A Clinical and Immunological Study on Late Onset Myasthenia Gravis

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Abstract

Myasthenia gravis is an autoimmune disorder of neuromuscular transmission caused by antibodies to the acetylcholine receptor and related components on the post synaptic membrane of the neuromuscular junction. Recent evidence has shown that the incidence of late-onset myasthenia gravis, defined as onset at more than 50 years of age, has been increasing. We sought to prospectively recruit patients newly diagnosed with myasthenia gravis and look at their clinical and immunological profile to see if there are any differences between early onset and late-onset myasthenia gravis.

Methods: This was a multicentre study across Nottingham, Birmingham and Oxford. We recruited 150 patients with myasthenia gravis across the three sites, newly diagnosed within the preceding 12 months. We did clinical examinations, completed MG composite scores (MGC), MG Quality of life scores (MG QOL), and blood tests including serum for antibodies, and whole blood for PBMC isolation and T-cell studies. These were repeated at annual follow up. The antibody studies were performed at Oxford by radioimmunoassay (RIA) and cell based assays (CBA) for acetylcholine receptor

antibodies (AChR), muscle specific kinase (MuSK) and lipoprotein-related protein 4 (LRP4) antibodies.

Results: We recruited 150 patients with myasthenia gravis, 76% of whom had LOMG, with a female to male ratio of 1:1.6. EOMG patients more frequently had ocular myasthenia compared to LOMG patients, 94.7% of patients were seropositive for either AChR, MuSK or LRP4 antibodies. T-cell studies showed that the pro-inflammatory and anti-inflammatory cytokine balance is disrupted in all MG patients with decreased Treg percentages and increased production of IL10, IL17 and TNF alpha, which is more pronounced in patients with AChR antibodies. The clinical presentation did not show any difference between the different antibody subgroups, but there was a milder, more indolent course in seronegative patients, and AChR + MuSK double positive patients were more likely to need steroids on generalisation. The majority of the patients responded well to treatment with improvement in MGC, MG QOL and AChR RIA titres with time.

Conclusions: To our knowledge, this is the largest prospective study on the clinical and immunological aspects of late-onset myasthenia gravis to date. Further studies on B cells in MG along with micro-RNAs as biomarkers in MG are being done.

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1 Introduction

1.1 What is myasthenia?

Myasthenia gravis (MG) is an autoimmune disorder of neuromuscular transmission caused by antibodies to the acetylcholine receptor (AChR) and related components on the post synaptic membrane of the neuromuscular junction. It is thought to affect approximately 1.5-17.9 per 100,000 of the population, as demonstrated in numerous epidemiological studies on different continents (Carr et al, 2010)(1).

1.2 History of myasthenia

Descriptions of a clinical syndrome similar to Myasthenia Gravis have been found in the ancient Indian Ayurvedic text *Charaka Samhita* as far back as the 2nd century BCE. Terms such as *'Khanja'* meaning 'limping' and *'Urustambha'* meaning 'thigh fatigue' have been used to describe the clinical presentation.

The first description of myasthenia gravis in Western literature was by Thomas Willis in 1672. An English translation of the works of Willis was made by Samuel Pordage in 1683. In his book *De Anime Brutorum* Willis describes in 'on the palsy': 'persons who in the morning are able to walk firmly, to fling about their arms hither and thither, or to take up any heavy

thing, before noon at the stock of spirits being spent, which had flowed into the muscles, they are scarce able to move the hand or foot'. Thomas Willis kept records of patients who presented with a chronic condition characterised by muscle fatigue with fluctuating progress, typically aggravated by physical effort and alleviated by rest. He also suggested the existence of a substance in the blood which would help muscle contraction and that the clinical presentation was due to fluctuations in the concentration of the substance in the blood. He writes 'it may be suspected, that not only de spirits themselves are in fault, but the impotency of local motion doth in some measure also depend upon the fault of the explosive copula, suffused everywhere from the blood, into the moving fibres' (Willis T Pordage, De anima brutorum, 1683). It was however, much later in 1903 that an English paediatrician Leonard George Guthrie (1858 to 1918) connected the descriptions by Thomas Wills with myasthenia gravis (Croitoru, et al, 2016)(2).

The next description of myasthenia gravis was by Samuel Wilks in 1877. His case was described as' bulbar paralysis; fatal; no disease found'. He went on to perform an autopsy on the patient and examined the medulla oblongata which he remarked as being healthy and normal.

In France, Amand Duchenne (1806 to 1875), Francois Aran (1817 to 1861), Jean Charcot (1825 to 1893), Pierre Marie

(1853 to 1940) and Joseph Dejerine (1849 to 1917) described muscular dystrophies and forms of motor neuron disease at the time.

In Germany, Heinrich Romberg (1795 to 1873), Carl Friedrich Westpal (1833 to 1890), and Nikolaus Freidrich (1825 to 1882) also made major advances in neuropathology. In 1879 Wilhelm Erb described cases of myasthenia gravis which appeared under the heading ‘Ueber einen eigentumlichen bulbrern (?) Symptomenkomplex’.

In the 19th century there were several single case reports of similar clinical presentations which were in keeping with myasthenia gravis. Samuel Goldflam (1852 to 1932) a Polish neurologist, gave a complete account of myasthenia gravis when he described three cases and reviewed papers of the previous neurologists. He gave an analysis of the varying presentation, severity and prognosis of the different cases (Hughes, 2005)(3).

In 1935, a Scottish physician called Mary Broadfoot Walker, who at the time was working at St Alfege’s hospital in Greenwich, described the dramatic improvement of myasthenia gravis through the administration of physostigmine and later on through Prostigmin (Neostigmine). She presented her findings at the clinical meeting of the Royal Society of medicine (neurology) at the National Hospital, Queen Square London on 17 February

1938 and this was published in detail. Dr Walker concluded that myasthenia gravis was the pathological effect of a substance that entered the circulation and caused abnormal fatigability via acetylcholine in the neuromuscular junction. She also used placebo (saline) to confirm the effect of physostigmine. The demonstration of the treatment was recorded in a movie produced in 1935 and which is accessible online (https://www.youtube.com/watch?v=uRoRsmvkhTI) (Carvalho et al, 2017)(4).

Although Dr Walker was thought to be the first to describe the use of Physostigmine, this was in fact first described by Dr Lazar Remen, a Polish doctor who described it's use in Myasthenia gravis in a paper published in 1932. He was studying the effects of Glycine at the time and the positive results of physostigmine on myasthenia were not given much importance (5).

In the early 1900s Campbell and Bramwell suggested that a toxin, possibly of microbial origin was the cause of myasthenia. Buzzard, in 1905 then suggested that this was an 'autotoxic agent' causing symptoms. Wilson and Stoner conducted animal experiments in 1944, and suggested that there was a blockage of transmission in neuromuscular junction in frogs. This was not confirmed by Lammers and Van Spijk, 1954. It was found that haemodialysis caused immediate but transient improvement in MG patients (Stricker et al, 1960) (6). In 1967, Parkes and

McKinna found changes in muscle contraction upon injection of globulin fractions from myasthenia serum. In 1973, Bergstrom et al studied the effect of thoracic duct drainage which again caused improvement in MG symptoms (7).

The first antibodies demonstrated were by Strauss and colleagues in 1960 who found the existence of anti-striated muscle antibodies (anti--SM); this is seen in 30% of all MG patients and nearly all patients with thymoma.

It was Prof Ian Simpson in 1964 who hypothesised that myasthenia gravis was an autoimmune disorder caused by an antibody to an endplate protein (8). Antibodies to the endplate protein i.e. acetylcholine receptors was first demonstrated by Almon, Andrew and Appel in 1974 (9). They found that serum globulins from MG patients could inhibit α bungarotoxin (α-BuTx) binding to solubilised rat AChR receptors.

Mittag, Kornfield, Tormay and Woo compared four different techniques of assessing AChR antibodies and found that immunoprecipitation of α-BuTx labelled AChR was the most effective. This was then described in detail by Lindstrom et al in 1976 (10). Over the years several different studies have been performed. Using subclass specific antisera, Vincent, Lang and Newsom-Davies found that most patients have anti-AChR within subclasses 1, and occasionally subclass 3 (11, 12).

Since then, a lot of work has been done on the diagnosis of myasthenia, including studies looking for antibodies to other components of the NMJ receptor complex, T cells and their pathophysiology, B cells and their receptors; and in the treatment of myasthenia including various immunosuppressants, monoclonal antibodies, thmectomy and other targeted therapies.

1.3 Pathophysiology of myasthenia

Myasthenia gravis is an autoimmune disease characterised by the presence of autoantibodies against components of the muscle membrane at the neuromuscular junction. The most common of these is the acetylcholine receptor antibody. Antibodies against other components of the postsynaptic membrane have also been described including MuSK, LRP4 and agrin, and to intracellular proteins such as ryanodine receptor and titin. The triggers for this autoimmune reaction are not yet clear. Many potentially autoreactive CD4+ T cells survive clonal deletion during development; their presence in normal subjects however does not result in clinically significant autoimmune responses. Clinical and epidemiological studies suggest that infections may be the triggering factors (Rose et al, 1998). It has been proposed that there may be molecular mimicry between a microbial epitope recognized by CD4+ T cells and a self antigen with a similar sequence.

Once the tolerance of the self- epitope is lost, T cells which recognise that epitope and which can secrete pro-inflammatory cytokines migrate into the tissues that contain the antigen. Here they can cause an inflammatory response and tissue destruction. The antigen presenting cells (APCs) can activate these CD4+ T cells and present new epitopes from the same antigens or even new antigens. This may become a self-maintaining process and causecasue chronic tissue destruction (13-15).

The neuromuscular junction (NMJ) is a chemical synapse between the motor neurons and the skeletal muscles. When the action potentials pass down the motor neuron, the terminals release acetylcholine which then activates the acetylcholine receptors (AChRs) present on muscle fibres. This triggers calcium release from the sarcoplasmic reticulum by polarising the muscle cell and this causes muscle contraction.

NMJs occupy less than 0.01-0.1% of the entire muscle surface. Positive factors such as agrin are secreted by the nerve terminals and initiate the concentration of the AChRs at the NMJ. Agrin promotes transcription of AChR subunit genes and the other proteins required for the NMJ function, AChR transport to the junction membrane, AChR clustering, anchoring and stability. ACh itself, conversely, is a negative signal and suppresses this mechanism is also released.

Acetylcholine receptors are found on the surface of muscle membranes but are concentrated at the neuromuscular junction where the nerve ending synapses with the muscle. The density of AChRs at the endplate is about 15,000-20,000 receptors/μm^2. The concentration of AChRs is 100 fold lower further away from the endplate (16). At a mature endplate, the half-life of AChRs is about 8-11 days. The old receptors are not recycled, but are internalised and degraded (17).

Nicotinic acetylcholine receptors (AChRs) are a family of acetylcholine gated cation channels. These receptors are seen in several parts of the human body; they are present in the postsynaptic region in the motor neurons of the skeletal muscles, and in autonomic ganglia. In the central nervous system they act presynaptically and extrasynaptically. They are also present in the skin, bronchial and vascular epithelium, and in other non-neuronal tissues where they mediate intercellular communication.

There are many subtypes of AChR depending on the subunits of which they are composed. All AChRs have five homologous subunits which are organised around a central cation channel. There are 17 AChR subunits α 1-10, β 1-4, γ, δ and ε.

AChR is made up of 5 protein chains- 2αβεδ in adults and 2αβγδ in the foetus. They are arranged longitudinally to form a channel across the cell membrane. The acetylcholine binds to the α

chains on the external side close to but distinct from the immunogenic region which is recognised by the AchR Ab. Upon binding of the acetylcholine to the 2 α chains, the channel opens by a change in shape of the receptor. This allows positively charged ions to move intracellularly; end plate potentials are generated leading to muscle contraction (18).

LRP4 (low density lipoprotein receptor - related protein 4) is a member of the LDL receptor family. Agrin released by the presynaptic terminal binds to LRP4, which in turn activates MuSK. This leads to AChR clustering and NMJ formation. MuSK activation requires Dok7 which is an adapter like protein. Dok7 is important for MuSK activity and NMJ formation.

MuSK activity is also regulated by proteins which interact with LRP4- Tid 1, β amyloid precursor protein (APP) and mesoderm development candidate 2 (Mesdc2).

The intracellular pathways downstream of MuSK are not very well understood, most of them are possibly modulatory except for rapsyn which is important for clustering of AChR. Rapsyn is thought to anchor AChR to the cortical cytoskeleton and could be a signalling protein and not just an adapter protein.

ACh-binding protein (AChBP) has been identified which under the right circumstances causes suppression of synaptic cholinergic transmission. AChBP can diminish or stop

acetylcholine response or raise basal AChBP concentration such that subsequent responses to acetylcholine are decreased (Smit et al, 2001) (19). Other proteins identified in the post synaptic membrane and which play a role in synaptic transmission are utrophin and other dystrophin-related complex proteins.

In MG patients with AChR Ab, there is reduction of AChR at the NMJ junction, and disruption and simplification of the synaptic folds visible on electron microscopy of muscle biopsies. The primary mechanism seems to be activation of the complement pathway with generation of the membrane attack complex. There may also be AChR blocking. There is some compensation by active synthesis of different AChR subunits.

The pathogenesis of AChR MG is due to autoantibodies of the IgG1 and IgG3 sub classes (12, 20). They induce myasthenia through three mechanisms: (i) complement mediated postsynaptic membrane damage (21) (ii) cross-linking by bivalent IgG1 and IgG3 molecules (antigenic modulation) which causes internalisation of AChRs and depletion of its surface pools (22) and (iii) competition with ACh on binding sites of AChRs preventing activation and opening of the ion channels (23). There are also hypotheses which suggest that some antibodies may physically block the ion channel pore of the

AChR, but this has not been supported by experimental evidence (24).

The muscle type nicotinic AChR with the five subunits has what is called the main immunogenic region or MIR. Epitope mapping shows that more than half of the different AChR autoantibodies bind to a distinct part of the AChR α subunit which is the MIR (25, 26).

Some parts of the β and γ subunits adjacent to the MIR were also found to be immunogenic (27). Autoantibodies against the ε subunit have been reported which causes slow channel myasthenia due to delayed closure of the AChR ion channel (28).

The AChR MIR is mainly located around a loop of amino acids 66 to 76 on the α-1 subunit (25, 29). The epitope spreading hypotheses proposes that the initial epitope target of antibodies does not remain fixed but it extends to other epitopes within the same protein or even to other closely associated proteins (30).

The IgG subclasses seen in MG are IgG1 and IgG3 (12, 21) which have high affinity for Fc receptors on immune cells and they are also potent complement activators in contrast to IgG2 and IgG4. Complement factor deposition is seen at the NMJ in MG (31, 32). Complement consumption increases during exacerbation of MG with deficiency of C3 and C5 in EAMG (33,

34). Antigenic modulation seen in AChR MG is due to the functional bivalency of IgG1 and IgG3 (22, 35).

In contrast to AChR MG, MuSK MG is associated with IgG4 autoantibodies (36). IgG4 antibodies are unable to activate complement and they have a low affinity for Fc receptors on immune cells (37, 38). The likely mechanism for pathology could be related to the epitope to which the antibodies bind. Epitope mapping showed that most of the antibodies in MuSK recognise epitopes within the first two extracellular IgG-like domains (39, 40). As this interferes with Wnt receptor and signalling which is essential for AChR clustering, the hypotheses is that MuSK antibodies induce myasthenia through (i) antigenic modulation and internalisation of surface MuSK (ii) inhibition of MuSK dimerisation and (iii) interference with MuSK binding partners. The findings of a recent study also showed that MuSK autoantibodies can prevent the interaction between MuSK and Col Q (41).

In AChR MG the loss of AChR clusters is compensated by upregulation of the presynaptic ACh release through retrograde signalling (42). In MuSK MG this compensatory upregulation is not seen. This may be because the retrograde signalling is upregulated by the N-terminal domains of MuSK (43)(Huijbers et al) (41).

The thymus is known to be a site of the antigen AChR. Myoid cells in the human thymus can yield muscle cells which bear AChR. The thymic epithelial cells also contain AChR. The rates of anti-AChR synthesis in culture do not suggest that the thymus is a major site for anti-ACHR production; the highest rates produced by thymus would be around 20 pmoles per 24 hours which is less than 1% of the synthesis required to maintain serum anti-ACHR levels of 20 nmol per litre, assuming a t1/2 of 20 days. It seems unlikely that the thymus makes more than a small contribution to the whole body production of antibodies. This suggests that the clinical benefit from thymectomy does not depend only on the removal of the antibody production site. The rate of antibody synthesis by thymic cells is also higher with longer duration of disease. This suggests that the thymic production of AChR is a secondary event. PBMCs can also synthesise anti-ACHR antibodies (Vincent et al) (44).

Both T and B lymphocytes originate in the bone marrow, but only B lymphocytes mature there; T lymphocytes mature in the Thymus as described below. These are the primary lymphoid organs. The secondary lymphoid organs are the lymph nodes, the spleen and the mucosal lymphoid tissues. In the lymph nodes the B cells are localised in follicles in the outer cortex and the T cells are present more diffusely in the paracortical areas or T cell zones. In the spleen, the lymphocytes surround an arteriole which is called the periarteriolar lymphoid sheath and

they are mainly T cells. B cells are present in the interspersed lymphoid follicles. In each of the lymphoid organs, the T cells and B cells interact along the marginal zones. The peripheral lymphocytes are mature lymphocytes comprising of both activated cells and naïve cells and continuously circulate between the periphery and the lymphoid organs.

The thymus is a primary lymphoid organ which is environmentally complex. T lymphocytes develop from a lymphoid progenitor in the bone marrow, some of which migrate to the thymus. Here, they receive a signal through the Notch1 receptor which instructs the precursor to commit to the T cell lineage rather than the B cell lineage. Notch signalling is also important in the CD4 Vs CD8 decision. The progenitor cells lack most of the surface molecules seen in mature T cells. The first cell surface marker expressed is CD2; this lacks both CD4 and CD8 and is called a ‘double-negative’ thymocyte. After several steps of gene rearrangements, the thymocytes express both CD4 and CD8 receptors, called ‘double-positive’ thymocytes. As they go through the positive selection process (described below), they lose one of the receptor molecules, becoming ‘single-positive’ thymocytes which are either CD4+ or CD8+.

The thymus is needed for T-cell maturation and differentiation as they migrate from the cortical to the medullary compartments. This is brought about by an interaction between the thymic

epithelial cells and the T cells but also with other cells including dendritic cells, fibroblasts and myeloid cells (45, 46). During the first differentiation in the cortex, the immature T cells gradually become double positive for CD4 and CD8 receptors and they acquire a complete T-cell receptor (TCR). Further differentiation occurs after interaction of the TCR with the major histocompatibility complex (MHC) on the stromal cells. A large majority of the thymocytes are eliminated due to this positive selection step ie if the TCR-MHC interaction is too weak. In the medulla, the thymocytes are eliminated by negative selection ie if the TCR-MHC interaction is too strong. This is the basis of central tolerance based on the ability of TECs to express tissue specific antigens (TSAs) presented to T cells. The expression of the tissue-specific antigens is monitored by the autoimmune regulator AIRE or the transcription factor FEZ family zinc finger (47, 48). The AIRE expression has been shown to be down regulated by oestrogen which would explain the female predisposition to autoimmunity including myasthenia gravis (49). Thymic myeloid cells also display functional AChR. The Thymic epithelial cells (TECs) are involved in the selective induction of natural regulatory T cells (50).

In early-onset myasthenia gravis, thymic hyperplasia is observed in 50 to 60% of patients and thymoma is detected in approximately 15% of AChR+ GMG. In other cases, the thymus is atrophic or involuted with adipose tissue and residual areas of

thymic parenchyma. In the thymus in myasthenia gravis patient, no changes are observed with the frequency of CD4+ and CD8+ T cells. However the natural regulatory T cells that are differentiated in the thymus are less functional; which is also seen, but to a lesser degree, in the periphery (51, 52). The other abnormality seen is that the effector T cells from the thymus of MG patients are resistant to suppression by T reg cells which is likely due to the inflammatory thymic environment. There are changes in the expression of pro-inflammatory cytokines by the T cells such as IL-17 and increase in interferon gamma, IL 21 and TNF alpha expression in both the Tregs and effector T cells (53).

B cells are seen at low levels in normal thymic tissue and are seen mainly in the medulla and perivascular spaces. In the thymus of an AChR antibody positive myasthenia gravis patient, there are increased numbers of B cells, often in germinal centres (GCs). There is a difference in the age and gender of the patients, with the younger patients displaying higher degrees, with three or more GC's, and the older patients displaying lesser degrees, with fewer than two GC's per thymic section of follicular hyperplasia; 80% of the patients with thymic hyperplasia are women (54, 55).

An increasing number of T follicular helper cells have been described in the periphery and in the thymus of MG patients.

Truffault et al showed that patients with AChR antibody positivity and thymic hyperplasia have higher titres of AChR antibody than patients with thymoma or involuted thymus, and there is a clear correlation between the degree of thymic hyperplasia and serum levels of anti-ACHR antibodies (54).

In the thymus of MG patients there is increased number of high endothelial venules (HEV). Chemokines are expressed by HEVs and this is dysregulated in the MG thymus, including CCL 19, CCL21, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13 and RANTES/CCL5. CXCL13 is the most potent chemoattractant for B cells. Inflammation of the thymus is required to reveal the chemotactic properties of CXCL13. The inflammation following a pathogen infection appears to be important in optimising the recruitment of mature lymphocytes. Interferon I that is released during infection could favour cell motility (56-58).

The presence of poliovirus infected macrophages and Epstein-Barr virus (EBV) infected B cells have been seen in MG thymuses. This supports the hypotheses that the innate immune system may promote, exacerbate and/or maintain the autoimmune condition (59, 60). Toll -like receptors (TLRs) are important in innate immunity. In an MG thymus there is overexpression of TLR3, TLR4, TLR6, TLR7, TLR8 and TLR9 (61).

IFN- I has been implicated in myasthenia gravis- clinical reports demonstrating development of MG after interferon-alpha or interferon beta therapies (62), antibodies against IFN alpha are found in MG patients, mainly those with thymoma (63), and IFN beta is overexpressed in MG thymuses (64, 65). IFN beta induces the expression of α-AChR by TECs and also increases TEC death. IFN-β triggers the expression of CXCL13 and CCL21 by TEC and lymphatic endothelial cells. It induces the expression of B cell activating factor (BAFF) which favours B cell survival and is overexpressed by TECs in MG thymus. In myasthenia patients, IFN beta is overexpressed long after disease onset and this is suggestive of the presence of a pathogenic agent (66).

MiRNA are small RNAs that are post transcriptional regulators of gene expression. They interact with mRNAs leading to degradation or inhibition of translation and cause decreased protein expression. The differential expression of some MiRNAs in the peripheral blood mononuclear cells are observed in MG patients (Cron et al, 2017) (50).

1.4 Clinical presentation of myasthenia

The signs and symptoms in myasthenia gravis result from fluctuating strength of voluntary muscles. The degree of

weakness is partly dependent on the exertion of the muscle but can vary spontaneously over time for no apparent reason. Myasthenia Gravis (MG) may present with a variety of symptoms including ptosis, diplopia, drooping of the neck, difficulty with chewing, dysarthria, dysphagia, orthopnoea, and limb weakness. This can be classified clinically into Ocular Myasthenia Gravis (OMG), Bulbar Myasthenia Gravis or Generalised Myasthenia Gravis (GMG). Myasthenia Gravis can be classified into seropositive or seronegative based on the presence or absence of antibodies in the serum. Further classification is based on whether the serum is positive for AChR Abs, MuSK Abs or LRP4 Abs.

MG can also be classified based on the severity of presentation. The medical scientific advisory board of the myasthenia gravis foundation of America formed a task force in 1997 to come up with a universally accepted classification grading system and method of analysis for patients undergoing therapy for myasthenia gravis. This classification is designed to identify subgroups of patients with myasthenia gravis who have similar clinical features which may indicate different prognoses and response to therapy (Appendix 1).

The MGFA (Myasthenia Gravis Foundation of America) grades MG from Class I to Class V; Class I being the least symptomatic

with purely ocular symptoms and Class V where patients need ventilatory support (323).

In about 15% of patients with MG, the symptoms can remain confined to the ocular muscles. Ocular symptoms are the most frequently seen in MG. It is not clear if diplopia or ptosis is the more frequent. Any extraocular muscle can be involved leading to various presentations of diplopia- horizontal, vertical or diagonal.

Bulbar involvement is that which affects muscles innervated by cranial nerves V, VII, IX, X, XI, and XII. Speech difficulty usually manifests as a nasal voice or difficulty with articulation. This may be isolated or accompanied by dysphagia or difficulty with chewing. The symptoms may occur in bouts. If dysarthria is due to palatal dysfunction, then nasal regurgitation of fluids may occur. Upper pharyngeal muscle weakness gives a sensation of food sticking in the throat. There may be preference for cold foods in patients with dysphagia (may be due to improvement of neuromuscular transmission due to muscle cooling). If there is severe weakness of the muscles of mastication, the jaw sags open and the patient may need to hold the mouth closed. Most patients with chewing problems also have weakness of neck flexion/extension. An important correlate with dysphagia is weight loss. Weakness of facial muscles may be present which may be misdiagnosed as Bell’s palsy. This may manifest as

inability to whistle, kiss, difficult eating soup with a spoon or by difficulty pronouncing ‘p,f and s’ .

Weakness in arms, hands or legs as the first presentation is seen in 15- 20% of patients. Patients may complain of vague symptoms of tiredness or heaviness in their limbs or they may complain of difficulty with specific tasks such as hanging out laundry, washing their hair, hammering a nail, etc. Pain in the back and girdle is seen with weakness of postural muscles.

In most patients with generalised MG who have not reported any respiratory symptoms, there is decrease in vital capacity; even in 40% of pure ocular myasthenics, there is decrease in vital capacity (Reuther et al)(67). Vital capacity is decreased to a greater extent than forced expiratory volume.

Focal muscle atrophy may be seen in 6-10% of MG patients (Oosterhuis et al, Osserman et al, Schimrigk et al, Simpson et al) (68, 69).

There have been suggestions that MG patients have cognitive symptoms including abnormalities of visual attention and reaction time (70, 71) but this has been refuted by other authors (72). Currently there is insufficient data to confirm this. AChR Abs do not bind to AChR extracted from human brain making it unlikely that central cholinergic receptors are affected in MG.

There may be exacerbating factors which may unmask MG including infection with fever, psychological stress, hypo or hyperthyroidism, drugs such as quinine, chloroquine, aminoglycosides, beta blockers and D-penicillamine. The effect of pregnancy on MG has been described in thirds: no change in a third, improvement in a third and deterioration in a third of women with MG. Most patients seem to improve in the second half of pregnancy (Plauche et al, 1979) (341).

Myasthenia may be misdiagnosed as other neuromuscular conditions and vice versa. LEMS should be considered in seronegative patients with fluctuating weakness. The other diagnoses to be considered with more or less fluctuating limb weakness are motor neurone disease, polymyositis, endocrine myopathies and mitochondrial myopathies. In all these conditions, there may be a slight benefit with choline esterase inhibitor treatment. With bulbar symptoms, motor neurone disease should be thought of and in isolated dysphagia, achalasia (disturbance in the parasympathetic innervation to the oesophagus) should be considered as the differential diagnosis.

1.4.1 Unusual clinical presentations of MG

As described above, myasthenia gravis most commonly presents with a combination of ocular symptoms of diplopia or

ptosis, bulbar symptoms of dysarthria, dysphagia, chewing difficulties, neck weakness with head drop, limb weakness and respiratory difficulties.

Limb girdle myasthenia is an uncommon clinical picture; patients have predominantly proximal muscle weakness with no ocular, bulbar or facial deficit. This could be mistaken for myopathies and make diagnosis difficult (Vecchio et al) (73).

Fearon et al published a case report of a gentleman with a history of distal arm myopathy. He had predominant weakness of finger extension and mild involvement of finger flexion, wrist flexion and wrist and finger extension. He did not have any ocular or bulbar symptoms and there was no fatiguability with his limb symptoms. He later developed ocular symptoms of diplopia and ptosis after which he tested positive for myasthenia gravis (74).

Sih et al retrospectively looked at a cohort of MG patients and found that of 146 generalised MG patients, 15 had head drop. These patients were older at onset and predominantly men (75).

Respiratory involvement in myasthenia gravis may be more common than previously thought. A Tiawanese study published in 2015 (Yeh et al) prospectively looked at 58 patients with myasthenia gravis without respiratory symptoms and tested for sleep-disordered breathing (SDB) with a watch-PAT (Peripheral

Arterial Tone) and concomitant recording of the MG score. This showed that the prevalence of SDB in Myasthenia patients with mild and moderate weakness was high when using the watch PAT. The predictive factors predisposing to the development of SDB were patients' age, male gender and use of azathioprine (76).

Nikolic et al looked at a cohort of MuSK positive and ACHR antibody-positive myasthenia patients. They did a clinical examination, EMG recording and proton magnetic resonance spectroscopy. Myopathic EMG was more frequent in MuSK compared to ACHR MG patients. In the ACHR MG patients myopathic EMG in facial muscles was more frequent after long-term corticosteroid treatment. In the MuSK MG patients, facial and tongue muscle atrophy was seen in 23% patients. This was associated with longer disease duration. Intramyocellular lipid deposition in the tongue was present in 85% of MuSK and 20% of ACHR MG patients. Myopathic changes in EOMG were more common with female MuSK patients and signs of Intramyocellular lipid deposition in the tongue were also more common in female compared to male patients (77).

Cartwright et al wrote a case report of a patient who presented with worsening dyspnoea with anti-MuSK antibody positivity. This patient had atrophy of the diaphragm which is an unusual

finding, atrophy of the tongue being more common. There was improvement with plasmapheresis and steroids (78).

Other rare presentations include ophthalmoparesis with unilateral finger flexor muscle weakness (Cordeiro Sousa, et al) (79). Focal predominant triceps muscle weakness is another unusual presentation; in the case report by Abraham et al, this was predominantly in African-American males (80).

There have been case reports of cognitive fatigue in patients with myasthenia gravis. Jordan et al published a paper in 2017 which looked at 33 myasthenia patients with stable generalised disease and compared the data with 17 healthy controls. They had repeated testing of attention and concentration and paced auditory serial addition test. The fatiguability was based on the calculation of linear trend (LT). MG patients showed a negative LT for testing of attention and concentration indicating cognitive fatiguability. This was significantly different from controls with a p of <0.05. Paced auditory serial addition test did not show any difference (71).

Olfactory and gustatory dysfunction in myasthenia gravis has been reported. MG patients showed low olfactory and gustatory scores studied with sniffin' sticks test and taste strip test. Tekeli et al also showed that olfactory loss correlated with the severity of the disease and treatment did not influence the results (81).

There is some evidence that acetylcholine receptors on the outer hair cells of the ear may also be affected in myasthenia gravis causing progressive loss of AChR on the outer hair cells decreasing their electromotility. This has been postulated as a useful additional tool for diagnosis and monitoring. Recording clinical hearing alterations before the onset of clinically evident hearing loss was suggested (Ralli, et al) (82).

There have been suggestions also of sensory disturbance associated with myasthenia gravis. This is thought to be because the presynaptic, synaptic and post synaptic mechanisms of AChR formation, action and termination are found in virtually all cells; widespread sensory dysfunction would be explainable. Leon Sarmiento et al tested tactile perceptual thresholds which were found to be higher in MG patients measured by grating orientation tasks. Tactile thresholds of corneal perception measured with asthesiometer were also significantly higher in MG patients. Symptoms similar to Restless Legs Syndrome were found in up to 43.8% of MG patients. Numbness and tingling were seen in 10% of patients and often found at disease onset. Pain scores of moderate or high severity were seen in 50% of patients. Coexisting sensory neuropathy and neuronopathy have also been reported in a number of MG cases. Somatosensory input processed within the somatosensory cortex and tested using somatosensory evoked potentials have been found to be impaired in myasthenia

gravis. There have been abnormalities in latency and amplitude of P100 response during visual evoked potentials which improved with anti-cholinesterase inhibitors. Orthostatic dizziness, abnormal thermoregulatory, sweat testing, impaired gastrointestinal function and severe pan-autonomic failure have been reported. Patients in myasthenic crisis have also been shown to have a wide heart rate and fluctuations of blood pressure. Cholinergic transmission is the mechanism used by almost all sensory organs. The human skin has the highest concentration of free acetylcholine (1000 pmol/ gram). The nicotinic receptors play a key role in cell cycle progression, apoptosis and differentiation of keratinocytes; muscarinic receptors are also involved (83).

1.4.2 Associations of MG with other disorders

There have been several case reports of amyotrophic lateral sclerosis coexisting with myasthenia gravis. In a study by Pasqua et al in 2016, they went through a prospective registry of all ALS incidences from 2009 to 2014. 671 patients were diagnosed with ALS of which five patients were also affected by myasthenia. Patients with ALS and myasthenia frequently had bulbar onset and a rapidly progressive course. They suggested the possibility of a shared immunological dysfunction (84). The findings were reflected by a Norwegian study by Gotaas et al

who suggested that the prevalence and incidence of myasthenia and ALS was far more than expected if the disorders were unrelated. This suggested immunological mechanisms in the neuromuscular junction which were relevant in ALS pathogenesis (85). Similarly, a Chinese study by Tai et al suggested that most patients had limb onset ALS, and myasthenia symptoms mainly affected the ocular and bulbar muscles (86). A study by Amador et al from France also suggested the same (87).

There have been case reports of myasthenia gravis and Lambert-Eaton overlap syndrome (MLOS). A review was undertaken by Shin Oh, published in 2016, who looked at 55 possible case reports of MLOS. Of these, 39 cases met the diagnostic criteria for myasthenia gravis and LEMS. Analysis of clinical data showed that the patients had common MG and LEMS symptoms of ocular and bulbar paresis, response to anticholinesterase for MG, and limb weakness and decreased or absent reflexes for LEMS. The RNS studies showed low compound muscle action potentials and incremental response in more than 60%. Eight patients had combined AChR antibodies or MuSK antibodies and voltage gated calcium channel (VGCC) antibodies (88).

Myasthenia gravis can coexist with other neurological disorders including nemaline myopathy (Cao et al) with positive

acetylcholine receptor and Titin antibodies. The pathogenesis may be related to AChR antibody and Titin antibody in adult onset nemaline myopathy with myasthenia (89).

There have also been case reports of myasthenia gravis associated with Morvan's syndrome and positive contactin associated protein like 2 antibodies.

Coexistence of MG and neuromyelitis optica (NMO) has been described. In most cases MG predated NMO, and most patients had a prior history of thymectomy at an early age. This suggests that the thymus gland is possibly protective against the development of NMO (Gotterer et al) (90).

Myasthenia gravis can be associated with pathologies in other organ systems, heart and skeletal muscles being the most commonly reported. This can be asymptomatic ECG changes, to giant cell myocarditis with ventricular tachycardia, conduction disorders, heart failure and sudden death. Myocardial involvement can also appear as Takotsubo Disease. Ryanodine, Titin, B1 and B2-Adrenergic Receptors could be the possible autoimmune targets in Myasthenia. With heart involvement, elevation of Troponin has been reported. Takotsubo Stress Cardiomyopathy with Myasthenia seems to be due to emotional or physical stress and high levels of circulating catecholamines. This could be helped with treatment for myasthenia gravis including plasma exchange.

In a study by Kubiszewska in 2016 the prevalence of autoimmune thyroid diseases was measured in a cross-sectional study of 343 consecutive patients with myasthenia gravis. Autoimmune thyroid diseases were diagnosed in 92 myasthenia patients (26.8%), including Grave's disease, Hashimoto's thyroiditis and antithyroid antibodies only. Grave's disease patients had ocular symptoms more often than those with antithyroid antibodies or Hashimoto's thyroiditis. The prevalence was comparable in early and late-onset. Immunosuppressive therapy was less frequently needed in patients with myasthenia and thyroid problems which indirectly indicated a possible milder course of disease. Amongst autoimmune diseases, autoimmune thyroid disorders seem to be the most associated pathology with myasthenia gravis (91).

It has previously been reported that the frequency of poly immunity in MG is anywhere between 11.6 and 32%. Duarte et al looked at all MG patients in a Portuguese tertiary centre. They found other autoimmune disorders in 37 patients with MG i.e. 17%. The frequency of the second autoimmune disorder was highest for females with EOMG at 68%. 78% had GMG and 78% had AChR antibody positivity. 51% had thymectomy with thymic hyperplasia being the most common (92).

Berrih-Aknin also wrote about autoimmune disorders in MG. It has previously been shown that familial autoimmunity was

common but it was variable. In EOMG, about 40% of patients had relatives with autoimmune disorders whereas in LOMG it was 20%. Only 4% of individuals had relatives with MG. In EOMG females, the known risk allele of the immune regulatory gene PTPN22 appears to be more common in patients with a second autoimmune disease or autoimmune relatives than in those without a second autoimmune disease (93).

Myasthenia gravis with proteinuria is a very rare disorder. A paper published by Tsai et al in 2016 reported 39 cases in literature thus far. The most commonly associated disorder being minimal change disease. Both these conditions are related to dysfunction of T lymphocytes and hence can be connected. Treatment for the myasthenia also lowered the proteinuria of minimal change disease (94). There is one reported case of concomitant myasthenia gravis and Anderson-Tawil syndrome. This is an autosomal dominant multisystem channelopathy characterised by periodic paralysis, ventricular arrhythmias and distinctive dysmorphic facial or skeletal features.

1.5 Literature review

1.5.1 Epidemiology of MG

Epidemiological studies done since the 1950s have shown an increasing incidence rate and prevalence rate of myasthenia gravis with time, especially in the older population. Comparison across the different studies has been difficult because of heterogeneity, different ethnicities and different methods of data collection. Before the advent of antibody tests for myasthenia gravis, the diagnostic criteria for myasthenia differed in each study, being defined by the authors. The age cut off for LOMG and EOMG also differed between studies. Since the advent of antibody testing in the 1980s, the incidence rates and prevalence rates of MG have approximately doubled. The methodology and the findings for some of the studies are described below; every study has a different method of data collection including: AChR Ab assays from national or regional immunology labs; discharge diagnosis, antibody tests and pyridostigmine used; health-insurance reviews and assessment; prospective MG registers; Medicare beneficiaries; cross-sectional study; complete enumeration approach; clinical records and pyridostigmine prescription registers; prescription of pyridostigmine alone; and hospital discharge register. The majority of the studies are retrospective, with only very few

conducted prospectively. The prospective study detailed below was based on clinical database and pyridostigmine registers rather than follow up of newly diagnosed myasthenia patients (Santos et al) (95). Despite the differing methods and heterogeneity, when Carr et al did a meta-analysis in 2010, they were able to calculate crude estimates which still showed a rising incidence rate and prevalence rate.

In 1900, when Campbell and Bramwell surveyed the literature and added one case of their own, they identified 60 cases of myasthenia gravis, three of which involved patients who were older than 50 years at the onset of the disease. By 1953 Schwab and Leland reported that 62% of women and 27% of men with MG were younger than 30 years at the onset of the disease. The corresponding figures reported by Simpson et al in 1966 were 49% women and 23% men. In both studies the disease was uncommon among younger men with the majority of male patients being older than 60 years. Men with MG formed two groups, one with the peak age at onset between 25 and 35 years, and the other between 60 and 70 years. These observations were reflected in the standard textbooks of neurology at the time (Aarli et al, 1999)(96, 97).

In 1996 Lawrence Phillips et al published a literature review of evidence of increasing prevalence of myasthenia gravis. They included 33 studies from 1952 to 1995 and found that

prevalence and incidence rates increased over time but the regression line for prevalence significantly exceeded that for incidence. The mortality rates had declined slightly over time. They concluded that the prevalence of MG had increased over the past 45 years, probably because patients with the disease had a longer lifespan owing to improvement in treatment. (Phillips et al,1996)(98).

In 2003 Angela Vincent et al published a paper on underdiagnosis of MG in older patients. They identified patients only by a positive AChR antibody test using data of all UK centres registered for the assay during 1997 to 1999. 3183 individuals had positive AChR antibody tests giving an annual incidence of 1.8 per 100,000. The age-specific incidence in both sexes rose steeply between the ages of 45 and 74 reaching 9.9 per 100,000 in men and then fell with a sharp decline above the age of 80. In the prevalence study based on community controls, there was only one serum from individuals aged 60 to 74 years which was positive for AChR antibodies (0.12%); Sera from 8 individuals aged ≥75 years were positive (0.7%), only one had a previous clinical diagnosis of myasthenia gravis but others had histories of stroke or transient ischaemic attacks. This suggested that either myasthenia gravis has a temporary peak in incidence in the older age groups which then falls off, or that the diagnosis is being missed in many older individuals,

particularly in those over 80 years of age (Vincent et al, 2003)(99).

In 2010 Carr et al(1) published a meta-analysis of all studies done to date on the incidence rate (IR), prevalence rate (PR) and mortality rate (MR) of myasthenia gravis. Population-based epidemiological studies of myasthenia gravis were included and cases without a defined denominator population were excluded. 55 studies were selected for inclusion in the review; these included 8033 cases from 1.7×10^{9} person-years studied. The time period studied ranged from 1950 to 2007. There was a wide geographical distribution of studies with representation of all continents except Australia. The incident rates ranged from 1.7 to 21.3 cases per million person years. AChR MG IR ranged from 4.3 to 18 per million. Only two epidemiological studies had been performed to date on MuSK MG in Holland and Greece. In Holland IR was 0.1 per million person years and in Greece it was 0.32 per million person years.

Linear regression of IR against the final year of study suggested a significant correlation equivalent to 3% increase per year (p= 0.0001). The average incidence rate after 1976 (eIR = 6.5 per million person years) was significantly higher (p = 0.0001) than before 1976 (eIR = 3.5 per million person years) corresponding to an approximate doubling. A bimodal distribution of IR in females was observed in 5 of 14 studies. IR in both sexes

increased with age peaking between 60 to 80 years in all but two studies with apparent male predominance in the older age group. The Asian study by Yu et al (100), who looked at the point prevelance of MG in the whole territory of Hong Kong, stood out, in that the proportion of childhood onset MG (onset less than 15 years) appeared to be higher in this population.

The observed prevalence rates (PR) ranged from 15 to 179 per million persons. The prevalence of AChR MG ranged from 70.6 to 163.5 per million persons. The observed prevalence for MuSK MG in southern Holland is 1.9 per million persons representing 2% of prevalent MG cases in the region; MuSK MG PR in Greece is higher at 2.9 per million persons.

The mortality rate (MR) ranged from 0.06 to 0.89 per million person years. The MR for AChR MG in Greece lies within this range: 0.43 per million person years. No trend was observed for MR with year of study.

The heterogeneity across the studies in this review was marked. Although there was a huge degree of dissimilarity, crude estimates were possible. They showed all MG eIR of 5.3 per million person years with the range of 1.7 to 21.3, ePR of 77.67 cases per million person years, AChR MG eIR of 7.3 per million person years- range: 4.3 to 18, PR range 70.6 to 163.5 per million, MuSK MG IR range of 0.1 to 0.32 per million person years, PR range 1.9 to 2.9 cases per million. An increasing trend

in IR and PR over time was observed. PR was on average 15 fold higher than IR across studies. It is notable that no detail was available on seronegative MG (Carr et al, 2010).

An Italian study looking at cases retrospectively (using the complete inumeration process by examining all possible sources of MG cases) from the Ferrara province in Italy between 1985 and 2007 showed a mean annual IR of 18 per million without any significant temporal trend. The incidence rates in the period 1985 to 1990 were 14 per million persons both for early and LOMG. Thereafter a significant increase in incidence of LOMG ($P < 0.05$) and a decrease in early-onset MG were detected ($P < 0.01$). These findings were related to non-thymomatous MG. The median age at onset of the disease steadily increased over time. A changing pattern of MG incidence with an increase in frequency of LOMG and a decrease of EOMG was found in the last years giving a significant shift to older age at onset of the disease. (Casseta, 2010)(101).

Another epidemiological study published in 2011 in Trento, Italy used data from sources including discharge diagnosis, antibody tests and anti-choline esterase drugs and was a replicate of the previously published study by Casseta et al looking at the same region/population group. These were analysed and the incidence was calculated from 2005 to 2009. The incidence and prevalence were greatly increased in comparison with the 1981

to 1990 data. The prevalence rate increased from 82.9 in 1990 to 129.6 per million population whereas the average annual incidence had increased from 7.4 per million person-years in 1981-1990 to 14.8 in 2005 – 2009. This increase was mainly due to increase in patients with late onset MG. (Pallaver et al, 2011)(102).

A Taiwanese (retrospective) study published in 2010 identified cases from the National health insurance research database from January 2000 to December 2007. IR of males to females was 0.68. The average annual IR was 2.1 per 100,000 population per year, MG occurred in all age groups, with higher incidence in older individuals and lower incidence in the 10 to 14-year-olds for both sexes. Among the 5211 cases 12% had a neoplasm of the thymus. The prevalence increased steadily during the study period from 8.4 per 100,000 in 2000 to 14 per 100,000 population in 2007 (Lai et al, 2010)(103).

A Japanese nationwide (retrospective) survey of myasthenia gravis was published in 2011. This showed a prevalence of 11.8 per 100,000. Elderly onset myasthenia gravis, which they defined as onset over the age of 65 years, accounted for 7.3% in 1987 but this had increased to 16.8% in 2006. Infantile onset MG accounted for 10.1% in 1987 and was still as high as 7% in 2006. The rate of ocular MG was highest (80.6%) in infantile onset and lowest (26.4%) in early onset disease but the rate

rose again in the late onset group. Anti-acetylcholine receptor antibodies were positive in only 50% of infantile onset but nearly 90% of elderly onset patients. Only 0.7% of cases had a family history of MG while 3% had a family history of autoimmune disease. The frequency of familial MG was high (2.38%) in the infantile onset group but did not reach statistical significance. (Murai et al, 2011)(104).

An epidemiological study of myasthenia gravis was performed retrospectively in Australia and published in 2012. They utilised prescriptions for pyridostigmine in 2009 from a national prescribing database to estimate the incidence and prevalence of symptomatic myasthenia gravis and treated disease. They found that in 2009 there were 2574 prevalent cases of symptomatic treated myasthenia gravis corresponding to an annual crude prevalence rate of 117.1 per 1 million residents. There were 545 incident cases yielding a crude incidence rate of 24.9 per 1 million residents. The crude incidence in women and men was estimated to be 27.9 and 21.9 per 1 million respectively (105).

Prevalence and incidence rates were higher in women than men between the ages of 15 and 64 years and were higher in men than women in those older than 65 years. Rates peaked between the ages of 74 and 84 years declining thereafter. Compared with women, incidence was higher in men for cases

younger than 15 years of age. There were 1.9 female cases for every male case in those aged younger than 35 years of age. The ratio of female to male cases in those aged between 35 and 64 years was 1.5:1 and there were 1.1 male cases for every female case in those over the age of 65 years. They suggested that the increasing prevalence and incidence of myasthenia observed in recent years likely reflected the improved longevity of populations in the developed countries. The declining rates in the very old were thought to reflect under-ascertainment. Myasthenia gravis in the elderly may be mistaken or masked by conditions such as stroke and myopathy, general frailty, symptoms of fatigue and weakness in non-neurological conditions such as heart failure and anaemia. (Gattelari et al, 2012)(105).

An Austrian epidemiological study of myasthenia gravis was published in 2012. They retrospectively looked at the yearly inpatient prevalence of myasthenia gravis from 1992 to 2009. The inpatient prevalence of 2009 was calculated as 8.0 and the population prevalence as 15.69 per 100,000 population. They observed a 2.2 fold increase in the inpatient prevalence between 1992 and 2009, which was mainly due to a rise in the number of older patients (> 50 years). This could partly be accounted for by an ageing of the population as a whole and a rise in the age of hospitalised patients. However, after adjusting for demographic

factors an unexplained average yearly rise of 3.7-3.9% remained (Cetin et al, 2012) (106).

A Danish (retrospective) study published in 2005 recorded the incidence of myasthenia gravis from 1970 through to 1999. The annual incidence rate of early-onset myasthenia gravis was constant at 3.5 per million, in late-onset myasthenia gravis the rate increased from 4.7 to 20.8 per million. The author hypothesised that late onset non thymomatous anti-acetylcholine receptor antibody seropositive MG may be provoked by environmental factors (Somnier, 2005)(107).

However, a further Danish study published in 2013 differed in their conclusion. They retrospectively looked at data from 1996 to 2009 using a combination of diagnosis and prescription data from nationwide registers. They found an IR of 9.2 per million person-years overall, and 29.9% were classified as early onset and 70.1% as late-onset MG. Women predominated in the early onset (70.5%) but not in the late-onset group (44.4%). The incidence rate of EOMG was 4.2 and LOMG 18.9 per million person-years and it did not vary over time in the study period (P values for trend 0.54 and 0.15 respectively). They found that LOMG comprised a large proportion of all incident cases in Denmark, was more common in men than women, and it occurred with a stable incidence in the 14 year study period (Pedersen et al, 2013)(108).

The incidence of AChR antibody positive myasthenia gravis in South Africa was published in 2014. They were calculated from retrospectively collected positive AChR antibody laboratory data between 2011 and 2012 using the 2011 population census data. 890 individuals were seropositive, giving an annual incident rate of 8.5 per million. Age standardised IR for early onset (less than 50) and late-onset MG were 4.1 and 24 per million respectively and 4.3 per million for juveniles. IRs may be higher among children with African genetic ancestry (Mombaur et al, 2014)(109).

A retrospective prevalence and cost study of myasthenia gravis in Medicare beneficiaries sample published in the United States of America in 2015 estimated that the US prevalence of MG was 20 per 100,000 between the years 2011 to 2013. The male and female prevalences were 68 and 87 per 100,000 respectively. On average male patients were older than females by 2.66 years ($p = 0.01$) and were less likely to be receiving Medicare disability benefits. Female MG patients were more frequently in the highest category for inpatient stays and ER visits (Gordon et al, 2015)(110).

A Portuguese study published in 2016 looked at the northern region of Portugal. They used two complementary approaches to identifying patients: one was a hospital clinical database and/or clinical records of neurologists of the participating

hospitals and second was a computerised database of pyridostigmine prescriptions by the GPs working in the North region. The data was collected prospectively; all patients who had disease onset and diagnosis of MG through the end of the study period were included. In December 2013 they found that the point prevalence was 111.7 patients per million population. The highest prevalence was observed in the >65 years age group especially in men. During 2013 they estimated an incidence rate of 6.3 per million per year. Among women, the incidence rate was highest in the 15 to 49 year age group; in men, incidence increased with age up to 22.1 per million in those >65 years. The MG related MR was 0.5 per million (Santos et al, 2016)(95).

A Canadian (retrospective) study was published in 2016. They performed a population-based epidemiological research study in Ontario. In 2013 there were 3611 prevalent cases of myasthenia gravis in Ontario. Crude prevalence rate was 32 per 100,000 population. The age and sex standardised prevalence rates rose consistently over time from 16.3 in 1996 to 26.3 in 2013. Standardised incidence rates remained stable between 1996 at 2.7 per 100,000 and 2013 at 2.3 per 100,000. Incidence was highest in younger women and older men and geographic variation was evident throughout the province (Breiner et al, 2016)(111).

A retrospective study published in 2016 estimated the incidence and prevalence rates of myasthenia gravis using the Korean national health insurance claims between 2010 and 2013. In 2011 there were 1236 incident cases, the standardised incidence rate was 2.44 per 100,000 person-years, the standardised prevalence rates was 9.67 and 10.66 per 100,000 persons in 2010 in 2011 respectively. The incidence and prevalence rates peaked in the elderly population aged 60 to 69 years for both sexes (Park et al, 2016)(112).

Another Korean study (also retrospective) published in 2016 looked at health insurance review and assessment data from 2010 to 2014. They quoted a prevalence of MG rate of 10.42 per 100,000 population in 2010 and this increased every year to 12.99 per 100,000 in 2014. The average incidence of MG between 2011 and 2014 was 0.69 cases per 100,000 person-years. The prevalence and incidence was higher in the older (≥50 years) age group than in the younger (< 50 years) age group (Lee et al, 2016)(113).

A cross sectional study published in 2017 looked at myasthenia patients from Norway and Netherlands. They looked at the prevalence and clinical aspects of immigrants with myasthenia gravis. They found no marked differences in prevalence between immigrants and native ethnic groups. MG with MuSK antibodies and MG with thymoma were more frequent in Asian

immigrants compared with other ethnic groups (8% versus 0 - 4%; and 21% versus 6 - 10%) respectively. (Boldingh et al, 2017)(114).

In 2017, epidemiological data was published from the Catalan County of Osona in Spain. The data from a prospective MG register were looked at retrospectively for the year 2013. They showed a prevalence of myasthenia gravis at 32.89 per 100,000 inhabitants. The standardised prevalence was 35.47 per 100,000; the ratio of women to men was 1.3. Overall the group of patients older than 65 years accounted for 62.75% of all cases. The prevalence of myasthenia gravis increased considerably in older age groups, no cases were registered among patients under 25 years, prevalence was 21.87 per 100,000 in the 25 to 64 age group and prevalence in patients over 65 years increased to 122.35 per 100,000. The figures showed the highest prevalence rate reported to date in Spain and the highest prevalence was due to the rate observed among patients older than 65 years (Aragones et al, 2017)(115).

A retrospective nationwide epidemiological study of myasthenia gravis in Latvia was published in 2017. They looked at data from the Central statistics bureau between January 2010 and December 2014. Myasthenia gravis as a diagnosis was confirmed in 99 new presenting patients of whom 70% were women and 30% were men. 61.1% of the patients were 50 or

more years old at the time of onset. The total crude incidence was 9.7 per million person years, for women this was 12.5 and for men it was 6.1. Crude as well as standardised incidence rates were significantly higher for women than for men. The incidence of late-onset myasthenia gravis was significantly higher than the incidence of early onset myasthenia gravis, 15.2 and 6.2 respectively. The incidence for women was significantly higher than that for men, 10 and 2.5 respectively. There was no significant difference in the incidence between the genders in late onset MG group, 15.7 and 14.4 for women and men respectively (Zieda et al, 2017)(116).

A retrospective Israeli study published in 2017 looked at the differences in clinical presentation of myasthenia gravis in different ethnic origins. They found that the frequency of age of MG onset was distributed in a bimodal fashion in the female patients and increased gradually over time, with a peak around 70 years of age in male patients. Ocular MG was more frequent in males and Ashkenazi (ASH) patients. Male patients had a higher proportion of positive serum AChR antibodies than female patients, with no ethnic differences in the rates of anti-AChR or MuSK antibodies. Comorbidity with another autoimmune disease was more frequent among female patients with late onset MG and non-Ashkenazi (NASH) patients. Male MG patients tended to have more malignant comorbidities than female MG patients (Asmail et al, 2017)(117).

1.5.2 Genetics in MG

The first report of HLA association with MG was published in 1976 (118). Since then, there have been several studies showing HLA associations in MG: DQB1*05:02 (119), DRB1*03, DRB1*04, DQB1*02 and DQB1*03 (120, 121). In Chinese population DRB1*09 was associated with MG while the DRB1*08 was protective (97, 122).

A genome wide association study (GWAS) published in 2012 on North Europeans identified a class I SNP rs7750641 as the strongest signal in MG. It also identified HLA-B*08 as a major risk allele. There was a risk association for HLA-C *07:01. The same study also showed a strong LD between HLA-C*07:01 and HLA-B*08 (123).

Age of onset effects of HLA in MG have shown mixed results. Multiple studies have reported the extended HLA haplotype namely A1-B8-DRW3-DQ 2 as being associated with EOMG in European ancestry; however it is unclear whether the signal maps in class I or class II genes (124-126). SNP rs1800629 has been linked to higher expression level and higher serum levels of TNF α in MG (127). In a Norwegian population the DRB1*13:01 was found to be protective for EOMG (128),

whereas a GWAS performed in the European population found a peak of association for EOMG and DQ A1(129).

The Norwegian study identified DRB1*15:01 as being associated with the risk of LOMG whilst DRB1*13:01 was found to be protective in LOMG. In an Italian cohort, DQB1*05:02 and DRB1*16 were associated with LOMG and Renton et al found an association for LOMG at HLA-DQA1(130).

Four studies found an association of DQ5 with MuSK positive MG (131-134) and another Turkish study found an association with the DRB1*14 and DRB1*16 in MuSK MG (133) whereas DRB1*13 was found in a Serbian study (134) (Misra et al) (135).

A Chinese study showed an increased risk of EOMG with PTPN22R620W polymorphism (Xiong et al) (136).

A genome wide Association study (GWAS) of MG was published in 2015. DNA was obtained from 1032 white caucasians from North America who had AChR receptor positive MG and 1998 race/ethnicity matched controls were also recruited. The samples were genotyped. An independent cohort of 423 Italian patients and 467 Italian controls were also used. They identified association signals at CTLA4, HLA-DQ 1, and TNFRSF11A. The findings were duplicated for CTLA4 and HLA-DQA1 in the Italian cases and controls. Further distinct but overlapping disease associated loci were seen in EOMG and LOMG. In EOMG there

were two associated peaks- one in TNFRSF11A and another in the MHC on chromosome 6p21 (HLA-DQ1). Association with the major histocompatibility complex (MHC) was also observed in EOMG cases-HLA-DQ1. The single nucleotide polymorphisms were different in EOMG compared to LOMG (129).

In 2016, Seldin et al published a GWAS on single nucleotide polymorphisms (SNPs) in 532 AChR Ab positive LOMG patients and 2,128 controls. Their data confirmed the association of LOMG with TNSFRF11A, and identified a novel candidate gene, ZBTB10. Other SNPs which were thought to be significant were rs2476601 encoding the PTPN22 R620W variant in EOMG. EOMG associated SNPs in TN1P1 did not show any association with LOMG. Many SNPs in the MHC region showed strong associations in LOMG but not in EOMG, and the associations were in opposite directions.The MHC regions showed three distinct peaks for LOMG corresponding to (a) MHC class II (DQA1) (b) HLA-A and (c) MHC class III SNPs (137).

1.5.3 Familial MG

Familial occurrence of myasthenia gravis is estimated at approximately 1 to 4%. Familial MG (FMG) tends to occur at a younger age and elderly onset FMG has been rarely reported. Ramanujam et al estimated that the MG concordance is

between 30% and 40% in monozygotic twins compared with 4% to 5% in dizygotic twins (138). Similar rates of concordance were found in a survey done in Europe and USA.

Salvado et al from Spain investigated the presence of familial cases in 462 MG patients. 16 cases from eight unrelated pedigrees were identified. The prevalence of FMG was 3.6% and the age of onset was 57.8 yrs, with six ocular patients, four MGFA class IIA, four IIB, one IIIA and one IIIB. Two patients had thymomas. The MGFA PIS did not differ from sporadic autoimmune myasthenia gravis. There was interfamilial heterogeneity and also members of the same family affected with FMG presented at different ages of onset, severity and thymus involvement (139).

Hirunagi et al reported a case of two siblings with elderly onset myasthenia which they defined as being over the age of 65 years. Both the patients and one unaffected sibling shared the same HLA haplotype. Patients developed generalised MG with elevated serum anti-AChR antibodies in their 70s. No other autoimmune disease was reported in the family and serum AChR antibody titres of the other members was normal (140). In the case presented by Hirunagi et al, unaffected siblings also had HLA-DR15 as did the patients, implying that the disease susceptibility is defined not only by HLA-DR but also by other genetic factors including gene-gene interactions.

Previous FMG studies have shown an earlier age of onset, slower progression and lower incidence of peripheral muscle involvement compared with sporadic MG. In a previous study in the US, 52/134 FMG patients presented with symptoms after the age of 18 years (141). In a nationwide epidemiological survey of MG in Japan, 2.4% of infantile onset patients had a family history of MG wheras only 0.4% of elderly onset patients had a family history (104). HLA-DR15 was significantly increased in patients with late-onset MG compared with patients with early onset MG in a Japanese cohort and HLA15, HLA-DRB1 * 15:01 was seen in late-onset MG patients in a Norwegian cohort.

Liu et al published a population-based family study from Taiwan. the relative risk (RR) for MG for patient siblings was 17.85, parents was 5.334, offspring was 5.82, and spouses without genetic similarities was 1.42 (142).

There was a case report by Chung et al of monozygotic twins who had mirror-image myopic anisometropia with ocular myasthenia gravis (143).

1.5.4 Viral infection and MG

Viruses have long been thought to have a role in inducing autoimmunity in MG. Cavalcante et al studied EBV virus in thymoma associated MG. They looked for EBV markers in MG;

EBV DNA and EBV-encoded small nuclear RNA 1 (EBER) transcript were detected in 14/26 i.e. 52.8% and 22/26 i.e. 84.6% of MG thymoma and only 3/14 i.e. 21.4% in non-MG thymomas. Latent EBNA2 and late gp350/220 lytic transcripts were undetectable in all but one thymoma. EBER 1 and 2 positive cells were detected in MG but not in non-MG thymomas, as well as cells expressing EBV latency proteins (EBNA one, LMP1, LMP A) that were mainly of the cell phenotype indicating EB association with MG rather than the thymoma. TLR 3 transcriptional levels were higher in MG than non-MG thymomas and positively correlated with EBER 1 levels suggesting a role for EBER in TLR 3 activation. The findings showed that EBV is commonly present in thymoma infiltrating B cells of myasthenia patients indicating a contribution of EBV to B cell-mediated autoreactivity e.g. in MG associated with thymoma (144).

The same group also published data on toll -like receptors 7 and 9 in MG and showed that TLR7 and TLR9 mRNA levels were significantly higher in EBV positive MG compared to the EBV negative normal thymus (60).

In contrast, a Chinese study by Jing et al showed that in a study of 30 MG thymic specimens, all were negative for both EBV-encoded small RNA 1 and EBV latent membrane protein 1.

Their results did not support the role of thymic EBV infection in MG pathogenesis (145).

Greco et al looked at seropositivity for West Nile virus (WNV) antibodies in MG patients. They looked at 29 patients with confirmed MG with AChR antibodies and screened them for anti-WNV antibodies. They found positive signals for anti-WNV IgG in 17% of MG patients although no clinical manifestations related to WNV infection were reported. They postulated that in predisposed individuals WNV infection could represent an additional risk factor for MG initiation (146).

Hsu et al looked at a Chinese cohort of patients to assess the risk of MG in patients with scabies which is an infectious and inflammatory pruritic skin disease. They found that scabies patients had a significantly increased risk of MG and proposed that prompt diagnosis and treatment of scabies may decrease the risk of MG (147).

Seok et al reviewed MG patients in Korea to look at the effect of influenza infection and vaccination on exacerbation of MG. In patients who had influenza-like illness, 40% had aggravation of MG symptoms, whereas only 1.5% had aggravation of MG symptoms following influenza vaccination. The rate of symptom aggravation was significantly higher in patients who had influenza -like illess than in those with common cold. The results suggested that the potential risk of aggravating autoimmune

diseases is higher for influenza-like illness than for influenza vaccination which suggests that influenza vaccination can be offered to patients with MG (148).

1.5.5 Demographics in MG

For a long time and it was thought that myasthenia gravis affected young adults and that it was uncommon after the age of 50 years. During the 1990s it became clear that myasthenia gravis was being diagnosed more often in older patients. In 1980 Compston and colleagues postulated two categories of non-thymoma myasthenia patients, one with presentation at less than 40 years of age and one after 40 years of age. Those who were younger were more often female, and had HLA-A1, B8, and DRW3 positive antigens. In the older age group there was a significant association with male gender and the presence of HLA-A3, B7 and/or DRW2 (97). In 1991 Somnier and co-workers reported a bimodal appearance for both sexes with one peak in the early onset group and another in the late onset group. On the basis of this, they proposed that the separation between early onset and late onset should be at the age of 50 years rather than 40 years. They found that in early-onset male patients, the onset was approximately 10 years later than in females, while in the late onset group the peak was at the same time in years in both sexes.

Diagnosis of myasthenia gravis in the elderly is difficult. Ageing causes sagging of the eyelids, particularly in men, and the size of the total eyelid fissure shortens in older age. Vision deteriorates because of other causes such as cataracts and macular degeneration making it difficult for patients to pick up on diplopia. Cerebrovascular disease is more commonly diagnosed when patients present with symptoms of dysarthria or dysphagia. Existing conditions such as cardiac or respiratory disease and hypertension worsen the condition (96).

Limburg and colleagues (149) and Mantegazza and colleagues (150) found an association between AChR antibody levels and age of onset. Late-onset was associated with lower concentrations of AChR antibodies and they also had antibodies to striated muscle. The younger patients had the same level of AChR antibodies irrespective of when they were examined. Patients without thymoma and age of 40 years had lower values of AChR titres. In MG with thymoma, the concentration of AChR Abs was higher.

Most data indicate that MUSK antibodies are more common among younger MG patients. Evoli and co-workers found a disease range of onset from 6 to 68 years. The mean age of onset was similar, however MUSK positive disease was more frequent in younger patients with 56.8% presenting at under 40 years of age (151). This was not reflected in all studies however;

Deymeer and colleagues showed that the age of onset was similar between seronegative ACHR and MUSK groups (152). Stickler and co-workers showed that MUSK antibody positive patients were more frequently female, younger and African-American and they seem to have a more limited distribution of single fibre EMG abnormalities (153).

Titin antibodies are seen very commonly in late-onset myasthenia gravis without thymoma. This is seen in about 50% of patients and they are extremely rare in early-onset myasthenia gravis. They have never been reported in seronegative myasthenia patients. In the early onset group Titin abs serve as a marker for thymic neoplasia.

In Johan Aarli's paper of 2008, he has suggested that late-onset myasthenia is seen only slightly more often in men than women, with a ratio of female : male of 3:1 in the early onset and 1:1.1 in late onset, whereas Evoli and colleagues stated a ratio of female: male of 1:1.9 in the late onset group.

1.5.6 Clinical presentation of antibody subtypes in MG

A review of the literature suggests that the clinical presentation may vary depending on the presence of antibodies and the type of antibody. Guptill et al (2010) studied 110 patients with MuSK Ab positive generalised myasthenia gravis. This was a

retrospective study of all the patients with MuSK MG from two clinics in Rome and USA. The mean follow up was 11 years for the patients from Rome and 5.3 years for those from USA. Of the AChR negative patients, , 39-49% had anti MuSK Ab;85% of the MuSK MG patients were female, with onset in the fourth decade. 79% of the MuSK positive patients had ocular and/or bulbar involvement at onset of the MG. 36% had ocular symptoms only at onset but all generalised (NB: some within 2 weeks and some after 4 years). 85% had MGFA classification of III or above, 28% had myasthenic crisis, 93% showed improvement with plasma exchange and 61% showed improvement with iv Immunoglobulins (iv Ig). Thymectomy was done in 36% of the patients and only one had a thymoma. Half of the thymectomised patients had a MGFA-PIS of MM or better (These patients were also on immunosuppressants) (154). This was a large cohort retrospective study but was not unbiased or population based. This gives a good clinical description of the generalised MuSK MG patients; but, only generalised myasthenia patients were included. Where patients were classed as having ocular symptoms only at onset, there was no clear diagnositic criteria for ocular myasthenia. Whilst thymectomy was reported to be beneficial, there was no comparison between those thymectomised and those that were not. MuSK Abs were tested on RIA, but not on CBA, potentially missing some of the MuSK positive GMG patients.

Zivkovic et al (2012) (155) described the characteristics of LOMG in a retrospective cohort of 174 patients, all seen in the neuromuscular clinics in one centre. Patients with thymoma were not included. Out of 174 patients, 66% were LOMG patients, AChR Ab were positive in 78% (65% in EOMG and 85% in LOMG), anti MuSK Ab were positive in 38% (similar in EOMG and LOMG). 13% of patients had myasthenic crisis and this was the same in EOMG and LOMG groups. Ocular myasthenia gravis (OMG) was found to be more common in LOMG vs EOMG (40% vs 18%). Although a large cohort study, there is no description of how the patients were selected, and is unlikely to be unbiased. Patients with thymoma were excluded, which as we know from other published literature, is more common in LOMG. This would suggest that the data is potentially skewed, missing out on the older MG patients with thymomas.

Suzuki et al in 2011(156) retrospectively looked at 260 Japanese patients with myasthenia gravis. They found that OMG was more frequent in LOMG than EOMG (67% vs 33%) and 68% of the EOMG patients were female with 58% of LOMG patients being male. Of the 260 patients, 62 had thymoma and they were divided into early onset and late-onset groups with a cut-off of 50 years. Thymoma was more frequent in the late onset compared to early-onset group. Anti-MuSK positivity was seen in 2 to 3% of the AChR negative patients and there was no

statistical difference with the presence of HLA-DRB1. Once again, a large cohort study from two big centres in Japan, but it is unclear if all consecutive patients were included and whether the catchment reflected the population in the area. It does however show a trend towards OMG in LOMG patients.

Klein et al (2013) (157) analysed serum/plasma and thymic samples from 226 EOMG, 97 LOMG and 150 TAMG patients. The samples were obtained from other overlapping studies in the UK, thymic tissue from patients referred for thymectomy in Germany, and clinical information was obtained retrospectively. They found that EOMG patients more commonly had relatives with other autoimmune disorders (40%), less so in LOMG patients (20%) and even less in patients with Thymoma associated Myasthenia Gravis (TAMG) (8%). The patient groups were from different cohorts, but the study had large numbers and provided good data about associated autoimmune conditions.

Huijbers et al (2014) (41) describe the different phenotypic presentations with various antibodies. AChR Abs were positive in 85% of MG patients, they had more ocular involvement, and generalised in a cranio-caudal distribution. MuSK Abs were positive in 8% of patients and they more commonly had Generalised MG with bulbar and respiratory involvement. LRP4

Abs were positive in 5% of patients and they more commonly had Generalised MG, sometimes with bulbar involvement.

Blum et al (158) looked at 165 Australian patients with myasthenia gravis. The patients were recruited via a survey of 303 patients with myasthenia who were asked to fill in a questionnaire. Only 198 responded and 33 of these were excluded due to lack of information. They found that patients with early onset myasthenia, which they defined as less than 40 years of age, were frequently female and the elderly patients were more frequently male. Occurrence of other immune - related diseases was seen in about 54% of patients. It is difficult to draw any conclusions from this study as it did not represent the prevalence or incidence of myasthenia.

Teo et al published in 2017 (159) looking at conversion rates of ocular to generalised myasthenia gravis in Singapore. It was a retrospective case series of patients diagnosed with OMG at one neuro-opthalmology centre. OMG was defined as purely ocular symptoms ‘initially’, but no time limit was stated. Patients who generalised within one month of presentation were excluded. Follow up was for 2 years. They found that the conversion rates of ocular myasthenia to generalised myasthenia in Asian patients was low with a rate of 10.6% at median follow-up and at two-year follow-up it was 7.7%. This was predicted by the presence of acetylcholine receptor

antibodies, presence of thymoma and positive RNS studies. Given that the patients were all seen at one neuro-opthalmology centre, and that OMG was defined as ocular symptoms for ≥1month, one would assume that the patients who did not present to hospital until much later with generalised symptoms, or who had early severe GMG were not included in the study. This would suggest that the data is skewed towards the milder phenotypes.

A Chinese retrospective study of 41 patients in 2007 by Chan et al (160) showed that late onset patients were characterised by male predominance, absence of thymic follicular hyperplasia and higher striated muscle Ab positivity. There was no statistically significant difference in the clinical severity and outcome or response to treatment between the early-onset and late-onset groups. These patients were all AChR Ab positive, had GMG for at least 3 years and did not have radiological or histological thymoma. The recruitment was very restrictive which means that the results would not be applicable to LOMG as a whole.

In 2017 Evoli et al published a paper with retrospective evaluation of 82 MuSK myasthenia patients with GMG who had ocular symptoms. Ocular manifestations were seen in 96.4% and it was the presenting symptom in 58.5%. They found that in myasthenia gravis with antibodies to MuSK, ocular

manifestations were as frequent as in other disease subtypes. Symmetrical ophthalmoparesis with conjugate gaze limitation was common and a proportion of these patients developed chronic eye muscle paresis (161). Evoli et al studied 78 patients with SNMG all of whom had GMG, using seropositive MG controls and healthy controls. Thymic tissue was available from 29 patients. MuSK Abs were detected in 37/78 (47.4%). Myasthenia with anti-MuSK antibodies were reported to be characterised by prevalence of female patients, age of onset between 6 - 68 years with 56.8% presenting under 40 years of age. They had a similar pattern of muscle weakness with predominantly cranial and bulbar muscle involvement and frequency of respiratory crises. The limb muscles were less severely involved. They did not find any association of thymic abnormalities in the study (Evoli et al, 2003). Most patients had developed permanent facial and pharyngeal weakness with some atrophy of facial muscles (151). Both these studies provide useful clinical phenotyping of MuSK MG, but do not comment on ocular to generalisation rates. Also, the MUSK antibody tests were performed using immunoblot (later confirmed on RIA in the study by Guptill et al), but no CBAs were done.

There have been reports of double positive myasthenia gravis patients with positivity to both ACHR and MuSK antibodies. One study published by Zouvelou et al looked at the five year follow-

up of one such patient; this patient had mainly ocular and bulbar symptoms with MGFA classification of IIIB and responded quickly and appropriately to prednisolone. The patient was in pharmacological remission (PR) at four months (162).

Antibodies to clustered AChR on cell based assays were first described by Leite et al in 2008. The study was done on previously collected serum samples from 65 patients with generalised myasthenia gravis. These were retested for AChR and MuSK antibodies using RIA. Of these, 24 patients were seronegative. They also studied thymic tissue from 14 seronegative myasthenia gravis patients who had been thymectomised. Amongst these patients, clustered AChR Abs were present in 11/14. For the seronegative myasthenia patients, and for those with AChR MG low samples, there was a significant correlation between binding to AChR clusters and the percentage of thymic tissue with infiltrates. Seronegative myasthenia gravis patients who were positive on clustered cell based assay had a similar antibody mediated disease to those with AChR MG on RIA, and also frequently had thymic changes (163).

Further studies on antibodies to clustered AChR in ocular and generalised myasthenia gravis were done by Jacob et al and published in 2012. This was a retrospective analysis of previously obtained serum samples from 16 patients with seronegative ocular myasthenia gravis and a further 28 patients

with seronegative myasthenia, 14 of whom had ocular myasthenia gravis. Eight of the 16 patients with seronegative ocular myasthenia gravis had clustered adult AChR antibodies similar to those in patients with GMG. The paper suggested that OMG was more common in patients with positive CBA compared to positive RIA. The numbers were small and the samples retrospective, but appropriate controls were used and they demonstrated a trend towards milder disease in patients with clustered AChR Abs (164).

Devic et al studied 37 patients with seronegative myasthenia gravis from a French database, and analysed the samples using cell based assays. They found 16% to be positive for antibodies to clustered AChR. In half of these patients, ocular symptoms were predominant; bulbar involvement was seen in half of the patients, but never predominant. The maximum severity of MG classification ranged from MGFA II to III. No atrophy was found in the tongue or axial/limb muscles. Two out of the six patients were positive for AChR antibodies and had early-onset myasthenia gravis, which in this study was described as below 40 years of age. One of these had a thymectomy which showed hyperplasia. Three patients had late-onset myasthenia gravis with thymic involution, and one patient had minimal ocular symptoms. The patients responded to variable levels of immunosuppression and all of them responded to intravenous immunoglobulin (165). The paper does not comment on whether

the database was all inclusive or not; if inferred that the data was inclusive, then this study provides useful data about positivity to AChR on clustered CBA in SNMG.

Rodriguez Cruz et al in their paper of 2015 suggest that patients with clustered AChR antibodies generally have a relatively mild disease, and a high proportion have predominantly ocular myasthenia. This was based on the paper published by Jacob et al, and on unpublished observations. Patients with MuSK antibodies on cell-based assays only with negative RIA were predominantly young females with a median age of onset of 21 years. Predilection for bulbar symptoms was noted at follow-up, but with MGFA grade < II predominantly, suggesting a milder phenotype (166).

Rodriguez Cruz et al published a further paper which analysed sera on 138 patients retrospectively. They all had a diagnosis of seronegative myasthenia gravis for both AChR and MuSK antibodies on RIA. The diagnosis was based on clinical presentation and response to treatment, with or without electromyographic evidence. Out of the 138 patients, 51 had an uncertain diagnosis, and 45 had other diagnoses. 16 patients had positive AChR antibodies on clustered cell based assays. Of the 16, 10 were female, and 62.5% were children. The presentation was predominantly ocular in 62.5% with no generalisation during the follow-up period. Only 25% had bulbar symptoms and none had respiratory weakness. Thymectomy

was performed in one patient which showed no evidence of thymoma or lymphocytic infiltration. When the clinical characteristics of the 26 patients with seronegative myasthenia gravis with negative CBA results were compared to those with clustered AChR antibody positivity, the patients with clustered AChR antibody positivity had a younger age of onset and a trend towards milder disease, and a higher proportion attained clinical remission (167). The study included both paediatric and adult patient samples, and does not comment on EOMG/LOMG. The sample size is large in number, but tested retrospectively from stored sera.

Huda et al published a paper in 2017 on their study of cell based assays for MuSK antibodies in seronegative myasthenia gravis. They studied the sera from 69 MuSK RIA positive patients, 169 patients negative for MuSK RIA and AChR RIA, and 35 healthy controls, along with 16 NMDA receptor antibody-positive disease controls. They found that MuSK antibody CBA positive and RIA negative patients were predominantly female and presented at the median age of 25 years. In eight of these patients the disease was confined to the ocular muscles. When MuSK Ab CBA positive patients were compared with MuSK RIA positive patients (defined in the paper as definite MuSK MG), female preponderance was noted in both groups but the median age at onset was later in the MuSK RIA MG patients at 40 years of age compared with 25 years for the CBA positive patients.

Generalised myasthenia gravis was seen in all 69 patients with definite MuSK MG compared with 8/13 of MuSK CBA positive patients and neurophysiology was only positive in half of the patients examined in the MuSK CBA patients. Patients with MuSK CBA responded better to pyridostigmine with 75% showing a good response compared to 20% of those with definite MuSK MG, but immunotherapy appeared to be equally effective in both the groups. Their findings would suggest a milder clinical phenotype in patients who are MuSK RIA negative but CBA positive (168).

There are several published studies on clinical phenotypes with different antibodies. All the studies are retrospective, many of them include large numbers with clear inclusion criteria and matched controls. The studies have shown that with AChR Abs, the presentation can be varied, and there is a trend towards OMG being more common in LOMG. MuSK Abs are more commonly seen in young females and they more commonly have bulbar symptoms. Patients who are seronegative on RIA but are positive on CBA are likely to have milder disease compared to those who are positive on RIA; this has been seen for both AChR and MuSK Abs. Whilst there is a large amount of literature on antibodies, what is lacking is a prospective study with long term follow up with unselected patients.

1.5.7 Ocular MG

Ocular myasthenia gravis (OMG) is the most common form of MG and varying rates of secondary generalisation have been reported. Ocular muscles demonstrate relatively reduced safety factor and complement regulation, simplified postsynaptic structures, and increased susceptibility to toxins- all of which have been postulated as the reasons for predominantly ocular involvement in myasthenia gravis. Typically between 50 and 80% of patients will develop generalised symptoms in the first two years and for this reason an arbitrary minimum duration of two years of isolated ocular symptoms is considered a reasonable limit for diagnosing of OMG. Oosterhuis suggested a minimum of three months as a limit for purely ocular symptoms before classifying a patient as having OMG (169). Similarly Sommer et al (170) and Monsul et al (171) also suggested purely ocular symptoms for at least three months from symptom onset to class them as OMG.

There have been several studies looking at the effect of prednisolone on the progression of OMG to GMG. Monsul et al looked retrospectively at 56 patients, 27 in the prednisolone treated group and 29 in the untreated group. The patient selection was from databases of the Yale neuromuscular and ophthalmology services. OMG was defined as purely ocular symptoms for at least 3 months from symptom onset. The

treatment group was defined as patients who had at least 3 months of oral prednisolone. Patients who had treatment for a lesser duration were included in the non treated group. At two years their data suggested that significantly fewer patients in the treated group progressed to generalised myasthenia compared to the untreated group. They suggested that use of steroids may decrease progression of ocular to generalised myasthenia gravis (171). This was a retropsective study, and the patients in the two groups were age and sex matched; however, it does not tell us why some patients with OMG were treated whilst others were not. Some of the patients in the 'non-treated' group had some form of immunosuppression albeit for less than 3 months duration.

These findings are reflected in another study by Zach et al who looked retrospectively at 44 patients (172) from one neuromuscular clinic. Their criteria to define OMG was purely ocular symptoms for at least one month. However, another study by Nagia et al looked retrospectively at 158 patients from a neuro-ophthalmology clinic and they found that the conversion rates from ocular into generalised myasthenia gravis may be lower than previously reported in both immunosuppressed and non-immunosuppressed patients (173). They also defined OMG as purely ocular symptoms for ≥1 month. There is potentially selection bias here as the patients were recruited from a neuro-opthalmology clinic, and it is likely that the more severe/

symptomatic patients would have been referred to the neurology/neuromuscular clinics.

A study by Kamarajah et al (including the present author) looked at 93 patients from symptom onset. The study reflected the natural history of the condition as the patients were steroid naïve before generalisation. Of these, 46% of patients developed generalised symptoms during the study period which was 11 years. The median time to generalisation was seven months. Time to generalisation was earlier in patients who were positive for AChR antibodies, had bilateral ptosis at onset and were younger than 50 years at disease onset (174). Whilst the patients were seen by a neurologist in a neuro-opthalmology clinic, it was not an unselected cohort.

A study by Imai et al showed that a lower dose of oral prednisolone regimen with early combination of other treatment options may ensure an early achievement of the treatment target in GMG (175).

The EPITOME study which was an RCT designed to look at the efficacy of prednisolone for the treatment of ocular myasthenia was not completed as planned because of failure of recruitment of enough patients. They suggested that low-dose prednisolone with gradual escalation appeared to be a safe and well tolerated treatment for all MG (176).

In 2016, Wong et al published a paper on 101 patients with OMG recruited from two neuro-ophthalmology centres in London. They defined OMG as purely ocular symptoms for ≥3 months.They proposed three predictors for generalisation of OMG: seropositivity (higher Ab titres had greater risk), presence of comorbidities (unrelated to age or autoimmunity) and thymic hyperplasia (weak association). A risk score (0-3) was calculated for each predictive variable and summed to give the overall risk score for the patient. The positive predictive value using this score was 38% and negative predictive value was 91% (177). The patient recruitment was retrospective and the definition of OMG arbitrary as with all the studies so far.

There have been no completed RCTs on the use of prednisolone in OMG. There have been no prospective clinical studies either. There is no consensus on the definition of OMG. Traditionally, OMG has been defined as ocular symptoms only for at least two years, but published studies have used a cut off of between 1 and 3 months.

1.5.8 Investigations in MG

Other than the standard diagnostic test for MG which is the serum antibody test, there are several other supportive diagnostic tests which can be performed.

The icepack test to diagnose MG was first published in 1979 by Saavedra et al. The possible physiological mechanisms of how cooling improves neuromuscular transmission are: (i) increased postsynaptic receptor sensitivity to ACh (178) (ii) facilitated transmitter replacement in the presynaptic terminal (179) (iii) efficient utilisation of ACh (180) (iv) decreased hydrolysis of ACh by acetylcholine esterase allowing sustained action of the transmitter already released from the axon terminal (181) and (v) reduced rate of removal of calcium ions from the nerve terminal following stimulation (182) (Yamamoto et al) (183).

Michael Benatar published a paper in 2006 after conducting a systematic review of all diagnostic methods used in MG. He found 3 studies describing icepack test for the diagnosis of OMG and 5 in GMG, all of which used case control study design The pooled estimates of sensitivity were 0.944 for OMG and 0.82 for GMG, specificity were 0.974 for OMG and 0.96 for GMG (184).

Yamamoto et al performed trigeminal nerve RNS, excitation-contraction coupling assessment, and bite force measurement before and after cooling of the masseters in 25 MG patients and normal controls. Of the MG patients, 4 had OMG, 21 GMG; 20 were AChR positive, 1 MuSK positive; 11 were treatment naïve and 14 had exacerbation of MG symptoms despite steroids. Ice pack test on masseters was done initially and assessed subjectively using MG- ADL scores. The bite was increased

significantly after cooling in icepack positive MG patients, the acceleration and acceleration ratio of jaw movement increased significantly after cooling of the masseters in icepack positive MG patients compared to negative patients and normal controls. The prolonged effect of cooling continued until the end of the recording even though decremental response to RNS had returned to baseline value (183). Patient selection was not explained, but the patients and controls were age and sex matched.

Park et al performed the icepack test on 26 patients with MG and 38 controls and assessed response of ptosis. The test was repeated two times on separate days. Repeated ice test results showed an agreement of 61.5% in MG and 97.4% in non-myasthenic ptosis. Repeated ice test increased the rate of sensitivity by 34.6% compared to a single test (185). The patients were recruited from one neuro-ophthalmology centre and patient selection was not explained.

Michael Benatar in his review of 2006 identified a single study examining the diagnostic accuracy of Tensilon test. There was poor methodology and the sensitivities reported 0.92 and 0.88 for ocular and generalised MG respectively; Specificities were 0.97 (184).

Tsunoda et al have assessed the improvement of voice in one patient with MG using voice spectroscopy after injection of IV

edrophonium and found that there was a marked improvement confirming a diagnosis of MG (186).

Various other diagnostic tools have been used including single breath count, electroglottography, ECG, oculo vestibular evoked myogenic potentials, diaphragmatic function testing and muscle strength tests.

1.5.8.1 Neurophysiology

Neurophysiological tests including repetitive nerve stimulation (RNS) and single fibre EMG (SFEMG) have been useful tools in diagnosing myasthenia gravis. In 2001 the American Association of electrodiagnostic medicine published a literature review on the usefulness of RNS and SFEMG in the evaluation of patients with MG or LEMS. They found that the results of the studies utilising RNS showed that a 10% decrement in amplitude from the first to fourth or fifth intravolley waveform while stimulating at 2 to 5 Hzs is valid for the diagnosis of MG. The degree of increment needed for the diagnosis of LEMS is at least 25% but most accurate when greater than 100%. Abnormal jitter or impulse blocking are the appropriate criteria for the diagnosis of NMJ disorders when using SFEMG. They also said that SFEMG was more sensitive than RNS for the diagnosis of NMJ transmission disorders; however, they may be

less specific and not easily available. Because of this, RNS was thought to be the preferred initial test for MG and LEMS (187).

