, rhinitis without asthma and controls, revealed that the majority of transcriptional factor genes (FOXP2,A1,A2, NKX2-1 and GATA6) that are involved in lung development were differently expressed between healthy upper and lower airways (42) which supports the hypothesis that the different embryologic origins of the nose and bronchi genes might be

responsible for the absence of remodeling in the nose in rhinitis compared to the bronchi in asthma (1).

Our result are also in line with recent data that showed treating chronic sinonasal disease does not improve asthma control (43,44). Hence, our results might support the notion that either the upper airways respond differently to allergic inflammation than the lower airways or that there are factors other than eosinophils or Th2 cytokines which drive the response in the lower airways.

In conclusion, our data suggests that despite the presence of Th2 allergic inflammation in persistent allergic rhinitis, there is no upper airway structural remodeling compared to healthy subjects. The impact of our findings is that in contrast to asthma, structural changes and remodeling are not features of allergic rhinitis. This questions the current dogma on the relation between the upper and lower airways and argues that in contrast to asthma, targeting remodeling in allergic rhinitis may not be appropriate as a therapeutic target.

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Legends:

Figure 1: Th2 cytokine (IL-4, IL-5, IL-13) levels from nasal lavage and sub-mucosal eosinophils comparing persistent allergic rhinitis and healthy controls. PAR: Persistent allergic rhinitis. Horizontal bars represent median values, p value: <0.05: statistical significance, *Mann-Whitney test*.

Figure 2: Blood and lymphatic vessels stained using immunohistochemistry, and PAS stained glandular structures. A-B): The percentage total area of nasal sub-mucosal glands comparing persistent AR and controls. C-D): Percentage total area of blood vessels stained using $CD31^{+}$. E-F): Percentage total lymphatic vascular area in nasal sub-mucosa stained using D2-40 antibody. PAR: Persistent allergic rhinitis. Horizontal bars represent medians.

Figure 3: Immunoreactivity and localization of matrix metalloproteinases-7 and 9 (MMP-7,9), and tissue inhibitor metalloproteinases-1 (TIMP-1) in the nasal sub-mucosa of persistent allergic rhinitis and healthy controls. No significant different in the expression of MMP-7 (A-B), MMP-9 (C-D) and TIMP-1 (E-F) in persistent allergic rhinitis compared to healthy controls nasal sub-mucosa. Horizontal bars represent medians.

Figure 4: Markers of collagen deposition comparing persistent allergic rhinitis and healthy controls are shown in A to F. The collagen chaperone heat shock protein (HSP)-47^{+} fibroblasts and α-smooth muscle actin (α-SMA-expressing myo-fibroblasts) (as positive stained cells per mm^2) are shown in A through D, respectively. Fibroblasts were fusiform with elongated nuclei. Picrosirius red stained sections of nasal mucosa from persistent

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allergic rhinitis and healthy controls assessed for total collagen deposition using polarised light microscopy are shown in E-F. Horizontal bars represent medians.

Table 1: Demographic features of participants

	Persistent allergic rhinitis *n*=46	**Healthy control *n*=19**	***P value***
Age yrs	30 (23-40)	30 (26-45)	0.47
Gender F/M	24/22	11/8	0.68
TNSS	6 (4-9)	0 (0-1)	<0.0001
TVAS	45 (23-67)	1 (0-7)	<0.0001
Total RQoL	2 (1-3.4)	0 (0-0.2)	<0.0001
Eczema %	21 (*n:10*)	- (*n*:0)	<0.0001
Asthma %	30 (*n:13*)	- (*n*:0)	<0.0001
sIgE grass IU/mL	11 (3.7-34.4)	0 (0)	<0.0001
sIgE HDM IU/mL	15.7 (3-36)	0 (0)	<0.0001
Total IgE IU/mL	157 (58-404)	15 (4-49)	<0.0001

Definition of abbreviations: TNSS= Total nasal symptoms score, TVAS= Total visual analogue score, RQoL= Rhinitis quality of life, sIgE= Specific IgE, *n*= Number of subjects.

Median (IQR:25-75 %)

P: Mann-Whitney test comparing persistent allergic rhinitis and control

Table 2: Summary of airway remodeling markers in upper airways in severe persistent allergic rhinitis and healthy controls

