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MULTIFOCAL MOTOR NEUROPATHY:
A TRIAL OF THERAPEUTIC COMPLEMENT INHIBITION, AND INVESTIGATION OF SEROLOGICAL FACTORS

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A thesis submitted in fulfilment of the requirements of the University of Glasgow for the degree of Master of Research,
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University of Glasgow

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Abstract

Immune-mediated neuropathies cause inflammation of the peripheral nerve, with disruption of the axon, myelin sheath or both. In the acute setting, immune-mediated neuropathy can lead to respiratory muscle weakness, in the group of Guillain-Barré syndrome (GBS). In the chronic setting, immune-mediated neuropathies, which can be sensorimotor (CIDP, MADSAM), sensory predominant (anti-MAG neuropathy, others) and purely motor (MMN), cause permanent and progressive disability and impairment in activities of daily living.

Anti-gangliosides antibodies have been detected with varying frequencies in the immune-mediated neuropathies, with the highest prevalence being anti-GQ1b antibodies in Miller-Fisher syndrome and anti-GM1 antibodies in MMN and AMAN (axonal variant of GBS). There is evidence that the inflammatory potential of these antibodies is reliant upon complement activation, and the resultant formation of the MAC (membrane attack complex). In experimental models of anti-ganglioside mediated neuropathy, inhibition of the complement cascade results in the complete prevention of inflammatory damage, and preserved nerve function.

Multifocal motor neuropathy is a chronic, progressive purely motor neuropathy which causes weakness and wasting. IgM anti-GM1 antibodies are found in between 50 – 80% of affected cases. The only current treatment for MMN is high dose IVIg (intravenous immunoglobulin). The response rate to IVIg is around 80%, and cases who are antibody negative can also respond to this treatment. However, the effect is temporary, and further doses are usually re-administered at around 4 weekly intervals. Since it is a human blood product which is pooled from donated blood products, it is in short supply and does carry some important side effects.

The main focus of this study was to test a novel therapy for immune-mediated neuropathy. The treatment tested was the first complement inhibitor licensed for human use, eculizumab. In this study it has been tested in the treatment of MMN, in patients who may also be receiving treatment with IVIg. The aim was to collect safety information regarding the concurrent use of these biological products, and to test for any neutralising effect between them. Any beneficial effect of complement inhibition in
MMN was investigated by various outcome measures, clinical, functional and electrophysiological.

The results of the clinical trial showed that eculizumab treatment was associated with a higher rate of adverse events, in patients who were or were not receiving IVIg. Most adverse events were mild to moderate in severity, none were unexpected, and more occurred during the induction phase of treatment than during the maintenance phase. The most common adverse event was headache, which 69% of patients experienced at any time. Two thirds of all headaches occurred in the induction phase. IVIg did lower the serum concentration of eculizumab, however eculizumab activity was not compromised.

There were significant changes to subjective scores overall, and some objective scores also displayed significant improvement. However repeated IVIg doses were still required by those who were regularly using it prior to the study, albeit perhaps at slightly longer intervals. Electrophysiology showed small significant improvement in two parameters in keeping with improved nerve conduction. Overall it was felt that complement inhibition was associated with some potential benefits in MMN however did not substitute the therapeutic mechanism of action of IVIg. Aspects of the study design meant that evidence of efficacy could not be concluded from this study, and further trials are necessary to elucidate this.

In addition, this thesis presents a laboratory-based study in which further information about the binding characteristics of the IgM GM1 antibody were sought using different methods than the standard ELISA technique. Using a combinatorial glycolipid microarray, MMN sera were screened against a large range of glycolipid pairs, to test for novel epitopes in the ‘antibody negative’ MMN patients without anti-GM1 antibody. It was found that in patients who did not have an antibody to GM1 or any other single ganglioside on ELISA or microarray, there was presence of an antibody to the glycolipid pair, GM1:GalC. It was shown that the IgM GM1 antibody in MMN is also inhibited from binding to GM1 in a solid phase and live membrane due to the local presence of GD1a. These findings lead to greater understanding of the pathogenesis of MMN and possibility of a more sensitive diagnostic test.
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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APB</td>
<td>abductor pollicis brevis</td>
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<tr>
<td>AchR-Ab</td>
<td>acetylcholine receptor antibody</td>
</tr>
<tr>
<td>AE</td>
<td>adverse event</td>
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<tr>
<td>AIDP</td>
<td>acute inflammatory demyelinating polyradiculoneuropathy</td>
</tr>
<tr>
<td>AMAN</td>
<td>acute motor axonal neuropathy</td>
</tr>
<tr>
<td>CB</td>
<td>conduction block</td>
</tr>
<tr>
<td>CIDP</td>
<td>chronic inflammatory demyelinating polyradiculoneuropathy</td>
</tr>
<tr>
<td>CMAP</td>
<td>compound muscle action potential</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CTB</td>
<td>Cholera toxin B subunit</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMC</td>
<td>Data Monitoring Committee</td>
</tr>
<tr>
<td>DML</td>
<td>distal motor latency</td>
</tr>
<tr>
<td>DP</td>
<td>deterioration point</td>
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<tr>
<td>EMG</td>
<td>electromyography</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EFNS</td>
<td>European Federation of Neurological Societies</td>
</tr>
<tr>
<td>EQ5D</td>
<td>European Quality of life scale</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>FcRn</td>
<td>neonatal Fc receptor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GBS</td>
<td>Guillain-Barré syndromes</td>
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<tr>
<td>HAHA</td>
<td>human anti-human antibody</td>
</tr>
<tr>
<td>HNLPP</td>
<td>hereditary neuropathy with liability to pressure palsy</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin-G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin-M</td>
</tr>
<tr>
<td>IU</td>
<td>intensity units</td>
</tr>
<tr>
<td>IQR</td>
<td>inter-quartile range</td>
</tr>
<tr>
<td>IVIg</td>
<td>intravenous immunoglobulin</td>
</tr>
<tr>
<td>LOS</td>
<td>lipo-oligosaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>MADSAM</td>
<td>multifocal acquired demyelinating sensory and motor neuropathy</td>
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<tr>
<td>MAG</td>
<td>myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MBP</td>
<td>mannose-binding proteins</td>
</tr>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>MFU</td>
<td>mean fluorescence unit</td>
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<tr>
<td>MFS</td>
<td>Miller-Fisher syndrome</td>
</tr>
<tr>
<td>MMN</td>
<td>multifocal motor neuropathy</td>
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<tr>
<td>MSF</td>
<td>muscle strength (force)</td>
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<tr>
<td>NCS</td>
<td>nerve conduction studies</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>9HPT</td>
<td>nine-hole peg test</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ONLS</td>
<td>Overall Neuropathy Limitation Scale</td>
</tr>
<tr>
<td>PNH</td>
<td>paroxysmal nocturnal haemoglobinuria</td>
</tr>
<tr>
<td>PD</td>
<td>pharmacodynamic</td>
</tr>
<tr>
<td>PK</td>
<td>pharmacokinetic</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RI</td>
<td>run-in period</td>
</tr>
<tr>
<td>RID0</td>
<td>run-in day zero</td>
</tr>
<tr>
<td>SEFR</td>
<td>Self Evaluated Functional Rating Scale</td>
</tr>
<tr>
<td>SAE</td>
<td>serious adverse event</td>
</tr>
<tr>
<td>TD</td>
<td>temporal dispersion</td>
</tr>
<tr>
<td>TESS</td>
<td>treatment emergent signs or symptoms</td>
</tr>
<tr>
<td>TP</td>
<td>treatment period</td>
</tr>
</tbody>
</table>
Acknowledgements

Professor Hugh Willison, who supervised all aspects of this project, was a great source of inspiration, encouragement and guidance. Firstly he provided me with great opportunity to run this project, and throughout has nurtured, with patience and understanding, my development as a researcher. He has been, and will remain, a valuable mentor to me in both clinical and academic medicine.

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Finally I am grateful to my close friends and my family, for their providing wonderful perspective from outside the field, and their support and encouragement throughout this period of research.
Declaration of Authorship

I declare that the work presented here is my own, unless where acknowledged otherwise.

Dr Amanda Fitzpatrick, BMedSci (Hons), MBChB

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Chapter 1. Introduction

1.1 Overview of peripheral nervous system

1.1.1 Peripheral nerve structure and function

The peripheral nerves control motor, sensory and autonomic functions of muscles and organs, relaying afferent and efferent information from the brain to the rest of the body, such as the stimulus for muscle contraction or sensation of pain. The peripheral nerves comprise 12 cranial nerves and 31 spinal nerves in pairs. Each nerve is composed of bundles of nerve fibres, which themselves comprise multiple axons lying adjacent to each other. The cell bodies of these axons reside in the ganglia, within the gray matter of the spinal cord (or the brain stem for cranial nerves). The dorsal root ganglion contains the cell bodies of sensory nerves; the anterior horn cell at the ventral root contains the cell bodies of the motor nerves.

With the exception of small (less than 1µm diameter) fibres, all peripheral nerves are myelinated. Myelin is produced by the Schwann cells, a type of glial cell which exists in the endoneurium of the peripheral nerves. Myelin is composed of around 80% protein and 20% lipid, a large amounts of which are glycosylated, that is, they bear sugar moieties on their surface. The major function of myelin is to insulate axons, allowing faster conduction of nerve impulses. At the distal end of the nerve fibre is the interface with the effector tissue. For motor nerves this is called the motor end plate, or the nerve terminal. Here the axons emerge from the myelin-encasing and communicate with the muscle unit by release of neurotransmitter. Each axon stimulates one motor unit within a muscle, and parallel axon firing results in summation of individual motor units contracting simultaneously and thus results in muscle contraction (Shin J. Oh, 2003a).

1.1.2 Blood-nerve barrier

The function of the blood-nerve barrier, similarly to the blood brain barrier, is to protect excitable neural tissue from potentially toxic substances in the circulation, and maintenance of the ionic homeostasis the endoneural space. It is formed by
the specialised endothelium of blood vessels which pass through peripheral nerves, lined by cells that are non-fenestrated, sealed by tight junctions, lined by a basement lamina and coated with negatively charged glycolipids that electrostatically repel many proteins (Kiernan, 1996).

1.1.3 Electrophysiology

Nerve impulses, generated in the cell body, are propagated distally by conduction of action potentials along the axonal membrane, by the depolarisation of the cell membrane, which is driven by movement of sodium and potassium ions. The role of the myelin sheath is to accelerate the journey of electrical charge, by insulating the axon and therefore preventing the repolarisation while the impulse travels its length. There are gaps between the myelinated nerve segments at intervals along the axon, known as the nodes of Ranvier, and at these non-myelinated points the axon then can repolarise. In this way, the nerve impulse jumps from node to node, known as ‘saltatory’ conduction (Shin J. Oh, 2003a).

Nerve conduction and muscle contraction properties are studied in the field of electrophysiology, and the techniques employed are essential tools for the clinical and experimental study of peripheral nerve disease.

1.1.3.1 Nerve conduction studies (NCS)

An electrode is applied to the skin at a known anatomical location of a peripheral nerve route, for example the median nerve at the wrist. A sensor is applied to the muscle supplied by that nerve, in the example of the median nerve (Figure 1.1) that muscle would be the abductor pollicis brevis (APB), and measures the compound muscle action potential (CMAP) voltage (mV) which results from the nerve impulse applied. The distal motor latency (DML) is the time taken for impulse to travel from the distal stimulation point (wrist) to the muscle (APB). By stimulating this same muscle from two separate anatomical points along the nerve (wrist and elbow), the conduction velocity of the nerve segment between those two points can be calculated (Shin J. Oh, 2003b).
To measure nerve conduction velocity of the proximal, and thus inaccessible to testing, nerve segments, the late response, or F-wave, is calculated. Using supramaximal stimulation of the distal nerve segment, the F-wave latency records the time for the impulse to travel from the point of stimulation, proximally to reach the spinal nerve root, and a small reflection of the impulse to travel distally back to the motor end plate. From this the velocity of conduction from the point of stimulation to the spinal cord can be derived (Shin J. Oh, 2003b).

The main objective of nerve conduction studies is to 1) identify the anatomical pattern of nerve dysfunction and 2) differentiate between axonal and demyelinating pathology. NCS features of axonal neuropathy are reduced or absent distal CMAPs, with normal conduction velocity and distal motor latencies. On the contrary, demyelinating neuropathies cause delayed conduction, with prolonged DML, slowed conduction velocities, and prolonged F-wave latencies (Hughes, 2002).

1.1.3.2 Electromyography (EMG)

Using a needle electrode inserted into the muscle belly, muscle activity is recording during at rest and during active contraction against resistance. In the resting phase, any spontaneous (abnormal) activity can be recorded, such as fibrillation, which is a signal of ongoing denervation. In the contraction phase, the motor unit activity can be visualised (and heard) as an interference pattern, comprising characteristic waveforms for each motor unit, repeated with increasing frequency as the force of contraction increases (Shin J. Oh, 2003b).

1.1.4 Classification of peripheral neuropathy

The term neuropathy is generally understood to mean peripheral neuropathy, in which there is disruption of the peripheral nervous system. The clinical features depend on the anatomical and functional distribution of the disruption, and include motor symptoms (weakness, muscle wasting), sensory symptoms (numbness, pain, paraesthesia) and autonomic symptoms (nausea, postural hypotension and urinary retention). The population prevalence of neuropathy is
about 2400 per 100 000 (2.4%), rising with age to 8000 per 100 000 (8%) (Martyn et al., 1997).

Conditions affecting the peripheral nerves can be classified by different features (Poncelet, 1998)

1) Type of nerve involved: purely motor, purely sensory, sensorimotor, or autonomic.

2) Sites of nerve damage: proximal, distal, symmetrical, multifocal, mononeuropathy, and polyneuropathy

3) Aetiology: toxic, infective, autoimmune, diabetic, paraneoplastic, nutritional, mechanical, hereditary.

The most common neuropathy in the developed world is diabetic neuropathy, which tends to be a distal symmetrical sensory polyneuropathy (Llewelyn, 1995). Autonomic and cranial (third nerve palsy) neuropathies are also encountered in diabetes. In developing countries, infective causes are prevalent, mostly related to HIV infection (and the medications used to treat HIV), causing a painful distal sensorimotor polyneuropathy (Gonzalez-Duarte et al., 2008). Neuropathy caused by Mycobacterium leprae (leprosy) remains the most common treatable neuropathy in the world, although its prevalence is declining worldwide, the number of new cases worldwide in 2006 was 250,000, mainly found in areas of extreme poverty (Scollard et al., 2006).

1.2 Immune-mediated neuropathy

Immune-mediated neuropathies are characterised by inflammatory pathology, and have an immune-related pathogenesis. Immune-mediated neuropathies can first be divided into acute and chronic.

Acute immune-mediated neuropathy is better known as the Guillain-Barré syndrome (GBS), and is characterised by acute onset symmetrical limb weakness and sensory change, which reaches a nadir within less than 4 weeks (Hadden et al., 1998). Typically, the weakness is ascending, however in the Miller
Fisher variant this is typically descending. The two main disease groups in GBS are AIDP (acute inflammatory demyelinating polyradiculoneuropathy) and AMAN (acute motor axonal neuropathy). The overall incidence of GBS worldwide is rare, at around 1.5/100,000 per year (Alshekhlee et al., 2008), however it is the leading cause of neuromuscular paralysis and is potentially life threatening (mortality 2-3%). Although GBS is self remitting, the average recovery to walking unaided time without treatment is 100 days, and 20% of cases (despite treatment) are left with permanent disability at 2 years (Rees et al., 1998), or can progress to a chronic form.

In the chronic immune-mediated neuropathy group, there are CIDP (chronic inflammatory demyelinating polyradiculoneuropathy), MMN (multifocal motor neuropathy), MADSAM (multifocal acquired demyelinating sensory and motor neuropathy), anti-MAG neuropathy and paraproteinaemic neuropathies.

CIDP is a sensorimotor polyneuropathy which reaches it nadir in more than 8 weeks, and includes progressive and relapsing phenotypes. The prevalence is 3-4/100,000 with equal numbers of men and women affected. Unlike GBS, respiratory muscle weakness is uncommon. Electrophysiology shows widespread features of demyelination. First line treatment in CIDP remains corticosteroids, and between 65 – 95% of patients respond favourably to steroids. Other treatment options are IVIg (intravenous immunoglobulin) and plasma exchange, which all show similar efficaciousness to corticosteroids (van Schaik et al., 2002).

MADSAM, originally known as Lewis-Sumner syndrome, is thought to be a variant of CIDP, and is similar to MMN due to the presence of conduction block in motor nerves. However, in MADSAM there are also significant sensory abnormalities (Verschueren et al., 2005).

Anti-MAG neuropathy is characterised by a slowly progressive sensory neuropathy, with an upper limb tremor and unsteadiness of gait. It is usually associated with IgM paraprotein directed against myelin-associated glycoprotein (MAG). Treatment with IVIg, plasma exchange and corticosteroids have not
shown great benefit, however recently rituximab has shown significant short and long-term benefit (Dalakas, 2010).

1.3 Multifocal Motor Neuropathy

1.3.1 History

It is impossible to explain the background to MMN without introducing the concept of conduction block. The phenomenon, of inability of nerve impulse propagation beyond a certain point in the nerve, has been described in nerves following trauma since around 1860, and experimentally reproduced (using tourniquets) since 1944, following which electrophysiology of conduction block was investigated (Trojaborg, 1978). It was initially thought that conduction block was a reversible lesion which resolved in less than 2 months, however later studies described conduction block lasting months following trauma (Harrison, 1976; Trojaborg, 1977), and at common compression points, in the condition now known as ‘hereditary neuropathy with liability to pressure palsies’ (HNLPP) (Magistris et al., 1985).

In succession, came the discovery of conduction block in motor nerves of patients who were thought to have a lower motor neuron variant of motor neurone disease (MND) (Roth et al., 1986). Various neurologists in the late 1980’s described similar findings, where conduction block of motor, but not sensory nerves, was found in patients presenting with asymmetrical, predominantly upper limb weakness and wasting, and absence of sensory symptoms (Parry et al., 1988). Initially it was presumed that this was a motor-predominant variant of CIDP, but when treatment with high dose corticosteroids, known to improve CIDP, failed to show benefit in this motor-variant, it was suspected that this disease was in fact a separate entity (Pestronk et al., 1988). In addition, immunological testing of patients with this condition showed that IgM antibodies against GM1 and other gangliosides existed in the majority of cases (50 – 80%). Since then hundreds of cases of MMN have been described, however it remains rare, with an estimated prevalence of between 1 to 2 per 100,000 population.
1.3.2 Clinical features

Multifocal motor neuropathy (MMN) is distinct from other chronic immune-mediated neuropathies, due to its characteristic clinical and electrophysiological features. Distal motor nerve paralysis usually involves the upper limbs initially, with a stepwise, asymmetric progression to involve motor nerves at other sites. Muscle atrophy occurs later in the course of the condition and usually involves distal small muscles first (Biessels et al., 1997; Bouche et al., 1995; Krarup et al., 1990). Sensory nerves are rarely affected although some patients may have minor sensory symptoms (Krarup et al., 1990). Conduction block across a motor nerve segment is the electrophysiological hallmark, and essential criterion for diagnosis (Bouche et al., 1995; van Schaik et al., 2006).

1.3.3 Electrodiagnostic criteria

As described in the previous section, nerve conduction studies are used to measure the velocity of nerve impulses, and the magnitude of effector response in the muscles innervated by motor nerves. Figure 1.1 demonstrates the electrophysiological phenomenon of conduction block, where stimulation of the median nerve at the wrist results in a CMAP in the APB muscle of the hand, however when the median nerve is stimulated more proximally, no CMAP is detected in the APB muscle. This means that between the stimulation points at the wrist and the elbow, conduction block exists, and in the case illustrated, this appears to be an almost 100% diminution. The degree of conduction block is expressed as the % CMAP amplitude (or area) reduction, calculated by ((distal CMAP – proximal CMAP/distal CMAP) x 100%).
Figure 1.1 Motor nerve conduction study showing conduction block in the right median nerve (left panel), and a schematic drawing (right) showing the anatomical origin of this. Nerve stimulation at the anatomical locations of the median nerve 1) wrist 2) elbow and 3) axilla, and the corresponding CMAP recorded from the abductor pollicis brevis muscle in the hand. The CMAP amplitude is reduced to almost zero when the nerve is stimulated at the elbow, showing that conduction block occurred in the median nerve between the wrist and the elbow. The CMAP recorded from stimulation at the axilla is temporally dispersed and therefore no statement regarding conduction block can be made about this nerve segment, although this dispersion in itself may represent demyelination in this nerve segment.

American (Olney et al.,2003) and European (van Schaik et al.,2006) consensus criteria exist for the diagnosis of MMN, and the electrodiagnostic criteria differ slightly in the definition of ‘probable’ conduction block, but are otherwise similar. Figure 1.2 presents the electrodiagnostic criteria of the European Federation of Neurological Societies (EFNS), originally released in 2006. In nerves where there is significant temporal dispersion, there can be the mistaken appearance of conduction block due to interphase cancellation, and therefore at the most ‘probable’ CB can be diagnosed from such a nerve.
Figure 1.2. Electrodiagnostic criteria in MMN, adapted from European Federation of Neurological Societies/Peripheral Nerve Society guideline on management of multifocal motor neuropathy, 2006 (van Schaik et al., 2006).

<table>
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<tr>
<th></th>
<th>Negative CMAP area reduction</th>
<th>Negative CMAP duration (temporal dispersion)</th>
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<tr>
<td><strong>Definite Conduction Block</strong></td>
<td>≥50%</td>
<td>≤30%</td>
</tr>
<tr>
<td><strong>Probable Conduction Block</strong></td>
<td>≥30%</td>
<td>≤30%</td>
</tr>
<tr>
<td>or</td>
<td>≥50%</td>
<td>≥30%</td>
</tr>
</tbody>
</table>

**Additional criteria**
1. Negative CMAP amplitude on stimulation of the distal part of the segment with motor CB must be >20% of the lower limit of normal and >1 mV (baseline negative peak).
2. Evidence for CB must be found at sites distinct from common entrapment or compression syndromes.

