universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„Establishment of a new diagnostic method for the detection of Lrp4 autoantibodies in patients with Myasthenia gravis“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2020 / Vienna, 2020

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	UA 066 834
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Molekulare Biologie UG2002
Betreut von / Supervisor:	Assoc. Prof. Priv.-Doz. Dr. Romana Höftberger

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Abstract

Myasthenia gravis (MG) is a disorder of the neuromuscular junction (NMJ). Patients with MG experience fluctuating weakness of the skeletal muscle, and it often affects selected muscle groups. In most cases, the skeletal muscle acetylcholine receptor (AChR) is the target of the autoimmune attack. If test results for anti-AChR, anti-MuSK, or anti-Lrp4 antibodies are not positive, patients are characterized as having seronegative MG. Lrp4-seropositive MG patients are predominantly female, where the age at onset is 46.5 years on average (later than for other MG patient groups). In Pevzner et al.'s study, moderate to severe weakness was manifested in 86% of Lrp4 MG patients, which makes it indistinguishable from AChR-antibody-positive patients.[15] Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are standard routine assays used for the detection of autoantibodies. For the detection of AChR and MuSK antibodies with higher sensitivity, cell-based assays (CBA) have been introduced. However, not all myasthenic antibodies can be detected with these assays.

This study intended to establish a new diagnostic test for the detection of Lrp4 autoantibodies in MG patients.

The patients' sera were first analyzed for the presence of MuSK/AChR autoantibodies. A total of 98 patients' sera were tested during the study. After analysis, the results showed that 44 samples were seronegative for AChR and MuSK autoantibodies. In the total number of seropositive sera11 were AChR positive, 16 MuSK positive, and 1 case was double-positive.
Usage of the HEK293 cell line showed that Lrp4 is not expressed on the surface in live cells regardless of the amount of DNA used, as well as the incubation period, but it was expressed in fixed and permeabilized cells. Therefore, another approach was needed. The second choice was to use NIH3T3 cell line instead the HEK293 cell line. Although the NIH3T3 cell line did not completely meet the expectations, this cell line could be a solution to the Lrp4 surface expression problem, and that requires further modification of the protocol presented in this work.

Abstrakt

Myasthenia gravis (MG) ist eine Erkrankung der neuromuskulären Synapse (NMS). Patienten mit MG erfahren eine fluktuierende Schwäche der Skelettmuskulatur, die häufig ausgewählte Muskelgruppen betrifft. In den meisten Fällen ist der Skelettmuskel Acetylcholinrezeptor (AChR) das Ziel des Autoimmunangriffs. Wenn die Testergebnisse für Anti-AChR-, Anti-MuSK- oder Anti-LRP4-Antikörper nicht positiv sind, dann haben die Patienten seronegative MG. LRP4-seropositive MG-Patienten sind überwiegend weiblich, wobei das Alter zu Beginn der Erkrankung im Durchschnitt 46,5 Jahre beträgt (später als bei anderen MG-Patientengruppen). In der Studie von Pevzner et al. zeigte sich bei 86% der LRP4-MG-Patienten eine mäßige bis schwere Schwäche, was damit keine Unterscheidung zu AChR-Antikörper-positiven Patienten zulässt. Der Radioimmunoassay (RIA) und der Enzymimmunoassay (ELISA) sind Standard-Routineuntersuchungen zum Nachweis von Autoantikörpern. Für den Nachweis von AChR- und MuSK-Antikörpern mit höherer Sensitivität wurden zellbasierte Untersuchungen (CBA) eingeführt. Allerdings können nicht alle myasthenischen Antikörper mit diesen Untersuchungen nachgewiesen werden.

Diese Arbeit versucht einen neuen diagnostischen Test für den Nachweis von Lrp4-Autoantikörpern bei MG-Patienten zu etablieren.

Die Seren der Patienten wurden zunächst auf das Vorhandensein von MuSK/AChR-Autoantikörpern untersucht. Während der Studie wurden insgesamt 98 Patientenseren getestet. Die Ergebnisse der Analyse zeigte, dass 44 Proben seronegativ für AChR- und MuSK-Autoantikörper waren. Von allen seropositiven Seren waren 11 AChR-positiv, 16 MuSK-positiv und 1 Fall war doppel-positiv.

Die Verwendung der HEK293-Zelllinie zeigte, dass Lrp4 unabhängig von der verwendeten DNA-Menge oder der Inkubationszeit nicht auf der Oberfläche lebender Zellen erschien, sondern in fixierten und permeabilisierten Zellen exprimiert. Daher war ein anderer Ansatz erforderlich. Die zweite Möglichkeit war es, anstelle der HEK293-Zelllinie die NIH3T3-Zelllinie zu verwenden.

Obwohl die NIH3T3-Zelllinie die Erwartungen nicht vollständig erfüllte, könnte diese Zelllinie eine Lösung für das Lrp4-Oberflächenexpressionsproblem darstellen, was eine weitere Änderung des in dieser Arbeit vorgestellten Protokolls erfordert.

Acknowledgments

I would like to express deep gratitude to my supervisor, Mag. Inga Koneczny, DPhil, for her guidance, encouragement, and gracious support throughout the course of my work, for her expertise in the field that motivated me to work in this area, and for her faith in me at every stage of this research.
I also wish to thank our lab team leader, Assoc.Prof.Priv.-Doz.Dr. Romana Höftberger, and all the lab members for their meaningful insights and troubleshooting for this research.

Finally, I would like to thank my family, especially my mother, father, and sister for their moral and financial support to finish this study.

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List of abbreviations

AChR – Acetylcholine receptor
BSA – Bovine serum albumin
CBA – Cell-based assay
C2C12 – Immortalized mouse myoblast cell line
DAPI – 4',6-diamidino-2-phenylindole
DMEM – Dulbecco's modified eagle medium
DMSO – Dimethyl sulfoxide
EDTA – Ethylenediaminetetraacetic acid
FACS – Fluorescence-activated cell sorting
FBS – Fetal bovine serum
FGF – Fibroblast growth factor
HEK 293 – Human embryonic kidney 293
HRP – Horseradish peroxidase
Lrp4 – Low-density lipoprotein receptor-related protein 4
L6 – Immortalized rat skeletal myoblast cell line
MG – Myasthenia Gravis
MuSK – Muscle specific kinase
NIH3T3 – Immortalized embryonic fibroblast cell line
NMJ – Neuromuscular Junction
PAGE – Polyacrylamide gel electrophoresis
PBS – Phosphate-buffered saline
Pen-Strep - Penicillin-Streptomycin
PFA – Paraformaldehyde
PIC – Protease Inhibitor Cocktail
PLL – Poly-L-Lysine
RT – Room temperature
SDS – Sodium dodecyl sulfate
TE671 – Human rhabdomyosarcoma cell line
TEMED – N,N,N',N'-tetramethylethylenediamine
WB – Western Blot

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1. Introduction

1.1 Physiology of the Neuromuscular Junction

A protracted postnatal period of maturation and life-long maintenance characterize the vertebrate neuromuscular junction (NMJ). It presents a peripheral synapse between motoneuron and skeletal muscle. Disruptions of NMJ maturation and/or maintenance are frequently observed in neuromuscular disorders. In these patients, defective neuromuscular transmission associated with structural and molecular abnormalities at the pre- and post-synaptic membranes, as well as at the synaptic cleft has been reported[2].

In neuromuscular transmission, the nerve synthesizes acetylcholine (Ach), which is stored in synaptic vesicles. When an action potential stimulates the distal motor nerve, voltage-gated calcium ion (Ca2+) channels open, and give rise to a rapid increase in nerve terminal Ca2+ concentration. This elevated concentration triggers fusion of ACh-containing synaptic vesicles to the presynaptic membrane, where they release ACh into the synaptic cleft that separates the nerve from the muscle. Here, ACh binds to nicotinic acetylcholine receptors (AChRs) on the surface of the postjunctional muscle membrane[2-4]. These ligand-gated ion channels are nonselective caption channels in the postjunctional membrane that allow influx of Na+. This results in endplate depolarization and muscle contraction[3] (Figure 1).

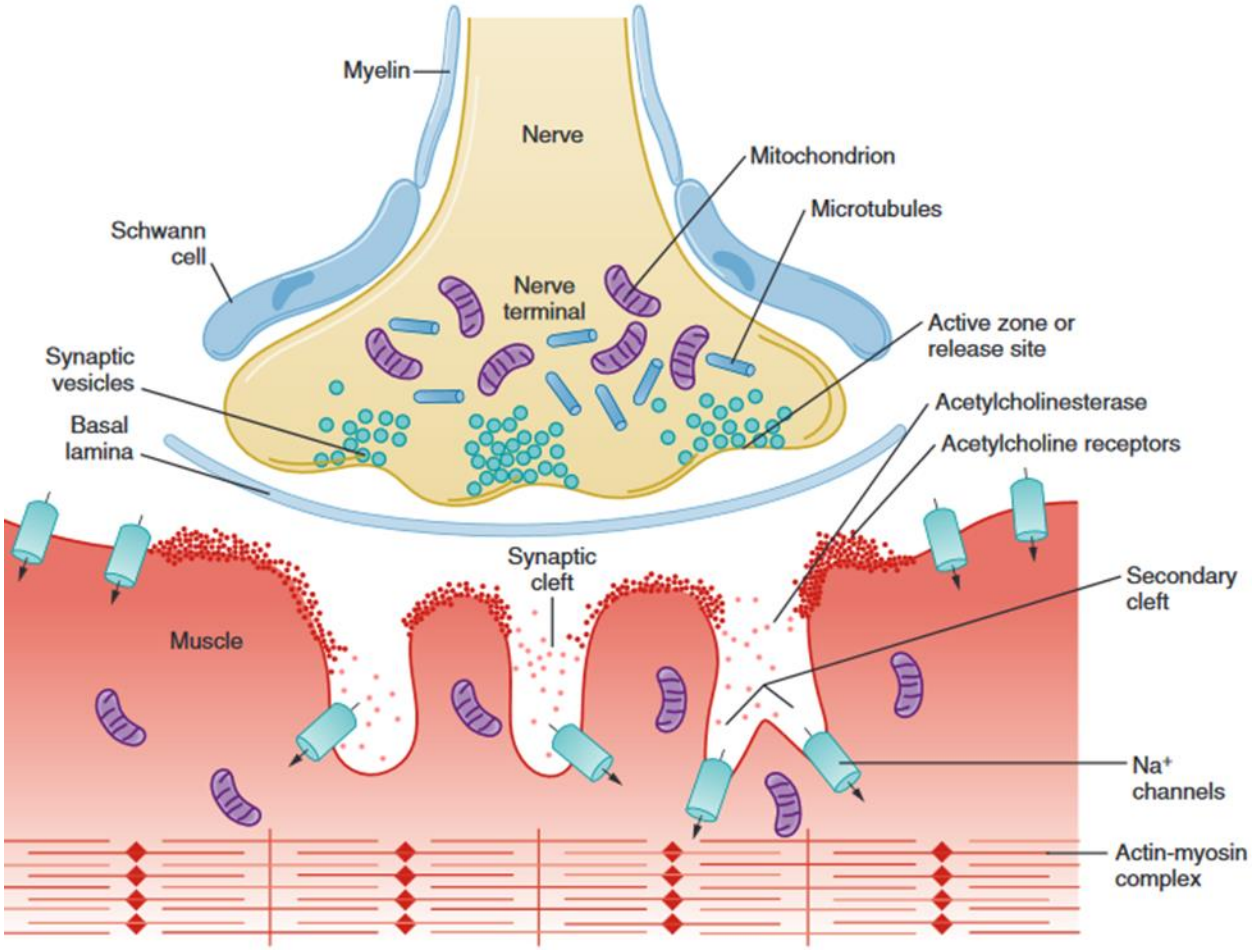

Figure 1. Structure of the neuromuscular junction. Extracted from[5]

Structure of the neuromuscular junction, composed of the motor nerve terminal, muscle fiber, and Schwann cell. As the nerve approaches the muscle fibers, it loses its myelin sheath and divides into branches that innervate many individual muscle fibers. The motor nerve without myelin is covered by the Schwann cell. The nerve terminal contains synaptic vesicles clustered about membrane thickenings, the active zones, at the synapse, and also at mitochondria and microtubules which are located further away from the synapse. A synaptic gutter or cleft, made up of a primary and many secondary clefts, separates the nerve from the muscle. The muscle surface is corrugated, and dense areas on the shoulders of each fold contain acetylcholine receptors. Sodium channels are present at the bottom of the clefts and throughout the muscle membrane. Acetylcholinesterase, as well as other proteins and proteoglycans that stabilize the neuromuscular junction, are present in the synaptic clefts.[5(413)]

The clustering of AChR is formed and maintained by post-synaptic signal pathways and it is a key mechanism for the proper function of the NMJ. Some of the known key proteins involved in the AChR clustering are agrin, rapsyn, and MuSK which forms complex with Dok7 and LRP4[5-7].

For the NMJ to function correctly, the activation of MuSK is crucial. The activation of MuSK is triggered by the binding of agrin to Lrp4 on the extracellular domain. The Lrp4-agrin binary complex dimerizes and forms an agrin-Lrp4 tetramer. This increases the affinity for MuSK and leads to the conformational change of MuSK and consequent autophosphorylation[8]. The activation of MuSK additionally requires interaction with the Dok7 protein. This protein contains a phosphotyrosine-binding domain and a C-terminal region with tyrosine residues for phosphorylation – the proteins are vital[9]. Mice deficient in MuSK, agrin, Lrp4 or Dok7 die prematurely from respiratory failure, due to having no postsynaptic NMJ. AChR clusters are accumulated and stabilized after MuSK is activated, and the downstream signaling pathway is triggered[6,7]. AChR-rapsyn interaction is boosted by the agrin stimulation which promotes linkage of AChR to the cytoskeleton. Rapsyn is crucial for the stabilization and clustering of AChR, and it interacts with it directly[10].