When Michael Benatar published his review in 2006 he found that there were several studies on RNS and SFEMG, however they were very heterogeneous and not comparable with each other (184).

Liik et al looked at RNS in all consecutive patients with severe GMG at one hospital.They found that of the nine patients with acute onset GMG (<4 weeks duration), only one patient had abnormal decrement, whereas of the 32 patients with slow onset MG (≥4 weeks duration), 26 patients i.e. 84% had decrement. Concentric needle EMG jitter was abnormal in all patients. The AChR Ab status was comparable whereas the MGFA class was higher in the acute onset group. They concluded that RNS is frequently normal in cases of acute severe GMG including myasthenic crisis. The pathophysiology of this remains unclear. Concentric needle electrode jitter analysis was a much more useful tool in these cases (188).

Bou Ali et al conducted RNS on 45 prospectively recuited MG patients from one centre; they tested 12 muscles bilaterally and found that the global sensitivity of RNS was 82% and specificity was 100%. The sensitivity in MG subgroups showed that in ocular MG it was 67%, oculobulbar MG it was 86%, and generalised MG it was 89%. The most sensitive muscles were

found to be the anconeus in the ocular group, orbicularis oculi or nasalis in the oculobulbar group, and trapezius in the generalised group. The maximum sensitivity was obtained by exploring the orbicularis oculi, trapezius and anconeus bilaterally. They suggested bilateral exploration of at least three muscles; a facial muscle, trapezius and anconeus (189).

Nikolic et al published their results on the electrophysiological findings in patients with LRP4 positive MG in 2016. They prospectively recruited patients already diagnosed with MG. They conducted RNS and jitter analysis using concentric needle electrode in 17 LRP4 positive MG patients and compared it with 31 MuSK positive patients and 28 AChR positive patients. They found that RNS was negative in almost all patients of the LRP4 group, the seronegative group and in the LRP4 and MuSK double positive groups. It was positive most frequently in AChR MG group especially those who were not double positive for LRP4. The presence of anti-LRP4 antibodies was connected to lower decremental values whilst the independent presence of anti-AChR or anti MuSK antibodies was connected to higher decremental values. The lowest jitter was recorded in patients with LRP4 or seronegative MG. Highest percentage was present in MuSK and AChR MG patients. The mean consecutive difference (MCD) did not change in anti-LRP4 MG whilst it was high in AChR and MuSK MG. They concluded that LRP4 MG subgroup have rarely any pathological electrophysiological test

results (190). The study was not unselected, but good number of patients and controls were recruited.

Abraham et al published a retrospective chart review of 75 MG patients. They found that high jitter and decremental values were associated with more severe disease, manifested by more frequent symptomatic bulbar and limb muscle weakness, more frequent ocular and limb muscle weakness on examination, higher quantitative MG scores and generalised disease. They found that electrophysiological assessment correlated with disease severity and the presence of generalised disease (191). Similarly, Sanders et al retrospectively reviewed the jitter and outcome data from all MG patients over a 32 year period, who had at least two jitter measurements in the extensor digitorum or frontalis muscle. Of the 789 patients with MG who had neurophysiological tests, 279 had at least 2 SFEMG studies. They found that absolute and percentage change in mean values of consecutive interval differences were equally accurate in predicting clinical change and were more accurate than change in the proportion of fibre pairs with blocking or normal jitter (192).

Machado et al published a prospective study on 33 MG patients on the diagnostic accuracy of concentric needle electrode myography (CNEMG) jitter. They found that CNEMG jitter yielded high positive rates for ocular MG of 92.3% and GMG of

100% and showed high sensitivity and specificity rates (193). OMG was defined as patients who remained ocular for at least 2 years.

Nikolic et al looked at classical and quantitative EMG in 31 MuSK and 28 AChR positive patients. It revealed the presence of myopathic changes more frequently in MuSK MG compared to AChR MG, especially in the facial muscles (194).

1.5.8.2 Imaging

Plain chest x-ray does not have a role in MG diagnosis due to low accuracy. Computed tomography (CT) is the imaging modality of choice although differentiation between a small thymoma and thymic lymphoid hyperplasia (TLH) may not be possible as they both appear as soft tissue masses. Magnetic resonance imaging (MRI) is not usually used but it could be useful when a CT scan is equivocal. MRI with DWI can differentiate lipid-poor normal/hyperplastic thymus from thymoma. Positron emission tomography (PET)-CT is not really helpful in distinguishing early from advanced thymoma, but can help to differentiate between thymic carcinoma from thymoma (Priola et al) (195).

1.5.9 Antibodies in MG

The first antibodies demonstrated in MG were by Strauss and colleagues who found the existence of anti-striated muscle antibodies (anti--SM). This is seen in 30% of all MG patients and nearly all patients with thymoma.

Antibodies to the endplate protein i.e. acetylcholine receptors was first demonstrated by Alman, Andrew and Appel in 1973. They found that serum globulins from MG patients could inhibit α bungarotoxin (α-BuTx) binding to solubilised rat AChR receptors.

Mittag, Kornfield, Tormay and Woo compared four different techniques of assessing AChR antibodies and found that immunoprecipitation of α-BuTx labelled AChR was the most effective (196). This was then described in detail by Lindstrom et al (197). Over the years several different studies were performed. Using subclass specific antisera, Vincent, Lang and Newsom-Davies found that most patients have anti-AChR with subclasses 1, and occasionally subclass 3.

A systematic study of AChR antibodies in MG was published by Jon Lindstrom in 1976 (10). The assay was based on immunoprecipitation by the patient's IgG antibodies of detergent solubilised muscle AChR which were mainly obtained from amputated limb muscle and which had been labelled with

radioactive α bungarotoxin, a toxin that binds irreversibly to AChR. The assay is now available commercially for AChR autoantibody and MuSK autoantibodies where a carefully balanced mixture of detergent solubilised foetal and adult forms of the receptor are labelled with radioactive iodine labelled α bungarotoxin (Vincent) (11, 197).

The other form of testing AChR antibodies is by using the ELISA method. Nguyen et al first published a paper in 1999 describing an enzyme-linked immunosorbent assay (ELISA) for AChR Ab detection. They use the $AChR_{TE671}$ as the antigen. The test was shown to be specific and was able to detect AChR antibodies at concentrations as low as 3 nmol/l (198). Nonradioactive fluorescent immunoprecipitation assay (FIPA) has also been done with good results (yang et al) (199).

The disadvantage of using a radioimmunoassay or RIA is that RIA cannot discriminate between antibodies to different channel components and cannot discriminate between extracellular and intracellular antigens.

With the advent of MuSK testing in 2001(200), 70% of the patients who were negative for AChR Ab were found to be positive for MuSK. The remaining GMG patients were classed as seronegative MG (SNMG) patients. However there was increasing evidence that SNMG was similar to AChR MG in clinical picture, response to immunosuppressive treatment and

thymic pathology. The muscle biopsies in these patients also indicated loss of AChR and complement deposition similar to AChR MG. The possible explanation for this was that the RIA did not detect AChR antibodies because of loss of antigenic determinants in the solubilised AChR, or because the AChR antibodies have low affinity/ability for the soluble AChR.

Leite et al in 2008 hypothesised that antibodies in SNMG patients could be detected by binding to AChRs on the cell membrane, particularly if they were clustered at the high-density that is found at the NMJ. They expressed recombinant AChR subunits with the clustering protein Rapsyn in human embryonic kidney cells (HEK) and these were tested for binding of antibodies by immunofluorescence. They detected AChR antibodies to Rapsyn clustered AChR in 66% of sera which were previously negative for binding to AChR in solution. These antibodies were mainly IgG1 subclass and they showed the ability to activate complement. They also used cell based assays to detect MuSK antibodies. These were mainly IgG4 but with partially IgG1 subclasses which were capable of activating complement bound to MuSK on the cell surface (163).

Rodriguez Cruz et al looked at clustered cell-based assays in 138 retrospectively recruited MG patients, mainly paediatric patients, and found that clustered AChR antibodies were

detected in 38.1% of RIA negative patients with MG with 100% specificity (167).

Huda et al published a paper in 2017 on cell-based assays in MuSK positive MG patients. They looked at 69 MuSK positive patients (on RIA) with MG, 169 patients negative for MuSK RIA and AChR RIA i.e. SNMG, and 35 healthy controls. They also used 16 NMDA receptor antibody patients as disease controls. Initially, the MuSK CBAs showed low specificity with high positive rates in healthy controls and in the disease controls. This was thought to be due to anti-IgG (H + L) detection of IgM which bound non-specifically to MuSK. They then used an IgG Fc gamma specific secondary antibody to eliminate the IgM. Repeat CBAs showed that MuSK antibodies were detected in 99% of definite MuSK MGs and in 8% of seronegative MG, and in none of the healthy controls or disease controls. This showed increased sensitivity with high specificity (168).

Cell-based assays provide an excellent diagnostic tool, however they are relatively costly and time-consuming compared to RIA. Additionally, culture facilities and expertise with performing and interpreting the assays is required. CBAs do not provide a titre but are subject to measure of positivity based on visual interpretation and for this reason titres of antibodies cannot be used for monitoring disease activity as can be done with RIA (Rodriguez Cruz et al) (166).

Jacob et al retrospectively studied 16 patients with seronegative OMG to look for binding to clustered AChR. This was correlated with their SFEMG results. They found that about 50% of patients with previous SN-OMG had complement fixing IgG1 clustered AChR antibodies. The IgG binding and complement deposition correlated with the mean jitter on SFEMG. When they did their CBAs, they found that most of the clustered AChR antibodies in OMG were directed against the adult form of AChR and did not bind the foetal form which was different to most patients with AChR antibody-positive GMG who usually bind foetal forms. They suggested that complement fixation is likely to be an important pathogenic mechanism in patients with OMG (164).

The French study by Devic et al looked at sera from 37 patients with SNMG using CBAs. 16% of the SNMG patients were found to have antibodies to clustered AChR. These included all clinical subtypes-EOMG, LOMG and TAMG (165).

There have been papers suggesting that the relative lack of intrinsic complement regulators and the differential expression of foetal AChRs on extraocular muscles may be the reason why these muscles are more susceptible to autoimmunity in MG.

It is not entirely clear why some MG patients develop only ocular symptoms and why extraocular muscle weakness usually precedes generalised muscle weakness. This is often explained by the increased susceptibility of extraocular muscles due to

their reduced endplate safety factor and lower complement inhibitor expression. Recent studies in animal models suggest that additional factors may be implicated. In EAMG studies, when AChR receptors carrying confirmational epitopes were injected into wild-type mice, this caused severe GMG whereas injection with recombinant unfolded AChR subunits containing linear epitopes induced ptosis without generalisation or mild generalised muscle weakness.

A similar milder picture with ocular symptoms was seen in mice that were given deficient T-helper cell mediated antigen with recombinant AChR subunits or whole native AChR pentamer. Wu et al published their study results in 2017 and hypothesised that ocular symptoms observed in earlier stages of MG may be triggered by linear and non-conformational AChR epitopes which are expressed by the thymic cells or by microorganisms. This initial stage of autoimmunity may be managed by T-cell independent and B-cell mediated mechanisms which yields low affinity AChR antibodies. These antibodies are capable of inducing muscle weakness only in extraocular muscles which have increased vulnerability due to their inherent biological properties. After the initial attack, as AChR bearing immune complexes are formed, and when the immune system is able to access native AChR in muscle and thymus, a more robust anti-AChR autoimmunity develops which then produces high affinity

AChR antibodies and germinal centre formation in the thymus causing severe generalised muscle weakness (201).

Koneczny et al looked at MuSK myasthenia gravis. They used archived samples from therapeutic plasmapheresis in MG patients. They found that total IgG, IgG4 or IgG 1 to 3 MuSK antibodies were not endocytosed unless they were cross-linked by divalent antihuman IgG. The MuSK IgG4 fab fragments and IgG4 inhibited the binding of LRP4 to MuSK and reduced agrin induced AChR clustering in C2C12 cells. IgG 1 to 3 antibodies did not inhibit LRP4/MuSK binding but they did inhibit agrin induced clustering. Both IgG4 and IgG1 to 3 dispersed agrin-independent AChR clusters in Dok7 overexpressing C2C12 cells. They concluded that interference by IgG4 antibodies of the LRP4/ MuSK interaction was one of the pathogenic mechanisms of MuSK antibodies but IgG1 to 3 MuSK antibodies also contribute to the reduced AChR density and NMJ dysfunction in MG patients with MuSK antibodies (202). Similar findings were reported by Huijbers and colleagues in 2013 when they looked at passive transfer studies in mice (203).

Koneczny et al looked at IgG4 MuSK antibodies and fab arm exchange in 51 MG patients using serum samples and plasma from plasmapharesis, all of whom had been immunosuppressed. One of the key features of IgG4 antibodies is their ability to exchange fab arms with other unrelated IgG4

molecules. This can make the IgG4 molecule potentially monovalent for that particular antigen. They applied exchange-inducing conditions to MuSK antibodies and studied these. They found that at least 50% of patients had IgG4 but not IgG1 to 3 MuSK antibodies that could undergo fab arm exchange in vitro. They also found that in vivo all MuSK antibodies were divalent i.e. more specific for MuSK. This did not prevent the inhibitory effect of the serum derived MuSK antibodies on AChR clustering in C2C12 myotubes. This suggests that a considerable proportion of MuSK IgG4 could already be fab arm exchanged in vivo and demonstrates that fab arm exchanged antibodies are pathogenic (204). It is not clear how immunosuppression may have affected the results. If they were being plasmapheresed it is likely that they had marked symptoms.

Otsuka et al studied the physiological significance of binding ColQ to MuSK and the block of this binding by MuSK IgG in 5 patient samples. They showed that passive transfer of MuSK IgG to ColQ knockout mice attenuated AChR clustering indicating that lack of ColQ is not the key event causing defective clustering of AChR in MuSK MG. They found that AChE/ColQ complex blocked MuSK LRP4 interaction and it oppressed agrin/LRP4/MuSK signalling. Quantitative analysis showed that MuSK IgG suppressed agrin/LRP4/MuSK signalling to a greater extent than ColQ (205).

Ishikawa et al published a paper on two cases of MG who were double positive for AChR and LRP4 antibodies and also had invasive thymoma. After treatment they found that AChR antibody levels were markedly decreased whereas there was no change in the LRP4 antibodies in one case and slight decrease in the second case. They suggested that the patients with double seropositivity with AChR and LRP4 were likely to present with more severe symptoms than those with LRP4 MG alone. They concluded that the main affecting course for the symptoms in MG in their case was probably AChR antibodies and the LRP4 antibodies may have been an exacerbating factor (206).

Zouvelou et al published a paper with two case reports of patients with anti-LRP4 antibodies. The first had isolated neck extensor weakness and the second ocular, bulbar and cervical weakness; both had mild to severe MG and responded to pyridostigmine. One of them also had follicular hyperplasia of the thymus (162).

Zisimopoulou et al published a paper with a comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 MG. They screened 800 MG patients from 10 countries for LRP4 antibodies. They found that the overall frequency of LRP4 MG in SNMG was 18.7% with variations among different populations with a range of 7 to 32.7%. They found double positive sera in 8/107 anti-AChR positive patients and 10/67

anti-MuSK positive patients. They looked at the clinical history for these LRP4 positive patients; 81% of patients had mild symptoms with MGFA grade 1 or II. Some with thymic changes-32 with hyperplasia but no thymomas. They felt that the double positive patients either with AChR or MuSK had more severe symptoms at onset compared with single positive group. Of the OMG patients who were seronegative to start off with, 27% had LRP4 antibodies. LRP4 antibodies were predominantly IgG1 and IgG2 subtypes. The prevalence was found to be higher in women than men with a ratio of 2.5:1 and an average disease age of 33.44 years in females and 41.94 years in males. The treatment response was similar to AChR MG (207).

Hong et al looked at 432 Chinese MG patients and tested them for antibodies against AChR, MuSK, Titin and ryanodine receptors antibodies. ACHR Abs were found in 82.2%, MuSK antibodies in 2.3%, Titin antibodies in 28.4% and Rynodine antibodies in 23.8%. Thymoma MG patients had higher frequencies of AChR, Titin and Rynodine antibodies. Titin and Rynodine antibodies were also present more frequently in LOMG patients. They found that patients with Titin and Rynodine antibodies tended to have more severe disease and worse outcome and they may need more active suppressive treatment (208).

Huda et al looked at the characteristics of AChR antibody negative MG patients in a South African cohort. The samples were tested using RIA and some CBAs for AChR, MuSK and LRP4. AChR antibodies were detected in 4/53 and MuSK Abs in 24%; 60% were triple seronegative. The MuSK MG patients had a younger age of onset, were likely to be of African genetic ancestry, and had a fourfold higher odds of reaching MGFA grade IV/V compared to triple SNMG. They were also ninefold more likely to reach at least minimum manifestations after ≥12 months of therapy (209).

Chang et al looked at a cohort of 113 patients from Sri Lanka recuited both prospectively and retrospectively, and they found that the clinical characteristics were similar to other populations (210).

Cordts et al screened 45 AChR negative and 55 AChR positive patients who already had a diagnosis of MG for LRP4, agrin and Titin antibodies. They found that in 55 AChR antibody positive patients, 7% were also positive for LRP4, 5% for agrin, 53% for Titin. In 45 AChR antibody negative patients, they had 2% positive for MuSK, 2% positive for LRP4, 2% for agrin, and 27% for Titin. The phenotype of Titin MG depended on the AChR antibodies- if AChR antibody negative, patients presented with mostly mild limb weakness but if AChR antibody positive, patients had more severe symptoms including crises with bulbar

predominance and could be associated with thymoma. Additional autoimmune disease was detected in 32% of the MG patients, most frequently Hashimoto's thyroiditis in 21% (211).

Stergiou et al also looked at Titin antibodies using a sensitive radioimmunoprecipitation assay (RIPA) in 667 MG patients, 372 triple seronegative patients, 121 healthy controls, 90 myopathy patients and 193 patients with other neurological disorders using stored samples.. They found that AChR MG patients had the highest frequency of Titin antibodies of 40.9%, in MuSK MG of 14.6%, and in LRP4 MG of 16.4%. 13.4% of the triple seronegative MG patients also had Titin antibodies. None of the healthy controls or myopathy controls had Titin antibodies and only 3.6% of other neurological patients were positive (212).

Berger et al looked at 44 patients with paraneoplastic neurological syndrome (PNS) to see whether Antititin antibodies were significant. They found that in a small proportion of patients with PNS they could have Antititin antibodies without a predictive relevance for MG or thymoma (213).

Patients with MG can have antibodies against skeletal muscle Rynodine receptor (RyR), which is the sarcoplasmic reticulum calcium release channel, and plays a crucial role in excitation contraction coupling. Skeie et al studied the role of Rynodine receptor antibodies in MG. Their data suggested that the RyR sequence defined by residues 799 - 1172 is involved in the

regulation of RyR function and dysregulation could be functionally affected in vivo in patients with MG (214).

Romi et al similarly suggested that the occurrence of Rynodine receptor (RyR) antibodies increases the risk of severe MG. They looked at MG in 152 patients. They found that patients with RyR antibodies had the highest rate of bulbar, respiratory and neck involvement at MG onset. They also had the highest frequency of non-limb MG symptoms. Weakness occurred in 40%. Patients with Titin antibodies with or without RyR antibodies had respiratory difficulties at onset (215).

Buckley et al looked at levels of Titin, IFN alpha and IL-12 antibodies using RIA in 191 MG patients and 82 controls. They found that Titin antibodies were uncommon in EOMG; however in LOMG, Titin antibodies had similar prevalence and levels to those with MG and thymoma. These antibodies were uncommon in patients between 40 and 60 years of age without tumour. Cytokine antibodies were more common in patients with thymoma than in patients without thymoma and increased substantially if the thymoma recurred. They concluded that measurement of Titin antibodies has limited use in predicting the presence of a tumour unless the patient is less than 60 years of age but the measurement of IFN alpha and IL-12 antibodies could be helpful in identifying patients with thymoma recurrence (216).

Similarly, Szczudlik et al looked at Antititin antibodies in EOMG and LOMG. They found that Antititin antibodies have high positive predictive value and negative predictive value for thymoma in EOMG. In MG without thymoma, antititin antibodies could be considered as markers of LOMG but not of a severe course (217).

Illa et al looked at Cortactin, and Cortactin antibody positivity in MG patients. By using a protein array to screen reactivity to 9000 human proteins, they identified Cortactin, an intracellular protein which interacts with agrin/MuSK leading to AChR aggregation. They identified this as a new antigen in double seronegative MG (dSNMG). In the second part of the study they looked at Cortactin antibodies, screening 250 patients with MG. They found that 23.7% of SNMG and 9.5% of AChR positive MGs had Cortactin antibodies. The patients with the SNMG cortactin positive MG presented with ocular or mild generalised MG without bulbar symptoms. They concluded that Cortactin antibodies were biomarkers of MG which when present suggested that the disease would be mild (218).

Tu et al studied 70 MG patient sera and screened them for collagen XIII autoantibodies using ELISA. This was then further analysed with CBA and western blotting. They found that 5/70 MG patients had autoantibodies against collagen XIII. All five were young women with negative or low levels of AChR

antibodies. It was not clear whether collagen XIII autoantibodies were pathogenic (219).

Zhang et al studied autoantibodies to agrin in MG. They found that agrin was another autoantigen in patients with MG, and agrin autoantibodies may be pathogenic through inhibition of agrin/LRP4/MuSK signalling at the NMJ (220).

Thymus and Antibodies in MG:

Normally, functional AChR is only expressed by several skeletal muscles and thymic myoid cells. In addition, unfolded AChR subunits are expressed by medullary thymic epithelial cells, partly under the control of the autoimmune regulator (AIRE). Autoimmunity against self proteins including AChR is kept in check by thymic deletion of autoreactive T cells. Since deletion is not 100% efficient, the normal human T cell repertoire contains a few potentially AChR reactive effector T cells that are kept under control due to T cell anergy, or in the periphery by regulatory T cells. This small proportion of potentially AChR reactive effector T cells is likely to be involved in the pathogenesis of EOMG (64, 221-226).

In EOMG, normal numbers of T regs with impaired function and down regulated Fox P3 are seen in the thymus and peripheral blood. In thymomas, there are reduced numbers of intra-tumourous T regs irrespective of MG status, and the number of

naive autoreactive effector CD4+ T cells exported from the tumour predicts the MG risk (51, 227-230).

10 to 20% of MG patients have a thymoma, and about 30% of thymoma patients have thymoma associated MG. There is a ‘gray zone’ in clinical, immunological and pathological terms between the ages of 40 and 60 years when patients may suffer either from ‘late EOMG’ or ‘early LOMG’. Some LOMG thymuses have shown lymphoid follicles like in EOMG even in patients over 60 years of age. The lymphoid epithelial tissue of the ageing thymus is replaced with fat, but residual parenchyma may continue exporting some T cells. In LOMG these T cells may rarely show signs of expansion and even infiltration. Thymic myoid cells are sparse in LOMG and decline with age, and there is also a reduction in the number of AIRE positive cells. There is however no difference between LOMG thymuses and age controlled matches (128, 222, 231-234).

There is a lot of similarity between LOMG patients and patients with TAMG, with (i) autoantibodies against Titin in 70% of patients (ii) neutralising antibodies against IFN alpha and or IL-12 in 40% (iii) expansion of Vβ T cell subset in more than 50% and (iv) expansion of peripheral blood CD8+T cells particularly CD45RA+ subset (231, 235-239).

It is postulated that because the immunological similarities between LOMG and TAMG are so close, it appears that

aberrations in the aged thymus in LOMG mimics thymoma behaviour without frank neoplasia which leads to export, and possibly even activation of non-tolerant T cells. There is a remote possibility that (i) a small thymoma could have regressed spontaneously before the diagnosis of MG (ii) that a small population of highly potent AChR and Titin reactive T cells generated in the absence of myoid cells inside a AIRE negative atrophic thymus could become activated after exposure to the periphery and to trigger LOMG and (iii) that the pathogenic T cell population derived from atrophic myoid cell-poor and AIRE-low thymus has accumulated in the periphery over a long period before the outbreak of LOMG similar to the thymoma patient who develops TAMG years after thymoma removal. Once initiated, LOMG could become self-perpetuating by stimulatory AChR/autoantibody complexes in muscle draining lymph nodes (222, 225, 229, 240).

MuSK MG in a thymoma patient is rarely reported and the pathogenic link is questionable. A direct link of the role of a non-neoplastic thymus in MuSK MG has not been shown, and with a few exceptions, thymectomy has not been beneficial in these patients. Similarly, the role of thymus in LRP4 MG is not entirely clear; given the high proportion of LRP4 positivity in otherwise seronegative caucasian MG patients, and the high prevalence of thymic hyperplasia in double seronegative MG patients, it is

assumed that a proportion of LRP4 MG patients may show inflammatory thymic changes (154, 226, 241-245).

1.5.10 B cells in MG

AChR MG is a CD4 dependent B-cell mediated disease and hence the interaction of the follicular helper (Tfh), the follicular regulatory (Tfr), and B cells are critical in the development of MG. CD4 T cells differentiate into Tfh cells upon expression of master transcription factor B cell lymphoma 6 (BCL 6) and surface marker CXCR5. This allows migration into the germinal centre to help differentiation of B cells into memory B cells and antibody secreting plasmablasts and plasma cells. In MG thymuses, the frequency of the Tfh cells and B cells is increased, and the expression of Tfh associated markers i.e. IL 21, PD 1 and ICOS is also increased on thymocytes. Tfr cells are counterparts to Tfh cells and they suppress Tfh and B-cell interactions in the germinal centre. The cells are derived from natural T reg precursors and express Fox P3 and BLIMP 1 (246-251).

B cells differentiate into plasma cells; immune response produces short lived and long-lived plasma cells. Initially blood plasma cells are rapidly formed in secondary lymphoid organs after antigen encounter where they undergo apoptosis after a

few days. A small proportion of plasma cells survive prolonged periods to maintain long-term in immunity, called long-lived plasma cells and these may play a role in autoimmunity.

AChR expression by TACs and myoid cells, increased pro-inflammatory cytokines and defective T reg cells is seen in some MG thymuses. B cells present in hyperplastic thymuses express markers of activation and display functional signs of activation. They are often organised in the hyperplastic thymuses within tertiary lymphoid organs. However these characteristics are not applicable to the entire MG population, given that in approximately 30% of patients with AChR MG the thymuses are not hyperplastic. The T cell subset in the thymic tissue which is responsible for the detectable AChR antibodies has not been precisely defined, but spontaneous production of AChR antibodies was demonstrated as most likely due to resident plasma cells and possibly plasmablasts. Other studies have demonstrated that the AChR autoantibody production by thymic lymphocytes can occur spontaneously or with mitogen stimulation, suggesting that heterogeneous B cell populations make such contributions. AChR autoantibody producing T cells can be found in circulation and in the lymph nodes, and have also been identified in the bone marrow (53, 252-261).

Circulating B cell repertoire was characterised through analysis of over 500,000 unique sequences and minor deviations from normal controls were evident. This indicated that pathogenic B

cells make small changes to the global repertoire which may not be obvious without very large datasets. The thymus of patients with MuSK MG appears different to AChR MG making one infer that MuSK antibodies may develop and reside in a compartment other than the thymus (245, 262).

The difference in frequency of B cell populations between AChR MG and healthy controls is unremarkable and there is no evidence of a general defect in B cell differentiation in patients with MG. CD 27^- IgD^- B cells do not appear to be altered in AChR MG. There appears to be an increase in CD5+ B cells in a subset of patients with AChR MG that may be associated with autoantibody production and regulation, although their roll in immunobiology is not unambiguously defined (263-266).

Next-generation deep sequencing allows for comprehensive evaluation of the B cell receptor repertoires in health and disease in up to 10^{11} B cells in humans. Using this approach, naive B-cell compartments in patients with AChR and MuSK MG showed repertoire features that were not observed when B-cell tolerance functioned properly (262, 267).

B lymphocytes augment immune responses by producing antibodies and activating T cells by antigen presentation. Studies have highlighted that a specific and functionally significant B-cell subset which could downregulate excessive immune and inflammatory responses to inhibitory cytokines

such as IL-10 and TGF β. The subset is called regulatory B cells (Bregs). IL-35 producing Bregs also play a role in downregulating immunity. They exert regulatory function mainly through suppressing the differentiation of Th1/Th17 cells and promoting T cell expansion. Reduced presence of the Bregs is associated with progressive neuroimmunological disorders (Han et al) (268).

Karim et al showed that patients with MG had relatively lower percentages of CD19+CD5+CD1d+ Bregs cells compared to healthy controls. The production of IL-10 and TGF β1 was relatively less in patients with MG than healthy controls and was linked with more severe MG disease status. The reduction of cytokine production was more significant for IL-10 than TGF β1 (269).

Lu et al looked at the significance of B10 cells in patients with thymoma and MG. A subset of the regulatory B cells (Bregs) have been identified- B10 cells, which function by secreting IL-10. In the study, 156 patients with thymoma were looked at and they analysed the percentage of the Bregs/CD19+ B cells and CD19+ B cells/PBMC. They also looked at gene and protein expressions of CD19 and IL-10. They found that thymoma patients without MG mainly had types A and AB thymoma and thymoma patients with MG mainly had type B- B1, B2 and B3 thymoma. ACHR antibody in TAMG group was highest. With the

progress of the disease the percentage of the Bregs/CD19+ B cells increased and the B10/CD19+ B cells decreased. They showed that B10 had the greatest significance for clinical directivity of TAMG with a cut-off of 0.55%. In accordance with the condition, in thymoma and TAMG group, the content of CD19+IL-10+ B10 cells gradually increased. The levels of CD19 and IL-10 also gradually increased. They found that as the severity of MG increases, the function of the Bregs (B10 cells) in peripheral blood decreases (270).

B-cell activating factor (BAFF) is a member of the TNF superfamily. It is a potent survival factor for B cells and plays an essential role in peripheral B-cell homeostasis. The expression of BAFF in secondary lymphoid tissues is essential for sustaining the long-term survival of mature B cells in vivo.There are three functional receptors for BAFF, B-cell maturation antigen (BCMA), transmembrane activator and cyclophilin ligand interactor (TACI) and BAFF receptor (BAFF-R). The BAFF-R is the main receptor which mediates BAFF signals in naïve B cells. Following activation, and during differentiation, BAFF-R expression is downregulated while TACI expression is upregulated. BCMA expression is upregulated in the terminal stages of B cell differentiation. Excess BAFF promotes the survival, growth and maturation of autoreactive B cells thereby breaking immune self-tolerance (Berrih-Aknin et al) (271).

Animals that express high levels of BAFF also suffer from a number of autoimmune manifestations including high levels of circulating autoantibodies, immune complexes in serum and kidneys, and proteinuria due to immune complex-mediated glomerulonephritis. Older BAFF transgenic mice also show hallmarks of the autoimmune disorder Sjogren's syndrome. Interestingly, a causal relationship between BAFF overexpression and human autoimmune disease is also suggested by the high levels of serum BAFF found in patents with Sjogren's syndrome and the association between high serum BAFF and autoantibody production in several other autoimmune diseases. Why age makes autoimmunity more common is not clear.

AIRE plays a role in both the central and peripheral immune self-tolerance mechanisms for T cells. AIRE deficiency leads to higher numbers of antigen presenting cells. AIRE-deficient mice also have higher serum levels of BAFF than wild-type mice, and this is associated with increased expression of membrane-bound BAFF on the surface of dendritic cells. Aging AIRE-/- mice have a similar phenotype to BAFF transgenic mice. As shown recently, AIRE-/- mice are also susceptible to the induction of EAMG, and this appears to be age related.

The ability of BAFF overexpression to rescue self-reactive B cells from deletion is limited to those cells normally deleted

relatively late in their maturation. The ability of self-reactive B cells to be rescued by BAFF is most likely determined by their expression of BAFF-R, which peaks around the point during B cell maturation where BAFF-mediated rescue begins to operate. It is probable that the expression of BAFF-R is delayed during B cell maturation to ensure that B cells with strong self-reactivity will not reach the point where they express this pro-survival receptor. If this were not the case then the autoimmunity associated with increased BAFF expression could well be significantly more catastrophic than it is (272).

Three different studies have shown that serum BAFF levels in patients with MG are significantly higher than in non-MG controls. There is no association between serum BAFF level and the extent or severity of the disease. There is a trend for BAFF levels to be higher in patients seropositive for AChR antibodies (273-275).

Lee et al found that the frequency of polyreactive and autoreactive B cells receptors (BCRs) was higher in both the AChR and MuSK MG patients compared to healthy controls. This indicates that both the MG subtypes have defects in central and peripheral B-cell tolerance checkpoints (276).

Yi et al looked at B10 cell frequencies in MG. B10 cells are known to inhibit B and T cell inflammatory responses in animal models. They found reduced B10 cell frequencies in AChR MG

patients which inversely correlated with disease severity. The disease severity also affected the function of B10 cells, as B10 cells in the moderate/severe group of MG patients were less effective in suppressing CD4 T-cell proliferation. They suggested that B10 cell frequencies may be a useful biomarker of disease severity (277).

Stathopaulos et al looked at the B-cell compartments in MuSK MG patients who were treated with rituximab. Autoantibody expressing CD27+ B cells were observed within the reconstituted repertoire during relapse but not during remission or in the controls. They demonstrated that antibody secreting $CD27^{hi}CD38^{hi}$ B cells (plasmoblasts) contribute to the production of MuSK autoantibodies during relapse (278).

1.5.11 T cells and cytokines in MG

The immune system is very organised, and proper functioning depends on adequate balance between the pro-inflammatory and anti-inflammatory signals, and the responses of the cellular system- the pro-inflammatory T cells (T helper 17 cells or TH 17 cells) and the anti-inflammatory cells- the regulatory T cells (T regs cells). Defective balance is seen in a lot of autoimmune conditions (279-283). There is an increased production of IL-17 which is a cytokine and is expressed by TH 17 cells. Several

studies have shown that in thymic hyperplasia there is increased production of pro-inflammatory cytokines like IL-6, IL-1β, interferon-γ, and TNFα. There is also increased production of the anti-inflammatory cytokine TGF or transforming growth factor β-1. The balance between T reg cells and TH 17 cells in MG is disrupted. T regs are shown to be defective in their regulatory function and can start expressing markers of TH 17 cells, while the effector T cells or T eff cells (CD4+25-) become resistant to suppression (51, 284-287).

T reg cells (CD4+CD25+FoxP3+) can be divided into natural T reg cells, also called thymic T reg cells, and peripheral T reg cells, or induced T reg cells. Whilst natural Treg cells consistently express FoxP3 and CD25, the peripheral T reg cells only express FoxP3 and CD25 after they have been activated, and this requires the presence of TGF β (288-290).

T reg cells regulate the effects/suppress the effector T-cell proliferation by cell-cell contact through the expression of CTLA4, by depriving IL-2, by expressing anti-inflammatory cytokines like TGF β and IL-10 and by expressing granzyme A and activating the perforin pathway (291, 292).

Autoimmune diseases could be caused by a deficiency in the number of T reg cells and/or defective suppression by the T reg cells. There have been several contradictory studies regarding the numbers and function of T reg cells, some of which show

that the T reg cell numbers are reduced in MG patients compared to healthy controls (227, 293, 294), whereas other studies showed no difference in the percentage of CD4+FoxP3+ T reg cells in the thymus and peripheral blood (295, 296). Most studies report that the T reg cells have reduced suppressive activity. A decreased expression of Foxp3 in MG thymus and in the peripheral blood could be one of the reasons for the impaired suppressive activity (51, 52, 265).

More recent studies have shown that there is another subpopulation of T cells known as T follicular regulatory cells or Tfr which are CD4+CXCR5+FoxP3+. These are derived from thymic T reg cells and they control the follicular helper cells or Tfh cells (297).

Wen et al looked at a large cohort of 79 MG patients with an established diagnosis of MG. Of these, 59 were immunosuppression naïve, and 13 went on to have steroids during the study. They found that Treg levels were lower in the untreated group compared to those who were given steroids. They also found significantly lower percentage of CD4+CXCR5+FOXP3+ Tfr-like cells and higher percentage of CD4+CXCR5+FOXP3- Tfh-like cells in the peripheral blood of untreated MG patients compared to treated patients and healthy controls. In the 13 treatment naïve patients who went on to have

steroids, the Treg levels and Tfr-like cells increased and Tfh-like cells decreased (298).

TH 17 cells are CD4+ T cells which have the phenotype RORγT+CCR6+IL-23R+IL-17+. The TH 17 cells are considered pathogenic when they differentiate in the presence of IL- 23 and overexpress IL-17. IL-17 induces several cytokines such as CXCL1, CXCL13, CCL2, CCL 7 and CCL20. It also induces pro-inflammatory cytokines such as IL-6, TNF α, IL-1 β and G-CSF. Overexpression of IL-17 family is seen in hyperplastic thymuses in MG (287, 299, 300).

A study on 11 patients with a prior diagnosis of MG by Cao et al showed that AChR reactive CD4+ T cells from MG produce high levels of IFN γ and IL-17 which suggests a mixed phenotype of TH 1/TH 17 cells. The differentiation of these two subtypes is linked to each other. When there is low IL-23, or high IL-12 and TNF α, the TH 17 cells can become IFN γ expressing cells. These TH 1-like cells derived from TH 17 cells are more pathogenic than the original TH 17 cells (301). This was a study on a small number of patients with a mixture of treatment naïve and immunosuppressed patients.

There have been other studies which have not reported any significant differences in the pro-inflammatory cytokines, and similar levels of plasma IL-17 in AChR+ MG patients and healthy controls. Another study showed no difference in serum IL-17

and IFN-γ between MG patients and healthy controls (Villegas et al) (302).

A study on EAMG by Yang et al which was recently published looked at the association of B lymphocyte induced maturation protein 1 (blimp1) and T reg cells. The loss of blimp1 has been shown to double the number of follicular regulatory T cells (Tfr). The study showed that Tfrs indirectly inhibited the activation and differentiation of B cells by negatively regulating follicular helper T cells which in turn lowers the secretion of antibody. Lack of blimp1 makes immune suppression function of Tfr cells impaired in vitro. When tested in vivo, Tfrs reduced immune responses in germinal centres and improved myasthenia symptoms in EOMG (303).

Zhang et al published a study on 45 patients with an established diagnosis of AChR+ MG. They were a mixture of GMG and OMG and about half had thymic abnormalities. They studied TIPE2, which is a tumour necrosis factor α induced protein 8 like 2. This is a member of the TNF family and is a negative regulator of innate and adaptive immunity. The study demonstrated that the expression of TIPE2 mRNA and protein was reduced in MG compared to healthy controls, being lower in GMG than in OMG. There is a negative correlation with serum levels of IL-6, IL-17 and IL-21 in GMG and all MG patients. TLR4 activation caused down-regulation of TIPE2 and

expression of RORγ T and production of IL-6, IL-17 and IL- 21 were increased. Their results indicate that TIPE2 participates in the development of MG through negative regulation of TLR4 mediated autoimmune T helper 17 cells (304). The patients had not been given immunomodulatory treatment for the three months preceding recruitment; however, there is no mention of what treatment the patients had had prior to this which makes interpreting the results tricky.

Another Chinese study by Yang et al shows the correlation of neutrophil to lymphocyte ratio (NLR) in myasthenia patients. They looked at 172 MG patients and 207 healthy controls and found that patients with MG had a significantly higher NLR compared to healthy control group. This was higher in MG patients with severe disease compared to milder disease and statistically higher in myasthenic crisis. The study suggested that elevated NLR was an independent predictor of severe disease activity (305). The study did not categorise patients based on whether they were immunosuppressed or not, which could potentially change how the data is interpreted and it is not clear if the changes are related to MG or to treatment.

Alahgholi-Hajibehzad et al found in their retrospective study of 78 MG patients that decrease of FoxP3 was associated with lower phosphorylation of STAT 5, and vitamin D3 increased suppression and can have modulatory effects (306).

A Chinese retrospective study by Zhang et al looked at the expression of P2X7 receptor in PBMCs and in myasthenia gravis. P2X7 is an activator of innate and adaptive immune responses. The study looked at 12 GMG and 20 OMG patients and 22 healthy controls. Their results showed increased expression of P2X7 mRNA and protein in PBMCs with high expression in GMG than in OMG. There was a correlation with clinical severity and serum levels of IL-1 β, lL-6, IL-17 and IL-21 in MG. In cultured MG PBMCs, LPS challenge led to upregulation of P2X7 expression with increased production of the cytokines and its blockade significantly attenuated the LPS induced production of cytokines. This suggests an involvement in the pathogenesis of MG by promoting TH 17 immune response (307). As with the previous study by the same group, immunomodulatory treatment had not been used in the three months preceding recruitment; but the study does not outline what treatments were used prior to this.

Luo et al looked at the role of the IncRNA IFNA-AS1 in MG. They found that the IFNA-AS1 is abnormally expressed in MG patients and is associated with positive AChR antibody levels (308).

Chuang et al looked at immunological correlations between LOMG and thymoma associated MG (TAMG) based on the fact that both have AChR antibodies, Titin antibodies, antibodies to

ryanodine receptor, type I interferons or IL-12. They looked for association with CTLA4$_{hi/gain\ of\ function}$+49A/A genotype which is seen in TAMG In a total of 152 historically collected patient samples. In contrast with TAMG, they found that there was CTLA4$_{low}$+49G+ genotype more frequently in LOMG patients with age of onset ≥ 60 years compared to healthy controls. They found that thymic export of naïve T cells from non-neoplastic thymus in LOMG patients was lower at diagnosis. They suggested that there were distinct initiating mechanisms in TAMG and LOMG and that the abberant immune regulation was in the periphery in LOMG (309).

Zhang et al looked at CD19+Tim-1+ B cells in 34 patients with MG recruited retrospectively. They included both treatment naïve and immunosuppressed patients. T-cell immunoglobulin mucin domain one (Tim-1) is thought to be essential for optimal regulatory B cell function and maintaining immune tolerance. Their study showed that mRNA and protein expression of B cell Tim-1 in both GMG and OMG were significantly lower than those in healthy controls with lower expression in GMG compared to OMG. This negatively correlated with clinical severity, plasma cell frequency, serum TH 17 related cytokines and anti-ACHR antibody levels. This indicated that aberrant expression of Tim-1 exists on B cells and this may contribute to the TH 17 polarisation and antibody secreting plasma cell differentiation in MG patients (310).

Zhang et al looked at circulating CD4+CXCR5+PD-1+ Tfh cells in OMG in GMG without thymic abnormalities. They showed that circulating Tfh cells are significantly enriched in GMG patients but not in all MG patients compared with healthy controls, and the proportion of follicular T reg cells was decreased. The frequency of plasma cells and B cells was higher and serum levels of IL-6 and IL-21 were elevated in MG patients. The results suggested that circulating Tfh cells may act in autoreactive B cells and contribute to the development of MG in patients without thymic abnormalities.

Alahgholi-Hajibehzad et al retrospectively looked at the effect of IL-21 and CD4+CD25++T cells on cytokine production of CD4 + responder T cells in 20 patients with AChR+ GMG. They found that IL-21 increased the proliferation of the responder T cells (T*resp* cells) in T*resp*/T reg cocultures. T*resp* cells in MG patients secreted significantly lower levels of IL-2. In these patients IL-2 levels did not change with the addition of T regs to the cultures whereas it decreased significantly in controls. In T*resp*/Treg cocultures, IL-4, IL-6 and IL-10 production was increased in the presence of T regs in patients. IFN-γ was decreased and IL-17 was increased in both patient and control groups. This demonstrates that IL-21 enhances the proliferation of the T*resp* cells in the presence of T regs (311).

Jeong et al have found that serum IL-27 levels were significantly higher in 32 retrospectively recruited, treatment naïve MG patients compared to controls and it was significantly higher in EOMG (312).

Uzawa et al looked at high mobility group box 1 (HMGB1) which is an inflammatory mediator, in 70 retrospectively recuited MG pts who were positive to either AChR (60) or MuSK (10) Abs. They found that serum HMGB1 levels in patients with AChR antibody + MG were higher than in the healthy controls. This decreased after treatment with immunosuppressants. Anti-MuSK antibody + patients also showed higher serum HMGB1 levels than controls but not significantly. GMG patients showed higher levels than OMG patients and controls. Patients with thymoma showed higher levels than those without thymoma and controls (313).

Xie et al found that IL-17A was higher amongst MG patients than in healthy controls. EOMG women without thymoma showed greater elevations of IL-17 A. The absence of thymoma was thought to be the more significant determinant and levels were associated with more severe MG (314). This was a retrospective study on 69 treatment naïve MG patients which suggests that the patients may have milder MG and does not include the wide spectrum of presentations.

Yilmaz et al retrospectively looked at plasma levels of cytokines related to TH cell subtypes in 46 AChR MG, 23 MuSK MG and 42 healthy controls. Plasma levels of TH1, TH 2 and TH 17 related cytokines were overall not significantly different between the subtypes and controls. However in vitro stimulated PBMCs in MuSK patients but not in AChR showed a significantly increased secretion of TH 1, TH 17 and Tfh cells and related cytokines which are IFN γ, lL-17a and IL-21. They postulated that TH1 and TH17 immune reactions play a role in MuSK MG and immunosuppression attenuates the TH1 response in AChR MG and in MuSK MG, but it modulates immune responses differently in the two groups (315).

Uzawa et al retrospectively looked at serum levels of 24 inflammatory cytokines in 43 AChR+ MG and 25 healthy controls. They found that in MG serum levels of a proliferation inducing- ligand (APRIL), IL-19, IL-20, IL-28A and IL-35 were significantly increased as compared to controls. IL-20, IL-28A and IL-35 were significantly decreased after treatment (10 patients). APRIL and IL- 20 was increased in LOMG and IL- 28A was increased in patients with thymoma associated MG (316).

Cufi et al have shown that IFN β could play a central role in thymic events and lead to MG by triggering the overexpression of αAChR which probably leads to thymic dendritic cell

autosensitization, the abnormal recruitment of peripheral cells and germinal centre formation (66).

Dragin et al showed that oestrogens inhibited the expression of of AChR and HLA-DR in TECs which suggest that oestrogens may alter the tolerization process and favours an environment for autoimmune response (49).

Molin et al published a paper with the profiles of upregulated inflammatory proteins in MG patient sera. They looked at sera in 45 MG patients and investigated 92 proteins associated with inflammation. They found that 11 of the analysed proteins were significantly elevated compared to healthy controls of which the three most significant were: matrix metalloproteinase 10 (MMP-10), transforming growth factor alpha (TGF α) and extracellular newly identified receptor for advanced glycation end products binding protein (EN-RAGE) (also known as protein S100-A12). Levels of MMP-10, C-X-C motif ligand one (CXCL1) and brain derived neurotrophic factor (BDNF) differed between EOMG and LOMG (317).

1.5.12 miRNAs in MG

MicroRNAs (miRNAs) are small non-coding RNA molecules that bind to specific mRNA targets and regulate a wide range of important biological processes within cells. Circulating miRNAs

are released into the extracellular space and can be measured in most bio fluids including blood serum and plasma. As circulating miRNAs are easily accessible, they can be used as markers for different human disorders including autoimmune diseases (Punga et al) (318).

Punga et al published a paper on 34 MG patients who were treatment naïve and 37 patients who were immunosuppressed. They measured serum levels of miR-150-5p and miR-21-5p; these were higher in the MG treatment naive patients compared to healthy controls. MiR-150-5p levels were 41% lower and miR-21-5p levels were 25% lower in MG patients on immunosuppression compared to treatment naive patients. In autoimmune disease patients mean miR-150-5p and miR-21-5p were comparable with healthy controls. The suggestion was that the miRNAs have a disease-specific signature and could be used as biological markers of MG (319).

In another study by Punga et al, they looked at miRNAs in MuSK+ MG patients. They found elevated levels of let-7a-5p, let-7f-5p, miR-151a-3p and miR-423-5p. This profile differed from the previously observed AChR positive MG patients (320).

Zhang et al found that there was significant downregulation of miR-181c in PBMCs from MG patients compared with healthy controls. There was low expression in GMG patients than in OMG patients. MG patients also had increased serum IL-7 and

IL-17 levels. Serum IL-7 had a positive correlation with serum IL-17. MiR-181c levels negatively correlated with serum IL-7 and IL-17 in GMG and OMG patients. They suggested that miR-181c was a negative regulator of the pro-inflammatory cytokines IL-7 and IL-17 in MG patients (321).

Xin et al examined the role of miR-20b in the development of TAMG. They found that miR-20b acts as a tumour suppressor in the development of thymoma and TAMG. This could be due to inhibition of NAFT signalling my depression of NAFT5 and CMTA1 expression (322).

1.5.13 Treatment of MG

Although Dr Walker was thought to be the first to describe the use of Physostigmine, this was in fact first described by Dr Lazar Remen, a Polish doctor who described it's use in Myasthenia gravis in a paper published in 1932. He was studying the effects of Glycine at the time and the positive results of physostigmine on myasthenia were not given much importance (5). Myasthenia specific treatment was tried by Mary Broadfoot Walker in 1935 when she tried physostigmine and later prostigmine due to the similarity of myasthenia with curare poisoning. Since then treatment of myasthenia has improved dramatically with steroids being the mainstay of treatment even now. The myasthenia

gravis Association of British neurologists' management guidelines was published in 2015 and outlines the stepwise management of myasthenia gravis (323). This suggests starting treatment with pyridostigmine at a dose of 30mg QDS, and gradually increasing to 60mg QDS. The next step is introduction of prednisolone gradually, upto a maximum dose of 50mg on alternate days (AD) or equivalent once daily (OD) dose for OMG, and 100mg AD or equivalent OD dose for GMG. This is given for 2-3mths until symptom resolution and then gradually withdrawn. If unresponsive to prednisolone, or a relapse occurs on dose reduction of prednisolone, then an alternate immunosuppressant is introduced, Azathioprine being the most commonly used drug. Other immunosuppressants recommended are mycophenolate, methotrexate, cyclosporine and rituximab.The EFNS/ENS guidelines for the treatment of ocular myasthenia published in 2014 is also very similar to the ABN guidelines (324).

There have been two controlled trials looking into the efficacy of **corticosteroids** in GMG; one done by Howard et al looked at prednisolone 100 mg on alternate days versus placebo (325). Lindbergh et al looked at 20 patients with GMG who were given 2g IV methylprednisolone versus placebo. A significant improvement in the steroid treated group was seen, however, the trial was limited because the patients had all had thymectomy previously and some of them had received

corticosteroids previously (326). Several large-size retrospective analyses using oral prednisolone have all demonstrated good efficacy, the doses varying from 10 to 100 mg per day. The muscle study group trial showed that 75% of patients with mild to moderate MG responded well to 20 mg of prednisolone.

Pradas et al analysed several different variables to see which would predict response to corticosteroid therapy. These variables included age of onset, sex, disease duration and severity. Only age of onset was predictive of treatment response and showed that older patients responded better than younger patients. MuSK MG patients may have a lower response to steroids (327) .

Within the first 2 to 3 weeks after starting steroids, a small portion of patients may have a deterioration or exacerbation of their MG symptoms which is called a steroid dip (Gotterer et al) (90). The incidence ranges from between 21 to 44% with approximately 7 to 11% of patients having a severe exacerbation of symptoms. Steroid induced exacerbation is not predictive of poor long-term response to steroids. Older patients and those with bulbar symptoms and severe disease are more likely to develop exacerbation.

Bae et al studied Fifty-five consecutive patients with MG who were administered high doses of prednisolone (40–80 mg) for the first time in a tertiary medical centre in Seoul (328).

Prednisolone-induced exacerbation was defined as a significant reduction in a patient's Myasthenia Gravis Severity Scale (MSS) score within 4 weeks of prednisolone administration. Twenty-three patients (42%) experienced definite exacerbation after prednisolone therapy. Older age, predominantly severe bulbar symptoms, and low MSS score were found to be significant clinical predictors of exacerbation. A high daily dosage of prednisolone was found to be neither a predictor of exacerbation nor a predictor of early improvement.

The hypotheses for the mechanisms of 'steroid dip' are: (i) action of antibodies released from degrading lymphocytes, (ii) increased activity of cholinesterase at the NMJ, and (iii) an overall increase in immune reactions (329-331).

Some studies have found that a stepwise increase in the dose of Prednisolone is better than using a large initial dose in preventing steroid induced worsening (332-334). Other studies however found that using a large initial dose of prednisolone produces rapid improvement in MG symptoms (335, 336).

Certain centres prefer to use an alternate daily regime of prednisolone; the rationale being, limitation of dose-related side-effects, and possibly encouragement of indigenous steroid production on the 'off' days. There is limited data to suggest that daily prednisolone is more effective than alternate day dosing,

and patients treated with alternate day therapy often experience subjective and objective worsening during their ‘off’ day (337).

Pulsed IV MEP therapy at regular intervals has been tried in some series and has shown short rapid improvement with less exacerbation and fewer side effects. Oral dexamethasone 10 to 20 mg per day has also been shown to be beneficial in a small series (337, 338).

Myasthenic crisis during the tapering of prednisolone is uncommon; in one study 18% of patients had one or more significant exacerbation when the steroid dose was reduced, but no one had myasthenic crisis. Approximately 5 to 20% of patients who were on steroid monotherapy were able to discontinue the steroids successfully (339, 340). In one study three quarters of patients were thymectomized and 50% of these patients were able to discontinue steroids. Patients with MuSK antibody positive MG may be more refractory to weaning off the corticosteroids and they may require two or more forms of immunotherapy.

Approximately 5 to 20% of MG patients do not respond to corticosteroids. Side effects of corticosteroid treatment include weight gain, cushingoid features, easy bruising, cataracts, glaucoma, diabetes mellitus, hypertension, hypercholesterolaemia, osteoporosis, and rarely, avascular necrosis of the femoral head.

The other immunosuppressants which are usually introduced if myasthenic symptoms recur on prednisolone withdrawal include azathioprine, mycophenolate, methotrexate, cyclosporine and tacrolimus.

Azathioprine is a purine analogue which interferes with DNA synthesis. It interrupts the proliferation of B and T lymphocytes. Azathioprine has been used as a monotherapy for myasthenia since 1964, even before prednisolone was commonly used. When used as monotherapy, improvement is seen after 4 to 6 months. Two RCTs were done looking into azathioprine; the first showed no difference between azathioprine alone and azathioprine plus prednisolone (341) whilst the other study showed that 63% of the patients treated with combination treatment were able to discontinue prednisolone eventually (342). The maximum therapeutic benefit was seen after two years. Gradual azathioprine withdrawal can be considered in patients whose MG symptoms have settled. A minimum duration of treatment of three years is desirable, although there is not enough evidence. The starting dose of azathioprine is usually 50 mg per day and this is increased every two weeks up to a maximum dose of 2 to 3 mg per Kg per day.

Mycophenolate mofetil (MMF) is a reversible inhibitor of Ionosine monophosphate dehydrogenase. MMF blocks the de novo pathway of purine synthesis and inhibits proliferation of T

and B lymphocytes. It has been used for treatment of myasthenia gravis since 1998. Two RCTs show that MMF was ineffective and a third RCT showed mild improvement (343-345); however, widespread clinical experience supports the use of MMF for MG treatment. In a study of 85 patients, MMF led to improvement in symptoms in 73% and remission in 50%. Corticosteroid dose was reduced in 71% and discontinued in 13%. The starting dose of MMF is 500 mg to 1 g twice a day and the maintenance dose is usually 1 g to 1.5 g twice a day.

Hobson-Webb and team published data on mycophenolate mofetil withdrawal. They found that when patients relapsed during withdrawal, it was because the mycophenolate was tapered quickly. They suggested that tapering MMF appears to be safe after years of disease stability and the dose reduction needs to be done at a dose of only 500 mg per day every 12 months (346).

Cyclosporine inhibits the function of calcineurin and blocks the synthesis of interleukin-2 and interferon by helper T cells. There are several uncontrolled trials which show improvement in MG after 12 to 30 months of treatment. The maintenance dose is usually 5 mg per Kg per day. Renal toxicity and potential interaction with other medications makes cyclosporine a less preferred treatment choice (347-350).

Tacrolimus also inhibits calcineurin but it has the advantage of being less nephrotoxic than cyclosporine. In a study by Ponseti et al with 212 MG patients, they showed remission of symptoms in 87.5% of patients and steroid discontinuation in 95.1%. As tacrolimus also acts on the ryanodine receptor related sarcoplasmic calcium release, it may provide extra therapeutic benefit in patients with thymoma. Dosage of tacrolimus varies from 0.05 to 0.1 mg per Kg per day (351).

Methotrexate is a folate analogue that inhibits purine and pyrimidine synthesis and leads to decreased T-cell proliferation. This was first used in MG in 1969. In one RCT, 24 MG patients on steroids were randomised to azathioprine or methotrexate; after two months, the average dose of steroids was reduced by more than 50% in both groups without difference. The standard dose of methotrexate is 15 to 25 mg weekly (352).

Rituximab is a genetically engineered chimeric mouse/human IgG1: kappa monoclonal immunoglobulin containing murine light and heavy chain variable region sequences and human constant region sequences. Rituximab acts against the cell membrane marker CD20 and leads to B lymphocyte depletion. There are a growing number of case series which support its use in severe GMG which is refractory to multiple immunosuppressive agents. Rituximab is particularly useful for patients with MuSK MG. The

standard rituximab dose is 375 mg/m² per week for four weeks or 1 g every two weeks for two doses.

Rituximab is used for the treatment of refractory myasthenia gravis. Illa et al reported three patients with AChR positive MG and three with MuSK positive MG; all patients improved dramatically, antibody titres declined in all the patients, but the decline was significantly better in the MuSK positive MG patients (353).

Tandan et al reviewed the efficacy and safety of rituximab in 165 MG patients from case reports and series. 59% of these patients were AChR positive, and 34% were MuSK positive. After treatment with rituximab, patients achieved a MGFA PIS score (Appendix 1) of MM or better in 44% and a combination of PR and CSR in 27%. MM or better was achieved in 72% of MuSK MG, and in 30% of AChR MG. Relapses also reduced in MuSK MG. The predictors of response to rituximab were MuSK MG, less severe disease and younger age at treatment. There was a post treatment reduction in antibody titres- 82% in MuSK MG and 26% in AChR MG. Depleted CD20+ B cells were observed up to 16 weeks after four weekly infusions (354).

Jing et al have suggested that low-dose rituximab at a dose of 600 mg may be sufficient in depleting B cells and maintaining low counts with improvement of clinical symptoms in six months. Afanasiev et al also looked at 28 patients who received

rituximab treatment for MG. Based on their PIS score, they suggested that rituximab may be efficient in 50% of patients with MG resistant to immunosuppressants. They had one patient who developed progressive multifocal leukoencephalopathy (355).

Cyclophosphamide is an alkylating agent that interferes with DNA replication. It reduces the production of lymphocytes, monocytes and macrophages. An RCT by De Feo and colleagues with 23 patients with refractory MG compared IV cyclophosphamide versus placebo. There was an improvement in the extraocular, masticatory and bulbar muscles after 12 months and there was also a reduction in the average prednisolone dosages at six months. In another study with 22 refractory MG patients, half the patients achieved remission after an average treatment duration of 3.6 months (356).

IV immunoglobulins are used routinely in acute exacerbations of myasthenia gravis. Previous studies have shown an improvement in 70 to 80% of MG patients. It can also be used as maintenance therapy in refractory MG. Compared to ACHR MG, IVIG is less effective in MuSK MG; in some studies only 20 to 61% of MuSK MG patients improved with IVIG.

Takizawa et al looked at patients receiving IV immunoglobulins and they assessed the patients clinically and included QMG scores. They judged that IVIG was ineffective in 18% of MG

patients. Significant improvement in QMG was seen after one month for AChR MG, there was no significant change seen in seronegative patients within three months, for anti-MuSK MG the improvement of QMG was significant after two months. They suggested that a judgement regarding whether IVIG is ineffective for MG patients should be considered at least three months after IVIG administration (357).

Subcutaneous immunoglobulins have also been shown to be effective instead of IV immunoglobulins. The mechanism of action of IV immunoglobulins includes it's affect on: (i) antibodies, which are reduced (ii) compliment, IVIG inhibits complement consumption and intercepts MAC formation (iii) genes, IVIG causes an alteration of the tissue genes associated with inflammation, fibrosis tissue remodelling and regeneration and (iv) degenerative pro-inflammatory molecules and beta-amyloid (358).

IV immunoglobulin was first used in the 1950s as replacement therapy in immune deficiencies, but is now widely used for the treatment of autoimmune and inflammatory diseases.

Plasma exchange may also be used as maintenance therapy in MG. In a study by Triantafyllou et al, 11 patients with refractory MG were treated with Plex on a cyclical basis; all patients improved after the first week and stabilised after three months.

Plex is effective in MuSK MG and leads to rapid improvement in 53 to 93% of patients (359).

Studies comparing IV immunoglobulin with double filtration plasmapheresis (DFPP) and immunoadsorption (IA) showed that DFPP and IA showed better short-term clinical effectiveness than IVIg (Liu et al) (360). A small study by Alipour-Faz et al of 24 patients comparing IVIG with plasma exchange for patients requiring thymectomy showed that in the plasma exchange group, post-operative outcomes, which included duration of hospitalisation, ICU length of stay after surgery, intubation period and duration of surgery, were longer than in the IVIG group. They suggested that the Administration of IVIG may be more effective in preparation before thymectomy in MG patients (361).

Similarly, another study by Schneider Gold et al comparing immunoadsorption versus plasma exchange showed that semiselective IA in combination with Plex, and to a lesser extent IA alone, was associated with a shorter hospital stay and more pronounced reduction of the MG score than Plex. A study by Yamada et al looked at 153 MG patients who had plasma exchange. 12 of these were positive for anti-MuSK antibodies. They suggested that maintenance Plex may be an effective option for MuSK MG patients (362).

Thymectomy:

An international multicentre randomised study comparing thymectomy to no thymectomy in 126 Non-thymomatous myasthenia gravis patients receiving prednisolone (MGTX) was completed in 2016. The MGTX trial showed that QMG scores were significantly lower, with an estimated difference in mean scores of 2.85 in the thymectomy group. The alternate day prednisolone dose was 44 mg in the thymectomy group compared to 60 mg in the prednisolone only group which was also statistically significant. MM status was reached in 67% in the thymectomy group compared to 37% in the prednisolone only group. The study showed that routine thymectomy in patients with MG was helpful even without thymoma (363).

Extended trans-sternal thymectomy for ocular MG was reviewed in a study by Liu et al. They reviewed cases of 115 patients with MG. Of these, 92.2% had thymic hyperplasia. The results indicated that ETT was safe and effective in OMG, particularly in patients with a shorter duration of illness (364).

Kawaguchi et al studied the effects of thymectomy on late-onset MG without thymoma. They followed up 34 MG patients over two years. Of these, 20 patients underwent thymectomy; these patients had more severe disability at entry than the non-thymectomized patients but compared to the non-

thymectomized patients they showed a greater percentage of clinical remission (365).

As described in section 1.5.9, there is a ‘gray zone’ in clinical, immunological and pathological terms between the ages of 40 and 60 years when patients may suffer either from ‘late EOMG’ or ‘early LOMG’. Some LOMG thymuses have shown lymphoid follicles like in EOMG even in patients over 60 years of age. The lymphoid epithelial tissue of the ageing thymus is replaced with fat, but residual parenchyma may continue exporting some T cells. This could explain why thymectomy appears to be effective in LOMG patients.

Other treatments:

The REGAIN study which looked at the safety and efficacy of **Eculizumab** in ACHR antibody-positive refractory GMG published their results in 2017. The phase 2 study suggested that Eculizumab produced clinically meaningful improvements in these patients. In the phase 3 study, the primary analysis showed no significant difference between Eculizumab and placebo. Eculizumab is a humanised monoclonal antibody that specifically binds with high affinity to the human terminal compliment protein C5, inhibits enzymatic cleavage to the protein C5a and C5b which in turn prevents the C5b- induced chemotaxis of pro-inflammatory cells and formation of the C5b induced membrane attack complex (366).

Bortezomib is a proteasome inhibitor and is known to be effective in the elimination of malignant plasma cells in multiple myeloma and it causes a depletion of short lived and long-lived B cells. Schneider Gold et al published a case report on a patient with severe refractory MuSK antibody positive MG who was treated with Bortezomib. The patient achieved a significant and rapid improvement of the severe myasthenia symptoms (367).

Tocilizumab is a blocker of interleukin-6 signalling and has been trialled in myasthenia gravis. Jonsson et al published a case report of two patients with ACHR antibody myasthenia gravis who responded insufficiently to rituximab. The patients responded well to Tocilizumab (368).

There have been case reports where a patient with coexisting MuSK antibody positive myasthenia gravis and polycythaemia Vera was treated with **Ticlopidine and Ruxolitinib** which led to an improvement of myasthenic symptoms. Ruxolitinib is a selective inhibitor of JAK1 and JAK3.

Acetyl cholinesterase inhibitor is used for temporary symptomatic treatment of muscle weakness. There are no large randomised or quasi randomised trials for the drug in GMG. There was one crossover randomised trial using intranasal neostigmine which included 10 patients, three with OMG and

seven with GMG. Symptoms improved in nine out of the 10 participants after a two-week neostigmine treatment.

Ephedrine was trialled in four patients with AChR MG (Lipka et al). Ephedrine as an add-on treatment for MG showed a small but consistent reduction of symptoms and weakness in patients with moderate disease severity but did show a prolonged corrected QT interval (369).

Leflunomide treatment was trialled in corticosteroid dependent MG. Leflunomide is an immunosuppressant that blocks pyrimidine nucleotidase biosynthesis. 15 patients who had all undergone thymectomy were recruited. After six months, there was a significant improvement in QMG by three points or more and an improvement in ADL score in 10 participants. In 12 patients the dose of prednisolone was reduced from an average of 24.3mg to 12.3 mg per day. This was a small but promising pilot study which showed that leflunomide may be a safe steroid sparing immunosuppressant. A longer duration of follow-up and a further placebo-controlled study would be helpful (370).

Several other therapeutic options are being trialled and have been suggested. **Linarin** is a flavone glycoside in plants and is shown to have a potent AChE inhibitory activity. It may be a promising therapeutic agent in conditions such as myasthenia gravis, glaucoma, gastric motility and Alzheimer's disease (371).

Autologous haematopoietic stem cell transplantation has also been shown to be effective in isolated case reports. **Fingolimod and Siponimod** have been trialled in MG, and although there were subtle changes in T-cell responses, they had no significant effect on antibody titres or disease severity (372). There have been case reports of severe refractory MG being treated with high-dose vitamin D treatment (373). Rapamycin has been shown to reduce Th17 cells and increase the proportion of the Tregs in MG patients in Experimental autoimmune myasthenia gravis (EAMG) (374). In rat models, preconditioned human mesenchymal stem cells have been shown to improve MG symptoms (375). The selective Immunoprotease inhibitor ONX-0914 has been shown to ameliorate EAMG (376). Immature exosomes derived from micro-RNA-146a can work as antigen-specific therapy in MG and it is shown to be of benefit in EAMG (377). Hinge-deleted IgG4 blocking therapy has been shown to be successful in AChR receptor MG in rhesus monkeys (378). Delivery of miRNA 155 inhibitor by anti-CD20 single chain antibody into B cells reduced AChR receptor antibodies and ameliorated EAMG (Wang et al) (379).

Novel biological agents which are relevant and can be tested in MG include those that work on: (i) T cell intracellular signalling molecules such as anti-CD52, anti-IL-2 receptors, anti-costimulating molecules and JAK1 and JAK3 (ii) B cells and the

trophic factors directed against key B cell molecules (iii) complement C3 or C5 (iv) cytokines and cytokine receptors such as IL-6 or P 40 subunit of IL-12/IL23 and (v) T and B cell transmigration molecules (Dalakas) (380).

Secondary MG:

With the advent of immunotherapies, there have been significant advances in cancer treatments. One such treatment is the use of anti-programmed cell death 1 (anti PD-1) antibodies which is used for metastatic melanoma and other cancer entities. They act via blockade of PD-1 receptors and inhibit the T-cell effector mechanism that limits immune responses against tumours. Pembrolizumab and Nivolumab are the anti-PD 1 antibodies commonly used. Zimmer et al looked at the immune related adverse events associated with PD-1 antibodies; these were seen in 138 of 496 patients. In 77 of the 138 patients this affected the nervous system. The neurological side effects of anti-PD-1 therapy include polyneuropathy, seizures, cranial nerve palsies, GBS, meningo-radiculitis and myasthenia gravis (381).

The other immune checkpoint inhibitor commonly used is Iplimumab which is a humanised monoclonal antibody directed against the immune checkpoint cytotoxic T-lymphocyte antigen-4 (CTLA-4). In Makarious et al's paper of 2017 they reported that among the 23 reported cases of immune checkpoint

inhibitor associated MG, 2.7% were de novo presentations, 18.2% were exacerbations of pre-existing MG, and 9.1% exacerbations of subclinical MG. The average onset of symptoms was within six weeks of treatment initiation. There was no association with elevated ACHR antibody titres. There was a 30.4% MG specific related mortality (382).

There are case reports of myasthenia gravis developing with chronic graft-versus-host reaction in a patient with acute myeloid leukaemia who underwent allogenic haematopoietic stem cell transplant. There are other similar case reports in literature (Tsutsumi et al) (383).

Treatments for myasthenia gravis can also be associated with complications; steroids are commonly associated with a lot of side effects as described above. There has been a case report of pyridostigmine causing leucocytoclastic vasculitis as a hypersensitivity reaction. Petramala et al reported a case of a woman who had an adrenal mass and hypertension. She also had subclinical hypercortisolism. An adrenalectomy was done; five months later she presented with symptoms and signs of myasthenia gravis. It appears that the patient with the adrenocortical adenoma was self- treating with cortisol excess (384).

1.5.14 Outcomes in MG

Kupersmith retrospectively looked at the long-term follow-up database of 147 patients with EOMG who generalise to GMG, and suggested that prednisolone delayed the onset of GMG and had sustained benefit in reducing the incidence of GMG and controlling diplopia. Without steroids, GMG developed in 50% of all MG patients, usually within one year (385).

Heckman and team published a unique sub-phenotype of myasthenia gravis which they called ophthalmoplegic myasthenia gravis which most commonly affects acetylcholine receptor antibody positive patients with juvenile onset myasthenia gravis and African genetic ancestry. A few cases were found with MuSK antibodies and also in triple seronegative myasthenia (386).

Although myasthenia patients respond well to treatment including immunotherapy, a small proportion of patients become refractory. Refractory patients are defined as those who cannot lower their immunotherapy without clinical relapse and are clinically controlled on their immunotherapy regimen, or have severe side effects from immunosuppressive therapy. Suh et al looked at a retrospective cohort of 122 myasthenia patients who were referred to the tertiary neuromuscular clinic. The patients were classified as refractory or non-refractory based on predefined criteria and the clinical features were compared.

14.8% of these patients were classed as refractory.The refractory patients were more likely to be younger at onset, female, Thymomatous, and anti-MuSK antibody positive (387).

Myasthenia gravis may become life-threatening if patients have respiratory insufficiency or dysphagia, called life-threatening events (LTE's). Ramos-Fransi et al did a retrospective analysis of 648 patients who presented with MG. Of these, 62 patients, ie 9.56% had an LTE. 32 classified as class V according to the MGFA classification and 30 as class IVB. The median duration of disease before the LTE was 24 months. The most common related factor was infection. All patients received IV immunoglobulins, some requiring a second infusion and a further few patients receiving plasma exchange. Median time to weaning from ventilation was 12 days and was significantly shorter in late-onset myasthenia group. LTE improved in less than two weeks in 55.8%, in 20% of patients this took more than one month. Four patients died (388).

Hong et al looked at the prognosis of ocular myasthenia gravis in Korea as they conducted a retrospective analysis of 376 patients with a diagnosis of myasthenia gravis. Patients were classed as ocular myasthenia gravis at the time of symptom presentation. They looked at secondary generalisation which developed in 23.3%, mostly within the first six months, while the disease remained ocular throughout the follow-up duration

(which was a median of 11.8 months) in the remaining patients. AChR antibody positivity and abnormal repetitive nerve stimulation and thymoma were frequently observed in patients in the generalised group. The study also showed that oral prednisolone treatment significantly reduced the risk of secondary generalisation whereas abnormal AChR antibodies and thymoma were predictive of development of secondary generalisation. It is to be noted however that the follow-up duration for the study was quite short and the definition of ocular myasthenia gravis was at symptom onset rather than three months or two years (389).

Akaishi et al did a two-step cluster analysis of 923 consecutive MG patients. They looked at the period from the start of treatment until achievement of MM status. They found that patients who had ocular MG showed the best early-stage response to treatment and stability; this was followed by thymoma associated MG and AChR antibody positive MG without thymic abnormalities. They found that AChR antibody - negative MG showed the worst early-stage response to treatment (390).

The outcome of myasthenia gravis treatment can be measured using the MGFA post intervention status (PIS). This classifies patients as being in complete stable remission (CSR), pharmacological remission (PR), having minimum

manifestations (MM), having improved (I), remaining unchanged (U), becoming worse (W), have an exacerbation (E) or having died from MG (D) (391) (Appendix1).

Andersen et al gathered information from 268 myasthenia patients; of these, 64% had attained optimal outcome at two years of follow-up, 73% at five years and 75% after 10 years. This was more likely to be achieved in patients with late-onset in those who had thymectomy and those who had ocular-only disease. They concluded that prognosis was favourable for the majority of MG patients regardless of age, maximum disease severity or antibody status (392).

A Danish study by Hansen et al looked at all AChR antibody seropositive myasthenia patients between 1985 and 2005 and were followed up until 2009. Mortality rates (MR) and estimated mortality rate ratios (MRRs) were calculated. Of the 702 myasthenia patients, 302 died during follow-up. The overall mortality was higher for patients with myasthenia gravis compared to the control group with a mortality rate ratio of 1.41. In late-onset women and men, the MRRs were 1.64 and 1.02 respectively. The total MRR was highest during the first five years after diagnosis (393).

A Thai study from 2017 showed that pneumonia, being on a mechanical ventilator, and septicaemia were independent

factors associated with poor treatment outcomes in the elderly hospitalised myasthenia patients (394).

Citirak et al have shown that in patients with generalised myasthenia gravis, there is significant muscle weakness, and this is more pronounced in men than women. Shoulder abductors, hip flexors and neck muscles are the most affected muscle groups. The duration of disease or treatment intensity alone did not predict loss of muscle strength in GMG (395).

Myasthenic crisis can develop in 15 to 20% of myasthenia gravis patients and usually this happens in the first year of illness. This may be the first presentation in about 20% of patients. Women are twice as likely as men to be affected by crisis and the average age is around 59 years (Godoy et al). Myasthenic crisis is characterised by severe weakness of the bulbar muscles and/or respiratory muscles which causes inability to maintain adequate ventilation or permeability of upper airways causing respiratory failure and which requires artificial airway or ventilatory support. The usual predicting factors are respiratory infection in 40%, emotional stresses and micro aspirations in 10%, changes in medication regimen in 8%, and surgery or trauma (396).

There are many drugs that can exacerbate myasthenia gravis. This includes antipsychotics such as the phenothiazines, sulpiride, atypical antipsychotics such as clozapine;

neuromuscular blocking drugs such as succinylcholine and vecuronium; anticholinergic drugs; cardiovascular medications including lidocaine, procainamide, propranolol, quinidine, verapamil and statins; neurologic and psychoactive medications including chlorpromazine, lithium, phenytoin, carbamazepine, trihexyphenidyl and trimethadone; antibiotics including all aminoglycosides, ciprofloxacin, macrolides, erythromycin, clarithromycin, polymyxins and tetracycline; haematological and immunosuppressive medications such as chloroquine, penicillamine, occasionally prednisolone and interferons and other medications which include iodinated contrast agents, Magnesium, acetazolamide, methocarbamol, interferon-alpha, etc.

A retrospective Taiwanese study of 2016 identified 29 patients with myasthenia gravis with 49 admissions to hospital. Of these, 16 patients were admitted with myasthenia gravis and 13 with myasthenic crisis. There were several readmissions reported amongst the 15 patients with myasthenic crisis; 14 were admitted to the intensive care unit, 8 were intubated and put on mechanical ventilators; the median ICU stay was seven days and one patient died during hospitalisation and another during a further hospitalisation within two years. Most of the patients responded well to treatment, plasma exchange being the most common one used. Infection was the most common trigger of myasthenic crisis and was a significant cause of death. They

showed that despite significant morbidity and mortality in patients with myasthenic crisis, a favourable long-term outcome is possible with intensive treatment (397).

Records of Chinese GMG patients from 1997 to 2012 were reviewed and the findings published in 2015 by Lee et al. The median follow-up duration was 114 months and the patients had received a variety of immunosuppressive therapy including corticosteroids, azathioprine, mycophenolate, cyclosporin and thymectomy. Of these, 35 patients i.e. 28.5% had myasthenic crisis, 2 patients died. 78% patients had good outcome defined by MGFA PIS of ‘complete stable remission’, ‘pharmacological remission’ or ‘minimal manifestation’. 19.5% had intermediate outcome defined by MGFA PIS of ‘improved’, 2.4% had an MGFA PIS of ‘unchanged’, ‘worse’, ‘exacerbation’ or ‘died’. Azathioprine therapy was the only independent predictor of good outcome (398).

1.5.15 Pregnancy and MG

Myasthenia gravis is not associated with infertility but it can expose pregnant women to an increased risk of maternal and foetal complications. Approximately 30% of pregnant women have been reported to have experienced an improvement in their symptoms, and a third experienced worsening, especially

in the first trimester and during the postpartum period. An increased rate of exacerbation during pregnancy in the first 2 to 3 years after diagnosis has also been reported. There is no indication for a caesarean delivery as smooth muscle is not affected, however, during the third stage of labour, there is contraction of the voluntary striated muscles, and hence assistance during vaginal delivery may be required. Management of pre-eclampsia is particularly challenging because of the use of magnesium sulphate which can exacerbate myasthenia. When eclampsia is present, alternative medications such as phenytoin, phenobarbital, amobarbital or benzodiazepines should be used to control seizures. Neonatal myasthenia gravis affects 12 to 20% of newborn babies of affected mothers. This usually presents with a weak cry and swallowing and suckling difficulties and usually is apparent during the first hours of life. This usually improves within two months. Atypical forms of arthrogryposis multiplex congenita are reported to occur in 29% of patients with neonatal myasthenia gravis.

A Portuguese study of 25 pregnant patients with myasthenia gravis was reported by Costa Braga et al. Mean maternal age was 32.4 years, miscarriage rate was 6.7%, deterioration in myasthenia symptoms during pregnancy happened in 43.3%, and 46.4% occurred postpartum. There were no maternal or neonatal deaths. Mean gestation at time of delivery was 38.2

weeks. There were no cases of foetal growth restriction, pre-eclampsia, preterm delivery, or foetal demise. The caesarean section rate was 64.3%. Two newborn babies developed transient neonatal myasthenia. A high rate of clinical worsening of myasthenia in the mother was observed in this retrospective study (399).

Boldingh et al did a cross-sectional population-based based cohort study to look at the risk of clinical onset of myasthenia gravis during pregnancy. They found that 11.5% of Dutch and 18% of the Norwegian patients had their first myasthenia symptoms during pregnancy or postpartum period. Postpartum period was thought to be significantly associated with the onset of symptoms of myasthenia and the risk was highest after the first childbirth (400).

A Brazilian study by Ducci et al found that 50% of pregnant women with MG deteriorated, mainly during the second trimester, 30% improved, and 20% remained unchanged. The course of myasthenia in the second pregnancy was different from that in the previous pregnancy in 65.3% of cases. Obstetric complications were reported in 20, the most common being preterm premature rupture of membranes, and the more severe was abortion in 11.4% and foetal death in 2.9%. Most patients had a caesarean section (in 66.7%) and transient neonatal

myasthenia gravis was recorded in 12.9% of the liveborn children (401).

1.6 Hypothesis

The epidemiological studies done to date have shown an increasing incidence rate and prevalence rate of myasthenia gravis, especially in the older population. Two peaks in incidence have been described: in the younger females between 25 and 35 years of age, and the older males between 60 and 70 years of age. The majority of the studies are retrospective, and in the few done prospectively, the studies include patients with an established diagnosis of MG, on a variety of treatments and a few new patients diagnosed within the study period. Before the advent of antibody tests, the diagnostic criteria for myasthenia gravis differed in each study, being defined by the authors. The age cut-off for LOMG and EOMG also differs between the studies. The method of data collection differs and there is a lot of heterogeneity.

The reason for the increasing incidence has been attributed partly to better diagnosis and partly to reduced mortality. However, this still does not fully explain the increasing incidence of MG. Several hypotheses have been postulated including

vaccine exposure, cohort effect, and viral infections. None of these have been proven conclusively.

Studies on ocular myasthenia gravis are also very varied, mainly because of the criteria used to define OMG. Some studies define OMG as ocular symptoms only for at least two years, and some as ocular symptoms only for 3 months. A lot of studies define OMG as ocular at diagnosis, or ocular at recruitment which is completely arbitrary. These patients have different median follow-ups with no prospective data and there are no long-term longitudinal studies. The generalisation rates of ocular myasthenia gravis also defer because of the different criteria used.

Previous studies have shown clinical and immunological differences between late-onset and early-onset MG patients. In some studies, LOMG patients are reported to have low AChR antibody titres, and are more likely to have anti-striated muscle antibodies. In younger patients, AChR antibody levels are thought to remain stable and not change with time. AChR antibody levels are higher in patients with thymoma and lower in patients without thymoma, and who are less than 40 years of age. MuSK antibodies are more common in the younger female patients in some studies, whereas other studies show that MuSK seropositivity is the same as in seronegative myasthenia gravis and in EOMG and LOMG. Patients with MuSK antibody

positivity are thought to have a more severe clinical course with ocular, bulbar and respiratory symptoms, with the majority being classified as MGFA class III or more, and a quarter of them having myasthenic crises. The treatment response is also thought to be different between AChR antibody positive patients and MuSK antibody positive patients. LRP4 antibodies are thought to be associated with milder disease, more common in younger women, with double positive patients having a more severe course of MG.

The big drawback in interpreting the above is that all the studies on antibodies in MG are retrospective, with patients on a variety of different treatment modalities and disease durations.

Thymic hyperplasia is thought to be very uncommon in LOMG patients. In patients with MG with thymic hyperplasia, studies show raised IL-6, interferon-gamma and TNF alpha. Studies on Treg levels in MG are contradictory, where some studies report low Treg levels and others report normal levels compared to healthy controls. There are several studies which show that the Tregs are defective in their function. Almost all the studies are retrospective, with a handful of prospective studies; even in these studies, patients have an established diagnosis of MG for different durations, and have been on different treatment regimes.

Treatment response and outcomes in MG are reported variably, again all in retrospective studies. The outcome in ocular LOMG patients is better than the other subgroups, although most patients do well.

From the above, we gather that, firstly, there do seem to be differences between LOMG and EOMG, and we are trying to define this more accurately. Secondly, there is a lack of a large prospective cohort study with patients being recruited at diagnosis when they are treatment naïve, and lack of long term follow up to plot the natural course of the disease and treatment response. We are trying to do this by performing a unique, large, incident cohort study from diagnosis, with full follow-up, which has never been done before. From this careful work, it is then possible to make assumptions about LOMG compared to EOMG. We aim to do this by studying the clinical aspects, including MGC and MGQoL scores, antibody aspects, and immunological aspects including Treg cells.

By doing this, the following questions were addressed:

- Is the incidence of LOMG greater than EOMG?
- Could there be a cohort effect leading to increasing incidence of LOMG? Could this be attributed to environmental exposure, Immunisation or viral infections?
- Is there a difference in the sex distribution between LOMG and EOMG?

- Is there a difference in clinical presentation between LOMG and EOMG?
- Is OMG more common in LOMG or EOMG?
- Is the rate of generalisation of ocular symptoms different in LOMG and EOMG?
- Is there a difference in thymic abnormalities between LOMG and EOMG patients?
- Is antibody positivity different between LOMG and EOMG?
- Is there any difference in clinical presentation between the different antibody subgroups?
- Is there a difference in T reg levels and cytokine levels in the peripheral blood lymphocytes of MG patients compared to healthy controls and in LOMG patients compared to EOMG patients?
- Is the treatment response different in LOMG and EOMG?
- Is the clinical outcome different in LOMG and EOMG patients?

2 Methods

2.1.1 Patient recruitment

The Study was conducted at three sites: Nottingham (Queens' Medical Centre), Birmingham (Queen Elizabeth Hospital Birmingham) and Oxford (John Radcliffe Hospital). Dr Paul Maddison, Consultant Neurologist and the chief investigator supervised recruitment in Nottingham, Dr Saiju Jacob, Consultant Neurologist and co-Investigator supervised recruitment in Birmingham and Dr David Hilton-Jones, Consultant Neurologist and Co-Investigator supervised recruitment in Oxford. Ethics approval was obtained from the NRES committee West Midlands – South Birmingham, REC number 12/WM/0414, first of February 2013.

