



Analysis of nAChR Autoantibodies Against Extracellular Epitopes in MG Patients

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Myasthenia gravis (MG) is an autoimmune disorder caused by autoantibodies targeting components of the postsynaptic membrane of the neuromuscular junction (NMJ), leading to neuromuscular transmission deficiency. In the vast majority of patients, these autoantibodies target the nicotinic acetylcholine receptor (nAChR), a heteropentameric ion channel anchored to the postsynaptic membrane of the NMJ. Autoantibodies in patients with MG may target all the subunits of the receptor at both their extracellular and intracellular regions. Here, we combine immunoadsorption with a cell-based assay to examine the specificity of the patients' autoantibodies against the extracellular part of the nAChR. Our results reveal that these autoantibodies can be divided into distinct groups, based on their target, with probably different impacts on disease severity. Although our findings are based on a small sample group of patients, they strongly support that additional analysis of the specificity of the autoantibodies of patients with MG could serve as a valuable tool for the clinicians' decision on the treatment strategy to be followed.

Keywords: myasthenia, autoantibodies, anti-nAChR antibodies, cell-based assay, diagnosis of myasthenia, immunoadsorption

INTRODUCTION

Myasthenia gravis (MG) is a well-characterized autoimmune disorder caused by autoantibodies (autoAbs) targeting molecules of the neuromuscular junction (NMJ). In MG, the signal transduction caused by the neurotransmitter acetylcholine is impaired and muscle weakness and fatigability occur (1–4).

To date, various MG-specific autoAbs have been identified. One case is the autoAbs against the muscle nicotinic acetylcholine receptor (nAChR) (5), which act according to one of the following three pathogenic mechanisms: (a) activation of the complement at the NMJ, which causes destruction of the typical folds in the sarcolemma, (b) antigenic modulation, which leads to internalization and degradation of the surface nAChR, or (c) blocking of the acetylcholine binding and consequently of the channel opening (6). AutoAbs against the muscle-specific kinase (MuSK) (7) and low-density lipoprotein receptor-related protein 4 (LRP4) (8–10) block the interactions of MuSK and LRP4 and affect the maintenance of the NMJ (6, 11). In addition, other autoAbs with unknown pathogenicity directed against agrin, cortactin, titin, and ryanodine receptor have also been detected in patients' sera with MG (12–16).

The nAChR is a ligand-gated ion channel anchored to the NMJ (17). In humans, two subtypes of the muscle nAChR have been identified, the fetal and the adult subtype. Both the subtypes are heteropentamers composed of 4 subunits forming pentameric assemblies with a stoichiometry of $2\alpha1:\beta1:\epsilon:\delta$ (adult subtype) or $2\alpha1:\beta1:\gamma:\delta$ (fetal subtype) (18–20). Each subunit consists of a ~ 210 amino acid extracellular domain (ECD), bearing the epitopes for potential pathogenic autoAbs (21, 22). Although the $\alpha1$ subunit hosts the main immunogenic region, patients with MG also harbor autoAbs against the non- $\alpha1$ subunit-ECDs (22–25). AutoAbs against the $\alpha1$ subunit of the nAChR are characterized as more pathogenic than those against the $\beta1$ subunit (26). Furthermore, autoAbs against the γ subunit trigger arthrogryposis in newborns and recognize the fetal subtype of the nAChR on the extraocular muscle in adults (27–30). Thus, the subunit specificity of the anti-nAChR autoAbs seems to influence disease severity.

Currently, the gold standard technique for anti-nAChR autoAbs detection and quantification is a radioimmunoprecipitation assay (RIPA), performed with a mixture of solubilized fetal and adult human nAChR bound to the [125 I]-labeled antagonist α -bungarotoxin. RIPA is a reliable technique that provides an accurate estimation of the anti-nAChR autoAbs titer (5, 31). The anti-nAChR autoAbs titer does not correlate with disease severity when patients are compared, although fluctuations in the anti-nAChR autoAbs concentration in an individual patient have been reported to correlate with the severity of muscle weakness and to predict exacerbations. Thus, repeated testing for autoAbs can influence therapeutic decisions (2). Other techniques with good sensitivity and specificity for the detection of the anti-nAChR autoAbs, namely, ELISA, luciferase and fluorescence immunoprecipitation assays, exist. However, these assays have not been widely adopted in clinical practice (2, 32–34). Recently, cell-based assays (CBAs) for the detection of anti-nAChR autoAbs have been developed (35). In brief, CBA utilizes either transiently or stably co-transfected cells with plasmids encoding the five subunits of the nAChR and rapsyn. This co-transfection results in overexpression of the native nAChR on the cell membrane, mimicking the tightly clustered nAChRs on the NMJ. Thus, in addition to other techniques, CBA allows the detection of conformational dependent anti-nAChR autoAbs that recognize discontinuous epitopes and clustered nAChRs (36–41). It has been reported that 16–66% of seronegative patients with MG have autoAbs against the clustered nAChR, detected by CBA (42–46).

