Presence of Autoantibodies against the Autonomic Nervous System in Primary Achalasia

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ABSTRACT **Background:** Primary achalasia is a rare disorder but a significant cause of esophageal motor dysfunction. The pathophysiology of achalasia is still unknown, although an autoimmune etiology is suspected.

Objectives: To examine the presence of autoantibodies against autonomic nervous system receptors among primary achalasia patients.

Methods: In this observational cross-sectional study, we measure the levels of serum autoantibodies targeting G protein-coupled receptors of the autonomic nervous system, including adrenergic, muscarinic, endothelin, and angiotensin receptors. The study included 40 primary achalasia patients and 40 healthy controls without known history of achalasia, autoimmune diseases, or symptoms of an esophageal motility disorder.

Results: A statistically significant low level of autoantibodies against the M2 muscarinic receptor was observed in the serum of primary achalasia patients compared with the control group ($P < 0.009$). When exploring the two common achalasia types, a statistically significant low level of autoantibodies against type M1, M2, and M5 muscarinic receptors was observed among type 2 achalasia patients compared to patients with type 1 achalasia.

Conclusions: The finding of reduced levels of autoantibodies targeting the M2 muscarinic receptor in the serum of primary achalasia patients provides a valuable insight into the underlying pathogenesis of the disease.

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KEY WORDS: achalasia, autoantibodies, autonomic nervous system, muscarinic

Idiopathic (primary) achalasia is a rare esophageal motility disorder characterized by the loss of inhibitory enteric neurons, leading to impaired lower esophageal sphincter (LES) relaxation and absent peristalsis. Clinically, achalasia presents primarily with dysphagia to both solids and liquids, but may also include regurgitation, chest pain, weight loss, aspiration, and increased risk of esophageal carcinoma [1]. The disease severity is often assessed using the Eckardt score, which evaluates dysphagia, regurgitation, chest pain, and weight loss [2].

High-resolution manometry (HRM) is the gold standard for diagnosis, according to the Chicago Classification v4.0 [3]. However, the etiology remains unclear. Current hypotheses suggest autoimmune or allergy-related mechanisms, possibly triggered by neurotropic viral infection and sustained by chronic autoinflammatory responses in genetically predisposed individuals [1].

Supporting the autoimmune hypothesis, studies have identified associations with HLA class II alleles [4-6], inflammatory infiltrates in the myenteric plexus [7], and increased prevalence of other autoimmune diseases such as type 1 diabetes, Sjögren's syndrome, and autoimmune thyroiditis among achalasia patients [8]. Immune profiling of tissue samples has revealed cytotoxic lymphocyte infiltration, complement activation, and altered cytokine expression, including IL-17, IFN-γ, IL-22, and TGF-β1 [9].

Importantly, circulating autoantibodies targeting enteric neurons, especially anti-PNMA2 (Ma-2/Ta), are more prevalent in achalasia, particularly among patients with *HLA-DQA10103* and *DQB1*0603 alleles [10,11]. These autoantibodies may contribute directly to pathogenesis, as shown by their ability to induce changes in myenteric neurons in vitro [12].

Despite growing evidence implicating autoimmunity, the role of autoantibodies against autonomic G protein-coupled receptors remains unexplored. In this study,

we assessed circulating autoantibodies against muscarinic, adrenergic, endothelin, and angiotensin receptors in achalasia patients. We also examined correlations with clinical and manometric findings.

PATIENTS AND METHODS

STUDY POPULATION

This observational, prospective, single-center, cross-sectional cohort study was conducted at the Department of Gastroenterology, Tel Aviv Sourasky Medical Center, Israel, between 2021 and 2022. Eligible participants were 18–95 years of age and had a confirmed diagnosis of primary achalasia established by a gastroenterologist specializing in esophageal motility disorders and based on clinical evaluation and supporting diagnostic tests including HRM, esophagogastroduodenoscopy (EGD), and barium esophagram.

Exclusion criteria included: other swallowing or motility disorders not classified as achalasia; secondary achalasia due to infection, malignancy, myopathy, trauma, toxic/metabolic, or infiltrative causes; pregnancy; age under 18 or over 95 years; and significant cognitive impairment.

Clinical data were collected through structured interviews. Achalasia subtype was classified according to the Chicago Classification v4.0 [3] using HRM: Type I: absent esophageal pressurization, Type II: pan-esophageal pressurization, Type III: spastic achalasia. Disease severity was evaluated using the Eckardt Score [2], which includes dysphagia, regurgitation, chest pain, and weight loss.

