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ORIGINAL ARTICLE

Lower esophageal sphincter muscle of patients with achalasia exhibits profound mast cell degranulation

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Abstract

Background: Eosinophils and mast cells are key effectors of allergy. When they accumulate in the esophagus, their myoactive, pro-inflammatory, and cytotoxic products potentially could cause achalasia-like motility abnormalities and neuronal degeneration. We hypothesized that there is an allergy-mediated form of achalasia.

Methods: LES muscle samples obtained during Heller myotomy from patients with achalasia or EGJ outflow obstruction (EGJOO) and from organ donor controls were immunostained for tryptase. Eosinophil and mast cell density, and mast cell degranulation were assessed. LES muscle was evaluated by qPCR for genes mediating smooth muscle Ca^{2+} handling and contraction.

Key Results: There were 13 patients (7 men, median age 59; 10 achalasia, 3 EGJOO) and 7 controls (4 men, median age 42). Eosinophils were infrequent in LES muscle, but mast cells were plentiful. Patients and controls did not differ significantly in LES mast cell density. However, 12 of 13 patients exhibited profound LES mast cell degranulation involving perimysium and myenteric plexus nerves, while only mild degranulation was seen in 2 of 7 controls. Hierarchical clustering analysis of qPCR data revealed two "mototype" LES gene expression patterns, with all type II patients in one mototype, and type I and III patients in the other.

Conclusions & Inferences: LES muscle of patients with achalasia or EGJOO exhibits striking mast cell degranulation, and patients with different achalasia manometric phenotypes exhibit different LES patterns of expression for genes mediating Ca^{2+} handling and muscle contraction. Although these findings are not definitive, they support our hypothesis that achalasia can be allergy-driven.

KEYWORDS
allergy, eosinophilic esophagitis, esophageal motility disorder, gene expression, smooth muscle

Abbreviations: Ca^{2+}, calcium; EGJOO, esophago-gastric junction outflow obstruction; EoE, eosinophilic esophagitis; GERD, gastro-esophageal reflux disease; H&E, hematoxylin and eosin; HPF, high power field; IRP, integrated relaxation pressure; LES, lower esophageal sphincter; POEM, per-oral endoscopic myotomy; PPIs, Proton pump inhibitors; qPCR, quantitative real-time polymerase chain reaction; SEM, standard error of the mean; Th2, T helper 2.

Nelson and Zhang are Co-First authors.

1 | INTRODUCTION

There is a poorly understood association between achalasia and esophageal eosinophilia. Achalasia esophagectomy specimens and esophageal muscle biopsies taken from achalasia patients during Heller myotomy sometimes exhibit dense accumulations of eosinophils in the muscularis propria, and it has been proposed that cytotoxic products released by degranulating eosinophils might destroy esophageal neurons and thereby cause achalasia.[1–5] Moreover, reports of patients with eosinophilic esophagitis (EoE) who had achalasia-like motility abnormalities resolving with treatments that eliminated esophageal eosinophils suggest that neuro- and myo-active substances released by degranulating eosinophils might cause a reversible form of achalasia.[6,7] We recently proposed that EoE, like eosinophilic gastroenteritis, might have *mucosal*-predominant and *muscle*-predominant forms.[8] Since esophageal muscularis propria is not accessible during routine endoscopy, a muscle-predominant form of EoE that causes achalasia might go unrecognized.

EoE is regarded as an allergic disorder in which food antigens induce a type 2 immune response, with esophageal expression of Th2 cytokines leading to esophageal infiltration by eosinophils.[9,10] Like eosinophils, mast cells also are key effectors of allergic disorders.[11] Immunohistochemical staining of esophageal mucosal biopsies from EoE patients for mast cell tryptase regularly reveals increased numbers of intact and degranulated mast cells, often to the point that mast cell density (cells/mm^2) exceeds eosinophil density.[12,13] In addition, recent studies in children with EoE suggest that mast cells play an eosinophil-independent role in causing the clinical manifestations of the disease.[14]

Like eosinophils, mast cells produce many pro-inflammatory cytokines and toxic molecules capable of damaging neurons.[15,16] Mast cells have been implicated in the pathogenesis of neurodegenerative disorders such as Parkinson's and Alzheimer's disease,[17,18] and a recent report has described mast cell infiltration in lower esophageal sphincter (LES) muscle of achalasia patients, suggesting that mast cells might contribute to the neuronal degeneration of achalasia.[19] Mast cells produce many of the same neuro- and myo-active substances as eosinophils, and the arguments proposed above for how eosinophils in esophageal muscle might cause motility disturbances also pertain to mast cells. In mice with transgenic overexpression of IL-5, esophageal mast cell accumulation is associated with the achalasia-like abnormalities of hypercontraction and impaired relaxation of esophageal muscle, even in the absence of eosinophils.[20] In another EoE mouse model induced by nasal instillation of aspergillus, esophageal muscle hyperplasia and hypertrophy develop in wild-type but not in mast cell-deficient mice.[21] All this suggests that mast cell effects on esophageal muscle and its innervation might contribute importantly to motility abnormalities in an allergic disorder of the esophagus.

The hypercontraction and poor relaxation of LES muscle that characterize achalasia have been attributed primarily to loss of inhibitory neurons in the myenteric plexus.[22] However, there is reason to consider that intrinsic or acquired features of LES muscle itself contribute to achalasic motility disturbances, especially if there is an allergy-mediated form of the disease. A considerable body of data implicates mast cells in the altered airway smooth muscle (ASM) contraction and gene expression found in allergic asthma. Like LES muscle in achalasia, ASM in allergic asthma exhibits hypercontractility but, unlike achalasia, allergic asthma is not characterized by neuronal loss that might explain the hypercontractility. Rather, studies have documented alterations in asthmatic ASM itself that can contribute to hypercontraction (eg, remodeling of actin cytoskeleton, increases in cytosolic Ca^{2+}, augmented Ca^{2+} sensitization).[23] A study comparing bronchial biopsies from patients with asthma with those from patients with eosinophilic bronchitis (who have bronchial eosinophilia as in asthma but without airway hyperresponsiveness) found that mast cell numbers were dramatically higher in the asthmatic biopsies, suggesting that mast cells, not eosinophils, are primarily responsible for ASM dysfunction in asthma.[24] Furthermore, when human ASM cells are co-cultured with mast cells or treated with mast cell tryptase, the ASM exhibit increased secretion of transforming growth factor (TGF)-β1, increased expression of alpha-smooth muscle actin, and increased agonist-provoked contraction, suggesting that mast cells promote smooth muscle cell differentiation into a more contractile phenotype.[25] Mast cells in esophageal smooth muscle of patients with EoE express TGF- β1,[26] and

Key points

- We recently proposed that a form of allergy-mediated esophagitis that involves esophageal muscle predominantly might cause achalasia.
- To explore our hypothesis that there is an allergy-mediated form of achalasia, we stained for eosinophils and mast cells in LES muscle biopsies taken during Heller myotomy from 10 patients with achalasia and 3 patients with esophago-gastric junction outflow obstruction; control LES muscle was obtained from 7 heart-beating, deceased organ donors.
- Although eosinophil and mast cell numbers did not differ significantly among patients and controls, tryptase immunostaining revealed profound LES mast cell degranulation involving perimysium and myenteric plexus nerves in 12 of 13 patients.
- Hierarchical clustering analysis of qPCR data on genes mediating smooth muscle $Ca2^+$ handling and contraction revealed two "mototype" LES gene expression patterns, with all achalasia type II patients in one mototype.
- These data support our hypothesis that achalasia can be an allergy-mediated disease, and suggest that when patients acquire an allergic form of achalasia, their underlying genetic mototype might contribute to the resulting manometric phenotype.

treatment of human esophageal smooth muscle cells with TGF-β1 increases their expression of phospholamban (PLN), a protein that facilitates muscle contraction by preventing uptake of cytosolic calcium by the sarcoplasmic reticulum.[27] Taken together, these data suggest that mast cells can alter smooth muscle expression of Ca^{2+} handling and contractility genes to enhance contraction.

