

# Evaluation of immunoglobulin G and immunoglobulin G subclass levels in serum of anti-muscle specific kinase and anti-acetylcholine receptor antibody positive myasthenia gravis patients

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## Content

Due to policy boundaries, the results and conclusions of my thesis cannot be disclosed in this paper. Instead this paper will provide background information about myasthenia gravis (MG) and the role of immunoglobulin G (IgG) antibodies in autoimmune MG. Additionally, previous literature will be discussed to elucidate the aim and relevance of my internship project. During the internship, total IgG and IgG subclass (IgG1, IgG2, IgG3, and IgG4) concentrations were evaluated in serum of anti-muscle specific kinase antibody positive MG (MuSK-MG), anti-acetylcholine receptor antibody positive MG (AChR-MG) patients and compared with the total IgG and IgG subclass levels in healthy control serum.

## Keywords

Myasthenia gravis, muscle specific kinase, IgG, IgG4, IgG4-mediated diseases.

## Background

The ability of animals to make coordinated and precise movements of body parts is the consequence of the conveyance and information processing of electrical and chemical signals by cells of the nervous system which lead to muscle contractions (1, 2). Communication between neuronal cells is enabled by synapses, which are specialized contacts between neurons or between neurons and target cells (2, 3). The pre and postsynaptic cells of electrical synapses are continuous structures through which a bidirectional ion-current flows through gap-junctions. In contrast, chemical synapses are not continuous structures. In chemical synapses, a cleft of approximately 20-40 nm

separates the pre and postsynaptic cells. In these synapses, transmission of an electrical signal from the presynaptic cell towards the postsynaptic cell depends on exocytosis of neurotransmitters and diffusion of the neurotransmitter molecules across this cleft (3). Subsequently, the neurotransmitters bind to their receptors on the postsynaptic membrane, inducing a further response.

### The neuromuscular junction

Motor units consist of motor neurons and groups of muscle fibers, which innervate the contraction of all muscles in our body. The neuromuscular junction (NMJ) is the synapse located between the motor neuron terminal and the muscle. The NMJ is responsible for the transmission of signals arriving from the central nervous system (CNS) to the muscles (figure 1)(1, 4). The motor unit is frequently involved as a target for diseases whereby the cell body of a motor neuron, the axons of the motor neurons, the NMJ, or the muscles themselves are affected (1). An altered or disrupted transmission of the neurotransmitters between the motor neuron and muscle cells characterize disorders of the NMJ. Disorders of the NMJ can be divided into two categories. First, chemical transmission by neurotransmitters at the NMJ can be altered by defects in the presynaptic terminal. The second form involves the postsynaptic membrane, also termed the motor endplate (1). Several postsynaptic proteins are important for the functioning and maintenance of the NMJ, including the acetylcholine receptor (AChR), muscle specific kinase (MuSK), and low density lipoprotein receptor-related protein 4 (Lrp4). The AChR is responsible for signal transduction by binding the neurotransmitter acetylcholine (ACh). MuSK and Lrp4 are postsynaptic proteins essential in NMJ maintenance. The tyrosine kinase activity of MuSK plays a crucial role in an agrin-Lrp-MuSK signaling pathway that facilitates AChR clustering at the postsynaptic membrane. Hence, AChR clustering is necessary for signal transduction by depolarizing the muscle fibers after binding of ACh (figure 1) (4).

### Myasthenia Gravis

MG, derived from fusing the Greek terms for muscle (*mus*) and weakness (*sthenos*) with the Latin term for severe (*gravis*) (5), is a rare and often severe disorder of the NMJ and characterized by fatigable muscle weakness in skeletal muscles (6, 7). The symptoms usually occur after use of affected muscles and ameliorate after rest (6). Two major subsets of MG are currently recognized: congenital and autoimmune MG. Congenital MG is characterized by inherited defects in some components of the NMJ caused by mutations. Autoimmune MG is the most prevalent form of MG (1). In patients with autoimmune MG, antibodies are produced against extracellular components of the NMJ (6-8).

Approximately, 80-90% of MG patients have been diagnosed with AChR-MG affecting approximately 90 persons per million inhabitants in the Netherlands (9). In about 20% of the MG patients no antibodies directed to AChR are detected. Antibodies to other important proteins than the AChR have been identified during the last decade. Approximately, 50% of this group have been diagnosed with antibodies directed to MuSK, affecting approximately 1.9 persons per million inhabitants in the Netherlands (9). More recently, antibodies to Lrp4 have been identified. Together, antibodies directed against these three proteins currently characterize the three most distinct forms of MG (8).