The control group included ambulatory patients undergoing endoscopy for indications unrelated to motility disorders. Exclusion criteria for controls included any symptom or history of dysphagia, suspected or diagnosed esophageal motility disorders, autoimmune disease, or use of immunosuppressive/immunomodulatory therapy.

Table 1. Clinical characteristics of the study population

Characteristics	Values
ECKARDT, median grade	3
Achalasia type 1, n (%)	15 (37.5)
Achalasia type 2, n (%)	22 (55)
Achalasia type 3, n (%)	2 (5)
Diabetes type 1, n (%)	0 (0)
Hyperthyroidism, n (%)	2 (5)
Hypothyroidism, n (%)	3 (7.5)
Pernicius anemia, n (%)	0 (0)
Vitiligo, n (%)	0 (0)
Rheumatoid arthritis, n (%)	0 (0)
Systemic lupus erythematosus, n (%)	0 (0)
Scleroderma, n (%)	0 (0)
Eosinophilic esophagitis, n (%)	2 (5)
Food allergy, n (%)	5 (12.5)
Drug allergy, n (%)	8 (20)
Dust allergy, n (%)	4 (10)
Exposure to asbestos, n (%)	3 (7.5)
Family history of achalasia, n (%)	0 (0)
Family history of malignancy, n (%)	0 (0)
Personal history of malignancy, n (%)	10 (25)
Family history of autoimmune diseases, n (%)	3 (7.5)
Active smoking, n (%)	0 (0)
Former smoker, n (%)	8 (20)
Achalasia management**	
Taking medications for achalasia, n (%)	0 (0)
Pneumatic balloon dilatation, n (%)*	36 (90)
Number for dilatations, mean ± SD	1.55 ± 1.25
POEM, n (%)*	0 (0)
Botox to LES, n (%)*	4 (10)
Surgery for achalasia, n (%)*	7 (17.5)

LES = lower esophageal sphincter, POEM = per oral endoscopic myotomy, SD = standard deviation

*Patients may have had more than one type of therapy

**One patient had non-typeable achalasia

AUTOANTIBODY QUANTIFICATION

Blood samples (18 ml) were collected on the recruitment day, regardless of diagnosis date or treatment status. Samples were processed double-blind. After clotting at room temperature, samples were centrifuged at 2000g for 15 minutes. Sera were stored at -35°C.

Circulating autoantibodies against G protein-coupled receptors (GPCRs) were measured using ELISA kits (Cell-Trend GmbH, Germany) [13]. These included anti-adrenergic receptors: α1, α2, β1, β2; anti-muscarinic receptors: M1–M5; and anti-endothelin receptor type A and anti-angiotensin II type 1 receptor. Plates were coated with purified GPCR proteins. Buffers contained 1 mM CaCl2 to preserve conformational epitopes. Sera (1:100 dilution) were incubated at 4°C for 2 hours, followed by HRP-conjugated anti-human IgG (1:20,000). Standard curves were established using reference sera from known GPCR antibody-positive patients. Cut-off levels were determined via receiver operating characteristic analysis.

Table 2. Sera autoantibodies in achalasia vs. control

Antibody	Achalasia group	Control group	*P*-value
N	40	40	–
AT1R-Ab, median (IQR)	11.87 (10.06–15.24)	11.09 (9.55–13.76)	0.199
ETAR-Ab, median (IQR)	11.79 (9.59–16.47)	10.46 (9–13.89)	0.194
a1-adr-R-Ab, median (IQR)	9.53 (6.9–13.69)	8.62 (6.08–12.12)	0.281
a2-adr-R-Ab, median (IQR)	23.66 (14.36–38.89)	18.44 (13.23–28.33)	0.273
b1-adr-R-Ab, median (IQR)	12.17 (8–21.39)	11.25 (8.47–16.57)	0.630
b2-adr-R-Ab, median (IQR)	11.35 (7.78–22.91)	10.91 (6.51–16.76)	0.341
M1R Ab, median (IQR)	7.67 (4.69–15.69)	11.02 (6.96–18.26)	0.054
M2R Ab, median (IQR)	9.17 (6.29–17.44)	14.27 (8.81–23.25)	**0.009**
M3R Ab, median (IQR)	6.94 (4.97–9.6)	7.18 (4.98–9.86)	0.665
M4R Ab, median (IQR)	9.13 (7.18–14.91)	9.73 (6.76–12.7)	0.962
M5R Ab, median (IQR)	12.98 (9.73–20.3)	15.82 (11.97–25.8)	0.059