Approximately 40 patients per year (20 at Nottingham, 10 at Oxford, 10 at Birmingham) obtain a new diagnosis of LOMG, which would be sufficient numbers to detect significant differences in regulatory T-cell function, viral antibody levels, and also in V-beta T-cell receptor repertoires (additional to Dr Tackenberg's stored peripheral blood lymphocytes (PBL) samples). Power calculations were based on previous studies by Tackenberg B et al (237) which indicate that a minimum of 75 MG patients would be required to have a 90% chance of detecting (significant at the 5% level) a significant increase in even the most exceptionally expanded (+) T cell Vbeta receptors

(>8SD above the mean) compared to controls. Even smaller sample sizes would still pick up moderate or major (+) T cell Vbeta receptor expansions in LOMG patients compared to controls. Roughly 21% of healthy controls show a CD8 TCR Vbeta expansion of >3SD. Treg cells form between 1- 20% of the CD4+ cells (depending on gating used), and as there are twice as many CD4+ cells as there are CD8+ cells, the numbers required would be much less than 75. We therefore aimed to recruit 35 patients with LOMG and 35 patients with EOMG each year for the first two years of the study. In many respects, the size of the study in terms of patients was calculated pragmatically, the aim to include every single patient with new onset MG over the 2 to 3 years (certainly in the Nottingham region, where additional researchers, i.e. Dr Maddison, could help with the recruiting). Patients would be invited to join the study, and planned to be subsequently followed for up to 5 years (initially up to 3 years as part of the fellowship, and continued follow-up by the lead clinicians for a further 2 years).

Information about the study was sent to all the Neurologists in the three centres and in the peripheral hospitals connected to the three hospitals (In Nottingham this was extended to contacting Ophthalmologists and going through laboratory data for AChR/MuSK Ab test requests). They were requested to inform us of any newly diagnosed patients with Myasthenia who fit into the inclusion criteria below and to give the patients the

information sheets. The patients were then contacted by the clinical research fellow (me) or the investigating neurologist. Patients were seen at one of the three hospitals- Queen's Medical Centre, Nottingham, Queen Elizabeth Hospital Birmingham or John Radcliffe Hospital, Oxford. The study details were explained once again and a written consent obtained.

Whilst the aim of the study was to prospectively recruit as many newly diagnosed patients with myasthenia as possible across the three sites, the recruitment rates varied. It was most successful at Nottingham where all newly diagnosed patients were recruited making it possible to study demographics further. The Birmingham cohort of patients, whilst missing some patients, was unselected with no age, sex or race bias.

2.1.2 Inclusion criteria

Patients had to be above the age of 18 years and should have been able to understand the patient information sheet and provide informed consent.

Patients should have had a new diagnosis of Myasthenia Gravis (within 12 months of recruitment). On paper this would appear to skew the recruitment towards the more mildly affected patient as they did not require treatment immediately. However, we aimed

to recruit all newly diagnosed patients including those presenting within days of symptom onset. The recruitment would run over a period of 2 to 3 years, which would balance the recruitment of the milder and the more severely affected patient.

The diagnosis of Myasthenia Gravis was based on clinical presentation, positive antibodies in serum to either AChR, MuSK or LRP4 and/or electropysiological evidence of Myasthenia on Single fibre Electromyography or repetitive nerve stimulation, or have shown good response to pyridostigmine and/or steroids.

Patients should not have been on immunosuppressive treatment. They could be on Pyridostigmine. (Caveat to this was the newly diagnosed patient admitted either in crisis or as an emergency for iv Immunoglobulins, Plasma exchange or steroids- these patients would be included, but coded separately. This was to ensure that the whole spectrum of the disease was covered.)

2.1.3 Withdrawal criteria

If the patient lost the capacity to understand the nature of the study during the study period, they would be withdrawn from the study as advised by their attending Neurologist.

2.1.4 Proforma for clinical data collection

Patients were assessed at recruitment and then at year 1 and year 2, with an option to be followed up for a further 3 years by the recruiting neurologist after the completion of the study. Detailed history was taken and a proforma filled in. The questions included in the proforma were:

- Age, sex and race
- Date of diagnosis and time since symptom onset to recruitment
- What was their first symptom
- Do they/did they have ocular symptoms? Is the ptosis unilateral or bilateral, partial or complete, variable and fatigable
- Do they/did they have bulbar symptoms of difficulty with chewing, dysarthria, dysphagia; was it variable and fatigable
- Do they/did they have drooping of the neck
- Do they/did they have difficulty with breathing; is this exertional and/or do they have orthopnoea
- Do they/did they have limb weakness; is this proximal or distal, in the upper limbs or lower limbs
- Dates of onset of all the symptoms
- Any other symptoms such as dry mouth or other autonomic symptoms; weight loss
- Past medical history with duration; in particular, past medical history of autoimmune conditions

- Medication history; in particular whether they are or have been on statins with dose and duration
- Is there a family history of autoimmune conditions
- Smoking and alcohol history
- Details of examination findings
- Quality of Life (QoL) score and MG composite score are measured
- In pure ocular myasthenia patients, Ocular MG score and VFQ 25 scores.
- Treatment history during follow up
- Steroid and other immunosuppressant doses and duration at follow up
- MG PIS scores at follow up
- Details of hospital admissions and outcomes
- Time to generalisation of ocular onset myasthenia

2.1.5 Myasthenia Gravis (MG) composite score

The MGC is made up of 10 items including tests for ocular involvement (3 items), bulbar involvement (3 items), respiratory involvement (1 item), neck involvement (1 item) and limb involvement (2 items). Each of these is scored differently with higher scores for respiratory and bulbar involvement (Appendix 1).

The MG composite score as an assessment tool in MG was first proposed by Ted Burns and colleagues in 2010 (402). The MGC was constructed using the best performing items from the quantitative myasthenia gravis scale (QMG), MG manual muscle testing (MG-MMT), and MG activities of daily living (MG-ADL). The skills were selected for each of 10 functional domains i.e. talking, breathing and upper limb strength. Item performance from these three scales was based on each item's performance during two randomised controlled trials of patients with seropositive generalised MG.

Validity testing of the MGC was conducted in 2008 to 2009 at 11 neuromuscular centres, nine in the United States and two in Europe during the routine care of adults with MG. 175 MG patients were enrolled at 11 sites and 151 patients were seen at follow-up. During the validation study which included test-retest analysis, it was determined that a three point improvement in MGC scores reliably indicated clinical improvement. A three point improvement in MGC also appeared to be meaningful to the patient. Rasch analysis of the MGC confirmed that all 10 items belonged and could be summed to provide a total score and that the weight given to the response categories of the item were appropriate.

2.1.6 Myasthenia Gravis quality of life score (MG QoL)

The MG QoL score is a health related quality of life score and provides information of the patients' perception of impairment and disability from their illness. The MG QoL consists of 15 items related to everyday life and is scored from 'not at all' - 0 points to 'very much'- 4 points (Appendix 1).

The 15 item myasthenia gravis quality of life scale (MG-QOL 15) is a health related quality of life (HRQOL) evaluative instrument for patients with MG. This was designed to be easy to administer and interpret. Ted Burns and colleagues presented the study results in 2010 (403). This was a multicentre study which demonstrated the construct validity of the MG-QOL15 in practice setting. Score distributions were examined for test items in different MG patient groups that represented the clinical spectrum of the disease. Patients in remission more frequently scored test items as 'normal' than did patients in other groups. Patients with lower/better MG composite scores also more frequently scored items as normal than did patients with higher or worse scores. There was also appropriate correlation between the MG-QOL and the other MG specific scales studied. The study findings reflected what troubled MG patients. The MG-QOL 15 was found to have construct validity in the clinical practice setting and represented an efficient and valuable tool for assessing HRQOL for patients with MG.

In 2016, Burns and colleagues reviewed the use of the MG-QOL 15. They performed a rasch analysis on more than 1300 15 item MG-QOL completed surveys (404). These results were discussed with specialists and biostatisticians. The decision was made to revise three items and prospectively evaluate the revised scheme using either 3, 4 or 5 responses. Rasch analysis was repeated. The MG-QOL 15r performed slightly better than the MG-QOL 15. The three response option MG-QOL 15r demonstrated better clinicometric properties than the 4 or 5 option scales. Relative distributions of item and person location estimates showed good coverage of disease severity. They concluded that the MG-QOL 15r was now the preferred HRQOL instrument for MG because of improved clinicometrics and ease-of-use. This revision did not negate previous studies or interpretations of results using the MG-QOL 15.

As our study was set up in 2014, before the revised score was introduced, we continued to use the old MG QoL 15 scores in all patients at recruitment and follow-up.

2.1.7 Blood samples

2.1.7.1 Whole blood samples in Lithium heparin

20 mls of whole blood was collected in lithium heparin bottles. The samples were taken to the laboratory (either at the Queen's

medical Centre, Nottingham or at Birmingham University) within 4 hours of collection. Peripheral Blood Monocytic Cells (PBMCs) also called Peripheral Blood Lymphocytes (PBLs) were isolated as per the procedure detailed later in chapter 5. These were frozen for further studies of regulatory T cells, Th17 cells and cytokines using flow cytometry.

2.1.7.2 Serum samples

8-16 mls of whole blood was collected in yellow/golden topped serum tubes. These were taken to the laboratory for spinning on the same day. The samples were centrifuged at 4^0C, 3000 rpm for 10 mins, at maximum acceleration and brake settings. The supernatant which is the sera was pipetted into 0.5 ml eppindorf tubes and stored at -80^0C. In the final year of the PhD, the samples were studied at the laboratory at the Department of Neurosciences, West wing, John Radcliffe Hospital, Oxford, to look for antibodies to AChR and MuSK using Radioimmunoprecipitation assays (RIA/RIPA), and AChR, MuSK and LRP4 antibodies using cell based assays. Additionally, stored serum samples were sent to Sweden to Dr Punga's lab for miRNA testing.

2.1.7.3 PAXgene samples

An aliquot of whole blood was collected in PAXgene tubes (Qiagen, Valencia, CA, USA). These were stored in the -80 freezer for further studies.

2.1.7.4 EDTA samples

EDTA samples were obtained and have been stored at -80 for future studies on HLA typing (P Gregersen et al, 2012; Renton et al, 2015).

2.1.8 Flow cytometry

Flow cytometry is the technology that is used to analyse physical and chemical characteristics of particles which are in a fluid state as it passes through at least one laser. The cell components are fluorescently labelled and then they are excited by the laser which in turn makes the cells/particles emit light at various wavelengths. The fluorescence is measured to determine various properties of the single particles, usually cells. Up to thousands of particles per second can be analysed as they pass through the liquid stream. Examples of the properties which can be measured include the cells' relative granularity, size and fluorescence intensity, as well as its internal complexity.

Flow cytometry is a standard laboratory tool which is used in the evaluation of haematopoietic cells including identifying subpopulations of cells referred to as immunophenotyping.

The cell populations can be characterised using a combination of antigens which are both on the surface and intracellularly. The practical applications include immunophenotyping, measuring intracellular cytokine production, cellular proliferation, assessing cell viability and analysis of cell type, stem cells and fluorescent proteins.

It is important to have correct controls to set up the flow cytometer and to compensate for any overlap in the emission of fluorescence as increasingly multiple antigen markers are used during flow cytometry. These controls are usually unstained cells, single colour controls and fluorescence minus one (FMO) where all antibodies in the panel are added to the cells removing a single antibody in turn.

T regs were officially identified as a distinct population of CD4+ T cells which express the α chain of the IL-2 receptor, CD25. However CD25 is also expressed by a significant proportion of CD45R+ CD45R- effector/memory CD4+ T cells. It was thought that 1 to 3% of circulating human CD4+ T cells were T regs, however this proportion varied between 1.4 and 20% in various studies depending on where the CD25 gate was placed. Fazekas de St Groth et al discovered that costaining for CD25

and CD127 (α chain of the IL-7 receptor) separated a distinct CD25+127 low population; these expressed very high levels of FoxP3 mRNA. Staining for these markers is what is commonly used for T reg cells although there is no universally accepted method (405).

As well as T reg cells, cytokine levels including IFNα, IFNγ, TNFα, IL17, IL4 and IL10 were studied. This was based on previously published literature on cytokine abnormalities seen in MG. IFNα however, was added on as part of the panel with IFNγ and TNFα, and the author acknowledges that this is unlikely to provide much information in this context.

2.1.9 Treatment

Patients recruited into our study were treated in the same manner as all other patients, starting with pyridostigmine and adding immunosuppressants as and when required. The treatment schedule strictly followed the UK myasthenia study group national guidelines published in Practical Neurology in 2015 (although this was a year after we started, because the principal supervisor was a co-author for these guidelines, we followed the schedule even before publication) (323). If patients diagnosed with myasthenia referred to us to be included in our study had already been started on pyridostigmine in the

meantime, we asked them about their quality of life and composite scores pre-pyridostigmine treatment.

2.1.10 Follow up

Patients were followed up on an annual basis (non-research related/clinical follow up in between times was as per individual patients' clinical indication) for two years with an option to be followed up for 5 years by the recruiting neurologist. At every follow up, the MG composite score and Quality of Life scores were repeated along with routine history taking and examination. Serum samples were taken to be tested for antibodies. These were compared with samples on recruitment to look at the effects of time and treatment on antibody levels. In 24 patients, whole blood was also taken at first year follow up for PBMC isolation and flow cytometric studies.

2.2 Preliminary Work

In a study supported by the Sir Halley Stewart Trust, Liete et al (including the principal supervisor) retrospectively analysed the case records of 636 MG patients, with onset of disease at ≥50 years, who had been studied at one of four UK Myasthenia Clinics (Oxford, Belfast, Nottingham and Glasgow), assessing

the clinical distribution of muscle weakness, results of serological tests and delays in the diagnosis (Leite et al, 2012).

They found an increasing male bias after onset-age 50; as many as 27% of patients had purely ocular symptoms during follow-up, with a rate of seronegative generalised MG of 13.6%. AChR antibodies were identified in more than 50% of the female ocular LOMG patients but only in 25% of the male ocular LOMG patients. Fifteen percent of patients were misdiagnosed at onset, most typically as stroke. In this population, thymoma occurrence was low at 3.5%. Sera on 38 of the seronegative patients was available for cell-based assay analysis, and found to be positive for binding to clustered AChRs (n=15) or MuSK (n=10).

2.2.1 Principal aims

There is accumulating evidence that late onset myasthenia gravis (LOMG, disease onset after the age of 50 years) is becoming more common for reasons that are not fully understood (1). From the initial clinical and immunological UK retrospective studies, it was found that myasthenia may present differently in this age group, with distinct initial diagnostic features, clinical outcomes and responses to treatment compared to patients with early onset disease. We aimed to

study a prospective cohort of patients with LOMG to establish the defining clinical and immunological features which may give us an insight into the pathogenesis in this age group, and some explanation for the rise in incidence.

2.2.2 Study outcome measures

This is an observational study of the clinical and immunological features of LOMG compared with EOMG.

2.2.2.1 Primary endpoint

To define a cohort of MG patients to assess:

1. Demographic characteristics (including sex ratios) in unbiased, unselected, consecutive groups of prospectively recruited patients
2. Associated conditions and family history of autoimmunity
3. Presenting clinical features: is LOMG worse/milder than EOMG at onset, diagnosis, worst stage?
4. Responses to medication, relapse rates, and outcomes in LOMG and EOMG: which drug combinations are beneficial?

2.2.2.2 Secondary endpoint

1. To Clarify the immunological features of LOMG to:

a. Determine the antibody profile to a number of neuromuscular targets (AChR MuSK, LRP4): are these antibodies predictive of disease outcome in LOMG, (and possibly thymoma development) compared to EOMG?

b. Assess the frequencies of functional regulatory T-cells: do they correlate with clinical severity at presentation in LOMG or autoantibody levels?

3 Clinical profile and Phenotypes

3.1 Introduction

Epidemiological studies done since the 1950s have shown an increasing incidence rate and prevalence rate of myasthenia gravis with time, especially in the older population. Initial studies show that early-onset myasthenia gravis was more common in women and late-onset myasthenia gravis was more common in men (96). Meta-analysis of all studies done by both Phillips et al (98) and Aarli et al (96) show an increasing prevalence and incidence rate of myasthenia gravis, particularly in the older population which was variably described as > 40 years of age, >45 years, >50 years and >60 years. Myasthenia Gravis was also thought to be underdiagnosed in the older patients given the other coexisting comorbidities (99). Since then several studies have been done across different countries and different continents showing an increasing incidence rate of myasthenia gravis. The majority of the studies were in patients with AChR antibody positivity with a couple of studies on MuSK MG. Familial MG was thought to occur in approximately 1 to 4% (138). Viruses are thought to have a role in inducing autoimmunity in MG and studies on EBV DNA and nuclear RNA in thymic tissue was shown to be increased in MG patients (60, 144) but these studies were not replicated by another Chinese

study (145). Similarly, West Nile virus was also thought to be an additional risk factor for MG initiation (146).

For a long time it was thought that myasthenia gravis affected young adults and that it was uncommon after the age of 50 years. During the 1990s it became clear that myasthenia gravis was being diagnosed more often in older patients. In 1980 Compston and colleagues (97) postulated two categories of non-thymoma myasthenia patients, one with presentation at less than 40 years of age and one after 40 years of age. Those who were younger were more often female, and had HLA-A1, B8, and DRW3 positive antigens. In the older age group there was a significant association with male gender and the presence of HLA-A3, B7 and/or DRW2. In 1991 Somnier and co-workers reported a bimodal appearance for both sexes with one peak in the early onset group and another in the late onset group (406). On the basis of this, they proposed that the separation between early onset and late onset should be at the age of 50 years rather than 40 years. They found that in early-onset male patients, the onset was approximately 10 years later than in females, while in the late onset group the peak was at the same time in years in both sexes.

Ocular MG has been difficult to define with various cut-off points for diagnosis of ocular MG being used. Sommer et al (170) and Monsul et al (171) suggested purely ocular symptoms for at

least three months from symptom onset to class them as ocular MG. There have been several studies looking at the effect of prednisolone on the progression of ocular to generalised MG. Several studies (171, 172, 175, 385, 389) have suggested that the conversion from ocular to generalised MG was lower in patients who were treated with steroids, however Nagia et al (173) suggested that conversion rates from OMG to GMG was similar in both immunosuppressed and non-immunosuppressed patients.

Patients with LOMG were thought to be more likely to be male and did not have thymic hyperplasia (160).

Clinical presentation of MG is thought to be different between different antibody subtypes. Patients with MuSK antibody positivity were commonly female, young, and had ocular, bulbar and respiratory symptoms. They responded better to plasma exchange compared to iv immunoglobulins (151, 154, 407). Zivkovic et al (who looked at the clinical presentation of LOMG in a retrospective cohort of 174 patients) (155) and Suzuki et al (156) suggested that OMG was more common in LOMG compared to EOMG; both groups had the same rate of myasthenic crisis in EOMG and LOMG.

Although the treatment regime for myasthenia gravis is different across different countries, in the UK, the treatment regime is usually pyridostigmine first, followed by steroids, usually

prednisolone, followed by other immunosuppressants if necessary. The ABN guidelines for management of myasthenia gravis were published in 2015; however, since our principal investigator was one of the authors of these guidelines, we followed the same treatment plan for all of our patients recruited into the study (323). Along with corticosteroids, the other immunosuppressants which have been shown to be useful in myasthenia include azathioprine, methotrexate, mycophenolate, iv immunoglobulins, plasma exchange, and rituximab in refractory cases. Several other monoclonal antibodies are also being studied.

There have been several studies on the benefits of thymectomy in patients with thymic enlargement; however, the MGTX trial compared thymectomy versus no thymectomy in non-thymomatous myasthenia gravis patients who were receiving prednisolone. This showed a significant improvement in QMG scores and also dose reduction of prednisolone in the patients who were thymectomised (363). This was also reflected in other studies (364, 365).

The refractory patients are thought to be more likely to be younger at onset, female, thymomatous and anti-MuSK antibody positive (387). The most common reason for patients developing myasthenic crisis was thought to be an infection. They showed good response to IVIG and plasma exchange; the median time

to weaning of ventilation was thought to be shorter in LOMG (388).

Studies looking at the start of treatment until achievement of MM status found that OMG patients showed the best early-stage response, followed by TAMG and AChR MG without thymic abnormalities. AChR antibody negative MG patients showed the worst early-stage response to treatment (390). Previous studies (408) have also shown that the majority of MG patients had a favourable prognosis regardless of age, maximum disease severity and antibody status although LOMG patients were more likely to achieve optimal outcome.

Mortality rates in MG have been shown to be higher compared to control groups and this is highest during the first five years after diagnosis (393).

The majority of the studies are retrospective and in the few done prospectively, the studies included patients with an established diagnosis of MG, on a variety of treatments and a few patients newly diagnosed within the study period. Before the advent of antibody tests, the diagnostic criteria for myasthenia gravis differed in each study, being defined by the authors. The age cut-off for LOMG and EOMG also differs between the studies. The method of data collection differs and there is a lot of heterogeneity. Studies in ocular MG are also very varied mainly because of the criteria used to define OMG.

The drawback in interpreting the above data from the previous studies is that the majority of the epidemiological studies, clinical studies, and all of the antibody studies were done retrospectively, with patients on a variety of different treatment modalities and disease durations.

We tried to perform a unique large incident cohort study from diagnosis with full follow-up which has never been done before. By doing this, we sought to answer the following questions:

- Is the incidence of LOMG greater than that of EOMG?
- Could there be a cohort effect leading to increasing incidence of LOMG? Could this be attributed to environmental exposure, Immunisation or viral infections?
- Is there a difference in the sex distribution between LOMG and EOMG?
- Is there a difference in clinical presentation between LOMG and EOMG?
- Is OMG more common in LOMG or EOMG?
- Is the rate of generalisation of ocular symptoms different in LOMG and EOMG?
- Is there a difference in thymic abnormalities between LOMG and EOMG patients?

- Is there any difference in clinical presentation between the different antibody subgroups?
- Is the treatment response different in LOMG and EOMG?
- Is the clinical outcome different in LOMG and EOMG patients?

3.2 Methods

This study was a multicentre prospective study conducted across three sites: in Nottingham at the Queen's medical Centre, Birmingham at the Queen Elizabeth Hospital Birmingham, and in Oxford at the John Radcliffe Hospital. The patients were prospectively recruited as soon as possible after a diagnosis of myasthenia gravis, but within 12 months of diagnosis. Patients were referred to the specialist Neuroimmunology/ Neuromuscular clinic at one of the three sites. Referrals from other neurologists and GPs were the primary source of recruitment, but patients were also recruited after inpatient admissions, after email advertisements to ophthalmology colleagues, neurophysiology colleagues and general neurologists across the region. The study was also advertised on the Myaware website. We went through laboratory requests for ACHR and anti-MuSK antibody testing in the

Nottinghmashire and Derbyshire regions (excluding the northern districts) which ensured 100% recruitment rates.

Whilst the study could not predict the incidence and prevalence of MG in all the areas except for the Trent region (as mentioned above) (NB: see Table 3), we think that it was powered enough to answer the primary and secondary endpoint questions.

A total of 150 patients with a new diagnosis of myasthenia gravis where recruited to the study. All patients were seen and assessed by the author at recruitment. During annual follow-ups, the patients were reviewed mostly by the author, but also by the chief investigator, or by the local investigating officers who clinically assessed the patients and performed blood tests and questionnaires including the MG composite scores and MG QoL scores. Other than for 15 patients, the remaining 135 patients were treatment naïve at the point of recruitment. The 15 patients (one EOMG and 14 LOMG) who were immunosuppressed had been either admitted to hospital, or needed urgent immunomodulatory treatment because of crisis or bulbar/respiratory symptoms. Most received iv immunoglobulins and oral prednisolone (8 patients), others prednisolone only (5 patients) and two received iv immunoglobulins, prednisolone and Azathioprine. Nearly all the patients were recruited during their initial admission to hospital, or within a few days of receiving immunosuppression.

Previous literature has shown that EOMG and LOMG may differ in severity. Our patient recruitment was unselected, so patients of all ages with a diagnosis of MG within the preceding 12 months were recruited. This ensured that the realtively mildly symptomatic patients (in either group) were also recruited. We do not think that the differing severity of illness in EOMG and LOMG has influenced case ascertainment.

Patients were followed up on an annual basis for research purposes at which time they had a clinical assessment, medications were reviewed, MG composite scores and QoL scores were repeated, and they had blood tests for serum. 24 patients, half of whom had been immunosuppressed since recruitment and the other half who had not, also had whole blood taken at first year follow up for PBMC testing. Home visits were done for a few of the patients to ensure good follow-up rates. Of the 150 patients, 4 patients were lost to follow-up and 6 patients had died upto the end of July 2017.

Patients with thymic abnormalities on imaging were offered thymectomy. Patients under the age of 50 years who did not have imaging abnormalities were also offered thymectomy (although not in all patients) if they had symptoms for less than three years. We found that, over the course of the study, we were approaching more people with milder symptoms because

of the availability of VATS thymectomy. Not all the patients opted to have surgery.

The data included in the current study are from 1st August 2014 up to 31st July 2017. At the end of this time, the mean follow-up time was 354 days, the median time being 330 days. Of the 150 patients, 120 patients had had their first year follow-up at the time the analysis was done and 65 patients had had a second year follow-up.

Some of the clinical data, including for ocular myasthenia gravis (OMG), and the antibody profiles was updated in May 2018 with further follow up data to include those patients who remained ocular at 2 years.

All the data was analysed by the author using GraphPad Prism version 7.01.

3.3 Results

3.3.1 Demographics

During the period from August 2014 to July 2017, the patients recruited from Birmingham and Oxford were selective, but every single patient with myasthenia from a study population covering the counties of Nottinghamshire and Derbyshire, UK, excluding the northern districts, were included.

Of the 150 patients with MG in our cohort, 26% belonged to the EOMG group and 74% to the LOMG group. In EOMG, 23 of the 39 patients were female i.e. 59%, and the remaining 16 of 39 i.e. 41% were male. In the LOMG group 68/111 i.e. 61.3% were male and 43/111 i.e. 38.7% were female (Figure 1). The total number of female patients in the whole cohort was 66/150 i.e 44%; of these, 23 i.e. 34.85% were early onset, and 43 i.e 65.15% were late onset. There were a total of 84 male patients (56%), of these 68 i.e. 80.95% were late onset and 16 i.e. 19.05% were early onset (Figure 2).

The mean age of female patients was 57.6 years; in EOMG this was 36.17 years and in LOMG this was 66.8 years. The mean age of male patients was 61.24 years; in EOMG this was 40.42 years and in LOMG this was 66.74 years.

Racial distribution showed no significant difference when compared to the national statistics for the area, except for under-representation of Asians in the south-east area including Oxford which is most likely due to small numbers (Tables 1&2).

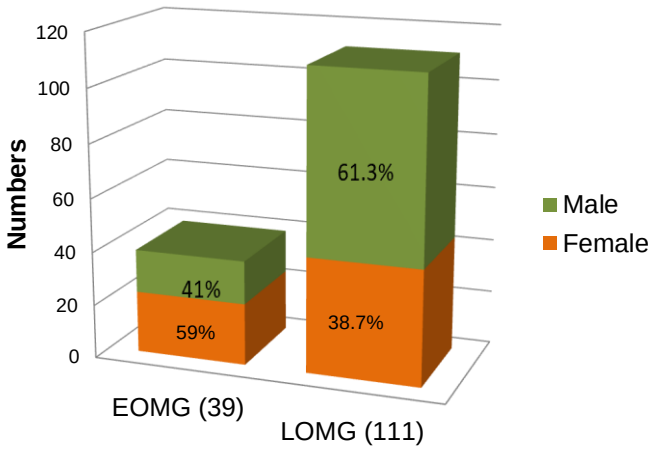


Figure1 Male:Female ratio in EOMG and LOMG

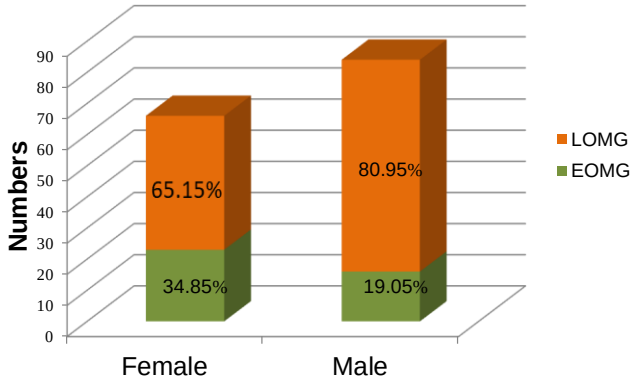


Figure 2 EOMG Vs LOMG in Males and Females

Table 1 Race distribution

Study	Afro-Carribean	Asian (and chinese)	Caucasian
Birmingham	3	3	41
Nottingham	2	4	89
Oxford	1	0	7
Total	6	7	137
Percentage	4%	4.67%	91.33%

Table 2 Racial distribution compared to national statistics (EM- East Midlands, WM- West Midlands, SE- South East)

Afrocarribean		p value
Study Vs national statistics	4% Vs 3.3%	0.6313
Study Vs EM statistics	2.11% Vs 1.8%	0.7823
Study Vs WM statistics	6.38% Vs 3.33%	0.0373
Study Vs SE statistics	12.5% Vs 1.6%	<0.0001
Asian		
Study Vs national statistics	4.67% Vs 7.5%	0.1882
Study Vs EM statistics	4.21% Vs 6.5%	0.2553
Study Vs WM statistics	6.38% Vs 10.8%	0.0811
Study Vs SE statistics	0% Vs 5.2%	0.0041
Caucasian		
Study Vs national statistics	91.33% Vs 86%	0.0599
Study Vs EM statistics	93.68% Vs 89.3%	0.0827
Study Vs WM statistics	87.23% Vs 89.3%	0.4121
Study Vs SE statistics	87.5% Vs 90.7%	0.1772

All p value were calculated using the Mann Whitney test

Data collection for the study was continued after the study period included in this thesis (August 2014 to July 2017). In March 2019, we had data on 213 patients in total. The clinical and immunological profiles of the patients in Nottingham (with 100% recruitment rates) and Birmingham/Oxford were compared (Table 3). There were no staistically significant differences between the two groups. This shows that there was no bias in the study population between the unselected

Nottingham patients (complete case recruitment) and the more selected Birmingham/Oxford group.

Table 3 Comparison of clinical and demographic data between the Nottingham region and Birmingham/Oxford regions

	Nottingham MG patients	Birmingham/Oxford MG patients	Univariate analysis
Number	158/213 (74.2%)	55/213 (25.8%)	
Median age in years (range) at MG diagnosis	64 (18-89)	64 (28-89)	P=0.92
Age ≥50 years (LOMG)	123/158 (77.8%)	40/55 (73%)	P=0.46
Proportion female	75/158 (47.5%)	22/55 (40%)	P=0.35
Ocular symptoms only at 3 months	90/158 (57%)	27/55 (49%)	P=0.34
Median MG composite score at diagnosis	7 (4-12)	7 (4-13.3)	P=0.86
Median MG QoL score at diagnosis	19 (6-35)	25 (11.5-34)	P=0.13
Thymoma	11/158 (7%)	5/55 (9%)	P=0.76
AChR RIA positive at diagnosis	[1]75/94 (80%)	48/55 (87%)	P=0.27

[1]64 Nottingham patients still needed AChR antibodies assaying (RIA)

3.3.2 Ocular myasthenia gravis defined

Ocular myasthenia gravis (OMG) is the most common form of MG and varying rates of secondary generalisation have been reported. Typically between 50 and 80% of patients will develop generalised symptoms in the first two years and for this reason an arbitrary minimum duration of two years of isolated ocular

symptoms is considered a reasonable limit for diagnosing OMG. Oosterhuis suggested a minimum of three months as a limit for purely ocular symptoms before classifying a patient as having OMG (169). Similarly Sommer et al and Monsul et al also suggested purely ocular symptoms for at least three months from symptom onset to class them as OMG (170, 171).

During the course of the study, we first categorised Ocular myasthenia Gravis (OMG) in those patients who had ocular symptoms at first symptom onset. We then categorised OMG as those patients who had purely ocular symptoms at diagnosis. However, during final analysis we focused mainly on those patients who had purely ocular symptoms at three months since symptom onset. This was based on the previous literature as above but also on our own findings of 'time of symptom onset'. We found that the median time for onset of bulbar, generalised and respiratory symptoms was less than three months. The recruitment was unselected, so we do not think that there was a selection bias towards the more unwell/ rapid generalisers.

In total we had 79/150 patients (52.67%) who had OMG at three months of whom 27 were EOMG (69.23% of all EOMG) and 52 were LOMG (46.84% of all LOMG) ($p = 0.0163$). Amongst the younger MG group, 14 were female and 13 male; in the LOMG group 19 were female and 33 male (Figure 3).

Mean time to generalisation was 457.2 days, and the median time was 331.5 days. The shortest time to generalisation was 91 days and the longest time was 1825 days. At the time of analysis, 11 of the early onset OMG patients i.e. 40.74% had generalised and 21 of the 52 LOMG patients i.e. 42% had generalised (p=0.1353) (Figures 6 and 8). The total generalisation rate was 40.51%. (NB: This statistic is different to that depicted on Figure 14 because on the graph, all MG patient data is included, ie those with OMG only at 3 months, which is the data shown here, and GMG, showing when patients first developed generalised symptoms. There is an argument then to classify some of the GMG patients who generalised quite early on in their illness as ‘early generalisers’, those that generalised just after 3 months as ‘intermediate generalisers’ and those that generalised after 2 years as ‘late generalisers’. For the purposes of this thesis, this distinction has not been made).

The majority of the patients generalised in under one year with a rate of 20.25% in total, 14.81% in EOMG and 23.08% in LOMG (p=0.2764) (Figure 8). 12 patients generalised between one and two years from symptom onset which was 15.19%; the distribution was 18.5% in EOMG and 13.46% in LOMG. As the data after two years was limited in this analysis, the numbers were small; generalisation rate between 2 to 3 years when the data was analysed was 1.27% and over three years was 3.8%.

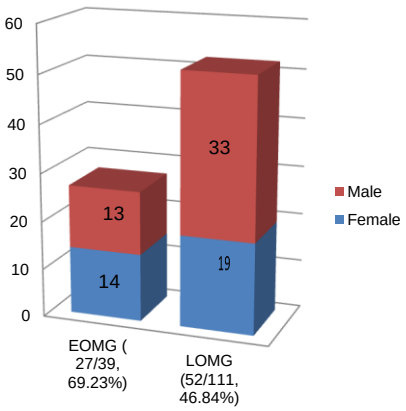


Figure 3 OMG (3 mths) with EOMG and LOMG distribution OMG in EOMG Vs LOMG p=0.0163

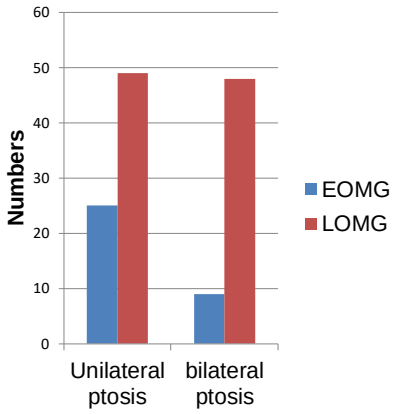


Figure 4 Unilateral and bilateral ptosis in EOMG and LOMG

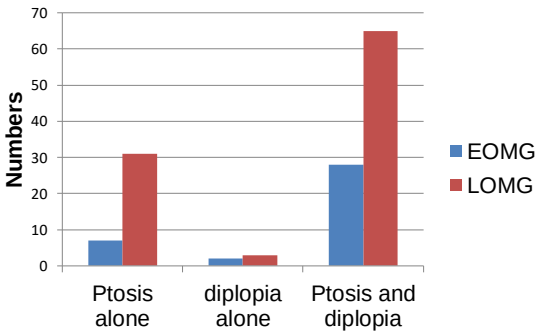


Figure 5 Ptosis and diplopia in EOMG and LOMG

In our cohort we found that 25 EOMG patients had unilateral ptosis and 9 had bilateral ptosis; 49 LOMG patients had unilateral ptosis and 48 had bilateral ptosis (Figure 4 and 5). There was no significant difference between time to generalisation between patients with unilateral ptosis and bilateral ptosis, both in the EOMG group and the LOMG group. Time to generalisation with ptosis only in EOMG versus LOMG showed a significant difference, with generalisation occurring much quicker in the LOMG group with a median of 35 days,

whereas with EOMG this was 608 days (p value =0.0385). It is not clear why this difference was noticed, but numbers with ptosis only in the EOMG group was small (3) and could be one of the reasons for this (Figure 7). There was no difference in time to generalisation when the ptosis only group was compared to patients who had both ptosis and diplopia.

Ocular QMG scores were recorded in 34 patients with ocular myasthenia at recruitment, VFQ 25 scores were recorded in 28 and VFQ 10 supplement was recorded in 27 patients. In EOMG, the median of the ocular QMG score was 4, the VFQ 25 was 68.5 and VFQ 10 supplement was 24. In LOMG, ocular QMG was 4, VFQ 25 was 45, and VFQ 10 supplement was 24. There was no statistically significant difference between Ocular QMG scores between early onset and late-onset groups (Figure 10). There was no difference in VFQ 25 scores between LOMG and EOMG, with a p value of 0.0727, the scores being higher in the LOMG group (Figure 11). There was no difference between VFQ 10 supplement between EOMG and LOMG groups.

The survival curve of ‘time to generalisation’ in OMG in patients when given steroids pre-generalisation did not show any statistical difference compared to when patients were not given steroids pre-generalisation (Figure 9). There was no statistical difference between time to generalisation of MG between the early-onset and late-onset groups with steroids either.

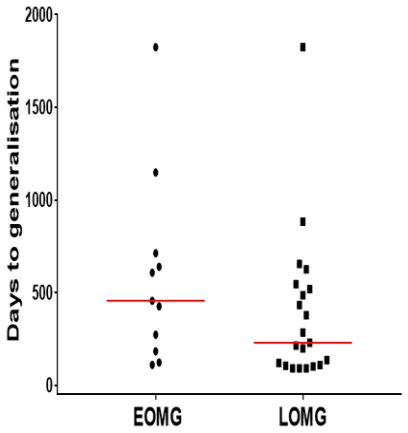


Figure 6 Time to generalisation in days of OMG (with medians) in EOMG and LOMG p-0.1353

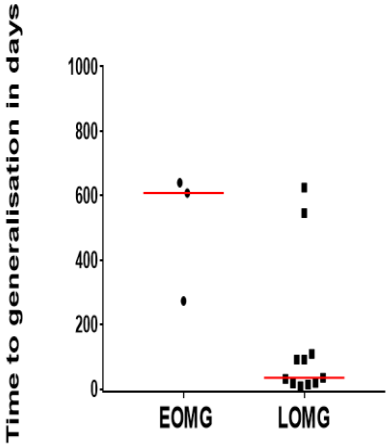


Figure 7 Time to generalisation (with median) with ptosis in EOMG and LOMG p=0.0385

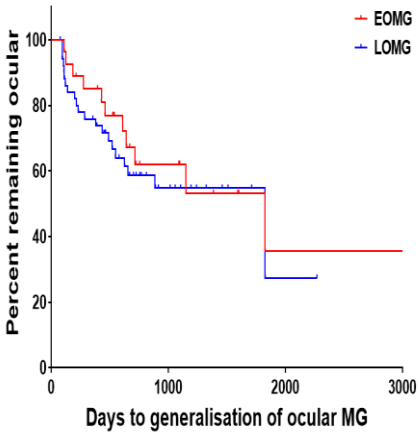


Figure 8 Survival curve for time to generalisation (in days) EOMG Vs LOMG p-0.2764

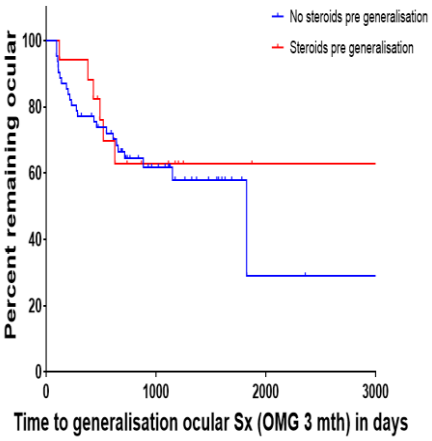


Figure 9 Survival curve for time to generalisation of OMG in patients given steroids pre-generalisation and those not given steroids

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed

Survival curve data was analysed using the Log Rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test)

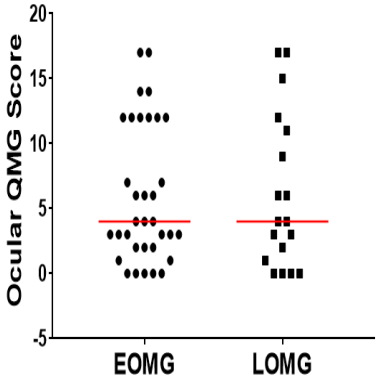


Figure 10 Ocular QMG scores (with medians) in EOMG and LOMG

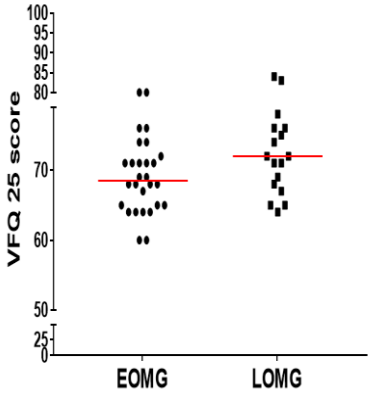


Figure 11 VFQ 25 scores (with medians) in EOMG and LOMG p=0.0727

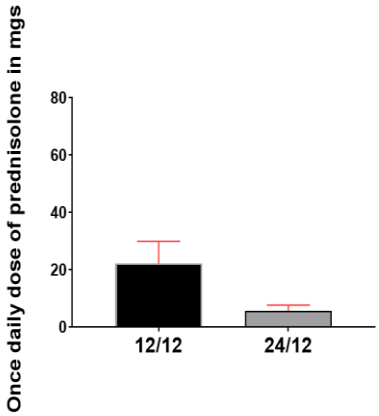


Figure 12 Steroid doses in EOMG at first and second year follow up (with standard error) p=0.0117

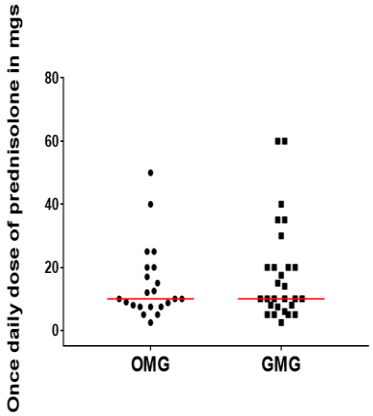


Figure 13 Comparison of steroid doses at first year follow up in OMG and GMG

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed

When we compared the steroid doses required in patients with EOMG at first year follow up and second year follow-up, there was a significant difference in the average doses required. The median dose was 10 mg at first year follow up and 7 mg at second year follow-up, p = 0.0117 (Figure 12). When the steroid

dose in OMG patients was compared with generalised MG at first year follow-up, there was no statistically significant difference, similarly at second year follow-up (Figure 13).

3.3.3 Symptom onset in myasthenia gravis

The most common first symptom on presentation was ocular, including either ptosis alone, diplopia alone or a combination of the two. Of the total of 150 patients, 116 (77.33%) had ocular symptoms at onset; this was either purely ocular or in combination with bulbar and generalised symptoms. 23 patients (15.32%) had bulbar symptoms, 21 patients (14%) had generalised symptoms and 2 patients (1.33%) had respiratory symptoms at onset. Comparison between the early-onset group and late-onset group showed a difference in ocular and generalised presentation, being more commonly ocular in younger patients and more commonly generalised in the older patients but this was not statistically significant (Table 4). Although the data is presented in a table form, the comparisons made were between individual parameters, e.g, OMG in EOMG compared to LOMG. However, when Bonferroni correction was used, the p values were below statistical significance.

Table 4 First symptom onset, either on their own or as a combination with other symptoms

	All pts (150)	Percentage of total	EOMG (39)	Percentage of EOMG	LOMG (111)	Percentage of LOMG	p value of EOMG Vs LOMG
Ocular	116	77.33%	34	87.18%	82	73.87%	0.0887
Bulbar	23	15.33%	4	10.25%	19	17.12%	0.3073
Generalised	21	14%	2	5.13%	19	17.12%	0.0643
Respiratory	2	1.33%	0	0%	2	1.80%	0.4005

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed; two individual parameters were compared

The times of onset of each of the symptoms was recorded for every patient recruited and updated during follow-up. This was analysed and plotted on a graph as below. Median time of onset of ptosis and diplopia was 0 days, dysphagia was 37 days, dysarthria 39 days, difficulty with chewing 55 days, limb weakness 21 days, neck weakness 70.5 days, and respiratory symptoms 87 days (Figure 14 A).

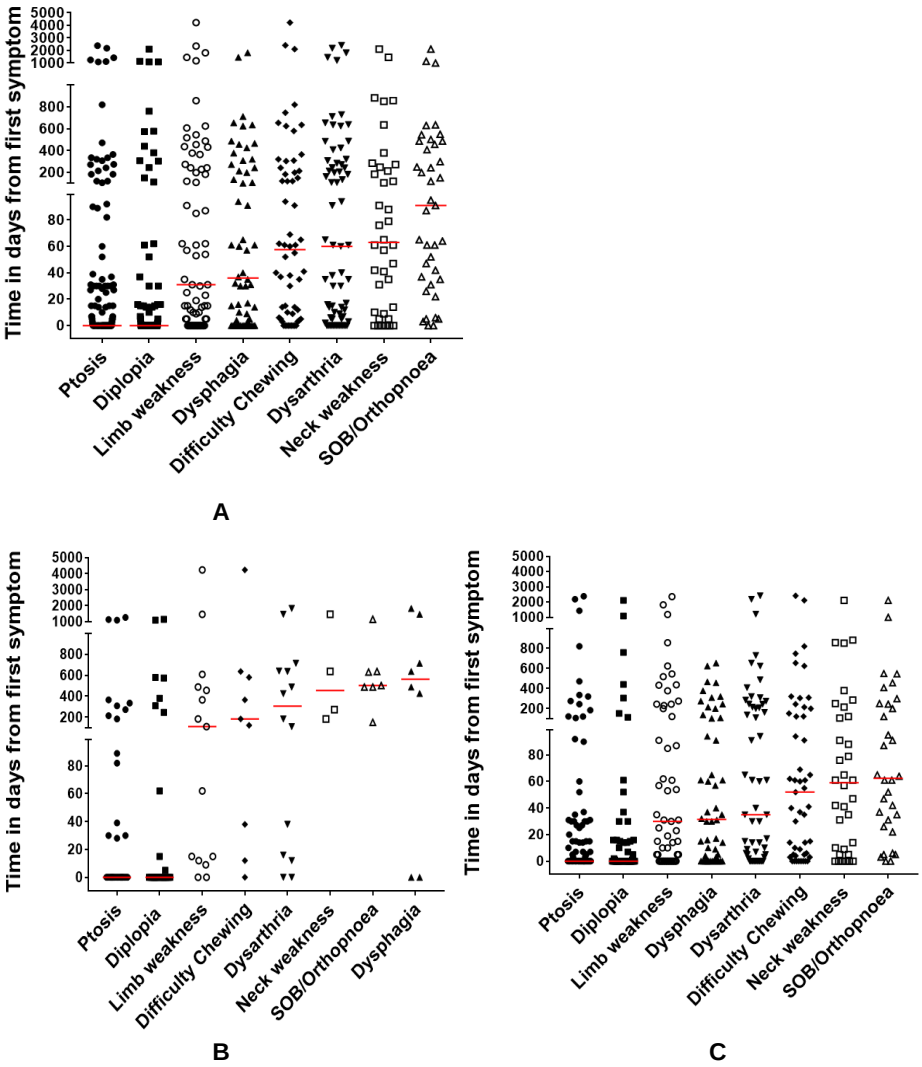


Figure 14 Time of onset of MG symptoms in days with medians. A: all patients, B: EOMG, C: LOMG

Medians were used as these were easier to compare. All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

On comparison of the EOMG and LOMG groups, the order in which patients generalised was different for the EOMG and LOMG groups. In EOMG, the order of median times of symptom onset was: ptosis, diplopia, limb weakness, chewing difficulties, dysarthria, neck weakness, shortness of breath and dysphagia (Figure 14 B); whereas in the LOMG patients this was: ptosis,

diplopia, limb weakness, dysphagia, dysarthria, chewing difficulties, neck weakness and shortness of breath (Figure 14C).

The median length of time for generalisation was not different (p=0.0581) in the younger and older patients when all the medians for the various symptoms were compared. When the last to generalise symptoms were compared, again, there was no difference; in the younger patients the longest median time for generalisation was 561.5 days (dysphagia) whereas for the older patients this was 62.5 days (SOB) (p=0.0862).

Dysphagia appears to affect the younger patients at a significantly later stage. On comparison of the time of onset of dysphagia in EOMG and LOMG, there was a significant difference in median times, 636 days in early-onset and 31 days in late-onset, p = 0.0037 (Figure 15A). Onset of dysarthria was also once again significant, with median onset time in days of 35 in late onset and 304.5 in early onset, p = 0.0331 (Figure 15 B).

There was no statistically significant difference between difficulty chewing, with median time of onset in young patients of 184 days and late onset of 46.5 days, p = 0.1033. Median time of onset of neck weakness in younger patients was 636 days and in older patients it was 59 days, p = 0.0063 (Figure 15C). There was no difference in the median time of onset of limb weakness; in younger patients it was 110 days and in older patients it was

30 days, p = 0.14364. There was a significant difference in the onset of respiratory symptoms with a median of 502 days in younger patients and 62.5 days in older patients with p = 0.0009 (Figure 15D). When Bonferroni correction was applied (adjusted alpha level or t* of 0.00625), time of onset of dysphagia and respiratory symptoms were still significantly different between EOMG and LOMG.

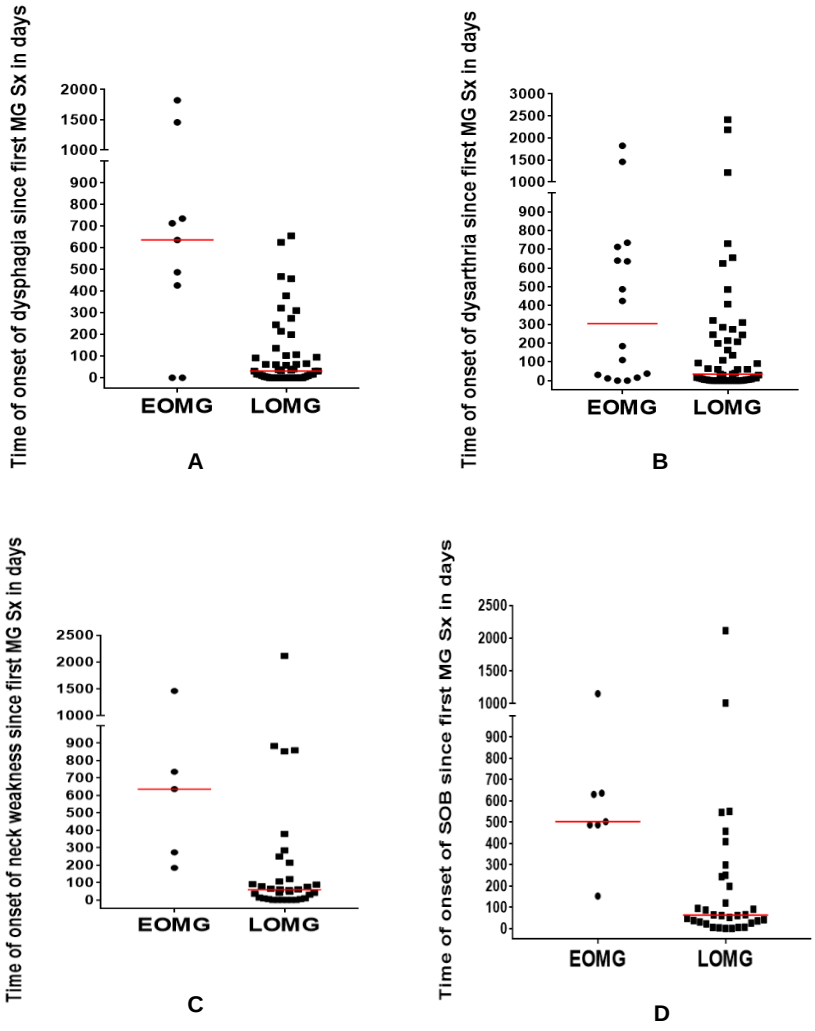


Figure 15 Comparison of time of onset of (A) dysphagia p=0.0037, (B) dysarthria p=0.0331, (C) neck weakness p=0.0063 and (D) SOB in EOMG and LOMG p=0.0009

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

3.3.4 Clinical phenotypes in early onset myasthenia versus late onset myasthenia gravis

Bulbar symptoms were seen in 13/39 i.e. 33.33% of EOMG patients and 68/111 i.e. 61.26% of patients in LOMG. The difference was statistically significant, p=0.0027. Limb weakness and shortness of breath were present in varying proportions in the EOMG and LOMG subgroup as detailed in Table 5 below. The frequency of limb weakness in EOMG was 38.46% and 49.54% in LOMG, p = 0.2344; there was no significant difference seen between the males and females in either group.

Table 5 Presence of limb weakness and shortness of breath (SOB) in EOMG and LOMG

Limb weakness in EOMG: 15/39= 38.46%
Limb weakness in EOMG Male: 5/16 = 31.25%
Limb weakness in EOMG Female: 10/23= 43.47%
Limb weakness in LOMG: 55/111= 49.54%
Limb weakness in LOMG Male:32/68= 47.05%
Limb weakness in LOMG Female: 23/43= 53.48%
SOB EOMG: 7/39
SOB LOMG: 32/111
SOB EOMG Female: 5/23
SOB EOMG Male: 2/16
SOB LOMG Female: 13/43
SOB LOMG Male: 19/68

Shortness of breath was seen in 17.9% of EOMG patients and 20.83% of LOMG patients, once again not statistically significant with p = 0.1842 (Table 6). There was no difference between the males and females in either group. Patients with limb weakness presented with varying combinations of distal and proximal

upper or limb weakness, most commonly both upper and lower limb proximal weakness.

Table 6 p values of number of patients with limb weakness and shortness of breath (SOB) between the various subgroups

	EOMG Vs LOMG	EOMG Male Vs EOMG Female	LOMG Male Vs LOMG Female	EOMG male Vs LOMG male	EOMG female Vs LOMG female
Limb weakness	0.2344	0.4465	0.4981	0.2579	0.4328
SOB	0.1842	0.4654	0.7962	0.2021	0.464

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

We looked at the occupations for all of our patients and there was no particular occupation that was more common than the other.

All patients had repeat MG composite scores during follow-up; the MG composite scores were also repeated when and if there was clinical worsening, or if they were admitted to hospital with worsening symptoms or crises. The first MG composite score at symptom onset and at their worst (whether at presentation, recruitment, follow up or clinical worsening) were recorded, along with the time from first symptom onset. The median time to worst MG composite score was 91 days with the minimum

being at symptom onset and the maximum at 4354 days (Figure 16). On subgroup analysis, the median time to worst composite score in EOMG was 109 days and in LOMG was slightly earlier at 90 days. This was not statistically significant with a p value of 0.1339 (Figure 17).

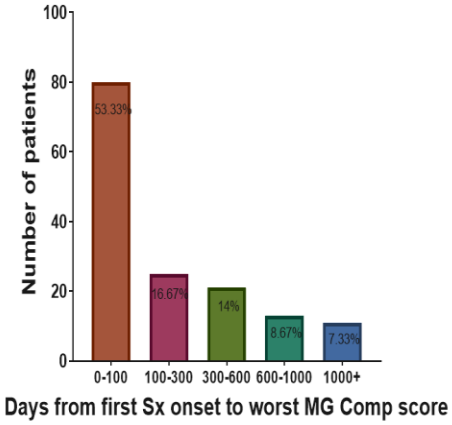


Figure 16 Time in days from first MG symptom onset and worst MG composite score

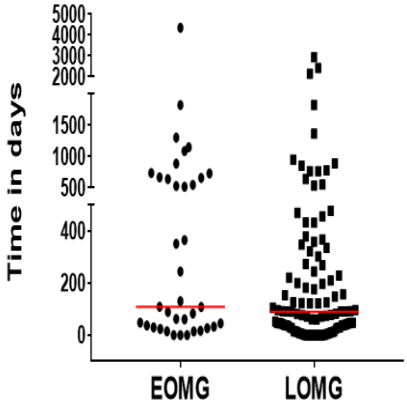


Figure 17 Time in days (with medians) from first symptom onset to worst MG composite scores, EOMG Vs LOMG p=0.1339

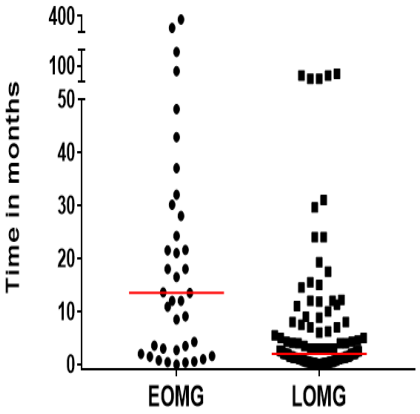


Figure 18 Time from Symptom onset to diagnosis in months (with medians), EOMG Vs LOMG p<0.0001

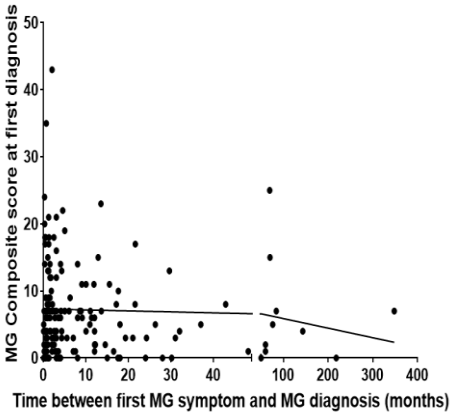


Figure 19 Correlation between diagnostic delay and first MG composite score

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Figure 19: Simple linear regression used

Time taken from initial MG symptom onset to MG diagnosis and recruitment was also recorded. The median time from MG symptom onset to MG diagnosis in months was 3 months, from diagnosis to recruitment was 2.48 months, and time from symptom onset to recruitment was 7.895 months. There was a significant difference in time to diagnosis from symptom onset between EOMG (median of 13.5 mths) and LOMG (median of 2 mths), older patients being diagnosed quicker than EOMG, $p<0.0001$ (Figure 18). This is different to previously published data (99).

There was no significant difference in MG composite scores between patients diagnosed early or late (Figure 19). The other more commonly reported symptoms in our cohort were: dribbling of saliva, snarl on smiling, difficulty moving the tongue, numb/swollen tongue, difficulty pursing lips, facial weakness, difficulty opening the mouth, urinary Sx, constipation, dry mouth, increased lacrimation, body ache, generalised fatigue, dizziness, decreased appetite, weight loss and falls/unsteadiness.

The more common of these were: dry mouth: 13/150= 8.67%, urinary Sx: 4/150= 2.67%, generalised fatigue: 9/150= 6%, and weight loss: 11/150= 7.33%.

3.3.5 Clinical phenotypes with AChR, MuSK, LRP4 Ab positivity and seronegative patients

All 150 of our patients had blood tests looking for anti-AChR, anti-MuSK and LRP4 antibodies. Of these, 142 patients were positive for one or more antibodies. We had 8 seronegative patients (NB: one of these 8 had positive AChR Abs on RIA when recruited, but repeat tests including CBAs were negative). AChR single positivity was seen in 107 patients, MuSK single positivity in 6 patients, LRP4 single positivity in 2, AChR and MuSK double positivity in 15, AChR and LRP4 double positivity in 9, MuSK and LRP4 double positivity in 1 and seronegativity in 8. Two patients were positive for all antibodies but the conclusion for these patients was that the MuSK and LRP4 antibodies were possibly non-specifically binding and they were likely to be single positive for AChR. The distribution of the subtypes is in Table 6.

A significant difference was seen in AChR single positivity between females in the early-onset and late-onset groups. In early-onset this was 11/23 and late-onset it was 32/43, p= 0.0319. Similarly, younger female patients were more likely to be double positive for AChR & MuSK antibodies compared to the older female patients, but this was not statistically significant with p = 0.0824. There was no significant difference between

antibody positivity in any of the other subgroups (Tables 7 and 8).

Table 7 Antibody positivity in the different subgroups

			Numbers	Percentage
AChR single +	EOMG	Female (23)	11	47.80
		Male (16)	12	75
	LOMG	Female (43)	32	74.42
		Male (68)	52	76.46
MuSK single +	EOMG	Female (23)	2	8.69
		Male (16)	0	0
	LOMG	Female (43)	1	2.32
		Male (68)	3	4.41
AChR & MuSK double +	EOMG	Female (23)	5	21.74
		Male (16)	1	6.25
	LOMG	Female (43)	3	6.98
		Male (68)	6	8.82
AChR & LRP4 double +	EOMG	Female (23)	2	8.69
		Male (16)	1	6.25
	LOMG	Female (43)	3	6.98
		Male (68)	3	4.41
Seronegative	EOMG	Female (23)	1	4.34
		Male (16)	1	6.25
	LOMG	Female (43)	3	6.98
		Male (68)	3	4.41

Table 8 Comparison of antibody positivity between subgroups, p values

	AChR +	MuSK +	AChR & MuSK	ACHR & LRP4	Seronegative
Total number of positives	107	6	15	9	8
EOMG female Vs LOMG female	0.0319	0.2398	0.0824	0.8039	0.6708
EOMG Male Vs LOMG male	-	0.3952	0.7394	0.7572	0.7572
EOMG female Vs EOMG male	-	0.2321	0.1930	0.7812	0.7928
LOMG female Vs LOMG male	-	-	-	0.5615	0.565

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Thymic abnormalities were seen in a total of 23 patients who had either thymic mass (possibly thymoma) or thymic enlargement (possibly hyperplasia). The only significant difference in antibody positivity in these patients was in those

who had LRP4 antibodies. They were more likely to have thymic enlargement/hyperplasia compared to AChR single positivity with a p value of 0.0057 (Table 9). However, it is to be borne in mind that we only had 2 LRP4 single positives in our cohort.

Ocular myasthenia gravis was seen in all antibody subtypes without a significant difference; the notable difference being between patients who were AChR single positive compared to AChR & LRP4 double positive (p = 0.0938), OMG being more common in double positives (Table 10).

Table 9 Comparison of thymic abnormalities between AChR single positive and the other antibody subgroups, p values.

		AChR +	MuSK +	LRP4 +	AChR & MuSK +	AChR & LRP4 +	MuSK & LRP4 +	Seronegative
Thymoma	Total numbers	107	6	2	15	9	1	8
	AChR +				0.5399			0.9734
Thymic hyperplasia	AChR +			0.0057	0.7390	0.4081		

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Table 10 Comparison of OMG occurrence in different antibody subtypes, p values.

	AChR +	MuSK +	LRP4 +	AChR & MuSK +	AChR & LRP4 +	MuSK & LRP4+	Seronegative
Total numbers	107	6	2	15	9	1	8
AChR +		0.3903	0.9688	0.7342	0.0938		0.2155

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

There was no difference in first presenting symptoms amongst the different antibody subtypes. Over the course of the disease, 100% of the patients who were MuSK Ab single positive, LRP4 Ab single positive, AChR & MuSK double positive and AChR & LRP4 double positive had ocular symptoms of ptosis and/or diplopia. In AChR single positive patients this was 92.5%, in seronegative patients ocular symptoms were seen at a slightly lower rate of 87.5%.

There was no difference in the presence of limb weakness. Seronegative patients were more likely (but non-significant) to have limb symptoms at 62.5% compared to 47.7% with AChR single positivity (p=0.4212) and 33.3% with AChR and LRP4 double positivity (p=0.2433).

Bulbar symptoms were seen with equal frequency in AChR single+ (58.9%), MuSK single+ (50%) and AChR and MuSK double + (60%) patients. However, seronegative patients were much less likely to have bulbar symptoms compared to AChR+ ($p=0.0112$) and compared to AChR & MuSK double+ ($p=0.0323$) but there was no difference compared to MuSK+ or LRP4+ patients (Table 11).

There was no difference in respiratory symptoms in any of the antibody subgroups including in MuSK MG (Table 12).

There was no difference in steroid requirements pre-generalisation in any of the antibody subgroups but there was a significant difference in steroid requirement post-generalisation in the antibody subgroups. This was most pronounced in AChR and MuSK double positivity with 80% of patients requiring steroids compared to 25.2% with AChR single positivity ($p<0.0001$). AChR & MuSK double positive patients were more likely to require steroids post- generalisation compared also to the MuSK single positives ($p = 0.0011$), AChR & LRP4 double positives ($p = 0.0065$) and seronegative group ($p = 0.0024$). There was no difference between AChR & MuSK double positives and MuSK & LRP4 double positives, $p = 0.0833$ (Table 13).

Table 11 Comparison of bulbar symtoms in the antibody subtypes with p values.

	Numbers with bulbar Sx	AChR +	MuSK +	LRP4 +	AChR & MuSK +	AChR & LRP4 +	MuSK & LRP4+	Seronegative
Total numbers		107	6	2	15	9	1	8
AChR +	63		0.6683	0.0962	0.9356	0.3997	0.2365	0.0112
MuSK +	3							0.1386
LRP4 +	0							0.6171
AChR & MuSK +	9							0.0323
AChR & LRP4 +	4							0.1621
MuSK & LRP4 +	0							0.7231
Seronegative	1							

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Table 12 Comparison of respiratory symptoms in antibody subtypes with p values.

	Numbers with resp Sx	AChR +	MuSK +	LRP4 +	AChR & MuSK +	AChR & LRP4 +	MuSK & LRP4+	Seronegative
Total numbers		107	6	2	15	9	1	8
AChR +	30		0.5477	0.4962	0.2281	0.7346	0.5355	0.8557
MuSK +	1							
LRP4 +	1							
AChR & MuSK +	2							
AChR & LRP4 +	3							
MuSK & LRP4 +	0							
Seronegative	2							

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Table 13 Comparison of steroid requirement pre and post-generalisation in different antibody subtypes, p values.

		AChR +	MuSK +	LRP4 +	AChR & MuSK +	AChR & LRP4 +	MuSK & LRP4+	Seronegative
Steroids pre generalisation	Total numbers	107	6	2	15	9	1	8
	AChR +		0.4100	0.6337	0.2719	0.2795	0.7360	0.8451
	MuSK +							
	LRP4 +							
	AChR & MuSK +							
	AChR & LRP4 +							
	MuSK & LRP4 +							
Steroids post generalisation	Seronegative							
	AChR +		0.1606	0.4153	<0.0001	0.8424	0.5640	0.4213
	MuSK +							
	LRP4 +							
	AChR & MuSK +		0.0011			0.0065	0.0833	0.0024
	AChR & LRP4 +							
	MuSK & LRP4 +							
	Seronegative							

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

In comparison, the requirement for alternate immunosuppressants in the AChR single positives compared to AChR & MuSK double positives was not significant, p = 0.1592.

Table 14 Comparison of number of hospital admissions in different antibody subtypes, p value.

	AChR +	MuSK +	LRP4 +	AChR & MuSK +	AChR & LRP4 +	MuSK & LRP4+	Seronegative
Total numbers	107	6	2	15	9	1	8
AChR +		0.4151	0.3286	0.5767	0.5180	0.4888	0.0535
MuSK +							
LRP4 +							
AChR & MuSK +		0.3180			0.3806	0.4386	
AChR & LRP4 +							
MuSK & LRP4 +							
Seronegative							

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

We compared the number of patients requiring hospital admissions based on antibody subtypes. There was no significant difference in any of the subgroups (Table 14). None

of our 8 seronegative patients required admission to hospital with myasthenic crises or worsening symptoms when compared to patients with AChR single positives, p value was 0.0535. There was no difference in bulbar symptoms between AChR+ and MuSK+ patients, and although LOMG patients more commonly had bulbar symptoms compared to EOMG patients, AChR positivity (including OMG and GMG) was similar in EOMG and LOMG. This does not differentiate between LOMG patients with GMG who were the ones more likely to be admitted to hospital, and who were more likely to be AChR+ compared to EOMG.

The seronegative patients included had the following clinical presentation: (NB: Of the 8 seronegatives described above, 1 had positive AChR RIAs when recruited, but repeat tests including CBAs were negative. This patient's details were included in the sero-positive group when neurophysiology comparisons were made) (Table 15).

1.NHS LRP4 +, fatiguable ptosis, no response to pyridostigmine

2.NHS MuSK CBA +, Thymic hyperplasia, no MG symptoms

3.Treatment response to pyridostigmine

4.MG post pembrolizumab treatment

5.Treatment response to steroids

6.NHS AChR RIA +, on no treatment, typical ptosis & diplopia, IBM

7.NHS AChR RIA +, typical ocular MG which then generalised

Table 15 Clinical information for seronegatives on study samples (7 patients)

Early onset	2
Late onset	5
Ocular Sx at onset	6
Ocular MG at 3 mths	4
Generalised MG	2
Asymptomatic	1
Bulbar Sx	1
Limb weakness	5
Respiratory Sx	2
Neurophysiology done	5 (all normal)

3.3.6 Effect of co-morbidities in myasthenia gravis

Along with the clinical presentation of myasthenia, a detailed history of other past medical history, comorbidities, smoking history, use of statins, alcohol, occupation, preceding infections prior to the onset of myasthenia, family history of autoimmune conditions, and medication history were obtained in detail. Details of smoking, alcohol and preceding infections are included under the section on MG composite and Qol scores.

Our cohort of patients had several different associated autoimmune conditions, Asthma being the most common, seen in nearly half the patients, followed by hypothyroidism in nearly a

quarter. The full list is in Table 16. 43.58% of EOMG patients had associated autoimmune conditions compared to 34.23% of LOMG patients, p = 0.2989. 43.93% of all the females in the study had associated autoimmune conditions compared to 30.95% of all males, p = 0.1027. 47.82% of female EOMG patients had associated autoimmune conditions compared to 37.5% of male EOMG patients, p = 0.5280.

Table 16 Frequency of other autoimmune conditions in the MG cohort

AI condition	Total	Percentage
Asthma	27	49.09%
Hypothyroidism	13	23.63%
Hyperthyroidism	4	7.27%
Eczema	2	3.63%
Pernicious anaemia	2	3.63%
Primary biliary Cirrhosis	2	3.63%
Type I DM	2	3.63%
Vasculitis-various	2	3.63%
Lupus	1	1.81%
Multiple Sclerosis	1	1.81%
Polymyalgia Rheumatica	1	1.81%
Psoriasis	1	1.81%
Scleritis/Episcleritis	1	1.81%
Urticaria	1	1.81%
Vitiligo	1	1.81%

There was a family history of autoimmune conditions in 40% of all our patients, 51.28% in EOMG and 36.03% in LOMG (p=0.0956). Hypothyroidism was the most common (15/60) followed by myasthenia gravis in 10/60 (2 of these patients belong to EOMG and 8 LOMG). All the affected relatives with

MG bar one were first-degree relatives (Table 17). There was no correlation between thymic abnormalities and autoimmune conditions, either in patients or in their families.

Table 17 Frequency of autoimmune conditions in family members

Conditions	Numbers
Hypothyrodism	15
Myasthenia Gravis	10
Rheumatoid arthritis	9
Multiple Sclerosis	7
Asthma	6
Type I DM	6
Lupus	5
Coeliac disease	2
Hyperthyroidism	2
Inflammatory Bowel disease	2
Pernicious anaemia	2
Polycythaemia	2
Ptosis	2
Aplastic anaemia	1
Macular degeneration	1
MND	1
Myelofibrosis	1
Polymyalgia Rheumatica	1
Pulmonary fibrosis	1

Other than for autoimmune conditions, there was no recurring theme with other comorbidities, other than the most commonly seen comorbidities in an elderly population including hypertension and diabetes. There were a few patients who also had cancers; 5 patients in our cohort had breast cancer- 4 were positive for AChR antibodies, and one was double positive for AChR & MuSK; 1 patient with AChR antibody had thyroid

cancer; 1 patient with gastric cancer was AChR & MuSK+; 2 patients with colorectal cancers were positive for AChR antibodies; 1 patient with lung cancer developed MG after Pembroluzimab infusion and was seronegative, and 1 AChR antibody-positive patient with prostate cancer. We also had two patients with inclusion body myositis of whom 1 was seronegative for MG antibodies and the other positive for both MuSK and LRP4.

We compared AChR titres in patients who were on statins with those who were not on statins; this did not show any statistically significant difference p = 0.630. As salbutamol is used for the treatment of Congenital Myasthenia, we were interested to see whether usage of salbutamol inhalers for asthma made any difference to ACHR titres. There was no significant difference when compared to patients who did not use salbutamol, p = 0.8137; there was no significant difference in their MG composite scores either. (NB: Most patients used salbutamol on an ‘as needed basis’ and not regularly).

3.3.7 Neurophysiological findings

Neurophysiological studies in our cohort consisted of repetitive nerve stimulation (RNS) and/or single fibre electromyography (SFEMG). Some patients also had routine nerve conduction

studies (NCS) and/or electromyography (EMG). When this data was analysed at the end of July 2017, a total of 55 patients had neurophysiology studies done; 18 EOMG patients and 37 LOMG patients. The subdivision was 10 female EOMG and 8 male EOMG, 14 female LOMG and 23 male LOMG. The total number of SFEMGs performed was 49 and of these 14 were normal. Of the 35 abnormal SFEMGs, 33 showed increased jitter and 21 showed conduction blocks. RNS was done in 38 patients, of these, 22 were normal and 16 abnormal, all of which showed a decrimental response. 4 patients also had routine EMGs, 3 of which were normal and 1 showed myopathic changes. 2 patients had NCS, one of which was normal and one showed neuropathic changes. The breakdown of the neurophysiology results along with the antibody titres for these patients is in Table 18.

Table 18 Results of neurophysiological studies. N: Normal, D: Decremental response, J: Increased Jitter, B: Conduction Block, nd : not done

		Patient	RNS	SFEMG	SFEMG	AChR RIA$_{as}$ x10^{-10}mol/l 0/12	AChR CBA 0/12	MuSK CBA 0/12	LRP4 CBA 0/12
EOMG	Female	1	D	J	B	16.46	2.5	2	0.5
		2	N	J	B	1217.73	3	0	0
		3	nd	N		0.72	1	1.5	2.5
		4	nd	N		-0.16	1.5	0	0
		5	nd	J	B	1.70	0	1.5	0
		6	D	J		1855.07	3	0	0
		7	N	N		0.07	2	0	0.5
		8	D	J		-3.65	3	1	0
		9	N	J	B	161.20	2.5	0	0
		10	nd	J	B	25.98	1	0.5	0
	Male	11	D	J	B	6.07	2.5	0	0
		12	nd	J		6.03	2	0	0

		13	nd	N		0.69	0.5	0	0
		14	nd	N		2.20	0	0	0
		15	N	J		4.67	2	0	0
		16	N	N		4.82	0	0	2
		17	nd	J		20.08	2.5	0	0
		18	N	J		91.75	3	0	0
LOMG	Female	19	D	J	B	1640.42	3	0	0
		20	N	J		300.85	3	0	0
		21	N	N		0.77	0.5	0	0
		22	nd	N	B	0.97	0	0	0
		23	D	J	B	374.94	3	0	1
		24	D	J		9.41	0	0.5	0
		25	D	nd		941.02	3	0	0
		26	N	nd	B	0.94	0	0	0
		27	D	J		1.79	3	0	0
		28	N	N		386.55	3	0	0
		29	D	nd		34.45	2	1.5	0

		30	N	nd		1239.29	3	0	0
		31	N	J	B	281.37	2.5	0	0
		32	N	N		58.87	2	0	0
	Male	33	N	nd		177.32	2	0	0
		34	nd	J	B	304.12	2.5	0	2
		35	N	N		191.20	2.5	0	0
		36	N	N		-0.04	0	0	0
		37	nd	J	B	122.61	2.5	0	0
		38	N	N		2.67	0	0	0
		39	N	J	B	185.67	3	0	0
		40	nd	J		68.17	2	0	0
		41	D	J	B	46.74	2.5	0	0.5
		42	D	J	B	15.38	3	0	0
		43	D	nd		12.38	3	2	0
		44	D	nd		312.84	2.5	0	0
		45	nd	N		14.32	2	0	0
		46	N	J	B	451.32	3	2	0

		47	N	N		706.82	2.5	2	2.5
		48	nd	J	B	0.85	0	1	0
		49	D	J	B	69.11	2.5	0	0
		50	nd	J	B	1237.67	2.5	0.75	0
		51	N	J		986.15	2.5	0	0
		52	D	J	B	97.98	3	0	0
		53	N	J		300.32	3	0	0
		54	nd	J		34.03	1.5	0.5	1.5
		55	nd	J		291.82	2.5	0	0

Five of our eight seronegative patients had neurophysiology studies done. (NB: one of these 8 had positive AChR RIAs when recruited, but repeat tests including CBAs were negative and was included in the sero-positive group when neurophysiology comparisons were made).

3.3.8 The Thymus gland in myasthenia

Of our 150 patients, 137 patients had their thymus imaged, and almost all of them were CT scans. Of these, EOMG patients

were 36/39 and LOMG 101/111. Abnormalities were found in a total of 32/137; EOMG 18/36 (50%) and in LOMG 14/101 (13.86%). Thymic abnormalities in EOMG versus LOMG: $p<0.0001$. NB: Not all patients were imaged, and not all patients who had thymic abnormalities on imaging had a histological diagnosis.

Thymic enlargement or hyperplasia (possible hyperplasia on CT or confirmed on histology) were seen in 61.11% of EOMG patients with thymic abnormalities (11/18). The proportion of thymic enlargement/hyperplasia in EOMG when compared with all EOMG patients who had their thymus imaged was 30.55% (11/36), thymoma(possible thymoma on CT or confirmed on histology) was 16.67% (3/36). None of the LOMG patients had thymic enlargement/hyperplasia, EOMG Vs LOMG for thymic enlargement/hyperplasia was significant, $p<0.0001$. Thymoma/thymic mass in all LOMG patients imaged was 10.89% (11/101), compared with EOMG this was not statistically significant ($p = 0.664$). There were 4 EOMG and 3 LOMG patients who had residual thymic tissue, also reported as an abnormality in the statistics above. This classification of thymic abnormalities has been used by Klimiec et al in their paper on the accuracy of routine imaging in predicting thymic pathology (409).

The number of EOMG patients who had a histologically confirmed diagnosis of thymic hyperplasia was 8 and thymoma was 2. The total number of scan abnormalities in EOMG (excluding a confirmed diagnosis of thymoma) was 16 i.e. 43.24%. If we were to assume that, by definition, all EOMG patients have thymic abnormalities, this is perhaps indicative of the numbers in whom the thymus is enlarged enough to be picked up on imaging.

AChR RIAs were compared in patients with thymoma/thymic mass in the LOMG and EOMG groups; there was no statistically significant difference (p = 0.2799) and this still remained statistically insignificant when the seronegatives were taken out of the calculation, p = 0.6593 (Table 19 and Figure 20).

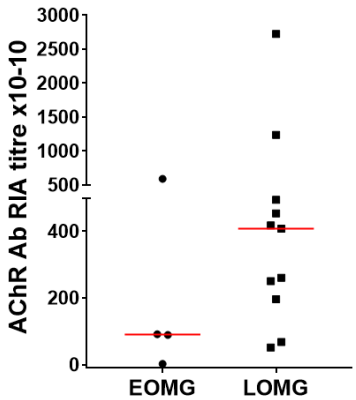


Figure 20 AChR RIA titres (with medians) in EOMG and LOMG with thymoma p=0.2799

Table 19 Thymic abnormalities with antibody titres and scores

Thymic abnormality			AChR RIA as $x10^{-10}$mol/l	Average AChR RIA as $x10^{-10}$mol/l (minus negatives)	AChR CBA	MuSK CBA	LRP4 CBA
EOMG	Thymic hyperplasia	Surgery	16.46	Mean: 149.57 (271.81) Median: 16.46 (65.11)	2.5	2	0.5
			2.75		0	0	0
			1217.73		3	0	0
			23.55		2	0	0
			2.24		0.75	0	1.5
			4.82		0	0	2
			242.9		2.5	0	1
			25.98		1	0.5	0
		CT (or surgery done but no histology as yet)	2.0		0	2	0
			2.64		1	0	0
			104.24		3	0	0
	Thymoma	Surgery	592.88	Mean: 503.29	3	1.5	0
			91.75	Median: 91.75	3	0	0
		CT	89.59		2.5	0	0
	Residual thymic	Surgery					
		CT	0.72	Mean: 6.80	1	1.5	2.5

	tissue		22.46	(15.08)	2	0	0
			7.70	Median: 4.21 (15.08)	0	0	1
			-3.65		3	1	0
LOMG	Thymic hyperplasia	Surgery	-		-	-	-
		CT	-		-	-	-
	Thymoma	Surgery	494.18	Mean: 596.89	3	0	0
			260.85		1.5	0	0
			408.02	Median: 408.02	3	0	0
			68.17		2	0	0
			2725.15		3	0	0
			51.56		2.5	0	0
		CT (or surgery done but no histology as yet)	453.64		3	0	0
			250.66		2.5	0	0
			1239.29		3	0	0
			417.90		2.5	0	0
			196.38		3	0.5	0
	Residual thymic tissue	Surgery	197.27	Mean: 282.55	3	0	0
			34.69		2.5	0	0
		CT	615.69	Median: 197.27	2.5	0	0

3.3.9 MG composite scores

The MG composite score is a clinical questionnaire based on clinical examination and history taking. The minimum score is zero (asymptomatic) and the maximum 50 (severe MG, on mechanical ventilation). We filled in MG composite scores on all patients at recruitment and at annual follow-up (f/u). When we compared the MG composite scores in all patients at point of recruitment with first year follow-up, there was a significant change in the medians (6 at recruitment and 0 at 1st year f/u) with $p<0.0001$ (Figure 21A and B). This was reflected in both the EOMG group where medians were 6 at recruitment and 0.5 at first year follow-up, $p<0.0001$; and in the late onset group with a median of 7 at recruitment and 0 at first year follow-up, $p<0.0001$.

We further analysed the MG composite scores in patients who were immunosuppression naive at recruitment and immunosuppressed at first year follow-up. There was a significant difference in medians of 7 at recruitment and 0 at first year follow-up, $p< 0.0001$.

When the MG composite scores were compared between EOMG and LOMG groups this was significantly different,

p=0.0287; the median for EOMG was 6 and for LOMG was 7 (Figure 23).

We compared MG composite scores in patients who had a history of preceding infections prior to the onset of MG with those who did not give any history of preceding infections. There was no significant difference in their MG composite scores. This was the same in both EOMG and LOMG groups.

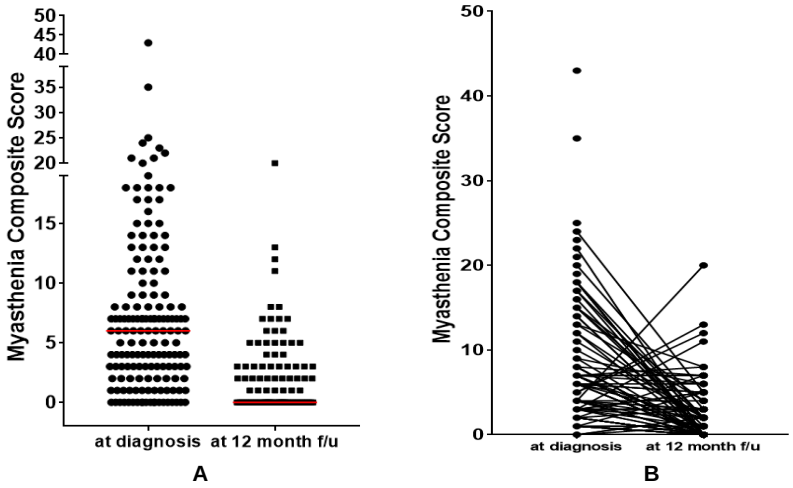


Figure 21 MG composite scores at recruitment and first year follow up in all patients A: with medians, B: paired p<0.0001

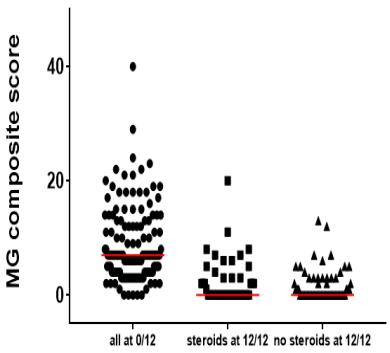


Figure 22 MG composite scores (with medians) at recruitment and at first year follow up, subdivided into those who were given steroids in the first year to those who were not

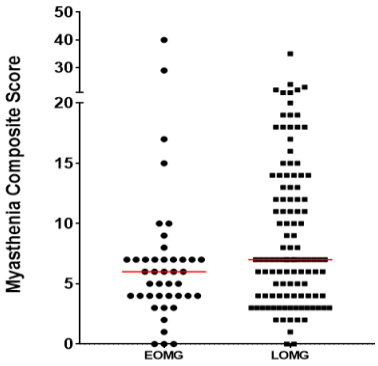


Figure 23 Comparison of diagnositic MG composite scores between EOMG and LOMG (with medians) p=0.0287

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

MG composite scores were compared between patients with a smoking history and non-smokers; there was no significant difference, p = 0.9698, and was similar in the EOMG and LOMG groups. The MG composite scores in the male smokers compared to the female smokerswas also not statistically significantly different, p = 0.0803 (Figure 24).

Patients with a history of alcohol intake were divided into groups- ‘consuming no alcohol’, ‘drinking alcohol rarely’, ‘moderate amounts of alcohol’ and ‘history of alcohol excess’. They were compared using the one way ANOVA test. There was no significant difference amongst the groups.

MG composite scores were compared between patients who used salbutamol inhalers for asthma with those that did not; there was no statistical significance between the groups.

MG composite scores at point of recruitment were compared between those patients who were immunosuppressed at recruitment with those patients who were immunosuppression naive at recruitment. The median was 17 for the immunosuppressed patients and 6 in the non-immunosuppressed patients with $p < 0.0001$ (Figure 25).

The MG comp scores of patients who were immunosuppression naive at recruitment but were immunosuppressed at first year follow-up were compared; the median for first year follow-up was

0 and at recruitment was 1 and there was no statistical significance, p = 0.7099 (Figure 22). Similarly at the second year follow-up there was no statistically significant difference between patients who were immunosuppression naive at recruitment but had been immunosuppressed at second year follow-up.

