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1 Modified radioimmunoassay versus ELISA to quantify anti-acetylcholine 2 receptor antibodies in a mouse model of myasthenia gravis

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20
21
22 **Keywords.** EAMG; autoantibodies; myasthenia gravis; ELISA; RIA.

23 24 **Abstract**

25 In mouse models of myasthenia gravis (MG), anti-acetylcholine receptor (AChR) antibodies can
26 be quantified to monitor disease progression and treatment response. In mice, enzyme-linked
27 immunosorbent assay (ELISA) is the gold standard to quantify these antibodies. However, this
28 method requires antigen purification, which is both time-consuming and expensive. In
29 humans, radioimmunoassay (RIA)—which is more sensitive than ELISA—is commonly used to
30 quantify AChR antibodies. At present, however, no commercial RIA kits are available to
31 quantify these antibodies in mice. The aim of this study was to compare a modified
32 commercial human RIA kit to two ELISA methods to detect AChR antibodies in an experimental
33 autoimmune mouse model of MG (EAMG). C57BL/6J mice were immunized with purified AChR
34 from *Tetronarce californica* (T-AChR). Serum samples were analyzed by RIA and two ELISAs (T-
35 AChR and purified mouse AChR peptide [m-AChR]). The modified RIA showed excellent
36 sensitivity (84.1%) and specificity (100%) for the detection of AChR antibodies. RIA showed a
37 good agreement with T-AChR ELISA ($\kappa = 0.69$) but only moderate agreement with m-AChR
38 ELISA ($\kappa = 0.49$). These results demonstrate the feasibility of modifying a commercially-
39 available RIA kit to quantify AChR antibodies in EAMG. The advantage of this technique is that
40 it eliminates the need to develop the entire methodology in-house and reduces inter and intra-
41 laboratory variability.

44

45 **Introduction**

46 Myasthenia gravis (MG) is a highly heterogeneous disease characterized by fluctuating muscle
47 weakness and fatigability. MG is an antibody-mediated autoimmune disorder in which
48 antibodies are directed against post-synaptic antigens at the neuromuscular junction (NMJ),
49 thereby impairing signal transmission from motor neurons to the muscle[1]. Most patients (\approx
50 85%) present nicotinic acetylcholine receptor (AChR)-specific autoantibodies [2].

51 The most common animal model of MG is the experimental autoimmune myasthenia gravis
52 (EAMG). In this model, MG is induced by administering purified AChR from *Tetronarce*
53 *californica* (T-AChR). The administration of purified AChR in complete Freund's adjuvant (CFA)
54 induces AChR antibody production, an accumulation of immunoglobulin G (IgG) and
55 complement deposits at the NMJ, and lysis of the postsynaptic muscle membrane, leading to
56 muscle weakness[3]. In EAMG mice, quantifying AChR antibodies is a useful biomarker of
57 disease progression and treatment response[4–6].

58 Two different types of assay can be used to detect AChR antibodies, either radioimmunoassay
59 (RIA)[7] or enzyme-linked immunosorbent assay (ELISA)[8]. Although RIA is more sensitive,
60 ELISA is normally used because it does not require the use of radioactive reagents[8–11]. While
61 most groups use purified m-AChR as the antigenic source to coat the ELISA plates, others use
62 purified AChR from *T. californica* electric organ (T-AChR) or cultured cell lines[10–13]. Given
63 the lack of commercial RIA kits for the quantification of AChR antibodies in mice, laboratories
64 that want to use this more sensitive test must develop their own RIA kit in-house, which can
65 be both time-consuming and expensive. In this regard, there is a clear need for a standardized
66 RIA kit to reduce inter and intra-laboratory variability when quantifying AChR antibodies in
67 mouse models.

68 In this context, the aim of this study was to compare a modified commercial RIA kit to two
69 ELISA methods broadly used for the detection of AChR antibodies in serum samples obtained
70 from EAMG mice. We also assessed the correlation between each assay and clinical outcomes
71 (treadmill scores and weight change in the mice).

