Received: 15 November 2019 | Revised: 25 December 2019 | Accepted: 30 December 2019

DOI: 10.1111/nmo.13804

Check for updates

Neurogastroenterology & Motility NGM WILEY

ORIGINAL ARTICLE

Triosephosphate isomerase, carbonic anhydrase, and creatinine kinase-brain isoform are possible antigen targets in patients with achalasia

Diego F. Hernández-Ramírez[1] | Elizabeth Olivares-Martínez[1] | Carlos A. Nuñez-Álvarez[1] | Enrique Coss-Adame[2] | Miguel A. Valdovinos[2] | Fidel López-Verdugo[1] | Janette Furuzawa-Carballeda[1] | Gonzalo Torres-Villalobos[3,4]

[1]Department of Immunology and Rheumatology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico, Mexico

[2]Department of Gastroenterology, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico, Mexico

[3]Department of Experimental Surgery, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico, Mexico

[4]Department of Surgery, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico, Mexico

Correspondence
Gonzalo Torres-Villalobos and Janette Furuzawa-Carballeda, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán. Vasco de Quiroga No. 15, Col. Belisario Domínguez Sección XVI, CP 14080 Mexico, CDMX, Mexico.
Email: torresvgm@yahoo.com.mx (G. T.-V.) and jfuruzawa@gmail.com (J. F.-C.)

Funding information
The study has not financial support.

Abstract

Background: Idiopathic achalasia is an uncommon esophageal motor disorder. The disease involves interaction between inflammatory and autoimmune responses. However, the antigens related to the disease are still unknown.

Aim: To identify the possible antigen targets in muscle biopsies from lower esophageal sphincter (LES) of achalasia patients.

Methods: Esophageal biopsies of patients with type I and type II achalasia and esophagogastric junction outflow obstruction (EGJOO) were analyzed. Lower esophageal sphincter muscle biopsy from a Healthy organ Donor (HD) was included as control for two-dimensional gel electrophoresis. Immunoblotting of muscle from LES lysate with sera of type I, type II achalasia, or type III achalasia, sera of EGJOO and sera of healthy subjects (HS) was performed. The target proteins of the serum were identified by mass spectrometry Matrix-assited laser desorption/ionization time-of-flight (MALDI-TOF).

Key Results: The proteomic map of muscle from LES tissue lysates of type I, and type II achalasia, EGJOO, and HD were analyzed and divided into three important regions. We found a difference in the concentration of certain spots. Further, we observed the serum reactivity of type I achalasia and type II achalasia against 45 and 25 kDa bands of type I achalasia tissue. Serum of type III achalasia and EGJOO mainly recognized 25 kDa band. Bands correspond to triosephosphate isomerase (TPI) (25 kDa), carbonic anhydrase (CA) (25 kDa) and creatinine kinase-brain (CKB) isoform (45 kDa).

Conclusions and Inferences: We identify three antigen targets, TPI, CA, and CKB isoform, which are recognized by sera from patients with achalasia.

KEYWORDS

achalasia, carbonic anhydrase, creatinine kinase-brain, lower esophageal sphincter muscle, mass spectrometry MALDI-TOF, proteomic, triosephosphate isomerase

Diego F. Hernández-Ramírez, Elizabeth Olivares-Martínez and Carlos A. Nuñez-Álvarez contributed equally to this study.

 © 2020 John Wiley & Sons Ltd

1 | BACKGROUND

Achalasia is an uncommon esophageal motor disorder characterized by impaired swallow-induced lower esophageal sphincter (LES) relaxation and absence of peristalsis in the esophageal body. These changes are caused by the loss of ganglion cells in the myenteric plexus of the esophagus. The clinical manifestations include dysphagia for solids and liquids, regurgitation of esophageal contents, and chest pain.[1,2]

The pathophysiological mechanism of achalasia remains unknown. However, several studies suggest that it involves an interaction between inflammatory and autoimmune responses, such as viral infections, in genetically susceptible individuals.[3,4] The proposed pathogens in triggering the inflammatory response in achalasia, include herpes simplex virus (HSV), varicella-zoster, measles, and human papillomavirus.[3,5] On the other hand, the autoimmune etiology of achalasia is supported by association with class-two major histocompatibility complex haplotypes (HLA-DQ and HLA-DR),[6-8] the presence gelatinase B/Matrix Metalloproteinase-9 as immune effector molecule,[9] antimyenteric antibodies, and inflammatory T cell infiltrates in the myenteric plexus, which are associated with damage to nerve plexus/ganglia, degeneration of neurons and ganglion cell loss.[3,10] Furthermore, there is evidence of an association of the diverse autoimmune diseases with achalasia. In a previous study, 11.9% of the idiopathic achalasia patients had an autoimmune disease such as type 1 diabetes, hypothyroidism, Sjögren's syndrome, and systemic lupus erythematosus.[11] Similarly, we found that 16.7% of idiopathic achalasia patients had an autoimmune disease, hypothyroidism was the most common diagnosis and 68.4% of the achalasia patients with autoimmune disease had a history of familial autoimmunity.[12] Other authors have also documented the association with autoimmune thyroid disease.[12,13]

Regarding the identification of antigen targets in achalasia patients, there are few studies that have analyzed it.[14,15] Thus, this study aimed to identify the possible antigens recognized by achalasia patients.

