REVIEW

Epithelial-stromal crosstalk and fibrosis in eosinophilic esophagitis

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Abstract Eosinophilic esophagitis (EoE) is a food allergen-induced inflammatory disorder. EoE is increasingly recognized as a cause of swallowing dysfunction, food impaction and esophageal stricture. Inflammation of the esophageal mucosa involves immune cell infiltrate, reactive epithelial changes and fibroblast activation, culminating in robust tissue remodeling toward esophageal fibrosis characterized by excess collagen deposition in the subepithelial lamina propria. Fibrosis contributes to a unique mechanical property of the EoE-affected esophagus that is substantially stiffer than the normal esophagus. There is a great need to better understand the processes behind esophageal fibrosis in order to foster improved diagnostic tools and novel therapeutics for EoE-related esophageal fibrosis. In this review, we discuss the role of esophageal inflammatory microenvironment that promotes esophageal fibrosis, with specific emphasis upon cytokines-mediated functional epithelial-stromal interplays, recruitment and activation of a variety of effector cells, and tissue stiffness.

We then explore the current state of clinical methodologies to detect and treat the EoE-related esophageal stricture.

Abbreviations

α-SMA	α-Smooth muscle actin
AV	Autophagy vesicles
BAPN	B-aminopropionitrile
BCH	Basal cell hyperplasia
CTGF	Connective tissue growth factor
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
EndoFLIP	Endoluminal functional lumen imaging probe
EoE	Eosinophilic esophagitis
HCQ	Hydroxychloroquine
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
IL-13R	IL-13 receptor
LOX	Lysyl oxidase
MMP	Matrix metalloproteinase
PAI-1	Plasminogen activator inhibitor 1
PPI	Proton pump inhibitor
ROS	Reactive oxygen species
TGF-β	Transforming growth factor-β
Th2	T helper-type 2
TNF-α	Tumor necrosis factor-α
TSLP	Thymic stromal lymphopoietin

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Introduction

Eosinophilic esophagitis (EoE) is an allergic chronic inflammatory disorder affecting $\sim$ 4 in 10,000 individuals in the United States. EoE involves transmural esophageal inflammation and subepithelial fibrosis, leading to esophageal stricture, the most serious clinical consequence of epithelial and lamina propria tissue remodeling events in EoE [1–3]. Clinical manifestations include dysphagia and food impaction, which impact patients' quality of life and health care costs [4, 5]. The natural history of the disease is unknown, but retrospective studies suggest that the most common clinical symptoms reflect the progression and worsening of untreated esophageal inflammation and fibrosis [6]. While the pathogenesis of EoE involves food antigen exposure, genetics, and eosinophil migration into the esophageal epithelium, it remains unclear how these interactions contribute to fibrosis. Dietary elimination and steroids remain the standard of care for EoE; however, 40–50% of EoE patients, despite age, are refractory to first line treatments [4]. Approximately, 10% of EoE patients show extremely narrow-caliber esophagus and are difficult to treat [7]. At the time of diagnosis up to 67% of adults and 16% of children already have fibrostenotic disease [8]; once there is fibrosis, decreasing inflammation alone may not provide symptomatic relief.

Food and environmental allergens trigger a diverse esophageal inflammatory response, leading to a pathologic cycle of tissue damage and repair. Active EoE is diagnosed by the presence of intramucosal eosinophilia ($\geq$ 15/hpf peak eosinophil count) following administration of proton pump inhibitor (PPI) for 8 weeks to rule out PPI-responsive esophageal eosinophilia [9]. The role of eosinophils in EoE remains elusive. Active EoE features cytolytic degranulation of eosinophils to release their granule proteins in the esophageal epithelium that entrap bacteria and fungi [10]. Thus, eosinophils may have a host defense role in the context of impaired mucosal barrier functions in EoE; however, the pathogenic role of eosinophils has been implicated in inflammation-related fibrosis of a variety of organs including the heart, the airway, and the skin [11]. While eosinophils are the most noticeable infiltrating cell population, there is actually a mixed inflammatory cell infiltrate comprising mast cells, basophils and lymphocytes [12–14]. In a mouse model of EoE induced by ova albumin, basophils have been implicated as a major effector in EoE-related inflammation [13].

The vigorous inflammatory state and progressive tissue damage promote esophageal fibrosis. Esophageal fibrosis is defined as excessive extracellular matrix (ECM) deposition, most notably collagen fibers, in the esophageal lamina propria. Fibroblasts are the major effector cells in fibrosis.