AT1R-Ab = autoantibodies directed against angiotensin type I, ETAR-Ab = endothelin type A, a1-adr-R-Ab = α1-adrenergic receptors autoantibodies, a2-adr-R-Ab = α2-adrenergic receptors autoantibodies, β1-adr-R-Ab = β1-adrenergic receptors autoantibodies, β2-adr-R-Ab = β2-adrenergic receptors autoantibodies, M1R Ab =M1 muscarinic receptors autoantibodies, M2R Ab = M2 muscarinic receptors autoantibodies, M3R Ab = M3 muscarinic receptors autoantibodies, M4R Ab = M4 muscarinic receptors autoantibodies, M5R Ab = M5 muscarinic receptors autoantibodies, IQR = interquartile range

Bold signifies significance

Table 3. Baseline characteristics achalasia type 1 vs. achalasia type 2

Baseline characteristics	Achalasia type 1 (n=15)	Achalasia type 2 (n=22)	*P*-value
Age in years, mean ± SD	70 (48–74)	67.5 (50–72)	0.757
Male, n (%)	10 (66.7)	10 (45.5)	0.438
ECKARDT, median grade (IQR)	3 (2–4)	2.5 (0.75–4.25)	0.501
Duration of illness, in years	11 (7–20)	7 (5.25–9.5)	**0.047**
Diabetes type 1, n (%)	0 (0)	0 (0)	–
Hyperthyroidism, n (%)	0 (0)	2 (9.1)	0.305
Hypothyroidism, n (%)	1 (6.7)	1 (4.5)	0.864
Pernicius anemia, n (%)	0 (0)	0 (0)	–
Vitiligo, n (%)	0 (0)	0 (0)	–
Rheumatoid arthritis, n (%)	0 (0)	0 (0)	–
Systemic lupus erythematosus, n (%)	0 (0)	0 (0)	–
Scleroderma, n (%)	0 (0)	0 (0)	–
Eosinophilic esophagitis, n (%)	1 (6.7)	1 (4.5)	0.864
Food allergy, n (%)	2 (13.3)	2 (9.1)	0.738
Drug allergy, n (%)	1 (6.7)	5 (22.7)	0.223
Dust allergy, n (%)	2 (14.3)	1 (5)	0.591
Asbestos exposure, n (%)	0 (0)	2 (9.1)	0.083
Family history of achalasia, n (%)	0 (0)	0 (0)	0.065
Family history of malignancy, n (%)	5 (33.3)	5 (22.7)	–
Personal history of malignancy, n (%)	4 (26.7)	5 (25)	0.772
Family history of autoimmune disease, n (%)	0 (0)	3 (14.3)	0.152
Active smoking, n (%)	0 (0)	0 (0)	–
Former smoker, n (%)	3 (20)	4 (18.2)	0.659
Medications usage for achalasia, n (%)	0 (0)	0 (0)	–
Pneumatic balloon dilatation, n (%)	15 (100)	18 (81.8)	0.085
Per oral endoscopic myotomy (POEM), n (%)	0 (0)	0 (0)	–
Botox to LES, n (%)	1 (6.7)	2 (9.1)	0.818
Surgery for achalasia, n (%)	0 (0)	5 (22.7)	**0.001**

LES = lower esophageal sphincter, POEM = per oral endoscopic myotomy, SD = standard deviation

Bold signifies significance

Table 4. Sera autoantibodies level in achalasia type 1 vs. achalasia type 2

Antibody	Achalasia type 1	Achalasia type 2	*P*-value
N	15	22	–
AT1R-Ab, median (IQR)	11.55 (10.18–16.99)	11.85 (9.82–14.78)	0.68
ETAR-Ab, median (IQR)	11.73 (9.52–16.82)	11.93 (9.43–17.9)	0.915
a1-adr-R-Ab, median (IQR)	10.44 (7.1–13.71)	9.53 (6.83–13.27)	0.593
a2-adr-R-Ab, median (IQR)	25.92 (20.3–41.57)	17.88 (11.7–29.8)	0.52
b1-adr-R-Ab, median (IQR)	11.41 (8.8–23.36)	12.17 (7.61–21.09)	0.551
b2-adr-R-Ab, median (IQR)	14.1 (6.8–25.27)	10.55 (7.88–23.99)	0.658
M1R Ab, median (IQR)	11.06 (6.48–17.89)	5.02 (4.07–8.59)	**0.015**
M2R Ab, median (IQR)	12.65 (8.67–20.9)	7.36 (4.88–10.9)	**0.004**
M3R Ab, median (IQR)	6.88 (5.13–10.37)	7.01 (4.76–9.71)	0.68
M4R Ab, median (IQR)	9 (7.14–14.92)	9.14 (7.29–14.98)	0.795
M5R Ab, median (IQR)	15.83 (11.81–23.13)	11.24 (8.55–14.72)	**0.028**

