Sensitivity and specificity of a commercial ELISA test for anti-MAG antibodies in patients with neuropathy

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Abbreviations: BTU, BÜHLMANN Titer units; MAG, Myelin-Associated Glycoprotein; CIDP, Chronic Inflammatory Demyelinating Polyradiculoneuropathy; DADS, Distal Acquired Demyelinating Symmetric Neuropathy; WESTERN BLOT, Western Blot; POEMS, Polineuropathy, Organomegaly, Endocrinopathy, M-protein, Skin; ALS, Amyotrophic Lateral Sclerosis; PPV, Positive Predictive Value; NPV, Negative Predictive Value.

ABSTRACT

For the diagnosis of anti-MAG polyneuropathy the commercial ELISA manufacturer currently recommends a cut-off of 1000 Bühlmann Titer units (BTU). We analyzed sera from 80 anti-MAG neuropathy patients and 383 controls (with other neuropathies or healthy controls) to assess the ELISA sensitivity and specificity at different thresholds. A better combination of sensitivity/ specificity was found at a threshold >1500 BTU than at >1000 BTU. The best value of specificity was obtained at threshold >7000 BTU. There was a diagnostic grey area between 1500-7000 BTU in which the clinical phenotypes as well as electrophysiological studies need to be carefully assessed particularly to differentiate CIDP and anti-MAG neuropathy.

1. Introduction

Anti-MAG neuropathy is a disorder of the peripheral nervous system linked to the presence of antibodies against the myelin-associated glycoprotein (MAG). It was originally reported in a patient with neuropathy and IgM monoclonal gammopathy of undetermined significance (MGUS) whose IgM bound to an antigen in myelin later identified as MAG (Latov et al., 1980). Anti-MAG neuropathy typically present as a distal acquired demyelinating symmetric (DADS) predominantly sensory neuropathy with ataxia and moderate motor impairment (Nobile-Orazio et al., 1994; Chaudry et al., 2017; Dalakas, 2018). It is a subtype of paraproteinemic neuropathy, a collective term used to refer to neuropathies associated with a serum monoclonal gammopathy (Gosselin et al., 1991; Nobile-Orazio, 2013).

Nowadays, the anti-MAG IgM ELISA from Bühlmann is the most frequently used commercial assay to detect these antibodies and was reported to be more sensitive even if less specific than previously ubiquitously used home-made or commercial Western Blot (Kuijf et al., 2009). The result of this test is expressed in Bühlmann Titer Units (BTU), with a recommended cut-off level for positivity of 1000 BTU. The cut-off for positivity for this assay is however debated with some studies reporting that some patients with BTU titers >1000 BTU may have an alternative diagnosis to anti-MAG neuropathy (Caudie et al., 2006; Kuijf et al., 2009).

The aim of our work was to define the ideal cut-off value for positivity from the analysis of our series of patients with anti-MAG neuropathy, other neuropathies and healthy subjects; and to correlate the results with those obtained by our home-made Western blot assay.

2. Patients and Methods

This was a retrospective, observational case-control diagnostic accuracy study. We analyzed the sera from 463 subjects, including 80 patients with anti-MAG neuropathy, 222 with CIDP

diagnosed according to the EFNS/PNS criteria (van den Bergh et al., 2010), 38 with POEMS syndrome (Dispenzieri et al., 2018), 30 with amyotrophic lateral sclerosis (ALS) (Brooks et al., 2000), 73 with other neuropathies (including diabetic, vasculitic, paraneoplastic, amyloidosis, multifocal motor neuropathy, hereditary, sensitive neuropathy of undefined origin) and 20 healthy subjects. Patients with a diagnosis of anti-MAG neuropathy had a chronic progressive demyelinating neuropathy associated with IgM monoclonal gammopathy (Nobile-Orazio et al., 1994) fulfilling the EFNS/PNS electrodiagnostic criteria for demyelination (Hadden et al., 2006) and increased titers of anti-MAG IgM antibodies by Western blot (Nobile-Orazio et al., 2008). In all patients, blood was taken at the time of our first neurological assessment and the same sample of serum was used for ELISA and Western blot.