	Natural exposure (severe persistent allergic rhinitis-PAR)		
	Healthy controls	**PAR**	***P values***
Epithelial Mucus % Area	10 (3-22)	17 (4-38)	0.43
RBM Thickness	9 (7-10)	8 (6.5-10)	0.50
Sub-mucosal Glandular % Area/ mm^2	34(21-43)	32(17-45)	0.98
CD31$^+$ Cells /mm^2	333 (180-566)	367 (213-472)	0.68
CD31$^+$ % Vascular Area/ mm^2	1.7 (1.1-3.9)	1.5 (0.4-3.4)	0.38
Number of Blood vessels/mm^2	41.3 (18-58)	37.7 (29-51)	0.65
Mean size blood vessels/µm^2	532 (270-1010)	372 (131-931)	0.12
D2-40$^+$ Cells/ mm^2	0 (0-51)	0 (0-38)	0.76
D2-40$^+$ % Vascular Area/ mm^2	0 (0-0.2)	0 (0-0.5)	0.69
Number of Lymphatic vessels/mm^2	0 (0-6)	0 (0-6)	0.74
Mean size Lymphatic vessels/µm^2	0 (0-494)	0 (0-601)	0.70
D2-40$^+$/CD31$^+$ vessels/mm^2 ratio	0 (0-0.19)	0 (0-0.1)	0.84
D2-40$^+$/CD31$^+$ % vascular area/mm^2 ratio	0 (0-0.1)	0 (0-0.15)	0.87
VEGF-A$^+$ cells/ mm^2	80 (0-129)	52.1 (0-121)	0.59
VEGF$^+$ % vascular Area/ mm^2	0.2 (0-0.7)	0.2 (0-1.2)	0.99
Number of VEGF$^+$ vessels/mm^2	10 (0-21)	10 (0-19)	0.68
Mean size VEGF$^+$ vessels/µm^2	45 (0-177)	40 (0-277)	0.90
MMP-7$^+$ cells/ mm^2	1 (0-60)	0 (0-8)	0.20
MMP-9$^+$ cells/ mm^2	6.7 (0-27.7)	7 (0-21)	0.95
TIMP-1$^+$ cells/ mm^2	0 (0-3)	0 (0-4)	0.91

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HSP-47^{+} Fibroblast/mm^{2}	0 (0-15)	0 (0)	0.06
α-SMA/ mm^{2}	130 (62-217)	105 (56-185)	0.45

Definition of abbreviations: α-SMA= Alpha smooth muscle actin, TIMP= Tissue inhibitor metaloproteinase, MMP= Matrix metalloproteinase, HSP= Heat shock protein, VEGF= Vascular endothelial growth factor, CD31= Vascular endothelial cells, D2-40= Lymphatic endothelial cells, RBM= Reticular basement membrane, PAR= Persistence allergic rhinitis. Median (25%-75%).

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Fig 1:

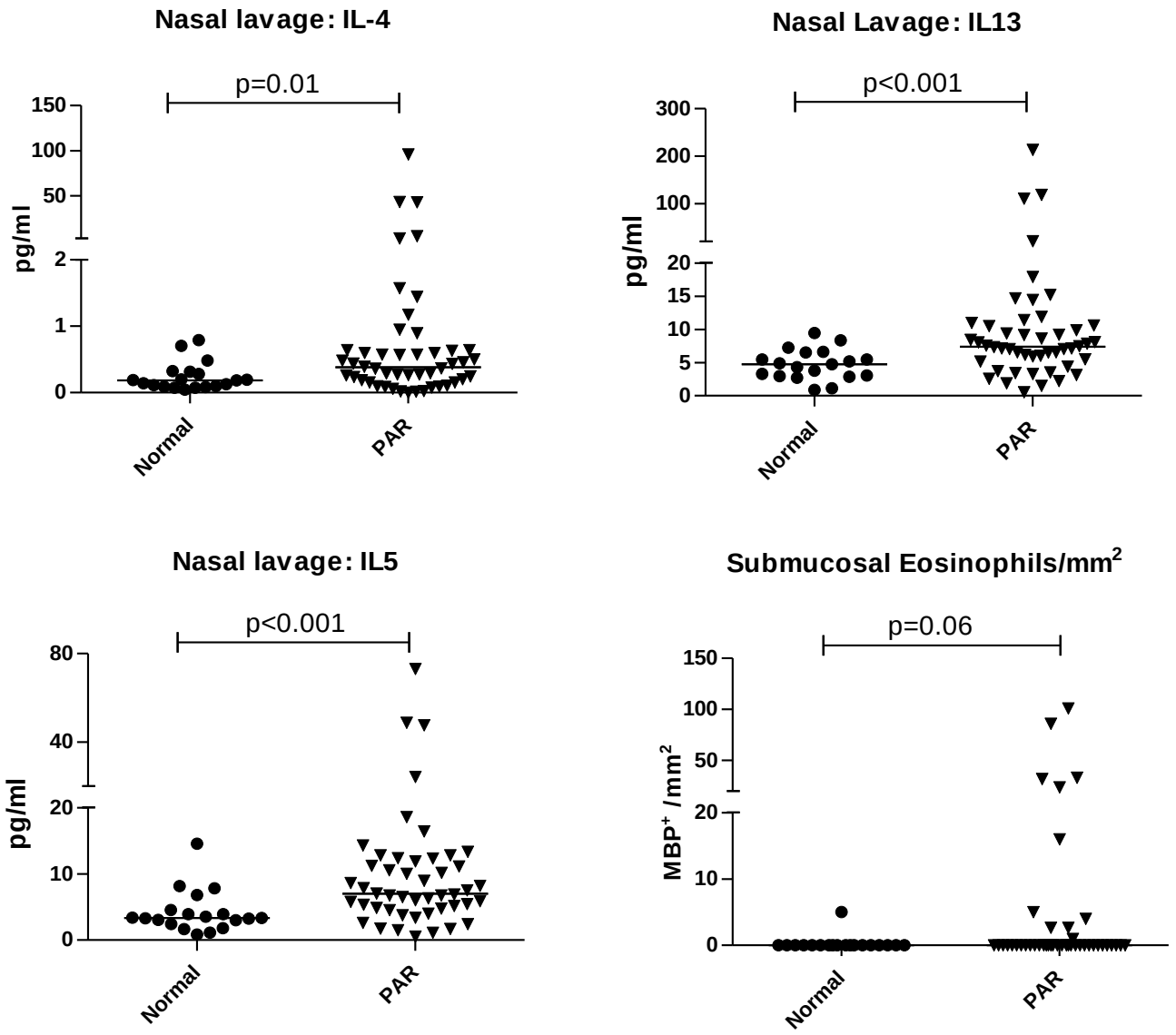

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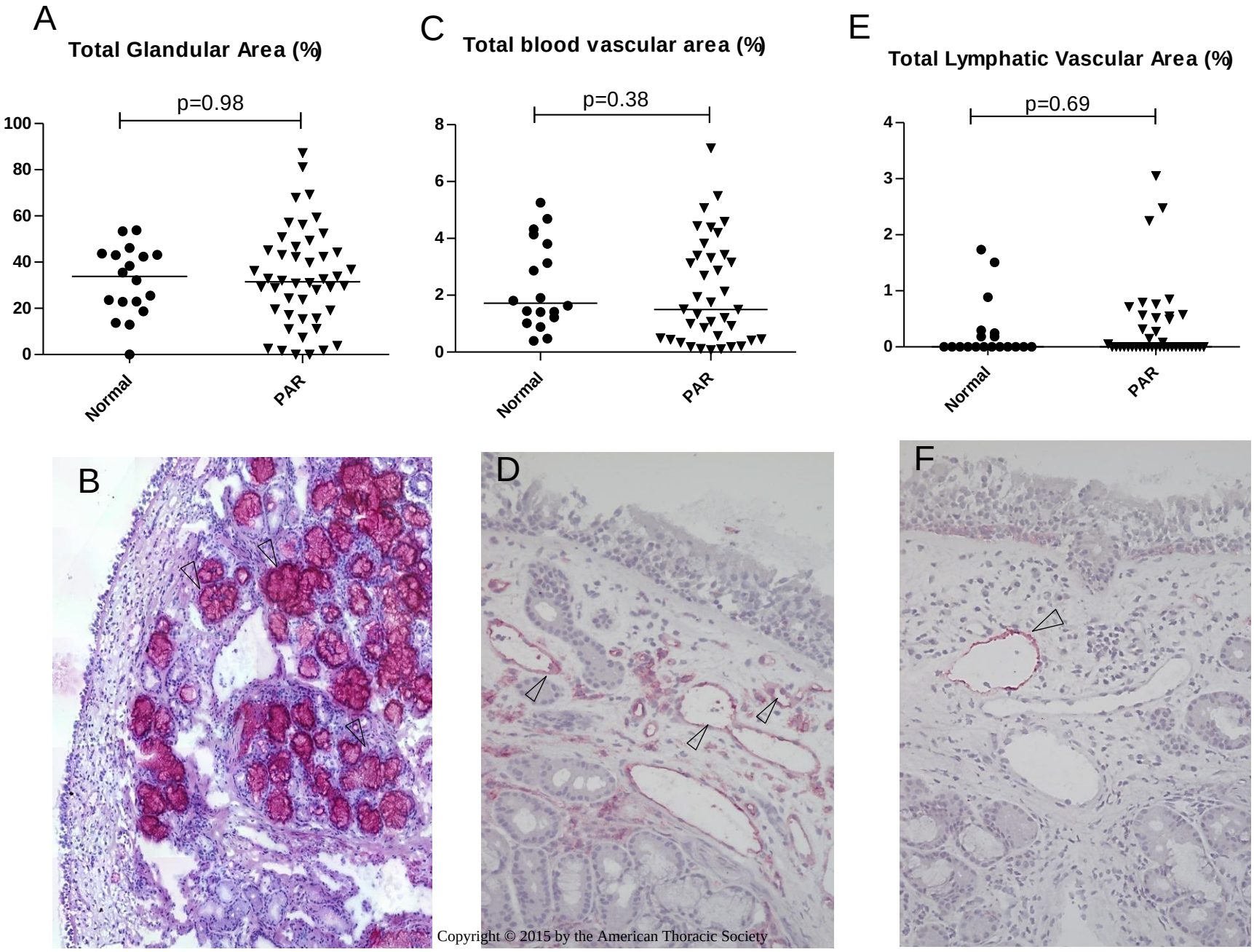
Fig 2