Here, we studied the specificity of the anti-nAChR autoAbs in sera, derived from a group of 20 anti-nAChR positive patients with MG at different time points. First, we investigated how many of these patients possess autoAbs against extracellular parts of the nAChR by CBA. We were surprised to find that 7 out of the 20 patients with MG were CBA negative (CBA $-$), which suggests that they mainly have autoAbs against intracellular parts of the receptor, since these patients were RIPA positive against the native nAChR. Then, only for the CBA positive sera (CBA $+$), we tested by immunoadsorption the subunit specificity of the autoAbs. Following the immunoadsorption of autoAbs against

specific subunit-ECDs, we tested the remaining autoAbs by: (a) RIPA to quantify the percentage of the unbound autoAbs and (b) CBA to test if all the autoAbs against extracellular parts of the nAChR were depleted. Based on our findings, we could divide the tested patients with MG into four groups, according to the target of their autoAbs, which possibly reflects differences in their clinical phenotype.

MATERIALS AND METHODS

Patients

Sera from patients with nAChR-MG, confirmed by RIPA, were provided by the diagnostic department of the Hellenic Pasteur Institute (HPI). The sera samples used were collected from at least two different time points for most of these patients. In total, 55 sera were collected from 20 patients with MG (Table 1). Clinical data from 9 patients are available and given in Table 1.

Statement of Ethics

The studies involving human participants were reviewed and approved by HPI Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Immobilization of Purified Recombinant Proteins on CNBr-Sepharose Beads

The expressed, in yeast *Pichia pastoris*, ECDs of the human $\alpha1$, $\beta1$, γ , δ , and ϵ nAChR subunits (47, 48) were immobilized on cyanogen bromide (CNBr)-sepharose beads, after their enzymatic deglycosylation and purification, as described previously (24, 49). In brief, 0.1 mg of ECD and 0.9 mg of bovine serum albumin (BSA) (used as a carrier) were immobilized on 0.1 g of CNBr-activated sepharose beads according to the manufacturer's protocol (GE Healthcare). Following the immobilization, the ECD-carrying beads were diluted in 12 ml phosphate-buffered saline (PBS) containing 0.02% NaN₃. As a control, BSA (1 mg) was immobilized on CNBr-activated sepharose beads.

Immunoabsorption

A total of 125 fmoles of anti-nAChR autoAbs, diluted in PBS/0.2% BSA (total volume: 40 μ l), were incubated with 120 μ l of sepharose-ECD or sepharose-BSA suspension, for 2 h at room temperature (RT). After centrifugation, supernatants from the immunoabsorption columns containing the unbound anti-nAChR autoAbs were tested by RIPA and CBA.

Radioimmunoprecipitation Assay

For the quantification of the unbound anti-nAChR autoAbs, the autoAb RIPA kit (RSR, UK), containing [125 I]- α -bungarotoxin-labeled human fetal and adult muscle nAChR preparations, was used, according to the manufacturer's instructions. From the 160 μ l immunoabsorption mix, duplicates of 30 μ l samples (containing ~ 25 fmoles in case of no depletion) were added to the reaction. The percentage of immunoabsorption was estimated using the equation: $100 \times \{[\Delta\text{cpmBSA}] -$

TABLE 1 | Results of the tested sera.

