RNA-sequencing reveals molecular and regional differences in the esophageal mucosa of achalasia patients

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Achalasia is an esophageal motility disorder characterized by the functional loss of myenteric plexus ganglion cells in the distal esophagus and lower esophageal sphincter. Histological changes have been reported in the esophageal mucosa of achalasia, suggesting its involvement in disease pathogenesis. Despite recent advances in diagnosis, our understanding of achalasia pathogenesis at the molecular level is very limited and gene expression profiling has not been performed. We performed bulk RNA-sequencing on esophageal mucosa from 14 achalasia and 8 healthy subjects. 65 differentially expressed genes (DEGs) were found in the distal esophageal mucosa of achalasia subjects and 120 DEGs were identified in proximal esophagus. Gene expression analysis identified genes common or exclusive to proximal and distal esophagus, highlighting regional differences in the disease. Enrichment of signaling pathways related to cytokine response and viral defense were observed. Increased infiltration of CD45+ intraepithelial leukocytes were seen in the mucosa of 38 achalasia patients compared to 12 controls. Novel insights into the molecular changes occurring in achalasia were generated in this transcriptomic study. Some gene changes observed in the mucosa of achalasia may be associated with esophagitis. Differences in DEGs between distal and proximal esophagus highlight the importance of better understanding regional differences in achalasia.
defensive responses. Furthermore, the epithelium is emerging as an important regulator of smooth muscle contraction in many tissues and organs through the release of epithelium-derived factors. Supporting this concept, in-silico simulations using a fully coupled computational model of the esophagus suggest that a highly compliant mucosa is essential for normal esophageal transport function. Some studies have reported histological changes of the esophageal mucosa of achalasia patients, including basal cell hyperplasia and esophagitis, but these studies rely entirely on histological analyses and there have been no studies of the human transcriptome in achalasia reported to date. To address this knowledge gap, we investigated whether esophageal mucosa of achalasia patients have distinct gene expression profiles compared to healthy subjects.

### Results

#### Subjects.
Overall, 22 controls, mean age 32.6 (25–48), 72.7% female, and 37 achalasia patients, mean age 50.3 (25–82), 59.4% female, were included. The controls all had normal endoscopy and 20 had normal motility on HRM. The achalasia cohort entailed 21 with type I achalasia (8/21, 38.0% with dilated esophagus) and 14 with type II achalasia (6/14, 42.9% with dilated esophagus). Table 1 describes subject characteristics by analysis.

### Transcriptomic analyses reveal changes in gene expression levels in the proximal and distal esophagus of achalasia patients.
We performed RNA-seq on esophageal mucosal biopsies from the proximal and distal esophagus of 14 achalasia and 8 healthy controls to identify gene targets that are dysregulated in achalasia (Table 1). Biopsies from the proximal and distal esophagus of these subjects were processed, sequenced, and analyzed separately to determine if regional differences are observed within subjects. Examples of the histology of the biopsies collected and processed are shown in Fig. 1. Differential gene expression was determined compared to healthy controls. Overall, 706 genes were significantly changed in the distal esophagus and 1896 genes were significantly changed in the proximal esophagus (false discovery rate-adjusted p value < 0.05). The gene expression profiles of each sample were visualized and compared using heat maps and volcano plots. PCA was used to evaluate similarities and differences between controls and achalasia patients, and to determine if each set of subjects can be grouped. As shown in Fig. 2, PCA plot shows apparent clustering of the achalasia subjects in the distal esophagus, separated from the controls. Data obtained from the proximal esophagus shows some clustering of achalasia patients and controls, despite more variability within each group.

### Transcriptomic analysis identifies pathways differentially regulated in achalasia subjects versus healthy controls.
After filtering differentially expressed genes (DEGs) between controls and achalasia patients using a threshold of fold change in expression of >0.9 or <0.9, we identified 65 DEGs in distal esophagus (31 up-regulated and 34 down-regulated) and 120 DEGs in proximal esophagus (81 up-regulated and 39 down-regulated). Gene Set Enrichment Analysis using Metascape and GSEA showed increased gene expression in 7 pathways including those related to cellular response to cytokine stimulus and defense response to virus, when compared to the controls. Decreased gene regulation in 6 pathways including those linked to skeletal muscle organ development, G alpha (i) signaling events and regulation of ERK1 and ERK2 cascade and axon development were observed in the distal esophageal mucosa from achalasia patients versus controls. On the other hand, mucosa from the proximal esophagus of achalasia had increased gene regulation in 12 pathways including those associated with leukotriene D4 metabolic process, degradation of extracellular matrix, NGF stimulated transcription, negative regulation of epithelial differentiation, positive regulation of tyrosine phosphorylation of STAT protein, angiogenesis, and Notch signaling pathway. Finally, down-regulated gene regulation in 4 pathways including ECM glycoproteins and matrisome associated were detected in proximal esophageal mucosa of achalasia patients.