There is some current debate about threshold for diagnosis of CB, as there are some case reports of patients in whom clinical evidence of MMN was not accompanied by conduction block adequate to qualify for probable CB, until later years when the disease progressed, and thus treatment has been delayed (Ghosh et al., 2005). In addition, there are cases where MMN is so advanced at presentation, that conduction block may be imperceptible, due to the degree of axonal degeneration, such that there is no appreciable difference between proximal and distal CMAP.
1.3.4 Pathology

The pathology of MMN is still largely unknown, and further work is being carried out to elucidate the pathophysiology. However, it has been classed as an immune-mediated neuropathy, and thought to be antibody mediated, mainly due to the clinical response in MMN to immune-modulating therapy with high dose immunoglobulins, the discovery of anti-GM1 antibodies in the majority of cases, and the evidence (explained further in this text) that anti-GM1 antibodies can cause conduction block in experimental models.

Multifocal motor neuropathy has originally been thought of as a demyelinating disorder, due to the initial pathological studies which showed demyelination at sites of conduction block. However, many studies have shown that axonal degeneration is an important feature, and is an independent predictor of weakness (Van Asseldonk et al., 2006). Until recently, no convincing histopathological studies existed in MMN, as nerves had been sampled outside the area of conduction block, and were relatively normal. However, Taylor et al performed nerve biopsy in 8 patients with MMN using intraoperative nerve conduction studies to localise the site of CB (Taylor et al., 2004). The predominant abnormality found was multifocal axonal degeneration. There was also some minor evidence of remyelination, however no overt features of demyelination. This is a surprising finding given that that conduction block is thought to be a feature of demyelination. Further pathophysiological studies are required.

1.3.5 Treatment of MMN

The finding of anti-ganglioside antibodies in MMN led to trials using immune modulating treatments. Intravenous immunoglobulin (IVIg) is pooled IgG extracted from the plasma of over one thousand blood donors. It is the gold-standard of treatment of MMN, and has been shown to cause significant muscle strength improvement in up to 80% of patients (Azulay et al., 1994; Leger et al., 2001; Van den Berg et al., 1995). However, its effects are short-lived and maintenance treatment is required. Maintenance regimes are individually tailored,
with most patients requiring cyclical dosing at 3-4 weekly intervals. Despite long
term therapy, disease progresses over time causing worsening disability
(Terenghi et al., 2004). All immunomodulating therapies were evaluated in a
recent Cochrane review (Umapathi et al., 2009). Corticosteroids and plasma
exchange are ineffective in MMN (Lehmann et al., 2008; Van den Berg et
al., 1997), and trials of various immunomodulatory therapies, including rituximab,
mycophenolate and interferon-gamma, have not shown sufficient benefit to allow
adoption in clinical practice. In some cases, corticosteroids have been shown to
cause drastic worsening of weakness (Donaghy et al., 1994) and are therefore
avoided in MMN.

1.3.6 Anti-ganglioside antibodies in MMN

Anti-ganglioside antibodies are widely thought to be responsible for the
pathogenesis in MMN, and IgM antibodies to GM1 ganglioside in particular are
found in between 50 – 80% of cohorts published. The finding of anti-GM1
antibody is supportive for the diagnosis, but its absence does not rule out the
disease.

The discovery of anti-GM1 antibody in MMN came parallel with the discovery
anti-ganglioside antibodies in other autoimmune neuropathies, and accumulating
evidence for their pathogenicity.

1.4 Anti-ganglioside antibodies in the pathogenesis of immune-mediated
neuropathy

1.4.1 Gangliosides

Gangliosides are glycosylated lipids, with a ceramide (lipid) backbone attached to
an oligosaccharide of varying structure, to which are attached sialic acid
residues. The family of gangliosides are named according to the Svennerholm
short-hand nomenclature system, in which M, D, T and Q refer to the number of
sialic acid residues i.e. mono-, di-, tri- and tetrasiialogangliosides, respectively, and the numbers 1, 2, 3 refer to the order of migration of the gangliosides on thin-layer chromatography. Figure 1.3 shows the basic structure of the most common gangliosides in the human nervous system. More than 100 different gangliosides have been characterised within vertebrates alone. Gangliosides are known to be abundant in the central and peripheral nervous system, where their main role is in cell signalling and adhesion, through which they regulate neural development and regeneration.

![Figure 1.3. Basic structure of major neural gangliosides. NeuAc is sialic acid, GalNAc is N-acetylgalactosamine.](image)

**1.4.2 Anti-ganglioside antibodies in neuropathy**

Anti-ganglioside antibodies are most firmly linked to pathogenesis of neuropathy in the Miller-Fisher syndrome (MFS), an acute, self-remitting variant of Guillain-Barré syndrome which manifests with acute onset of ophthalmoplegia, ataxia, and areflexia, and results in a descending paralysis (Fisher, 1956). Anti-GQ1b antibodies are detected in upwards of 90% of cases of this condition (Willison et al., 1993), with complete absence of anti-GQ1b IgG antibodies from normal and other disease control groups, indicating a high level of specificity for this disease. Additional evidence of this antibody as the pathogenic mediator is that antibody titres peak at clinical presentation, and decay rapidly with the course of clinical recovery (Mizoguchi, 1998). Also, ophthalmic nerves are known to be rich in GQ1b ganglioside (Chiba et al., 1997).
Further evidence of antiganglioside antibodies in neuropathy comes from the association between AMAN variant of Guillain-Barré syndrome and IgG antibodies to GM1, and GD1a; which are shown to exist preferentially in this variant rather than AIDP (Ho et al., 1999; Kuwabara et al., 1998). In cohorts of Guillain-Barré syndrome, the presence of IgG anti-GM1 antibodies is strongly correlated with axonal versus demyelinating neuropathy, predominant motor involvement, more severe weakness and previous infection with Campylobacter jejuni. The association with C. jejuni infection led to the theory of ‘molecular mimicry’ (Yuki et al., 2007), which there now exists substantial evidence regarding its major aetiological role in axonal variants of Guillain-Barré syndrome. ‘Molecular mimicry’ describes the phenomenon where antibodies which develop during infection with C. jejuni, can cross-react with GM1 ganglioside, and therefore act as an autoantibody. The C. jejuni and other gram-negative organisms, bear an external endotoxin, lipo-oligosaccharide (LOS), and these LOS have been shown to have ganglioside-like regions (Godschalk et al., 2007). Other infections which are linked with GBS are Mycoplasma pneumoniae, cytomegalovirus (CMV), Epstein-Barr virus (EBV), and more rarely influenzas (Jacobs et al., 1998). These organisms all bear carbohydrate structure on their surfaces which resemble peripheral nerve gangliosides (Yu et al., 2006), and there are some associations between certain antecedent infections and certain autoantibodies, for example there is a correlation between CMV antibodies and GM2 antibodies in serum of patients with GBS (Irie et al., 1996).

1.4.3 Evidence of pathogenic potential of anti-ganglioside antibodies

The pathogenic potential has been demonstrated in animal models for the major antibody-disease associations. Santoro et al showed this for anti-GM1 in 1996, by injecting the serum from a patient with MMN into rat sciatic nerve. The serum caused conduction block and pathological features of demyelination, in a complement dependent manner, where IgM and complement were localised to the nodes of Ranvier (Santoro et al., 1992). Rabbit models, inoculated with a bovine brain ganglioside mixture or isolated GM1, developed high anti-GM1 IgG antibody titres and acute flaccid paralysis (Yuki et al., 2001). Anti-GQ1b
antibodies, in an *in vitro* murine model of neuropathy, caused complement-mediated injury of peri-synaptic Schwann cells and caused disruption of pre-synaptic motor nerve terminals at the neuromuscular junction (*Halstead et al., 2004*). Numerous other *in vitro* and *in vivo* studies demonstrate that anti-ganglioside antibodies can mediate, in the presence of complement, impairment of nerve conduction and pathological changes of neuropathy (*Goodyear et al., 1999; Greenshields et al., 2009; O’Hanlon et al., 2003; Yuki et al., 2001*).

### 1.4.4 Complement system

The complement system a key component of the innate immune system, and functions to aid antibodies in immune clearance of pathogens and promote inflammation (*Walport, 2001*). It consists of over 30 proteins and pro-proteins, which are synthesised in the liver, and enzymatically activated in the serum in a sequential cascade-like manner.

The cascade consists of three pathways that are initiated following recognition of different antigenic targets, the classical pathway recognises antibody-antigen complex, the alternative pathway recognises C3b binding to cell membranes, and the lectin binding pathway which recognises mannose-binding proteins (MBP) on microorganisms.

The classical pathway begins with the activation of C1q via binding to the Fc region of the bound IgG (or IgM). This induces a conformational change in C1 and the systematic activation of proteins occurs. C3 is cleaved to C3a and C3b, which themselves have important roles in innate defence: C3a triggers mast-cell degranulation, and C3b is a potent opsonising agent. The next important step, in which C3b is vital, is the formation of C5 convertase, which binds to and cleaves C5 to C5a and C5b. From here the pathway is known as terminal complement, and comprised of proteins C5b – C9, which upon activation, assemble to form a pore-like structure, the membrane attack complex (MAC) (*Muller-Eberhard, 1985*). MAC inserts itself into the target cell membrane causing osmotic lysis, by the rapid influx of extracellular fluids, disruption of the ionic
gradient across the membrane, rendering the cell or pathogen destructed (Janeway CA, 2001).

Hereditary complement deficiencies are described for many of the complement factors, and result in an increased susceptibility to infections, in particular, from encapsulated bacteria. Bacteria which are protected by a polysaccharide capsule, including Neisseria meningitidis, Streptococcus pneumoniae, and Haemophilus influenzae, must be lysed by MAC for their destruction. In the absence of terminal complement activation, increased susceptibility to infection with encapsulated bacteria is seen (Figueroa et al., 1991).
**1.4.5 Complement as a therapeutic target in autoimmune neuropathy**

Complement fixation plays a major role in the antibody-mediated defence mechanism, via the classical pathway of complement activation, and has been shown to be active in many autoimmune diseases including renal, vascular, neurological disease.

With the knowledge that MAC could be detected in the target tissue of a variety of immune-mediated conditions such as polymyositis ([Morgan et al., 1984; Vanguri et al., 1988](#)), research has been directed towards demonstrating the complement-fixing properties of the antibodies found in these conditions ([Latov et al., 1981](#)). Much of the progress in this field has come from the study of neuropathies where antibodies have been isolated.

In the case of anti-ganglioside antibody mediated neuropathy, antibody and MAC deposits are found at nerve terminals and nodes of Ranvier of motor axons following anti-ganglioside antibody-induced damage with anti-GM1 ([Greenshields et al., 2009; Susuki et al., 2007; Uetz-von et al., 1998](#)) , anti-GD1a ([McGonigal et al., 2010](#)) and anti-GQ1b antibodies ([Halstead et al., 2004; Halstead et al., 2005b](#)). Pathogenic effect of these antibodies was shown to be completely inhibited in experimental animal models by the administration of terminal complement inhibitors including APT070 and rEV576 ([Halstead et al., 2005a; Halstead et al., 2008a; McGonigal et al., 2010](#)), both in pathological and electrophysiological parameters.

Recently, Halstead et al developed an in vivo mouse model of acute neuropathy, by the injection of anti-GQ1b antibody. These mice developed respiratory paralysis due to impaired phrenic nerve conduction, however the administration of eculizumab, an inhibitor of terminal complement, completely abrogated the paralysis ([Halstead et al., 2008b](#)).
1.5 **Eculizumab**

Eculizumab is the first targeted complement inhibitor licensed for use in humans.

It is a humanised IgG monoclonal antibody which specifically inhibits activation of the terminal complement system by binding to C5, thus preventing its cleavage into C5a and C5b, resulting in failure of MAC pore assembly. Since it acts at C5, it inhibits terminal complement regardless of the pathway of activation, and it leaves proximal complement, namely proteins C3a and C3b, preserving innate immunity.

Eculizumab was developed and tested mainly in the complement driven haematological disorder paroxysmal nocturnal haemoglobinuria (PNH) (*Rother et al.*, 2007). PNH is not an intrinsic autoimmune disorder, but is a condition caused by imbalanced complement activation at red cell surfaces. This occurs due to a genetic lack of the enzyme needed to make GPI anchor-protein, whose function is to anchor complement regulators, CD59 and CD55, to the cell surface, preventing inappropriate complement activation (*Parker et al.*, 2005). Uncontrolled MAC deposition on red blood cells leads red cell destruction and chronic anaemia in PNH. The only curative option is allogenic stem cell transplantation, and prior to trials of eculizumab, patients were managed by recurrent blood transfusions. In two randomised control trials of eculizumab in a total 184 patients with PNH, the median yearly blood transfusion requirement reduced from 10 to 0 with eculizumab treatment (*Brodsky et al.*, 2008; *Hillmen et al.*, 2006), and improvements were seen in fatigue and quality of life scores.

Eculizumab lends itself well to trials in other complement-mediated conditions, and recent efficacy has been presented in some case reports in haemolytic-uraemic syndrome associated with renal transplant (*Chatelet et al.*, 2010; *Zimmerhackl et al.*, 2010), results from larger trials for this indication are awaited.

A substantial amount of safety data exists for eculizumab, since approximately 820 unique patients to now have been exposed to eculizumab, in dosing
regimens ranging from one day to almost six years, over a range of conditions including renal transplant, idiopathic membranous glomerulonephropathy, rheumatoid arthritis, psoriasis, and asthma (Alexion Pharmaceuticals, 2010).

The most serious risk associated with eculizumab therapy is meningococcal septicaemia. Since eculizumab inhibits terminal complement, defence against encapsulated bacteria is reduced whilst on treatment. There have been three reported cases of Neisseria meningitis infection, and one of these was in an unvaccinated patient (Alexion Pharmaceuticals, 2010). This roughly equates to an incidence of 0.3% whilst on eculizumab.

Other potentially life threatening risk of eculizumab treatment relates to immunogenicity of monoclonal antibodies. Although eculizumab is humanised and therefore less immunogenic than murine antibodies, immune-mediated drug reactions can occur. The incidence of severe infusion reaction in eculizumab is estimated at around 8%, and in most cases successfully managed with pre-medication (corticosteroid) and slowing the rate of infusion (Dmytrijuk et al., 2008). Drug tolerance due to development of HAHAs (human anti-human antibodies) are low in frequency (3%) with eculizumab treatment and considered to be not clinically significant as similar frequency is of HAHA found in placebo.

Other, less serious side effects are more common, including headache, nasopharyngitis, back pain, nausea, and upper respiratory tract infections. In a large open-label study of 97 PNH patients receiving eculizumab, headache was the most common side effect, occurring in up to 56% of patients at the beginning of eculizumab treatment, and reducing to around 15% after 6 months of treatment (Brodsky et al., 2008). Further analyses of safety data show that the incidence of headache during the maintenance phase with eculizumab was the same as with placebo treatment and represents a 50% reduction as compared to the induction phase.

Eculizumab has not yet been given in patients also receiving intravenous immunoglobulin, and safety data does not exist for this.
1.6 Research aims

The major research aim of the project was to conduct the first trial of a complement inhibition in humans with an immune-mediated neuropathy. The complement inhibitor chosen was eculizumab, which is the first inhibitor of systemic terminal complement to be licensed for human use, and is now used widely in the treatment of paroxysmal nocturnal haemoglobinuria. A substantial amount of safety data exists for eculizumab, however, no trial has yet been published of terminal complement inhibition in any neurological condition, or of the co-administration of eculizumab with high-dose IVIg.

Therefore, the aim of this study was not to determine the general safety of the eculizumab, but to discover any additional safety information arising from its administration in patients with immune-mediated neuropathy, or from its co-administration with high dose intravenous immunoglobulin. For this reason an open-label, pilot study design was chosen as the initial trial design.

Multifocal motor neuropathy was chosen as the neuropathy to study as firstly there is clear evidence of a pathogenic antibody which is complement-fixing in neuropathy models, secondly it is a chronic condition which (in contrast to acute neuropathy) should be relatively stable over time and therefore lend itself study of intervention, and thirdly, patients are already known to be immune-responsive in MMN due to their responsiveness to IVIg.

Secondary aims of this clinical trial were to discover any beneficial therapeutic effect by inhibiting complement in MMN. This effect would be measured by the reduced requirement for IVIg therapy, and by the clinical and functional outcome measures employed.
2 Clinical trial: Methods

2.1 Patients

Patients were screened for eligibility from patients known to have immune-mediated neuropathy, who were currently attending the short-stay ward for regular intravenous immunoglobulin, or attending neurology outpatient clinics at the study centre (Institute of Neurological Sciences, Southern General Hospital, Glasgow).

2.2 Inclusion and exclusion criteria

Core criterion for entry to the study were

1) Fulfilment of electrodiagnostic criteria for diagnosis of MMN with conduction block, either with definite or probable conduction block in at least one nerve segment.

2) Documented clinical improvement with intravenous immunoglobulin.

3) Ability to complete weekly self-evaluation functional rating scales.

4) Agreement to be vaccinated against meningococcal disease.

5) Ability to give informed consent.

Core exclusion criteria were

1) Below the age of 18 years old.

2) Pregnancy, planned pregnancy or lactation.

3) Inability to comply with study related procedures or appointments.

4) Unresolved Neisseria meningitidis infection or history of meningococcal infection.

5) Known complement deficiency.

6) Any significant medical co-morbidity which was deemed to make the patient unsuitable for the trial.

Patients who met the criteria above were invited to join the trial, given verbal and written information (see Patient Information leaflet, appendix 1). They were
contacted at least 2 weeks following this to have further discussion, and then proceeded to give written consent.

2.3 Study drug

Eculizumab (Soliris™) was supplied by Alexion Pharmaceuticals, Cheshire USA, as a clear liquid in 300mg/30ml vials. The required dose was made up in 0.9% saline (Baxter™) to 5mg/ml, and infused via peripheral venous cannulae at a rate of up to 4ml (20mg) per minute. When eculizumab and IVIg were given on the same day, eculizumab was administered first, with a 2 hour interval before IVIg was given. Clinical trial nurses were employed to record patients’ vital signs before and during the infusion. Any signs of infusion reaction (hypotension, tachycardia, pyrexia, flushing, rash, bronchoconstriction, facial oedema, and stridor) were managed by stopping the infusion, clinical assessment of the patient, and administration of corticosteroids and antihistamine. The following dose could be given as scheduled if deemed safe by the investigator, but at a slower infusion rate, and following premedication with corticosteroids and antihistamine.

2.4 Study design

This was an open-label observational study. There were three trial periods, comprising an initial run-in period of maximum length 8 weeks, a 14 week treatment period, and an 8 week run-out period (Figure 2.1). At enrolment all patients were vaccinated with tetravalent meningococcal vaccine (ACWY Vax®, Glaxo Smith Kline).
The run-in period began on the date of a scheduled IVIg infusion, and for patients who were not receiving IVIg, at a mutually convenient date. On run-in day zero (RID0), full past medical history, clinical examination (vital signs, cardiovascular, respiratory, abdominal and neurological exam), and current medications were recorded in the patient clinical trial record. Clinical assessments (Table 2.1) were carried out in all patients. Electrophysiology was also performed at run-in day zero. IVIg infusion (Kiovig™) at dose of 1g/kg over 2-5 days was carried out as usual for each patient who already received IVIg.

### 2.5 Clinical assessments

Various trial assessments were employed. These were measured in all patients at baseline, treatment weeks 0, 4, 8 and 14, and finally at run-out week 8. Additional assessments were carried out in those patients receiving IVIg, as outlined below.
Muscle strength was assessed by two means, firstly a qualitative assessment using the widely clinically practiced MRC score. Five muscles or muscle groups, each from two affected limbs, were chosen and scored from 0 (no visible contraction) to 5 (full active power against resistance), giving a total maximum MRC sum score of 50. The 10 muscles or muscle groups were kept constant for each patient throughout each trial assessment. Quantitative measurement of muscle strength on a continuous scale was measured by myometry. Five clinically weak (MRC 3 or 4) muscles were chosen from each individual for myometry assessment, to be kept constant for each individual throughout the trial. A hand-held myometer (Lafayette™ manual muscle testing system, model 01163) was used to measure the maximum force (kg) during isometric contraction, using the ‘break’ technique, in which both the tester and subject exerted maximal opposing strength until either the subject or the tester broke the contraction. Three trials in each muscle were recorded.

<table>
<thead>
<tr>
<th>Table 2.1. Clinical Assessments</th>
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<tbody>
<tr>
<td>1. Medical Research Council (MRC) sum score: total of 10 muscle groups from 2 affected limbs</td>
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<tr>
<td>2. Muscle strength force (MSF) sum score: total of 5 muscle groups from 2 affected limbs, using myometry</td>
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<tr>
<td>3. Hand Grip Strength: using hydraulic dynamometer</td>
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<tr>
<td>4. Palm and pinch strength: using vigorimeter</td>
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<tr>
<td>5. Nine-hole peg test (9HPT): time to completion in seconds</td>
</tr>
<tr>
<td>6. 10 metre walk: time to completion in seconds</td>
</tr>
<tr>
<td>7. Self Evaluated Functional Rating Scale (SEFR)</td>
</tr>
<tr>
<td>8. Overall Neuropathy Limitation Scale (ONLS)</td>
</tr>
<tr>
<td>9. European Quality of life scale (EQ5D)</td>
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</table>
Overall hand grip strength was measured in kilograms using a hydraulic hand dynamometer (Jamar™). In addition, various-sized rubber balloons attached to a barometer (vigorimeter) were used to measure maximum palm and pinch strength (kiloPascals) in each hand.