	Date	Age	Onset	MGFA	anti-nAChR (nM)	Live CBA	anti- α 1 (%)	anti- β 1 (%)	anti- γ (%)
Live CBA negative									
P1	2017	n.a.	n.a.	n.a.	820	N	N	N	N
	2021	n.a.	n.a.	n.a.	496	A	N	N	N
P2	2011	n.a.	n.a.	n.a.	275	N	N	N	N
	2013	n.a.	n.a.	n.a.	450	A	N	N	N
	2018	n.a.	n.a.	n.a.	256	A	N	N	N
	2019	n.a.	n.a.	n.a.	480	A	N	N	N
P3	2017	n.a.	n.a.	n.a.	97	A	N	N	N
	2019	n.a.	n.a.	n.a.	39	A	N	N	N
	2020	n.a.	n.a.	n.a.	28	A	N	N	N
P4	2017	n.a.	n.a.	n.a.	65	N			
	2018	n.a.	n.a.	n.a.	46	A			
P5	2011a	28	Early	IIA	272	N			
	2011b	28		IIA	277	N			
	2018	35		PR	130	N			
P6	2010	33	Early	I	341	N			
	2019	42		I	310	N			
P7	1999	51	Early	I	144	N			
	2009	61		I	145	N			
Anti-α1 autoAbs									
P8	2018	79	Late	I	8.8	P	57.84 (\pm 6.07)	N	N
	2019	80		V	36	P	58.38 (\pm 8.83)	N	N
P9	2017	n.a.	n.a.	n.a.	32	P	38.38 (\pm 4.08)	N	N
	2018	n.a.		n.a.	36	P	27.55 (\pm 1.82)	N	N
P10	2016	n.a.	n.a.	n.a.	82	P	87.43 (\pm 0.08)	N	N
	2017	n.a.		n.a.	40	P	88.06 (\pm 2.16)	N	N
	2020	n.a.		n.a.	33	P	88.00 (\pm 1.06)	N	N
P11	2016	n.a.	n.a.	n.a.	16	P	90.70 (\pm 1.65)	N	N
	2017	n.a.		n.a.	9	P	93.20 (\pm 1.32)	N	N
	2020	n.a.		n.a.	18.7	P	85.47 (\pm 0.31)	N	N
P12	2011	n.a.	n.a.	n.a.	165	P	90.69 (\pm 0.67)	N	N
	2012	n.a.		n.a.	77	P	89.61 (\pm 0.12)	N	N
	2019	n.a.		n.a.	48	P	92.59 (\pm 0.13)	N	N
Non anti-α1 autoAbs									
P13	2016	73	Late	I	165	P	N	N	65.33 (\pm 2.48)
	2018	75		I	160	P	N	N	67.50 (\pm 3.28)
P14	2016	n.a.	n.a.	n.a.	26	P	N	76.45 (\pm 0.64)	N
	2020a	n.a.		n.a.	153	P	N	39.69 (\pm 1.43)	N
	2020b	n.a.		n.a.	89	P	N	41.52 (\pm 4.58)	N
	2021	n.a.		n.a.	43	P	N	51.90 (\pm 1.82)	N
P15	2013	n.a.	n.a.	n.a.	420	P	N	60.83 (\pm 2.21)	N
	2016	n.a.		n.a.	193	P	N	52.08 (\pm 2.33)	N
	2020	n.a.		n.a.	208	P	N	57.17 (\pm 1.53)	N
Anti-α1 and non anti-α1 autoAbs									
P16	2017	36	Early	IIA	98	P	27.97 (\pm 3.97)	N	16.15 (\pm 0.83)
	2020	39		IIA	246	P	25.07 (\pm 4.51)	N	15.83 (\pm 7.85)
P17	2007	61	Late	IVB	25.4	P	18.21 (\pm 5.59)	20.01 (\pm 0.73)	34.50 (\pm 4.48)
	2017a	71		IIA	3.5	P	17.51 (\pm 4.32)	14.03 (\pm 3.18)	63.50 (\pm 4.47)
	2017b	71		IIA	2.5	P	16.11 (\pm 1.73)	18.51 (\pm 5.06)	53.51 (\pm 2.13)
	2020	74		IIA	22	P	N	N	38.53 (\pm 5.43)

(Continued)

TABLE 1 | Continued

	Date	Age	Onset	MGFA	anti-nAChR (nM)	Live CBA	anti- α 1 (%)	anti- β 1 (%)	anti- γ (%)
P18	2011a	n.a.	n.a.	n.a.	65	P	37.37 (\pm 5.16)	N	36.51 (\pm 0.86)
	2011b	n.a.	n.a.	n.a.	14	P	38.38 (\pm 1.54)	N	34.93 (\pm 0.95)
	2017	n.a.	n.a.	n.a.	7.6	P	N	N	50.71 (\pm 0.99)
	2019	n.a.	n.a.	n.a.	12.7	P	59.66 (\pm 1.69)	N	18.26 (\pm 1.36)
P19	2007	48	Early	IVB	8.2	P	56.85 (\pm 6.70)	N	N
	2009	50		IIIB	5	P	N	50.94 (\pm 0.85)	N
P20	2015	34	Early	IVB	11	P	17.51 (\pm 5.96)	N	25.15 (\pm 3.74)
	2018	37		IIB	9.6	P	26.51 (\pm 6.01)	N	35.01 (\pm 7.07)
	2020	39		IIB	198	P	N	44.12 (\pm 4.94)	33.21 (\pm 3.17)

