


RESEARCH

Open Access



Characterization of lamina propria remodeling in pediatric eosinophilic esophagitis using second harmonic generation microscopy

Ezekiel J. Haugen^{1,2}, Andrea K. Locke^{1,3}, Hernán Correa⁴, Justin S. Baba^{1,2}, Anita Mahadevan-Jansen^{1,2} and Girish Hiremath^{1,2,5*} 

Abstract

Eosinophilic esophagitis (EoE) is a chronic inflammatory condition characterized by an intense infiltration of eosinophils into the esophageal epithelium. When not adequately controlled, eosinophilic inflammation can lead to changes in components of the extracellular matrix (ECM) of the lamina propria. Particularly, alterations to the collagen fiber matrix can lead to lamina propria fibrosis (LPF), which plays an important role in the fibrostenotic complications of EoE. Current approaches to assess LPF in EoE are prone to inter-observer inconsistencies and provide limited insight into the structural remodeling of the ECM. An objective approach to quantify LPF can eliminate inter-observer inconsistencies and provide novel insights into the fibrotic transformation of the lamina propria in EoE. Second harmonic generation (SHG) microscopy is a powerful modality for objectively quantifying disease associated alterations in ECM collagen structure that is finding increasing use for clinical research. We used SHG with morphometric analysis (SHG-MA) to characterize lamina propria collagen fibers and ECM porosity in esophageal biopsies collected from children with active EoE ($n = 11$), inactive EoE ($n = 11$), and non-EoE ($n = 11$). The collagen fiber width quantified by SHG-MA correlated positively with peak eosinophil count ($r = 0.65$, $p < 0.005$) and histopathologist scoring of LPF ($r = 0.52$, $p < 0.005$) in the esophageal biopsies. Patients with active EoE had a significant enlargement of ECM pores compared to inactive EoE and non-EoE ($p < 0.005$), with the mean pore area correlating positively with EoE activity ($r = 0.76$, $p < 0.005$) and LPF severity ($r = 0.65$, $p < 0.005$). These results indicate that SHG-MA can be utilized to objectively characterize and provide novel insights into lamina propria ECM structural remodeling in children with EoE, which could aid in monitoring disease progression.

Keywords Eosinophilic esophagitis, Fibrosis, Second Harmonic Generation Microscopy, Multiphoton Imaging, Extracellular matrix, Histopathology

*Correspondence:

Girish Hiremath

Girish.Hiremath@vumc.org

¹Vanderbilt Biophotonics Center, Vanderbilt University, Nashville, TN 37232, USA

²Department of Biomedical Engineering, Vanderbilt University, Nashville, TN 37232, USA

³Department of Chemistry, Vanderbilt University, Nashville, TN 37232, USA

⁴Division of Pediatric Pathology, Monroe Carell Jr. Children's Hospital at Vanderbilt, Vanderbilt University Medical Center, Nashville, TN 37232, USA

⁵Division of Pediatric Gastroenterology, Hepatology and Nutrition, Monroe Carell Jr. Children's Hospital at Vanderbilt, Vanderbilt University Medical Center, Nashville, TN 37232, USA



© The Author(s) 2024. **Open Access** This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

Background

Eosinophilic esophagitis (EoE) is an allergen-mediated chronic inflammatory condition of the esophagus estimated to affect 1 in 1500 children in the United States [1]. If left undiagnosed or ineffectively managed, EoE can lead to fibrostenotic complications such as dysphagia, esophageal narrowing, and esophageal food impaction that may require urgent endoscopic intervention [2–5]. An improved understanding of fibrostenosis in EoE and the development of objective methods to detect early fibrotic changes are needed to improve clinical outcomes.

The diagnostic hallmark of EoE is an intense influx of eosinophils into the esophageal epithelium, defined as ≥ 15 eosinophils/high-power field (eos/hpf). Prolonged eosinophilic inflammation can lead to lamina propria fibrosis (LPF), which is regarded as one of the key changes associated with fibrostenotic EoE and is a result of excessive deposition of extracellular matrix (ECM) components such as collagen (i.e., type I collagen) with increased fibroblast activation and proliferation [2, 6–8]. Currently, the assessment of LPF in biopsies collected from EoE patients relies on manual and subjective interpretation of lamina propria (LP) remodeling (e.g. increased collagen fiber size or deposition) by pathologists and researchers using traditional staining methods such as hematoxylin and eosin (H&E), trichrome and picrosirius red stains [6, 9, 10]. Regrettably, these approaches lack quantitative measurements of ECM remodeling that occurs with LPF and are prone to inter-observer inconsistencies, which limits the understanding of LPF pathobiology and can yield inconsistent diagnosis [11, 12]. As such, an unbiased approach is needed to better characterize the mechanisms of fibrostenosis in EoE and improve the clinical assessment of lamina propria health.

Second harmonic generation (SHG) microscopy is a label-free imaging technique that allows for accurate, unbiased, and automated characterization of fibrillar collagen in tissues. Second harmonic generation occurs when two photons simultaneously interact with a molecule and produce a photon of exactly twice the energy. Type I and II collagen provide significant SHG due to their highly non-centrosymmetric triple-helix structure and fibrillar alignment [13, 14]. Exploiting this effect, SHG microscopy is used for the optical characterization of collagen at submicron scales in many physiologic conditions and pathologic states [14, 15]. Morphometric analysis of SHG images can then be used to quantify alterations in the geometry and porosity of the collagen fiber matrix [16]. These properties can provide novel insights into collagen fibrogenesis, cross-linking, and degradation; cell migration and proliferation; and ECM biomechanical properties [16–19]. For the esophagus, SHG microscopy has been employed to characterize

alterations in subepithelial collagen content and morphology in Barrett's esophagus, squamous cell carcinoma and adenocarcinoma [20–22]. However, SHG microscopy has not been used to investigate LPF in EoE.

