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The Value of Anti-drug Antibody Detection in Discriminating Patients from Healthy Controls and Predicting the Gross Motor Functional State in Patients with Pompe Disease

Solmaz Aziz-Ahari[1], Mahdi Aminian[2], Aliasghar Rahimian[2], Shahriar Nafissi[3,4], Morteza Heidari[1], Ali Rabbani[5,6], Amir-Hassan Zarnani[7], Ali Mohebbi[5], Reza Shervin Badv[1], Nargess Saladjegheh[8], Sahar Mortezagholi[9], Safdar Masoumi[10], Zahra Hadipour[11], Reza Boostani[12], Mehran Beiraghi Toosi[13], Keyhan Sayadpour Zanjani[14], Ali Khajeh[15], Seyed Hassan Tonkaboni[16], Maryam Sadeghi[1], Farzad Fatehi[4], Elahe Vafaei[17], and Mahmoud Reza Ashrafi[1,3,5]

[1] Division of Pediatric Neurology, Pediatrics Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
[2] Department of Clinical Biochemistry, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
[3] Neuromuscular Research Center, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran
[4] Department of Neurology, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran
[5] Growth and Development Research Center, Pediatrics Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
[6] Department of Pediatric Endocrinology, Pediatric Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
[7] Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[8] Medical Laboratory, Social Security Organization, Tehran, Iran
[9] Department of Immunology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[10] Endocrine Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[11] Department of Clinical Genetics, Atieh Hospital, Tehran, Iran
[12] Department of Neurology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[13] Department of Pediatric Neurology, Ghaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran
[14] Division of Cardiology, Department of Pediatric, Pediatric Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
[15] Department of Pediatric Neurology, Children and Adolescents Health Research Center, Zahedan University of Medical Sciences, Zahedan, Iran
[16] Department of Pediatric Neurology, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[17] Fetal and Pediatric Cardiovascular Research Center, Pediatric Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran

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ABSTRACT

Anti-recombinant human acid α-glucosidase (anti-rhGAA) antibody formation is a major challenge in patients with Pompe disease receiving enzyme replacement therapy (ERT). The clinical significance of these antibodies and their detection methods remain uncertain. This study aimed to evaluate the diagnostic and functional relevance of anti-rhGAA antibodies in late- onset Pompe

Corresponding Author: Mahmoud Reza Ashrafi, MD
Division of Pediatric Neurology, Pediatrics Center of Excellence, Children's Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran. Tel/Fax: (+98 21) 6612 9262, Email; ashrafim@tums.ac.ir

disease (LOPD) and to compare the performance of ELISA and Western blot assays.

Fourteen patients with LOPD undergoing ERT and 14 age- and sex-matched healthy controls were studied. Serum anti-rhGAA antibodies and their IgG, IgM, and IgA isotypes were quantified using ELISA and verified by Western blot. Motor function was assessed using the Pompe Motor Function Levels Questionnaire, an adapted version of the GMFCS validated for Pompe disease.

Total and isotype-specific anti-rhGAA antibody levels were significantly higher in patients than in controls. ROC analysis showed excellent discrimination between groups. Strong agreement was observed between ELISA and Western blot results. However, antibody levels were not significantly correlated with motor function grade. Given the small sample size (n=14), this non-significant result may reflect limited statistical power rather than a true lack of association.

Anti-rhGAA antibody detection effectively distinguishes LOPD patients from healthy individuals. Western blot provides a reliable, low-cost alternative to ELISA, particularly useful in resource-limited settings. Nevertheless, the prognostic utility of antibody titers for functional outcomes remains uncertain and warrants larger, multicenter validation studies.

