Subepithelial collagen deposition, profibrogenic cytokine gene expression, and changes after prolonged fluticasone propionate treatment in adult eosinophilic esophagitis: A prospective study

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Background: Recent research shows that both pediatric and adult patients with eosinophilic esophagitis (EoE) experience esophageal remodeling marked by increased collagen deposition in which TGF-β plays an important role. However, limited data are available on the intensity and reversibility of fibrous remodeling in adults with EoE.

Objective: We sought to analyze differences in collagen deposition in the lamina propria (LP) and profibrogenic cytokine gene expression along with other changes induced by prolonged treatment with fluticasone propionate in adults with EoE.

Methods: Ten adults given consecutive diagnoses of EoE were studied prospectively. Deep esophageal biopsy specimens were obtained before and after 1 year of treatment with fluticasone propionate. Collagen deposition in the LP was assessed in tissue sections with the aid of the Masson trichrome technique. *IL5*, *TGFB1*, fibroblast growth factor 9 *(FGF9)*, and *CCL18* gene expression was quantified through real-time PCR. EoE results were compared among samples from 10 adult patients with gastroesophageal reflux disease and 10 control subjects with healthy esophagi.

Results: Patients with EoE showed a significant increase in subepithelial collagen deposition; this correlated positively with eosinophil density in the LP and the patient's age. Prolonged steroid treatment induced a nonsignificant reduction in subepithelial fibrosis, which remained significantly higher than in control subjects. Profibrogenic cytokine gene expression also increased in patients with EoE, with *IL5* ($P < .001$), *FGF9* ($P = .005$), and *CCL18* ($P = .008$) all significantly upregulated. After 1 year of treatment, a reduction was observed in gene expression; for *CCL18* expression, this decrease was statistically significant ($P < .001$).

Conclusions: Esophageal remodeling is associated with upregulated gene expression of profibrogenic cytokines in adults with EoE. Prolonged treatment with fluticasone propionate leads to a nonsignificant reduction in subepithelial collagen deposition accompanied by downregulation of profibrogenic cytokine gene expression, with that of *CCL18* being especially significant. (J Allergy Clin Immunol 2011;128:1037-46.)

Key words: *Eosinophilic esophagitis, fibrosis, lamina propria, IL-5, TGF-β1, fibroblast growth factor 9, CCL18, fluticasone propionate*

Eosinophilic esophagitis (EoE) is a chronic inflammatory clinicopathological entity characterized by the presence of large numbers of intraepithelial eosinophils in esophageal biopsy specimens.[1] A T_H2-type immunologic response similar to that found in bronchial asthma has been associated with EoE pathogenesis in that activated eosinophils release the cytotoxic content, including major basic protein (MBP), from their granules, leading to epithelial damage and a subsequent proliferative response.[2]

Eosinophilic inflammation has been widely explored in the airways, where it leads to structural changes in the bronchial wall known as bronchial remodeling. This involves hyperplasia of the submucosal glands, smooth muscle hypertrophy, angiogenesis, and subepithelial collagen deposition,[3] all of which impair respiratory function. Recently, researchers have also observed subepithelial fibrous remodeling in patients with EoE.[4-9] This has important clinical implications because it could be associated with dysphagia and might help explain and predict future esophageal strictures and dysmotility.[10] As with bronchial asthma, topical steroid treatment has proved effective both in resolving the esophageal eosinophilic infiltrate and in restoring esophageal histology in patients with EoE while simultaneously providing relief of symptoms.[11]

EoE-associated fibrosis is related to the extent of esophageal eosinophil activation, as evidenced by eosinophil degranulation, which can be determined through immunohistochemical staining for MBP.[5] EoE biopsy specimens show that eosinophil-released MBP increases the expression of fibroblast growth factor 9 (FGF-9),[12] a cytokine implicated in the proliferative response to injury,[13] which in turn correlates with EoE-associated basal cell hyperplasia. In a murine model of EoE, subepithelial fibrosis was found to be directly caused by tissue eosinophilia induced

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Abbreviations used
B2M: β_2-Microglobulin
EoE: Eosinophilic esophagitis
FGF-9: Fibroblast growth factor 9
GERD: Gastroesophageal reflux disease
HPF: High-powered field
LP: Lamina propria
MBP: Major basic protein
PPI: Proton pump inhibitor

by IL-5,[4] a T_H2-type cytokine the expression of which is also upregulated in human EoE.[13] Eosinophils also produce and secrete high amounts of CCL18,[14] a type 2 chemokine implicated in fibrous remodeling of the lungs[15] through the stimulation of fibroblast proliferation and collagen deposition.[16,17]

Research carried out on pediatric patients with EoE has shown that they have significant subepithelial fibrosis of the esophageal lamina propria (LP) in comparison with healthy control donors or children with gastroesophageal reflux disease (GERD). In these studies fibrosis was demonstrated by means of trichrome staining and was associated with increased expression in eosinophils of TGF-β and its signaling factor phosphorylated Smad2/3.[6] A 3-month course of budesonide therapy reduced epithelial eosinophil density and led not only to a significant reduction in esophageal remodeling but also to decreases in fibrosis and the number of TGF-β^+ cells.[18]

Recent studies assessing fibrous remodeling in adults with EoE and the effects of budesonide treatment have found that only half the biopsy specimens displayed representative LP tissue with a significantly higher fibrosis score than that of healthy donors.[7,8] Moreover, low-dose budesonide maintenance therapy did not significantly reduce the fibrosis score, which was associated with a slight nonsignificant decrease in the thickness of deeper esophageal wall structures, as assessed by means of endoscopic ultrasonography.[8]

Unfortunately, the fact that endoscopic biopsy specimens taken with the aid of standard biopsy forceps in the past did not systematically include esophageal LP constitutes an important obstacle to the study of the deep esophageal remodeling associated with EoE. Information on this phenomenon is thus limited. In addition, there are no consistent data on the intensity and reversibility of fibrous remodeling of the esophagus in adults with EoE. Furthermore, although there are no definitive data indicating that EoE in adults has its origin in childhood, because of the persistent eosinophilic inflammation they experience over many years, adults with EoE comprise the group most likely to experience structural changes.

The goal of this study is to determine the amount of collagen deposition in the LP of naive adults with EoE in comparison with their counterparts with GERD and healthy control subjects. In addition, changes in the tissue remodeling and gene expression of profibrogenic molecules induced by prolonged topical treatment with fluticasone propionate have been analyzed.