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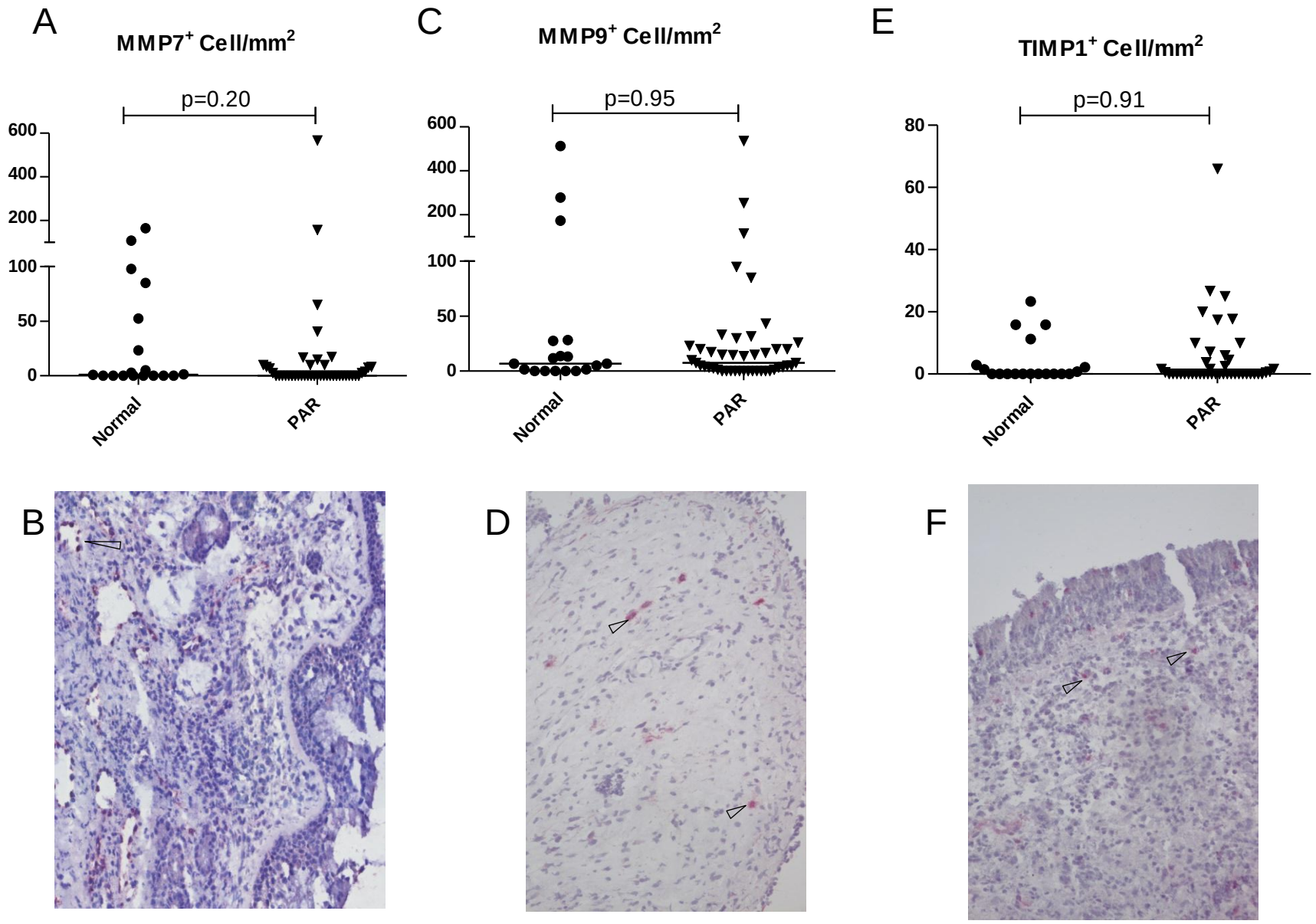