The nine-hole peg test comprised nine wooden pegs which were placed in corresponding holes in a wooden block. The time to completion of the test was recorded from contact with the first peg until the ninth peg was securely placed. Again, three trials each side were recorded at each assessment. For ease of interpretation, and to accommodate those who could not complete the test, this was converted to speed, expressed as percentage of test completed per second (100/seconds).

The ten-metre timed walk was performed on a flat level surface using the patient’s usual walking aid (if any). This was also converted to speed, as percentage of test completed in one second (100/seconds).

Quality of life scale (EuroQOL™ EQ5D, 1995) was used, which comprises a visual analogue scale from 0% (worst imaginable health) to 100% (best imaginable health), and a descriptive system which scores on five dimensions of living: mobility, self-care, usual activities, pain/discomfort, anxiety/depression, giving a score between 0 and 1, where 1 is the value for full health. The overall neuropathy limitation scale (ONLS) measured the limitation of activities of daily living usually affected by neuropathy, and provided a total score from 0 (no limitation) to 12 (full limitation, bed bound).

The self evaluated functional rating (SEFR) score was central to the longitudinal assessment of patients throughout the trial. At run-in day zero the patient and the investigator chose 5 tasks of daily living that were affected by the condition, for example, using knife and fork together or lifting cup with left hand. These tasks were graded from 0 (normal) to 5 (impossible) based on the level of difficulty with this task. Pre-printed SEFR score sheets were completed at the outset and tasks remained constant for the duration of the trial.
Typically MMN patients, who are on maintenance IVIg regimes, experience a cyclical change in muscle strength due to the half life of IVIg, with an improvement in weakness 5 to 10 days after infusion, plateau of increased strength for some weeks, then subsequent decline heralding requirement for further IVIg. The aim of using the SEFR score was to measure this effect, and allow its communication, in a standardised format, from the patient to the study team. It was expected that the SEFR score would be highest prior to IVIg dosing (indicating highest level of difficulty with chosen tasks), and lowest mid-cycle.

SEFR score sheets were collected by the investigator weekly, by fax, email or completed over the telephone.

2.6 Deterioration point criteria

For patients receiving IVIg, the run-in period length was determined by reaching the deterioration point.

Deterioration point criteria were defined (Table 2.2), the primary criteria being an increase in the SEFR score by at least 2 points above the baseline (RID0) score. This triggered clinical examination by the investigator, and if further deterioration point criteria were fulfilled, IVIg dose was scheduled. At this point, patients then entered the treatment period, and the first eculizumab dose was then given on day 0, prior to IVIg dosing.

<table>
<thead>
<tr>
<th>Table 2.2. Deterioration Point (DP) Criteria</th>
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<tbody>
<tr>
<td>1.  SEFR score increase by 2 points from baseline Plus at least one of the following (2) to (6)</td>
</tr>
<tr>
<td>2.  MRC sum score decrease by at least 1 point</td>
</tr>
<tr>
<td>3.  Pinch/palm grip decrease by at least 10% (either side)</td>
</tr>
<tr>
<td>4.  9 hole peg test time increased by at least 10%</td>
</tr>
<tr>
<td>5.  10m walk time increase by at least 2 seconds</td>
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<tr>
<td>6.  Patient/clinician feels deterioration has occurred</td>
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</table>
Patients not receiving IVIg also completed weekly SEFR score sheets, however did not require to reach deterioration point prior to entering the trial, all completed the maximum 8 week run-in period.

2.7 Electrophysiology

Motor nerve conduction studies and electromyography were carried out in at least one affected nerve segment and muscle group, at baseline (RID0) and again at the end of the treatment period. The trial neurophysiologist was not involved in any other trial assessments. Data for distal latency (DL), compound muscle action potential (CMAP) amplitude and duration, conduction velocity, and F-wave latency were collected. Needle electromyography recordings (videos) of voluntarily contracting muscle activity were blindly scored by a panel of five qualified neurophysiologists, to assess any difference in the motor unit recruitment density between baseline and the end-of-treatment period. The recordings were presented in pairs (pre and post treatment) and assessors were blinded to the patient details and the ordering of the recordings. This resulted in a total score of minus 5 (all 5 examiners scored the post-treatment EMG less dense than the pre-treatment) to plus 5 (all 5 examiners scored the post-treatment EMG more dense than the pre-treatment).

2.8 Pharmacological tests

Blood samples were collected at run-in day 0, then prior to every eculizumab dose (trough), 1 hour post dose (peak) and if IVIg was also given at same session, 1 hour into IVIg dose. Samples were immediately centrifuged at 3000 rpm at room temperature for 10 minutes, the resultant supernatant was decanted into cryotubes, and stored at -80°C until being shipped to Alexion laboratories, Cheshire USA. Here pharmacokinetic (PK) and pharmacodynamic (PD) assays were performed. The PK assay measured serum eculizumab concentration (µg/ml), while the PD assay measured serum haemolytic activity (% of chicken
red blood cells lysed by serum). In addition, serum was collected for detection of human-anti-human antibodies (HAHAs).

### 2.9 Detection of anti-ganglioside antibodies

Enzyme-linked immunosorbent assay (ELISA) was used to detect anti-glycolipid antibodies to GM1, GM2, GD1b and GA1 in patient serum prior to trial commencement. These were not re-measured at trial completion. For ELISA methodology see section 5.2.1.

### 2.10 Adverse events and safety reporting

At each scheduled trial visit, patients were asked to report any symptoms, medical encounters or changes to medications. During the treatment period, adverse event (AE) data was recorded weekly for the first 4 weeks, then fortnightly. During run-in and run-out periods, the AE collection interval varied between patients according to their visits, up to a maximum interval of 8 weeks from the previous visit. Adverse events were graded as mild, moderate or severe, and their attribution to study drug graded 0 (unrelated), 1 (unlikely), 2 (possibly), 3 (probably), 4 (definitely). Serious adverse events (SAEs) were reported within 24 hours to the local Pharmacovigilance office and to the Data Monitoring Committee (DMC). Adverse events were defined as serious if they were fatal, resulted in or lengthened hospital admission, led to persistent or severe disability, congenital anomalies of birth defects.

At enrolment, patients were issued with a detailed safety information card, which described the study and “alert” symptoms for meningococcal infection. Patients were counselled about the increased risk of developing meningococcal septicaemia whilst on eculizumab, and were asked to carry this safety card with them at all times during the treatment and run-out period. Alert symptoms were moderate to severe headache with nausea or vomiting, stiff neck or stiff back, fever, rash, confusion, severe myalgia with flu-like symptoms and photophobia. Clear instructions were given that patients should be seen by a physician at their
local emergency department without delay. 24 hour emergency contact numbers of the trial doctors were listed on this card, for specialist advice.

2.11 The role of the author

My involvement in this clinical trial began following acceptance of the trial protocol by the ethics panel, therefore I did not contribute to the original protocol design. However, my role began by putting into effect the approved protocol. I screened and recruited patients, designed the data collection sheets for the clinical research files, liaised with research and enterprise, pharmacy and NHS staff and briefed colleagues and the clinical research nurses which were allocated to the study.

Following recruitment, I collected all the baseline clinical data from patients. Thereafter, at each trial visit, I examined each patient as set out in section 2.5. Some of the timed assessments were also performed by the clinical research nurse(s), whose main roles were preparation the drug for intravenous infusion, performing measurements of vital signs (pulse, BP and temperature), logging information essential to drug accountability, and assisting with data collection and adherence to GCP (Good Clinical Practice) guidelines.

At each trial visit I collected information about any adverse events and made clinical assessments as indicated. I then evaluated the potential causal relationship between study drug and adverse effect. Due to the increased risk of meningococcal septicaemia I was ‘on-call’ for patient queries, and they carried a safety card with my 24 hr contact number, so that themselves or any doctor treating them in an emergency could contact me for advice about suspected adverse reaction.

Blood tests were taken at numerous time points which I then centrifuged in the lab to decant the serum for storage. Following completion of the trial period, I sent all the data to the Robertson Centre for Biostatistics, who performed data cleaning and analysis. At this stage my involvement was dealing with data queries and liaising with the trial statistician about the analyses to be undertaken.
Following this, I wrote the trial up for publication (see Appendix 2) and presented the findings at local, national, and international meetings.

### 2.12 Ethics and monitoring

The trial protocol and supporting documentation were approved by the regional ethical committee, and conducted in keeping with the Declaration of Helsinki. The trial is registered on EudraCT database (unique no. 2008-005748-18). The study was funded by Alexion Pharmaceuticals however remained an investigator-led trial, co-sponsored by the University of Glasgow and NHS Greater Glasgow and Clyde. A data monitoring committee (DMC) of three independent specialists was formed, who were provided with interim data and safety reports throughout the study. Only the DMC and the investigators had a steering role.

### 2.13 Statistics

Since the study was not designed primarily to test efficacy of this medication, no power calculation was performed, and the number of patients enrolled was merely a convenience sample based on the availability of eligible candidates with the condition.

Individual data was collected on clinical research forms (CRF), and carbon copies were sent to the Robertson centre for Biostatistics (University of Glasgow), then entered into a study-specific database which was maintained by the Biostatistics department.

Myometry or muscle strength/force (MSF) recordings were summed across all 5 selected muscles for each patient to result in a total MSF score. Electrophysiology measurements were transformed to z-scores (excluding conduction block). Z-scores were calculated for each nerve (anatomical location) by subtracting the mean and dividing by the standard deviation. Thus a difference of 1 in z-score is a difference of 1 standard deviation across nerves.
Task scores, speeds or values were summarized by median and inter-quartile range (IQR) values for each time point or period. The Wilcoxon signed-rank test was used to test whether the median differences in the intra-patient scores or speeds between the measurement points or periods and baseline (run-in day zero) were statistically significantly different from zero. The Mann-Whitney test was used to compare medians between unpaired groups. A p-value of 0.05 or less was considered to be significant. Statistical software packages used were R software version 2.9.0 (R Development Core Team, 2009), used by trial statistician where indicated in the text, and Minitab version 16 (Minitab Solutions), used otherwise by the author. Analyses performed by the trial statistician are acknowledged in the figure legends and text.
### 3 Results

#### 3.1 Patients

25 patients with a prior diagnosis of MMN were screened, and 22 were considered eligible according to EFNS electrodiagnostic criteria and other inclusion criteria (see Figure 1.2 and Section 2.2). One patient was excluded from recruitment since he required air travel to reach the hospital. Another patient was diagnosed with metastatic cancer of unknown primary during the screening period and was therefore was not enrolled. 7 out of 20 patients declined enrolment due to a) potential risks of the trial drug and/or b) already receiving perceived full benefit from IVIg.

The remaining 13 patients, who fulfilled all the additional criteria, were recruited to the study with informed consent. Basic clinical data are listed (Table 3.1).

85% of patients in the trial were male (n = 11), and the mean age at entry was 56 years (± SD 10 years). The mean length of disease by the start of the trial was 19 years (± SD 10 years), and mean duration of IVIg treatment, in those receiving IVIg (n = 10, 77%), was 8.5 years (± SD 5 years). The median IVIg inter-treatment interval was 4.0 weeks (IQR 3.0 – 4.8 weeks), and the mean dose in the year prior to the trial was 16.2 g/wk (IQR 5.8 – 19.2g/wk).

Eleven patients (85%) had upper limb onset of weakness. The most common nerves affected were right median (77%), left ulnar (69%), left median (69%) and right radial nerve (62%). Six patients (46%) also had lower limb involvement, with common peroneal nerve involvement in 6, and tibial nerve involvement in 3 patients (23%). Five patients (38%) had sensory symptoms (numbness, paraesthesia), and 4 (31%) had minor sensory abnormalities on nerve conduction studies. Four (31%) had diminished reflexes in affected limbs only, whilst five (38%) had global hyporeflexia.
Table 3.1. Clinical Features (n = 13)

<table>
<thead>
<tr>
<th>Feature</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>11 (85%)</td>
</tr>
<tr>
<td>Age at start of trial</td>
<td>55 years (IQR 51 - 65)</td>
</tr>
<tr>
<td>Years affected by start of trial</td>
<td>19 years (IQR 10 - 29)</td>
</tr>
<tr>
<td>Upper limb onset</td>
<td>11 (85%)</td>
</tr>
<tr>
<td>Lower limb involvement</td>
<td>6 (46%)</td>
</tr>
<tr>
<td>Sensory symptoms</td>
<td>5 (38%)</td>
</tr>
<tr>
<td>IgM anti-GM1 antibody positive</td>
<td>9 (69%)</td>
</tr>
<tr>
<td>IgM anti-GM1 antibody titre</td>
<td>1/9000 (IQR 1/1275 – 1/12500)</td>
</tr>
<tr>
<td>Current IVIg treatment</td>
<td>10 (77%)</td>
</tr>
<tr>
<td>Duration of IVIg treatment</td>
<td>9 years (IQR 5 – 14)</td>
</tr>
<tr>
<td>Current IVIg inter-treatment interval</td>
<td>4 weeks (IQR 3.0 – 4.75)</td>
</tr>
<tr>
<td>Median IVIg dose per year</td>
<td>840 g/yr (IQR 300 – 1000)</td>
</tr>
</tbody>
</table>

Seven patients had been tested for paraprotein (at some point in the past), and one had IgM paraprotein, kappa light chain, with high titres of IgM anti-GM1 antibody. Anti-GM1 IgM antibodies were detected in 9 patients (69%), of which all were also reactive against GA1, and 6 (46%) against GD1a. Anti-GM2 IgM antibody was detected in 1 patient (8%) who also had reactivity against GM1.

### 3.2 Safety

No patient discontinued the study medication due to an adverse event. One patient had an aborted infusion due to an allergic response, with bronchoconstriction, tachycardia and rash, which was managed with prophylactic steroid and antihistamine before subsequent doses. This patient had a history of allergic response to IVIg, and routinely received pre-medication for this.
Figure 3.1. Study progress diagram. Each patient is represented by a horizontal line (numbered 1 – 14 in vertical axis, note no patient 10 exists as this patient number was allocated to a patient who never reached enrolment). The shaded area in the middle shows the 14 week treatment period, whilst the unshaded areas to the left and right show the run-in and run-out periods. Events (IVIg given, adverse events) are marked at the corresponding week number.
There were four serious adverse events (SAEs) in total: two in the treatment period, and two in the run out period. All four were episodes of moderate headache accompanied by nausea (and dizziness in one case), which prompted these patients, in keeping with the high alert for symptoms of meningococcal disease, to seek medical attention. All patients were kept in overnight for observation. One case was investigated with lumbar puncture, revealing mild CSF lymphocytosis but no bacterial growth, in keeping with aseptic meningitis. Both SAEs which occurred during the treatment period, occurred 2 days following administration of IVIg and eculizumab together, however these patients reported that they had had similar headaches and nausea in the past with usual IVIg treatment, and would never usually report this or consult medical attention, but due to high alert state for meningococcal disease throughout the trial, they were obliged to do so. In both cases, symptoms were resolved fully within 48 hours. The two SAEs at week 8 run out period were in the same patient, for two separate hospital admission with moderate-severe headache, nausea, photophobia and dizziness. This patient had a previous diagnosis of labyrinthitis, and this diagnosis was made. Symptoms improved with medical management.

There were 5 adverse events in total during the run-in period, and these were reported in a total of 2 patients. In the treatment period there were 52 adverse events in total, reported by 11 patients. The treatment period was divided into two periods when reviewing the adverse event data, the induction phase TP 0-3 (eculizumab given weekly) and the maintenance phase TP 4-13 (eculizumab given fortnightly). There was a higher rate of adverse events reported in the induction phase of eculizumab treatment, with 7.25 AEs/week, compared to the maintenance phase, 2.3 AEs/week.

To account for the inter-patient variation in run-period duration, the AE rate was expressed as the proportion of weeks per each period during which an AE was experienced by a patient (table 3.2). During the run-in period, the median proportion was 0% of weeks (IQR 0 – 0%), during the treatment period the median proportion was 14% of weeks (IQR 7 – 21%), and during the run-out period the median proportion was 0% of weeks (IQR 0 – 3%). This means that
during the treatment period, on average a patient experienced an adverse event during 14% of weeks (2 weeks) meaning that the remaining 86% (12 weeks) were on average, free from adverse events. There was a significant increase in the AE rate during the treatment period compared to the run-in period ($p = 0.004$, Wilcoxon signed rank test) and run-out period ($p = 0.007$, Wilcoxon signed rank test). There was no significant difference between the AE rate during run-in and run-out periods ($p = 0.79$, Wilcoxon signed rank test).

Of all 52 adverse events recorded during the treatment period, all except one (patient 8, diarrhoea, also experienced during run-in period) were treatment emergent signs or symptoms (TESS). TESS are events which started following the administration of the study medication, such that any adverse event which had been present prior to receiving the medication could not be treatment-emergent. The classification of an event as a TESS does not indicate the causality of the study drug to the event.
<table>
<thead>
<tr>
<th></th>
<th>RI</th>
<th>TPW0-W3</th>
<th>TPW4-W13</th>
<th>RO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of patients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>13</td>
<td>13</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td><strong>Number of patients with AEs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>2</td>
<td>11</td>
<td>7</td>
<td>3</td>
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<tr>
<td><strong>Number of AEs</strong></td>
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<td></td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>29</td>
<td>23</td>
<td>5</td>
</tr>
<tr>
<td><strong>Number of patients experiencing AEs and receiving IVIg</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1</td>
<td>8</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td><strong>Number of TESS events</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Yes</td>
<td>0( 0.0%)</td>
<td>29(100.0%)</td>
<td>22( 95.7%)</td>
<td>0( 0.0%)</td>
</tr>
<tr>
<td>No</td>
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<td>0( 0.0%)</td>
<td>1( 4.3%)</td>
<td>5(100.0%)</td>
</tr>
<tr>
<td><strong>Serious</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>0( 0.0%)</td>
<td>2( 6.9%)</td>
<td>0( 0.0%)</td>
<td>2( 40.0%)</td>
</tr>
<tr>
<td>No</td>
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<td>27(93.1%)</td>
<td>23(100.0%)</td>
<td>3( 60.0%)</td>
</tr>
<tr>
<td><strong>Duration (days)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N(missing)</td>
<td>5(0)</td>
<td>29(0)</td>
<td>23(0)</td>
<td>5(0)</td>
</tr>
<tr>
<td>Mean(SD)</td>
<td>61.60(77.72)</td>
<td>12.45(35.21)</td>
<td>12.00(25.62)</td>
<td>3.80(1.79)</td>
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<tr>
<td>Median(IQR)</td>
<td>47.00(14.00,48.00)</td>
<td>3.00(100.50,5.00)</td>
<td>3.00(2.00,11.50)</td>
<td>3.00(3.00,3.00)</td>
</tr>
<tr>
<td>Range</td>
<td>3(196)</td>
<td>(0.145)</td>
<td>(0.125)</td>
<td>(3.7)</td>
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<td><strong>Ongoing at end of study</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>1( 20.0%)</td>
<td>2( 6.9%)</td>
<td>1( 4.3%)</td>
<td>0( 0.0%)</td>
</tr>
<tr>
<td>No</td>
<td>4( 80.0%)</td>
<td>27(93.1%)</td>
<td>22(95.7%)</td>
<td>5(100.0%)</td>
</tr>
<tr>
<td><strong>Time after most recent IVIg treatment (days)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>N(missing)</td>
<td>1(0)</td>
<td>13(0)</td>
<td>11(0)</td>
<td>3(0)</td>
</tr>
<tr>
<td>Mean(SD)</td>
<td>4.31(6.07)</td>
<td>11.18(7.85)</td>
<td>4.67(4.04)</td>
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<td>Median(IQR)</td>
<td>0.00(0.00,0.00)</td>
<td>2.00(1.00,4.00)</td>
<td>8.00(5.50,18.00)</td>
<td>7.00(3.50,7.00)</td>
</tr>
<tr>
<td>Range</td>
<td>0(0.20)</td>
<td>(3.25)</td>
<td>(0.7)</td>
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</tr>
<tr>
<td><strong>Time after most recent study drug treatment (days)</strong></td>
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<td>23(0)</td>
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<tr>
<td>Mean(SD)</td>
<td>1.93(1.62)</td>
<td>5.04(3.96)</td>
<td>38.40(33.41)</td>
<td></td>
</tr>
<tr>
<td>Median(IQR)</td>
<td>2.00(1.00,3.00)</td>
<td>4.00(1.50,8.00)</td>
<td>14.00(14.00,75.00)</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0(0.6)</td>
<td>(0.12)</td>
<td>(14.75)</td>
<td></td>
</tr>
<tr>
<td><strong>Severity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>4(80.0%)</td>
<td>19(65.5%)</td>
<td>19(82.6%)</td>
<td>2(40.0%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>1(20.0%)</td>
<td>10(34.5%)</td>
<td>4(17.4%)</td>
<td>3(60.0%)</td>
</tr>
<tr>
<td>Severe</td>
<td>0( 0.0%)</td>
<td>0( 0.0%)</td>
<td>0( 0.0%)</td>
<td>0( 0.0%)</td>
</tr>
<tr>
<td><strong>Causality</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not related</td>
<td>5(100.0%)</td>
<td>2( 6.9%)</td>
<td>0( 0.0%)</td>
<td>2( 40.0%)</td>
</tr>
<tr>
<td>Unlikely</td>
<td>0( 0.0%)</td>
<td>8(27.6%)</td>
<td>7(30.4%)</td>
<td>3(60.0%)</td>
</tr>
<tr>
<td>Possibly</td>
<td>0( 0.0%)</td>
<td>15(51.7%)</td>
<td>13(56.5%)</td>
<td>0( 0.0%)</td>
</tr>
<tr>
<td>Probably</td>
<td>0( 0.0%)</td>
<td>2( 6.9%)</td>
<td>3(13.0%)</td>
<td>0( 0.0%)</td>
</tr>
<tr>
<td>Definitely</td>
<td>0( 0.0%)</td>
<td>2( 6.9%)</td>
<td>0( 0.0%)</td>
<td>0( 0.0%)</td>
</tr>
</tbody>
</table>

**Table 3.2. Summary table of adverse event characteristics.** Brackets contain the number of AEs in that category, as a percentage all AEs in that time period. (Table produced by trial statistician)
Causality of adverse events to the study drug were graded by the investigator, and there were 2 AEs which were ‘definitely’ attributed to the study drug (infusion reaction, and subsequent rash), and 5 AEs which were ‘probably’ attributed to study drug (headache (n = 3), skin rash (n = 1) and pharyngitis (n=1). Overall during the treatment period, 28 AEs (54% overall) were classed as possibly related, 15 (29%) were unlikely to be related, and 2 (4%) were unrelated.