METHODS

Ten adults consecutively given diagnoses of EoE on the basis of their esophageal symptoms were studied. Diagnostic criteria for EoE[1] included the following: (1) infiltration of esophageal epithelium by 15 or more eosinophil leukocytes per high-powered field (HPF) at ×400 light microscopy; (2) absence of eosinophilic infiltration in biopsy specimens of the gastric and duodenal mucosa; (3) elimination of gastroesophageal reflux as a cause of eosinophilia through either ambulatory 24-hour pH-metry or persistence of eosinophilic infiltration after an 8-week treatment with omeprazole (20 mg/twice a day); and (4) elimination of drug intake, parasites, esophageal caustications, hematologic neoplasm, or other events in the patient's medical history as possible causes of esophageal eosinophilia. All patients included in this study were naive to topical or systemic steroid therapy for EoE to determine initial fibrous remodeling precisely. However, all of them had previously received proton pump inhibitor (PPI) drugs.

Control samples were obtained endoscopically from 20 patients (12 men and 8 women) who consecutively underwent endoscopy under sedation during the study period. Ten of the control subjects had GERD, as characterized by typical chronic (>1 year) symptoms and compatible endoscopic lesions. It is worth noting that these patients had a positive clinical response to PPI therapy during follow-up. The remaining 10 patients had dyspepsia or a suspected gastroduodenal ulcer. Subjects in this last group exhibited a normal esophagus in which symptoms of GERD, hiatus hernia, incompetent cardias, and esophageal peptic lesions were ruled out; they also had a normal endoscopic appearance and microscopic analysis result. The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the institutional review board of the La Mancha Centro Hospital. Informed consent was obtained from all patients and control subjects.

Endoscopic and biopsy-sampling procedure

All endoscopic examinations were carried out by the same endoscopist (A. J. L.) and were performed under conscious sedation with a flexible 9-mm-caliber Pentax EG-2770K gastroscope (Pentax of America, Inc, Montvale, NJ) with a 2.8-mm work channel. Biopsy specimens were taken with the aid of a commercially available, reusable, single-side, open-type endoscopic biopsy forceps (Olympus FB-11-K-1; Olympus Medical Systems Corp, Tokyo, Japan). This forceps, devised to facilitate biopsy through a tangential approach, was capable of sampling deep esophageal tissue (epithelial and LP), reaching down to the muscularis mucosae (Fig 1). The average tissue volume obtained in each sample was approximately 2.5 mm^3.

Endoscopic biopsy specimens were taken in the upper and lower esophageal thirds of patients with EoE to compare topographic differences in subepithelial collagen deposition. In contrast, patients in both control groups were sampled only in the middle third to minimize risks and discomfort, as well as to avoid interference with peptic erosions in patients with GERD. A minimum of 5 specimens were taken per location. These were then fixed in 4% formalin and routinely processed for histopathological analysis.

Three additional endoscopic samples from the middle esophageal third of each patient with EoE and control subject were also collected during the same endoscopic procedure. These were preserved in an RNA stabilization solution (RNAlater; Ambion, Inc, Austin, Tex) at −20°C until processing. No specific complications were observed in any patients after the biopsy procedure despite the high fragility of the esophageal wall described in patients with EoE. The only problem was occasional slight chest discomfort after endoscopy, especially when swallowing, which disappeared completely after 2 days.

Treatment and follow-up period

All patients with EoE received topical treatments of a liquid suspension of 400 μg of fluticasone propionate in a 2-mL volume (Flixonase 0.4, nasal drops; GlaxoSmithKline, Durham, United Kingdom) during a 12-month period. Patients were told to swallow the liquid twice a day after breakfast and dinner and to avoid eating or drinking in the subsequent 3 hours. No PPI treatment was administered during this period. Every 2 months, the subjects visited the gastroenterology clinic, where they were encouraged not to withdraw from therapy and were given renewals for their prescriptions at the appropriate time intervals. This suggests an adherence to therapy. No dietary restrictions or changes in environment or medication between the baseline and follow-up biopsy specimens were mandated. Patients were also