Patients with myasthenia gravis (MG) are grouped by autoAbs specificity. The year of the sample collection, the age of the patients, and the time of disease onset are listed. The distribution and severity of myasthenic weakness were classified according to the MG Foundation of America (MGFA) grading system. The titer of the anti-nAChR autoAbs is given as estimated by RIPA. All the sera were tested for the presence of anti-nAChR autoAbs targeting the extracellular part of the receptor by CBA. The sera were also tested for the presence of the autoAbs against each ECD of the five subunits of the receptor and the percentage of immunoadsorption presented here was estimated as described in "Materials and Methods" section. The average percentage of immunoadsorption from three experiments is presented. In parenthesis, the numbers refer to the \pm SD of the immunoadsorption percentage between the different experiments (there was no depletion of autoAbs after the treatment with δ and ϵ ECD sepharose beads and thus these are not shown in the table).

N, negative; A, ambiguous; P, positive; n.a., not available; PR, pharmacology remission.

$[\Delta\text{cpmECD}]/[\Delta\text{cpmBSA}]$, where Δcpm is the cpm of [^{125}I]- α -bungarotoxin-labeled nAChR (provided in the RSR kit) precipitated by the serum minus that precipitated by a control normal human serum and ΔcpmBSA and ΔcpmECD are the corresponding Δcpm values for samples incubated with immobilized BSA or nAChR-ECD, respectively.

Cell-Based Assay

The CBA was performed as described by Leite et al. (35). Briefly, HEK293T cells were transiently co-transfected with the plasmids encoding for human α 1, β 1, γ , δ , and ϵ nAChR subunits and for rapsyn in a ratio of 2:1:1:1:1, respectively. Transfection was performed with polyethylenimine (Polyplus). After 48 h, the transfected cells were incubated with serum (20 fmoles of anti-nAChR autoAbs) or supernatant from the immunoadsorption mixture (30 μ l containing \sim 20 fmoles if no depletion occurred) for 1 h at RT. Afterwards, cells were fixed in 10% formalin solution (Sigma-Aldrich) for 10 min at RT. Patients' anti-nAChR autoAbs were detected after incubation of the cells for 1 h at RT with Alexa Fluor-555 conjugated anti-human IgG Ab (Life Technologies, Invitrogen) in 1:750 dilution. The presence of nAChR on the cell surface was verified by staining with Alexa Fluor-488 labeled α -bungarotoxin (Life Technologies, Invitrogen) in 1:1,000 dilution. Cells were examined under an Olympus IX51 fluorescence microscope by 2 observers.

RESULTS

Detection of AutoAbs Against the Extracellular Parts of the nAChR

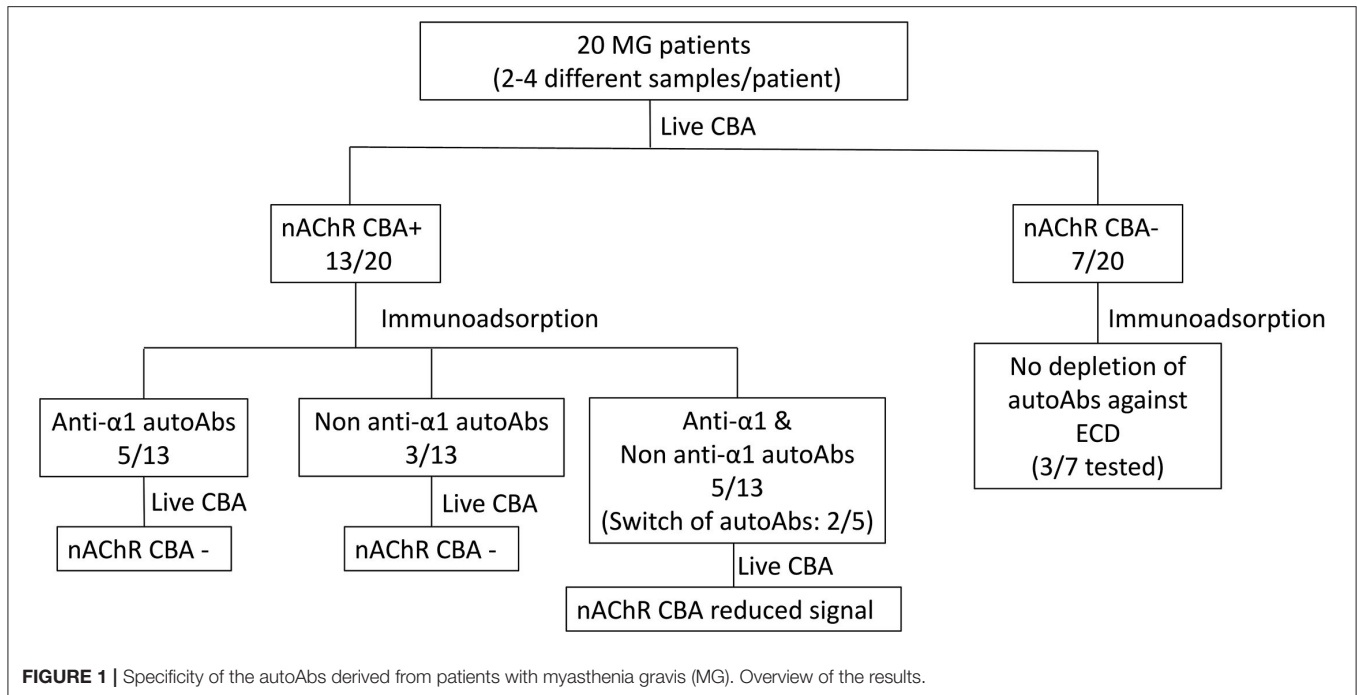
We used sera from patients who were tested positive for the presence of anti-nAChR autoAbs at the diagnostic department of the HPI. We chose 20 patients with MG, from whom a recent and at least one previous serum sample were available (55 sera in total). In addition, clinical data for 9 out of the 20 patients were available (Table 1). The anti-nAChR autoAbs titer