72

73 **Materials and Methods**

74 *Induction of EAMG*

75 Pathogen-free C57BL6/J mice were purchased from Charles River (Saint Germain Nuelles,
76 France) and housed (five mice per cage) in the animal facility at the Institut de Recerca-
77 Hospital de la Santa Creu i Sant Pau (IRHSCSP). Mice were fed a regular chow diet. The
78 experiments were performed on 5- to 6-week-old male and female mice after 7 days of
79 quarantine. The study was approved by the IRHSCSP Ethics Committee for animal
80 experimentation. All research staff has performed an animal experimentation course certified
81 by the local government. All experiments were conducted according to local government
82 guidelines and regulations.

83 The mice were randomly allocated to one of two groups: 1) EAMG or 2) a non-immunized
84 control group. The disease was induced by administering two consecutive immunizations of
85 purified T-AChR with CFA (Sigma; St Louis, MO, USA) on days 0 and 28. T-AChR was purified
86 from electroplaque tissue from *T. californica* (Aquatic Research Consultants; San Pedro, CA,
87 USA) using low-pressure affinity chromatography as described elsewhere[14]. Briefly, EAMG

88 was induced by injecting 20 µg of purified T-AChR diluted in phosphate buffered saline (PBS)
89 solution and emulsified in CFA (1:1 vol) to a final volume of 200 µL. Next, 50 µL of the emulsion
90 was injected intradermally into both foot pads in the lower limbs and subcutaneously at the
91 site of the scapulae of the two upper limbs. Twenty-eight days later, a second immunization
92 was performed by injecting 50 µL of the emulsion in both shoulders and both thighs.
93 Immunizations, blood extraction and euthanasia were performed under anesthesia using
94 isoflurane. Animals were euthanized the day 53 or if weakness impaired normal feeding (not
95 necessary in any case)[14].

96 *Clinical assessment*

97 The general condition of the mice was evaluated daily and the mice were weighed weekly.
98 Strength and fatigability were assessed using a treadmill controller device (Columbus
99 Instruments; Columbus, OH, USA)[15]. Briefly, mice were forced to run to exhaustion on the
100 treadmill as the speed was gradually increased. An electric rod placed at the end of the belt
101 emitted an electric discharge to force the mice to keep running. We evaluated the number of
102 electric discharges and total distance run (in centimeters).

103 *AChR antibody quantification by RIA*

104 Blood samples were collected on day 0 (before the first immunization) and on day 53. Serum
105 samples obtained from the mice were tested for anti-AChR-antibodies by radioimmunoassay
106 (DLD Diagnostika GmbH; Hamburg, Germany) according to the manufacturer's instructions.
107 This kit uses a human rhabdomyosarcoma cell line as the AChR source. However, to precipitate
108 mouse IgG, anti-human IgG was replaced by recombinant protein G agarose (Invitrogen Inc;
109 Carlsbad, CA, USA). Counts per minute of radioactivity were quantified using an automatic
110 gamma counter (Wizard, PerkinElmer, Singapore). The readings were then converted to nM of
111 anti-AChR antibodies. Following the manufacturer's recommendations, all values < 0.25 nM
112 were considered negative.

113 *Anti-AChR antibody ELISAs using T-AChR and m-AChR for coating*

114 ELISA experiments were carried out on serum samples in 96 well plates coated with 0.5 µg/mL
115 of T-AChR or 0.5 µg/mL of subunit alpha m-AChR peptide (AP73672; Biovalley, Nanterre,
116 France) diluted in a 10 mM NaHCO₃ buffer (pH 9.6) and stored overnight at 4°C. Wells were
117 blocked with PBS (10% fetal calf serum) for 2h30 at 37°C. Samples were diluted in 0.1% bovine
118 serum albumin (BSA)-PSA (1/400,000 for T-AChR and 1/200 for m-AChR ELISAs) and incubated
119 at 37°C for 1h30. Next, samples were incubated with 100 µL of biotinylated rabbit anti-mouse
120 IgG 0.04µg/ml (E0413, Dako, Courtaboeuf, France) at 1/1800 for 1h30 and 100 µL of
121 streptavidin-horseradish peroxidase 0.1µg/ml (S911, ThermoFisher Scientific, Villebon-sur-
122 Yvette, France) at 1/10000 for 30 minutes. Tetramethylbenzidine was used for color
123 development; the reaction was stopped with H₃PO₄ 1M; optical density (OD) at 450 nm was
124 measured with the SPARK 10M microplate reader (TECAN France, Lyon, France). Between each
125 step, wells were washed four times with 200µl of PBS 0.05% Tween 20. OD values > the mean
126 OD + 2 standard deviation (SD) in non-immunized mice were considered positive.

