Eosinophilic esophagitis: Epithelial mesenchymal transition contributes to esophageal remodeling and reverses with treatment

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Background: Mechanisms underlying esophageal remodeling with subepithelial fibrosis in subjects with eosinophilic esophagitis (EoE) have not been delineated.

Objectives: We sought to explore a role for epithelial mesenchymal transition (EMT) in subjects with EoE and determine whether EMT resolves with treatment.

Methods: Esophageal biopsy specimens from 60 children were immunostained for epithelial (cytokeratin) and mesenchymal (vimentin) EMT biomarkers, and EMT was quantified. Subjects studied had EoE (n = 17), indeterminate EoE (n = 15), gastroesophageal reflux disease (n = 7), or normal esophagus (n = 21). EMT was analyzed for relationships to diagnosis, eosinophil counts, and indices of subepithelial fibrosis, eosinophil peroxidase, and TGF-β immunostaining. EMT was assessed in pretreatment and posttreatment biopsy specimens from 18 subjects with EoE treated with an elemental diet, 6-food elimination diet, or topical corticosteroids (n = 6 per group).

Results: TGF-β1 treatment of esophageal epithelial cells *in vitro* for 24 hours induced upregulation of mesenchymal genes characteristic of EMT, including N-cadherin (3.3-fold), vimentin (2.1-fold), and fibronectin (7.5-fold). EMT in esophageal biopsy specimens was associated with EoE (or indeterminate EoE) but not gastroesophageal reflux disease or normal esophagus and was correlated to eosinophil counts ($r = 0.691$), eosinophil peroxidase ($r = 0.738$), and TGF-β ($r = 0.520$) immunostaining and fibrosis ($r = 0.644$) indices. EMT resolved with EoE treatments that induced clinicopathologic remission with reduced eosinophil counts. EMT decreased significantly after treatment by 74.1% overall in the 18 treated subjects with EoE; pretreatment versus posttreatment EMT scores were 3.17 ± 0.82 versus 0.82 ± 0.39 ($P < .001$), with similar decreases within treatment groups. Pretreatment/posttreatment EMT was strongly correlated with eosinophil counts for combined ($r = 0.804$, $P < .001$) and individual treatment groups.

Conclusions: EMT likely contributes to subepithelial fibrosis in subjects with EoE and resolves with treatments that decrease esophageal inflammation, and its resolution correlates with decreased numbers of esophageal eosinophils. (J Allergy Clin Immunol 2012;129:1387-96.)

Key words: *Eosinophil, esophagitis, epithelium, remodeling, fibrosis, mesenchymal, vimentin, cytokeratin*

Eosinophilic esophagitis (EoE) has emerged as an increasingly recognized immune-mediated food allergy– or aeroallergen-associated chronic inflammatory disorder of the esophagus.[1,2] Prolonged unbridled esophageal inflammation can lead to structural and functional changes, including thickening of the mucosa and muscularis, dysmotility, decreased compliance, food impaction, and strictures.[3-5] A variety of clinical presentation patterns ranging from feeding difficulties in toddlers to solid-food dysphagia and food impaction in adolescents and adults suggests that structural and functional changes might be part of the natural history of EoE.[6] This is further supported by pediatric studies showing that subepithelial fibrosis occurs in greater than 50% of

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Abbreviations used
ED: Elemental diet
EMT: Epithelial mesenchymal transition
EoE: Eosinophilic esophagitis
EPX: Eosinophil peroxidase
GERD: Gastroesophageal reflux disease
hpf: High-power field
PPI: Proton pump inhibitor
RT-Q-PCR: Reverse-transcriptase quantitative polymerase chain reaction
SFED: Six-food elimination diet
TC: Topical corticosteroid

children with EoE.[7,8] Understanding the mechanisms leading to subepithelial fibrosis in patients with EoE could lead to identification of novel therapeutic targets.

Epithelial mesenchymal transition (EMT) describes a series of events during which epithelia lose many epithelial characteristics, including polarity, expression of epithelial markers, and tight junctions, and acquire properties of mesenchymal cells, including motility, loose cell adhesion through N-cadherin, and depolarized cytoskeletal arrangements, such as vimentin.[9] EMT facilitates the development of tissue fibrosis in different organ systems in response to injury and chronic inflammation and is associated with the development of fibrosis in the kidney, lung (idiopathic pulmonary fibrosis and asthma), liver, heart (cardiac fibrosis), and gastrointestinal tract (Crohn disease).[10-12] Whether EMT occurs in the esophagus and contributes to subepithelial fibrosis and remodeling in patients with EoE has not been explored.

The purposes of this study were to determine whether EMT occurs in children with EoE and, if successful, whether treatment of EoE (symptoms and histologic remission) results in resolution of EMT. Results demonstrate that EMT occurs to a significantly greater degree in the esophageal tissues of children with EoE compared with those of children with gastroesophageal reflux disease (GERD) or those with normal esophageal tissue. The degree of EMT correlates with traditional measures of esophageal inflammation and remodeling in patients with EoE, including eosinophil number, expression of remodeling factors (TGF-β), and extent of subepithelial fibrosis. EMT resolves in patients with EoE in response to treatments that decrease esophageal inflammation, as characterized by decreases in eosinophil burden.

METHODS

Cell culture and induction of EMT *in vitro*

Human esophageal epithelial HET-1A cells (American Type Culture Collection, Manassas, Va) were maintained in Bronchial Epithelial Growth Media without gentamycin-amphotericin B (Lonza/Clonetics, Walkersville, Md). For mRNA analysis, cells at confluence in 6-well plates were cultured an additional 24 or 48 hours in fresh media with or without 5 ng/mL TGF-β1 (R&D Systems, Minneapolis, Minn). Expression of mRNAs encoding adhesion and cytoskeletal proteins representative of epithelial cells (E-cadherin, cytokeratin 8, and cytokeratin 14) and mesenchymal cells (N-cadherin, vimentin, and fibronectin) as biomarkers of EMT were analyzed in total RNA by using real-time quantitative RT-PCR (RT-Q-PCR).

RT-Q-PCR

Total RNA was prepared by using QIAshredder columns and the RNeasy Mini RNA Isolation Kit (Qiagen, Valencia, Calif), and cDNA was synthesized by using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, Calif).[13] Briefly, from 500 ng of RNA, gene transcripts were assessed by using TaqMan Gene Expression Assay FAM dye-labeled TaqMan MGB probes (Applied Biosystems; see Table E1 in this article's Online Repository at www.jacionline.org) and ABsolute Blue QPCR ROX MasterMix (Thermo Scientific, Surrey, United Kingdom). Thermocycling and analysis was performed with an ABI-7300 system. Data were normalized to 18S expression and calculated as relative quantity ($2^{-\Delta\Delta Ct}$, where Ct is defined as the cycle threshold).

Study populations and design: Clinical biopsy specimens

A retrospective analysis of 890 archived esophageal biopsy specimens from pediatric subjects at Children's Hospital Colorado from 2006 was performed. Of these, tissue sections from 60 subjects' biopsy specimens with more than 2 mm of lamina propria were analyzed based on the following: (1) availability of sufficient formalin-fixed, paraffin-embedded tissue and (2) the subject's diagnosis of EoE, indeterminate EoE, GERD, or normal esophagus. Diagnostic criteria were as follows: active symptomatic EoE, 15 or more eosinophils/high-power field (hpf) and other causes excluded; indeterminate symptomatic EoE, less than 15 eosinophils/hpf and clinical features suggestive of EoE or clinical features of EoE and 15 or more eosinophils/hpf without documented treatment with proton pump inhibitors (PPIs) or pH probe to exclude GERD[1,14]; GERD–PPI-responsive esophagitis, less than 15 eosinophils/hpf; and healthy control subjects, subjects undergoing clinically indicated endoscopy but with an endoscopically and histologically normal esophagus.