They become activated in the setting of injury to provide the ECM proteins needed for wound healing. ECM proteins serve as the scaffolding for re-epithelialization and wound closure. In EoE, fibroblasts residing in the subepithelial lamina propria express markers of activated myofibroblasts such as α-smooth muscle actin (α-SMA) under inflammatory conditions. Chronic inflammation leads to continuous fibroblast activation, proliferation, and survival with excessive secretion of ECM components, increasing esophageal stiffness [15, 16]. Tissue stiffness is associated with esophageal dysfunction as evident clinically by dysphagia, food impaction, and stricture. Thus, understanding the mechanisms underlying fibrosis in EoE and identifying novel pharmacologic targets aimed at decreasing tissue stiffness and matrix remodeling are paramount to improving patient outcomes.

In this review we seek to define how inflammation promotes esophageal fibrosis in EoE. We will examine the effects of inflammation and tissue stiffness on esophageal remodeling. We will discuss the chemical microenvironment mediated by inflammatory cytokines that facilitates the functional interplays between epithelial cells and stromal fibroblasts. Additionally, we will discuss the mechanical microenvironment associated with tissue stiffness. Finally, we will evaluate the effects of tissue remodeling clinically, looking at the evolution of esophageal fibrosis in EoE and the methods we use to detect it.

Mechanisms of fibrosis in EoE

Fibroblasts, immune cells, epithelial cells and the interactions of these key players via secreted inflammatory cytokines are all likely responsible for fibrosis in EoE. In addition to the rich chemical changes, tissue stiffness contributes to the progression of fibrosis. Herein we outline the complex cellular interplays in the inflammatory milieu, the stiffness of the esophagus, and the mechanism by which these factors drive fibroblast activation.

Th2 immunity and fibrosis in EoE

Multiple inflammatory cytokines play critical roles in EoE pathobiology. The inflammatory milieu in EoE is dominated by T helper-type 2 (T_h2) lymphocytes characterized by production of interleukins (IL)-4, 5, and 13 [17]. Chronic T_h2 inflammation leads to tissue fibrosis and end organ dysfunction [18, 19]. In EoE, it is believed that the food allergen-exposed esophageal epithelium releases cytokines thymic stromal lymphopoietin (TSLP) [20, 21] and IL-33 [22], potent enhancers of T_h2-mediated immunity and trigger the inflammatory cascade (Fig. 1). Induction of T_h2 inflammation leads to production of
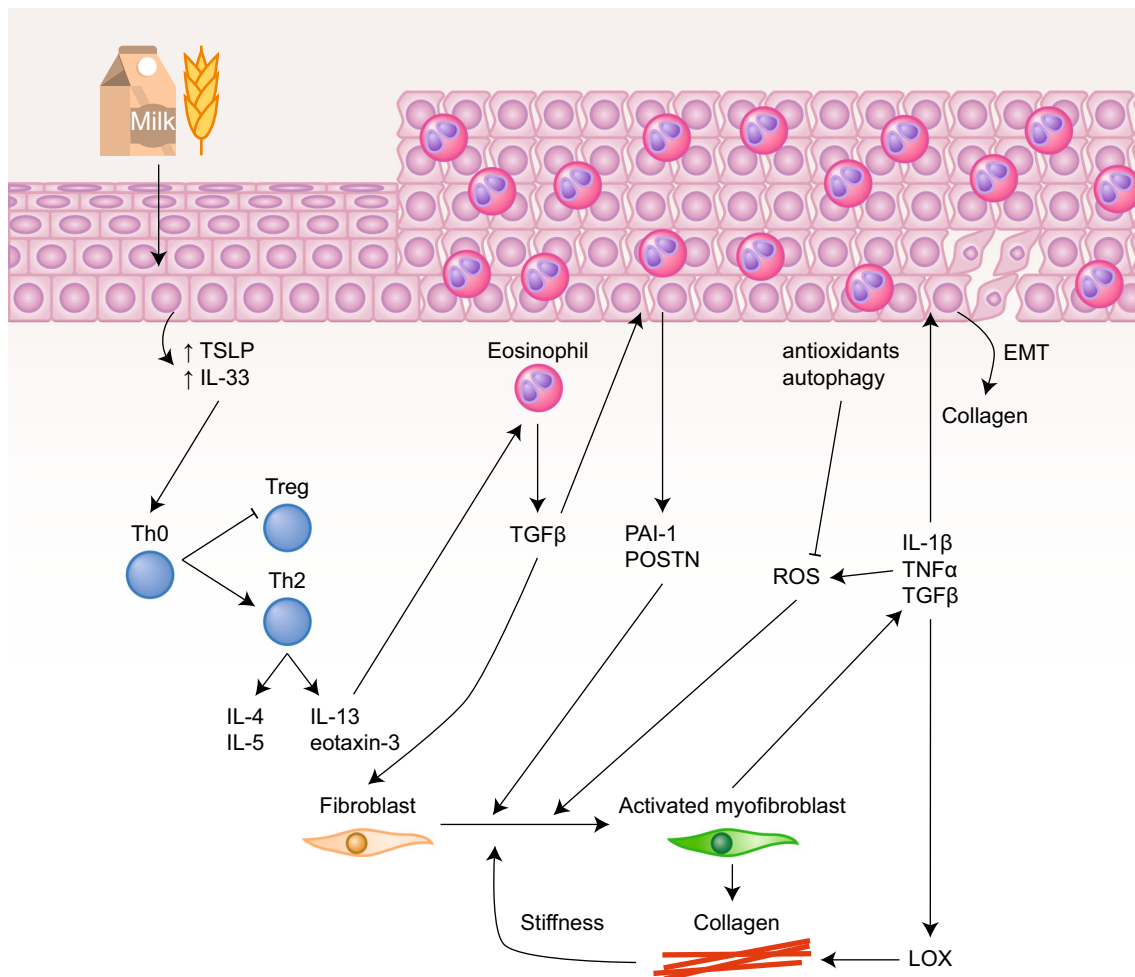
Fig. 1 Food allergens trigger epithelial cell production of pro-inflammatory cytokines such as TSLP and IL-33 which drive T_h2-predominant inflammation, recruiting eosinophils via T_h2 cytokines such as IL-13, IL-5 and eotaxin3. These cytokines may stimulate esophageal epithelial cell proliferation while delaying terminal differentiation, resulting in basal cell hyperplasia (BCH). BCH may be associated with diminished epithelial characteristics via EMT with decreased epithelial barrier functions, leading to the aggravated inflammatory milieu. Multiple inflammatory cytokines trigger ROS production which is subjected to regulation by antioxidants and autophagy. ROS are also essential in EMT. Limited autophagy flux in the epithelial cells may promote EoE inflammation, BCH and fibrosis. EoE-relevant cytokines may recruit and stimulate fibroblasts to induce activated myofibroblasts. Activation of myofibroblasts also involves epithelial-stromal crosstalk mediated by cytokines and growth factors as well as tissue stiffness increased via collagen cross-linking