We hypothesize that SHG is an accurate and objective method to quantify structural changes in the esophageal lamina propria of EoE patients. As such, in this study, SHG microscopy with morphometric analysis (SHG-MA) was employed to examine the relationship between markers of structural remodeling in the lamina propria and EoE activity status. Additionally, we investigated the association between SHG-MA parameters and conventional histopathology scoring of EoE-based tissue changes assessed by a pathologist. Lamina propria collagen fiber width, fiber SHG/TPEF (two-photon excitation fluorescence) ratio, and extracellular matrix porosity were found to correlate with EoE diagnosis, the severity of eosinophilic inflammation, and LPF as assessed by an independent pathologist (gold standard). These findings provide new insights into lamina propria ECM remodeling in EoE and indicate that SHG-MA has potential to be a valuable tool for accurately characterizing LPF to aid in monitoring disease progression.

Methods

Study subjects and clinical assessment

Esophageal biopsies with adequate lamina propria, as determined by an expert pathologist (H.C.), were analyzed from 33 children (≤ 18 years) who had undergone esophagogastroduodenoscopy (EGD) with biopsies at Monroe Carell Jr. Children's Hospital at Vanderbilt University Medical Center for their clinical care between 2017 and 2019. During EGD, esophageal mucosal biopsies (3–4 each from the proximal and distal esophagus) were obtained using conventional biopsy forceps (Endo-Jaw, Olympus Medical Systems Corp, Tokyo, Japan) and submitted for H&E staining per the institutional protocol.

The clinical H&E sections of the biopsies were further analyzed as approved by the Vanderbilt University Medical Center Institutional Review Board (IRB #202459). The sections were assessed by an expert pathologist (H.C.) for peak eosinophil count (PEC) per high-power-field (hpf= 0.237mm^2) and for the grade (or the severity) of the involvement of esophageal tissue per the validated EoE Histology Scoring System (EoEHSS) [9]. The EoEHSS assesses eight EoE-relevant histologic features: eosinophilic inflammation (EI), basal zone hyperplasia (BZH), eosinophilic abscess (EA), eosinophilic surface layering (ESL), dilated intercellular spaces (DIS), surface epithelial alteration (SEA), dyskeratotic epithelial cells (DEC), and LPF. Each feature was scored on a 4-point scale (0–3), with 0 representing normal features and 3 denoting the most severe or extensive pathology. The mean score of

the number of features assessed yields the total EoEHSS score.

The study groups were defined per the 2011 Consensus statement [23]. Children with active EoE (aEoE) had one or more symptoms of esophageal dysfunction and a PEC of ≥ 15 eos/hpf in at least one of the multiple esophageal biopsies acquired, after excluding other causes of esophageal eosinophilia. Those with a previous diagnosis of EoE and a PEC < 15 were classified as having inactive EoE (iEoE). The non-EoE control group (controls) consisted of children without a prior diagnosis of EoE but with symptoms of esophageal dysfunction and not meeting the histologic criteria for EoE (PEC < 15).

Second harmonic generation imaging

The H&E sections (5 μm thick) from 33 esophageal biopsies were scanned under a multimodal nonlinear microscope previously described [24]. Briefly, a femtosecond laser (Insight DS+, Spectra-Physics, USA) tuned to 940 nm was used for excitation, with ~ 12.5 mW average power on the specimen (Fig. 1a). Images (1024 \times 1024 pixels; 381 \times 381 μm) were collected via galvanometric scanning and a 20X objective (Olympus UPLXAPO, 20 \times 0.8 NA). The images were stitched using Mosaic] in FIJI/ImageJ [25, 26] across the entire lamina propria and used for quantitative analysis. A blue filter (447/60

nm | Semrock, USA) was used to isolate SHG emission (470 nm) in the forward and backward directions to visualize collagen. A red filter (625/90 nm | Semrock, USA) was used to collect two-photon excitation fluorescence (TPEF), which allowed for visualization of the epithelium and underlying muscularis mucosa (if present). The signals were collected using photomultiplier tubes (Amplified PMT, Thorlabs, USA) with 5–10 μs pixel dwell times, with the intensities of each image being normalized to its dwell time. A digital H&E image and its corresponding SHG and TPEF stitched images from a patient biopsy section are shown in Fig. 1b and c, respectively.

Quantitative image analysis

Stitched images from each sample were batch processed in MATLAB (Natick, MA, USA) and ImageJ. First, the images were segmented such that only the LP was present for further analysis. A 1-pixel radius Gaussian blur was applied to each image to reduce shot noise. Morphometric features in the lamina propria, including collagen fiber width and ECM porosity, were then quantified. The SHG images were converted to 8-bits so that the gray values were within the accepted range for the ridge detection algorithm in Fiji/ImageJ [27, 28] (Fig. 1d). The ridge detection algorithm was then applied with the linewidth set to 6 pixels (~ 2.23 μm) [27, 29] (Fig. 1e).