Headache was the most common AE, accounting for 33% of all AEs during the treatment period. Almost two-thirds (11/17; 65%) of the headaches were in the first 4 weeks of treatment. Nine patients (69%) experienced headache at least once during the treatment period. Two out of the three patients who were not receiving IVIg, also experienced headache during the treatment period.

Respiratory and coryzal symptoms (rhinorrhea, pharyngitis) were responsible for 7 (13%) of AEs during the treatment period, musculoskeletal (myalgia and arthralgia) accounted for 6 (12%), gastrointestinal symptoms (nausea, diarrhoea, constipation, and abdominal pain) accounted for 4 (8%) and skin rashes accounted for 4 (8%).

The adverse event profile in patients not receiving IVIg was similar to those receiving IVIg. The most common was headache (n = 7). The others included joint pains (n = 3), malaise (n = 3), rhinorrhea (n = 2), abdominal pain (n = 2), excess sweating, diarrhoea, leg cramps, bruising and hypertension (all n = 1).

No unexpected treatment emergent signs or symptoms were noted. No bacterial or other infections were encountered.

The majority (73%) of AEs during the treatment period were classed as mild, and the remainder moderate (27%). No adverse event was classed as severe. The median duration of AE was 3 days (IQR 1 – 5). During the treatment period, AEs occurred at a median of 2 days post-eculizumab dose, and 2 days post-IVIg treatment. The three patients who were not receiving IVIg also experienced adverse events.
3.3 Pharmacological studies

Median serum eculizumab concentration had increased to above the 35µg/ml minimum therapeutic level by treatment period week 1, and was maintained above this level throughout the treatment period. Pharmacodynamic (PD) analysis measured the terminal complement activity of patient serum, by its ability to haemolyse chicken red blood cells in vitro. A value of 100% haemolysis signifies that the collected serum has equal haemolysing ability as control human serum, therefore full terminal complement activity. Before Eculizumab treatment the median % haemolysis was 95% (IQR 77.5-98), whilst by week 1 this had reduced to 5% (IQR 2.75-19.5).

Patients receiving IVIg had significantly lower median eculizumab concentration (78.7 µg/ml, IQR 55 - 108) compared to those not receiving IVIg (119.7µg/ml, IQR 95 - 147). Importantly however, complete terminal complement inhibition in serum was achieved, with no difference between the median haemolytic complement activity in both groups (2% and 1% respectively). The serum measurements were taken prior to Eculizumab or IVIG infusion, and therefore reflect the plateau state. There was no difference between the haemolytic complement activity pre-trial in patients receiving IVIg (median 98% haemolysis) and not receiving IVIg (median 94%) signifying that IVIg maintenance treatment did not affect terminal complement activity measured at the end of the IVIg cycle.
Figure 3.2. Pharmacological data depicted by boxplots, showing median value and interquartile range. Panel A. PK data: serum eculizumab concentrations (µg/ml). Panel B. PD data: serum haemolytic activity, % activity compared to control pooled normal human sera. Panel C and Panel D. Comparison of PK and PD data depending on patients receiving IVIg or no IVIg. Outliers shown by asterisks, were not excluded from analysis. Significance testing by Mann Whitney test. (Panel A and B produced by trial statistician)
### 3.4 Intravenous Immunoglobulin Requirements

During the treatment period, 9 out of 10 patients receiving IVIg as maintenance therapy continued to require IVIg at regular intervals throughout the treatment period, which was qualified by reaching deterioration point.

IVIg requirement was measured as the inter-treatment interval (in days) (Figure 3.3). In the year prior to trial commencement, the median interval was 28 days (IQR 21 – 37). During the run-in period, the median interval was 30.5 days (IQR 22 – 46). During the treatment period, the median interval was 35 days (IQR 23 – 46), which was not significantly different to the run-in interval (p = 0.64) but was significantly different to the pre-trial interval (p = 0.006).

![Figure 3.3. Intravenous immunoglobulin requirements. Boxplots showing median inter-treatment interval (days) across the time points: prior, year preceding trial; RI, run-in period; TP, treatment period; RO, run-out period. Significance testing carried out by Wilcoxon signed rank test.](image)

Figure 3.3. Intravenous immunoglobulin requirements. Boxplots showing median inter-treatment interval (days) across the time points: prior, year preceding trial; RI, run-in period; TP, treatment period; RO, run-out period. Significance testing carried out by Wilcoxon signed rank test.
During the run-out period, the median IVIg interval was 31 days (IQR 22 – 44), this was neither significantly different to the treatment period (p = 0.15) nor the run-in period (p = 0.44). The run-in and run-out periods were not significantly different from the pre-trial interval (p = 0.44 and 0.53 respectively), however the treatment period interval was significantly longer than the pre-trial interval (median difference 6.5 days, p = 0.006). Summarised, there was no significant difference between the IVIg inter-treatment interval in any of the trial periods, RI, TP and RO. There was no significant lengthening of the interval between pre-trial and RI, however there was a significant difference between pre-trial and treatment period, indicating a small net increase between RI and TP that was not statistically significant.

The median of the average IVIg dose in the year preceding the trial, across all 13 patients, was 16.2g/week (IQR 5.7 - 19.2), which is very similar to the median of the average weekly dose during run-in 16.3g/week (IQR 9.4 – 21.5) (p = 0.42). The average dose during the treatment period was 15g/week (IQR 0 – 23), but this was not significantly decreased from the run-in dose (p = 0.31). The run-out period dose was increased to 18.5g/week (IQR 9.5 – 2.5), but this was not statistically significant (p = 0.15).

Patient 012, who did not require IVIg at all during the treatment period, had been receiving IVIg at 10 week intervals for 2 years prior to the trial. During the run-in period, the 8 week maximum length was reached without deterioration, so patient 012 entered the treatment period without receiving his next cycle of IVIg. During the treatment period, deterioration point criteria were not reached, in fact, SEFR score dramatically reduced (Figure 3.4), but during the run-out period increased to beyond baseline levels and IVIg was given again at week 7 of the run-out period due to reaching deterioration point. For the purpose of the analysis, patient 12 is included in the IVIg treatment interval analysis below, with a run-in interval of 8 weeks, treatment interval of 14 weeks, and a run-out interval of 8 weeks, however the actual IVIg interval was 207 days (29 weeks). A sensitivity analysis was carried out excluding and including patient 012, and there was no overall change to the significance of the comparisons of IVIg inter-treatment intervals.
Figure 3.4. SEFR score of patient 012, who did not require IVIg between time points RID0 (run-in day zero) and ROW8 (run-out week 8), giving an IVIg interval of 30 weeks which spanned the three study periods.

3.5 Muscle strength

The MRC sum score did not differ significantly between any of the trial periods (Figure 3.5 Panel A). MRC sum score was also compared at successive deterioration points during the treatment period, and showed a non-significant trend to increasing score, from a median score of 23 (IQR 23 – 32) at the first DP to a median score of 28.5 (IQR 25 – 30) by the fourth DP (figure 3.6).
Figure 3.5 Muscle strength measurements. Panel A, MRC sum score (out of total possible score 50). Panel B, MSF (muscle strength force/myometry) sum (kg). Boxplots displaying median score at individual assessment points. Significance testing compares to baseline (RIDO), Wilcoxon signed-rank test (Trial statistician).
Figure 3.6. MRC sum score at successive deterioration points during the treatment period in patients receiving IVIg. Median MRC sum score across patient (n = 9). Significance testing by Wilcoxon signed rank test.

The MSF (myometry) score steadily increased at each successive assessment point (Figure 3.5 Panel B), and was significantly higher than baseline (median 35kg, IQR 25 - 42) at treatment week 8 (median 43kg, IQR 38 – 52, p = 0.01) and week 14 (median 44kg, IQR 31 – 59, p = 0.01), and run-out week 8 (median 44kg, IQR 30 – 48, p = 0.02). The increase between run-in period and treatment week zero was non-significant (p = 0.13). There was no significant difference between treatment period overall, and run-out week 8 (p = 0.73).

To control for any ‘learning’ effect between RID0 and TPW0, assessments were also compared to TPW0, rather then RID0, and the similar increase was found, also significant.

Median pinch grip strength (vigorimetry) showed small but significant changes at week 4 in both sides, but this difference was not seen at any other time point (Figure 3.7 Panels A and B). There were no significant changes in median palm grip strength throughout all periods (Figure 3.7 Panels C and D). Whole hand grip
(dynamometry) similarly did not show any overall trends, and there were no significant differences at any assessment points.

Figure 3.7. Pinch and palm grip (vigorimetry). Boxplots of median pinch (A and B) and palm (C and D) grips for each side at study time points. Significance testing compares to baseline (RIDO), Wilcoxon signed-rank test (Trial statistician).
3.6 Timed assessments

Figure 3.8. Timed assessments. Panel A shows timed 10m walk expressed as speed in percentage of total completed in 1 second (100/s). Panels B and C display the speed of completion of 9 hole peg test, expressed as percentage of test completed in 1 second. Significance testing compares to baseline (RIDO), Wilcoxon signed-rank test (Trial statistician).

The timed walk speed did not differ from baseline throughout the treatment period (Figure 3.8 Panel A), save for a slight dip at treatment week 0, which was non-significant (p = 1.0). Patient 006 at baseline took 21 seconds (speed 4.8/sec) to
complete 10m (and was using a stick for balance), at week 14 completed this in 14 seconds (speed 7.14/sec) without walking aids. This improvement in timed walk did not vary with IVIg dosing, and had further improved to 12 seconds by the end of the run-out period.

In the nine hole peg test (9HPT) the right hand was faster than the left hand at baseline (right hand 2.9/sec, left hand 1.8/sec) (Figure 3.8 Panels B and C). There was a significant increase in 9HPT speed of right hand between baseline and all treatment time points (including TPW0), equating to an overall 21% increase in the speed of completion by week 14 (p = 0.01). There was no significant change from treatment to run-out period (p = 0.50). The left hand 9HPT speed was significantly different only at week 4. There was a significant increase right, but not left 9HPT speed from run-in day zero to the start of the treatment period (median increase 0.4/sec, p = 0.05). Analysis using TPW0 as the baseline time point shows speed increases in both hands which were smaller and less significant.

3.7 Subjective assessments

Overall, there were improved SEFR scores (i.e. numerically reduced) week by week throughout the treatment period. This trend can be seen in Figure 3.9. The SEFR score was significantly improved from baseline at week 8 (median decrease 1 point, p = 0.03) and week 14 (median decrease 3 points, p = 0.02). There was no significant difference between run-in period and run-out period SEFR score, indicating that once eculizumab treatment finished, patient rated difficulty with daily tasks increased again.
Baseline median EQ5D analogue scale response was 70% (IQR 60 – 81) and significantly increased to 75% (IQR 70 – 90) at week 4 (p = 0.04), and was equally, but non-significantly, raised at later treatment time points (Figure 3.10 Panel A). The EQ5D health utility showed no change in the median score throughout the treatment period compared to baseline (median 0.7 throughout) (Figure 3.10 Panel B).

The median score ONLS score remained at 4 (IQR 4 -5) throughout all time points, the minimum and maximum scores showing no variation (Figure 3.10 Panel C).
Figure 3.10. Subjective assessments. Panel A shows EQ5D visual analogue scale representing perceived health in percentage at study assessment points. Panel B shows EQ5D health utility score, for description see text. Panel C shows ONLS (Overall Neuropathy Limitation Scale) total score at study assessment points. Significance testing compares to baseline (RID0), Wilcoxon signed-rank test (Trial statistician).
3.8 Responders versus non-responders

In a post hoc exploratory analysis, patients that had a minimum SEFR score during the treatment period which was at least 2 points lower than the minimum SEFR score during the run-in period (i.e. subjectively improved with daily tasks during eculizumab treatment) were retrospectively classed as ‘subjective responders’. Using this criterion, 7 of 13 (54%) of patients were subjective responders: of these 5 of 7 had subsequent increase in SEFR score by at least 2 points during the run-out period, indicating that this SEFR score decrease was only seen whilst on eculizumab in these patients.

The seven subjective responders included 2 patients not receiving IVIg. Comparison of trial assessments at baseline and at time points between responders and non-responders reveals a difference in the measurements between these two groups. No statistical analysis has been performed since this was a post-hoc descriptive analysis, but the pattern suggests that those who had subjectively responded to treatment with eculizumab also had higher MRC score and myometry recordings at baseline (Figure 3.11). They also had greater hand functionality with faster 9HPT speeds and greater pinch-grip strengths at the outset. The responder group appears to have had an increased IVIg inter-treatment interval from run-in period to treatment period, while the non-responder group shows a slightly decreased interval.
**Figure 3.11. Responders vs non-responders.** Boxplots displaying values for two groups of patients, no (clear box) = subjective non-responders \( n = 6 \), and yes (shaded box) = subjective responders \( n = 7 \), at time points RI (run-in day zero) and TP (values averaged throughout treatment period). Panel A shows IVIg inter-treatment interval. Panel B shows MRC sum score and MSF sum score showing responders. Panel shows pinch grip for right and left hands. Panel D shows nine hole peg test (time to complete in seconds). Asterisks represent outliers in the data.

### 3.9 Electrophysiology

Approximately two motor nerves from each patient were studied by nerve conduction studies. The median conduction block (expressed as % CMAP amplitude reduction) at run-in was 36% (IQR 27 – 53), and at treatment week 14 had significantly reduced by median 6.5% (IQR 2.5 – 11.5, \( p = 0.05 \)) (Figure 3.12). The distal latency Z-score also showed a small but significant decrease at the end of treatment, by median 0.16 standard deviations (\( p = 0.05 \)). This equated to a decrease in distal motor latency from 5.5 seconds (IQR 3.6 – 6.1) to
4.7 seconds (IQR 3.7 – 5.9). There was no change in the conduction velocity, proximal CMAP, distal CMAP or F-wave latencies between nerves.

Figure 3.12. Electrophysiology. Interval plots showing individual values for each nerve studied (total of 22 nerves from 13 patients) motor nerve conduction studies. Panel A shows conduction block expressed as the percentage decrease in voltage between distal CMAP to proximal CMAP, across a nerve segment. Panel B, distal CMAP amplitude expressed in mV. The two time points for the studies were RI (run-in day zero) and TP (end of the treatment period).

To test whether conduction block and distal CMAP had changed over time in this group, 1 nerve segment showing conduction block was selected from each patient from trial baseline studies, and values for percentage conduction clock and distal CMAP amplitude were compared with those from the last available historical study of that nerve segment. The median interval in this comparison was 5 years (IQR 2 – 6), and the median percentage conduction block was 51% in both the previous and the current studies (IQR 41 – 87 previous; IQR 37 – 83
There was a non-significant trend to lower median distal CMAP with time, (4.0mV previous; 2.7mV current, p = 0.17).

**Figure 3.13. Comparison of conduction block and distal CMAP amplitude changes over the pre-trial period.** Line plots showing individual values for conduction block (left panel) and distal CMAP amplitude (right panel) across 11 nerves with conduction block from this cohort of patients, CB 1 and CMAP 1 values refer to the last available nerve conduction studies prior to trial inclusion. CB 2 and CMAP 2 values refer to the baseline studies recorded for this trial. Significance testing by Wilcoxon signed rank test.

Electromyography studies of a maximum voluntary contraction were compared by being assessed blindly by a panel of 5 neurophysiologists. 22 muscle groups (2 from each patient, n = 11, 2 patients excluded as recordings were not available at time of assessment). Out of 22, only 6 muscle groups received unanimous scorings, four showing increased motor unit recruitment and two showing decreased recruitment following treatment.
4 Discussion

4.1 Safety

This trial did not set out to further clarify the absolute safety of eculizumab, as this has already been done, but was designed to assess if a complement inhibitor could be co-administered in patients receiving IVIg, and whether there were any unexpected adverse events with this combination. Overall, this study observed a significant increase in the rate of adverse events during eculizumab treatment, compared with run-in period. Around 10% of these were classed as definitely or probably related to the study drug. Most of the adverse events were mild, the remainder moderate in severity. None were severe, and no patient discontinued treatment due to adverse events. There were two serious adverse events whilst on treatment, which became classed as ‘serious’ adverse events due to heightened awareness of meningococcal meningitis, leading to reporting of ‘alert’ symptoms (headache), and the subsequent need for investigation and hospitalisation for these two episodes. Overall, the most common adverse event was headache, which made up 33% of the adverse event profile, and occurred at least once in 69% of patients. The rate of occurrence of headache was higher than the TRIUMPH study (Schubert et al., 2008), a prominent phase III randomised control trial of eculizumab in PNH, which found 44% of patients had headache during eculizumab treatment. Perhaps the increase seen in this trial is due to the co-administration of IVIg, as headache is also the most side effect of IVIg treatment. The other most common adverse events encountered in this study were (in descending order) respiratory, musculoskeletal, gastrointestinal and dermatological. This compares well to data from other studies with eculizumab, and there were no unexpected AEs which were thought to be possibly related to treatment. There were no cases of meningococcal septicaemia in this group, and no other bacterial infections noted. Furthermore, no patients experienced a worsening of disease, an important point given that corticosteroids are known to be deleterious in MMN.
However, there were some difficulties with the interpretation of the safety data findings of this study. Firstly, this trial was not placebo-controlled, and therefore we do not know how frequently these adverse events would be reported in this same group of patients whilst not receiving eculizumab. Secondly, the heightened alert for meningococcal disease led to increased reporting of headaches. Thirdly, the frequency of adverse event reporting was not constant throughout the three phases of this trial, as the reporting in the run-in period was from 3 up to 8 weeks, however in the treatment period, investigators and patients met at least weekly, and thus there was greater prompting and opportunities for reporting of mild events.

Overall, in this short duration open label study, it was observed that eculizumab treatment was associated with an increase in adverse events, of which none were unexpected side effects, and were roughly in keeping with safety data from previous studies. There is evidence thus, that co-administration of eculizumab and IVIg, may be associated with slightly higher rate of adverse events, but is well tolerated.

**4.2 Pharmacokinetic and pharmacodynamics**

Pharmacological assays demonstrated that terminal complement function was fully inhibited in all patients, regardless of concomitant IVIg use, and thus there was no neutralizing effect of IVIg upon eculizumab. However it was found that patients receiving IVIg had lower median eculizumab concentrations. Eculizumab is expected to be eliminated via the same pathways as are native human antibodies, being too large to be renally excreted, even in the context of nephrotic syndrome, they are taken up into endothelial pinocytotic vesicles containing FcRn receptors, also known as the ‘Brambell’ receptor. The Brambell hypothesis is precisely this, that IgG homeostasis is regulated by the FcRn receptor which salvages IgG (but not IgA or IgM) and prolongs the life-span of IgG, and regulates its recycling. (BRAMBELL et al.,1964). Mice genetically lacking expression of FcRn demonstrated IgG hypercatabolism and faster IgG elimination (Ghetie et al.,1996).
The pharmacokinetics of IgG immunoglobulins is known to be non-linear, displaying concentration-dependant elimination \((Lobo \ et \ al.,2004)\) and this has been shown for therapeutic monoclonal antibodies also \((Tabrizi \ et \ al.,2006)\). The rate of catabolism of immunoglobulins is increased when higher concentrations are present, and this is thought to explained by the Brambell hypothesis, as at lower concentrations, a larger proportion can be ‘salvaged’ by the FcRn receptor, but at higher concentrations there are less available receptors and thus IgG remains in the circulation where it is more likely to be catabolised. It has previously been thought that patients receiving high dose intravenous immunoglobulin will show higher rates of catabolism of IgG antibodies, due to saturation of FcRn receptors \((Yu \ et \ al.,1999)\). Animal models where monoclonal antibodies were administered, followed by high dose IVIg (2g/kg), resulted in an increased rate of excretion of the monoclonal antibody \((Bleeker \ et \ al.,2001)\), and extrapolated to humans, this predicts a 25% increase in elimination of the monoclonal antibody within 3 to 4 weeks.

A highly possible explanation for the finding in this study, of lower eculizumab concentrations in patients receiving high dose IVIg, is due to concentration-dependent elimination, and therefore further supports the Brambell hypothesis. However it is clear from the data presented here that although this occurs, pharmacodynamics and this serological efficacy of this antibody were not compromised.

It is thought that perhaps this effect this also accounts for some of the therapeutic effect of IVIg in antibody-driven autoimmune conditions, by increased elimination of the autoantibody, as a result of saturation of the salvaging mechanism by exogenous IVIg. In myasthenia gravis and stiff-person syndrome, IVIg treatment results in the decline in AchR-Ab \((Illa,2005)\) and anti-GAD antibodies respectively \((Dalakas,2005)\). However the same does not hold true for MMN \((Piepers \ et \ al.,2010)\), most likely because the antibody is IgM isotype, and therefore is not regulated by the FcRn receptor.
The MMN response to IVIg, but not to other immunomodulatory methods, may be due to the complement regulating effects. Antibodies against several components of the classical complement pathway have been identified in IVIg. They include antibodies against C1, C3a, C3b and C4 (Jacob et al., 2009). In addition, high doses of IVIg are thought to enhance the degradation of C3b (Lutz et al., 1996). It has now been shown in vitro (using ELISA) that IVIg decreases complement deposition by IgM-GM1 antibodies in vitro, and results in decreased classical pathway activation in MMN patients receiving IVIg (Piepers et al., 2010).