AT1R-Ab = autoantibodies directed against angiotensin type I, ETAR-Ab = endothelin type A, a1-adr-R-Ab = α1-adrenergic receptors autoantibodies, a2-adr-R-Ab = α2-adrenergic receptors autoantibodies, β1-adr-R-Ab = β1-adrenergic receptors autoantibodies, β2-adr-R-Ab = β2-adrenergic receptors autoantibodies, M1R Ab =M1 muscarinic receptors autoantibodies, M2R Ab = M2 muscarinic receptors autoantibodies, M3R Ab = M3 muscarinic receptors autoantibodies, M4R Ab = M4 muscarinic receptors autoantibodies, M5R Ab = M5 muscarinic receptors autoantibodies, IQR = interquartile range

Bold signifies significance

STATISTICAL ANALYSIS

Normality was tested using the Shapiro-Wilk test. Due to non-normal distribution, non-parametric tests were used. Continuous variables were presented as median (interquartile range [IQR]) and compared with Mann-Whitney U tests. *P*-values for antibody titers were corrected for multiple comparisons using Bonferroni correction. Categorical variables were compared using chi-square tests. A *P*-value < 0.05 was considered statistically significant. Statistical analyses were performed using IBM Statistical Package for the Social Sciences statistics software, version 23 (SPSS, IBM Corp, Armonk, NY, USA).

ETHICS CONSIDERATIONS

All participants provided informed consent. The study was approved by the institutional ethics committee (Tel Aviv Medical Center; approval no. 0433-21-TLV) and conducted in accordance with the Declaration of Helsinki.

RESULTS

BASELINE CLINICAL CHARACTERISTICS

The study included 40 patients with primary achalasia and 40 controls without swallowing disorders. The achalasia group had a mean age of 64±15.2 years (55% female), and the control group 59.4±16.1 years (62.5% male), with no significant age or sex differences. Among achalasia patients, 37.5% had type I, 55% type II, and 5% type III. One patient (2.5%) had non-typeable achalasia, as he was unable to tolerate high-resolution manometry and was therefore diagnosed based on esophagogastroduodenoscopy and barium esophagram. The median Eckardt score IQR was 3/12. Co-morbidities included thyroid disease (12.5%), eosinophilic esophagitis (5%), allergies (42.5%), and autoimmune family history (7.5%). Detailed medical history and co-morbidities are presented in Table 1.

TITERS OF AUTOANTIBODIES IN ACHALASIA PATIENTS

Autoantibody titers against angiotensin type I, endothelin type A, adrenergic receptors (α1, α2, β1, β2), and muscarinic receptors (M1–M5) were measured in both achalasia patients and controls [Table 2]. A significantly lower level of anti-M2R autoantibodies was found in achalasia patients compared to controls (median 9.17, IQR 6.29–17.44 vs. 14.27, IQR 8.81–23.25, $P = 0.009$). In addition, borderline lower levels were observed for anti-M1R ($P = 0.054$) and anti-M5R ($P = 0.059$) in the achalasia group. No significant differences were found for autoantibodies targeting angiotensin I, endothelin A, adrenergic (α1, α2, β1, β2), or muscarinic M3/M4 receptors between groups [Table 2].

CLINICAL CHARACTERISTICS OF PATIENTS WITH PRIMARY ACHALASIA TYPE 1 AND TYPE 2

Patients were stratified by type of achalasia according to the Chicago Classification IV. The cohort included 15

patients with type I achalasia and 22 with type II. The average age was 70 years (range 48–74) in the type I group and 67.5 years (range 50–72) in the type II group. Both groups included 10 males (66.7% vs. 45.5%, respectively).

There was no significant difference in disease severity based on the Eckardt score (median 3, IQR 2–4 vs. median 2.5, IQR 0.75–4.25; $P = 0.501$). However, disease duration was significantly longer in the type I group (11 years, IQR 7–20) compared to type II (7 years, IQR 5.25–9.5; $P = 0.047$). No differences in co-morbidities or prior treatments were noted, except for Heller myotomy, which was significantly more common in type II patients (22.7% vs. 0%; $P = 0.001$) [Table 3].

TITERS OF AUTOANTIBODIES IN THE SERA OF TYPE 1 AND TYPE 2 ACHALASIA PATIENTS

Significantly lower levels of anti-M2R autoantibodies were observed in type II achalasia patients compared to type I (median 7.36, IQR 4.88–10.9 vs. 12.65, IQR 8.67–20.9, P=0.004). Similarly, type II patients had significantly reduced levels of anti-M1R (median 5.02 vs. 11.06, P=0.015) and anti-M5R autoantibodies (median 11.24 vs. 15.83, P=0.028) compared to type I. No significant differences were found between groups in titers of autoantibodies against angiotensin I, endothelin A, adrenergic receptors (α1, α2, β1, β2), or muscarinic M3/M4 receptors [Table 4].