Key points

- Idiopathic achalasia is an uncommon esophageal motor disorder. The loss of myenteric plexus associated with inflammatory infiltrates and autoantibodies support the hypothesis of an autoimmune mechanism. However, the antigens related to the disease are still unknown.
- This is the first study to identify the antigen targets in muscle biopsies from lower esophageal sphincter (LES) of achalasia patients by mass spectrometry MALDI-TOF.
- Three antigen targets, triosephosphate isomerase, carbonic anhydrase, and creatinine kinase-brain isoform were identified in LES of achalasia patients.

2 | MATERIALS AND METHODS

2.1 | Patients

Lower esophageal sphincter biopsies from 10 achalasia patients (5 type I and 5 type II), 5 patients with esophagogastric junction outflow obstruction (EGJOO) and one organ donor biopsy (control) were included. Sera from 22 achalasia patients (9 type I achalasia; 7 type II achalasia; and 6 type III achalasia), 6 EGJOO patients and 9 healthy subjects (HS) were analyzed (Table 1). All patients were recruited from the Outpatient Clinics of Gastroenterology and Surgery of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (a tertiary referral center in Mexico City, Mexico). Esophagram, high-resolution manometry (HRM) and endoscopy were performed for the diagnosis of achalasia and EGJOO.[16,17] Patients were excluded from the study if they presented any of the following pathologies: Chagas disease, eosinophilic esophagitis, esophageal stricture, gastric or esophageal cancer, scleroderma, hiatal hernias, gastroesophageal reflux disease with erosive esophagitis, human immunodeficiency virus (HIV), or hepatitis C virus (HCV) infections.[16,17] Demographic, clinical, and laboratory information were collected (Table 1). Lower esophageal sphincter muscle sample from one organ donor was included as tissue control. This donor had no known history of metabolic, inflammatory, neoplastic, or autoimmune diseases. No viral infections with cytomegalovirus, HCV, hepatitis B virus, and HIV were detected. Tests for syphilis and serum antinuclear antibodies were negative.

2.2 | High-resolution esophageal manometry

Esophageal HRM was performed in every patient at baseline and before being referred for surgery. A solid-state HRM probe with 36 circumferential sensors was used (Given Imaging). Having the patient in a sitting position and at 45 degrees, stationary esophageal HRM was performed. After a 12-hour fasting period, the probe was inserted trans-nasally until passing the EGJ, assessed visually on the computer screen. Ten water swallows of 5 mL separated by 30 seconds were provided. Analyses were performed using Manoview 2.0 (Given Imaging), and patients were classified according to latest Chicago classification v3.0[18] into four groups: (a) type I achalasia (without pressurization within the esophageal body); (b) type II (with >20% panpressurization); (c) type III (spastic); and (d) EGJOO (elevated median integrated relaxation pressure [IRP]-15 mm Hg-with enough evidence of peristalsis). The classification was performed by two gastroenterologists (MAV, EC-A) experts in esophageal HRM.

2.3 | HRM data analysis

The analysis was performed with ManoView software (Given Imaging). Four key metrics of HRM, the distal contractile integral,

TABLE 1 Demographic, clinical, and laboratory variables

Tissue	HD (n = 1)	EGJOO (n = 5)	Type I achalasia (n = 5)	Type II achalasia (n = 5)	*P* value
Demographics					
Age (years),					
Mean ± SD	51	33.6 ± 14.6	38.0 ± 10.2	44.6 ± 9.4	NS
Median		35.0	36.0	43.0	
Range		19-54	23-48	34-57	
Sex					
Female/male	0/1	1/4	3/2	4/1	
Disease evolution (mo), mean ± SD	NA	9.6 ± 8.2	48.8 ± 34.3	19.6 ± 11.1	.026 (EGJOO vs type I achalasia)
Median		7.0	48.0	18.0	
Range		3-24	12-100	7-36	
Clinical variables					
Dysphagia n, (%)	NA	5 (100)	5 (100)	5 (100)	NS
Regurgitation n, (%)	NA	4 (80)	5 (100)	5 (100)	NS
Weight loss n, (%)	NA	4 (80)	4 (80)	5 (100)	NS
Pyrosis (%)	NA	3 (60)	2 (40)	4 (80)	NS
Antinuclear antibodies n, (%)	0 (0)	0 (0)	3 (60)	3 (60)	<.05

Serum	Healthy subjects (n = 9)	EGJOO (n = 6)	Type I achalasia (n = 9)	Type II achalasia (n = 7)	Type III achalasia (n = 6)	*P* value
Age (y)						
Mean ± SD	33.8 ± 15.3	37.8 ± 16.7	38.7 ± 19.6	47.1 ± 18.8	48.7 ± 17.8	NS
Median	25.5	37.5	43.0	48.0	43.5	
Range	21-62	19-59	18-79	19-67	26-77	
Sex Female/male	4/5	5/1	5/4	5/2	4/2	NS
Disease evolution (mo) mean ± SD Median Range	NA	10.0 ± 7.4 7.0 3-24	40.1 ± 30.7 36.0 5-100	29.3 ± 17.7 18.0 12-48	49.7 ± 93.5 12.0 4-240	NS
Clinical variables						
Dysphagia n, (%)	NA	6 (100)	9 (100)	7 (100)	6 (100)	NS
Regurgitation n, (%)	NA	5 (83)	7 (78)	7 (100)	4 (67)	NS
Weight loss n, (%)	NA	5 (83)	7 (78)	6 (86)	4 (67)	NS
Pyrosis (%)	NA	3 (50)	3 (33)	6 (86)	2 (33)	NS
Antinuclear antibodies n, (%)	NA	0 (0)	5 (56)	3 (43)	5 (83)	<.05

Note: Disease evolution: onset of symptoms.