127 *IgG deposits*

128 The presence of IgG deposits in muscle plaques was assessed. Briefly, muscle tissue obtained
129 from the quadriceps after euthanasia was frozen in methylbutane at -150 to -160°C and cooled
130 with liquid nitrogen. Next, cross-sections (thickness: 8 µm) were obtained and fixed with
131 acetone for 5 minutes. The sections were washed in PBS and blocked with goat serum (Gibco;
132 Life Technologies, Auckland, New Zealand) for 30 minutes and incubated with goat anti-mouse
133 IgG conjugated with Alexa Fluor 488 (Invitrogen; Eugene, OR, USA) for 1 hour. The sections
134 were washed with PBS and incubated with alpha-Bungarotoxin Alexa fluor 555 (Invitrogen) for

135 1 hour. The sections were then washed again, mounted, and observed under a fluorescent
136 microscope (Axioskop 2 plus; Zeiss, Göttingen, Germany). To acquire images, we use the
137 software ZEN from Zeiss. To avoid bias when analyzing sections, we used the auto contrast
138 tool. This highlights positive signal vs background.

139

140 *Statistical analysis*

141 The Kolmogorov–Smirnov test was used to test data distribution normality. All variables are
142 reported as means \pm SD. Correlation analyses were carried out with Pearson’s or Spearman’s
143 correlation according to the Gaussian distribution. Cohen’s kappa coefficient was used to
144 measure agreement between the RIA and ELISA results. Fisher’s exact test was used to
145 compare the sensitivity and specificity of the tests. The SPSS statistical software (V.21.0) was
146 used to determine the Cohen’s kappa coefficient. GraphPad Prism 7 was used to perform the
147 Fisher’s exact test and correlations. P-values $<$ 0.05 were considered significant.