Eighteen pediatric subjects with EoE were randomly selected from the EoE patient database at Children's Memorial Hospital in Chicago who had achieved histologic remission after treatment with an elemental diet (ED), empiric 6-food elimination diet (SFED), or topical corticosteroids (TCs) to assess the effect of treatment on EMT (n = 6 per group). Diagnostic criteria for EoE were as above, with histologic remission defined as 10 or fewer eosinophils/hpf. Posttreatment biopsy specimens were obtained from the mid and distal esophagus after at least 6 weeks of treatment and pretreatment and posttreatment tissue sections immunostained for EMT.

Assessment of EMT: EMT index

Three-color immunofluorescence and confocal microscopy were used to identify and evaluate epithelial and mesenchymal cells by using cytokeratin (epithelial), vimentin (mesenchymal), and 4′-6-diamidino-2-phenylindole dihydrochloride (nuclear) stains in esophageal biopsy tissue sections. A 6-point scale was developed to score the amount of EMT, assessing the presence, location, and degree of vimentin-positive mesenchymal cells and loss of cytokeratin staining of epithelial cells in the context of hyperplastic changes in epithelial architecture (Fig 1). Confocal microscopy was used to acquire fluorescent images of 18 to 25 hpfs covering the entirety of each tissue section. Confocal images of stained sections were analyzed in a blinded manner by 2 independent observers (N.A. and K.R.P.) and scored for EMT; mean EMT indices per hpf were calculated.

Assessment of eosinophil counts in biopsy specimens

Eosinophils in hematoxylin and eosin–stained slides were quantified in hpfs (area, 0.26 mm^2) by counting the 5 most densely populated regions of the tissue, and peak (highest in single section) and mean values were recorded.

Anti–eosinophil peroxidase immunohistochemistry: Eosinophil peroxidase index

Sections from esophageal biopsy specimens were stained with anti-eosinophil peroxidase (EPX) mAb (hybridoma MM25-82.2.1; Mayo Clinic, Phoenix, Ariz).[15] On the basis of the presence of eosinophils, evidence of

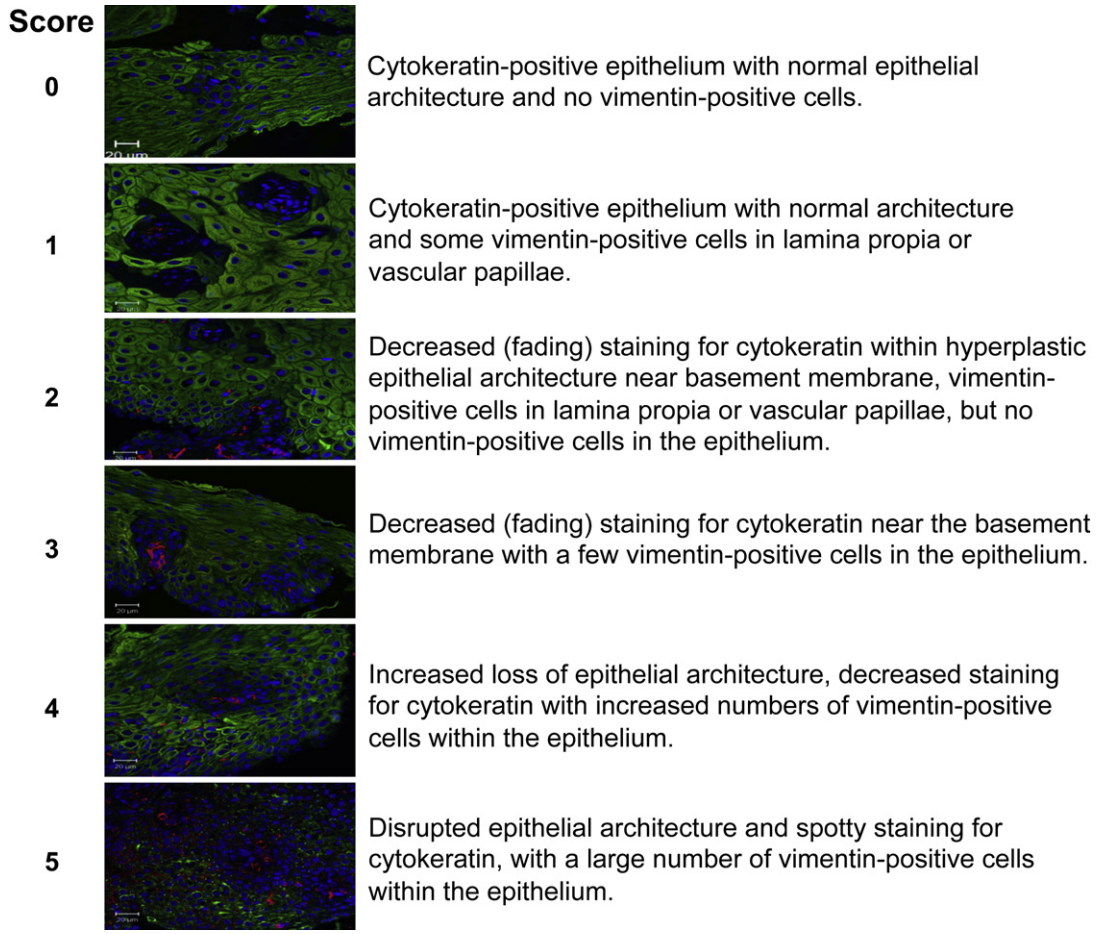

FIG 1. Six-point EMT assessment scale for quantitation in esophageal biopsy specimens. Representative merged confocal images of immunofluorescent staining for cytokeratin *(green)* and vimentin *(red)* with 4′-6-diamidino-2-phenylindole dihydrochloride–stained nuclei *(blue)* is shown. The EMT score is indicated on the *left,* with descriptions of the characteristics of EMT biomarker staining relative to changes in epithelial architecture on the *right.* The score incorporates (1) the location and amount of vimentin-positive (mesenchymal marker) cell staining within the epithelium and (2) decreased cytokeratin (epithelial marker) staining in hyperplastic epithelium.

eosinophil degranulation, and extent of eosinophil infiltration and/or degranulation, an EPX index was assigned (by C.A.P.) to each subject's biopsy specimen, as previously described.[15]

Fibrosis index

Hematoxylin and eosin–stained sections were used to assess the degree of fibrosis in esophageal biopsy specimens. A fibrosis score of 0 to 2 was assigned by 3 independent blinded observers (S.A.W., V.M., and J.C.M.) based on the number of fibroblasts, thickness, and the character of collagen bundles and collagen accumulation, as previously described.[8] A fibrosis score of 0 indicated loose, lacy individual collagen fibrils; a score of 1 indicated more densely packed collagen fibrils along the basal lamina with loss of individual laciness but further away from the basal lamina normalized to lacy individual fibrils; and a fibrosis score of 2 indicated tightly packed collagen fibrils with individual fibrils no longer evident (see Fig E1 in this article's Online Repository at www.jacionline.org).

TGF-β1 immunohistochemistry: TGF-β1 index

Sections from esophageal biopsy specimens were stained with anti–TGF-β1 antibody (catalog no. 500-M66; Peprotech, Rocky Hill, NJ), as previously described,[16] and scored by 3 independent blinded observers (S.A.W., V.M., and J.C.M.). A 3-point scoring system was used based on staining of the epithelium. A score of 1 (mild staining) corresponded to blue/light brown epithelium, a score of 2 (moderate staining) corresponded to darker brown epithelium, and a score of 3 (severe staining) corresponded to dark brown staining throughout the entire epithelium.

Statistical analyses

Data were analyzed by using ANOVA with the Bonferroni multiple comparisons test or the 2-tailed Student *t* test. Differences between means were considered significant at a P value of less than .05. Relationships between the EMT index, eosinophil counts, and staining indices for EPX, fibrosis, and TGF-β were analyzed by using the Pearson test; correlation coefficients (r values) were considered significant at a P value of less than .05.