Abbreviations: EJOO, Esophageal junction outflow obstruction; HD, healthy donor; NA, not applicable; NS, not significant.

the basal EGJ pressure, the intrabolus pressure (IBP), and the IRP were used to assess the pressure motor activity of the esophageal body and EGJ.

Achalasia was defined by IRP > 15 mm Hg and aperistalsis. Type I achalasia was defined by absent peristalsis with no compartmentalization of IBP. Type II achalasia was defined as panesophageal pressurization present in at least 20% of swallows in a 30-mm Hg isobaric contour. Type III achalasia was defined by premature contractions with shortened distal latency (<4.5 seconds) in at least 20% of swallows ≥450 mm Hg.s.cm.

2.4 | Tissue biopsies

Biopsies of EGJOO and achalasia patients were taken during laparoscopic Heller myotomy. After the myotomy was completed

with no use of energy devices, a full-thickness muscle biopsy, 2 mm wide and 2 cm long, was obtained by cutting with scissors and was immediately preserved.[19] For healthy tissue control, a biopsy from an organ donor was included. The EGJ was obtained during organ procurement, with previous signed informed consent from the family. The EGJ with 3 cm of the esophagus and 2 cm of the stomach was taken. The tissue was transported at 4°C in Bretschneider's (Custodiol) solution within a period of 4-6 hours. Subsequently, a full-thickness biopsy of the muscle (thus including the myenteric plexus) of the esophagus was obtained.

2.5 | Esophageal tissue lysate

Esophageal tissue lysates were pooled. For the above, tissues were mechanically disintegrated in lysis buffer (10 mmol/L HEPES pH 7.9, 10mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L dithiothreitol [DTT]), incubated 10 minutes on ice and centrifuged. The supernatant was recuperated and dialyzed against H_2O for 24 hours. Protein concentration was determined using the bicinchoninic acid method. Samples were stored at −80°C until use.

2.6 | Two-dimensional gel electrophoresis

After thawing, acetone was added to each sample at a 1:4 proportion and was incubated overnight at −20°C. Samples were next centrifuged at 18 500× g for 30 minutes. Pellets were washed two times with acetone (−20°C). Each pellet was vacuumed dried at room temperature and finally rehydrated in ultrapure water and concentration adjusted at 1 mg/mL. One-hundred µg of esophageal tissue lysate was mixed with rehydration buffer (8 mol/L urea, 100 mmol/L DTT, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate [Bio-Rad], 0.5% carrier ampholytes pH 4-7, 0.01% bromophenol blue and 40 mmol/L Tris) to a final volume of 200 µL and then incubated for 1 hour at room temperature. This mixture was then applied to a ready strip (7 cm, pH 4-7; Bio-Rad) and actively rehydrated at 20°C for 14 hour. Esophageal tissue lysate was isoelectrically focused at 50 V for 15 minutes, followed by a first linear 50-100 V gradient for 15 minutes. This was then slowly ramped up to 4000 V for 2 hours and then maintained at 4000 V for a total of 12 000 V per gel. The ready strips were equilibrated in two steps for 15 minutes in an equilibration buffer containing 6 mol/L urea, 540 mmol/L Tris-HCl pH 8.8, 30% glycerol, 4% sodium dodecyl sulfate (SDS), and 130 mmol/L DTT. Then, they were equilibrated for 20 minutes in the same solution without DTT but containing 135 mmol/L iodoacetamide. Strips were rinsed in tris-glycine SDS running buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% SDS pH 8.8) applied to the top of a 12% polyacrylamide gel and run at 300 V for 4-5 hours. Gels were visualized using silver staining (Invitrogen).

2.7 | Sera samples

A 10 mL sample of venous blood was obtained in SST BD Vacutainer tubes and allowed to stand for 20 minutes at room temperature. Subsequently, the blood samples were centrifuged at 400 g for 20 minutes at 4°C. The sera were obtained under sterile conditions, aliquoted and stored at −70°C until use. They were only thawed once.

2.8 | Immunoblotting

The samples of esophageal tissue lysate were separated on 12% SDS-PAGE and then transferred to Polyvinylidene fluoride (PVDF), blocked with 3% non-fat milk in PBS/Tween, and incubated with human serum at a 1:100 dilution overnight at 4°C. Membranes were washed three times with PBS-Tween and incubated with Immunoglobulin G (IgG) antihuman conjugated with alkaline-phosphatase. After a further wash, membranes were developed with fast red (SIGMA) and naphthol-phosphate (SIGMA).

2.9 | Mass spectrometric analysis

The spots were subjected to in-gel trypsinization according to the manufacturer's protocol (Promega). The resulting peptides were placed into the Liquid chromatography-mass spectrometry (LC-MS) system consisting of a nanoflow pump EASY-nLC (Thermo-Fischer Co.) coupled to a mass spectrometer LTQ-Orbitrap Veils (Thermo-Fischer Co.) with a nano-electrospray ionization system type. Peptides were fragmented by the collision-induced dissociation and high energy collision dissociation methods. The spectrometric data were analyzed and compared in the Uniprot human database through the Sequest search system using the Discoverer 1.4 program.