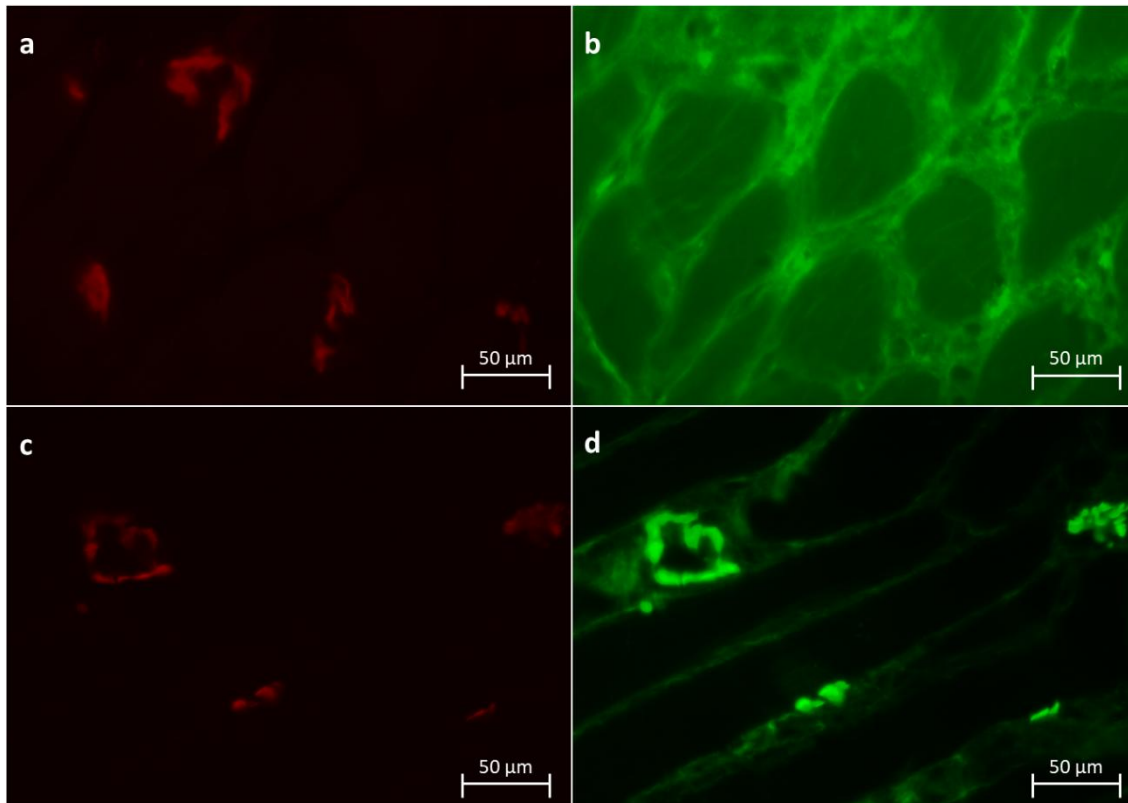
148

149

150 **Results**

151 *RIA validation*

152 We evaluated AChR antibody levels in serum samples obtained from 104 mice (52 non-
153 immunized and 52 immunized). In the immunized mice, blood was extracted on day 53 (25
154 days post-immunization). The mean (SD) concentration was 0.02 (\pm 0.04) nM for the non-
155 immunized mice and 3.42 (\pm 2.5) nM for the immunized mice. The concentration was $>$ 0.25
156 nM (3.7 ± 2.4 nM) in 48 of the 52 immunized mice (92%). Immunofluorescence testing on
157 muscle sections revealed no IgG deposits in the non-immunized mice; by contrast, IgG deposits
158 were detected in the neuromuscular endplate of all 48 mice who had AChR antibodies
159 detected by RIA. Two of the four immunized mice with nM levels $<$ 0.25 nM also had IgG
160 deposits (Figure 1; Supplementary table 1).



161

162 **Figure 1. IgG deposits in the neuromuscular plate.** Representative image of the
 163 immunofluorescence with alpha-bungarotoxin (red) and anti-mouse IgG (green) in muscle of a
 164 non-immunized (a-b) and an immunized mouse (c-d).

165

166 Next, we assessed the reproducibility of RIA for the quantification of mouse AChR antibodies.
 167 We selected two mouse samples at different concentrations for the intra-assay coefficient of
 168 variability (CV) and three mouse samples at different concentrations for the inter-assay CV.
 169 The results of those tests are shown in Table 1.

170

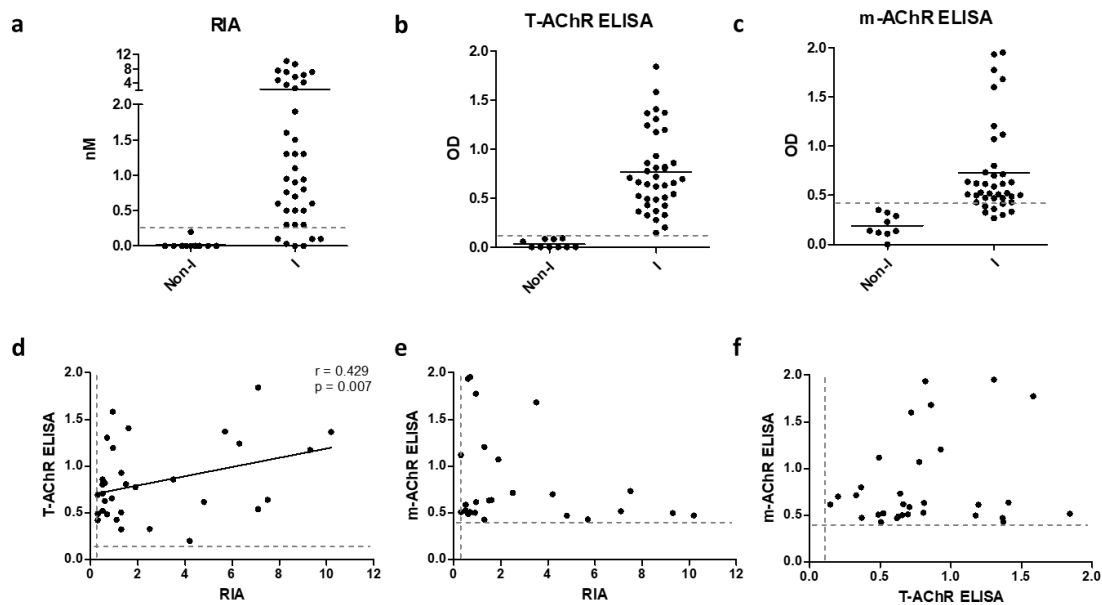
171 **Table 1. Reproducibility of the RIA assay to quantify mouse AChR antibodies**

Sample	Replicates	Mean (nM)	SD	CV (%)
Intrassay				
Mouse 1	9	1.9	0.18	9.44
Mouse 2	9	4.9	0.11	2.19
Interassay				
Mouse 1	9	1.02	0.14	14.8
Mouse 2	9	4.6	0.6	14.3
Mouse 3	9	7.4	0.9	11.7

172 **Abbreviation:** SD, standard deviation; CV, coefficient of variability.