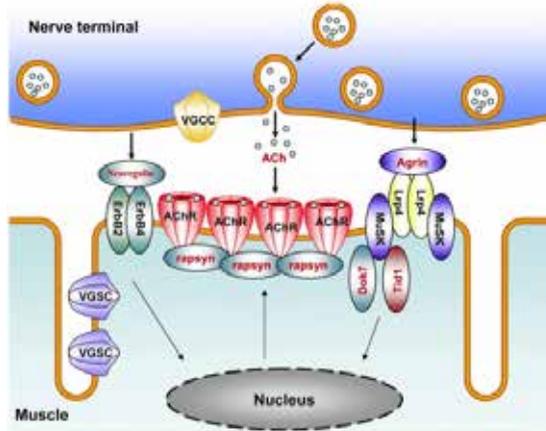
Different clinical subtypes of MG have been described which can be distinguished from each other according to age of onset and topography of the altered NMJ transmission (6). In 67% of all MG patients, the ocular muscles are initially affected causing symptoms as diplopia and ptosis. In a small subgroup of patients, this can be the only manifestation of the disease. However, in other MG patients the symptoms can become more generalized. The ocular symptoms are usually followed by affected bulbar and limb muscles (6). MG with anti-MuSK antibodies often involves fluctuating weakness of bulbar and respiratory muscles. When respiratory muscles are affected, the disease becomes more life threatening. During a crisis, the patients might require intensive care and mechanical ventilation (10). Diagnosis of MG currently relies on serological and electrodiagnostic tests. Therapies currently in use include anti-cholinesterase drugs, immunosuppression, immunomodulation, and a novel drug named rituximab (6).

It has been suggested that environmental and/or genetic factors play a role in the susceptibility to develop the disease. The prevalence of MuSK-MG differs in different geographic regions and ethnic groups. For example, the prevalence of MuSK-MG is low in Taiwan and Denmark, whereas it is high in North American, British and Italian populations (9, 11). Furthermore, MuSK-MG has been associated with the human leukocyte antigen (HLA) DR14-DQ5 haplotypes (12) whereas AChR-MG has been associated with among others HLA-B8DR3 (8, 13).

#### **Antibodies and effector mechanisms in autoimmune myasthenia gravis**

Autoimmune MG is an antibody-mediated autoimmune disorder fulfilling the Witebsky postulates, since (i) antibodies are present at the NMJ, (ii) immunoglobulins from MG patients cause MG symptoms in rodents, and (iii) therapies that remove antibodies decrease the severity of the MG symptoms (15). Different mechanisms have been previously described by which the antibodies might interfere with AChR, MuSK, and the

functioning of other postsynaptic proteins. Effector mechanisms by which antibodies can cause clinical symptoms include: (i) complement activation via binding of the antibody to C1q, resulting in damage to the muscle endplate via membrane attack complexes (MAC), (ii) competition with ligand binding sites, (iii) cross-linking of proteins resulting in endocytosis of the antigen (also called antigenic modulation), and (iv) steric hindrance, inhibiting binding to proteins (8, 16). The effector mechanisms of the antibodies involved in the pathogenic mechanisms in MG are dependent on the IgG subclass. A typical immunoglobulin molecule consists of four polypeptide chains. Among these four, two are identical light chains and the other two are identical heavy chains. The N-terminal regions of the light and heavy chains form the antigen-binding site of the immunoglobulin molecule (variable region (V) regions). The C-terminal regions (constant (C) regions) form the tail, which mediate different functional properties of an immunoglobulin molecule. Five major immunoglobulin isotypes can be distinguished in mammals, each with different biological properties: IgA, IgD, IgE, IgG, and IgM, with their own heavy chain class,  $\alpha$ ,  $\delta$ ,  $\epsilon$ ,  $\gamma$ , and  $\mu$  respectively. Moreover, next to the different heavy chain classes, two different indistinguishable light chain classes,  $\kappa$  and  $\lambda$ , can be distinguished (17). From all immunoglobulin isotypes, IgG is predominantly found in serum covering approximately 75% of all isotypes (17). The different IgG subclasses exhibit different functional properties. Antibody flexibility and functional affinity are affected by differences in the constant regions of the heavy chains (17). IgG 1 - 3 are able to bind C1q and thereby activating complement fixations, whereas IgG4 is not able to bind C1q (18-20). Some properties of different IgG subclass molecules are summarized in table 1. Complement activation and cross-linking of the AChR are most commonly involved in the pathogenesis of AChR-MG. This mechanism leads to removal of AChRs and destruction of the muscle membrane morphology (6, 8, 16). Anti-MuSK antibodies have only been discovered in 2001 (21). MuSK-MG differs in several aspects from AChR-MG. In contrast to the IgG1 and IgG3 subclass that predominate in AChR-MG, IgG4 predominates in the anti-MuSK antibodies in MuSK-MG (8). MuSK specific IgG4 antibodies are believed to cause MG by inhibiting Lrp4-MuSK signaling resulting in destroyed AChR clusters (20, 22, 23). However, a contribution of IgG1, IgG2, and IgG3 have also been previously described. These IgG subclass molecules are thought to affect the NMJ and reduce AChR clusters (23).