2.10 | Ethical Considerations

This work was performed according to the principles expressed in the Declaration of Helsinki. The ethical committee from our institution approved the study and written informed consent was obtained from all subjects (ref. no. 1522).

3 | RESULTS

3.1 | Patients' characteristics

Demographic, clinical variables, and laboratory data from the HS (sera), tissue control (organ donor), patients with EGJOO and patients with achalasia are described in Table 1.

3.2 | Esophageal tissues lysates 2DGE analysis

Two-dimensional gel electrophoresis (2DGE-PAGE) analysis of the different study groups revealed differences in both, the number of spots protein, and the concentration. To facilitate the analysis, the 2DGE protein profiles of esophageal tissue lysates were divided into three regions (Figure 1).

In region 1, six spots were observed; in type I achalasia, its concentration was considerably diminished compared with achalasia type II and healthy donor tissue. In EGJOO, these spots were not observed.

In region 2, 22 spots were observed; in type I achalasia only 9 spots were detected; 2 spots were exclusive of type II achalasia (21 and 22); in EGJOO spots 13, 14, 16, and 17 were increased (9.5, 42.0, 3.9, and 2.5-fold, respectively) compared with healthy donor tissue.

In region 3, 56 spots were observed; in type I achalasia and EGJOO, spot 38 was highlighted, while in healthy donor tissue and type II achalasia this was not observed. In type I achalasia, type II achalasia, and EGJOO, spot 35 was decreased compared with healthy donor tissue. Spots 15, 16, 17, and 18 were decreased in type I achalasia, type II achalasia (3.0, 1.1, 9.0, and 13.5-fold, respectively) compared healthy donor tissue; spots 10, 11, and 12 were not detected in type I achalasia and EGJOO compared with healthy donor tissue. Finally, spots 1, 2, 3, 4, and 5 were found to be diminished in type I achalasia (3.6, 1.5, 3.7, 2.5, and 2.7-fold, respectively) and type II achalasia (1.9, 13.0, 3.2, 59.0, and 1.7-fold, respectively) compared healthy donor tissue.

3.3 | |Reactivity of the sera against a lysate of esophageal tissue

More than 56% of the sera of HS had reactivity against the 25 and 45 kDa proteins of the lysed LES muscle from type I but not type II achalasia (Figure 2, Table 2). Although the reactivity of sera of HS was observed against the 25 and 45 kDa proteins, the intensity of the bands is conspicuously lower compared with that observed in patients with achalasia (Figure 2).

More than 50% of the sera of patients with EGJOO had reactivity against the 25 kDa protein of the lysed LES muscle from patients with EGJOO, and patients with type I and type II. While 67% of EGJOO sera had reactivity against the 45 kDa protein of type I and II achalasia tissues (Figure 2; Table 2).

The 56% of the sera of patients with type I achalasia had only reactivity against the 25 kDa protein of the lysate LES muscle from type I achalasia.

More than 71% of the sera of patients with type II achalasia had reactivity against the 25 kDa protein of the lysate LES muscle from HD, EGJOO, type I, and type II achalasia. While 86% of the sera had reactivity against the 45 kDa protein of the lysate muscle LES from type I and type II achalasia. The sera with the highest reactivity were those of patients with achalasia type II (Figure 2; Table 2).

More than 50% of the sera of type III achalasia reacted against the 25 and 45 kDa proteins of the healthy donor tissue and type I achalasia lysate (Figure 2; Table 2).

The lysate tissue that showed the highest reactivity was the type I achalasia, where the 25 kDa protein was recognized by 89%-100% of the sera (Figure 2; Table 2). The above suggests that it could have a higher content of antigenic proteins due to one or several post-translational modifications and/or a greater antigenic exposure, which would correlate with a long time of disease evolution.

3.4 | |Identification of proteins by mass spectrometry

Bands 25 and 45 kDa were identified by mass spectrometry MALDI-TOF as triosephosphate isomerase (TPI), carbonic anhydrase (CA) for 25 kDa band and creatinine kinase bran isoform (CKB) for 45 kDa band (Table 3).

4 | DISCUSSION

Although achalasia has been considered a multifactorial disease with autoimmune etiology, there are few studies focused on the search antigenic targets. To our knowledge, there are at least 2 studies regarding proteomic analysis. One of them has been used monkey cerebellum extract in an immunoblot commercial kit for the detection of antineuronal antibodies. It has been found bands of 52/53 and 49 kDa. Nonetheless, the proteins were not identified. It has been only determined the presence of anti-Hu, anti-Yo, and anti-Ri antibodies.[14] Another proteomic analysis has been performed in the serum of patients with achalasia, finding elevation of complement proteins (C3, C5, and C4b5), CDK5, transthyretin, and alpha-2 macroglobulin.[15] Herein, we analyzed the proteomic profile of the lysates of esophageal tissue of patients with EGJOO and sub-classified achalasia as type I and type II. Of the three regions analyzed in the proteomic map of type I achalasia and type II achalasia, we observed 50 and 47 spots, respectively. The main difference was the concentration of certain proteins, two spots in region 3 draw attention; in type I achalasia the spot 35 is diminished and the spot 38 is increased compared with type II achalasia. On the other hand, healthy donor tissue versus achalasia has a higher concentration of most spots. However, spot 38 is decreased in HD esophageal tissue compared with achalasia tissue of type I achalasia and EGJOO. Unfortunately, due to the amount of esophageal tissue biopsy, it was not possible to identify all proteins. The decrease in the number of proteins in 2DGE may be due to the alteration of the extracellular matrix components, such as protein cross-linking, proteolysis, post-translational modification, etc.[20]