RESULTS

EMT is induced by TGF-β in cultured esophageal epithelial cells

To determine whether esophageal epithelium has the capacity to undergo EMT, we analyzed the ability of TGF-β1 to induce

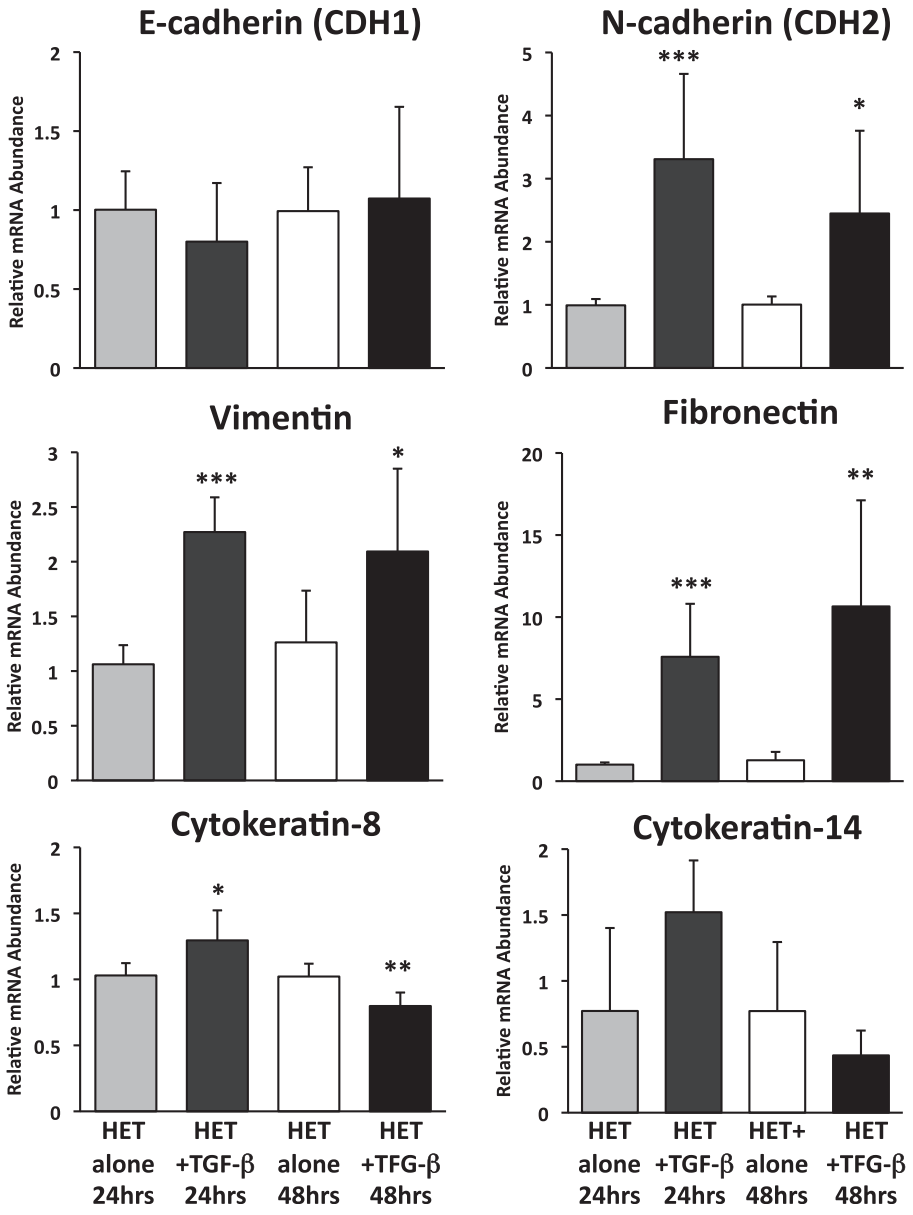

FIG 2. Induction of mesenchymal genes in esophageal epithelial cells in culture: evidence for EMT *in vitro*. Analysis of adhesion molecules and cytoskeletal component expression representative of epithelial cells (E-cadherin, cytokeratin 8, and cytokeratin 14) and mesenchymal cells (N-cadherin, vimentin, and fibronectin) in HET-1A cells after 24 and 48 hours of culture with TGF-β1 (5 ng/mL) is shown. Data are expressed as mean ± SD relative mRNA abundance compared with that in untreated control subjects, as determined by using RT-Q-PCR. Statistical significance was assessed by using the Student *t* test compared with untreated control subjects at 24 and 48 hours. *$P < .05$, **$P < .01$, and ***$P < .001$ (n = 5-7 per group).

EMT *in vitro* in the HET-1A esophageal epithelial cell line. Culture of HET-1A cells with TGF-β1 decreased gene transcription for epithelial biomarkers, including adhesion proteins and cytoskeletal components representative of the epithelial phenotypes cytokeratin 8 (22% decrease, $P < .01$) and cytokeratin 14 (44% decrease, $P = .27$; Fig 2). Correspondingly, increased mRNA expression for a number of biomarkers representative of the mesenchymal phenotype, including N-cadherin (adhesion; 3.3-fold, $P < .001$), vimentin (cytoskeletal; 2.1-fold, $P < .001$), and fibronectin (extracellular matrix; 7.5-fold, $P < .001$), was detected (Fig 2), all of which are gene expression changes characteristic of metastable EMT.[17-19]

EMT is present in esophageal tissue from subjects with active EoE

Clinical characteristics of the 60 subjects studied based on diagnostic criteria are shown in Table I. Subjects ranged from 8 months to 22 years old with a duration of symptoms ranging from 2 months to 7 years. Treatment histories for subjects with active EoE included PPIs, TCs, and elimination of allergenic foods, whereas subjects with normal esophagus, GERD, and indeterminate EoE had only been treated with PPIs. Pretreatment and posttreatment biopsy specimens from an additional 18 randomly selected pediatric subjects with EoE were analyzed to determine the effect of treatments for EoE on EMT. Patients ranged in age from 6 to 13

TABLE I. Clinicopathologic characteristics of the subject study groups

	Patients with normal esophagus	Patients with GERD	Patients with EoE (indeterminate)*	Patients with EoE
No. (n = 60)	21	7	15	17
Sex (male/female)	10/11	7/0	8/7	10/7
Age range	8 mo-15 y	1-18 y	22 mo-21 y	1-15 y
Duration of symptoms	3 mo-6 y	2 mo-2 y	1 d-3 y	1-7 y
Previous treatments	Zantac (ranitidine), Prevacid (lansoprazole), Prilosec (omeprazole), Reglan (metoclopromide), Zyrtec (cetirizine), Miralax (polyethylene glycol)	Zantac, Prevacid, Prilosec, Protonix (pantoprazole)	None, unknown, Zantac, TUMS, Prevacid	Zantac, Prevacid, Prilosec, swallowed Flovent (fluticasone), Singulair, elimination of foods identified as allergic
No. of eosinophils (peak/hpf range)	0-2	0-30	0-87	7-123

Demographic and clinical features of the 60 subjects evaluated for EMT, fibrosis, TGF-β, EPX index, and eosinophil counts. The subject groups include patients undergoing a clinically indicated endoscopy with biopsy but with an otherwise histologically normal esophagus and those with recorded diagnoses of GERD, indeterminate EoE, and EoE. *EoE (indeterminate) represents subjects who had many clinical features of EoE but lacked the threshold number of greater than 15 eosinophils/hpf or had no documented treatment with acid suppression.[1,14]