1.2 Myasthenia Gravis

Myasthenia gravis (MG) is a disorder of the neuromuscular junction (NMJ). It is a relatively rare disease, affecting approximately 20 per 100,000 people globally[2], but over time, prevalence has increased. This could be because of the improved diagnosis of MG, and an increasing longevity of the population[12,45]. It is affected by sex and age, where women are affected three times more often than men in early adulthood, and after 50 years of age, MG is higher in men. In Europe and North America, childhood MG is uncommon (10-15% cases), where it is much more common in Asian countries (50% of patients have disease onset aged <15 years)[10].

Patients with MG experience fluctuating weakness of the skeletal muscle and it often affects selected muscle groups. In most cases, the nicotinic AChR which is expressed in skeletal muscle is the target of the autoimmune attack. Non-AChR components of the neuromuscular junction (the muscle-specific receptor tyrosine kinase (MuSK), lipoprotein-related protein 4 (Lrp4), or agrin) can also be targeted[11]. Dysfunction of muscle endplate and muscle weakness are a result of the attack. Patients can experience different degrees of axial weakness, diplopia, dysphagia, dysarthria, ptosis, dyspnea, fatigable limb, or facial weakness, and they often note that weakness worsens with activity, and improves with rest[12].

The origin of the autoimmune response in MG is unknown, but there is a wide range of clinical presentations that allow classification of MG into subtypes based on autoantibody profiles,

disease distribution, thymic abnormalities, and age at onset. In order to determine prognosis and management strategies, it is necessary to make an appropriate recognition of the subtype.

1.2.1 AChR-associated MG

The identification of several MG clinical subtypes is allowed by differences in clinical presentation (Table 1). Patients can be divided into early-onset and late-onset disease. Early-onset MG is usually defined as beginning at <40 years[12]. These patients are usually female, have anti-AChR antibodies, and enlarged, hyperplastic thymus glands. In addition, other autoantibodies might be present, and patients might be affected by other autoimmune diseases. It can be associated with HLA-DR3, HLA-B8, as well as other autoimmune risk genes[12,13] In early-onset MG, antibodies to non-AChR muscle components are not typically present. Standard diagnostic testing is used for the detection of serum AChR antibodies, and patients usually respond to thymectomy[13].

In late-onset MG, patients have their first onset of symptoms after the age of 50 years. It is slightly more frequent in males, and there are weak HLA associations that occur with HLA-DR2, HLA-B7, and HLA-DRB1*15:01[14,15]. In this sub-type, thymoma is not evident on imaging, serum AChR antibodies are present, and thymic hyperplasia occurs only rarely. These patients most often do not respond to thymectomy[13,14,16.]

Table 1. Clinical subgroups of myasthenia gravis. Extracted from[11]

MG Subgroup	Clinical Characteristics	Antigen	Thymus Pathology	IgG Subclass
Early onset MG (EOMG)	Age of onset <50, sex ratio (F:M) 3:1, genetic association with HLA-B8, A1, and DRw3	AChR	Thymic lymphofollicular hyperplasia	IgG1, IgG
Late onset MG (LOMG)	Age of onset >50, sex ratio (F:M) 1:1.5, genetic association with HLA-A3, B7, and DRw2	AChR	Normal thymus (age-related thymus atrophy)	IgG1, IgG3
Thymoma associated MG (TAMG)	Paraneoplastic MG, non-pathogenic antibodies against striated muscle, titin, ryanodine receptor	AChR	Thymoma	IgG1, IgG3
Ocular MG (OMG)	Restricted to ocular muscles, low AChR titres	AChR	Variable, no lymphoid follicles	IgG1, IgG3
MuSK MG	Severe phenotype, respiratory, and bulbar muscle weakness, sex ratio (F:M) up to 9:1, genetic association with HLA-DR14-DQ5	MuSK	Normal thymus	IgG4
Lrp4 MG	Mild phenotype, sex ratio (F:M) 2.5:1	Lrp4	Variable (normal, thymoma, thymic lymphofollicular hyperplasia)	IgG1, IgG2
Agrin MG	Generalized weakness, often also additional AChR, MuSK, or Lrp4 antibodies, associated with severe weakness	Agrin	No thymoma (few studies)	N/A
Transient neonatal MG (TNMG)	Mild symptoms, onset at birth, remission after days to months	AChR, MuSK		maternal IgG
Fetal myasthenia gravis	Reduced fetal mobility, arthrogryposis congenital (AMC), very severe, risk of fetal death	Fetal AChR γ subunit		maternal IgG

1.2.2 MuSK-associated MG

MuSK is a protein expressed in the postsynaptic muscle membrane and it has vital importance in forming and maintaining of NMJ[11,17]. It is functionally linked to AChR and it is necessary to maintain AChR function. 1–4% of MG patients have serum MuSK antibodies, but with increasingly sensitive test assays, more cases will probably be identified. MuSK and AChR antibodies rarely coexist in the same patient. Usually, it is reported in adults, and rarely in the very old or in children[11]. Patients usually do not respond to thymectomy and there are no thymus pathological changes[18,19,20].

In the pathogenesis, IgG4 antibodies play an important role. Preventing Lrp4 from binding MuSK by binding to the first Ig-like domain in MuSK, IgG4 antibodies inhibit Agrin-stimulated MuSK phosphorylation. This likely causes disruption of the structure of the synapse, compromising synaptic transmission and causing MG[21]. Cranial and bulbar muscles are predominantly involved in MuSK-associated MG unlike in other MG subgroups[18,20]. About a third of the patients experience ptosis and diplopia[16].

Bulbar weakness is the first symptom that is experienced by more than 40% of patients with MuSK-associated MG. Facial, tongue and pharyngeal weakness is often associated with neck and respiratory involvement. Ocular muscles are often unaffected, and limb weakness is also uncommon. Muscle atrophy might occur, and patients reported little variation in muscle strength during the day[22,23]. Autoantibodies against Lrp4 interfere with the agrin: Lrp4:MuSK complex, thus leading to muscle weakness[23].

1.2.3 LRP4-associated MG

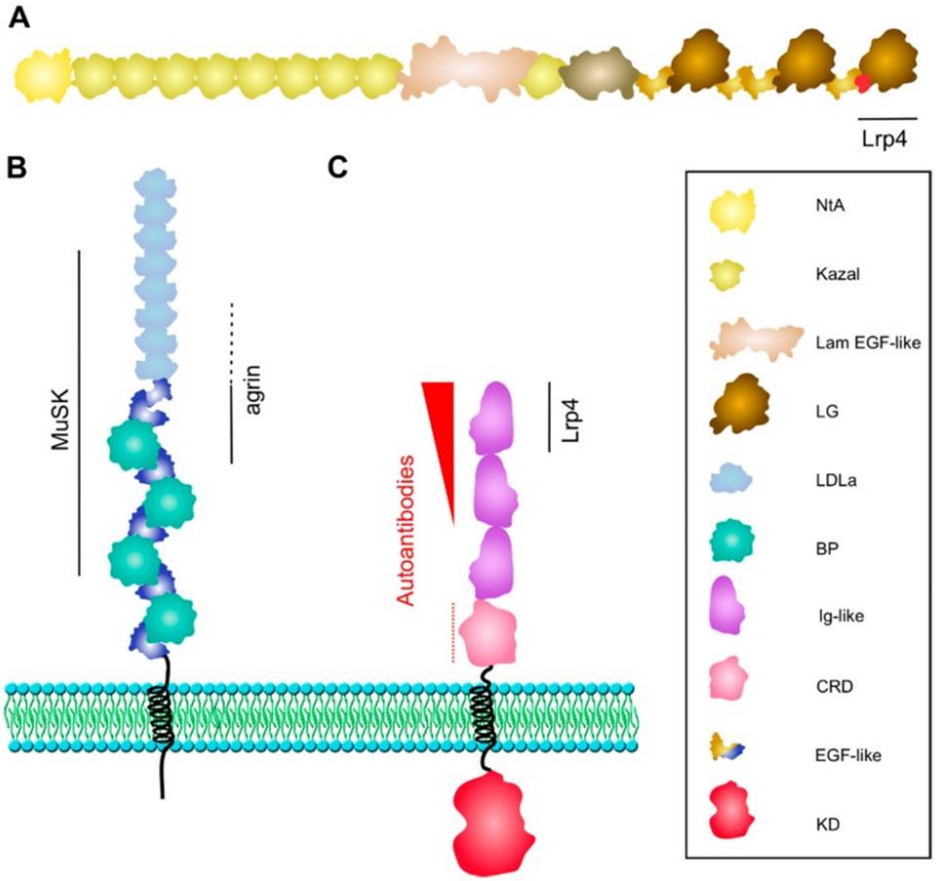

Figure 2. Structure of Lrp4. Extracted from[16]

Lrp4 has large extracellular domain which contains eight low-density lipoprotein receptor domain class A (LDLa) repeats, two EGF-like domains, and four β-propeller (BP) domains, each of which is fused together with an EGF-like domain (Figure B). Neuronal Agrin binds predominantly to the first BP domain, although the last few LDLa repeats contribute to Agrin-binding. The crystal structure of the Agrin-Lrp4 complex showed that the central part of BP1 represents a six-bladed β-propeller domain (conserved in the LDLR family) with two slightly concave surfaces perpendicular to the β-propeller's central axis. One surface is enclosed by the two flanking EGF-like domains, the second surface is involved in Agrin binding via the B/Z loop. Crystal structures of close relatives of Lrp4 such as Lrp6 demonstrated the use of the same surface for ligand binding. The Agrin–Lrp4 dimers further assemble into a tetrameric complex via interfaces between Agrin and Lrp4 (no Lrp4 dimers were observed), which is required for MuSK activation. The third BP domain as well as the fourth and fifth LDLa repeats are critical for binding of Lrp4 to MuSK (via the first Ig-like domain). The transmembrane and intracellular domains are not required for MuSK activation by Agrin.[16(8)]

LRP4 is expressed in the postsynaptic muscle membrane. It is a receptor for nerve-derived agrin, an activator of MuSK, and necessary for maintaining AChR function[12,24]. In 2-27% of patients with MG without AChR and MuSK antibodies, LRP4 antibodies have been detected, mostly in females. Most LRP4-associated MG patients experience ocular or generalized mild MG, and approximately 20% of patients experience only ocular weakness for more than 2 years. Except in a subgroup with additional MuSK antibodies, respiratory insufficiency occurs very rarely.Two-thirds of patients were reported hyperplasic[24].

This group of patients usually have milder symptoms, and very rarely they experience a myasthenic crisis where respiratory support is needed. A combination of anti-AChR, anti-MuSK, and anti-LRP4 antibodies is reported in few patients, where thymoma is not reported[23].

1.2.4 Seronegative MG

If test results for anti-AChR, anti-MuSK, or anti-LRP4 antibodies are not positive, patients are characterized as having seronegative MG[25,26]. They constitute about 10% of generalized MG patients[26,27]. Seronegative MG group includes patients with a too low concentration of antibodies to detect, patients with antibodies against relevant antigens that have not yet been defined and cannot be tested for, and patients with myasthenic symptoms not mediated by antibodies, including late-onset genetic forms associated with mutations in rapsyn or other relevant muscle proteins[24].
The existence of self-reactive T-cells or pathogenic autoantibodies, reproduction of the disease by the direct transfer of autoantibody, and some clinical circumstantial evidence are required by the Witebsky postulate. This postulate is fulfilled by AChR and MuSK MG, so that a disease is classified as autoimmune[28].

1.3 Pathogenic mechanism of MG antibodies

MG antibodies belong to the IgG class. IgG can be divided into the Fc and the Fab region. The Fab region is the part of the antibody and it contains the variable regions of the heavy and light chains (VL, VH), which binds the antigen and the first constant domain (CH1, CL). Fc region determines a class and subclass of the antibody and the effector mechanisms of the antibody. It comprises most of the constant region of the two heavy chains of an antibody (CH2-CH3)[18].
There are three main mechanisms in which the AChR antibodies induce pathogenicity: (1) Activation of the classical complement cascade. (2) Binding and cross-linking of AChRs by antibodies (3) The direct inhibition of the function of the AChRs by preventing the binding of ACh or blocking the channel[18,29,9].

MuSK antibodies behave different from AChR antibodies. Belonging to IgG4 subclass, due to structural differences in the Fc region, it does not activate the classical complement system or immune cells[10]. These antibodies can exchange half-molecules with other IgG4 half-molecules. This process is known as Fab-arm exchange that can generate IgG4 antibodies with two different antigen-binding fragments, meaning that they are unable to cross-link antigens in order to induce endocytosis or form immunocomplexes[30]. The Agrin-Lrp4-MuSK-Dok-7 signaling axis is interrupted by the presence of MuSK antibodies, causing reduced densities of AChRs at the synapse and defects in neuromuscular transmission[8,21].

LRP4 itself is also a target for autoantibodies. Whether LRP4, Agrin, and ColQ antibodies are pathogenic is not clear yet. It is speculated that LRP4 autoantibodies interfere either with binding to Agrin or with binding to MuSK[16,18,21].

1.3.1. Detection of antibodies

Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) are standard routine assays used for the detection of autoantibodies[31]. For the detection of AChR and MuSK antibodies with higher sensitivity, cell-based assays (CBA) have been introduced[32]. In some patients, with increased antibody concentration, epitope spreading, or increased test sensitivity, repeated testing can lead to a diagnostic revision from the seronegative MG subgroup to one of the other subgroups[27].

In some MG patients, autoantibodies against intracellular antigens, including autoantibodies against striational antigens, are also found. Even though they are considered non-pathogenic, they might be relevant as biomarkers[33].

1.4 Problem Statement and aims

There are several standard routine assays used for the detection of autoantibodies. Cell-based assays are used for higher sensitivity detection of antibodies. However, not all myasthenic antibodies can be detected using these methods.

This study aims to establish a new diagnostic method for the detection of Lrp4 autoantibodies in patients with Myasthenia gravis. To achieve this, we intend to modify existing methods and to optimize cell-based assays using live HEK293 cells.

2. Materials and Methods

2.1 Materials

See table 2.1 and table 2.2.