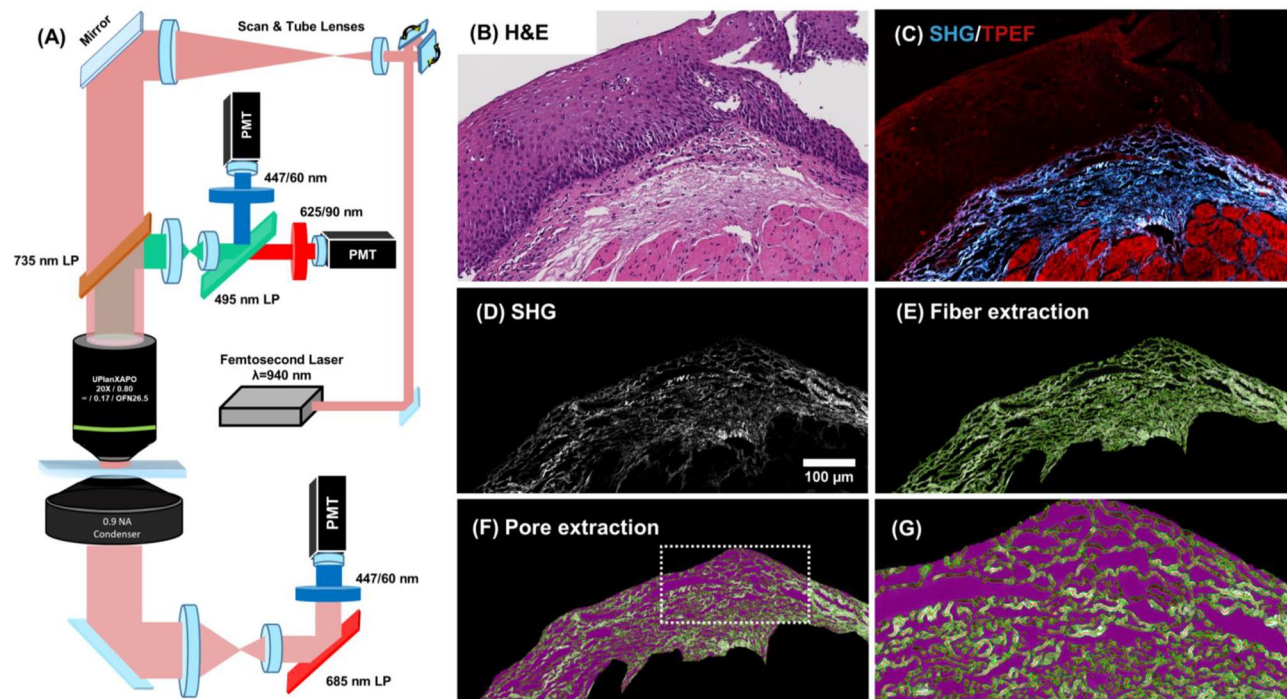


Fig. 1 Nonlinear microscopy and morphometric analysis of esophageal lamina propria in pediatric biopsies. **a** A nonlinear microscope is used to scan **b** 5 μm thick H&E sections, providing **c** two-photon excitation fluorescence images (red) and second harmonic generation (SHG) images with collagen fiber contrast (blue). The images are linearly contrast adjusted for visualization (Scale bar: 100 μm). **d** Backward SHG image depicts the collagen fiber distribution in the lamina propria. **e** A ridge detection algorithm (Steger method) is used for automated fiber segmentation. **f** Regions not containing fibers, termed pores (magenta), are extracted using Moore-Neighbor tracing and the white rectangle is magnified in panel **g**

The output lists of identified fibers, with their respective widths, were used to calculate each patient sample's mean LP fiber width (μm). The fibers detected from the ridge detection algorithm and a median threshold of the SHG signal within the LP were combined to generate a binary mask of collagen fibers. Through visual inspection, this method was found to robustly separate collagen fibers from pores (regions absent of collagen). The percentage of lamina propria taken up by collagen fibers termed the fiber coverage, was then calculated for each specimen. Additionally, the SHG/TPEF ratio and the forward-to-backward (F/B) SHG ratio were calculated for pixels within the binary mask of fibers. For porosity quantification, the fiber mask was inverted to provide a binary image of the pores (Fig. 1g). Each pore was then identified using Moore-Neighbor tracing [30]. The list of pores (with their respective areas) for each patient specimen was used to calculate the mean pore area (μm^2). The number of pores, along with the total area of the lamina propria, was used to calculate pore density (# pores / mm^2).

Statistical analysis

To determine if the fiber and porosity metrics were distinct between patients with aEoE, iEoE, and controls, analysis of variance (ANOVA) was used with a multiple comparison test using Tukey's criterion [31]. A Spearman correlation was then used to quantify the relationships between the fiber and porosity metrics from SHG-MA and conventional histopathology metrics (including PEC, BZH, LPE, and total EoEHSS grade). Lastly, to determine the diagnostic accuracy of detecting EoE using the most significant SHG-MA metric we employed receiver operating characteristic (ROC) curve analysis.

Results

Cohort characteristics

Overall, 22 children had EoE (aEoE=11 and iEoE=11), and 11 were controls. The cohort's median (interquartile range) age was 8 (4–12) years. Expectedly, PEC was significantly different in aEoE compared to iEoE and non-EoE controls (Table 1). Similarly, aEoE had higher LPF scores compared to iEoE and controls. The EoEHSS grade, indicating the overall severity of the esophageal tissue involvement, was also higher for aEoE compared to iEoE and controls (Table 1).

Correlating SHG-MA fiber and porosity features with EoE diagnosis

Second harmonic generation images of the lamina propria revealed altered morphology that was dependent on EoE status (Fig. 2a). The lamina propria collagen fiber width was significantly greater in aEoE compared to controls [(mean \pm standard deviation): $2.12 \pm 0.06 \mu\text{m}$ vs. $2.04 \pm 0.04 \mu\text{m}$; $p=0.006$] (Fig. 2b) but not iEoE ($2.09 \pm 0.07 \mu\text{m}$; $p=0.5$). The fiber coverage was lower for aEoE compared to iEoE ($56.2 \pm 2.1\%$ vs. $58.1 \pm 1.0\%$; $p=0.02$) but was not significantly different from controls ($57.5 \pm 1.5\%$; $p=0.6$) (Fig. 2c). The pore area was significantly higher for aEoE compared to iEoE ($50 \pm 10 \mu\text{m}^2$ vs. $33 \pm 5 \mu\text{m}^2$; $p<0.005$) and controls ($32 \pm 6 \mu\text{m}^2$; $p<0.005$) (Fig. 2d). Lastly, lower pore density was observed in aEoE compared to iEoE (9950 ± 2200 pores/ mm^2 vs. $13,300 \pm 2300$ pores/ mm^2 ; $p<0.005$) and controls ($13,900 \pm 2400$ pores/ mm^2 ; $p<0.005$) (Fig. 2e). Ultimately, the most significant SHG-MA metric for detecting aEoE samples was the pore area. Using ROC analysis the pore area was found to differentiate aEoE from iEoE samples with 81.8% sensitivity and 100% specificity and aEoE from nonEoE controls with 81.8% sensitivity and 90.9% specificity, using a threshold of $42.8 \mu\text{m}^2$ (Fig. 3b).