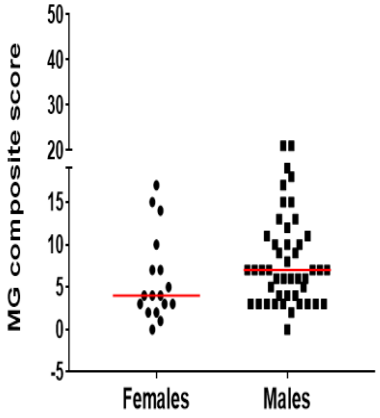


Figure 24 Comparison of diagnostic MG composite scores between female and male smokers (with medians) p=0.0803

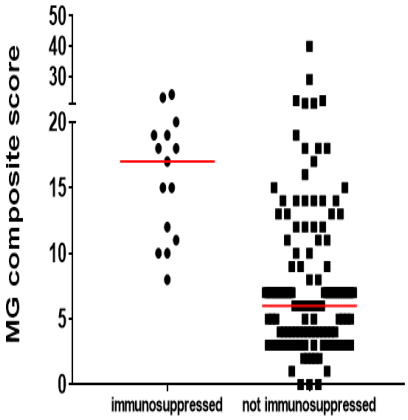


Figure 25 Comparison of diagnostic MG composite scores (with medians) in patients immunosuppressed at recruitment with those who were immunosuppression naïve p<0.0001

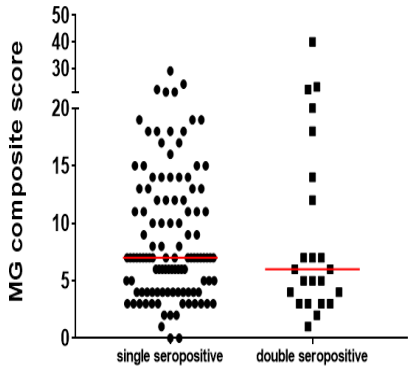


Figure 26 Comparison of MG composite scores (with medians) in single Ab positive patients with double seropositive patients p=0.4694

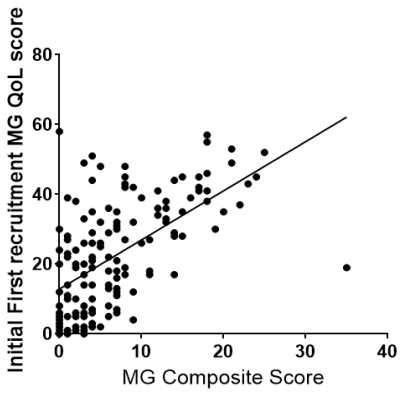


Figure 27 Correlation between MG Composite scores and MG QoL at recruitment p<0.0001 and R^2 of 0.3309

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Figure 17: Simple linear regression used

MG composite scores were compared in patients who were seropositive for a single antibody compared to all double seropositives in any combination; there was no statistically significant difference between their medians, p = 0.4694 (Figure 26).

We compared the MG composite and QoL scores at recruitment; there was a significant correlation, with p<0.0001 and R^2 of 0.3309 (Figure 27).

3.3.10 Quality of life scores

MG quality of life score (MG-QoL 15) measures how the patients perceive their illness and how they feel that myasthenia impacts on their lifestyle. We measured MG-QoL scores at recruitment and at annual follow-up. The minimum score obtainable is 0, meaning the patient was happy, and the maximum is 60, meaning the patient had a very poor quality of life. We compared the MG-QOL scores at recruitment and at annual follow-up; there was a significant difference in the scores, median at recruitment was 22 and at first year follow-up was 5, p<0.0001 (Figure 28A and B, Figure 33). This difference was more significant in the LOMG group with a median of 22 at recruitment and 5 at first year follow up, p<.00001 (Figure 31). For the EOMG patients the difference was just below

significance with medians of 18 at recruitment and 4.5 at first year follow-up, p = 0.0594 (Figure 30).

There was a significant difference in the MG-QOL in patients who were treatment naïve at recruitment and were given immunosuppression at first year follow up (median 27.5 at point 0 and 10 at year one follow-up, p <0.0001) (Figure 32).

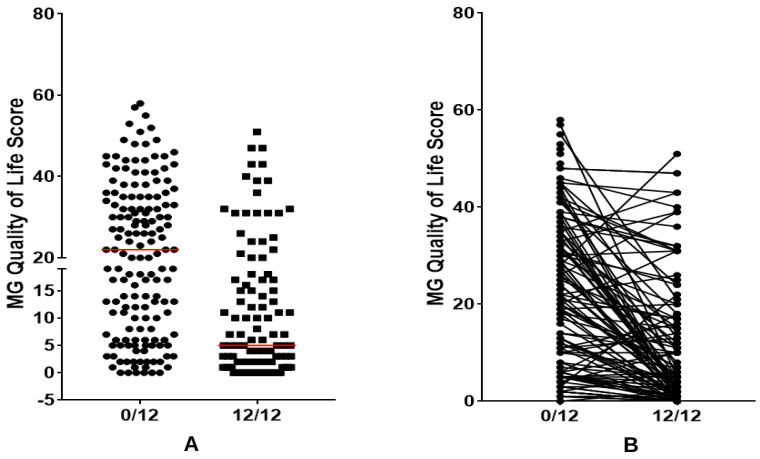


Figure 28 Comparison of QoL scores at recruitment and first year follow up in all patients; A: with medians, B: paired p<0.0001

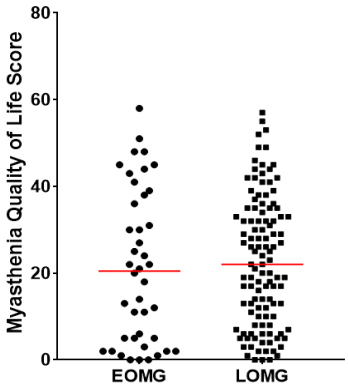


Figure 29 Comparison of MG QoL (with medians) between EOMG and LOMG at recruitment p=0.4629

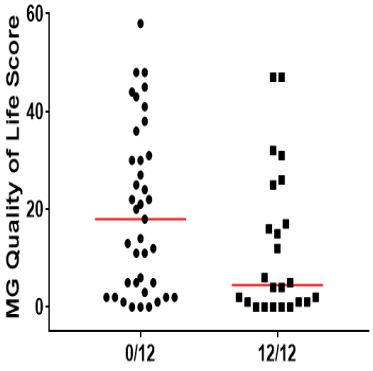


Figure 30 Comparison of QoL scores (with medians) at recruitment and first year follow up in EOMG patients p=0.0594

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

We looked at correlation between changes in composite score and QoL from point of recruitment to 1st year follow-up; the trend was improvement in both parameters with a few outliers (Figure 34).

There was no difference between MG-QOL scores between EOMG and LOMG patients at recruitment ($p = 0.4629$) (Figure 29); there was no significant difference in MG-QOL in patients who had preceding infections prior to MG onset compared to those who did not, and this was the same in both the EOMG and LOMG groups.

There was no difference in the MG QoL between smokers and non-smokers, $p = 0.3311$; this was the same in the EOMG and LOMG subgroups. There was no difference in MG-QOL scores between the male and female smokers either. There was no difference between the MG-QOL scores between patients who drank no alcohol, compared to the other categories which were ‘rarely drinks alcohol’ ‘moderate alcohol’ and ‘excess alcohol’.

There was no statistically significant difference between MG-QOL in patients who were on statins compared to those who were not on statins.

There was no difference in MG-QOL scores between patients who were positive for a single antibody compared to those who were positive for more than one antibody in any combination.

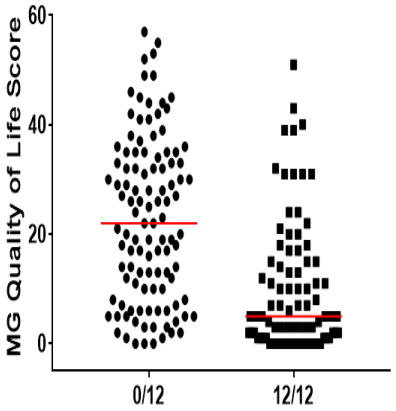


Figure 31 Comparison of QoL scores (with medians) at recruitment and first year follow up in LOMG patients p<0.0001

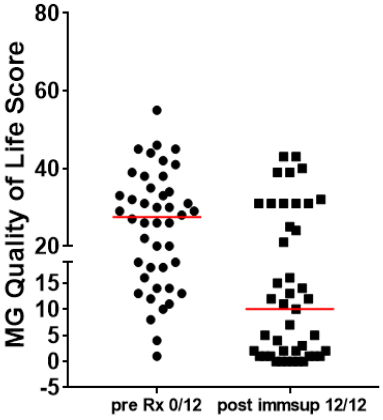


Figure 32 Comparison of QoL scores (with medians) at recruitment and follow up in patients who were immunosuppression naive at recruitment and were treated with steroids in the first year p<0.0001

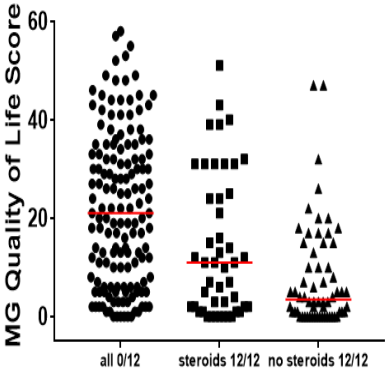


Figure 33 Comparison of MG QoL scores (with medians) at recruitment (treatment naive) with first year follow up - on immunosuppression and without immunosuppression

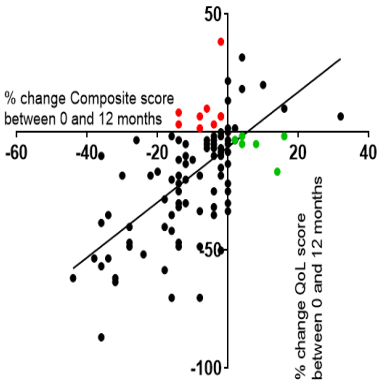


Figure34 Correlation between percentage change MG composite scores and QoL scores over the first year

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Figure 34: Simple linear regression used

3.3.11 Myasthenia related admissions to hospital

We analysed the data up to the end of July 2017 looking at patients who were admitted to hospital during the period of the research study over three years. 6/39 EOMG patients (15.38%) and 40/111 LOMG patients (36.03%), p = 0.025, were admitted to hospital (Table 20). The total number of admissions also differed between the younger and older patients, older patients frequently requiring more than one admission (EOMG Vs LOMG admissions, p = 0.0036).

Table 20 MG related admissions (Percentages are of total admissions, 7 for EOMG and 15 for LOMG)

		EOMG (Patients 6/39=15.38%)		LOMG (Patients 40/111=36.03%)	
		Number	Percentage	Number	Percentage
Reason for admission	Never known MG diagnosed at admission	2	28.57%	15	30%
	Admission for another reason, diagnosed with MG during admission	0	0%	1	2%

	Elective admission for immunosuppression	2	28.57%	14	28%
	Admission for exacerbation of MG Sx	3	42.86%	13	26%
	Admission for non MG reasons	0	0%	5	10%
	Admission with side effects of MG Rx	0	0%	2	4%
	Total admissions	7	17.95%	50	45.05%
Treatment given during admission	Pyridostigmine only	1	14.29%	1	2%
	Steroids only	2	28.57%	13	26%
	ivIg only	0	0%	4	8%
	Plex only	0	0%	1	2%
	ivIg and Steroids (and additional immunosuppressant in some)	4	57.14%	23	46%
	ivIg, steroids and	0	0%	1	2%

	PLEX				
	Steroids and other immunosuppressant	0	0%	2	4%
	Other Rx (eg. Antibiotics)	0	0%	5	10%
	PEG/NG fed	1	14.29%	2	4%
	ITU admission	1	14.29%	2	4%
	Good outcome	3	50%	37	92.5%
Outcome	Moderate outcome	3	50%	2	5.12%
	Death	0	0%	1	2.56%

There were several different reasons for admission; some patients were admitted with myasthenic symptoms but without a known diagnosis at that point, but most admissions were because of exacerbation of myasthenia symptoms or elective

admissions for initiation of steroids. There were a couple of admissions because of side-effects of myasthenia treatment. More than half the patients required a combination of IV immunoglobulins and steroids (and also an additional immunosuppressant in some patients) in both the EOMG and LOMG groups. About a quarter of the patients required steroids only, 3 patients in total required ITU admission- 1 EOMG and 2 LOMG patients. The majority of patients in the LOMG group had a good outcome; half the patients had a good outcome in the EOMG group and the other half were categorised as moderate outcome. There was one death in the LOMG group during admission.

The overall mortality over the three-year period was 4/150 (2.6%), all of them in the LOMG group. One female patient died from aspiration pneumonia; of the three male patients, one had cardiac failure and dilated cardiomyopathy with AF and was thought to have passed away from non-myasthenia related problems. One patient had acute coronary syndrome and respiratory arrest which was thought to have been contributed to by myasthenia, the fourth patient had a malignant thymoma, although the exact cause of death was not clear. Overall, the mortality rate was in keeping with the published data of around 2.2%.

3.3.12 Treatment and effect of early immunosuppression on disease progression

Of our 150 recruits, 15 patients were immunosuppressed at the time of inclusion into the study. This was because of bulbar or respiratory symptoms and/or myasthenic crisis. 14 of the 15 belonged to the LOMG group, 4 females and 10 males; and 1 was a younger female. Of the 15, 14 had generalised MG at diagnosis and one had OMG. The majority of patients were older- this could be due to several factors including other co-existing comorbities which meant that they sought or were referred to hospital earlier than younger patients, or as described earlier in the chapter, LOMG patients had a higher MG composite score at recruitment compared to EOMG, perhaps indicating a more symptomatic onset in these patients. All 15 patients were given steroids, 10 patients had IVIG, and none of the patients had plasma exchange or IVIG on its own. 8 patients had a combination of IVIG and prednisolone, 2 patients had a combination of IVIG, prednisolone and were started on azathioprine, and 5 patients had prednisolone only. The dosage of IVIG used was 0.4g/Kg/day for five days. In all patients prednisolone was started at either 5 mg or 10 mg a day (or equivalent alternate day dosing) and increased gradually to between 25 mg a day and 60 mg a day. One of the patients was admitted to ITU, needed mechanical ventilation, had a respiratory arrest and an acute coronary event and died.

Amongst the remaining 135 patients, all patients were treatment naïve at recruitment which ensured a realistic comparison of treatment response. We divided the daily dose of steroids required into categories: <5 mg, 5 to 9.9 mg, 10 to 14.9 mg, 15 to 19.9 mg, 20 to 29.9 mg, 30 to 39.9 mg, 40 to 49.9 mg and ≥50 mg. The distribution of numbers and the comparison of doses are listed in Table 21. NB: during analysis of the data, less than half the patients (65) had had a second year follow-up.

The daily dosage of steroids required in all patients was compared at first year follow-up and second year follow-up; there was a significant difference in dosage ($p = 0.0053$) (Figure 35A and B). There is no set way to measure steroid requirements. We documented what dose of steroids the patient needed at the time of assessment (yearly follow ups) for symptom control and compared these doses.

Table 21 Distribution of steroid doses- number of patients

Steroid doses OD	<5MG	5-9.9MG	10-14.9MG	15-19.9MG	20-29.9MG	30-39.9MG	40-49.9MG	≥50MG
Ocular MG Steroids 12/12	1	8	5	2	4	0	1	1
Gen MG steroids 12/12	1	8	7	2	4	3	1	2

All MG 12/12	2	16	12	4	8	3	2	3
Ocular MG steroids 24/12	6	3	5	0	1	0	0	0
Gen MG steroids 24/12	1	3	3	2	1	0	0	0
All MG 24/12	7	6	8	2	2	0	0	0
EOMG steroids 12/12	0	1	0	3	1	0	0	1
LOMG steroids 12/12	2	15	12	1	7	3	2	2
EOMG steroids 24/12	1	2	1	0	0	0	0	0
LOMG steroids 24/12	7	4	6	2	2	0	0	0

There was a significant difference in the steroid doses in EOMG (OMG and GMG) at first and second year follow-up, the median being 16 mg at 1st year follow-up and 6.25 mg at second year follow-up, p = 0.0333 (Figure 35C). There was a difference also in steroid doses in LOMG (OMG and GMG) at first and second year follow-up, median of 10 mg at first year follow-up and 7 mg at second year follow-up, P = 0.0195 (Figure 35 D).

There was no significant difference in the steroid doses between EOMG and LOMG at first year follow-up, p = 0.2515 (Figure 36A) or at second year follow-up, p = 0.5290 (Figure 36B).

Steroid doses in OMG at first and second year follow-up were significantly different, p = 0.0117, with a median of 10 mg at first year follow-up and 7 mg at second year follow-up (Figure 36A). The average steroid dose in GMG at first and second-year follow-up did not show any difference, the medians being 10 mg in both cases, p = 0.2502 (Figure 36B).

There was no difference in steroid dose between OMG and GMG at first year follow-up p = 0.6862; there was no statistically significant difference in steroid dosages in OMG and GMG at second year follow-up, 7 mg in OMG and 10 mg in GMG, p = 0.1674 (Figure 38).

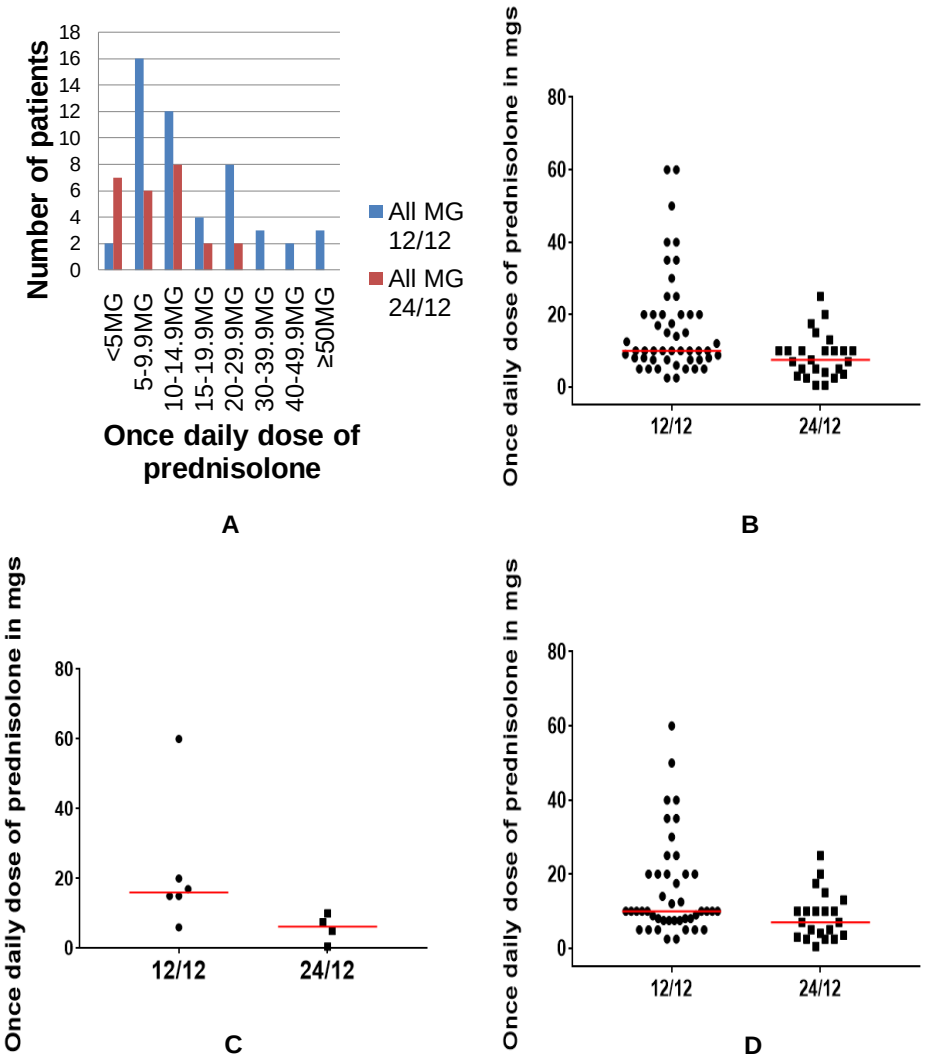


Figure 35 Comparison of steroid doses at 1st and 2nd year follow up (with medians). A: number of patients in each category, B: in all patients p=0.0053, C: in EOMG p=0.0333 D: in LOMG p=0.0195

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

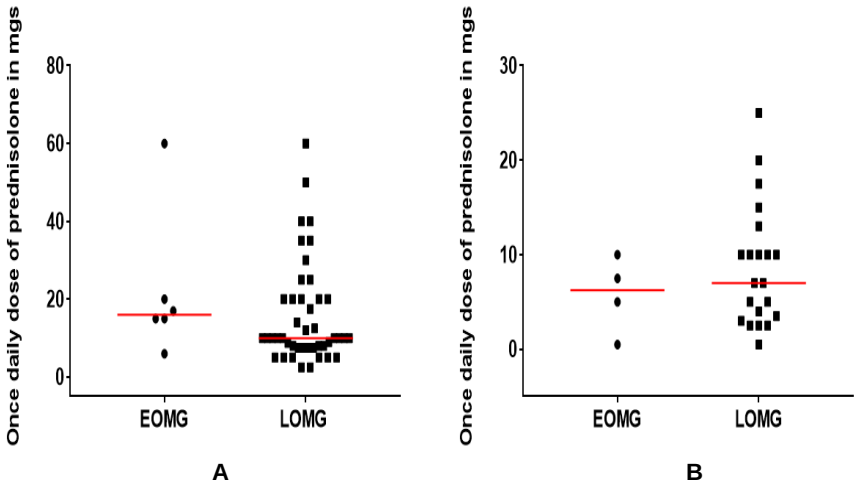


Figure 36 Steroid doses (with medians) in EOMG and LOMG at (A) first year p=0.2515 and (B) second year follow up p=0.5290

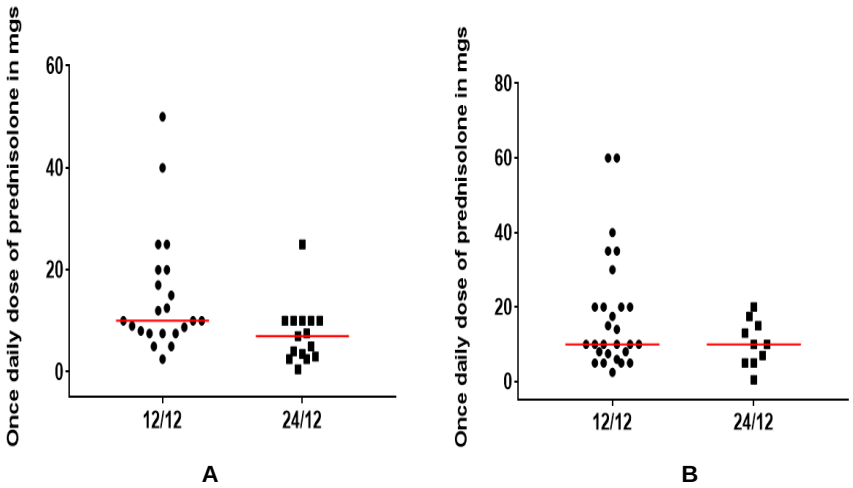


Figure 37 Comparison of steroid doses (with medians) at 1st and 2nd year follow up in (A) OMG p=0.0117 and (B) GMG p=0.2502

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

We compared the data for conversion rates for OMG to GMG. The survival curve did not show a significant difference. When the medians were assessed using the Mann Whitney-U test, there was no difference in P value either. There was no

difference in time to generalisation between LOMG and EOMG (p = 0.1353) (Figure 40).

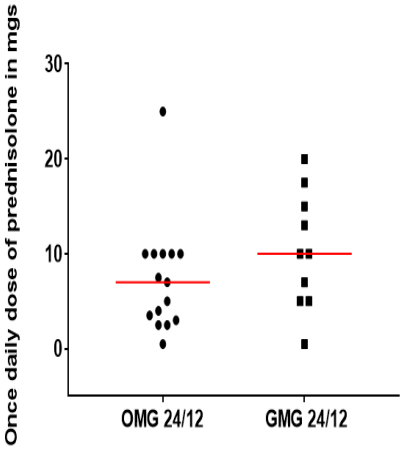


Figure 38 Comparison of steroid doses (with medians) between OMG and GMG at 2nd year follow up p=0.1674

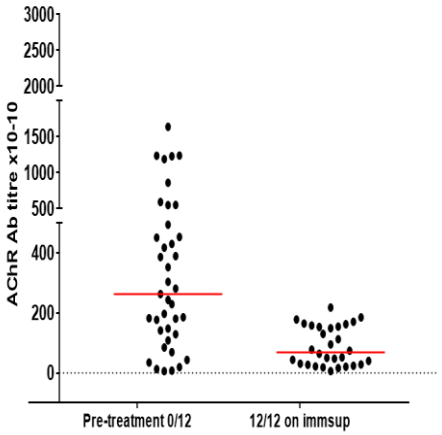


Figure 39 Comparison of AChR RIA titres (with medians) pre-treatment at recruitment, with titres at 1st year follow up on immunosuppression p<0.0001

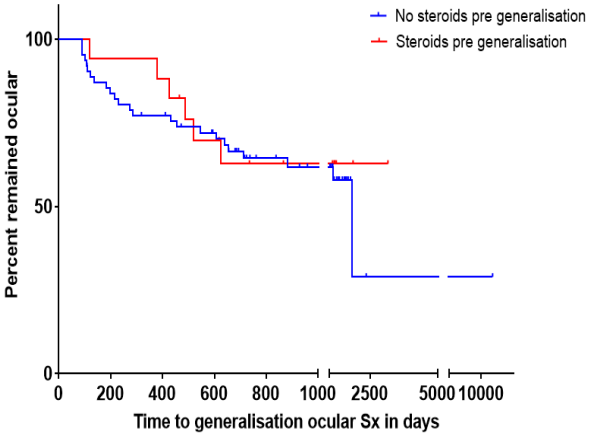


Figure 40 Survival curve for time to generalisation of OMG with and without steroids pre-generalisation p=0.1353

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Survival curve data was analysed using the Log Rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test)

MGFA PIS scores were calculated for all patients at annual follow-up. When the MGFA PIS scores were compared at first year follow-up between EOMG and LOMG, there was a difference, LOMG patients did better than EOMG, similarly at second year follow-up (Figure 42). When all patient categories were compared, there was no significant difference in their PIS scores at first and second year follow-up (Figure 41).

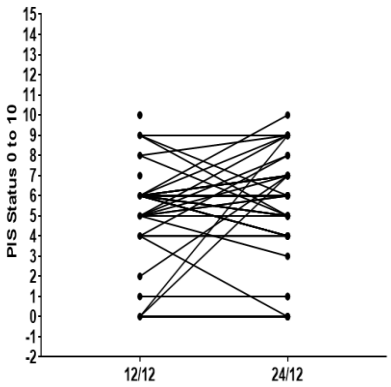


Figure 41 MGFA-PIS in all patients at 1st and second year follow up. 0: CSR, 1: PR, 2: MM-0, 3: MM-1, 4: MM-2, 5: MM-3, 6: I, 7: U, 8: W, 9: E, 10: D

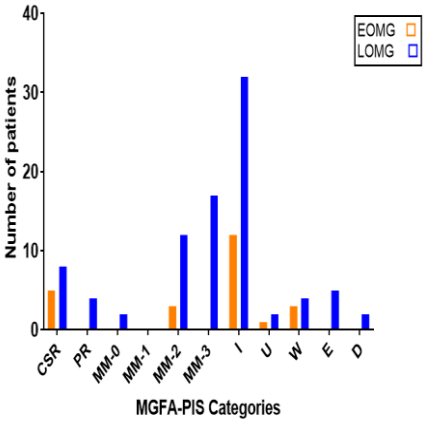


Figure 42 Comparison of MGFA-PIS in EOMG and LOMG at 1st year follow up

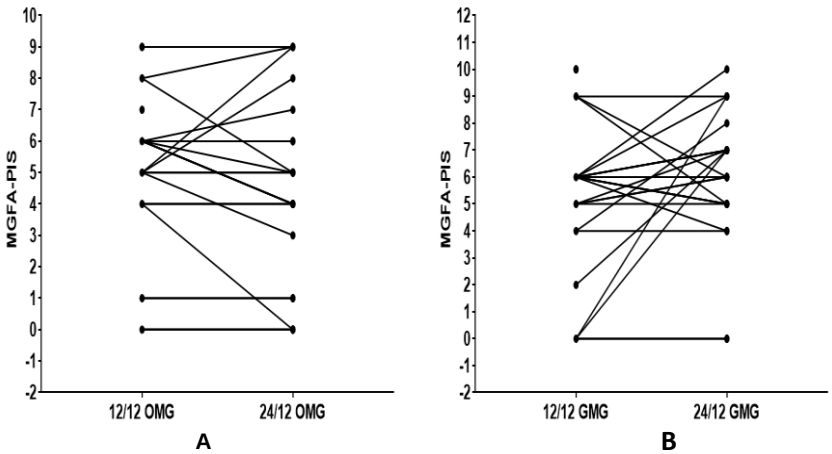


Figure 43 MGFA-PIS (paired) patients at 1st and second year follow up in (A) OMG and (B) GMG. 0: CSR, 1: PR, 2: MM-0, 3: MM-1, 4: MM-2, 5: MM-3, 6: I, 7: U, 8: W, 9: E, 10: D

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

When MGFA PIS scores in OMG patients was compared at first and second year follow-up, there was an improvement (Figure 43A). When MGFA PIS in GMG were compared at first and second year follow-up, there was no statistically significant difference (Figure 43B).

Clinical improvement as measured by the MG composite score had also improved from point of recruitment to first year follow-up; and although there was no statistically significant improvement between first and second year follow-up, the trend was towards improvement. Similarly with the QOL scores, there was an improvement between scores at recruitment and first year follow-up and although patients continued to improve, there was no statistically significant difference between first and second year follow-up scores.

AChR RIA titres also fell significantly from point of recruitment to follow-up, with a significant improvement in the first year, and a further slight reduction in the second year (Figure 39).

3.4 Discussion of clinical findings

In the introductory chapter, we listed a set of questions which we sought to answer with our prospective study. We were able to answer almost all these questions based on the results above.

Is the incidence of LOMG greater than that of EOMG?

Epidemiological studies in the last 65 years have shown that late onset myasthenia gravis is becoming increasingly more common. In our cohort of 150 MG patients, more than three quarters of the patients (76%) were over the age of 50 years, in keeping with published data. The mean age of female patients was 57.6 years and in males was 61.24 years, again depicting the more frequent incidence in late-onset group. We found a difference in the medians of peak age of 4 years in male and female EOMG patients (lower in females) which is less than that reported by Somnier et al but the peak age in LOMG males and females is no different, which is consistent with that previously reported (406). Whilst our study recruited all the newly diagnosed patients in the Trent region, this was not the case with Birmingham and Oxford and so we could not comment on increasing incidence rates of LOMG overall, but we have shown that that LOMG is more common than EOMG.

Could there be a cohort effect leading to increasing incidence of LOMG? Could this be attributed to environmental exposure, Immunisation or viral infections?

From our detailed questionnaire (listed in chapter 2), we were unable to ascertain any environmental, infectious, occupational or medication triggers in our patients in either EOMG or LOMG. This analysis was based purely on the questionnaire and no

laboratory or immunological tests were done to look into this. We did not check for EBV infection for example which has previously been shown to have some association with MG (144).

Is there a difference in the sex distribution between LOMG and EOMG?

In Jon Aarli's paper of 2008 he found that the female to male ratio was 1:1.1 for LOMG, and 3:1 in EOMG (96). Evoli and colleagues stated a female to male ratio of 1:1.9 in LOMG (151). Our data has shown a female to male ratio of 1:1.6 in LOMG and 1.4:1 in EOMG. Our study has shown that late-onset myasthenia gravis in male patients has increased even further whilst the number of female EOMG patients has dropped.

There have been a few studies on racial distribution in MG. Population-based studies in several different countries and across continents show a similar incidence and prevalence rate and similar distribution of EOMG and LOMG patients. A study by Oh and colleagues in Alabama, USA showed that AChR Ab positivity was more common in white americans (WA) compared to African-americans (AA), SNMG AA patients were more likely to be MuSK Ab positive, three quarters of OMG AA patients were seronegative, and disease onset was earlier and more common in females amongst AA patients compared to WA patients in whom it was later in onset and more common in males (410). Whilst this was large study with 235 patients and

good follow up, it was a retrospective study looking at patienst treated in a single neuromuscular clinic, and may not reflect the true incidence or prevalence. Another study in Norway and Netherlands looked at the prevalence between the native and emigrant population and did not find any difference. They noted that the incidence of MuSK MG and MG with thymoma was higher in the emmigrant population compared to the native population (114). In our cohort, there was no significant difference in racial distribution across the three regions where patients were recruited i.e. West Midlands, East Midlands and the South East England. Whilst recruitment in Birmingham and Oxford was selective, we recruited all patients with a diagnosis of myasthenia across Nottinghamshire and Derbyshire excluding the northern districts, and hence these results are truly representative of the racial distribution of myasthenia in the area.

Is OMG more common in LOMG or EOMG? And, Is the rate of generalisation of ocular symptoms different in LOMG and EOMG?

Ocular myasthenia gravis (OMG) has been more difficult to define because of varying time limits applied across several different studies. Defining OMG based on ocular symptoms at diagnosis or recruitment is completely arbitrary and does not provide any useful clinical information about disease

progression (172, 173). Oosterhuis suggested a minimum of three months as a limit for purely ocular symptoms before classifying a patient as having OMG (169). Similarly Sommer et al and Monsul et al also suggested purely ocular symptoms for at least three months from symptom onset to class them as OMG (170, 171).

We defined our cohort of OMG patients as all patients who had purely ocular symptoms up to and including three months from symptom onset. This was based on previous literature but also on our own findings of 'time of symptom onset'. We found that the median time for bulbar, generalised and respiratory symptom onset was less than three months. There is an argument to classify some of the GMG patients who generalised quite early on in their illness as 'early generalisers', those that generalised just after 3 months as 'intermediate generalisers' and those that generalised after 2 years as 'late generalisers'. For the purposes of this thesis, this distinction has not been made

Whilst previous studies have shown that OMG was more common in LOMG compared to EOMG (155, 156) neither of the papers mentions how they defined OMG. This means that interpreting this data is almost impossible and difficult to compare. Our study has shown that younger patients have ocular myasthenia gravis more frequently compared to LOMG,

whereas the older patients more frequently have generalised myasthenia gravis. Generalisation of OMG has not been reported in an entirely treatment naïve cohort of patients before. We found that the generalisation time from OMG is similar in both EOMG and LOMG patients. It appears that the majority of the patients generalise in under a year, more frequently in LOMG than EOMG. We had reviewed 120 patients for their first year follow-up and 65 patients for their second year follow-up at the time of data analysis, and although this data was not complete at the time of writing, the numbers are still significant enough to reflect realistic generalisation rates. As there are no large-scale studies looking at treatment naive patients over a course of time, we did not have any published data to compare this against. The one study by Kamarajah et al which studied 93 MG patients from symptom onset over a period of 11 years (and for which the author and chief investigator of this research study are co-authors), looked at the natural history of ocular patients who were not treated with immunosuppression. They found that 46% of the patients during the study period developed GMG. They also showed that the median time to generalisation was earlier in patients who were AChR antibody-positive, had bilateral ptosis at onset, and were younger in age (174).

In our own cohort, there was no difference in presentation with unilateral or bilateral ptosis in the EOMG and LOMG patients, and there was no significant difference between time to

generalisation between unilateral ptosis and bilateral ptosis in either group. In patients who had ptosis only, there was a difference between the EOMG and LOMG groups with generalisation occurring more quickly in the older patients (perhaps due to smaller numbers in the EOMG group who had ptosis only); but when generalisation rates were compared between the ptosis only group and patients who had both ptosis and diplopia, there was no statistically significant difference. It is not entirely clear whether presenting with ptosis only in an older patient is an independent risk factor for generalisation.

There have been several studies looking at the effect of prednisolone on the progression of OMG to GMG. Several of the studies suggest that early treatment with steroids decreases the progression of OMG to GMG. The EPITOME study which was the only RCT designed to look prospectively at steroid response in patients with OMG was not completed as planned although they did suggest that low dose prednisolone appeared to be safe and well tolerated for treatment for all types of MG (176). In our cohort, when we looked at the survival curve for time to generalisation in OMG patients who were given steroids pre-generalisation against those who were not given steroids pre-generalisation, there was no difference. There was no difference between the EOMG and LOMG groups either. There was no difference in time to generalisation of OMG between LOMG and EOMG; however, LOMG patients as a whole developed

generalised symptoms earlier than EOMG, which was just under statistical significance, p=0.0581 (described below). From our data, it appears that treating an OMG patient with steroids does not change generalisation rates. This is important in clinical practice; however, this was not a drug trial. In order to confirm or refute this, an RCT would be required. Given that the well designed, international EPITOME trial failed to recruit patients, this may be difficult to do, but should nonetheless be tried.

There was no significant difference between VFQ 25 scores VFQ 10 supplement scores in EOMG and LOMG groups.

Essentially, our data shows that OMG is more common in EOMG than LOMG, which is different to previously published data. This difference is perhaps in part due to different definitions of OMG used, and partly because our study was a prospective longitudinal study whilst the published papers were retrospective. However, there is no difference in the type of ocular presentation or generalisation rates of OMG in EOMG and LOMG. This is novel data that has not been published before.

Is there a difference in clinical presentation between LOMG and EOMG?

Because of the prospective nature of our study, recruiting patients when they were treatment naïve, and within the first

year of diagnosis, we were able to plot the onset of all MG symptoms from point zero and compare the median times of symptom onset for all the different symptoms. This has not been done before and is novel data. The median time of symptom onset for all symptoms when compared shows that the longest median time of symptom onset is less than three months at 87 days. This was one of the reasons why we defined ocular MG as ocular symptoms only for more than 3 months.

Although the general order of symptom onset seems to be similar in all patients, with ocular symptoms occurring first, followed by other generalised symptoms, there was a difference in the order of symptom onset between EOMG and LOMG groups. Neither presentation with ocular symptoms (more common in EOMG) nor generalisation rates (quicker in LOMG) were significantly different. EOMG patients presented with dysphagia, dysarthria, neck weakness and respiratory symptoms at a much later stage than LOMG patients which was statistically significant. The median time of onset of chewing difficulties and limb weakness was not significantly different between the two groups.

During the course of the disease, bulbar symptoms were seen much more commonly in LOMG patients compared to EOMG patients. There was no difference in limb weakness in the EOMG and LOMG groups, nor with respiratory problems. There

was no difference in symptom presentation in males and females in any of the groups.

We looked at the timing of the worst MG composite scores from symptom onset in all patients and found that the majority of the patients had the worst MG composite score during the first year, in nearly 3/4 of them, and most of them within the first 100 days, in 53.33%. This would suggest that patients are at their worst with MG-related symptoms during the first year and subsequently improve, with or without treatment. On subgroup analysis, there was no difference in time to worst MGC between EOMG and LOMG.

We compared MG composite scores in patients who were diagnosed early compared to those in whom there had been a diagnostic delay and there was no difference. There was a significant difference in time to diagnosis from symptom onset between EOMG (median of 13.5 mths) and LOMG (median of 2 mths), older patients being diagnosed quicker than EOMG. This is different to previously published data (99). The study by Vincent et al was a very large prospective study using positive AChR Ab test results from all UK centres. This was not a clinical study and was not longitudinal. Our study on the other hand is prospective with high quality recruitment including referrals from other Neurologists, GPs, Ophthalmologists, Neurophysiologists and Laboratory data. The patients were recruited from regions

where there was a neurology service which ensured better pick up rates and reduced selection bias. Also, LOMG patients more commonly present with GMG, are more likely to have other comorbid conditions, and more commonly require admission to hospital, which may be why we see this difference.

Older patients were also more likely to require admission to hospital. This could be multiple times compared to younger patients. There are contradictory reports in literature, where some studies suggest that MG crises are more common in younger females, whilst other show no difference (390) (392, 394). It is to be noted that, in our cohort, amongst the LOMG patients needing admission, nearly three quarters were not for MG crises (Table 19), whereas nearly half the admissions in EOMG patients were with MG crises. So, whilst the total number of admissions is greater in LOMG patients, within the total MG admissions, MG crises were more common in younger patients. One could argue that neurologists have a lower threshold for admitting older patients to hospital as they are more likely to have other co-morbidities making them more susceptible to deterioration/ steroid dips, whereas younger patients are admiited when they are clinically much worse and/or in crisis.

MG composite scores, which we used as our tool for clinical assessment for patients at recruitment and follow-up, showed a significant change in median scores at first year follow-up

compared to at recruitment in both EOMG and LOMG. Similarly, this difference was seen in patients who were treatment naïve at recruitment and were immunosuppressed at first year follow-up. There was also a significant difference in the MG composite scores between EOMG and LOMG groups, being higher in LOMG compared to EOMG. This suggests that patients do better with time, and that older patients have a more severe illness at onset.

The MG composite scores for the 15 patients who were immunosuppressed at recruitment compared to the patients who were immunosuppression naïve at recruitment showed a significant difference. This was likely to be reflective of the fact that patients who required immunosuppression at recruitment were at the severe end of the disease spectrum, requiring admission to hospital in crises or bulbar/respiratory symptoms. MG composite scores in patients who were immunosuppression naïve at recruitment but were immunosuppressed at first year follow-up did not show any statistical significance and there was no difference at second year follow-up either.

The MG composite scores and QOLs seem to have a linear correlation. There was no difference between MG QOL between EOMG and LOMG patients at recruitment. The MG QOL in our cohort also improved when recruitment scores were compared with first year follow-up and this difference was more significant

in the LOMG group compared to the EOMG group. The QoL scores in the older patients may have been influenced by other co-existing medical problems. There was also a significant difference in MG QOL in patients who were treatment naïve at recruitment and those who were immunosuppressed in the first year. LOMG patients had worse MG composite scores and QOL scores at recruitment but seemed to respond well to treatment clinically and in quality of life, doing much better than EOMG patients.

There was no difference in MG composite or MG QoL scores in patients who had preceding infections prior to the onset of MG symptoms to those who did not; there was no difference in both the scores between smokers and non-smokers. Alcohol intake and salbutamol inhalers did not make any difference to their scores either. Although unlikely, the reasoning behind asking about inhalers was to see if Salbutamol, which is used in some forms of congenital MG, made any difference to the symptoms. This is of course not a direct comparison, as the doses used and methods of delivery are different in the two conditions. There was no difference in MGC or MG QOL in patients who were single positive for one antibody compared to double positives.

There were other associated symptoms seen in our patients, including dry mouth in 8.6% urinary symptoms in 2.67%, fatigue in 6%, and weight loss in 7.33%. This is similar to reported

literature, although none of the studies were longitudinal cohort studies (71, 83, 411). Whether urinary symptoms are related to MG or to the use of pyridostigmine is unclear.

Other autoimmune diseases associated with myasthenia have been reported to be very common, the most common being autoimmune thyroid disorders (91). It has also been reported that the frequency of second autoimmune disorders is higher in females and EOMG group who are more likely to be AChR antibody-positive and have GMG (93). In our cohort we found that asthma was the most common other autoimmune disorder followed by hypothyroidism. Comparison between EOMG and LOMG patients did not show any difference at 43.58% and 34.23% respectively; 43.93% of all the females in the study and 30.95% of all the males in the study had associated autoimmune conditions. There was no difference between the younger female and younger male patients either. This may be partly because asthma was not included as an AI condition in the other papers, and this may have narrowed the difference in our own cohort.

Previous literature has reported that familial autoimmunity in patients with MG is common and has been seen in 40% of EOMG patient relatives and 20% of LOMG relatives (93). 4% of the relatives had MG. In our cohort we found that family history of autoimmunity was 40% overall, more common in EOMG at

51.28% and 36.02% in LOMG in keeping with literature; however, this was not statistically significant. Hypothyroidism in the family seems to be the most common autoimmune disease followed by myasthenia gravis in 10/60 patients (16.67%) and a rate of 6.67% overall of familial MG. Studies on the effects of HLA on the age of onset of MG have shown mixed results. Different HLA haplotypes have been linked to EOMG and LOMG, but it is not clear why familial MG and indeed of other autoimmune conditions is more common in EOMG (129-142). One explanation could be that although HLA haplotypes may be shared between siblings, disease susceptibility could be defined not only by HLA-DR but also by other genetic factors including gene-gene interactions. Why this would be different in younger patients compared to older patients is unclear.

Other than for autoimmune conditions, there was no correlation with other comorbidities in our MG cohort. We had two patients with inclusion body myositis in our cohort, one of whom was seronegative and one who was double positive for MuSK and LRP4.

There have been several case reports in literature associating statin use with myasthenia gravis (412). We compared AChR titres, MG composite scores and MG QOL in all patients who were on statins with those who were not on statins and there was no significant difference. The rationale for looking into this

was to see if patients on statins had a more severe illness/ had worse MGC scores.

Previous studies and literature have shown that single fibre EMG (SFEMG) has more sensitivity and specificity compared to RNS; however, because of the easy availability of RNS this was the diagnostic test recommended by AAEM (413). In a study by Punga and colleagues (188) they showed that RNS was normal in patients with severe GMG and they recommended using concentric needle electrode myography instead. In our cohort, SFEMG was abnormal in 66.67% compared to 43.24% of RNS abnormalities; this was not statistically significant, although in keeping with literature. Only four patients had routine EMG of which one showed myopathic changes. Of the 8 seronegative patients, 5 had neurophysiology of which two were abnormal showing blocks on SFEMG, the other three were normal.

Our data shows that LOMG patients are more likely to have bulbar and respiratory symptoms than EOMG patients, and they also develop these symptoms significantly earlier than EOMG patients. Age seems to be the factor here rather than sex, as there was no difference between male and female patients. LOMG patients have worse MG composite scores and QOL scores at recruitment but seem to respond well to treatment clinically and in quality of life, doing much better than EOMG patients. The rates of autoimmunity were the same in EOMG

and LOMG, but family history of autoimmunity was more common in EOMG than LOMG.

Is there any difference in clinical presentation between the different antibody subgroups?

Of our 150 patients, the majority were single positive for AChR antibodies, with a small proportion positive for MuSK antibodies and LRP4 antibodies. We had a large number of patients who were double positive for AChR and MuSK, AChR and LRP4, or MuSK and LRP4, and 8/150 who were seronegative. Two patients were triple positive but were thought to be single positive for AChR with non-specific binding for the other two. The reason for this differentiation between double postives and triple positives was that in the double positives, the binding was to the expressed receptors- either EGFP tagged AChR or MuSK or untagged LRP4 receptors, whereas in triple positives, the binding was to the cell surface/ other proteins as well as the expressed receptors.

There was a significant difference in AChR single positivity in the females in EOMG and LOMG in our cohort, being much more frequent in LOMG patients. This is different to previously published data by Burke and colleagues who suggested that LOMG patients had lower AChR titres and were more likely to have striated muscle antibodies (414). Although more younger female patients in our cohort were double positive to AChR and

MuSK compared to older female patients, this was not statistically significant. Previous studies by Zisimopoulou et al looked at double positivity with LRP4 Abs. They found that it was more common in young females, and they had a more severe illness than those with single positivity to LRP4. Our study has found this with MuSK and AChR; whilst this cannot be directly compared, we can infer that as both these antibodies are pathogenic at different targets, together they can cause a more severe disease (207). MuSK antibody positivity was also not statistically significant in our EOMG or LOMG groups or between males and females, which is different to published literature which suggest that MuSK positivity is more commonly seen in young females (151, 154). This is discussed in more detail in the antibodies chapter. Ocular myasthenia gravis was also seen at the same rate in all antibody subtypes, and although OMG was more common in AChR and LRP4 double positivity, this was not significant. This is again different to previously published data which suggests that AChR and LRP4 double seropositivity is more likely to be associated with bulbar symptoms and a more severe course (206). It is not entirely clear why this difference was seen, but the number of LRP4 Ab positive patients in our cohort was relatively less than that in the study.

There was no difference in first presenting symptom in our cohort amongst any of the different antibody subtypes. Over the

course of the disease, all patients (100%) who had antibodies to MuSK, LRP4, MuSK and AChR double positive, and AChR and LRP4 double positive had ocular symptoms, whereas AChR single positive patients had ocular symptoms in 92.5% and seronegative patients had ocular symptoms in 87.5%; neither of which were statistically significant. This would suggest that AChR Abs and seronegative patients can have ocular sparing MG, whereas this is not the case with the other Abs.

Previous literature has suggested that patients with MuSK antibodies are more likely to be female, younger, without thymoma and more likely to have bulbar and respiratory involvement (154, 161). They were also found to remain immunosuppression dependent and needing rituximab. In our cohort we did not find any difference in bulbar symptoms amongst patients who had antibodies to MuSK, AChR, and MuSK and AChR double positive. However, seronegative patients were much less likely to have bulbar symptoms compared to AChR single positives, and compared to AChR and MuSK double positives. This difference was not seen between seronegatives and MuSK and LRP4 single positive patients. One could assume that seronegative patients have an as yet unidentified Ab that is less pathogenic than AChR. However, since they are clinically more similar to patients with MuSK and LRP4 Abs, this does not make sense. Any interpretation of this should be made with caution as the number of single positive

LRP4 patients was small, and there were only 8 seronegative patients. The subgroups did not show any difference in the frequency of limb weakness. There was no difference in respiratory symptoms in any of the antibody subgroups including in MuSK MG. Whilst there was a trend towards MuSK MG patients being more commonly female and having more bulbar and respiratory symptoms, this was not statistically significant. Whether this is a reflection of the fact that this was a prospective study with follow up data for two years in the majority of patients, and whether these patients would deteriorate later on in their course of illness is unclear. This is discussed further in the antibodies chapter.

We did not find any difference in thymic abnormalities in the different antibody subgroups except for patients with LRP4 antibodies who were more likely to have thymic hyperplasia compared to AChR single positivity. However, it is to be borne in mind that we only had 2 LRP4 single positive patients in our cohort. Also to be borne in mind, the data was based on scan findings of thymic enlargement with subsequent tissue diagnosis in most of these patients. This presumes that a normal scan excludes thymic abnormality which may not be the case, particularly in EOMG.

Steroid requirements pre-generalisation was no different in any of the antibody subgroups; but, post generalisation i.e. in GMG

patients with AChR and MuSK double positivity, the steroid requirement was much higher (80%) compared to AChR single positives (25.2%), MuSK single positives, AChR and LRP4 double positives, and the seronegatives. When compared to the MuSK and LRP4 double positive group, it was still higher but not statistically significant. In comparison, the requirement for alternate immunosuppressants in AChR single positive compared to AChR and MuSK double positive was not significant. It has been reported in literature that MuSK MG follows a more severe course, and patients were more likely to require alternate immunosuppression- more commonly plasma exchange or rituximab. We did not see this difference in our cohort, except for higher rates of steroid requirement in AChR and MuSK double positives. This would suggest that in our cohort, although the clinical presentation between the AChR and MuSK subgroups was not significantly different, the double positives were harder to treat implying perhaps a more brittle myasthenia?

Our data of the clinical phenotypes in the different antibody subgroups suggests that there is no difference in bulbar, limb, respiratory and ocular symptoms in any of the subgroups, except for seronegative patients who are less likely to have bulbar symptoms. AChR Ab positivity is seen more commonly in older females compared to younger females; all the other antibody subgroups are comparable between younger and older

patients and females and males. AChR and MuSK double positive patients require steroids more frequently in GMG compared to other antibody subgroups. Seronegative patients in our cohort did not require admission to hospital suggesting that they may have a less severe illness and a more indolent course compared to the antibody positive subgroups.

Is there a difference in thymic abnormalities between LOMG and EOMG patients?

Previous reports in literature have shown that thymic hyperplasia is seen in 50-60% of EOMG patients and is not seen in LOMG patients (51, 52, 415).

Of our 150 patients, 137 had imaging to look for thymic abnormalities. The total number of abnormalities, including thymoma/thymic mass, thymic hyperplasia/enlagment and thymic remnants, are more commonly seen in EOMG patients, in 50% compared to LOMG, 13.86%. Thymic enlagment/hyperplasia (depending on CT findings or confirmed on histology) was much more commonly seen in EOMG patients in 30.55% compared to 0% in LOMG. Thymomas/thymic mass (depending on CT findings or confirmed on histology) were seen in 16.67% of EOMG patients, compared to 10.89% in LOMG patients which was not significantly different. We did not find any difference in AChR titres amongst patients with thymoma in the LOMG and EOMG group. Overall, in our cohort, younger

patients appear to have more frequent thymic hyperplasia/enlargement and thymoma/thymic mass compared to LOMG. The data was based on scan findings of thymic enlargement with subsequent tissue diagnosis in most of these patients. This presumes that a normal scan excludes thymic abnormality which may not be the case, particularly in EOMG. This means that our data is not directly comparable to published studies, and this would require categorising patients into histologically normal and abnormal groups; however, our data suggests that thymic hyperplasia is much more common in EOMG than LOMG.

Is the treatment response different in LOMG and EOMG? And, Is the clinical outcome different in LOMG and EOMG patients?

Previous studies have reported that 14.8% of MG patients are refractory to treatment and they are more likely to be young female patients with anti-MuSK antibodies and with thymomas. Life-threatening events have been shown to occur in 9.56% of MG patients (387, 388). In our cohort, we found that 15.38% of EOMG patients and 36.03% of LOMG patients required admissions to hospital for MG related reasons. The older patients were also more likely to be admitted more frequently compared to younger patients. The majority of admitted patients were older- this could be due to several factors including other

co-existing comorbities which meant that they sought or were referred to hospital earlier than younger patients, or as described earlier in the chapter, LOMG patients had a higher MG composite score at recruitment compared to EOMG, perhaps indicating a more symptomatic onset in these patients. Previous studies have shown that prognosis is favourable in all MG patients, but LOMG patients are more likely to achieve an optimal outcome (392). In keeping with this, the majority of the patients in our cohort responded well to treatment with 92.5% of LOMG patients attaining a good outcome compared to 50% in EOMG patients. 2.56% of patients, all LOMG, died during an admission to hospital. It would seem then, contradictory to say that LOMG patients had a better outcome. This is explained by the fact that the majority of patients admitted to hospital had a proportionately good outcome compared to the EOMG patients. The overall mortality rate over the three-year period in our cohort was 4/150 i.e. 2.6%, all of them in the LOMG group. Of these, three were thought to have been caused by or contributed to by myasthenia gravis. This is similar to previously published literature (393).

Most of the literature which compares steroid doses in MG patients has been in those who have had thymectomy versus those who have not had thymectomy. There was no comparable data in literature for steroid doses in OMG and GMG patients as a natural cohort.

When we compared the daily dose steroid requirement in all patients at first year follow-up with second year follow-up (patients being treatment naïve at recruitment), there was a significant difference in doses, with patients at second year requiring less average daily dose steroids compared to the first year, both in EOMG (16mg Vs 6.25mg) and in LOMG (10mg Vs 7mg). There was no significant difference in the steroid doses between EOMG and LOMG at first year follow-up or at second year follow-up, but the dose requirements at first year follow up in LOMG is slightly less than EOMG. This would be in contrast to the clinical presentation of worse disease in LOMG with more GMG. This could be explained by the fact that LOMG patients required earlier initiation of steroids during the first year and doses were tapered down by their first year follow up. It could also suggest that LOMG patients responded more rapidly to treatment.

The steroid doses in OMG at first year were higher than at second year; however, in GMG there was no difference between first and second year steroid dosages. On direct comparison between OMG and GMG, there was no difference in steroid doses at first year follow up, or at second year follow up. It appears that OMG patients respond more quickly to steroids than GMG, leading to clinically significant improvement and reduced steroid doses with time.

When we compared the MGFA-PIS scores at first year follow-up, LOMG patients did better than EOMG patients and this trend was seen even at second year follow-up. When the whole cohort of patients was compared at first and second year follow-up, there was no difference in their PIS scores. The MGFA PIS in OMG patients was better at second year follow-up compared to first-year, but not inin GMG patients. The AChR RIA titres also fell significantly from point of recruitment to follow up with a significant improvement in the first year and a further slight reduction in the second year.

In conclusion, our data shows that LOMG is more common than EOMG, and EOMG is more common in females and LOMG more common in males, although the difference between younger males and females is becoming less significant. OMG is more common in EOMG than LOMG; however, there is no difference in the type of ocular presentation or generalisation rates in EOMG and LOMG. LOMG patients are more likely to have bulbar and respiratory symptoms than EOMG patients, and they also develop these symptoms significantly earlier than EOMG patients. Age seems to be the factor here rather than sex, as there is no difference between male and female patients. LOMG patients have worse MG composite scores and QOL scores at recruitment but seem to respond well to treatment clinically and in quality of life, doing much better than EOMG patients. The rates of autoimmunity were the same in EOMG

and LOMG, but family history of autoimmunity was more common in EOMG than LOMG. Clinical phenotypes in the different antibody subgroups suggests that there was no difference in bulbar, limb, respiratory and ocular symptoms in any of the subgroups, except for seronegative patients who were less likely to have bulbar symptoms. AChR Ab positivity was seen more commonly in older females compared to younger females; all the other antibody subgroups were comparable between younger and older patients and females and males. AChR and MuSK double positive patients required steroids more frequently in GMG compared to other antibody subgroups. Seronegative patients in our cohort did not require admission to hospital suggesting that they may have a less severe illness and a more indolent course compared to the antibody positive subgroups. OMG patients respond more quickly to steroids than GMG, leading to clinically significant improvement and reduced steroid doses with time. One could argue that this would suggest that OMG is a milder disease than GMG. It has been postulated that ocular muscles are more susceptible to being affected by Abs in MG, which is why OMG is more common than GMG; hence one explanation would be that ocular muscles respond more quickly and effectively to steroids compared to muscles elsewhere. Taken together, this would suggest that patients present with their worst MG symptoms during the first year, more often in the first three

months, there is an improvement in all scores including MG composite scores, QOL scores and PIS scores in parallel with improvement in AChR RIA titres. The overall improvement appears to be much more marked in the LOMG patients although they are more likely to have GMG compared to the EOMG patients.

4 Antibodies in myasthenia

4.1 Introduction

The first antibody tests for myasthenia gravis were demonstrated in 1973 by Almon, Andrew and Appel (9) and the detailed immunoprecipitation assay using α bungarotoxin labelled AChR was described by Lindstrom et al (10). Later studies using subclass specific antisera showed that anti-AChR subclasses 1, 2 and 4, and occasionally subclass 3 were seen. AChR antibodies were found to be positive in between 85-90% of GMG patients and 75% of OMG patients (44). AChR Ab titres were reported to be higher in patients with thymic hyperplasia (54).

The assay now available commercially for AChR autoantibodies and MuSK autoantibodies is a carefully balanced mixture of detergent solubilised foetal and adult forms of the receptor labelled with radioactive iodine labelled α bungarotoxin (12, 44). Testing of AChR antibodies using ELISA was first described in 1999 (198).

Anti MuSK antibodies were first detected and found to be pathogenic in 2001. Radioimmunoassay for testing of anti-MuSK antibodies were described in the same paper by Hoch et al (200). 70% of the patients who were negative for AChR antibodies were positive for MuSK. Despite this, a fair number of

patients continued to remain seronegative. These patients behaved similarly to ACHR MG clinically, in their response to immunosuppressive treatment, and in thymic pathology. In 2008 Leite et al hypothesised that antibodies in SNMG could be detected by binding to ACHRs on the cell membrane, particularly if they were clustered at the high-density that is found at the NMJ. They described the clustered cell based assays for AChR and MuSK antibodies. These antibodies were mainly IgG1 subclass for ACHR and showed the ability to activate complement in cell based assays; in MuSK antibodies IgG4 was the main pathogenic subclass and partially IgG1 subclass (163). Clustered ACHR antibodies were detected in 38.1% of RIA negative patients with MG with 100% specificity (167). Clustered AChR antibodies were seen in 16% of seronegative MG patients in a study by Devic et al, including both EOMG and LOMG (165).

In 2017 Huda et al described clustered cell based assays to detect MuSK antibodies using an additional IgG Fc gamma specific secondary antibody to eliminate the IgGM making the assay much more specific. By doing this, they detected MuSK antibodies in 99% of definite MuSK MGs (positive on RIA) and in 8% of SNMG (168).

Higuchi et al described the pathogenic properties of LRP4 antibodies in 2011 along with Pevzner et al in 2012 and Zhang et al in 2012 (243, 244, 416).

Zisimopoulou et al published a comprehensive analysis of the epidemiology and clinical characteristics of anti-LRP4 MG. They found an overall frequency of LRP4 MG in SNMG of 18.7% with a range of 7 to 32.7%. They found double positivity with ACHR and LRP4 in 8/107 (7.45%) and LRP4 and anti-MuSK in 10/67 (14.92%). They felt that double seropositive patients had more severe symptoms at onset compared to single positive patients. Patients who have pure LRP4 positivity had milder symptoms with MGFA grade I or II; some had thymic hyperplasia but no thymomas (207).

Cordts et al described double seropositivity with ACHR and LRP4 of 7%, ACHR and agrin of 5%, and ACHR and Titin in 53%. In the seronegative group they found 2% positivity for MuSK, 2% for LRP4 and 2% for agrin (211).

Several other autoantibodies have also been described in MG including Titin antibodies in 28.4% and Rynodine antibodies in 23.8%. Thymoma MG patients had higher frequencies of AChR, Titin and Rynodine antibodies. Titin and Rynodine antibodies were also present more frequently in LOMG patients. They found that patients with Titin and Rynodine antibodies tended to

have more severe disease and worse outcomes and they may need more active suppressive treatment (208, 417).

Stergiou et al found that ACHR MG patients had the highest frequency of Titin antibodies compared to MuSK MG and LRP4 MG (212). Titin antibodies were uncommon in EOMG unless associated with thymoma in LOMG patients. Titin antibodies had similar prevalence and levels as those with MG and thymoma (216, 217).

Cortactin antibodies have also been found to be pathological in MG; they were found in 23.7% of SNMG and 9.5% of ACHR positive MG. These antibodies were thought to be biomarkers of MG and which when present suggested that the disease would be mild (218).

Agrin antibodies are also thought to be pathogenic through inhibition of agrin/LRP4/MuSK signalling at the NMJ (220). Collagen XIII autoantibodies have been found in MG but it is not clear whether these antibodies are pathogenic or not (219).

Antibodies in myasthenia gravis and the assays for them have been well established with good sensitivity and specificity; however, there have been no large-scale prospective cohort studies on treatment naive patients with long-term follow-up. Our study was a unique chance to check antibodies from a diagnostic cohort longitudinally. This would then give us a

chance to assess the utility of RIA and cell-based assays in parallel. It would also give us an opportunity to test patient sera for antibodies to other targets even when they are positive for ACHR antibodies. It would also be a good opportunity to see if there are any differences in antibody positivity, titre levels and response to treatment in EOMG and LOMG patient groups.

In testing for AChR, MuSK and LRP4 antibodies, we sought to answer the following questions:

- Is antibody positivity different in EOMG and LOMG?
- Is there a difference in the antibody subgroups between EOMG and LOMG?
- Is there a difference in antibody titres in EOMG and LOMG?
- Does antibody positivity and titre change with time and treatment?

4.2 Methods

All the experiments to look for MG antibodies were done at the laboratories at the Nuffield Department of Clinical Neurosciences, Oxford. All RIA analysis for AChR and MuSK Abs were done by the author. For high positive titres, serial dilutions were done until the Counts Per Minute or CPM value

dropped by half and then the titres were calculated. VGCC Ab RIAs were done by Dr Bethan Lang and Ms Selina Tomsen.

All 150 MG patients in the study had serum samples taken at recruitment and 120 patients had serum samples at first year follow-up. ACHR RIA was tested on all patient samples at recruitment and first year follow-up. MuSK RIA was tested on all 150 of the recruitment patient samples. VGCC antibodies on RIA were tested in 139 patients at recruitment.

General principles for RIA:

Radioimmunoassay (RIA) or radioimmunoprecipitation assay (RIPA) involves precipitating radioactively labelled antigen and antibody complex. In the first step patient sera is incubated with I^{125} labelled antigen. Any specific antibody in the sera binds to the antigen. In the second incubation step the antigen-antibody complexes are precipitated using a precipitation agent. The precipitate is washed with buffer. After centrifugation and decanting of the supernatant, radioactivity in the precipitate is counted using a gamma counter. The intensity of the radioactivity is proportional to the concentration of specific antibody in the patient serum. The antibody concentration is evaluated quantitatively using a calibration curve (418).

RIA method:

Serum samples which were stored either at -20°C or -80°C were left to thaw at room temperature for a few minutes. The first assays were done using test serum (patient samples) with a concentration of 5 µL which was diluted to 250 µl using PTX. We obtained our AChR assay kit from RSR laboratories. 50 µL of I^{125} α bungarotoxin ACHR was added to each of the test samples. This was briefly mixed and left in the fridge overnight. The next day, human IgG serum of 50 µL diluted up to 250 µL using PTX was added to each test serum. This was left to precipitate for 30 minutes and then centrifuged. The supernatant was discarded and the pellets were washed a couple of times. After the second wash, the eppindorfs were placed in the gamma counter to count radiation in counts per minute (CPM).

For samples with very high CPM counts, i.e. equal to or more than the control serum, the samples were tested with serial dilutions starting with 2.5 µl and progressively more dilute samples up to a concentration of 0.1 µL were tested. When the CPM counts dropped by 50%, this was taken to be the correct titre for the sample. The titres were then converted into 10^{-10} moles per litre using the formula below. Values of ≥5 X 10^{-10} moles per litre were considered abnormal.

$$\text{nmol/L AChR} = \frac{(\text{CPM test sample} - \text{CPM negative control}) \times A}{C \times K \times BX\ 2.22}$$

A – Decay factor between receptor labelling day and day of assay

B - Counter efficiency (which was 80% on the machine in the Oxford lab)

C- Volume of serum used in the assay

K - Specific activity of ^{125}I at the time it was used.

A, C and K were on the sheet included with every bottle of ^{125}I AChR.

These titres were multiplied by 10, to give the values in 10^{-10} mols/L

For MuSK RIA assays, the procedure was the same as for ACHR including for the dilutions. The kit used was RSR I^{125} MuSK, and the formula to calculate the titres was the same as for ACHR. Values of ≥0.5 X 10^{-10} moles per litre were considered abnormal.

CBA Method

For CBAs, the human embryonic kidney (HEK) 293 cell line was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 100 units per ML each of penicillin G and streptomycin at 37°C in an atmosphere of 5% CO_2 to 50% confluence on 13 mm glass cover slips which were

placed in six well cell culture plates. For the clustered AChR assay, the cells were transiently cotransfected using polyethylenimine with the plasmids encoding for subunits of human adult AChR and Rapsyn with EGFP. A total of 3 µg of DNA was used in a proportion of α:β:δ:ε:Rapsyn of 2:1:1:1:1, respectively. For MuSK CBA, 3 µg of MuSK-EGFP DNA was transfected. For LRP4 CBA, LRP4 CASPR and LRP4 AP were transfected in a proportion of 5:1 and a total of 6 µg of DNA was used for transfection. For each of the assays, the medium was changed 16 hours post transfection.

After 24-hours, the cover slips were transferred into a 24 well cell culture plate. To these cells, human sera (patient samples and controls) diluted to 1:20 using DMEM, HEPES and bovine serum albumin (BSA) was added. This was incubated for one hour at room temperature. After washing, this was fixed with 3% formaldehyde. After further washing, the secondary Fc antibody (blocking) (we used Invitrogen goat anti-human IgG Fc cross-adsorbed secondary antibody) was added in a dilution of 1 in 750. This was incubated for 45 minutes at room temperature in the dark. After further washing, the tertiary antibody (blocking) (we used Alexa fluor 568 donkey anti-goat IgG) in 1 in 750 dilution was added. This was incubated for a further 45 minutes at room temperature in the dark. After final washing the cover slips were mounted on the mounting media using 1% DAPI. The

slides were allowed to dry for at least an hour before reading using an Axion Zeiss inverted fluorescent microscope.

The assays were read based on the degree of cell-surface fluorescence and co- localisation as per Dr Leite's paper of 2008 (163).

(0) = no labelling

(0.5) = very weak labelling of very few transfected cells with no obvious co-localisation

(1) = weak labelling of some of the transfected cells, with co-localisation

(2) = moderate labelling of some (approximately 20 to 50%) of transfected cells, with precise co-localisation

(3) = moderate/strong labelling of approximately 50 to 80% of the transfected cells, with perfect co-localisation

(4) = strong labelling of virtually all transfected cells with perfect co-localisation.

4.3 Results

4.3.1 Anti Acetyl choline receptor antibodies (AChR Abs)

We performed radioimmunoassays (RIA) for AChR, MuSK and VGCC antibodies. We also performed cell based assays (CBA) for AChR, MuSK and LRP4 antibodies. All 150 patient samples were tested for AChR and MuSK on RIA. Cell based assays were performed on all 150 recruitment samples for AChR and MuSK and 147 samples were tested for LRP4. 139 patient samples were also tested for VGCC on RIA. The AChR RIA titres are reported as X 10^{-10} moles per litre.

During follow-up, serum samples were collected at annual follow-up; 120 patient samples were collected at first year follow-up, all of which were tested for AChR on RIA and CBA. 36 patient samples were collected at second follow-up which were once again tested for AChR on RIA and CBA. We had three patient samples for year three follow-up which were tested for AChR on RIA and CBA.

In OMG, positivity for AChR was seen in 75% in EOMG and 80% in LOMG, P = 0.6985. When tested on CBA, the number of LOMG patients positive was still 80% whereas the EOMG patients increased in number to 87.5%. The difference was not statistically significant. Seropositivity on RIA in younger GMG patients was low at 56.5% compared to LOMG patients where it

was 91.4%, p< 0.0001; with cell based assays, positivity in the younger GMG patients increased to 82.6% and in the older patients to 93.8% (p=0.0372). Overall, AChR positivity in EOMG (85.05%) and LOMG (86.9%) are comparable (p=0.7725). All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

The breakdown of all positive cell based assays is as below, with 8 seronegatives (5.32%) (Figure 44). NB: one of these 8 patients seronegative on CBA was positive on an initial ACHR RIA.

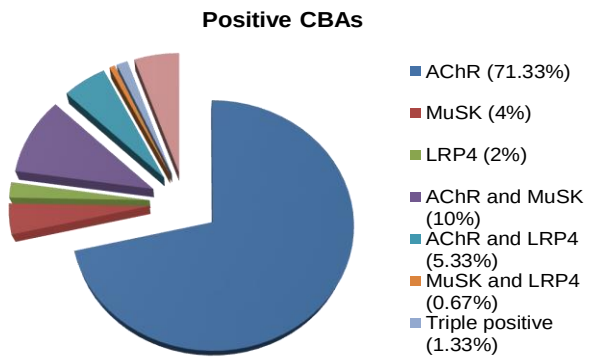


Figure 44 Breakdown of all positive CBAs

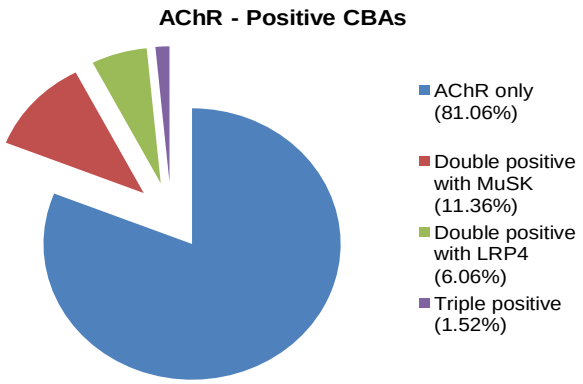


Figure 45 Breakdown of AChR positives on CBA

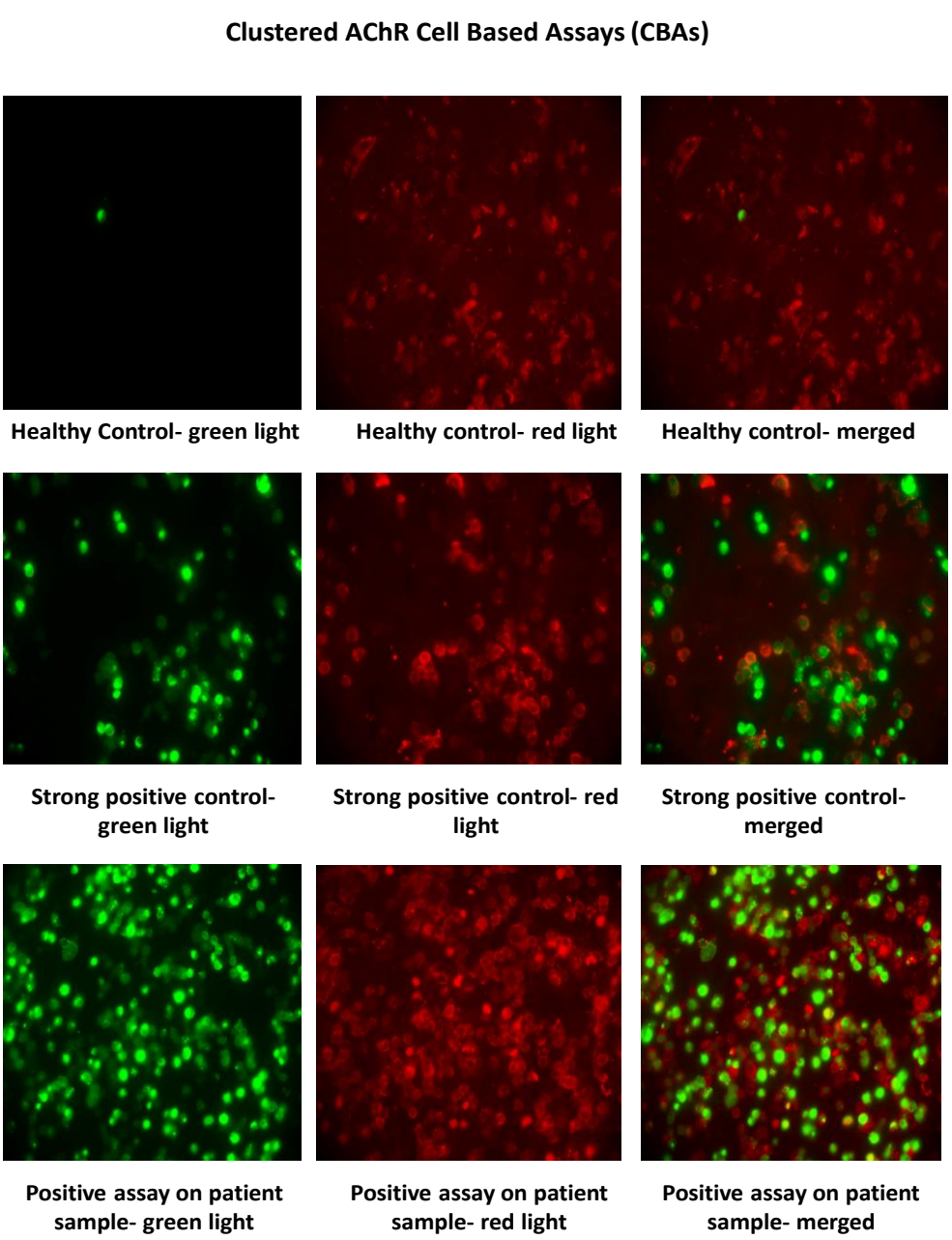


Figure 46 Photographs of the Clustered AChR CBA; top row shows the healthy control, middle row the strong positive control and the bottom row shows the patient sample

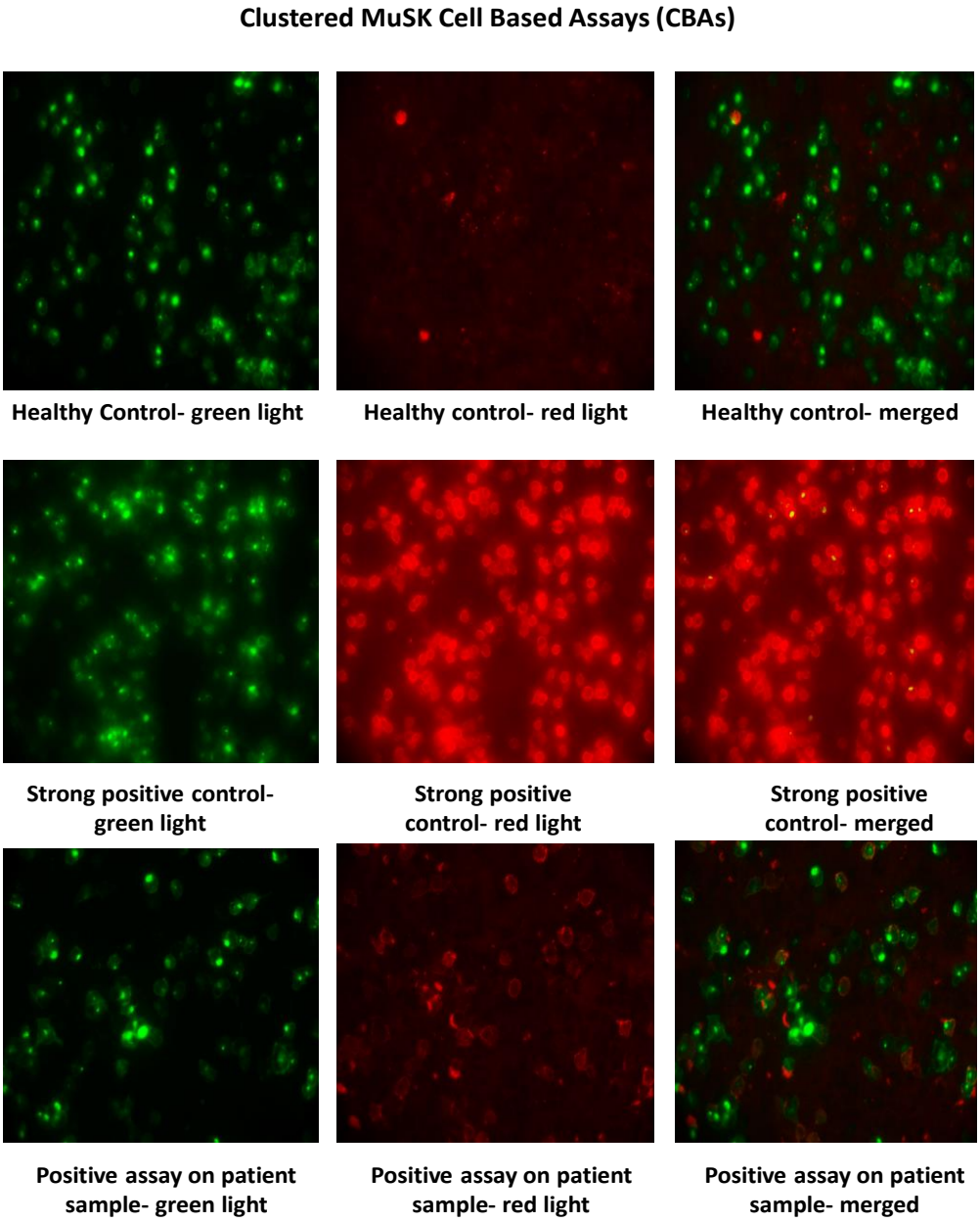


Figure 47 Photographs of the Clustered MuSK CBA; top row shows the healthy control, middle row the strong positive control and the bottom row shows the patient sample

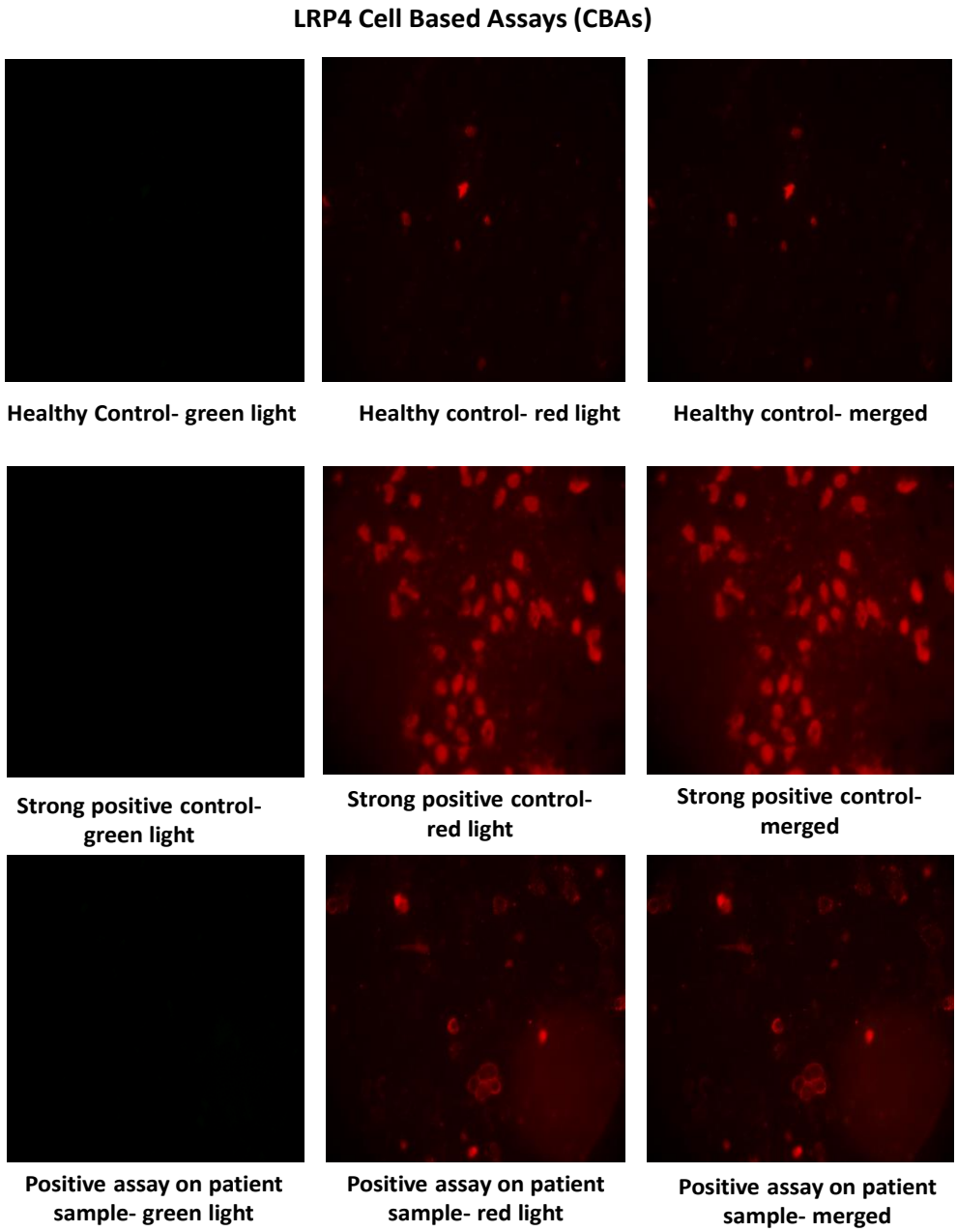


Figure 48 Photographs of the LRP4 CBA; top row shows the healthy control, middle row the strong positive control and the bottom row shows the patient sample

The breakdown of the positive AChR CBA results is as per figure 45. Of all the positive AChRs on cell based assays, we had 107 who were positive for AChR alone i.e. 81.06%, 15

double positive with MuSK i.e. 11.36%, 8 double positive with LRP4 i.e. 6.06%, and 2 patients i.e. 1.52% were positive for all three antibodies. The triple positives were thought to represent AChR single positives with non-specific binding to the other antibodies. The reason for this differentiation between double postives and triple positives was that in the double positives, the binding was to the expressed receptors- either EGFP tagged AChR or MuSK or untagged LRP4 receptors, whereas in triple positives, the binding was to the cell surface/ other proteins as well as the expressed receptors.

There was a significant drop in AChR RIA titres when the recruitment samples were compared to the first year follow-up $P< 0.0001$ (Figure 49A). This was also seen when CBAs were analysed (Figure 49B). There was a significant difference when AChR RIAs were compared between patients who were treatment naïve at recruitment and the immunosuppressed at first year follow-up. Although there was a drop in titres in patients who remained immunosuppression naïve at first year follow-up, this was no longer statistically significant. There was linear correlation between AChR RIA at recruitment with their MG composite scores with $p=0.0011$ and R^2 of 0.0671 (Figure 50A). Similarly, there was linear correlation between MG QOL and AChR RIA titres at recruitment, $p=0.0070$ and R^2 of 0.04623 (Figure 50B) ACHR RIA titres were compared at recruitment between the EOMG and LOMG groups and there was a

significant difference with a median of 20.08 $x10^{-10}$mol/l in EOMG and 176.8 $x10^{-10}$mol/l in LOMG, P=0.0005; when the seronegatives were taken out of the equation so as to not skew the data, the difference was still statistically significant at p = 0.0498 with a median for EOMG of 91.75 $x10^{-10}$mol/l and for LOMG 196.8 $x10^{-10}$mol/l (Figure 51).