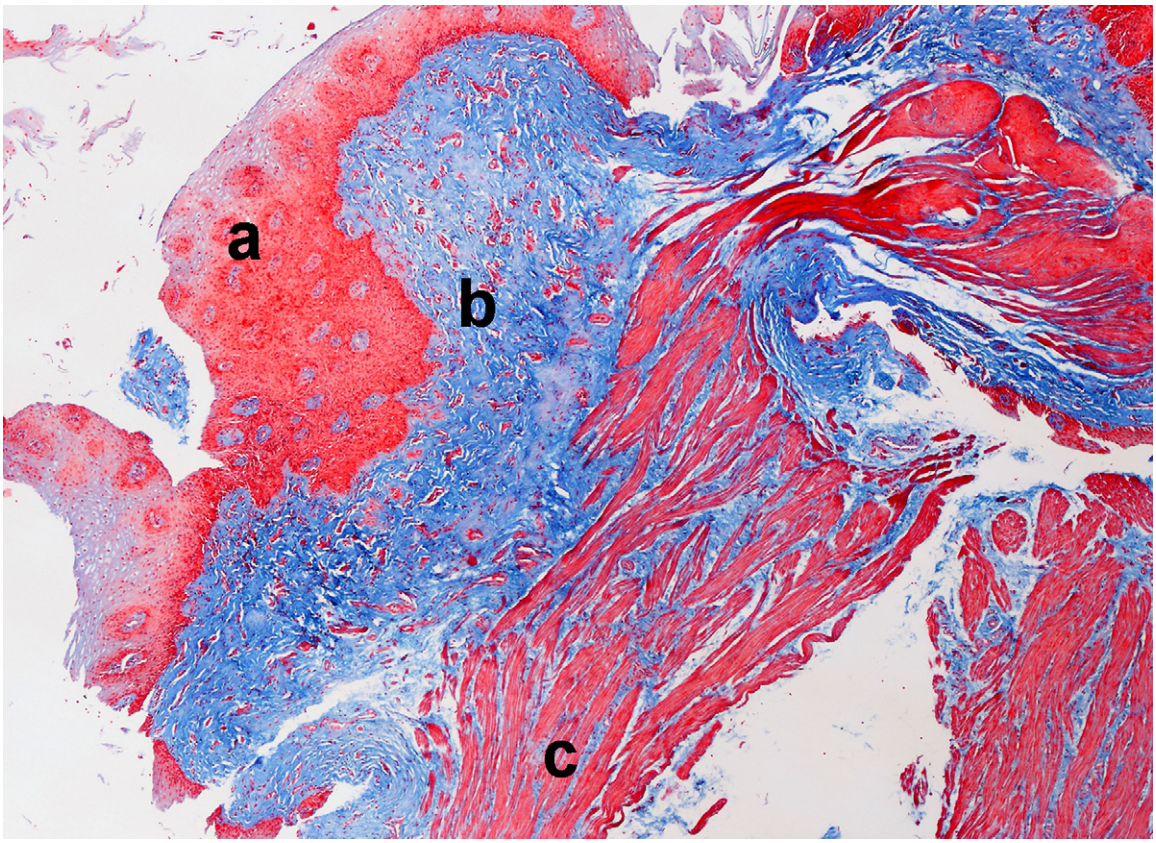

FIG 1. Masson trichrome staining of an endoscopic biopsy sample from an adult with EoE. The biopsy specimen was obtained with a single-side, open-type endoscopic biopsy forceps (Olympus FB-11K-1). We were able to sample deep esophageal tissue, including the epithelial stratum *(a)*, LP *(b)*, and up to the muscularis mucosae *(c)*, where longitudinal smooth muscle fibers can be observed (original magnification ×100).

advised not to self-restrict any food. In cases of asthma exacerbation, only anticholinergic and antihistaminic drugs were allowed.

After 1 year, the endoscopic and esophageal biopsy sampling procedures were repeated as described above.

Histologic study

All the digestive mucosal samples fixed in formalin were routinely processed: sections (5-μm thick) were cut from formalin-fixed, paraffin-embedded blocks and then placed on microscope slides and stained with hematoxylin and eosin. The histologic stains were analyzed by a researcher blinded to the patients' biopsy identities. The peak number of eosinophils was counted in the most densely inflamed areas with the aid of Nikon Eclipse 50i (Nikon Corp, Tokyo, Japan) light microscopy in 3 HPFs at ×400 (the HPF area measured 0.212 mm^2). The mean eosinophil count per HPF was calculated in both epithelial and LP strata by averaging the eosinophil counts in 3 HPFs at the 2 esophageal levels. Results were expressed as cells per square millimeter to provide a standard measure.

The degree of fibrosis was evaluated after tissue staining with the Masson trichrome technique. A semiquantitative estimation of esophageal remodeling in the LP was carried out through individual scoring on a 0- to 3-point scale of several histologic features (Table I) by adapting the method previously described by Aceves et al.[6] Although the full thickness of the LP was analyzed, a more detailed evaluation was made in the subepithelial strata, 150 μm immediately below the basal membrane, which was the area we considered least affected by the biopsy procedure.

We evaluated (1) the amount of collagen bundles, (2) the thickness of such bundles, and (3) the degree of cell loss in fibrotic areas or sclerosis. The partial scores for each feature were added, and the sum was then divided by 3 to establish an overall index (Table I). All biopsy specimens were analyzed by 2 blinded expert pathologists (J. L. Y.-C. and R. A. C.) experienced in studying EoE biopsy samples, who assigned the marks based on consensus.

TABLE I. Fibrosis score measured in esophageal LP

	Fibrosis score (0-3)		
Patients	**Amount of bundles**	**Thickness of bundles**	**Cell density in LP**
0	Normal	Normal	Normal
1 (+)	Lightly increased	Lightly enlarged	Lightly reduced
2 (++)	Moderately increased	Moderately enlarged	Clearly reduced
3 (+++)	Highly increased	Severely enlarged	Near acellular or sclerosis

Two blinded investigators scored fibrosis on a scale from 0 to 3 points on the basis of the amount of collagen bundles, the thickness of such bundles, and the degree of cell loss. The overall index was obtained after averaging the partial scores.

RNA extraction and real-time PCR

Total RNA was extracted in TriReagent (Molecular Research Center, Inc, Cincinnati, Ohio), according to the manufacturer's instructions. After treatment with DNAse I, 1 μg of total RNA was reverse transcribed with random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif) for 1 hour at 42°C.

Quantitative analysis of human *IL5*, *TGFB1*, *FGF9*, and *CCL18* gene expression levels was performed by means of real-time PCR amplification of

TABLE II. Clinical characteristics of adults with EoE included in our study

Patient no.	Age (y)	Sex	Symptom evolution (mo)	Symptoms	Endoscopy		Family history of atopy	Personal history of atopy
					Caliber	Mucosal appearance		
1	48	M	36	Repeated food impaction	R	LF	Father: BA	DS, AR
2	37	M	24	Weekly self-limited food impaction	N	LF, C	Brother: BA, AR, FA	BA, AR
3	36	F	60	Chronic dysphagia self-limited food impaction	N	LF, WP	Sister: AR	BA, AR
4	29	M	300	Repeated food impaction	R	LF	No	BA, AR
5	31	M	48	Food impaction over a stenosis	N	N	No	AR
6	19	M	5	Retrosternal pain and dysphagia	N, Schatzky ring	LF	No	BA, AR
7	18	M	12	Chronic dysphagia and intermittent impaction	N	LF, WP	Brother, ND	AR
8	33	M	120	Long evolution dysphagia	R	C, WP	No	BA
9	23	M	10	Intermittent dysphagia and food impaction	N	LF, C	Brother: BA	No
10	25	M	12	Epigastric pain and chronic dysphagia, frequent food impaction	S	C, WP	3 brothers: BA	BA, AR

AR, Allergic rhinitis; *BA*, bronchial asthma; *C*, crêpe-paper appearance; *DS*, drug sensitivity; *F*, female; *FA*, food allergy; *LF*, longitudinal furrows; *M*, male; *N*, normal; *ND*, not determined; *R*, rings; *S*, stricture; *WP*, white plaques.

the resulting cDNAs in a Light Cycler with FastStart DNA Master SYBR Green I (Roche, Penzburg, Germany). The primers used were as follows: β_2-microglobulin *(B2M)* forward, 5′-CCA GCA GAG AAT GGA AGG TC-3′; *B2M* reverse, 5′-GAT GCT GCT TAC ATG TCT CG-3′; *IL5* forward, 5′-GCT TCT GCA TTT GAG TTT GCT-3′; *IL5* reverse, 5′-CCG TCA ATG TAT TTC TTT ATT AAG-3′; *TGFB1* forward, 5′-GGT GGA AAC CCA CAA CGA AAT C-3′; *TGFB1* reverse, 5′-AAT TCC CCT CCA CGG CTC AAC-3′; *CCL18* forward, 5′-ACA AAG AGC TCT GCT GCC TC-3′; *CCL18* reverse, 5′-CCC ACT TCT TAT GGG GTC A-3′; *FGF9* forward, 5′-AAG GAC TGC GGC CTG ATG-3′; and *FGF9* reverse, 5′-TTT GCT TTA AGT TCA CTG CGA TG-3′.

