Transglutaminase 6 autoantibodies in neurological gluten-related affection suspected patients: validation as a diagnostic biomarker

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FINAL DEGREE PROJECT

Bachelor's degree in Biochemistry and Molecular Biology

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Tarragona, June 2022

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SUMMARY

IgA autoantibodies against transglutaminase 6 (TG6-Ab) have been suggested as a diagnostic biomarker for neurological dysfunctions related to gluten sensitivity like gluten ataxia. The importance of gluten sensitivity diagnosis behind neurological disorders relies on a gluten-free diet treatment shown to improve the symptoms. The present study aims to determine TG6-Ab levels in neurological affection-suspected patients and coeliac disease (CD) patients, to validate the test for its use in clinical practice. Methodology includes an Enzyme-linked Immunosorbent Assay. Results recapitulate the prevalence of TG6-Ab in CD and neurological patients, although resulted higher in healthy controls. TG6-Ab follow-up determinations seem to be a helpful diagnostic marker for neurological gluten-related dysfunctions together with previous clinical evaluation and classical gluten sensitivity tests, especially in patients with negative results for commonly used markers of gluten sensitivity and without enteropathy.

Key words: transglutaminase 6, gluten sensitivity, biomarker, neurological disorders, autoantibodies.

LIST OF ABBREVIATIONS

AGA	Anti-gliadin antibodies	HLA	Human leukocyte antigen
CD	Coeliac disease	RSD	Relative standard deviation
CNS	Central nervous system	RV	Reference values
ELISA	Enzyme-linked Immunosorbent Assay	TBS	Tris-buffered saline
EMA	Anti-endomysial antibodies	TMB	3, 3', 5, 5'-tetramethylbenzidine
GA	Gluten ataxia	tTG-Ab	Tissue transglutaminase antibodies
GFD	Gluten-free diet	TG6	Transglutaminase 6
HC	Healthy control	TG6-Ab	Transglutaminase 6 antibodies

1. INTRODUCTION

1.1. DEFINITIONS

Gluten is a mixture of wheat structural proteins. Its toxic fraction includes gliadins and glutenins (Sapone et al., 2012). Coeliac Disease (CD) is “an immune-mediated systemic disorder triggered by dietary gluten intake and other related prolamines in genetically susceptible individuals”. Features characterizing CD are the presence of a combination of clinical manifestations, a specific serum antibodies response, Human Leukocyte Antigen (HLA)-DQ2/DQ8 haplotypes and enteropathy (Román et al., 2020; Husby et al., 2012). Physiological sensitivity to gluten is known to exist in other forms. For this reason, the umbrella “gluten-related disorders” is frequently used. It includes other immune-mediated disorders, wheat allergy and non-coeliac gluten sensitivity (Leffler et al., 2015).

Gluten ataxia (GA) is defined as apparently sporadic ataxia with positive serological markers for gluten sensitivity. Gluten neuropathy is defined as apparently sporadic idiopathic neuropathy in the absence of an alternative etiology and in the presence of serological evidence of gluten sensitivity. They are the two most common neurological manifestations of gluten sensitivity (Hadjivassiliou et al., 2010).

1.2. EPIDEMIOLOGY

CD prevalence in Catalonia was seen to be 1:204, and five times higher in children (1:71) than in adults (1:357) (Mariné et al., 2011). The global seroprevalence of CD is estimated to be 1.4 %. It is sex, age and geographic location-dependent, varying due to genetic, environmental and wheat-consumption factors (Singh et al., 2018).

There is a lack of epidemiological studies considering the spectrum of gluten sensitivity. Overall, the global prevalence of gluten-related disorders is reckoned to be approximately 5 % (Taraghikhah et al., 2020). Some extraintestinal manifestations of gluten sensitivity are common in both adults and children, like musculoskeletal manifestations, which prevalence varies among studies in a

range of 10-50 % (Leffler et al., 2015). Paedriatric extraintestinal manifestations are related to growth disorders, like short stature and delayed puberty. Some extraintestinal manifestations are less common in childhood, like anaemia, present in 15 % of adults with CD, skin manifestations like dermatitis herpetiformis, and neurological manifestations (Durazzo et al., 2022).

The prevalence of neurological manifestations of gluten sensitivity has not been estimated accurately. Particularly, GA is observed in up to 6 % of the patients with gluten sensitivity. The onset of GA occurs at a mean age of 48 years, usually following a rapid progression of the disease (Patel et al., 2021). Among all ataxias, GA has a prevalence of 15 %, and 40 % among idiopathic sporadic ataxias (Hadjivassiliou et al., 2015).

1.3. CLINICAL MANIFESTATIONS AND CLASSIFICATION

Extraintestinal manifestations of gluten sensitivity encompass a wide spectrum of symptoms. The first comprehensive report of neurological manifestations in CD was published in 1966. It was then assumed that vitamin deficiencies secondary to malabsorption caused these presentations. In 1996, Hadjivassiliou et al. studied the prevalence of gluten sensitivity in patients with neurological dysfunction. The results showed a 16-times higher prevalence of CD in these patients than in the healthy population. Hence, neurologists became interested in the possible link between gluten sensitivity and particular neurological manifestations (Hadjivassiliou et al., 2010).

GA and gluten neuropathy are the two most common gluten-sensitivity related neurological presentations. Further rare neurological manifestations include epilepsy, autism spectrum disorder, schizophrenia, myopathy, myelopathy, multiple sclerosis, stiff-man syndrome and myoclonic ataxia (Hadjivassiliou et al., 2010), besides other neuropsychiatric associations like anxiety, depression and attention-deficit or hyperactivity disorders (Leffler et al., 2015).

1.4. ETIOLOGY

Transglutaminases (2.3.2.13) are a aminoaciltransferase family that catalyze the formation of a isopeptide bond between a γ-carboxamide group of peptide-bond glutamine residues, which acts as acyl donor, and a primary amine of peptide-bond lysine residues, which acts as acyl acceptor. Transglutaminase activity leads to the formation of intra- and inter-molecular N6-(5-glutamyl)-lysine crosslinks (Hadjivassiliou et al., 2010).

The most well characterized family member is tissue transglutaminase (tTG or TG2). It is known to be the autoantigen of CD. Epidermal transglutaminase (TG3) is the autoantigen of dermatitis herpetiformis, and mainly expressed in the skin. Neuronal transglutaminase or transglutaminase 6 (TG6) is the autoantigen typically found in patients with neurological manifestations, and preferentially expressed in the Central Nervous System (CNS). These isozyms share structural similarities, enzymatic activity and 65 % homology as they are phylogenetically close related (Grenard et al., 2001; Hadjivassiliou et al., 2021b).

The primary mechanism for neurological dysfunction due to gluten is thought to be triggered by antibodies against TG6 (TG6-Ab). Although TG6 is not expressed in small intestine cells, it can be expressed in mucosal antigen-presenting cells, this is, macrophages or dendritic cells in lamina propria (Hadjivassiliou et al., 2008). This could explain the gut-located onset of gluten-dependant neurological diseases, regardless of the mainly brain-located expression of TG6.