was estimated in all the sera by RIPA (Table 1), which detects autoAbs targeting both the extra- and intracellular parts of the nAChR, since solubilized intact nAChRs are used (5, 31). We tested samples containing 20 fmoles of anti-nAChR autoAbs from all sera by CBA. This assay detects only the potential pathogenic autoAbs against adult and fetal subtypes of the nAChR extracellular part (38). We found 18 sera, derived from 7 patients with MG, negative or ambiguous by CBA (Figure 1 and Table 1). This suggests that these patients with MG do not harbor autoAbs targeting extracellular parts of the nAChR or that these autoAbs could not be detected by this method, due to their low concentration in the serum. Interestingly, 3 out of these 7 patients, of whom the clinical data were available, belong to the I-IIA scale according to the MG Foundation of America (MGFA) clinical classification (Table 1).

Depletion of AutoAbs Against the Extracellular Domain of the nAChR Subunits

To characterize the autoAbs' subunit-ECD specificity, depletion of autoAbs against the various nAChR ECDs from serum samples was achieved by immunoadsorption (Figure 1). For the following experiments, we used immunoadsorption protocols previously established in our laboratory (24, 49). Each immunoadsorption column contained sepharose beads with immobilized either one of the ECDs of the five nAChR subunits (α 1, β 1, γ , δ , or ϵ) or only BSA (23, 24, 49). To ensure that all the autoAbs incubated with the immobilized ECDs could be depleted, we used 0.125 pmoles antibodies (Abs)/mg of ECD-sepharose beads, since the capacity of the columns was determined in previous studies to be 1.5 pmoles Abs/mg of immobilized α 1-ECD sepharose beads and 5 pmoles Abs/mg of β 1-ECD sepharose beads (24, 49).

All the CBA+ MG patients' sera were incubated with the 6 proteins (α 1-, β 1-, γ -, δ -, ϵ -ECD, and BSA) immobilized on sepharose beads; unbound autoAbs were then quantified by RIPA and the percentage of immunoadsorption by each column was



calculated (**Figure 1** and **Table 1**). From the group of CBA– MG patients, only 3 out of the 7 patients were chosen to be tested by immunoabsorption to verify the absence of any extracellular autoAbs. As expected, there was practically no depletion of autoAbs after incubation with columns containing beads with immobilized nAChR ECDs (**Figure 1** and **Table 1**), confirming the CBA result.

After immunoabsorption, the depleted sera from CBA+ MG patients were further qualified by CBA. More specifically, we investigated if all the autoAbs directed against extracellular epitopes of the nAChR were removed. Based on these experiments, we divided the MG patients tested into the three distinct groups, as given in **Figure 1** and described below:

(a) Patients With MG Harboring autoAbs Against the $\alpha 1$ Subunit.