Keywords: Anti-rhGAA antibody; ELISA; Enzyme replacement therapy; Motor function; Pompe disease; Western blot

INTRODUCTION

Pompe disease, also known as glycogen storage disease type II or acid maltase deficiency, is a rare genetic disorder caused by mutations in the *GAA* gene.[1] This gene provides instructions for the production of an enzyme called acid α-glucosidase (GAA), which is essential for breaking down glycogen, a complex sugar stored in muscles for energy.[2] When GAA is deficient or absent, glycogen accumulates in cells, particularly in muscles, impairing their function.[3] Pompe disease can be classified into three main types on the basis of the age of onset and the severity of symptoms: 1) infantile-onset Pompe disease (IOPD), which manifests within a few months of birth and manifests as muscle weakness, hypotonia, cardiomegaly, respiratory difficulties, and failure to thrive; 2) late-onset Pompe disease (LOPD), which manifests in childhood, adolescence, or adulthood with muscle weakness, respiratory issues, and mobility problems but typically does not have severe heart problems; and 3) juvenile-onset Pompe disease, which appears in early childhood and has symptoms that are less severe than those of IOPD but more severe than those of LOPD.[4,5] Pompe disease is diagnosed through a combination of clinical evaluation, biochemical tests, genetic testing, and sometimes muscle biopsy. In this context, measuring the activity of the GAA enzyme in blood, skin, or muscle, identifying mutations in the *GAA* gene, and examining muscle tissue for glycogen accumulation are the fundaments of definitive diagnosis.[6] While there is no cure for Pompe disease, treatments aim to manage symptoms and improve quality of life.[7,8] The main treatment is enzyme replacement therapy (ERT) with alglucosidase alfa, which provides a synthetic form of the GAA enzyme.[9] ERT has been shown to improve heart and muscle function, especially in IOPD, and slow disease progression in LOPD patients.[10] In addition to ERT, some supportive care has also been examined, including respiratory support, physical therapy, occupational therapy, nutritional support, and cardiac care.[11]

Since 2006, ERT with recombinant human GAA (rhGAA; alglucosidase alfa) has become the standard of care in the treatment of PD patients. ERT has considerably changed survival, motor function, daily activities, and quality of life in IOPD patients.[12] Additionally, ERT in patients with LOPD significantly improved their walking distance on the six-minute walk test, but no improvement was found in respiratory function or muscle strength.[13] In contrast, other LOPD patients have displayed constant respiratory capacity and enhanced motor function with ERT.[14] In contrast to the significant advantages of ERT, there is a spectrum of clinical responses to ERT because of many factors, such as age at ERT, dose of ERT, extent of present pathology, and development of anti-rhGAA antibodies.[15] In the context of Pompe disease, anti-rhGAA antibodies are a significant concern, particularly for patients undergoing ERT with alglucosidase alfa.[16] Anti-rhGAA antibodies can develop as the body's immune system recognizes the

infused enzyme as foreign and mounts an immune response against it. This immune response can potentially reduce the efficacy of treatment and lead to adverse effects. The presence of anti-rhGAA antibodies can neutralize the therapeutic enzyme, decreasing its effectiveness.[17] Anti-rhGAA antibodies can also cause infusion-related reactions that can complicate the administration of ERT and may require premedication or adjustments in the infusion protocol.[18] In total, if ERT is less effective due to anti-rhGAA antibodies, patients may experience more rapid progression of muscle weakness, respiratory issues, and other disease-related symptoms.[19] Some risk profiles have been introduced for the development of anti-rhGAA antibodies, such as *GAA* gene mutations and immune system hyperactivity, in Pompe disease patients.[20] Interestingly, there was no obvious effect of anti-rhGAA antibodies on ERT for most LOPD patients with high anti-rhGAA antibodies titres, whereas a minority of LOPD patients experienced clinical deterioration associated with anti-rhGAA antibodies in response to ERT. Therefore, these patients may need immunotolerance induction (ITI) for the purpose of prevention or treatment.[21]

Overall, early detection of anti-rhGAA antibodies might be meaningful in reducing clinical challenges with some interventions. We hypothesized that anti-rhGAA antibodies could be detected by Western blot in patients on ERT. This study aimed to establish a method to determine the anti-rhGAA antibodies in LOPD patients on ERT and to assess the relationship between the rates of anti-rhGAA antibodies and ERT functional outcomes.