Transglutaminases' role in disease development has been mainly studied on tTG. It selectively modifies gliadin peptides by deamidation. This fact increases their affinity for HLA-DQ2/DQ8, potenciating T-cell response. Besides, crosslinking with gliadins and consequently TG-gliadin peptide complex formation leads to haptenisation of self-antigens, increasing immunogenicity of gluten peptides and having an effect on autoantibodies development (Hadjivassiliou et al., 2010). The autoantigen is recognized and internalized by the B cell together with the TG-specific autoantibody. TG-specific B-cells present to T cells not only

transglutaminase-derived peptides themselves, but also TG-deamidated gliadin complex through the HLA molecules, on which relies genetic susceptibility to gluten sensitivity. The most important genetic risk factors are the HLA-DQ2 –most frequent haplotypes being DQ2.5 and DQ2.2– and DQ8, and are carried by ~30% of the population. Other environmental and genetic factors are suggested to be associated with the disease predisposition (Bourgey et al., 2007). CD4+ T cells recognise the deamidated gliadin peptides and activate TG-specific B cells for its proliferation and autoantibody production.

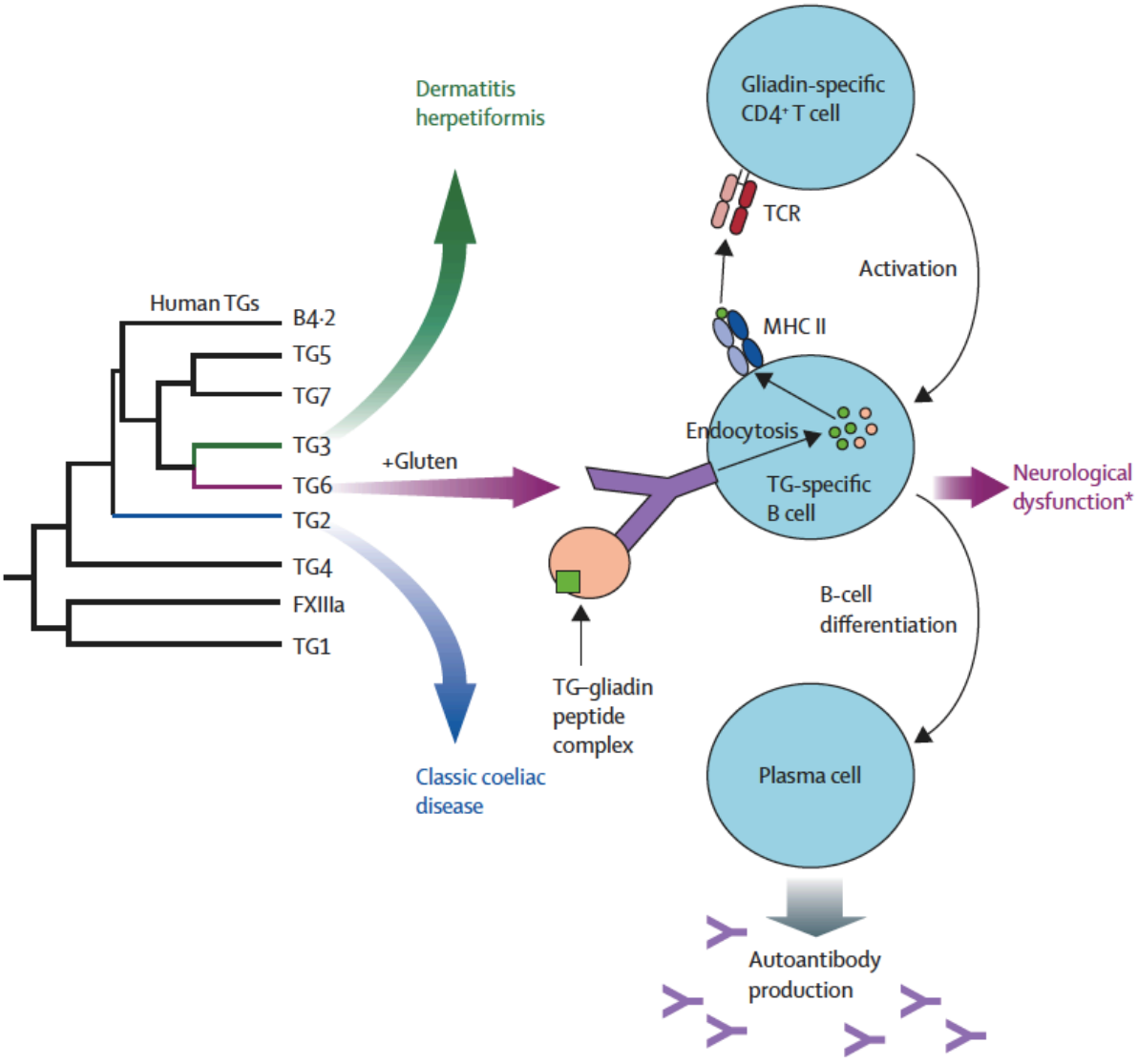

Figure 1. The role of transglutaminases in humoral response, linked to different manifestations of gluten sensitivity. From Hadjivassiliou et al. (2010).

Neurological damage can be explained through vasculature-centered inflammation. Events taking place are the deposition of IgA TG6-Ab in blood vessels around the brain and pathological perivascular cuffing of inflammatory cells. IgA deposits could either derive from circulation and accumulate in the periphery of vessels due to vascular leaking, or from production of stimulated gliadin-reactive CD4+ T cells. Regardless, these findings might compromise the blood-brain barrier permeability and expose the CNS to autoantibodies (Hadjivassiliou et al., 2010).

Neuropathology of GA involves ultimately the loss of Purkinje cells with Bergmann gliosis. This means that the Purkinje layer of the cerebellum, containing Purkinje cells and Bergmann glia, is affected (Hadjivassiliou et al., 2010). Clark et al. (2022) summarize that “when inflammatory changes are absent, it is difficult to separate GA cases from other hereditary or sporadic ataxias by neuropathological findings alone”.

1.5. DIAGNOSIS, TREATMENT AND PROGNOSIS

CD is an underdiagnosed condition with a diagnosis at a mean age of 45 years (Durazzo et al., 2022). Amongst the most accepted diagnostic criteria for CD are those proposed by Catassi et al. (2010). At least 4 out of 5 criteria should be satisfied: typical symptoms of CD, positive for serum CD IgA class autoantibodies at high titer, HLA-DQ2 and/or DQ8 genotype determination, enteropathy compatible with CD at small bowel biopsy, and response to gluten-free diet (GFD).

Neurological dysfunction has been apparent in two-thirds of gluten-sensitive patients at the time of diagnosis (Durazzo et al., 2022). GA diagnosis starts with the identification of typical neurological dysfunction. The Scale for the Assessment and Rating of Ataxia is frequently used by neurologists as a tool to semiquantitatively evaluate cerebellar ataxia by impairment of motor and coordination items (Schmitz-Hübsch., 2010). Genetic testing is used to discard hereditary ataxia. Moreover, an imaging technique that can help diagnosis is Magnetic Resonance Spectroscopy of the cerebellum. The N-acetyl

aspartate/creatine or N-acetyl aspartate/choline ratio is measured. These ratios reflect the metabolic and functional state of neurons and therefore are an indicator of neuronal viability (Patel et al., 2021). Magnetic Resonance Imaging showed cerebellar atrophy in up to 60 % of patients with GA, supporting cerebellar neuronal physiology differences between healthy and GA patients (Wilkinson et al., 2005).

Once apparently sporadic ataxia is diagnosed, Hadjivassiliou et al. (2010) proposed a diagnosis flow chart of neurological dysfunction associated with gluten sensitivity. Patients presenting neurological manifestations of gluten sensitivity mostly do not suffer from enteropathy. Neither do some patients with CD. Thus, clinical cannot be the only basis for the diagnosis of gluten sensitivity. Diagnostic tests may help reach an accurate diagnosis. It is noteworthy that patients should be on a normal diet. Gluten ingestion is required for initial serology in order to detect anti-TG2 IgA and IgG antibodies (tTG-Ab), antigliadin antibodies and anti-DGP antibodies. If positive, enteropathy might be assessed by duodenal biopsy, despite it is not a precondition for gluten sensitivity.