### Table 1.
Clinical characteristics of enrolled patients.

|                  | Healthy (n=8) | Achalasia (n=14) | Healthy (n=4) | Achalasia (n=23) | Healthy (n=10) | Achalasia (n=23) |
|------------------|--------------|------------------|--------------|------------------|--------------|------------------|
| Age, years, mean ± SD | 33 ± 8.0     | 49.9 ± 16.1      | 29.3 ± 2     | 50.6 ± 15        | 33.6 ± 4.4   | 50.6 ± 15        |
| Achalasia subtype, n (%) |              |                  |              |                  |              |                  |
| Type I           |              | 5 (35.7)         | 16 (70)      | -                | 16 (70)      |                  |
| Type II          |              | 9 (64.3)         | 7 (30)       | -                | 7 (30)       |                  |
| Dilation status, n (%) |              |                  |              |                  |              |                  |
| Non-dilated      |              | 7 (50.0)         | 15 (65.2)    | -                | 15 (65.2)    |                  |
| Dilated          |              | 7 (50.0)         | 8 (34.8)     | -                | 8 (34.8)     |                  |
| Sex, n (%)       |              |                  |              |                  |              |                  |
| Male             | 2 (25.0)     | 3 (21.4)         | 4 (100)      | 12 (52)          | 4 (40)       | 12 (52)          |
| Female           | 6 (75.0)     | 11 (78.6)        | 0 (0)        | 11 (48)          | 6 (60)       | 11 (48)          |

Dilated – 7 (50.0) – 8 (34.8) – 8 (34.8)
Non-dilated – 7 (50.0) – 15 (65.2) – 15 (65.2)

|                  | Achalasia subtype, n (%) | Healthy (n=8) | Achalasia (n=14) | Healthy (n=4) | Achalasia (n=23) | Healthy (n=10) | Achalasia (n=23) |
|------------------|--------------------------|--------------|------------------|--------------|------------------|--------------|------------------|
| Type I           | 5 (35.7)                 |              |                  |              |                  |              |                  |
| Type II          | 9 (64.3)                 |              |                  |              |                  |              |                  |
| Dilation status, n (%) |                  |              |                  |              |                  |              |                  |
| Non-dilated      | 7 (50.0)                 |              |                  |              |                  |              |                  |
| Dilated          | 7 (50.0)                 |              |                  |              |                  |              |                  |

Achalasia subtype and dilation status were determined by endoscopy in all subjects.
Regional differences in gene expression are observed in the proximal versus distal esophagus of achalasia patients. Our RNA-seq analyses showed differences in the number of DEGs in the distal and proximal esophageal mucosa from achalasia subjects (Figs. 2d and 3d) resulting in differential enrichment of pathways in each region (Figs. 2e,f, 3e,f). As shown in Fig. 4a, only 23 DEGs were found to be common between the distal and proximal esophagus of achalasia patients. 13 of these genes were up-regulated (Fig. 4b) and 10 were down-regulated (Fig. 4c). Examples of common DEGs include CPA3, MAMDC2, and CAPN6. 42 DEGs were exclusive to the distal esophageal mucosa of achalasia patients (Fig. 4a): 18 of these DEGs were up-regulated (Fig. 4b) and 24 were down-regulated (Fig. 4c). The most significantly DEGs only in the distal esophagus include IL-33, IFNε, LOX, and JUN. On the other hand, 97 genes were uniquely differentially expressed in the proximal esophageal mucosa of achalasia patients (Fig. 4a): 68 of these genes were up-regulated (Fig. 4b) and 29 were down-regulated (Fig. 4c). This includes change in expression in CDH16, HES5, IGFBP3 and FGF14.