inflammatory cytokines IL-4, 5, and 13. As the major effector cytokine in EoE [23, 24], IL-13 stimulates epithelial production of eotaxin-3 (aka CCL26), a potent chemoattractant for eosinophils and basophils [25–27], and promote tissue eosinophilia [28, 29]. Additionally, tumor necrosis factor (TNF)-α, IL-4 and IL-13 act synergistically to induce eotaxin-3. The induction of eotaxin-3 occurs not only in the esophageal epithelium but also in esophageal fibroblasts via transcription factor STAT6 activated by T_h2 cytokines [26, 30]. These cytokines cooperate to promote fibrosis via trans-differentiation of fibroblasts into activated myofibroblasts, the key effector cells in fibrosis [31, 32]. Additionally, fibroblasts are stimulated by eosinophil-derived factors such as transforming growth factor (TGF)-β

and IL-1β [33]. Once activated, myofibroblasts secrete extracellular matrix components including collagen, pro-liferate, migrate, and become contractile. These abilities allow for normal wound healing in the setting of injury but in the pathologic state, robust and constant activation promote tissue stiffness, causing dysphagia, food impaction and esophageal stricture in EoE.

IL-13 and fibrosis

Besides granulocyte recruitment, IL-13 mediates fibrotic tissue remodeling in murine models of fibrotic disorders. Treatment with anti-IL-13 (Tralokinumab) decreased air-way fibrosis in mice [34]. In mice, lung-targeted transgenic