Standard curves for target mRNA expression were generated by using serial dilutions (1/10) of known quantities of amplified products of the studied genes. Quantification of *IL5*, *TGFB1*, *FGF9*, and *CCL18* gene expression was obtained with the aid of Light Cycler system software (Roche) through interpolation into the respective standard curves. RNA molecules estimated from this quantification were normalized to *B2M* gene expression by calculating the ratio between specific mRNA molecules per microliter of cDNA and molecules of B2M per microliter of cDNA.

Statistical analysis

Results were represented as means ± SDs. Pairwise comparisons between groups were performed with nonparametric tests: the Mann-Whitney *U* test for quantitative variables and the Fisher exact test for nominal variables. For comparison before and after fluticasone treatment, the nonparametric paired Wilcoxon signed-rank test was used. Spearman correlation (rho) was used to test for correlations among variables. All significance tests were 2-sided, with a *P* value of less than .05 indicating statistical significance.

Analyses and summaries were produced with the PASW statistical program, version 18.0 (SPSS, Inc, Chicago, Ill).

RESULTS

We examined a total of 10 patients with EoE (9 men and 1 woman) between 18 and 48 years of age (average, 29.9 years) who had exhibited esophageal symptoms for a mean period of 62.7 months (range, 5-300 months; Table II). The healthy control subjects consisted of 6 men and 4 women averaging 29.6 years of age (range, 15-52 years). The GERD control subjects included 5 men and 5 women averaging 35.3 years of age (range, 25-55 years). After the 1-year treatment period, all patients with EoE reported clinical improvement, with no episodes of food impaction during this time. Esophageal caliber and mucosal appearance in the endoscopy carried out after prolonged fluticasone propionate treatment were normal in all patients with EoE.

Intraepithelial and LP eosinophilic infiltrate and changes after topical steroid treatment

Eosinophil densities in the esophageal epithelium and LP of patients with EoE were significantly higher than those encountered in the GERD and healthy groups (Table III). Statistical differences in eosinophil densities between the upper and lower esophageal thirds were not observed (Table IV). After topical steroid treatment, eosinophil densities were significantly reduced, both intraepithelially and in the LP (Fig 2).

Subepithelial fibrous deposition in patients with EoE and changes induced by topical steroid treatment

All analyzed biopsy samples displayed a representative amount of LP tissue. Subepithelial collagen deposition in patients with EoE was similar in both the upper and lower esophageal thirds and significantly more intense ($P < .001$) than that found in either control group (Tables III and IV). The degree of subepithelial fibrosis was positively and significantly correlated with patient age (Spearman rho, 0.71; $P = .032$), but not with symptom duration (rho, 0.18; $P = .63$).

After prolonged treatment with fluticasone propionate, a small reduction in subepithelial fibrosis was observed, but the differences found were not statistically significant ($P = .086$, Figs 3 and 4).

Multiple linear regression was used to analyze the association between age and treatment-induced changes in collagen deposition in both esophageal thirds. The initial fibrosis being equal, age maintained its association with a positive and

TABLE III. Age and sex of patients, degree of fibrosis, density of eosinophils in esophageal epithelia and LP, and cytokine expression in adults with EoE, patients with GERD, and healthy control subjects

Characteristics	Patients with EoE	Patients with GERD	Healthy subjects	*P* value
Mean age (SD; rank)	29.9 (9.2; 18-48)	35.3 (11.2; 25-55)	29.6 (12.3; 15-52)	.37*
Sex (M/F)	9/1	5/5	3/7	.02*
Intraepithelial eosinophils:				
Cells/mm^2 (SD)	308.0 (117.3)	1.5 (1.9)	0	<.001†
Cells/HPF (SD)	65.29 (24.86)	0.318 (0.41)	0	
Eosinophils in LP				
Cells/mm^2 (SD)	67.8 (32.6)	0.3 (0.7)	0	<.001†
Cells/HPF (SD)	14.37 (6.91)	0.06 (0.15)	0	
Fibrosis score	1.6 (0.3)	0.2 (0.3)	0	<.001†
IL5/B2M	1.46 (3.07)	0	0	<.001†
TGFB1/B2M	1.62 (2.48)	0.20 (0.17)	0.11 (0.17)	.11†
CCL18/B2M	185.0 (258.6)	11.4 (21.9)	1.0 (0.9)	.008†
FGF9/B2M	131.8 (175.0)	48.7 (56.9)	1.1 (1.6)	.005†

*Nonparametric paired Wilcoxon signed-rank test.
†Kruskal-Wallis test.

TABLE IV. Individual data regarding the effect of fluticasone propionate over densities of eosinophils in the esophageal epithelia and LP and fibrosis scores in the upper and lower esophageal thirds expressed as means (SDs)

	Upper esophageal third	*P* value*	Lower esophageal third
Intraepithelial eosinophils			
Baseline			
Cells/mm^2 (SD)	277.5 (175.0)	.58	338.6 (207.0)
Cells/HPF (SD)	58.83 (37.09)		71.78 (43.88)
P value†	.005		.005
After treatment			
Cells/mm^2 (SD)	3.5 (5.0)	.27	7.4 (10.4)
Cells/HPF (SD)	0.742 (1.05)		1.56 (2.21)
Eosinophils in LP cells/mm^2 (SD) and cells/HPF (SD)			
Baseline			
Cells/mm^2 (SD)	67.8 (29.6)	.78	64.5 (49.6)
Cells/HPF (SD)	14.37 (6.28)		13.67 (10.52)
P value†	.005		.008
After treatment			
Cells/mm^2 (SD)	1.7 (2.9)	.04	12.5 (17.2)
Cells/HPF (SD)	0.36 (0.62)		2.65 (3.53)
Fibrosis score			
Baseline	1.6 (0.4)	.24	1.7 (0.4)
P value†	0.33		0.058
After treatment	1.2 (0.7)	.09	1.2 (0.6)

F, Female; *M*, male.
*Nonparametric paired Wilcoxon signed-rank test.
†Kruskal-Wallis test.

significant increase in collagen deposition at the end of treatment ($P = .025$ for the lower third and $P = .035$ for the upper third).