Table 1 Peak eosinophil count (PEC) and histopathology scores for esophageal biopsies

	Cohort (n = 33)			P ^a	
	nonEoE (n = 11)	aEoE (n = 11)	iEoE (n = 11)	aEoE vs. nonEoE	aEoE vs. iEoE
PEC (eos/hpf)	0 (0–0)	62 (44–116)	3 (0–4)	<0.005	<0.005
Eosinophilic Inflammation	0 (0–1)	2 (2–3)	1 (0–1)	<0.005	<0.005
Basal Zone Hyperplasia	0 (0–1)	3 (3–3)	1 (0–1)	<0.005	<0.005
Eosinophilic Abscess	0 (0–0)	1 (0–3)	0 (0–0)	<0.005	<0.005
Eosinophil Surface Layering	0 (0–0)	1 (0–3)	0 (0–0)	<0.005	<0.005
Dilated Interstitial Spaces	0 (0–0)	3 (2–3)	0 (0–1)	<0.005	<0.005
Surface Epithelial Alteration	0 (0–0)	0 (0–2)	0 (0–0)	0.02	0.03
Dyskeratotic Epithelial Cells	0 (0–0)	0 (0–1)	0 (0–0)	0.02	0.02
Lamina Propria Fibrosis	0 (0–0)	2 (2–3)	0 (0–1)	<0.005	<0.005
EoEHSS Grade	0 (0–0.13)	1.63 (1.13–2.25)	0.13 (0.13–0.50)	<0.005	<0.005

Results given as median (interquartile range)

^aAnalysis of Variance with Tukey multiple comparison tests

aEoE Active EoE, iEoE Inactive EoE

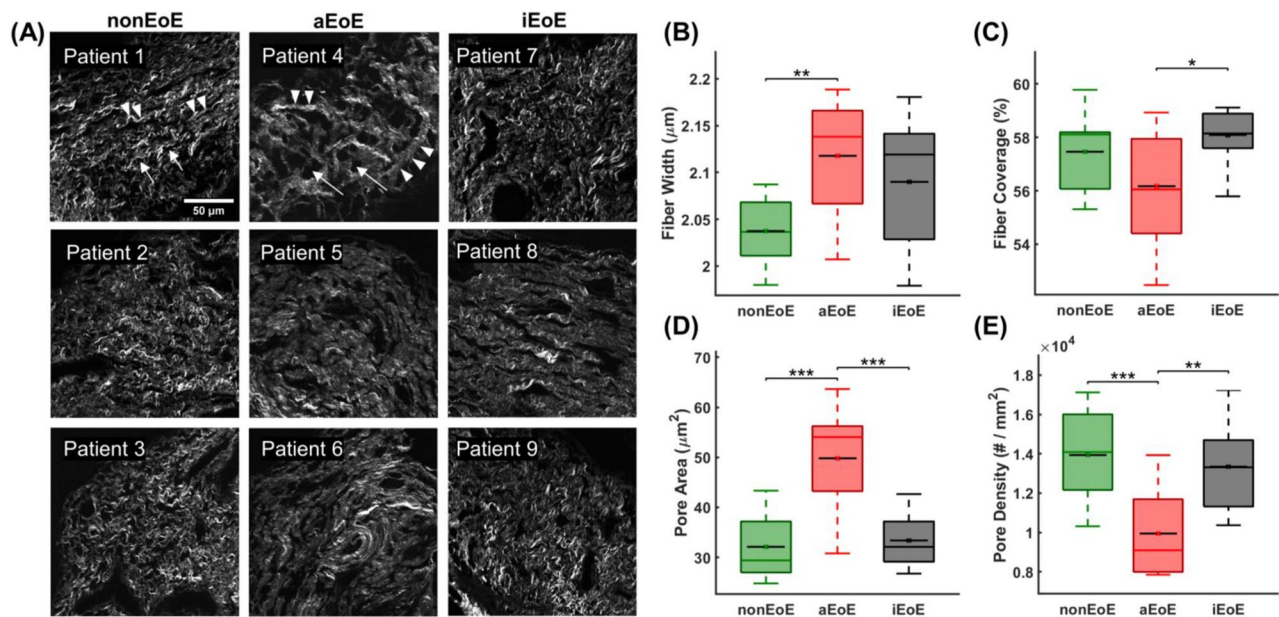


Fig. 2 Dependence of esophageal lamina propria morphology on eosinophilic esophagitis (EoE) status. **a** Mean normalized second harmonic generation images of esophageal lamina propria from nine patients depict morphological differences in the collagen fiber matrix that are dependent on EoE activity status (Scale bar: 50 µm). Arrows point to pores and arrow heads point to fibers. **b** Esophageal lamina propria fiber width (µm) extracted using a ridge detection algorithm is significantly higher in active EoE (aEoE) compared to non-EoE controls. **c** Fiber coverage (%) is significantly higher in inactive EoE (iEoE) compared to aEoE. **d** Lamina propria pore area (µm²) and **e** pore density (# pores / mm²) are significantly different in aEoE compared to iEoE and non-EoE controls. Box plot displays median line across the box and mean line in black with a center mark, while the top and bottom of the box represent the 25th and 75th percentiles. The whiskers represent the farthest data points not considered an outlier (1.5x the respective interquartile range). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$)