Quantitative polymerase chain reaction (qPCR) validates DEGs in achalasia when compared to controls. To validate the DEGs identified by RNA-sequencing, we performed quantitative PCR (qPCR) on esophageal mucosal samples in a distinct cohort from that used for RNA-seq (4 controls, 23 achalasia patients). Table 1 shows the clinical characteristic of the enrolled subjects. Among the genes identified by RNA-sequencing, we selected 5 genes to be validated by qPCR: the mast cell specific protease carboxypeptidase (CPA3), the cytokine interleukin 33 (IL-33), the type 1 interferon family member interferon epsilon (IFNε), the antiviral response gene MAM domain containing 2 (MAMDC2) and the cell adhesion molecule cadherin 16 (CHD16). As shown in Fig. 5a, CPA3 mRNA expression levels were significantly up-regulated in the distal mucosa of achalasia subjects compared to controls, but not in the proximal esophagus. On the other hand, the cytokine IL-33, known to activate target cells such as mast cells and type 2 innate lymphoid cells13, had significant increased mRNA expression levels in the proximal esophageal mucosa of achalasia patients, but this change was not significant in the distal esophagus (Fig. 5b). We also observed that achalasia patients had a strong increase in mRNA expression levels of the type I interferon IFNε, in both the distal and proximal esophageal mucosa compared to controls (Fig. 5c). Interestingly, a significant increase in CDH16, an atypical member of the cadherin family16, was seen in both the proximal and distal esophageal mucosa of achalasia subjects compared to controls (Fig. 5d). Finally, we observed a significant decrease in MAMDC2 in the proximal esophageal mucosa of achalasia patients compared to controls, but this change was not significant in the distal esophagus (Fig. 5e).

Infiltration of intraepithelial leukocytes is detected in the esophageal epithelium of achalasia patients. Our RNA-seq and qPCR analyses showed changes in many genes associated with inflammation and immune cell infiltration in achalasia. We first examined the presence of immune cells by performing immunofluorescence to detect the leukocyte marker CD45 in human esophageal mucosal biopsies from achalasia and healthy subjects. As shown in Fig. 6, immunostaining and scoring for CD45 showed increased infiltration of intraepithelial leukocytes in the distal esophageal mucosa of achalasia subjects (Fig. 6b,c), compared to controls.
A significant increase in the recruitment of intraepithelial leukocytes was also observed in the proximal esophageal mucosa of achalasia patients (Fig. 6e,f) compared to controls (Fig. 6d,f).

Distinct changes in gene expression and differentially regulated pathways are observed in the proximal and distal esophagus of type I versus type II achalasia patients. Given that type I achalasia is usually a later phase of disease progression, we next determined differential gene expression between type I or type II achalasia and healthy controls, in both proximal and distal esophagus. As shown in Fig. 7a, we identified 501 DEGs (240 up-regulated and 261 down-regulated) in the distal esophagus of type I achalasia
compared to healthy controls. For type II achalasia, we found 144 DEGs in distal esophagus; 77 of these DEGs were up-regulated and 67 DEGs were down-regulated (Fig. 7b). In the proximal esophagus of type I achalasia, we identified 329 DEGs (209 up-regulated and 120 down-regulated) (Fig. 8a). On the other hand, the type II achalasia had 294 DEGs (191 up-regulated and 103 down-regulated) in proximal esophagus (Fig. 8b). We then determined the number of DEGs that were common or exclusive to type I and type II achalasia. In distal esophagus, we found 86 DEGs to be common between type I and type II achalasia (Fig. 7c). A total of 415 DEGs were exclusive to type I achalasia (Fig. 7c): 194 DEGs were up-regulated (Fig. 7d) and 221 were down-regulated.
rolling, negative regulation of canonical Wnt signaling and regulation of leukocyte proliferation (Fig. 7g). In the
decreased gene regulation of genes associated to pathways related to extracellular matrix, leukocyte tethering or
immune response and focal adhesion were enriched in the distal esophagus of type 1 achalasia. We also found
ated to lymphocyte activation, regulation of cell adhesion, smooth muscle contraction, positive regulation of
immune response and focal adhesion were enriched in the distal esophagus of type 1 achalasia. We also found
decreased gene regulation of genes associated to pathways related to extracellular matrix, leukocyte tethering or
rolling, negative regulation of canonical Wnt signaling and regulation of leukocyte proliferation (Fig. 7g). In the
distal esophagus of type II achalasia, we observed increased gene regulation in pathways related to intermedi-
ate filament organization and negative regulation of wound healing (Fig. 7f) and decreased expression of genes
related to pathways such as fat cell differentiation, NGF stimulated transcription and regulation of system process
(Fig. 7g). Analyses performed in proximal esophagus showed the enrichment of pathways related to matrisome,
delta Np63 pathway, positive regulation of signaling receptor activity in type I achalasia (Fig. 8f). Decreased gene
regulation of genes associated to reactive oxygen species metabolic process, defense response to fungus, matri-
some associated and PLC beta mediated events was seen (Fig. 8g). In type II achalasia, increased expression of
genres related to peptide cross-linking, organic hydroxy compound transport and transport of small molecules
was observed (Fig. 8f). Analyses also showed decreased gene regulation of genes associated to pathways related
to negative regulation of secretion, smooth muscle contraction and regulation of angiogenesis (Fig. 8g).