MATERIALS AND METHODS

Patients and Sampling

Fourteen Pompe patients (ten males, four females) who underwent ERT were included in this study. These patients were from different medical centers throughout the country. Fourteen sex- and age-matched normal control subjects were recruited to serve as healthy control individuals (ten males, four females). Initially, a questionnaire containing demographic information and clinical history was designed. The study protocol was subsequently thoroughly explained to the patients and their families, and written informed consent was obtained from all the patients prior to the study. The study was approved by the ethics committee of Tehran University of Medical Sciences. A sample of venous blood (5 mL) was collected following proper blood sampling procedures. The blood was collected in clot tubes, and the serum samples were stored at -70 °C until testing. All patients included in this study were diagnosed with late-onset Pompe disease (LOPD). In all cases, the onset of clinical symptoms occurred after the first year of life, consistent with the definition of LOPD. All patients received enzyme replacement therapy (ERT) with recombinant human acid α-glucosidase (alglucosidase alfa; Myozyme) at a standard dose of 20 mg/kg body weight, administered biweekly (every two weeks). This dosing regimen was consistent across all patients in the study and adhered to the international standard treatment protocol for Pompe disease.

Detection of Anti-rhGAA Antibodies in Serum

An ELISA was used to examine the presence of antibodies against recombinant human α-glucosidase enzyme (rhGAA) in patient sera. In the initial step, Myozyme (rhGAA) at a concentration of 5 μg/mL was applied to coat 96-well plates (Nunc, USA), which were subsequently incubated at 4 °C for 16 hours. Following the incubation period, the wells were washed three times with 0.05% PBS-Tween 20. Next, the wells were blocked with 3% skim milk at room temperature for three hours. Subsequent to another round of washing, the prepared dilutions (1:500) from patient and control serum samples were added to the wells, and incubation was continued at room temperature for 90 minutes, followed by additional washing steps. HRP-conjugated sheep anti-human Ig (Sina Biotec, Iran) at a dilution of 1:2000 was added to the wells and incubated at room temperature for 60 minutes. Following the washing step, TMB was applied for 15 minutes, and the process was stopped by the addition of 1 N HCl. Negative control wells in which the coating layer of serum was omitted were included. The optical density was measured at 450 and 630 nm via an ELISA reader (BioTek, United States).

Determining the Isotype of Patients' Antibodies

To determine the antibody isotype for each patient, three wells were used. ELISA was performed as described above, but HRP-conjugated anti-human IgG, IgA or IgM (all from Diazist, Iran) was added as the detection antibody at a dilution of 1:2000. Optical densities (ODs) were read as described above.

Western Blotting

The reactivity of patients' antibodies against the reduced form of Myozyme was assessed by Western

blotting. A total of 20 µg of Myozyme in sample buffer containing 10% 2-mercaptoethanol (2ME) was loaded into the wells of a 12% polyacrylamide gel. Electrophoresis was conducted at 90 V for 20 minutes, and subsequently, the voltage was increased to 150 V for an additional 60 minutes. The resolved proteins were then transferred onto a nitrocellulose membrane (Roche, Germany) at 200 V for 120 minutes. After the membranes were blocked for 16 hours at 4 °C in 4% skim milk in 0.05% TBS-Tween (TBS-T), the blocked strips were washed three times in TBST buffer. The membranes were probed with patient serum (1:20) in blocking solution for 120 minutes at 37 °C on a shaker. After being washed with TBS-Tween, the membranes were incubated with secondary HRP-conjugated rabbit anti-human Ig (1:1000) (Sina Biotech) for 60 minutes at 37 °C, followed by washing three times with TBST and two times with TBS. Finally, the bands were developed by incubating the membranes for 1 minute in DAB solution (Sigma, USA). To establish a threshold for band positivity in Western blotting, we used negative control serum samples. The cut-off was defined as the mean band intensity of background signal from these negative controls plus three standard deviations (mean + 3SD), assuming a normal distribution of background noise. Given the lack of a commercially validated positive control, we selected a patient-derived positive control based on the following criteria: (i) confirmed diagnosis of Pompe disease, (ii) evidence of clinical resistance to enzyme replacement therapy, and (iii) consistent presence of a strong immunoreactive band against rhGAA on Western blot. Negative controls consisted of healthy individuals without a history of Pompe disease or exposure to recombinant enzyme therapy. All Western blot experiments were performed in triplicate. Densitometric analysis of the band intensities was conducted and results were expressed as mean ± standard deviation (SD). The coefficient of variation (%CV) was calculated for replicate runs, and values below 10% were considered acceptable for intra-assay variability. This method allowed for object ive differentiation between true positive and background signals. Representative blots are included in Supplementary Figure 1.