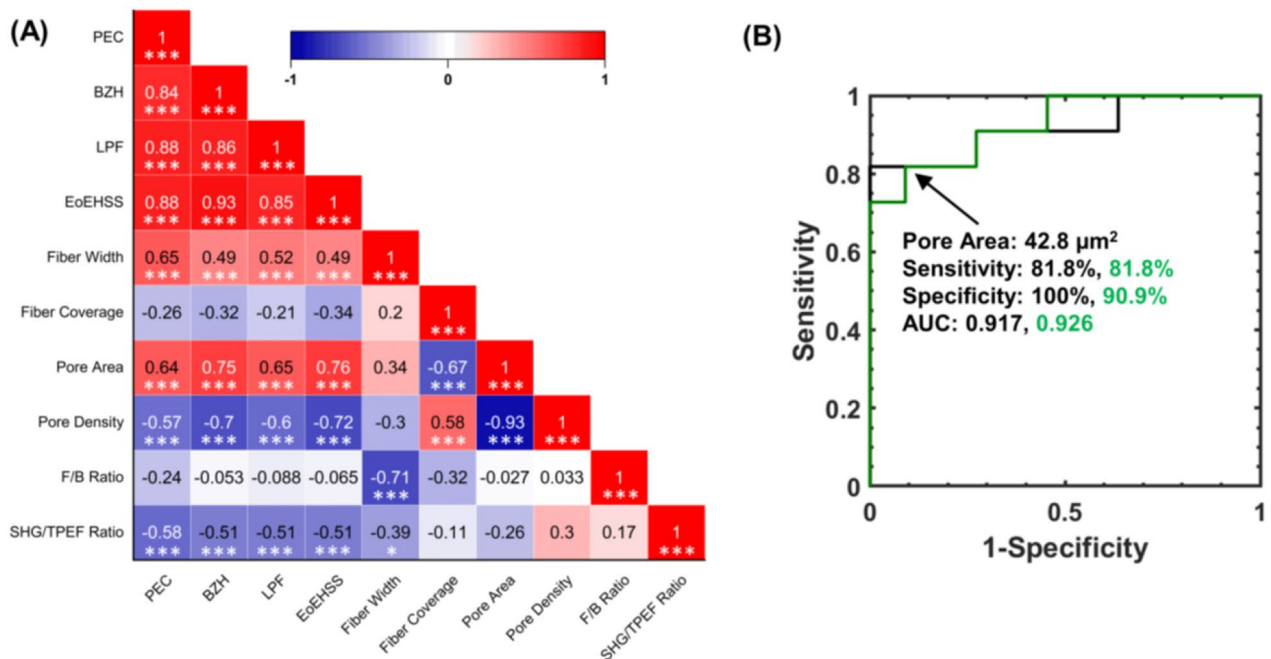


Fig. 3 a Correlation between second harmonic generation image parameters and eosinophilic esophagitis features assessed by a pathologist. Coefficient values are given by a Spearman's rank correlation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$). **b** Receiver operating characteristic (ROC) curve analysis to determine the diagnostic accuracy of the mean pore area in differentiating active EoE from inactive EoE samples (black line) and active EoE from nonEoE control samples (green line)

Correlating SHG-MA features and histopathologist scoring

The lamina propria collagen fiber and porosity metrics strongly correlated with the gold standard histopathology scores (Fig. 3a). The lamina propria collagen fiber width had a significant positive relationship with PEC ($r=0.65, p<0.005$), BZH ($r=0.49, p<0.005$), LPF ($r=0.52, p<0.005$) and total EoEHSS ($r=0.49, p<0.005$). Likewise, the mean pore area had a significant positive correlation with PEC ($r=0.64, p<0.005$), BZH ($r=0.75, p<0.005$), LPF ($r=0.65, p<0.005$) and total EoEHSS ($r=0.76, p<0.005$). Conversely, pore density had a significant negative correlation with PEC ($r=-0.57, p<0.005$), BZH ($r=-0.7, p<0.005$), LPF ($r=-0.6, p<0.005$), and total EoEHSS ($r=-0.72, p<0.005$). The F/B ratio of the collagen fibers did not correlate with histopathology scoring but had a strong negative correlation with the fiber width ($r=-0.71, p<0.005$). Lastly, the SHG/TPEF ratio of the collagen fibers had a significant negative correlation with PEC, BZH, LPF, and total EoEHSS ($p<0.005$).

Discussion

Understanding the health of esophageal lamina propria has important clinical, diagnostic, and prognostic implications in EoE. Current approaches to characterize the esophageal lamina propria are largely based on visual cues that include increased fiber thickness and density in stained sections [6, 9, 10]. Unfortunately, these approaches are prone to inconsistencies as they are subjective and rely on the training and experience of the pathologist [11]. We present the application of SHG-MA to objectively quantify changes in the lamina propria and observed that the properties of ECM collagen fibers and porosity strongly correlated with EoE activity as determined by an expert pathologist. Specifically, the fiber width and SHG/TPEF ratio, and the pore area and density most significantly correlated with EoE activity status, PEC, BZH, LPF, and total EoEHSS grade. These findings provide novel insights into the structural changes of collagen fibers in esophageal lamina propria affected by EoE. Additionally, they support the application of SHG-MA as an unbiased and automated approach to quantify markers of LPF in EoE.