Further serological tests to help make an accurate diagnosis are TG6-Ab. Patients with neurological manifestations and without enteropathy can lack serum tTG-Ab, yet have TG6-Ab. Genetic testing for HLA DQ2 or DQ8 variants is also useful in uncertain cases, as a negative result for those variants regularly is enough to exclude gluten sensitivity diagnosis. If gluten sensitivity is confirmed, final diagnosis may be GA (Hadjivassiliou et al., 2010).

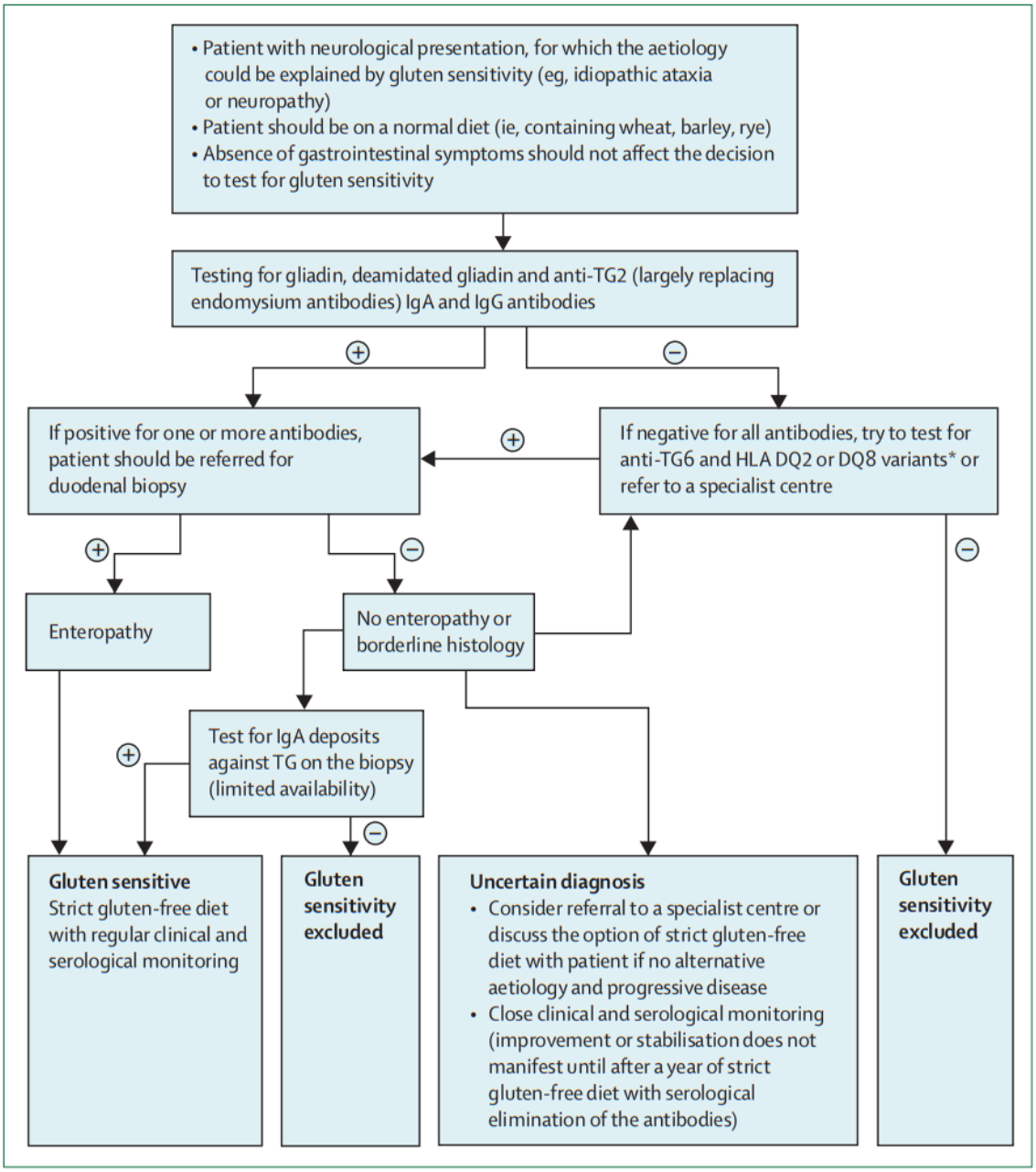

Figure 2. Flow chart of recommended approach to confirm or exclude a diagnosis of neurological dysfunction associated with gluten sensitivity. From Hadjivassiliou et al. (2010).

Results from several studies show the presence of TG6-Ab in sera from patients with GA. A study conducted by Hadjivassiliou et al. showed positive results of TG6 IgA in 40 % of patients with GA and enteropathy, and in 47 % of patients with GA without enteropathy (Hadjivassiliou et al., 2008). Another study concluded that the prevalence of TG6-Ab was 73 % in patients with GA, 32 % in patients with CD, and 4 % in healthy controls (Hadjivassiliou et al., 2013).

Of note, CD patients can also test positive for IgA TG6-Ab. Hadjivassiliou et al. estimated that a 45 % of CD patients are positive for TG6-Ab as well as for tTG-Ab (Hadjivassiliou et al., 2008). However, these observations can variate due to geographical differences. The prevalence of TG6-Ab in patients newly diagnosed with CD was 32 % in the UK cohort and 14 % in the Finnish cohort. Whether those patients are prone to develop neurological disorders after prolonged gluten intake is unclear (Hadjivassiliou et al., 2013).

The main treatment for GA is GFD. After strict GFD exposure, studies demonstrated an increase in N-acetyl aspartate/creatine area ratios in most of the patients. This fact denotes the effectiveness of the treatment even without enteropathy (Patel et al., 2021). A prospective cohort study showed that TG6-Ab titers are significantly reduced after one year of GFD (Hadjivassiliou et al., 2013). It has also been proposed a treatment based on intravenous immunoglobulins for GFD-resistant GA patients (Bürk et al., 2001) or along with the GFD start (Newrick et al., 2021).

2. AIM

The present study involves the determination of serum IgA Transglutaminase 6 autoantibodies in coeliac disease patients and neurological affection suspected patients. The latter could develop neurological gluten-related disorders. Together with the medical history, Enzyme-linked Immunosorbent Assay (ELISA) of TG6-Ab could have clinical relevance in certain diagnoses as GA. The aim of the study is the determination of IgA TG6-Ab titers as a validation to apply the technique in clinical practice.

3. HYPOTHESIS

IgA transglutaminase 6 antibodies are specific for neurological gluten-related disorders like GA and have clinical relevance to complement other serological markers for gluten sensitivity.

4. MATERIALS AND METHODS

4.1. STUDY DESIGN AND SAMPLE COLLECTION

Patients selection was based on blood tests at Reus' Sant Joan Hospital during the months of June and July 2021, analysed at Laboratori de Referència Camp de Tarragona i Terres de l'Ebre. Subjects were divided into two group: CD and neurological affection suspected. A healthy control (HC) group was also included in the study.

CD patients (n=20) were positive for tTG-Ab (>10 U/mL). Neurological affection suspected patients (n=10) were driven by neurologists who asked for a tTG-Ab determination. Patients included in the HC group (n=10) were negative for tTG antibodies, derived from Primary Health Care or Paediatrics Service. Exclusion criteria for healthy controls were: patients driven by gastroenterologists, neurologists or autoimmune disease physicians, or patients with gastrointestinal or neurological symptoms.