The results presented here show that IVIg maintenance therapy did not reduce the haemolytic activity (MAC lysis effect) in MMN patient serum, as this was >90% in patients regardless of IVIg therapy when patient serum was tested at baseline, prior to eculizumab treatment. However, the samples from this assay were collected immediately prior to the next dose of IVIg, and thus perhaps since the effect of IVIg has since declined, and any changes to complement have returned to baseline. It would be interesting to test MMN patient serum mid-cycle for complement levels. This effectively did happen during the eculizumab treatment period, but complement was already fully suppressed by eculizumab, and remained inhibited even at the end of the dosing window. Hence, even if IVIg does cause a decrease in terminal complement activity in MMN, by replacing this effect more robustly with eculizumab, there still remained a need for IVIg. From this it could be concluded that the overriding IVIg therapeutic effect in MMN is mediated by factors other than complement activity. There are many other immunomodulatory roles of IVIg: interference anti-idiotypic antibodies interfere with the binding of autoantibodies to their target ligand, reducing the formation of immune complexes, modulation of macrophages function via modulation of Fc receptors, and modulation of cytokines and cell signalling molecules in the humoral and cellular immune reaction.

Another possible reason for lack of IVIg replacement by eculizumab could be that terminal complement inhibition was not fully achieved in the endoneurial compartment, since this is separated from plasma by the blood-nerve barrier (BNB), and we do not know what proportion of eculizumab was able to cross this.
Schwann cells and macrophages are known to synthesise C5 locally (Ramaglia et al., 2009), thus the combination of lower intraneural eculizumab concentration, and higher C5 concentration, may reduce the pharmacodynamic properties of eculizumab in this compartment. In addition, Schwann cell expression of complement regulators such as CD59 may also be fully up-regulated in MMN to the extent that additional terminal complement inhibition may have little effect on the ongoing injury.

Regarding the passage of immunoglobulins across the BNB, in animal models of Guillain-Barré syndrome, radiolabelled immunoglobulin was increased 3-4 days into the disease, but declined by day 8. MMN is a chronic condition, in this group the median disease duration was 19 years, and there are multifocal lesions of different ages throughout various motor nerves. Perhaps the permeability of the blood-nerve barrier is increased in the acute lesion, but returns to baseline in the chronic phase, and thus eculizumab and other immunoglobulins cannot enter all sites of conduction block. This however still leaves the question of why IVIg works effectively in MMN.

### 4.3 Secondary outcome measures

Although complement inhibition did not replace the requirement for IVIg, some improvements in secondary outcomes were observed. There was an overall trend towards improvement in patient-rated subjective scores whilst receiving eculizumab, and in approximately half of the group eculizumab treatment decreased their difficulty with tasks of daily living more than usual with IVIg. Eculizumab also benefited two patients who were not receiving IVIg, displayed by changes to SEFR scores in these two patients.

There was an overall trend towards increased muscle strength as measured by myometry, which did not diminish during the run-out period. The maintenance of muscle strength during the run-out period could have represented longer lasting benefit from eculizumab, which could be proposed to occur through remyelination of some nerve fibres.
Speed of 9HPT completion significantly increased in the right hand with treatment; although this could have been a practice effect, and was not seen in the left hand. However, other objective clinical measurements did not show any clear trends, even when responders and non-responders were considered separately.

Half of the patients who were subjective responders, although still requiring IVIg, reported the pre-treatment deterioration points (DPs) were less severe, as scored by SEFR, and the MRC sum score improved during these DP compared to their previous DPs. One patient who routinely received 10 weekly IVIg did not reach deterioration point at all during eculizumab treatment, making the inter-treatment interval 30 weeks in total, and whilst the SEFR scoring greatly improved, no improvements in objective measurements were noted in this patient.

There are some difficulties in the evaluation of MMN as a condition, the most prominent reason is due small numbers of patients with this condition. Additional difficulties are introduced due to the multifocal nature of this neuropathy, resulting in a slightly different anatomical pattern and severity distribution in each patient, and thus there is no global set of assessments that can be broadly compared across the heterogeneous group of patients. Discrepancies between subjective and objective outcome trends, has been previously noted in MMN trials (Leger et al., 2001). Few articles have been published on the best outcome measures to evaluate patients with MMN, and those selected here were based on clinical experience with neuropathy, knowledge of tools used well other published MMN trials, for example SEFR score (Van den Berg et al., 1995). Since MMN is an upper limb predominant disease, multiple measures of hand strength were employed in this study, and dexterity was also measured by the 9HPT. However, due to same hand side being compared across the whole patient group, unaffected hands in some patients were compared with affected hands in other patients, which could cause the collective data to mask smaller changes in affected muscles. As proposed during the ENMC (European Neuromuscular Centre) International workshop on selection of outcome measures for peripheral neuropathy clinical trials (Merkies et al., 2006), modified sum scores are used
here where possible, choosing the most affected muscles in each patient. This can explain the observation in this study, that myometry and SEFR scores were those to show a convincing trend, since these tests was individualized for each patients depending on their most affected muscle groups and functions.

Outcome measures in this study were also likely to be affected by the inter-test variability introduced by random fluctuations in patient performance and operator or device measurement errors. In a series of MMN and CIDP patients, controls limits analysis was performed on myometry data with the aim of describing the variability within this group, and found that almost 90% of patients had variability of up to 30% (Lough et al., 2000). Coupled with the suggestion that the minimally clinically important difference in a measure could be as low as 8% (Jaeschke et al., 1989), the chances of discovering this within data from neuropathy trials could be extremely low due to the high levels of inter-test variability.

An additional level of variability introduced in this study was IVIg therapy, as this is known to cause cyclical changes to muscle strength (Van den Berg et al., 1998), which were also noted in some, but not all, patients in this trial. Due to fixed assessment points in this study design, patients were assessed at varying points in their cycle, so intra- and inter-patient comparisons were also likely to be difficult to interpret. Due to this, outcomes measures were compared, in a separate analysis, at successive deterioration points only, which were thought to represent a fixed ‘trough’ level of function as assessed by patient and clinician. No significant differences in measures compared at deterioration points were found, although there was a pattern of increasing MRC sum score at deterioration points.

The finding that subjective responders had a higher level of pre-trial function and muscle strength in comparison with subjective non-responders suggests that patients who were less severely affected were more likely to experience an eculizumab-related treatment response. It should be equally recognised that the eculizumab non-responders still remained responsive to IVIg, and so these patients were not treatment-resistant. Motor axon loss is an important
determinant of weakness in MMN and the degree of axonal degeneration increases with disease duration (Van Asseldonk et al., 2006; Van den Berg-Vos RM et al., 2002a). As has also been shown in CIDP (Iijima et al., 2005) and MMN (Nobile-Orazio et al., 2002), muscle atrophy and electrophysiological evidence of axon loss, are more likely in patients who are not responsive to IVIg. Therefore it would be not only intuitive, but also evidence-based, to aim this and other novel therapies towards less advanced cases where more scope for a treatment response exists.

4.4 Electrophysiology

Electrophysiological measurements are important here as they represent the only objective assessments collected in the study that were independent of patient-related performance bias. There were improvements in individual measurements in some patients, and deterioration in others, but with an overall trend in favour of improvement, by a small but significant net decrease in the degree of conduction block across all nerves studied. It would not be expected that conduction block would change appreciably over time, and the median conduction block had remained stable in these nerves studied for a median of 5 years before the study, yet within 14 weeks of treatment, there was a significant change. The effect of IVIg on conduction block remains unresolved, as some studies show improvement in conduction block with IVIg treatment (Van den Berg-Vos RM et al., 2002b; Vucic et al., 2004), whilst in others no electrophysiological changes were found, despite improvements in muscle strength in by up to 80% (Leger et al., 2001). This could be explained by an improvement in nerve conduction at sites not measured by nerve conduction, such as those more proximal or more distal to the segment studied. There was a significant change in distal motor latency, which suggests that distal conduction was slightly faster. This may well represent resolution of distal conduction block at micro sites in the small nerve fibres in the hands and feet. It has been proposed that this occurred in 4 out of 18 nerves studied with long term IVIg treatment, and is thought to be due to remyelination (Van den Berg-Vos RM et al., 2002b). However, this effect was not observed in the nerves as a whole, as there was no significant change to
conduction velocity, or F wave latency, other parameters which would be expected to improve with nerve remyelination, and thus this effect may have been length dependent.
4.5 Conclusion and future work

Overall, in this short duration open label study, it can be concluded that co-administration of eculizumab and IVIg did not highlight any additional safety concerns, and IVIg did not reduce the pharmacological effectiveness of eculizumab. There was some evidence of small positive benefits of eculizumab treatment in MMN. This was observed in some, but not all, of the subjective and objective measurements used. The outcome measures that were most tailored to assess each individual showed the most significant changes. In addition, truly objective measurement using electrophysiology, showed some small but significant improvements in nerve conduction. It is difficult to come to any overall conclusion about the efficacy of eculizumab in multifocal motor neuropathy, due to the uncontrolled study design and the lack of dramatic response seen (such as large reduction in amount of IVIg required), however it can be viewed as encouraging for further studies of complement inhibition in MMN and other immune-mediated neuropathies.

Ideally, a longer term study of terminal complement inhibition should be performed in MMN, looking for a more gradual cumulative effect or an arrest of disease progression. When considering future studies, placebo controlled trials designs would be clearly be the next step for the investigation of this drug, in order to control for test performance variability, learning effects, and operator and patient-related assessment bias.

Perhaps more pressing than future trials in MMN, would be a trial in acute, severe neuropathy such as Guillain-Barré syndrome in which early aggressive treatment has a greater capacity to majorly influence short and long-term morbidity, and more evidence for the beneficial effect of complement inhibition from animal models exists. Now that safety data regarding the co-administration of IVIg and eculizumab exists, a trial of complement inhibition in Guillain-Barré syndrome is a high priority.
5 Laboratory work: Investigating the serological factors in MMN

5.1 Introduction

The knowledge that up to 50% of patients in MMN do not possess antibodies to GM1, may place some doubt over the theory that MMN is an antibody driven disease (Nobile-Orazio et al., 2005), although it remains clear that MMN is an immune-mediated disease since antibody-negative patients are also largely immune-responsive.

5.1.1 Anti-GM1 antibody in MMN

There are some alternative theories to explain the significance of GM1 antibodies in this disease, for example, that the GM1 antibody is a biomarker for disease, produced when inflammation of the nerves exposes GM1-rich areas of nerve to immune cells and thus antibodies develop. A similar explanation is that GM1 antibodies are present as markers of previous infection with Campylobacter jejuni or other organisms which may have similar cross-reactivity with GM1. However, much evidence exists to support the theory that immune attack of nerves is mediated by anti-GM1 antibodies, via complement fixation, causing destruction of peripheral nerve structure and function. This has been demonstrated in vivo and ex vivo for not only the anti-GM1 antibody (Greenshields et al., 2009; Nobile-Orazio et al., 2005; Yuki et al., 2001), but for anti-GD1a antibodies (Goodfellow et al., 2005; McGonigal et al., 2010) and anti-GQ1b antibodies (Halstead et al., 2005a; Halstead et al., 2008a; Halstead et al., 2008b). As support for the antibody-driven theory, the anti-GM1 antibodies found in MMN are demonstrated in vitro to be specific for motor nerves, though sensory nerves do contain GM1 gangliosides, the antibodies from MMN sera do not bind (Corbo et al., 1992). It is perhaps no coincidence that MMN is a predominantly motor condition. The binding site on the GM1 antibody found in MMN perhaps therefore has specificity for a particular structural arrangement of GM1 which seems to be specific for GM1 in motor nerves although GM1 ganglioside is found in non-neural tissue types within the body.
The fluid mosaic model of the cell membrane helps us to understand that lipids and proteins diffuse easily in a bilaminar phospholipid layer (Singer et al., 1972), and thus the conformation of the gangliosides in this environment are not fixed, although it is known that gangliosides exist in lipid clusters (Fujita et al., 2007). Current methods of antibody detection, ELISA and combinatorial microarray both described in this paper, are conducted in the ‘solid phase’, and therefore detect antibodies which bind to glycolipids which have been immobilised on a fixed plane.

5.1.2 Glycolipid complex microarrays

Current work in the investigation of autoimmune neurological conditions uses combinatorial microarrays to assess the effect of glycolipid pairings on antibody binding in the solid phase, thereby imitating the effect of glycolipid clustering on the cell membrane. Using this method, novel antibody sensitivities have been discovered, for example, sulfatide:ganglioside interactions in CSF from patients with multiple sclerosis (Brennan et al, unpublished data), and many ganglioside:ganglioside pairings in serum from patients with GBS (Kaida et al., 2004; Kaida et al., 2007). The explanation for this new reactivity is that the pairing of the two lipids creates a novel epitope which matches the binding site of an antibody already present in serum from the disease population. This may reflect a configuration of lipids on the nerve membrane within the lipid clusters via which that antibody exerts pathogenic effects. Previous work within our research group has demonstrated that the ability of anti-GM1 antibodies to bind to target tissue is affected by its local glycolipid environment, and that GM1 is often inaccessible due to the presence of terminal sialic acid residues on neighbouring gangliosides (Greenshields et al., 2009). This work has primarily involved two mouse IgG monoclonal antibodies (DG1 and DG2) both with similar binding affinities for GM1. DG1 was developed by harvesting of the immunogenic response to lipopolysaccharide isolated from Campylobacter jejuni, and DG2 using a GM1-bearing liposome as the immunogen. DG2 bound to the live ex-vivo nerves and exerted pathogenic effects via MAC deposition, whilst DG1 did not, unless the nerves were treated with neuraminidase which altered the ganglioside
composition of the nerve membrane by removing terminal sialic acid residues (see Figure 5.1). Greenshields also used a monoclonal IgM antibody cloned from a patient with MMN (Willison et al., 1994), and this showed similar binding requirements as DG1.

Figure 5.1. Schematic illustration of the effect of neuraminidase on gangliosides with terminal sialic acid. Neuraminidase cleaves the terminal sialic acid residue, leaving a terminal ganglioside configuration identical to GM1.

5.1.3 PC12 cells

PC12 phaeochromocytoma cells are clonally derived from rat adrenal medullary tumour cells, originally generated by Greene and Tischler in 1976 (Greene et al., 1976). PC12 cells are small round cells, diameter 6-14µm (Tischler et
al., 1978), which is comparable in size with human red blood cells. Grown in vitro they have morphologic and cytochemical features in common with normal chromaffin cells, synthesising and storing catecholamines and acetylcholine, and bearing sympathetic nervous system receptors (Tischler et al., 1978). Further to the development of the cell line, it was discovered that PC12 cells also bear nerve growth factor (NGF) receptors, and when this is added to culture, it causes cessation of cell division and development on neuronal cell qualities, with branched processes (neurites), electrical excitability via activation of Ca\(^{2+}\) and Na-K\(^{+}\) channels, and development of synapses between cells. Therefore, the cells undergo morphological and physiological changes with the addition of NGF which transforms them into neurones similar to those of the sympathetic nervous system (Fujita et al., 1989). Sialic acid bearing gangliosides account for 10% of the lipid weight of the PC12 cell, and this approximately doubles with NGF-induced differentiation. Tri- and tetra-sialogangliosides make up the biggest proportion of gangliosides, with mono and di-sialogangliosides also present (Walton et al., 1988; Wu et al., 1988). Although the total amount of gangliosides increases with neural differentiation, the ganglioside repertoire and their relative amounts do not change (Walton et al., 1988).

### 5.1.4 Neuraminidase

Neuraminidases are naturally occurring enzymes that cleave the glycosidic linkages of neuraminic (sialic) acids (CARUBELLI et al., 1962). The most characterised is its role as a virulence factor in influenza, and this has led to the development of therapeutic neuraminidase inhibitors, such as oseltamivir (Tamiflu™) to prevent virus mobility and penetrance (Jefferson et al., 2010). It has been demonstrated to cleave terminal sialic acid groups, thus converting GD1a and GT1b to de novo GM1 (Figure 5.1), in a variety of ganglioside-containing tissues, including red blood cells (Ackerman et al., 1980), neural tissue (Wang et al., 2009) and PC12 cells (von et al., 2001).
5.1.5 *Cholera toxin B subunit*

Cholera toxin is a heat-labile enterotoxin secreted by diarrhoeal bacterium *Vibrio cholerae*, and less classically, by *Escherichia coli* (Van Heyningen et al.,1976). It is composed of two subunits A and B. Each complex contains 5 B-subunits arranged in a pentamer, and one A-subunit with a long tail that sits within the central pore of the B-subunit pentamer-complex. Cholera toxin binds to intestinal epithelium via B-subunit binding GM1 on the cell surface. Upon binding the A-subunit is released and enters the cells, resulting in increased adenylate cyclase activity, cAMP production and massive secretion of water and electrolytes leading to profuse watery diarrhoea (Bennett et al.,1975).

Cholera toxin B subunit (CTB) is widely used experimentally as a reliable probe for GM1 ganglioside, and the binding affinity is remarkably high with dissociation constants ($K_D$) in the $10^{-10}$ to $10^{-12}$ M range (Kuziemko et al.,1996) (which is $10^4$ to $10^6$ fold higher than the anti-GM1 monoclonal antibodies used in the following experiments). Regarding the specificity of the CTB-ganglioside interaction, there is some evidence to suggest that CTB may cross react with other gangliosides, in particular asialo-GM1 (GA1) and GD1b, however with low avidity and requirement for high concentrations of these other gangliosides (Cumar et al.,1982; Lauer et al.,2002).

5.1.6 *Fluorescence-activated cell sorting (FACS) analysis*

FACS analysis uses flow cytometry to detect the fluorescence and structural characteristics of individual cells (or particles) within heterogeneous mixtures, and thus has potential to sort the cells into different groups by their characteristics or different fluorescent labels. Flow cytometers are custom built machines which propel the cells or particulate mixture in a focused stream, via an arrangement of lasers, and fluorescence detectors, which are perpendicularly arranged and detect direct light emittance (forward scatter) and deflected light (side scatter), giving information about the size and the complexity of the cell structure (Fulwyler,1980).
5.1.7 Research aims

The aim of this experiment was to screen a database of MMN sera, to determine the different binding characteristics which may exist within this seemingly homogenous group and through this perhaps elucidate more clues to the antibody's pathogenicity. First the aim to was discover any anti-GSC (ganglioside complex) antibodies in MMN patients, through the use of novel, miniaturised solid phase immunoabsorption study (microarray), which could facilitate the screening of larger cohorts of membrane lipids than traditional ELISA techniques.

Secondly, the binding characteristics of IgM anti-GM1 antibody in MMN sera were sought, in particular, looking for an inhibitory effect of local GD1a as was discovered with human monoclonal antibody from an MMN patient.

5.2 Methods

5.2.1 ELISA

Ganglioside stock solutions for ELISA were made by diluting gangliosides in methanol to 2µg/mL. 96 well ELISA plates (Immulon 2HB) were ‘coated’ with gangliosides by adding 100µL per well, and for the negative control methanol only was added to a number of wells per ELISA plate. Subsequently, the plate was left to air dry for at least 2 hours in a fume hood. Plates were kept at 4°C for at least 1 h prior to further use.

Plates were blocked with 2% BSA/PBS for 1 h at 4°C. Primary samples were diluted in 0.1% BSA/PBS. Then 100µL of the diluted solution was applied to each coated well of the ELISA plate. Incubation was for 12 h at 4°C.

The primary solution was discarded, and the plates immersed in cold PBS were then discarded for five cycles. IgM peroxidase-labelled secondary antibody was, diluted 1:3000 in 1% BSA, and 100µL applied to the wells and incubated for 1 h at 4°C. The plates then underwent the same wash protocol as for the primary solution. Detection was performed with an o-phenylenediamine dihydrochloride
solution. The reaction was terminated with 50µL of 4 M H₂SO₄. Optical density at 492 nm was detected by an automated plate reader (Ascent Multiscan, Labsystems, GMI, USA). Background (methanol) optical density (OD) values were subtracted to give final OD values.

5.2.2 Microarray

Screening of combinatorial lipid arrays was performed as previously described (Rinaldi et al., 2010). Sera from 33 patients with MMN were screened on a microarray panel comprising lipid membrane components GM1, GM2, sulphated galactosylerceramide (sulfatide), galactocerebroside (GalC), phosphatidylserine (PS), GD1a, GT1b, GA1, sulfoglucuronyl paragloboside (SGPG) and sialosyl-lactoneotetraosylceramide (LM1) (Sigma, UK or Avanti Polar Lipids, Alabaster, AL). These were solubilised in methanol and immobilized on a PVDF membrane (Invitrogen, UK) alone and in combinations (a 1:1 v/v mixture comprising 10ng of each lipid per spot) using an automated TLC arrayer (Camag Linomat Autosampler). Membranes were blocked with 2 % bovine serum albumin (BSA) in PBS (10 mM Na₂HPO₄, 150 mM NaCl, pH=7.4) for 1h. Membranes were then incubated with 250 µl of serum diluted in 1 % BSA in PBS for 1 h at 4°C. After a further wash cycle, membranes were incubated with 250 µl of a 1:25000 diluted HRP-labelled anti-human IgM antibody for 30mins and washed twice in 1% BSA in PBS. The membranes were dried for 10 minutes at room temperature and chemiluminescence signals were detected (ECLplus, GE Healthcare) by autoradiography. Exposure time was 1 minute.