The collagen fiber width, quantified using SHG-MA, correlated positively with EoE activity and the gold standard histopathology assessment of LPF, which is based on a visual evaluation of the ECM fiber width. The SHG images indicated that the growing fiber width was due to the merging of normal smaller collagen fibers into larger bundles in aEoE (Fig. 2a). This observation aligns with studies that have noted an increase in collagen cross-linking enzymes in aEoE patients [32]. Morphometric analysis revealed that the fiber width and the pore area significantly increased with EoE activity (Fig. 3a), which has been seen in subepithelial tissues undergoing natural

(e.g. pregnant cervix remodeling) or pathological (e.g., colon cancer) collagen remodeling characterized by cross-linking [16, 19]. Excessive collagen deposition, often mentioned when describing LPF in EoE [7], was not visually evident in the SHG images. In fact, the quantified percentage of lamina propria taken up by collagen (fiber coverage) had a slight but non-significant negative correlation with EoE severity (Fig. 3a). However, this analysis does not consider total LP volume or thickness, due to the inherent limitation of biopsy sampling, which would be needed to assess total volumetric collagen deposition. Moreover, this result does not preclude an increase in overall ECM fibrous content [33] but instead raises the possibility of the presence of other non-SHG-producing ECM fibers, as indicated by the decreasing fiber SHG/TPEF ratio with EoE. Overall, the results demonstrate that SHG-MA quantification of fiber width alone has significant correlation with the conventional method of manually assessing esophageal LPF in children with EoE.

Novel to our study was the identification of altered ECM porosity during LP remodeling in EoE. Specifically, the mean pore area increased, and the pore density decreased with EoE activity and returned to normal values with inactive EoE. We postulate that the increased pore space, devoid of type I collagen, may be filled with cells, fluid, blood vessels, and other biomolecules that do not produce SHG signals (elastin, soluble collagen, non-fibrillar collagen, etc.). For example, the known increase in eosinophils and lymphoid follicles within the lamina propria of EoE samples [34] may contribute towards the formation of larger pores. Likewise, enhanced cell migration in EoE (i.e., leukocyte and myofibroblast trafficking) may contribute towards increased ECM pore size, which could be further studied by developing bioengineered tissues within the range of quantified fiber and porosity values [7, 17, 35, 36]. However, additional assays will be necessary to identify the most significant driver of altered lamina propria ECM porosity and how it relates to epithelial profibrotic markers (such as transforming growth factor β , α -smooth muscle actin, and periostin) in children with EoE.

Along with the new insights into LP remodeling observed in this study, the significant correlation between fiber and porosity metrics with gold standard histopathology and diagnosis has potential clinical implications. This is evidenced by the significantly greater pore area and lower pore density in aEoE compared to iEoE and control patients (Fig. 2d, e), leading to high diagnostic accuracy in detecting aEoE (Fig. 3b). These findings suggest that the quantitative features extracted from SHG-MA could aid physicians and provide improved intra-observer agreement on LPF progression and treatment efficacy, which could improve patient outcomes. Likewise, SHG-MA could provide objective metrics for

relating microscopic changes of the LP to macroscopic changes in esophageal distensibility, as assessed by the endoluminal functional lumen imaging probe [2, 37].

Overall, the results from this study highlight that SHG-MA can provide objective metrics that significantly correlate to gold standard EoE and LPF severity assessments. However, future studies are needed to address the limitations of this work. Methodologically, images were collected from fixed biopsy sections, which does not overcome the clinical limitations of conventional tissue processing and sectioning artifacts, including improper section orientation. As such, future work will focus on scanning fresh bulk biopsies, which is an important advantage of SHG microscopy that can address this pitfall. Another limitation, related to the *ex vivo* SHG-MA approach is that about 50% of the esophageal biopsies collected using conventional biopsy forceps from pediatric EoE patients have inadequate lamina propria [38]. As such, this method may not be applicable in certain *ex vivo* situations. Despite these weaknesses, our study has several strengths. This is the first study to apply SHG-MA to objectively quantify the fibrotic transformation of lamina propria in patients with EoE. This approach can complement existing methods to better understand the pathobiology of lamina propria fibrosis and remodeling in EoE. Future studies will look to combine SHG with other multiphoton modalities (e.g. third harmonic generation [39] and stimulated Raman microscopy [40]) to quantify changes in the lamina propria, beyond collagen, toward improved sensitivity to EoE and LPF. Ultimately, the results from this study lay the foundation for the development of an *in vivo* endoscopic nonlinear imaging probe for the assessment of the health of esophageal lamina propria in children with EoE. This *in vivo* approach has the potential to overcome the limitation associated with inadequate sampling of the esophageal lamina propria using conventional biopsy forceps and could in the future serve as a modality for real-time assessment of the esophageal lamina propria in EoE.

Conclusions

Using SHG-MA, we objectively characterized and quantified esophageal lamina propria remodeling in children with and without EoE. This study adds to our understanding of esophageal LPF in EoE and demonstrates that SHG-MA holds promise to serve as an accurate, unbiased, and automated approach to quantify markers of LPF. Ongoing studies aim to investigate how the SHG-MA metrics correlate with epithelial profibrotic markers, esophageal distensibility assessed by the endoluminal functional lumen imaging probe, and EoE therapies. In the future, the use of SHG-MA for *in vivo* endoscopic detection of LPF in EoE will be explored.

Abbreviations

EoE	Eosinophilic esophagitis
LPF	Lamina propria fibrosis
ECM	Extracellular matrix
H&E	Hematoxylin and Eosin
SHG	Second harmonic generation
SHG-MA	Second harmonic generation with morphometric analysis
TPEF	Two photon excitation fluorescence
EGD	Esophagogastroduodenoscopy
IRB	Institutional review board
PEC	Peak eosinophil count
hpf	high-powered field
eos	eosinophils
EI	eosinophilic inflammation
BZH	basal zone hyperplasia
EA	eosinophilic abscess
ESL	eosinophilic surface layering
DIS	dilated intercellular spaces
SEA	surface epithelial alteration
DEC	dyskeratotic epithelial cells
aEoE	active eosinophilic esophagitis
iEoE	inactive eosinophilic esophagitis
F/B	forward-to-backward
ANOVA	analysis of variance
EoEHSS	eosinophilic esophagitis histology scoring system

Acknowledgements

We acknowledge Regina Tyree's (RN) help with clinical data management and procurement of pathology slides.