IL-13 overexpression leads to epithelial hyperplasia, angiogenesis, eotaxin production, and subepithelial fibrosis with increased collagen deposition in the esophagus [35]. Furthermore, when IL-13 was overexpressed in an eosinophil deficient mouse, there was continued remodeling despite lack of eosinophilic infiltration [35], suggesting that IL-13 may drive esophageal fibrosis in a manner independent of its role in eosinophil recruitment. The mechanism by which IL-13 enhances fibroblast activation and collagen deposition in EoE is not completely understood. In a murine model of pulmonary fibrosis, IL-13 has been shown to induce fibroblast migration via IL-13-mediated enhanced formation of lamellipodia, cytoskeletal projections at the leading edge of the cells, as well as enhanced matrix metalloproteinases (MMPs) activity [36]. The role of IL-13 receptor (IL-13R)-mediated signaling has been tested in genetically engineered mice with defective IL-13Rα2, a decoy receptor competing with IL-13R for IL-13 [35], suggesting. Loss of *IL-13Rα2* resulted in enhanced IL-13R-mediated signaling and esophageal fibrosis in mice with transgenic IL-13 expression [35], suggesting a role for IL-13R in fibrotic tissue remodeling during the inflammatory process.

TGFβ and fibrosis

The inflammatory infiltrate in EoE consists of eosinophils, mast cells and basophils. Activation of the T_h2 responses leads to invasion of these granulocytes and robust production of the cytokine TGFβ leading to tissue damage and fibrotic tissue remodeling [12, 37]. We have previously shown that TGFβ stimulation of primary esophageal fibroblasts leads to enhanced expression of α-SMA, collagen and fibronectin [38]. Furthermore, esophageal biopsies of EoE patients demonstrate increased expression of TGFβ and phosphorylation of its downstream transcription factor SMAD2/3 compared with gastroesophageal reflux disease and normal control patients. There was co-localization of phosphorylated-SMAD2/3 with eosinophil granule proteins, suggesting that the eosinophils are the major source of TGFβ production [37].

A mouse model of EoE supports the role of canonical TGFβ signaling in EoE. In an ova albumin-induced model of murine EoE with impaired TGFβ signaling via Smad3 deficiency resulted in a decreased collagen deposition and angiogenesis, but not eosinophilia [31]. Thus, even with ongoing inflammation, inhibition of TGFβ leads to attenuated fibrosis. TGFβ also induces a number of other profibrotic tissue remodeling factors including MMPs, plasminogen activator inhibitor 1 (PAI-1) and Periostin. MMP2 and MMP14 are upregulated in EoE and are reduced in patients responding to corticosteroid therapy [39]. PAI-1 has also been found to be upregulated in active EoE patient biopsies and its

expression correlates with lamina propria fibrosis. PAI-1 inhibition leads to diminished gene expression of profibrotic α-SMA [40]. Periostin is a glycoprotein expressed in both epithelial cells and fibroblasts that has been shown to be upregulated in the EoE transcriptome [41, 42]. In the setting of IL-4 and IL-13 stimulation, Periostin binds to integrins on the cell surface leading to fibroblast proliferation, activation, and production of collagen [43, 44]. However, IL-13 may activate tissue fibrosis in a TGFβ independent manner since IL-13 activated the fibrogenic machinery in mice with the impaired TGFβ signaling cascade [45]. Other TGFβ target genes essential in fibrosis and extracellular matrix remodeling, yet to be studied in the context of EoE, include connective tissue growth factor (CTGF) [46], insulin-like growth factor binding protein (IGFBP)-3 [47] and lysyl oxidase (LOX) [48]. In particular, LOX catalyzes collagen cross-linking. Besides, TGFβ stimulates smooth muscle hypertrophy, increasing tissue stiffness [12], which may increase with the EoE disease progression [49].

Epithelial contributions to fibrosis in EoE

While the inciting events in EoE are unknown, damage to the epithelial barrier leading to T_h2 immune response is likely an early initiator. EoE inflammation involves reactive epithelial changes leading to epithelial barrier defects [50]. The normal stratified squamous epithelium of the esophagus comprises a single layer of proliferative basal cells (keratinocytes) that exit cell division cycle in the suprabasal cell layers to undergo terminal differentiation and desquamate eventually into the esophageal lumen. This differentiation gradient is disrupted in EoE by basal cell hyperplasia (BCH) [51], an expansion of basaloid cells (> 20% of epithelial height) as well as edematous dilatation of the intercellular spaces (spongiosis), and the retention of nuclei in the superficial cell layer (parakeratosis) [52]. BCH contributes to barrier defect via downregulation of epithelial junction proteins. EoE-relevant cytokines promote epithelial barrier defects by downregulating desmoglein-1, which mediates cell–cell junction formation [41, 53]. In addition to its role in propagating inflammation, esophageal epithelial cells can act as effector cells in fibrosis. Conditioned media from esophageal epithelial cell culture stimulate esophageal fibroblasts to produce profibrotic cytokines IL-1β and TNF-α [32]. TNF-α and TGF-β stimulate epithelial-mesenchymal transition (EMT), by which epithelial cells take on characteristics of mesenchymal cells including collagen production, migration, and contraction [32, 54–56]. In normal esophageal keratinocytes, impaired squamous-cell differentiation triggers EMT [57]. BCH lesions express EMT markers in EoE [32, 54, 56]. Diminishing cell adhesion, EMT contributes to barrier defects [54, 58]. Additionally, keratinocytes that