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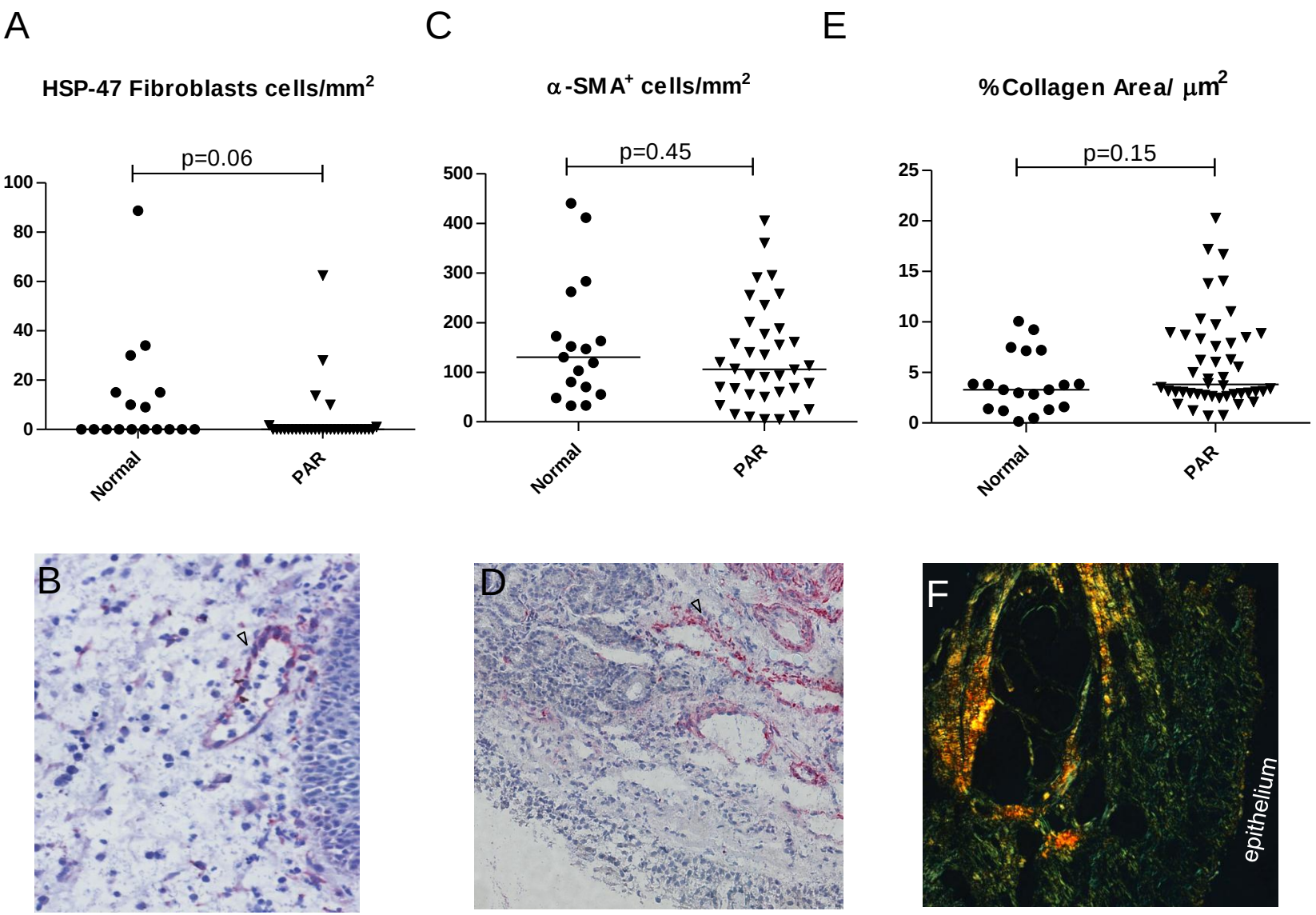
Fig 4.



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Severe persistent allergic rhinitis: inflammation but no histologic features of structural upper airway remodeling.

Online Supplement

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Methods:

Study Subjects

Subjects aged 18-55 years with clinical history of moderate-severe persistent allergic rhinitis according to ARIA (E1), with a history of seasonal allergic rhinoconjuctivitis (May-July) and/or perennial rhinitis to perennial allergens such as house dust mite with duration of at least two years or more requiring treatment were included. All atopic subjects had a positive skin response (wheal diameter ≥ 3 mm) and a positive specific IgE against aeroallergens (≥ 0.73 kU/ml). Non- atopic healthy controls were subjects without any nasal symptoms, negative skin test and no specific IgE to aeroallergens. Other inclusion criteria were no clinically relevant findings on physical examination and willingness to comply with this study. Patients were excluded if they smoked, used systemic steroid treatment for 6 weeks or topical steroid treatment for 2 weeks prior to the start of the study, had a history of anaphylaxis, family history of bleeding, currently used non-steroidal anti-inflammatory drugs or immunosuppressive medication, had other nasal or systemic chronic diseases, severe asthma or specific immunotherapy within the last 5 years. All subjects were recruited via advertising in print and on-line from the general public, Imperial College and Royal Brompton Hospital staff .The study was approved by the ethics committee of The Royal Brompton and Harefield Hospitals NHS Trust and was performed with the subjects' written informed consent.

Study design

Each subject was required to make 2 separate visits. After the screening visit eligible participants were scheduled for a second visit during the pollen season to provide symptom scores, rhinitis quality of life visual analogue score and nasal lavages to determine inflammatory response remodeling markers immediately prior to the nasal biopsies as shown in E-Fig 1.

***Skin prick test*:**

Skin prick testing was performed with a sterile stainless steel lancet (ALK Abello, Denmark) and standardized reagents [Timothy grass *Phleum Pratense* extract, mixed grass pollen, birch pollen, mixed tree, cat hair, dog hair, house dust mite (*Dermatophagoides pteronyssinus*), mixed weeds, *Aspergillus fumigatus, Cladosporium herbarum, Alternaria alternata*, Soluprick; ALK Abello]; a wheal of >3 mm compared with the diluent (negative control) was considered positive.