Regarding sera from achalasia patients, it is important to note that sera from type I, type II, and type III achalasia reacted mainly against lysate of type I achalasia tissue. Type I achalasia

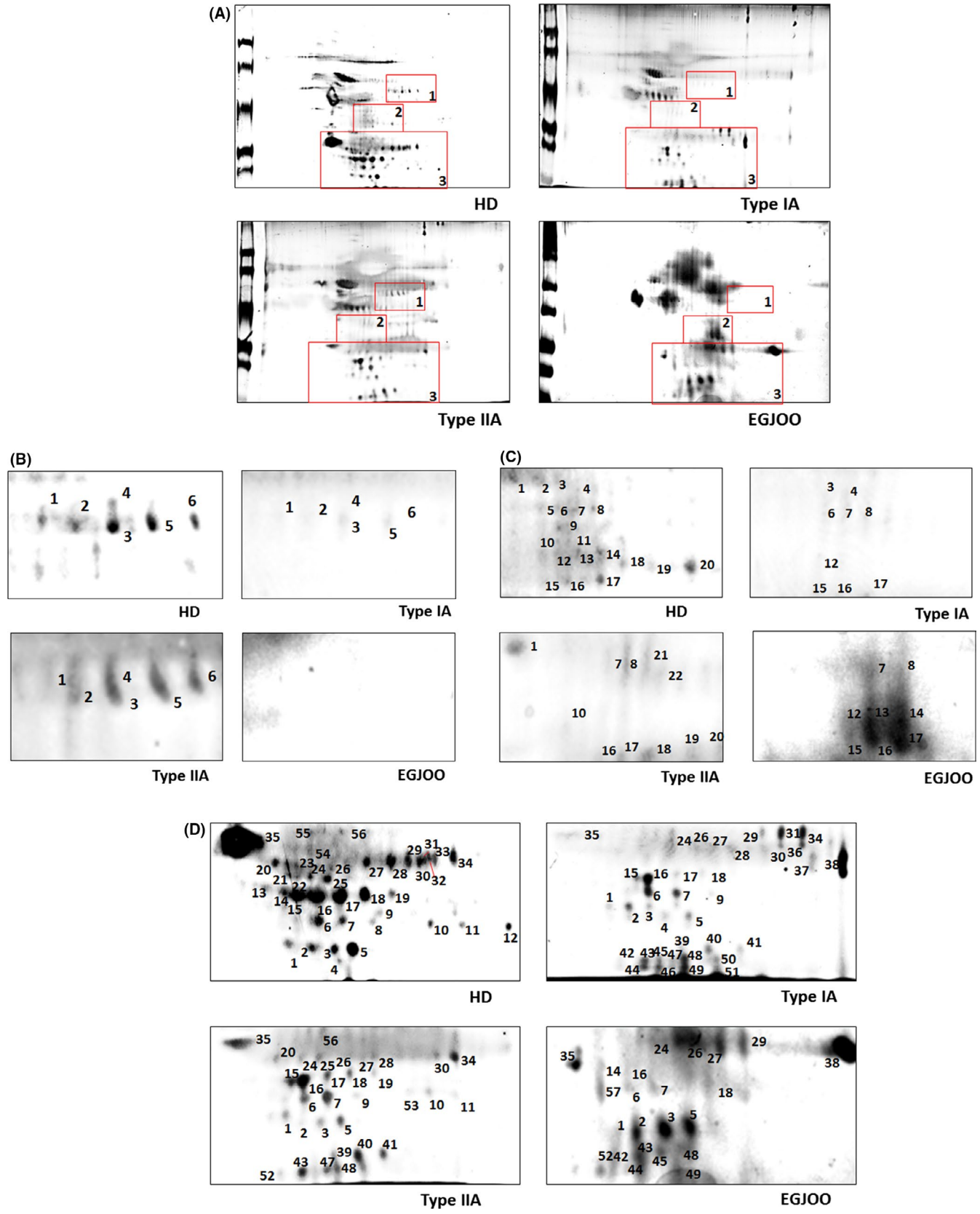

FIGURE 1 2DGE-PAGE of esophageal tissue lysate: (A) proteomic map of healthy donor tissue (HD), type I achalasia (Type IA), type II achalasia (type IIA), and esophagogastric junction outflow obstruction (EGJOO). 2DGE was divided into three regions; (B) Region 1. Comparison of spots of HD, Type IA, Type IIA, and EGJOO; (C) Region 2. Comparison of spots of HD, Type IA, Type IIA, and EGJOO; (D) Region 3. Comparison of spots of HD, Type IA, Type IIA, and EGJOO