have undergone EMT display increased collagen production [56]. Thus, epithelial changes may influence the tissue microenvironment to facilitate fibrosis in a non-cell autonomous manner.

Oxidative stress, redox homeostasis and fibrosis

Reactive oxygen species (ROS) are produced via cellular metabolism and are essential in physiological processes including cell signaling, proliferation, differentiation and metabolic adaptation [59, 60]. ROS modulate the balance between proliferation and differentiation in a variety of tissues types, including the esophagus [61]. EMT involves ROS and requires proper redox regulation in esophageal keratinocytes [62]. ROS have been implicated in immune cell mobilization and eosinophils generate ROS [63]. Multiple EoE-relevant cytokines including IL-5, IL-13, TNF-α and TGF-β induce ROS in esophageal keratinocytes [64]. While excessive ROS or impaired redox homeostasis cause oxidative stress to play a pathogenic role in inflammation and fibrosis [65–67], the role of ROS and their regulation in EoE remain elusive. For example, excessive ROS damage cellular components such as mitochondria, major cellular sources of ROS. Dysfunctional mitochondria further generate ROS [68]. ROS are scavenged by many cellular antioxidant enzymes (e.g. superoxide dismutase, catalase) and non-enzymatic scavengers (e.g. vitamin E and glutathione). The antioxidant-activating transcription factor NRF2 is suppressed in EoE [61]. Besides antioxidants, ROS trigger autophagy, an adaptive response that degrades intracellular components such as damaged dysfunctional mitochondria under oxidative stress [69]. Impaired autophagic flux contributes to hepatic and intestinal fibrosis, the latter found in Crohn's disease [70, 71]. We have demonstrated autophagy-mediated redox regulation in EoE. Autophagy-related gene products including ATG7 and LC3 regulate the formation of autophagy vesicles (AV), and ATG7 is an independent tissue biomarker for EoE inflammation in pediatric patients [72]. AV accumulation was further demonstrated in esophageal epithelia of EoE patients and mice with EoE-like inflammation [64]. Pharmacological inhibition of autophagy flux by hydroxychloroquine (HCQ) in EoE-bearing mice resulted in exacerbated oxidative stress, BCH and eosinophil infiltrates [64], indicating a protective role for autophagy in EoE [64]. Damaged mitochondria may undergo mitochondria-targeted autophagy, termed mitophagy [73]. The E3 ubiquitin ligase Parkin protein (PARK2 gene product) recruits damaged mitochondria to autophagic machinery [73–76]. Impaired Parkin-mediated mitophagy may have a role in the pathogenesis of pulmonary fibrosis [77]. Therefore, it is plausible that epithelial redox homeostasis and mitochondria-targeted autophagy may limit EoE-related fibrosis.

Mechanical environment and fibrosis

In addition to the chemical environment impacting the behavior of fibroblasts, the mechanical environment also stimulates fibroblast activation. Fibroblasts are mechanosensitive, thus they sense the stiffness of their environment and react to it. We have recently shown that primary esophageal fibroblasts, when cultured in a relatively soft environment (1–3 kPa), display quiescent features. However, when placed in a stiff environment, the fibroblasts display enhanced proliferation, contractility, cell spreading, and α-SMA expression [38] even in the absence of exogenous cytokine stimulation. Autocrine TGF-β signaling has been implicated as SMAD3 phosphorylation was induced in fibroblasts as a function of stiffness. Fibroblasts, therefore, can differentiate into activated myofibroblasts without an inflammatory milieu in the setting of a stiff environment suggesting that even in the setting of disease remission, fibroblasts may continue to perpetuate matrix remodeling in a stiff environment.