Discussion
Over the past decades, transcriptome profiling has been a common approach used to characterize the molecular
changes occurring in human disease, which has led to the identification of numerous molecular biomarkers and
new therapeutic targets. This is the first study to evaluate transcriptomic changes in the esophageal mucosa of
achalasia patients compared to healthy controls. We identified 65 DEGs in the distal esophageal mucosa and
120 DEGs in the proximal esophageal mucosa of achalasia patients. Among the differentially expressed genes,
we identified distinct changes in cellular response induced by cytokine stimulus in distal esophageal achalasia
biopsies. These findings are consistent with our knowledge of the pathophysiology of achalasia and its connec-
tion to inflammation. Persistent esophageal distension leads to food stasis and liquid residue that sometimes
ferments causing bacterial overgrowth. Over time this predisposes to chronic inflammation and increases the risk
of developing esophageal squamous cell cancer. Furthermore, our study confirms the presence of esophagitis
previously reported in histological studies of the esophageal mucosa in achalasia, which we confirmed in
our study as well.

Among the inflammation-related genes, our study indicates significant up-regulation of the mast cell pepti-
dase CPA3 in the mucosa of achalasia patients. Interestingly, previous studies showed significant mast cell
infiltration or a change in their distribution and degranulation in the LES muscle of achalasia patients compared
to controls. However, the significance of mast cell degranulation and infiltration in the LES of achalasia
patients, as well as the function of intraepithelial mast cells compared to LES mast cells needs to be determined.
Nonetheless, this suggests a potential role for mast cells in the pathogenesis of achalasia. Further supporting
this idea, we observed increased expression of the cytokine IL-33, an alarmin generally expressed by esophageal
epithelial cells, fibroblasts and endothelial cells. IL-33 is released extracellularly upon stimulus and can activate
mast cells, eosinophils and basophils and type 2 innate lymphoid cells. IL-33 plays a role in sensing damage
caused by inflammation and is reportedly important in the pathogenesis of eosinophilic esophagitis and

![Figure 4](https://doi.org/10.1038/s41598-022-25103-7)
reflux esophagitis[^30]. Although our findings suggest an association between CPA3+ mast cells and IL-33 in the esophageal mucosa of achalasia, the lack of significant increase of CPA3 in proximal esophagus also implies a role for IL-33 on other immune cell types in achalasia.

**Figure 5.** Using qPCR, mRNA expression levels of DEGs were determined in a validation cohort of achalasia. (a) CPA3 is enriched in the esophageal mucosa of achalasia subjects in distal esophagus. *P < 0.02. (b) IL-33 mRNA expression levels are increased in the proximal esophagus of achalasia subjects. **P < 0.005. (c) Increased IFNγ is seen in both proximal and distal esophageal achalasia. **P < 0.002, ***P < 0.001. (d) The proximal and distal esophageal mucosa of achalasia is enriched for CDH16. *P < 0.05, ***P < 0.001. (e) Decreased MAMDC2 is observed in the proximal esophageal mucosa of achalasia. *P < 0.05. n = 4 healthy control and 23 achalasia.

[^30]: K. Takaishi, et al., *Gastroenterology*, 133(3), 849-858, 2007.
[^31]: S. Ueki, et al., *Gastroenterology*, 141(5), 1433-1443, 2011.
Our transcriptomic analyses also showed that genes associated with the defense response to virus are enriched in the distal esophageal mucosa of achalasia patients. Interestingly, a possible association between achalasia, the destruction of myenteric neurons and viral infection has been proposed, indicating a possible association between achalasia and viral infection. Potential viruses associated with achalasia include herpes simplex virus (HSV)-1, varicella-zoster virus (VZV), measles, mumps, and human immunodeficiency virus (HIV)32. Among the up-regulated genes associated to viral response was IFNε, a unique member of the type I interferon family which plays crucial role in innate and adaptive responses to viral infection33. In the lung mucosa, IFNε induced the infiltration of a functional/cytotoxic CD8+CD4+ T cell population subset34. In the esophageal mucosa of achalasia patients, CD4+ T cells are shown to be a predominant immune cell population17,19. The function of IFNε, and its impact on intraepithelial leukocyte activation in the esophagus and in achalasia is yet to be determined.