Anti-MAG reactivity was determined by ELISA using a commercial available system (Anti-MAG ELISA; Bühlmann Laboratories, Switzerland) according to manufacturer's instructions. Results are expressed in arbitrary units (Bühlmann titer units, BTU) and the cut-off of positivity (ELISA's manufacturer established) is 1000 BTU. All the sera were tested in duplicated. In all the measurements, sera from two positive patients were included to ascertain the reproducibility of the data and the variation of the results that was always within a 10%. In patients with moderately increased levels (1000 to 10000 BTU) the measurement was also repeated at least once to confirm the data.

All patients with anti-MAG neuropathy and 206 patients of other subgroups (32 with CIDP, 11 with ALS, 9 with POEMS, 54 other neuropathies, 20 healthy controls) were tested for anti-MAG IgM antibodies by Western blot after electrophoresis of human brain myelin (normal value up to 1:3200) according to our previously described procedures (Nobile-Orazio et al., 1989 and 2008). Antibody titers were determined by two-fold serum dilution until disappearance of the visible band

of MAG. Illustrative examples of the results of Western blot were previously reported (Nobile-Orazio, et al., 2009, Nobile-Orazio, 2013)

We calculate sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of ELISA method using different cut-off values: >1000 BTU, >1500 BTU, >3000 BTU, >6000 BTU, >7000 BTU, >10000 BTU. Correlation between variables was explored with Pearson correlation index. All analyses were performed with Stata15 software (StataCorp LLC, USA).

3. Results

Table 1 summarize results of sensitivity and specificity of ELISA method at different cut-off values. With a cut-off of >1000 BTU, twenty-three patients would have been false positive: twenty CIDP patients, two POEMS patients and one with another neuropathy (sensitivity 100%, specificity 93.99%). We then used the >1500 BTU cut-off level on the base of a previous study which established that the recommended cut-off of 1000 BTU was too low and should be increased to 1500 BTU (Kuijf et al., 2009). At this value, we maintained 100% sensitivity with a reduction of false positive patients (10 with CIDP and one with POEMS, specificity 97.13%).

3.1 Optimal cut-off for Sensitivity

Among the 80 anti-MAG neuropathy patients, four had BTU values below 3000 (at 1524, 1722, 2183, 2962 BTUs) and two were between 3000 and 6000 BTU (3170, 5206 BTUs). This meant that any value above 1500 BTU yielded a threshold value with <100% sensitivity, with the sensitivity declining to 95% for cut-off of 3000 BTU and 92.5% for a cut-off of 6000 BTU (Table 1). The sensitivity remained the same for a cut-off level of 7000 BTU.

3.2 Optimal Cut-Off for Specificity

At a cut-off of 1500 BTU, the specificity was at its lowest, at 97.13% (Table 1). With a positivity cut-off of 3000 BTU, we had 95% sensitivity and 98.96% specificity, since we had four false negative and four false positive patients (four CIDP). At the threshold of 6000 BTU, we had 92.5% sensitivity (six false negative patients) and 99.74% specificity with only one false positive (one CIDP patients with a BTU 6963). The specificity raised therefore to 100% using the 7000 BTU cut-off. Using the cut-off of 10000 BTU, there were more false negative patients (n=10) with a sensitivity of 87.5%.

Table 2 shows the value of sensitivity, specificity, PPV and NPV obtained analyzing anti-MAG patients vs CIDP patients as control sample. The values obtained have the same trend of previous analysis, with the best sensitivity at >1500 BTU and better specificity and PPV at >7000 BTU.

3.3 Relationship between BTU values and MAG titers form Western Blot

Overall, there was a good correlation between the presence of increased titers of anti-MAG antibodies by ELISA and Western blot. Comparing however the level of these antibodies by ELISA and Western blot results, there was only a slight positive correlation (r=0.24), indicating that there was only a moderate correspondence between these titers (Figure 1). The correlation increased to r=0.30, when we limited the analysis to patients with Western blot titers between 1:6400 and 1:25600 (inclusive, n=42 patients), but was still not statistically significant (Figure 2). The comparison of the level of anti-MAG antibodies in each patient with anti-MAG neuropathy is reported in the Supplementary table.