To explore our hypothesis that there is an allergy-mediated form of achalasia, we quantitated eosinophil and mast cell infiltration in LES muscle biopsies taken during Heller myotomy for achalasia or esophago-gastric junction outflow obstruction (EGJOO). We also assayed LES muscle for hypertrophy, hyperplasia, or atrophy, and for expression of genes involved in Ca^{2+} handling and smooth muscle contraction. For controls, we used LES muscle from the esophagus of heart-beating, deceased organ donors with no history of esophageal disease.

2 | MATERIALS AND METHODS

2.1 | Study subjects, controls, and LES muscle sample acquisition

All patients undergoing Heller myotomy for achalasia or EGJOO performed by our surgical investigators (E.P., S.L., M.W.) at Baylor University Medical Center October 2017-August 2019 were invited to participate. Achalasia diagnosis was based on a combination of typical clinical, endoscopic, and radiographic features, with high-resolution manometry showing absent peristalsis and elevated IRP (>15 mmHg); achalasia type was determined by post-swallow changes in esophageal body pressures per Chicago classification v3.[28] EGJOO diagnosis was based on the same features as for achalasia patients, except that some peristalsis was preserved. Demographic and clinical data including age, gender, type and duration of symptoms, medication use for allergic disorders, prior surgeries and achalasia treatments, and Eckardt score were recorded. During Heller myotomy, a 1 cm × 0.5 cm × 0.5 cm segment of LES muscularis propria was excised, placed on ice, and transported to our laboratory.

Esophagi from heart-beating deceased organ transplant donors with no known history of esophageal disease were used as controls. From March 2018 through August 2019, when E.P. (thoracic surgeon and lung transplant team member) finished harvesting the heart and/or lungs from such donors, he dissected the esophagus and proximal stomach from surrounding tissues, and transected the proximal esophagus between linear staple loads; the stomach was transected in a similar fashion several centimeters distal to the EGJ.

This study was approved by Baylor Scott and White Research Institute IRB and by Southwest Transplant Alliance. All patients provided signed informed consent, and all persons authorizing esophagus organ donation per Revised Uniform Anatomical Gift Act signed the Southwest Transplant Alliance authorization form.

2.2 | Tissue handling and histopathologic evaluation

For human donor esophagi, full-thickness sections across the EGJ were taken to provide an LES muscle segment similar in location to that obtained from study patients, but also with accompanying mucosa. Full-thickness sections of the LES segment from organ donors and LES muscle from study patients were fixed in 10% buffered formalin; if available, LES muscle was also snap frozen and stored in liquid nitrogen for RNA analyses. Formalin-fixed LES specimens were oriented longitudinally and placed into cassettes prior to paraffin embedding (ensuring that cuts would contain both longitudinal and circular muscle with intervening myenteric plexus), and serial 5 μm sections were cut and mounted on glass slides. Slides were stained with H&E for routine histologic evaluation. Tryptase and CD117 immunostaining for mast cells and their degranulation products and Ki-67 immunostaining were performed on a Ventana BenchMark Ultra autostainer using Optiview DAB Detection Kit (cat no. 760-700, Roche Diagnostics, Indianapolis, IN) and Ultra Cell Conditioning 1 (cat no. 950-224, Roche Diagnostics, Indianapolis, IN) with pH 8.5 retrieval solution for pre-diluted mouse monoclonal anti-human tryptase [clone G3 (cat no. 760-4276); Roche Diagnostics], rabbit monoclonal anti-human CD117 [clone YR145 (cat no. 117R-15-ASR), 1:100; Cell Marque, Rocklin, CA], or mouse monoclonal anti-human Ki-67 [clone MIB-1 (cat no. M7240), 1:160; Dako, Carpinteria, CA]. Slides were incubated in primary antibody for tryptase for 16 minutes, CD117 for 24 minutes, or Ki-67 for 48 minutes. DAB (3,3'-diaminobenzidine) was used for visualization. Positive and negative controls for tryptase, Ki-67, and CD117 are described and shown in Figure S1. Two gastrointestinal pathologists (RMG, KT) independently evaluated histology specimens from patients and controls. Any discrepancies between the two were resolved later in group meetings. Esophageal specimens from controls were evaluated for evidence of mucosal disease (eg GERD, eosinophilic esophagitis). Quantitative assessment of eosinophil and mast cell density was performed by counting peak number of eosinophils or mast cells per high power field (HPF, 40× = 0.238 mm^2) in each of the 5 most representative and best-oriented tissue sections, and these 5 values were averaged. Mast cell degranulation was scored on a 0–10 scale corresponding to the pathologists' estimate of percentage of degranulated mast cells (ie 0 = no degranulation, 10 = 100% degranulation) in the most representative and best-oriented tissue sections (Figure S2).

2.3 | Muscle cell morphometry

Morphometry was assessed by computer-assisted image analysis on H&E stained slides. A grid with 10 μm squares was overlaid on tissues section of LES muscle, and diameter of individual LES muscle cells (in μm) was measured across the nucleus in a minimum of 250 cells in at least 4 representative and well-oriented tissue sections.[29] The fraction of cells with diameters of <15 μm or ≥15 μm was calculated for each sample and then averaged.

TABLE 1 Clinical features of the study patients

ID no.	Age/ Sex	ManometricDiagnosis	Atopy History	IRP (mmHg)	EckardtScore	Medications (at time of myotomy)	Symptom Duration (months)	Eos in Pre-Op Epithelial Biopsies	Prior Treatment for Achalasia
1	55 F	Achalasia I	Asthma, Eczema, Allergic rhinitis	NA	5	PPI	360	None	None
2	52 M	Achalasia I	None	25	6	PPI, ranitidine, NTG	120	None	Pneumatic dilation
3	68 M	Achalasia I	Allergic rhinitis	24	3	PPI, NTG	60	None	Pneumatic dilation
4	62 M	Achalasia II	None	22	7	None	9	Scattered	Pneumatic dilation
5	62 M	Achalasia II	None	NA	5	PPI	2	None	TTS balloon dilation
6	70 F[a]	Achalasia II	Allergic rhinitis	22	7	PPI, ranitidine, NSAID	18	None	None
7	26 M	Achalasia II	None	57	5	PPI, NSAID	12	None	TTS balloon dilation
8	32 F	Achalasia II	None	29	6	None	120	None	Pneumatic dilation
9	77 F	Achalasia III	None	21	8	PPI	108	None	TTS balloon dilation
10	67 F	Achalasia III	Asthma, allergic rhinitis	29	6	PPI, ranitidine, opioid, albuterol, fluticasone	60	Scattered	Pneumatic dilation
11	59 F	EGJOO	None	26	7	PPI	8	None	TTS balloon dilation
12	53 F	EGJOO	Asthma	23	5	PPI, albuterol	36	None	TTS balloon dilation
13	55 M	EGJOO	None	19	10	PPI	3	None	TTS balloon dilation

Abbreviations: IRP, integrated relaxation pressure; EGJOO, esophago-gastric junction outflow obstruction; PPI, proton pump inhibitor; NTG, nitroglycerin; NSAID, non-steroidal anti-inflammatory drug; TTS, through-the-scope.