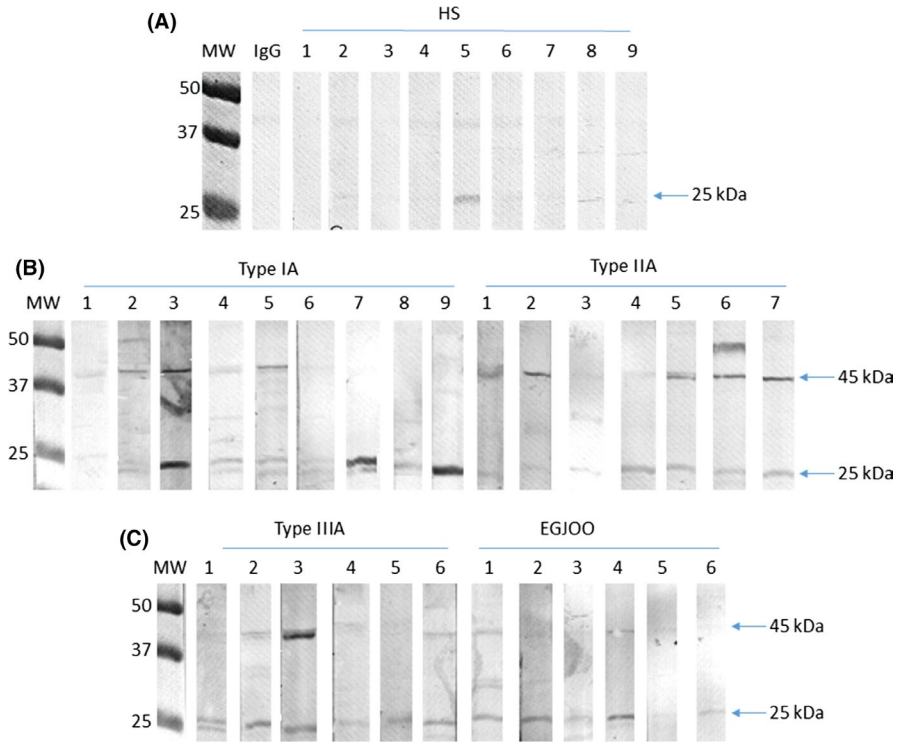

FIGURE 2 Representative immunoblotting of esophageal tissue lysate against the 25kDa and 45kDa proteins: (A) serum of healthy subjects HS; (B) serum of type I achalasia (Type IA) and type achalasia II (Type IIA), and; (C) Serum of type achalasia III (Type IIIA) and esophagogastric junction outflow obstruction EGJOO

TABLE 2 Percentage of sera that recognize achalasia tissue 25 and 45 kDa proteins

	HD tissue		EGJOO tissue		Type I achalasia tissue		Type II achalasia tissue	
Band	45 kDa (%)	25 kDa n (%)	45 kDa n (%)	25 kDa n (%)	45 kDa n (%)	25 kDa n (%)	45 kDa n (%)	25 kDa n (%)
Serum								
HS (n = 9)	0 (0)	1 (11)	0 (0)	4 (44)	5 (56)	9 (100)	0 (0)	2 (22)
EGJOO (n = 6)	2 (33)	5 (83)	2 (33)	5 (83)	4 (67)	6 (100)	4 (67)	3 (50)
Type I achalasia (n = 9)	5 (56)	6 (33)	1 (11)	2 (22)	5 (56)	8 (89)	0 (0)	4 (44)
Type II achalasia (n = 7)	5 (71)	6 (88)	0 (0)	5 (71)	6 (86)	7 (100)	0 (0)	5 (71)
Type III achalasia (n = 6)	4 (67)	6 (100)	1 (17)	1 (17)	3 (50)	6 (100)	2 (33)	2 (33)

Abbreviations: EGJOO, esophageal junction outflow obstruction; HD, healthy donor; HS, healthy subjects.

TABLE 3 Proteins identified by mass spectrometry

Band (kDa)	Accession no.	Protein ID	Protein description	MW kDa
25	Q53HE2_HUMAN (+1)	TPI	Triosephosphate isomerase	27
25	CAH1_HUMAN (+1)	CA	Carbonic anhydrase	29
45	AOAOC4DFV3_HUMAN (+1)	CKB	Creatinine kinase-brain isoform	45

Note: UniProt accession number and protein ID (www.uniprot.org).

is considered to be a later stage of the disease and therefore the tissue may have neoantigens or higher content of antigenic proteins due to one or several post-translational modifications and/or greater antigenic exposure.[20] These could be recognized by the immunoglobulins of the sera from healthy individuals, as well as achalasia patients, in contrast to earlier stages of the disease such as achalasia type II and type III where antigen expression is not abundant and/or varied.

On the other hand, we identified two 25 and 45 kDa bands as possible targets, derived from esophageal tissue lysate, which were recognized by the autoantibodies of patients with achalasia, and EGJOO, were identified as TPI and CA for the 25 kDa band and CKB for the 45 kDa. Triosephosphate isomerase and CA have been identified by other authors as targets antigens in several autoimmune diseases.

Triosephosphate isomerase is a glycolytic enzyme that interconverts D-glyceraldehyde-3 phosphate and dihydroxyacetone phosphate. TPI is recognized by 24.7% of the tested serum samples from patients with osteoarthritis.[21] Triosephosphate isomerase is also involved in the stability of neuronal microtubules, which are potential receptors of TPI. Studies showed that the inhibition of TPI activity influences microtubule stabilization and induces neuronal death in cultured murine cortical cells.[22] Autoantibodies anti-TPI in cerebrospinal fluid is associated with neuropsychiatric lupus[23,24] and MRL/MpJ-Fas^{lpr} mouse.[25] Recent studies have shown to the association of anti-TPI antibodies and neurological lupus, as well as a possible mechanism through the formation and deposition of immune complexes in regions near the ventricles, and the hippocampus choroid plexus that could contribute to brain damage and behavioral deficit.[23,24] In the present study, we show that the antibodies of achalasia patients recognized TPI. Similarly, a mechanism could be occurring in the esophageal myenteric plexus neurons, where the accumulation of TPI-anti-TPI immune complex could affect, in this case, the esophageal motility.