Five out of 13 CBA+ patients (P8–P12) had anti- $\alpha 1$ autoAbs (**Figure 1** and **Table 1**). After immunoabsorption with the immobilized $\alpha 1$ -ECD, these sera were found negative or ambiguous by CBA (**Figures 2A–F**), suggesting that the vast majority of autoAbs targeting extracellular epitopes were depleted by immunoabsorption. Also, data from P8 revealed that an increase of the anti- $\alpha 1$ autoAbs attributed to a higher MGFA score (**Table 1**).

(b) Patients With MG Harboring autoAbs Against the non- $\alpha 1$ Subunits.

Three out of 13 CBA+ patients had non-anti- $\alpha 1$ autoAbs (**Figure 1** and **Table 1**); one patient had anti- γ autoAbs (P13) and two had anti- $\beta 1$ autoAbs (P14, P15). After immunoabsorption, all sera of the P13 that had been incubated with the γ - immobilized ECD and all sera of the P14 and P15, incubated with the

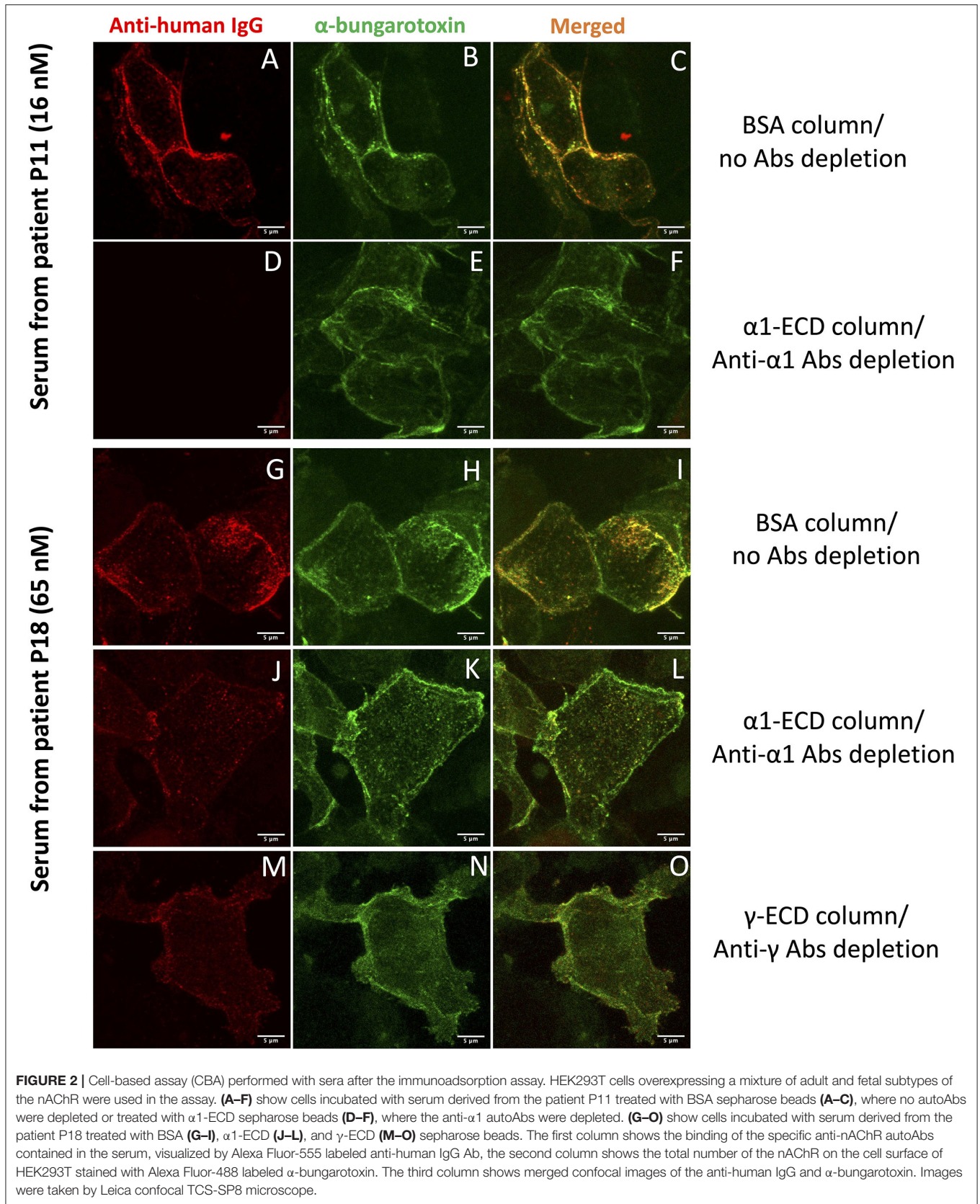
$\beta 1$ -immobilized ECD were negative or ambiguous by CBA. Moreover, P13 who harbors anti- γ autoAbs belongs to the MGFA-I clinical classification, indicating ocular MG (**Table 1**).