Relationship between eosinophil density and degree of fibrosis

Before treatment, a weak positive, although nonsignificant, correlation between the degree of subepithelial fibrosis and the intraepithelial eosinophil density ($r = 0.577$, $P = .104$) was observed; this was not the case for LP eosinophil density ($r = 0.117$, $P = .764$). The relationship between eosinophils in the LP and the degree of fibrosis was stronger and reached significance after treatment ($r = 0.741$, $P = .014$).

Expression of profibrogenic cytokines and changes induced by prolonged steroid treatment

Patients with EoE showed a significant upregulation in the gene expression of *IL5* ($P < .001$), *FGF9* ($P = .005$), and *CCL18* ($P = .008$) compared with that seen in healthy control subjects. Expression of *TGFB1* also increased, although not significantly ($P = .11$), in comparison with that seen in the healthy control subjects and patients with GERD (Table III and Fig 5).

After prolonged treatment with fluticasone propionate, downregulation of gene expression was observed for the 4 cytokines analyzed, but only *CCL18* mRNA levels were reduced significantly ($P < .001$, Fig 5).

In addition, associations between various parameters were explored in patients with EoE. Thus, at basal conditions, the densities of eosinophils in the LP of patients with EoE strongly and directly correlated with gene expression levels of both *IL5* ($r = 0.952$, $P = .0001$) and *TGFB1* ($r = 0.786$, $P = .021$). A mild association with CCL18 gene expression was also observed ($r = 0.643$, $P = .086$).

Baseline gene expression of *IL5* strongly correlated with *TGFB1* ($r = 0.881$, $P = .004$) and *FGF9* ($r = 0.762$, $P = .028$) gene expression. Furthermore, a strong positive correlation was observed between *CCL18* and *FGF9* gene expression ($r = 0.81$, $P = .015$).

DISCUSSION

This work analyzes, for the first time, esophageal subepithelial fibrosis in adults with EoE, along with the changes induced by prolonged topical steroid treatment affecting collagen deposition and profibrogenic cytokine gene expression. Previous studies had demonstrated that children with EoE already exhibited a significant deposition of subepithelial collagen.[5,6] Our work shows that adults with EoE, who presumably but not always have had a longer evolution of eosinophilic inflammation in the esophagus, also present a subepithelial collagen deposition significantly higher than that observed under normal conditions or in patients affected by GERD.

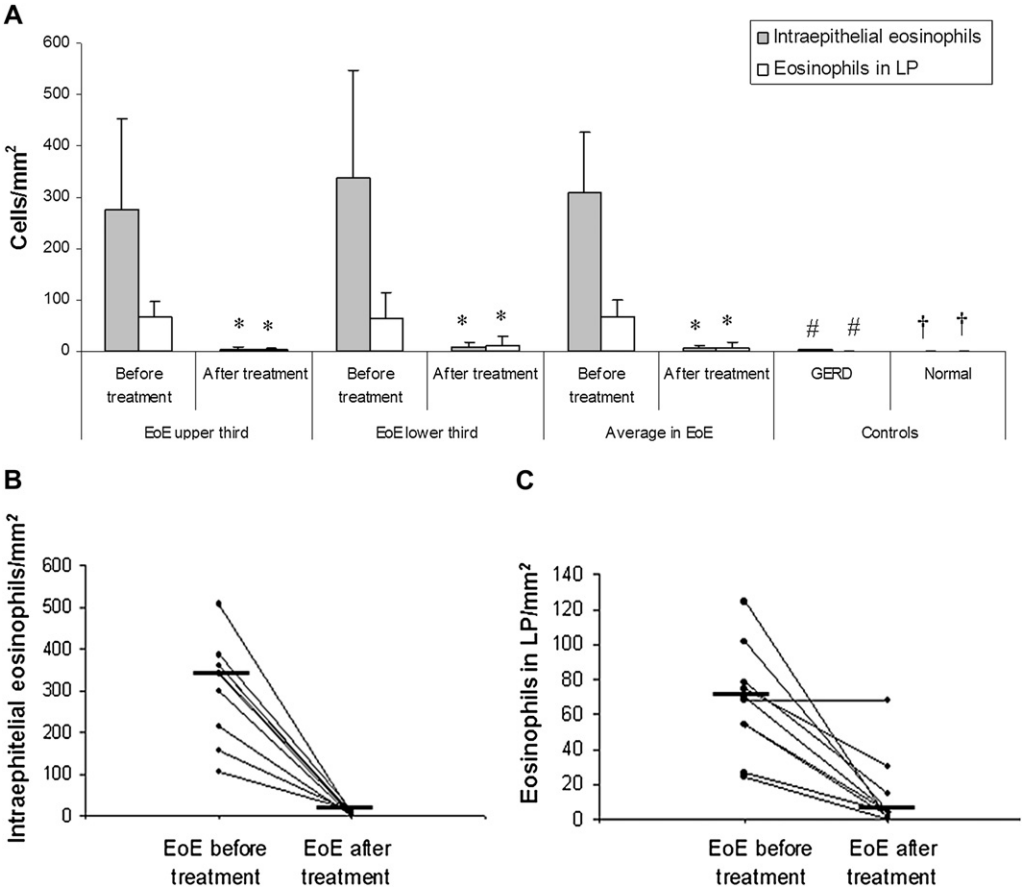

FIG 2. A–C, Density of intraepithelial and LP eosinophils in patients with EoE before and after treatment with fluticasone propionate compared with that seen in patients with GERD and healthy subjects expressed as an average ± SD. Because the data followed a normal distribution, they were checked with the Shapiro-Wilk test. *Statistically significant differences ($P < .05$) before and after treatment in patients with EoE. #Statistically significant differences ($P < .05$) between patients with EoE before treatment and patients with GERD. †Statistically significant differences ($P < .05$) between patients with EoE before treatment and normal esophagi.