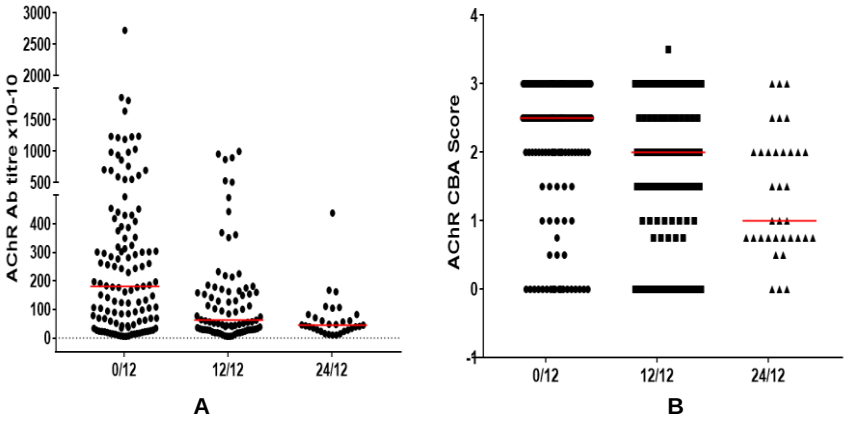


Figure 49 Comparison of AChR (with medians) at point 0, 1st and 2nd year follow up p<0.0001; A: on RIA, B: on CBA

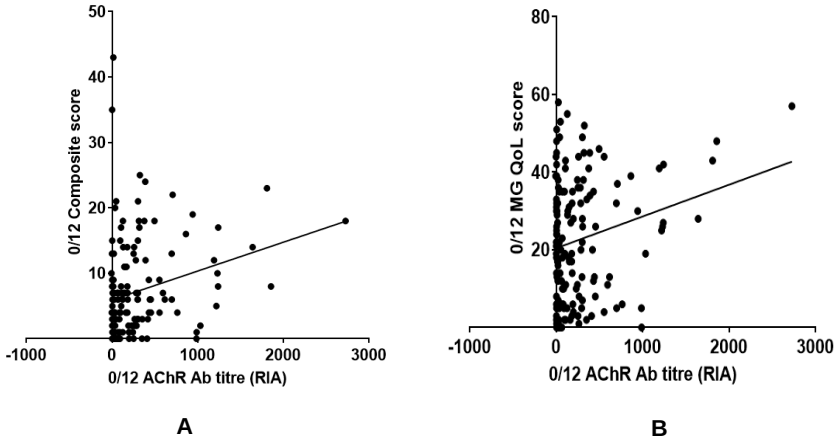


Figure 50 Correlation between AChR RIAs at recruitment with (A) MG composite scores p=0.0011, R^2 of 0.0671 and (B) MG QoL scores at diagnosis 0.0070, R^2 of 0.04623

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Figure 50 A and B: Simple linear regression used

ACHR RIAs in EOMG and LOMG patients with thymic abnormalities were compared; there was no significant difference in patients with thymoma or thymic hyperplasia. There was no difference between patients who were reported to have residual thymus, with medians being lower in the EOMG patients compared to LOMG, p value of 0.0571 (Figure 52). NB: The data was based on scan findings of thymic enlargement with subsequent tissue diagnosis in most of these patients. This presumes that a normal scan excludes thymic abnormality which may not be the case, particularly in EOMG.

Our cell-based assays were not performed using serial dilutions so AChR RIA titres were not directly comparable with the cell based assays, however we did plot both of these on GraphPad Prism and there was a linear correlation between the readings, $p<0.0001$ and R^2 of 0.1656 (Figure 53) There was no significant difference in AChR RIA titres between patients on statins and those who were not on statins; similarly there was no significant difference between the AChR RIAs in patients who used salbutamol inhalers and those who did not use salbutamol inhalers.

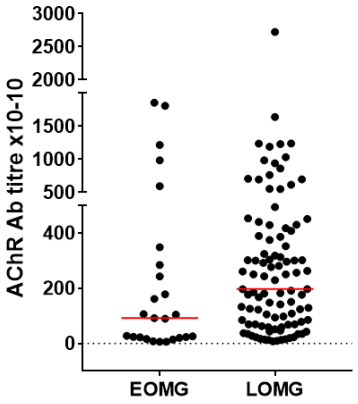


Figure 51 Comparison of AChR RIAs (with medians) without seronegatives at recruitment between EOMG and LOMG p=0.0498

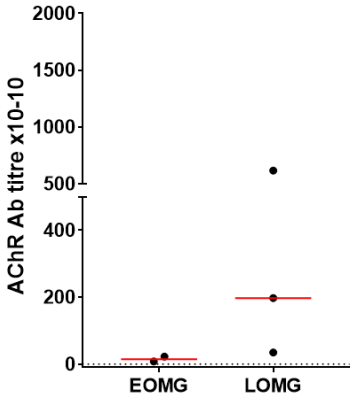


Figure 52 Comparison of AChR RIAs (with medians) in patients reported to have thymic remnants in EOMG and LOMG p=0.0571

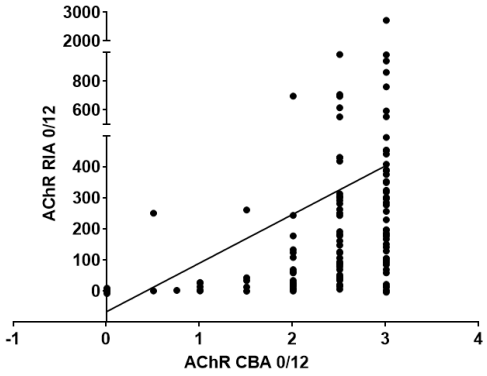


Figure 53 Correlation between AChR RIAs and AChR CBAs at recruitment p<0.0001, R^2 of 0.1656

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Figure 53: Simple linear regression used

4.3.2 Anti Muscle specific kinase antibodies (MuSK Abs)

Of the 150 patient samples tested at recruitment, MuSK RIA was positive in one patient. Clustered cell based assay for MuSK Ab was positive in a total of 24 patients. Of these, 6 patients (4%) were single positive for MuSK Ab, 15 patients

(10%) double positive for AChR and MuSK antibodies, 1 patient (0.67%) was positive MuSK and LRP4 antibodies. 2 patients were positive for all three antibodies but as mentioned in the previous section, the conclusion was that the antibodies were non-specifically binding to MuSK and LRP4.

All positive CBAs were repeated to confirm results. In the case of MuSK antibodies, because of the high rate of positivity and double positivity, most samples were assayed 3 times and the results read by three different observers including the author. Low positive results with a score of 0.5 and 0.75 were excluded from the analysis, and only scores of 1 or more were included. The scoring was based on previous literature (163) and laboratory practices where a score of 1 is reported as positive and anything below as possible/ maybe.

Table 22 Breakdown of MuSK seropositivity in EOMG and LOMG (O: Ocular, G: Generalised, F: Female, M: Male, T: Total)

		O +ve	O + %	G +ve	G + %
EOMG	F	4	28.6	4	44.4
	M	1	7.7	0	0
	T	5	18.5	4	33.3
LOMG	F	2	10.5	3	12.5
	M	6	18.2	4	11.4
	T	8	15.4	7	11.9

Total number of patients in each subgroup ie denominators were: OMG in EOMG- 27, with 14 female and 13 male; OMG in LOMG- 52, with 19 female and 33 male; GMG in EOMG- 12, with 9 female and 3 male; GMG in LOMG- 59, with 24 female and 35 male

Amongst the total MuSK positives, single positivity was seen in 25%, double positivity with ACHR was 62.5%, double positivity with LRP4 was 4.1%, and triple positivity was 8.33% (Figure 54). 33.3% of the younger patients with GMG were positive for MuSK antibodies on CBA compared to 18.5% in the OMG patients.

Table 23 MuSK seropositivity in subgroups with p values.

	Ocular	Generalised	P value
EOMG	18.5%	33.3%	0.3174
LOMG	15.4%	11.9%	0.5924
P value	0.7262	0.0638	

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

MuSK positivity in the GMG patients in EOMG was 33.3% and LOMG 11.9%, this was not significantly different, p=0.0638. Similarly, younger female patients were more likely to be MuSK positive (30.43%) compared to younger male patients (6.25%), but the difference was not statistically significant, p=0.0694 (Tables 22 and 23). When female EOMG and LOMG patients were compared, although the trend was towards the younger females being more likely to be positive (30.43% Vs 11.62%), this was just under statistical significance (p=0.0610). The clinical presentations with MuSK Ab positivity has been described in the previous chapter.

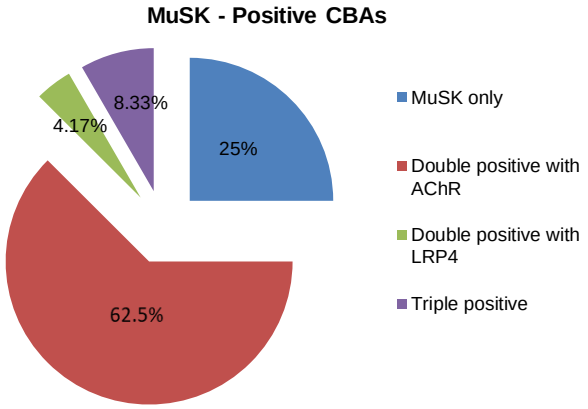


Figure 54 Breakdown of all MuSK positives on CBA

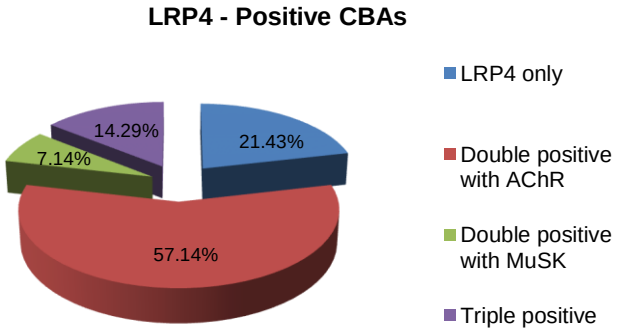


Figure 55 Breakdown of all LRP4 positives on CBA

4.3.3 Anti LRP 4 antibodies

Cell-based assays for LRP4 antibodies were done in 147/150 patient samples at recruitment. Of these, 14 patients were positive for LRP4 antibodies. 3 were positive purely for LRP4, 8 were double positive for ACHR and LRP4 and 1 patient was double positive for MuSK and LRP4. Two patients were positive for all three antibodies (see NB in above section). Amongst the LRP4 positives, 21.43% were single positive, 57.14% were

double positive for ACHR, 7.14% double positive for MuSK and 14.29% triple positive (Figure 55).

Table 24 Breakdown of LRP4 seropositivity in EOMG and LOMG (O: Ocular, G: Generalised, F: Female, M: Male, T: Total)

		O +ve	O + %	G +ve	G + %
EOMG	F	4	28.6	0	0
	M	1	7.7	1	33.3
	T	5	18.5	1	8.3
LOMG	F	2	10.5	2	8.3
	M	2	6.1	2	5.7
	T	4	7.7	4	6.8

Total number of patients in each subgroup ie denominators were: OMG in EOMG- 27, with 14 female and 13 male; OMG in LOMG- 52, with 19 female and 33 male; GMG in EOMG- 12, with 9 female and 3 male; GMG in LOMG- 59, with 24 female and 35 male

The division of positives amongst the subgroups is listed in tables 24 and 25. 18.5% of the ocular EOMG patients were positive for LRP4 antibodies as opposed to 8.3% of generalised EOMG patients. In LOMG, the OMGs positive for LRP4 antibodies were 7.7%, and in GMG were 6.8%.

Table 25 LRP4 seropositivity in subgroups with p values.

	Ocular	Generalised	P value
EOMG	18.5%	8.3%	0.4209
LOMG	7.7%	6.8%	0.8556
P value	0.1545	0.8543	

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

4.3.4 Seronegative myasthenia

Of our cohort of 150 patients, 8 were seronegative (5.33%). One of the 8 patients however was positive on AChR RIA when first diagnosed, RIA when repeated during the study, and CBA were negative.

The distribution of negatives in the subgroups is shown in table 26. There was no statistically significant difference amongst the subgroups.

Table 26 Subgroups in triple seronegative patients

			Numbers	Percentage
Seronegative	EOMG	Female (23)	1	4.34
		Male (16)	1	6.25
	LOMG	Female (43)	3	6.98
		Male (68)	3	4.41

In seronegative patients, ocular symptoms were seen slightly less at 87.5% compared to antibody positives. Seronegative patients were more likely to have limb weakness at 62.5% compared to 47.7% with AChR single positivity and 33.3% with AChR and LRP4 double positivity.

Table 27 Clinical features in Seronegative patients (7 patients)

Clinical features	Numbers
Early onset	2
Late onset	5
Ocular Sx at onset	6
Ocular MG at 3 mths	4
Generalised MG	2
Asymptomatic	1
Bulbar Sx	1
Limb weakness	5
Respiratory Sx	2
Neurophysiology done	5 (all normal)

4.3.5 Does immunosuppression change the Ab profile?

When we compared AChR RIA titres in all patients at recruitment with first year follow up samples, there was a significant drop in titres $p<0.0001$. We then analysed the samples without seronegatives so that the data was not skewed, and there was still a significant drop in titres at first year follow up, $p=0.0002$ (Figure 56).

We performed subgroup analysis comparing AChR RIA titres in patients who were treatment naïve at recruitment and were

treated with immunosuppression (mainly steroids) in the first year, with their titres at first year follow up. There was a significant drop in titres, p<0.0001 (Figure 58). Titres in the patients who remained immunosuppression naïve at first year follow up also showed a drop, but not statistically significant, p=0.0891 (Figure 57).

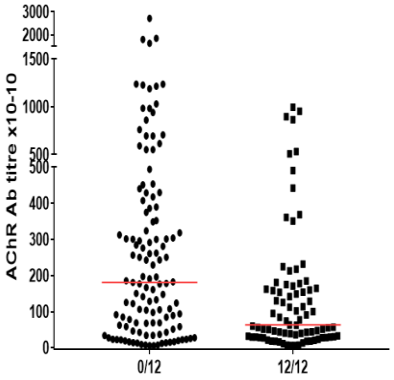


Figure 56 Comparison of ACHR RIAs (with medians) in all patients at recruitment and first year follow up p=0.0002

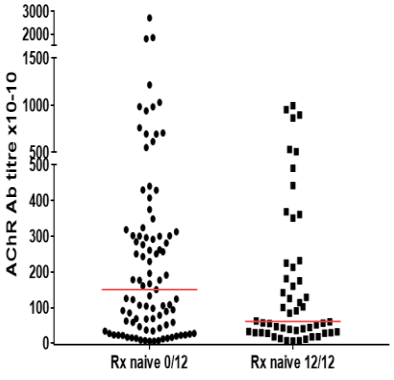


Figure 57 Comparison of ACHR RIAs (with medians) in immunosuppression naïve patients at recruitment and still immunosuppression naive at first year p=0.0891

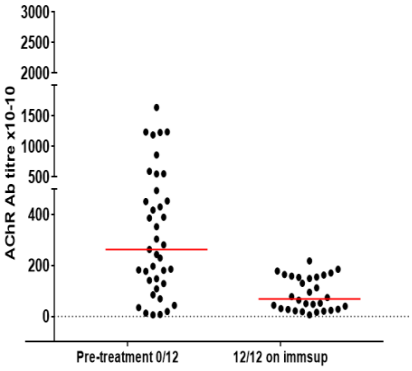


Figure 58 Comparison of ACHR RIAs (with medians) in treatment naïve patients at recruitment and after receiving steroids at first year follow up p<0.0001

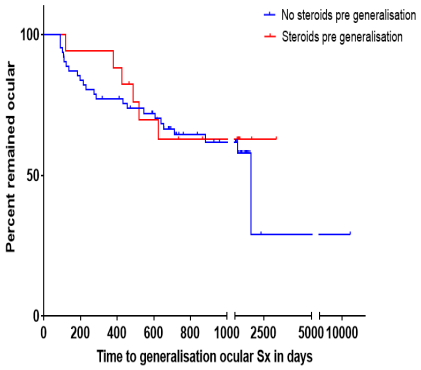


Figure 59 Survival curve for time to generalisation in OMG comparing patients who received steroids pre-generalisation to those who did not

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Survival curve data was analysed using the Log Rank (Mantel-Cox) test and Gehan-Breslow-Wilcoxon test)

From this data, it appears that immunosuppression does lead to a fall in AChR RIA titres. As mentioned in the chapter on clinical phenotypes, steroids do not seem to alter the generalisation rates in OMG significantly in our cohort (Figure 59).

4.4 Discussion

We performed radio immunoassays (RIA) for AChR, MuSK and VGCC antibodies on patient samples at recruitment. AChR and MuSK was tested on all 150 patient samples, and for VGCC in 139 patient samples. We also did cell based assays for AChR, MuSK and LRP4 antibodies, AChR and MuSK were done on all 150 recruitment samples and 147 samples were tested for LRP4. First year follow-up samples in 120 patients were tested for AChR on RIA and CBA. 36 patient samples at second year follow-up were tested for AChR on RIA and CBA and 3 third year follow-up samples were also tested for AChR on RIA and CBA.

It has been reported in previous literature that AChR positivity is seen in 85-90% of GMG patients and between 50-75% of OMG patients (44, 419). There is wide variability in MuSK antibody positivity, with initial reports of 7%, but other papers reporting anywhere between 3.8 and 47.4% (151, 200, 420). Most studies have shown that AChR titres are lower in LOMG patients with no

thymic abnormalities (96, 97, 149, 150, 421), patients with thymoma had higher titres (no age correlation) and EOMG patients with thymic hyperplasia had higher titres (10). A study by Iwasa et al differed slightly when they found raised AChR titres amongst MG patients in Japan for a brief period of time; this was more pronounced in LOMG patients (422).

Is antibody positivity different in EOMG and LOMG? And, Is there a difference in the antibody subgroups between EOMG and LOMG?

In our study, we found that in OMG, AChR positivity was seen in 75% in EOMG and 80% in LOMG on RIA and 87.5% and 80% respectively on CBA. The difference in positivity on RIA and CBA was not statistically significant. The positivity on RIA in younger GMG patients was low at 56.5% compared to the LOMG patients at 91.4%; however with CBAs the positivity in EOMG GMG patients increased to 82.6% and in LOMG patients to 93.8%, which was still statistically significantly different (p=0.0372).

Zivkovic et al have published similar results in their retrospective study where they found AChR positivity to be more common in LOMG than in EOMG (p=0.0026). They did not differentiate between OMG and GMG, but mention that OMG is much more common in LOMG than in EOMG. Jacob et al suggest that patients with AChR antibodies positive only on clustered cell based assays more commonly have OMG. This is different to

what our study suggests; however, the study by Jacob et al was a small retrospective study, and whilst showing a trend, the results were not conclusive (155, 164).

It appears that the EOMG patients in our cohort, particulary those with GMG, may have low affinity antibodies to AChR which were not detected in the solution phase (used in RIA), but were detectable on CBAs. As has been shown previously, low affinity Abs are still pathogenic in vivo against the AChR clusters at the NMJ (163). It appears that EOMG patients have highly pathogenic, but low affinity AChR Abs compared to LOMG.

Of our 150 patient samples, when tested for all three antibodies on RIA and CBA, we had 8 seronegative patients (5.32%) (one of these patients was initially positive for AChR on RIA). The majority of patients were positive for AChR (71.33%). The rates of AChR single positivity in our cohort was lower than previously published, however, we had a further 15.33% who were double positive, bringing the total AChR positivity to 86.66% which is more in keeping with published literature. In routine clinical practice, serum is tested for AChR antibodies first and if this is negative, they are tested for MuSK antibodies on RIA followed by clustered CBA. It is possible that there are a small number of patients in the community who have been diagnosed with AChR MG, but who may well have a second antigenic target which has not been tested for.

The rates of LRP4 (2%) and MuSK MG (4%) was in keeping with published literature.

We had quite a few patients who were double positive. AChR and MuSK double positivity was seen in 10%, which is slightly less than previously published data of 12.5% (423). AChR and LRP4 double positivity was seen in 5.33%, again, less than previously published rates of 7.45% (207). MuSK and LRP4 double positivity was seen in 0.67%, this is much less than previously published rates of between 14.92% and 19.8% (207, 423). The previously published data is mainly from a multinational retrospective study of 904 stored serum samples. Two patients were positive for all three antibodies, although the conclusion was that the MuSK and LRP4 antibodies were probably non-specifically binding. This was thought to be the case as the binding with these stains was not just to the expressed MuSK/LRP4 receptors, but to other cell wall proteins as well. Triple seropositivity has also been described before at a similar rate (423).

There was no difference between MuSK positivity in EOMG and LOMG in ocular patients. MuSK seropositivity in GMG in EOMG patients compared to LOMG was not significantly different although there was a trend towards higher positive rates in EOMG. There was no significant difference in MuSK seropositivity amongst the EOMG group when OMG and GMG

were compared, neither was there any difference in the LOMG subgroup. There was a trend towards higher numbers of female EOMG patients being positive to MuSK Abs (30.43%) compared to male EOMG patients (6.25%), but this was not significantly different. Similarly, when female EOMG and LOMG patients were compared, although the trend was towards the younger females being more likely to be positive (30.43% Vs 11.62%), this was not significantly different (p=0.0610). This is different to previously published data which report that MuSK Abs are more common in younger females (151, 154, 407). The study by Guptill et al was a large cohort retrospective study; however, all the antibody tests were done on RIA, and not on CBAs. It is possible that lower affinity MuSK Abs, which may well be what is seen in LOMG patients, were missed (NB: the majority of our MuSK positives were on CBA alone), skewing the data towards the younger females. In the study by Evoli et al, who noticed a striking female preponderance, MuSK Abs were tested using immunoblot. The study by Huda et al showed that although female preponderance was seen with MuSK CBA+ RIA- patients, the age of onset was less, and they were more likely to have OMG, suggesting a milder phenotype in these patients. Our MuSK cohort was predominantly RIA- CBA+. This could be one of the reasons why our results differ from published literature. For future studies, this subgroup will need to be looked into more carefully by recruiting higher numbers of

patients who are MuSK RIA+, and comparing them with those who are MuSK CBA+ RIA-.

LRP4 seropositivity in our cohort was similar to published data. There was no difference between OMG and GMG in EOMG and LOMG, and no difference between EOMG and LOMG (both for OMG and GMG) either. This is different to previously published data which report that LRP4 single positivity is associated with milder disease and double positivity with more severe disease (207). Our LRP4 cohort was predominantly double positive with less than a quarter of them being single positive. This is perhaps why, clinically, as a group, the phenotype was not dissimilar to the other subgroups.

In our cohort of 150 patients, 8 patients were seronegative, as explained previously, one of these patients was positive on an initial AChR RIA when diagnosed and all the repeat testing on RIA and CBA were negative. Among the seronegative patients, ocular symptoms were seen slightly less frequently at 87.5% compared to antibody positive patients; however, this was not statistically significant.

Does antibody positivity and titres change with time and treatment?

There have been no large prospective studies looking at AChR titres with longitudinal data and long term follow up of patients.

Our study provides novel data on AChR titres. We compared the titres of AChR RIA in patients at recruitment and at annual follow-up. We found a significant drop in titres over the first year. The drop in titres was seen mainly in patients who were treatment naïve at recruitment and who were immunosuppressed at first year follow-up, and although there was a drop in titres in patients who remained immunosuppression naïve throughout the first year, it was not statistically significant. There was a comparable drop in scoring on AChR CBA as well when recruitment samples were compared with first year and second year follow-up samples. Both AChR RIA titres and MG composite scores fell during the first year, with linear correlation; similarly with MG QOL. It appears that with a clinical response to treatment, there is a corresponding fall in AChR titres. This would make sense theoretically. It appears that in an individual patient, falling titres may indicate clinical improvement, and vice versa. However, functional studies were not done.

Is there a difference in antibody titres in EOMG and LOMG?

There was a significant difference between AChR RIA titres in EOMG and LOMG, with the titres being much higher in LOMG. Compston et al found in their retrospective study of stored serum samples, that patients with thymoma had the highest titres of AChR Abs, followed by the EOMG patients, and then

the LOMG patients. They used 40 years as the age cut off. This was similar to data published by Lindsburg et al and Mantegazza et al. Somnier et al found lower concentrations of AChR in LOMG, but non significantly. In contrast, Lindstrom et al did not find any correlation with age. Neither Linstrom nor Somnier divided the groups into thymomatous and non-thymomatous. Our analysis also did not differentiate between thymomatous and non-thymomatous patients. As explained in previous chapters, the relationship between thymic abnormalities and LOMG is not entirely clear. It is postulated that abberations in the aged thymus in LOMG mimics thymoma behaviour without frank neoplasia, or, a small thymoma could have regressed spontaneously before the diagnosis of MG. It is therefore possible that our LOMG cohort reflects this immunological similarity with TAMG with high AChR titres.(97, 149, 150, 421).

We did not find a difference in AChR RIAs in EOMG and LOMG patients with thymic abnormalities. This could be because we did not thymectomise all EOMG patients; it is possible that a proportion of patients with no radiological abnormalities had histological abnormalities which were not picked up, and so a proportion of the data may be missing. (10, 97).

There was no significant difference in AChR RIA titres in patients who were on statins compared to those who were not

on statins. Similarly, there was no difference between patients who used salbutamol inhalers and those who did not. There is no previous literature on this.

In conclusion, our study has shown a lower rate of AChR single positivity, but when double positives were added, the positivity rates were similar to published data. EOMG patients were less likely to be positive on RIA, possibly due to lower affinity antibodies. AChR titres were higher in LOMG compared to EOMG. The titres dropped after treatment on annual review. There was no difference in AChR titres in patients with (mainly radiological) thymic abnormalities. There was no difference in MuSK or LRP4 positivity in the different subgroups, and we had a lower percentage of double seropositivity compared to published literature.

5 Regulatory T cells

5.1 Introduction

Myasthenia gravis is the best characterised antibody mediated autoimmune disease where patients have autoantibodies against nicotinic acetylcholine receptor at the neuromuscular junction. Although the condition is antibody mediated, anti-AChR-T cells have a crucial role in the pathogenesis because they permit and modulate the synthesis of the high affinity antibodies. Activation of potentially self reactive CD4+ T cells may be the primary event in the pathogenesis of myasthenia; this may occur because of cross-reactivity of self reactive CD4 T cells with microbial antigens or because of the action of microbial super antigens (Oldstone, Brocke et al) (424, 425).

The immune system is very organised, and proper functioning depends on adequate balance between the pro-inflammatory and anti-inflammatory signals, and the responses of the cellular system- the pro-inflammatory T cells (T helper 17 cells or TH 17 cells) and the anti-inflammatory cells the regulatory T cells (T regs cells). Defective balance is seen in a lot of autoimmune conditions (279-283). The balance between T reg cells and TH 17 cells in MG is disrupted. T regs are shown to be defective in their regulatory function and can start expressing markers of TH 17 cells while the effector T cells or T eff cells (CD4+25-) become resistant to suppression (51, 284-287).

One large cohort study of 79 MG patients with an established diagnosis of MG, 59 of whom were immunosuppression naïve, and 13 of whom went on have steroids during the study, found that Treg levels were lower in the untreated group compared to those who were given steroids. In the 13 treatment naïve patients who went on to have steroids, the Treg levels and Tfr-like cells increased and Tfh-like cells decreased (298).

Autoimmune diseases could be caused by a deficiency in the number of T reg cells and/or defective suppression of the T reg cells. There have been several contradictory studies regarding the numbers and function of T reg cells, some of which show that the T reg cell numbers are reduced in MG patients compared to healthy controls (227, 293, 294), whereas other studies showed no difference in the percentage of CD4+FoxP3+ T reg cells in the thymus and peripheral blood (295, 296). Most studies report that the T reg cells have reduced suppressive activity. A decreased expression of Foxp3 in MG thymus and in the peripheral blood could be one of the reasons for the impaired suppressive activity (51, 52, 265).

There is an increased production of IL-17 which is a cytokine and is expressed by TH 17 cells. Several studies have shown that in thymic hyperplasia there is increased production of pro-inflammatory cytokines like IL-6, IL-1 β, interferon-γ, and TNF α (250, 310). There is also increased production of the anti-

inflammatory cytokine TGF or transforming growth factor β-1. Serum IL-27 is a heterodimeric cytokine and is produced by APCs. It promotes Th1 differentiation and suppresses inflammation by inhibiting Th17 and Th2 cells. Serum IL-27 levels have shown to be significantly higher in MG patients compared to controls and it was significantly higher in EOMG suggesting that it could possibly contribute to MG pathogenesis or immunoregulation (312). Other studies have shown that IL-17A was higher amongst MG patients with EOMG, women without thymoma showing the greatest elevations of IL-17A (314).

In vitro stimulated PBMCs in MuSK Ab positive MG patients but not in AChR Ab positive MG patients have been reported to show a significantly increased secretion of IFN gamma, IL-17A and IL-21. It was postulated that TH1 and TH 17 immune reactions play a role in MuSK MG and immunosuppression attenuates the TH1 response in AChR MG and in MuSK MG, but it modulates immune responses differently in the two groups.

So far, except for a few, all studies on Tregs and cytokines in MG have been done on a small number of patient samples. What is particularly lacking is data on treatment naïve patient samples. There are no large scale prospective studies to date. Our study was a unique chance to look at the Immunological profile from a diagnostic cohort longitudinally. This would then

give us a chance to assess Treg levels, and some of the cytokines that are thought to be deranged in MG, compare EOMG with LOMG, and look for changes with time and treatment.

With our prospective study with long term follow up, we sought to answer the following questions:

- Is there a difference in T reg levels in the peripheral blood lymphocytes of MG patients compared to healthy controls?
- Is there a difference in T reg levels in the peripheral blood lymphocytes and in LOMG patients compared to EOMG patients?
- Is there a difference in the cytokines lFNα, IFNγ, TNFα, IL-10, IL-17 and IL-4 in the peripheral blood lymphocytes of MG patients compared to healthy controls?
- Is there a difference in the cytokines lFNα, IFNγ, TNFα, IL-10, IL-17 and IL-4 in the peripheral blood lymphocytes and in LOMG patients compared to EOMG patients?
- Is there a difference in Treg levels and cytokines in patients with thymic abnormalities?
- Is there a difference in Treg levels and cytokine levels in the different antibody subgroups?

5.2 Methods

Whole blood was collected from 135 MG patients and 8 LEMS patients at recruitment and 24 MG patients at first year follow up, 9 of whom had been immunosuppressed in the first year, 11 who were immunosuppression naïve at first year follow up and the remainder had been immunosuppressed at recruitment.

Peripheral blood monocytes/lymphocytes (PBMCs) were isolated using the method described below by the author within 4 hours of sample collection. The majority of the PBMC isolation was done in the laboratory at the Neurology research unit at the Queen's Medical Centre, Nottingham. Some of the samples collected at Birmingham and Oxford were processed at the University of Birmingham laboratories.

The method of PBMC isolation is the same as that used by the MS research team at Nottingham (Prof Constantinescu and Dr Gran) and is described here briefly. The detailed protocol and controls used for flow cytometry are described in Appendix 3.

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood which was drawn from patients in heparinised tubes. This was layered on histopaque and centrifuged for 20 minutes. The PBMCs were recovered from the interface, washed and viability checked using trypan blue. Cell counts

were noted and the PBMCs were preserved initially in the -80^{0}C refrigerator and then transferred to liquid nitrogen.

Table 28 Consumables and reagents used in PBMC staining

ITEM (Panel 1-Treg)	LOCATION	ORDERING
CD4 FITC	Fridge (4C)	BD biosciences (555346)
CD 25 PE	Fridge	BD biosciences (555432)
CD127 PE-Cy7		BD biosciences (560822)
FoxP3 Alexa Flour 647	Fridge	BD biosciences (560045)
Human FoxP3 Buffer Set BD biosciences		BD biosciences 560098
Live/Dead fixable blue dead cell stain kit, for UV excitation		L34962
OneComp eBeads Compensation beads		01-1111-42
FITC Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 555748
PE Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 555749
PE-Cy 7 Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 557872
Alexa Flour 647 Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 557714
ITEM (Panel 2-Th17)	LOCATION	ORDERING

INFγ APC		Biolegend 506510
INFα PE		BD biosciences 560097
TNFα PerCP-Cy5.5		Biolegend 502926
CD3 APC-Fire 750		Biolegend 344840
CD4 FITC	Fridge (4C)	BD biosciences 555346
CD8 PE-Cy7		Biolegend 301012
Fixation/Permeabilization solution Kit		BD GolgiStop 554715
Live/Dead fixable blue dead cell stain kit, for UV excitation		L34962
OneComp eBeads Compensation beads		01-1111-42
APC Mouse IgG1 κ Isotype Control		Biolegend 400142
PE Mouse IgG1 κ Isotype Control Clone MOPC-21		BD biosciences 555749
PerCP-Cy5.5 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400150
APC- APC/Fire 750 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400196
FITC Mouse IgG1, κ Isotype Control Clone		BD biosciences 555748

MOPC-21		
PE-Cy7 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400126
ITEM (Panel 3-Th2)	LOCATION	ORDERING
IL17 PE		Biolegend 512306
IL4 PerCP-Cy5.5		BD biosciences 561234
IL10 APC		Biolegend 506807
CD3 APC/Fire 750		Biolegend 344840
CD4 FITC	Fridge (4C)	BD biosciences 555346
CD8 PE-Cy7		Biolegend 301012
Fixation/Permeabilization solution Kit		BD GolgiStop 554715
Live/Dead fixable blue dead cell stain kit, for UV excitation		L34962
OneComp eBeads Compensation beads		01-1111-42
PE Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400140
PerCP-Cy5.5 Mouse IgG1 κ Isotype Control Clone MOPC-21		BD biosciences 550795
APC Rat IgG2a κ Isotype Control Clone R35-95		Biolegend 400512
APC/Fire 750 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400196
FITC Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 555748

PE-Cy7 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400126
ITEM	LOCATION	ORDERING
EDTA 100 mM (20 ul/tube)*7 tubes	Room temp	
Formaldehyde 4% (1 ml/tube)*7 tubes & 0.5% (0.4 ml/tube)*11 tubes (40% stock w/ isoton dil.)	Room temp	B8F77119 (Philips-Harris)
Isoton diluent	Room temp	8448011(Beckman-Coulter)
Aluminium foil (kitchen quality)	Room temp	Terinex
PBA (0.5%): BSA (0.5%) in PBS: (3 ml/tube)*4 tubes (0.25 ml 30% BSA, add PBS up to 15 ml)	Fridge	A7284 (Sigma)
PBS (diluents to BSA)	Room temp	LH-SIG2017E (MSS)

All of our samples were cryopreserved. The PBMCs were thawed in batches for the staining.

Once the PBMCs were thawed, they were washed and the cells counted again. They were placed into 96 well plates and stained with live/dead stain. We used three separate panels for staining. The stains used for each of the panels are listed in the table above. The first panel was for Treg cells. Panel two was for IFN gamma, IFN alpha and TNF alpha. Panel three was for IL-17, IL-

4 and IL-10. After extracellular staining, the well plates were covered in foil and incubated for 30 minutes.

After washing, Panel 1 was fixed and permeabilised with the FoxP3 buffer set. Panels two and three were also washed and fixed and permeabilised using the fixation and permeabilisation kit. Following this, intracellular stains were added and the plates were incubated for a further 30 minutes. These were washed and resuspended, in preparation for flow cytometry.

We used single colour controls for each colour used with compensation beads. Flow cytometry these days uses many different fluorochromes to analyse different proteins/ cells. The success of polychromatic flow cytometry depends on a number of hardware factors that need optimising and calibrating to obtain the best results. One of the ways to calibrate this is by using beads. The identification of cells on flow cytometry depends on the levels of background signals, which can arise from various sources. The cleanest method for assessing the background is by using compensation beads which are singly stained with the fluorochromes used in the experiment. This process reduces spreading error and increases the dynamic range of cell population determination (426).

In our experiment, 25 µL of compensation beads were added for each single colour control used; these were vortexed, covered in

foil and incubated for 15 minutes. They were then diluted with 250 µL of PBS.

During flow cytometry, the contents of each well were transferred into a FACS tube and run on the flow cytometry machine. The results were interpreted using the Kaluza software. For panel one, the cells were first gated and separated into live cells, then CD4+ cells. The next gating was CD4+/CD127low cells, and the final gating was CD4+/CD127low/CD25+/FoxP3+ cells. For panel two, the gating was CD3+ then CD3+CD4+CD8-. These were separated into interferon gamma+, TNF Alpha+, and interferon-alpha+. Further separate gating was used for CD3+CD4-CD8+ and again separated into the individual cytokines. For panel three the gating used was CD3+CD4+CD8- and then separated into IL-10 IL-4 and IL-17 and then separate gating used for CD3+CD4-CD8+ and separated into the individual cytokines.

All the results obtained on Kaluza were converted into an Excel spreadsheet and analysed using GraphPad Prism software.

The software provides results in both absolute numbers and percentages; Treg percentage represents the percentage of all CD4+ cells which express CD25 and FoxP3, and have low expression of CD127. As absolute numbers change with each sample tested, and depend on whether they are fresh or cryopreserved, percentages are a better way of comparing data.

Our cohort was very large with many samples which were tested in batches on different days, and we felt that expressing the results as percentages would make them more comparable.

5.3 Results

5.3.1 Treg cell population

T reg proportions were measured according to the method described above. Although in literature the percentage of T regs have been described to be anywhere between 1 to 10%, in our cohort, all had lower readings of less than 1% with a few outliers with higher percentages. The few outliers amongst the Treg patient samples who had much higher percentages than the median were looked at; there were 8 samples, 5 of whom had OMG- of these 3 generalised later on; 3 patients had GMG- 1 with associated malignant thymoma and another with associated MGUS.

After discussion with the immunologists, we concluded that as the same staining method and protocol was used for all patient samples and healthy controls, the results could still be compared, but comparison with published literature needed to be done cautiously.

The median for T regs in patient samples was 0.06% and in healthy controls it was 0.51%, P <0.0001 (Figure 60) (Table 28).

T regs in EOMG and LOMG at recruitment did not show any statistically significant difference; median for EOMG was 0.035% and LOMG was 0.065%, P = 0.1635 (Figure 61).

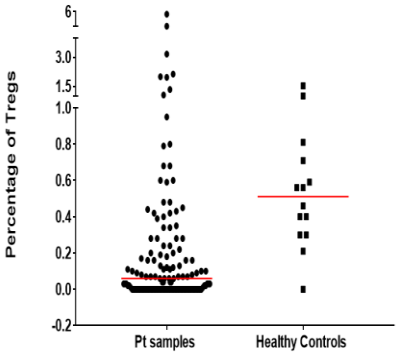


Figure 60 Comparison of T reg percentages (with medians) in patient samples and healthy controls p<0.0001

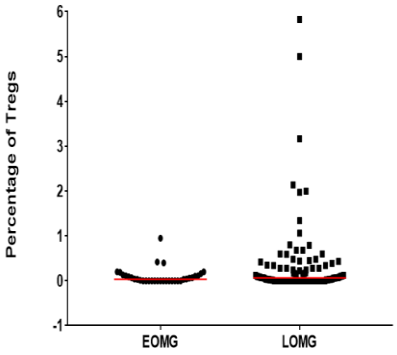


Figure 61 Comparison of T reg percentages (with medians) at recruitment in EOMG and LOMG p=0.1635

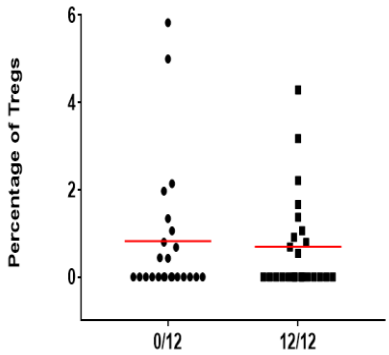


Figure 62 Comparison of T reg percentages (with medians) in paired patient samples at recruitment and first year follow up p-0.9591

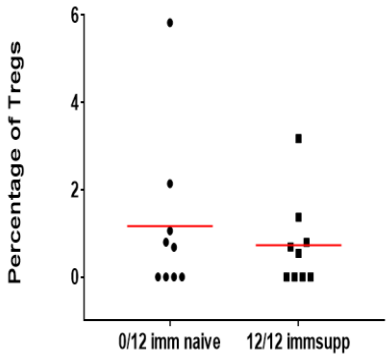


Figure 63 Comparison of T reg percentages (with medians) in paired patient samples who were immunosuppression naïve at recruitment and immunosuppressed at first year follow up p=0.0625

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

T regs compared between immunosuppression naïve patients and immunosuppressed patients at recruitment (non-paired samples) did not show any significant difference, median of 0.06% in the immunosuppression naïve patients and 0.015% in the immunosuppressed patients with P = 0.8004. For 24 patients, we obtained whole blood at first year follow up and isolated PBMCs. Of the 24 patients, 9 patients were treatment naïve at recruitment and were immunosuppressed at first year follow up, 11 patients were still treatment naïve at first year follow up, and the remainder had been immunosuppressed at recruitment and continued to be immunosuppressed at first year follow up.

When all the paired samples were compared at recruitment and first year follow up, the medians were 0% for both, p = 0.9591 (Figure 62).

Comparison of T regs in immunosuppression naïve patients at recruitment with their paired samples at first year follow up after being immunosuppressed did not show a difference, p = 0.0625 (Figure 63); the median for immunosuppression naive patients was 0.68% and immunosuppressed patients at first year follow up was 0.54%.

There was no significant difference in T reg levels in patients who were immunosuppression naïve at recruitment with their

paired samples at first year follow up in those patients who remained immunosuppression naïve, P = 0.6250.

The T regs in LOMG patients at recruitment compared to paired first year samples did not show any statistical difference, P =0.3223.

T regs at recruitment were compared in those patients with thymic abnormality (including histological abnormalities, and those with radiological abnormalities only) and those with normal thymuses; median for thymic abnormality was 0.03% compared to normal thymus 0.06%;this did not reach statistical significance p = 0.4035. There was no difference in T regs in patients with normal thymus and thymic hyperplasia p = 0.965 (Figure 64) and no difference in normal thymus versus thymoma either, P = 0.6792 (Figure 65). T reg percentages at recruitment were plotted against ACHR RIA titres at recruitment using simple linear regression, and there was no linear correlation.

When the recruitment samples of AChR Ab patient T regs were compared with T regs from healthy controls, there was a significant difference in numbers with the Treg count of 0.07% in AChR positive patients compared to 0.51% in the healthy controls P = 0.0005 (Figure 67). We did not analyse AChR RIA postives and CBA positives separately. There was a significant difference also when the T regs from healthy controls were compared with the T regs from all double seropositive patients,

median of the double positive being 0.06% and for healthy controls 0.51%, P = 0.0021. The Tregs were compared between AChR positive patients with the MuSK positive patients and there was no statistical statistically significant difference, P = 0.2591.

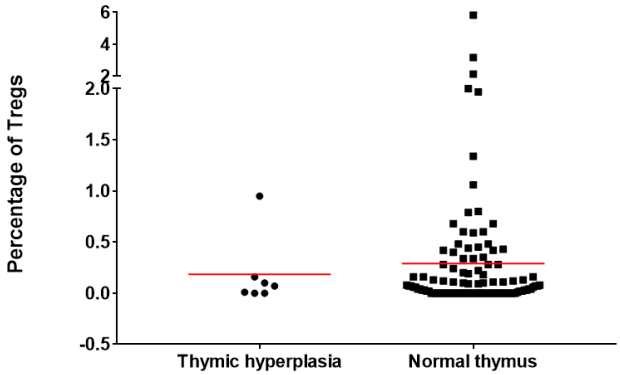


Figure 64 Comparison of T reg percentages (with medians) in patients with normal thymus and with thymic hyperplasia p=0.965

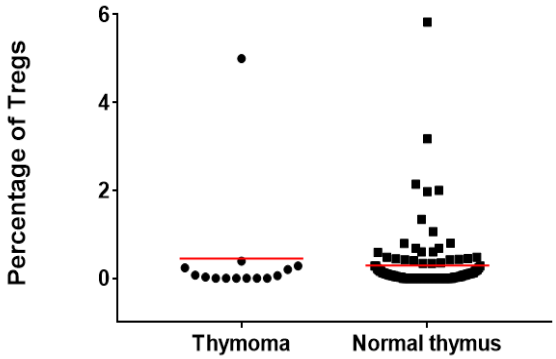


Figure 65 Comparison of T reg percentages (with medians) in patients with normal thymus and with thymoma p=0.6792

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

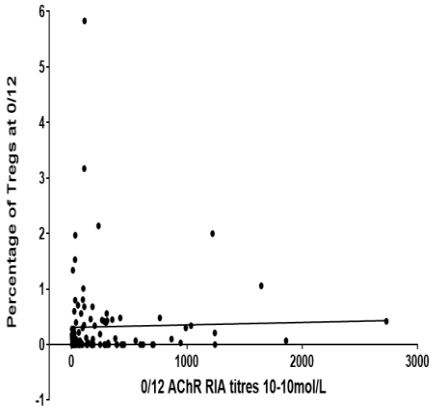


Figure 66 Correlation between T reg percentages and AChR RIAs in patient samples at recruitment p=0.7838 on simple linear regression

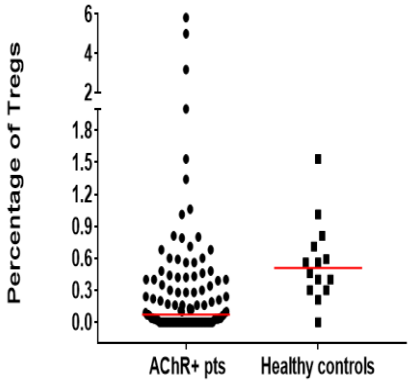


Figure 67 Comparison of T regs (with medians) at recruitment in AChR+ patients and healthy controls p=0.0005

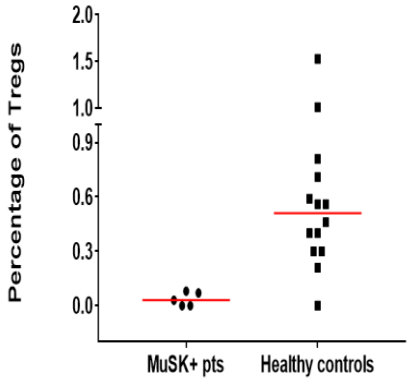


Figure 68 Comparison of Tregs (with medians) in MuSK+ patients and healthy controls p=0.0018

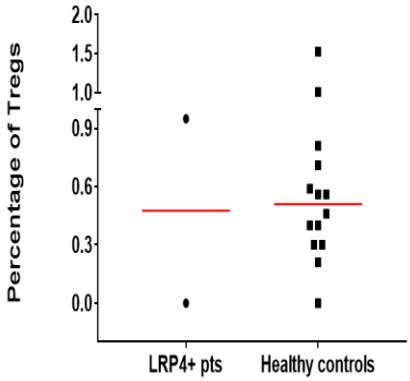


Figure 69 Comparison of Tregs (with medians) in LRP4+ patients and healthy controls p=0.8417

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Figure 66: Simple linear regression used

The T regs at recruitment in MuSK positive patients were compared with healthy controls. There was a statistically significant difference with median of 0.03% in the MuSK positive patients and 0.51% in the healthy controls, p = 0.0018 (Figure 68).

The Tregs in LRP4 positive patients was compared with healthy controls. There was no statistical significance with median of 0.475% in the LRP4 group and 0.5% in healthy controls, p=0.8417 (Figure 69).

5.3.2 Cytokines and Treg cells

In addition to the Treg panel as described above, we did a further 2 panels, the second panel looking at interferon alfa, IFN gamma, and TNF alpha production by CD4 and CD8 cells; and panel three looking at IL-17, IL-10 and IL-4 production by CD4 and CD8 cells. This was based on previously published literature on cytokine abnormalities seen in MG. IFNα however, was added on as part of the panel with IFNγ and TNFα; we acknowledge that this is unlikely to provide much information in this context and has not been included in the analysis. The staining methods and flow cytometry was as described above.

CD4 interferon-gamma in patient samples at recruitment compared to healthy controls did not show a significant difference, median for patient samples was 22.82% and for healthy controls was 15.62%, P= 0.1692 (Figure 70A) (Table 28).

CD4 interferon-gamma in patients who were treatment naïve at recruitment with those who were immunosuppressed at

recruitment showed no difference with median for immunosuppression naïve patients of 24.17% and immunosuppressed patients of 19.59%, P = 0.0749 (Figure 70B).

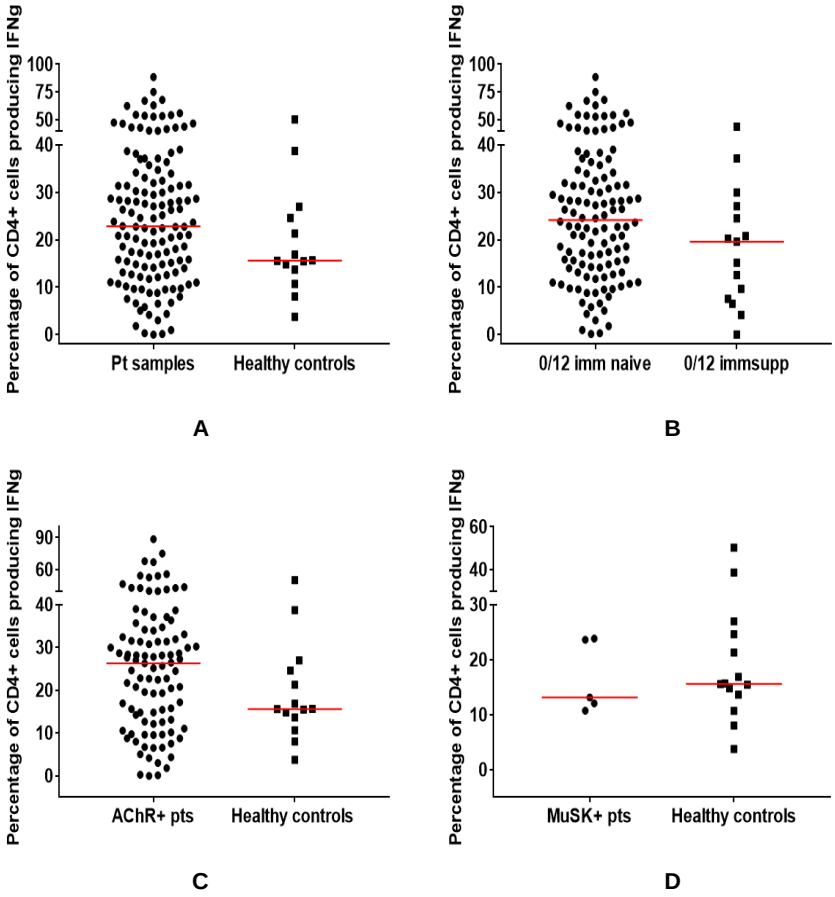


Figure 70 Comparison of CD4 IFN-γ (with medians) in (A) patient samples and healthy controls p=0.1692 (B) patients who were immunosuppressed and those who were not, at recruitment p=0.0749 (C) ACHR+ patients and healthy controls p=0.1453 and (D) MuSK+ patients and healthy controls p=0.6216

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Patients who were treatment naïve at recruitment and were given steroids in the first year did not show any significant difference in their interferon-gamma levels either, P = 0.9453. Patients who remained immunosuppression naïve through their first year also did not show any difference in CD4 interferon-gamma, P = 0.4609.

EOMG patients did not show any difference in their CD4 interferon-gamma levels at recruitment and first year follow-up, p> 0.9999. Similarly LOMG patients did not show a difference either with p>0.9999.

The percentage of CD4 interferon-gamma positive cells was compared between AChR positive patients and healthy controls; there was no statistically significant difference with median for AChR positive patients of 26.29% and healthy controls of 15.62%, P = 0.1453 (Figure 70C). There was no difference in the double seropositives compared to healthy controls either and there was no difference between AChR positive patients and MuSK positive patients; median for AChR positive patients was 26.29% and for MuSK positive patients 13.13%, P = 0.1826.

CD4 interferon-gamma in MuSK patients compared to healthy controls did not show any statistically significant difference; median for MuSK patients was 13.13% and in healthy controls was 15.62%, P = 0.6216 (Figure 70D).

CD8 interferon-gamma in patient samples at recruitment compared to healthy controls did not show a difference with median for patient samples of 41.13% and healthy controls of 26.08%, P = 0.0795 (Figure 71A).

There was a statistically significant difference between the percentage of CD8 interferon-gamma positive cells in EOMG and LOMG patients at recruitment, median of 30.49% in EOMG patients and 47.98% in LOMG patients, P = 0.0002 (Figure 71B).

There was no statistically significant difference between EOMG patient samples compared to EOMG healthy controls, P = 0.6917; nor was there a difference between LOMG patients & LOMG healthy controls, P = 0.0818.

There was no difference in CD8 interferon-gamma levels between patients who were immunosuppression naïve and those were immunosuppressed at recruitment, P = 0.6889.

When paired samples were compared for CD8 interferon-gamma, there was no difference between recruitment samples and first year samples, P = 0.7987. In patients who were immunosuppression naïve at recruitment and those who received steroids over the first year, there was no difference in CD8 interferon-gamma, p= 0.578. There was no difference when

patients remained immunosuppression naïve for the first year either, P = 0.4609.

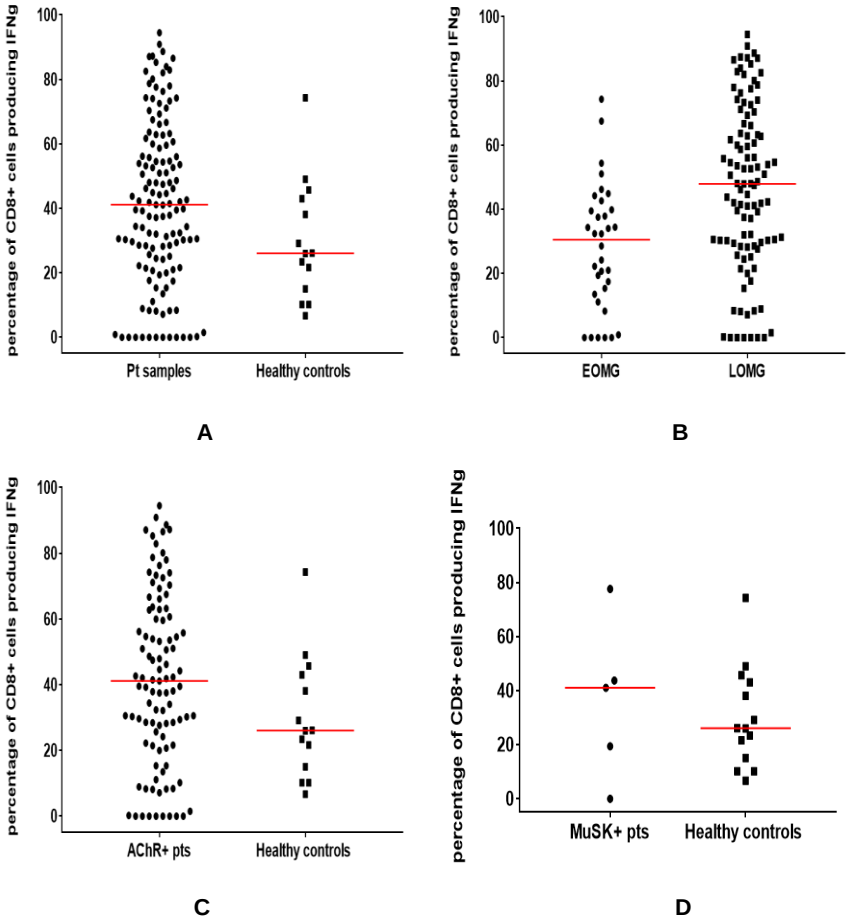


Figure 71 Comparison of CD8 IFN-γ (with medians) in (A) patient samples and healthy controls p=0.0795 (B) EOMG and LOMG at recruitment p=0.0002 (C) AChR+ patients and healthy controls p=0.0978 and (D) MuSK+ patients and healthy controls p=0.7539

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

There was no difference in EOMG patient samples at recruitment and at first year follow up with p>0.9999 and the LOMG patient samples were also not significant.

CD8 interferon-gamma was compared between AChR positive patients and healthy controls; there was no difference with median of 41.13% in AChR patients compared to 26.08% in healthy controls, p = 0.0978 (Figure 71C). There was no difference when this was compared between double seropositives and healthy controls, P = 0.2322; and there was no difference between AChR positive and MuSK positive patients, P = 0.6536.

CD8 interferon-gamma in MuSK positive patients compared to healthy controls was not statistically significant; median for MuSK patients was 41.03% and healthy controls was 26.08%, P = 0.7539 (Figure 71D).

The percentages of CD4 TNF alpha positive cells in recruitment patient samples compared to healthy controls were significantly different, median for patient samples was 63.52% and for healthy controls was 53.7%, P = 0.0237 (Figure 72A).

CD4 TNF alpha in EOMG patients was positive in 60.66% compared to LOMG patients 64.11%, this was not significantly different, P = 0.067 (Figure 72B). EOMG patient samples compared to healthy controls at recruitment did not show a statistically significant difference, P = 0.1499 nor did LOMG patients compared to healthy controls, p = 0.4065. This may be because the number of healthy controls was less.

CD4 TNF alpha levels between immunosuppressed and immunosuppression naïve patients at recruitment was not significantly different, P = 0.4130. CD4 TNF alpha levels in paired samples at recruitment and first year follow-up was not significantly different, p = 0.2726 (Figure 72C).

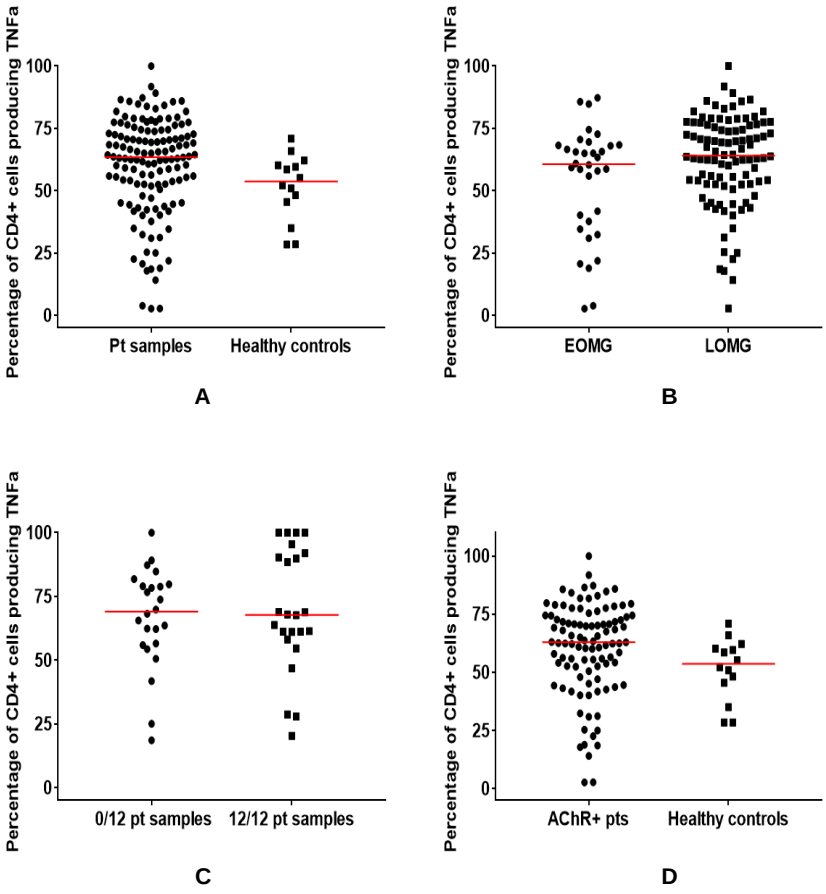


Figure 72 Comparison of CD4 TNFα (with medians) in (A) patient samples and healthy controls p=0.0237 (B) EOMG and LOMG p=0.0670 (C) paired patient samples at recruitment and first year follow up p=0.2726 and (D) AChR+ patients and healthy controls p-0.0279

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

In patients who were immunosuppression naïve at recruitment and given steroids over the first year, the CD4 TNF alpha levels were not significantly different, P = 0.3223. There was no significant difference between patients who remained treatment naïve over the first year either, P = 0.595.

CD4 TNF alpha in EOMG patients at recruitment and first year follow-up did not show any significant difference, P =0.2500. LOMG patients did not show a difference either, P = 0.6226.

CD4 TNF alpha in AChR positive patients compared to healthy controls were significantly different; median for AChR positive patients was 62.93% and for healthy controls was 53.7%, P = 0.0279 (Figure 72D); the difference was still significant in double seropositives, P = 0.0285. There was no statistically significant difference between the AChR positive and MuSK positive patients, P = 0.6156.

CD4 TNF alpha in MuSK positive patients compared to healthy controls was not statistically significant; median of 53.7% in MuSK patients and 68.23% in healthy controls (p=0.2566) (Figure 73A). This was different to the AChR antibody positive patients in whom there was a statistically significant difference. This could be because we only had 5 MuSK+ samples which were tested for TNFalpha.

The percentages of CD8 TNF alpha positive cells in patient samples at recruitment showed a difference compared to controls; median for patient samples was 44.4% and healthy controls was 28.86%, P = 0.0489 (Figure 74A). CD8 TNF alpha in EOMG and LOMG groups at recruitment was also significantly different, median for EOMG being 29.77% and LOMG 52.95%, P <0.0001 (Figure 74B). CD8 TNF alpha in EOMG patients at recruitment compared to EOMG healthy controls was not statistically significant, p> 0.9999; there was no difference in the LOMG patients at recruitment compared to the healthy control LOMG patients, p= 0.2336. This may be because the number of healthy controls was lower, and so, on subgroup analysis, the difference was not significant.

CD8 TNF alpha in patients who were treatment naïve at recruitment compared to those who were immunosuppressed at recruitment was not statistically significantly different, P = 0.4293.

Comparison of paired samples at recruitment and first year follow-up did not show any statistically significant difference in CD8 TNF alpha, P = 0.0858 (Figure 74C). There was no difference in patients who were treatment naïve at recruitment and immunosuppressed at first year, P = 0.8125; and there was no difference in patients who remained treatment naïve over the first year, P = 0.191.

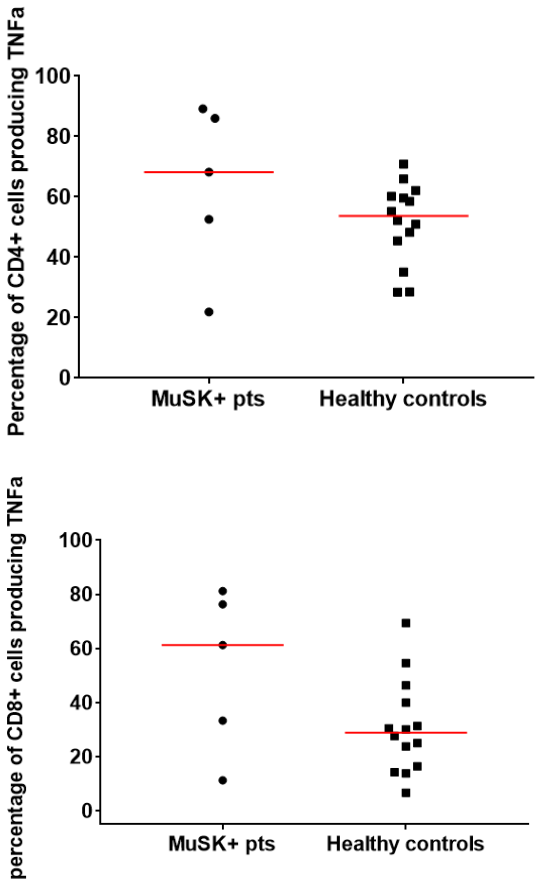


Figure 73 Comparison between (with medians) MuSK+ patients and healthy controls of (A) CD4 TNFa p=0.2466 and (B) CD8 TNFa p=0.1299

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

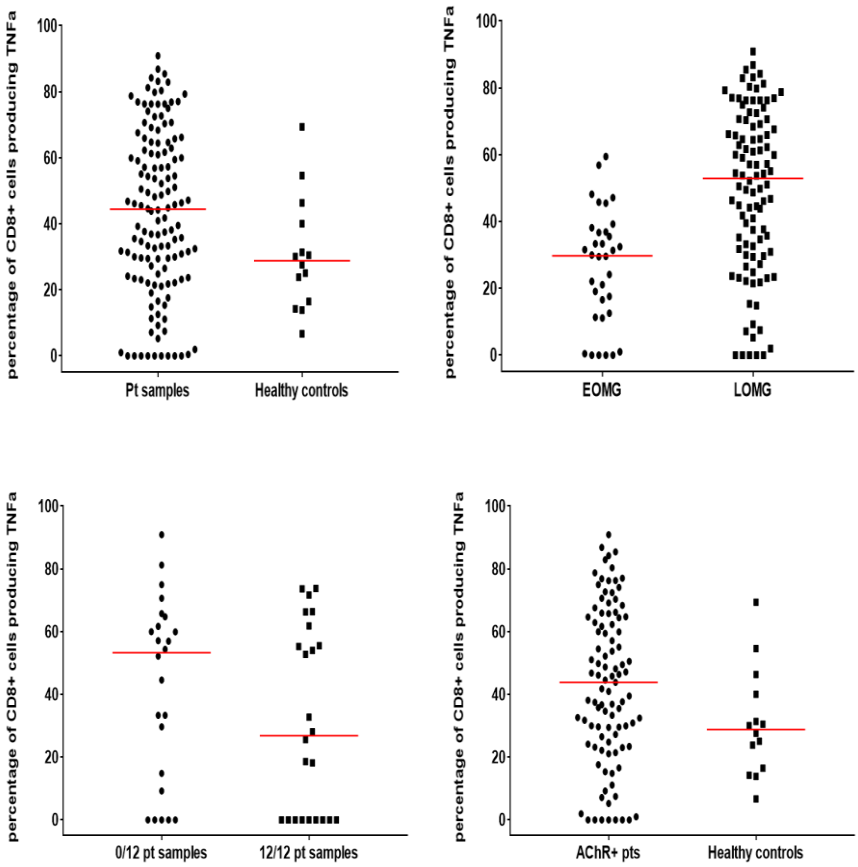


Figure 74 Comparison of CD8 TNFα (with medians) in (A) patient samples and healthy controls p=0.0489 (B) EOMG and LOMG p<0.0001 (C) paired patient samples at recruitment and first year follow up p=0.0858 and (D) AChR+ patients and healthy controls p=0.0636

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

The percentage of CD8 TNF alpha positive cells did not change statistically in the EOMG group from recruitment to first year follow-up, P = 0.5000 and they did not change in the LOMG group either, p= 0.2633.

CD8 TNF alpha compared between AChR positive patients and healthy controls did not show a difference, median being 43.86% in AChR positive patients and 28.86% in healthy controls, P = 0.0636 (Figure 74D). There was no difference when double seropositives were compared with healthy controls p = 0.1425, and there was no difference when AChR and MuSK positives were compared with each other.

CD8 TNF alpha in MuSK positive patients was also not significantly different from healthy controls; median for MuSK positive patients was 61.32% and healthy controls was 28.86%, P = 0.1299 (Figure 73B).

Percentage of **CD4 cells producing IL-10** were significantly greater in the patient samples compared to healthy controls at recruitment; the median for patient samples was 6.51% and healthy samples was 2.185%, P = 0.0082 (Figure 75A).

There was no difference in CD4 IL-10 between EOMG and LOMG at recruitment, p= 0.5642 (Figure 75B). When EOMG patient samples at recruitment were compared to EOMG healthy controls, there was a significant difference in CD4 IL-10, P = 0 (Figure 75C). There was no difference in LOMG patient samples at recruitment compared to LOMG healthy controls, P = 0.1899 (Figure 75D).

There was no difference in CD4 IL-10 between patients who were treatment naïve and those who were immunosuppressed at recruitment, P = 0.8728.

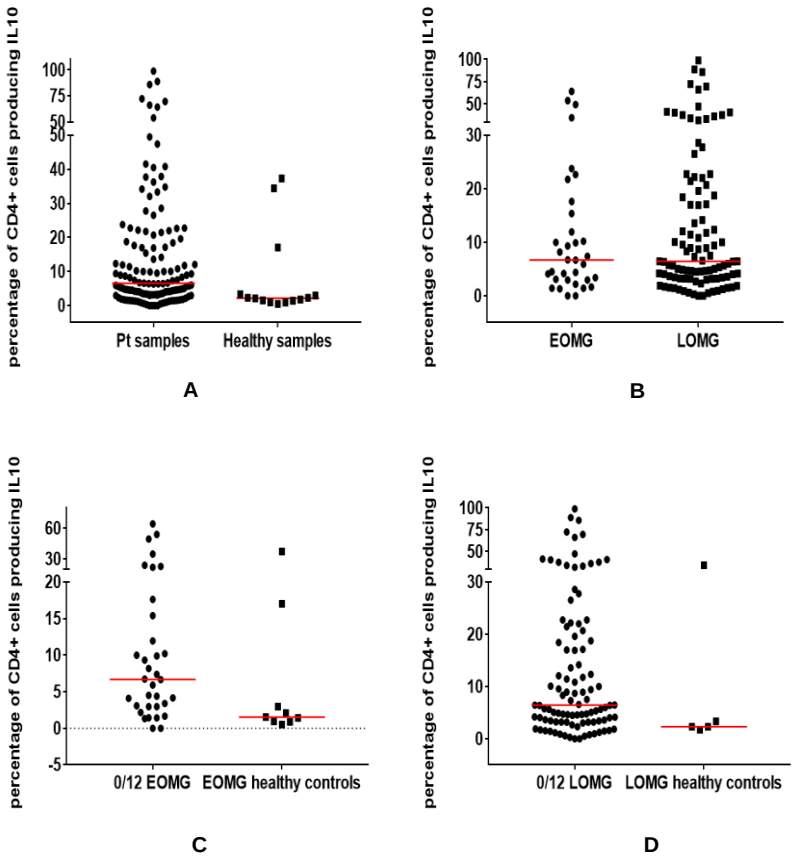


Figure 75 Comparison of CD4 IL10 (with medians) in (A) patient samples and healthy controls at recruitment p=0.0082 (B) EOMG and LOMG p=0.5642 (C) EOMG patient samples and EOMG healthy controls at recruitment p=0.0400 and (D) LOMG patient samples and LOMG healthy controls at recruitment p=0.1899

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

When paired samples at recruitment and first year follow-up were compared, there was no difference in CD4 IL-10, p=

0.7843. There was no difference in treatment naïve recruitment samples compared to their paired first-year follow-up samples when they were immunosuppressed either, p= 0.4961. There was no difference in CD4 IL-10 counts between immunosuppression naïve patients at recruitment who remained treatment naïve at first year follow-up, P = 0.5195. Similarly there was no difference in EOMG patients between recruitment and first year follow-up, p=0.2500, nor was there any change in LOMG patients from recruitment to first year follow-up, P = 0.70124.

CD4 IL-10 was compared between AChR positive patients and healthy controls; this showed a significant difference with median of 6.57% in AChR positive patients and 2.185% in healthy controls, P = 0.0098 (Figure 76A). It was still statistically significant when double seropositives were compared with healthy controls, P = 0.0196. There was no difference between MuSK and AChR positive patients.

CD4 IL-10 in MuSK positive patients was not statistically significantly different to healthy controls; median for MuSK patients was 5.64% and healthy controls was 2.185%, P = 0.4998 (Figure 76B).

CD8 IL-10 did not show significant difference between patient samples at recruitment compared to healthy controls; median for

patient samples was 2.785% and for healthy controls was 1.035%, P = 0.5097 (Figure 77A).

There was no significant difference between the EOMG and LOMG subgroup either at recruitment, P = 0.6988. This was no significant difference between EOMG patient samples at recruitment compared to EOMG healthy controls, P = 0.55930, and there was no difference in the LOMG patient samples at recruitment compared to LOMG healthy controls, P = 0.9677.

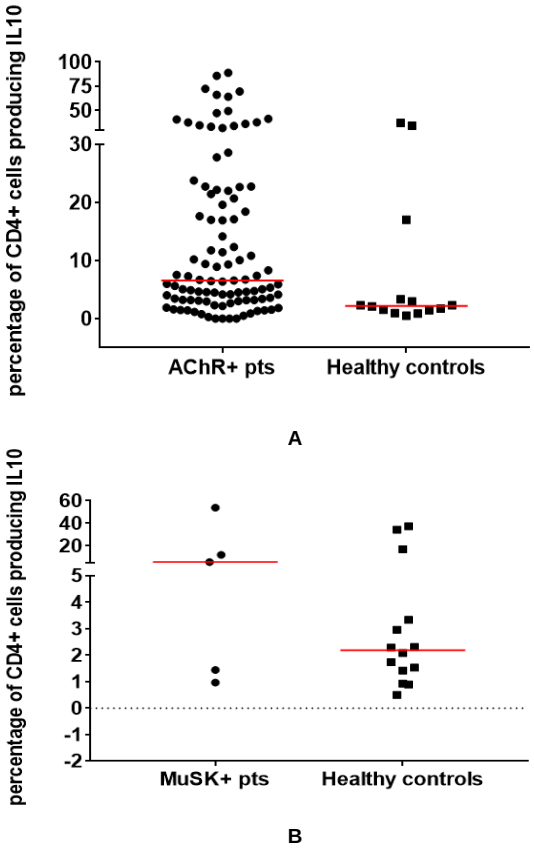


Figure 76 Comparison of CD4 IL10 (with medians) at recruitment in (A) AChR+ patients and healthy controls p=0.0098 and (B) MuSK+ patients and healthy controls p=0.4998

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

This was no statistically significant difference between the patients who were immunosuppression naïve compared to those who were immunosuppressed at recruitment, P = 0.8952.

When paired samples were compared at recruitment and first year follow-up, there was no significant difference in CD8 IL-10 counts, P = 0.8986. There was no significant difference between patients who were treatment naïve at recruitment and who were given steroids and first year follow-up, p=0.2500, and there was no difference in paired samples in patients who were treatment naïve at recruitment and stayed treatment naïve at first year follow-up, P = 0.4688.

Paired samples in LOMG at recruitment and first year follow-up did not show any significant difference in CD8 IL10, P = 0.8986.

There was no difference in CD8 IL-10 between AChR positive patients and healthy controls, median for AChR positive patients was 3.85% and healthy controls was 1.035%, P = 0.5063 (Figure 77B). There was no difference between double seropositives and healthy controls. There was no difference in CD8 IL 10 between AChR positive patients and MuSK positive patients, median for AChR positive patients being 2.85% and MuSK positive patients 1.05%; P = 0.0871 (Figure 77C). CD8 IL-10 in MuSK positive patients was not statistically significantly different from healthy controls; median for MuSK positive

patients is 1.05% and healthy controls was 1.035%, P = 0.5115 (Figure 77D).

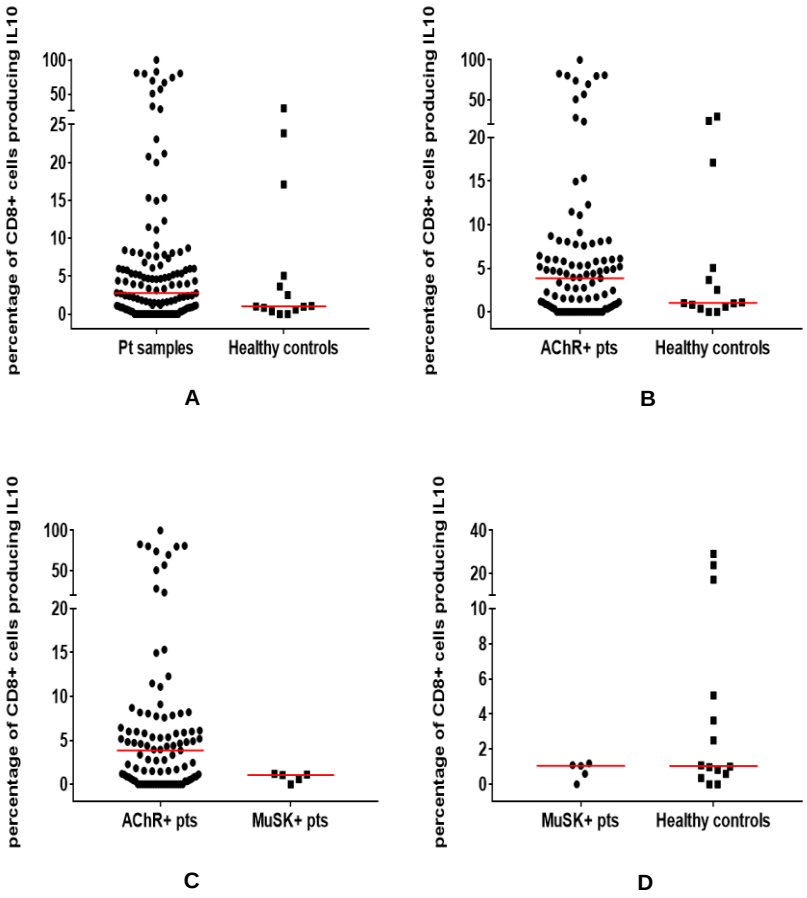


Figure 77 Comparison of CD8 IL10 (with medians) in (A) patient samples and healthy controls at recruitment p=0.5097 (B) AChR+ patients and healthy controls p=0.5063 (C) AChR+ and MuSK+ patients p=0.0871 and (D) MuSK+ patients and healthy controls p=0.5115

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

CD4 IL-17 in patient samples at recruitment compared to healthy controls showed a significant difference, median for patient samples was 1.525% and for healthy controls it was 0.705%, P = 0.0009 (Figure 78A).

There was no significant difference in CD4 IL-17 between the EOMG and LOMG patients at recruitment, P = 0.3159 (Figure 78B). There was a difference between EOMG patient samples at recruitment compared to EOMG healthy controls, P = 0.0159 (Figure 78C) but there was no statistically significant difference in the LOMG patient samples compared with LOMG healthy controls at recruitment, P = 0.2488 (Figure 78D).

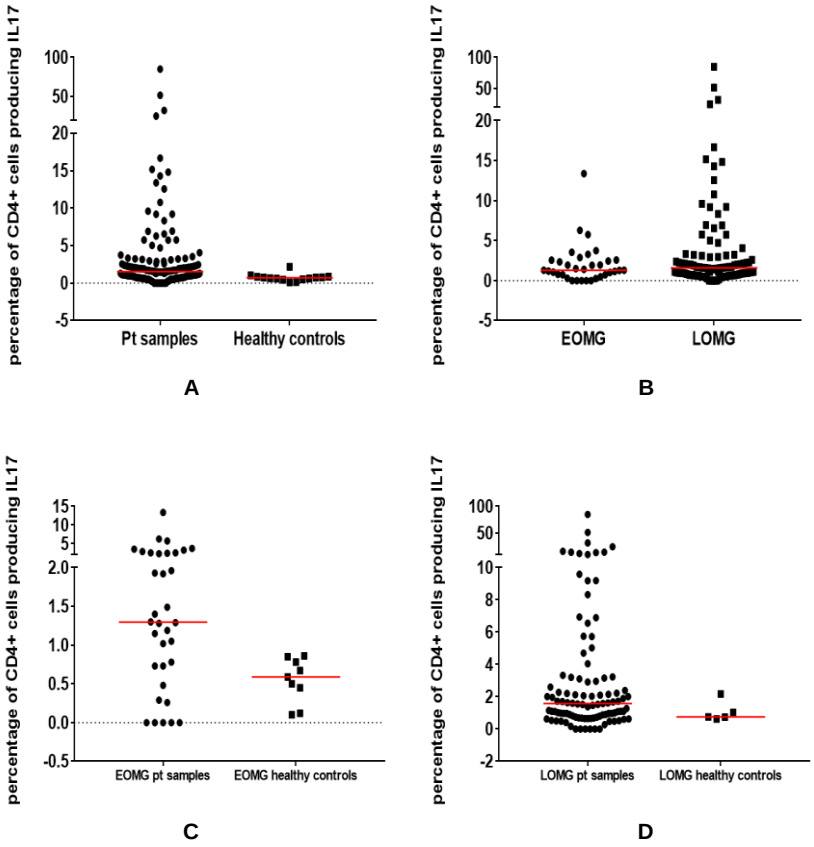


Figure 78 Comparison of CD4 IL17 (with medians) in (A) patient samples and healthy controls at recruitment p=0.0009 (B) EOMG and LOMG p=0.3159 (C) EOMG patient samples and EOMG healthy controls at recruitment p=0.0159 and (D) LOMG patient samples and LOMG healthy controls at recruitment p=0.2488

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

There was no difference in CD4 IL-17 in patients who were immunosuppression naïve and those who were immunosuppressed at recruitment, P = 0.2249.

CD4 IL-17 in paired samples at recruitment and at first year follow-up did not show a significant difference, P = 0.8695. Paired samples in patients who were treatment naïve at recruitment compared to after they were given steroids at the first year follow-up did not show any significant difference in CD4 IL-17, P > 0.9999 and there was no difference in immunosuppression naïve patients remained immunosuppression naïve at first year follow-up, P = 0.9453.

The EOMG patients also did not show a difference in CD4 IL-17 with their paired samples at recruitment and first year follow-up, P > 0.9999; similarly LOMG patients did not show a difference in CD4 IL-17 in their paired samples at recruitment and first year follow-up either, P = 0.9530.

There was a difference in the CD4 IL-17 levels between AChR positive patients and healthy controls with a median of 1.6% in AChR positive and 0.705% in healthy controls, P = 0.0014 (Figure 79A). The difference between double seropositives and healthy controls was also significant with P = 0.0008, and there was no statistically significant difference between AChR positive and MuSK positive patients, median being 1.6% in AChR and 0.97% in MuSK patients, P = 0.1728.

CD4 IL-17 in MuSK positive patients compared to healthy controls was not statistically significant with a median of 0.97% in MuSK positive patients and 0.705% in healthy controls, P = 0.5593 (Figure 79B). This was different to the AChR positive patients in whom there was a statistically significant difference in values.

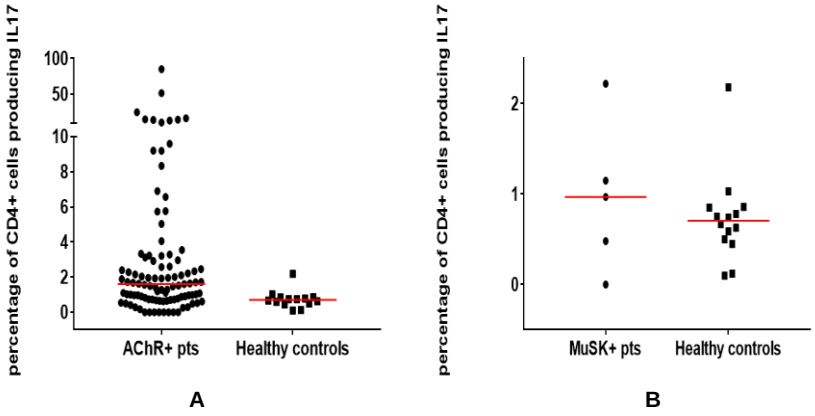


Figure 79 Comparison of CD4 IL17 (with medians) at recruitment in (A) AChR+ patients and healthy controls p=0.0014 and (B) MuSK+ patients and healthy controls p=0.5593

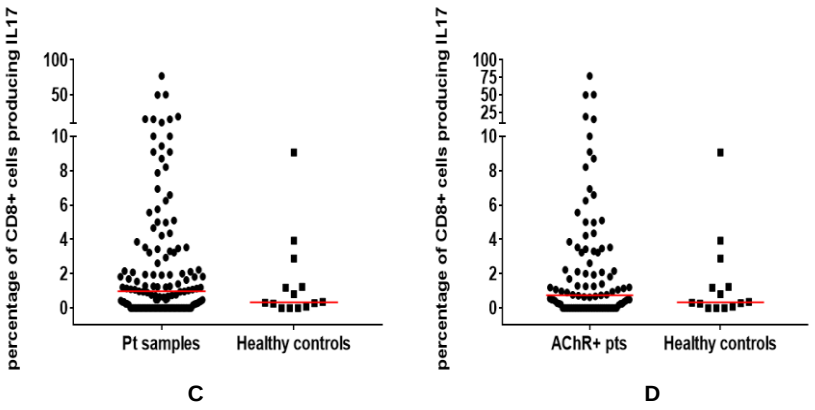


Figure 80 Comparison of CD8 IL17 (with medians) in (A) patient samples and healthy controls at recruitment p=0.4390 and (B) AChR+ patients and healthy controls p=0.6385

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

CD8 IL-17 in patient samples and at recruitment compared to healthy controls did not show a significant difference, median for samples was 0.965% and median for healthy controls was 0.33%, P = 0.439 (Figure 80A). There was no difference in EOMG and LOMG patient samples at recruitment either, P = 0.2283.

EOMG patient samples at recruitment compared to the EOMG healthy controls did not show any significant difference in CD8 IL-17, P = 0.4723 similarly there was no significant difference in LOMG patients at recruitment compared to LOMG healthy controls, P = 0.3500.

There was no significant difference between CD8 IL-17 in patients who were treatment naïve at recruitment compared to those immunosuppressed at recruitment, P = 0.2018.

Paired samples at recruitment and at first year follow-up did not show any significant difference in CD8 IL-17 either, P = 0.9799. This was not significant in patients who were treatment naïve at recruitment compared to after they had been immunosuppressed at first year either, P = 0.975. Treatment naive patients at recruitment and first year follow-up also did not show difference in CD8 IL-17, P = 0.8125. CD8 IL-17 in LOMG patients at recruitment and first year follow-up did not show a difference, P = 0.9799.

CD8 IL-17 in AChR positive patients and healthy controls showed a median of 0.74% in AChR patients and 0.33% in healthy controls, P = 0.6385 (Figure 80B). There was no significant difference between double positive patients compared to healthy controls and there was no difference between AChR and MuSK positive patients either.

There was no difference in the CD8 IL-17 counts between MuSK positive patients and healthy controls. Median for MuSK positive patients was 0.94% and healthy controls was 0.33%, P = 0.9467.

CD4 IL-4 in patient samples at recruitment compared to healthy controls did not show a significant difference, median for patient samples was 5.42%, and for healthy controls was 5.21%, P = 0.7063 (Figure 81A).

There was no significant difference between EOMG and LOMG subgroup, P = 0.5649. There was no difference in EOMG patients at recruitment compared to EOMG healthy controls, P = 0.5619 and similarly there was no difference in LOMG patient samples at recruitment compared to LOMG healthy controls either, P = 0.7131. Patients who were treatment naïve and those immunosuppressed at recruitment did not show a difference in their CD4 IL-4 either, P = 0.3293.

When CD4 IL-4 was compared between patients at recruitment to their paired samples at first year follow-up was not significantly different, P = 0.060 (Figure 81B). There was no difference in CD4 IL-4 in patients who were treatment naïve at recruitment and who were given steroids at first year follow-up, P = 0.1641. There was no difference in patients treatment naïve at recruitment and stayed immunosuppression naïve at first year follow-up either, P = 0.1230.