Venous blood was collected from each subject into the gel collection tubes. Phlebotomists performed the venipuncture in accordance with the established Standard Operating Procedure using BD Vacutainer® Safety LokTM winged collection set with 21G needle. All collection tubes were centrifuged at 1500 g for 10 min at 18-25ºC. Serum samples from all participants were stored at 3-8 ºC for several days. Then, samples were stored at -80ºC until the time of the study.

4.2. MATERIALS AND METHODS

Materials used within this project are listed in Suppl. Table 1 and Suppl. Table 2.

The reagent used in this work is the Kit TG-6-ab ELISA (IgA) from Zedira (Ref: E103). The kit contains one microtitre plate (96 wells pre-coated with TG6 antigen), sample buffer (Tris-buffered saline, bovine serum albumin, Tween and

Na-azide), wash buffer 10x-concentrate (Tris-buffered saline, Tween and bromonitrodioxane), standard calibrators, negative and positive controls, substrate solution (3, 3', 5, 5'-tetramethylbenzidine (TMB) and hydrogen peroxide), and STOP solution (0.2 M sulfuric acid). The kit can be stored at 2 - 8ºC. Before usage it must have reached room temperature.

Before each assay, samples are diluted 1/100 by mixing 10 µL sera and 990 µL sample buffer.

4.2.1. Assay principle

The test is based on three reactions. In the first reaction, IgA TG6-Ab from the sample bind specifically the immobilised antigen. The antigen-antibody complex is formed. In the second reaction, a secondary anti-human IgA HRP-conjugated antibody binds to the complex. It will bind the primary antibody proportionally. In the third reaction, the enzyme-labelled complex converts the TMB substrate into a blue product. TG6-specific antibodies present in samples can be colorimetrically quantified. Instead, samples without TG6-Ab remain colourless.

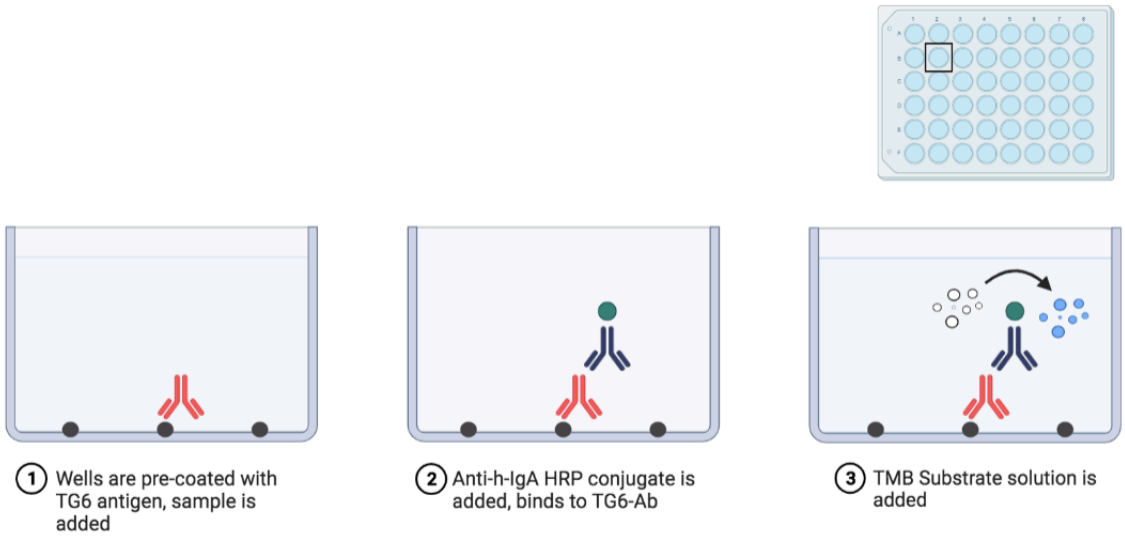

Figure 3. Assay principle.

4.2.2. Protocol configuration

As an automatized procedure, the protocol is set once and saved. Every assay is performed by opening the protocol file. The protocol setting suits the following parameters. Absorbance is measured at 405 minus 620 nm. Plate layout is set by choosing the blank, standard samples, positive control, negative control positions and unknown sample. Six standard sample concentration (U IgA TG6-Ab/mL) are set. According to the kit's calibrators, the standard input concentrations are specified in Table 1. Duplicate measurements were done.

Table 1. Standard samples concentration (U/mL).

#SD	Concentration (U/mL)
01	0.000
02	3.000
03	8.000
04	18.000
05	45.000
06	100.000

Shaking parameters are set as normal speed, 10 seconds shaking, before each measurement and without pause. Before each measurement, the plate will be shaken 10 seconds.

Calculation parameters are related to the quantitative analysis. Parameters are set as non-kinetic. Absorabce preprocess method is M1-M2, this is, 405-620 nm. Curve fitting follows the CubicSpline model. However, in this project the quantitative analysis of samples was made using a four parameter logistic curve.

Interpretation parameters are related to the qualitative analysis. Source is defined as Abs, con. Formula for threshold value "cutoff" is "*a x NC + b x PC + c"*, where "a", "b" and "c" are reagent control coficients. For this specific determination the absorbance borderline is determined as the absorbance of the positive control multiplyed for the "b" factor, which value is defined in each kit lot. "a" and "c" factors are set as zero.

4.2.3. Assay procedure

Firstly, wash buffer 10x-concentrate is diluted 10-fold with 900 mL deionised water and mixed thoroughly. The solid phase is washed once with 350 µL diluted wash buffer. An automated washer (Microplate washer, NeoBiotech) is used for that purpose. Wells are soaked for 10 seconds before the washing solution is removed. Washing steps must be done carefully to obtain the maximum signal-to-noise ratio. Next, 100 µL of calibrators, positive and negative controls, and diluted samples are dispensed into the microwells following the plate layout configuration. The plate is incubated for 30 minutes at room temperature. Then, wells are washed 4 times the same way as before. The anti-human-IgA conjugate is added, 100 µL per well, and the plate is incubated as before. Another four-times washing step is conducted. Afterwards, 100 µL per well of the substrate solution are rapidly added with an 8-channel pipette. After another incubation step, 100 µL per well of the STOP solution are added. Directly afterwards, the plate is shaken briafly and absorbance at 450 minus 620 nm is measured.

4.2.4. Data analysis

The qualitative analysis is related to the positivity of the samples for IgA TG6-Ab. The absorbance of the samples is compared with the borderline absorbance or cutoff. The borderline absorbance is determined by multiplying the absorbance of the positive control with the factor from the lot-specific certificate of analysis. In this case, the borderline factor was 0.17.

$$Abs_{borderline} = Abs_{positive\ control} * factor$$

Equation 1. Borderline absorbance calculation.

The ratio of the samples can be calculated in order to gain insight into how positive a given sample is. The ratio is calculated by dividing the mean absorbance of the sample by the borderline absorbance.

$$Ratio = \frac{Abs_{sample}}{Abs_{borderline}}$$

Equation 2. Positivity ratio calculation.

The ratio for the borderline absorbance is 1.0. Following the reagents manual, ratios between 0.89 and 1.14 are included in the equivocal range, and a positive result cannot be given. A ratio over 1.14 can be interpreted as a positive result for IgA TG6-Ab.

The quantitative analysis corcerns the serum concentration of IgA TG6-Ab of the samples. Quantitative evaluation was conducted using a Four-Parameter Logistic standard curve. Following the reagents manual, concentration values of TG6-Ab over 4.0 U/mL are considered a positive result. In contrast, concentration values under 2.6 U/mL are considered a negative result.