173 *RIA comparison with ELISA*

174 Based on the interquartile range of the RIA-positive samples (1.67 – 5.53 nM), we selected a
175 total of 48 samples (46 from the initial RIA validation experiments and 2 extra samples from
176 immunized mice without anti-AChR antibodies by RIA, to increase this group). Samples were
177 categorized as follows: negative < 0.25 nM (n=10 from non-immunized mice and n=6 from
178 immunized mice); low titer: 0.25-1 nM (n=14); medium titer: 1-5 nM (n=11); and high titer > 5
179 nM (n=7). Next, we analyzed those 48 samples with T-AChR and m-AChR ELISA and compared
180 the findings to the results obtained by RIA. The mean (+2SD) values in the non-immunized
181 mice were 0.121 OD units for the T-AChR ELISA and 0.422 for the m-AChR ELISA. The results of
182 the three assays are shown in Figure 2a-c and supplementary table 1.



183

184 **Figure 2. Detection of acetylcholine receptor (AChR) antibodies by RIA and two ELISA**
185 **protocols.** AChR antibodies concentration in samples obtained from non-immunized (Non-I) or
186 immunized (I) mice as measured by RIA (a) ELISA using AChR from *T. californica* (T-AChR) (b) or
187 from mouse muscle (m-AChR) (c). Correlation between AChR antibodies levels in immunized
188 mice quantified by RIA and T-AChR (d) or m-AChR ELISA (e), and between the two ELISA
189 protocols (f). Dotted lines indicate the cut-off for the various techniques. Spearman's
190 correlation was used for the statistical analysis.

191

192 To evaluate concordance between negative and positive results by RIA and the two ELISA we
193 analyzed the Cohen's kappa coefficient. Agreement between the RIA and T-AChR ELISA was $\kappa =$
194 0.69 and between the two ELISAs was $\kappa = 0.65$; however, only moderate agreement ($\kappa = 0.49$)
195 was observed between RIA and m-AChR ELISA. In the samples from the immunized mice
196 (n=38), the correlation between RIA and T-AChR ELISA was statistically significant (Figure 2d).
197 By contrast, RIA was not significantly correlated with m-AChR ELISA (Figure 2e). No correlation
198 was observed between the two ELISA protocols (Figure 2f).

199 Table 2 shows the sensitivity and specificity values for the three assays for all tested samples
200 (n=48).

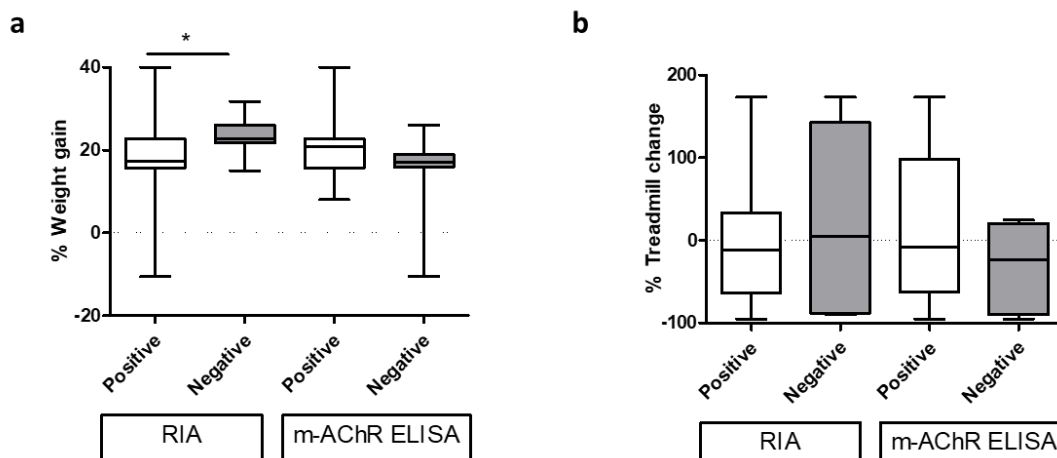
201 **Table 2. Comparison of sensitivity and specificity for RIA and ELISA in the detection of AChR**
 202 **antibodies**

	(a) RIA	(b) T-AChR ELISA	(a) vs (b) <i>p</i>	(c) m-AChR ELISA	(b) vs (c) <i>p</i>
Sensitivity (%)	84.2	100	0.02	81.6	0.01
Specificity (%)	100	100	ns	100	ns

203 **Abbreviation:** RIA, radioimmunoassay; ns, not significant.