Authors' contributions

E.J.H. contributed to conceptualizing the study, data acquisition, data analysis, statistical analysis, interpretation of data, drafting the manuscript and critical revision of the manuscript for important intellectual content. A.K.L. contributed to conceptualizing the study, interpretation of data and critical revision of the manuscript for important intellectual content. H.C. contributed to acquisition, analysis, and interpretation of data. J.S.B. contributed to critical revision of the manuscript for important intellectual content, interpretation of data, and study supervision. A.M.-J. contributed to critical revision of the manuscript for important intellectual content, statistical analysis, obtained funding, administrative, technical, and material support, and study supervision. G.H. contributed to conceptualizing the study, acquisition and interpretation of data, drafting the manuscript, critical revision of the manuscript for important intellectual content, obtained funding, administrative, technical, and material support, and study supervision. Each of the authors have approved the final draft submitted.

Funding

E.H. is supported by 1R01DK132338-01 from the NIH (awarded to A.M.-J.) and 1K23DK131341-01 (awarded to G.H.). A.L. is supported by 1R01DK132338-01 from the NIH (awarded to A.M.-J.). A.M.-J. is supported by 1R01DK132338-01 from the NIH. G.H. is supported by NIH 1R01DK132338-01, NIH 1K23DK131341-01, NIH 1R21AI168832-01 and the Consortium of Eosinophilic Gastrointestinal Diseases Researchers (NIH 1U54AI117804-01). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Data availability

All data collected during the study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval and consent to participate

This retrospective study was done under the approval of the Vanderbilt University Medical Center Institutional Review Board (IRB #202459).

Consent for publication

Not applicable.

Competing interests

None to report.