Significantly, our findings show molecular heterogeneity between proximal and distal mucosa in the esophagus of achalasia patients, with few shared genes that are differentially expressed when compared to healthy subjects. Thinking about the pathophysiology of achalasia, this heterogeneity makes sense. During disease progression, achalasia patients may develop massive dilatation of the esophageal body, subjecting the esophageal mucosa to significant mechanical stress. The distal esophageal mucosa gets more exposure to food stasis as the result of gravity. Given that the primary etiology of achalasia involves the lower esophageal sphincter and distal esophagus, studies characterizing changes in the esophageal mucosa in achalasia patients have focused mostly on the distal esophageal mucosa. However, our findings suggest that including separate analyses of the proximal esophageal mucosa could offer additional insights.

Our study also demonstrates transcriptomic differences between type I and type II achalasia, both in proximal and distal esophagus. Given that type I achalasia is believed to be a more advanced stage of achalasia compared to type II, with the presence of food stasis and dilatation6, it is not surprising that we observed a higher number of DEGs in type I achalasia compared to type II, when both compared to healthy controls. These changes in DEGs in type I achalasia also correlate with the enrichment of pathways associated to wound healing and the regulation of immune response. Furthermore, the gradual increase in a subset of genes and pathways from type II to type I achalasia strongly supports at the molecular level the concept of type I achalasia representing a progression of the disease from type II achalasia. These current results are based on our current cohort of 4 type I achalasia patients and 9 type II achalasia patients and give strong support to continue investigating the
molecular differences between type I and type II achalasia in a larger cohort, which will allow greater power to directly uncover the changes that occur in the progression from type I to type II achalasia.

One major limitation of studies involving patients with early-stage achalasia is the acquisition of esophageal biopsies, which are often limited to the epithelium. Furthermore, we have observed increased epithelial thickness in most achalasia subjects, resulting in partial absence of the basal cell layer in some of these biopsies, while others have presence of some lamina propria. This can introduce variability in gene expression. Furthermore, given the etiology of achalasia, performing analyses on LES and muscle biopsies in parallel with esophageal mucosal

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### Figure 7

Differential gene expression analysis comparing changes in distal esophageal mucosa of type I and/or type II achalasia versus controls. (a) The number of differentially expressed genes (DEGs) in type I achalasia versus healthy controls based on a log2 fold change of 0.9 and FDR < 0.05. (b) Differentially expressed genes between type II achalasia and healthy controls were selected based on a log2 fold change of 0.9 and FDR < 0.05. (c) Venn diagram showing overlap of total DEGs in type I and type II achalasia subjects. (d) Venn diagram showing overlap of up-regulated DEGs between type I and type II achalasia subjects. (e) The overlap of down-regulated DEGs between type I and type II achalasia subjects is shown in a Venn diagram. (f,g) DEGs from each comparison were used to identify enrichment of functional pathways by Gene Ontology analysis. (f) A heatmap of the top up-regulated pathways is shown. (g) A heatmap of the top down-regulated pathways is shown.
samples from the same patients would be extremely valuable and informative. Unfortunately, acquiring muscle tissue from achalasia subjects is usually done during myotomy surgery, thus limiting this option. Although bulk RNA-sequencing was helpful in providing an overview of gene expression analyses in the esophageal mucosa of type I and type II achalasia, it reflects the average gene expression across thousands of cells. Therefore, it is possible that cellular heterogeneity in the disease could be masked. Thus, combining our bulk transcriptomic findings with single cell transcriptomic analyses in the future could provide additional insights into the pathogenesis of achalasia.

In conclusion, our study demonstrated transcriptomic differences in the esophageal mucosa of type I and type II achalasia compared to control subjects. We also reported regional differences in the distal vs proximal esophageal mucosa of achalasia subjects.
Methods

Subject sample collection and processing. Thirty-seven patients with newly diagnosed type 1 or type 2 achalasia according to the Chicago Classification of esophageal motility disorders v4.0 encountered at the Esophageal Center at Northwestern were included. Type 3 achalasia patients were excluded because the spastic contractions in type 3 achalasia have unique physiology independent of bolus retention and sphincter function. Patients were also excluded if they had previously been treated for achalasia with pneumatic dilation, Heller’s myotomy, or PerOral Endoscopic Myotomy (POEM). All achalasia patients underwent endoscopy, HRM, barium esophagram, and functional luminal imaging probe (FLIP) Panometry. A subject’s esophagus was considered dilated when the esophageal width measured ≥ 5 cm on barium esophagram. All procedures using human tissue received approval from the Northwestern Institutional Review Board (STU00208111) and all methods were performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from all subjects/legal guardians.