204 *Association between RIA/ELISA and clinical scores*

205 We grouped the immunized mice according to the presence or absence of AChR antibodies.
 206 None of the immunized mice tested by T-AChR ELISA were negative and thus this ELISA was
 207 not included in the analysis. In the mice that tested negative for AChR antibodies by RIA, the
 208 body weight gain from day 0 (at immunization) to day 49 post-immunization was higher than
 209 in the mice who tested positive for AChR antibodies. However, when we grouped the mice
 210 according to the presence or absence of AChR antibodies by m-AChR ELISA (Figure 3a), there
 211 was no difference in body weight gain during the same time period. There were no differences
 212 in treadmill scores between mice with or without AChR antibodies detected by RIA or m-AChR
 213 ELISA (Figure 3b). There was no correlation between AChR antibodies titers (measured by RIA
 214 or the two ELISA protocols) and the percentage weight change or treadmill variation (data not
 215 shown).



216

217 **Figure 3. Differences in clinical scores between immunized mice with or without**
 218 **acetylcholine receptor (AChR) antibodies detected by RIA or ELISA.** Comparison of
 219 percentage weight gain (a) and percentage change in treadmill scores (b) of mice 49 days after
 220 immunization grouped according to the presence or absence of AChR antibodies by RIA or
 221 ELISA. Weight gain refers to the percentage of weight at day 49 post-immunization relative to
 222 the weight measured on day 0. Treadmill change refers to the centimeters completed on the
 223 treadmill at day 49 post-immunization relative to those on day 0. RIA positive (n=32). RIA
 224 negative (n=6). m-AChR ELISA positive (n=31). m-AChR ELISA negative (n=7). The Mann-
 225 Whitney test was used for the statistical analysis. * *p*<0.05.
 226

227 **Discussion**

228 The results of this study show that a commercial RIA kit originally designed for the diagnosis of
229 MG in humans can be modified to reliably detect AChR antibodies in mice. The advantage of
230 this approach is that it allows researchers to use an off-the-shelf RIA kit, thus eliminating the
231 need to develop the entire methodology in-house and may also reduce inter and intra-
232 laboratory variability.

233 RIA was the method of choice over other commercially available ones for human anti-AChR
234 antibody detection, such as the cell-based assay (CBA) or the ELISA, for different reasons. First,
235 RIA is the method validated in larger patient cohorts; second, ELISA has proven a lower
236 specificity than the RIA in humans; finally, fixed-CBA requires specialized training for result
237 interpretation to achieve high sensitivity[16]. Nevertheless, it would be advisable to choose
238 CBA in terms of sensitivity and specificity, but this is a more expensive and time-consuming
239 method.

240 AChR antibodies were detected by RIA in 92% of the 52 immunized mice and 96% had IgG
241 deposits. These findings are consistent with previous reports in EAMG models [13,14]. In
242 EAMG, induction of the humoral response can be assessed by using an assay such as ELISA to
243 detect AChR antibodies or through immunofluorescence techniques to detect IgG deposits
244 located at the NMJ [13,14]. We found good agreement between the modified RIA kit and T-
245 AChR ELISA ($\kappa = 0.69$) but only moderate agreement between RIA and m-AChR ELISA ($\kappa = 0.49$).
246 T-AChR and m-AChR ELISA showed moderate agreement. However, the RIA results were only
247 significantly correlated with T-AChR ELISA, but not with m-AChR ELISA.