(c) Patients With MG Harboring autoAbs Against the $\alpha 1$ and non- $\alpha 1$ Subunits.

Five out of 13 CBA+ patients had anti- $\alpha 1$ and non-anti- $\alpha 1$ autoAbs. More specifically, P16 and P18 had anti- $\alpha 1$ and anti- γ autoAbs, P17 and P20 had anti- $\alpha 1$, anti- $\beta 1$, and anti- γ autoAbs and P19 had anti- $\alpha 1$ and anti- $\beta 1$ autoAbs (**Figure 1** and **Table 1**). Interestingly, anti- $\beta 1$ autoAbs were detected in P19 and P20 for the first time at the second and third samples, respectively, while anti- $\alpha 1$ autoAbs were not detected at those time points (**Figure 1** and **Table 1**). The sera that were treated with the corresponding column when tested by CBA produced a reduced signal (**Figures 2G–O**). In some sera, the reduction of the signal in the CBA was higher than the percentage of immunoabsorption. This is probably due to the depletion of the autoAbs against extracellular epitopes of the receptor after immunoabsorption. The remaining autoAbs were still detected by RIPA but not CBA; this implies that they probably target intracellular epitopes. The clinical data for P16, P17, P19, and P20 indicate that the increase of the non-anti- $\alpha 1$ autoAbs correlates with a decrease in the MGFA score (**Table 1**).

DISCUSSION

Myasthenia gravis is an autoimmune disease caused mainly by autoAbs targeting the nAChR on the NMJ and results in the impairment of neuromuscular transmission and muscle weakness (4). Anti-nAChR autoAbs are heterogeneous and may target all the subunits of the receptor with demonstrated



different potency for inducing experimental autoimmune MG in rodent models (26, 50, 51). Moreover, epitope spreading against intracellular epitopes, may occur at later stages of MG, as shown in the experimental autoimmune MG rat model, probably due to tissue damage (52).

Here, we studied the specificity of the anti-nAChR autoAbs in sera derived from 20 anti-nAChR positive patients with MG at different time points. We found that 7 out of the 20 patients with MG were negative or ambiguous by CBA (**Figure 1** and **Table 1**). This suggests that the majority of the autoAbs found in these patients' sera is probably against the intracellular parts of the receptor, which are detectable only by RIPA. Interestingly, according to RIPA, these sera had high anti-nAChR autoAbs titer. On the other hand, one could claim that the negative signal of CBA could be attributed to a concentration of autoAbs against extracellular parts of the nAChR well-below the detection limits of the CBA. However, previous studies have also shown patients with MG to be positive by RIPA and negative by CBA (53). The majority of these patients did not have the clinical profile of a neuromuscular transmission disorder, implying that they had no pathogenic autoAbs, which probably recognized intracellular parts of the nAChR (53). Despite the absence of clinical data, we believe that 7 CBA–MG patients in this study fall into the same category as that other study.

Having detected CBA+ patients, we proceeded to further characterization of the autoAbs presented in their sera regarding their subunit specificity. We used immunoabsorption columns appropriate for the depletion of autoAbs targeting the nAChR subunits, as described previously (24, 49). In brief, the columns contained immobilized either the ECD of one of the five subunits of the nAChR ($\alpha 1$, $\beta 1$, γ , δ , or ϵ) or BSA. Each serum was incubated with each column and the unbound autoAbs were quantified by RIPA. Moreover, to qualify the unbound autoAbs which recognize extracellular parts of the nAChR, we performed CBA. It is worth mentioning that by testing each serum for the presence of autoAbs against all the five subunits, we also tested the specificity of the bound autoAbs. Based on the results from both techniques, we concluded that CBA+ MG patients tested here can be divided into the three distinct groups: (a) patients with MG harboring anti- $\alpha 1$ autoAbs (5/13), (b) patients with MG harboring non-anti- $\alpha 1$ autoAbs (3/13), and (c) patients with MG harboring anti- $\alpha 1$ and non-anti- $\alpha 1$ autoAbs (5/13) (**Figure 1** and **Table 1**). The CBA signal of all the sera after immunoabsorption was reduced in agreement with the immunoabsorption treatment (**Figures 2A–F**). In fact, in some sera, we observed a higher reduction in the CBA signal (**Figures 2G–O**), compared to the depletion of autoAbs detected in RIPA after immunoabsorption, e.g., although the immunoabsorption percentage of P8's serum after treatment with the immobilized $\alpha 1$ -ECD was only 58%, the CBA performed after the immunoabsorption produced no signal. This is probably due to the depletion of the autoAbs against the extracellular epitopes of the receptor after the immunoabsorption. The fact that the remaining autoAbs were detected by RIPA but not by CBA implies that they target intracellular epitopes. In other sera, the CBA signal