2.2 Methods

2.2.1 Cell culture

Cell culture refers to the cultivation of disaggregated cells and implies their maintenance in an artificially controlled environment that favors their growth. The establishment of cell culture was carried out under sterile conditions in a laminar flow hood. Human embryonic kidney 293 (HEK293) cell line, TE671, C2C12, L6, Phoenix Eco, and NIH3T3 cell lines were used during the experiments.

2.2.2 Maintenance of cell lines

Human embryonic kidney 293 (HEK293) cells were cultivated in HEK cell growth medium (DMEM – D6429, Sigma-Aldrich, supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (Pen-Strep)). The optimal temperature in the incubator was 37°C, with the optimal concentration of CO_2 gas (5.5%) and humidity of 95%. The cells were split at 70-80% confluency in the following steps: The growth medium was aspirated, the cell culture dish was briefly rinsed with 1x phosphate-buffered saline (PBS). The separation of cultured cells from the bottom of the plate was achieved by short-term exposure to trypsin-EDTA at 37°C. After the cells were detached, they were resuspended in DMEM. The cell suspension was centrifuged, and after the supernatant was discarded, the cell pellet was resuspended in a fresh medium. Usually, one-tenth of this cell suspension was transferred to a new plate with a fresh growth medium.

Cryo-preservation is a procedure by which cells can be kept in a frozen state for a long period of time. For freezing, a cryopreservation agent, 10% dimethyl sulfoxide (DMSO) was added to the cell suspension and transferred to a cryotube. The CoolCell® container was used to place cryo-tubes at -80°C for 24h and after it was stored in liquid nitrogen. Thawing of the cells suspension is done quickly – by immersing the tube with the cells in a water bath at 37°C until

most of the cell suspension was thawed. The thawed cells were directly transferred dropwise into a sterile falcon tube and warm DMEM was slowly pipetted to the solution. The cell suspension was centrifuged, the supernatant discarded, and the cell pellet was resuspended in fresh medium and added to a cell culture dish.

TE671, L6, Phoenix Eco, and NIH3T3 cell lines were maintained in the same way as HEK293 cells.

C2C12 mouse myoblasts were cultured in growth medium (DMEM supplemented with 15% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (Pen-Strep)). After the cell reached 70-80% confluence, the growth medium was replaced with 10ml of differentiation medium (DMEM supplemented with 2% horse serum (HS) and 1% penicillin-streptomycin (Pen-Strep). Differentiation to form myotubes lasted between 4 and 7 days. In most cases, on the sixth day evening, Agrin was added. The next day, cell lysates were prepared.

Table 2.1: Materials

Product	**Company**	**Product number**
Ampicilin	Sigma-Aldrich	A-9518
Ammonpersulfat (APS)	Sigma-Aldrich	A-3678
Aqua-Poly/Mount	PolySciences	18606-20
DC Protein assay	Bio-rad	500-0116
DMEM	Sigma-Aldrich	D6429
E.coli DH5α	Invitrogen	11319019
E.coli TOP10	Invitrogen	C404052
Glycine	MERCK	1042011000
HEPES	Sigma-Aldrich	H-3375
Kanamycin	Sigma-Aldrich	K1377
Lipofectamine 2000	Thermo Fisher Scientific	11668-019
LB Broth with agar	Sigma-Aldrich	L3147
Lauria Broth Base (LB)	Invitrogen	12795027
MaxiPrep	Qiagen	12663
Opti-Mem	Thermo Fisher Scientific	11058-021
PBS	Gibco	14190-094
Penicilin/Streptomycin	Sigma-Aldrich	P4333
Poly-L-Lysine	Sigma-Aldrich	P2636
Protease inhibitor coctail	Sigma-Aldrich	P8340
Tris(hydroxymethyl)aminomethane	MERCK	1083822500

Trypsin-EDTA	Sigma-Aldrich	T4049
Tween	Sigma-Aldrich	P-7949
SuperSignal West Pico Luminol/Enhancer Solution	Thermo Fisher Scientific	1856136
SuperSignal West Pico Stable Peroxide Solution	Thermo Fisher Scientific	1856135
Precision Plus Protein Dual Color Standard	Bio-Rad	1610374

Table 2.2: Antibodies

Antibody name	**Company**	**Product Number**	**Species**	**Application**	**Dilution**
Polyclonal Goat Anti-Mouse Immunoglobulins/HRP	Dako	P0447	Mouse	WB	1:1000
Purified (azide-free) anti-LRP4 (NH2 terminus)	BioLegend	832201	Mouse	WB	1:200
Monoclonal Anti-beta-Actin-Peroxidase	Sigma	A3854-200UL	Mouse	WB	1:50 000
Alexa Fluor® 594 AffiniPure Goat Anti-Human IgG	Thermo Fisher Scientific	A-11014	Human	CBA	1:750
Alexa Fluor® 594 AffiniPure Donkey Anti-Mouse IgG (H+L)	Jackson Immunoresearch	715-585-150	Mouse	CBA	1:750
Alexa Fluor® 488 AffiniPure Goat Anti-Human IgG	Jackson Immunoresearch	109-545-098	Human	CBA	1:750
Streptavidin-HRP	Dako	K0675	Mouse	WB	-
Streptavidin-HRP	Bio-Rad	STAR5B	Mouse	WB	1:1000

2.2.3 Transfection of HEK293 with AChR and MuSK

Human embryonic kidney 293 (HEK293) cells were cultivated in HEK cell growth medium (DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) under conditions 37°C, 5.5% CO2 in a humidified atmosphere. The cells were split at 70-80% confluency. The medium was aspirated and the Petri dish was rinsed with 1x PBS. Cells were trypsinized for five minutes in an incubator. The cells were trypsinized and resuspended in DMEM and transferred into the centrifuge tube. After centrifugation, the supernatant was decanted and cells were gently resuspended in the growth medium. Ten µL of the cells were added to the hemacytometer. After the cells were counted, they were plated at densities of $3x10^5$ per well onto glass coverslips coated with poly-L-lysine into a 6-well plate. HEK293 cells were plated and incubated at 37°C until the next day.

On the following day, cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific – Ref. 11668-019) as a transfection agent. AChR α, β, γ, δ, ε subunits and rapsyn-enhanced green-fluorescent protein in a ratio of 2:1:1:1:1:1 (2µg α:1µg β:1µg δ:1µg γ: 1µg ε:1µg rapsyn) were used for the transfection of HEK cells for AChR cell-based assays. HEK cells transfected with rapsyn- enhanced green fluorescent protein were used as a negative control.
Transfection of HEK cells for MuSK cell-based assays was done with 4µg of pIRES2-AcGFP1-MuSK per well and for the negative control 1µg of empty vector per well.

In both cases (AChR or MuSK cell-based assay), in one Eppendorf tube DNA was added to 250 µl of Opti-Mem. In the second tube, 10 µl of Lipofectamine were mixed with 250 µl of Opti-Mem (Thermo Fisher Scientific – Ref. 11058021). Both tubes were incubated for 5 minutes at room temperature. After incubation, Lipofectamine solution and DNA solution were mixed together and incubated for 20 minutes at room temperature. Incubation was followed by adding 500 µl of transfection mix into each well of the 6-well plate which is stored overnight at 37°C.
On the following day, cells were checked under the microscope. The presence of green fluorescence was an indicator that the transfection was done successfully, and the cell-based assay can be completed.

2.3 Cell based assay (CBA)

Sera from MG patients were diluted 1:40 in blocking buffer (DMEM supplemented with 1M HEPES and 1%BSA). The coverslips carrying the transfected cells were transferred into a 24-well plate. Into each well, diluted sera were added and incubated for one hour at 37°C. In the following step, cells were washed three times with 500µl of DMEM. There was incubation for 10 minutes at 37°C between each washing step. For the fixation, cells were incubated for 5 minutes, at room temperature in 250µl of 4% paraformaldehyde (PFA). Aspiration of PFA was followed by three times washing of cells with 500µl of DMEM, after which cells were incubated in secondary antibody Alexa Fluor® 594 AffiniPure Goat Anti-Human IgG (Thermo Fisher Scientific – Ref. A-11014) diluted 1:750 in blocking medium. The secondary antibody was incubated for 45 minutes at room temperature in the dark. Cells were briefly washed 3 times with 500µl of 1x PBS. The 40µl of the mounting medium (Aqua-Poly/Mount, PolySciences – Ref.18606-20) was placed on a glass slide and the coverslip was placed on top with the cell side down. The microscopic slides were stored at 4°C in the dark. The assay was analyzed by fluorescence microscopy. The analysis determined which samples were AChR/MuSK-Ab seropositive or seronegative.

2.3.1. Intracellular staining

In order to stain intracellular molecules, the cells need to be fixed and then permeabilized before the detection antibody is added. This allows the antibody to pass through the plasma membrane into the cell interior. After the cells were transfected successfully, they were transferred into a 24-well plate. Into each well, 250µl of 4% paraformaldehyde (PFA) was added and incubated for 10 minutes at RT. After incubation, PFA was aspirated, and the cells were washed three times with 500µl of DMEM. In the following step, the cells were permeabilized with 250µl of 0.3% Triton X solution and incubated for 10 minutes at RT. Afterward, the cells were washed 3 times with 500µl of DMEM and used for the cell-based assay.

2.3.2 Cell based assay with fixed and permeabilized cells

For the cell-based assay, fixed and permeabilized HEK293 cells were used. The cells were grown in a growth medium and transfected with 5µg of Lrp4-GFP per well, and for the transfection control, mock-transfected cells were used. After fixation/permeabilization treatment of the HEK293 cells, the patient sera diluted 1:40 in blocking medium were added into each well and incubated at 37°C for one hour. After, the cells were washed 3 times with DMEM. Each washing step was followed by 10 minutes incubation at 37°C. The secondary antibody Alexa Fluor® 594 AffiniPure Goat Anti-Human IgG (Thermo Fisher Scientific – Ref. A-11014) diluted 1:750 in the blocking medium was added and incubated for 45 minutes at RT in the dark. After the incubation, the cells were washed three times with 500µl of 1x PBS. For nuclear staining, cells were left in 250µl of DAPI for 10 minutes at RT. Finally, the cells were washed three times with 500µl of 1x PBS. The slides were mounted using 40µl of Aqua-Poly/Mount (PolySciences – Ref. 18606-20) medium. The assay was analyzed by fluorescence microscopy.

2.4 Plasmid amplification

10µl of Competent bacteria *E.coli* TOP10 were mixed with 1µl of plasmid DNA (Lrp4 from the laboratory of Doz. Dr. Ruth Herbst). In another tube, only bacteria were added. Both tubes were incubated for 10 minutes on ice. After incubation, the tubes were transferred into a water bath for 45 seconds at 42°C. In order to cool down the sample, the tubes were immediately placed on ice for 5 minutes. Thereafter 1ml of LB medium was added to the transformation mix and bacteria and they were incubated for 30 minutes at 37°C. After incubation, the tubes were spun down for 3 minutes at a speed of 12.000 g. 2/3 of the supernatant was removed, and the pellet was resuspended in 1/3 of the supernatant. The bacteria were pipetted on top of the LB-agar plate and stored overnight at 37°C. The next morning, the plates were checked for the colonies. If the transformation was successful, bacteria containing a plasmid with antibiotic resistance would grow in the presence of the antibiotic. The plates were stored at 4°C. Later that day, 5ml of LB medium and 5µl of Ampicillin was mixed with a colony selected from the plate.
The culture was incubated overnight in a bacterial incubator at 37°C, shaking around 300rpm. In the morning, if bacteria have grown, they were stored at 4°C. In 1l Erlenmeyer beaker 250ml LB medium, 250µl of Ampicillin and 5ml of bacterial culture were mixed together and left for

overnight incubation at 37°C shaking in a bacterial incubator. On the following day, bacteria were spun down for 15 minutes at 4°C at speed of 4.000 g. The supernatant was removed and the pellet was used for the Maxiprep. This protocol was performed according to the manufacturer's instructions.
The same protocol was used for the isolation of Lrp4-SBP.

2.5 Retroviral infection

Phoenix-Eco cells were maintained in HEK cell growth medium (DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin-streptomycin (Pen-Strep) at 37°C, 5.5% CO2. 24 hours after the cells were plated in the Petri dish, 20µg plasmid DNA was mixed with 750µl Opti-Mem (Thermo Fisher Scientific – Ref. 11058021) in one tube, and in another tube 30µl of Lipofectamine 2000 (Thermo Fisher Scientific – Ref. 11668-019) was mixed with 750µl of Opti-Mem. After 5 minutes of incubation, contents from both tubes were mixed together and left for another 20 minutes of incubation at RT. After incubation, the transfection mix was added to the cells. The presence of red fluorescence was an indicator that the transfection was done successfully.

On the fourth day after transfection, the supernatant was removed from Phoenix-Eco cells. NIH3T3 cells were infected with supernatant supplemented with polybrene 1:1000 (5µg/ml). After the incubation for 4 hours at 37°C, the supernatant was removed from NIH3T3 cells and replaced with HEK cell growth medium. After 24 hours of incubation in the incubator at 37°C, the sorting was done. Before the disposal of material used, everything was decontaminated for at least 15 minutes with vircon.

2.5.1 Fluorescence activated cell sorting assay (FACS)

Fluorescence-activated cell sorting (FACS) is a type of flow cytometry that provides physical separation of cells of interest. It is based upon light scattering and fluorescent characteristics of cells. Interests are first labeled with an antibody coupled to a fluorescent dye, and then each cell's fluorescence is measured. Small droplets (each containing a single cell with a negative or positive charge) are then formed by a vibrating nozzle. Differently charged droplets are then separated into different containers with respect to the cell surface molecule tagged along with the fluorescent antibody.

On the day of sorting (after 24 hours incubation) transfected and untransfected NIH3T3 cells were trypsinized and resuspended in 2ml of HGM-EDTA medium. The cells were stored on ice. Right after the sorting was done, cells were transferred into a fresh growth medium and stored at 37°C.