4. Discussion

Since the original report of anti-MAG IgM antibodies in a patient with neuropathy associated with serum IgM monoclonal gammopathy (Latov et al.. 1980), several laboratories reported the presence of these antibodies in approximately 50% of patients with neuropathy and IgM monoclonal gammopathy (reviewed in Nobile-Orazio, 2013). Different techniques were used to detect anti-MAG antibodies in these patients including ELISA or radioimmunoassay with purified MAG, Western blot after electrophoresis of brain or peripheral myelin proteins or of purified MAG, ELISA or overlay immunostaining after chromatography for reactivity with cross-reacting peripheral nerve glycolipids, indirect immunohistochemistry on peripheral nerve sections (reviewed in Nobile-Orazio, 2013) and more recently immunofluorescence for reactivity to HNK-1 (Matà et al., 2011). There was some variability in the detection of these antibodies using these different techniques (Nobile-Orazio, et al., 1989; Pestronk, et al., 1994; van den Berg, et al., 1996; Weiss et al., 1999; Jaskowski et al., 2004 & 2007; Matà et al., 2011) making it difficult a comparison of the results among different laboratories. This might also reflect differences in the specificity (Nobile-Orazio, et al., 1989; Pestronk, et ql., 1994; Fluri, et al., 2003) or affinity (Ogino, et al., 1994) of these antibodies or in their binding capability to the antigen using different substrate. This may also explain the lack of correlation between anti-MAG titers and disease severity observed in most centers. Most importantly, these methods were time consuming and required the preparation of reactive antigens from human autopsy tissues.

In early 2000' an new commercial ELISA procedure using human purified MAG was introduced to determine serum anti-MAG antibody reactivity. This technique was shown to be sufficiently sensitive and specific (Kuijf et al., 2009) and shortly became a diffuse method for the measurement of these antibodies avoiding the local preparation of tissues or antigens from autopsy. It also has the advantage of facilitating the interpretation and comparison of the results among different Centers. There is not however a clear consensus on the cut-off to be used for the best

combination of sensitivity and specificity in the detection of these antibodies in patients with anti-MAG neuropathy. Previous studies reported that the commercially proposed cut-off titer of >1000 BTU also included some patients without anti-MAG neuropathy (Caudie et al., 2006), so that a new cut-off of 1500 BTU was proposed (Kuijf et al., 2009). An even higher cut-off of 10,000 BTU was recently proposed (Svahn et al., 2018) even if how this cut-off was determined was not specified. Considering the presence of some variability in the assessment measures, the Italian Neuroimmunological Society, recently recommended that each Centre should calculate its own cut-off level for diagnostic confirmation (Franciotta et al., 2017).

We compared the results of anti-MAG antibodies using a commercial ELISA method and our currently used Western blot in patients with anti-MAG neuropathy and with other neuropathies including CIDP, POEMS and neuropathies of other causes, with ALS and in healthy subjects. We did not used a control population of patients with IgM monoclonal gammopathy without neuropathy that we previously analyzed by Western blot (Nobile-Orazio, et al., 2008) since we did not consider this population relevant in differential diagnosis with anti-MAG neuropathy.

With the recommended threshold value of 1000 BTU, we obtained 100% sensitivity for anti-MAG neuropathy, but also find 23 patients with other neuropathies including 20 with CIDP, two with POEMS and one with a sensory neuropathy of undefined etiology. Almost 10% of our tested CIDP patients therefore had values higher than the cut-off level. Using the cut-off value of 1500 BTU (Kuijf et al., 2009) we maintained a 100% sensitivity for anti-MAG neuropathy increasing the specificity from 94% to 97%. There were still however 10 positive patients with CIDP (5%) and one with POEMS. A further increase of the cut-off level to 7000 BTU resulted in a 100% specificity with only six false negative patients (92.5% sensitivity). There is therefore a grey area between 1500 and 7000 BTU where a small number of patients with anti-MAG neuropathy resulting negative (6 patients) were balanced by an even larger number of positive patients with CIDP (10

patients). Using this cut-off measure a few patients would therefore need further investigation to exclude other possible diagnosis unless the clinical phenotypes is typical for anti-MAG neuropathy. Our proposed threshold of 7000 BTU permitted to distinguish in our series, patients with anti-MAG neuropathy from other neuropathies with only a moderate loss of sensitivity.