was negative or ambiguous, suggesting that most autoAbs against the extracellular part of the nAChR were depleted by immunoabsorption. In agreement with previous works, in none of the samples anti- δ or anti- ϵ autoAbs were detected (23, 24). In general, we observed that the increase of the non-anti- $\alpha 1$ autoAbs correlates with improvement in the disease manifestation (**Table 1**).

The pathogenicity of the anti- $\alpha 1$ autoAbs is well-characterized. The $\alpha 1$ subunit is immunodominant and it can induce experimental autoimmune MG in rats (51). Accordingly, in the anti- $\alpha 1$ autoAbs positive P8 patient, we observed that the increase of the anti-nAChR autoAbs titer correlates with disease deterioration (**Table 1**). The pathogenicity of the anti- $\beta 1$ autoAbs is less studied and these are thought to be less pathogenic than the anti- $\alpha 1$ autoAbs (26). In fact, in P19 the MGFA score decreased when the autoAbs specificity switched from anti- $\alpha 1$ to anti- $\beta 1$ autoAbs. Interestingly, in the P20 patient, despite the great increase of the anti-nAChR autoAbs titer, there was no change in the patient's clinical profile upon decrease of the anti- $\alpha 1$ autoAbs and increase of the anti- $\beta 1$ autoAbs (**Table 1**). Although, the pathogenicity of the anti- γ autoAbs is proved in newborns, in adults they are less pathogenic and may recognize the fetal subtype of the nAChR presented on the extraocular muscle (29, 30, 54). Indeed, P13 who is positive for anti- γ autoAbs has a low MGFA score, which indicates ocular MG (**Table 1**). Moreover, the disease symptoms improve when the anti- γ autoAbs in P17 increase over the anti- $\alpha 1$ autoAbs. Also, there was no difference in the patient's clinical profile when the anti-nAChR autoAbs titer increased, probably due to the presence of only anti- γ autoAbs (**Table 1**). By these observations, we had previously reported a double positive MG patient (anti-nAChR and anti-MuSK autoAbs positive) who was presented with MuSK phenotype (25). This patient's clinical manifestation of the disease was not affected by the increase of the anti-nAChR autoAbs titer. After immunoabsorption, we showed that in all sera from different time points, the patient had relatively small amounts of anti- $\alpha 1$ autoAbs and the vast majority of autoAbs were directed against the $\beta 1$ and γ subunits. We concluded that the patient did not show any clinical deterioration, because the pathogenic anti- $\alpha 1$ autoAbs were always in low concentration, while the increase of the anti-nAChR autoAbs titer was attributed to the increase of only the less pathogenic anti- $\beta 1$ and anti- γ autoAbs (25).

Although we do not have a complete clinical profile of all patients, our results support the idea that additional analysis of the autoAbs of patients with MG can provide additional information to the clinicians about the patients' status. This study presents the importance of the CBA technique in the MG diagnosis. It seems that some anti-nAChR positive patients with MG do not harbor pathogenic autoAbs against the extracellular parts of the nAChR or their concentration is under the detection limit of CBA, something that may affect the decision of treatment's strategy. Moreover, we conclude that anti-nAChR positive MG patients can be divided into distinct groups, based on their autoAbs specificity. Consequently, we propose the combination of RIPA and CBA for the follow-up of the MG patients. The former is to be used for the quantification of the

autoAbs and the latter for the identification of the fluctuation of the pathogenic ones.

In future studies, we aim to enlarge our sample group and continue the study of the anti-nAChR autoAbs specificity in MG patients. Moreover, we plan to collect more clinical data from patients with MG and investigate in-depth the correlation of the clinical presentation with autoAbs specificity.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by HPI Ethics Committee. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MM conducted the CBAs and immunoadsorption experiments, wrote and edited the manuscript. VZ contributed to the concept of the study, provided the clinical data of the patients, and interpreted the data. MB performed the RIPA experiments. AH conducted confocal microscopy experiments and edited the manuscript. MZ expressed the nAChR ECDs and edited the manuscript. PZ designed the experiments, interpreted the data, and edited the manuscript. All the authors reviewed and approved the final version of the manuscript.

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