2.6. Preparation of cell lysates

The medium was removed from the adherent cell culture and the surface of the Petri dish was covered with 220µL of lysis buffer and 1:1000 dilution of Protease Inhibitor Cocktail (PIC). After incubation on ice for 15 minutes, cells were scratched off from the cell cultivation dish. Lysates were transferred to Eppendorf tubes and centrifuged for 15 minutes at +4°C at a speed of 12.000 g. The supernatant was placed into a fresh tube and froze at -20°C until use.

2.6.1 Protein concentration

The protein standard was prepared with BSA. 0.04g of BSA was mixed with 1ml of lysis buffer (40mg/ml stock). 0.1 ml from stock was added to 900µl of lysis buffer, then 1:2 dilution series were prepared, A-H, with H=100% lysis buffer. In the 96 well plate 5µl of the standard was added in duplicates. The reagent mix was prepared from the DC Protein assay kit in the following way: 20µl reagent S mixed with 1ml reagent A and 25 µl of reagent mix was added to each well. 200µl of reagent B was added in the same way. After 15 minutes of incubation at room temperature, the wells should turn blue. OD620 was measured with the plate reader.

The standard curve was measured by plotting OD620 on the x-axis vs protein concentration in mg/ml on the y-axis. A linear trendline was added with the equation being displayed (Y= bx -/+ a).

The protein concentration (mg/ml) of the samples was measured using the equation:

$$Y= b*valueOD620x -/+ a$$

With b = slope of a trendline, x is the mean value of OD620nm measured from the samples and a is the y-intercept, which is the expected mean value of y when all x variables are equal to 0. On a chart, it's the point where the trendline crosses the y- axis.

2.6.2 Western Blot

The Western blot method is a method used for the identification of specific proteins or the determination of important traits protein antigens from a protein solution extracted from cells. The method consists of three steps: polyacrylamide gel electrophoresis, protein transfer to the membrane, and protein immunodetection.

2.6.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is the process of protein separation based on differences in their mass. SDS (anionic detergent) gives a negative charge to all proteins in the presence of disulfide bond breaking reagents.
Reagents were prepared for the preparation of the separation gel (Table 2.3.) and about 5ml was added between glasses. The surface was covered with 70% ethanol and left to allow the polymerization process to proceed for 40 minutes. During the process of polymerization, a

solution for collection gel was prepared (Table 2.3.). After polymerization, ethanol was removed and the top gel was then poured on top and the comb was inserted. After 30 minutes, collection gel polymerized, the comb was removed and the electrophoresis system was ready for use. After adding a running buffer (Table 2.4) the process of applying samples to the wells was initiated. Cell lysates were premixed with 2X sample buffer in 1:2 ratio and heated for 4 minutes at 95°C and cool down 5 minutes on ice. In the first well, the Precision Plus Protein Dual Color Standard (Bio-Rad – Ref. 1610374) was added. In other wells, samples were pipetted carefully. Added volume does not exceed the volume of the well. Electrophoresis was performed at a voltage of 60V until the samples reached the limit of the collection gel (approx. 15 minutes), then a voltage of 150V was maintained until the sample buffer color front reached the lower edge of the gel (1h).

Table 2.3: Preparation of the gel

	Separation gel	Collection gel
Gel concentration	10 %	4 %
A. Bidest.	3,96 mL	2,97 mL
1,5 M Tris-HCl pH 8.8	2,50 mL	-
0,5 M Tris-HCl pH 6.8	-	1250 µL
Acrylamid/Bis	3,33 mL	670 µL
10% (w/v) SDS	100 µL	50 µL
10% APS	100 µL	50 µL
TEMED	10 µL	10 µL

Table 2.4: Preparation of buffer for applying samples to the gel

Stock-2xSB
250 µL 0,5 M Tris-HCl pH 6,8
360 µL Glycerin (18%)
270 µL 15% SDS (4%)
20 µL 0,5% (w/v) Bromphenolblau

2.6.4 Transfer to membrane

After electrophoresis, the gel was placed in a transfer buffer. Immobilon-P membrane (0.45 µm) and filter papers (4 pieces) were cut according to the dimensions of the gel. The membrane was incubated for 5 minutes in 100% methanol and then 10 minutes in transfer buffer.
Filter paper, membrane, and gel were immersed together in transfer buffer just before stacking on the sponge. To the page that will be turned according to the negative electrode, a well-soaked sponge is placed and 1 piece of moistened filter paper was added to the sponge. On filter paper, the gel was placed. On the gel, the membrane was carefully laid. Two pieces of moistened filter paper were used to cover the membrane, the sponge was added and the surface carefully pierced with a glass rod to expel air. The parts thus arranged are closed by the other side of the cassette which is then facing the positive electrode. The protein transfer vessel was filled with a cold transfer buffer. Protein transfer to the membrane was conducted at 100V for one hour.

2.6.5 Detection

After transferring the proteins to the membrane, the presence of the required proteins was determined using antibodies. Primary and secondary antibodies were used (Table 2.2). Primary antibodies are highly specific for the desired protein, and to them bind secondary antibodies that are in complex with the enzyme. The enzyme provides signal visibility and detection mode.

The membrane incubated in a blocking buffer (5% dry milk, 1× PBS) was stored in the fridge overnight. The next day, the membrane was incubated in the primary antibody Purified (azide-free) anti-Lrp4 (BioLegend – Ref. 832201) 1:200 diluted in blocking buffer for one hour. The primary antibody was diluted with blocking buffer in an antibody-dependent ratio (prepared according to manufacturer's instructions). After one hour of incubation, the membrane was washed in PBS Tween three times for 10 minutes. After washing, the membrane was incubated for one hour at room temperature with the secondary peroxidase-conjugated antibody: Polyclonal Goat Anti-Mouse Immunoglobulins/HRP (Dako – Ref. P0447) in dilution 1:1000 followed by washing 3x10 minutes in PBS Tween. Secondary antibodies used for Lrp4-SBP were Streptavidin-HRP (Bio-rad – Ref. STAR5B) in dilution 1:1000 and Streptavidin-HRP (Dako – Ref. KO675). SuperSignal-solution was made by mixing equal volumes of Luminol/Enhancer Solution and Stable Peroxidase Solution.

A CCD-camera (Bio-Rad Laboratories) is used for the detection of a signal from the substrate.

3. Results

This study aimed to establish a new diagnostic method for the detection of Lrp4 autoantibodies in patients with Myasthenia gravis. In order to optimize existing protocols, patients' sera were first analyzed for the presence of MuSK/AChR autoantibodies. A total of 98 patients' sera were tested during the study. After analysis, the results showed that 44 samples were seronegative for AChR and MuSK autoantibodies. In the total number of seropositive sera,11 were AChR positive, 16 MuSK positive, and 1 case was double-positive.

3.1 Screening of Mysthenia Gravis patient sera

Cell-based assays were used to evaluate the presence of autoantibodies against MuSK and AChR in the MG patients' sera. Visible clusters on the cell membrane (Figure 3a,3c) indicates AChR/MuSK-Ab-seropositive patient. Sera from healthy control were tested in cells transfected with AChR+Rapsyn and pG-MuSK (Figure 3b, 3d).

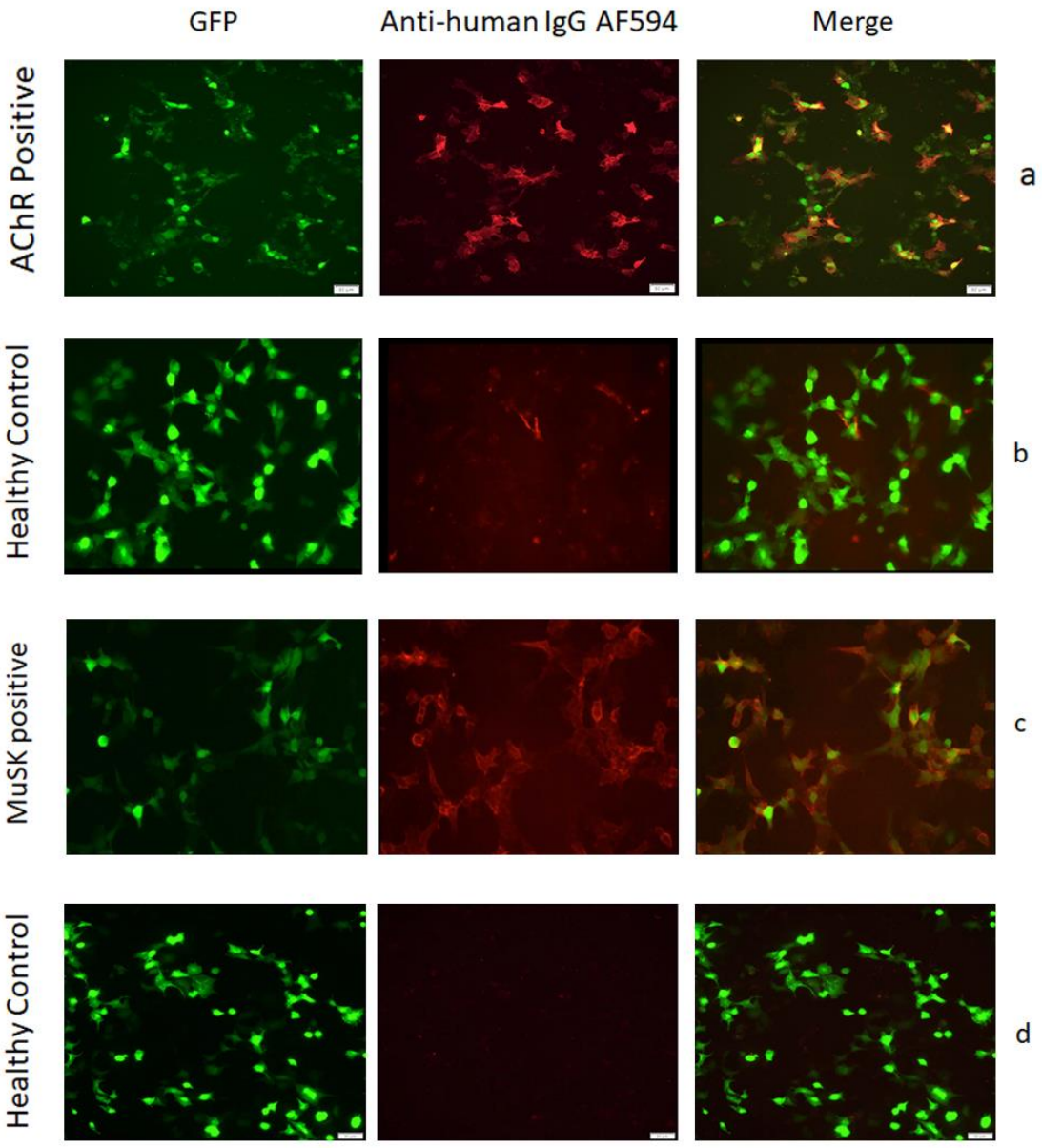

Figure 3. Fluorescent microscopy of cell-based assays in transfected HEK293 cells. Serum dilution 1:40. Left column: GFP transfected cells ; middle column: staining with anti-human IgG AF594; right column: merge of green and red fluorescence. **a.** Cells transfected with AChR subunits, incubated with AChR positive serum; **b.** GFP transfected cells incubated with healthy control serum; **c.**Cells transfected with MuSK, incubated with MuSK positive serum; **d.** GFP transfection, incubated with healthy control serum.

The presence of antibodies against AChR and MuSK was tested in almost 100 patients' sera (Supplementary Table 3.2). About 50 samples were double seronegative, 27 samples were seropositive, and 1 case was double seropositive (Table 3.1).

Table 3.1. Results from cell-based assays for AChR and MuSK

Total number of tested samples	98
AChR Positive:	11
MuSK Positive:	16
Double Positive:	1
Double Negative:	44

Table 3.2: Summary of sera screening

AChR Positive	MuSK Positive	AChR+MuSK Positive	Seronegative
4150/17	4341/17	5156/19	1862/17
3293/18	1917/18		1863/17
5156/19	2824/19		1864/17
5449/19	4655/19		1865/17
5506/19	5156/19		2099/18
6248/19	5782/19		574/19
7002/19	5851/19		2590/19
8198/19	6419/19		2591/19
350/20	6464/19		4203/19
631/20	6509/19		4398/19
1047/20	7444/19		4552/19
	8060/19		4557/19
	8130/19		5042/19
	64/20		5096/19
	933/20		5142/19
	975/20		5484/19
			5501/19

			6234/19
			6262/19
			6357/19
			6379/19
			6542/19
			6546/19
			6931/19
			7288/19
			7433/19
			7559/19
			7656/19
			7910/19
			90/20
			102/20
			312/20
			446/20
			519/20
			616/20
			841/20
			954/20
			1131/20
			1203/20
			1311/20
			1323/20
			1616/20
			1620/20
			1676/20

3.2 Detection of Lrp4 autoantibodies in sera of MG patients using CBAs

3.2.1 Lrp4 expression

To investigate whether the Lrp4 is expressed in the Human embryonic kidney 293 cells, the HEK293 cells were transfected with Lrp4-GFP. As a transfection control, MOCK transfected cells were used. The cells were incubated for 24 hours. Later, the cell-based assay was performed in live and in fixed, permeabilized cells. CBAs were analyzed by fluorescence micros-

copy. Fixed and permeabilized HEK293 cells transfected with Lrp4-GFP were visible with 594 donkey anti-mouse staining (Figure 4a). In contrast, there was no signal in live cells (Figure 5a).