[a]This patient had prior gastric bypass surgery.

2.4 | Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was isolated from LES muscle by RNeasy Mini Kit (Qiagen, Redwood City, CA) or TRIzol (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed using QuantiTect Reverse Transcription kit (Qiagen, Redwood City, CA) per manufacturer's instructions. The primer sequences (Table S1) were designed using PrimerBank - Harvard University (https://pga.mgh.harvard.edu/primerbank) and manufactured by Sigma (St. Louis, MO). qPCR for mRNA expression was carried out with QuantStudio 6 Flex Real-Time PCR System and SYBR Green mix (Applied Biosystems, Foster City, CA); GAPDH was used as reference gene. All qPCR assays were performed in triplicate for each study subject's sample.

2.5 | Bioinformatics analyses

The delta Ct (cycle threshold) method was used to normalize raw Ct values of target gene to reference gene (GAPDH, Table S2).[30] Further transformation was performed to obtain final delta Ct values by subtracting the original delta Ct from the maximum observed delta Ct value so that larger final delta Ct values would correspond to higher relative expression. Genes with undetermined raw Ct values had zero final delta Ct values. Hierarchical clustering of genes and samples was performed using Euclidean distance and complete linkage. Heat-maps were generated using NMF package.[31] The Rand index was used to measure accuracy between clustered groups and true sample labeling and was performed using ClusterR package.[32,33] Principal component analysis (PCA) was performed on centered and scaled data, and the first two principal components (PCs) were visualized using ggplot2.[34] All bioinformatics analyses were performed using R statistical software.

2.6 | Statistical analyses

Quantitative data are expressed as mean ± standard error of the mean (SEM). For multiple comparisons, a one-way ANOVA with post hoc Student-Newman-Keuls multiple-comparisons test was performed with Instat for Windows statistical software package (GraphPad). *p* values ≤0.05 were considered significant for all analyses.

3 | RESULTS

LES muscle specimens were obtained during Heller myotomy in 13 study patients whose clinical characteristics are shown in Table 1. There were 7 men, 6 women; median age 59 years, range 26–77 years. Ten patients had achalasia (3 type I, 5 type II, 2 type III), 3 had EGJOO (Note: For 2 achalasia patients, the motility catheter could not be advanced through the LES to obtain IRP values). Although the 3 EGJOO

TABLE 2 Clinical features, squamous epithelial features, LES muscle mast cell features, and LES muscle Ki-67 immunostaining for organ donor control patients

ID no.	Age/ Sex	Cause of Death	Squamous Epithelium	Max no. of Epithelial Eos	Max no. of Muscle Eos	Avg no. of Muscle Eos (5 Fields)	Max no. of Muscle Mast Cells	Avg no. of Muscle Mast Cells (5 Fields)	Mast Cell Degranulation	Ki–67 Muscle
1	53 M	CVA	Reflux Changes	0	0	0.0	57	33.0	0	0%
2	42 M	Anoxia	Normal	0	14	2.8	44	24.4	0	0%
3	45 F	Anoxia, Asphyxia	Normal	0	0	0.0	36	31.4	0	0%
4	38 F	CVA	Normal	0	0	0.0	14	11.0	2	0%
5	37 F	CVA	Normal	0	0	0.0	9	7.6	2	0%
6	48 M	CVA	Normal	0	1	0.2	48	22.9	0	0%
7	20 M	Head trauma	Reflux Changes	0	0	0.0	21	13.7	0	0%

Abbreviation: CVA, cerebrovascular accident; Eos, eosinophils.

patients clinically were indistinguishable from the 10 achalasia patients, patients with EGJOO recently were found to have a neuroimmunological profile distinct from that of achalasia.[35] Therefore, we analyzed our EGJOO patients as an independent group.

Age, sex, and cause of death for the 7 organ donors are listed in Table 2. There were 4 men, 3 women; median age was 42 years, range 20–53 years. No esophageal disease was mentioned in the limited clinical data available for any organ donor, and squamous epithelium in full-thickness specimens of their distal esophagus revealed no abnormalities other than changes of mild reflux esophagitis (basal cell and papillary hyperplasia) in 2 of 7 donors (Table 2).

3.1 | Eosinophils were rare and eosinophil numbers did not differ among LES muscle from achalasia patients, EGJOO patients, and controls

There were no eosinophils in preoperative biopsies of esophageal squamous epithelium in 8 of 10 achalasia patients and all 3 EGJOO patients; 2 achalasia patients had scattered epithelial eosinophils (<5/HPF) (Table 1). However, 11 of the 13 patients were taking PPIs, which can eliminate epithelial eosinophilia in EoE, and so EoE cannot be ruled out in those cases[36]; PPI effects on esophageal muscle eosinophilia are not known. No organ donor control had any eosinophils in esophageal squamous epithelium. Eosinophils were found infrequently in esophageal muscularis propria of both patients and controls (Tables 2 and 3). Among the 10 achalasia patients, 3 had eosinophils identified in esophageal muscle, but the average maximum number of muscle eosinophils for any patient was only 4.4/HPF (Figure 1A and B); no EGJOO patient had eosinophils in esophageal muscle. Only 2 of 7 controls had any eosinophils identified in esophageal muscle, and the average maximum number of muscle eosinophils for any control was only 2.8/HPF (Figure 1C). There were no significant differences between achalasia patients and controls in the average (0.84 ± 0.47 SEM versus 0.43 ± 0.40, respectively) or average maximum number (1.3 ± 0.80 SEM versus 2.1 ± 1.98, respectively) of eosinophils per HPF in LES muscle (Figure 1B and C).

3.2 | Mast cells numbers in LES muscle did not differ between achalasia or EGJOO patients and controls

Unlike eosinophils, mast cells were plentiful in LES muscle of achalasia patients and controls (Tables 2 and 3). In controls, we observed two patterns of mast cell distribution. In one pattern (Figure 1D, panel a), intact mast cells were numerous (up to 40–50 per HPF) and evenly distributed among muscle fibers. In the other pattern (Figure 1D, panel b), intact mast cells were confined almost exclusively to the perimysium surrounding muscle fiber bundles. We often observed both mast cell distribution patterns in different parts of LES muscle from the same patient. There were no significant differences between achalasia or EGJOO patients and controls in the average (17.8 ± 2.38 SEM or 15.7 ± 6.31 versus 20.6 ± 3.78, respectively) or average maximum number (21.7 ± 2.82 SEM or 19.7 ± 8.29 versus 32.7 ± 6.92, respectively) of mast cells per HPF in LES muscle (Figure 1E and F).