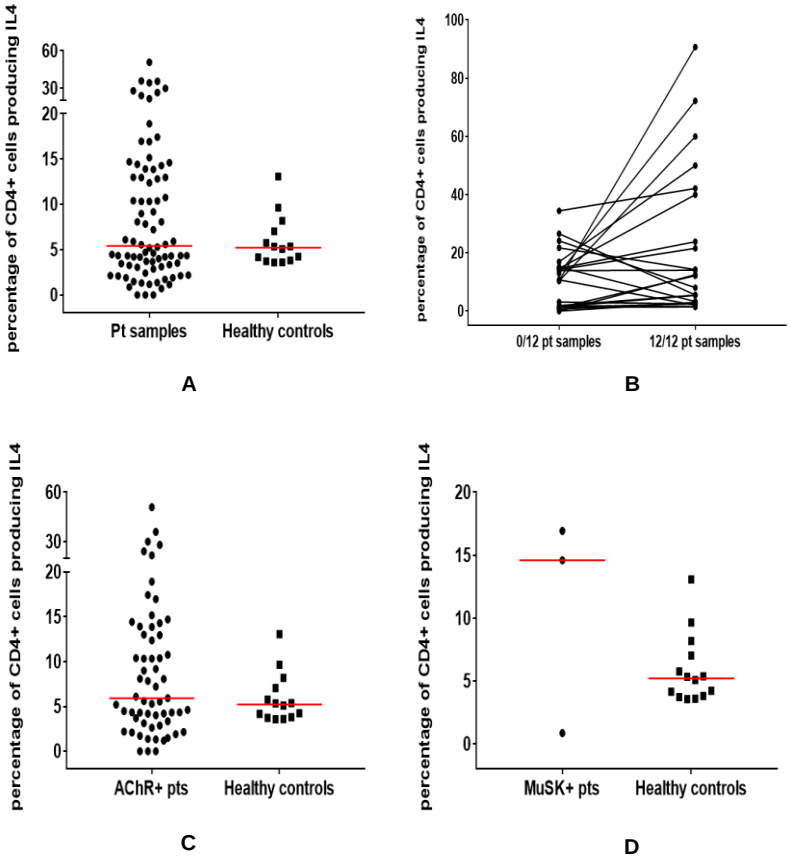


Figure 81 Comparison of CD4 IL4 (with medians) in (A) patient samples and healthy controls at recruitment p=0.7063 (B) paired patient samples at recruitment and first year follow up p=0.0600 (C) AChR+ patients and healthy controls p=0.4288 and (D) MuSK+ patients and healthy controls p=0.4320

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

Paired samples in EOMG patients at recruitment and first year follow-up did not show a difference in CD4 IL-4, P = 0.3750. LOMG patients did not show a difference in CD4 IL-4 at recruitment and first year follow-up either p= 0.1231.

CD4 IL-4 in AChR positive patients compared to healthy controls did not show any difference with a median of 5.92% in AChR positive patients and 5.21% in healthy controls, P = 0.4288 (Figure 81C). There was no difference in double seropositives compared to healthy controls and there was no difference between MuSK and AChR positive antibodies patients either.

CD4 IL-4 in MuSK positive patients was not significantly different to healthy controls; median for MuSK positive patients was 14.61% and healthy controls was 5.21%, P = 0.432 (Figure 81D).

The **CD8 IL-4** did not show a significant difference between patient samples at recruitment compared to healthy controls, P = 0.4905 (Figure 82A). There was no difference in EOMG and LOMG patient samples at recruitment with CD8 IL-4 of 1.95% in LOMG patients and 0.69% in EOMG patients, P = 0.0682 (Figure 82B).

There was no difference in CD8 IL-4 in EOMG patients at recruitment compared to EOMG healthy controls, P = 0.4346 and there was no difference in LOMG patients either at

recruitment compared to LOMG healthy controls, P = 0.2588. There was no difference in CD8 IL-4 in patients who were suppression naïve at recruitment compared to those who were immunosuppressed at recruitment, P = 0.9729.

CD8 IL-4 at recruitment compared to their paired samples at first year follow-up did not show significant difference, P = 0.1388. There was no difference in CD8 IL-4 in patients who were treatment naïve at recruitment and were immunosuppressed at first year follow-up, median for treatment naïve patients at recruitment was 0.95% and for immunosuppressed patients at first follow-up was 2.01%, p= 0.0547 (Figure 82C).

There was no difference in CD8 IL-4 in patients who were treatment naïve at recruitment and remain treatment naïve at first year follow-up, P = 0.4688. There was no difference in the LOMG samples at recruitment compared to their paired samples at first year follow-up, P = 0.1388.

CD8 IL-4 in AChR positive patients compared to healthy controls did not show statistically significant difference, median for AChR positive patients was 1.65% and healthy controls was 1.605%, P = 0.5449 (Figure 82D). There was no statistically significant difference between double seropositives and healthy controls and between AChR and MuSK positive patients.

There was no statistically significant difference between CD8 IL-4 in MuSK positive patients and healthy controls; median for MuSK positive patients was 0.36% and healthy controls was 1.605%, P = 0.204.

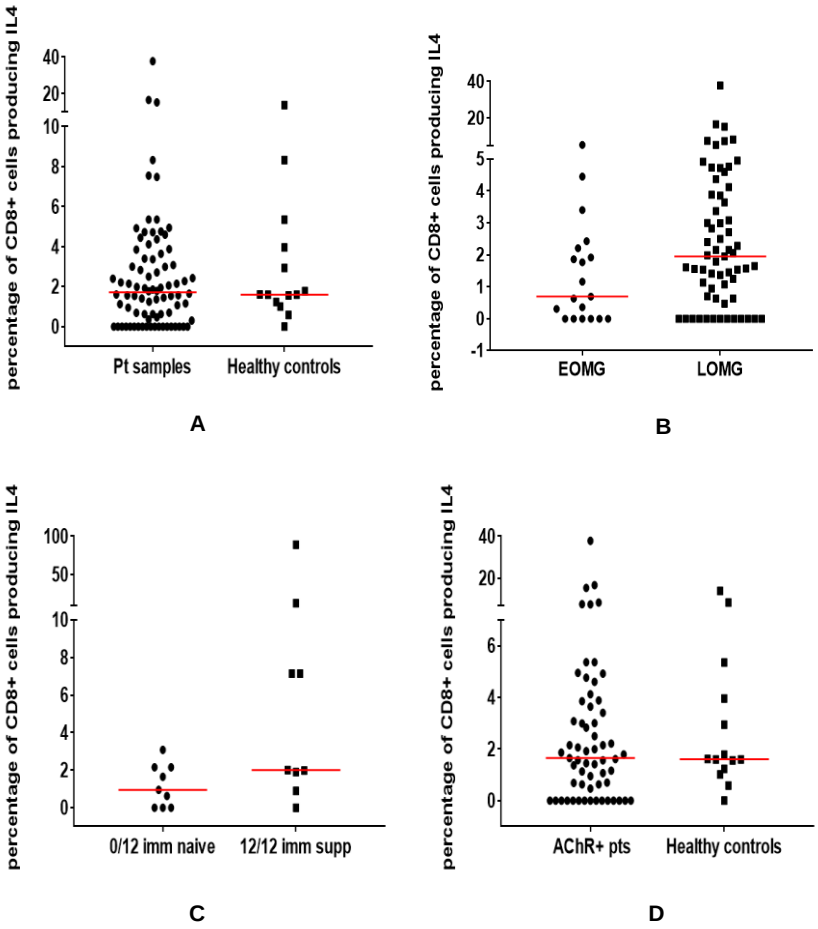


Figure 82 Comparison of CD8 IL4 (with medians) in (A) patient samples and healthy controls at recruitment p=0.4905 (B) EOMG and LOMG p=0.0682 (C) paired patient samples at recruitment when immunosuppression naïve with first year follow up after treatment with steroids p-0.0547 and (D) AChR+ patients and healthy controls p=0.5449

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

T cells and Thymic abnormalities

We looked at the Treg proportions and CD4 and CD8 production of interferon-alpha, interferon-gamma, TNF alpha, IL-10, IL-17, and IL-4. We then compared these in patients who had normal thymuses with those who had thymic abnormalities (this includes patients with thymoma, thymic remnant and thymic hyperplasia). NB: Not all patients were imaged, and not all patients who had thymic abnormalities on imaging had a histological diagnosis. The number of EOMG patients who had a histologically confirmed diagnosis of thymic hyperplasia was 8 and thymoma was 2. The total number of scan abnormalities in EOMG (excluding a confirmed diagnosis of thymoma) was 16 i.e. 43.24%. If we were to assume that, by definition, all EOMG patients have thymic abnormalities, this is perhaps indicative of the numbers in whom the thymus is enlarged enough to be picked up on imaging.

CD4 interferon-gamma in normal thymus as compared to thymic abnormalities was not significant, P = 0.4540; it was not significant in normal thymus as compared to thymoma either, P = 0.4802 and similarly with thymic hyperplasia compared to normal thymus, P = 0.3970. CD8 interferon-gamma in normal thymus compared to thymic abnormalities did not show any significant difference, P = 0.1701; there was no significant difference between normal thymus and thymoma, P = 0.4474,

but it was significant in normal thymus compared to thymic hyperplasia with a median of 21.01% in thymic hyperplasia and 42.22% in normal thymus, P = 0.0235 (Figure 83).

CD4 TNF alpha in thymic abnormalities compared with normal thymus was not significant, P = 0.8382; normal thymus compared to thymoma was not significant, P = 0.6248, and normal thymus compared to thymic hyperplasia was not significant either, P = 0.3132.

CD8 TNF-alpha in thymic abnormalities compared to normal thymus does not show any difference with a median of 31.67% in thymic abnormalities compared to 46.25% in normal thymus, P = 0.0778 (Figure 85A). Normal thymus compared to thymoma was not significant, P = 0.2586. There was a significant difference in CD8 TNF alpha in thymic hyperplasia compared to normal thymus; median for thymic hyperplasia was 29.57% and for normal thymus was 46.25%, P = 0.012 (Figure 85B).

CD4 IL-10 in thymic abnormalities compared to normal thymus was not significant, P = 0.8219; it was not significant in thymoma compared to normal thymus either, P = 0.2336; and it was not significant in thymic hyperplasia compared to normal thymus, P = 0.6496.

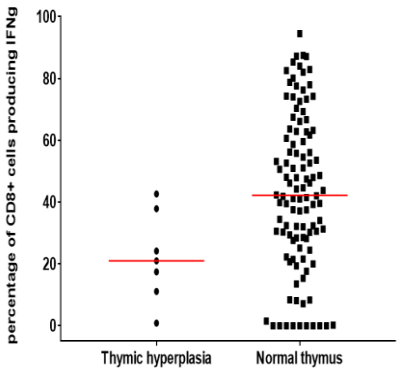


Figure 83 Comparison of CD8 IFNγ (with medians) in patients with normal thymus and thymic hyperplasia p=0.0235

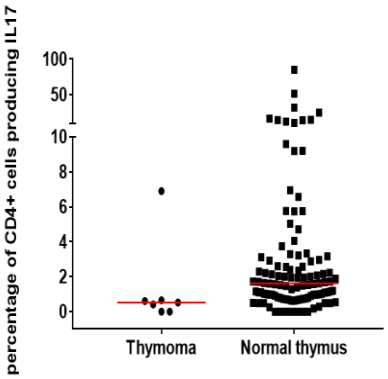


Figure 84 Comparison of CD4 IL17 (with medians) in patients with normal thymus and thymoma p=0.0086

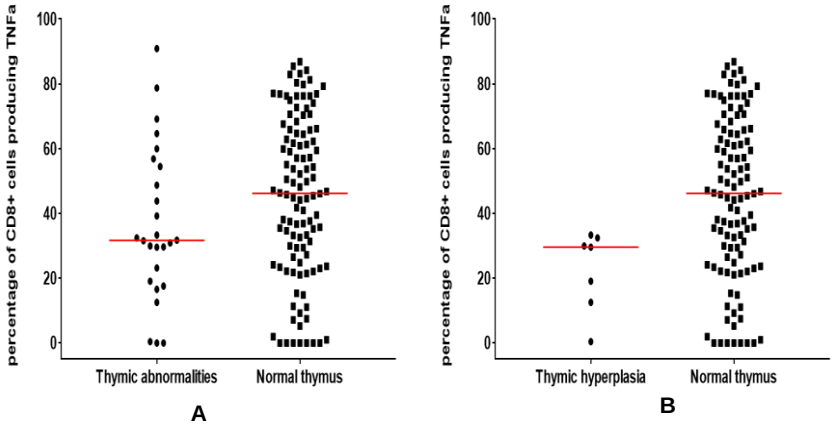


Figure 85 Comparison of CD8 TNFα (with medians) in (A) patients with normal thymus and all thymic abnormalities p=0.0778 and (B) in patients with normal thymus and thymic hyperplasia p=0.012

All p values were calculated using the Mann-Whitney test as the comparison was between medians of two independent groups and not normally distributed.

CD8 IL-10 in thymic abnormalities compared to normal thymus was not significant, P = 0.7352; was not significant in thymoma compared to normal thymus, P = 0.4769 and it was not significant in thymic hyperplasia compared to normal thymus, P = 0.6231.

CD4 IL-17 in thymic abnormalities compared to normal thymus was not significant, P = 0.1801; however it was significantly different in thymoma compared to normal thymus, median for thymoma was 0.52% and for normal thymus was 1.61%, P = 0.0086 (Figure 84). It was not significant in normal thymus compared to thymic hyperplasia, P = 0.9157.

CD8 IL-17 in thymic abnormalities compared to normal thymus was not significant, P = 0.8329, in thymoma to normal thymus it was not significant, P = 0.6315, and not significant in normal thymus and thymic hyperplasia, p=0.6917.

CD4 IL-4 in thymic abnormalities compared to normal thymus was not significant, P = 0.7218; it was not significant in normal thymus compared to thymoma, P = 0.9606 and was not significant in thymic hyperplasia compared to normal thymus, P = 0.7259.

CD8 IL-4 was not significantly different in thymic abnormalities compared to normal thymus, P = 0.3074, not significant in thymoma compared to normal thymus, P = 0.440, and in thymic hyperplasia compared to normal thymus it was not significant, P = 0.4339.

Table 29 Summary of flow cytometry results

Median or p value	Tregs	CD4 IFNg	CD4 TNFa	CD8 IFNg	CD8 TNFa	CD4 IL10	CD4 IL17	CD4 IL4	CD8 IL10	CD8 IL17	CD8 IL4
healthy Controls	0.51	15.62	53.7	26.08	28.86	2.185	0.705	5.21	1.035	0.33	1.605
0/12	0.07	22.82	63.52	41.13	44.4	6.51	1.525	5.42	2.785	0.965	1.71
12/12	0	22.89	67.8	40.82	26.86	17.62	0.675	10.12	2.845	0.255	1.715
Pt samples Vs controls	<0.0001	0.1692	0.0237	0.0795	0.0489	0.0082	0.0009	0.7063	0.5097	0.4394	0.4905
EOMG Vs LOMG	0.1635	0.7884	0.0674	0.0002	<0.0001	0.5642	0.3159	0.5649	0.6988	0.2283	0.0682
EOMG pts Vs EOMG Controls	0.0001	0.2812	0.1499	0.6917	>0.9999	0.0400	0.0159	0.5619	0.5593	0.4723	0.4346
LOMG pts Vs LOMG Controls	0.0131	0.3491	0.4065	0.0818	0.2336	0.1899	0.2488	0.7131	0.9677	0.3500	0.2588
0/12 imm naïve Vs 0/12 imm sup	0.8004	0.0749	0.4130	0.6889	0.4293	0.8728	0.2249	0.3293	0.8952	0.2018	0.9729
0/12 pts Vs 12/12	0.3223	0.7987	0.2726	0.7987	0.0858	0.7843	0.8695	0.0604	0.8986	0.9799	0.1388

pts, paired											
0/12 imm naïve Vs 12/12 imm sup	0.0625	0.9453	0.3223	0.5781	0.8125	0.4961	>0.9999	0.1641	0.2500	0.9375	0.0547
0/12 imm naïve Vs 12/12 imm naïve	0.6250	0.4609	0.5195	0.4609	0.1914	0.5195	0.9453	0.1230	0.4688	0.8125	0.4688
0/12 EOMG Vs 12/12 EOMG	n/a	>0.9999	0.2500	>0.9999	0.5000	0.2500	>0.9999	0.3750	n/a	n/a	n/a
0/12 LOMG Vs 12/12 LOMG	0.3223	>0.9999	0.6226	>0.9999	0.2633	0.7012	0.9530	0.1231	0.8986	0.9799	0.1388
Thymic abn Vs normal thymus	0.4035	0.4540	0.8382	0.1701	0.0778	0.8219	0.1801	0.7218	0.7352	0.8329	0.3074
Thymoma Vs normal thymus	0.6792	0.4802	0.6248	0.4474	0.2586	0.2336	0.0086	0.9606	0.4769	0.6315	0.4340
Thymic	0.9651	0.3970	0.3132	0.0235	0.0124	0.6496	0.9157	0.7259	0.6231	0.6917	0.4339

hyperp Vs normal thymus											
0/12 pts Vs AchR RIA	0.7838 (NS)	0.3798 (NS)	0.1740 (NS)	0.2438 (NS)	0.1381 (NS)	0.8878 (NS)	0.3235 (NS)	0.6404 (NS)	0.7646 (NS)	0.3709 (NS)	0.3969 (NS)
AChR+ Vs controls	0.0005	0.1453	0.0279	0.0978	0.0636	0.0098	0.0014	0.4288	0.5063	0.6385	0.5449
MuSK+ Vs controls	0.0018	0.6216	0.2566	0.7539	0.1299	0.4998	0.5593	0.4324	0.5115	0.9467	0.2044
Double+ Vs controls	0.0021	0.2490	0.0285	0.2322	0.1425	0.0196	0.0008	0.5186	0.8222	0.3375	0.4358
AChR+ Vs MuSK+	0.2591	0.1826	0.6156	0.6536	0.3897	0.7034	0.1728	0.6942	0.0871	0.6569	0.3579

5.3.3 Does immunosuppression change Treg function?

When we compared the samples of patients at recruitment and at their first year follow-up in patients who were immunosuppression naïve at recruitment and immunosuppressed during the first year (9 patients), there was no significant difference in any of the interferons, TNF or interleukins.

Recruitment samples in patients who were treatment naïve and the 15 who were immunosuppressed during recruitment did not show any difference in T regs, CD4 TNF alpha, CD8 interferon-gamma, CD8 TNF alpha, CD4 IL-10, CD4 IL-17, CD4 IL-4, CD8 IL-10, CD8 IL-17 and CD8. The only difference was seen in CD8 interferon-alpha.

Comparison of T regs in immunosuppression naïve patients at recruitment with their paired samples at first year follow up after being immunosuppressed in the first year did not show a difference, p = 0.0625; the median for immunosuppression naive patients was 0.68% and immunosuppressed patients at first year follow up was 0.54%.

CD4 interferon-gamma in patients who were treatment naïve at recruitment with those who were immunosuppressed at recruitment did not show a difference with median for immunosuppression naïve patients of 24.17% and immunosuppressed patients of 19.59%, P = 0.0749.

There was no difference in CD8 IL-4 in patients who were treatment naïve at recruitment and were immunosuppressed at first year follow-up, median for treatment naïve at recruitment was 0.95% and for immunosuppression at first year follow-up was 2.01%, p= 0.0547. It could be assumed that patients remaining treatment naïve at first year follow up have a milder disease, and their immune markers would be different to those

receiving immunosuppression. Although the difference in T regs between treatment naïve and immunosuppressed patients was not significant, there was a tendency towards improvement of T reg levels after immunosuppression. CD8 IL-4 also showed a tendency towards improvement with increasing percentages after immunosuppression. CD8 interferon-alpha in patients who were immunosuppressed at recruitment was much lower than treatment naïve patients showing that immunosuppression reduces levels of CD8 interferon-alpha. Similarly CD4 interferon-gamma also appears to be suppressed with lower levels in patients who received steroids at recruitment. CD4 interferon-alpha significantly improved with time with no immunosuppression.

5.4 Discussion

The Treg proportions we found in our study were much lower than those reported in literature where they have been shown to be anywhere between 1 and 10% (405), whereas in our cohort we found that the readings were much lower than 1%. There were a few outliers amongst these patient samples with higher percentages. However, we found that the median for both the patient samples and our healthy controls was much less than published in literature and concluded that this was due to our gating mechanism. We did not think that this would affect results

as the same staining methods and gating were used for the patient samples and healthy controls. Stringent controls were used during the experiment to ensure that the staining methods were appropriate. The proportions in the controls were as normally expected and therefore lower levels but the proportions remain plausible.However, we accept that our data needs to be interpreted with caution because of the low percentages, and because of this, may not be directly comparable to published literature.

Is there a difference in T reg levels in the peripheral blood lymphocytes of MG patients compared to healthy controls, and in LOMG patients compared to EOMG patients?

The percentage of T regs in our patients was significantly lower than healthy controls. There was no statistically significant difference between EOMG and LOMG at recruitment. The EOMG patient samples compared with EOMG healthy controls shows a significant difference; LOMG patients compared to LOMG healthy controls also showed a difference but to a slightly lesser degree. Due to our large sample size, 135 of which were treatment naïve samples, we think that these results are indicative of the immunopathogenesis in MG, but accept that the percentages are too small to make accurate predictions. Published data so far have been contradictory, some showing reduced Treg numbers, and others no difference in Treg numbers in MG. The data on Treg in aging (and thus corresponding to EOMG vs LOMG) are also contradictory, with

some studies showing an increase and others a decrease (51, 52, 265). What most papers agree on is that there is a functional defect in the Tregs, irrespective of numbers/percentages. We did not perform functional studies on our patient samples, and so this information is not available. Initial immunosuppression at recruitment does not seem to change the Treg percentages as seen when we compared the 15 immunosuppressed patients at recruitment with the 135 immunosuppression naive patients. There was no difference in the Treg levels at recruitment and at first year follow up in the 24 patients whose samples were tested at recruitment and a year later; and, although there was a further drop in Treg levels in patients who went to have immunosuppression, this was not statistically significant. The paired LOMG samples did not show any difference either. We were unable to compare the EOMG patient samples as there was no mean difference between the two values.This is different to the one study by Wen et al which looked at 59 treatment naïve patients, 13 of whom then had immunosuppression, where they found that Treg levels in treated patients was significantly higher than untreated patients and the levels improved after treatment (298). It can also be assumed that if the patients were on low dose steroids, the differences in Treg may be minor. Braitch et al have shown a modestly increased percentage in Tregs in MS after iv methylprednisolone, but after 6 weeks they were back to baseline levels (427).

Is there a difference in Treg levels in patients with thymic abnormalities?

T regs showed a trend towards being lower in patients with thymic abnormalities (radiologically abnormal with many histologically confirmed) as a whole compared to patients with a normal thymus, but it was not significantly different. There was no difference in T regs in patients with normal thymus and thymic hyperplasia/enlargement nor was there a difference in normal thymus compared to thymoma/thymic mass. Previous studies have shown contradictory results on Treg numbers in MG with thymic abnormalities, with no consensus. Once again, with low Treg percentages, interpretation of our data is difficult. We analysed our data on radiologically abnormal thymuses (most of whom also had a histological diagnosis). This does not take into account those patients who may have a histological abnormality with normal radiological appearances, particularly in EOMG. Perhaps if this is looked into in more detail in future studies, we may get data that is comparable to published literature.

Is there a difference in Treg levels levels in the different antibody subgroups?

There was a significant difference in the Tregs in AChR antibody-positive patients compared to healthy controls and in MuSK positive patients compared to healthy controls, but there

was no difference between Tregs in LRP4 positive patients compared to HCs. NB: We had fewer LRP4+ patients compared to the other subgroups. There was a significant difference also when T regs were compared between double seropositive patients and healthy controls. There was no difference however when AChR antibody-positive patients' T regs were compared to MuSK positive patients' T regs. Although there is some literature on cytokines in the antibody subgroups, there is no large scale data on Treg levels in the different antibody subgroups.

Is there a difference in the cytokines IFNγ, TNFα, IL-10, IL-17 and IL-4 in the peripheral blood lymphocytes of MG patients compared to healthy controls and in LOMG patients compared to EOMG patients?

Although IFNa was tested on the flow cytometry panel, we realise that this is unlikely to add to the current study and so has not been included in this discussion. When cytokine levels (using the percentage of expressing change as a surrogate for the levels) were compared between recruitment patient samples and HC, the proinflammatory cytokines TNFa and IL-17 were higher, and IFNg and IL-4 did not show any difference. The anti-inflammatory cytokine IL-10 was higher in patient samples compared to HC. For cytokines IL-17 and IL-10, this difference was seen only in EOMG and not in LOMG patients. Previous studies have shown an increase in several pro-inflammatory

cytokines in MG including IFNγ, TNFα and IL-17 (250, 310). Our study has shown an increase in IL-17 and TNFa, and although there is a trend towards higher IFNg in the MG patients, this was not significant. In keeping with literature, this increase was seen mainly in EOMG patients (314).

IFNg levels were lower in treatment naïve patients compared to those who were immunosuppressed at recruitment. There are no studies comparing cytokine levels in treatment naïve and immunosuppressed patients. It could be postulated that treatment naïve patients have milder disease and so their inflammatory markers are less than those with more severe disease needing immunosuppression.

When EOMG was compared to LOMG, the only significant difference was in IFNg levels which were higher in LOMG patients, IL-4 levels were slightly higher in LOMG compared to EOMG. There are no large studies comparing cytokines in EOMG and LOMG and this is novel data. As mentioned before in the chapter on clinical presentation and antibodies, the LOMG patients in our cohort seem to have more severe disease, and higher levels of inflammatory cytokines would be in keeping with this.

Is there a difference in cytokine levels in the different antibody subgroups?

In AChR MG patients, the pro-inflammatory cytokine IFNg was slightly higher, TNFa was higher, IL-17 was higher and the anti-inflammatory cytokine IL-10 was higher than HC. In contrast, MuSK MG patients had no increase in the pro-inflammatory cytokines or anti-inflammatory cytokines. This is different to previously published data which reports that MuSK MG patients have higher IFNg and IL-17 levels, and not AChR MG patients, in direct contrast to our study results (315). The majority of the patients in the published study were on immunosuppression, some on two or more drugs. We did not perform subgroup analysis on patients who were immunosuppressed in each antibody category.

Double sero-positive patients had cytokine levels similar to AChR MG with lower IFNa levels, higher TNFa, IL-17 and IL-10 levels, but no increase in IFNg compared to HC.

When we compared AChR and MuSK MG patients, IL-10 was no different in AChR MG compared to MuSK MG,.

Our T reg and cytokine analysis in the antibody subgroups shows a mixed anti-inflammatory and pro-inflammatory picture. The percentage of CD8 cells producing cytokines (which we used as surrogate markers instead of measuring cytokines), was

not significantly altered other than slightly increased pro-inflammatory cytokines IFNγ and TNFα in AChR Ab + patients.

In AChR MG, the main cytokine producing T cell appears to be the CD4+ cells, which shows increased production of the pro-inflammatory cytokines TNFα and IL-17. This, along with reduced T regs indicates a ‘pro-inflammatory’ environment. At the same time, there is increased production of the anti-inflammatory cytokine IL-10 indicating an ‘anti-inflammatory’ environment. This kind of defective balance is seen in a lot of autoimmune conditions.

In contrast to AChR MG, in MuSK MG, Tregs are reduced indicating a ‘pro-inflammatory’ environment only.

Is there a difference in cytokines in patients with thymic abnormalities?

There was no difference in cytokines between patients with normal thymuses and thymic abnormalities as a whole; CD4 IL-17 is significantly lower in patients with thymoma/thymic mass compared to patients with normal thymuses, CD8 interferon-γ and CD8 TNF α are significantly lower in patients with thymic hyperplasia/enlargement compared to normal thymuses. This is different again to previous literature which has shown increased pro-inflammatory cytokines in thymic hyperplasia (250, 310). We accept that our study results are not conducive to a direct

comparison, as not all patients had a histological diagnosis, and comparison was made between radiologically abnormal (with histological conformation in many), and radiologically normal thymuses.

In conclusion, in our patient cohort, IL-10 which is an anti-inflammatory cytokine is increased in patient samples. This suggests an anti-inflammatory environment. At the same time, Treg cell population is reduced in patient samples both in EOMG and LOMG, and the pro-inflammatory cytokine TNFα is increased in patient samples, as is IL-17. This mixed picture would suggest that there is a defective balance in immune regulation in MG.

6 Conclusion

6.1 Summary of findings and General discussion

To our Knowledge, this is the largest prospective study on the clinical and immunological aspects of late-onset myasthenia gravis to date. It is also the only study which has 100% recruitment rates from Nottinghamshire and Derbyshire over the three-year recruitment period. Between August 2014 and July 2017, we recruited 150 patients newly diagnosed with myasthenia gravis across the East Midlands, West Midlands and south-east England.

6.1.1 Demographics

Epidemiological studies in the last 65 years have shown that late onset myasthenia gravis is becoming increasingly more common. In our cohort of 150 MG patients, more than three quarters of the patients (76%) were over the age of 50 years in keeping with published data with a mean age of 57.6 years in females and 61.24 years in males. We found a difference in the medians of peak age of 4 years in male and female EOMG patients (lower in females) which is less than that reported by Somnier et al but the peak age in LOMG males and females is no different, which is consistent with that previously reported (406). Whilst our study recruited all the newly diagnosed

patients in the Trent region, this was not the case with Birmingham and Oxford. However, we have shown that there was no bias in patient recruitment amongst the different regions (Table 3), there was no difference in the racial distribution compared to the national statistics for the regions, and that the incidence of LOMG in the Nottingham area is increasing. From this, we can infer that our study shows that the incidence of LOMG is increasing in all the regions.

Previous studies have suggested that the incidence of LOMG may be rising because of an ageing population as a whole, declining mortality rates, and possibility of better ascertainment of cases that were previously misdiagnosed. All these factors did not fully explain the percentage of rise in incidence of LOMG; environmental factors were postulated, but not proven (98, 99, 106).

From our detailed questionnaire (listed in chapter 2), we were unable to ascertain any environmental, infectious, occupational or medication triggers in our patients in either EOMG or LOMG. This analysis was based purely on the questionnaire and no laboratory or immunological tests were done to look into this. We did not check for EBV infection for example which has previously been shown to have some association with MG (144).

In Jon Aarli's paper of 2008 he found that the female to male ratio was 1:1.1 for LOMG, and 3:1 in EOMG (96). Evoli and

colleagues stated a female to male ratio of 1:1.9 in LOMG (151). Our data has shown a female to male ratio of 1:1.6 in LOMG and 1.4:1 in EOMG. Our study has shown the same trend, and that late-onset myasthenia gravis in male patients has increased even further whilst the number of female EOMG patients has dropped. Why there is a male bias in LOMG is not clear; in most conditions where oestrogen has a role to play, incidence between males and females equalises as they get older. It is possible that different HLA haplotypes play a dominant role in disease susceptibility in males and females in LOMG.

There have been few studies on racial distribution in MG. Population-based studies in several different countries and across continents show a similar incidence and prevalence rate and similar distribution of EOMG and LOMG patients. A study by Oh and colleagues in Alabama, USA showed that AChR Ab positivity was more common in white americans (WA) compared to African-americans (AA), SNMG AA patients were more likely to be MuSK Ab positive, three quarters of OMG AA patients were seronegative, and disease onset was earlier and more common in females amongst AA patients compared to WA patients in whom it was later in onset and more common in males (410). Whilst this was a large study with 235 patients and good follow up, it was a retrospective study looking at patients treated in a single neuromuscular clinic, and may not reflect the true incidence or prevalence. Another study in Norway and

Netherlands looked at the prevalence between the native and emigrant population and did not find any difference. They noted that the incidence of MuSK MG and MG with thymoma was higher in the immigrant population compared to the native population (114). In our cohort, there was no significant difference in racial distribution across the three regions where patients were recruited i.e. West Midlands, East Midlands and the South East England. Whilst recruitment in Birmingham and Oxford was selective, we recruited all patients with a diagnosis of myasthenia across Nottinghamshire and Derbyshire excluding the northern districts, and hence these results are truly representative of the racial distribution of myasthenia in the area.

6.1.2 Ocular Myasthenia Gravis

Ocular myasthenia gravis (OMG) has been more difficult to define because of varying time limits applied across several different studies. Defining OMG based on ocular symptoms at diagnosis or recruitment is completely arbitrary and does not provide any useful clinical information about disease progression (172, 173). Oosterhuis suggested a minimum of three months as a limit for purely ocular symptoms before classifying a patient as having OMG (169). Similarly Sommer et al and Monsul et al also suggested purely ocular symptoms for

at least three months from symptom onset to class them as OMG (170, 171).

We defined our cohort of OMG patients as all patients who had purely ocular symptoms up to and including three months from symptom onset. This was based on previous literature but also on our own findings of 'time of symptom onset'. We found that the median time for bulbar, generalised and respiratory symptom onset was less than three months. There is an argument to classify some of the GMG patients who generalised quite early on in their illness as 'early generalisers', those that generalised just after 3 months as 'intermediate generalisers' and those that generalised after 2 years as 'late generalisers'. For the purposes of this thesis, this distinction has not been made

Whilst previous studies have shown that OMG is more common in LOMG compared to EOMG (155, 156) neither of the papers mentioned how they defined OMG. This means that interpreting this data and comparing our study with theirs is not possible. Our study has shown that younger patients have ocular myasthenia gravis more frequently compared to LOMG, whereas the older patients more frequently have generalised myasthenia gravis.

Generalisation of OMG has not been reported in an entirely treatment naïve cohort of patients before. We found that whilst

the generalisation time from OMG is similar in both EOMG and LOMG patients, with the majority of the patients generalising in under a year, generalisation occurred more frequently in LOMG than EOMG. We had reviewed 120 patients for their first year follow-up and 65 patients for their second year follow-up at the time of data analysis, and although this data was not complete at the time of writing, the numbers are still significant enough to reflect realistic generalisation rates. As there are no large-scale studies looking at treatment naive patients over a course of time, we did not have any published data to compare this against.

In our own cohort, there was no difference in presentation with unilateral or bilateral ptosis in the EOMG and LOMG patients, and there was no significant difference between time to generalisation between unilateral ptosis and bilateral ptosis in either group. In patients who had ptosis only, there was a difference between the EOMG and LOMG groups with generalisation occurring more quickly in the older patients (perhaps due to small numbers in the EOMG group); but when generalisation rates were compared between the ptosis only group and patients who had both ptosis and diplopia, there was no statistically significant difference. It is not entirely clear whether presenting with purely ptosis in an older patient is an independent risk factor for generalisation.

Ocular myasthenia gravis was also seen at the same rate in all antibody subtypes, and, although OMG was more common with

AChR and LRP4 double positivity, this was not significant. This is different to previously published data which suggests that AChR and LRP4 double seropositivity is more likely to be associated with bulbar symptoms and a more severe course (206). It is not entirely clear why this difference was seen, but the number of LRP4 Ab positive patients in our cohort was relatively less. There was no difference in first presenting symptom in our cohort amongst any of the different antibody subtypes. Over the course of the disease, all patients (100%) who had antibodies to MuSK, LRP4, MuSK and AChR double positive, and AChR and LRP4 double positive had ocular symptoms, whereas AChR single positive patients had ocular symptoms in 92.5% and seronegative patients had ocular symptoms in 87.5%; neither of which were statistically significant. This would suggest that AChR Abs and seronegative patients can have ocular sparing MG, whereas this is not the case with the other Abs. Again, it is not clear why this difference is seen between the antibody subgroups; one would assume that as ocular muscles are more susceptible to circulating MG antibodies, they should be equally vulnerable to all subtypes. Is it possible that the IgG1 subclass of AChR Abs has a slightly less pathogenic effect on ocular muscles compared to IgG4 subclass of MuSK Abs?

6.1.3 Clinical Presentation

Because of the prospective nature of our study, recruiting patients when they were treatment naïve, and within the first year of diagnosis, we were able to plot the onset of all MG symptoms from point zero and compare the median times of symptom onset for all the different symptoms. This has not been done before and is novel data. The median time of symptom onset for all symptoms when compared shows that the longest median time of symptom onset is less than three months at 87 days. This was one of the reasons why we defined ocular MG as ocular symptoms only for more than 3 months.

Although the general order of symptom onset seems to be similar in all patients, with ocular symptoms occurring first, followed by other generalised symptoms, there was a difference in the order of generalised symptom onset between EOMG and LOMG groups. There was no significant difference in presentation of ocular symptoms (although more common in younger patients), nor in development of generalised symptoms (although quicker in older patients). EOMG patients presented with dysphagia, dysarthria, neck weakness and respiratory symptoms at a much later stage than LOMG patients. The median time of onset of chewing difficulties and limb weakness was not significantly different between the two groups.

During the course of the disease, bulbar symptoms were seen much more commonly in LOMG patients compared to EOMG

patients. There was no difference in limb weakness or respiratory symptoms in the EOMG and LOMG groups. There was no difference in symptom presentation in males and females in any of the groups.

Previous literature has suggested that patients with MuSK antibodies are more likely to be female, younger, without thymoma and more likely to have bulbar and respiratory involvement (154, 161). They were also found to remain immunosuppression dependent and needing rituximab. In our cohort we did not find any difference in bulbar symptoms amongst patients who had antibodies to MuSK, AChR, and MuSK and AChR double positives. However, seronegative patients were much less likely to have bulbar symptoms compared to AChR single positives, and compared to AChR and MuSK double positives. This difference was not seen between seronegatives and MuSK and LRP4 single positive patients. One could assume that seronegative patients have an as yet unidentified Ab that is less pathogenic than AChR. However, since they are clinically more similar to patients with MuSK and LRP4 Abs, this does not make sense. Any interpretation of this should be made with caution as the number of single positive LRP4 patients was small, and there were only 8 seronegative patients.The subgroups did not show any difference in the frequency of limb weakness. There was no difference in respiratory symptoms in any of the antibody subgroups including

in MuSK MG. Whilst there was a trend towards MuSK MG patients being more commonly female and having more bulbar and respiratory symptoms, this was not statistically significant. Whether this is a reflection of the fact that this was a prospective study with follow up data for two years in the majority of patients, and whether these patients would deteriorate later on in their course of illness is unclear.

We looked at the timing of the worst MG composite scores from symptom onset in all patients and found that the majority of the patients had the worst MG composite score during the first year, in nearly three quarters of them, and most of them (53.33%) within the first 100 days. This would suggest that patients are at their worst with MG-related symptoms during the first year and subsequently improve, with or without treatment. On subgroup analysis, there was no difference in time to worst MGC between EOMG and LOMG.

We compared MG composite scores in patients who were diagnosed early compared to those in whom there had been a diagnostic delay and there was no difference. There was a significant difference in time to diagnosis from symptom onset between EOMG (median of 13.5 mths) and LOMG (median of 2 mths), older patients being diagnosed quicker than EOMG. This is different to previously published data (99). The study by Vincent et al was a very large prospective study using positive AChR Ab test results from all UK centres. This was not a clinical

study and was not longitudinal. Our study on the other hand is prospective with high quality recruitment including referrals from other Neurologists, GPs, Ophthalmologists, Neurophysiologists and Laboratory data. The patients were recruited from regions where there was a neurology service which ensured better pick up rates and reduced selection bias. Also, LOMG patients more commonly present with GMG, are more likely to have comorbid conditions, and more commonly require admission to hospital, which may be why we see this difference.

Older patients were also more likely to require admission to hospital. This could be multiple times compared to younger patients. There are contradictory reports in literature, where some studies suggest that MG crises are more common in younger females, whilst others show no difference (390) (392, 394). It is to be noted that, in our cohort, amongst the LOMG patients needing admission, nearly three quarters were not for MG crises (Table 19), whereas nearly half the admissions in EOMG patients were with MG crises. So, whilst the total number of admissions is greater in LOMG patients, within the total MG admissions, MG crises were more common in younger patients. One could argue that neurologists have a lower threshold for admitting older patients to hospital as they are more likely to have other co-morbidities making them more susceptible to deterioration/ steroid dips, whereas younger patients are admitted when they are clinically much worse and/or in crisis.

Previous studies have reported that 14.8% of MG patients are refractory to treatment and they are more likely to be young female patients with anti-MuSK antibodies and with thymomas. Life-threatening events have been shown to occur in 9.56% of MG patients (387, 388). In our cohort, we found that 15.38% of EOMG patients and 36.03% of LOMG patients required admissions to hospital for MG related reasons. The older patients were also more likely to be admitted more frequently compared to younger patients. The majority of admitted patients were older- this could be due to several factors including other co-existing comorbidities which meant that they sought or were referred to hospital earlier than younger patients, or, as we saw earlier, LOMG patients had a higher MG composite score at recruitment compared to EOMG, perhaps indicating a more symptomatic onset in these patients. Previous studies have shown that prognosis is favourable in all MG patients, but LOMG patients are more likely to achieve an optimal outcome (392). In keeping with this, the majority of the patients in our cohort responded well to treatment with 92.5% of LOMG patients attaining a good outcome compared to 50% in EOMG patients. 2.56% of patients, all LOMG, died during an admission to hospital. This is slightly contradictory to saying that LOMG patients had a better outcome. This is explained by the fact that the majority of LOMG patients admitted to hospital had a proportionately good outcome, compared to the EOMG patients.

The overall mortality rate over the three-year period in our cohort was 4/150 i.e. 2.6%, all of them in the LOMG group. Of these, three were thought to have been caused by or contributed to by myasthenia gravis. This is similar to previously published literature (393).

When we compared the MGFA-PIS scores at first year follow-up, LOMG patients did better than EOMG patients and this trend was seen even at second year follow-up. When the whole cohort of patients was compared at first and second year follow-up, there was no difference in their PIS scores. The MGFA PIS in OMG and GMG patients at second year follow-up compared to first-year was not significantly different. The AChR RIA titres fell significantly from point of recruitment to follow up with a significant improvement in the first year and a further slight reduction in the second year. MG composite scores showed a significant change in median scores at first year follow-up compared to at recruitment in both EOMG and LOMG. Similarly, this difference was seen in patients who were treatment naïve at recruitment and were immunosuppressed at first year follow-up. There was also a significant difference in the MG composite scores between EOMG and LOMG groups, being higher in LOMG compared to EOMG. This suggests that patients do better with time, and that older patients have a more severe illness at onset.

The MG composite scores and QOLs seem to have a linear correlation. There was no difference between MG QOL between EOMG and LOMG patients at recruitment. The MG QOL in our cohort improved when recruitment scores were compared with first year follow-up and this difference was more significant in the LOMG group compared to the EOMG group. The QoL scores in the older patients may have been influenced by other co-existing medical problems. There was also a significant difference in MG QOL in patients who were treatment naïve at recruitment and those who were immunosuppressed in the first year. LOMG patients had worse MG composite scores and QOL scores at recruitment but seemed to respond well to treatment clinically and in quality of life, doing much better than EOMG patients.

There was no difference in MG composite or MG QoL scores in patients who had preceding infections prior to the onset of MG symptoms to those who did not; there was no difference in both the scores between smokers and non-smokers. Alcohol intake and salbutamol inhalers did not make any difference to their scores either. Although unlikely, the reasoning behind asking about inhalers was to see if Salbutamol, which is used in some forms of congenital MG, made any difference to the symptoms. This is of course not a direct comparison, as the doses used and methods of delivery are different in the two conditions. There

was no difference in MGC or MG QOL in patients who were single positive to one antibody compared to double positives.

There have been several case reports in literature associating statin use with myasthenia gravis (412). We compared AChR titres, MG composite scores and MG QOL in all patients who were on statins with those who were not on statins and there was no significant difference. The rationale for looking into this was to see if patients on statins had a more severe illness/ had worse MGC scores.

There were other associated symptoms seen in our patients, including dry mouth in 8.6% urinary symptoms in 2.67%, fatigue in 6%, and weight loss in 7.33%. This is similar to reported literature, although none of the studies were longitudinal cohort studies (71, 83, 411). Whether urinary symptoms are related to MG or to the use of pyridostigmine is unclear.

Other autoimmune diseases associated with myasthenia have been reported to be very common, the most common being autoimmune thyroid disorders (91). It has also been reported that the frequency of second autoimmune disorders is higher in females and EOMG group who are more likely to be AChR antibody-positive and have GMG (93). In our cohort we found that asthma was the most common other autoimmune disorder followed by hypothyroidism. Comparison between EOMG and LOMG patients did not show any difference at 43.58% and

34.23% respectively; 43.93% of all the females in the study and 30.95% of all the males in the study had associated autoimmune conditions. There was no difference between the younger female and younger male patients either. This may be partly because asthma was not included as an AI condition in the other papers, and this may have narrowed the difference in our own cohort.

Previous literature has reported that familial autoimmunity in patients with MG is common and has been seen in 40% of EOMG patient relatives and 20% of LOMG relatives (93). 4% of the relatives had MG. In our cohort we found that family history of autoimmunity was 40% overall, more common in EOMG at 51.28% and 36.02% in LOMG in keeping with literature; however, this was not statistically significant. Hypothyroidism in the family seems to be the most common autoimmune disease followed by myasthenia gravis in 10/60 patients (16.67%) and a rate of 6.67% overall of familial MG. Studies on the effects of HLA on the age of onset of MG have shown mixed results. Different HLA haplotypes have been linked to EOMG and LOMG, but it is not clear why familial MG and indeed other autoimmune conditions are more common in EOMG (129-142). One explanation could be that although HLA haplotypes may be shared between siblings, disease susceptibility could be defined not only by HLA-DR but also by other genetic factors including

gene-gene interactions. Why this would be different in younger patients compared to older patients is unclear.

Other than for autoimmune conditions, there was no correlation with other comorbidities in our MG cohort. We had two patients with inclusion body myositis in our cohort, one of whom was seronegative and one who was double positive for MuSK and LRP4.

6.1.4 Effects of Immunosuppression

There have been several studies looking at the effect of prednisolone on the progression of OMG to GMG. Several of the studies suggest that early treatment with steroids decreases the progression of OMG to GMG. The EPITOME study which was the only RCT designed to look prospectively at steroid response in patients with OMG was not completed as planned (176). In our cohort, when we looked at the survival curve for time to generalisation in OMG patients who were given steroids pre-generalisation against those who were not given steroids pre-generalisation, there was no difference. There was no difference between the EOMG and LOMG groups either. There was no difference in time to generalisation of OMG between LOMG and EOMG; although LOMG patients as a whole developed generalised symptoms much earlier than EOMG, this was not significantly different. From our data, it appears that treating an

OMG patient with steroids does not change generalisation rates. This is important in clinical practice; however, this was not a drug trial. In order to confirm or refute this, an RCT would be required. Given that the well designed, international EPITOME trial failed to recruit patients, this may be difficult to do, but should nonetheless be tried.

Most of the literature which compares steroid doses in MG patients has been in those who have had thymectomy versus those who have not had thymectomy. There was no comparable data in literature for steroid doses in OMG and GMG patients as a natural cohort.

When we compared the daily dose steroid requirement in all patients at first year follow-up with second year follow-up (patients being treatment naïve at recruitment), there was a significant difference in doses, with patients at second year requiring less average daily dose steroids compared to the first year, both in EOMG (16mg Vs 6.25mg) and in LOMG (10mg Vs 7mg). There was no significant difference in the steroid doses between EOMG and LOMG at first year follow-up or at second year follow-up, but the dose requirements at first year follow up in LOMG was slightly less than EOMG. This would be in contrast to the clinical presentation of worse disease in LOMG with more GMG. This could be explained by the fact that LOMG patients required earlier initiation of steroids during the first year and doses were tapered down by their first year follow up. It

could also suggest that LOMG patients responded more rapidly to treatment.

The steroid dose in OMG at first year was higher than at second year; however, in GMG there was no difference between first and second year steroid dosages. On direct comparison between OMG and GMG, there was no difference in steroid dose at first year or at second year. It appears that OMG patients respond more quickly to steroids than GMG, leading to clinically significant improvement and reduced steroid doses with time.

Steroid requirements pre-generalisation were no different in any of the antibody subgroups; but, post generalisation i.e. in GMG patients with AChR and MuSK double positivity, the steroid requirement was much higher (80%) compared to AChR single positives (25.2%), MuSK single positives, AChR and LRP4 double positives, and the seronegatives. When compared to the MuSK and LRP4 double positive group, it was not significantly different. The common factor in both appears to be positivity to MuSK Abs. In comparison, the requirement for alternate immunosuppressants in AChR single positives compared to AChR and MuSK double positives was not significant. It has been reported in literature that MuSK MG follows a more severe course, and patients were more likely to require alternate immunosuppression- more commonly plasma exchange or rituximab. We did not see this difference in our cohort, except for

higher rates of steroid requirement in AChR and MuSK double positives. This would suggest that in our cohort, although the clinical presentation between the AChR and MuSK subgroups was not significantly different, the double positives were harder to treat, implying perhaps a more brittle myasthenia?

The MG composite scores for the 15 patients who were immunosuppressed at recruitment compared to the patients who were immunosuppression naïve at recruitment showed a significant difference. This was likely to be reflective of the fact that patients who required immunosuppression at recruitment were at the severe end of the disease spectrum, requiring admission to hospital in crises or bulbar/respiratory symptoms. MG composite scores in patients who were immunosuppression naïve at recruitment but were immunosuppressed at first year follow-up did not show any statistical significance and there was no difference at second year follow-up either.

Initial immunosuppression at recruitment does not seem to change the Treg percentages as seen when we compared the 15 immunosuppressed patients at recruitment with the 135 immunosuppression naive patients. There was no difference in the Treg levels at recruitment and at first year follow up in the 24 patients whose samples were tested at recruitment and a year later; and although there was a further drop in Treg levels in patients who went on to have immunosuppression, this was not statistically significant. The paired LOMG samples did not show

any difference either. We were unable to compare the EOMG patient samples as there was no mean difference between the two values. This is different to the one study by Wen et al which looked at 59 treatment naïve patients, 13 of whom then had immunosuppression, where they found that Treg levels in treated patients was significantly higher than untreated patients and the levels improved after treatment (298).

IFNg levels were lower in treatment naïve patients compared to those who were immunosuppressed at recruitment. There are no studies comparing cytokine levels in treatment naïve and immunosuppressed patients. It could be postulated that treatment naïve patients have milder disease and so their inflammatory markers are less than those with more severe disease needing immunosuppression.

6.1.5 Neurophysiology

Previous studies and literature have shown that single fibre EMG (SFEMG) has more sensitivity and specificity compared to RNS; however, because of the easy availability of RNS this was the diagnostic test recommended by AAEM (413). In a study by Punga and colleagues (188) they showed that RNS was normal in patients with severe GMG and they recommended using concentric needle electrode myography instead. In our cohort, SFEMG was abnormal in 66.67% compared to 43.24% of RNS

abnormalities; this was not statistically significant, although in keeping with literature. Only four patients had routine EMG of which one showed myopathic changes. Of the 8 seronegative patients, 5 had neurophysiology of which two were abnormal showing blocks on SFEMG, the other three were normal.

6.1.6 Immunological profile

It has been reported in previous literature that AChR positivity is seen in 85-90% of GMG patients and between 50-75% of OMG patients (44, 419). There is wide variability in MuSK antibody positivity, with initial reports of 7%, but other papers reporting anywhere between 3.8 and 47.4% (151, 200, 420). Most studies have shown that AChR titres are lower in LOMG patients with no thymic abnormalities (96, 97, 149, 150, 421), patients with thymoma had higher titres (no age correlation) and EOMG patients with thymic hyperplasia had higher titres (10). A study by Iwasa et al differed slightly when they found raised AChR titres amongst MG patients in Japan for a brief period of time; this was more pronounced in LOMG patients (422).

Of our 150 patients, the majority were single positive for AChR antibodies, with a small proportion positive for MuSK antibodies and LRP4 antibodies. We had a large number of patients who were double positive for AChR and MuSK, AChR and LRP4, or MuSK and LRP4, and 8/150 who were seronegative. Two

patients were triple positive but were thought to be single positive for AChR with non-specific binding for the other two. The reason for this differentiation between double postives and triple positives was that in the double positives, the binding was to the expressed receptors- either GFP tagged AChR or MuSK or untagged LRP4 receptors, whereas in triple positives, the binding was to the cell surface/ other proteins as well as the expressed receptors.

Of our 150 patient samples, when tested for all three antibodies on RIA and CBA, we had 8 seronegative patients (5.32%) (one of these patients was initially positive for AChR on RIA). The majority of patients were positive for AChR (71.33%). The rates of AChR single positivity in our cohort was lower than previously published, however, we had a further 15.33% who were double positive, bringing the total AChR positivity to 86.66% which is more in keeping with published literature. In routine clinical practice, serum is tested for AChR antibodies first and if this is negative, they are tested for MuSK antibodies on RIA followed by clustered CBA. It is possible that there are a small number of patients in the community who have been diagnosed with AChR MG, but who may well have a second antigenic target which has not been tested for.

The rates of LRP4 (2%) and MuSK MG (4%) was in keeping with published literature.

We had quite a few patients who were double positive. AChR and MuSK double positivity was seen in 10%, which is slightly less than previously published data of 12.5% (423). AChR and LRP4 double positivity was seen in 5.33%, again, less than previously published rates of 7.45% (207). MuSK and LRP4 double positivity was seen in 0.67%, this is much less than previously published rates of between 14.92% and 19.8% (207, 423). The previously published data is mainly from a multinational retrospective study of 904 stored serum samples. Two patients were positive for all three antibodies, although the conclusion was that the MuSK and LRP4 antibodies were probably non-specifically binding. Triple seropositivity has also been described before at a similar rate (423).

In our cohort of 150 patients, 8 patients were seronegative. Among the seronegative patients, ocular symptoms were seen slightly less frequently at 87.5% compared to antibody positive patients; however, this was not statistically significant.

In our study, we found that in OMG, AChR positivity was seen in 75% in EOMG and 80% in LOMG on RIA, and 87.5% and 80% respectively on CBA. The difference in positivity on RIA and CBA was not statistically significant. The positivity on RIA in younger GMG patients was low at 56.5% compared to the LOMG patients at 91.4%; however with CBAs the positivity in EOMG GMG patients increased to 82.6% and in LOMG patients to 93.8%, which was still statistically significant.

Zivkovic et al have published similar results in their retrospective study where they found AChR positivity to be more common in LOMG than in EOMG (p=0.0026). They did not differentiate between OMG and GMG, but mention that OMG is much more common in LOMG than in EOMG. Jacob et al suggest that patients with AChR antibodies positive only on clustered cell based assays more commonly have OMG. This is different to what our study suggests; however, the study by Jacob et al was a small retrospective study, and whilst showing a trend, the results were not conclusive (155, 164).

It appears that the EOMG patients in our cohort, particularly those with GMG, may have lower affinity antibodies to AChR which were not detected in the solution phase (used in RIA), but were detectable on CBAs. As has been shown previously, low affinity Abs are still pathogenic in vivo against the AChR clusters at the NMJ (163). It appears that EOMG patients have highly pathogenic, but low affinity AChR Abs compared to LOMG.

There was a significant difference in AChR single positivity in the females in EOMG and LOMG in our cohort, being much more frequent in LOMG patients. This is different to previously published data by Burke and colleagues who suggested that LOMG patients had lower AChR titres (NB: titres, not positivity) and were more likely to have striated muscle antibodies (414). There was tendency towards younger female patients in our cohort being double positive to AChR and MuSK compared to

older female patients, however this was not statistically significant. Previous studies by Zisimopoulou et al looked at double positivity with LRP4 Abs. They found that it was more common in young females, and they had a more severe illness than those with single positivity to LRP4. Our study has found this with MuSK and AChR; whilst this cannot be directly compared, we can infer that as both these antibodies are pathogenic to different targets, in combination, they can cause a more severe disease (207). MuSK antibody positivity was also not statistically significant in our EOMG or LOMG groups or between males and females, which is different to published literature which suggest that MuSK positivity is more commonly seen in young females (151, 154).

There was no difference between MuSK positivity in EOMG and LOMG in ocular patients. MuSK seropositivity in GMG in EOMG patients was 33.3% compared to 11.9% in LOMG patients, which was not significant. There was no significant difference in MuSK seropositivity amongst the EOMG group when OMG and GMG were compared, neither was there any difference in the LOMG subgroup. Female EOMG patients positive to MuSK Abs (30.43%) compared to male EOMG patients (6.25%) did not show a statistically significant difference. When female EOMG and LOMG patients were compared, although the trend was towards the younger females being more likely to be positive (30.43% Vs 11.62%), this was not significant (p=0.0610). This is

different to previously published data which report that MuSK Abs are more common in younger females (151, 154, 407). The study by Guptill et al was a large cohort retrospective study; however, all the antibody tests were done on RIA, and not on CBAs. It is possible that lower affinity MuSK Abs, which may well be what is seen in LOMG patients, were missed (NB: the majority of our MuSK positives were on CBA alone), skewing the data towards the younger females. In the study by Evoli et al, who noticed a striking female preponderance, MuSK Abs were tested using immunoblot. The study by Huda et al showed that although female preponderance was seen with MuSK CBA+ RIA- patients, the age of onset was less, and they were more likely to have OMG, suggesting a milder phenotype in these patients. Our MuSK cohort was predominantly RIA- CBA+. This could be one of the reasons why our results differ from published literature. For future studies, this subgroup will need to be looked into more carefully by recruiting higher numbers of patients who are MuSK RIA+, and comparing them with those who are MuSK CBA+ RIA-.

LRP4 seropositivity in our cohort was similar to published data. There was no difference between OMG and GMG in EOMG and LOMG, and no difference between EOMG and LOMG (both for OMG and GMG) either. This is different to previously published data which report that LRP4 single positivity is associated with milder disease and double positivity with more severe disease

(207). Our LRP4 cohort was predominantly double positive with less than a quarter of them being single positive. This is perhaps why, clinically, as a group, the phenotype was not dissimilar to the other subgroups.

There have been no large prospective studies looking at AChR titres with longitudinal data and long term follow up of patients. Our study provides novel data on AChR titres. We compared the titres of AChR RIA in patients at recruitment and at annual follow-up. We found a significant drop in titres over the first year. The drop in titres was seen mainly in patients who were treatment naïve at recruitment and who were immunosuppressed at first year follow-up, and although there was a drop in titres in patients who remained immunosuppression naïve throughout the first year, it was not statistically significant. There was a comparable drop in scoring on AChR CBA as well when recruitment samples were compared with first year and second year follow-up samples. Both AChR RIA titres and MG composite scores fell during the first year, with linear correlation; similarly with MG QOL. It appears that with a clinical response to treatment, there is a corresponding fall in AChR titres. This would make sense theoretically. It appears that in an individual patient, falling titres may indicate clinical improvement, and vice versa. However, functional studies were not done and although this can be inferred, it cannot be confirmed.

There was a significant difference between AChR RIA titres in EOMG and LOMG with the titres being much higher in LOMG. Compston et al found in their retrospective study of stored serum samples, that patients with thymoma had the highest titres of AChR Abs, followed by the EOMG patients, and then the LOMG patients. They used 40 years as the age cut off. This was similar to data published by Lindsburg et al and Mantegazza et al. Somnier et al found lower concentrations of AChR in LOMG, but non significantly. In contrast, Lindstrom et al did not find any correlation with age. Neither Lindstrom nor Somnier divided the groups into thymomatous and non-thymomatous patients. Our analysis also did not differentiate between thymomatous and non-thymomatous patients. As explained in previous chapters, the relationship between thymic abnormalities and LOMG is not entirely clear. It is postulated that abberations in the aged thymus in LOMG mimics thymoma behaviour without frank neoplasia, or, a small thymoma could have regressed spontaneously before the diagnosis of MG. It is therefore possible that our LOMG cohort reflects this immunological similarity with TAMG with high AChR titres (97, 149, 150, 421).

The Treg proportions we found in our study were much lower than those reported in literature where they have been shown to be anywhere between 1 and 10% (405), whereas in our cohort we found that the readings were much lower than 1%. There

were a few outliers amongst these patient samples with higher percentages. However, we found that the median for both the patient samples and our healthy controls was much less than published in literature and concluded that this was due to our gating mechanism. We did not think that this would affect results as the same staining methods and gating were used for the patient samples and healthy controls. However, we accept that our data needs to be interpreted with caution because of the low percentages, and because of this, may not be directly comparable to published literature.

The percentage of T regs in our patients was significantly lower than healthy controls. There was no statistically significant difference between EOMG and LOMG at recruitment. The EOMG patient samples compared with EOMG healthy controls shows a significant difference; LOMG patients compared to LOMG healthy controls also showed a difference but to a slightly lesser degree. Due to our large sample size, 135 of which were treatment naïve samples, we think that these results are indicative of the immunopathogenesis in MG, but accept that the percentages are too small to make accurate predictions. Published data so far have been contradictory, some showing reduced Treg numbers, and others no difference in Treg numbers in MG (49, 50, 264). What most papers agree on is that there is a functional defect in the Tregs, irrespective of

numbers/percentages. We did not perform functional studies on our patient samples, and so this information is not available.

When EOMG was compared to LOMG, the only significant difference was in IFNg levels which were higher in LOMG patients, IL-4 levels were slightly higher in LOMG compared to EOMG. There are no large studies comparing cytokines in EOMG and LOMG and this is novel data. The LOMG patients in our cohort seem to have more severe disease, and higher levels of inflammatory cytokines would be in keeping with this. We did not compare immune markers between patients with similar disease severity in EOMG and LOMG to see if the difference was still seen, and if age was a factor here along with disease severity. This is perhaps something that could be done in future studies.

When cytokine levels were compared between recruitment patient samples and HC, the proinflammatory cytokines TNFa and IL-17 were higher, and IFNg and IL-4 did not show any difference. The anti-inflammatory cytokine IL-10 was higher in patient samples compared to HC. For cytokines IL-17 and IL-10, this difference was seen only in EOMG and not in LOMG patients. Previous studies have shown an increase in several pro-inflammatory cytokines in MG including IFNγ, TNFα and IL-17 (250, 310). Our study has shown an increase in IL-17 and TNFa, and although there is a trend towards higher IFNg in the

MG patients, this was not significant. In keeping with literature, this increase was seen mainly in EOMG patients (314).

There was a significant difference in the Tregs in AChR antibody-positive patients compared to healthy controls and in MuSK positive patients compared to healthy controls, but there was no difference between Tregs in LRP4 positive patients compared to HCs. NB: We had fewer LRP4+ patients compared to the other subgroups. There was a significant difference also when T regs were compared between double seropositive patients and healthy controls. There was no difference however when AChR antibody-positive patients' T regs were compared to MuSK positive patients' T regs. Although there is some literature on cytokines in the antibody subgroups, there is no large scale data on Treg levels in the different antibody subgroups. As mentioned earlier, our Treg percentages were small and so this data needs to be interpreted with caution.

In AChR MG patients, the pro-inflammatory cytokine IFNg was slightly higher, TNFa was higher, IL-17 was higher and the anti-inflammatory cytokine IL-10 was higher than HC. In contrast, MuSK MG patients had no increase in the pro-inflammatory cytokines or anti-inflammatory cytokines. This is different to previously published data which reports that MuSK MG patients have higher IFNg and IL-17 levels, and not AChR MG patients, in direct contrast to our study results (315). The majority of the patients in this published study were on immunosuppression,

some on two or more drugs. We did not perform subgroup analysis on patients who were immunosuppressed in each antibody category.

Double sero-positive patients had cytokine levels similar to AChR MG with lower IFNa levels, higher TNFa, IL-17 and IL-10 levels, but no increase in IFNg compared to HC.

When we compared AChR and MuSK MG patients, there was no difference in IL-10.

Our T reg and cytokine analysis in the antibody subgroups shows a mixed anti-inflammatory and pro-inflammatory picture. The percentage of CD8 cells producing cytokines (which we used as surrogate markers instead of measuring cytokines), was not significantly altered other than slightly increased pro-inflammatory cytokines IFNγ and TNFα in AChR Ab + patients.

In AChR MG, the main cytokine producing T cell appears to be the CD4+ cells, which shows increased production of the pro-inflammatory cytokines TNFα and IL-17. This, along with reduced T regs indicates a ‘pro-inflammatory’ environment. At the same time, there is increased production of the anti-inflammatory cytokine IL-10 indicating an ‘anti-inflammatory’ environment. This kind of defective balance is seen in a lot of autoimmune conditions.

In contrast to AChR MG, in MuSK MG, Tregs are reduced indicating a ‘pro-inflammatory’ environment only.

6.1.7 The Thymus

Previous reports in literature have shown that thymic hyperplasia is seen in 50-60% of EOMG patients and is not seen in LOMG patients (51, 52, 415). Of our 150 patients, 137 had imaging to look for thymic abnormalities. The total number of abnormalities, including thymoma/thymic mass, thymic hyperplasia/enlagment and thymic remnants, are more commonly seen in EOMG patients in 50% compared to LOMG in 13.86%. Thymic enlagment/hyperplasia (depending on CT findings or confirmed on histology) was much more commonly seen in EOMG patients in 30.55% compared to 0% in LOMG. Thymomas/thymic mass (depending on CT findings or confirmed on histology) was seen in 16.67% of EOMG patients compared to 10.89% in LOMG patients, which was not statistically significant. We did not find any difference in AChR titres amongst patients with thymoma in the LOMG and EOMG group. Overall, in our cohort, younger patients appear to have more frequent thymic hyperplasia/enlargement and thymoma/thymic mass compared to LOMG. The data was based on scan findings of thymic enlargement with subsequent tissue diagnosis in most of these patients. This presumes that a normal scan excludes thymic abnormality which may not be the case,

particularly in EOMG. This means that our data is not directly comparable to published studies, and this would require categorising patients into histologically normal and abnormal groups; however, our data suggests that thymic hyperplasia is much more common in EOMG than LOMG.

We did not find a difference in AChR RIAs in EOMG and LOMG patients with thymic abnormalities. This could be because we did not thymectomise all EOMG patients; it is possible that a proportion of patients with no radiological abnormalities had histological abnormalities which were not picked up, and so a proportion of the data may be missing. (10, 97).

We did not find any difference in thymic abnormalities in the different antibody subgroups except for patients with LRP4 antibodies who were more likely to have thymic hyperplasia compared to AChR single positivity. However, it is to be borne in mind that we only had 2 LRP4 single positive patients in our cohort. Also to be borne in mind, the data was based on scan findings of thymic enlargement with subsequent tissue diagnosis in most of these patients.

There was no difference in T regs in patients with normal thymus and thymic hyperplasia/enlargement nor was there a difference in normal thymus compared to thymoma/thymic mass. Previous studies have shown contradictory results on Treg numbers in MG with thymic abnormalities, with no

consensus. It is not clear why these studies differ but it could be due to the different staining and gating used and the different patient mix. Once again, with low Treg percentages, interpretation of our data is difficult. We analysed our data on radiologically abnormal thymuses (most of whom also had a histological diagnosis). Perhaps if this is looked into in more detail in future studies, we may get data that is comparable to published literature.

There was no difference in cytokines between patients with normal thymuses and thymic abnormalities as a whole; CD4 IL-17 is significantly lower in patients with thymoma/thymic mass compared to patients with normal thymuses, CD8 interferon-γ and CD8 TNF α are significantly lower in patients with thymic hyperplasia/enlargement compared to normal thymuses. This is different again to previous literature which has shown increased pro-inflammatory cytokines in thymic hyperplasia (249, 309). We accept that our study results are not conducive to a direct comparison, as not all patients had a histological diagnosis, and comparison was made between radiologically abnormal (with histological conformation in many), and radiologically normal thymuses.

6.1.8 Summary

Our data shows that the incidence of LOMG is increasing, and EOMG is more common in females and LOMG more common in males, although the difference between younger males and females is becoming less significant. OMG is more common in EOMG than LOMG; however, there is no difference in the type of ocular presentation or generalisation rates in EOMG and LOMG. LOMG patients are more likely to have bulbar and respiratory symptoms than EOMG patients, and they also develop these symptoms significantly earlier than EOMG patients. Age seems to be the factor here rather than sex, as there is no difference between male and female patients. LOMG patients have worse MG composite scores and QOL scores at recruitment but seem to respond well to treatment clinically and in quality of life, doing much better than EOMG patients. The rates of autoimmunity were the same in EOMG and LOMG, but family history of autoimmunity was more common in EOMG than LOMG. Clinical phenotypes in the different antibody subgroups suggests that there is no difference in bulbar, limb, respiratory and ocular symptoms in any of the subgroups, except for seronegative patients who are less likely to have bulbar symptoms.

LOMG patients have worse MG composite score and QOL scores at recruitment but seem to respond well to treatment clinically and in quality of life, doing much better than EOMG

patients. The rates of autoimmunity were the same in EOMG and LOMG, but family history of autoimmunity was more common in EOMG than LOMG.

AChR Ab positivity is seen more commonly in older females compared to younger females; all the other antibody subgroups are comparable between younger and older patients and females and males. AChR and MuSK double positive patients require steroids more frequently in GMG compared to other antibody subgroups. Seronegative patients in our cohort did not require admission to hospital suggesting that they may have a less severe illness and a more indolent course compared to the antibody positive subgroups. AChR Ab positivity is seen more commonly in older females compared to younger females; all the other antibody subgroups are comparable between younger and older patients and females and males. AChR and MuSK double positive patients require steroids more frequently in GMG compared to other antibody subgroups. OMG patients respond more quickly to steroids than GMG, leading to clinically significant improvement and reduced steroid doses with time. One could argue that this would suggest that OMG is a milder disease than GMG. It has been postulated that ocular muscles are more susceptible to being affected by Abs in MG, which is why OMG is more common than GMG; hence an alternative explanation would be that ocular muscles respond more quickly and effectively to steroids compared to muscles elsewhere.

Our study has shown a lower rate of AChR single positivity, but when double positives were added, the positivity rates were similar to published data. EOMG patients were less likely to be positive on RIA, possibly due to lower affinity antibodies. AChR titres were higher in LOMG compared to EOMG. The titres dropped after treatment on annual review. There was no difference in AChR titres in patients with (mainly radiological) thymic abnormalities. There was no difference in MuSK or LRP4 positivity in the different subgroups, and we had a lower percentage of double seropositivity compared to published literature.

In our patient cohort, IL-10 which is an anti-inflammatory cytokine is increased in patient samples. This suggests an anti-inflammatory environment. At the same time, Treg cell population is reduced in patient samples both in EOMG and LOMG, and the pro-inflammatory cytokine TNFα is increased in patient samples, as is IL-17. This mixed picture would suggest that there is a defective balance in immune regulation in MG.

Our study data shows that there are clinical differences between EOMG and LOMG with differences in onset, severity, generalisation, MGC scores, and outcomes. There are also immunological differences with rates of seropositivity and AChR titre levels. Despite the differences, patients in both groups respond well to treatment and show an improvement with time, both clinically and immunologically.

6.2 Future research

After the completion of the present study in July 2017, we have continued the project by recruiting more newly diagnosed patients with MG in the East Midlands area. We have continued to collect follow up data on the 150 patients initially recruited and should have two year follow- up data for all 150 patients by the middle of 2019.

We plan to continue this work with further research:

- To isolate B cells from PBMCs and identify AChR producing plasma cells.
- Determine levels of membrane-bound B-cell activating factor and its receptor (BAFF/BAFF-R) in peripheral blood lymphocytes (PBL).
- Determine the antibody profile to Titin, ryanodine receptor, Agrin and ColQ
- Undertake molecular HLA typing to expand current UK datasets contributing to an ongoing genome-wide association study.
- Perform studies on viral and auto-antibodies on frozen sera and PBL to be stored from every case; also on V-beta T-cell receptor repertoires, and correlate with clinical data, viral serological results and HLA profile.

We sent some of our serum samples to Dr Punga's lab in Sweden, where she and her colleagues have evaluated the serum for circulating microRNAs.

The papers that we have published so far are:

Circulating microRNA miR-21-5p, miR-150-5p and miR-30e-5p correlate with clinical status in late onset myasthenia gravis.

Sabre L, Maddison P, Sadalage G, Ambrose PA, Punga AR.J Neuroimmunol. 2018 Aug 15;321:164-170. doi: 10.1016/j.jneuroim.2018.05.003. Epub 2018 May 8.PMID: 29804819

miR-30e-5p as predictor of generalization in ocular myasthenia gravis.

Sabre L, Maddison P, Wong SH, Sadalage G, Ambrose PA, Plant GT, Punga AR.Ann Clin Transl Neurol. 2019 Jan 24;6(2):243-251. doi: 10.1002/acn3.692. eCollection 2019 Feb.PMID: 30847357

False-positive acetylcholine receptor antibody results in patients without myasthenia gravis.

Maddison P, Sadalage G, Ambrose PA, Jacob S, Vincent A.J Neuroimmunol. 2019 Jul 15;332:69-72. doi: 10.1016/j.jneuroim.2019.04.001. Epub 2019 Apr 3.PMID: 30959340

A Prospective Study of the Incidence of Myasthenia Gravis in the East Midlands of England.

Maddison P, Ambrose PA, Sadalage G, Vincent A.Neuroepidemiology. 2019;53(1-2):93-99. doi: 10.1159/000500268. Epub 2019 May 8.PMID: 31067543

Ocular presentation of myasthenia gravis: A natural history cohort.

Kamarajah SK, Sadalage G, Palmer J, Carley H, Maddison P, Sivaguru A.Muscle Nerve. 2018 Apr;57(4):622-627. doi: 10.1002/mus.25971. Epub 2017 Oct 6.PMID: 28881457

7 Appendices

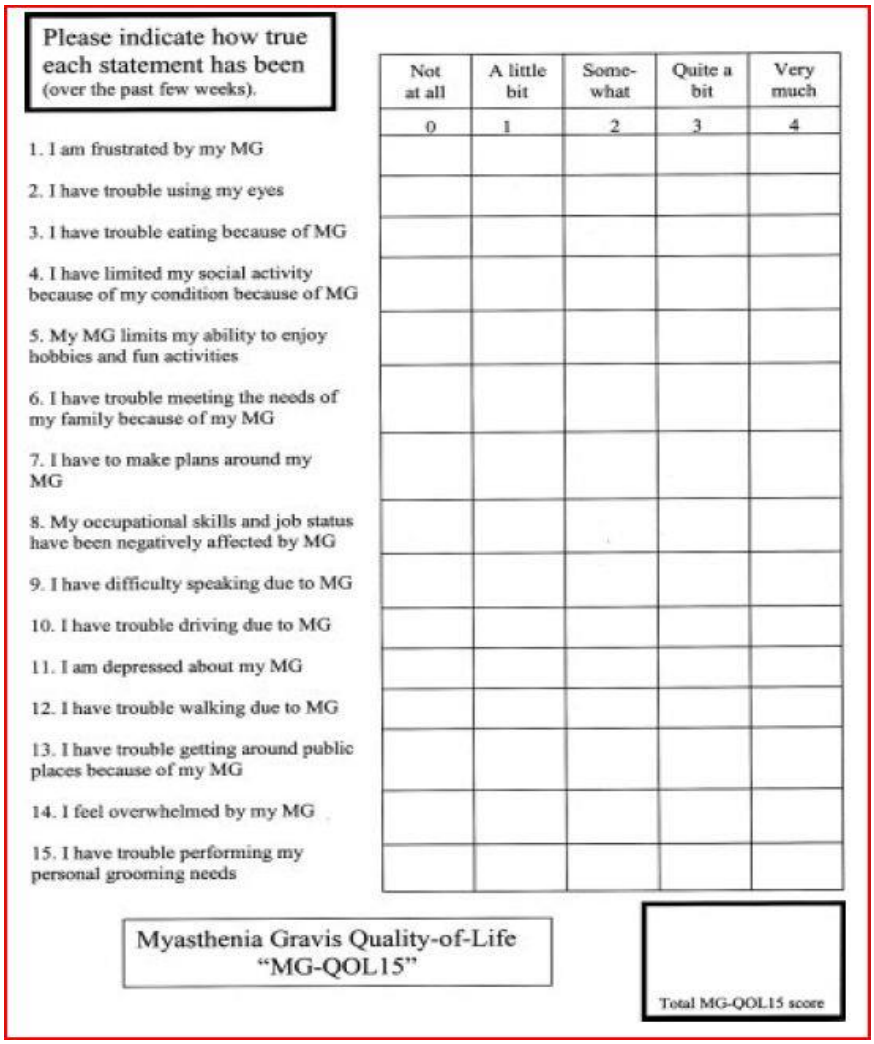
7.1 Appendix 1 Scoring sheets and Classifications

Table 1 The Myasthenia Gravis Composite scale

Ptosis, upward gaze (physician examination)	>45 seconds = 0	11-45 seconds = 1	1-10 seconds = 2	Immediate = 3
Double vision on lateral gaze, left or right (physician examination)	> 45 seconds = 0	11-45 seconds = 1	1-10 seconds = 3	Immediate = 4
Eye closure (physician examination)	Normal = 0	Mild weakness (can be forced open with effort) = 0	Moderate weakness (can be forced open easily) = 1	Severe weakness (unable to keep eyes closed) = 2
Talking (patient history)	Normal = 0	Intermittent slurring or nasal speech = 2	Constant slurring or nasal but can be understood = 4	Difficult to understand speech = 6
Chewing (patient history)	Normal = 0	Fatigue with solid food = 2	Fatigue with soft food = 4	Gastric tube = 6
Swallowing (patient history)	Normal = 0	Rare episode of choking or trouble swallowing = 2	Frequent trouble swallowing, e.g. necessitating changes in diet = 5	Gastric tube = 6
Breathing (thought to be caused by MG)	Normal = 0	Shortness of breath with exertion = 2	Shortness of breath at rest = 4	Ventilator dependence = 9
Neck flexion or extension (weakest) (physician examination)	Normal = 0	Mild weakness = 1	Moderate weakness (i.e., ~50% weak, ±15%) = 3[a]	Severe weakness = 4
Shoulder abduction (physician examination)	Normal = 0	Mild weakness = 2	Moderate weakness (i.e., ~50% weak, ±15%) = 4[a]	Severe weakness = 5
Hip flexion (physician examination)	Normal = 0	Mild weakness = 2	Moderate weakness (i.e., ~50% weak, ±15%) = 4[a]	Severe weakness = 5

[a]Moderate weakness for neck and limb items should be construed as weakness that equals roughly 50% ±15% of expected normal strength. Any weakness milder than that would be mild and any weakness more severe than that would be classified as severe.

Figure 1. Myasthenia Gravis Composite Score (MGC)



Please indicate how true each statement has been (over the past few weeks).

	Not at all	A little bit	Some-what	Quite a bit	Very much
	0	1	2	3	4
1. I am frustrated by my MG					
2. I have trouble using my eyes					
3. I have trouble eating because of MG					
4. I have limited my social activity because of my condition because of MG					
5. My MG limits my ability to enjoy hobbies and fun activities					
6. I have trouble meeting the needs of my family because of my MG					
7. I have to make plans around my MG					
8. My occupational skills and job status have been negatively affected by MG					
9. I have difficulty speaking due to MG					
10. I have trouble driving due to MG					
11. I am depressed about my MG					
12. I have trouble walking due to MG					
13. I have trouble getting around public places because of my MG					
14. I feel overwhelmed by my MG					
15. I have trouble performing my personal grooming needs					

Myasthenia Gravis Quality-of-Life "MG-QOL15"

Total MG-QOL15 score

Figure 2. Myasthenia Gravis Quality of Life Score (MG-QoL)

Class	Clinical symptoms
I	Any ocular weakness
II	Mild Weakness. May also have ocular muscle weakness of any severity
II A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
II B	Predominantly affecting orорpharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
III	Moderate weakness affecting other than ocular muscles. May also have ocular muscle weakness of any severity
III A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
III B	Predominantly affecting orорpharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
IV	Severe weakness affecting other than ocular muscles. May also have ocular muscle weakness of any severity
IV A	Predominantly affecting limb, axial muscles, or both. May also have lesser involvement of oropharyngeal, respiratory muscles or both
IV B	Predominantly affecting orорpharyngeal, respiratory muscles, or both. May also have lesser or equal involvement of limb, axial muscles or both
V	Defined by intubation, with or without mechanical ventilation, except when employed during routine postoperative management

doi:10.1371/journal.pone.0114060.t001

Figure 3. MGFA classification of weakness

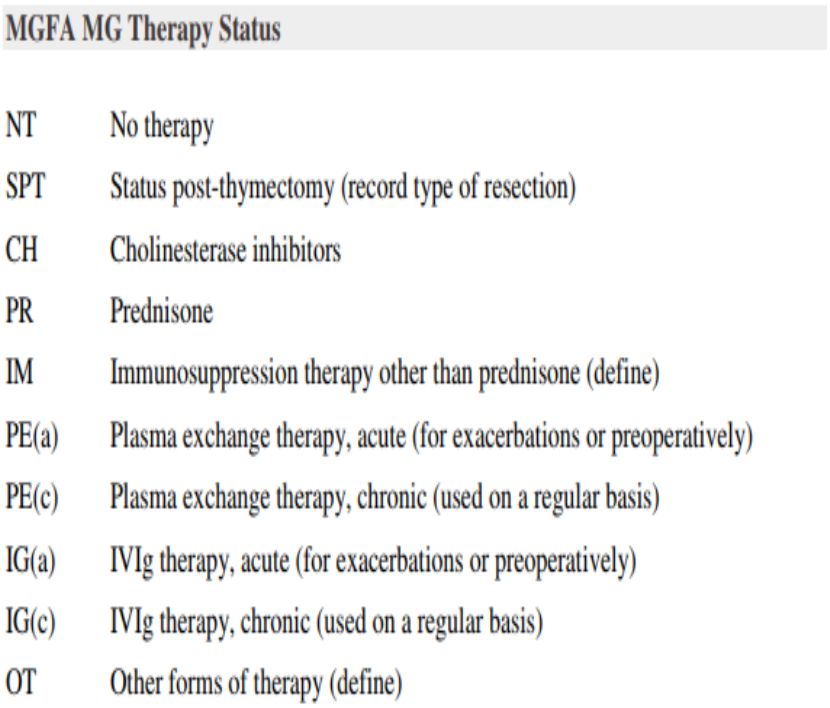


Figure 4. MGFA classification of treatment/therapy status

MGFA Post-intervention Status (MGFA-PIS)

Complete Stable Remission (CSR)	The patient has had no symptoms or signs of MG for at least 1 year and has received no therapy for MG during that time. There is no weakness of any muscle on careful examination by someone skilled in the evaluation of neuromuscular disease. Isolated weakness of eyelid closure is accepted.
Pharmacologic Remission (PR)	The same criteria as for CSR except that the patient continues to take some form of therapy for MG. Patients taking cholinesterase inhibitors are excluded from this category because their use suggests the presence of weakness.
Minimal Manifestations (MM)	The patient has no symptoms of functional limitations from MG but has some weakness on examination of some muscles. This class recognizes that some patients who otherwise meet the definition of CSR or PR do have weakness that is only detectable by careful examination.
MM-0	The patient has received no MG treatment for at least 1 year.
MM-1	The patient continues to receive some form of immunosuppression but no cholinesterase inhibitors or other symptomatic therapy.
MM-2	The patient has received only low-dose cholinesterase inhibitors (<120 mg pyridostigmine/day) for at least 1 year.
MM-3	The patient has received cholinesterase inhibitors or other symptomatic therapy and some form of immunosuppression during the past year.
Change in Status	
Improved (I)	A substantial decrease in pretreatment clinical manifestations or a sustained substantial reduction in MG medications as defined in the protocol. In prospective studies, this should be defined as a specific decrease in QMG score.
Unchanged (U)	No substantial change in pretreatment clinical manifestations or reduction in MG medications as defined in the protocol. In prospective studies, this should be defined in terms of a maximum change in QMG score.
Worse (W)	A substantial increase in pretreatment clinical manifestations or a substantial increase in MG medications as defined in the protocol. In prospective studies, this should be defined as a specific increase in QMG score.
Exacerbation (E)	Patients who have fulfilled criteria of CSR, PR, or MM but subsequently developed clinical findings greater than permitted by these criteria.
Died of MG (D of MG)	Patients who died of MG, of complications of MG therapy, or within 30 days after thymectomy. List the cause (see Morbidity and Mortality table).

Figure 5. MGFA Post-intervention status (MGFA-PIS)

7.2 Appendix 2 Methods for Antibody assays

Radioimmunoprecipitation assay (RIA/RIPA)

All the experiments to look for MG antibodies were done at the Oxford labs at the Nuffield Department of Clinical Neurosciences. All RIA analysis for AChR and MuSK Abs were done by the author. For high positive titres, serial dilutions were done until the CPM value dropped by half and then the titres were calculated. VGCC Ab RIAs were done by Dr Bethan Lang and Selina Tomsen.

All 150 MG patients in the study had serum samples taken at recruitment and 120 patients had serum samples at first year follow-up. ACHR RIA was tested on all patient samples at recruitment and first year follow-up. MuSK RIA was tested on all 150 of the recruitment patient samples. VGCC antibodies on RIA were tested in 139 patients at recruitment.

General principles for RIA:

Radioimmunoassay (RIA) or radioimmunoprecipitation assay (RIPA) involves precipitating radioactively labelled antigen and antibody complex. In the first step patient sera is incubated with I125 labelled antigen. Any specific antibody in the sera binds to the antigen. In the second incubation step the antigen-antibody complexes are precipitated using a precipitation agent. The precipitate is washed with buffer. After centrifugation and

decanting of the supernatant, radioactivity in the precipitate is counted using a gamma counter. The intensity of the radioactivity is proportional to the concentration of specific antibody in the patient serum. The antibody concentration is evaluated quantitatively using a calibration curve (246).

RIA for AChR antibodies

Day 1

1. Serum samples taken out of the -20^{0}C/-80^{0}C freezer and left to thaw at room temperature for a few minutes

2. When thawed, the eppindorfs were agitated to mix the serum and prevent layering (using the small mechanical mixer)

3. These were aligned in a row on a rack along with one healthy control (HC) and one strongly positive (SP) serum for AChR antibody

4. New eppindorfs were labelled for each corresponding serum sample and placed on the rack parallel to the test samples

5. PTX solution was poured into a large universal tube/sterile port

6. 225 µL of PTX was pipetted into each of the new eppindorfs

7. One pipette was placed into each new eppindorf for ease of pipetting and also to make it easier to draw up the test serum

8. 25 µL of the each test serum, HC and SP samples pipetted into the corresponding Eppendorf containing 225 µL of PTX making up to 250 µL in total

9. Another set of eppindorfs placed on a new rack for each corresponding sample

10. 50 µL of serum plus PTX mix was pipetted into the labelled eppindorf. The pipette tips used in the earlier step could be reused for each individual sample. This would equal 5 µL of serum.