Collagen deposition increases with patient age but not with symptom duration. It is also not significantly reduced after a 1-year treatment with fluticasone propionate. To date, research on pediatric patients with EoE has shown no correlations between symptom duration and any markers of remodeling in children in which the average duration of symptoms was 3.2 years before correct diagnosis of EoE.[6] Moreover, no association was found between duration of symptoms before diagnosis and response to therapy.[18]

Technical limitations in obtaining tissue samples, including LP, in esophageal biopsies performed in patients with EoE have until now restricted systematic studies about the fibrous remodeling caused by the chronic eosinophilic inflammation that characterizes this disease. Until now, research has mainly consisted of short case series retrospectively selected from histologic sample collections because they displayed LP tissue; prospective studies in adults with EoE have displayed representative LP tissue in only half of the biopsy samples.[7,8] Moreover, standard endoscopic biopsy forceps only allow sampling of the most superficial strata of the esophageal mucosa because the opening of the forceps is parallel to the organ lumen. Our study is the first to make use of a single-side, open-type endoscopic biopsy forceps capable of systematically sampling deep esophageal tissue, reaching down to the muscularis mucosae in each case. This facilitates a more precise analysis of the subepithelial tissue.

With these techniques, we were able to observe that eosinophilic infiltrate permeates the LP in adults with EoE, although its density there is approximately one fifth of that documented in the epithelium. Nevertheless, eosinophils at this level seem capable of inducing fibroblast proliferation and collagen deposition[19]; eosinophil density in the epithelium and especially in the LP tended to correlate directly with the subepithelial fibrosis score. Moreover, eosinophil density in the LP directly correlated with the gene expression of profibrogenic cytokines.

Recently published research on children with EoE has explored the effects of treatment with topical steroids[19,20] or elimination diets[20] on subepithelial fibrous remodeling. In a retrospective study Aceves et al[18] observed that children whose epithelial eosinophilia was resolved after at least 3 months of budesonide therapy showed significantly reduced esophageal remodeling, as well as decreases in fibrosis, TGF-$\beta 1^+$ and phosphorylated Smad2/3$^+$ cells, and vascular activation compared with those patients in whom the eosinophilic infiltrate persisted. Our study found similar results in adults, in whom a positive relationship between

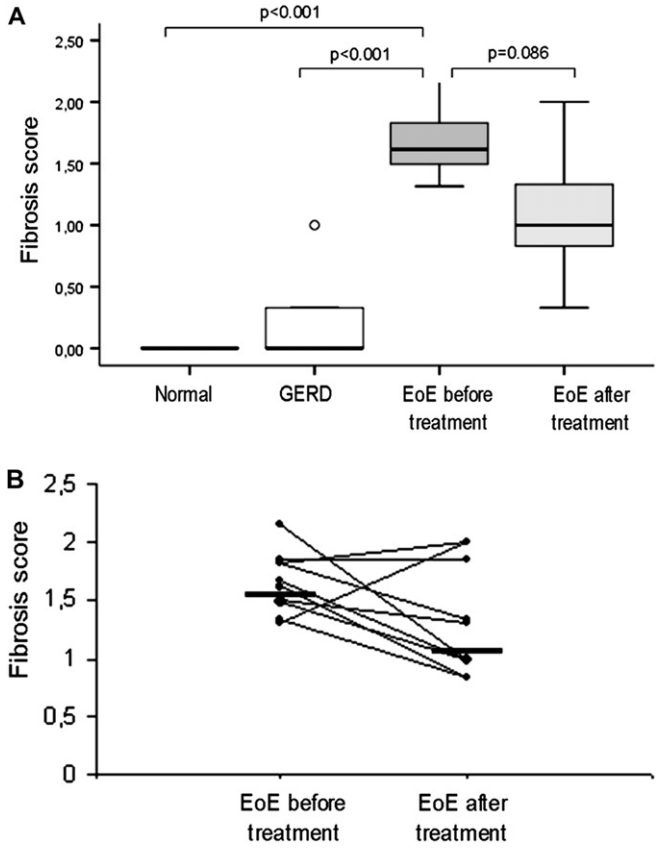

FIG 3. Mean fibrosis scores in healthy subjects, patients with GERD, and patients with EoE before and after 1 year of topical treatment with fluticasone propionate. **A**, Median and interquartile ranges are represented in the boxes, with whiskers *(vertical lines)* extending to a limit of ± 1.5 interquartile range. **B**, Individual changes in fibrosis scores induced by prolonged fluticasone propionate treatment in patients with EoE. *Bars* represent means.

eosinophil density and fibrosis score was observed both before and after treatment.

Furthermore, recent research carried out on adults with EoE has shown decreases in TGF-β1 protein expression after long-term treatment with topical budesonide.[7,8] However, as we observed, fibrous remodeling tended to persist over time.[7,8] These results, which seem contradictory at first, will be discussed in more detail below.

Our study also analyzed the gene expression of several molecules with profibrogenic effects in patients with EoE. To date, only 1 study developed in a murine model of EoE has explored the function of IL-5 in esophageal fibrous remodeling.[4] The expression of this cytokine, which plays a central role in all phases of eosinophil biology, directly and significantly correlated with the expression of the other cytokines studied. Likewise, until now, the only profibrogenic cytokine studied in human EoE has been TGF-β1, which exhibits increased expression, as evidenced by the immunohistochemistry of EoE biopsy specimens from pediatric and adult patients,[6-8] together with Smad2/3, its immediate downstream signaling molecule. TGF-β1 promotes the migration and proliferation of fibroblasts, foments collagen synthesis, and reduces the degradation of the extracellular matrix.