5.2.3 PC12 cell culture

Culture flasks were coated with poly-l-lysine (Sigma-Aldrich) diluted to 4mg/ml in PBS. Culture medium was made as follows: for each 100ml, 7.5ml FBS (foetal bovine serum, Sigma), 7.5ml horse serum, 1ml penicillin/streptomycin (GIBCO 15140), 1ml L-glutamine 200mM (GIBCO), and remainder, 83ml DMEM (Dulbecco’s modified eagle medium, GIBCO). The serum-enriched culture medium was filtered and stored at 4°C until use. PC12 cells were grown in culture
flasks at 37°C in water jacketed CO₂ incubator until around 80% confluence was reached, with culture medium changed every 48-72 hours.

5.2.4 Immunohistochemistry

Culture medium was removed from the flasks and flasks were rinsed with PBS (pre-warmed to 37°C), before being trypsinised at 3 7°C for 1-2mins to detach cells from the coated surface of the flasks. Serum-enriched culture medium was added to the flasks to suspend trypsinisation, and the cell-containing medium was added to sterile universal containers, centrifuged at 900rpm for 5 minutes. The supernatant was discarded and the cell pellet re-suspended in 5ml culture medium using a 23 gauge needle. Cells were then counted under the microscope to give cell concentration per ml. 20,000 cells were pipette onto the centre of poly-l-lysine coated glass cover slips, and once cells had adhered to cover slip, wells were filled with culture medium, and placed in the 37°C for 24 hours.

Neuraminidase (Sigma-Aldrich) was made up to concentration of 2 units/ml (1 unit = 128µg) in serum-free DMEM, and warmed to 37°C prior to adding to the culture wells, which had been pre-rinsed with serum-free DMEM. Cell-coated cover slips were incubated with neuraminidase for 30 mins at 37°C. Following this, cover slips were rinsed with 4°C PBS, and kept on ice.

The immunostaining step follows. In preliminary experiment, to establish the presence of GM1 ganglioside on neuraminidase treated and untreated cells, FITC-labelled cholera toxin B subunit (Vibrio cholera, Sigma-Aldrich C9903) was made up to concentrations of 250ng, 500ng, 1µg and 2µg and incubated in darkness at 4°C for 60 mins. In further experiments, unlabelled CTB was incubated as above, at concentration 1µg/ml, prior to incubation with monoclonal antibodies (DG1, DG2 and SM1) 10 µg/ml, or MMN sera at 1:10, 1:50 and 1:100 dilutions. Following this, FITC-labelled secondary antibodies were applied at 1:300 concentrations for 30mins; for DG1 and DG2 goat anti-mouse IgG3-FITC (Southern Biotech) and for MMN, control sera and SM1 mAb, goat anti-human IgM-FITC (Southern Biotech).
Cover slips were then washed in 4°C PBS, and then fixed with 4% paraformaldehyde, left for 15mins at room temperature, then Triton-X100 0.1% was added to permeabilise the cells. Cover slips were rinsed in PBS and mounted on microscope slides with addition of DAPI mounting medium for nuclear staining (Vectashield, Vector). Image acquisition was performed using Carl Zeiss software, exposure time was kept constant for each image.

5.2.5 FACS analysis

Following the trypsinisation step above, cells were re-suspended in serum free DMEM and 5 x 105 cells were added to 12mm diameter polyester round bottom tubes (BD Falcon). Neuraminidase treatment was carried out as above. Following this, FACS tubes were centrifuged at 4°C 500rcf in FACS buffer (recipe) for rinsing, prior to addition of 5µg unlabelled CTB (initially 2µg but increased later in experiment due to lack of blocking of sera – see results). Cells were rinsed twice in FACS buffer between CTB incubation, and addition of MMN sera, concentration 1:50. This was the optimum concentration found on cells staining (above) to have detectable binding to cells with low background binding. Cells were washed 3 times in FACS buffer by resuspension of the pellet between successive centrifugations, before incubation with secondary antibody (FITC-labelled as above, 1:3000). All FACS analyses were carried out on the same FACSscalibur machine, each sample recorded at least 10,000 cells. Using FlowJo software, mean fluorescence intensities were calculated.
5.3 Results

5.3.1 Combinatorial microarray

MMN sera from 33 patients were screened on a 10 x 10 combinatorial microarray composed of the lipids GM1, GM2, GD1a, GT1b, GA1, GalC, SGPG, Sulphatide, LM1 and phosphatidylyserine (PS), giving a total of 45 lipid complexes, each duplicated in a mirror image against a diagonal line of methanol only as shown in figure 5.2.

**Figure 5.2. Combinatorial glycolipid microarray.** Two microarray images are shown with superimposed layout grids for ease of interpretation. Boxes marked ‘X’ represent spots where methanol only, no lipid, was spotted onto the PVDF. Along the outer horizontal and vertical rows are the single lipid spots, duplicated in a mirror image across the methanol only line. All other spots represent lipid combinations as per the corresponding row/column headers. The microarray depicted on the left side is a typical anti-GM1 positive patient, who has binding detectable to GM1 single, which is seen to be diminished by combinations with GD1a, GT1b and LM1, whilst enhanced in combinations with GalC notably. Whilst on the right, a typical anti-GM1 negative patient is depicted, showing no binding to any single lipid, but binding to the following combinations of lipids: GM1:GalC, GM2:GalC, GA1:GalC and SGPG:GA1.
5.3.2 ELISA vs microarray

The widely employed threshold in ELISA above which a value is regarded positive is 0.1 OD (which is derived from screening a large pool of healthy control serums at intervals and in-house quality control checks) (Willison et al., 1999). Two methods for positive value threshold were proposed. First, the median and 95% confidence interval of GM1 single in the healthy control population was calculated by Wilcoxon signed rank test (data was not normally distributed) giving an estimated median of 460 intensity units (IU), with a 95% confidence interval of 341 to 654 IU. Thus any values above 654 IU were considered to be positive for GM1 single on the microarray. As can be seen from figure 5.3, there was concordance between ELISA and microarray results such that ELISA negative sera were microarray negative, and positives likewise, except for one patient who was positive on the microarray (mean intensity 3996 ± 1086) whilst negative on the ELISA (mean OD 0.09 ± 0.03).

Figure 5.3. Correlation between ELISA and microarray for GM1 single. Both panels display the same scatterplot of individual values which are mean binding intensity (of repeated experiments) for 32 individual patient sera in ELISA and microarray testing for GM1 single epitope. In both plots, regression analysis was performed, and the solid line represents the best fit line, whilst the dotted lines represent reference lines on the corresponding axes, where value above the line would be considered positive. In both panels, the reference line on the x-axis (ELISA) is 0.1 OD, the origin of this value is explained in the text. The two
scatterplots here differ by the choice of two different reference lines for microarray method. Panel A uses the value 654 IU, which is the upper 95% confidence interval of the median binding intensity to GM1 single by the cohort of 27 healthy controls. Panel B uses the value 4365 IU, which is the predicted value of binding intensity which corresponds to an ELISA value of 0.1 OD, calculated by the regression equation presented in the text.

There was a linear relationship between ELISA and microarray values, such that the regression equation was \( \text{MICROARRAY (IU)} = 387 + (39780 \times \text{ELISA (OD)}) \), on linear regression analysis the p value of the correlation coefficient was < 0.001, and \( R^2 \) was 59.2%, indicating a good fit of this regression line. From this equation the corresponding microarray value to the widely used positive cut-off value of 0.1 OD on ELISA can be predicted. Thus a value of 0.1 OD would be \((387 + (39780 \times 0.1) =) 4365\) IU on microarray testing. Since an OD of 0.1 is the upper limit reference point for the diagnostic lab, then perhaps the corresponding microarray value should also be used as the cut-off for a positive result. If we do this, the yield of GM1 positives is reduced, to 19/33, and as can be seen from figure 5.3 panel B, there are now two samples which had been positive on the ELISA but were negative on the microarray.

5.3.3 GM1 binding in complex with other glycolipids

Using the former method, the upper 95% confidence interval of the median as the cut-off, there are 25 sera which are positive on the microarray for GM1 single, equating to 76% of the MMN population screened. The median signal intensity of GM1 single epitope was 13394 IU (IQR 5213 – 28801). When GM1 was in complex with other gangliosides or glycolipids, the signal intensity increased, decreased or was unchanged as shown in figure 5.4. The complex-inhibiting lipids were (in order of inhibition) LM1, GD1a, and GT1b. The complex-enhancing lipids were (in order of enhancement) GalC, SGPG and sulphatide.
Figure 5.4. GM1:glycolipid complex binding Boxplots are drawn, each depicting the median change in binding intensity from GM1 single and the corresponding GM1 complex. Shaded boxes represent interquartile range, whiskers represent the remaining upper and lower 25% of the data, while outliers are represented by circles with diagonal crosses through the centre. Outliers were not excluded from any analyses. Boxplots where the majority of the data falls below the zero line are considered to be complex inhibited, while those which lie above the zero line are considered to be complex-enhanced.

5.3.4 GM1:GalC association with disease

Binding to GM1:GalC lipid combination was found in 100% of MMN sera, regardless of reactivity to GM1. ANOVA of the significance of the difference between MMN (all sera) and healthy controls reveals that the combination GM1:GalC is the most significantly different in the MMN group compared with the control group, with a p-value of 6.38x10^{-17} was GM1:GalC. As can be seen in figure 5.5, this epitope had the highest fold change from the healthy control group, and was more sensitive than even GM1 single epitope (since it was present in all MMN sera regardless of GM1 positivity).
Figure 5.5. Glycolipid moieties associated with MMN disease vs healthy controls

Scatterplot where individual dots depict the statistical data for the epitope (single or paired glycolipids) comparing the binding intensity of MMN sera to healthy control sera for that epitope. Dots are plotted at x values representing the inverse size of the p-value, where the negative value of x is the number in scientific notation of power-of-ten, thus a larger x-value means a smaller decimal of p-value, thus stronger significance. The fold change value (y-axis) is calculated by the binding intensity in the MMN sera divided by the binding intensity in the healthy control sera.

Examining the GM1:GalC complex further, there was clear complex enhancement with GalC in the GM1 positive sera. The mean intensity of the complex was significantly higher by 8797 IU (95% confidence interval 2538 – 15056), than if the intensity of each single spot was added together (GM1:GalC complex vs. GM1 single + GalC single, paired t-test, p=0.008). In the GM1 negative group this complex enhancement is greater, at 10734 (95% CI 2595 – 18873). The above can be seen illustrated in figure 5.6.
5.3.5 Immunohistochemistry of PC12 cells

Immunohistochemistry was performed as a qualitative experiment to test the antibody and serum binding to the cell membrane. First the cells were incubated with fluorescence-labelled cholera toxin B subunit, to demonstrate GM1 epitope presence on the cells. This showed that GM1 was present in both the untreated and treated PC12 cells.
Figure 5.7. **Fluorescent-labelled Cholera toxin B in neuraminidase treated cells.** Images showing immunohistochemistry staining of PC12 cells. DAPI staining (blue) is specific for the cell nuclei, and FITC-staining (green) appears to be binding to the cell membrane, and in this case is conjugated with cholera-toxin B subunit used as a probe for GM1 ganglioside on the cell surface. Different concentrations of FITC-labelled CTB were applied (500ng left side; 1µg right side) to cells which were neuraminidase treated (row B) and those which were not (row A). CTB can be seen to bind the cells regardless of CTB treatment, and this is effective at both concentrations of CTB.

Next monoclonal antibodies were studied, human IgM GM1 antibody (SM1) and mouse IgG monoclonal antibodies DG1 and DG2. SM1 been shown on ganglioside-complex microarray, to be 100% inhibited from binding to GM1 by being in complex with GD1a or GT1a, similarly too, DG1 reacted in this pattern, whilst DG2 binding was not complex inhibited and bound to GM1 in the microarray in a complex independent manner. Figure 5.8 shows that both DG1
and SM1 did not bind untreated cells, however did bind to the cell surface following removal of sialic acid residues by neuraminidase treatment, and this binding was blockable by incubating treated cells with unlabelled cholera toxin B. It is shown, by the binding of labelled CTB and DG2 antibody to untreated PC12 cells, that GM1 is present, and potentially bindable, on the cell surface before neuraminidase treatment. As can be seen in figure 5.8, binding of DG2 was also blockable by unlabelled CTB.
Figure 5.8. Binding of anti-GM1 monoclonal antibodies to PC12 cells treated with neuraminidase. Immunohistochemistry images with nuclei stained blue (DAPI) and green fluorescent staining (FITC) corresponds to binding of the monoclonal antibody depicted in columns (left to right DG1, DG2 and SM1). In row A the cells are untreated and have had no incubation with cholera toxin, simply incubated with monoclonal antibody then with secondary FITC labelled antibody. Row B shows cells which have been treated with neuraminidase prior to incubation with mAbs and labelled secondary antibody. Row C shows cells which have been treated with neuraminidase then incubated with unlabelled cholera toxin B prior to being incubated with the monoclonal and labelled secondary antibody.

Using unpurified human serum from MMN patients, binding to PC12 cells was investigated, and similarly found that sera only bound to cells which had been neuraminidase treated, however binding did not seem to be ‘blockable’ by CTB. Serum from patients with MMN who were IgM GM1 antibody negative were also studied, and this revealed no binding to either treated or untreated cells.
Figure 5.9. Binding of MMN sera to PC12 cells treated with neuraminidase. As before, PC12 cells are stained with DAPI (blue) nuclear staining, and FITC-labelled (green) secondary antibody binds to the primary antibody (MMN sera). ‘MMN 10’ and ‘MMN 24’ (first two columns) are sera from patients who are known to be IgM-anti-GM1 positive on ELISA and microarray. ‘MMN neg serum’ (final column) is serum from a patient known to be IgM-anti-GM1 negative. As before, cells in the second and third rows have been treated with neuraminidase, and only the cells in the final row have been pre-incubated with unlabelled cholera toxin B subunit.
5.3.6 FACS analysis

Fluorescence activated cell sorting was employed as a quantitative measure of the above experiments. Essentially, the same experiments as shown in the immunohistochemistry above were repeated using FACS methodology. Using the monoclonal antibodies, antibody binding to the cells was able to be quantified in terms of the fluorescence of a cell population. Mean fluorescence intensities (mean fluorescence unit, MFU) were calculated from cell populations of 10,000 (~8000 after gating), and divided by the background fluorescence intensity to provide a proportion of background fluorescence. For DG1 and SM1, there was no binding above background levels of fluorescence therefore can be expressed as 0 MFU. Both DG1 and SM1 were demonstrated to bind to neuraminidase treated PC12 cells, with mean fluorescence rising to 890 MFU (± SD 142) and 991 MFU (± SD 159), which is between 14 and 18.5 times the background fluorescence or the binding to untreated cells. Antibody binding to treated cells was completely blocked by pre-incubation with cholera toxin B-subunit, with mean fluorescence in this cell population (minus background fluorescence) of 0 MFU, for both DG1 and SM1.

DG2 showed a different pattern, and was able to bind to untreated cells with intensity up to 20 times higher than background, with mean fluorescence intensity of 1298 MFU (± SD 207). The binding of DG2 to neuraminidase-treated PC12 cells was over double the binding to untreated cells (3068 FU ± SD 398 vs. 1298 MFU untreated), which was fully blockable by cholera toxin to fluorescence intensity roughly equal to that of the untreated cell population (1023 MFU ± SD 163). For this monoclonal, the effect of cholera toxin on untreated cells was investigated, and this blocked just over 50% of the binding of DG2 to untreated cells (580 MFU ± SD 85).
Figure 5.10. FACS analysis of neuraminidase-treated PC12 cells

Histograms overlaid on the same graphs are drawn for each monoclonal antibody tested on Flow cytometry. The x-axis is the mean fluorescence intensity, which is a direct measure of the antibody binding to the cell (and thus to GM1 ganglioside on the cell surface). The y-axis displays the number of cells which were counted in the corresponding fluorescence range, standardised across all three cell groups overlayed on the same graph, as the percentage of the maximum number of cells counted across all three groups. In all three panels, the green line is the histogram for fluorescence intensity of untreated cells, the red line is that of neuraminidase treated cells, and the blue line is the histogram of neuraminidase treated and cholera toxin pre-incubated cells. Where a line shift to the left we can say that binding in that population was lower, whereas line shifted to the right represents higher binding.

Following this initial experiment, the next step was to investigate the binding of MMN sera to these cell populations (neuraminidase treated PC12 cells), with the aim to characterise whether the binding of the anti-GM1 antibody in MMN sera could be enhanced by neuraminidase treatment. Sera from 16 patients, which were known from microarray and ELISA to be IgM GM1 antibody positive, were tested. SM1 was used as a standard during each run of the experiment to ensure there was concordance between the cell populations and the efficacy of neuraminidase treatment. Thus the MFI value for SM1 binding to neuraminidase-treated PC12 cells was used as the ‘maximum binding intensity’ for each run.
Differences between cell cultures were corrected for by expressing the fluorescence as a percentage of this maximum binding intensity for each run. The mean maximum binding intensity across all runs was 1052 IU, 95% confidence interval 690 – 1413 IU.

Overall, the binding intensity (as % of maximum) increased from 13% to 26%, a significant increase of 12.7% (95% confidence interval 1.8 to 23.7, p = 0.02). There was a mean fold change for treated vs untreated cells of 2.0 (95% confidence interval 1.3, 2.8), thus there was on average a doubling of binding intensity when treated with neuraminidase.
Figure 5.11. The effect on neuraminidase treatment of MMN sera binding to PC12 cells. Binding intensity is expressed as percentage of the maximum binding intensity for that run of experiments, which was SM1 binding to neuraminidase treated cells. Panel A and B show the mean percentage binding intensity for the 3 cell groups overall, and the 95% confidence intervals of the mean, in anti-GM1 positive (Panel A) and anti-GM1 negative patients (Panel B). In panel A, significance testing by paired t-test, showed significant difference between untreated cells and treated or untreated cells and treated plus CTB pre-incubated. In panel B significance testing revealed no significant differences between cell groups. Panel C shows the binding intensity of 16 anti-GM1 positive MMN sera with lines showing the trend between cell groups for each individual serum tested, where ‘treated’ means neuraminidase treatment and ‘CTB’ means cells were incubated with cholera toxin prior to addition of the serum.

Incubation of cholera toxin B following treatment with neuraminidase but prior to incubation with patient sera did not show the full blocking effect that was
demonstrated with the monoclonal antibodies, as can be seen in figure 5.11 panel A, the binding intensity remained as high as the treated cells without cholera toxin. Significance testing between the two groups, treated and treated plus CTB incubated, unsurprisingly revealed no difference between groups of CTB incubation or no incubation. (p = 0.69, paired t-test).

By looking at the individual trend for each patient sera (figure 5.11 panel C) we can see that there was one in particular (subject ID MMN 10) which behaved similarly to DG2 and SM1, in that binding increased with neuraminidase treatment (from 14% untreated to 98% treated). It was partially blocked by subsequent cholera toxin binding (42%). This gives a mean fold change for this individual of 6.9 (± SD 3.9).

We divided the mean fluorescence intensity (MFU) of the CTB incubated cells, by the MFI of the non-CTB cells, giving a ratio, where a value of less than 1 meant that CTB blocked some of the binding of the sera to the neuraminidase treated cells. This data was not normally distributed, and Wilcoxon signed rank test was used to calculate the estimated median ratio to be 1.06, with a 95% confidence interval 0.8 to 1.6. 5 sera had a ratio of less than 0.8 and therefore we can say were partially blocked, whilst 3 sera had ratio above 1.6, displaying further increase of sera binding with incubation of CTB.

A control group of MMN sera known to be negative for IgM anti-GM1 antibody (figure 5.11 panel B) showed low degree of binding to untreated cells, which did not increase with neuraminidase treatment (p = 0.7, paired t-test).

5.3.7 Correlation between solid-phase and fluid phase membrane binding characteristics

Correlation studies were performed using data from the glycolipid complexes analysis, to search for any relationship between the increase in binding with neuraminidase treatment (measured fold change mean fluorescence in treated cells over the mean fluorescence of untreated cells) and the percentage inhibition
of solid phase GM1 binding when in complex with GD1a. This revealed that an inverse relationship existed, where the regression equation was ‘fold change = 12.39 – 0.1 x % inhibition’, such that that the lower the inhibitory effect of GD1a on GM1 binding, the higher the increase in binding to PC12 cells with neuraminidase treatment. This is the converse of what was logically suspected. The p-value for this correlation was significant (0.027) and the R-squared value was positive but less than 50%, indicating that the residuals were highly variable from this line. This suggests that there may be a weak association, but as can be seen from the figure 5.12 panel A, there are a few data points at the lower end of the GD1a inhibition, and therefore there may be excessive variability within the data to support this relationship.

Similarly we looked for a relationship between the amount of inhibition by cholera toxin B, and the presence of other serum factors, such as GA1 antibody binding. The fitted regression line was almost horizontal, with an R-squared of 0.0%, and therefore there was no relationship between the amount of GA1 binding and the degree of CTB blocking in the treated cells (Figure 5.12 panel B). It should be noted also, of that the two sera which display the highest degree of CTB blocking (around 0.5 fold change); one is GA1 negative, whilst the other is strongly positive.
Figure 5.12. Relationship between PC12 binding and combinatorial microarray binding. Scatterplots are drawn with individual data point representing values for individual patient serum from 16 anti-GM1-positive MMN patient sera. Panel A plots the neuraminidase-increased binding effect (expressed as fold change treated over untreated cells) against the percentage of inhibition seen on the microarray lipid complex of GM1:GD1a compared to GM1 alone. It was theorised that the greater the inhibitory effect of GD1a on binding to GM1, the greater the increase in binding with neuraminidase. However, as displayed by the best fit line panel A, there is a pattern towards the converse relationship although this relationship may be weak. Panel B displays the degree of inhibition of serum binding to cells by pre-incubation with cholera toxin B, which was expressed as fold change CTB over no CTB groups (both neuraminidase treated). This showed a horizontal line fit with no correlation between these two variables.
Three different approaches to the detection of antibody-ligand interaction in MMN sera were used in this study.