3.3 | There were striking differences between patients with achalasia and EGJOO versus controls in the distribution and degranulation status of mast cells in LES muscle

Although mast cell numbers in LES muscle did not differ between patients and controls, there were striking differences in

TABLE 3 Clinical features, LES muscle mast cell features, and LES muscle Ki-67 immunostaining for achalasia and EGJOO patients

IDno	Age/Sex	Manometric Diagnosis	Max no. of Muscle Eos	Avg no. of Muscle Eos (5 Fields)	Max no. of Muscle Mast Cells	Avg no. of Muscle Mast Cells (5 Fields)	Mast Cell Degranulation	Ki−67 Muscle
1	55 F	Achalasia I	0	0.0	40	32.8	7	0%
2	52 M	Achalasia I	0	1.4	22	19.8	8	0%
3	68 M	Achalasia I	0	0.0	31	25.8	10	0%
4	62 M	Achalasia II	5	2.4	17	14.2	6	0%
5	62 M	Achalasia II	0	0.0	21	17.2	9	0%
6	70 F	Achalasia II	0	0.0	10	7.8	3	0%
7	26 M	Achalasia II	1	0.2	17	11.6	8	0%
8	32 F	Achalasia II	7	4.4	26	21.4	6	0%
9	77 F	Achalasia III	0	0.0	21	16.5	9	0%
10	67 F	Achalasia III	0	0.0	12	10.6	6	0%
11	59 F	EGJOO	0	0.0	14	12.2	5	0%
12	53 F	EGJOO	0	0.0	36	28.0	6	0%
13	55 M	EGJOO	0	0.0	9	7.0	8	0%

Abbreviations: EGJOO, esophago-gastric junction outflow obstruction; Eos, eosinophils.

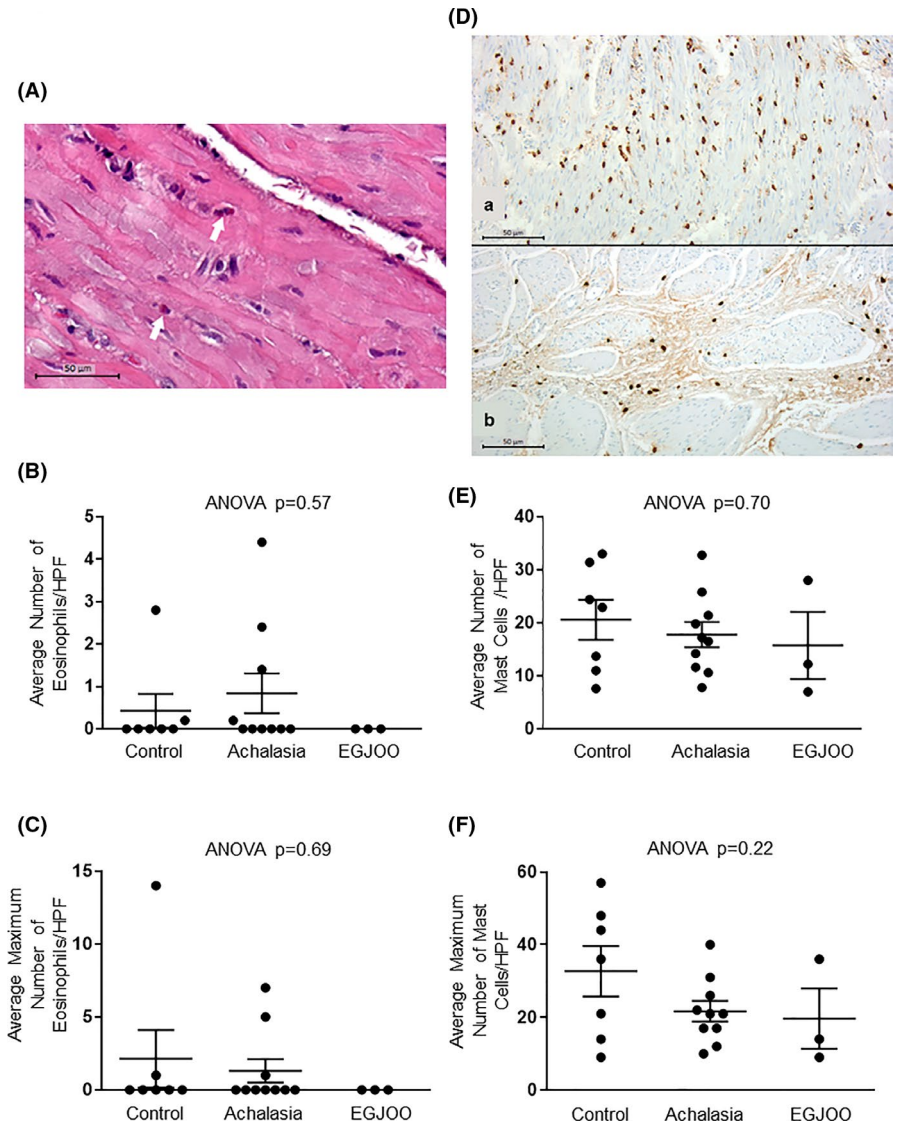


FIGURE 1 Histopathologic evaluation of LES muscle reveals no difference between controls and achalasia patients in numbers of eosinophils and mast cells. (A) Arrows point to rare fragments of eosinophils within muscle fibers of an achalasia patient. H&E, Scale bar = 50 µm. (B) Average number of eosinophils per HPF. (C) Average maximum number of eosinophils per HPF. Solid lines depict the mean ± SEM from 7 controls, 10 achalasia patients, and 3 EGJOO patients. (D) Immunohistochemical staining for mast cell tryptase revealed two patterns of mast cell distribution in LES muscle of control subjects. In pattern a, numerous intact mast cells were evenly distributed among muscle fibers. In pattern b, intact mast cells were confined almost exclusively to the perimysium surrounding muscle fiber bundles (diffuse brown color in endomysium represents non-specific staining). Scale bar = 50 µm. (E) Average number of mast cells per HPF. (F) Average maximum number of mast cells per HPF. Solid lines depict the mean ± SEM from 7 controls, 10 achalasia patients, and 3 EGJOO patients

the distribution and degranulation status of mast cells. Only 2 of 7 controls showed evidence of degranulation, which was mild in both cases (2/10); mean mast cell degranulation for all 7 controls was 0.57 ± 0.37 SEM (Table 2 and Figure 2A). In contrast, all 10 achalasia patients exhibited mast cell degranulation in LES muscle that was profound (≥5/10) in all but 1 patient; EGJOO patients also displayed profound mast cell degranulation (Table 3). Mean mast cell degranulation for achalasia and EGJOO patients was 7.2 ± 0.65 and 6.3 ± 0.88, respectively (Figure 2A). We observed two patterns of mast cell degranulation in achalasia patients. In one pattern (Figure 2B, panel a), virtually no intact mast cells were recognizable, and large quantities of tryptase-positive granules were seen streaming within endomysium and perimysium. In the other pattern (Figure 2B, panel b), most mast cells were degranulating, but they retained their shape and the granules remained close to cells. We often observed both mast cell degranulation patterns in different parts of LES muscle from the same patient.

We also observed differences between patients and controls in the pattern of mast cell distribution and degranulation around nerves in the LES myenteric plexus. In controls (Figure 2C, panel a), intact mast cells were detected in perineural sheaths, but virtually never within nerve tissue itself. In contrast, we observed profound mast cell degranulation in the LES myenteric plexus of achalasia patients,