Assessing the Motor Functional State

To assess motor function in patients with Pompe disease, we utilized the Pompe-adapted Gross Motor Function Classification System (GMFCS) framework as proposed in the Advance study by van der Ploeg et al.[22] The questionnaire classified participants into five levels analogous to GMFCS levels but adapted for Pompe disease: Level I, walkers; Level II, supported walkers; Level III, supported standers (deemphasizing device-based ambulation relative to GMFCS level III and allowing crawling for most indoor mobility); Level IV, sitters (omitting GMFCS IV's optional ambulation items); and Level V, restricted antigravity movement. This classification, also referred to as the Pompe Motor Function Level Questionnaire, is increasingly used in clinical research and provides a functional stratification more suitable than the original GMFCS, which was designed for cerebral palsy.[22]

Statistical Analysis

The results were analyzed via SPSS software (version 23). The information is reported in two descriptive and analytical sections. For descriptive variables, the mean and standard deviation were used for quantitative variables, and the number and percentage were used for qualitative variables. For the analysis, scaling scores were compared between the two groups via the independent t test or the Mann–Whitney U test if needed. The comparison of qualitative parameters was performed via the chi-square test. ROC curve analysis was used to assess the value of total anti-rhGAA antibodies and their subtypes in discriminating patients with disease from healthy individuals. In all tests, the significance level was $p<0.05$. A post-hoc power analysis was conducted to evaluate the statistical power of the study to detect differences between patients and control groups. The analysis was based on an independent two-sample t-test, assuming a moderate effect size (Cohen's d=0.5), a significance level of α=0.05, and a sample size of 14 participants per group. The calculated power was approximately 24.7%, which is significantly below the conventional threshold of 80% required to confidently detect moderate effects. This suggests a high risk of Type II error and implies that the non-significant findings in the study, particularly regarding the correlation between anti-rhGAA antibody levels and GMFCS scores, may be due to insufficient sample size rather than the absence of a true association (Supplementary Table 1).

RESULTS

In total, 14 patients (10 male and 4 female) with Pompe disease and 14 sex- and age-matched healthy subjects (10 male and 4 female) were included in the study. All patients were treated with Myozyme at standard dosages and completed their treatment regimen. The average ages of the participants were 19.21 ± 9.61 years and 18.57 ± 9.21 years, respectively (p=0.585). Table 1 presents the values of total and IgG, IgA or IgM isotypes of antibodies in both the patient and healthy groups. In this regard, the levels of total anti-rhGAA antibodies and their subtypes were significantly greater in the patient groups than in the healthy groups.

According to the ROC curve analysis (Figure 1), assessing the level of all antibody isotypes could differentiate Pompe disease patients from healthy controls with different cut-off values. Overall, the sensitivity of antibody assessment for discriminating Pompe disease patients from healthy controls ranged from 71.4% to 92.9%, and the specificity ranged from 78.6% to 92.9% (Table 2).