**Figure 1.** Schematic illustration of the NMJ. The presynaptic and postsynaptic membrane are separated by a synaptic cleft. Transmission of signals from the CNS are mediated by the neurotransmitter ACh, which is released from synaptic vesicles. ACh diffuses into the synaptic cleft where it binds to its receptor (AChR) which subsequently induces the opening of other voltage- dependent Na<sup>+</sup> ion-channels. Other proteins that are present at the postsynaptic membrane include MuSK, Lrp4, and Rapsyn, which are important in signaling cascades that promote AChR clustering. Figure adapted from Gomez et al. *Autoimmunity* (2010) (14).

### Unique properties of IgG4 subclass molecules

IgG4 is a unique IgG subclass with unique biological properties and structure. First, IgG4 is not able to bind C1q and to fixate the complement system. Second, an interesting difference in the sequence of IgG1-3 and IgG4 is located in the hinge region (18, 19). A consequence of this difference is the increased susceptibility for chemical reduction of the disulfide bonds at the hinge region allowing heavy chains with attached light chain (half-antibody) to separate from each other (18). This distinctive feature is the basis of the Fab arm exchange reaction, a unique property of the IgG4 molecule. Over the last decade,



**Figure 2.** Schematic representation of Fab arm exchange reaction in IgG4 molecules.

researchers revealed that the half antibodies could randomly re-associate with other half-antibodies, thereby becoming a bispecific IgG4 molecule (figure 2). Consequently, Fab arm exchange

is believed to attribute IgG4 antibodies with anti-inflammatory activity. Because these bispecific antibodies are monovalent for a specific antigen, they are unable to form immune complexes with an antigen, a property that the other IgG subclasses (IgG1, IgG2, and IgG4) do possess (18, 19).

#### **igg4 antibody production in MuSK-MG and other IgG4 mediated diseases**

IgG levels have been investigated in pemphigus patients, revealing increased levels of the IgG4 subclass exclusively (24, 25). Increased IgG4 levels have also been found in other IgG4 mediated diseases (26). In healthy individuals, IgG4 levels increase after repeated exposure to an antigen. Yet, little is known about the immunologic factors that underlie the production of antigen specific IgG4 molecules. Interactions between T-lymphocytes and B-lymphocytes are crucial for the production of antibodies with a high affinity for its antigen. Particular cytokines regulate class switch gene rearrangements and influence the immunoglobulin subtypes that are being produced. Different T helper (Th) cells secrete these cytokines (27, 28). The result of activated B-lymphocyte exposure to a particular cytokine is differentiation of the B-lymphocytes into an antibody producing plasma cell (27). Like IgG4, IgE production is regulated by the secretion of Th2 cytokines (e.g. IL-4 and IL-13). In contrast to these cytokines, IL-10, IL-12, and IL-21 shift the balance towards IgG4 production (29-31). It has been hypothesized that chronic exposure to an allergen provides a strong stimulus that induces a switch to IgG4 under exposure of IL-4 in the germinal centers. Furthermore, B-lymphocytes that express IgE would not survive herein (32). It also has been suggested that T-regulatory (Treg) cells may be involved in IgG4 production (44). IL-10 produced by these Treg cells are thought to suppress IgE production and induce allergen-specific IgG4 producing plasma cells (33). Several studies investigated cytokine profiles and the involvement of Th cells in MuSK-MG (45, 46), but it remains controversial which Th cells subset (Th1, Th2 or Th17) are involved in the development of MG. However, recently it has been suggested that the Th1 and Th17 subsets play a role in MuSK-MG (34). Moreover, a decreased B10-lymphocyte percentage has been found in MuSK-MG patients. It is suggested that the loss of B10-lymphocytes and B10 derived IL-10 is involved in creating a permissive environment for autoantibody production and Th1 and Th17 immune responses, due to loss of self-tolerance (35). The role of T and B-lymphocytes in the pathogenesis have yet to be elucidated. Future studies are needed to elucidate how antigen specific IgG4 production is regulated and induced in MuSK-MG.