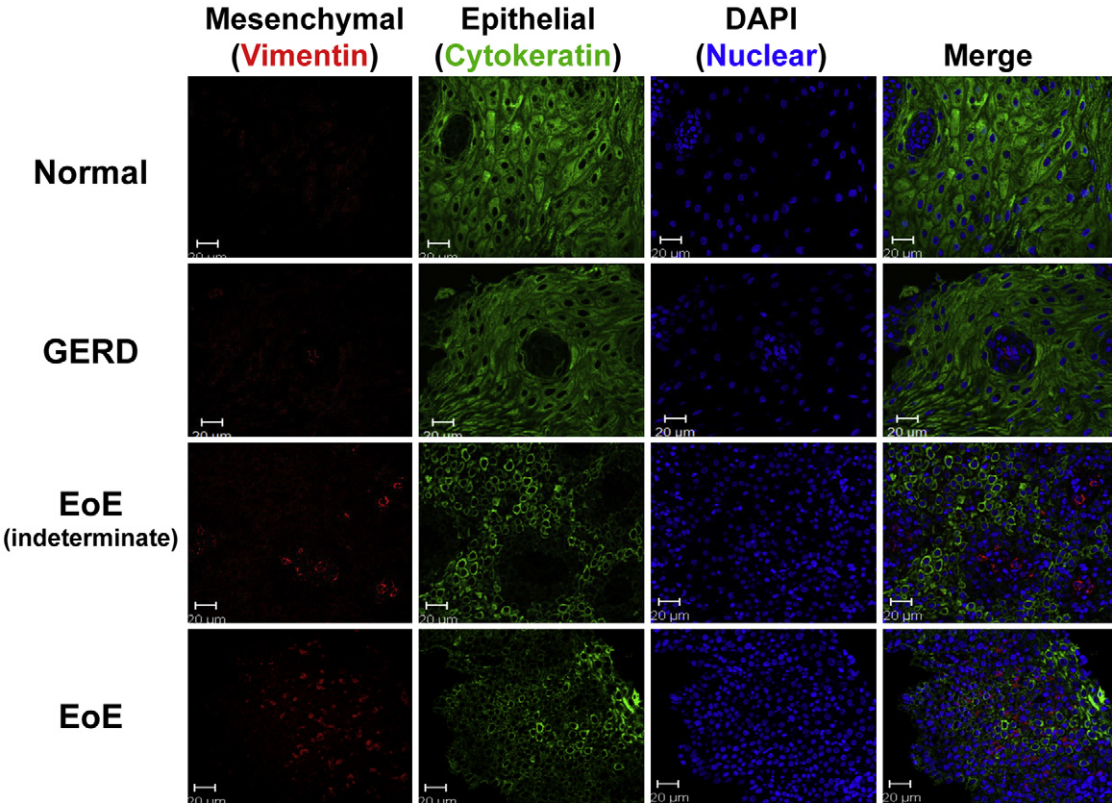

FIG 3. Immunofluorescent staining for EMT in subject study groups. Immunofluorescent staining for cytokeratin (epithelial marker, *green*) and vimentin (mesenchymal marker, *red*) in the epithelium with 4′-6-diamidino-2-phenylindole dihydrochloride nuclear counterstaining *(blue)* is shown. Representative confocal images from esophageal biopsy specimens of subjects with EoE, indeterminate EoE, GERD, and normal esophagus are shown. Quantitative assessment of the EMT index for these subject groups is shown in Fig 4.

years and had been treated with ED, SFED, or TCs (n = 6 per treatment). Subjects' clinical symptoms had resolved, and histopathologic remission of their EoE was defined as 10 or fewer eosinophils/hpf with normalization of epithelial hyperplasia.

Tissue sections were dual stained for the EMT biomarkers cytokeratin (epithelial) and vimentin (mesenchymal) to determine whether EMT occurred in the esophageal mucosa of subjects with EoE (Fig 3). Vimentin-positive cells were present within the lamina propria, which is consistent with fibroblasts, myofibroblasts, or both. Importantly, vimentin-positive cells were visualized within the hyperplastic epithelium (Fig 3), which is consistent with the process of EMT.[17,19] A small number of vimentin-positive cells were also cytokeratin positive, suggesting cells in transition between epithelial and mesenchymal phenotypes, a characteristic feature of EMT, but these were infrequent and more difficult to visualize (see Fig E2 in this article's Online

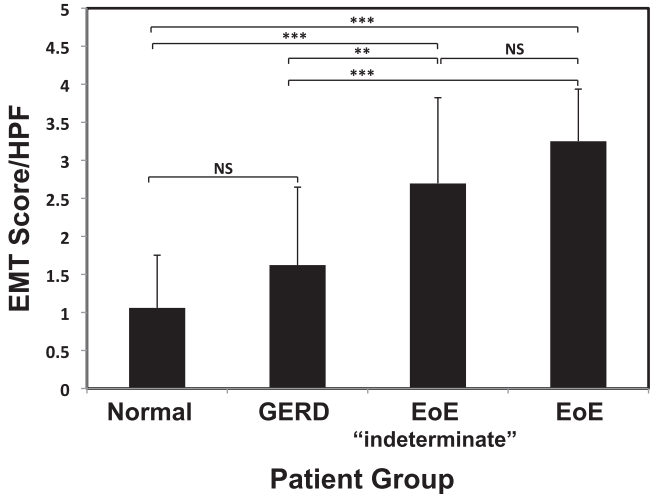

FIG 4. Quantitative assessment of EMT in subjects with EoE, GERD, and normal esophagus. Mean ± SD EMT scores for the 4 subject groups are shown. The highest EMT index was associated with EoE, followed by indeterminate EoE > GERD > normal esophagus. The mean for the normal esophagus control group was right skewed; that is, more values were closer to an EMT score of zero, whereas the mean for EoE was slightly left skewed. The level of EMT in subjects with EoE was not significantly different from that seen in the indeterminate EoE group, whereas both of these subject groups had significantly greater EMT scores than the GERD and normal esophagus groups. Comparative EMT scores for the 2 independent observers are shown in Table E2. *NS*, Not significant. ** $P < .01$ and *** $P < .001$.

Repository at www.jacionline.org). Epithelium-localized, vimentin-positive, mesenchymal-like cells were found most commonly in tissues from subjects with active EoE (12/17 [70.6%]) and indeterminate EoE (11/15 [73.3%]) compared with tissues from subjects with GERD (1/8 [12.5%]) or normal esophagus (0/21, Fig 3). Tissues from subjects with active EoE or indeterminate EoE, but not GERD or normal esophagus, had markedly decreased epithelial staining for cytokeratins coupled with vimentin-positive cells within the epithelium, a characteristic appearance of EMT (Fig 3) and representative of a score of 5 on the 6-point EMT scale (Fig 1). EMT scores were significantly higher in tissues of subjects with active EoE (3.08 ± 0.25) and indeterminate EoE (2.70 ± 0.31) compared those of subjects with GERD (1.71 ± 0.33) or normal esophagus (1.28 ± 0.15, Fig 4 and see Table E2 in this article's Online Repository at www.jacionline.org).

EMT correlates with measures of eosinophilic inflammation

Because eosinophils are potent sources of remodeling factors associated with EMT, such as TGF-β, we quantitated the eosinophil burden associated with active EoE and correlated it with EMT scores. Consistent with previous studies, mean ± SEM eosinophil counts and EPX scores from tissues of subjects with active EoE (46.2 ± 6.9 eosinophils/hpf and 43.4 ± 1.9 EPX score) and indeterminate EoE (31.5 ± 7.9 eosinophils/hpf and 40.9 ± 2.1 EPX score) were significantly greater than those from subjects with GERD (5.0 ± 4.2 eosinophils/hpf and 14.6 ± 5.9 EPX score) and normal esophagus (0.2 ± 0.1 eosinophils/hpf and 2.7 ± 1.2 EPX score, see Table E2). Comparison of subjects' EMT scores with peak eosinophil counts per hpf (Fig 5, *A*, top) and EPX index (Fig 5, *A*, bottom) identified significant correlations for both eosinophils ($r = 0.691$, $P < .01$) and EPX index ($r = 0.738$, $P < .01$).

EMT correlates with esophageal subepithelial fibrosis

We analyzed correlations between EMT scores and indices of subepithelial fibrosis and TGF-β1 expression in esophageal biopsy specimens from the EoE and other study groups. The mean ± SEM fibrosis index was significantly greater in tissues from subjects with EoE (1.67 ± 0.14) and indeterminate EoE (1.47 ± 0.19) compared with that seen in subjects with GERD (0.29 ± 0.3) and normal esophagus (0.26 ± 0.13; $P < .001$, EoE vs GERD; $P < .001$, EoE vs normal esophagus; $P < .01$, indeterminate EoE vs GERD; and $P < .001$, indeterminate EoE vs normal esophagus; see Table E2). Of note, EMT scores were significantly correlated with the fibrosis index ($r = 0.644$, $P < .01$; Fig 5, *B*, top). Similarly, the TGF-β index was significantly higher in subjects with active EoE (2.33 ± 0.16) and indeterminate EoE (2.0 ± 0.24) compared with that seen in subjects with GERD (1.14 ± 0.34) or normal esophagus (1.09 ± 0.14; $P < .01$, EoE vs GERD; $P < .001$, EoE vs normal esophagus; not significant for indeterminate EoE vs GERD; and $P < .01$, indeterminate EoE vs normal esophagus; see Table E2). In addition, EMT scores were significantly correlated with the TGF-β index ($r = 0.520$, $P < .01$; Fig 5, *B*, bottom).