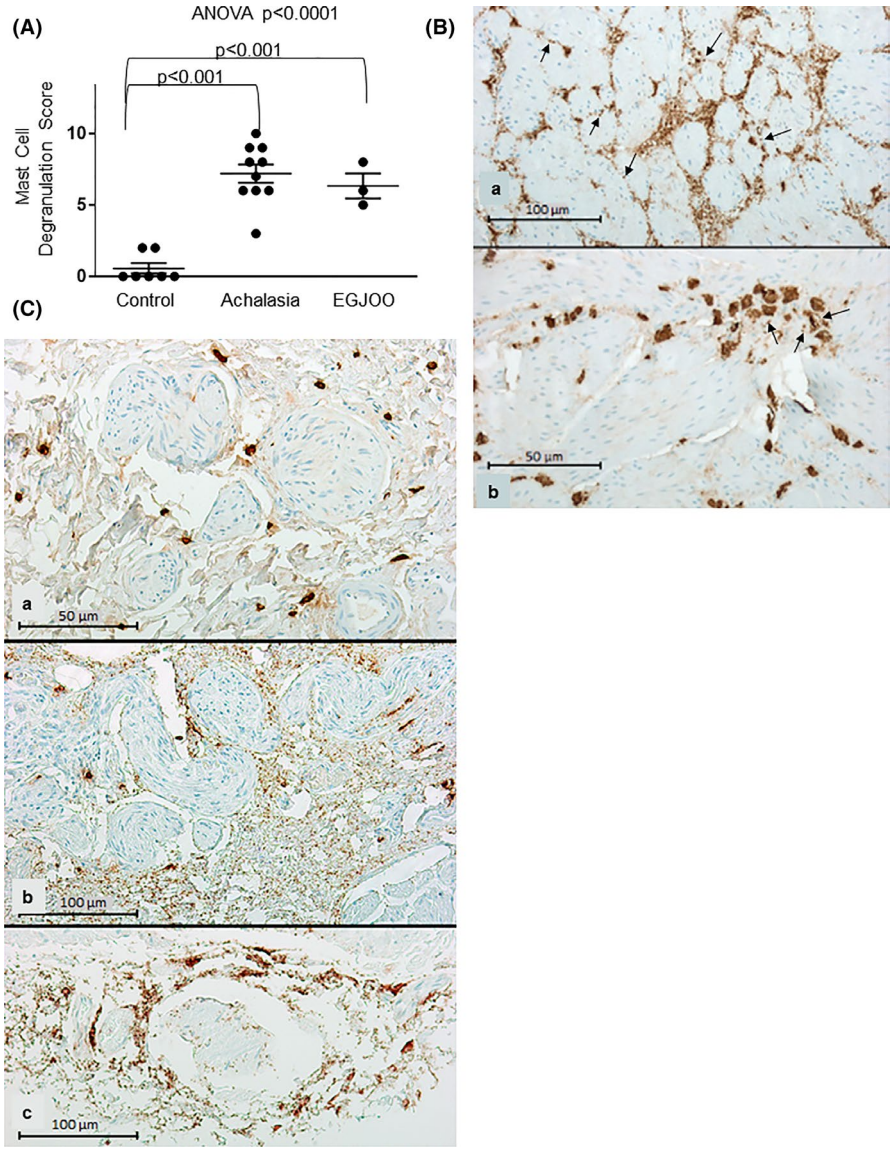


FIGURE 2 Histopathologic evaluation of LES muscle biopsies stained for mast cell tryptase demonstrates striking differences between controls and achalasia patients in distribution and degranulation status of mast cells in muscle and myenteric plexus. (A) Mast cell degranulation score. Solid lines depict the mean ± SEM from 7 controls, 10 achalasia patients, and 3 EGJOO patients. (B) Immunohistochemical staining for mast cell tryptase revealed two patterns of mast cell degranulation. In pattern a, virtually no intact mast cells are recognizable, and large quantities of tryptase-positive granules (black arrows) are seen streaming within the endomysium and perimysium (Scale bar = 100 µm). In pattern b, most mast cells are degranulating, but the cells retain their shape and the granules (black arrows) remain close to the cells (Scale bar = 50 µm). (C) Myenteric plexus of LES muscle stained for mast cell tryptase. Panel a shows the myenteric plexus of a control subject, with intact mast cells found in perineural sheaths but virtually never within the nerve tissue itself (Scale bar = 50 µm). Panels b and c show the myenteric plexus of achalasia patients exhibiting profound mast cell degranulation, with tryptase-positive granules surrounding and often penetrating into the nerve tissue (Scale bar = 100 µm)

with tryptase-positive granules surrounding and often penetrating into nerve tissue (Figure 2C, panels b and c).

Many clinical laboratories perform immunohistochemical staining for CD117 (c-kit) rather than for tryptase to evaluate for mast cells.[37] Since CD117 is a transmembrane tyrosine kinase receptor found primarily on the cell surface, we wondered if CD117 immunostaining would be as effective as immunostaining for tryptase for identifying mast cell degranulation. To explore this issue, we performed immunostaining for both tryptase and CD117 on LES muscle from 3 achalasia patients. The profound mast cell degranulation identified by tryptase immunostaining was not apparent by immunostaining for CD117 (Figure S3).

3.4 | LES muscle cells of achalasia patients showed no evidence of hyperplasia and were smaller than control muscle cells

Immunohistochemical staining of LES muscle for Ki-67, which identifies proliferating cells, was performed to seek evidence of muscle