LACK OF CORRELATION BETWEEN THE ECKARDT SCORE AND ANTI-M2R AUTOANTIBODIES LEVEL

Pearson's correlation analysis between Eckardt score and anti-M2R autoantibody levels (Units/ml) revealed a weak positive correlation (r = 0.116). However, this correlation was not statistically significant ($P = 0.477$), with a 95% confidence interval ranging from -0.203 to 0.412, encompassing zero. This finding suggests that no meaningful linear relationship exists between symptom severity and anti-M2R titers in this cohort, and the observed correlation may be due to chance.

DISCUSSION

In this study, we evaluated the presence of circulating autoantibodies targeting autonomic nervous system receptors in patients with primary achalasia. We report, for the first time, a statistically significant reduction in serum levels of anti-M2R autoantibodies in achalasia patients compared to age- and sex-matched controls ($P < 0.009$). However, no significant correlation was found between anti-M2R titer and clinical severity (Eckardt score), which is likely to reflect effective symptom control through therapeutic intervention. In addition, the Eckardt score may be limited in capturing disease progression, as some patients with advanced esophageal dilation may report minimal symptoms until complications arise.

We also observed a non-significant trend toward reduced levels of anti-M1R and anti-M5R autoantibodies in achalasia patients. Importantly, when stratifying by achalasia subtype (Chicago Classification v4.0), significantly lower titers of anti-M1R, anti-M2R, and anti-M5R autoantibodies were observed in type II compared to type I patients ($P = 0.015$, $P = 0.004$, and $P = 0.028$, respectively), suggesting immunological distinctions between subtypes.

Muscarinic receptors, particularly M2 and M3, are abundantly expressed in esophageal smooth muscle and play essential roles in regulating esophageal motility via acetylcholine-induced calcium signaling [14,15]. While M2 receptors are more prevalent in esophageal tissue, M3 is more potent in triggering smooth muscle contraction. M1 receptors, though less dominant in the esophagus, are expressed in various tissues, including salivary glands [16,17].

Autoantibodies targeting muscarinic receptors have been implicated in several autoimmune diseases. For example, anti-M3R, M1R, and M2R antibodies are found in Sjögren's syndrome and are associated with gastrointestinal symptoms in postural orthostatic tachycardia syndrome (POTS) [18,19]. In Chagas disease-a parasitic condition that mimics achalasia pathophysiology-anti-M2R autoantibodies have been identified in patients with esophageal involvement [20]. These antibodies have been shown to exert functional effects on smooth muscle and myocardial tissues, suggesting a role in neuromuscular dysfunction and dysmotility [20].

We hypothesize that higher M2R expression in the lower esophageal sphincter may lead to preferential autoantibody binding, disrupting nitric oxide-mediated relaxation. This may contribute to the pathogenesis of primary achalasia. The observed borderline reductions in anti-M1R and anti-M5R titers further support this notion, as all five muscarinic receptor subtypes are expressed in esophageal tissue [12].

Despite a small cohort size (n=40), our findings align with prior studies and are consistent with the rarity of the disease. Future research in larger, multi-center cohorts is needed to further explore the mechanistic and diagnostic relevance of anti-muscarinic autoantibodies in achalasia.

CONCLUSIONS

Our finding of reduced levels of autoantibodies targeting the M2 muscarinic receptor in the serum of primary achalasia patients in our study provides a potential invaluable insight into the underlying pathogenesis of the disease. Furthermore, our study sheds light on potential distinctions in pathophysiological mechanisms among different subtypes of achalasia. This finding emphasizes the need for further research to evaluate the role of anti-muscarinic autoantibodies in primary achalasia, in addition to examination of potential dysfunctional activities of these antibodies, both in vitro and in vivo. Such investigations could potentially unveil therapeutic targets, with the goal of halting the progression of the disease in its early stages and averting permanent damage.

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The real voyage of discovery consists not in seeing new landscapes, but in having new eyes.
Marcel Proust (1871–1922), French novelist, literary critic, and essayist

My own experience and development deepen every day my conviction that our moral progress may be measured by the degree in which we sympathize with individual suffering and individual joy.
George Eliot (Mary Ann Evans) (1819–1880), English novelist, poet, journalist, translator, and one of the leading writers of the Victorian era