Treatment of EoE resolves EMT

EMT was quantitated in esophageal biopsy specimens before and after treatments known to induce clinicopathologic remission to determine whether treatment affects esophageal EMT in children with EoE (Fig 6, *A*, and see Table E3 in this article's Online Repository at www.jacionline.org). After treatment, EMT scores decreased significantly in all subjects (3.17 ± 0.17 before treatment vs 0.82 ± 0.09 after treatment). Analysis of EMT with respect to individual treatments (n = 6 subjects per group) showed similarly decreased EMT scores for subjects treated with TCs (2.77 ± 0.92 before treatment vs 0.88 ± 0.61 after treatment, a 68.2% decrease; $P < .001$), SFED (3.49 ± 0.71 before treatment vs 0.99 ± 0.25 after treatment, a 72.8% decrease; $P < .001$), and ED (3.25 ± 0.76 before treatment vs 0.61 ± 0.18 after treatment, an 81.2% decrease; $P < .001$).

EMT scores were compared with peak eosinophil counts in biopsy specimens obtained from all 18 subjects before and after treatment to determine whether resolution of EMT was associated with a decrease in eosinophil counts. There was a strong positive correlation of EMT with subjects' peak eosinophil counts per hpf for the combined EoE treatment groups ($r = 0.804$, $P < .001$, n = 36; Fig 6, *B*) and within individual treatment groups (TCs, $r = 0.868$; SFED, $r = 0.857$; ED, $r = 0.820$; all $P < .001$; all n = 12; Fig E3 and see Table E4 in this article's Online Repository at www.jacionline.org).

DISCUSSION

Because esophageal tissue can demonstrate significant epithelial basal zone hyperplasia and subepithelial fibrosis in subjects with EoE, we hypothesized that EMT might be one of the processes associated with these remodeling events. Our results

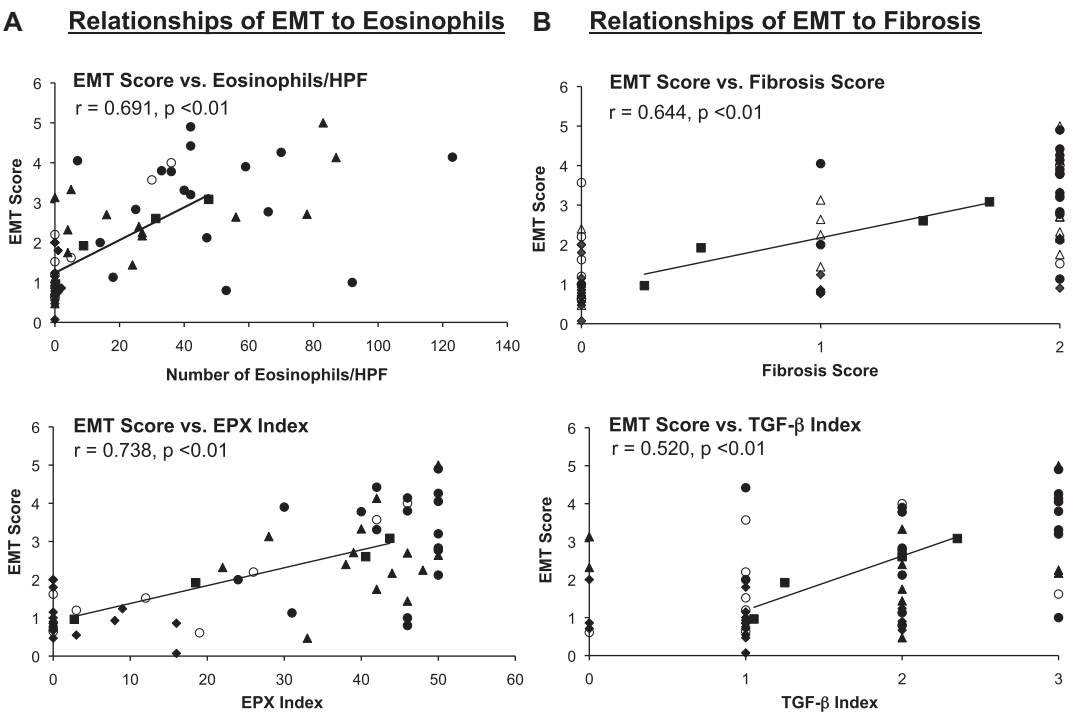

FIG 5. EMT scores correlate with measures of esophageal eosinophil counts and staining for EPX and with subepithelial fibrosis and staining for TGF-β. EMT scores per hpf were analyzed for relationships to subjects' peak numbers of esophageal eosinophils/hpf *(top)* and staining index for EPX (*bottom*; **A**) and fibrosis index *(top)* and TGF-β staining index (*bottom*; **B**) for all 60 subjects in the subject groups. The Pearson correlation coefficient *(r)* and its associated statistical significance are shown for all 60 subjects (dot plot with symbols) comprising the subject groups, including EoE *(solid circles)*, indeterminate EoE *(solid triangles)*, GERD *(open circles)*, and normal esophagus *(solid diamonds)*. The trend line for the mean indices *(solid squares)* is also shown *(solid line)*.

identified the presence of EMT in children with active EoE and showed that treatments that resolve eosinophilic inflammation and epithelial hyperplasia reverse EMT and subepithelial fibrosis. We also showed that the degree of EMT and its resolution in subjects with EoE are strongly correlated with the load of tissue eosinophils within the esophagus.

Esophageal remodeling with subepithelial fibrosis occurs in both children and adults with EoE.[6-8,20,21] Histologically, the subepithelial space is occupied by increased collagen deposition, and by using endoscopic ultrasonography, several studies have demonstrated significant thickening of the mucosa, submucosa, and muscularis that is suggestive of fibrosis.[4,22,23] Most recently, Straumann et al[24] used endoscopic ultrasonography to show that there was marked thickening of these esophageal layers in adolescents and adults with long-term EoE (symptoms for 9.3 ± 5.2 years), leading to speculation that in some patients the chronic unbridled inflammation seen with EoE results in fibrosis and remodeling. Molecular support for remodeling arises from studies showing that there is increased expression of TGF-β1 and its downstream signaling molecules phospo-SMAD2/3 in association with subepithelial fibrosis and esophageal stricture formation.[8,25,26] TGF-β1–expressing cells include eosinophils[8] and mast cells.[25] Thus current evidence suggests that increased expression of and signaling by profibrotic TGF-β1 is key to the induction of esophageal fibrosis in subjects with EoE.[6]

The origins of mesenchymal cells (fibroblasts and myofibroblasts) participating in tissue repair during chronic inflammation and tissue damage, notably fibrosis, are still poorly understood. Emerging evidence from fields including allergic diseases and asthma suggests that EMT contributes to the genesis of disease-related fibroblasts and myofibroblasts and development of tissue fibrosis, representing a significant source of these fibrogenic cells.[9,11,27,28] We demonstrated the presence of vimentin-positive mesenchymal-like cells in the context of loss of normal epithelial architecture and decreased expression of cytokeratins within hyperplastic epithelium in a majority of our subjects with active EoE compared with those with GERD or normal esophagus. We also showed that these changes, which are characteristic of EMT, are directly proportional to the eosinophil load in esophageal biopsy specimens.