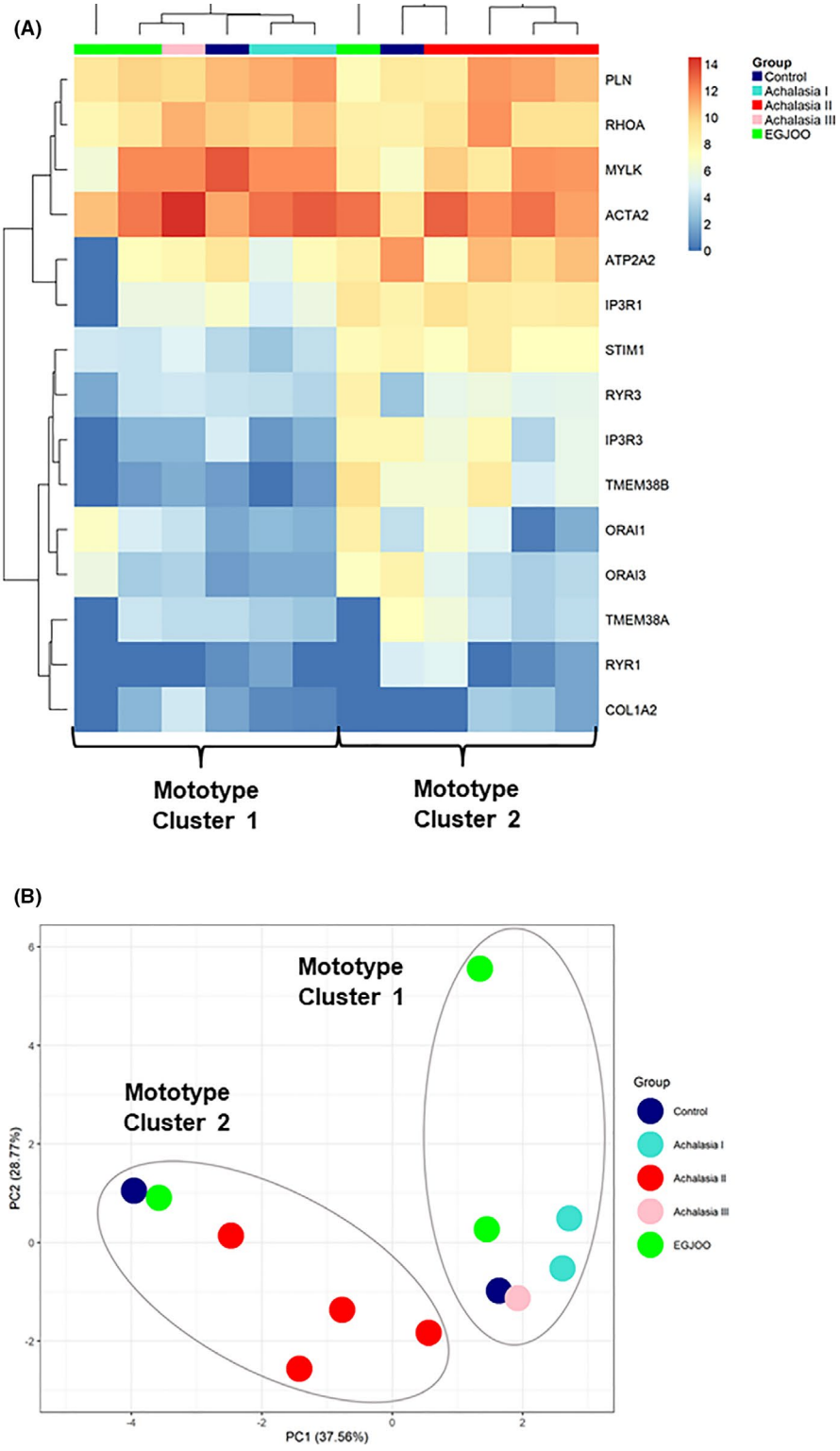


FIGURE 3 A panel of genes known to mediate cytosolic Ca^{2+} handling and contraction in smooth muscle identifies two "mototype" clusters that distinguish patients with achalasia type II from achalasia types I and III. (A) Heat-map and hierarchical clustering with dendrogram generated from qPCR gene expression data of LES muscle for 2 control subjects, 7 achalasia patients, and 3 EGJOO patients reveals clustering of achalasia types I and III in mototype 1, and achalasia type II into mototype 2. Red color corresponds to high relative expression and blue color corresponds to low relative expression. (B) Principal component (PC) analysis of gene expression data confirms mototype clustering of achalasia types 1 and III distinct from achalasia type II

hyperplasia. No Ki-67-positive muscle cells were observed in any sections of LES muscle from achalasia or EGJOO patients and controls (Tables 2 and 3 and Figure S4A-C). To seek evidence of muscle hypertrophy or atrophy, we measured the diameter of individual LES muscle cells in 10 achalasia and 3 EGJOO patients, and 6 of 7 controls; 1 control tissue section was technically inadequate for muscle cell measurements. LES muscle cells of achalasia patients were significantly smaller in diameter than control LES muscle cells. In controls, 80.3 ± 0.16% SEM of LES muscle cells had diameters ≥15 μm, while only 11.3 ± 0.05% of LES muscle cells in the achalasia patients and none of the 3 EGJOO patients had diameters ≥15 μm ($p < 0.0001$ for achalasia or EGJOO patients compared with controls).

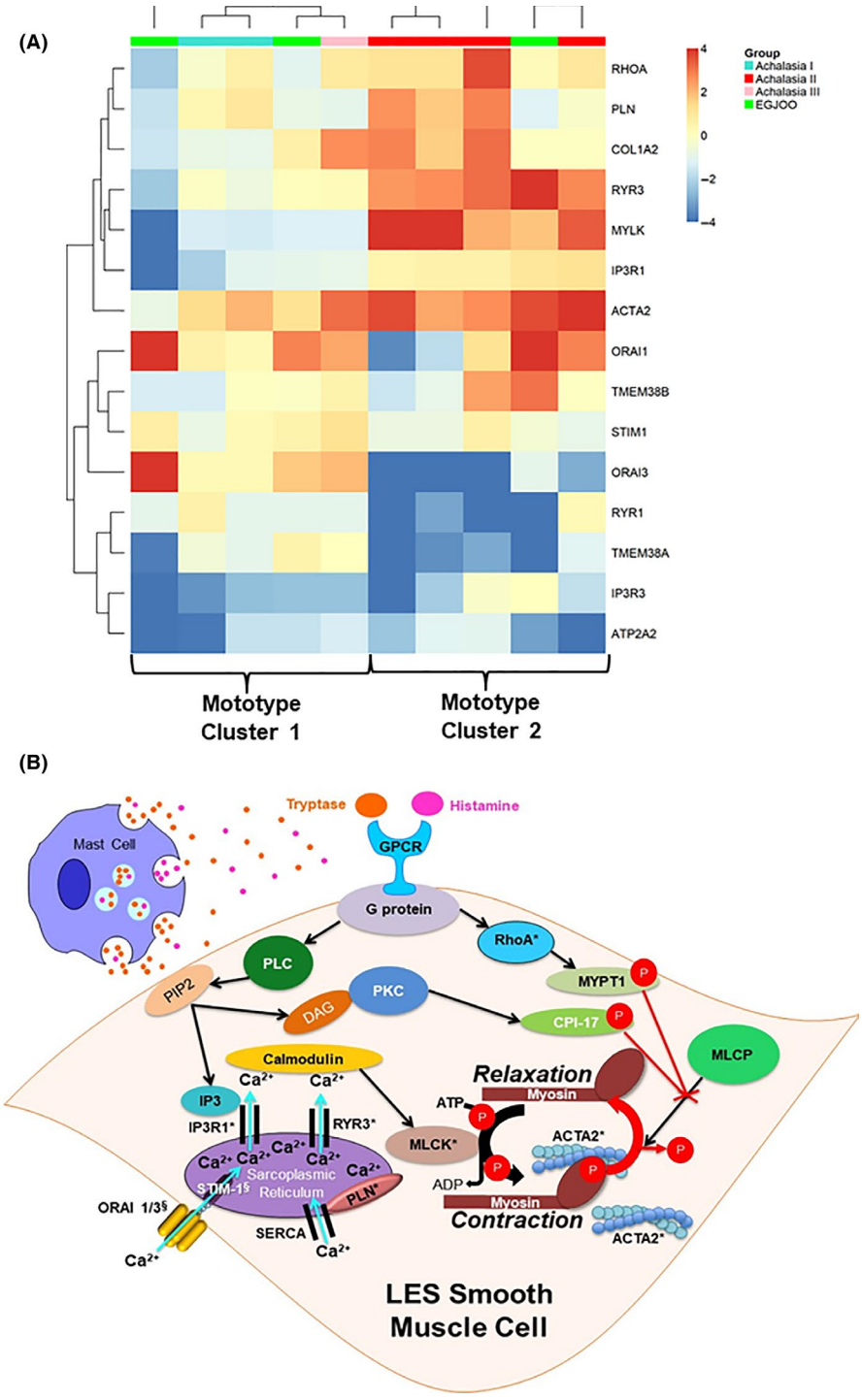
3.5 | A genetic "mototype" of LES muscle distinguishes manometric phenotypes in achalasia associated with mast cell degranulation