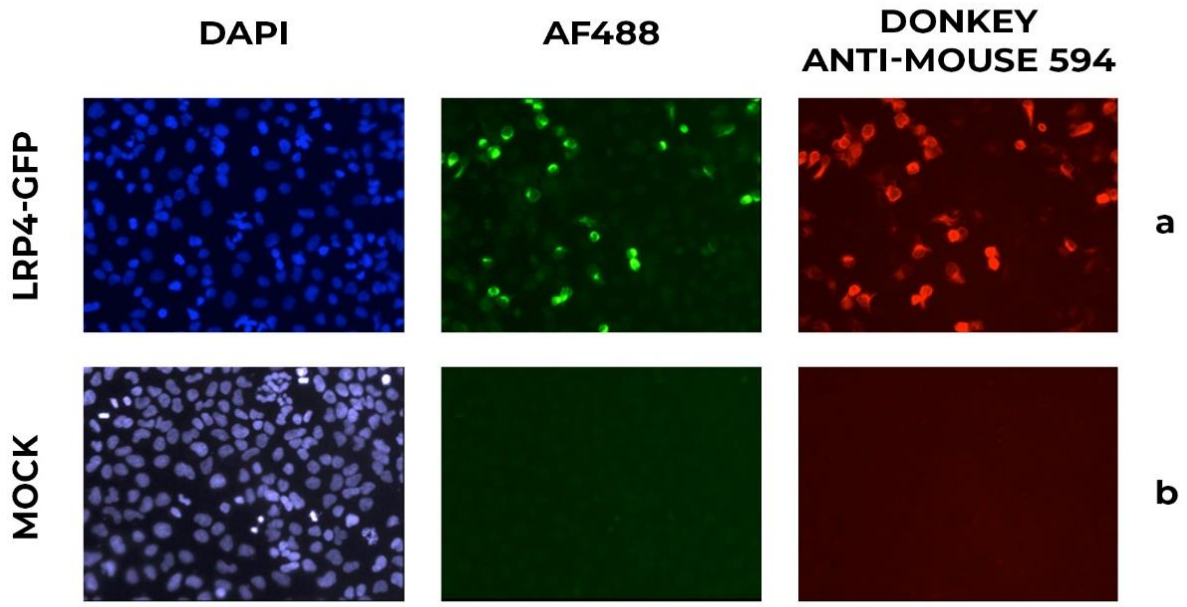

Figure 4. Fluorescent microscopy of CBAs with fixed and permeabilized HEK293 cells. In blue, DAPI is visible. In green, the anti-human 488 antibody is visible; in red the donkey anti-mouse 594 antibody is visible. **a.** Fixed HEK293 cells transfected with Lrp4-GFP; **b.** MOCK transfected cells, no expression.

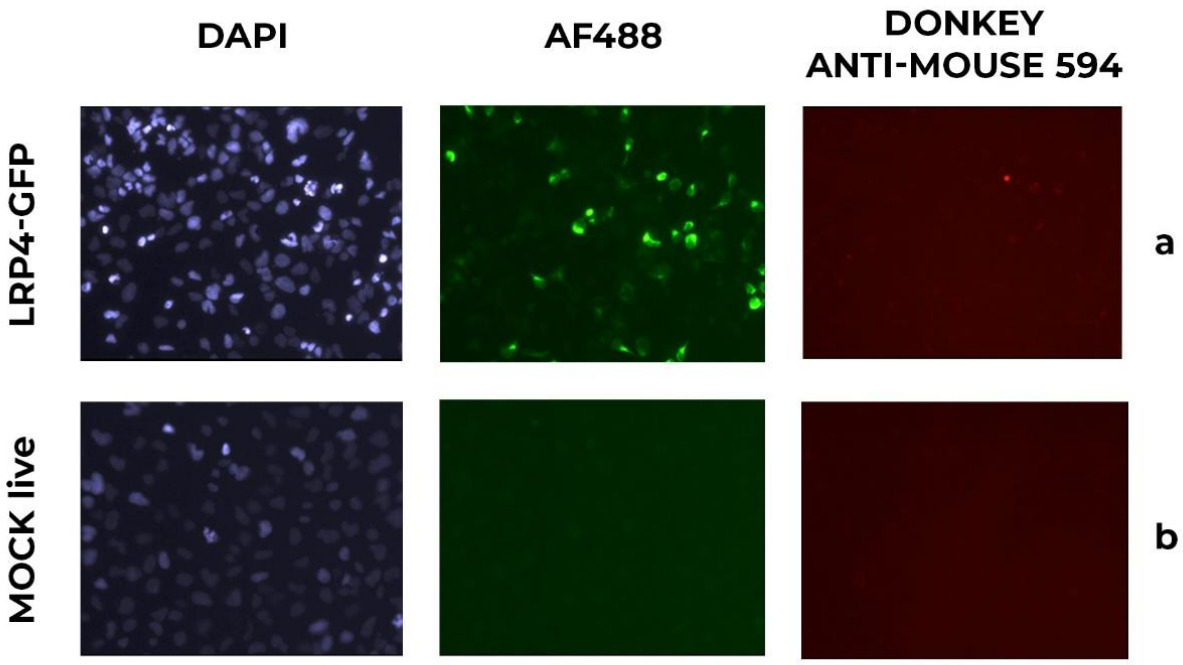

Figure 5. Fluorescent microscopy of CBAs with live Lrp4-GFP transfected HEK293 cells. **a.** Live HEK293 cells transfected with Lrp4-GFP; **b.** MOCK transfected cells, no expression.

3.2.2 Titration of Lrp4-Caspr2+Lrpap

Previous experiments have shown that HEK293 cells are expressing Lrp4, but it is not expressed on the cell surface. We wanted to ensure that the surface expression of Lrp4 is in a concentration-dependent manner. Accordingly, the DNA was titrated to determine if the larger amount of DNA used for transfection will result in surface expression. In parallel, the same procedure was done with fixed and live cells. HEK cells were transfected with 5µg DNA of Lrp4-Caspr2+Lrpap. On the following day, transfection efficiency was checked by fluorescence microscopy (Figure 6). The Lrp4-Caspr2 + Lrpap expressed well with 5µg DNA per well in a 6-well plate. Figure 6. shows that the expression was not dependent on the volume of DNA. Equal expression with decreasing the amount of DNA was observed in the fixed and permeabilized cells. In contrast, surface expression was not detected from the live cells (Figure 7).

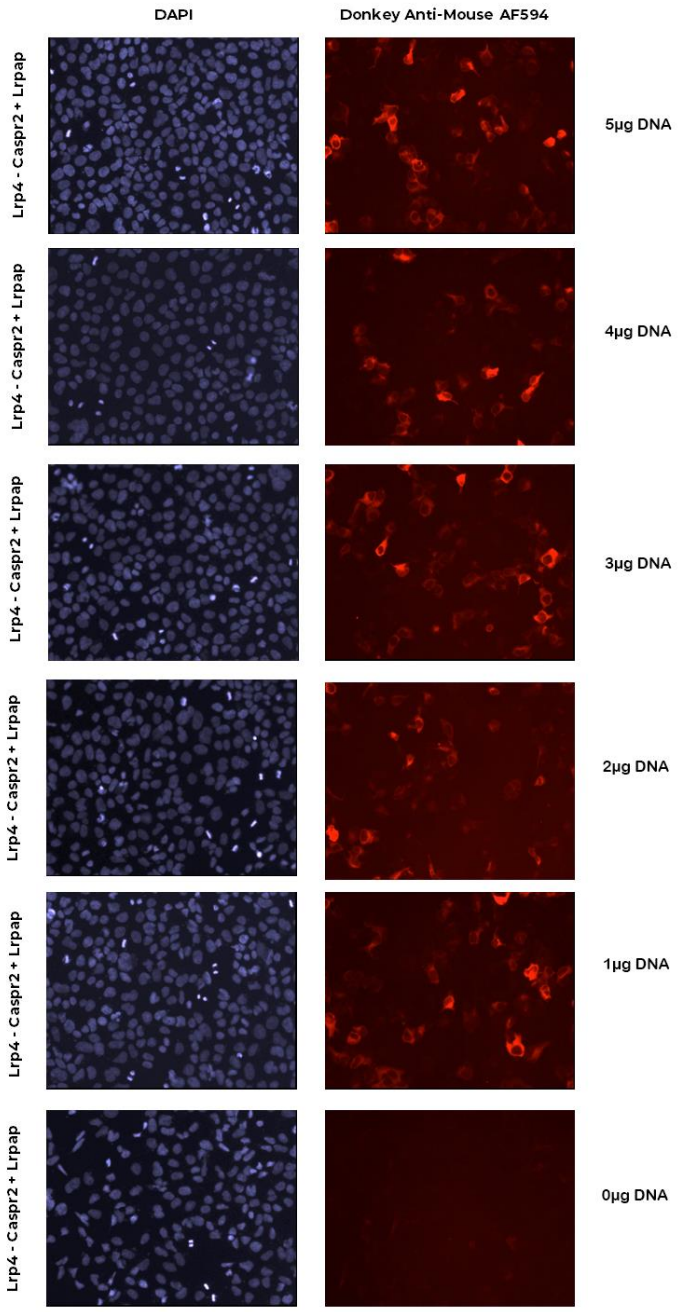

Figure 6. Titration of Lrp4-Caspr2+Lrpap in fixed HEK293 cells. Left column: DAPI is visible; right column: Lrp4-Caspr2+Lrpap with donkey anti-mouse 594 staining is visible. 5µg of Lrp4-Caspr2+Lrpap was starting concentration. The amount of DNA used was in descending order ending with 0µg.

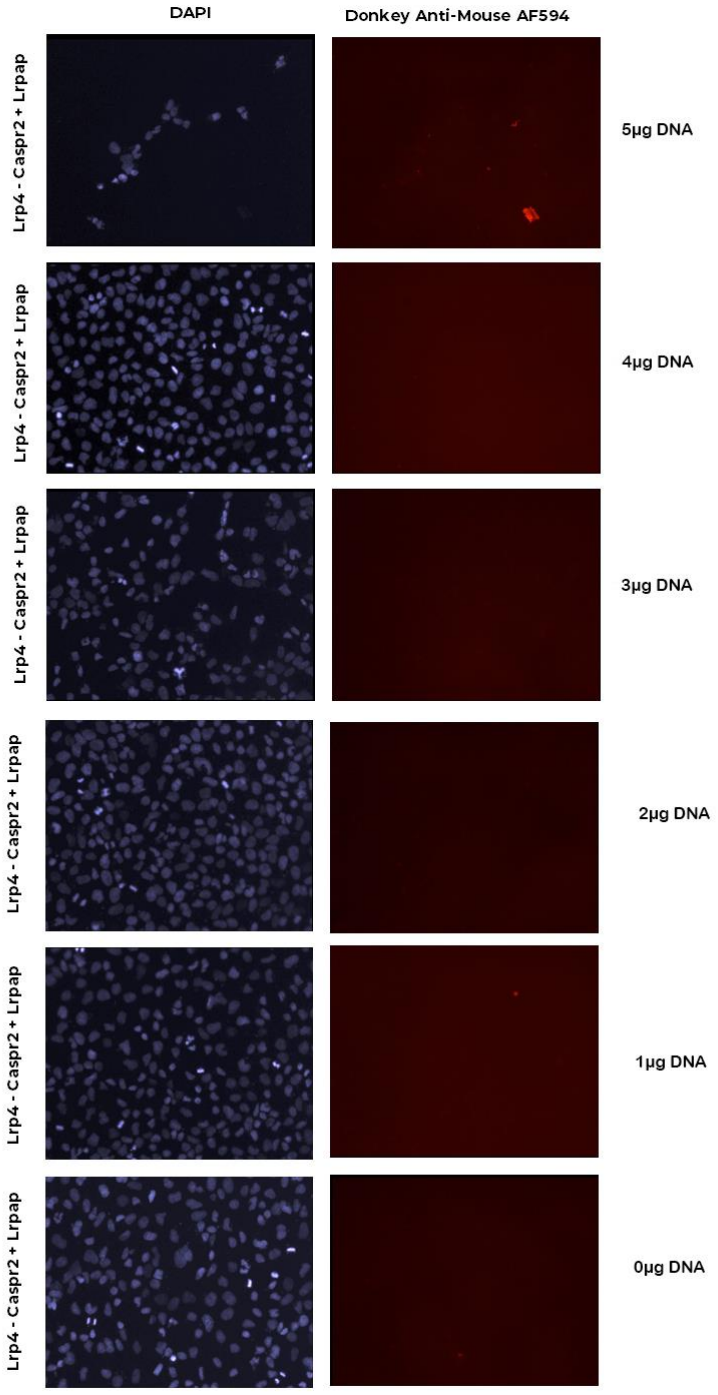

Figure 7. Titration of Lrp4-Caspr2+Lrpap in live HEK293 cells. Left column: DAPI is visible; right column: Lrp4-Caspr2+Lrpap with donkey anti-mouse 594 staining is visible. 5µg of Lrp4-Caspr2+Lrpap was starting concentration. The amount of DNA used was in descending order ending with 0µg. Lrp4-Caspr2+Lrpap surface expression was not observed.

3.2.3 Time – course

As shown in the previous experiment, the surface expression of Lrp4-Caspr2+Lrpap in live cells does not depend on the amount of DNA used for transfection. We wanted to check how time affects it, and the time-course experiment was done. As a control, HEK293 cells transfected only with Lrpap were used. Transfection efficiency was checked by fluorescence microscopy one day after transfection (Figure 8) and continued every day for the next six days. The Lrp4-Caspr2+Lrpap was almost equally expressed on the first and the last day after transfection in fixed cells. Again, no surface expression of Lrp4-Caspr2+Lrpap in live cells was observed (Figure 9).