First, it was demonstrated that novel solid-phase antibody detection assay, PVDF microarray, correlated well with the standard method of antibody detection, ELISA, which is used widespread in clinical diagnostic labs for anti-glycolipid antibody testing. The major advantage of using the PVDF glycolipid microarray, or ‘microarray’, is that using smaller amounts of all materials, larger cohorts of samples can be screened against expanded panels of ligands, allowing more opportunities to screen for antibody interaction with pairs of glycolipids. The microarray array method may have been more sensitive for detection of antibody binding to GM1 single lipid which was not already detected by ELISA, however it depends on the value employed for upper limit of normal, and this may have to be decided by screening a larger cohort of healthy controls as had been done for the GM1 ELISA. Using the microarray, antibody binding to a novel epitope was found to be the most specific for MMN, composed of GM1 ganglioside paired with galactocerebroside (GalC), a major glycosphingolipid of the myelin sheath in both central and peripheral nervous system (Lisak et al., 1980). From our cohort of 33 MMN sera tested, all sera were reactive against the GM1:GalC complex, including those that were not reactive to either GM1 single or GalC single. This finding has great impact upon the understanding to the immunopathogenesis of MMN, as patients who were previously considered to be ‘antibody negative’ despite having no discerning clinical features from the antibody positive group, are now known as also having serological factors which react against glycolipids. Thus a more unifying theory of antibody-driven pathogenesis may be plausible in this neuropathy.

In addition, in all MMN sera in this cohort it was demonstrated that GalC pairing increases the intensity of antibody binding to GM1, thus we have another example of enhancement of glycolipid pairing in a neurological clinical disease group. It is known that GM1 is present mostly on the axonal surface and GalC on
the myelin. It could be assumed that GM1:GalC pairing may occur at the paranodal loops, where the myelin and the axon juxtaposed. Given that conduction block in MMN is thought to occur due to disruption at the nodes of Ranvier, it is an attractive idea that GM1:GalC antibodies bind to the paranodal loops, fix complement and form MAC pores, leading to conduction block.

Findings from the microarray reveal that the anti-GM1 antibody in MMN is inhibited by GD1a and GT1b, gangliosides with terminal sialic acid groups, which have been proposed to ‘mask’ the GM1 within lipid rafts in the cell membrane, thus shielding it from antibody binding.

It has previously been demonstrated, using mouse and human monoclonal antibodies that there are (at least two identified so far) different types anti-GM1 antibodies, separated by their ability to bind GM1 in the locale of GD1a, and that the monoclonal antibody from an MMN patient was of the GD1a-inhibited type, such that it could only bind GM1 when GD1a had been removed by sialidase treatment (Greenshields et al., 2009). Results from the microarray clearly show that this also holds true for a cohort of MMN sera, that all the anti-GM1 antibodies in MMN are complex inhibited by local GD1a in solid phase immunoassays. This seemingly is a unifying feature of MMN, and a further clue towards the pathogenesis of this condition.

Furthering the biological relevance of these findings, MMN sera was applied to live cell cultures of PC12 cells known to contain mono-, di- and poly-sialogangliosides. Cholera toxin avidly bound to GM1 on the cells regardless of sialidase treatment, thus native GM1 was present in abundance enough that anti-GM1 recognising factors could bind to untreated cells. Further to that, we repeated the experiments using mouse mAbs DG1 and DG2, and human monoclonal SM1 from MMN patient to confirm the presence of the two different types of anti-GM1 antibodies.

Using sera from MMN patients, we showed that anti-GM1 antibody negative sera did not bind to PC12 cells, regardless of sialidase treatment. However, almost all anti-GM1 positive sera displayed the same binding properties as DG1 and SM1:
binding did not occur on untreated cells, but with removal of terminal sialic acid residues, converting GD1a and GT1b to de novo GM1, IgM in the patient sera bound to the cell membranes. This leads on from the findings that GD1a and GT1b were inhibitory on the microarray analysis, and allows us to see that this is relevant in a biological membrane.

DG2 was shown to bind to treated cells up to 45 times the background level, its binding would be to both native and de novo GM1 since this antibody is shown not to be GD1a inhibited. DG1 (and SM1) on the other hand, bound up to around 20 times the background level, which is roughly the difference between DG2 binding to treated and untreated cells, and thus these antibodies may be binding to the de novo GM1. However we would expect the full binding potential of the GD1a inhibited antibodies to be equal to DG2, since they should also be able to bind native GM1 with the removal of neighbouring sialic acid residues. This was addressed by Greenshields et al, who first blocked the native GM1 with unlabelled cholera toxin, then treated with neuraminidase, and demonstrated that this reduced the binding potential of DG1, showing that DG1 also binds to native GM1, which has been exposed by sialidase treatment {Greenshields, 2009 47 /id}. It may be that DG1 and SM1 are unable to bind to the de novo GM1, and only bind to the unmasked native GM1, however if this was so, then the pre-blocked then treated cells would have no binding sites available for DG1, yet DG1 does bind in this condition. We must conclude that DG1 and SM1 can bind to both native and de novo GM1, but do not bind all the potentially available GM1 lipids in either group. There may be other factors which are inhibitory to the binding of these antibodies which remain to be discovered, and further expansion of the combinatorial arrays to include not just glycolipids, but other cell membrane components such as phospholipids, cholesterol and glycoproteins, may further elucidate the subtle interactions of neighbouring epitopes on the binding of antibodies to glycolipids.

A shortcoming of the results presented here was that cholera toxin B did not effectively block the ability of MMN sera to bind to treated PC12 cells, although CTB at this concentration demonstrated full blocking activity of the monoclonal
SM1, under identical experimental conditions. This implies that there is another factor in the MMN serum that binds to PC12 cells, but only when they have been sialidase treated. We know that sialidase treatment converts GD1a and GT1b to not only GM1 but also to GA1, and that the anti-GM1 antibody has cross reactivity (via the terminal Gal-GalNAc epitope) with GA1 and GD1b. A potential explanation for the lack of CTB blocking could be that sialidase treatment unblocks GA1 and MMN patient sera binds to both de novo GM1, exposed GM1, and exposed GA1. Since there is no reason to suspect that cholera toxin would bind to GA1, then this could explain the high mean fluorescence despite CTB pre-incubation. However there are shortcomings of this theory, firstly that we would then expect CTB to cause a reduction but not complete blocking of MMN sera binding, and this was only demonstrated in roughly one-third of patient sera. Secondly, there was no relationship between GA1 binding intensity on the microarray and the inhibitory potential of CTB. Thirdly, the same phenomenon should perhaps be expected to occur with the MMN mAb, since this in theory, should bind the terminal Gal-GalNAc epitope of GA1, however the mAb binding to treated cells was fully blocked by CTB. Further work in this series of experiment could use TLC to separate the lipid components of neuraminidase treated cells and determine, using ganglioside standards, the factor to which MMN serum is binding. However, what may be highlighted by this experiment is the inefficient nature of working with unpurified human serum, and that further work to develop monoclonal cell lines should be continued, to further discover the characteristics of MMN antibody binding.

Regarding the discovery of the anti-glycolipid-complex antibody (anti GM1:GalC) as specific for MMN, the next logical step would be to validate this finding in a larger cohort of MMN patients. If this revealed that this epitope was highly sensitive and specific for MMN, then new diagnostic tests should be developed for accessible testing of this antibody in potential cases of MMN. This would aid the diagnostic yield, and reduce the number of cases of MMN with delayed diagnosis and delayed treatment due to diagnostic uncertainty. ELISA could easily be used for this purpose, with mixing of the gangliosides prior to coating of
the wells, and correlation studies between ELISA glycolipid complex binding and microarray binding should be carried out to test whether this test can potentially be used. This may herald a more sensitive and specific diagnostic tool for MMN, and opens up the potential of screening other autoimmune conditions for complex antibodies, both within the nervous system and beyond.
6 Conclusion of Thesis

This project, in its entirety, has made multiple advances in the field of autoimmune neuropathy. Firstly, it has been the first human trial of complement inhibition in an immune-mediated neurological disease, and should herald the beginning of others. It has demonstrated that eculizumab can be given in patients with MMN and certainly did not worsen their condition, nor interfere with the beneficial effect of IVIg in this condition. There was however, an increase in side effects, especially headache. Many of the side effects relate the drug being a biological agent. Some of the side effects could potentially be avoided with the development of non-biological complement inhibitors, as some of the experimental compounds, which demonstrated inhibition of nerve disruption in animal models, may soon be developed for use in humans. The risk of meningococcal septicaemia would remain even with these non-biological compounds however, and therefore therapeutic complement inhibition will never be without significant risks. However no patients in this trial had bacterial infection. Overall, the safety profile was felt to be acceptable, and certainly all patients tolerated the drug.

In this study, it has been shown that concurrent administration of high dose IVIg caused a decrease in the monoclonal antibody concentration, which provides the first human evidence of this effect. Despite this, eculizumab retained its pharmacological activity, and therefore can be given concurrently with IVIg without reduced effect. Within the limitation of the open-label study design, there were some indicators of a possible benefit of complement inhibition, which were superadded to the IVIg benefit. This sheds some light on the pathogenesis of the condition, as although there perhaps some response the complement inhibition, the necessity for the other immunomodulatory effects of IVIg remained. It is suspected that this has much to do with the chronic nature of this neuropathy, and it is most likely that complement inhibition may be more efficacious in the prevention of the initial inflammatory injury, as has been demonstrated widely in the animal models. This leads to future studies of intervention, in which complement inhibitors could be aimed at patients with new or recent diagnoses of
MMN. It is suspected that complement inhibition could prevent progression of disease. A longer treatment period is necessary, as the small changes in neurophysiology could increase with time, even in chronic lesions, with removal of complement injury which may allow remyelination and axonal repair, a process which is likely to take longer than 14 weeks. However the difficulty with running trials in immune-mediated neuropathies, given the rarity of cases and difficulties with finding sensitive outcome measures, may hinder trials. In the acute immune-mediated neuropathy setting, complement inhibition is predicted to be more efficacious, and evidence presented here should support this.

In addition to the clinical trial, the experimental laboratory work presented here elucidates further the nature of the antibody-basis to this disease. Firstly, it has been the first study to identify an immunological target in patients who have been demonstrated so far to be antibody negative. Validation studies in larger cohorts of MMN patients are required, and if this epitope is widely associated with MMN, then this could potentially open up the field of immunodiagnostics in the testing towards antigen-pairs or antigen-complexes. These theories could be extrapolated to many other autoimmune conditions, and contribute to the understanding of pathogenesis, diagnostics and therapeutics in many body systems. Further advances are being made within this research group, to miniaturise the immunoassays further, allowing screening of large libraries of lipid complexes, which in time could be used to analyse the combinative effects of the various components of cell membranes, in particular looking at the lipid rafts, and their interplay with serological factors in disease.
Patient Information Sheet (written by Professor H Willison)

Version 1, 25/11/08

SAFETY AND TOLERABILITY OF ECULIZUMAB IN THE TREATMENT OF MULTIFOCAL MOTOR NEUROPATHY: A SINGLE CENTRE OPEN LABEL STUDY

We would like to invite you to take part in a research study. Before you decide you need to understand why the research is being done and what it would involve for you. Please take time to read the following information carefully. Talk to others about the study if you wish. Ask us if there is anything that is not clear or if you would like more information.

Part 1 This part provides details on the purpose of this research study

1. Who is conducting the research?

The research is being carried out by Professor Hugh Willison from the Department of Neurology.

2. What is the purpose of the study?

The main purpose is to see how safe and how well tolerated the anti-inflammatory drug called eculizumab is, when used for treatment of patients with multifocal motor neuropathy (MMN). Eculizumab has never been used to treat MMN before. Eculizumab is currently available in the UK for use in patients for a different condition and has been used to treat approximately 1000 patients worldwide with a range of different autoimmune diseases. The secondary purpose of this study is to gather information on the clinical effectiveness of
eculizumab in patients with MMN, and to see whether being treated with eculizumab reduces your requirement for intravenous immunoglobulin (IVIg).

3. Why have I been invited?

You have been invited to take part in this study as you have MMN and are already receiving treatment with IVIg or have responded to IVIg treatment in the past.

4. Do I have to take part?

No, you do not. It is up to you to decide. Taking part in this study is entirely voluntary. We will describe the study and go through this information sheet, which we will then give to you. You will be asked to sign a consent form to show you have understood our explanation and have agreed to take part. You are free to withdraw from the study at any time, without giving reason. This will not affect the standard of care you receive or your future treatment.

5. What does taking part involve?

Vaccination

In order to protect you against infection with meningococcus you will be required to be vaccinated against Neisseria meningitides at least 2 weeks before you begin the study. Eculizumab works by blocking the action of proteins called complement. In the body, the complement system acts to cause inflammation and helps the body fight infection. Neisseria meningitides, is a bacteria which causes meningitis and can be contracted in patients whose complement system is not working fully. A very small number (less than 1%) of patients receiving eculizumab have developed meningitis. We will provide the vaccine for you in our clinic before you start treatment with eculizumab. The vaccine is given as a single injection.

You will be given a study card which you should carry with you at all times whilst you are receiving treatment with eculizumab and for 3 months after your last treatment. You should show this card to any doctor or other health care
professional eg. nurse, pharmacist, or dentist that is involved in your treatment. The card also lists the signs and symptoms that you should be alert for. They are:

- Headache with nausea and/or vomiting
- Headache and fever
- Headache with a stiff neck or back
- Fever of 103°F / 39.4°C or higher
- Fever and a rash
- Confusion
- Severe muscle aches combined with flu-like symptoms
- Sensitivity to light

If you experience any of these symptoms then you should contact the study doctor immediately or if you cannot reach the doctor, you must go to the nearest Accident and Emergency department and show them the study card.

Women of childbearing potential

If you are a woman of childbearing potential then you will be required to have a blood test before you start on eculizumab treatment to confirm that you are not pregnant. You must use adequate contraception during treatment with eculizumab and for 5 months after treatment. This is because it can take several months for the body to fully eliminate eculizumab and the effect of eculizumab on unborn children is not yet known. The study doctor will discuss contraception with you.

Treatment and monitoring

There will be two treatment phases to your involvement, a run in period up to 8 weeks long, then a 14 week treatment period, followed by an 8 week run out period. In all these periods you will receive regular assessments and examinations.

Run –In Period
You will be given your normal IVIg treatment and asked to complete self-evaluation questionnaires in the first instance. This will last for a maximum of 8 weeks and will be followed by a physical examination and some muscle strength tests. You will be asked to complete the self evaluation test once a week. Once the study doctor has reviewed the information provided, you will begin treatment with the study drug.

**Treatment Period**

Following the run-in period, you will be given the study drug Eculizumab by a drip through a tube directly into one of your veins before your IVIg treatment. This treatment will last for 14 weeks in total. You will receive eculizumab infusion as follows:

- **Week 0, 1, 2 and 3** – one infusion containing 600mg of eculizumab
- **Week 4** – one infusion containing 900mg of eculizumab
- **Weeks 6, 8, 10 and 12** – one infusion containing 900mg of eculizumab

Each infusion of eculizumab will take approximately 25 to 45 minutes, but may take longer. Following each infusion you will be monitored for one hour.

During the treatment period you will continue to receive IVIg if it is required. If your motor function does not get worse then you will not be given IVIg during this period.

Prior to, and just after the first infusion periods, a small volume (10mls; equivalent to 2 teaspoonfuls) of your blood will be checked for levels of the drug. This will require 2 additional blood tests per infusion.

**Run-out Period**

Following the treatment period there will be a period of 8 weeks where you will not receive Eculizumab and in which time your IVIg treatment will be put back to its usual interval.
6. Expenses and Payments

You will not be paid for your involvement in this study. You will be given money to cover the costs of your travel.

7. What will I have to do?

The trial protocol follows a carefully structured series of visits to hospital in which the trial drug and any IVIg you require is administered, and in which a series of observational measurements are made on your muscle performance. These will be described to you in detail by the trial doctors. You will need to attend hospital on a weekly basis for the first 4 weeks to receive your infusion of Eculizumab and on a fortnightly basis for the next 10 weeks. During some of your visits, measurements of your strength and performance on particular tasks will be made. At some visits, blood tests may also be taken. At home, on a weekly basis, you will complete a short self evaluation questionnaire - this will let us know whether you require further treatment with IVIg. If you are currently involved in another research study please discuss this with one of the study doctors whose names are at the back of this sheet.

You should bring along all medicines that you are currently taking including any that you have obtained without a prescription eg. herbal or over-the-counter medicines to each study visit.

8. What drug is being tested?

Eculizumab is being tested. It is one of a new class of drugs referred to as monoclonal antibodies. It has not previously been used to treat patients with MMN but has been used to treat other diseases. Many similar drugs are now used to treat a wide range of diseases, including inflammation and cancer. The drug will be administered by intravenous drip.

9. What are the possible advantages and disadvantages of taking part in this study?

Advantages
It is hoped that treatment with Eculizumab will have a positive effect on the symptoms of MMN and that in this study it will reduce the need for IVIg infusions. The results of this study will be made public and provide information to both the medical and patient communities. Eventually, if this and future studies demonstrate that Eculizumab has a positive effect, it may be possible to introduce it into routine clinical practice for MMN, although this may take time and is not guaranteed.

**Disadvantages**

There is a very small risk of contracting bacterial infections, including a form of meningitis. Steps have been taken to reduce this risk further by vaccination, and to ensure that any infection is treated promptly. After receiving the meningitis vaccine, the most commonly reported adverse effects were pain and redness at the injection site. Most of these were reported within 48 hours following vaccination. Other common side effects are headache, drowsiness, nausea, vomiting and diarrhoea and loss of appetite, but these should not last long.

It is also possible that you may develop other side effects from treatment with Eculizumab which may be mild, moderate or severe. Possible side effects include allergic reactions, which may require additional treatment. Other known side effects, which were very common (occurring in about 1 in 10 patients) that have been reported include headache, dizziness, runny nose or sore throat, nausea, diarrhoea, back pain, pain in joints, fever and bruising. Other common side effects include itchy skin, rash, cold sores, infections, and abdominal pain. If you notice any side effects that you are concerned about you should discuss these with the study doctor.

There is also an inconvenience factor as you will have to complete forms and examinations, and attend hospital more frequently than usual during the course of the study.

10. **What happens to me when the study is finished?**
When the study is finished you will go back to receiving your normal IVIg treatment. It is normal practice for drug companies to conduct larger trials before making long term recommendations on introduction of a new treatment. However, if both the investigators and the patient agree that Eculizumab appears to have been beneficial, over and above the benefit achieved by routine IVIg therapy alone, it may be possible for you to continue on the treatment on a named patient basis until further studies on larger groups of patients have been conducted, and agreement to fund the treatment has been received from the NHS. This arrangement will be discussed with you at the end of the study.

11. What happens to the information?

Your identity and personal information will be completely confidential and known only to the research team and NHS Greater Glasgow and Clyde staff or UK regulatory authority staff who monitor research studies. The information obtained will remain confidential and stored within a locked filing cabinet. The data are held in accordance with the Data Protection Act, which means that we keep it safely and cannot reveal it to other people, without your permission.

This completes part of the patient information sheet. If the information in part 1 has interested you please continue to read the additional information in Part 2 before making a decision to participate.

Part 2, This part provides more information.

12. What if relevant new information becomes available?

If we receive new information about the study drug during your participation the study doctor will tell you about this and discuss whether or not you should continue. If you want to continue we may ask you to sign an updated consent form. If the study is stopped for other reasons we will tell you and arrange your continuing care.

13. What will happen to me if I don’t want to continue with the study?
You are allowed to withdraw from this study at any time. Your data will be used up to the time you withdraw unless you inform the study doctors that it shouldn’t be used. We will destroy all identifiable samples if you wish.

14. Involvement of your family doctor.

We will notify your GP of your involvement in this study.

15. What will happen to the samples I give?

Your samples will be analysed in the laboratory for routine clinical tests, for antibody and complement activity and for anti-nerve antibodies. There will be no genetic testing of your samples.

16. Will my taking part in this study be kept confidential?

All information relating to your participation in this study will remain confidential and be stored in keeping with the Data Protection Act 1998.

17. What will happen to the results of this study?

The study results will be published in a clinical neurology journal and disseminated at neurology meetings.

18. Who is organising and funding this research?

NHS Greater Glasgow and Clyde and The University of Glasgow are sponsoring this study. Alexion Pharmaceuticals is providing funding to support this research.

21. Who has reviewed the study?

This study has been reviewed by the West Glasgow (1) Research Ethics Committee.

22. If you have any further questions?
We will give you a copy of the information sheet and signed consent form to keep. If you would like more information about the study and wish to speak to someone not closely linked to the study, please contact Dr O'Leary, Consultant Neurologist at the Southern General Hospital (Tel: 01412011100).

23. Contacts:

Professor H Willison, Dr J Overell, Dr A Fitzpatrick are contactable at the Southern General Hospital on 0141 201 2474, 201 2461 and 201 2096.

24. What if there is a problem?

In the event that something does go wrong, you are harmed during the research and this is due to someone’s negligence then you may have grounds for a legal action for compensation against Glasgow University & Greater Glasgow and Clyde NHS. You may have to pay your legal costs.

25. If you have a complaint about any aspect of the study?

If you are unhappy about any aspect of the study and wish to make a complaint, please contact the study doctors in the first instance through the Southern General Hospital (Telephone: 0141 201 2474, 201 2461 and 201 2096). Any complaint about the way you have been dealt with during the study will be addressed. The normal NHS complaint mechanisms are also available to you and details for this can be obtained from the hospital.