Symptoms scores, Visual analogue score and quality of life

Symptoms scores were evaluated using a four-point scoring system: 0 (no symptoms) to 3 (severe symptoms) for each rhinitis symptom (sneezing, nasal discharge, itching and nasal obstruction). Subjects were asked to score their symptoms when they were most severe during the last week. The total score of all four rhinitis symptoms was termed the total nasal symptom score (TNSS) (maximum 12 points). Total nasal symptom scores were recorded before collection of nasal lavage and biopsy procedures. Quality of life in relation to symptoms was assessed using mini Rhinitis Quality of Life Questionnaires (mRQLQs). Participants were asked to mark a visual analogue scale (scale 0-100 mm) documenting the overall severity of their allergic rhinitis symptoms during the past week.

Nasal Lavage procedure and measurement of nasal lavage cytokine levels

Subjects were seated in a forward-flexed neck position to prevent fluid from reaching the nasopharynx. To ensure adequate washing, the lavage fluid (5 ml of PBS) was passed slowly into the nasal cavity via an olive consisting of an oval, hollow, stainless steel device that was used to obstruct the nostril. The fluid was withdrawn into the syringe and gently flushed back into the nasal cavity 20 times over 1 minute. Samples were centrifuged (4^0C, 10 minutes at

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400 *g*), and the separated supernatant was aliquoted and frozen (−80^{0}C). For the electrochemiluminescence studies, the levels of IFN-γ, IL-4, IL-5, IL-10, and IL-13 in the nasal lavage samples were analyzed in duplicates using the Human TH1/TH2 10-plex Ultra-Sensitive kit from Meso Scale Discovery (MSD, Gaithersburg, MD). Assays were conducted according to standard manufacturer's protocols. MSD plates were analysed on the Sector Imager 6000 using MSD Discovery workbench software (v 3.0.17.3). According to manufacturer's protocol, the lower limit of detection for IL-4:0.31 pg/mL, IL5: 0.076 pg/mL, IL-13: 1.8 pg/mL, IL10: 0.36 pg/mL, and IFN-γ: 0.39 pg/mL.

Nasal Biopsies, immunohistochemistry staining and quantification

Nasal biopsies specimens (2.5 mm) were taken from the under surface of the inferior turbinate using Gerritsma forceps with lidocaine 5% and phenylephrine 0.5% mixture nasal spray and 10% cocaine as local anaesthetic as described previously (E-2). Tissue specimens were fixed in 4% paraformaldehyde for 2 hours, then dehydrated in 15% sucrose for 1 h and then again overnight, before being embedded in OCT and snap frozen. Five-micron sections were cut and slides were dried overnight at 37^{0}C and then stored at -80^{0}C.

Immunohistochemistry staining and quantification

Immunohistochemistry was performed using the Vectastatin avidin/biotin complex-alkaline phosphatase (ABC-AP) method as described previously (E-2). Briefly, sections were incubated with 20% horse serum in PBS for 30 minutes to reduce non-specific binding. Mouse monoclonal primary antibodies were applied to slides using pre-determined dilutions (E-Table 1) and incubated overnight at 4^{o}C. Sections were washed with buffer followed by incubation with biotinylated horse anti-mouse secondary antibody and then ABC-AP

complex at room temperature (E-Table1). The reaction was developed with Fast Red substrate and counterstained with Harris Haematoxylin.

Biopsy sections were stained using monoclonal antibodies against eosinophils (MBP), mast cells (AAI) and basophils (2D7). Upper airway remodeling was assessed by staining vascular (CD31) and lymphatic endothelial (D2-40) cells, and vascular endothelial growth factor-A (VEGF-A). Furthermore, extracellular matrix activation was evaluated by staining heat shock protein-47 (HSP-47, marker of fibroblasts), alpha-smooth muscle actin (α-SMA-those expressing myo-fibroblasts) matrix metalloproteinase-7 and 9 (MMP7 & 9) and tissue inhibitor of metalloproteinase-1 (TIMP-1). Monoclonal CD3 antibodies and mouse IgG isotypes (DakoCytomation, Cambridgeshire, United Kingdom) were used as positive and negative controls respectively.

Reticular basement membrane and epithelial integrity were assessed using haematoxylin stained slides. Epithelial goblet cells and the glandular mucus area were evaluated using PAS staining. The collagen content was assessed by picrosirius red staining (E-3). Biopsies were coded and evaluated blindly by three investigators without the knowledge of clinical characteristics.

Nasal mucosal inflammatory cells, were quantified using a BH-2 Olympus microscope. Epithelial integrity was scored on 0-4 scale, with 4 being an intact epithelium. Vascular and lymphatic staining was assessed using computer assisted image analysis (Image Pro-Plus 7.0 system, Media Cybernetics, Inc). The whole specimen was captured at x200 magnification, using Nikon 80*i* light microscope coupled to colour camera (QImaging, Media Cybernetics, Inc) that transmits the image to a computer. Images can be magnified up to 200% which allows clear identification of stained cells, blood and lymphatic vascular structures. Images were captured and examined manually. Epithelial, glandular, total sub-mucosal and vascular area was measured separately using computer Image analysis (Image Pro-Plus 7.0 system,

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Media Cybernetics, Inc). Reticular basement membrane thickness was measured as proposed by Sullivan et al (E-4). Epithelial and sub-mucosal cell counts were expressed as the number of positively stained nucleated cells per mm^2.