4.2.5. Statistical analysis

Statistical calculations and graph representations were made using the Statistical Package for Social Sciences (SPSS 24.0, Chicago, IL, USA) and GraphPad Prism 9.3.1 (GraphPad Software, San Diego, CA, USA). Quantitative data were shown as medians and interquartile range, and assessed by the Kruskal-Wallis test. Qualitative comparisons in order to estimate whether exists relation between a positive TG6-Ab test and GFD adherence were made using the Chi-squared test. Statistical significance was set at $p < .05$. The prevalence of positive TG6-Ab for each group was calculated following Equation 3.

$$Prevalence = \frac{Number\ of\ patients\ positive\ for\ IgA\ TG6\text{-}Ab}{Total\ number\ of\ patients} * 100$$

Equation 3. Prevalence of positive TG6-Ab.

5. RESULTS

Table 2 shows clinical characteristics of patients from the CD group. Specific information for each patient can be found in Suppl. Table 1.

Table 2. CD group.

Index	Age	Sex	tTG-Ab (U/mL)	On GFD
1	6	F	20	No
2	2	F	19	No
3	3	F	14	Yes
4	7	F	13	Yes
5	24	F	11	Yes
6	8	F	11	Yes
7	15	M	11	Yes
8	1	F	21	No
9	14	F	11	No
10	32	F	18	Yes
11	12	F	30	Yes
12	7	F	52	No info
13	53	F	123	Yes
14	31	F	30	No
15	12	F	>128	No
16*	6	F	2	No
17	7	F	30	Yes
18	8	M	63	Yes
19	14	F	34	Yes
20	12	F	30	Yes

M: Male. F: Female. *patient #16 presented a negative tTG test when this study was conducted. However, four years before was diagnosed with potential coeliac disease due to positive tTG, positive EMA and carrying HLA variant DQ2. Moreover, patient 16 anti-TG6 levels were interesting to determine owing to its clinical presentation including epilepsy and motor disorders. For this reason, patient 16 was included in the CD group.

Table 3 shows clinical characteristics of patients from the neurological group.

Table 3. Neurological group.

Index	Age	Sex	tTG-Ab (U/mL)	Clinical presentation
21	8	F	<0.1	Nonspecific headache
22	3	M	<0.1	Unsteadily walk, Global Developmental Delay
23	4	M	1.9	S. Down
24	10	M	<0.1	S. Down
25	11	M	<0.1	Nonspecific Tension-type headache
26	7	F	<0.1	Cephalea, migraine
27	12	F	<0.1	Cephalea
28	11	M	<0.1	Cephalea, migraine, learning disorder, gait incoordination
29	2	F	<0.1	S. Down
30	7	F	<0.1	S. Down

M: Male. F: Female.

Table 4 shows clinical characteristics of patients from the HC group.

Table 4. HC group.

Index	Age	Sex	tTG-Ab (U/mL)
31	17	M	<0.1
32	40	M	<0.1
33	21	M	<0.1
34	56	F	<0.1
35	9	M	<0.1
36	9	M	<0.1
37	9	F	<0.1
38	21	F	<0.1
39	2	F	<0.1
40	20	F	<0.1

M: Male. F: Female.

Duplicate measurements were done. Evaluation of the samples was performed using mean absorbances. Raw absorbances, standard deviation and coefficient of variation values of each sample are listed in Suppl. Table 4.

Figure 4 shows the Four-Parameter Logistic standard curve. Standard #2 with a TG6-Ab concentration of 3 U/mL was discarded to obtain a better curve fitting (R^2=0.9948).

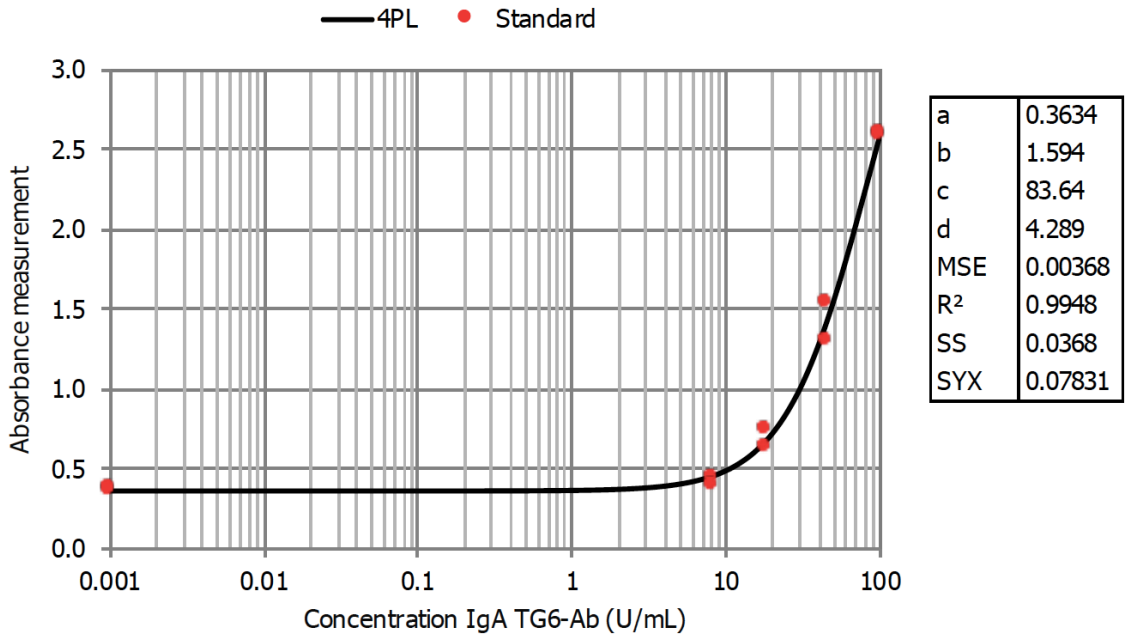

Figure 4. Four-Parameter Logistic curve.

Table 5 outlines the mean absorbances, concentration of TG6-Ab and positivity ratios of the samples.

Table 5. Mean Absorbance values, concentration values and positivity ratios of CD, neurological and HC groups samples, standard samples, positive control and negative control.

Sample	Index	Mean Absorbance	Qualitative evaluation (Ratio)	Quantitative evaluation (U TG6-Ab/mL)
CD group	1	0.235	1.35	<2,6
CD group	2	0.302	1.74	<2,6
CD group	3	0.182	1.05	<2,6
CD group	4	0.411	2.37	5.26

CD group	5	0.232	1.34	<2,6
CD group	6	0.312	1.80	<2,6
CD group	7	0.870	5.02	25.25
CD group	8	0.332	1.91	<2,6
CD group	9	0.456	2.63	8.09
CD group	10	0.337	1.95	<2,6
CD group	11	0.333	1.92	<2,6
CD group	12	0.410	2.37	5.22
CD group	13	0.668	3.85	17.68
CD group	14	0.512	2.96	10.99
CD group	15	0.481	2.77	9.42
CD group	16	0.319	1.84	<2,6
CD group	17	0.735	4.24	20.27
CD group	18	0.386	2.23	3.288
CD group	19	0.301	1.73	<2,6
CD group	20	0.344	1.99	<2,6
Neurological group	21	1.330	7.67	41.44
Neurological group	22	0.375	2.16	2.11
Neurological group	23	0.439	2.53	7.11
Neurological group	24	0.589	3.40	14.47
Neurological group	25	0.718	4.14	19.62
Neurological group	26	0.788	4.55	22.25
Neurological group	27	0.387	2.23	3.35
Neurological group	28	0.344	1.98	<2,6
Neurological group	29	0.394	2.27	4.00
Neurological group	30	0.497	2.87	10.25
HC group	31	0.412	2.38	5.33
HC group	32	0.422	2.44	6.04
HC group	33	0.386	2.23	3.26
HC group	34	0.440	2.54	7.17
HC group	35	0.426	2.46	6.27
HC group	36	0.315	1.82	<2,6