We found that *TGFB1* gene expression was upregulated in adults with EoE in comparison with that seen in both control groups. However, the difference did not reach statistical significance, probably because of the small number of our series and the wide individual variations in gene expression levels. Previous studies carried out on pediatric patients and adults with EoE conceded to TGF-β1 a central role in the origin of fibrous remodeling after observing significant differences in TGF-β1 immunostaining between patients with EoE and control subjects.[6-8,18] This does not necessarily contradict our results, however, especially when one takes into account that these previous studies used different techniques to evaluate TGF-β1 expression (demonstrating the transcription of the *TGFB1* gene vs the actual presence of the translated protein). At any rate, TGF-β1 is secreted in a latent form, and its activation is mediated by different factors, including matrix metalloproteases, tissue inhibitor of metalloproteases, and other proteins implicated in fibrosis and tissue remodeling. Additionally, some cytokines, such as IL-13, have been demonstrated to activate a mechanism of tissue fibrosis that is completely independent from TGF-β1.[21] Further study is thus necessary, especially because IL-13 seems to play a central role in the pathophysiology of EoE.[22,23]

We also examined, for the first time, the gene expression of other profibrogenic molecules that have not been studied previously in conjunction with fibrous remodeling in patients with EoE. Thus gene expression of FGF-9, a molecule implicated in tissue repair after the epithelial lesion associated with EoE and that is regulated by eosinophil-derived MBP,[12] was upregulated in patients with EoE in comparison with that seen in the control groups ($P < .005$) and was relevantly reduced after treatment. Baseline *FGF9* gene expression significantly correlated with *IL5* and *CCL18* gene expression, suggesting that there are common regulatory pathways for these cytokines. The most significant results, however, were for *CCL18*. This chemokine has been related to bronchial asthma,[24,25] atopic dermatitis,[26] and other chronic fibrosing diseases, such as scleroderma[27] and encapsulating peritoneal sclerosis,[28,29] but until now, its role in esophageal diseases was unknown. We have observed that *CCL18* was the most highly upregulated gene in patients with EoE when compared with both control groups ($P = .008$), with a significant downregulation after long-term topical steroid treatment ($P = .01$). *CCL18* constitutes a stimuli for fibroblast proliferation[29] and collagen synthesis by pulmonary fibroblasts[16,30] through a mechanism that seems to be independent from *TGFB1*.[31] In fact, we observed no significant association between the gene expression of these 2 molecules ($r = 0.381$, $P = .352$). Our results showed a significant reduction in *CCL18* gene expression after treatment, which was, in turn, associated with a reduction in the number of eosinophils in the infiltrate, especially at the level of the LP, while fibrosis persisted. A nonsignificant reduction in collagen staining was also detected. It is possible that a longer follow-up period is needed to detect a significant reduction of fibrosis in the esophagus. In any case our study suggests that the esophageal remodeling phenomenon observed in patients with EoE is complex and involves different molecules interacting with fibroblasts to achieve the final goal of repairing the tissue damage caused by the eosinophil-mediated inflammatory response.

Our study was specifically designed to analyze the effect of long-term therapy with topical steroids on collagen deposition in the LP of adults with EoE. Thus adults with EoE naive to both steroid treatment and long-term therapy were needed. Subepithelial collagen deposition in adults with EoE was not found to be

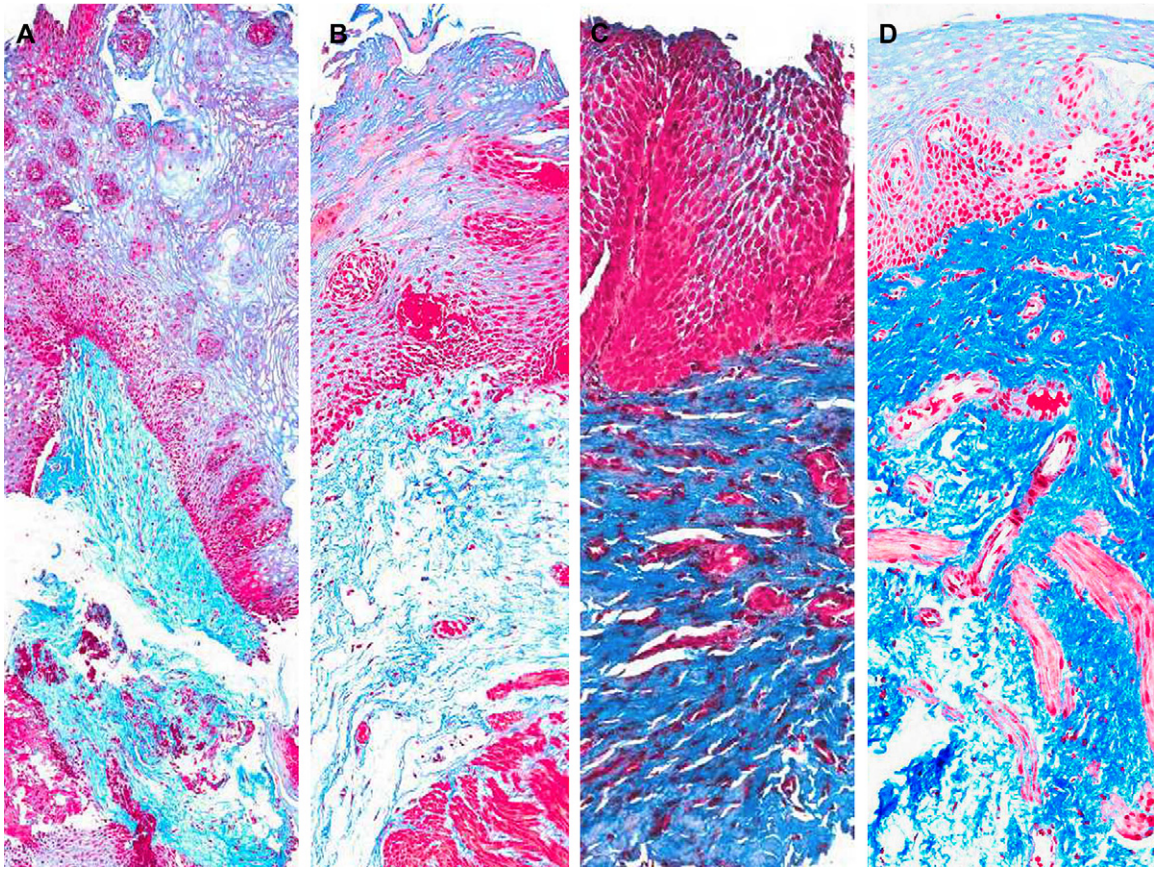

FIG 4. Masson trichrome staining of esophageal biopsies obtained from a subject with a normal esophagus **(A)**, a patient with GERD **(B)**, and a patient with EoE before **(C)** and after **(D)** 1 year of topical treatment with fluticasone propionate.

significantly reduced after treatment in our study despite the marked reduction in eosinophil density in both the epithelium and the LP, along with the downregulation of profibrogenic cytokines. These results are similar to those obtained in studies on patients with bronchial asthma treated with inhaled topical steroids,[32-34] in which collagen deposits persisted in spite of treatment.