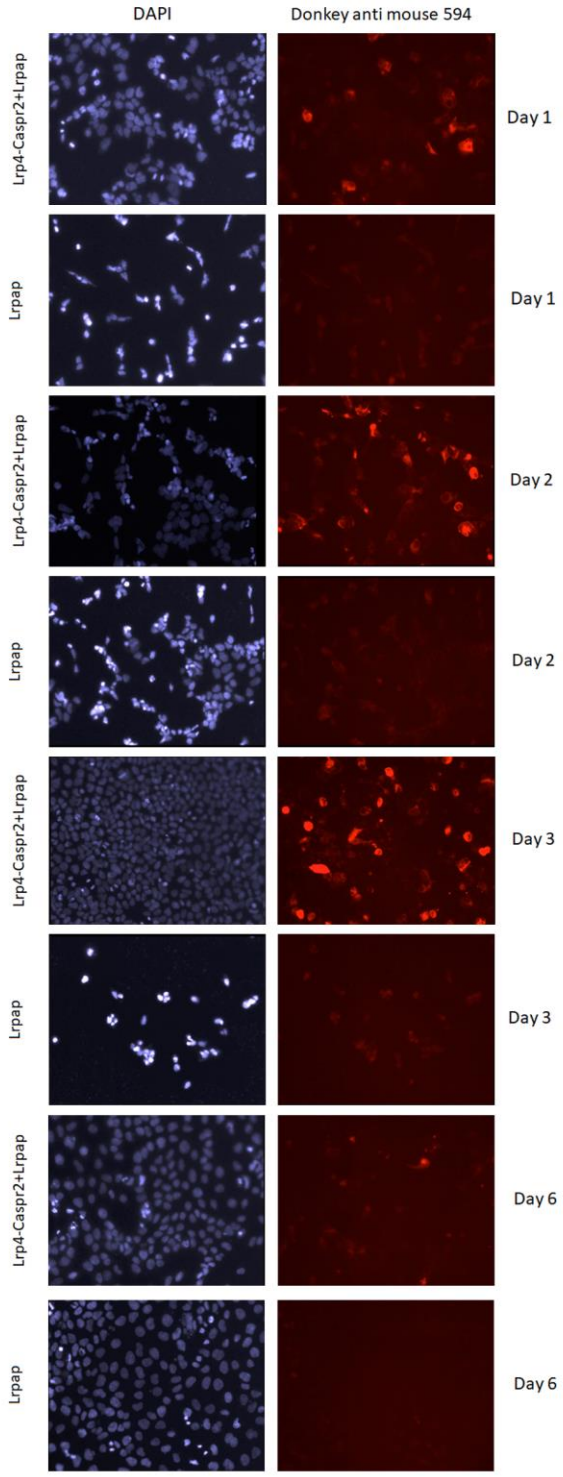

Figure 8. Time-course of Lrp4-Caspr2+Lrpap in fixed and permeabilized HEK293 cells. Left column: DAPI is visible; right column: Lrp4-Caspr2+Lrpap with donkey anti-mouse 594 staining is visible.

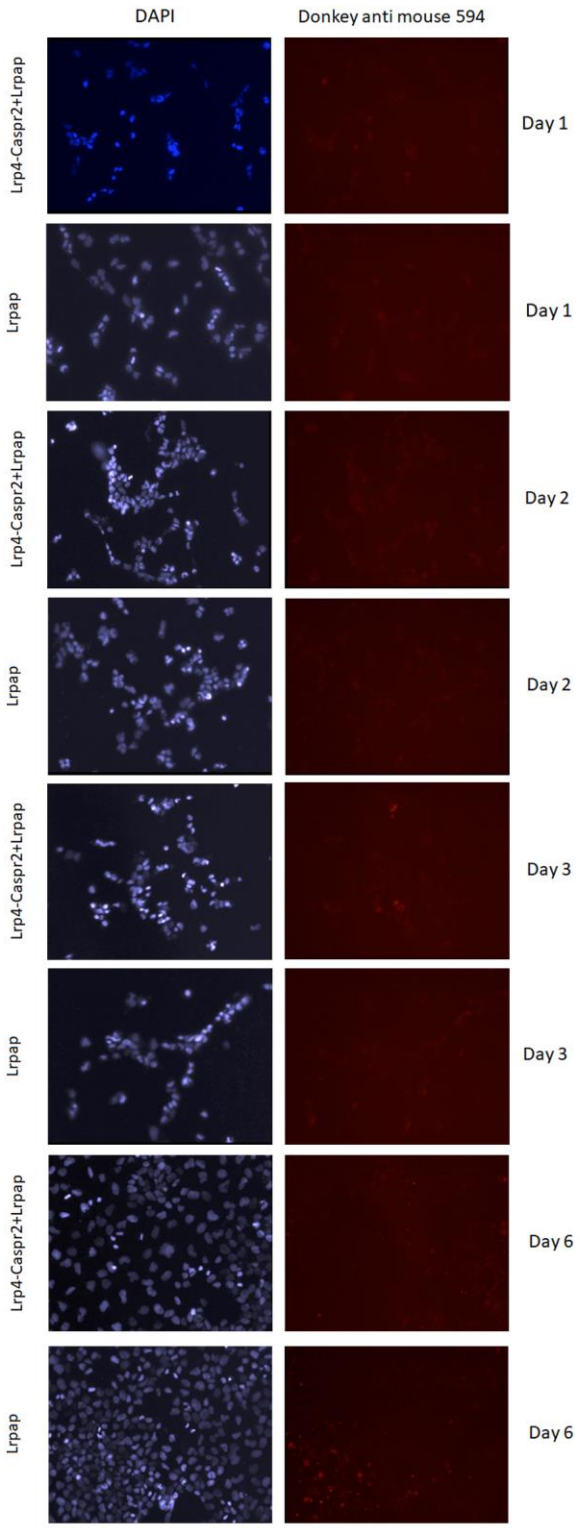

Figure 9. Time-course of Lrp4-Caspr2+Lrpap in live HEK293 cells. Left column: DAPI is visible; right column: Lrp4-Caspr2+Lrpap with donkey anti-mouse 594 staining is visible.

3.2.4 CBAs with Lrp4

In this assay, live and fixed HEK293 cells were used. The HEK293 cells were transfected with Lrp4-Caspr2 and Lrp4-GFP. After the transfection was done successfully, sera from MG patients, healthy individuals diluted 1:40 in blocking medium were incubated with transfected cells. Secondary staining was done with Alexa Fluor® 594 AffiniPure Donkey Anti-Mouse antibody (Jackson Immunoresearch – Ref. 715-585-150). In summary, figure 10a and 10b show the expression of Lrp4 in fixed and permeabilized HEK293 cells, but the cells were too dense followed with the high background. Again, no surface expression of Lrp4 in the live HEK293 cells was observed (Figure 10c, 10d). Therefore, we conclude that another approach is necessary to solve the problem of Lrp4 expression on the cell surface.

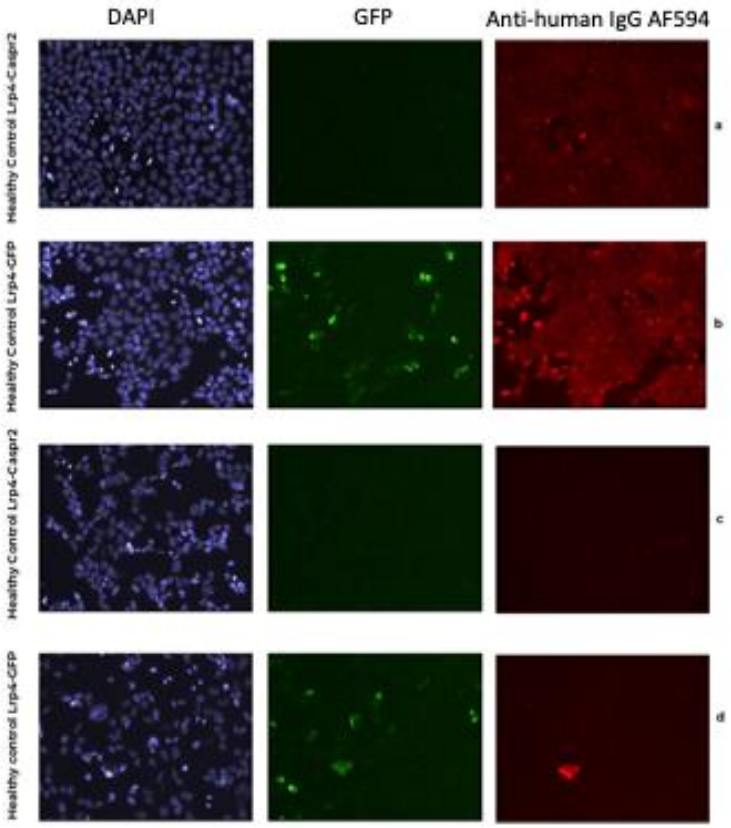

Figure 10. Fluorescent microscopy of cell-based assays in transfected HEK293 cells with Lrp4; a. Fixed cells transfected with Lrp4-Caspr2, incubated with healthy control serum; b. Fixed cells transfected with Lrp4-GFP, incubated with healthy control serum; c. Live cells transfected with Lrp4-Caspr2, incubated with healthy control serum d. Fixed cells transfected with Lrp4-GFP, incubated with healthy control serum.

3.3 Epression of Lrp4 using NIH-3T3 cell line

After the previous experiment failed and Lrp4 expression on the cell surface was not possible with the HEK293 cell line, a new approach was needed. Instead of using the HEK293 cell line, the NIH-3T3 cell line was used. In order to perform this experiment, Flag-Lrp4-mCherry/pMXs was used. The plasmid DNA was used for plasmid amplification followed by maxi prep which is done according to the manufacturer's instructions. Using this method of isolating plasmid DNA we ensure higher DNA yield. The obtained DNA was used for the transfection of Phoenix-Eco cells. Flag-Lrp4-mCherry/pMXs was introduced into NIH3T3 cells with a retrovirus (derived from Phoenix Eco cells). The cells were incubated for three days at 37°C. Successful infection, determined by red fluorescence of m-Cherry, was verified by fluorescence microscopy (Figure 10) and on the fourth day after transfection, retroviral infection of NIH3T3 cells was done. On the following day, NIH3T3-Lrp4 cells were sorted by FACS (Figure 11). After FACS, cells were transferred into a fresh growth medium and stored at 37°C. Figure 11 shows that a very small amount (0.5%) of the cells was sorted successfully which was not enough to continue with optimizing the new protocol for Lrp-4 expression on the cell surface.

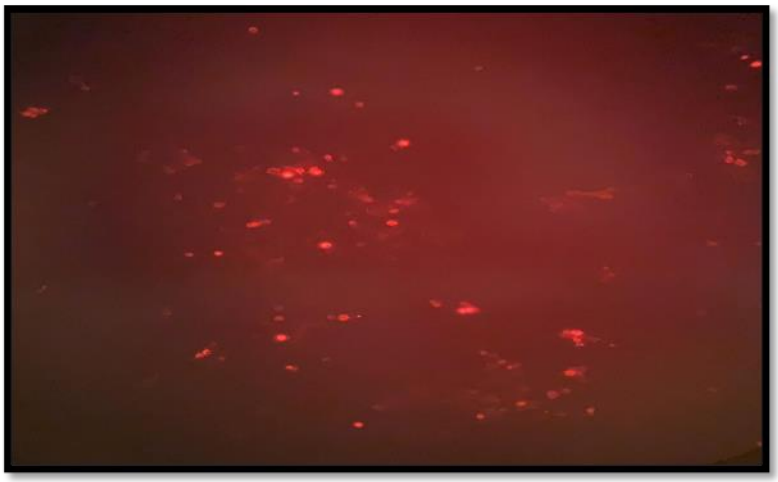

Figure 10. Fluorescent microscopy of Phoenix-Eco cells three days after transfection.

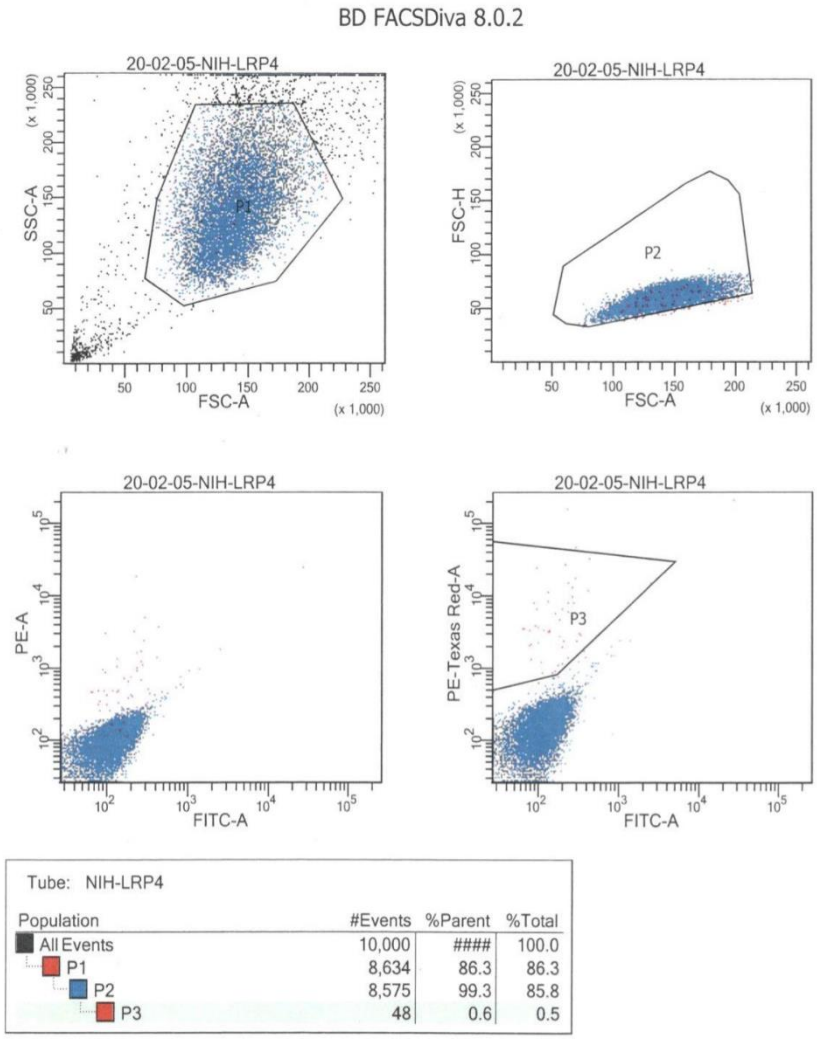

Figure 11. Fluorescence activated cell sorting assay of NIH3T3-Lrp4 cells. Each dot on the blot represents an individual particle that has passed through the laser. A gate has been applied to identify a specific population, in this case NIH3T3-Lrp4 cells. The picture shows that after FACS we had 0.5% of successfully sorted cells.