Further, we identified the type I CAI in the esophagus lysate, as other antigen recognized by achalasia serum. The CA is a metalloenzyme that catalyzes the reversible hydration of carbon dioxide to bicarbonate, and it is essential for the regulation of acid-base balance. Carbonic anhydrase functions in many physiological and pathological processes, such as transport, bone resorption, calcification, and tumorigenesis are demonstrated. Thus, 16 different isozymes that differ from each other with tissue distribution, cell localization, catalytic activity, and resistance to inhibitors have been described.[26] CAI and CAII antibodies have been identified in various autoimmune and idiopathic diseases; Grave's disease,[27] metabolic syndrome, polycystic ovary syndrome,[28] acute anterior uveitis, and Behcet's disease.[29] Besides CAII autoantibodies have been reported in systemic lupus erythematosus,[30,31] rheumatoid arthritis,[32] primary biliary cirrhosis, Sjögren's syndrome,[30] and acute anterior uveitis.[33] Both CA isoforms have high homology, so it is expected to found some cross-reactivity between them.

Finally, the CKB, other antigen recognized by achalasia patients in this study, predominates in the brain, but is also ubiquitous in smooth muscle and comprises the major fraction of CK found in urinary bladder, lung, prostate, uterus, thyroid, pancreas, stomach, smooth muscle, and kidney homogenates. The liver, spleen, salivary glands, and bile contain a varying but small amount of CKB. Anti-CKB antibody was present in the sera from paraneoplastic cerebellar degeneration with bladder cancer, small cell lung cancer, non-Hodking lymphoma.[34]

In summary, we identified three possible antigenic targets recognized by patients with achalasia; it is the first time that anti-TPI, anti-CA, and anti-CKB antibodies have been described in patients with achalasia. We recognize the limitation of the study: It is necessary to verify the reactivity against these antigens in a larger sample of patients and by another technique that allows elucidating the role of antibodies, anti-TPI, anti-CA, and anti-CKB, which in this study was by immunoblotting. Furthermore, these findings add evidence to the hypothesis that achalasia has an autoimmune component.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

Guarantor of the article: Gonzalo Torres-Villalobos, MD, PhD, and Janette Furuzawa-Carballeda, MSc, PhD. CAN-Á, DFH-R, and EO-M involved in conceptualization, data curation, formal analysis, investigation, methodology, writing—original draft, and writing—review and editing, EC-A, MAV, and FL-V involved in data curation, formal analysis, and writing-review and editing, and JF-C and GT-V involved in conceptualization, data curation, formal analysis investigation, methodology supervision, writing—original draft, writing—review and editing.

ORCID

Janette Furuzawa-Carballeda https://orcid.org/0000-0001-5804-7221

REFERENCES

1. Walzer N, Hirano I. Achalasia. *Gastroenterol Clin North Am*. 2008;37(4):807-825.
2. Vaezi MF, Pandolfino JE, Vela MF. ACG clinical guideline: diagnosis and management of achalasia. *Am J Gastroenterol*. 2013;108(8):1238-1249.
3. Furuzawa-Carballeda J, Aguilar-León D, Gamboa-Domínguez A, et al. Achalasia—an autoimmune inflammatory disease: a cross-sectional study. *J Immunol Res*. 2015;2015:729217.
4. Furuzawa-Carballeda J, Torres-Landa S, Valdovinos MÁ, Coss-Adame E, Martín del Campo LA, Torres-Villalobos G. New insights into the pathophysiology of achalasia and implications for future treatment. *World J Gastroenterology*. 2016;22(35):7892-7907.
5. Boecxstaens GE. Achalasia: virus-induced euthanasia of neurons? *Am J Gastrenterol*. 2008;103(7):1610-1612.
6. Verne GN, Hahn AB, Pineau BC, Hoffman BJ, Wojciechowski BW, Wu WC. Association of HLA-DR and -DQ alleles with idiopathic achalasia. *Gastroenterology*. 1999;117(1):26-31.
7. Gockel I, Becker J, Wouters MM, et al. Common variants in the HLA-DQ region confer susceptibility to idiopathic achalasia. *Nat Genet*. 2014;46(8):901-904.
8. Furuzawa-Carballeda J, Zuñiga J, Hernández-Zaragoza DI, et al. An original Eurasian haplotype, HLA-DRB1*14:54-DQB1*05:03, influences the susceptibility to idiopathic achalasia. *PLoS ONE ONE*. 2018;13(8):e0201676.
9. Furuzawa-Carballeda J, Boon L, Torres-Villalobos G, et al. Gelatinase B/matrix metalloproteinase-9 as innate immune effector molecule in achalasia. *Clin Transl Gastroenterol*. 2018;9(11):208.
10. Clark SB, Rice TW, Tubbs RR, et al. The nature of the myenteric infiltrated in achalasia: an immunohistochemically analysis. *Am J Surg Pathol*. 2000;24(8):1153-1158.
11. Booy JD, Takata J, Tomlinson G, Urbach DR. The prevalence of autoimmune disease in patients with esophageal achalasia. *Dis Esophagus*. 2012;25(3):209-213.