To further visualize the distribution and magnitude of differences in anti-rhGAA antibody levels between patients and controls, a log-transformed box-and-whisker plot was generated. The log scale allowed better representation of the wide dynamic range observed among patients and reduced visual compression of lower OD values in controls. A horizontal dashed line representing the ELISA cut-off value (0.223 OD) was included for reference. This plot clearly illustrates the marked separation between groups, with minimal overlap near the diagnostic threshold (Supplementary Figure 2).

To examine the value of Western blot versus ELISA for assessing the anti-rhGAA antibody, we tested the correlation of the value of this antibody with two tools, which indicated strong agreement between the two tests (r coefficient of 0.765, p<0.001) (Figure 2).

With respect to the relationship between the functional grade (GMFCS) and the value of the anti-rhGAA antibody, we did not observe a significant change in the level of the anti-rhGAA antibody with increasing GMFCS grade (p=0.374) (Figure 3).

Table 1. The mean values of antibody isotypes in patients and healthy controls.

Antibody	Patients group	Healthy group	*p* value
Total AAG	1.48 ± 1.21	0.14 ± 0.04	p<0.001
IgG isotype	0.88 ± 0.74	0.10 ± 0.04	p=0.001
IgM isotype	0.10 ± 0.04	0.03 ± 0.01	p<0.001
IgA isotype	0.05 ± 0.03	0.02 ± 0.01	p=0.001

AAG: anti-acid α-glucosidase

Table 2. The area under the ROC curve analysis results.

Antibody	AUC (95%CI)	Cut-off point	Sensitivity	Specificity
Total AAG	0.974 (0.920 – 1.029)	0.20	92.9%	92.9%
IgG isotype	0.980 (0.940 – 1.020)	0.16	85.7%	92.9%
IgM isotype	0.959 (0.894 – 1.024)	0.05	85.7%	92.9%
IgA isotype	0.865 (0.732 – 0.997)	0.02	71.4%	78.6%

AAG: anti-acid α-glucosidase; AUC: area under the curve; CI: confidence interval; ROC: receiver operating characteristic

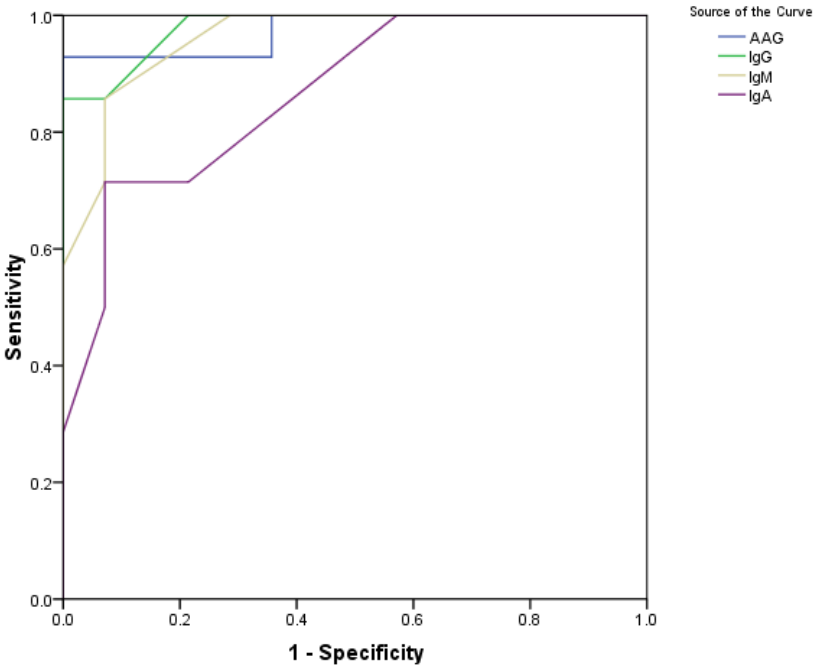

Figure 1. ROC curve analysis for discriminating Pompe disease patients from healthy controls.