3.4 Lrp4 expression in TE671, L6, HEK293 and C2C12 cell lines

3.4.1 Expression of Lrp4-GFP and Lrp4-Caspr2+Lrpap in the HEK293 cell line

In order to analyze the expression levels of Lrp4 in different cell lines, protein extracts from cells were prepared in lysis buffer and characterized by SDS-PAGE and Western blot as described above. Firstly, we wanted to ensure the expression of Lrp4 in the HEK293 cell line. Accordingly, the cell lysates were prepared from HEK293 cells transfected with Lrp4-GFP and Lrp4-Caspr2+Lrpap. To detect the Lrp4 protein, anti-Lrp4 antibodies were used as the primary antibodies and anti-mouse IgG HRP as the secondary antibody. The analysis showed that both, Lrp4-GFP and Lrp4-Caspr2+Lrpap were expressed in the HEK293 cell line and that their size is between 150 – 250kDA (Figure 12).

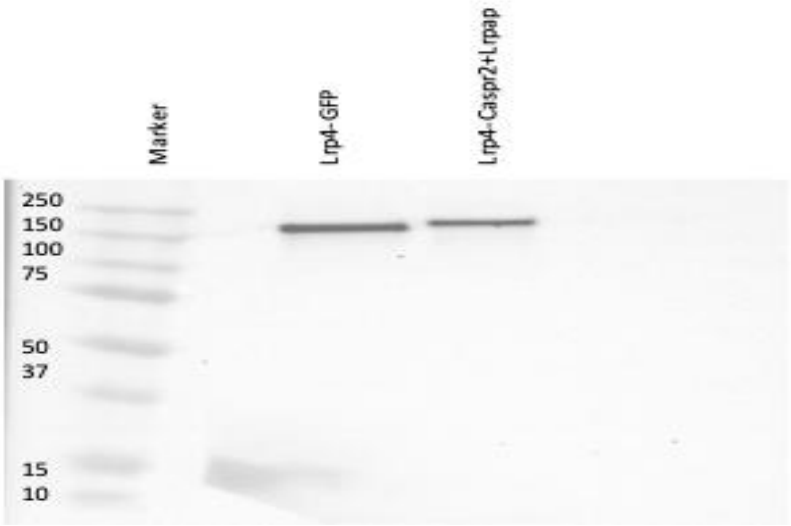

Figure 12. Expression of Lrp4-GFP and Lrp4-Caspr2+Lrpap in HEK293 cell line. Samples were subjected to SDS-PAGE and Western Blotting.

3.4.2 Expression of Lrp4-SBP

The main idea was to develop a diagnostic enzyme-linked immunosorbent assay (ELISA) test for measuring the Lrp4 antibodies in clinical sera from patients with Myasthenia gravis.
In order to do this, we decided to produce and purify a recombinant Lrp4-SBP. The Streptavidin-Binding Peptide (SBP)-Tag is a 38-amino acid sequence. It can be engineered into recombinant proteins which, containing the SBP-Tag, have the property of binding to streptavidin. This property can be used in specific purification, immobilization, or detection strategies. Lrp4-SBP was used for the transfection of the HEK293 cells. The cell supernatant was subjected to SDS-Page and Western blot. The visualization is achieved by immunoblotting of SDS-Page. Figure 13a shows the membrane that was incubated with anti-Lrp4 primary antibody and later on, it was incubated with secondary anti-mouse IgG HRP. The bands were detected successfully.
The same protocol was used for streptavidin-peroxidase conjugate to detect SBP (Streptavidin-Binding Peptide). Streptavidin is covalently linked to horseradish peroxidase. This time, no signal was obtained (Figure 13b).

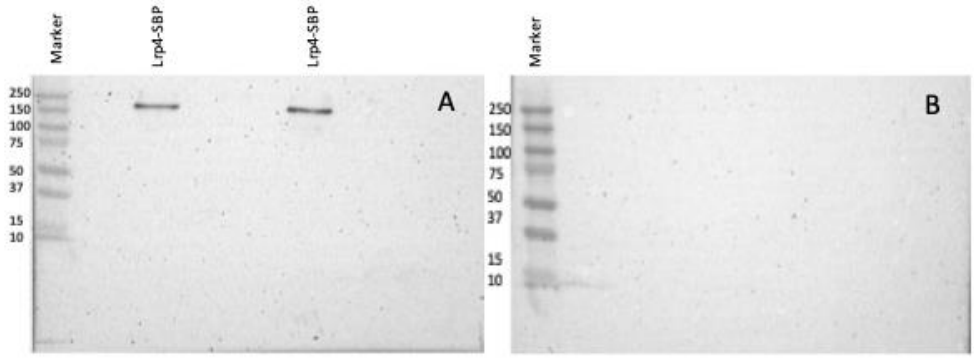

Figure 13. **a.** Lrp4-SBP bands were visible on the membrane incubated with anti-Lrp4 primary antibody and anti-mouse IgG HRP secondary antibody. **b.** Lrp4-SBP bands were not visible on the membrane incubated with Streptavidin-HRP.

Again, the whole procedure was repeated with one modification. This time, instead of using 1ml of Streptavidin-HRP, the membrane was incubated for one hour with Streptavidin-HRP diluted in blocking buffer. Figure 14b shows that even with this secondary antibody no signal was detected.

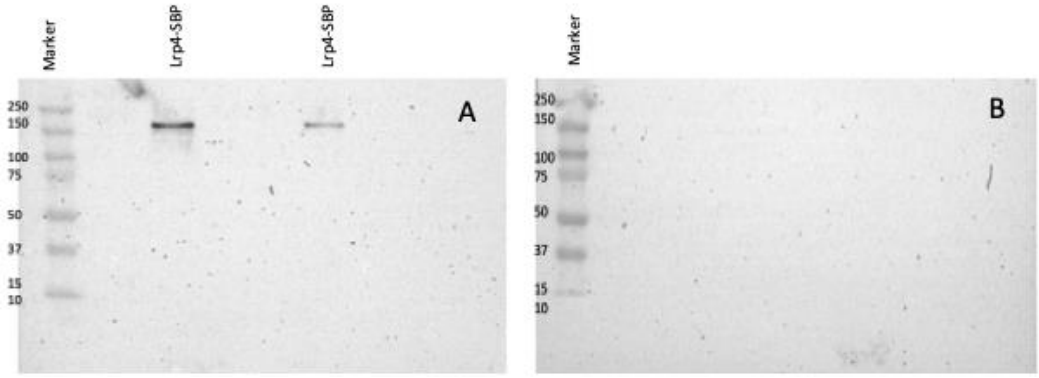

Figure 14. **a.** Lrp4-SBP bands were visible on the membrane incubated with anti-Lrp4 primary antibody and anti-mouse IgG HRP secondary antibody. **b.** Lrp4-SBP bands were not visible on the membrane incubated with Streptavidin-HRP.

If the bands of Lrp4-SBP were fine for Strep-HRP and anti-Lrp4 we would continue with the experiment and we would elute Lrp4-SBP from lysates of transfected HEK293 cells by affinity chromatography on Streptavidin-Agarose-Column. For the confirmation that we have our protein of interest, we would perform SDS-Page and Western blot with the supernatant, lysate, flow-through, and the elution where we suspected our protein of interest.

Since the bands were not visible with streptavidin-HRP we proceed with making fresh supernatant. In parallel, we tried to make fresh plasmid. Unfortunately, both experiments failed.

3.4.3 Expression of Lrp4 in TE671, L6, HEK293 and C2C12 cell lines

In order to analyze the expression levels of Lrp4 in different cell lines, protein extracts from cells were prepared in Lysis buffer and characterized by SDS-PAGE and Western blot as described above. Our results show that Lrp4 is expressed in HEK293 transfected with Lrp4, L6, and C2C12 cell lines. Lrp4 was hardly detectable in myoblasts, but its expression was detected as myotubes matured. No bands were visible in the TE671 cell line.

Table 3.4: Loading of the samples to the gel

Cell line	Protein concentration mg/ml	WB
HEK 293 untransfected	2.66	7.8 µl
HEK293 transfected with Lrp4-GFP	2.5	7.5 µl
L6	2.6	7.6 µl
TE671	3.1	6.4 µl
C2C12 myoblasts	3.5	5.7 µl
C2C12 myotubes	2.5	7.9 µl

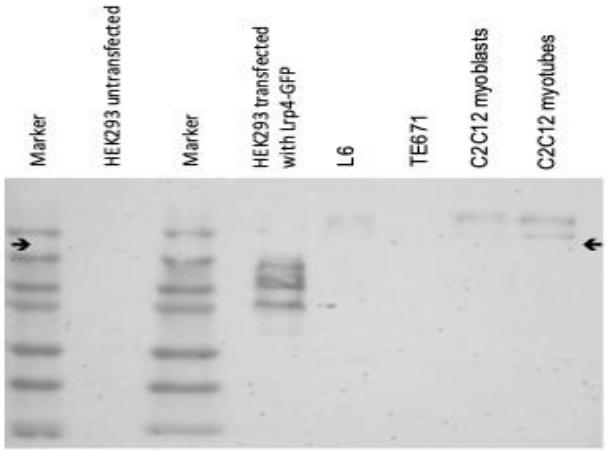

Figure 15. Expression analysis of Lrp4 in L6, TE671, HEK293 and C2C12 cell line. Samples were subjected to SDS-PAGE and Western Blotting. The arrow indicates where Lrp4 bands should be seen.

4. Discussion

The cell-based assay is used for live cells expressing MuSK and AChR autoantibodies in the diagnostic of MG patients. Our goal was to develop a diagnostic test for the detection of Lrp4 autoantibodies in patients with Myasthenia gravis. During the study, a diagnosis was made for nearly 100 patients with autoantibodies against MuSK and AChR in the sera. The presence of autoantibodies against NMJ was tested from the clinically diagnosed MG patients' sera stored at NeuroBiobank located at the Medical University of Vienna, in the Department of Neuropathology and Neurochemistry in Vienna, Austria.
Diagnostics is done for 98 patients from which 44 samples were seronegative for AChR and MuSK autoantibodies. In the total number of seropositive sera, 11 were AChR positive, 16 MuSK positive, and 1 case was double-positive.
Guided by the principle of cell-based assay used for diagnostics against MuSK and AChR autoantibodies, the idea was to create a diagnostic test that works on the same principle but for the detection of Lrp4 autoantibodies in MG patients.

In order to create this test, it was necessary to determine if the Lrp4 was expressed on the cell surface. In parallel, the cell-based assay was done for live and fixed, permeabilized HEK293 cells. The protocol that is used for the presence of MuSK and AChR autoantibodies, was used for the live cells, while a modified protocol was used for fixed, permeabilized HEK293 cells. We have tried several modifications to the existing diagnostic assay. DNA titration was done to determine whether the DNA concentration itself affects Lrp4 surface expression. The time-course experiment was done to check if the surface expression of Lrp4 depended on the time. No significant difference was observed in Lrp4 surface expression with different amounts of DNA used for transfection, nor with a longer incubation period. Lrp4 was equally expressed in fixed and permeabilized HEK293 cells, while the surface expression of Lrp4 was not observed in the live HEK293 cells.
Since this experiment did not give expected results, we decided to try instead of using the HEK293 cell line, to use the NIH3T3 cell line.

In parallel, we wanted to isolate rat Lrp4-SBP with affinity chromatography on Streptavidin-Agarose-Column, which would be used in a new diagnostic ELISA test for measuring the Lrp4 antibodies in clinical sera from patients with Myasthenia gravis. In order to do this, we decided to produce and purify a recombinant Lrp4-SBP. HEK293 cells were transfected with Lrp4-SBP. The cell supernatant was subjected to SDS-Page and Western blot. The visualization is achieved by immunoblotting of SDS-Page.. After the incubation of Lrp4-SBP with the primary antibody, Lrp4-SBP was incubated with Streptavidin-HRP. No signal was detected. Since the bands were not visible, we proceed with making fresh supernatant and we tried to make fresh plasmids. Unfortunately, both experiments were not successful. A possible reason for this was the low concentration of plasmid DNA (around 300µg/mL).

At the same time, the expression of Lrp4 was tested in L6, TE671, C2C12 cell lines using SDS-PAGE and Western blot. Our results show that Lrp4 is expressed in HEK293 transfected with Lrp4, L6 and C2C12 cell line. Lrp4 was hardly detectable in myoblasts, but its expression was detected as myotubes matured. No bands were visible in the TE671 cell line.

The titration experiment showed that the surface expression of Lrp4 in live HEK293 cells does not depend on the amount of DNA used for transfection and the time-course experiment showed that even six days after transfection no surface expression of Lrp4 was detected. We conclude that the HEK293 cell line was unsuitable for Lrp4 surface expression, both in the live and in the fixed, permeabilised HEK293 cells. Although it did not give the expected results, with certain modifications NIH-3T3 cell line could solve the problem of surface expression of Lrp4.

4.1 Antibody diagnostics

For diagnostics of patients with MG, it is necessary to perform an analysis of patients' sera for the detection of the presence of autoantibodies, against the antigen of interest. Detection of autoantibodies against AChR and MuSK is easily feasible today because there are tests that give relevant results and these are ELISA and radioimmunoassays. This does not apply to all patients as there is a subgroup of patients in which antibodies recognize clustered AChR and autoantibodies against AChR cannot be detected. In this case, another detection method is used, the cell-based assay[21,34,35,36]. Live cell-based assays were used to detect the presence of antibodies against antigens due its higher sensitivity in clustered AChR and MuSK assays[37, 38,39]. The methods used for the purpose of detecting autoantibodies are already well known

when it comes to autoantibodies against AChR, MuSK and clustered AChR. However, not all myasthenic antibodies can be detected by this test. That is the case with Lrp4. The main problem with Lrp4 is its inability to express on the cell surface. Since the cell-based assay works on the principle of surface binding of antibodies to antigen, this is not possible with Lrp4. The studies have shown that depending on the method used for the detection of autoantibodies in patients without MuSK and AChR antibodies the presence of Lrp4 MG varies between 2-50%[34, 40,41,42].