*Thank-you for your time and co-operation*
Appendix 2. Clinical Trial publication

RESEARCH REPORT

An open label clinical trial of complement inhibition in multifocal motor neuropathy

Amanda M. Fitzpatrick1,2, Cameron A. Mann3, Sarah Barry4, Katie Brennan1,2, James R. Overell5, and Hugh J. Willison1,2

1 Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK; 2 Department of Neurology and 3 Department of Clinical Neurophysiology, Institute of Neurological Sciences, Southern General Hospital, Glasgow, UK; and 4 Robertson Centre for Biostatistics, University of Glasgow, Glasgow, UK

Abstract Human and animal studies on antibody-mediated neuropathy implicate complement in pathogenesis. In animal models complement inhibition is therapeutically beneficial. The monoclonal antibody, eculizumab (SolirisTM, Alexion Pharmaceuticals, Cheshire, CT), prevents cleavage of C5 and thus inhibits terminal complement activation. In an open label study, 13 multifocal motor neuropathy patients received eculizumab for 14 weeks, 10 of whom were concomitantly receiving intravenous immunoglobulin. The primary outcome was safety of eculizumab, and the secondary outcomes included change in intravenous immunoglobulin (IVig) dosing frequency, performance, and electrophysiological parameters. Adverse events were minor during the study. Nine of 10 patients on IVig maintenance continued to require IVig. IVig dosing interval was not different between the run-in and the treatment period. There were improvements in patient-rated subjective scores and selected clinical and electrophysiological measurements. Overall, a small treatment effect occurred in some patients that appeared supplementary to and independent of the IVig treatment effect, and occurred more frequently in patients with higher baseline motor function.

Key words: clinical trial, complement, intravenous immunoglobulin, membrane attack complex, multifocal motor neuropathy, therapy

Introduction

Intravenous immunoglobulin (IVig) remains the only therapeutic option in multifocal motor neuropathy (MMN); however, its effects are temporary and weakness slowly progresses despite regular therapy (Terenghi et al., 2004). No other immunomodulatory therapies have shown enough benefit to be routinely recommended alone or as an adjunct to IVig.

Pre-clinical research has demonstrated that the pathogenic effect of anti-GM1 antibodies, found in up to 80% of MMN cases, is complement mediated, and inhibition of complement factors prevents nerve damage (Santoro et al., 1992; van Schaik et al., 1995; Goodyear et al., 1999; Yuki et al., 2001; O’Hanlon et al., 2003; Halstead et al., 2004; Greenshields et al., 2009). The monoclonal antibody eculizumab (SolirisTM) binds and neutralises human complement factor C5 preventing terminal complement activation and membrane lysis via membrane attack complex (MAC) (Rother et al., 2007).

The safety and efficacy of eculizumab in complement-mediated disorders have been shown in clinical trials of patients with paroxysmal nocturnal haemoglobinuria (Hillmen et al., 2006). However, eculizumab has yet to be trialed in any putatively MAC-dependent neurological disorders, and its
administration and safety in patients who are concurrently receiving intravenous immunoglobulin have not yet been studied. In this study, we sought to find out whether co-administration of eculizumab with high dose IVig was safe and tolerable in MMN patients, and whether eculizumab remained pharmacologically active during co-administration with IVig. Secondary aims were to assess patients for possible therapeutic benefit of eculizumab.

Materials and Methods

Patients

Patients fulfilled European Federation of Neurological Societies (EFNS) electrodiagnostic criteria for MMN (Joint Task Force of the EFNS and the PNS, 2006; van Schaik et al., 2006). Patients had a current or past history of IVig responsiveness and met other inclusion and exclusion criteria (Table 1). At enrollment, all were vaccinated with tetravalent meningococcal vaccine (ACWY Vax®, Glaxo Smith Kline, Uxbridge, Middlesex, UK). The trial protocol and supporting documentation were approved by the Regional Ethics Committee and conducted in keeping with the Declaration of Helsinki. The trial is registered on EudraCT database (unique no. 2008-005748-18). The study was investigator led and co-sponsored by the University of Glasgow and NHS Greater Glasgow and Clyde. Financial support and supply of drug were provided by Alexion Pharmaceuticals, Cheshire, CT, USA.

Study design

Patients entered the trial at baseline visit, run-in day zero (Fig. 1), which was timed to coincide with their next scheduled IVig infusion. Patients not receiving regular IVig entered the trial at a convenient date. Baseline clinical, functional, and electrophysiological assessments (Table 2) were measured immediately prior to IVig infusion in those receiving IVig. The next dose of IVig was withheld until criteria for reaching their individualised deterioration point (DP) met self-evaluated functional rating (SEFR) score increase by 2 points from baseline plus at least one of the following: Medical Research Council (MRC) sum score decrease by at least 1 point; pinch/palm grip decrease by at least 10%; 9-hole peg test time increase by at least 10%; 10-m walk time increase by at least 2 s or patient/clinician feels deterioration has occurred. This allowed determination of the IVig inter-treatment interval. Upon reaching this DP, the run-in period ended and the 14-week treatment period began. If no DP was

Table 1. Inclusion and exclusion criteria.

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
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<tbody>
<tr>
<td>1. Slowly progressive or stepwise progressive, asymmetric limb weakness, or motor involvement having a motor nerve distribution in at least two nerves, for more than 1 month.</td>
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<tr>
<td>2. Absent or minor sensory symptoms or objective abnormalities.</td>
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<tr>
<td>3. Predominant upper limb involvement.</td>
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<td>4. Documented conduction block in at least one nerve segment, classified by EFNS criteria.</td>
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<tr>
<td>5. Clinical improvement following IVig treatment (current or historical).</td>
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<tr>
<td>6. Able to complete the self-evaluation functional rating scale weekly.</td>
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<tr>
<td>7. Agreement to vaccination against meningococcal infection.</td>
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<tr>
<td>8. Willing and able to give written informed consent.</td>
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</table>

<table>
<thead>
<tr>
<th>Exclusion criteria</th>
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<tbody>
<tr>
<td>1. Upper motor neuron signs.</td>
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<tr>
<td>2. Prominent focal or diffuse sensory impairment on routine sensory testing.</td>
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<tr>
<td>3. Below the age of 18.</td>
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<tr>
<td>4. Pregnancy, planned pregnancy, or lactation.</td>
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<tr>
<td>5. Inability to comply with study related procedures or appointments.</td>
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<tr>
<td>6. Unresolved Neisseria meningitidis infection or history of meningococcal infection.</td>
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<tr>
<td>7. Any condition that in the opinion of the investigator could increase the patient’s risk by participating in the study or confound the outcome of the study.</td>
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<tr>
<td>8. Known hypersensitivity to eculizumab, murine proteins, or to any of the excipients.</td>
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<tr>
<td>9. Known or suspected hereditary complement deficiencies.</td>
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</table>

IVig, intravenous immunoglobulin.

![Figure 1. Study flow chart.](image-url)
Table 2. Assessments and deterioration point criteria.

<table>
<thead>
<tr>
<th>Clinical and functional assessments</th>
<th>Deterioration point (DP) criteria</th>
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<tbody>
<tr>
<td>1. Medical Research Council (MRC) sum score: total of 10 muscle groups from two affected limbs</td>
<td>SEFR score increase by 2 points from baseline Plus at least one of the following (a)–(e)</td>
</tr>
<tr>
<td>2. Muscle strength force (MSF) sum score: total of five muscle groups from two affected limbs, using myometry</td>
<td>(a) MRC sum score decrease by at least 1 point</td>
</tr>
<tr>
<td>3. Hand grip strength: using hydraulic dynamometer</td>
<td>(b) Pinch/palm grip decrease by at least 10% (either side)</td>
</tr>
<tr>
<td>4. Palm and pinch strength: using visiometer</td>
<td>(c) 9-hole peg test time increased by at least 10%</td>
</tr>
<tr>
<td>5. Nine-hole peg test: time to completion in seconds</td>
<td>(d) 10-m walk time decrease by at least 2 s</td>
</tr>
<tr>
<td>6. 10-m walk time to completion in seconds</td>
<td>(e) Patient/clinician feels deterioration has occurred</td>
</tr>
<tr>
<td>7. Self-evaluated functional rating scale (SEFR)</td>
<td></td>
</tr>
<tr>
<td>8. Overall neuropathy limitation scale (ONLS)</td>
<td></td>
</tr>
<tr>
<td>9. European quality of life scale (EQoL)</td>
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</tbody>
</table>

reached or the patient did not receive IVlg, then the run-in period ended at week 8. In the treatment period, all patients received eculizumab by intravenous infusion 600 mg at weeks 0, 1, 2, and 3, then 5 doses at 900 mg every 2 weeks, up to week 12. IVlg was given at DP, as assessed by the investigator. Clinical assessments were repeated at weeks 4, 8, and 14 and electrophysiology repeated at week 14.

Study outcomes

The primary outcome was safety as assessed by the frequency of adverse events (AEs) and serious adverse events (SAEs) occurring during the treatment period compared with the run-in and run-out period. The secondary outcomes were: (1) IVlg inter-treatment interval (in days), excluding the three patients who were not receiving IVlg; (2) clinical and functional efficacy measurements between the baseline (run-in day zero) and treatment and run-out periods (Table 2); and (3) electrophysiology parameters at baseline and treatment period week 14.

Self-evaluated functional rating scale (SEFR)

In addition to investigator-assessed functional scorings, a self-rated score was completed by patients weekly (Leger et al., 2001). Each patient identified five tasks of daily living affected by MMN. Performance on these tasks was graded on the perceived level of difficulty from 0 (normal) to 5 (impossible). The baseline SEFR score was used as a reference point throughout the trial. An increase in SEFR score by 2 points beyond this baseline score indicated a DP and triggered clinical assessment by the investigator to assess if DP criteria were reached (Table 2).

Electrophysiology

Motor nerve conduction studies and electromyography were carried out in at least one affected nerve segment and muscle group, respectively, at baseline and again at the end of the treatment period. Temperature of the limbs was maintained above 30°C. The trial neurophysiologist was not involved in any other trial assessments. Data for distal latency (DL), compound muscle action potential (CMAP) amplitudes and durations, conduction velocity, and F-wave latency were collected. Needle electromyography recordings of voluntarily contracting muscle activity were scored by a panel of five qualified neurophysiologists to assess any difference in the recruitment density pattern between baseline and the end-of-treatment period. The recordings were presented in pairs (pre- and post-treatment) and assessors were blinded to the patient details and the ordering of the recordings.

Pharmacological monitoring

Blood tests were taken prior to each eculizumab dose, and assays were performed for pharmacokinetics (serum drug concentration) and pharmacodynamics, as measured by terminal complement functional assay (percentage of red cell lysis using patient serum as complement source). Anti-GM1 glycolipid antibodies were measured at baseline by enzyme-linked immunosorbent assay (ELISA) (Willison et al., 1999).

Statistics

Because the study was not designed to test efficacy, no power calculation was performed, and the number of patients enrolled was a convenience sample. Task scores, speeds or values were summarised by median and inter-quartile range (IQR) values for each time point or period, and presented as box-and-whisker plots. The myometry or muscle strength/force (MSF) recordings were summed across all five selected muscles for each patient to make a total MSF score. All electrophysiology results were transformed to z-scores, except % conduction block. The Wilcoxon signed-rank test was used to test whether the median differences in the intra-patient scores or speeds between the measurement points or periods and baseline (run-in day zero) were statistically significantly different from zero. The Mann-Whitney U-test was used to compare the medians between unpaired groups. A p-value < 0.05 was considered to be significant and all tests were two-sided.
Results

Patients

Twenty-two patients with a diagnosis of MMN were screened and considered eligible. One patient was excluded from recruitment as he required air travel to reach hospital. One other patient was diagnosed with metastatic cancer of unknown primary during the screening period and was excluded from recruitment. Seven of 20 patients declined enrollment due to (1) perceived risks of the trial drug and/or (2) already receiving perceived full benefit from IVlg.

The remaining 13 patients were recruited to the study with informed consent. Basic clinical data are listed in Table 3. At the time of inclusion, 10 of 13 patients were regularly attending for cycles of intravenous IVlg 1 g/kg administered over 1–5 days and repeated on average at 4 weekly intervals (Table 3). The remaining three patients were not currently on treatment.

Primary outcome – safety and tolerability

Adverse events

No patient discontinued the study medication due to an AE. One patient had an aborted infusion due to an allergic response, which was managed with prophylactic steroid and anti-histamine before subsequent doses. No unexpected treatment emergent signs or symptoms were noted. No bacterial or other infections were identified.

There were 52 AEs during the treatment period (Table 4), which were either mild (73%) or moderate (27%). Headache was the most common AE, accounting for 33% of all AEs during the treatment period. Almost two-thirds (11 of 17; 65%) of the headaches were in the first 4 weeks of eculizumab treatment.

The rate of AEs (expressed as the proportion of weeks per period where an AE was experienced) was significantly higher (median 14%; IQR 7%–21%) during the treatment period than in either the run-in (median 0%; IQR 0%–0%; p = 0.004) or the run-out periods (median 0%; IQR 0%–3%; p = 0.011).

There were two SAEs during the treatment period comprising headache and vomiting following co-administration of IVlg and eculizumab treatment. These led to overnight admission for medical management. One was diagnosed as aseptic meningitis and the other as treatment-related headache.

Table 4. Adverse event data.

<table>
<thead>
<tr>
<th>Adverse events</th>
<th>Run-in</th>
<th>Treatment</th>
<th>Run-out</th>
</tr>
</thead>
<tbody>
<tr>
<td>All AEs</td>
<td>5 (2)</td>
<td>52 (11)</td>
<td>5 (3)</td>
</tr>
<tr>
<td>Headache</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
<tr>
<td>Gastrointestinal symptoms</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Respiratory/crural symptoms</td>
<td>0</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Musculoskeletal</td>
<td>1</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Skin rash</td>
<td>1</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

AEs, adverse events.

Pharmacological studies

Median serum eculizumab concentration had increased to above the 35 μg/ml minimum therapeutic level by treatment period week 1, and it was maintained above this level throughout the treatment period (Fig. 2A). Before eculizumab treatment the median % haemolysis was 95% (IQR 77.5–98), whilst by week 1 this had reduced to 5% (IQR 2.75–19.5) (Fig. 2B).

Patients receiving IVlg had significantly lower median eculizumab concentration (78.7 μg/ml, IQR 55–106) compared with those not receiving IVlg (119.7 μg/ml, IQR 95–147) (Fig. 2C). Complete terminal complement inhibition in serum was achieved in both groups, with no difference between the median haemolytic complement activity in both groups (2% and 1%, respectively) (Fig. 2D).

Secondary outcomes – efficacy

Intravenous immunoglobulin requirements

During the treatment period, 9 of 10 patients receiving IVlg as maintenance therapy continued to require IVlg at regular intervals, as indicated by reaching their DP. Patient 012, who did not require IVlg during the treatment period, had been receiving IVlg at 10 week intervals for 2 years prior to the trial.

Table 3. Clinical features.

<table>
<thead>
<tr>
<th>Clinical features, n = 13</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>11 (85%)</td>
</tr>
<tr>
<td>Age at start of trial</td>
<td>55 years (IQR 51–65)</td>
</tr>
<tr>
<td>Years affected by start of trial</td>
<td>19 years (IQR 10–29)</td>
</tr>
<tr>
<td>Upper limb onset</td>
<td>11 (85%)</td>
</tr>
<tr>
<td>Lower limb involvement</td>
<td>6 (46%)</td>
</tr>
<tr>
<td>Sensory symptoms</td>
<td>5 (38%)</td>
</tr>
<tr>
<td>Diminished reflexes</td>
<td></td>
</tr>
<tr>
<td>Affected limbs only</td>
<td>4 (31%)</td>
</tr>
<tr>
<td>All limbs</td>
<td>5 (38%)</td>
</tr>
<tr>
<td>IgM anti-GM1 antibody</td>
<td>9 (69%)</td>
</tr>
<tr>
<td>positive</td>
<td></td>
</tr>
<tr>
<td>IgM anti-GM1 antibody</td>
<td>1/8,000 (IQR 1/1,275–1/12,500)</td>
</tr>
<tr>
<td>titre</td>
<td></td>
</tr>
<tr>
<td>Current IVlg treatment</td>
<td>10 (77%)</td>
</tr>
<tr>
<td>Duration of IVlg treatment</td>
<td>9 years (IQR 5–14)</td>
</tr>
<tr>
<td>Current IVlg</td>
<td>4 weeks (IQR 3.0–4.75)</td>
</tr>
<tr>
<td>inter-treatment interval</td>
<td></td>
</tr>
<tr>
<td>Average IVlg dose in year before trial</td>
<td>840 g/year (IQR 300–1,000)</td>
</tr>
</tbody>
</table>

IgM, Immunoglobulin M; IVlg, IQR, inter-quartile range; intravenous immunoglobulin.
The three patients not receiving IVIg were excluded from this analysis.

There was no significant difference between the IVIg dosing interval between the run-in period (median 30.5 days), the treatment period (median 34.5 days), or the run-out period (median 34 days). There was a difference when comparing the historical pre-trial IVIg interval (median 28 days) to the treatment interval ($p = 0.006$) (Fig. 3).

**Muscle strength measurements**

The MRC sum score did not differ significantly in any of the trial periods (Fig. 4A). The MSF (myometry) score steadily increased at each successive
significantly higher than baseline (median 35 kg, IQR 25–42) at treatment week 8 (median 43 kg, IQR 38–52) and week 14 (median 44 kg, IQR 31–59), and run-out week 8 (median 44 kg, IQR 30–48). This suggests a generalised increase in patient muscle strength with eculizumab treatment, which was sustained into the run-out period. Median pinch grip strength showed small but significant changes at week 4 in both sides, but this difference was not seen at any other time point. There was no significant change in median palm grip strength throughout all periods.

Timed assessments

The timed walk speed did not differ from baseline throughout the treatment period. There was significant increase in the speed of completion of 9-hole peg test using the right hand at all time points, and the left hand at week 4 only. This suggests a possible learning effect for the right hand.

Subjective measures

Overall, there were improved SEFR scores (i.e., numerically reduced) week by week throughout the treatment period. There was a significant improvement from the baseline SEFR only at week 8 (median change −1 point, p = 0.03) and week 14 (median change −3 points, p = 0.02). There was no significant change in SEFR between the treatment and run-out periods. In a post hoc exploratory analysis, patients that had at least a 2-point decrease in the lowest SEFR score at any time during the treatment period compared with the run-in period were retrospectively classed as “subjective responders.” Using this criterion, 7 of 13 patients were subjective responders: of these 5 of 7 had subsequent worsening of SEFR by at least 2 points (i.e., score increase) during the run-out period.

In the EQ5D visual analogue scale, the median baseline response was 70% (IQR 60–81) and significantly increased to 75% (IQR 70–90) at week 4 only. The EQ5D health utility showed no change in the median score throughout the treatment period compared with baseline (median 0.71 throughout). The overall neuropathy limitation scale (ONLS) remained the same throughout all time points (median 4 points).

Electrophysiology

There was a small yet significant net decrease in the median percentage conduction block across all nerves studied of 6.5% (IQR 2.5–11.5) (Fig. 5). There was no significant change in the median distal CMAP amplitudes or conduction velocity of the whole group.

Electromyographical assessment of a maximum voluntary contraction was carried out in 22 muscle groups. Of 22, only 6 muscle groups received unanimous scorings, 4 showing increased motor unit...
Figure 5. Nerve conduction studies. Interval plots motor nerve conduction studies, each data point represents the median value for each patient in that parameter, and the bold horizontal line shows the median value overall. (A) Conduction block expressed as the percentage decrease in voltage between distal CMAP to proximal compound muscle action potential (CMAP) across a nerve segment. (B) Distal CMAP amplitude expressed in mV. The two time points for the studies were RI (run-in day zero) and TP14 (end of the treatment period). There was a significant decrease in conduction block by 6.5% between RI and TP14 (p = 0.05).

Discussion

The primary aim of this open label single arm study was to test the safety and tolerability of eculizumab in MMN patients receiving IVlg. We found a significant increase in AE incidence during eculizumab treatment when compared with the run-in period. Many of the AEs reported are known side effects of IVlg treatment (Orbach et al., 2005). The three patients not receiving IVlg experienced mild AEs, indicating that eculizumab alone is associated with side effects. None of the AEs throughout the study were severe, bacterial infections were not encountered in this study, and no patients experienced a worsening of disease.

Pharmacological assays demonstrated that terminal complement function was fully inhibited in plasma in all patients, regardless of concomitant IVlg use, and thus there was no major neutralising effect of IVlg upon eculizumab. However, we do not know whether terminal complement inhibition was completely achieved within the endoneurial compartment, which is separated from plasma, relatively protected by the blood-nerve barrier, and may contain cells that synthesise C5.

This study shows a continuing benefit from IVlg treatment, even while complement blockade in the plasma has been achieved, as 9 of 10 patients continued to require and respond to IVlg during the treatment period. Thus, the acute mechanism of IVlg action in MMN is likely due to immunomodulatory or pharmacological mechanisms other than terminal complement inhibition over this short period of time.

We did observe some improvements in secondary outcomes. Overall, there was a trend towards an improvement in patient-rated subjective scores and increased muscle strength as measured by myometry. However, objective clinical measurements did not show any clear trends, even when responders and non-responders were considered separately in post hoc analysis.

There were improvements on individual electrophysiological measurements in some patients and deterioration in others. When considered as a group there was a small but significant net decrease in the degree of conduction block across all nerves studied. Following only 14 weeks of eculizumab treatment, a small improvement is noteworthy.

Overall, in this short duration open label study we observed that eculizumab was well tolerated and safe in MMN when administered in conjunction with IVlg. A small improvement was seen in selected objective motor performance measures and in conduction block. Ideally, a longer term study of terminal complement inhibition, looking for a more gradual cumulative effect or an arrest of disease progression,
should be conducted in MMN and related disorders. When considering future studies, placebo-controlled trial designs would be preferable to the current unblinded study in order to control for test performance variability, learning effects, and operator and patient-related assessment bias.

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References

Reference List