The implication of defining a clear-cut distinction in antibody titers between anti-MAG neuropathy and other neuropathy is important considering that POEMS syndrome is also associated with a monoclonal gammopathy and that the DADS phenotype of CIDP closely resembles anti-MAG neuropathy. All but one of our positive patients with CIDP had a typical CIDP and improved after therapy with intravenous immunoglobulins (IVIg) which is unusual for anti-MAG neuropathy (Lunn & Nobile-Orazio, 2016). Beside supporting the pathogenetic distinction between anti-MAG neuropathy and CIDP within the boundaries of chronic immune mediated neuropathies (van den Bergh et al., 2020), this distinction may also allow to avoid the not so infrequent misdiagnosis of anti-MAG neuropathy in patients with CIDP and moderately increased antibodies. This has relevant therapeutic implication, since patients with CIDP often respond to steroids and IVIg that are usually ineffective in anti-MAG neuropathy (Lunn & Nobile-Orazio, 2016). This data might also help defining the high level of anti-MAG antibodies that the EFNS/PNS guidelines recommended to exclude the diagnosis of CIDP in the diagnostic criteria (van den Bergh et al., 2006).

Similarly to what we previously reported comparing the sensitivity of ELISA and Western in the detection of anti-MAG antibodies (Nobile-Orazio, et al., 1989; Jaskowski et al., 2004 & 2007; Kuijf et al., 2009), there was not a strict correlation between anti-MAG levels by Western blot and ELISA. This applied to the entire cohort of patients (r-value 0.24) and to patients with Western blot titters between 1:6400 and 1:25600 (r-value 0.29). As already mentioned this may reflect difference in the specificity or affinity of these antibodies for MAG (Nobile-Orazio, et al., 1989; Pestronk, et al., 1994; van den Berg, et al., 1996; Weiss et al., 1999; Matà et al., 2011) or in their access to the

antigen using different substrate for the analysis. The use of a fixed serum dilution of 1.1000 in the ELISA techniques may also allow the binding of lower affinity antibodies that might disappear with higher serum dilution.

In conclusion, we think that more stringent criteria for the definition of positivity and some caution in the interpretation moderately increased anti-MAG antibody levels, should be used for this otherwise accurate and easy to use procedure.

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Author contribution

ENO, GL, CG and BPS conceived, organized and designed the study, reviewed and commented on the statistical analysis, wrote the first draft of the report and reviewed the report. EM performed statistical analysis. FG, FT, PED, FM, DC, MF, GA, GC, GAM, AMC, GL, TR, GC,

contributed to the conception, organization, and execution of the research project, reviewed and commented on the statistical analysis and the report.

Ethical approval

The study was conducted according to the declaration of Helsinki and was approved by the local ethics committee. All the patients gave written informed consent.

Declaration of Competing Interest

E.N.O. reported personal fees for Advisory or Scientific Board from Kedrion, Italy, Baxter, Italy, Novartis, Switzerland, CSL-Behring, Italy, LFB, France, Astellas, the Netherlands, outside the submitted work and travel grants to attend Scientific Meeting from Baxter, Grifols, Kedrion, and Novartis, Italy. P.E.D. reported travel grants to attend scientific meetings from CSL Behring and Kedrion. G.L. reported travel grants to attend scientific meetings from CSL Behring and Kedrion. D.C. reported honoraria for lecturing from Shire, CSL Behring, and Kedrion and travel grants to attend scientific meeting from Shire, Kedrion, and CSL Behring. E.P. reported travel grants to attend scientific meetings from CSL Behring. G.C. reported travel grants to attend scientific meetings from CSL Behring and Kedrion. L.S. reported personal fees for scientific events from CSL Behring and has received travel grants to attend scientific meetings from CSL Behring and Kedrion. F.M. reported personal fees for scientific events from CSL Behring and has received travel grants to attend scientific meetings from CSL Behring and Kedrion. G.C. reported honoraria for lecturing and travel grants to attend scientific meetings from Kedrion. M.F. has served on scientific advisory boards for CSL Behring and Sarepta Therapeutics and has received travel grants from Sanofi Genzyme, Kedrion, Baxter and CSL Behring to attend scientific meeting. G.A.M. reported consulence fees and travel fundings from CSL Behring, Kedrion, Shire and Grifols. G.A. reported