We hypothesized that intrinsic or acquired abnormalities in LES muscle itself might contribute to the hypercontraction and poor relaxation of achalasia LES muscle that traditionally have been attributed solely to loss of inhibitory innervation. To explore genetic factors that might underlie such intrinsic abnormalities, we evaluated LES muscle by qPCR for a panel of genes known to mediate smooth muscle Ca^{2+} handling and contraction (Figure 3). qPCR was performed on frozen LES muscle specimens from 2 controls, 3 EGJOO, and 7 achalasia patients (2 type I, 4 type II, 1 type III); 5 controls and 2 achalasia patients did not have frozen tissues available, and RNA quality was poor in 1 patient. Hierarchical clustering analysis revealed two discrete clusters, which we term "mototype" gene patterns, with unexpected groupings of achalasia phenotypes within mototypes (Figure 3A). The dendrogram demonstrates: Mototype Cluster 1 comprising 2 achalasia type I patients, 1 achalasia type III patient, and 2 EGJOO patients; and Mototype Cluster 2 comprising all 4 achalasia type II patients and 1 patient with EGJOO; one control subject grouped within each cluster. Principal component analysis recapitulated the clustering of mototypes that separated achalasia types I and III from achalasia type II (Figure 3B). The Rand Index (RI), a measure of accuracy between clusters and true sample labeling with values ranging between 0 (0% accurate) and 1 (100% accurate), was 0.621. To assess if this RI was merely due to chance, we generated 10,000 random permutations of the gene expression data (Figure S5) and recalculated the RI each time to obtain an empirical null distribution; we found that the observed RI (0.621) was indeed significant ($p = .027$) (Figure S6). Finally, we evaluated the patients' gene expression relative to the corresponding control in each mototype. This revealed relative upregulation of cytosolic calcium handling genes PLN, RYR3, IP3R1, and smooth muscle contraction and contractility genes RHOA, COL1A2, MYLK, and ACTA2 in mototype 2 patients, while mototype 1 patients have relative upregulation of calcium handling genes ORAI 1/3 and STIM-1 (Figure 4A). These findings show that the genetic mototype of LES muscle can distinguish manometric phenotypes in achalasia associated with mast cell degranulation and suggest that when patients acquire this achalasia, the underlying genetic mototype might contribute to the resulting manometric phenotype. A schematic model summarizing potential mechanisms whereby allergy-mediated mast cell degranulation in LES muscle might cause contractile disturbances in the gene expression mototypes is provided in Figure 4B.

4 | DISCUSSION

We found that normal LES muscle is largely devoid of eosinophils, and we found no significant difference between patients and controls in LES muscle eosinophil density. In contrast, mast cells are plentiful in LES muscle. Although we saw no significant difference between patients and controls in mast cell density, we observed striking differences in the distribution and degranulation status of LES mast cells between those groups. Only 2 of 7 control subjects showed evidence of mast cell degranulation, which was mild in both cases, while 9 of 10 achalasia patients and all 3 EGJOO patients exhibited profound mast cell degranulation with copious amounts of tryptase-positive granules in LES muscle and around myenteric plexus nerve tissue. Immunohistochemical staining for Ki-67 revealed no evidence of muscle proliferation in patients or controls, while morphometric

FIGURE 4 (A) Heat-map and hierarchical clustering with dendrogram generated from qPCR gene expression data of LES muscle from 7 achalasia and 3 EGJOO patients relative to their corresponding control highlights differences between the two mototype clusters. Note the relative upregulation of the cytosolic calcium handling genes PLN, RYR3, IP3R1, and the smooth muscle contraction and contractility genes RHOA, COL1A2, MYLK, and ACTA2 in mototype 2 patients, while mototype 1 patients have relative upregulation of the calcium handling genes ORAI 1/3 and STIM-1. (B) Conceptual model whereby allergy-mediated mast cell degranulation in LES muscle might cause contractile disturbances in the gene expression mototypes. Tryptase and histamine released by mast cells can bind a G-protein coupled receptor (GPCR), leading to activation of phospholipase C (PLC), which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) in the membrane into diacyl glycerol (DAG) and inositol trisphosphate (IP3). IP3 binds its receptor IP3R on the sarcoplasmic reticulum, with subsequent activation of the ryanodine receptor 3 (RYR3), triggering Ca^{2+} release from the sarcoplasmic reticulum into the cytosol. Cytosolic Ca^{2+} activates calmodulin, which in turn activates myosin light chain kinase (MLCK) to phosphorylate myosin, enabling the binding of ACTA2, which ultimately induces muscle contraction. Relaxation occurs when myosin light chain phosphatase (MLCP) dephosphorylates myosin. However, GPCR activation by tryptase and histamine also results in MLCP inhibition through activation of Rho, which phosphorylates protein subunit 1 (MYPT1) to block MLCP, and through phosphokinase C (PKC)-mediated phosphorylation of CPI-17, which also blocks MLCP. Relaxation also can occur when cytosolic Ca^{2+} is pumped back into the sarcoplasmic reticulum by SERCA. However, phospholamban (PLN) prevents Ca^{2+} reuptake via SERCA. Ca^{2+} stores in the sarcoplasmic reticulum also can be replenished when store depletion is sensed by stromal-interacting molecule-1 (STIM-1), which enables the entry of extracellular calcium through Orai1/3. *indicates genes upregulated in Mototype 2 (RhoA, PLN, RYR3, MYLK, ACTA2),[§]indicates genes upregulated in Mototype 1 (Stim1, ORAI1/3)



analysis showed that the diameter of LES muscle cells from patients was smaller than that of control subjects. Finally, our analyses of LES muscle for mRNA expression of genes known to mediate cytosolic Ca^{2+} handling and contraction in smooth muscle revealed two "mototype" gene patterns that could distinguish among achalasia manometric phenotypes, with all 4 achalasia type II patients segregating into one mototype, and patients with achalasia type I (2 patients) and III (1 patient) segregating into the other.

Esophageal muscularis propria is accessible only to invasive procedures such as per-oral endoscopic myotomy (POEM) and esophageal surgery, and esophageal muscle rarely is biopsied for clinical purposes. Consequently, histological studies of LES muscle in achalasia have been limited to infrequent evaluations of esophagectomy specimens from patients with end-stage achalasia,[3] and to several studies of esophageal muscle biopsies taken during Heller myotomy[2,4,5,38-40] or POEM.[19,40,41] Furthermore, most of those studies either included no control tissues,[3,5,40,41] or used the suboptimal control of muscle in esophagi resected because of cancer.[2,4,19,38,42] Few studies have done as we did,[35,39,43,44] comparing LES muscle samples taken from achalasia patients undergoing Heller myotomy

with LES muscle from organ donors with no important esophageal disease.

We undertook this investigation to explore our hypothesis that there is a muscle-predominant form of allergen-driven esophagitis (EoE), and initially we thought there would be differences between achalasia patients and controls in the degree of eosinophil infiltration of LES muscle.[8] In fact, we found very few eosinophils in LES muscle of patients and controls. There are wide discrepancies among reports on the frequency with which eosinophils are found in achalasia LES muscle. Goldblum found eosinophils in esophageal muscle in 22 (52%) of 42 achalasia patients at University of Michigan[3] and in 6 (55%) of 11 at Cleveland Clinic,[4] and Tøttrup found eosinophil cationic protein in LES muscle from all 9 (100%) of his 9 achalasia patients undergoing Heller myotomy.[2] In contrast, Nakajima described no eosinophils in POEM biopsies of esophageal muscle from 22 achalasia patients,[41] and Sodikoff found an eosinophil-predominant infiltrate in the myenteric plexus in only 1 (2%) of 46 achalasia patients who had LES muscle biopsied during Heller myotomy.[5] The reason for this wide variation among studies is not clear, but might be related to differences in patient populations and biopsy techniques. Another potential confounder relates to PPI use, which is not described specifically in most of these reports. Eleven of our 13 patients were taking PPIs at the time of LES muscle biopsy. The diagnosis of EoE presently is confirmed by esophageal mucosal biopsy and, since PPIs can eliminate mucosal eosinophils, EoE cannot be excluded unless PPIs are stopped prior to endoscopy.[36] The effect of PPIs on eosinophils in esophageal muscle is not known, but it is conceivable that eosinophils would have been present in our patients' LES muscle had PPIs been stopped prior to myotomy.