Healthy, asymptomatic (ie, free of esophageal symptoms including dysphagia, heartburn, and chest pain), adult volunteers, “controls”, were enrolled. Potential subjects were excluded for a previous diagnosis of esophageal, autoimmune, or eating disorders. Additional exclusion criteria included use of antacids or proton pump inhibitors, body mass index greater than 30 kg/m², or a history of tobacco use or alcohol abuse. The controls underwent endoscopy, HRM, and FLIP, as previously described.

For both patients and controls, esophageal mucosal biopsies were collected during sedated endoscopy through the Digestive Health Foundation Biorepository. Biopsies were collected from the distal and proximal esophagus, at 5 and 15 cm above the squamocolumnar junction, respectively. Briefly, for histology and immunostaining studies, tissue was fixed in neutral buffered formalin (Fisher Scientific, Hampton, NH) for 24 h, embedded in paraffin, and 4-μm sections were applied to positively charged slides. Slides were stained with hematoxylin and eosin, and images were captured on a Nikon Eclipse Ci-E microscope with a Nikon DS-Ri2 camera and NIS Elements software. For RNA studies, tissue was stored in RNA later and AllProtect (Thermo Fisher Scientific, Pittsburgh, PA) and stored at ~80 °C until RNA extraction.

RNA isolation and quantitative PCR. Esophageal mucosal biopsies were homogenized prior to RNA extraction in RLT lysis buffer (Qiagen). Total RNA was extracted using the RNasy kit according to the manufacturer’s instructions (Qiagen, Germantown, MD). The Maxima First-Strand complementary DNA Synthesis reaction was performed in accordance with the relevant guidelines and regulations. Informed consent was obtained from all subjects/legal guardians.

RNA-seq was deposited in Gene Expression Omnibus (GEO #201699) and can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201699.

Immunohistochemistry and immunofluorescence. Heat antigen retrieval (2100 Antigen Retriever, Electron Microscopy Sciences, Hatfield, PA) was performed for paraffin-embedded esophageal sections and slides were incubated with the following antibodies: 1:500 rabbit anti-CD45 (AB10558, Abcam, Cambridge, MA), 1:500 rabbit anti-CPA3 (HPA008689, Sigma). Species-specific secondary antibodies were added, and detection was performed as previously described. For fluorescent labeling, Alexa Fluor™ 488 (#A32814, Thermo Fisher Scientific) was used. Dapi was used as a nuclear stain. CD45 staining was scored based on (1) the percent surface area covered by positive cells on a scale from 0 to 4 (0 = none, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%) and (2) localization of positive cells in the epithelium on a scale from 1 to 4 (1 = localized around the papilla, 2 = slightly away from papilla, 3 = away from papilla and at another location in epithelium, 4 = throughout the epithelium). Scoring for CPA3 was done by counting the number of positive cells per high power field. Scoring was done by two blinded investigators. Images were captured on a Nikon Eclipse Ci-E microscope with a Nikon DS-Ri2 camera and NIS Elements software.

Statistical analyses. Results are expressed as mean ± SEM, with statistical differences performed on normalized values between experimental conditions established at 95% confidence. The Welch’s t-test was used to indicate statistical difference between groups. All statistics were performed using Graph Pad Prism version 9.2.0 (Graph Pad software, San Diego, CA).

Data availability RNA-seq was deposited in Gene Expression Omnibus (GEO #201699) and can be accessed at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE201699.
References

1. Sanoff, S. et al. Incidence and prevalence of achalasia in Central Chicago, 2004–2014, since the widespread use of high-resolution manometry. Clin. Gastroenterol. Hepatol. 15, 366–373. https://doi.org/10.1016/j.cgh.2016.08.030 (2017).

2. Nenishi, R. et al. The cost of achalasia: Quantifying the effect of symptomatic disease on patient cost burden, treatment time, and work productivity. Surg. Innov. 17, 291–294. https://doi.org/10.1177/1553530610376392 (2010).

3. Porschen, R., Molsberger, G., Kuhn, A., Sarbia, M. & Borchard, F. Achalasia-associated squamous cell carcinoma of the esophagus: Flow-cytometric and histological evaluation. Gastroenterology 108, 545–549. https://doi.org/10.1016/S0016-5085(99)90084-5 (1995).

4. Streitz, J. M. Jr., Ellis, F. H. Jr., Gubb, S. P. & Heatley, G. M. Achalasia and squamous cell carcinoma of the esophagus: Analysis of 241 patients. Ann. Thorac. Surg. 59, 1604–1609. https://doi.org/10.1016/S0003-4975(94)00971-9 (1995).

5. Goldblum, J. R., Whyte, R. L., Orringer, M. B. & Appelman, H. D. Achalasia: A morphologic study of 42 resected specimens. Am. J. Surg. Pathol. 18, 327–337 (1994).