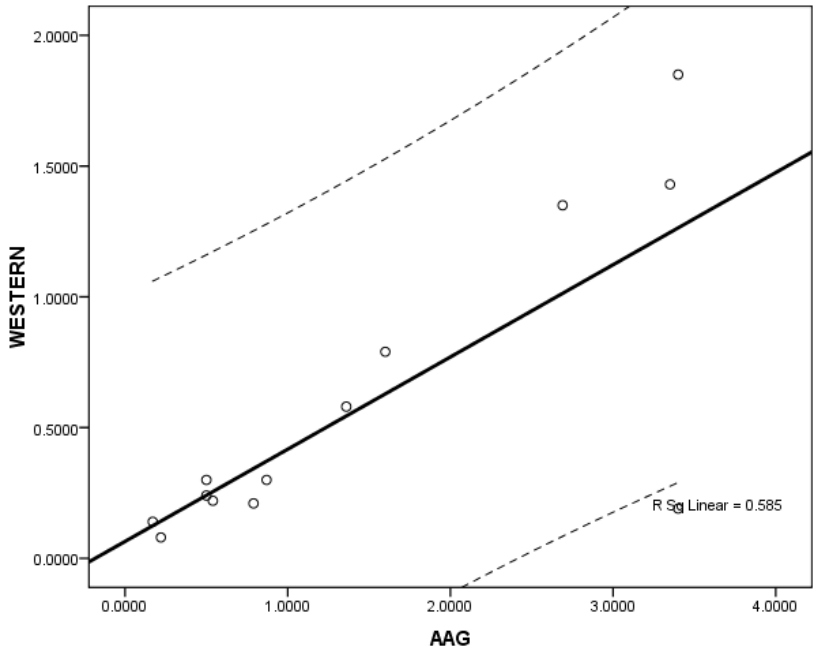

Figure 2. Association of Anti-rhGAA(Anti-recombinant human AlGlucosidase Alpha) antibodies assessed by ELISA (EnzymeLinked Immunosorbent Assay) and western blot techniques

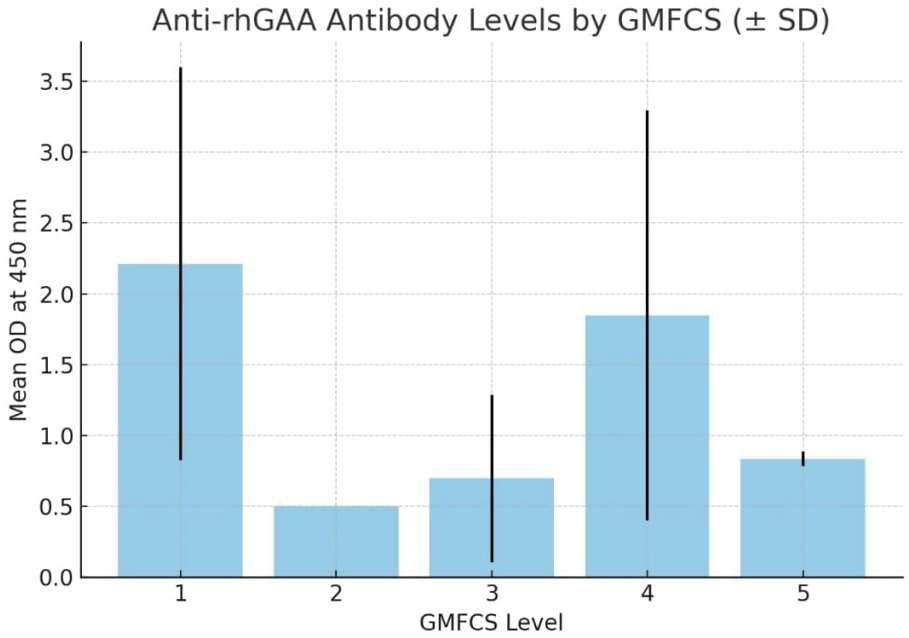

Figure 3. Mean anti-rhGAA antibody levels (OD at 450 nm) across GMFCS (Gross Motor Function Classification State) levels in 14 patients with late-onset Pompe disease. Error bars represent ± standard deviation. Higher antibody levels were observed in several patients with both lower and higher functional status, though no statistically significant trend was observed due to limited sample size.