EMT participates in the genesis of tissue and organ fibrosis in the kidney, liver, and lung in response to chronic injury and repair by contributing to the population of disease-related fibroblasts and myofibroblasts that overproduce extracellular matrix.[9,29,30] These responses are regulated in part by exogenous sources, autocrine sources, or both and signaling by TGF-β in epithelial cells.[18,29] The presence of EMT in subjects with EoE was highly correlated with the index of subepithelial fibrosis, eosinophil presence, and state of activation (determined based in both cell-associated and secreted expression of EPX) and the presence of TGF-β1 in the biopsy specimens, thus providing compelling support that EMT contributes to the genesis of subepithelial fibrosis in subjects with EoE. A number of growth factors induce or regulate the development of EMT, primarily TGF-β, with others being more variable and context dependent, such as fibroblast

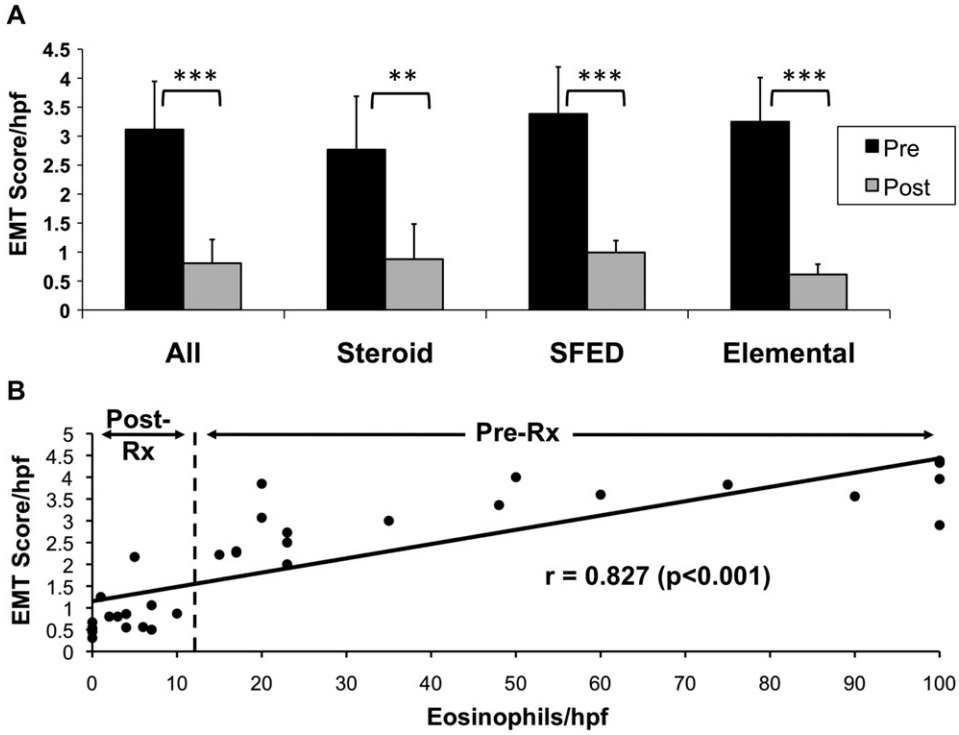

FIG 6. Resolution of esophageal EMT in subjects with EoE after treatment: correlation with eosinophil load. **A,** The presence/amount of EMT was scored in subjects with EoE treated with 3 different modalities that reduce epithelial eosinophilic inflammation. Mean ± SD EMT scores before and after treatment are shown for all treated subjects combined (n = 18) and for individual treatment groups (n = 6 per group; see Table E3 for the percentage reduction in EMT). **B,** Resolution of esophageal EMT was directly correlated with the number of esophageal eosinophils before and after treatments that reduced the esophageal eosinophil burden *(Rx)*. The correlation coefficient *(r)* and associated significance is shown for the relationship between all 18 EoE subjects' pretreatment and posttreatment EMT scores and peak eosinophil counts (combined n = 36). The *vertical dashed line* delineates pretreatment from posttreatment eosinophil counts/EMT scores (see Table E4 and Fig E3 for correlations within each of the individual treatment groups). $^{**}P < .01$ and $^{***}P < .001$.

growth factor 2, epidermal growth factor, insulin-like growth factor 2, and hepatocyte growth factor.[18,31] Of these, TGF-β, either induced by autocrine expression within epithelial cells themselves in response to tissue damage or from a paracrine inflammatory cell source, plays a key role in inducing EMT and is vital to expression of the EMT proteome.[18,19,32]

We showed here that TGF-β1 induces changes in gene expression *in vitro* in an esophageal epithelial cell type, HET-1A cells, in a manner consistent with EMT. Importantly, TGF-β1 potently induced transcription of mesenchymal genes (N-cadherin, vimentin, and fibronectin) and downregulated expression of cytokeratins in HET-1A esophageal cells, which are characteristic findings for the induction of EMT in primary and epithelium-derived cell lines from other tissues and organs.[18,29,33,34] Previous studies of TGF-β family–induced EMT in culture showed that although phospho-Smad signaling occurs rapidly, induction of transcriptional repressors associated with induction of EMT does not occur until 48 hours, and subsequent repression of epithelial markers, such as E-cadherin, at both the mRNA and protein levels takes even longer (up to 72 hours).[35] Loss of epithelial phenotype with dissolution of tight polarized cell-cell adhesion might be a gradual or even reversible process[36] or might be regulated posttranslationally by proteases, including metalloproteases, as shown for EMT-processes associated with tumorigenesis.[37] A number of studies showed simultaneous expression of both epithelial and mesenchymal adherens junction proteins in primary and metastatic tumors, but functional studies suggest the metastatic invasive phenotype of mesenchymal N-cadherin prevails over stable polarized E-cadherin when they are coexpressed.[38,39] Thus although HET-1A cells coexpressed epithelial and mesenchymal markers in the current study, the mesenchymal phenotype might prevail, allowing this gradual EMT process to contribute to the development of subepithelial fibrosis in the esophagus in subjects with EoE.

Eosinophils, in addition to direct contribution of TGF-β for induction of EMT, can induce expression of EMT- and fibrosis-relevant remodeling factors in epithelial cells themselves, including TGF-β and others (endothelin-1, TGF-α, platelet-derived growth factor AB, epidermal growth factor receptor, matrix metalloproteinase 9, IL-6, IL-11, fibronectin, and tenascin), through secretion of their granule cationic proteins (major basic protein 1 and EPX)[40,41] or cytokines (IL-13).[42,43] Increased TGF-β1 expression, previously shown in pediatric subjects with EoE,[8,44] was also demonstrated in the current study, providing further evidence for its role and that of EMT in inducing esophageal fibrosis in subjects with EoE. Importantly, the relevant cellular sources of TGF-β (eosinophils, mast cells, epithelial cells, and fibrocytes), the mechanisms by which TGF-β in the esophagus in subjects with EoE becomes activated from its latent form (through integrin αvβ6-mediated[11,45] or proteolytic pathways), and the

downstream signaling pathways relevant to induction of EMT[9,18] and its resolution[46] remain to be determined in subjects with EoE.

For the 18 children with active EoE who were successfully treated with 3 different treatment modalities (TCs, SFED, and ED), their decreased posttreatment EMT scores were significantly correlated with their reduced esophageal eosinophil load. However, we were unable to assess a corresponding posttreatment decrease in the levels of fibrosis in these subjects with EoE because there was insufficient lamina propria (<2 mm) present in many of the esophageal biopsy specimens to allow quantitation of the fibrosis index. However, several studies previously showed that TC treatment decreases or completely resolves esophageal fibrosis in children with EoE.[6,25] Thus in addition to these earlier reports of decreased subepithelial fibrosis with steroid treatment, the present study shows a corresponding decrease in the amount of EMT that likely contributes to the fibrogenesis characteristic of subjects with EoE.