honoraria for lecturing from Kedrion and Sanofi-genzyme, travel grants from Kedrion, Sanofi-Genzyme and LJ Pharma. G.M. reported consulence fees and travel fundings from CSL Behring, Kedrion, Shire and Grifols. The other authors declare no conflict of interest.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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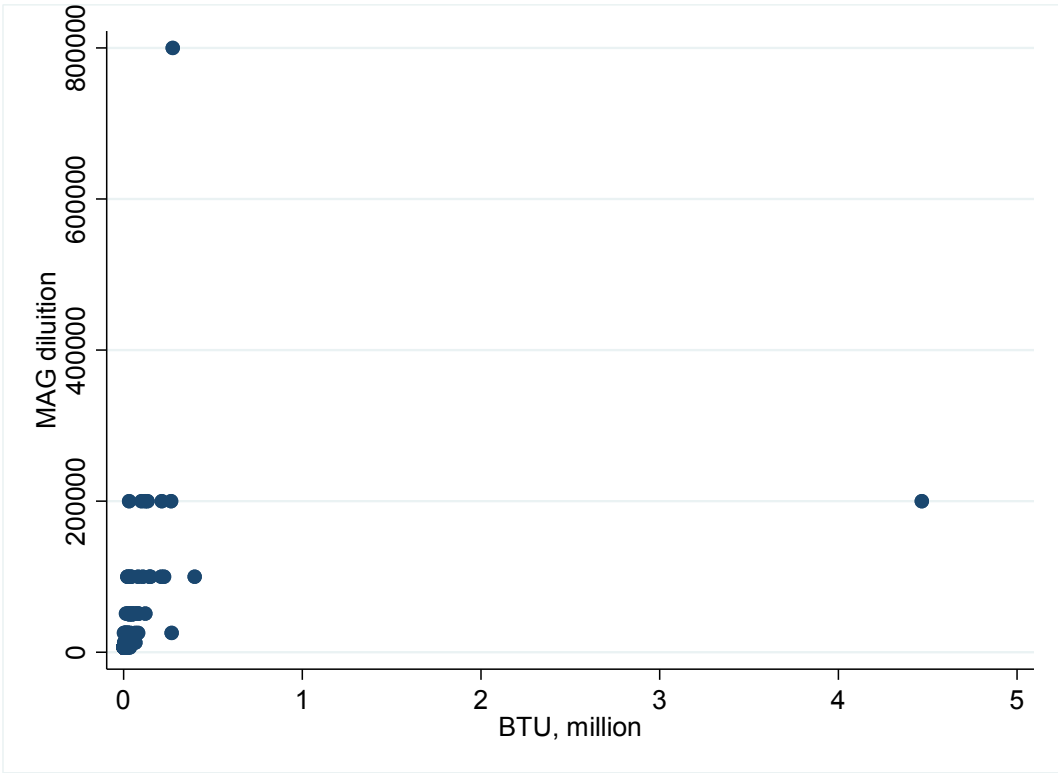

Figure 1: correlations between Western Blot (entire cohort, on the y-axis) and ELISA results (on x-axis). Analysis showed a low correlation (r=0.24).