HC group	37	0.283	1.63	<2,6
HC group	38	0.264	1.52	<2,6
HC group	39	0.237	1.37	<2,6
HC group	40	0.635	3.67	16.38
Standard 1	SD1	0.377	-	0.00
Standard 2	SD2	0.422	-	3.00
Standard 3	SD3	0.427	-	8.00
Standard 4	SD4	0.694	-	18.00
Standard 5	SD5	1.424	-	45.00
Standard 6	SD6	2.605	-	100.00
Positive control	PC	0.112	5.88	30.52
Negative control	NC	1.019	0.64	<2,6

Concentration values from samples with absorbances outside the range of the standard samples could not be further determined and are listed as <2.6 U/mL. Concentration values over 4.0 U/mL, considered as a positive result, are highlighted in orange.

Neurological patients were classified in three groups according to their clinical presentation (Table 3): abnormal gait, cephalea and Down syndrome.

- Abnormal gait: patients #22 and #28. Both TG6-Ab results are negative.
- Cephalea or migraine: patients #21, #25, #26, #27 and #28. Three out of five TG6-Ab results are positive.
- Down syndrome: patients #23, #24, #29 and #30. Three out of four TG6-Ab results are positive.

Table 6 shows the prevalence of positive TG6-Ab for CD, neurological and HC groups.

Table 6. Prevalence of positive TG6-Ab for CD, neurological and HC group.

	Positive TG6-Ab
CD group	8/20 (40%)
Neurological group	6/10 (60%)
HC group	5/10 (50%)

To assess the results and compare them to clinical features, concentration values are used. For a comprehensive comparison of sample distribution within the three groups, TG6-Ab concentration values were represented in a violin plot (Figure 5). Medians and interquartile range for TG6-Ab concentrations were 2.60 (2.60-9.09) U/mL for the CD group, 8.68 (3.16-20.28) U/mL for the neurological group and 4.29 (2.6-6.49) U/mL for the HC group. The Kruskal-Wallis test was performed with p-value= .247. The difference between the groups is not significant.

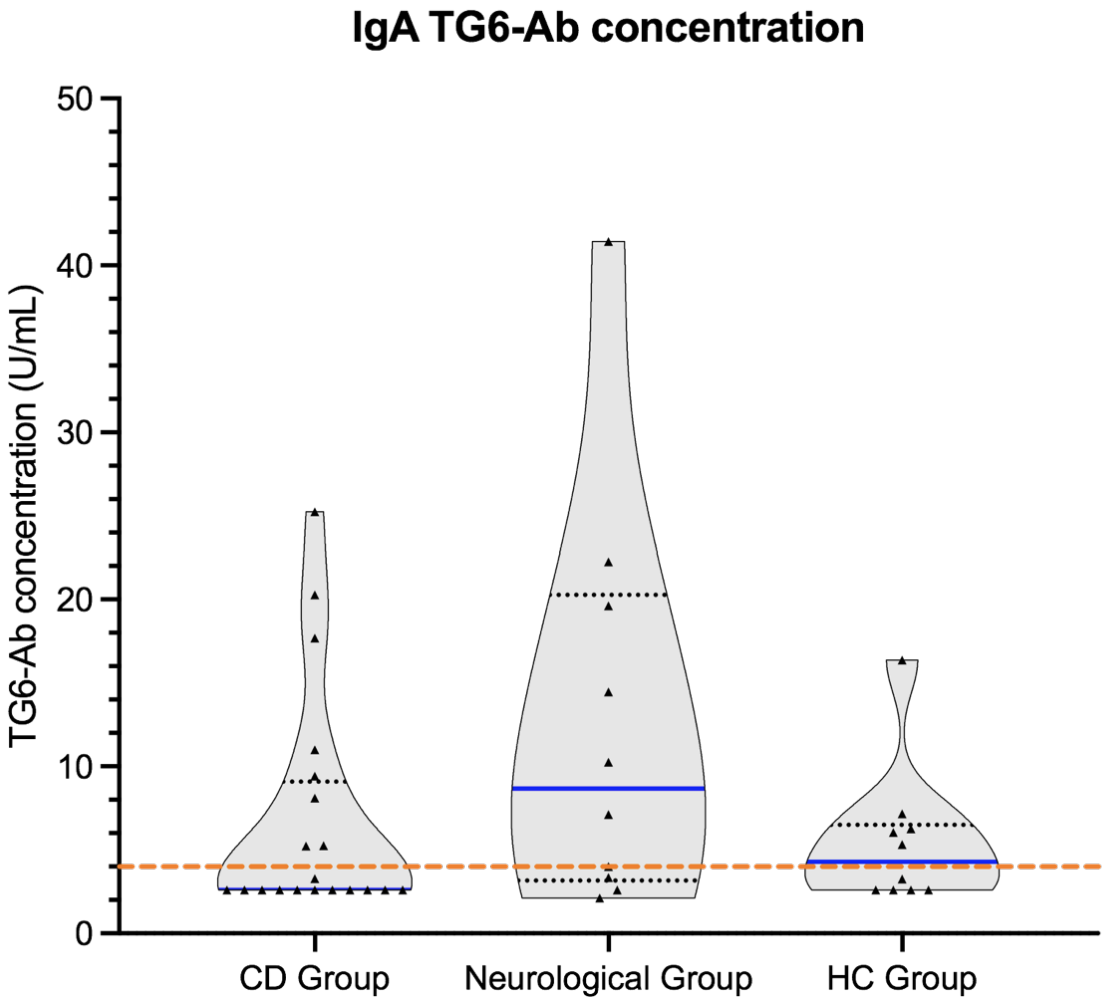

Figure 5. TG6-Ab concentration (U/mL serum) for CD, neurological and HC groups graphed as a violin plot. All values are shown as black triangles. The median of each group corresponds to the blue lines. Interquartile range is indicated between the black dotted lines. Threshold for a positive result is set as 4 U/mL and represented with an orange dotted line. Concentration values below 2.6 U/mL could not be determined by curve interpolation, thus they were represented as 2.6 U/mL. A truncated violin plot was chosen over an extended violin plot to reduce the violin to the minimum TG6-Ab concentration that could be obtained.

Cross-tabulation for TG6-Ab positivity in CD patients and attachment to GFD is shown in Table 7.

Table 7. Contingency table of CD group.

Attatched to GFD / TG6-Ab	No	Yes	Total
Negative	4 (57,14%)	8 (66,67%)	12 (63,16%)
Positive	3 (42,86%)	4 (33,33%)	7 (36,84%)
Total	7 (100%)	12 (100%)	19 (100%)

Patient #12 was discarded for the chi-squared test due to little clinical information about adherence to GFD.

X^2 (degress of freedom, N = sample size) = chi-square statistic value, p = p-value: X^2 (1, N=19) = 0.172, p = .678

As p>.05, alternative hypothesis is rejected. There is no significant association between adherence to GFD and a positive test for TG6-Ab.

6. DISCUSSION

GA is one of the most common neurological manifestations of gluten sensitivity. Previous studies have found that TG6-Ab are a gluten-dependent sensitive and specific marker for the diagnosis of GA (Hadjivassiliou et al., 2013). The present study aimed to verify the usefulness of the technique on a wider neurological spectrum disorders suspected to be related to gluten intake, in terms of whether TG6-Ab determination can be related to a clinical presentation of GA or to other neurological dysfunctions related to gluten sensitivity.