EoE is a chronic inflammatory disorder,[21] and remission of EoE inflammation results in resolution of EMT (current study) and fibrosis.[44] Current therapies, either pharmacologic with TCs or dietary restrictions (SFED and ED), are effective for inducing disease remission, but maintaining remission long-term is difficult because disease recurs once the treatment is discontinued. For example, TCs are effective in many but not all subjects with EoE in inducing clinicopathologic remission, but relapse rates are high once the corticosteroid is discontinued.[47] Currently, there are no recommendations for low-dose maintenance therapy with TCs that will maintain subjects in remission and thereby prevent fibrosis.[1] A recent study in adults showed that low-dose maintenance treatment with budesonide was well tolerated, and 50% of patients were maintained in remission after a 50-week treatment period.[24] However, after 50 weeks of low-dose budesonide, submucosal and muscularis propria thickening still persisted, fibrosis scores were increased slightly, and TGF-β and tenascin C levels were still increased. Additionally, long-term low-dose budesonide therapy was associated with significant reductions in overall mucosal but not epithelial thickness, and esophageal remodeling showed only a trend toward normalization.[24] Finally, 1 year of topical fluticasone treatment of adults with EoE led to a nonsignificant reduction in subepithelial fibrosis.[20] Taken together, these findings suggest that low-dose continuous TCs might be unable to prevent the progression of esophageal fibrosis, supporting the need to explore alternative long-term treatment modalities to block fibrogenesis of the esophagus in subjects with EoE.

Finally, we analyzed a group of pediatric patients with an indeterminate diagnosis of EoE and found their EMT scores to be virtually identical to those of patients with confirmed EoE. Clinical experience is identifying an increasing number of these kinds of children and adults with features highly suggestive of EoE but who do not reach the requisite eosinophil threshold number.[1] Reasons for this might include limitations in biopsy sampling, a later more chronic stage in the inflammatory process, a different EoE phenotype, or a more fibrotic phenotype of GERD. Future studies that provide additional clinical and molecular characterization will help clarify this patient population more fully.

In conclusion, correlations of EMT with esophageal eosinophil counts, their state of activation, and measures and mediators of fibrosis suggest that EMT contributes significantly to the subepithelial fibrosis characteristic of EoE. Thus treatments affecting esophageal eosinophilia in subjects with EoE can alter the natural history of the disease in terms of reversing esophageal remodeling. Prospective studies are needed to extend these findings to further define the profibrotic mediators and signaling cascades that propagate esophageal epithelial reactions in subjects with EoE, leading to EMT and factors, such as bone morphogenic protein 7, that might be involved in its resolution with treatment.[48,49]

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Key messages

- **Esophageal epithelial cells can undergo TGF-β–induced EMT, a process associated with tissue and organ fibrosis, including the subepithelial fibrosis associated with airway remodeling in asthmatic subjects.**
- **Fibrosis-associated EMT occurs in the esophagi of children with active EoE but not in children with other esophageal diseases, such as GERD.**
- **The degree of EMT in the esophagus in subjects with EoE is highly associated with the amount of subepithelial fibrosis, numbers and measures of activation of esophageal eosinophils, and levels of remodeling factors, such as TGF-β.**
- **Esophageal EMT resolves with EoE treatments that significantly decrease the esophageal burden of eosinophils.**
- **EoE treatments that significantly reduce esophageal eosinophil counts are likely to alter the natural history of this food-induced allergic disease by reversing EMT-associated fibrogenesis.**

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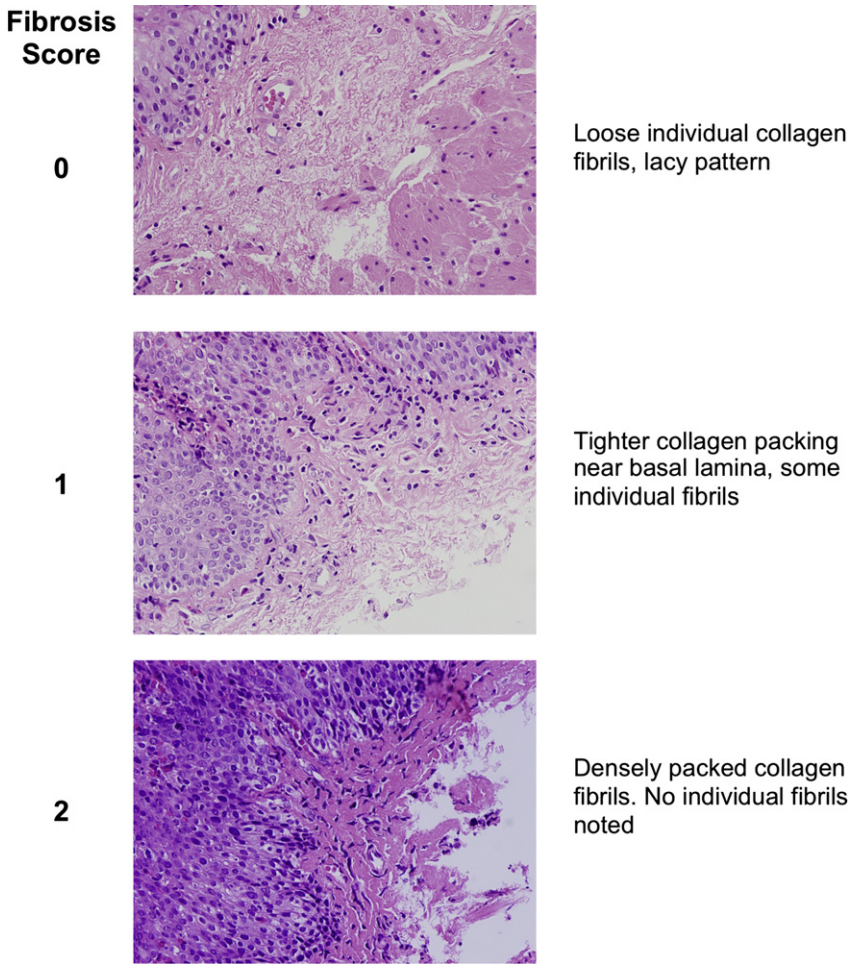

FIG E1. Three-point fibrosis assessment scale in esophageal biopsy specimens. Representative hematoxylin and eosin staining of esophageal mucosa. The fibrosis score (0-2) is indicated on the *left,* with descriptions of the scoring system on the *right*.

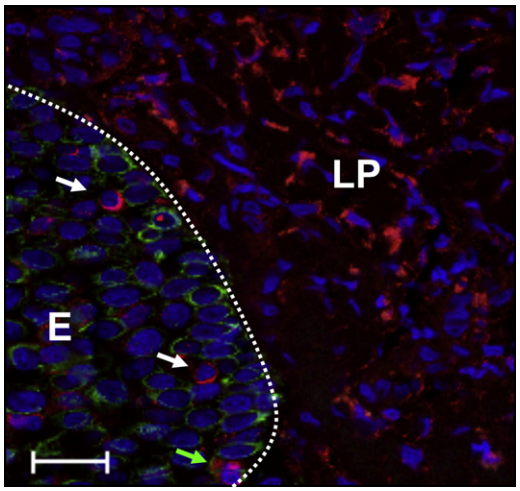

FIG E2. Evidence for EMT in subjects with EoE: presence of mesenchymal (vimentin-positive) cells in the hyperplastic epithelium. Tissue section from a subject with active EoE stained for cytokeratin (epithelial marker, *green*) and vimentin (mesenchymal marker, *red*) by using immunofluorescence, with 4′-6-diamidino-2-phenylindole dihydrochloride nuclear counterstaining *(blue)*. The *dashed white line* delineates the epithelial basement membrane/lamina propria boundary. The *white arrows* show abnormally present vimentin-positive cells within a region of epithelium *(E)* near the basal zone showing decreased expression of cytokeratin, a characteristic feature of EMT. The *green arrow* indicates a rare cell stained for both vimentin and cytokeratin. There are numerous vimentin-positive cells (fibroblasts) in the lamina propria *(LP)*. The *bar* indicates 20 μm in this ×630 confocal image.