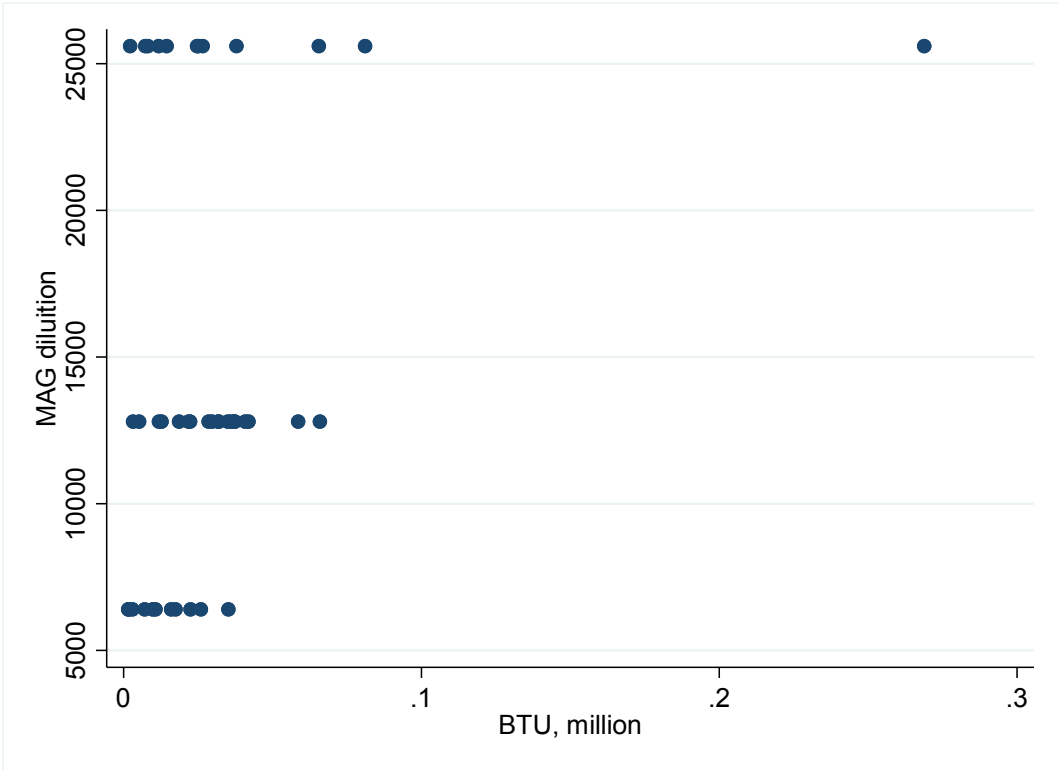

Figure 2: correlations between Western Blot (patients with titers between 1:6400 and 1:25600) on y-axis and ELISA results (on x-axis). Analysis showed a low correlation (r=0.30).

Threshold	Sensitivity	Specificity	PPV	NPV	Accuracy
>1000 BTU	100%	93.99%	77.67%	100%	95.03%
>1500 BTU	100%	97.13%	87.91%	100%	97.26%
>3000 BTU	95%	98.96%	95%	98.96%	98.27%
>6000 BTU	92.5%	99.74%	98.67%	98.45%	98.49%
>7000 BTU	92.5%	100%	100%	98.46%	98.7%
>10000 BTU	87.5%	100%	100%	97.46%	97.84

Table 1: values of sensitivity, specificity, PPV, NPV, accuracy at different threshold of BTU (anti-MAG patients vs entire cohort of control sample). The best value of Sensitivity was found at >1500 BTU, the best value of Specificity and Accuracy at >7000 BTU.

Threshold	Sensitivity	Specificity	PPV	NPV	Accuracy
>1000 BTU	100%	90.99%	80%	100%	93.38%
>1500 BTU	100%	95.5%	88.89%	100%	96.69%
>3000 BTU	95%	98.2%	95%	98.2%	97.35%
>6000 BTU	92.5%	99.55%	98.67%	97.36%	97.68%
>7000 BTU	92.5%	100%	100%	97.37%	98.01%
>10000 BTU	87.5%	100%	100%	95.69%	96.69

Table 2: values of sensitivity, specificity, PPV, NPV, accuracy at different threshold of BTU (anti-MAG patients vs CIDP cohort as control sample). Similarly to previous analysis, the best value of Sensitivity was found at >1500 BTU, the best value of Specificity and Accuracy at >7000 BTU.