11. The remaining serum and serum plus PTX mix placed on the rack in the cold room, ideally covered with foil

12. The test samples taken to the radioactive zone

13. Diluted the 125I labelled α bungarotoxin AChR, recommended to make up to 1.3 mls, but can be diluted up to 2.5 mls if a new batch, and the gieger counter reads very high counts. Titrated counts to approximately 15,000 CPM per 50 µL using the gamma counter

14. 50 µL of the 125I α BuTX AChR added to the test samples. This was mixed very briefly on the vortex mixer.

15. The whole rack was placed in the fridge in the radioactive zone overnight

Day two

16. Taken the test samples (with I125αBuTX-AChR) out of the fridge

17. Antihuman IgG serum-this was diluted up to 5 times i.e. 1 ml was made up to 5 ml by adding PTX

18. 50 µL of antihuman IgG was needed for every 5 µL of serum of being tested

19. After dilution, 250 µL of antihuman IgG plus PTX was added to each test serum eppindorf.

20. The solution became cloudy and started precipitating

21. After approximately 30 minutes at room temperature there was a clear layer at the top with a precipitate at the bottom

22. Added PTX, approximately 600 µL per tube

23. Centrifuged this (balanced properly) at 11 G, room temperature for three minutes

24. Pellets formed at the bottom

25. The supernatant was sucked out using the suction device taking care to not disturb the pellet

26. More PTX was added to each tube till it was full, using a squeezy bottle for ease

27. This needed to be left for a few minutes, then the supernatant was suctioned as before

28. Washed a second time as before, PTX was left for approximately 10 minutes to ensure that any excess I125 was washed off

29. After the second wash and suctioning of the supernatant, the lids were cut off from the eppindorfs, ensuring a clean cut so that they could sit snugly in the gamma counter rack

30. Placed the tubes in the order of how the results should print out i.e. first used the rack marked ‘I counter’, placed the first tube in the first lot on the side opposite to ‘I counter’ sign going from the left to the right. The ‘stop counter’ rack was the last one to go in with the other racks placed parallel in between the two if needed

31. These racks were placed on the right-hand side of the counter, perpendicular to the orientation of the space with the ‘I counter’ sign to the right and the ‘I counter’ rack furthest away from the front.

32. The results were printed out in order as counts per minute (CPM)

33. When the tests were done, the eppindorfs were taken out of the ranks using tweezers/scissors, and then thrown in the radioactive bin

34. Before and after working in the radioactive zone, the gieger counter was run over all of the areas including the sink to check for spillages. Filled in the sheets (X3) making note of I125 used, batch number and disposal (solid and drain)

Serial dilutions

Day two

1. For results which were highly positive i.e. close to or higher than the high positive control, the samples were serially diluted and retested.

2. The sera was diluted from 5 µL to 2.5 µL (100 µL of the remaining 200 µL of PTX per serum mix was pipetted into another eppindorf and a further 100 µL of PTX was added. 50 µL of this was pipetted into another eppindorf to get a serum concentration of 2.5 µL)

3. Similarly after each dilution, 100 µL of the remaining PTX and serum mix from the earlier step was diluted with 100 µL of PTX to get concentrations of 1.25 µL, 0.625 µL, 0.3125 µL and 0.15625 µL.

4. The rest of the process was similar to before

5. Ideally when using lower test serum concentrations, this caused a lower ratio of serum to 125I and to antihuman IgG. To avoid this, the remaining concentration of serum made up by adding HC serum. For example, for 1.25 µL concentration of the test serum, added 2.5 µL of healthy control serum.

6. For 2.5 µL test serum dilution, no need to add extra HC serum; for the other dilutions added 2.5 µL of HC serum each.

7. For example, if using 16 samples, diluted 40 µL of healthy control serum with 360 µL of PTX to make up 400 µL. Then used 25 µL of this mix (i.e. 2.5 µL of HC serum) for each test sample. This was added at the same time as adding the antihuman IgG.

8. Antihuman IgG was used in the same concentration and amount as used in the initial assay.

Calculation of titres:

For all our RIA assays, we used the RSR kit. The calculation of titres were done using the formula provided by the RSR company along with the information sheet provided for each sample of 125Iα BuTX AChR.

$$\text{nmol/L AChR} = \frac{(\text{CPM test sample} - \text{CPM negative control}) \times A}{C \times K \times BX\ 2.22}$$

A – Decay factor between receptor labelling day and day of assay

B - Counter efficiency (which was 80% on the machine in the Oxford lab)

C- Volume of serum used in the assay

K - Specific activity of 125I at the time it was used.

A, C and K are on the sheet included with every bottle of 125I AChR.

These titres were multiplied by 10, to give the values in 10-10 mols/L

For MuSK RIA assays, the procedure was the same as for AChR including dilutions. The assay kit used was RSR125I MuSK and the formula to calculate the titres was the same as AChR.

RIAs for VGCC Abs were performed by Dr Bethan Lang and Selina Tomsen in the Oxford laboratories. Dr Lang prepared the radioactive label for the solubilised cells freshly for each batch of VGCC tested.

Cell based assay

All CBAs for clustered AChR, MuSK and LRP4 Abs were performed and interpreted by the author. All positive assays

were repeated at least once. In the case of positive MuSK CBA assays, most samples were assayed 3 times (twice by the author and once by Dr Mark Woodhall) and interpreted independently by the author and 2 others- Dr Mark Woodhall and Dr Isabel Leite.

The list of consumables, media, reagents and antibodies used for CBAs are listed in the table below.

Table 30 Consumables, media, reagents and antibodies used for CBAs

Media/Reagents/Consumables	Supplier
HEPES	Sigma
Dulbeccos's Modified Eagle Medium (DMEM)	Sigma
Foetal Calf Serum (FCS/ PAA)	Sigma
Antibiotic/antimycotic solution (Penicillin, Streptomycin, Amphotericin, PSA)	Invitrogen
Trypsin-EDTA solution	Gibco
Poly-L lysine (PLL) solution	Sigma
Trptan blue solution (0.4%)	Sigma
Corning 175 cm (large) Tissue Culture (TC) flask	APPW
Corning 75 cm (medium) TC flask	APPW
Corning 6-well TC multi-well plate	APPW
Corning 24-well TC multi-well plate	APPW
Glass coverslips	VWR
Glass slides	VWR

Vectrashield mounting media	Vectrashield
DAPI	
Bovine serum albumin (BSA)	
Formaldehyde 4% (made up from 36%)	
Phosphate buffered saline (PBS)	
Goat anti-Human IgG	Invitrogen
Donkey anti-Goat IgG Alexa Fluor 568	Invitrogen
HEK 293 cells	TC flasks
AChR DNA (α, β, δ, ε and rapsyn with tagged EGFP subunits)	
MuSK DNA (with tagged EGFP)	
LRP4 (LRP4 AP and LRP4 caspr subunits)	

All CBAs for clustered AChR, MuSK and LRP4 Abs were performed and interpreted by the author. All positive assays were repeated at least once. In the case of positive MuSK CBA assays, most samples were assayed 3 times (twice by the author and once by Dr Mark Woodhall) and interpreted independently by the author and 2 others- Dr Mark Woodhall and Dr Isabel Liete.

Preparation of solutions

1. DMEM-HEPES: 500 ML of the DMEM +2.3 g of HEPES (no BSA)
2. DMEM-HEPES-1% BSA: To 500 ML of DMEM solution, added 2.3 g of HEPES and 5 g of BSA and mixed.

3. PBS: to 100 mls of distilled water added one tablet of PBS and mixed till it dissolved

4. 4% formaldehyde: 36% solution in the chemical area- added 10 mls of this to 90 mls of PBS.

5. DAPI + mounting media: 15 ml of fluorescent mounting medium from the fridge and added15 µL (i.e. 1 µL per ml) of DAPI (kept covered in foil in the fridge)

6. Trypsin:

- Thawed five ml aliquot of trypsin solution stored in -20° fridge

- in 50 ml Falcon tube added 45 ml PBS then 5 ml trypsin solution and mix

- The solution was stored in the fridge until required

7. PLL:

- Thawed 1 ml aliquot of PLL stored at -20°C fridge

- in 150 ml container added 99 ml PBS or water then 1 ml PLL solution and mixed

- The solution (0.01% in PBS) was stored in the fridge until required

8. Tissue culture media:

- Took 500 ML of DMEM from the cold room and brought to room temperature in the tissue culture hood

- Thawed 50 ML FCS and 5 ML BSA on the bench in the tissue culture

- Sprayed the lids of each solution with ethanol before placing in the TC hood

- Added 15 ML of FCS to DMEM and mix

- Added 5 ML PSA to DMEM + FCS and mixed, additional reagents as necessary for individuals cell lines were added at this point

- The solution was in the fridge until required

CBA assay procedure:

Day one

Preparation

1. Prepared the tissue culture media, trypsin solution, PLL solution as described in the previous steps.

2. 3 or 4 cover slips placed into each well of a 6 well plate using fine forceps

3. Added 3 ML of PLL solution and checked the cover slips were not floating and were covered in the PLL

4. Left the PLL on for 15 to 20 minutes, up to 2 days if necessary

5. Aspirated the PLL and made sure the cover slips were separated from each other. Left to dry in the TC with the lid open for more than an hour

Cell counting:

6. From the incubator took a T-175 flask which was previously seeded with HEK 293 T cells, now 80 to 95% confluent. Checked that the HEK 293 T cells were healthy under the inverted light microscope. (They are healthy if not moving, not all are rounded in shape and if the medium looks clear without any growth)

7. Aspirated the medium from the flask, added 2 mls of trypsin and left at 37°C for one minute. Removed the flask and

shaken until almost all the cells had detached. If using a small flask, used 1 ML of trypsin.

8. DMEM-FCS-PSA medium added to the flask to make up a final volume of 10 ML and transfered the contents to a 20 ML University bottle. For example, if using 2 ML is of trypsin then 8.5 ML of DMEM/FCS/PSA and if using 1 ML of trypsin added 9.5 ML of medium.

9. In an eppindorf tube mixed 20 or 50 µL of the cell suspension obtained above with either 20 or 50 µL trypan blue solution (one in two dilution), mixed well by vortexing and carefully loaded 50 µL of the sample into a haemocytometer making sure to not overfill.

10. Cells counted and calculated the number of cells per ML: The outer squares i.e. the 4 squares made up of 16 small squares each, divided this number by 4, and multiplied by 2 X 10^4. This was the number of cells in million per ML. If counting the middle squares (the 25 smaller squares) counted on both sides of the haemocytometer, multiplied by 2 X 10^4. This is the number of cells in million per ML. When counting cells on the edges of the squares counted only two edges of the square and not all four edges.

11. Centrifuged the cell suspension in the universal tube at 1000 rpm for five minutes whilst the cells were being counted

12. Carefully aspirated the supernatant from the universal and thoroughly resuspended in 10 ML DMEM-FCS-PSA, being careful to not aspirate the pellet

13. Seeded out 600,000 cells per well into 2 ML of DMEM-FCS-PSA, making sure that all the cover slips were immersed in media. (Calculated the volume required according to the cell count above, making up enough solution for all wells plus one extra for ease of pipetting) (If a T175 flask was 100% confluent, you would expect between 40 and 50 million cells in total, from a T75 flask between 20 and 30 million).

14. Incubated at 37°C and 5% CO2 overnight.

15. Made up a new flask of cells with the remaining cell mixture. Taken a 20th of what I started off with for each new flask, mixed 0.5 ML with 35 ML of tissue culture media if using a large flask, or 0.4 ML and 18 ML of medium if using a small TC flask. Checked the flask under the microscope to make sure that there were cells in it and put this is back in the incubator.

16. When making up a new flask of cells, they last for a maximum of 4-5 days; to last for the required number of days when seeding, added in the following proportions: for 3 days added 1: 12, 4 days added 1:24, for 5 days added 1:48. Splitting cells i.e. making new cells could be done only after day 3 ideally,

if after 2 days, then 1: 4 or1 : 5, and if desperate after one day, 1:3.

Day two

Transfection:

1. Checked that the cells had attached and looked healthy under the inverted light microscope

2. Changed the medium in the 6 well plate before transfection.

3. Prepared the DNA/polyethylene amine (PEI) mixture in the following order in the TC hood

- 50 µL DMEM without supplements/well
- 3 µg DNA/ well (2.8 µg specific DNA untagged and 0.2 µg EGFP) (the EGFP had to be 7% or less than the DNA)
- 15 µg PEI/well
- For AChR assays the ratio of DNA for each subunit used was- 2α:1β:1δ:1ε:1rapsyn, making a total concentration of DNA 3 µg per well; Rapsyn was already EGFP tagged
- For MuSK assays, a total of 3 g of MuSK DNA was used per well, this was also already tagged with EGFP

- For LRP4 assays, the ratios of subunits were- 5 LRP4 Caspr : 1 LRP4 AP. This needed to be added as 6 µg of DNA in total, ie 5µg of LRP4 Caspr and 1µg of LRP4 AP.

4. It was recommended that we made a master mix of DNA/polyethylene amine and preparing an extra well to account for pipetting error

5. Incubated the mixture at room temperature for 10 minutes

6. While the DNA/PEI mixture was incubating, made sure that none of the cover slips were overlapping.

7. Added the DNA (~50 µL) to each well and left in the 37° C incubator (5% CO2) overnight (less than 16 hours).

Day three

1. Changed the medium with 2 ML per well of tissue culture medium within 16 hours of transfecting.

Day four

Cell-based assays

1. For AChR, MuSK and LRP4, used patient serum in one in 20 concentration i.e. 12.5 µL of test serum made up to 250 µL of medium for each well.

2. Diluted the serum samples one in 20 in DMEM-HEPES-1% BSA and the controls in the same manner.

3. Transfered the samples to a 24 well plate and added a coverslip to each well with the cell side up and left to incubate at room temperature for one hour- this was put on the see-saw mixer after covering with either foil or foil box.

4. The wells on the 24-well plate were marked A 1-6 etc, but the lids were also labelled.

5. Aspirated the supernatant and washed the coverslip three times in 250 µL of DMEM-HEPES. A serial dispenser used for this

6. Added 250 µL of 4% formaldehyde in PBS and incubated for 1 to 10 minutes- put in a dark place i.e. covered with foil and put back on the see-saw mixer

7. Aspirated the solution and washed three times in 250 µL of DMEM-HEPES

8. Added 200 µL of the secondary antibody which was an Fc Ab, Goat anti-Human IgG Fc cross adsorbed secondary antibody. This was added diluted to 1 in 750 in DMEM-HEPES-1% BSA. The plates were incubated for 45 minutes at room temperature on the see-saw mixer with foil on it.

9. Aspirated the secondary antibody and washed it three times in 250 µL DMEM-HEPES

10. Added the tertiary antibody which was the Donkey anti-Goat IgG Alexa Flour 568. Once again, this was made up into 1 in 750 dilution using DMEM-HEPES-1% BSA. This was incubated for 45 minutes at room temperature, with foil, on the see-saw mixer

11. Aspirated the solution and washed three times in 250 µL of DMEM-HEPES and two times in 250µL of PBS. Left the cells in PBS.

12. To check that the cells had been transfected with a fluorescent green dye, one of the coverslips from the culture was put facedown on a slide and this was looked at under the microscope. If the lighting for EGFP could be seen, then the cells were transfected.

13. Placed a drop, approximately 20 µL of the DAPI + mounting solution on the microscope slide and place the coverslip on top with the cell side down. Tapped the slide gently on its side on a tissue so that it was not too wet. Six coverslips per slide were used and the slides were labelled appropriately.

14. Left it to dry in the dark for at least 30 minutes before looking at the cells under the microscope.

15. Examined the slides using a fluorescent microscope with filters for DAPI, red and green. Scored the slides according to the scoring system detailed below.

16. All positive samples and any borderline samples were repeated.

17. The slides were stored at 4°C if further examination was required at a later date.

Scoring CBAs

- 0 = no labelling
- 0.5 = weak labelling of a few transfected cells with no obvious EGFP co-localisation
- 1 = weak labelling of some of the transfected cells, with precise co-localisation
- 2 = moderate labelling of some (approximately 20 to 50%) of the transfected cells, with precise co-localisation
- 3 = moderate/strong labelling of approximately 50 to 80% of the transfected cells, with precise co-localisation
- 4 = strong labelling of virtually all transfected cells, with precise co-localisation.

The DNA used for the AChR subunits, MuSK and LRP4 subunits had been prepared by Mark Woodhall. Further DNA was also prepared by the author.

DNA master mix preparation

Working solutions:

L broth:

1 L distilled water in a 2 L glass bottle +10 g triptone +5 g of yeast extract +5 g of salt. This was shaken until it was mixed properly. This was autoclaved overnight on day one

Also on day one, made up more L broth and poured 600 ML into a big flask. This was autoclaved overnight or first thing on day two.

Agar Plates:

Agar satchets came with different antibiotics mixed in it, checked which one was required for the particular DNA being prepared

AChR α, β, δ and ε required Ampicillin Agar, Rapsyn tagged with EGFP required Kanamycin, MuSK with EGFP required Kanamycin, LRP4 Caspr and AP subunits required Ampicillin, if using EGFP plasmid separately this also required Ampicillin.

This description is for MuSK DNA which required Kanamycin as explained below. Poured the contents of the satchet into a glass jar which was small enough to fit into the microwave and added 200 ML of distilled water. Mixed and microwaved for 21/2 minutes with the lid loose, gave it a shake and microwaved for a further 30 seconds; they were mixed uniformly.

Took this agar mixture into the TC room to a sterile hood. Here poured 20 ML each into petri dishes. The mixture was cool enough to handle but not so cool that it set in the bottle. Left it in the petri dishes for a couple of minutes to form a gel. Closed the lids, turned them over and labelled with the antibiotic- example kanamycin on the bottom and what the gel is and the date if needed. Closed the petri dishes and sealed with paraffin paper. Put these in the cold room. These could be used for up to 3 weeks.

DNA

You would need some DNA to start off from previous preparations. This was usually in µg/µL concentration for ready use. In order to make more DNA in the maxi prep, this was diluted by 10 times i.e. 1 µL is made up to 10 µL by adding 9 µL of nuclease free water. This gave a concentration of 100ng/µL.

Day one

1. This was done in the workspace rather than TC hood

2. Cleaned the area with alcohol spray to avoid contamination

3. Kept a Bunsen burner to hand to keep things sterile while transferring samples. This needed to be switched on and the screw knob turned so that the flame was blue

4. Turned on the water bath with a thermometer in it; the temperature needed to be at 42°C

5. Brought ice from the washroom in a thermocol box

6. Got competent cells from the -80° freezer. These were small green top tubes. Put this on ice. Needed 50 µL for every DNA prepared. If using a small amount from that, placed a red dot on the lid to indicate that some had been used.

7. Nuclease free water. This was part of the DNA kit

8. Turned on the Bunsen burner and worked underneath the blue flame

9. Added 1.5 µL of master DNA, for example MuSK into a labelled eppindorf and 1.5 µL of nuclease free water into another eppindorf labelled ‘water control’ (WC)

10. Then added 50 µL of competent cells into each eppindorf and put straight on ice for 20 minutes. Flicked it to mix

11. Turned off the Bunsen burner

12. Heat-shock: after 20 minutes on ice, the eppindorfs were put into the water bath on thermocol eppindorf holders for 45 seconds then put back on ice for two minutes; flicked to mix

13. Turned off the water bath

14. Under the Bunsen burner, aliquoted some of the L broth prepared into a universal tube, took out the eppindorfs from the ice and added 150 µL of L broth into each eppindorf. This was left at room temperature

15. Placed the eppindorfs into the eppindorf rack of the shaking incubator for 45 minutes at 37°C

16. Brought the agar plates out of the cold room and labelled them appropriately; for example, if preparing MuSK DNA, labelled three agar plates as MuSK 150, MuSK 30 and WC (water control). 150 meant using 150 µL of sample and 30 meant 30 µL.

17. Turned on the Bunsen burner

18. After 45 minutes in the shaking incubator, brought out the eppindorfs

19. Turned the gel plates right side up and pipetted 150 µL and 30 µL of the DNA/competent cell/L-broth into the corresponding agar plates and water control into the third plate.

20. Using a spreader, which was a blue plastic hockey-stick shaped stick; held the plate with the left-hand and rotated gently whilst moving the spreader back and forth

21. Once evenly spread, closed the lid and placed it in an incubator overnight.

Day two

1. Prepared 600 ML of L-broth in a flask (one for each DNA) and autoclaved first thing in the morning if not done on day one

2. Brought out the agar plates from the incubator

3. Got 2 universal tubes for each DNA and labelled them, for example MuSK 1 and 2

4. Added 5ml of L-broth into each tube using a mechanised pipette

5. Got appropriate antibiotics from the -20° freezer; in case of MuSK this was kanamycin

6. Antibiotics added in the ratio of 1:1000

7. Worked under the blue flame of the Bunsen burner again

8. Pipetted 5 µL of antibiotic into the L-broth in the universal tube

9. Examined the colony formation on the agar plates and chose the plate which had discreet colonies visible, either from MuSK 150 or MuSK 30

10. One colony needed per universal tube

11. Used an inoculator which was a blue plastic stick with a hoop like a bubble blower, quickly scraped one of the colonies to catch one discreet colony and stirred this into the L broth in the universal tube.

12. Two universal tubes were used per DNA i.e. one was used as backup

13. Put the Universal tubes in the shaking incubator at 37° for 5 hours

14. Made sure to book a place on the shaking incubator in advance

15. After five hours, the tubes were cloudy, chose the cloudiest of the two and used this one

16. Got more antibiotics, for example kanamycin from the freezer

17. Worked under the Bunsen burner

18. Again, used antibiotics in 1:1000 ratio

19. For 600 ML L-broth in the flask, needed 600 µL of antibiotics

20. Pipetted the antibodies into the universal tube, gave it a mix and poured this into the flask with the L broth. Gave it a swirl

21. Put the flasks in the shaking incubator balanced appropriately at 37° overnight.

Day three

1. Took the flask out of the shaking incubator

2. 2 plastic bottles of 500 ML with lids per flask used

3. Poured the contents of the flask roughly divided into the two bottles

4. Placed the bottles into centrifuge tubes- these were black and looked like Thermos flasks kept in the big centrifuge

5. Placed the centrifuge tubes with the bottles in them on the weighing scales and transferred the L broth around (poured extra) so that they were balanced.

6. The centrifuge needed to be pre-cooled to 4°C and a place booked for use

7. The big rotor was needed, placed the tubes in this, the extra tubes were left in place to balance the centrifuge

8. The settings were: Rotor D-JLA, speed-5000 rpm, time-10 minutes, temperature: 4°C

9. Switched on

10. In the meantime, prepared the columns

11. Three blue-and-white columns needed per DNA, these were plastic tubes from the DNA kit

12. Placed these one on top of the other, blue on top of the white and there was a filter inside; this was fit on top of the taps after opening the cover on the vacuum pump. Opened the vacuum pump by turning the knob anticlockwise.

13. Three washing buffers were needed from the plasmid maxi prep kit

- Cell suspension solution (CRA)
- neutralisation solution (NSB)
- cell lysis solution (CLA)-checked if there were precipitates and if present incubated them to get rid of it

14. Three more plastic tubes about the size of Falcon tubes and with lids on were required

15. Once centrifuged, took the bottles out and replaced the large rotor with the small rotor

16. There was a small pellet at the bottom of the bottles, decanted the liquid back into the flask

17. Resuspended the pellet using 9 ML of CRA per bottle, 18 ML in total

18. If there were many bottles they were put in the shaking incubator for 5 minutes to resuspend, if not, done with a pipette

19. Poured 6 ML each into the three smaller plastic tubes and divided any excess equally

20. Added 12 ML of lysis buffer CLA into each bottle and mixed them by inverting 10 to 15 times. Occasionally white precipitates were seen, this was normal; the solution became viscous and could become stringy

21. Left at room temperature for three minutes

22. After three minutes added 12 ML of neutralisation buffer into each bottle; mixed by inverting 15 to 20 times gently as otherwise the genomic DNA would break

23. Precipitates were seen- this needed to be centrifuged. Used balance if odd number of bottles were used

24. Big centrifuge- same as before, but with a smaller rotor, settings were rotor ID-JA 25.50, speed-14,000 RCF, time-25 minutes, temperature: 4°C

25. After centrifuging, took the tubes out

26. Turned on the pump mentioned earlier

27. Turned the left gauge to between 300 and 400 bars

28. Poured the supernatant from the centrifuge tubes one into each of the blue with white columns

29. The plastic tubes used couldbe reused so were washed-soaked in virkon first

30. Whilst the liquid was filtering in the columns, prepared solutions if not already done

31. Column wash-added 350 ML of 95% ethanol to the column wash

32. Endotoxin removal wash- added 5 to 7 ML of isopropanol

33. Once the liquid had filtered in the columns, DNA was left bound to the white filter at the bottom

34. Added 5ml of endotoxin removal wash to the white columns after taking out the blue column; allowed this to filter through

35. Once filtered, added 20 ML of column wash to each column and wait for this to filter through

36. Need three eppindorfs labelled with the date and type of DNA

37. Needed three eluator devices from the DNA kit which was ‘Promega purefield ‘plasmid maxi prep system’

38. Put one eppindorf into each eluator device, kept the lids of the eppindorfs open

39. 1 ml pipette tip with filter and nuclease free water was needed

40. Left the columns for 10 more minutes after all the liquid has filtered so that it was dry

41. Detached the column from the pump, tapped it on some tissue and fit in on top of the eluator device; fit on the vacuum pump again

42. Pipetted 1 ML of nuclease free water into the filter, made sure the whole surface was wet, this dripped into the eppindorf. Left for 10 minutes to make sure everything dripped in; 1 ML collection was very good

43. Put the eppindorfs on ice straight after

44. The eluator devices could be reused - put them back after use.

Measuring DNA

1. Needed: 10 µL pipette, 10 µL pipette tip, nuclease free water or TE buffer

2. Used the nano drop machine which was a small cuboid structure with a metallic arm on top connected to a laptop.

3. Clicked on ‘nucleic acid’ on the computer software

4. Opened the arm on the machine

5. Placed 1 µL of nuclease free water i.e. one drop upon the black spot without any bubbles

6. Clicked ‘okay’ on the computer

7. Made sure sample type said ‘DNA 50’

8. Placde another microlitre drop in the black spot with nuclease free water, clicked on ‘blank’

9. Wiped this clean, then placed the DNA- either 1 or 1.5 µL drop

10. Clicked on ‘measure’

11. Concentration came up on the screen in nanograms per microlitre

12. Wrote this on top of the eppindorf converted into micrograms per microlitre concentration

13. Measured all the eppindorfs

14. Made a note of the purity- this was the column saying 260/280. Anything above 1.8 was good

15. In one of the columns it said 260/230, anything above 2 was good

16. 260 was the wavelength of DNA and 280 that of other proteins, similarly 230 was a different wavelength of other proteins.

17. Made sure to close the metal arm with a tissue between as cushioning

18. Stored the DNA eppindorfs in the -80° freezer.

7.3 Appendix 3 Methods of PBMC isolation and study using Flow cytometry

Whole blood was collected from 135 MG patients and 8 LEMS patients at recruitment and 24 MG patients at first year follow up, 9 of whom had been immunosuppressed in the first year, 11 who were immunosuppression naïve at first year follow up and the remainder had been immunosuppressed at recruitment.

Peripheral blood monocytes/lymphocytes (PBMCs) were isolated using the method described below by the author within 4 hours of sample collection. The majority of the PBMC isolation was done in the laboratory at the Neurology research unit at the Queen's Medical Centre, Nottingham. Some of the samples collected at Birmingham and Oxford were processed at the University of Birmingham laboratories.

The method of PBMC isolation was the same as that used by the MS research team at Nottingham (Prof Constantinescu and Dr Gran) and was adapted by the author into simple steps as below.

The equipment and reagents used were:

Table 31 Equipment and reagents used for PBMC isolation

ITEM	LOCATION	ORDERING
Green top vacutainer tubes (lithium heparin)	Room temp	Via Sandra Lever
Sterile Universal tubes (50ml) (better resolution) or Falcon tubes (3 or 4)	Room temp	LS-M0144E (MSS)
Histopaque (same as blood volume)	Fridge	H8889 (Sigma)
Kwill filling tube (1)	Room temp	UN888 (Universal Hospital Supplies)
10/20ml syringe (1)	Room temp	DC-M0157E / DC-M0160E (MSS)
Pasteur pipette or 1ml micropipette with its sterile tip	Room temp	LS-M0212E (MSS)
PBS (~100 ml)	Room temp	LH-SIG2017E (PBS)
Trypan blue (90 ul)	Room temp	T8154 (Sigma-Aldrich)
Haemocytometer with cover glass (1)	Room temp (drawer by microscope)	Superior Marienfeld Germany

Microscope (1)	Room temp	Leica
Aliquot (Eppendorf) tube (1) (0.5 ml)	Room temp	LS-M0280E (MSS)
Marker pen	Room temp	163932 (Fisher Scientific)
Centrifuge	Room temp	Eppendorf centrifuge 5810 R
Pipette tips (blue)	Room temp	LS-M0223E (MSS)

PBMC isolation

1. Blood- heparinised in green tubes. This was mixed gently by inverting the tube 5-10 times
2. The tubes were kept at room temp until ready for processing
3. To Work in tissue culture hood- sterile environment. Tubes and reagents taken into the hood
4. 1:3 :: Trigene: water; prepared in a jar
5. Area and test tube rack sprayed with 70% ethanol
6. 10mls of histopaque (1.0771) poured into the bottom of sterile universal tubes or falcon containers (blue head) × 3 using sterile pipettes
7. Equal amount of histopaque to blood
8. Used pipettes put in a jar of trigene/ water mixture (prepared in advance)

9. The histopaque bottle wiped with paper before closing the lid
10. Kwill filling tube and sterile 20 ml syringe needed
11. Kwill filling tube attached to the syringe
12. This was used to draw blood; alternatively, disposable plastic pipettes used
13. Blood put on top of the histopaque; to layer it, not mix it, not to push too hard, holding Kwill along the side of the Falcon container and dripping in
14. Histopaque and blood needed to be in 2 layers
15. New Kwill tube and syringe used for each bottle of blood unless samples of the same patient
16. The remaining blood could be diluted with PBS in order to not lose any cells and this was added to the blood with histopaque
17. Falcon containers- Centrifuged at 5810R, temp: room temp 25^0C, speed: 2000rpm, time: 20 mins, acceleration: 9, brake: 0
18. Braked slowly to keep layers intact and not mixed with each other
19. Samples balanced equally in the centrifuge
20. After 20 mins + 3 mins to break, the sample was in 4 layers: top serum, next cloudy (WCC + platelets) which was what we needed, next histopaque, then RBCs at the bottom
21. There should not be any blood spatters
22. Using a disposable pipette or a Pasteur pipette with 1 ml microtip, PBMCs were extracted. This was the white/cloudy layer.

23. Samples collected from all 3 Falcon containers that were in the centrifuge, then put into another 50 ml labelled falcon container
24. Dulbecco's phosphate buffered saline (PBS) used at room temperature. This was poured into the Falcon container holding the PBMC extract to make up to 50ml total.
25. Shaken slightly
26. Centrifuged again (first wash); temp: 20^0 to 25^0C, speed: 1700 rpm, time: 10 mins, acceleration: 9, brake: 9 (this differs with different machines) (1600-1700 rpm for 5-10 mins, acceleration was not important)
27. After the centrifuge, supernatant poured out into the trigene jar
28. There was a pellet at the bottom which is WCCs. Tapped gently to loosen it
29. Using a plastic pipette, 10mls of PBS poured to this to dilute it
30. Tryptan blue (careful as dangerous) used. This was needed to look at cells under the microscope, If the cells were alive, the dye would not penetrate and there would be a rim of blue around the cells
31. Tryptan blue:PBMCs::9:1
32. Using a mechanised pipette set at 90 microlitres, first the tryptan blue was drawn and put it into an Eppendorf aliquot tube
33. The mechanised pipette was set to 10 microlitres and the PBMCs drawn up. This was also poured into the same Eppendorf tube
34. The pipette used to mix them up slightly

35. Haemocytometer and cover glass used
36. Using the mechanised pipette, 10 microlitres of the tryptan blue/PBMC mixture drawn up
37. This wsa dropped slowly between the cover glass and the haemocytometer
38. The number of cells counted to see if the harvest was good enough!
39. Haemocytometer placed under the microscope and switched on. magnification of x10, occasionally x40 used
40. Counting cells: the number of cells in the four large corner squares (each has 16 smaller squares) (better on x40 lens) counted, and total number claculated. The numbers in the 4 large squares added and divided by 4. This was the cell count in million cells per ml
41. After counting, the remaining PBMCs mixed with more PBS to make up to 50mls
42. Centrifuged again (second wash), same settings as first wash; temp: 20^0 to 25^0C, speed: 1700 rpm, time: 10 mins, acceleration: 9, brake: 9
43. After the centrifuge, supernatant poured out
44. There was a pellet at the bottom again, disturbed gently by tapping
45. Nutrient medium needed which is 10%FCS in RPMI
46. Nutrient medium added in a proportion of

    Nutrient medium (ml)= total PBMC (million)/12 (million)

47. 1ml of the PBMC/FCS mix drawn up using a mechanised pipette (with a maximum setting of 1000 microlitres) and this was poured into another falcon tube
48. A further 11mls of FCS (nutrient medium) added to make upto 12 mls
49. This was divided into 11 FACS tubes labelled before use
50. Further nutrient medium added to the rest of the cells to make up to 30 mls
51. Centrifuged again (third wash); temp: 20^0 to 25^0C, speed: 1200 rpm, time: 10mins, acceleration: 9, brake: 9
52. After the third wash, supernatant poured off
53. Cells at the bottom loosened by gentle tapping
54. 3mls (1 ml for every 10-15 million cells) of freezing medium added and gently mixed
55. Freezing medium (Fetal calf serum FCS 9 parts and dimethyly sulfoxide DMSO 1 part)
56. Cryovials which come in 1ml, 1.5ml and 2ml sizes used. Each ml would contain 10-15 million cells
57. Using a mechanised pipette, 1ml of the PBMC/freezing medium mixture transferred into each of the 4 cryovials. If there was any leftover, it was divided equally between the vials
58. The vials needed to be cooled
59. The vials were put into Mr Frosty/ Cool cell purple box (or between 2 polystyrene blood tube holders if nothing else available- idea was to freeze slowly)

60. The purple box was placed in the -80^{0}C freezer for 5 days to be cooled by 1^{0}C per hour
61. After 5 days, the vials were put into liquid nitrogen for cryostorage
62. Labelling checked and a note made of where the cryovials were in the canister
63. The used containers were put into the yellow bins to be burnt
64. If the PBMCs were going to be put into the flow cytometer straight away, FACS tubes to be used in step no. 49 (we did not so this)
65. This needed further staining and/or stimulation prior to flow cytometry

All of our PBMC samples were cryo-stored and analysed in the final year of the study. The protocol for the flow cytometry was set up with the help of Dr David Onion, Immunologist at the QMC, Nottingham. The author performed the staining on all samples. The samples were put through the flow cytometer by Dr David Onion and team who provided the raw data. The results were analysed on 'Kalusa' software. The gating was set up with the help of the immunologists for the first two samples and all subsequent samples were analysed by the author.

The study was divided into three panels- the first panel for Treg cells, the second panel for the cytokines- IFNα, IFNγ and TNFα, the third panel for the cytokines- IL-10, IL-17 and IL-4.

The panel was set up on a 96 well plate and was as below.

Panel (T Reg) 1

Table 32 Design for 96 well plate for Tregs, panel 1

US	SCC										
FMO	FMO	FMO	FMO	FMO							
IC	FS	IC	FS	IC	FS						
IC	FS	IC	FS	IC	FS						
IC	FS	IC	FS	IC	FS						
IC	FS	IC	FS	IC	FS						
IC	FS	IC	FS	IC	FS						
IC	FS	IC	FS	IC	FS						

Panel 2 **Panel 3**

Table 33 Design for 96 well plate for Th17, panel 2 and Th2, panel 3

US	FMO	FMO	FMO			SCC	FMO	FMO	FMO		
FMO	FMO	FMO	FMO			FMO	FMO	FMO	FMO		
IC	FS	IC	FS	IC	FS	IC	FS	IC	FS	IC	FS
IC	FS	IC	FS	IC	FS	IC	FS	IC	FS	IC	FS
IC	FS	IC	FS	IC	FS	IC	FS	IC	FS	IC	FS
IC	FS	IC	FS	IC	FS	IC	FS	IC	FS	IC	FS
IC	FS	IC	FS	IC	FS	IC	FS	IC	FS	IC	FS
IC	FS	IC	FS	IC	FS	IC	FS	IC	FS	IC	FS

Single Colour Compensation (SCC) in FACS Tubes

CD4	FoxP3	TNF α	IL 17
CD25	INF γ	CD3	IL 4
CD127	INF α	CD8	IL 10

The consumables and reagents used are as below:

Table 34 Consumable needed for PBMC thawing * and stimulation**

ITEM	LOCATION	ORDERING
RPMI (~43.5 ml)	Fridge	LH-SIG2034E (MSS)
FCS (5 ml)	Fridge / -20	LH-SIG-2022E (MSS) p.cod: F9665
Pen / strep (0.5 ml)	Fridge / -20	LH-SIG2031E (MSS)
Hepes (0.5 ml)	Fridge / -20	H0887 (Sigma)
Glutamine (0.5 ml)	Fridge / -20	LH-SIG2024E (MSS)
Frozen (or fresh) PBMCs	LN2	N/A
15ml tubes *	Room temp	LS-M0145E (MSS)
Pasteur pipette	Room temp	LS-M0212E (MSS)
BSA 0.5% (from 30% stock) diluted with PBS (50ml* dilution)	Fridge	A7284 (Sigma)
PBS	Room temp	LH-SIG2017E (MSS)
FACS tubes (sterile)	Room temp	LOT 2399001 2015-04 No. D-51588 (SARSTEDT)
96-Well Plates		

PDB (20 ng/ml tube) ** (1 mg/ml DMSO) stock (take 2 ul)+ RPMI (add up to 200 ul) (total 6 ul dilution needed)	Fridge / -20	P code: 1001419806 (Sigma)
Ionomycin (0.5 ug/ml tube) (1mg/ml DMSO) stock (take 5 ul) + RPMI (45 ul) (total 15 ul dilution needed)**	Fridge	Product code: 10643-1mg (Sigma)
Brefeldin A (10 ug/ml tube) (5mg/ml DMSO) stock (take 10 ul) + RPMI (40 ul) (total 50 ul dilution needed)**	Fridge	Product code: B7651-5mg (Sigma)
CO2 incubator (5% CO2, 37 C)	Room temp	Sanyo CE® model MCO-17AIC Serial no. 00303091
Falcon (universal) tubes (3 or 4) (50 ml)	Room temp	LS-M0144E (MSS)
Water bath or incubator*		
FACS lids	Room temp	ETN: 240112 (Elkay)
Pipette tips (white)	Room temp	LH-M0226 (MSS)
FACS stand	Room temp	

Table 35 Consumables and reagents used in PBMC staining

ITEM (Panel 1-Treg)	LOCATION	ORDERING
CD4 FITC	Fridge (4C)	BD biosciences (555346)
CD 25 PE	Fridge	BD biosciences (555432)
CD127 PE-Cy7		BD biosciences (560822)
FoxP3 Alexa Flour 647	Fridge	BD biosciences (560045)
Human FoxP3 Buffer Set BD biosciences		BD biosciences 560098
Live/Dead fixable blue dead cell stain kit, for UV excitation		L34962
OneComp eBeads Compensation beads		01-1111-42
FITC Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 555748
PE Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 555749
PE-Cy 7 Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 557872
Alexa Flour 647 Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 557714
ITEM (Panel 2-Th17)	LOCATION	ORDERING
INFγ APC		Biolegend 506510
INFα PE		BD biosciences 560097

TNFα PerCP-Cy5.5		Biolegend 502926
CD3 APC-Fire 750		Biolegend 344840
CD4 FITC	Fridge (4C)	BD biosciences 555346
CD8 PE-Cy7		Biolegend 301012
Fixation/Permeabilization solution Kit		BD GolgiStop 554715
Live/Dead fixable blue dead cell stain kit, for UV excitation		L34962
OneComp eBeads Compensation beads		01-1111-42
APC Mouse IgG1 κ Isotype Control		Biolegend 400142
PE Mouse IgG1 κ Isotype Control Clone MOPC-21		BD biosciences 555749
PerCP-Cy5.5 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400150
APC- APC/Fire 750 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400196
FITC Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 555748
PE-Cy7 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400126

ITEM (Panel 3-Th2)	LOCATION	ORDERING
IL17 PE		Biolegend 512306
IL4 PerCP-Cy5.5		BD biosciences 561234
IL10 APC		Biolegend 506807
CD3 APC/Fire 750		Biolegend 344840
CD4 FITC	Fridge (4C)	BD biosciences 555346
CD8 PE-Cy7		Biolegend 301012
Fixation/Permeabilization solution Kit		BD GolgiStop 554715
Live/Dead fixable blue dead cell stain kit, for UV excitation		L34962
OneComp eBeads Compensation beads		01-1111-42
PE Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400140
PerCP-Cy5.5 Mouse IgG1 κ Isotype Control Clone MOPC-21		BD biosciences 550795
APC Rat IgG2a κ Isotype Control Clone R35-95		Biolegend 400512
APC/Fire 750 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400196
FITC Mouse IgG1, κ Isotype Control Clone MOPC-21		BD biosciences 555748
PE-Cy7 Mouse IgG1 κ Isotype Control Clone MOPC-21		Biolegend 400126
ITEM	LOCATION	ORDERING

EDTA 100 mM (20 ul/tube)*7 tubes	Room temp	
Formaldehyde 4% (1 ml/tube)*7 tubes & 0.5% (0.4 ml/tube)*11 tubes (40% stock w/ isoton dil.)	Room temp	B8F77119 (Philips-Harris)
Isoton diluent	Room temp	8448011(Beckman-Coulter)
Aluminium foil (kitchen quality)	Room temp	Terinex
PBA (0.5%): BSA (0.5%) in PBS: (3 ml/tube)*4 tubes (0.25 ml 30% BSA, add PBS up to 15 ml)	Fridge	A7284 (Sigma)
PBS (diluents to BSA)	Room temp	LH-SIG2017E (MSS)

Protocol for Three Panel T cell studies for Flow Cytometry

Prep

1. **Medium**

   500ml RPMI + 50ml FCS + 5ml Pen/Strep + 5ml Glutamine

   (FCS, Pen/Strep and Glutamine come in bigger bottles and had to be aliquoted into the required quantities and re-frozen)

2. FCS thawed to use in the staining buffer
3. Pipette tips- various sizes, 5ml, 10ml, 25 ml and 1000ul, 200ul and 20ul

Morning

1. Switched on the water Bath- checked level of H2O and temp (37 C) (Press black button to check temp)
2. Placed bottle of media into the water bath
3. Once media was warmed, poured 10-15 mls into labelled falcon tubes- one for each pt. (ideally if using more than one cryovial for one patient, they were pipetted into different falcon tubes, but to save time, one used)
4. Ice obtained in a thermocol box and the cryovials to be tested taken from liquid N2 and placed onto the ice (If doing many samples, placed in order in a box on ice)
5. Cryovials thawed individually by holding them in the water bath for a minute or so. Shaken to see if the ice had melted
6. The cryovials taken to the hood. Lids opened slightly to release the pressure
7. With mechanised 5 ml (or 10 ml) pipette few drops of medium taken from the labelled falcon tube and added a few drops into the cryovial. Taken care to not overfill. Mixed a couple of times and pipetted the whole lot into the falcon tube. The pipette emptied gently.
8. When all the samples were thus thawed, placed the falcon tubes in the centrifuge for washing at 1200 rpm, RT for 10 mins
9. Poured out the supernatant, agitated the pellet and re-suspended in 10ml of medium. Mixed by pipetting up and down

10. Taken 10 ul of this in a labelled eppindorf for cell counting. 90 ul of trypan blue needed to be added and cells counted the same way as with PBMCs ie outer 4 (4X4) squares divided by 4, or inner (5X5) square.
11. Centrifuged the Falcon tubes again at RT 1200 rpm for 10 mins
12. Poured out the supernatant and agitated the pellet
13. Re-suspended the cells in 10ml medium (Less if wanted to use less of the cell stimulation cocktail- we diluted to 3ml)
14. Added 2 ul of cell stimulation cocktail (plus protein transport inhibition) for every 1ml dilution, so if diluting to 10 ml, needed 20 ul (6ul if diluted to 3ml)
15. Poured the contents of the falcon tubes into labelled 75 cm2 culture flasks. These could be left in the Falcon tubes if there were many samples
16. Placed these vertically in the incubator (37^0C) for 4 ½ hours

Afternoon

1. Took the flasks/falcon tubes out of the incubator after 4 ½ hrs
2. If in flasks, pipetted into labelled falcon tubes
3. Filled this up with PBS
4. Centrifuged at 1200 RPM, 10 mins, RT
5. Discarded supernatant, agitated the pellet
6. Re-suspended this with more PBS to fill the falcon tubes
7. Centrifuged again, same settings 1200 rpm, 10 mins, RT
8. Discarded supernatant, agitated the pellet

9. Whilst the centrifugation was taking place, marked out the layout of the stains on the 96 well plate. For the 3 panel assay, used one plate for the FoxP3 assay (Panel 1) and another one for Panels 2 and 3.
10. Dilutions now depended on how many wells each sample needed to be put into. For the control samples- ideally only one control sample, but as this uses a lot of cells, used 2 (or 3) control samples, but ensured that the same control used for each panel. Control samples diluted more using PBS (calculated as 50 ul per well). For the other samples, which only went into Isotype Control (IC) and Fully Stained (FS) wells for that particular sample, only 300 ul were required (for all 3 panels). Usually, when pellets were agitated, there was some liquid left after the wash. This usually sufficed. Mixed well and pipetted into the wells of a 96 well plate.

11. Next step was Live/Dead staining

12. This was kept in the freezer- when new, the vial needed to be made up by adding 50 ul of DMSO provided in the kit and vortexing. Covered this in aluminium foil
13. Worked with the hood lights off for the next steps
14. Diluted some of the prepared L/D stain in a 1:10 ratio. Made up 100 ul in total (which would be sufficient for 100 wells) with 10 ul of L/D stain and 90 ul of PBS

15. Looked at the planned panel layout to see which wells require L/D stain and add 1 ul per well (not to be added to the Unstained (US), IC and FMO for L/D, but in our experiment, used L/D in IC wells in Panel 3)
16. Covered this with foil and left to incubate for 30 mins at RT

17. Prepared the staining buffer
18. Poured the buffer into a plastic reservoir for ease of use
19. Needed a multichannel mechanised pipette
20. Once the plates had incubated for 30 mins, pipetted 100ul of the staining buffer into the well plates and centrifuged for 5 mins at 1200 rpm at RT
21. Discarded the supernatant by tipping it once into the sink (to not tap it into the sink), then gently tapped onto some tissue paper
22. Pipetted 200 ul of staining buffer into the wells and repeated the wash at 1200 rpm, RT, 5 mins
23. Tapped out as above

24. Next step was Surface/ Extracellular staining

25. Followed the list below for the extracellular staining for all three panels- 9 in all, 5 colours and 4 isotype controls.

Stain 1: 20ul of CD4-FITC panel 1, 2 and 3

Stain 2. 20ul of CD25-PE panel 1

Stain 3. 5ul of CD127-PE cy7 panel1

Stain 4. 5ul of CD3-APC fire 750 panel 2 and 3

Stain 5. 20ul of CD8-PE cy7 panel 2 and 3

Stain 6. 20ul of IC-FITC mouse (CD4) panel 1, 2 and 3

Stain 6. 20ul of IC-FITC mouse (CD4) panel 2 and 3

Stain 7. 5ul of IC-PE mouse (CD25) panel 1

Stain 8. 5ul of IC-PE cy 7 (CD127 & CD8) panel 1

Stain 8. 5ul of IC-PE cy 7 (CD127 & CD8) panel 2 and 3

Stain 9. 5ul of IC-APC fire (CD3) panel 2 and 3

26. Incubated covered in foil at RT for 30 mins
27. Prepared the Fox P3 buffers A and C for Panel 1 (as below)
28. Prepared the BD Perm wash buffer for Panel 2 & 3 (as below)
29. From this point on, Panel 1 and Panels 2 and 3 were washed, stained and incubated differently
30. Panel 1 (FoxP3): Washed with Staining buffer, 200 ul first and centrifuged at 1500 rpm for 5 mins at RT
31. Emptied the wells and tapped on tissue as before
32. Now added Buffer A 200 ul/well of Panel 1
33. Incubated covered in foil at RT for 10 mins
34. Whilst Panel 1 was incubating, washed panels 2 and 3 with 200 ul staining buffer per well
35. Centrifuged at 1500 rpm RT for 5 mins

36. Next step was permiabilisation

37. Added Fix Perm solution 100 ul per well (This was ready to use and came as part of the kit)
38. Incubated covered in foil for 20 mins in the fridge
39. Whilst Panel 2 and 3 were incubating, and once Panel 1 had incubated for 10 mins after step 33, centrifuged Panel 1 at 1500 rpm at RT for 5 mins
40. Emptied the supernatant and tapped dry as before
41. Added 200 ul per well of the staining buffer
42. Centrifuged at 1500 rpm, RT for 5 mins

43. Next step was permiabilisation

44. Added Buffer C (Fox P3 Buffer set, prepared as below) 100 ul per well
45. Incubated at RT covered in foil for 30 mins
46. Whilst Panel 1 was incubating and once Panel 2 and 3 had incubated for 20 mins after step 38, added 100ul per well of Perm Wash (prepared as below) to Panels 2 and 3
47. Centrifuged at 1500 rpm RT for 5 mins
48. Emptied and tapped
49. Added 200 ul per well of Perm Wash to Panels 2 and 3
50. Centrifuged at 1500 rpm RT for 5 mins
51. Emptied and tapped

52. Once Panel 1 had incubated for 30 mins after step 45, added 100 ul of staining buffer per well and centrifuged at 1500 rpm at RT for 5 mins
53. Emptied and tapped on tissue
54. Added 200 ul per well of staining buffer and centrifuged again at 1500 rpm RT for 5 mins
55. Emptied and tapped on tissue
56. **Next Step was intracellular staining**

    Stain 1. 20ul of FoxP3 AlexaFluor 647 panel 1

    Stain 2. 2.5ul of IFNγ panel 2

    Stain 3. 20ul of IFNα PE, panel 2

    Stain 4. 5.5ul of TNFα PerCP cy 5.5, panel 2

    Stain 5. 5ul of IL 17 PE, panel 3

    Stain 6. 5ul of IL 4 PerCP-cy5.5, panel 3

    Stain 7. 5ul of IL 10 APC, panel 3

    Stain 8. 20ul of IC Alexa Fluor 64, panel 1

    Stain 9. 5ul of IC APC mouse IgG1 (IFNγ), panel 2

    Stain 10. 20ul of IC PE mouse IgG1 (IFNα & IL 17), panel 2 and 3

    Stain 11. 5ul of IC PerCP-cy5.5 (TNFα & IL 4), panel 2 and 3

    Stain 12. 5ul of IC APC rat Ig (IL 10), panel 3

57. Once intracellular staining was done, covered both the well plates in foil and incubated for 30 mins- Panel 1 at RT and Panels 2 and 3 in the fridge

58. Whilst the panels were incubating, prepared the Single Colour Controls (SCC) using compensation beads
59. The beads were kept in the fridge. Mixed by vortexing
60. Each drop ~ 50ul, so added enough drops into an eppindorf tube (25 ul needed for each SCC- 12 in total, so at least 6 drops needed)
61. Pipetted well to mix and added 25 ul into each FACS tube or another new well plate
62. Added 2 ul of each colour into the tubes/plate as below except for CD8 PE cy 7 (used 0.5 ul)

1. **CD4 FITC**
2. **CD25 PE**
3. **CD127 PE-cy7**
4. **FoxP3 Alexa Flour 647**
5. **IFN γ APC**
6. **IFN α PE**
7. **TNF α PerCP cy 5.5**
8. **CD3 APCfire750**
9. **CD8 PE cy7 (0.5 ul)**
10. **IL 17 PE**
11. **IL 4 PerCP cy 5.5**
12. **IL 10 APC**

63. Vortexed to mix

64. Incubated covered in foil for 15 mins in the fridge
65. Once incubated, added 250ul of PBS per tube/well
66. Ready for Flow cytometry
67. Covered in foil and left in the fridge overnight
68. Once the incubation after intracellular staining for Panels 1 and 2 & 3 was complete, washed
69. For Panel 1, used staining buffer- 100 ul per well, then centrifuged at 1500 rpm RT for 5 mins, emptied and tapped on tissue, then 200 ul per well added, centrifuged at 1500 rpm RT for 5 mins, empty and tapped on tissue
70. For Panel 2 &3, used Perm Wash buffer and centrifuged twice same as above
71. Re-suspended both with staining buffer 200 ul per well
72. Ready for flow cytometry
73. Left all wells covered in foil overnight in the fridge

Buffers

Staining Buffer

50 mls PBS + 1ml FCS (needed three times this if doing more samples)

BD Perm Wash Buffer (for Panel 2 and 3)

This buffer needed to be used for all the washes after fixation, ie 4 washes

1:10 dilution of buffer from the kit with either distilled water or PBS

Would need ~ 80mls of buffer in total for 4 washes for all the wells in a 96 well plate- calculated accordingly. So for 80 ml, used 8ml of buffer from the kit and added 72 ml of H2O or PBS.

Fox P3 Buffer

Diluted Buffer A: From the kit in 1:10 dilution with H2O or PBS. This would be used as a diluent to make Buffer C also, so prepared enough quantities.

For all the wells in a 96 well plate, would need to make up ~30mls of Buffer A in total, ie 3ml of Buffer A from the kit and 27 ml of H2O/PBS

Buffer C: This was made by diluting Buffer B from the kit with the diluted Buffer A from above in a 1:50 dilution.

For all the wells in a 96 well plate, would need 10 mls in total of Buffer C, ie 200ul of Buffer B from the kit and 9.8ml of Diluted Buffer A

Flow cytometry:

Our gating was applied as per the pictures below. For controls, we used both single colour controls (SCC) for all the antibodies used and Flow minus one (FMO) where all antibodies except

one was added. We also did isotype controls (IC) for every patient sample tested.