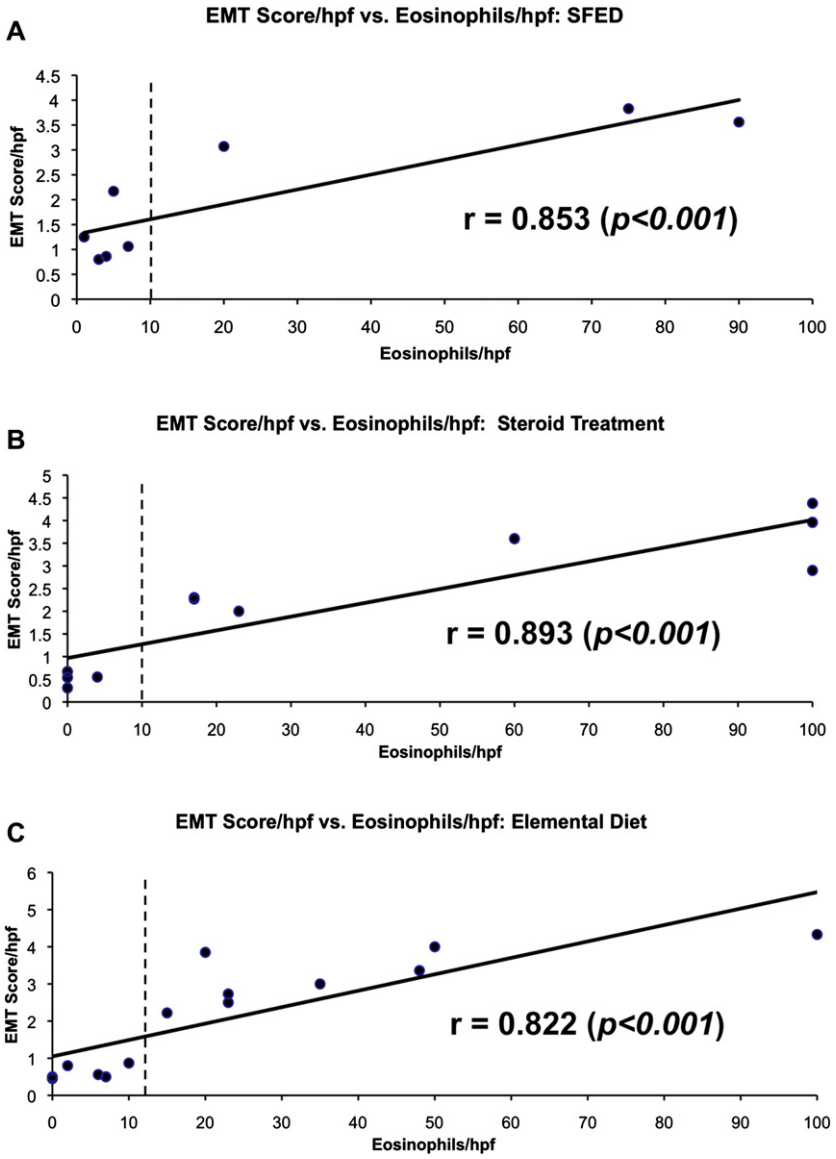

FIG E3. The resolution of esophageal EMT correlates with the number of esophageal eosinophils before and after treatments that reduced the esophageal eosinophil burden in subjects with EoE. Pearson correlation analysis *(r)* and associated significance (*P* value) are shown for EMT scores versus peak eosinophil counts (n = 6 pretreatment and n = 6 posttreatment values in each group) for subjects treated with swallowed corticosteroids **(A)**, SFED **(B)**, and ED **(C)**. The *vertical dashed lines* delineate pretreatment *(right of line)* from posttreatment *(left of line)* eosinophil counts/EMT scores.

TABLE E1. TaqMan probes used for RT-Q-PCR of EMT biomarker genes

Gene name	Protein	TaqMan probe ID
CDH1	E-cadherin	Hs01023895_m1
CDH2	N-cadherin	Hs00169953_m1
VIM	Vimentin	Hs00958112_g1
FN	Fibronectin	Hs00365058_m1
KRT8	Cytokeratin 8	Hs01595539_g1
KRT14	Cytokeratin 14	Hs00265033_m1

The indicated EMT biomarker mRNA transcripts were assessed by using the TaqMan Gene Expression Assay FAM dye-labeled TaqMan MGB probes (Applied Biosystems) and ABsolute Blue QPCR ROX MasterMix (Thermo Scientific).

TABLE E2. Quantitative indices for EMT, fibrosis, TGF-β, EPX, and eosinophils in the subject and control groups

Parameter	Control subjects with normal esophagus	Subjects with GERD	Subjects with EoE (indeterminate)	Subjects with EoE (confirmed)
No. (n = 60)	21	7	15	17
EMT scores				
Observer 1	1.06 ± 0.15	1.62 ± 0.39	2.70 ± 0.33	3.08 ± 0.31
Observer 2	1.41 ± 0.17	1.79 ± 0.27	2.60 ± 0.28	3.08 ± 0.26
Mean EMT	1.28 ± 0.15	1.71 ± 0.33	2.7 ± 0.31	3.08 ± 0.25
Eosinophils (peak)*	0.2 ± 0.1	5.0 ± 4.2	31.5 ± 7.9	46.2 ± 6.9
EPX index†	2.7 ± 1.2	14.6 ± 5.9	40.9 ± 2.1	43.4 ± 1.9
Fibrosis index‡	0.26 ± 0.13	0.29 ± 0.29	1.47 ± 0.19	1.67 ± 0.14
TGF-β index§	1.09 ± 0.14	1.14 ± 0.34	2.0 ± 0.24	2.33 ± 0.16

Mean ± SEM indices are shown for quantitative measurements of EMT levels, subepithelial fibrosis, EPX index (eosinophils and secreted EPX), TGF-β index, and peak eosinophil counts. For the EMT index, evaluations by 2 independent observers using the 6-point EMT scoring system (Fig 1) are shown. The EoE (indeterminate) group represents subjects who had many clinical features of EoE but lacked the threshold number of greater than 15 eosinophils/hpf or had no documented treatment with acid suppression.[1,14] Statistical comparisons between means are as follows (for EMT, see Fig 4).

*Eosinophils/hpf: not significant, normal esophagus vs subjects with GERD; $P < .001$, normal esophagus versus indeterminate EoE; $P < .001$, normal esophagus versus subjects with EoE; not significant, GERD versus indeterminate EoE; $P < .001$, GERD versus EoE; not significant, indeterminate EoE versus EoE.

†EPX index: $P < .05$, normal esophagus versus GERD; $P < .001$, normal esophagus versus indeterminate EoE; $P < .001$, normal esophagus versus EoE; $P < .001$, GERD versus indeterminate EoE; $P < .001$, GERD versus EoE; not significant, indeterminate EoE versus EoE.

‡Fibrosis index: not significant, normal esophagus versus GERD; $P < .001$, normal esophagus versus indeterminate EoE; $P < .001$, normal esophagus versus EoE; $P < .01$, GERD versus indeterminate EoE; $P < .001$, GERD versus EoE; not significant, indeterminate EoE versus EoE.

§TGF-β index: not significant, normal esophagus versus GERD; $P < .01$. normal esophagus versus indeterminate EoE; $P < .001$, normal esophagus versus EoE; not significant, GERD versus indeterminate EoE; $P < .01$, GERD versus EoE; not significant, indeterminate EoE versus EoE.

TABLE E3. Comparison of EMT scores before and after treatment of subjects with EoE

Treatment (no. of patients)	EMT scores			
	Pretreatment	Posttreatment	Decrease in EMT (%)	*P* value
All (18)	3.17 ± 0.82	0.82 ± 0.39	74.1	<.001
Steroid (6)	2.77 ± 0.92	0.88 ± 0.61	68.2	<.01
SFED (6)	3.49 ± 0.71	0.95 ± 0.25	72.8	<.001
Elemental (6)	3.25 ± 0.76	0.61 ± 0.18	81.2	<.001

Mean ± SD EMT scores are shown for all subjects combined and individual treatment groups before and after treatment. The percentage decrease in the EMT score is indicated, as is the *P* value for the difference between the pre- and post-EMT means.

TABLE E4. Correlations between EMT scores and peak eosinophil counts before and after treatment in subjects with EoE

Treatment	*r* value	*P* value
All (18)	0.827	<.001
Steroids (6)	0.853	<.001
SFED (6)	0.893	<.001
Elemental (6)	0.822	<.001

Pearson correlation coefficients *(r)* and their associated statistical significance (*P* values) are shown for all subjects combined (graphically presented in Fig 6) and for the individual treatment groups for subjects' pretreatment (n = 6) and posttreatment (n = 6) EMT scores and corresponding peak eosinophil counts (shown graphically in Fig E3).