The total numbers of $CD31^+$ and $D2\text{-}40^+$cells in the sub-mucosa were counted to a depth of 200 μm below the epithelium. Blood and lymphatic vessel numbers, the area of each vessel, and the total selected area of sub-mucosa were determined by capturing the entire section using Image Pro-Plus 7 software. The area of each vessel was measured along the inner luminal margin of positively stained endothelial basement membrane and automatically calculated using the Image Pro-Plus 7 software. Total vascular area is the sum of the individual CD31 or D2-40 vessel areas within a given region (the sub-mucosa to a depth of 200μm below the basement membrane). The software measurements of the area of positive immunostaining in the region of interest were transferred to an Excel spreadsheet for statistical analysis and determination of the total number of vessels, vascular area and number of positive cells.

For each sample the percentage vascular area, the mean vessel size (mm^2), and the vessel number (per mm^2) within the total area of assessed sub-mucosa was evaluated. Percentage vascular area was calculated as [(total vascular area/sub-mucosal area) x 100]; number of blood vessels/mm^2 (vessels density) as (total number of blood vessels/ total sub-mucosal area); and mean vessel size/mm^2 (total vascular area/total number of blood vessels). Furthermore, the relative lymphatic vessel density was studied by calculating the ratio of D2-40/CD31 positive vessels/mm^2.

Picrosirius stained collagens were quantified as previously described (E-3). Briefly, colour images of two sections from each biopsy were acquired at x200 magnification (with a polarizing lens for Picrosirius red–stained sections; Leica QWin version 3). Subsequently, images were converted to grayscale, and the area of positive staining that includes reticular

basement membrane and whole of sub-mucosa was determined at a preset pixel intensity by using the Leica QWin software. The mean area of collagen and total sub-mucosal area was used to calculate the percentage area of collagen in each nasal biopsy.

Statistics

Values are presented as median [IQ 25-75 range], unless otherwise specified. Comparisons for quantitative variables were performed by non-parametrical analysis, Mann–Whitney *U* test for non-related samples. Differences between three groups were assessed using Kruskal-Wallis test (nonnormal distribution) followed by Dunn's multiple comparisons test. Within-group comparisons were performed by using the Wilcoxon matched-pairs signed-rank test. Interobserver reproducibility within 2 standard deviation was measured using Bland-Altman test. All tests were two-tailed. Significance was set at $P<0.05$. GraphPad Prism 5 Project software was used for analysis. Further to these tests, we performed limited principal component analysis (PCA) using XLSTAT statistical software for Microsoft Excel on the data set of allergic rhinitis patients who also had asthma comparing 6 variables (TIMP1, MMP-9, Basement membrane thickness, collagen area, total blood and lymphatic vascular area) that might be associated with remodeling features. Analysis showed low variability as determined by eigenvalue, no correlation between the variables, and no positive contribution on allergic rhinitis with asthma as analysed by eigenvectors.

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Legends:

Supplement Figure 1: Flow chart of study design. *Definition of abbreviations*: TNSS= Total nasal symptom score, VAS= Visual analogue score, RQoL= Rhinitis quality of life, sIgE= Specific IgE. IHC: Immunohistochemistry, SPT: Skin prick test, V1/2: Visit 1 & 2, *: Time points.

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Table 1 Supplement: Primary and secondary antibodies used for Immunohistochemistry staining.

Primary antibody	Clone/Cat. No	Source	Host species-Monoclonal	Working Dilution	Secondary antibody
CD31	M0823	Dako	Mouse	1:100	Horse Anti- Mouse
D2-40	M3619	Dako	Mouse	1:150	Horse Anti- Mouse
VEGF-A	sc-7269	Santa Cruz	Mouse	1:75	Horse Anti- Mouse
MMP-7	Ab3205	Abcam	Mouse	1:20	Horse Anti- Mouse
MMP-9	Ab51203	Abcam	Mouse	1:75	Horse Anti- Mouse
TIMP-1	Ab1827-250	Abcam	Mouse	1:50	Horse Anti- Mouse
HSP-47	SPA470	Stressgen	Mouse	1:800	Horse Anti- Mouse
α-SMA	M0851	DAKO	Mouse	1:800	Horse Anti- Mouse
AA1	M7052	DAKO	Mouse	1:50	Horse Anti- Mouse
MBP	MCA5751	Bio-RAD	Mouse	1:50	Horse Anti- Mouse
2D7	B136194	Biolegend	Mouse	1:50	Horse Anti- Mouse

Definition of abbreviations: CD31: Vascular endothelial cells, D2-40: Lymphatic endothelial cells, VEGF-A: Vascular endothelial growth factor, MMP: Matrix metalloproteinase, α-SMA= Alpha smooth muscle actin, TIMP= Tissue inhibitor metalloproteinase, HSP= Heat shock protein, MBP: Major basic protein, 2D7: Basophils marker, AA1: Mast cells marker