6. Pandolfo, J. E. & Gawron, A. J. Achalasia: A systematic review. JAMA 313, 1841–1852. https://doi.org/10.1001/jama.2015.2906 (2015).

7. Beyder, A. In pursuit of the epithelial mechanosensitivity mechanisms. Front. Endocrinol. (Lausanne) 9, 804. https://doi.org/10.3389/fendo.2018.00804 (2018).

8. Brooks, J. W., Parton, R. G., Yap, A. S. & Duszyc, K. In Tight junctions (ed. González-Mariscal, L.) 27–50 (Springer International Publishing, 2022).

9. Li, J. et al. The strength of mechanical forces determines the differentiation of alveolar epithelial cells. Dev. Cell 44, 297–312 e295. https://doi.org/10.1016/j.devcel.2018.01.008 (2018).

10. McGinn, J. et al. A biomechanical switch regulates the transition towards homeostasis in oesophageal epithelium. Nat. Cell Biol. 23, 511–525. https://doi.org/10.1038/s41556-021-00679-w (2021).

11. Ruan, Y. C., Zhou, W. & Chan, H. C. Regulation of smooth muscle contraction by the epithelium: Role of prostaglandins. Physiol. (Bethesda) 26, 156–170. https://doi.org/10.1152/physiol.00363.2010 (2011).

12. Kou, W., Pandolfo, J. E., Kahrilas, P. J. & Patankar, N. A. Simulation studies of the role of esophageal mucosa in bolus transport. Biomech. Model. Mechanobiol. 16, 1001–1009. https://doi.org/10.1007/s10237-016-0867-1 (2017).

13. Bektas, A. et al. Flow cytometric DNA analysis, and immunohistochemical p53, PCNA and histopathologic study in primary achalasia: Preliminary results. Hepatogastroenterology 48, 408–412 (2001).

14. Kjellin, A. P., Ost, A. E. & Pope, C. E. I. Histology of esophageal mucosa from patients with achalasia. Dis. Esophagus 18, 257–261. https://doi.org/10.1111/des.12014 (2005).

15. Cayrol, C. & Girard, J. P. Interleukin-33 (IL-33): A nuclear cytokine from the IL-1 family. Immunol. Rev. 281, 154–168. https://doi.org/10.1111/imr.12619 (2018).

16. Wendeler, M. W., Jung, R., Himmelbauer, H. & Gessner, R. Unique gene structure and paralogy define the 7D-cadherin family. Biomech. Model. Mechanobiol. 17, 122–131. https://doi.org/10.1007/s10237-016-0867-1 (2017).

17. Dohla, M. et al. Intramuscular interstitial cells of Cajal associated with mast cells survive nitrergic nerves in achalasia. Neurogastroenterol. Motil. 33, e14055. https://doi.org/10.1111/nmo.14055 (2021).

18. Zárate, N. et al. Intramuscular interstitial cells of Cajal associated with mast cells survive nitrergic nerves in achalasia. Neurogastroenterol. Motil. 18, 556–568. https://doi.org/10.1111/1365-2982.2006.00788.x (2006).

19. Martin, N. T. & Martin, M. U. Interleukin 33 is a guardian of barriers and a local alarmin. Nat. Immunol. 17, 122–131. https://doi.org/10.1038/nri3370 (2016).

20. Jouli, R., U'Azqi, F. E., Valittuti, S. & Espinos, E. IL-33 fine tunes mast cell degranulation and chemokine production at the single-cell level. J. Allergy Clin. Immunol. 140, 497–509 e10. https://doi.org/10.1016/j.jaci.2016.09.049 (2017).

21. Bourfi, C. et al. IL-33 markedly activates murine eosinophils by an NF-kappaB-dependent mechanism differentially dependent upon an IL-4-driven autinflammatory loop. J. Immunol. 191, 4317–4325. https://doi.org/10.4049/jimmunol.1301465 (2013).

22. Kim, B. S. & Artis, D. Group 2 innate lymphoid cells in health and disease. Cold Spring Harb. Perspect. Biol. 7, a016337. https://doi.org/10.1101/cshperspect.a016337 (2015).

23. Judd, L. M. et al. Elevated IL-33 expression is associated with pediatric eosinophilic esophagitis, and exogenous IL-33 promotes eosinophilic esophagitis development in mice. Am. J. Physiol. Gastrointest. Liver Physiol. 310, G13–G25. https://doi.org/10.1152/ajpgi.00290.2015 (2016).