DISCUSSION

The present study pursued three main objectives. First, can the detection of anti-rhGAA antibodies and their isotypes via ELISA distinguish between Pompe disease patients and healthy individuals? As a second hypothesis, is Western blot like ELISA capable of detecting anti-rhGAA antibody is such patients? Third, can monitoring antibody levels help predict clinical functional status, disease severity, and outcomes? In response to the first question, we found that the anti-rhGAA antibody andtheir IgG, IgM, and even IgA isotypes have a high capacity to distinguish patients from healthy individuals. Indeed, the presence of these antibodies has been fully accounted for in the pathophysiology of this disease. According to previous studies, the development of immunological responses through the production of anti-rhGAA antibodies constitutes the pathophysiological basis of Pompe disease. In this context, different mechanisms have been developed to generate anti-rhGAA antibodies in Pompe disease. First, the infused enzyme in ERT (recombinant human acid α-glucosidase or rhGAA) is recognized by the immune system as a foreign protein, especially in patients who produce little or no endogenous GAA due to severe mutations. This triggers an immune response, leading to the production of anti-rhGAA antibodies.[22] Additionally, the development of anti-rhGAA antibodies involves the activation of B cells and helper T cells. B cells produce antibodies, while helper T cells support this process and enhance the immune response. In some cases, anti-rhGAA antibodies can also involve the formation of memory B cells, which can lead to a persistent antibody response.[23] In this context, the impact of the generation of anti-rhGAA antibodies on disease pathophysiology can be described. First, these antibodies bind to the enzyme in such a way that it inhibits its enzymatic activity, directly reducing the efficacy of ERT. Second, these antibodies bind to the enzyme without directly inhibiting its function but may still contribute to faster clearance of the enzyme from the bloodstream, reducing its availability. The findings of the present study emphasized that the early detection of this antibody can be beneficial in both the early detection of patients and the rate of response to standard treatment.

Second, the Western blot technique may be as useful and sensitive as ELISA for detecting GAA antibodies to detect Pompe disease. In this study, Western blotting was used for the detection of GAA antibodies for the

first time in Iran. We detected high agreement values between the levels detected by ELISA, the gold standard tool, and those detected by Western blotting. As previously described, Western blotting is a technique commonly used to detect and quantify specific proteins in a sample, and it can also be applied to the detection of anti-rhGAA antibodies in Pompe disease. When investigating anti-rhGAA antibodies, Western blotting can help identify the presence and specificity of antibodies that a patient has developed against the therapeutic enzyme used in enzyme replacement therapy (ERT).[24] Western blotting can confirm the presence of antibodies against the recombinant human acid α-glucosidase (rhGAA) enzyme by detecting the binding of these antibodies to the enzyme.[25] In addition, this technique allows the identification of which specific regions (epitopes) within the rhGAA enzyme that are recognized by the patient's antibodies.[25] In total, western blotting can confirm the presence of anti-rhGAA antibodies detected by other methods, such as ELISA. It also helps in understanding the immunogenicity of the therapeutic enzyme and the specificity of the immune response. In addition, the regular use of Western blotting can help monitor the development of and changes in anti-rhGAA antibodies levels over time, informing adjustments in treatment protocols. However, this technique is more labor intensive and time consuming than other methods, such as ELISA. Moreover, while specific, it may not be as sensitive as some other quantitative methods.