**Table 1.** Properties of IgG subclasses. Table adapted from Nirula et al. *Curr Opin Rheumatol* (2011) (18).

	Proportion of total IgG in serum (%)	Complement activation (C1q binding)	Half- life (days)	Biological target	
<b>IgG subclass</b>	<i>IgG1</i>	43 – 75	++	21	Protein antigen
		16 - 48	+	21	Carbohydrate antigen
	<i>IgG2</i>	1.7 – 7.0	+++	7	Protein antigen
	<i>IgG3</i>	0.8 - 11.7	-	21	Protein antigen
	<i>IgG4</i>				

## Relevance of the study

Despite a lot is known about AChR-MG, important knowledge on MuSK-MG is still lacking. Recently, it has been reviewed that some important similarities in HLA associations, pathogenic mechanisms and epitope binding exist in IgG4 mediated autoimmune disorders (20). Disorders evaluated in this review included MuSK-MG, Guillain-Barré syndrome, limbic encephalitis and non-neurological diseases like pemphigus. In IgG4 mediated diseases, an overlap has been found with the DQ5 and DR14 HLA haplotypes. Furthermore, it was found that all antigens in these IgG4 mediated diseases are N-linked glycoproteins (20). It has therefore been suggested that there might be a common underlying aetiology in IgG4 mediated diseases. IgG and IgG subclass concentrations have been previously described for AChR-MG. Elevated total IgG and IgG subclass (IgG1, IgG2, IgG3, and IgG4) concentrations were found in serum of non-immunosuppressed AChR-MG patients compared to the concentrations found in serum of healthy controls (36). However, little research has been done on IgG levels in serum of MuSK-MG patients. Therefore, it is currently unclear if total IgG and IgG subclass levels are also increased in serum of MuSK-MG patients like in AChR-MG patients or if the distribution of IgG and IgG subclass levels are different from the levels in AChR-MG and more comparable to the levels in other IgG4 mediated diseases. Studies that evaluated total IgG and IgG subclass levels in serum of pemphigus patients revealed an enrichment of IgG4 exclusively (24, 25). Therefore, total IgG and IgG subclass concentrations were analyzed and evaluated in serum of MuSK-MG, AChR-MG patients, and healthy controls. We aimed to elucidate if the distribution of total IgG and IgG subclass concentrations in serum of MuSK-MG patients was comparable to the concentrations in AChR-MG or pemphigus, another IgG4 mediated disease. Total IgG and IgG subclass concentrations were evaluated in serum using Enzyme-Linked Immuno Sorbent Assay (ELISA) and dot blot. Elucidating the total IgG and IgG subclass levels in

MuSK-MG patients will provide us with some new useful insights on MuSK-MG which will be helpful for future research in the field of MuSK-MG. These insights can provide us with more knowledge on the pathological mechanisms leading to the production of MuSK specific IgG antibodies and new therapeutic options for treating MuSK-MG. However, the complexity of the interactions between T and B-lymphocytes and cytokines involved in IgG4 production highlights the fact that here is still much more to understand about MuSK-MG.

## Role of the student

Sander de Haas was an undergraduate student Biomedical Sciences, major in Molecular Life Sciences, working under the supervision of I. Koneczny PhD and Dr. M. Losen (MHeNS) when the research described in this paper was performed. The topic was proposed by Inga Koneczny. The analysis of the serum samples by ELISA and dot blot, data analysis, and writing were done by the student.

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