Before discussing the results, a few factors have been weighed up. Firstly, the total IgA levels of the sample should be considered within the reference values (RV) when interpreting the results. Data can be found in Suppl. Table 3. The IgA TG6-Ab concentration should not be overestimated in case of total IgA levels above the upper reference limit. In this way, patient #8 and patient #24 presented

high levels of total IgA (112 mg/dL, RV 0-83; 222 mg/dL, RV 53-204; respectively). Patient #8 presented a negative TG6-Ab test result. However, patient #24 presenting a TG6-Ab concentration of 14.47 U/mL (Table 5) could actually have lower levels of IgA TG6-Ab. In contrast, total IgA levels below the lower reference limit can imply an actually higher IgA TG6 value. Patient #16 presented low levels of total IgA (38 mg/dL, RV 41-297) and a negative TG6-Ab test result, which could be underrated if total IgA values are not taken into account. All in all, samples with total IgA levels out of the reference range should be considered under careful discussion.

Besides, regarding the mean absorbance of the duplicates, relative standard deviation (RSD) values or coefficients of variation are considered high above 30 %. RSD values are specified in Suppl. Table 4. The quantitative evaluation of the samples was performed doing the mean of the duplicates and regardless of RSD values. However, some samples show analytical discordance in this respect. The reason might be a poorly homogenized serum sample at the moment of the assay, leading to a heterogeneous concentration of TG6-Ab between the duplicates. To solve this situation in clinical practice, a third measurement could be done, and either discard the outlier among the three measurements or take the mean of three measurements as a more reliable result.

This study determined the prevalence of positive TG6-Ab result as 40 % in CD patients, 60 % in neurological patients and 50 % in HC. The prevalence of TG6-Ab in the CD group recapitulates the results of previous studies (Hadjivassiliou et al., 2008; Hadjivassiliou et al., 2013). A GA cohort was also tested in previous studies, obtaining a prevalence of TG6-Ab of 40 to 73 %. In comparison, this study tested a more general neurological group that was not specific for GA, still the prevalence of TG6-Ab was high. This fact could potentially point towards TG6-Ab as a seric biomarker for gluten related neurological disorders. However this cannot be overstated since the prevalence of TG6-Ab in the HC group is also high.

In contrast, the prevalence of TG6-Ab in healthy controls was much higher in this study (50 %) than the reported by other authors (4 %) (Hadjivassiliou et al.,

2013). On one hand, the here defined exclusion criteria for healthy controls were stricter. While the HC group from Hadjivassiliou et al. comprised patients with no serologic evidence of gluten sensitivity, here healthy controls not only tested negative for tTG-Ab but also were not driven by gastroenterologists, neurologists or autoimmune disease physicians nor presented gastrointestinal or neurological symptomatology. On the other hand, some patients from the HC group could have developed gastrointestinal or neurological symptoms after the patients selection. This was a time-limiting factor yet the employed HC selection seemed at first a good strategy. Other factors could also explain the disparity between HC prevalence in this and previous studies, like geographical differences or different approaches for defining the positive cutoff.

Figure 5 shows the distribution of the values of TG6-Ab concentration for the different groups. The median is represented by the dotted blue line, and corresponds to the widest section of the plot, meaning that there is a higher probability that a particular determination is around that value. As a general overview, the width of the curves for CD and HC groups is below the positivity threshold. In contrast, a wider distribution in the neurological group is found above the positivity threshold. Moreover, TG6-Ab concentration values from the CD and HC groups are mainly clustered to the median. Instead, values from the neurological group are widespread and the range of values within this group is larger.

6.1. USEFUNESS OF TG6-AB DETERMINATION FOR GLUTEN ATAXIA DIAGNOSIS

It is difficult to discern whether the two patients with abnormal gait are related to a clinical picture of GA because all patients from the neurological group are paediatric population and GA onset occurs at a mean age of 48 years. Nonetheless, TG6-Ab as a marker can be diagnostically helpful when using the test in the appropiate population (Hadjivassiliou et al., 2021b). GA represents a 15% of all types of ataxia, yet it is the only type that can be easily treated. In light of a clinical presentation compatible with ataxia, a long term follow-up for gluten sensitivity markers could be appropiate to confirm or discard the diagnosis and

start the GFD. Accordingly, the TG6-Ab determination can be useful in the diagnosis of GA together with a prior neurological evaluation and gluten sensitivity tests like tTG and AGA antibodies.

Regarding the patients presenting headaches, Hadjivassiliou et al. (2021a) reported that two-thirds of CD patients had evidence of neurological dysfunction, which evolved in an overall reduction in headache prevalence but an increase in imbalance and incoordination. TG6-Ab tests were positive in 37 % of newly diagnosed CD patients at the baseline, and in 33 % of the same patients seven years later. Instead, the prevalence of classic gluten sensitivity markers like tTG-Ab, antigliadin antibody (AGA) and anti-endomysial antibody (EMA) decreased dramatically during the follow-up period and under GFD. This study suggests in some way that patients with headaches could develop incoordination over time while TG6-Ab would have the same prevalence. Therefore, a long-term follow-up of TG6-Ab levels could be interesting to do in patients with ongoing headaches together with serological tests of gluten sensitivity, in order to elucidate the cause. Furthermore, a case-series study of rapid-onset gluten ataxias also described headache as an initial symptom (Newrick et al., 2021).

Additionally, gluten encephalopathy is a less frequent neurological manifestation of gluten sensitivity with migraine as the most common symptom. In the same line, migraines are also common in CD. It has been reported that migraines ameilorate with the introduction of GFD (Beuthin et al., 2020). Hence, TG6-Ab determination could give a clue when migraines are triggered by gluten intake and subsequent humoral response affecting the CNS (Hadjivassiliou et al., 2010). It has also been suggested that TG6-Ab are not only related to the CNS, but also could play a role in the peripheral nerve function, and TG6-Ab might be helpful in the diagnosis of other neurological gluten-related pathologies like gluten neuropathy (Zis et al., 2017).

6.2. USEFUNESS OF TG6-AB DETERMINATION IN COELIAC DISEASE PATIENTS FOR GLUTEN-FREE DIET FOLLOW-UP

Hadjivassiliou et al. (2021a) argue that “patients with CD who do not adhere to a strict GFD and are serological positive are at risk of developing ataxia”. It was discussed that since a strict adherence to the GFD usually leads to elimination of gluten sensitivity antibodies, CD patients that developed GA, and had positive serological markers for gluten sensitivity, were not adhering properly to the GFD. Another study supporting the gluten dependence of TG6-Ab demonstrated that TG6-Ab titers were lowered after one year on GFD (Hadjivassiliou et al., 2013).

Thus, in this study, the possible relation between TG6-Ab and adherence to the GFD was qualitatively investigated. No significant association between adherence to GFD and a positive test for TG6-Ab was found. It can be deduced that the interdependence between the two variables is long-term. A discrete measurement of a gluten sensitivity serological marker might not be enough to verify adherence to GFD, and a follow-up study should rather be done. This way relative changes in serological markers of gluten sensitivity including TG6-Ab levels could be perceived, which emphasizes the importance of close and regular monitoring of the patient.