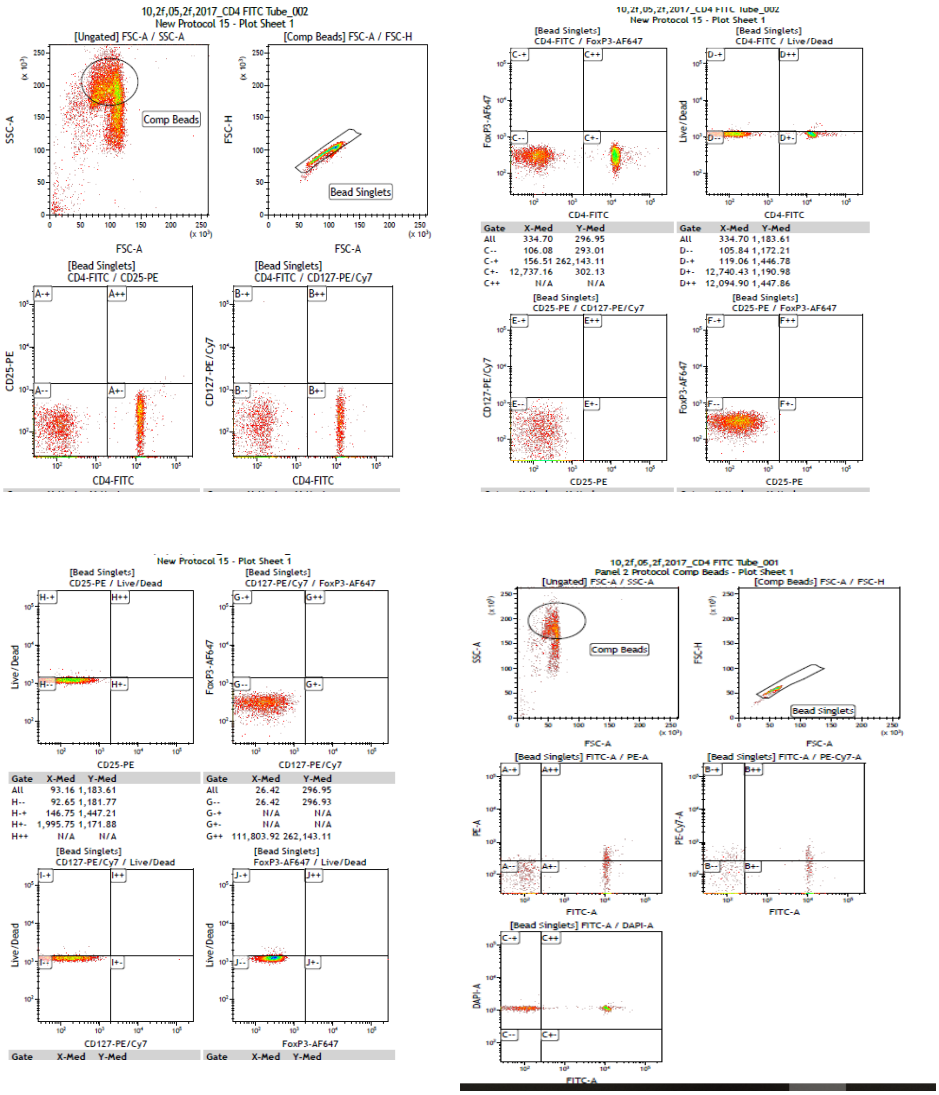


Figure 6A SCC for CD4 FITC

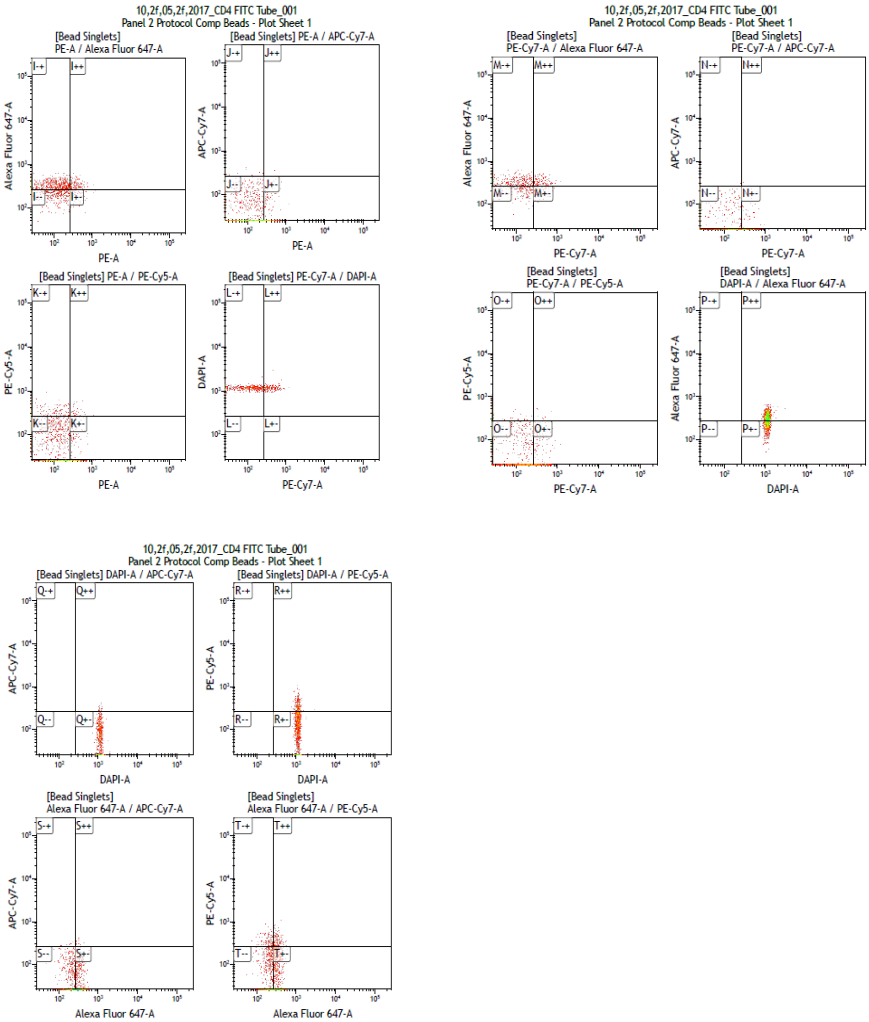


Figure 6B SCC for CD4 FITC

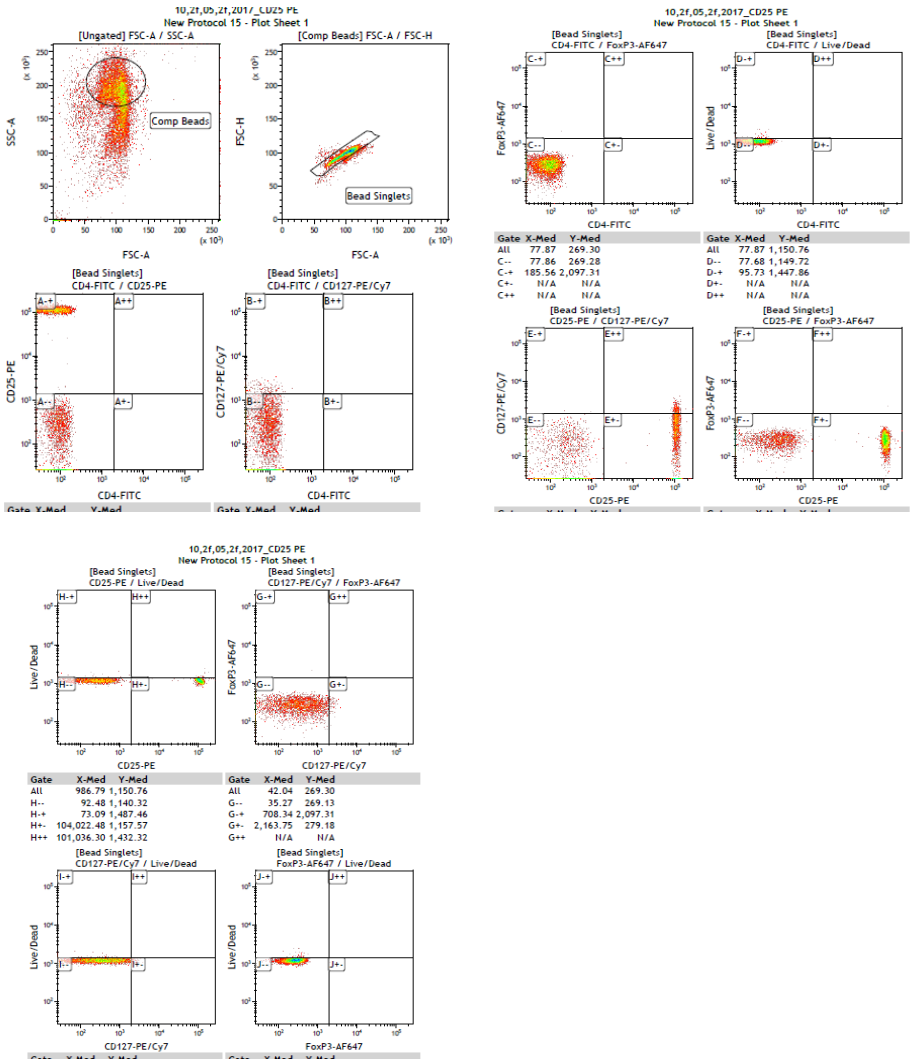


Figure 7 SCC for CD25 PE-

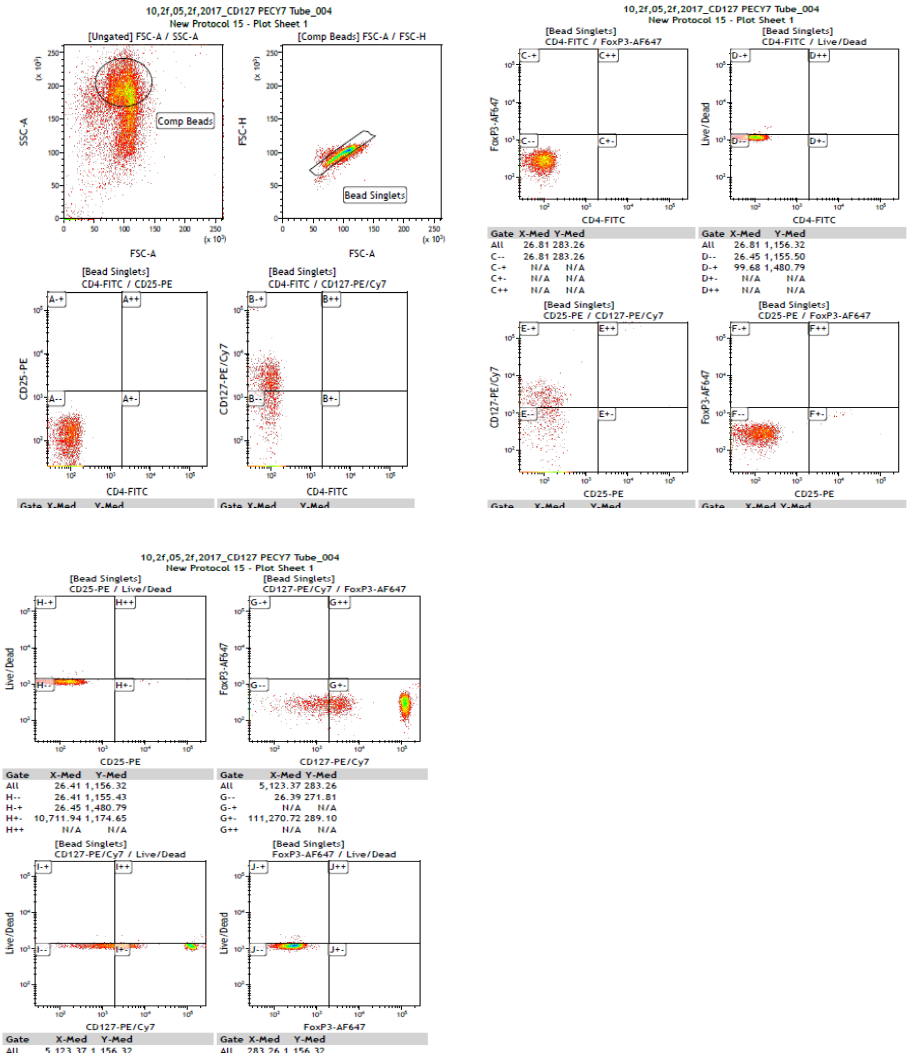


Figure 8 SCC for CD127 PEcy7

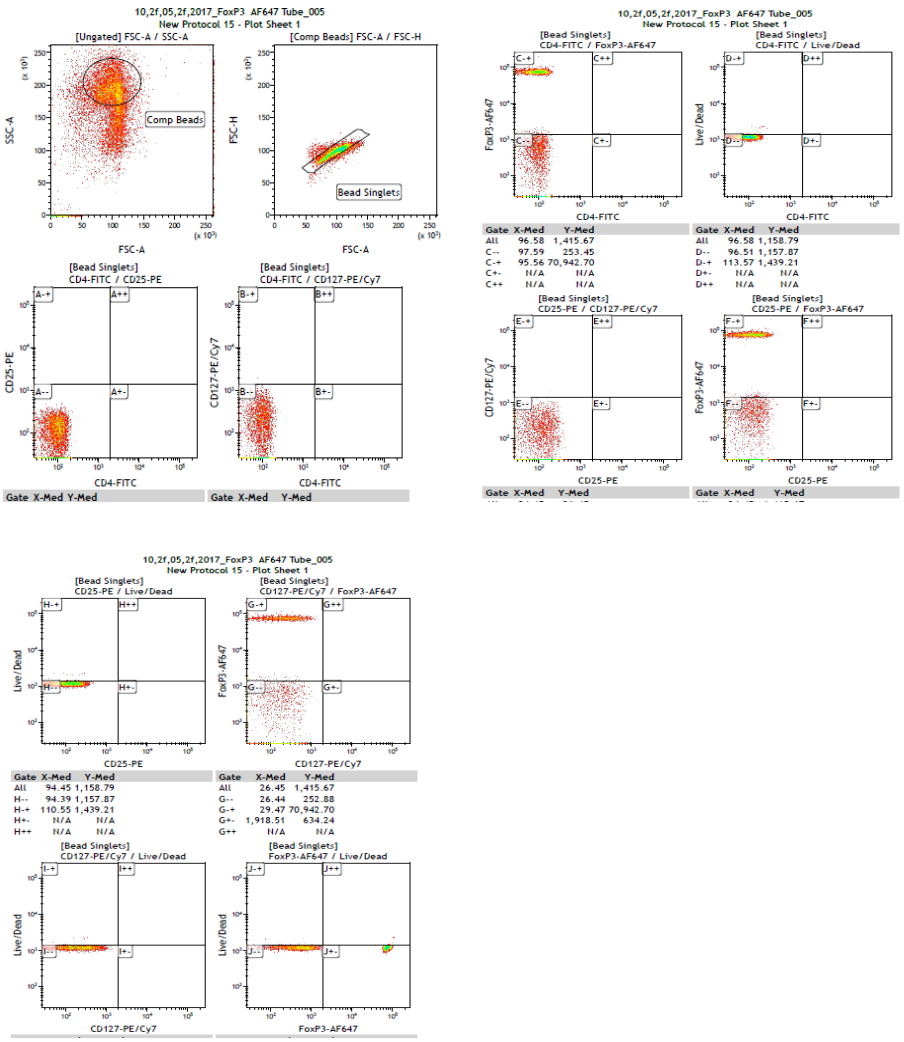


Figure 9 SCC for FoxP3 AlexaFluor647

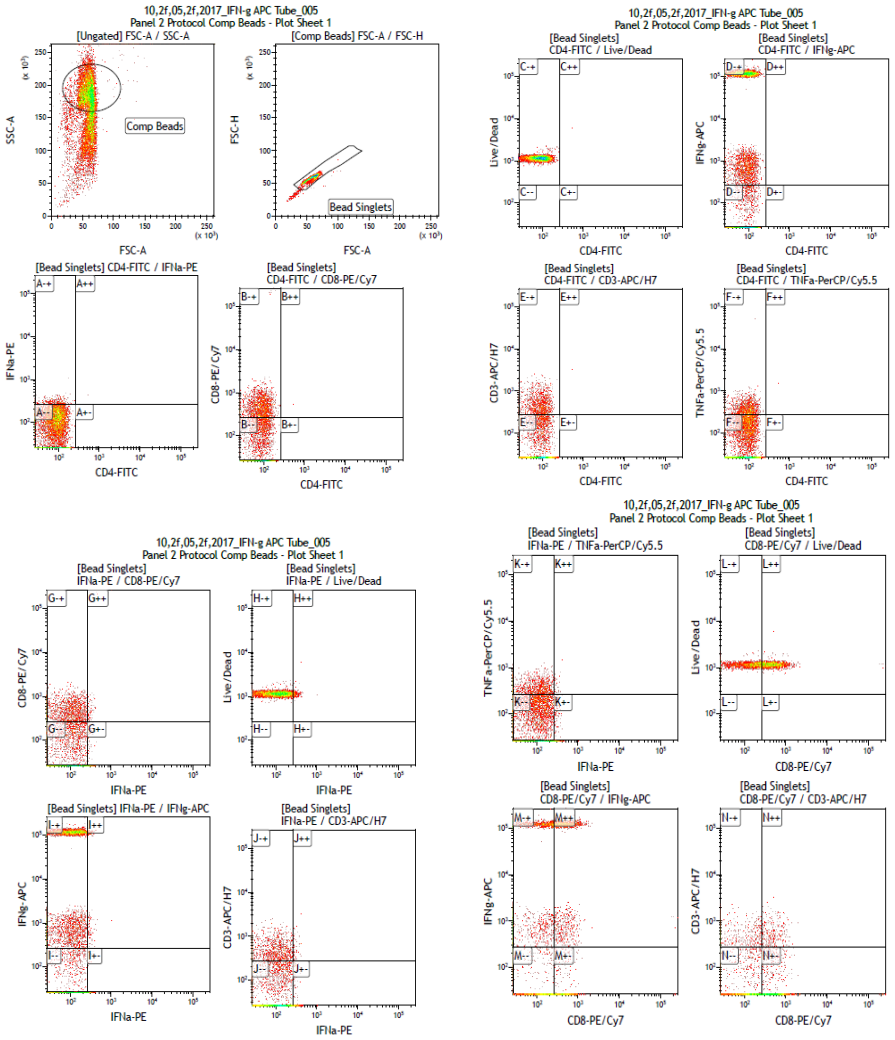


Figure 10 A SCC for IFNg APC

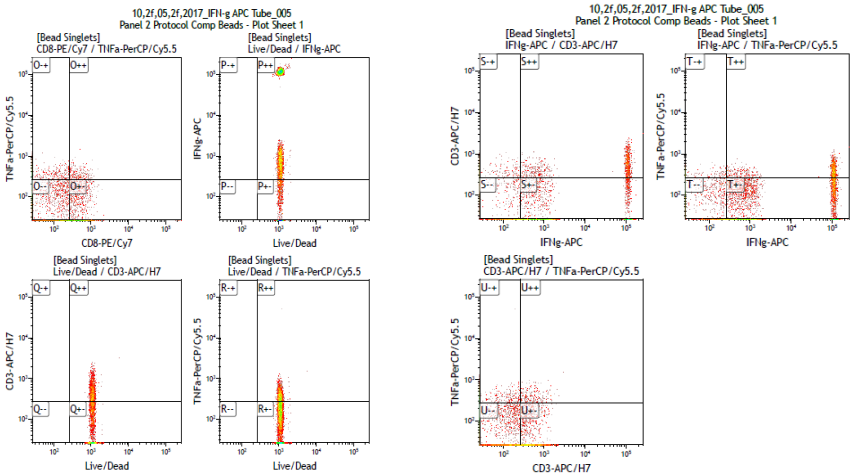


Figure 10 B SCC for IFNg APC

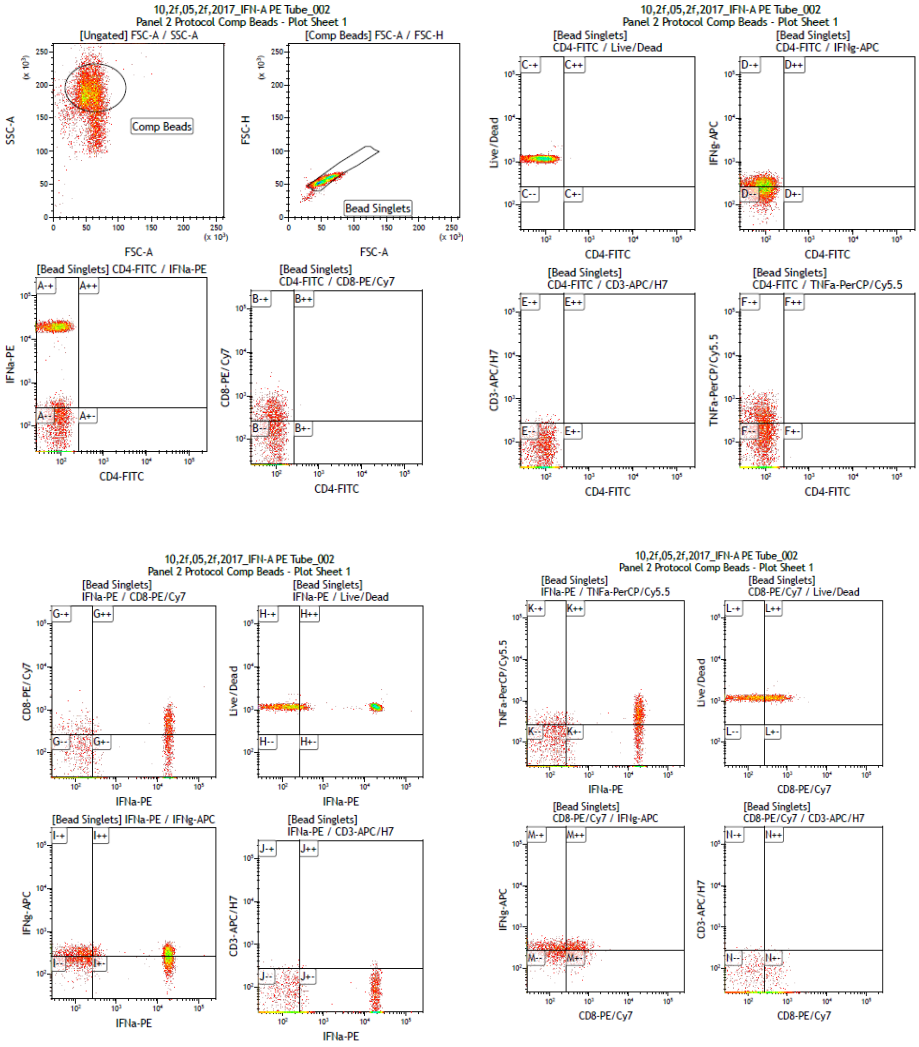


Figure 11A SCC for IFNa PE

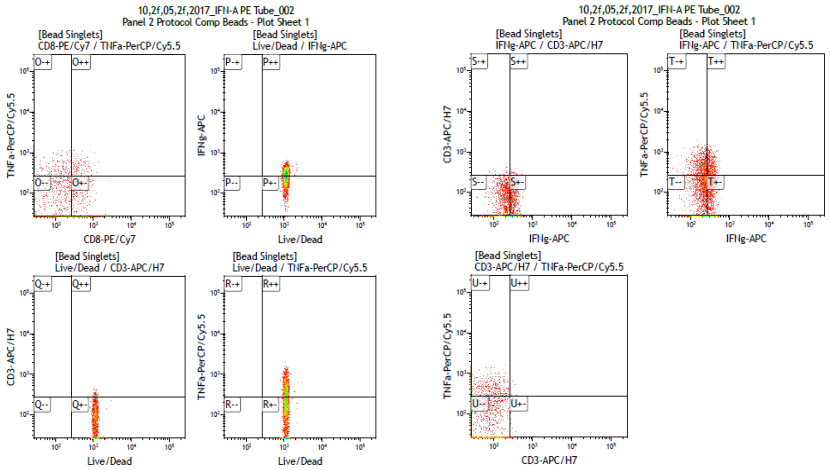


Figure 11B SCC for IFNa PE

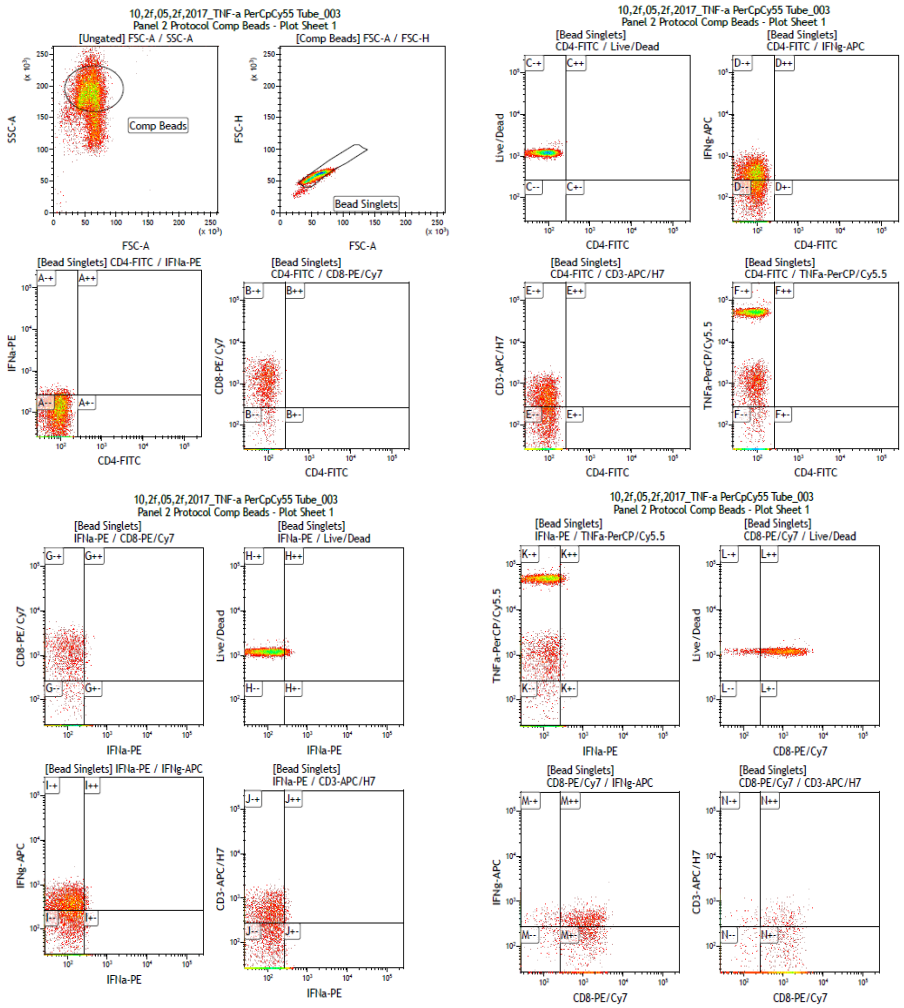


Figure 12A SCC for TNFa PerCPCy5.5

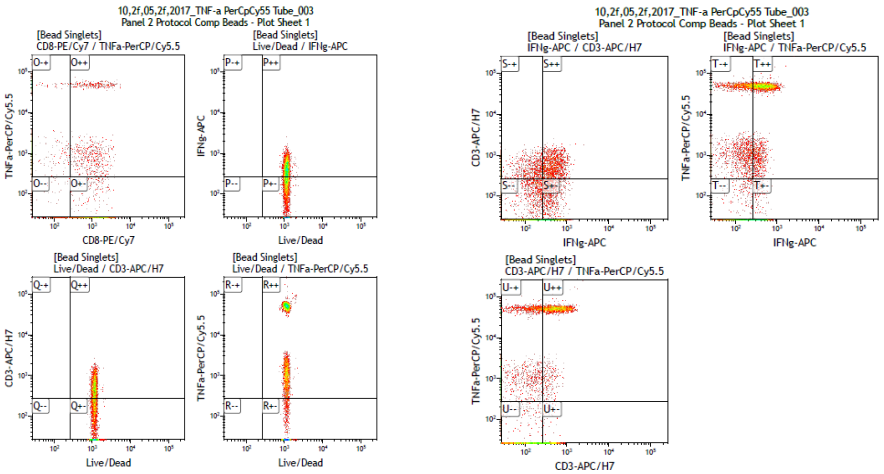


Figure 12B SCC for TNFa PerCPCy5.5

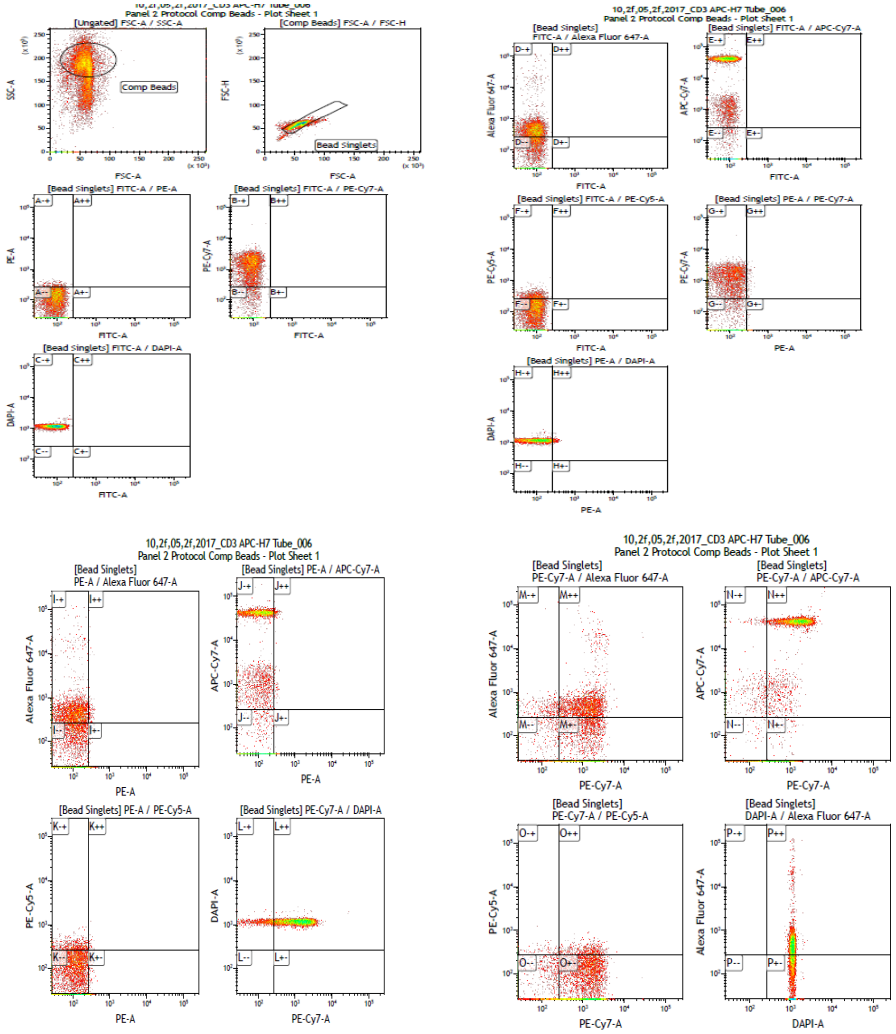


Figure 13A SCC for CD3 APC

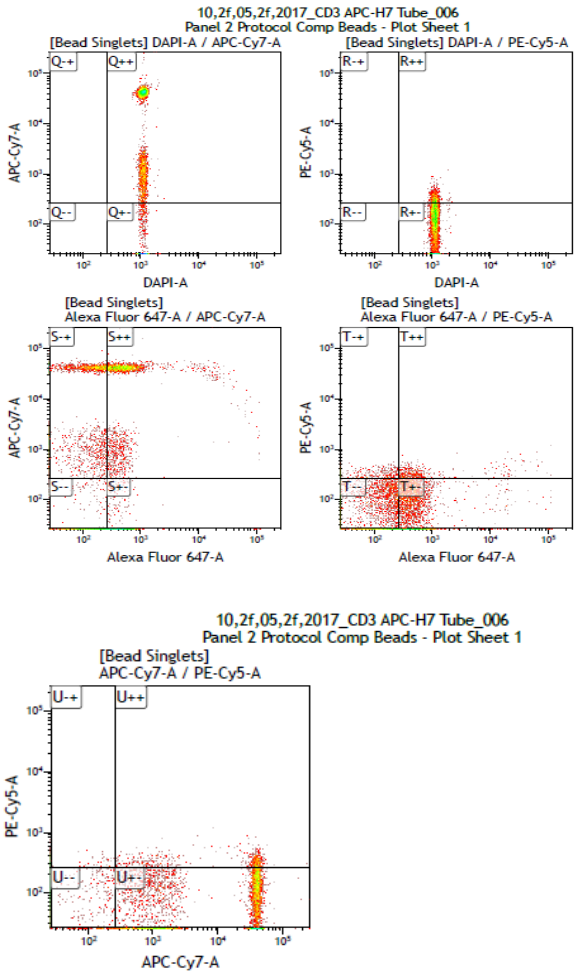


Figure 13B SCC for CD3 APC

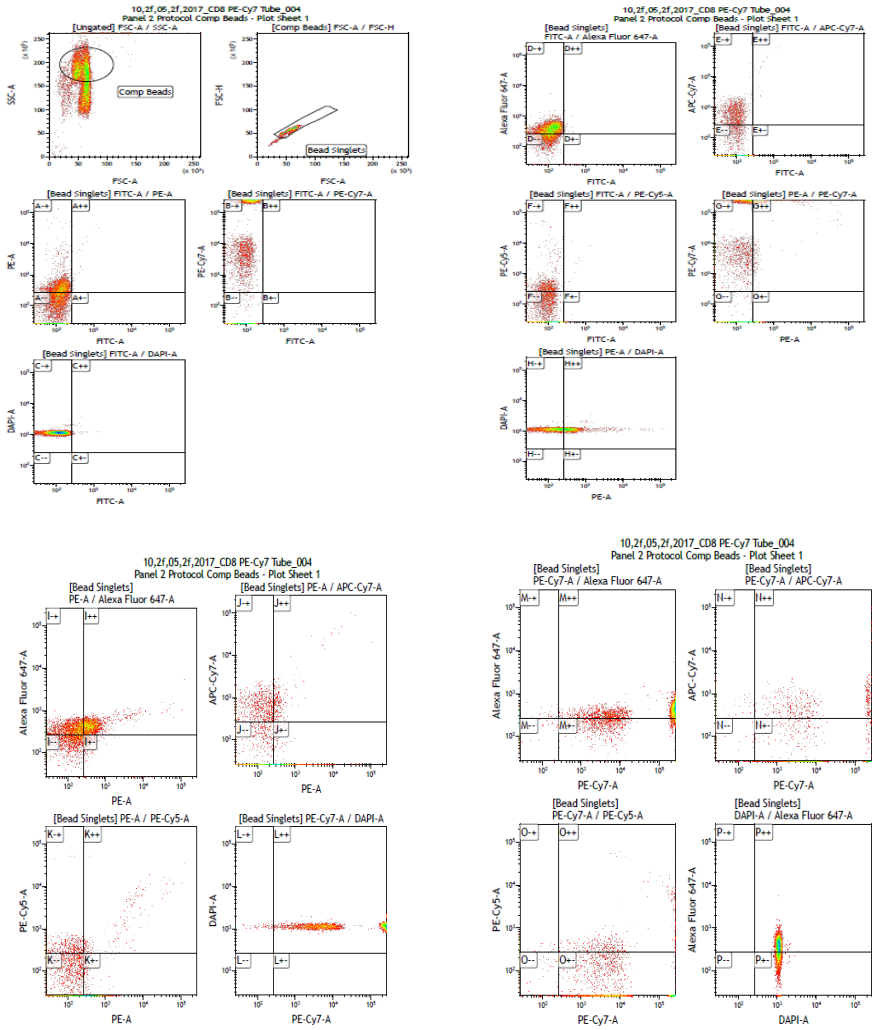


Figure 14A SCC for CD8 PEcy7

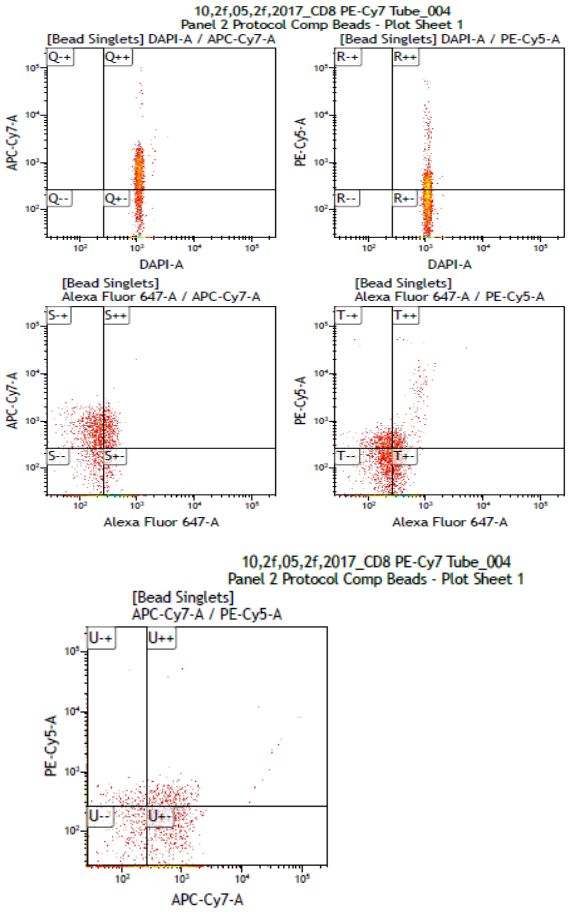


Figure 14B SCC for CD8 PEcy7

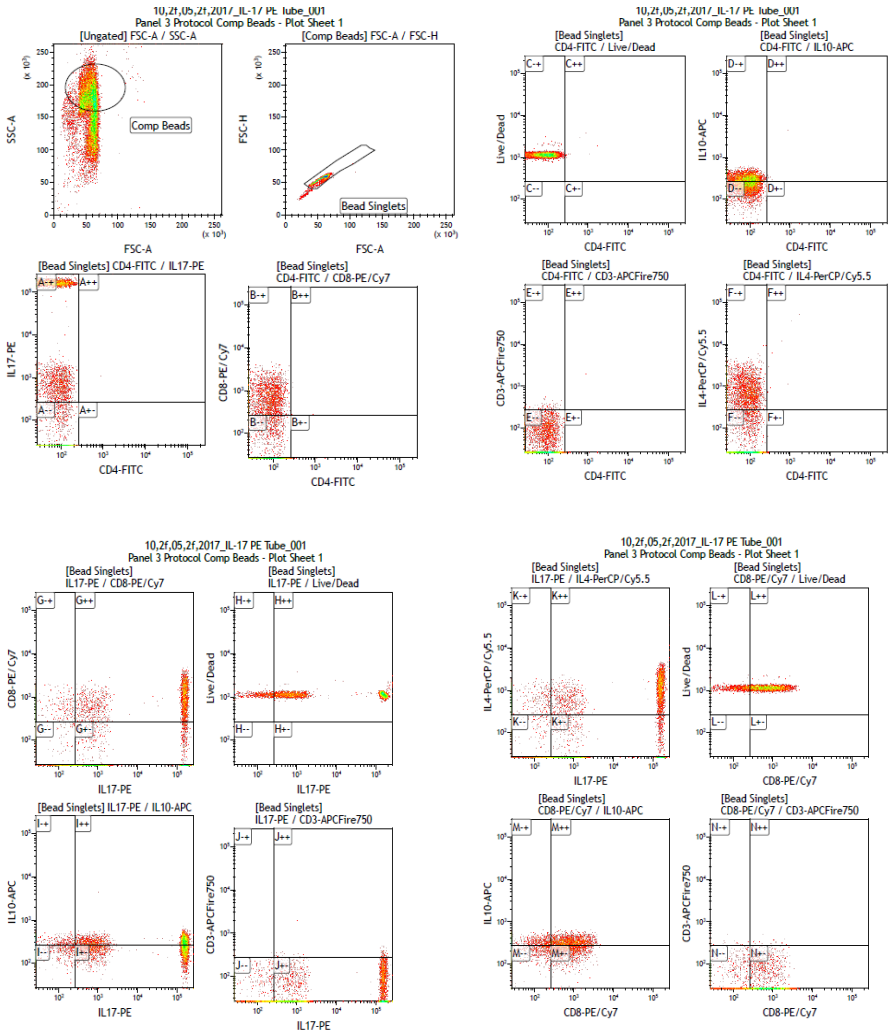


Figure 15A SCC for IL17 PE

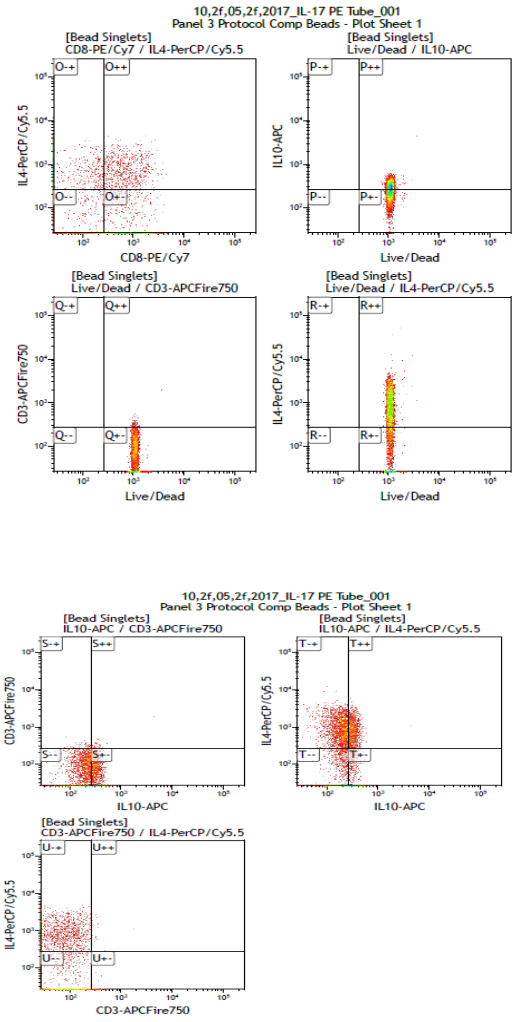


Figure 15BSCC for IL17 PE

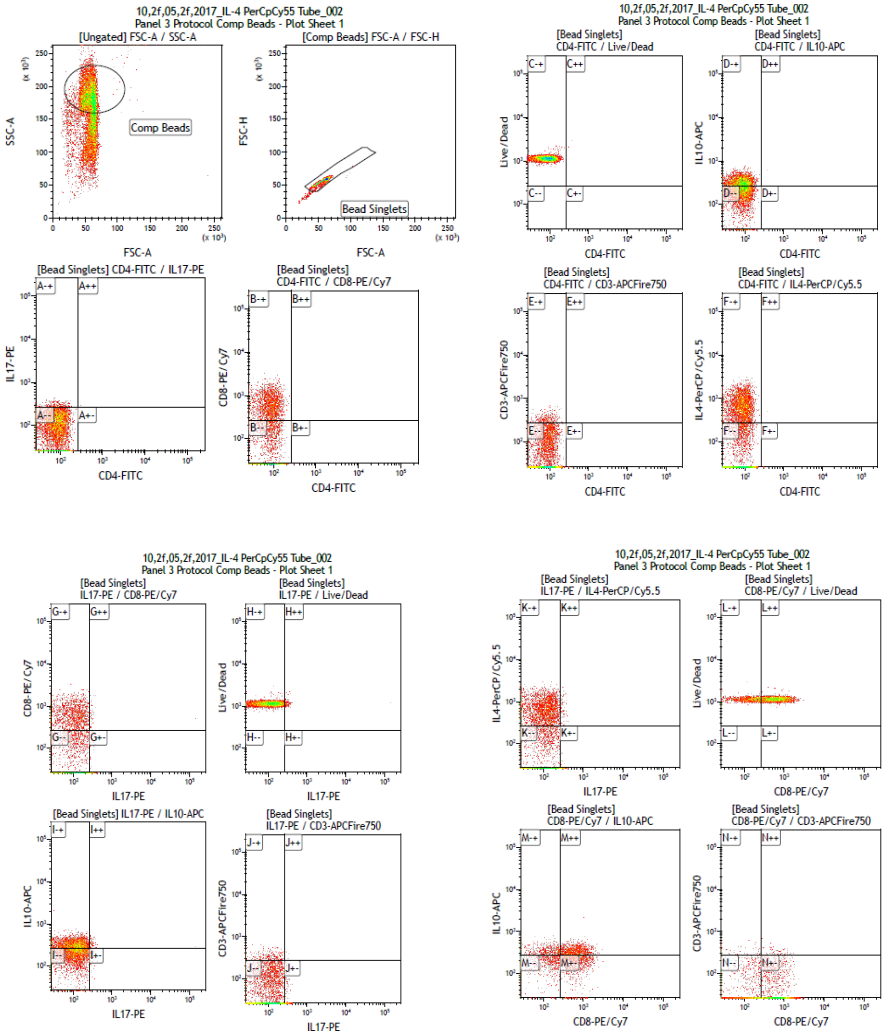


Figure 16A SCC for IL4 PerCPcy5.5

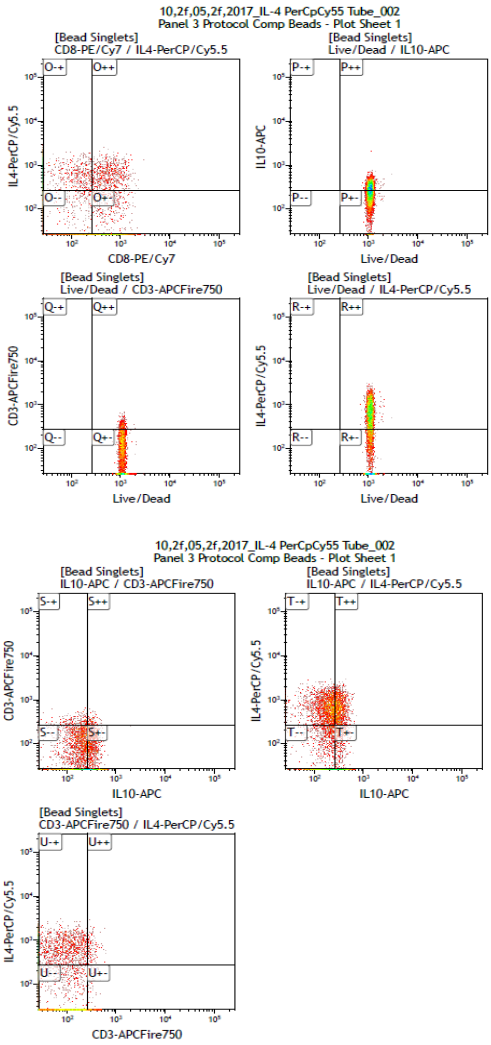


Figure 16B SCC for IL4 PerCPcy5.5

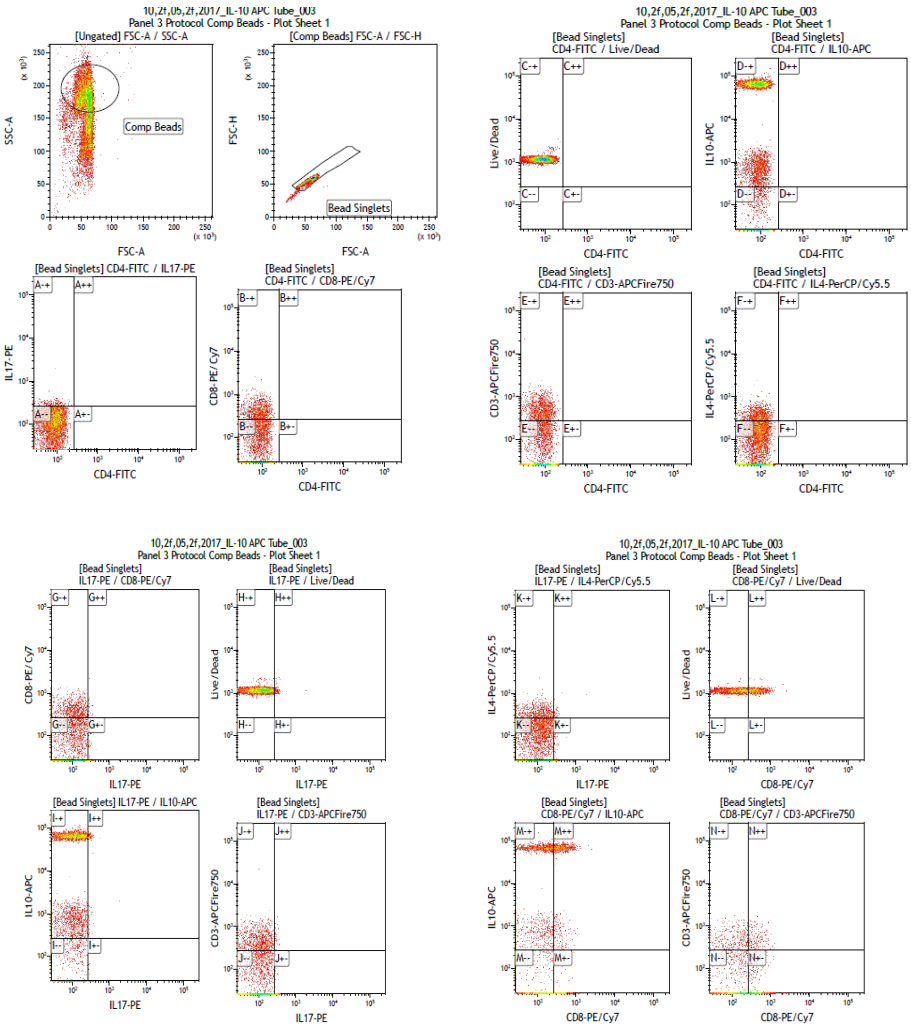


Figure 17A SCC IL10 APC

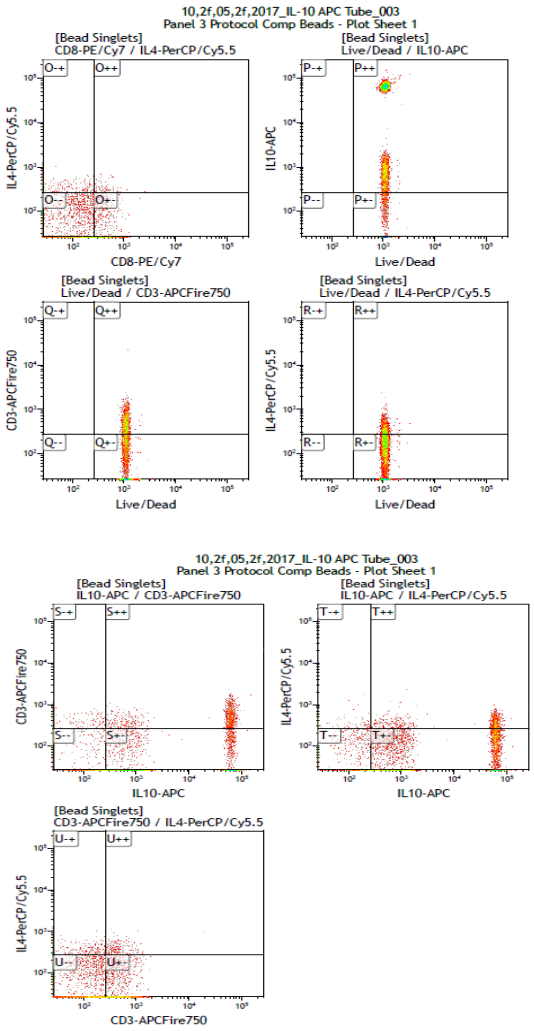


Figure 17B SCC IL10 APC

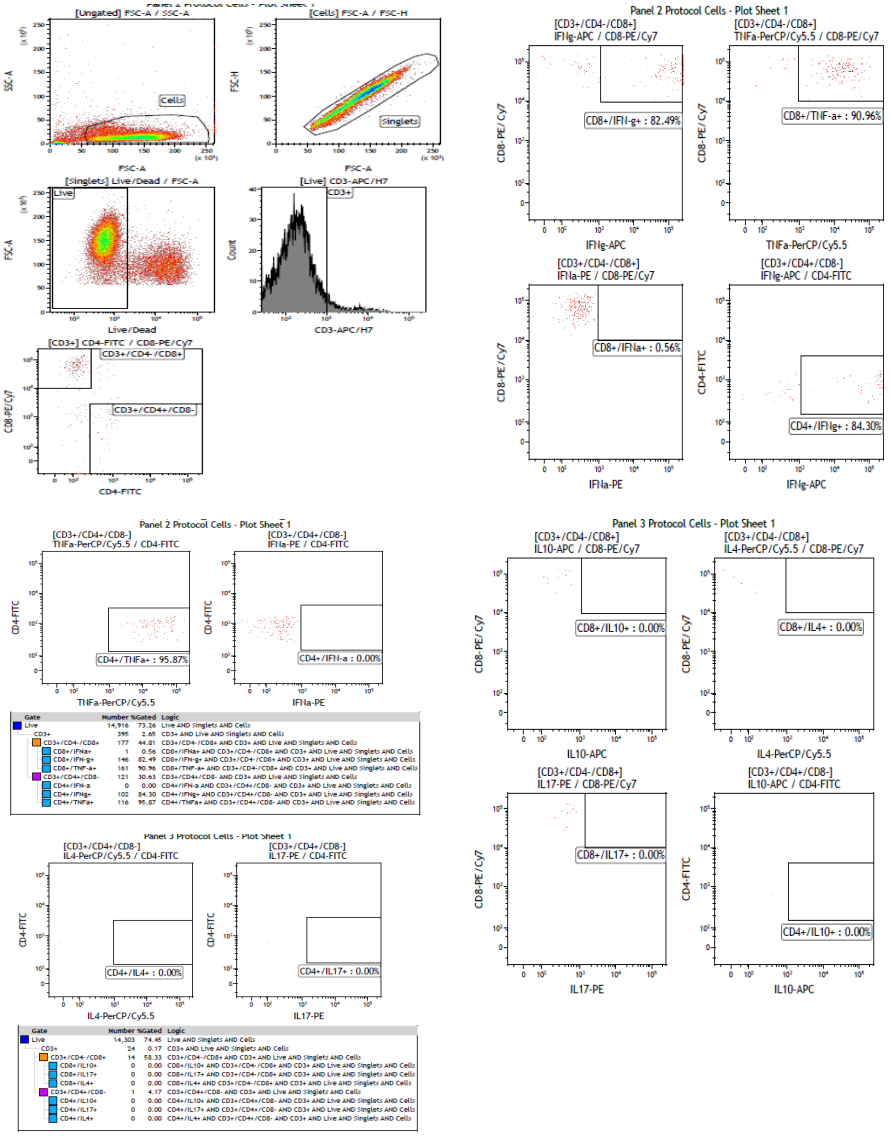


Figure 18 FMO for CD3 APC fire

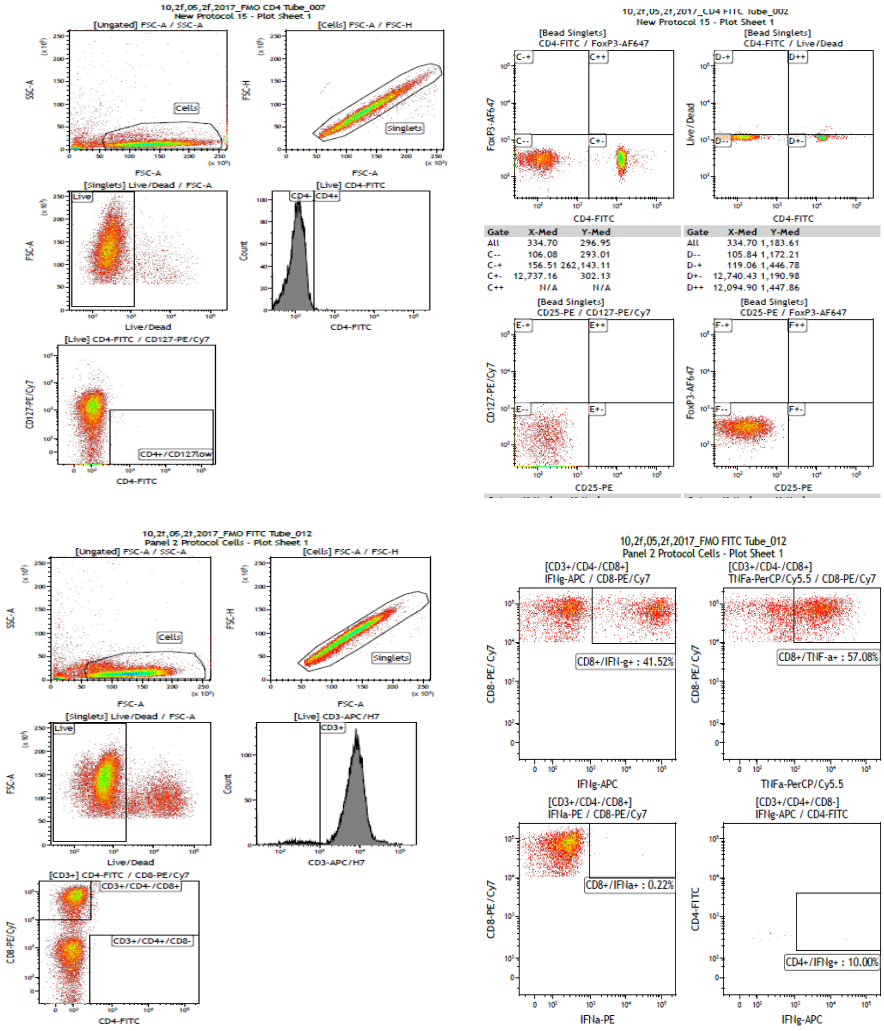


Figure 19A FMO for CD4 FITC

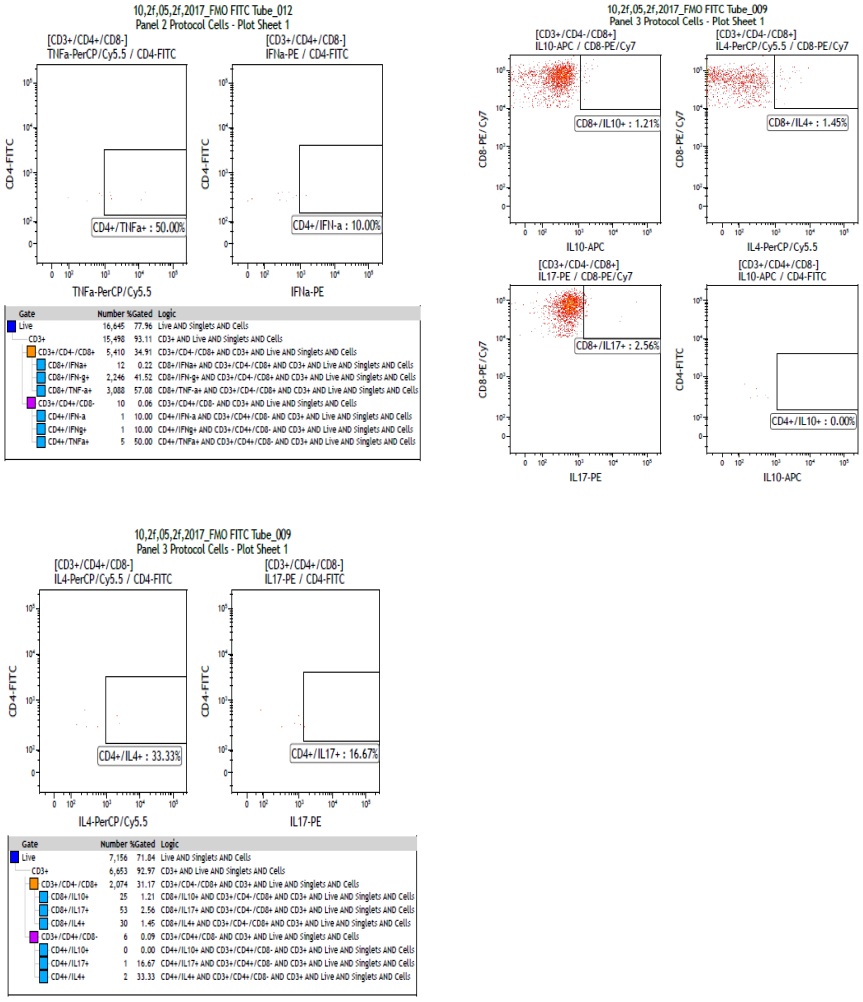


Figure 19B FMO for CD4 FITC

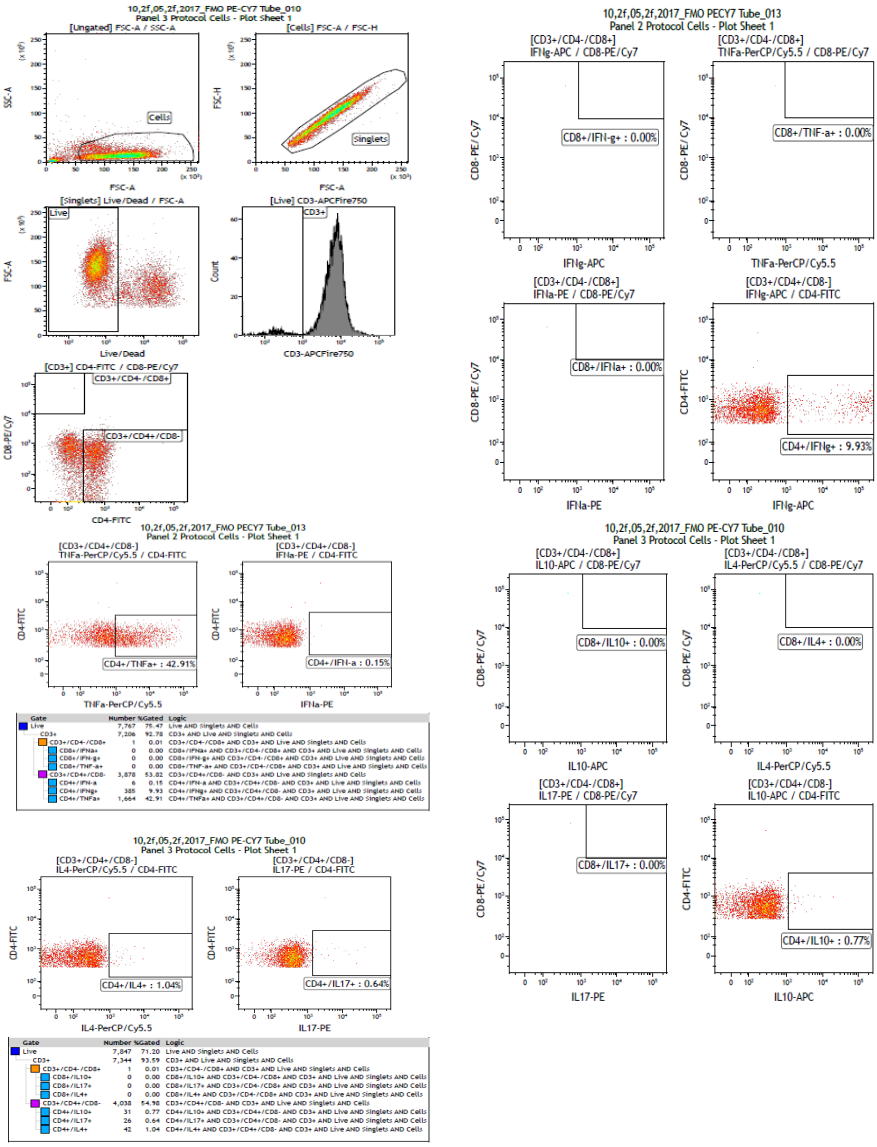


Figure 20 FMO for CD8 PE-cy7

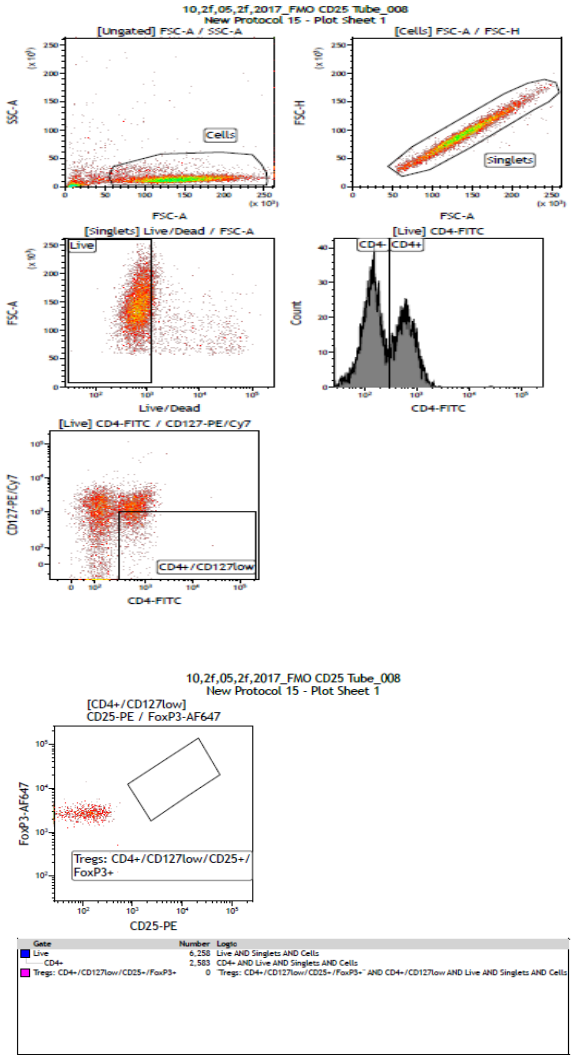


Figure 21 FMO for CD25 PE

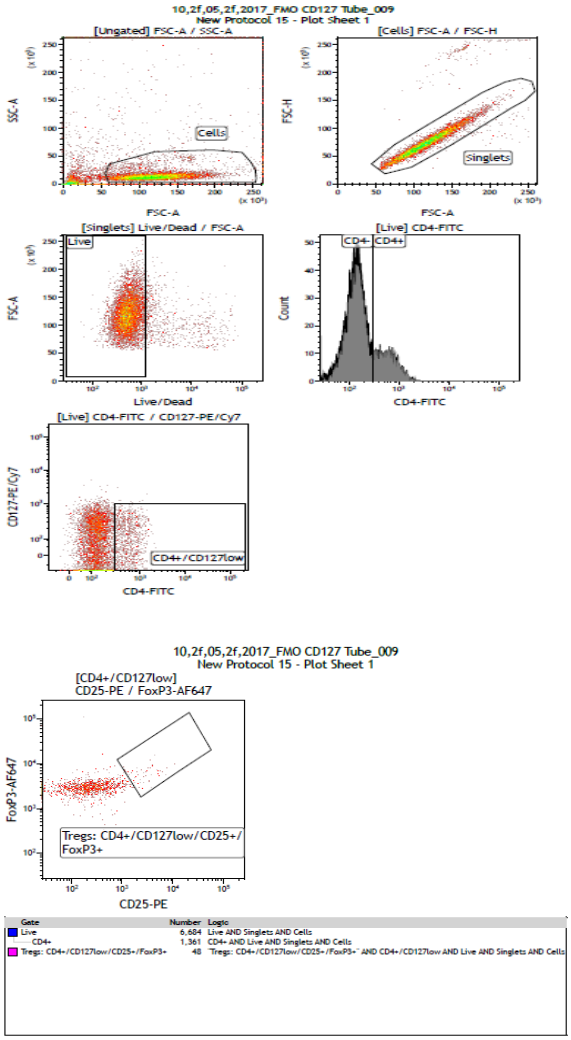


Figure 22 FMO for CD127 PEcy7

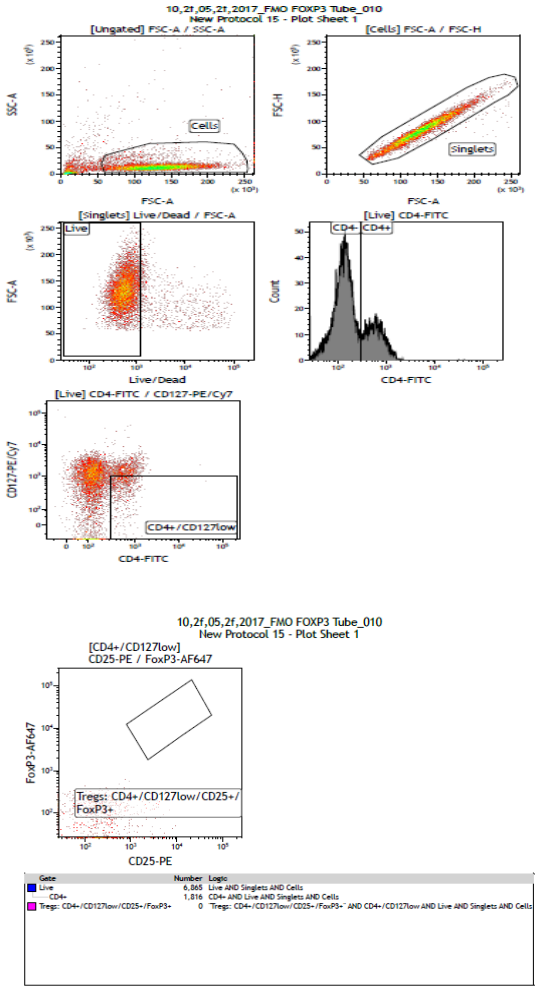


Figure 23 FMO for FoxP3 AF647

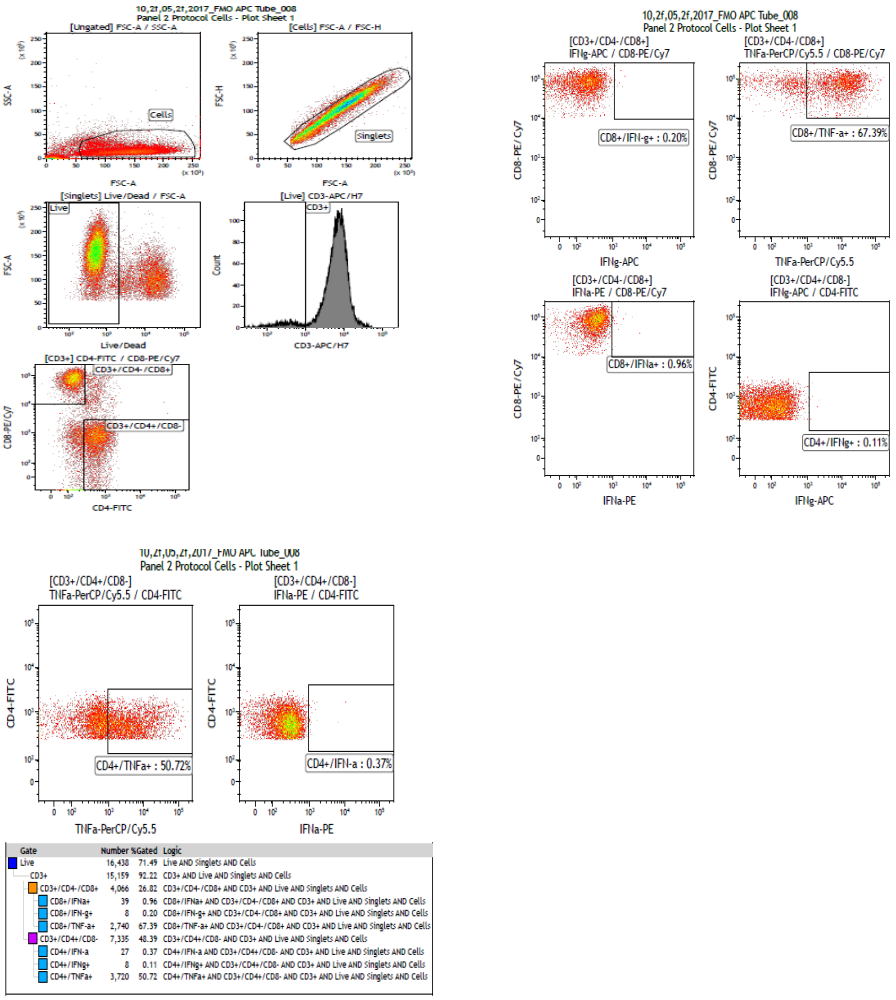
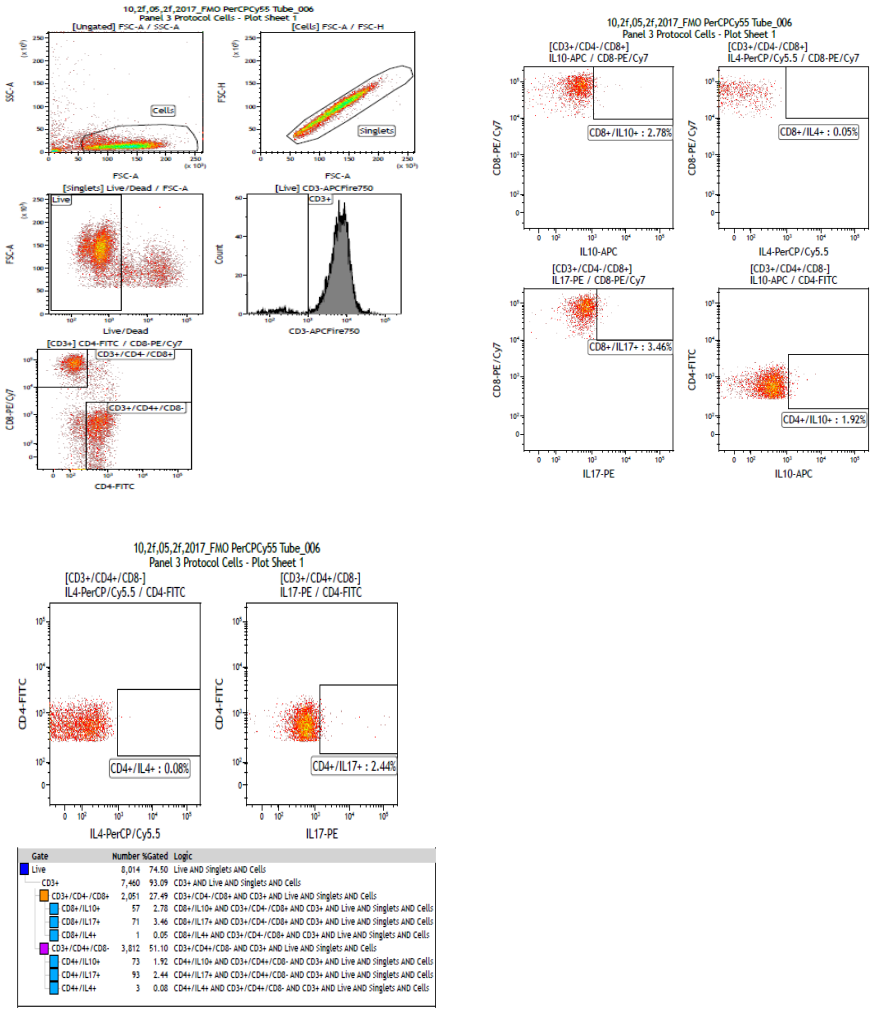


Figure 24 FMO for IFNg APC



Gate	Number	%Gated	Logic
Live	8,014	74.50	Live AND Singlets AND Cells
CD3+	7,460	93.09	CD3+ AND Live AND Singlets AND Cells
CD3+/CD4-/CD8+	2,051	27.49	CD3+/CD4-/CD8+ AND CD3+ AND Live AND Singlets AND Cells
CD8+/IL10+	57	2.78	CD8+/IL10+ AND CD3+/CD4-/CD8+ AND CD3+ AND Live AND Singlets AND Cells
CD8+/IL17+	71	3.46	CD8+/IL17+ AND CD3+/CD4-/CD8+ AND CD3+ AND Live AND Singlets AND Cells
CD8+/IL4+	1	0.05	CD8+/IL4+ AND CD3+/CD4-/CD8+ AND CD3+ AND Live AND Singlets AND Cells
CD3+/CD4+/CD8-	3,812	51.10	CD3+/CD4+/CD8- AND CD3+ AND Live AND Singlets AND Cells
CD4+/IL10+	73	1.92	CD4+/IL10+ AND CD3+/CD4+/CD8- AND CD3+ AND Live AND Singlets AND Cells
CD4+/IL17+	93	2.44	CD4+/IL17+ AND CD3+/CD4+/CD8- AND CD3+ AND Live AND Singlets AND Cells
CD4+/IL4+	3	0.08	CD4+/IL4+ AND CD3+/CD4+/CD8- AND CD3+ AND Live AND Singlets AND Cells

Figure 25 FMO for IL4 PerCPcy5.5

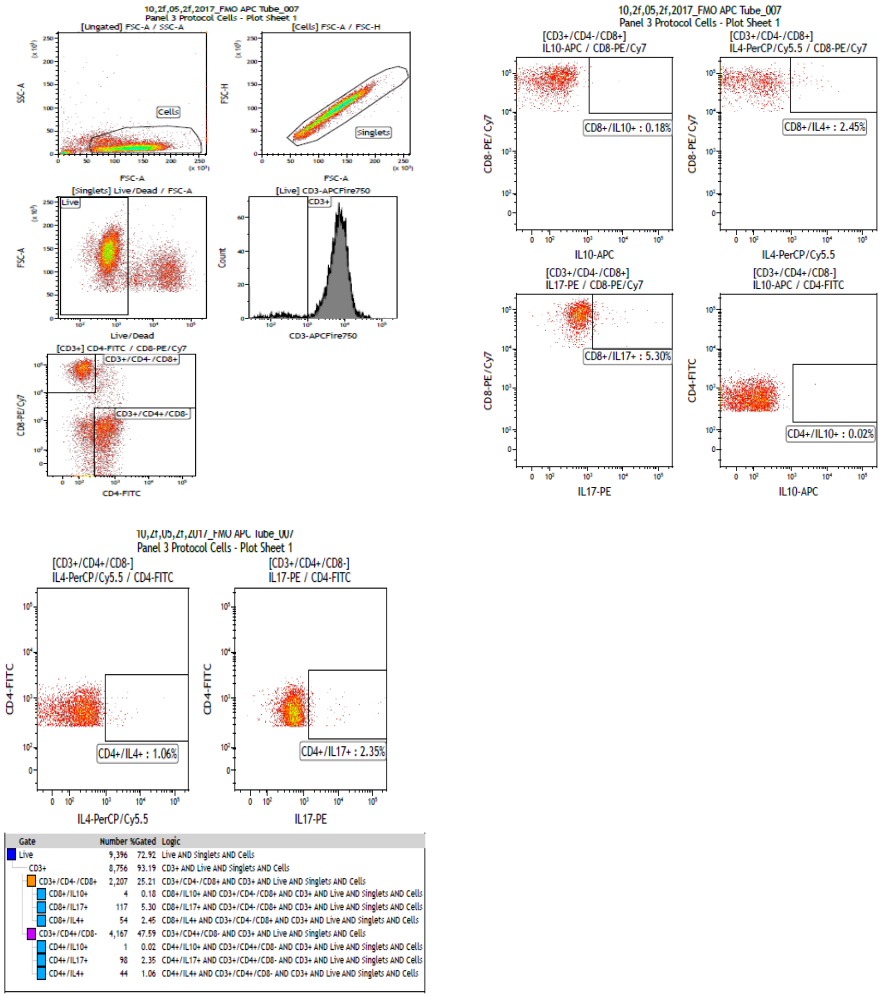


Figure 26 FMO for IL10 APC

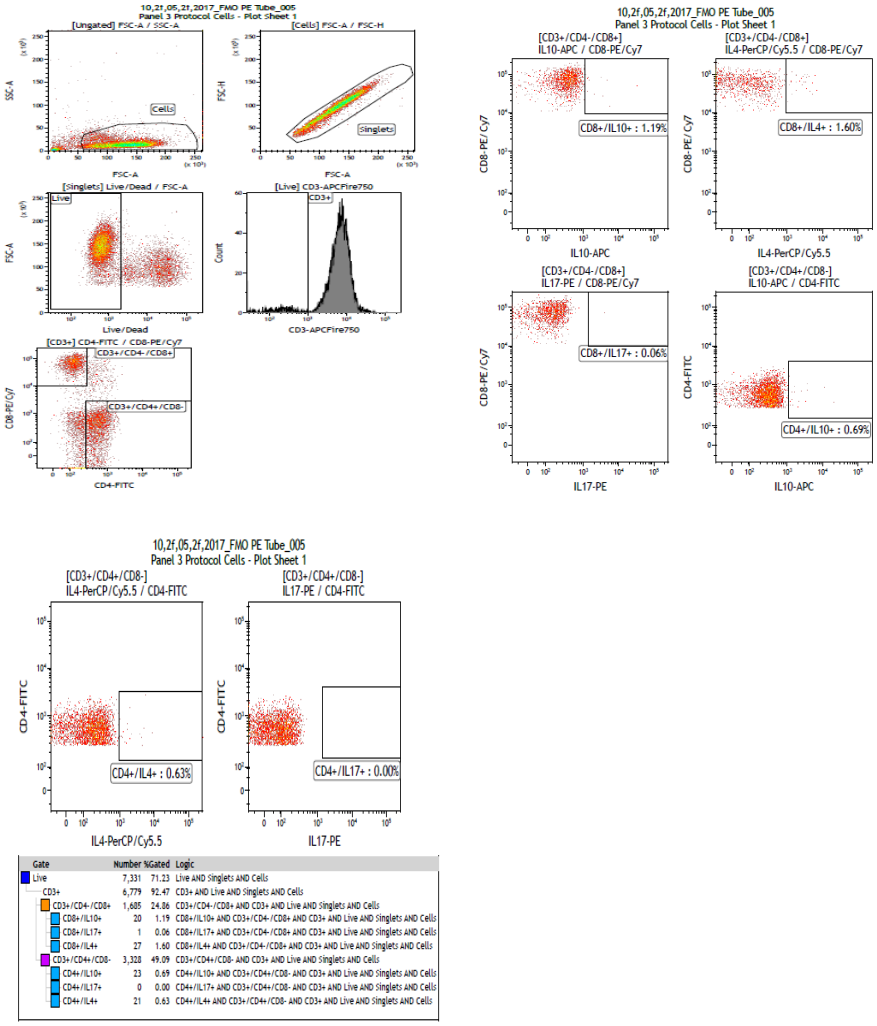


Figure 27 FMO for IL17 PE

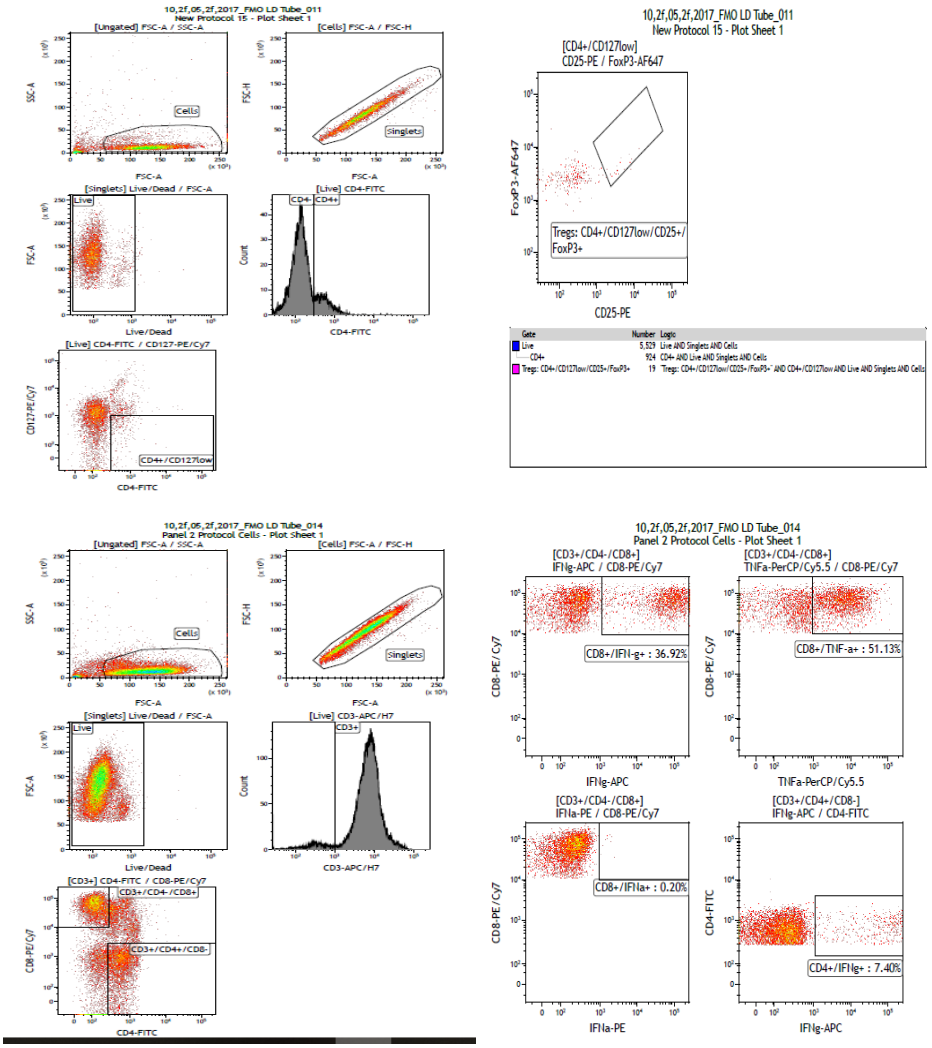


Figure 28A FMO L/D

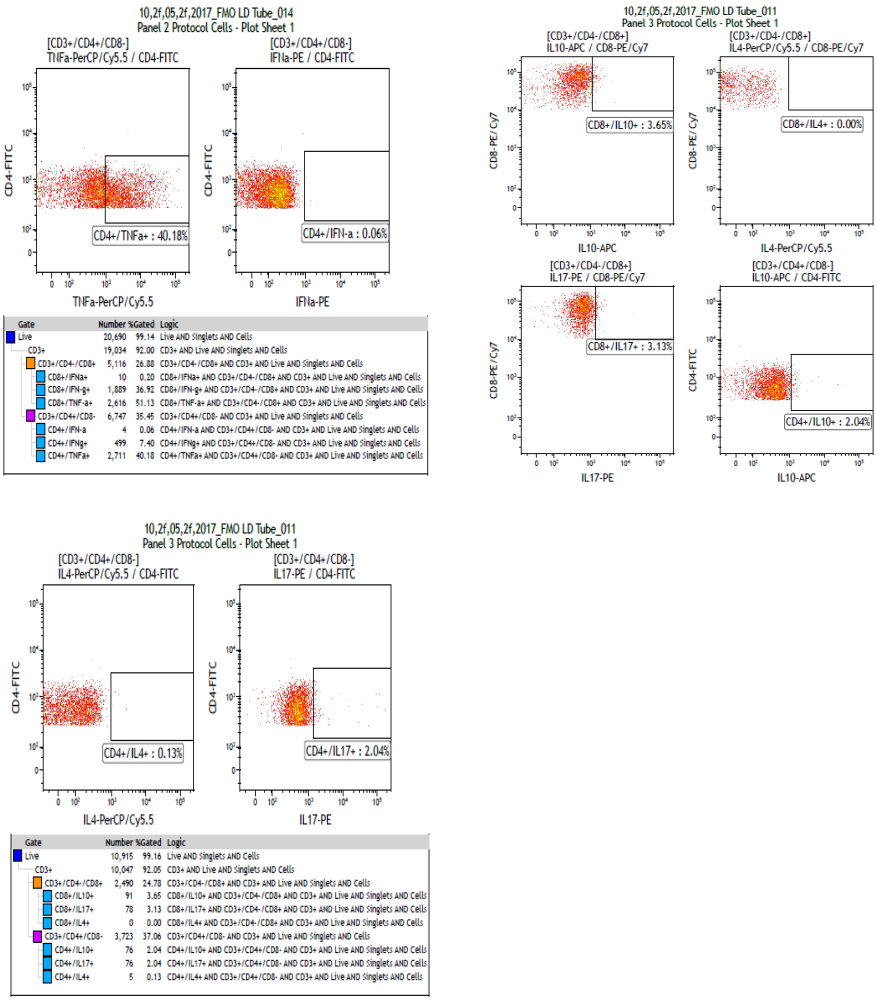


Figure 28B FMO L/D

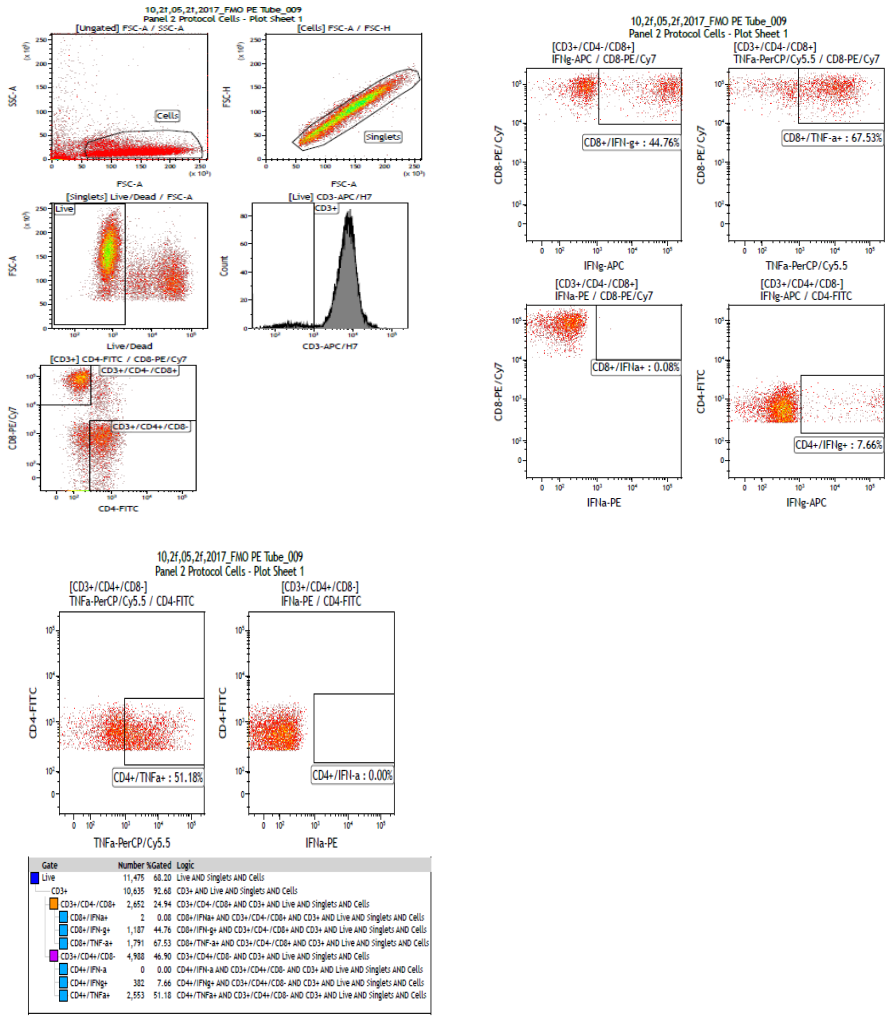


Figure 29 FMO IFNa PE

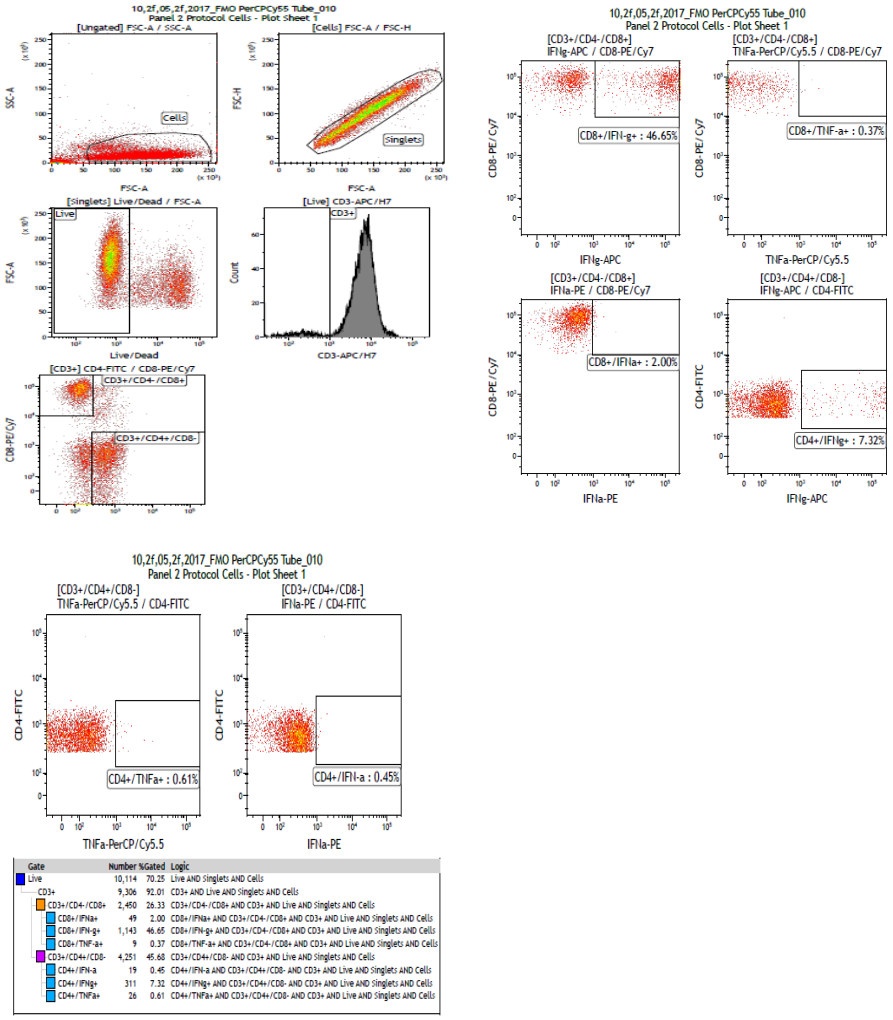


Figure 30 FMO for TNFa PerCPCy5.5

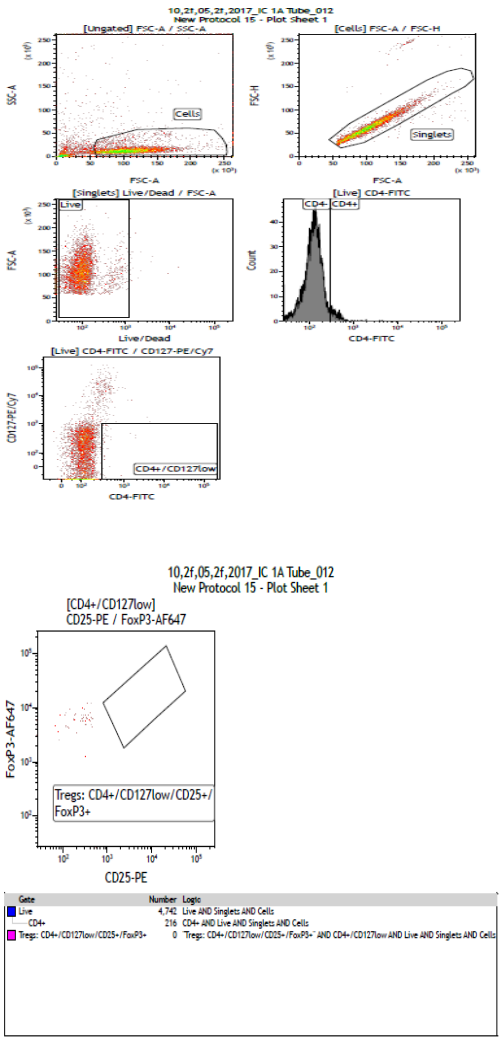


Figure 31 Isotype Control (IC) Panel 1

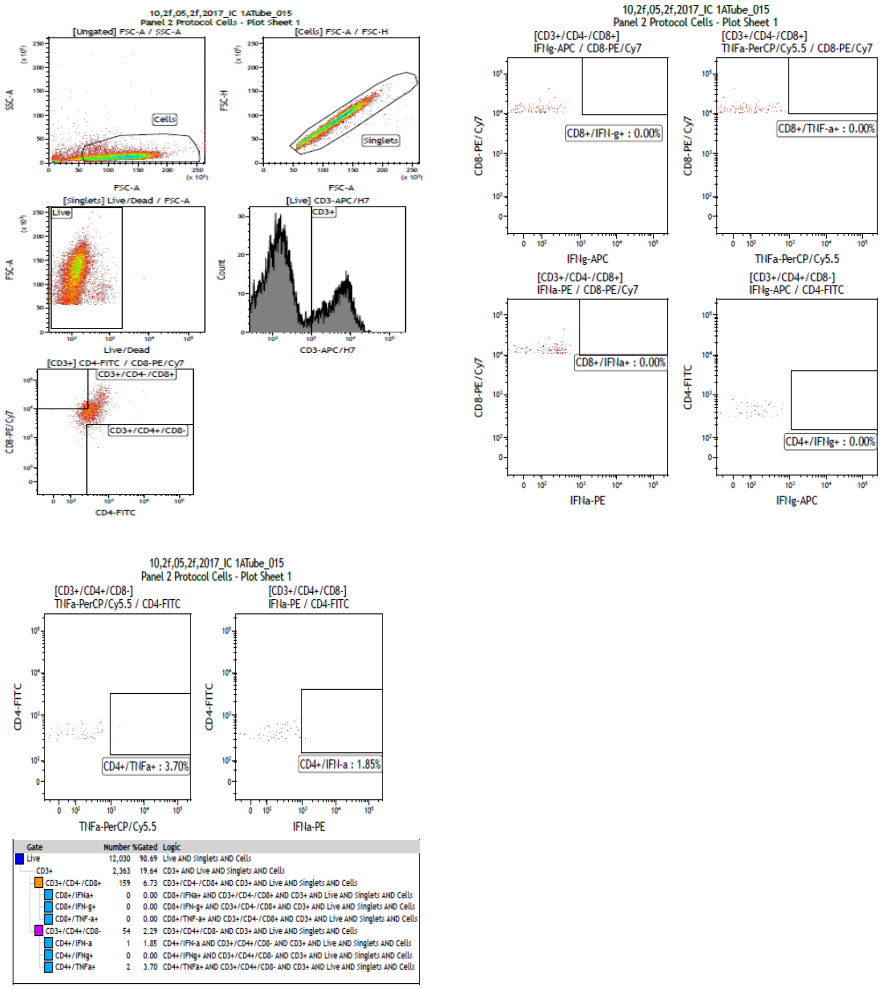


Figure 32 Isotype Control (IC) Panel 2

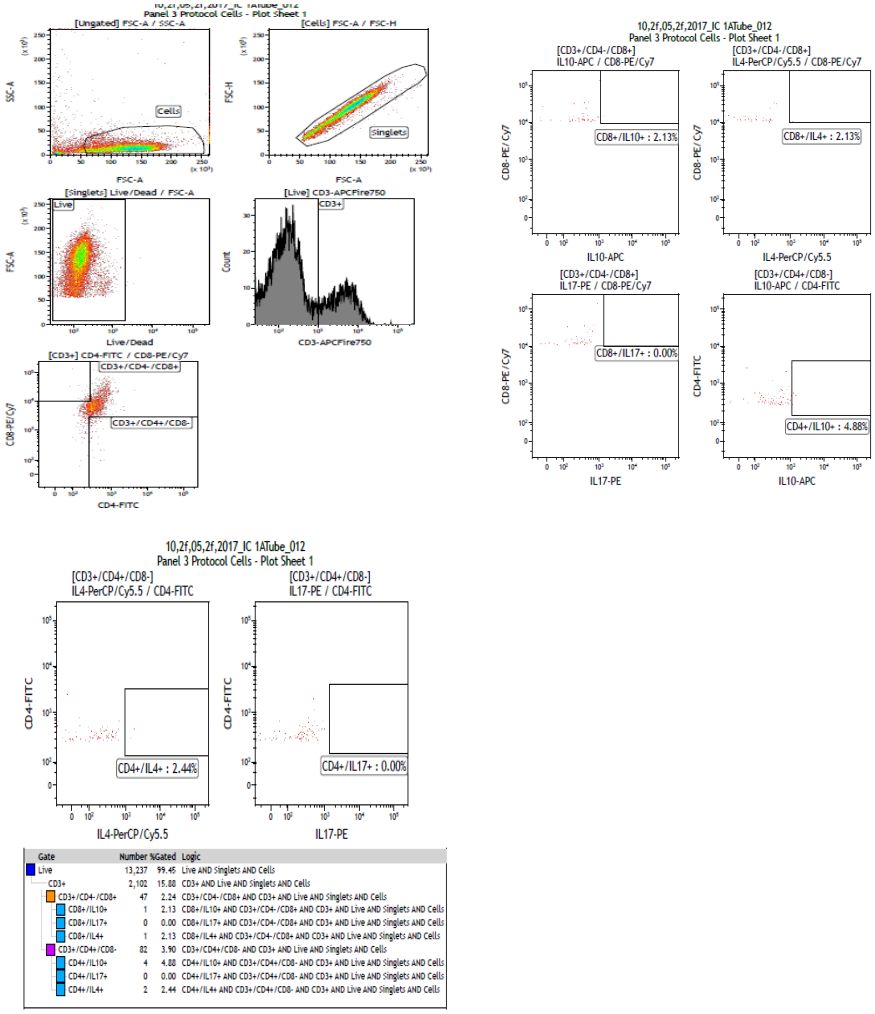


Figure 33 Isotype Control (IC) Panel 3

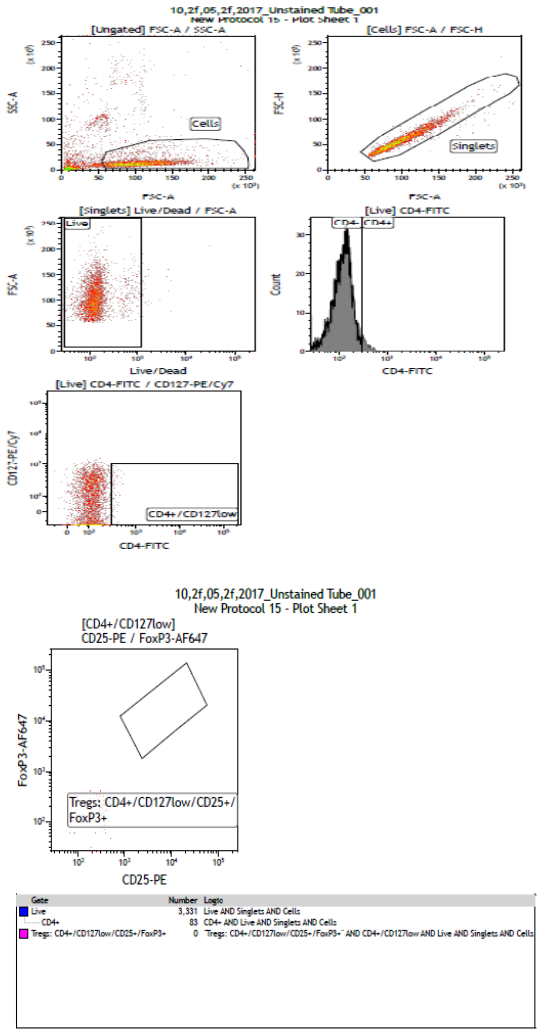


Figure 34 Unstained sample (US), Panel 1

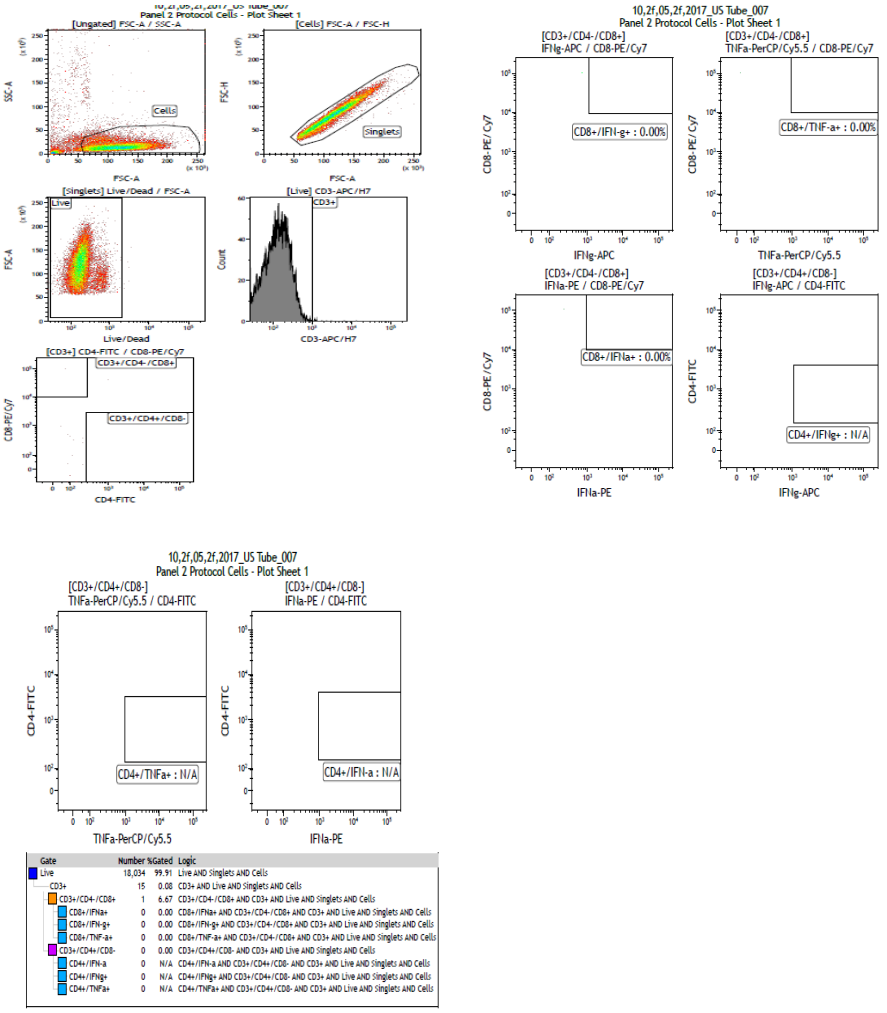


Figure 35 Unstained sample (US), Panel 2

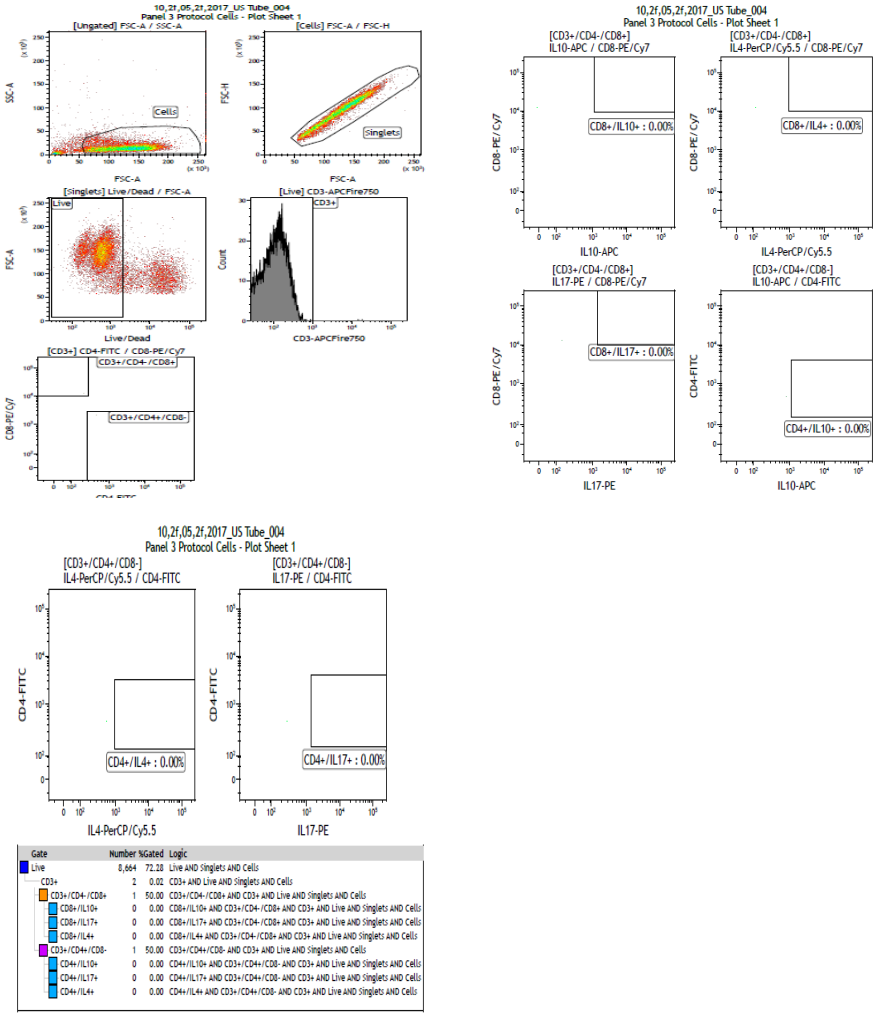


Figure 36 Unstained sample (US), Panel 3

For panel 1, firstly, live cells were identified using the live/dead stain and separated into ‘singlets’. These cells were then separated into CD4+ cells; the next gating was CD4+ CD127*low* cells, the next gating was for CD25+Fox P3+, so in the end we got the Treg cell population which we defined as CD4+CD127*low*CD25+FoxP3+ cells. The statistical analysis on Kalusa was used to calculate the number of live cells, CD4+ cells and Treg cells with percentages.

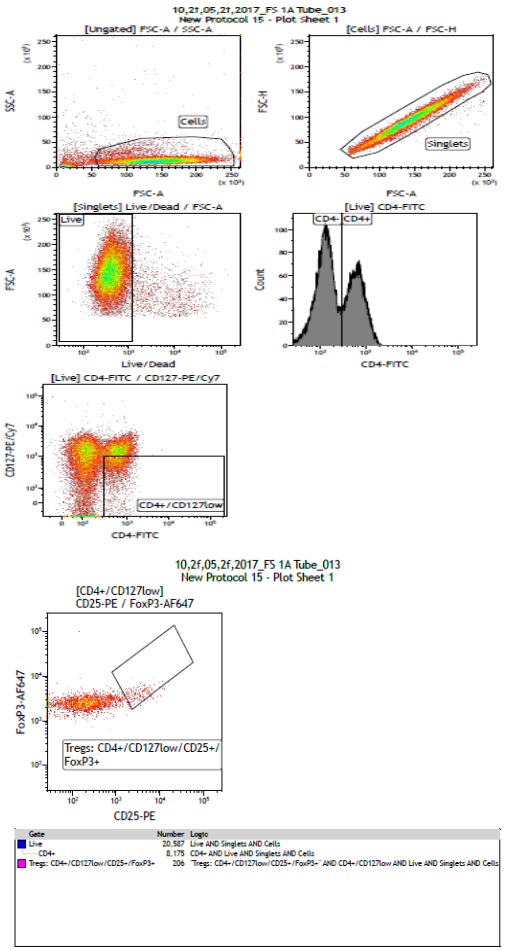


Figure 37 Fully Stained (FS) patient sample, Panel 1

For Panel 2, once again live singlet cells were isolated. The next gating used was for CD3+ cells. These were then separated into CD3+CD4-CD8+ and CD3+CD4+CD8- cells. The next few gates were for CD8+IFNγ, CD8+TNFα, CD8+IFNα, CD4+IFNγ, CD4+TNFα and CD4+IFNα. The statistical analysis on Kalusa was used to calculate the number of live cells, and number and proportions of CD8+IFNγ, CD8+TNFα, CD8+IFNα, CD4+IFNγ, CD4+TNFα and CD4+IFNα.

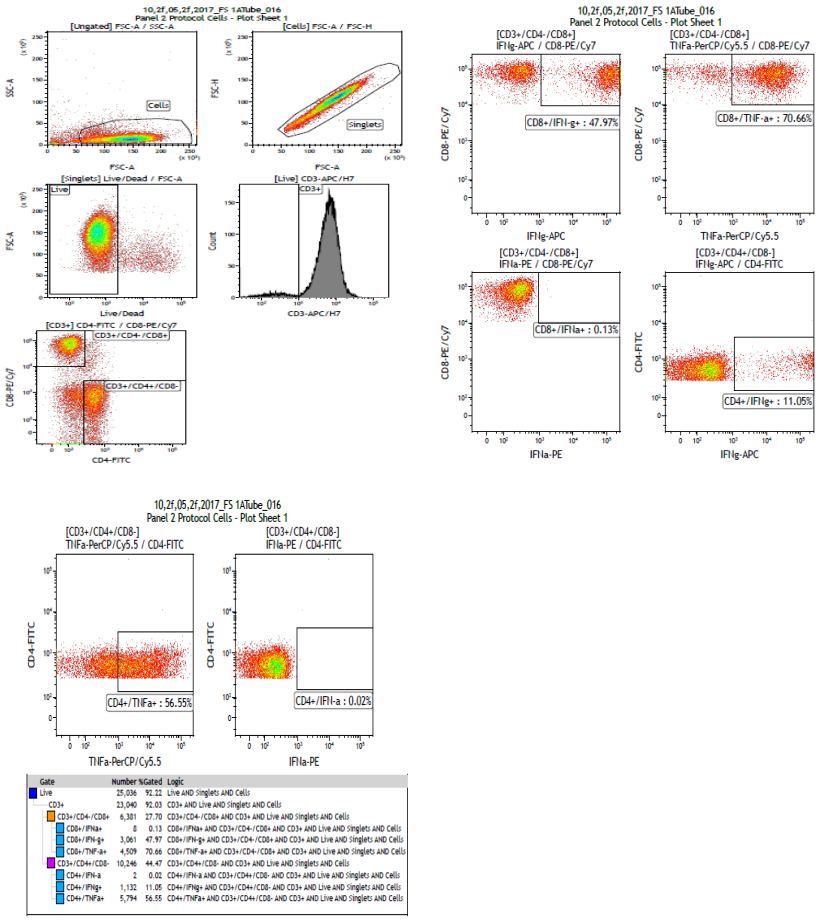


Figure 38 Fully Stained (FS) patient sample, Panel 2

For panel 3, the same protocol as panel 2 was used to isolate CD4+ and CD8+ cells, the next gates were used to identify CD4+IL10, CD4+IL4, CD4+IL17, CD8+IL10, CD8+IL4 and CD8+IL17. The statistical analysis on Kalusa was used to calculate the number of live cells, and number and proportions of CD4+IL10, CD4+IL4, CD4+IL17, CD8+IL10, CD8+IL4 and CD8+IL17.

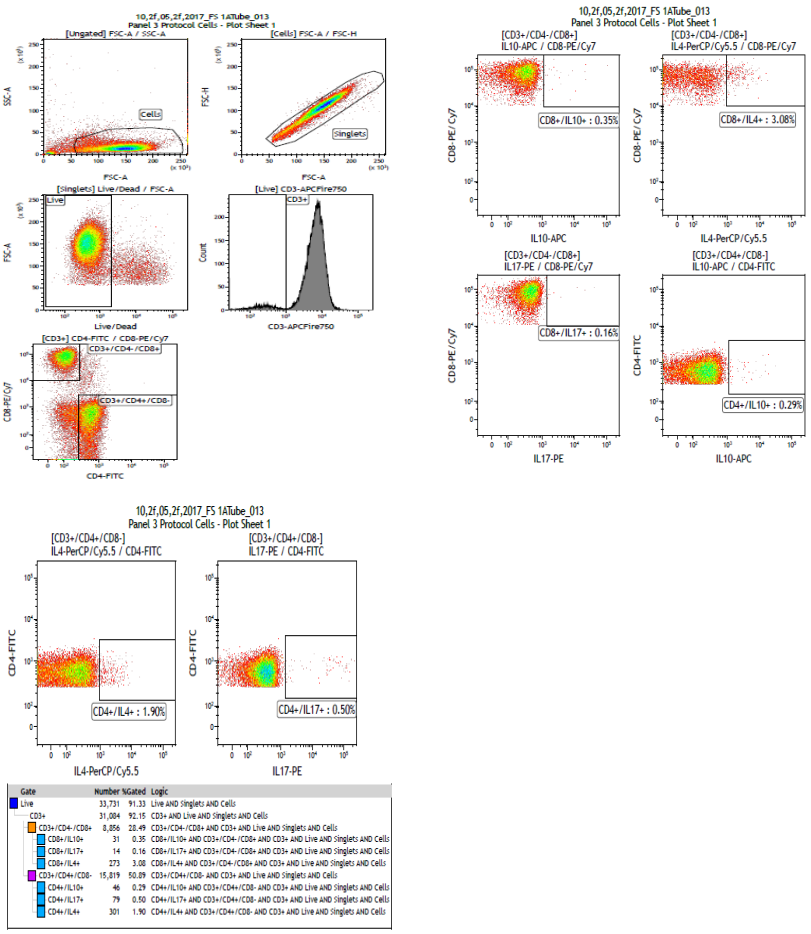


Figure 39 Fully Stained (FS) patient sample, Panel 3

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