Table 2 Supplement: Demographic features of participants

	Seasonal AR *n*=23	Perennial AR *n*= 23	Controls n=19	*p value*
Age yrs	28 (23-34)	31 (27-41)	30 (26-45)	0.29
Gender F/M	11/12	13/10	11/8	0.77
TNSS	9 (7-11)	4 (3-6)	0 (0-1)	<0.0001
TVAS	(24 13-45)	64 (44-71)	1 (0-7)	<0.0001
Total RQoL	3 (2-3.6)	1 (1-2)	0 (0-0.2)	<0.0001
Eczema %	30(*n*:7)	13(*n*:3)	-(*n*:0)	<0.0001
Asthma %	30 (*n*:7)	26 (*n*:6)	-(*n*:0)	<0.0001
sIgE Grass IU/mL	15 (5-34)	9 (1-61)	0 (0)	<0.0001
sIgE HDM IU/mL	1 (0-2.5)	16 (12-48)	0 (0)	<0.0001
Total IgE IU/mL	74 (28-195)	244 (117-687)	15 (4-49)	<0.0001

Definition of abbreviations: TNSS= Total nasal symptoms score, TVAS= Total visual analogue score, RQoL= Rhinitis quality of life, sIgE= Specific IgE.

Median (IQR:25-75 %)

***: p<0.0001, Kruskall wallis test comparing seasonal and perennial rhinitis with control.

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Table 3 Supplement: Summary of airway remodeling markers in upper airways in severe persistent allergic rhinitis and healthy controls.

	Natural exposure (severe PAR)				
	Normals	Perennial AR	In Season AR	Out-season AR**	*p**
Epithelial Mucus % Area	10 (3-22)	17 (6-38)	18 (2-33)	4 (1-18)	0.54
RBM Thickness	9 (7-10)	8 (7-11)	8 (6-9)	8 (6-10)	0.60
Submucosal Glandular % Area/ mm2	34(21-43)	32 (17-50)	31 (11-45)	20 (11-35)	0.99
$CD31^+$ Cells /mm^2	333 (180-566)	367 (193-480)	370 (217-470)	308 (214-480)	0.91
$CD31^+$ % Vascular area/ mm^2	1.7 (1.1-3.9)	1.5 (0.4-3.8)	1.7 (0.4-3.4)	1.7 (1.7-3.1)	0.68
Number of Blood vessels/mm^2	41.3(18-58)	34.3(28-49)	40(30-56)	41(30-61)	0.59
Mean size blood vessels/μm^2	532(270-1010)	306(112-1022)	406(171-673)	479(276-691)	0.30
$D2\text{-}40^+$ Cells/ mm^2	0 (0-51)	0 (0-28)	0 (0-85)	0 (0-22)	0.31
$D2\text{-}40^+$ % Vascular area/ mm^2	0 (0-0.2)	0 (0-0.4)	0 (0-0.6)	0 (0-0.1)	0.62
Number of Lymphatic vessels/mm^2	0 (0-6)	0 (0-3.5)	0 (0-11)	0 (0-5.8)	0.32
Mean size Lymphatic vessels/μm^2	0 (0-494)	0 (0-873)	0 (0-426)	0 (0-260)	0.86
$D2\text{-}40^+/CD31^+$ vessels/mm^2 ratio	0 (0-0.19)	0 (0-0.07)	0 (0-0.15)	0 (0-0.1)	0.59
$D2\text{-}40^+/CD31^+$ % area/mm^2 ratio	0 (0-0.1)	0 (0-0.14)	0 (0-0.2)	0 (0-0.1)	0.76
VEGF-A^+ cells/ mm^2	80 (0-129)	67.4 (0-116)	30.7 (0-121)	107 (0-179)	0.73
VEGF-A^+ % vascular Area/ mm^2	0.2 (0-0.7)	0.5 (0-1.5)	0.2 (0-0.8)	0.4 (0-1.5)	0.24
MMP-7^+ cells/ mm^2	1 (0-60)	0 (0-6)	0 (0-12)	0 (0-30)	0.40
MMP-9^+ cells/ mm^2	6.7 (0-27.7)	10 (3-30)	6.2 (0-16)	12 (3-49)	0.32
TIMP-1^+ cells/ mm^2	0 (0-3)	0 (0-10)	0 (0-1.6)	2 (0.8-8) §	0.61
HSP-47^+ Fibroblast/mm^2	0 (0-15)	0 (0-0.8)	0	0 (0-20)	0.11
α-SMA/ mm^2	130 (62-217)	124 (53-240)	92 (51-165)	81 (17-170)	0.49

Definition of abbreviations: α-SMA= Alpha-smooth muscle actin, TIMP= Tissue inhibitors of metalloproteinase, MMP= Matrix metalloproteinase, HSP= Heat shock protein, VEGF= Vascular endothelial growth factor, CD31= Vascular endothelial cells, D2-40= Lymphatic endothelial cells, RBM= Reticular basement membrane, AR= Allergic Rhinitis. Median (25%-75%).

*Kruskall wallis test: comparing in or out of season, perennial and healthy controls.

**Wilcoxon signed rank test: comparing between in season and out of season.

§ $p<0.01$, Significant statistical difference between in season and out of season..

E-Fig 1 Supplement. Flow chart of study design