6.3. LIMITATIONS OF THE STUDY

Finally, there are some limitations in the design of this study that future studies for additional validation of the technique could take advantage of. Firstly, patients selection criteria could be reassessed. For a more reliable validation to study particular neurological diseases, age filtering before patients selection seems reasonable. Because all neurological patients were paediatric while GA onset occurs at a mean age of 48 years, there are few studies with paediatric population to compare this data. Our results are not directly comparable to previous studies due to the unalike ages within the studied population. A more well-grounded comparison and discussion by testing middle-aged patients in future studies would be interesting. Nonetheless, the here presented findings serve as a starting

point that arises the matter of studying neurological dysfunction associated with gluten sensitivity in paediatric population for earlier detection.

Moreover, healthy controls clinics should be checked thoroughly before selection to ensure that the prevalence of TG6-Ab in the healthy population is, at least encompassing the province of Tarragona, unexpectedly high. Besides, some methodological criteria that were not fully controlled could have played a role. Sample storage time, which is related to stability, should not exceed 3 days in the fridge, while samples were stored for up to 5 days. This factor should also be considered in future clinical routine. Finally, further studies with a greater number of patients per group could help verify these results.

7. CONCLUSION

IgA autoantibodies against transglutaminase 6 have been suggested as a diagnostic biomarker for neurological dysfunctions related to gluten sensitivity like GA. As an additional marker for gluten sensitivity, previous studies have also determined TG6-Ab in CD patients. Here, TG6-Ab testing in neurological affection-suspected patients and in CD patients is validated, aiming to introduce the test to clinical practice.

As main results of this study, the prevalence of TG6-Ab in CD and neurological affection-suspected patients is similar to that in the literature. However, a higher prevalence has been found in healthy patients. Besides, the conducted testing showed that despite all neurological patients were negative for tTG-Ab, 6/10 neurological patients were positive for TG6-Ab. This suggests that gluten sensitivity detection may not be restricted to the gold standard tests.

TG6-Ab as a diagnostic biomarker specifically for GA could not be assessed in this study, but results open up the possibility of a wider application as a complementary test for neurological gluten-related disorders. After appropriate neurological evaluation, classical tests together with complementary determination of TG6-Ab can help anticipate gluten sensitivity, especially in patients with negative results for commonly used markers of gluten sensitivity and

without enteropathy. Noteworthy is also the informative value of follow-up determinations to assess antibody levels over time in a similar fashion as tTG-Ab.

To sum up, TG6-Ab seem to be a helpful diagnostic tool for neurological gluten-related dysfunctions. Future studies should corroborate these findings within our population.

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9. ANNEXES

Supplementary table 1. Devices, consumables and reagents used.

Type	Source	Reference
Microplate Reader with four standard filters: 405, 450, 492 and 630 nm	NeoBiotech	NB-12-0035
Optical filter 620 nm	NeoBiotech	NB-12-0035-24
NeoLine multichannel pipette 10-100 µL	NeoBiotech	NB-12-6003-8-4
Microplate washer	NeoBiotech	NB-12-0034
Graduate cylinder 1000 mL	N.A.	N.A.
Nitrile gloves, powder-free	AACHEN Fortis	N.A
Tips 100-1000 µL 5-200 µL 0.1-10 µL	Daslab (Deltalab)	 162222X 162001X 301-01

Supplementary table 2. List of software and data analysis tools used.

Software	Source	Reference
BioRender	N.A.	https://biorender.com/
Microplate Reader PC Software	NeoBiotech	NB-12-0035-27
Four Parameter Logistic Curve	MyAssays	https://www.myassays.com/four-parameter-logistic-curve.assay
Prism 9	GraphPad	https://www.graphpad.com/scientific-software/prism/
PSPP	GNU Project	https://www.gnu.org/software/pspp/

Supplementary table 3. Extended information about clinical characteristics of CD and neurological patients.

Index	Total IgA (mg/dL)	Other clinical information	Other confirmative CD tests
1			EMA
2			EMA, AGA
7			AGA, Compatible citometry, HLA DQ 8/2.2
8	High (112, RV 0-83)		
9		Epilepsy in family history	EMA, AGA
10		Dermatitis herpetiforme	
11		Growth delay	HLA DQ 2.5 homozygote, compatible citometry
14			Compatible citometry
15		Growth and puberty delay	EMA
16	Low (38, RV 41-297)	Epilepsy, motor and cognitive disorder after suffering from encephalitis	AGA, EMA, HLA DQ2 (2.5/7)
17		Muscle cramps, joint pains	EMA, HLA DQ 2 (2.5/7.5)
18			EMA
19			positive HLA
24	High (222, RV 53-204)		
25		Migraine and epilepsy in family history	

Blank cells refer to no relevant information. Patients not shown had normal levels of IgA and no relevant medical data.

Supplementary table 4. Raw absorbances, mean absorbance standard deviation and coefficient of variation values of each sample.

Index	Raw Abs	Mean Absorbance	STDEV	RSD (coefficient of variation)
Standard 1	0.375 0.378	0.377	0.0015	0.40
Standard 2	0.595 0.249	0.422	0.1730	41.00
Standard 3	0.450 0.404	0.427	0.0230	5.39
Standard 4	0.643 0.744	0.694	0.0505	7.28
Standard 5	1.542 1.306	1.424	0.1180	8.29
Standard 6	2.593 2.616	2.605	0.0115	0.44
Positive control	0.998 1.040	1.019	0.0210	2.06
Negative control	0.119 0.104	0.112	0.0075	6.73
1	0.215 0.254	0.235	0.0195	8.32
2	0.361 0.243	0.302	0.0590	19.54
3	0.207 0.156	0.182	0.0255	14.05
4	0.320 0.501	0.411	0.0905	22.05
5	0.220 0.243	0.232	0.0115	4.97
6	0.224 0.399	0.312	0.0875	28.09
7	1.232 0.508	0.870	0.3620	41.61

8	0.314 0.349	0.332	0.0175	5.28
9	0.401 0.511	0.456	0.0550	12.06
10	0.320 0.354	0.337	0.0170	5.04
11	0.365 0.300	0.333	0.0325	9.77
12	0.550 0.270	0.410	0.1400	34.15
13	0.646 0.689	0.668	0.0215	3.22
14	0.595 0.429	0.512	0.0830	16.21
15	0.293 0.668	0.481	0.1875	39.02
16	0.279 0.359	0.319	0.0400	12.54
17	1.148 0.321	0.735	0.4135	56.30
18	0.472 0.554	0.513	0.0410	7.99
19	0.291 0.310	0.301	0.0095	3.16
20	0.335 0.353	0.344	0.0090	2.62
21	0.307 2.352	1.330	1.0225	76.91
22	0.427 0.322	0.375	0.0525	14.02
23	0.586 0.292	0.439	0.1470	33.49
24	0.540 0.638	0.589	0.0490	8.32

25	0.784 0.651	0.718	0.0665	9.27
26	0.771 0.804	0.788	0.0165	2.10
27	0.487 0.286	0.387	0.1005	26.00
28	0.447 0.240	0.344	0.1035	30.13
29	0.379 0.409	0.394	0.0150	3.81
30	0.505 0.489	0.497	0.0080	1.61
31	0.443 0.380	0.412	0.0315	7.65
32	0.439 0.405	0.422	0.0170	4.03
33	0.457 0.314	0.386	0.0715	18.55
34	0.432 0.448	0.440	0.0080	1.82
35	0.399 0.452	0.426	0.0265	6.23
36	0.354 0.275	0.315	0.0395	12.56
37	0.295 0.271	0.283	0.0120	4.24
38	0.240 0.288	0.264	0.0240	9.09
39	0.254 0.219	0.237	0.0175	7.40
40	0.640 0.630	0.635	0.0050	0.79

Samples with relative standard deviation values higher than 30% are highlighted in red.