Received: 3 November 2023 / Accepted: 17 March 2024

Published online: 22 March 2024

References

- Dellon ES, Jensen ET, Martin CF, Shaheen NJ, Kappelman MD. Prevalence of Eosinophilic Esophagitis in the United States. *Clin Gastroenterol Hepatol*. 2014;12(4):589–e5961.
- Menard-Katcher C, Benitez AJ, Pan Z, Ahmed FN, Wilkins BJ, Capocelli KE, et al. Influence of age and eosinophilic esophagitis on esophageal distensibility in a Pediatric Cohort. *Am J Gastroenterol*. 2017;112(9):1466–73.
- Schoepfer AM, Safroneeva E, Bussmann C, Kuchen T, Portmann S, Simon H, et al. Delay in diagnosis of Eosinophilic Esophagitis increases risk for stricture formation in a time-dependent manner. *Gastroenterology*. 2013;145(6):1230–e12362.
- Furuta GT, Katzka DA. Eosinophilic Esophagitis. Ingelfinger JR, editor. *N Engl J Med*. 2015;373(17):1640–8.
- Etyreddy AR, Sink JR, Georg MW, Kitsko DJ, Simons JP. Association between Eosinophilic Esophagitis and Esophageal Food Impaction in the Pediatric Population. *Otolaryngol Neck Surg*. 2018;159(4):750–4.
- Chehade M, Sampson HA, Morotti RA, Magid MS. Esophageal Subepithelial Fibrosis in Children with Eosinophilic Esophagitis. *J Pediatr Gastroenterol Nutr*. 2007;45(3):10.
- Muir AB, Wang JX, Nakagawa H. Epithelial-stromal crosstalk and fibrosis in eosinophilic esophagitis. *J Gastroenterol*. 2019;54(1):10–8.
- Wynn TA. Common and unique mechanisms regulate fibrosis in various fibroproliferative diseases. *J Clin Invest*. 2007;117(3):524–9.
- Collins MH, Martin LJ, Alexander ES, Boyd JT, Sheridan R, He H, et al. Newly developed and validated eosinophilic esophagitis histology scoring system and evidence that it outperforms peak eosinophil count for disease diagnosis and monitoring: eosinophilic esophagitis pathology. *Dis Esophagus*. 2017;30:1–8.
- Pronio A, Covotta F, Pallotta L, Palma R, Badiali D, Sacchi MC, et al. Eosinophilic esophagitis: Cytokines expression and fibrotic markers in comparison to Celiac Disease. *Diagnostics*. 2022;12(9):2092.
- Vieira MC, Gugelmin ES, Percicote AP, Ribeiro MG, De Miranda RA, Vieira GG, et al. Intra- and interobserver agreement of histopathological findings in pediatric patients with eosinophilic esophagitis. *J Pediatr (Rio J)*. 2022;98(1):26–32.
- Thaker AI, Smith J, Pathak M, Park JY. Challenges in inter-rater agreement on Lamina Propria Fibrosis in Esophageal biopsies. *Pediatr Dev Pathol*. 2023;26(2):106–14.
- Cox G, Kable E, Jones A, Fraser I, Manconi F, Gorrell MD. 3-Dimensional imaging of collagen using second harmonic generation. *J Struct Biol*. 2003;141(1):53–62.
- Campagnola PJ, Dong CY. Second harmonic generation microscopy: principles and applications to disease diagnosis. *Laser Photonics Rev*. 2011;5(1):13–26.
- Mostaço-Guidolin L, Rosin N, Hackett TL. Imaging Collagen in scar tissue: developments in second Harmonic Generation Microscopy for Biomedical Applications. *Int J Mol Sci*. 2017;18(8):1772.
- Akins ML, Luby-Phelps K, Mahendroo M. Second harmonic generation imaging as a potential tool for staging pregnancy and predicting preterm birth. *J Biomed Opt*. 2010;15(2):026020–1.
- Miron-Mendoza M, Seemann J, Grinnell F. The differential regulation of cell motile activity through matrix stiffness and porosity in three dimensional collagen matrices. *Biomaterials*. 2010;31(25):6425–35.
- Raub CB, Unruh J, Suresh V, Krasieva T, Lindmo T, Gratton E, et al. Image Correlation Spectroscopy of Multiphoton Images Correlates with Collagen Mechanical properties. *Biophys J*. 2008;94(6):2361–73.
- Despotović SZ, Miličević ĐN, Krmpot AJ, Pavlović AM, Živanović VD, Krivokapić Z, et al. Altered organization of collagen fibers in the uninvolved human colon mucosa 10 cm and 20 cm away from the malignant tumor. *Sci Rep*. 2020;10(1):6359.
- Mehravar S, Banerjee B, Chatrath H, Amirsolaimani B, Patel K, Patel C, et al. Label-free multi-photon imaging of dysplasia in Barrett's esophagus. *Biomed Opt Express*. 2016;7(1):148.
- Chen WC, Chen YJ, Lin ST, Hung WH, Chan MC, Wu IC, et al. Label-free characterization of collagen fibers in cancerous esophagus tissues using ratiometric nonlinear optical microscopy. *Exp Biol Med*. 2020;245(14):1213–21.
- Liu NR, Chen GN, Wu SS, Chen R. Distinguishing human normal or cancerous esophagus tissue *ex vivo* using multiphoton microscopy. *J Opt*. 2014;16(2):025301.
- Liacouras CA, Furuta GT, Hirano I, Atkins D, Attwood SE, Bonis PA, et al. Eosinophilic esophagitis: updated consensus recommendations for children and adults. *J Allergy Clin Immunol*. 2011;128(1):3–e206.
- Adams WR, Mehl B, Lieser E, Wang M, Patton S, Throckmorton GA, et al. Multi-modal nonlinear optical and thermal imaging platform for label-free characterization of biological tissue. *Sci Rep*. 2021;11(1):8067.
- Thévenaz P, Unser M. User-friendly semiautomated assembly of accurate image mosaics in microscopy. *Microsc Res Tech*. 2007;70(2):135–46.
- Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. 2012;9(7):676–82.
- Steger C. An unbiased detector of curvilinear structures. *IEEE Trans Pattern Anal Mach Intell*. 1998;20(2):113–25.
- Wagner T, Hiner M. Ridge Detection 1.4.0 [Internet]. Zenodo; 2017. <https://doi.org/10.5281/zenodo.845874>.
- Wedowski RD, Farooq AR, Smith LN, Smith ML. High speed, multi-scale tracing of curvilinear features with automated scale selection and enhanced orientation computation. In: 2010 International Conference on High Performance Computing & Simulation. Caen, France: IEEE; 2010. p. 410–7.
- Gonzalez RC, Woods RE, Eddins SL. Digital Image processing using MATLAB. Pearson/Prentice Hall; 2004.
- Kramer CY. Extension of multiple range tests to Group means with unequal numbers of replications. *Biometrics*. 1956;12(3):307.
- Kasagi Y, Dods K, Wang JX, Chandramouleeswaran PM, Benitez AJ, Gambanga F, et al. Fibrostenotic eosinophilic esophagitis might reflect epithelial lysyl oxidase induction by fibroblast-derived TNF- α . *J Allergy Clin Immunol*. 2019;144(1):171–82.
- Hsieh LY, Chiang AWT, Duong LD, Kuo CC, Dong SX, Dohil R, et al. A unique esophageal extracellular matrix proteome alters normal fibroblast function in severe eosinophilic esophagitis. *J Allergy Clin Immunol*. 2021;148(2):486–94.
- Schoepfer AM, Simko A, Bussmann C, Safroneeva E, Zwahlen M, Greuter T, et al. Eosinophilic esophagitis: relationship of Subepithelial eosinophilic inflammation with epithelial histology, Endoscopy, Blood eosinophils, and symptoms. *Am J Gastroenterol*. 2018;113(3):348–57.
- Zhu S, Yuan Q, Yin T, You J, Gu Z, Xiong S, et al. Self-assembly of collagen-based biomaterials: preparation, characterizations and biomedical applications. *J Mater Chem B*. 2018;6(18):2650–76.
- Aceves SS, Ackerman SJ. Relationships between eosinophilic inflammation, tissue remodeling, and Fibrosis in Eosinophilic Esophagitis. *Immunol Allergy Clin North Am*. 2009;29(1):197–211.
- Nicodème F, Hirano I, Chen J, Robinson K, Lin Z, Xiao Y, et al. Esophageal distensibility as a measure of Disease Severity in patients with eosinophilic esophagitis. *Clin Gastroenterol Hepatol*. 2013;11(9):1101–e11071.
- Hiremath G, Choksi YA, Acra S, Correa H, Dellon ES. Factors Associated with adequate Lamina Propria Sampling and Presence of Lamina Propria Fibrosis in Children with Eosinophilic Esophagitis. *Clin Gastroenterol Hepatol*. 2021;19(9):1814–e18231.
- Van Huizen LMG, Kuzmin NV, Barbé E, Van Der Te Velde VS, Groot EA. Second and third harmonic generation microscopy visualizes key structural components in fresh unprocessed healthy human breast tissue. *J Biophotonics*. 2019;12(6):e201800297.
- Orringer DA, Pandian B, Niknafs YS, Hollon TC, Boyle J, Lewis S, et al. Rapid intraoperative histology of unprocessed surgical specimens via fibre-laser-based stimulated Raman scattering microscopy. *Nat Biomed Eng*. 2017;1(2):0027.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.