Potential therapeutic targets for fibrosis in EoE

Compared to other human diseases featuring fibrosis, there is a substantially large knowledge gap in the biological processes and profibrotic signaling pathways involved in the pathogenesis of EoE-related fibrosis. Despite extensive gene expression profiling in biopsies from EoE patients [42, 78–80], there is no reliable tissue biomarker of EoE-related esophageal fibrotic stricture. Potential therapeutic targets for EoE-related fibrosis include the biological processes, signaling pathways, cytokines and factors regulating recruitment and activation of fibroblasts, production and deposition of collagen and other ECM components and tissue stiffness. To this end, neutralizing antibodies for TGF-β [81] and other cytokines can be utilized. Small molecule inhibitors may suppress pertinent signaling pathways. Immunosuppressants such as rapamycin may activate autophagy to decrease oxidative stress. Rapamycin has been shown to attenuate fibrosis in the kidney and the liver [82, 83]. β-aminopropionitrile (BAPN) may suppress LOX-mediated collagen crosslinking [84]. Preclinical in vivo testing can be done in emerging murine models of EoE [13, 85, 86] although characterization of lamina propria fibrosis remains limited in these models.

Clinical evaluation of remodeling in EoE

EoE was first described in the early 90′s in a series of reports describing a population of patients with refractory reflux symptoms and difficulty swallowing who had increased esophageal epithelial eosinophilia [87, 88]. As more was learned about EoE, it was noted that there is an age dependent difference in presenting symptoms in EoE. Young children present with feeding disorders and vomiting while older children and adults present with dysphagia and food impaction [89]. Others have shown that duration of untreated disease and symptoms correlate with stricture formation and fibrostenosis [8, 90, 91]. Furthermore, fibrostenotic phenotype as defined as the presence of rings or stricture on esophagogastroduodenoscopy increases with age. For every 10 years gained in age, there is double the risk of having fibrostenotic disease [90]. Taken together, this data suggest that EoE is a progressive disease with unchecked chronic inflammation leading to increased stiffness and fibrosis. However, there have not been long-term prospective studies that follow patients through the maturation process from childhood into adulthood to confirm these observed trends. An alternative theory is that this is a disease comprising inflammatory and fibrostenotic types [92]. Longitudinal studies to evaluate the progression of disease and improved methods of detecting fibrosis are needed to better delineate the natural history.

Measurements of fibrostenotic disease

The current methods of evaluating for fibrostenosis include: (1) visual endoscopic evaluation, (2) esophagogram, and (3) histologic evaluation [93]. Visual inspection by endoscopy can identify rings and more severe narrow caliber esophagi, however esophagogram may be more sensitive at detecting more subtle esophageal narrowing [94]. Histologic evaluation of the lamina propria-the current gold standard is limited by sampling. This is especially true in pediatric samples where approximately 50% have adequate lamina propria for interpretation defined this as > 35 μm of thickness without crush effect [95]. Adult studies fare only slightly better with 61% with adequate lamina propria reported [96]. Taken together, this suggests that we are unable to assess fibrotic tissue remodeling in half of the EoE patients.

More recently, use of the endoscopic Endoluminal Functional Lumen Imaging probe (EndoFLIP) has provided information that the EoE esophagus is less distensible than the normal esophagus [49, 95, 97, 98]. Adult studies have shown that in EoE, patients with a history of food impaction have decreased distensibility and the distensibility is predictive of future food impaction [49, 98].

While all natural history studies show that fibrostenosis is less common in pediatrics, the pediatric esophagus in EoE is less distensible than non-EoE when controlling for age [95]. Importantly, decreased esophageal distensibility has been associated with the presence of lamina propria fibrosis in pediatric active EoE patients [95]. Moreover, pediatric patients with active EoE (> 15 eos per hpf) displayed decreased esophageal distensibility with a history of dysphagia or food impaction [95]. While the EndoFLIP is used largely as a research tool at this time, its use for clinical care is increasing. As more studies are done, this tool has significant promise as a method to detect subtle narrowing currently undetectable with other tools.

Conclusions

An improved understanding of the microenvironment in the EoE esophagus has provided insights into the development of fibrosis. The mixed inflammatory infiltrate and cytokine redundancy have led to therapeutic challenges [99, 100]. Because of high rates of non-response with current therapeutic options [4] and high rates of fibrostenosis at diagnosis, consideration of novel therapeutic strategies directed at the activated myofibroblast and collagen deposition may improve patient symptoms more effectively than current strategies. Currently, we are treating fibrostenotic disease after it has occurred. Future research to detect sub-clinical fibrosis prior to the onset of narrowing and dysphagia will greatly improve patient outcomes and decrease the burden of disease.

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