12. Romero-Hernández F, Furuzawa-Carballeda J, Hernández-Molina G, et al. Autoimmune comorbidity in achalasia patients. *J Gastroenterol Hepatol*. 2018;33(1):203-208.
13. Emami MH, Raisi M, Amini J, Daghaghzadeh H. Achalasia and thyroid disease. *World J Gastroenterol*. 2007;13(4):594-599.
14. Kallel-Sellami M, Karoui S, Romdhane H, et al. Circulating antimyenteric autoantibodies in Tunisian patients with idiopathic achalasia. *Dis Esophagus*. 2013;26(8):782-787.
15. Im SK, Yeo M, Lee KJ. Proteomic identification of proteins suggestive of immune-mediated response of neuronal degeneration in serum of achalasia patients. *Gut Liv*. 2013;7(4):411-416.
16. Vaezi MF. The American College of Gastroenterology's new guidelines on achalasia: what clinicians need to know? *Curr Gastroenterol Rep*. 2013;15(12):358.
17. Bredenoord AJ, Fox M, Kahrilas PJ, et al. Chicago classification criteria of esophageal motility disorders defined in high resolution esophageal pressure topography. *Neurogastroenterol Motil*. 2012;24(Suppl 1):57-65.
18. Kahrilas PJ, Bredenoord AJ, Fox M, et al. International High-Resolution Manometry Working Group. The Chicago Classification of esophageal motility disorders, v3.0. *Neurogastroenterol Motil*. 2015;27(2):160-174.
19. Torres-Villalobos G, Coss-Adame E, Furuzawa-Carballeda J, et al. Dor vs Toupet fundoplication after laparoscopic heller myotomy: long-term randomized controlled trial evaluated by high-resolution manometry. *J Gastrointest Surg*. 2018;22(1):13-22.
20. Cloos PAC, Christgau S. Post-translational modifications of proteins: implications for aging, antigen recognition, and autoimmunity. *Biogerontology*. 2004;5:139-158.
21. Xiang Y, Sekine T, Nakamura H, et al. Proteomic surveillance of autoimmunity in osteoarthritis: identification of triosephosphate isomerase as an autoantigen in patients with osteoarthritis. *Arthritis Rheum*. 2004;50(5):1511-1521.
22. Orosz F, Wagner G, Liliom K, et al. Enhanced association of mutant triosephosphate isomerase to red cell membranes and to brain microtubules. *Proc Natl Acad Sci USA*. 2000;97(3):1026-1031.
23. Sasajima T, Watanabe H, Sato S, Sato Y, Ohira H. Anti-triosephosphate isomerase antibodies in cerebrospinal fluid are associated with neuropsychiatric lupus. *J Neuroimmunology*. 2006;182(1–2):150-156.
24. Sato S, Yashiro M, Asano T, Kobayashi H, Watanabe H, Migita K. Association of anti-triosephosphate isomerase antibodies with aseptic meningitis in patients with neuropsychiatric systemic lupus erythematosus. *Clin Rheumatol*. 2017;36(7):1655-1659.
25. Sato S, Watanabe H, Shio K, Kobayashi H, Ohira H. Association of anti-triosephosphate isomerase antibody and MRL/MpJ-Fas^{lpr} mouse. *J Neuroimmunol*. 2010;226(1–2):110-115.
26. Gokcen T, Gulcin I, Ozturk T, Goren AC. A class of sulfonamides as carbonic anhydrase I and II inhibitors. *J Enzyme Inhib Med Chem*. 2016;31(suppl 2):180-188.
27. Alver A, Mentense A, Karahan SC, et al. Increased serum anti-carbonic anhydrase II antibodies in patients with Graves's disease. *Exp Clin Endocrinol Diabetes*. 2007;115(5):287-291.
28. Mentese A, Guven S, Sumer A, et al. Serum anti-carbonic anhydrase I and II antibodies and polycystic ovary syndrome. *Turk J Biochem*. 2013;38(1):43-48.
29. Mentese A, Alver A, Demir S, et al. Carbonic anhydrase I and II autoantibodies in Behcet's disease. *Acta Reumatol Port*. 2017;42(1):26-31.
30. Inagaki Y, Jinno-Yoshida Y, Hamasaki Y, Ueki H. A novel autoantibody reactive with carbonic anhydrase in sera from patients with systemic lupus erythematosus and Sjögren's syndrome. *J Dermatol Sci*. 1991;2(3):147-154.
31. Itoh Y, Reichlin M. Autoantibodies to carbonic anhydrase in systemic lupus erythematosus and other rheumatic disease. *Arthritis Rheum*. 1992;35(1):73-82.
32. Alver A, Şentürk A, Çakirbay H, et al. Carbonic anhydrase II autoantibody and oxidative stress in rheumatoid arthritis. *Clin Biochem*. 2011;44(17–18):1385-1389.
33. Turk A, Aykut M, Akyol N, et al. Serum anti-carbonic anhydrase antibodies and oxidant-antioxidant balance in patients with acute anterior uveitis. *Ocul Immunol Inflamm*. 2014;22(2):127-132.
34. Tetsuka S, Tominaga K, Ohta E, et al. Paraneoplastic cerebellar degeneration associated with an onconeural antibody against creatine kinase, brain-type. *J Neurol Sci*. 2013;335(1–2):48-57.

How to cite this article: Hernández-Ramírez DF, Olivares-Martínez E, Nuñez-Álvarez CA, et al. Triosephosphate isomerase, carbonic anhydrase, and creatinine kinase-brain isoform are possible antigen targets in patients with achalasia. *Neurogastroenterol Motil*. 2020;00:e13804. https://doi.org/10.1111/nmo.13804