Topical fluticasone propionate was extremely effective in reducing intraepithelial eosinophil density in all patients to the same levels observed under normal conditions (Fig 2, *B*). However, this effect was not the same in the LP because some degree of eosinophilic infiltration persisted in a subgroup of 3 patients despite prolonged treatment (Fig 2, *C*). This posttreatment persistence of a low degree of eosinophilic infiltration into the LP of some patients might constitute a continued stimulus for profibrogenic cytokine gene expression (as shown in Fig 5) and could lead to the persistence of fibrosis. We currently lack the data to determine whether a long-term systemic steroid treatment would be effective enough to achieve a deeper reversion of fibrous remodeling compared with a topically administered one, although our correlation data suggest this as a plausible hypothesis.

Remodeling phenomena alter the elastic properties of an organ's walls,[35] which could have important clinical repercussions in patients with EoE. We have limited data on the natural history of EoE, but it is accepted that although chronic eosinophilic inflammation of the esophagus persists over time, its symptoms can fluctuate.[35] Patients who have undergone long-term follow-up tend to show symptomatic impairment and eventually exhibit a worse response to therapy.[9] This could be the result of maintained or progressive fibrosis. In contrast, studies carried out on pediatric patients with EoE have shown a response to treatment characterized by remission of eosinophilic infiltrate and significant reduction of fibrosis.[18,20] A randomized blinded design including a control group receiving a placebo could clarify many questions about the progression of fibrosis in patients with EoE, but such a study is unfeasible because of ethical concerns.

Steroids are highly effective drugs in reducing inflammation in several diseases, including bronchial asthma[36] and EoE, in which they usually lead to clinical improvement and histologic normalization of epithelium.[10,37,38] However, lack of standardization in treatment duration, variability in prescribed dosages,[39-41] and, most importantly, technical limitations in assessing the deepest mucosal layers in both diseases have impeded our efforts to gain a better insight into the range of possibilities for reversing subepithelial fibrosis. In asthma the problems are due to the difficulties in obtaining biopsy specimens from the most distal and smallest bronchi, which are those most affected by fibrous remodeling, whereas in patients with EoE, they are due to limitations on the systematic sampling of deep biopsy specimens.

Results to date, however, seem to indicate that the fibrous remodeling associated with pediatric EoE might be initially

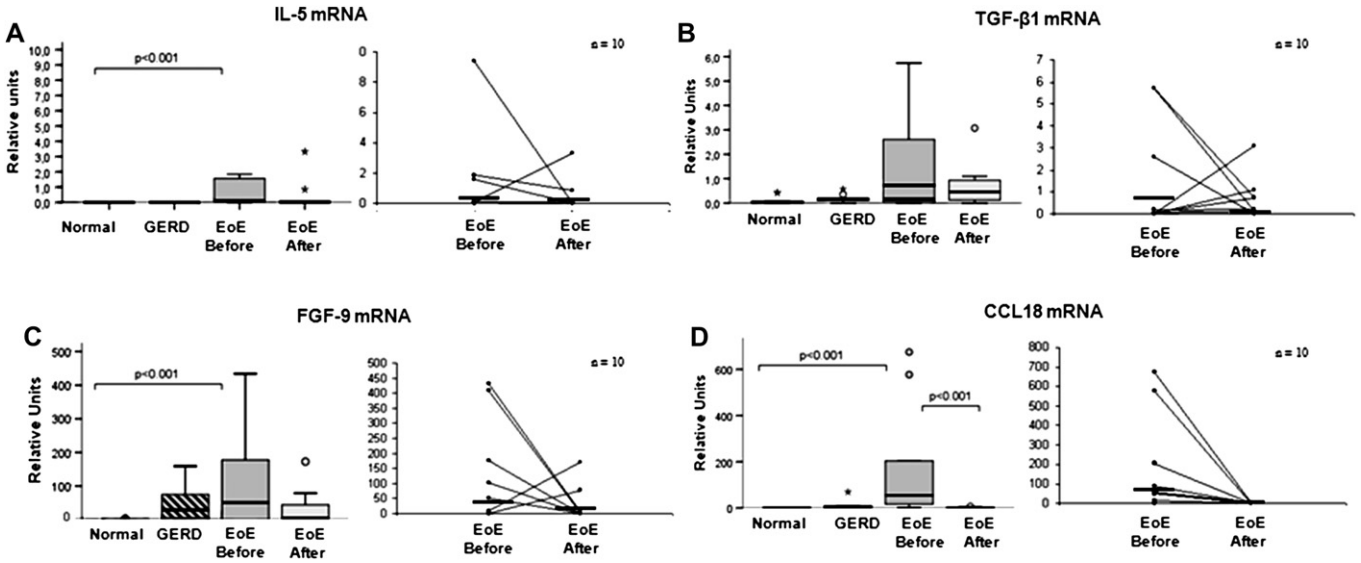

FIG 5. Expression of profibrogenic cytokines in patients with EoE, patients with GERD, and control subjects *(Normal)* and changes induced in patients with EoE by prolonged treatment with fluticasone propionate (EoE before *[EoE Before]* and EoE after *[EoE After]* fluticasone propionate). Median and interquartile ranges are represented in the boxes, with whiskers *(vertical lines)* extending to a limit of ± 1.5 interquartile range. Individual changes in cytokine gene expression are provided. *Horizontal bars* represent means. **A**, IL-5; **B**, TGF-β1; **C**, FGF-9; **D**, CCL18. *Individual extreme values.

reversible after topical steroid treatment. In due course, then, fibrosis in younger patients might be stabilized, whereas in adult patients prolonged anti-inflammatory treatment does not seem to reverse this remodeling. Further research, including appropriate biopsy specimens, should focus on the true natural history and long-term complications of this interesting disease.

Key messages

- **Adults with EoE exhibit a dense subepithelial collagen deposition that is significantly higher than that observed under normal conditions or in patients with GERD.**
- **Profibrogenic cytokine gene expression of *IL5*, *FGF9*, and especially *CCL18* was upregulated in biopsy specimens from adults with EoE, but no significant change was observed for *TGFB1* expression.**
- **The intensity of subepithelial collagen deposition is positively correlated with patient age but not with duration of symptoms.**
- **Prolonged fluticasone propionate treatment reversed intraepithelial and LP eosinophilic infiltration and downregulated profibrogenic cytokine gene expression. However, no significant change in subepithelial collagen deposition was observed.**

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