248 In the EAMG model, antibodies against T-AChR can destroy the NMJ, leading to epitope
249 spreading and the possible development of antibodies against m-AChR [17,18]. For this reason,
250 a mix of antibodies targeting T-AChR, m-AChR, and/or both may be present in EAMG mice.
251 Analysis using the Basic Local Alignment Search Tool (BLAST) shows that the AChR alpha
252 subunit in mice and humans share a 95.51% sequence homology, which is why human AChR is
253 an excellent antigenic source for the detection of pathogenic AChR antibodies in the EAMG
254 model. In this regard, since *T. californica* shares about 80% of the AChR alpha subunit peptide
255 sequence with humans and mice, the presence of some T-AChR antibodies might be irrelevant
256 to disease pathogenesis[14]. Therefore, it would seem to be more appropriate to measure
257 AChR antibodies using a source other than the one used in immunization, as we have done in
258 this study.

259 With regard to sensitivity and specificity, the most sensitive test was T-AChR ELISA followed by
260 RIA (Table 2). As expected, T-AChR ELISA was more sensitive because the mice were
261 immunized with T-AChR. In the study by Pachner et al., who compared T-AChR RIA to T-AChR
262 ELISA (both developed in-house), the sensitivity of both assays was high[8], a finding that
263 suggests that the differences in sensitivity observed in our study are most likely due to the
264 antigenic source rather than to the methodology. We used human AChR as the antigenic
265 source for the RIA assay, which would explain the difference in sensitivity compared to
266 Pachner et al., since our mice were immunized with T-AChR. The lower sensitivity of the m-
267 AChR ELISA could be explained by the fact that we used a peptide of the α -subunit of AChR
268 (rather than the whole receptor) to coat the wells, which means that antibodies directed
269 against conformational antigens or other AChR subunits may have remained undetected. The
270 suitability of human AChR to detect AChR antibodies in the EAMG model opens the possibility
271 of exploring other methodologies, such as CBA, that is among the gold-standard methods
272 more commonly used in the clinic.

273 In our study, AChR-negative mice (tested by RIA) experienced a significant increase in body
274 weight at day 49 compared to the AChR-positive mice. By contrast, when the mice were

275 grouped according to m-AChR ELISA test results (positive vs. negative), there was no difference
276 in the percentage of body weight gain. These findings are consistent with those reported by
277 Vaughan et al., who also observed that modified RIA is more accurate than ELISA
278 protocols[19]. Although Wu et al. found that pathogenic antibodies in EAMG models are cross-
279 reactive with m-AChR, we did not find any difference in the percentage of body weight gain
280 between mice with or without AChR antibodies detected by m-AChR ELISA[14]. Similarly, we
281 found no differences on treadmill test scores between mice with or without AChR antibodies
282 detected by either RIA or m-AChR ELISA. Other authors have found that only 50-70% of
283 immunized mice develop muscle weakness, even with the presence of clear neuromuscular
284 dysfunction[12]. In this regard, further experiments are needed to elucidate the pathogenicity
285 of AChR antibodies detected by the various assays. However, regardless of the pathogenicity of
286 these antibodies, testing may still be useful in EAMG models in order to confirm that a
287 humoral response to AChR has been established as part of the disease characterization and
288 follow-up.

289 To our knowledge, this study is the first to use human AChR as the antigenic source for the
290 detection of serum AChR antibodies in mice immunized with T-AChR. Crucially, we used the
291 whole human AChR, which is highly homologous to mouse AChR, with excellent sensitivity
292 (84.1%), specificity (100%), and reproducibility. As our results demonstrate, a commercially-
293 available RIA kit for human AChR antibodies can be successfully modified to detect AChR
294 antibodies in the EAMG model, thus eliminating the need to develop and validate an in-house
295 system.

296 **Author contributions.** Conceptualization AM and EG; Data curation CM, LG and AM; Formal
297 analysis AM; Funding acquisition BB and EG; Investigation AM, CM, LG, EM, CJ, LQ, RL and EG;
298 Supervision EG; Roles/Writing - original draft AM, RL, CJ and EG; and Writing - review & editing
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311