4.2 Modification of the Cell-Based Assays

In order to create a diagnostic test for the detection of Lrp4 autoantibodies, it was necessary to improve the already existing diagnostic test used for the detection of MuSK and AChR. As is already known, in cell-based assay the antigen is highly expressed at the cell membrane which enables the binding of the autoantibody to the antigen[39,44].

Before establishing a new diagnostic method for the detection of Lrp4 autoantibodies in MG patients, it was necessary to check the expression of Lrp4 in the cell. In parallel, live cells were transfected with Lrp4, as well as the fixed cells with one main difference. HEK293 cells were fixed with 4%PFA, in the following step the cells were permeabilized with 0.3% Triton X solution. This allowed the antibody to bind to the antigen, which was detected by fluorescence microscopy after staining with the secondary antibody. The result of this experiment showed that Lrp4 was not expressed in live HEK293 cells. In contrast, Lrp4 expression was observed in fixed and permeabilized HEK293 cells. Although the Lrp4 was expressed in fixed and permeabilized cells (modified from Pevzner et al.[23]), the cells were compact, with irregular morphological shape, and with a high background as shown in Figure 10. A possible reason for this result is the insufficient capacity of the HEK293 cell line. Other research groups as Zisimopoulou et al.[40] used fixed HEK293 for Lrp4 cell-based assay. Our goal was to use live cell-based assay, not fixed and we conclude that a new way of resolving Lrp4 expression was needed. We decided to use another cell line to achieve the desired result.

4.3 Usage of NIH-3T3 cell line in the surface expression of Lrp4

The Phoenix-Eco cells were transfected and the target NIH3T3 cells were infected with supernatant supplemented with polybrene. On the fourth day after transfection, the supernatant was removed from Phoenix-Eco cells. After the incubation for 4 hours at 37°C, the supernatant was removed from NIH3T3 cells and replaced with HEK cell growth medium. After 24 hours of incubation in the incubator at 37°C, sorting was done. FACS is done to determine the percentage of cells stably infected with the virus. According to the protocol of Dr. Ute Koch, transfected cells should be stored at 32°C which slows the rate of cell division without significantly decreasing viral production. Incubation at 32°C also increases the stability of the retroviral particles. Since the laboratory in which this experiment was performed is not equipped with an incubator that can be used at that temperature, we did this step at 37°C degrees. The other thing that could be done is the re-infection of the cells every 12h in order to improve infection efficiency. These steps were not done by us and this could be a possible reason why we had a low percentage of successfully sorted cells. However, improving the protocol presented in this study could be a solution for Lrp4 surface expression.

4.4 Expression of Lrp4 in cell lines

Cell lysates of HEK293 transfected with Lrp4, HEK293 untransfected, TE671, L6, C2C12 myoblast, and C2C12 myotubes were prepared. Protein concentration was measured using the Biorad kit at 620nm. Samples were subjected to SDS-PAGE and Western Blotting. Our results show that Lrp4 is expressed in HEK293 transfected with Lrp4, L6, and C2C12 cell lines. Lrp4 was hardly detectable in myoblasts, but its expression was detected as myotubes matured. This has been proven by Zhang et al.[43] No bands were visible in the TE671 cell line. As shown in figure 15, the bands look faint, and the signal is not strong, which could be improved by further analysis. One way is to load a higher concentration of protein, and the other solution could be to prepare always fresh lysates and prevent protein damage caused by frequent thawing and freezing.

4.5. Final considerations

This study aimed to establish a new diagnostic method for the detection of Lrp4 autoantibodies in patients with Myasthenia gravis. On the way to achieving this goal, certain difficulties arose that led this scientific work in another direction. The original plan based on the idea that the HEK293 cell line expresses the Lrp4 on its surface proved unsuccessful, so the final result was without Lrp4 surface expression. Although unsuccessful, the NIH-3T3 cell line leaves space for further modifications because if the conditions from Dr. Koch's protocol were met, the outcome would very likely be successful.

5.Bibliography

1. MedlinePlus. *Myasthenia gravis.* Available from: https://medlineplus.gov/genetics/condition/myasthenia-gravis/ [Accessed October 14, 2020].

2. Mori, S. *et al*. Immunization of mice with LRP4 induces myasthenia similar to MuSK-associated myasthenia gravis. *Exp. Neurol*. **297**, 158–167 (2017).

3. Gilhus, N. E. *et al*. Myasthenia gravis—autoantibody characteristics and their implications for therapy. *Nat. Rev. Neurol*. **12**, 259–268 (2016).

4. Martyn, J. A. J., Fagerlund, M. J. & Eriksson, L. I. Basic principles of neuromuscular transmission. *Anaesthesia* **64**, 1–9 (2009).

5. Bittner, E. A. & Martyn, J. A. J. *Neuromuscular Physiology and Pharmacology. Pharmacology and Physiology for Anesthesia* (Elsevier Inc., 2019). doi:10.1016/b978-0-323-48110-6.00021-1

6. Okada, K. The Muscle Protein Dok-7 Is Essential for Neuromuscular Synaptogenesis. *Science (80-.)*. **312**, 1802–1805 (2006).

7. DeChiara, T. M. et al. The Receptor Tyrosine Kinase MuSK Is Required for Neuromuscular Junction Formation In Vivo. *Cell* **85**, 501–512 (1996).

8. Li, L., Xiong, W.-C. & Mei, L. Neuromuscular Junction Formation, Aging, and Disorders. *Annu. Rev. Physiol*. **80**, 159–188 (2018).

9. Drachman, D. B., Adams, R. N., Stanley, E. F. & Pestronk, A. Mechanisms of acetylcholine receptor loss in myasthenia gravis. *J. Neurol. Neurosurg. Psychiatry* **43**, 601–610 (1980).

10. Shi, L., Fu, A. K. Y. & Ip, N. Y. Molecular mechanisms underlying maturation and maintenance of the vertebrate neuromuscular junction. *Trends Neurosci*. **35**, 441–453 (2012).

11. Carr, A. S., Cardwell, C. R., McCarron, P. O. & McConville, J. A systematic review of population based epidemiological studies in Myasthenia Gravis. *BMC Neurol*. **10**, (2010).

12. Meriggioli, M. N. & Sanders, D. B. Autoimmune myasthenia gravis: emerging clinical and biological heterogeneity. *Lancet Neurol.* **8**, 475–490 (2009).

13. Gilhus, N. E. & Verschuuren, J. J. Myasthenia gravis: Subgroup classification and therapeutic strategies. *Lancet Neurol*. **14**, 1023–1036 (2015).

14. Aarli, J. A. Late-Onset Myasthenia Gravis. *Arch. Neurol*. **56**, 25 (1999)

15. Marx, A., Ströbel, P. & Weis, C.-A.Thymoma-Associated Myasthenia Gravis. in *Myasthenia Gravis and Related Disorders* (eds. Kaminski, H. J. & Kusner, L. L.) 113–129 (Springer International Publishing, 2018). doi:10.1007/978-3-319-73585-6_8

16. Koneczny & Herbst. Myasthenia Gravis: Pathogenic Effects of Autoantibodies on Neuromuscular Architecture. *Cells* **8**, 671 (2019).

17. Bergamin, E., Hallock, P. T., Burden, S. J. & Hubbard, S. R. The Cytoplasmic Adaptor Protein Dok7 Activates the Receptor Tyrosine Kinase MuSK via Dimerization. *Mol. Cell* **39**, 100–109 (2010).

18. Zhang, W., Coldefy, A. S., Hubbard, S. R. & Burden, S. J. Agrin binds to the N-terminal region of Lrp4 protein and stimulates association between Lrp4 and the first immunoglobulin-like domain in muscle-specific kinase (MuSK). *J. Biol. Chem*.**286**, 40624–40630 (2011).

19. Leite, M. I. et al. Fewer thymic changes in MuSK antibody-positive than in MuSK antibody-negative MG. *Ann. Neurol*. **57**, 444–448 (2005).

20. Guptill, J. T., Sanders, D. B. & Evoli, A. Anti-MuSK antibody Myasthenia gravis: Clinical findings and response to treatment in two large cohorts. *Muscle and Nerve* **44**, 36–40 (2011).

21. Huijbers, M. G. *et al.* MuSK IgG4 autoantibodies cause myasthenia gravis by inhibiting binding between MuSK and Lrp4. *Proc. Natl. Acad. Sci.* **110**, 20783–20788 (2013).

22. Rodriguez Cruz, P. M., Huda, S., López-Ruiz, P. & Vincent, A. Use of cell-based assays in myasthenia gravis and other antibody-mediated diseases. *Exp. Neurol*. **270**, 66–71 (2015).

23. Pevzner, A. et al.Anti-LRP4 autoantibodies in AChR-and MuSK-antibodynegative myasthenia gravis. *J. Neurol*. **259**, 427–435 (2012).

24. Zhang, B. *et al.* Autoantibodies to agrinin myasthenia gravis patients. *PLoS One* **9**, 4–9 (2014).

25. Katarzyna, M. Z., Belaya, K., Leite, M., Patrick, W. & Beeson, D. Europe PMC Funders Group Collagen Q –A potential target for autoantibodies in myasthenia gravis. **348**, 241–244 (2018).

26. Yan, M. *et al.* Induction of Anti-agrin Antibodies Causes Myasthenia Gravis in Mice. *Neuroscience* **373**, 113–121 (2018).

27. Gasperi, C. *et al.* Anti-agrinautoantibodies in myasthenia gravis. *Neurology* **82**, 1976–1983 (2014).

28. Rose, N. R. & Bona, C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited). *Immunol. Today* **14**, 426–430 (1993).

29. Lee, C. W., Zhang, H., Geng, L. & Peng, H. B. Crosslinking-Induced Endocytosis of Acetylcholine Receptors by Quantum Dots. *PLoS One* **9**, e90187 (2014).

30. Koneczny, I. *et al*. IgG4 autoantibodies against muscle-specific kinase undergo Fab-arm exchange in myasthenia gravis patients. *J. Autoimmun*. **77**, 104–115 (2017).

31. Kaminski, H. J. Seronegative Myasthenia Gravis—A Vanishing Disorder? *JAMA Neurol*. **73**, 1055 (2016).

32. Rodriguez Cruz, P. M., Huda, S., López-Ruiz, P. & Vincent, A. Use of cell-based assays in myasthenia gravis and other antibody-mediated diseases. *Exp. Neurol*. **270**, 66–71 (2015).
33. Suzuki, S., Utsugisawa, K., Nagane, Y. & Suzuki, N. Three Types of Striational Antibodies in Myasthenia Gravis. *Autoimmune Dis*. **2011**, 1–7 (2011).
34. Vincent A., Huda S., Cao M., Cetin H., Koneczny I., Rodriguez-Cruz P., Jacobson L., Viegas S., Jacob S., Woodhall M., et al. Serological and experimental studies in different forms of myasthenia gravis. *Ann. N. Y. Acad. Sci*. **1413**, 143–153 (2018).
35. Rodriguez Cruz P.M., Al-Hajjar M., Huda S., Jacobson L., Woodhall M., Jayawant S., Buckley C., Hilton-Jones D., Beeson D., Vincent A., et al. Clinical Features and Diagnostic Usefulness of Antibodies to Clustered Acetylcholine Receptors in the Diagnosis of Seronegative Myasthenia Gravis. *JAMA Neurol.* **72**, 642–649 (2015).
36. Devic P., Petiot P., Simonet T., Stojkovic T., Delmont E., Franques J., Magot A., Vial C., Lagrange E., Nicot A.S., et al. Antibodies to clustered acetylcholine receptor: Expanding the phenotype. *Eur. J. Neurol*. **21**, 130–134 (2014).
37. Gilhus, N. E. *et al* Myasthenia gravis-Autoantibody characteristics and their implications for therapy. *Nat. Rev. Neurol*. **12**, 259–268 (2016).
38. Leite M.I., Jacob S., Viegas S., Cossins J., Clover L., Morgan B.P., Beeson D., Willcox N., Vincent A. IgG1 antibodies to acetylcholine receptors in 'seronegative' myasthenia gravis. *Brain*. **131**, 1940–1952 (2008).
39. Maddison, P., Sadalage, G., Ambrose, P. A., Jacob, S. & Vincent, A. False positive acetylcholine receptor antibody results in patients without myasthenia gravis. *J. Neuroimmunol.* **332**, 69-72 (2019).
40. Zisimopoulou, P., Evangelakou P., Tzartos J., Lazaridis K., Zouvelou V., Mantegazza R., Antozzi C., Andreetta F., Evoli A., Deymeer F., et al. A comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 in myasthenia gravis. *J. Autoimmun*.. **52**, 139-145 (2014).
41. Zhang, B., Tzartos J.S., Belimezi M., Ragheb S., Bealmear B., Lewis R.A., Xiong W.C., Lisak R.P., Tzartos S.J., Mei L. Autoantibodies to lipoprotein-related protein 4 in patients with double-seronegative myasthenia gravis. *Arch. Neurol*. **69**, 445-451 (2012).
42. Yan M., Xing G.L., Xiong W.C., Mei L. Agrin and LRP4 antibodies as new biomarkers of myasthenia gravis. *Ann. N. Y. Acad. Sci.* **1413**, 126–135 (2018).
43. Zhang, B., Luo S., Wang Q., Suzuki T., Mei L. Lrp4 Serves as a Coreceptor of Agrin. *Neuron.* **60**, 285-297 (2008).

44. Yan, C. *et al.* Cell-Based Versus Enzyme-Linked Immunosorbent Assay for the Detection of Acetylcholine Receptor Antibodies in Chinese Juvenile Myasthenia Gravis. *Pediatr. Neurol.* **98**, 74-79 (2019).

45. Grob, D., Brunner, N., Namba, T. & Pagala, M. Lifetime course of myasthenia gravis. *Muscle and Nerve* **37**, 141–149 (2008).