24. Venturelli, N. et al. Allergic skin sensitization promotes eosinophilic esophagitis through the IL-33-basophil axis in mice. J. Allergy Clin. Immunol. 138, 1367–1380. https://doi.org/10.1016/j.jaci.2016.02.034 (2016).

25. Shan, J. et al. Epithelial-derived nuclear IL-33 aggravates inflammation in the pathogenesis of reflux esophagitis. J. Gastroenterol. Hepatol. 30, 414–423. https://doi.org/10.1111/jgh.13988–1 (2015).

26. Shan, J. et al. Interferon gamma-induced nuclear interleukin-33 potentiates the release of esophageal epithelial derived cytokines. Manometr Int 11, e0151701. https://doi.org/10.1111/jpme.151701 (2016).

27. Wu, X. Y. et al. The etiology of achalasia: An immune-dominant disease. J. Dig. Dis. 22, 126–135. https://doi.org/10.1111/1751-2980.12973 (2021).

28. Marks, Z. R. C. et al. Properties and functions of the novel type I interferon epsilon. Semin. Immunol. 43, 101328. https://doi.org/10.1016/j.seminimmunol.2019.101328 (2019).

29. Xi, Y., Day, S. L., Jackson, R. J. & Ranasinghe, C. Role of novel type I interferon epsilon in viral infection and mucosal immunity. Mucosal Immunol. 5, 610–622. https://doi.org/10.1038/mi.2012.35 (2012).

30. Gwyal, C. P. et al. Chicago Classification update (V4.0): Technical review on diagnostic criteria for ineffective esophageal motility and absent contractility. Neurogastroenterol. Motil. 33, e14134. https://doi.org/10.1111/nmo.14134 (2021).

31. Carlson, D. A. et al. Classifying esophageal motility by FLIPpanometry: A study of 72 subjects with manometry. Am. J. Gastroenterol. 116, 2357–2366. https://doi.org/10.1038/ajg.2019.009 (2021).

32. Carlson, D. A. et al. Normal values of esophageal distensibility and distension-induced contractility measured by functional luminal imaging probe manometry. Clin. Gastroenterol. Hepatol. 17, 674–681 e671. https://doi.org/10.1016/j.cgh.2018.07.042 (2019).
38. Dobin, A. et al. STAR: Ultrafast universal RNA-seq aligner. Bioinformatics 29, 15–21. https://doi.org/10.1093/bioinformatics/bts635 (2013).
39. Anders, S., Pyl, P. T. & Huber, W. HTSeq—A Python framework to work with high-throughput sequencing data. Bioinformatics 31, 166–169. https://doi.org/10.1093/bioinformatics/btu638 (2015).
40. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. https://doi.org/10.1186/s13059-014-0550-8 (2014).
41. Zhou, Y. et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat. Commun. 10, 1523. https://doi.org/10.1038/s41467-019-09234-6 (2019).
42. Mootha, V. K. et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat. Genet. 34, 267–273. https://doi.org/10.1038/ng1180 (2003).
43. Subramanian, A. et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. U.S.A. 102, 15545–15550. https://doi.org/10.1073/pnas.0506580102 (2005).
44. Yang, Y., Goldstein, B. G., Nakagawa, H. & Katz, J. P. Kruppel-like factor 5 activates MEK/ERK signaling via EGFR in primary squamous epithelial cells. FASEB J. 21, 543–550. https://doi.org/10.1096/fj.06-6694com (2007).

**Author contributions**

C.K.P. and M.P.T. were involved in the collection, analysis, and interpretation of the data and in writing the report. N.B.H. and L.E.T. was involved in collection, analysis, and interpretation of the data. P.J.K., D.A.C. and J.E.P. were involved in the study design and writing the report. N.B.H. was involved in collection, analysis, and interpretation of the data. C.K.P., P.J.K., N.B.H., L.E.T., D.A.C., J.E.P. and M.P.T. have approved the final draft submitted.

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**Competing interests**

JEP, PJK, and Northwestern University hold shared intellectual property rights and ownership surrounding FLIP Panometry systems, methods, and apparatus with Medtronic Inc. DAC: Medtronic (Speaking, Consulting). PJK: Ironwood (Consulting); Reckitt (Consulting), Johnson & Johnson (consulting), Astra Zeneca (consulting). JEP: Endogastric Solutions (Consulting, Speaking), Ironwood (Grant, Consulting), Sandhill Scientific (Consulting, Speaking), Takeda (Speaking), Astra Zeneca (Speaking), Medtronic (Speaking, Consulting, Advisory Board, IP-Patent with Licensing), Torax/Ethicon (Speaking, Consulting). CKP, NBH, LET, MPT: nothing to disclose.

**Additional information**

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