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Mice	Sex	Weight			Treadmill score			IgG deposits	RIA		T-AChR ELISA		m-AChR ELISA	
		Day 0	Day 49	% gain 49 vs 0	Day 0	Day 49	% gain 49 vs 0		Nm	Conclusion	OD	Conclusion	OD	Conclusion
1	M	24	28	16,7	119,6	142,8	19,40	Yes	0,5	P	0,859	P	0,302	N
2	M	22	26	18,2	329,1	288,8	-12,25	Yes	0,3	P	0,422	P	0,267	N
3	F	19	22	15,8	177,1	374,6	111,52	Yes	0,5	P	0,805	P	0,528	P
4	M	23	29	26,1	134,5	6,7	-95,02	Yes	0,5	P	0,706	P	0,589	P
5	M	22	26	18,2	329,1	288,8	-12,25	Yes	0,3	P	0,695	P	0,511	P
6	M	22	27	22,7	329,1	33,5	-89,82	Yes	0,6	P	0,819	P	1,936	P
7	F	19	22	15,8	253,4	324,3	27,98	Yes	0,6	P	0,628	P	0,489	P
8	F	19	22	15,8	177,1	374,6	111,52	Yes	0,3	P	0,490	P	1,119	P
9	M	23	27	17,4	88,4	54,2	-38,69	Yes	0,76	P	1,306	P	1,955	P
10	M	22	27	22,7	172,8	419,1	142,53	Yes	0,8	P	0,522	P	0,523	P
11	M	23	27	17,4	88,4	54,2	-38,69	Yes	0,94	P	1,582	P	1,775	P
12	M	23	27	17,4	224,0	168,1	-24,96	Yes	0,9	P	0,656	P	0,502	P
13	M	23	27	17,4	78,2	24,8	-68,29	Yes	1,5	P	0,810	P	0,634	P
14	M	22	27	22,7	123,7	338,3	173,48	Yes	1,3	P	0,929	P	1,206	P
15	M	26	30	15,4	118,9	10,9	-90,83	Yes	0,95	P	1,196	P	0,616	P
16	M	25	29	16,0	155,8	194,3	24,71	Yes	1,1	P	0,428	P	0,388	N
17	M	23	29	26,1	134,5	6,7	-95,02	Yes	1,3	P	0,324	P	0,362	N
18	M	22	25	13,6	110,4	152,8	38,41	Yes	1,9	P	0,776	P	1,072	P
19	F	19	22	15,8	115,6	272,8	135,99	Yes	1,6	P	1,407	P	0,638	P
20	F	19	22	15,8	253,4	324,3	27,98	Yes	1,3	P	0,505	P	0,429	P
21	M	23	28	21,7	197,0	181,1	-8,07	Yes	3,5	P	0,859	P	1,682	P
22	M	24	28	16,7	132,4	85,4	-35,50	Yes	5,7	P	1,371	P	0,431	P
23	M	22	25	13,6	178,9	195,2	9,11	Yes	2,5	P	0,328	P	0,716	P
24	M	23	29	26,1	68,2	85,6	25,51	Yes	7,5	P	0,640	P	0,734	P
25	M	24	29	20,8	86,8	6,7	-92,28	Yes	10,2	P	1,367	P	0,473	P
26	M	22	25	13,6	146,4	95,8	-34,56	Yes	7,1	P	0,540	P	0,326	N
27	M	23	29	26,1	235,1	87,2	-62,91	Yes	4,8	P	0,618	P	0,471	P
28	M	25	27	8,0	70,1	24,5	-65,05	Yes	9,3	P	1,175	P	0,499	P
29	F	19	23	21,1	120,8	239,3	98,10	Yes	7,1	P	1,842	P	0,519	P
30	F	19	17	-10,5	242,2	-	-	Yes	6,3	P	1,242	P	0,416	N
31	M	22	27	22,7	172,8	419,1	142,53	Yes	0,1	N	0,663	P	0,620	P
32	M	20	28	40,0	-	-	-	Yes	0,7	P	0,486	P	0,509	P
33	M	22	27	22,7	-	-	-	Yes	4,2	P	0,201	P	0,702	P
34	M	22	29	31,8	85,4	230,5	169,90	Yes	0,1	N	0,364	P	0,801	P
35	M	22	27	22,7	87,3	33,5	-61,63	No	0	N	0,718	P	1,601	P
36	M	23	28	21,7	193,2	22,7	-88,25	Yes	0,03	N	0,278	P	0,332	N
37	M	19	25	31,6	108,4	152,3	40,49	Yes	0,1	N	0,146	P	0,617	P
38	F	20	23	15,0	332,0	349,2	5,18	No	0	N	0,368	P	0,474	P

372

373 **Supplementary table 1. Clinical data and anti-AChR antibodies result by RIA, T-AChR ELISA**
374 **and m-AChR ELISA. Nm=nanomolar; P=positive; N=negative.**