As a second hypothesis, we showed that tracking the level of GAA antibodies may not be applicable for the assessment of patients' functional status using the GMFCS grading system. It is important to note that, with a sample size of only 14 patients, post-hoc power calculation indicates less than 50% power to detect moderate correlations (r=0.5). Therefore, the lack of significant association may reflect type-II error rather than a true absence of effect. Further studies should incorporate additional validated outcome measures, such as the 6-minute walk test (6MWT) or pulmonary function (FVC % predicted), to enhance clinical correlation. In a systematic review by Ditters et al[26] which assessed 17 studies with the goal of examining the value of anti-rhGAA antibodies in predicting treatment outcome, no clear effect of anti-rhGAA IgG antibodies on treatment response was shown for the majority of LOPD patients with a high antibody titres. Although total IgG levels were measured and shown to dominate the humoral response in our cohort, IgG subclass differentiation (e.g., IgG1 vs. IgG4) was not performed. Given the role of specific IgG subclasses in modulating immune responses, especially in the context of enzyme replacement therapy, future studies should consider subclass profiling to better assess potential neutralizing effects, as suggested by Ditters et al.[26] Similarly, van Kooten in 2022[27] reported 91% of childhood-onset Pompe patients developed anti-rhGAA, a minority of whom had high antibody titres at repeated time points, which does not seem to interfere with the clinical outcome. Therefore, the detection of this antibody can be effective in monitoring clinical conditions, especially the performance of patients, and requires more clinical evaluations, especially with a variety of methods to check the functional status of patients and in longer-term follow-ups.

Clinical Implications

Our findings suggest that anti-rhGAA antibody measurement could support diagnostic confirmation in suspected LOPD cases and may be considered for treatment monitoring. In clinical settings with limited access to commercial ELISA kits, Western blot offers a cost-effective alternative. An acceptable trade-off between sensitivity and specificity (≥ 85%) could guide immunomodulation strategies. Routine anti-rhGAA antibodies screening might be integrated annually or at predefined ERT milestones, such as 6- and 12-months post initiation. Iranian guidelines for Pompe disease do not currently include anti-rhGAA antibodies screening, but incorporating such markers could refine clinical management.

One important limitation of our study is the inability to assess the cross-reactive immunologic material (CRIM) status of patients. Currently, CRIM testing is not available in our country, which precluded direct classification of patients as CRIM-positive or CRIM-negative. This is a relevant limitation, as CRIM-negative patients are known to be at higher risk for developing high sustained anti-rhGAA antibody titres and reduced treatment efficacy. Although CRIM status can sometimes be inferred through detailed genetic analysis of *GAA* mutations,[28] such genotype-phenotype correlations were not explored in our cohort. We recommend that future studies incorporate genetic sequencing and international collaboration to determine CRIM status, which may help in better stratifying immune responses and tailoring immunomodulatory interventions in Pompe disease management.

It can be concluded that first tracking GAA antibody and its isotypes is very useful for the early detection of Pompe disease and for distinguishing affected individuals from healthy controls. For the detection of such antibodies, Western blot technique is just as effective and useful as an ELISA. However, many questions remain about whether detection of these antibodies can predict functional improvement after treatment. In our study, we found no clear effect of the anti-Myozyme antibody level on the treatment response in the patients. On the basis of our findings, administration of exogenous enzymes can induce the expression of antibodies in most cases; however, the circulating antibody level may not be strongly related to the level of enzyme activity inhibition. A reasonable assumption is that an exogenous protein presents multiple epitopes to the immune system, only a few of which are related to the active site of the protein. Given the small sample size, our findings should be interpreted with caution. Further studies with larger and more diverse cohorts are warranted to validate and expand upon these results.

STATEMENT OF ETHICS

This study was approved by the medical ethical committee of Tehran University of Medical Sciences. [Ethics Number; IR.TUMS.VCR.REC.1399.079]

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

The datasets generated and analyzed during the current study are included in the article's Supplementary Materials. These materials contain anonymized raw ELISA absorbance values, representative Western blot images, and additional methodological details supporting the findings of this study. Further inquiries can be directed to the corresponding author upon reasonable request.

AI ASSISTANCE DISCLOSURE

Not applicable.

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