Our most striking finding was the profound mast cell degranulation we observed in LES muscle in 12 of our 13 patients. Mast cells are well known to play a pivotal role in allergic diseases in which IgE triggers mast cell degranulation.[11] We speculate that the numerous myoactive, neuroactive, pro-inflammatory, and cytotoxic products released by degranulating mast cells have the capacity to contribute to the dysmotility, myenteric inflammation, and neuronal degeneration of achalasia.[16] Our finding of profound mast cell degranulation in achalasia LES muscle provides support for our hypothesis that achalasia can be an allergy-mediated disease. However, by no means are our findings proof of underlying allergy, because mast cell degranulation can be triggered by physical stresses and a wide variety of non-allergic stimuli including neuropeptides, cytokines, chemokines, and other inflammatory products.[45] Thus, it is possible that the mast cell degranulation we observed in achalasic LES muscle was not the cause of inflammation and myenteric nerve destruction, but merely an effect of the myenteric inflammation triggered by another underlying condition.

Many clinical laboratories perform immunohistochemical staining for CD117 (c-kit) rather than for tryptase to evaluate for mast cells, and the two stains are generally considered equally valid for clinical purposes.[37] However, CD117 is primarily a cell surface marker and, as such, might not be expected to identify mast cell granules reliably. Indeed, we found that the profound mast cell degranulation identified by tryptase immunostaining of achalasia LES muscle was not apparent by immunostaining for CD117. If it is important to recognize mast cell degranulation for clinical or research purposes, our findings suggest that tryptase immunostaining is preferable to CD117 immunostaining.

Two prior studies specifically evaluated mast cells in achalasia LES muscle and, unlike our investigation, those studies found a greater number of mast cells in LES muscle of achalasia patients than in control patients who had esophageal cancer.[19,38] One study noted that mast cell infiltration was associated with the presence of interstitial cells of Cajal (ICCs, which are frequently lost along with ganglion cells in achalasia), and the authors speculated that mast cells might promote ICC survival by secreting stem cell factor.[38] In contrast, the other study found that mast cell infiltration was associated with decreased numbers of ICCs, and the authors speculated that mast cells mediated the neurodegeneration of achalasia.[19] Neither study described the profound mast cell degranulation that we observed. The reasons for differences between the findings of those studies and ours are not clear, but might be related to differences in patient populations, and their use of cancer patients for controls.

Epidemiologic data on achalasia are limited, but its frequency appears to be rising. One recent study suggested that, from 2004 to 2014, the incidence and prevalence of achalasia in central Chicago were twofold to threefold greater than estimates from earlier years would have predicted.[46] The investigators speculated that this apparent increase in achalasia frequency was spurious, due to increasing use of high-resolution manometry that has facilitated the diagnosis and increased awareness of achalasia. However, it is also possible that there has been a genuine increase in frequency of the disease. Allergic diseases, including EoE, have increased dramatically in frequency during this same period.[47,48] Our hypothesis that there is an allergic form of achalasia is consistent with the observation that achalasia is increasing in frequency.

Our genetic analyses of LES muscle revealed interesting gene expression differences among patients with different manometric phenotypes of achalasia. All 4 achalasia type II patients segregated into a mototype characterized by upregulation of genes that might increase cytosolic calcium levels (PLN, RYR3, IP3R1) and smooth muscle contraction (RHOA, COL1A2, MYLK, and ACTA2), whereas both achalasia type I patients and the one type III patient fell into another mototype in which there was upregulation of genes that might replenish calcium stores in the sarcoplasmic reticulum (ORAI 1/3 and STIM-1). Each of two control subjects fell into one of the two mototypes. Although we used statistical methods that support the significance of this finding of two distinct achalasia mototypes, the small sample size (sometimes limited to a single sample) limits the strength of conclusions that can be drawn from these results. While these data are not definitive and require validation in larger studies, they suggest that when patients acquire achalasia associated with mast cell degranulation, their underlying genetic mototype might contribute to the resulting manometric phenotype. Interestingly, mast cell tryptase is a potent activator of protease-activated receptor-2 (PAR-2)

that releases the intracellular calcium that mediates contraction in smooth muscle cells.[49] Further exploration of the mast cell degranulation-related transcriptome of achalasia LES muscle clearly is warranted. Such studies might provide insight into achalasia pathogenesis and reveal novel therapeutic strategies.

Another interesting feature of achalasia and EGJOO patients we observed was the small diameter of their LES muscle cells. Studies using endoscopic ultrasonography have described increased thickness of esophageal muscularis propria in a small number of achalasia patients,[50] but few studies have focused on the size of muscle cells themselves. Our findings that achalasia and EGJOO LES muscle cells exhibited small diameters and absent Ki-67 staining suggests that muscle hyperplasia and hypertrophy are not common features of these diseases.

A major limitation of our study is its relatively small sample size, especially regarding the mototype analyses that required frozen LES muscle specimens, which were available for only 2 controls, 3 EGJOO patients, and 7 achalasia patients (2 type I, 4 type II, 1 type III). Although the association we found between gene expression mototypes and achalasia manometric phenotypes was statistically significant despite the small sample size, further studies are needed to validate these findings. Another limitation is the lack of formal allergy testing in our achalasia patients to support our hypothesis that there is an allergy-mediated form of achalasia. Future studies on this issue should include such testing. A major strength of our study is the use of esophagi from organ donors as controls.

In conclusion, we have found that LES muscle of patients with achalasia and EGJOO exhibits profound mast cell degranulation with copious amounts of tryptase-positive granules in the perimysium and around nerve tissue in the myenteric plexus. Analyses of LES muscle for mRNA expression of a panel of genes known to mediate cytosolic Ca^{2+} handling and contraction in smooth muscle revealed two "mototype" gene patterns that could distinguish among achalasia manometric phenotypes. Although our findings are not definitive, they provide support for our hypothesis that achalasia can be an allergy-mediated disease, and they suggest that when patients acquire this form of achalasia, their underlying genetic mototype might contribute to the resulting manometric phenotype.

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DISCLOSURE

Nothing to disclose.

AUTHOR CONTRIBUTIONS

M.N.: study design; technical and material support; analysis and interpretation of data; critical revision of manuscript; important intellectual content; and drafting of manuscript. X.Z.: study design; technical and material support; analysis and interpretation of data; critical revision of manuscript; important intellectual content; and drafting of manuscript. R.M.G.: study design; technical and material support; analysis and interpretation of data; critical revision of manuscript; important intellectual content; and drafting of manuscript. K.T.: technical and material support; analysis and interpretation of data; critical revision of manuscript; important intellectual content; and drafting of manuscript. E.P.: study design; technical and material support; important intellectual content; and critical revision of manuscript. S.P.: technical and material support; important intellectual content; and critical revision of manuscript. J.C.: technical and material support; analysis and interpretation of data; critical revision of manuscript; important intellectual content; and drafting of manuscript. J.G.: technical and material support; analysis and interpretation of data; critical revision of manuscript; important intellectual content; and drafting of manuscript. S.L.: study design; technical and material support; important intellectual content; and critical revision of manuscript. M.W.: study design; technical and material support; important intellectual content; and critical revision of manuscript. A.N.: study design; important intellectual content; and critical revision of manuscript. V.K.: study design; technical and material support; important intellectual content; and critical revision of manuscript. G.F.: study design; important intellectual content; and critical revision of manuscript. Z.P.: study design; critical revision of manuscript; and important intellectual content. R.F.S.: study concept/design; analysis and interpretation of data; critical revision of manuscript; important intellectual content; and drafting of manuscript. S.J.S.: study concept/design; analysis and interpretation of data; critical revision of manuscript; important intellectual content; and drafting of manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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