THE CLINICAL AND GENETIC CHARACTERISTICS OF CONGENITAL MYASTHENIC SYNDROMES

Amina Chaouch

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Abstract

Congenital myasthenic syndromes (CMS) are inherited disorders in which the safety margin of the neuromuscular transmission is compromised. The clinical hallmark of CMS is fatigable weakness. To date, nineteen genes have been implicated in causing this disorder, with most mutations located in postsynaptic proteins. Nevertheless, a great proportion of patients remains with no molecular diagnosis and cannot therefore access optimum therapy. In this thesis, each topic is summarized in one chapter that corresponds to one or part of a selected journal publication, or a book chapter. Chapter 1 is dedicated to the review of our current understanding of the different CMS subgroups based on their underlying molecular defects. Chapter 2 focuses on the methodology used to acquire the data described in the subsequent chapters. Chapter 3 reports on the mutation distribution, clinical features and genotype phenotype correlation of CMS patients referred to one of the largest CMS diagnostic centres worldwide. The phenotype genotype correlation and response to treatment in specific CMS subgroups are refined in chapters 4 and 5, including slow channel CMS and GFPT1 associated CMS respectively. Chapter 6 focuses on the selection strategy of an undiagnosed CMS cohort for whole exome sequencing and reports on the candidate variants identified. Finally, in chapter 7 and 8, detailed clinical and biological data are shown to demonstrate the pathogenicity of novel *AGRN* and *SLC25A1* mutations identified using next generation sequencing.

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Amina Chaouch

Institute of Genetic Medicine, Newcastle University

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*Joint first author.

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Dedications

This thesis is dedicated to my parents

In loving memory of my sister Hala

1982-2013

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Abbreviations

3,4-DAP	3,4-diaminopyridine
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
ALG14	UDP-N-acetylglucosamine transferase subunit ALG14 homolog
ALG2	Alpha-1,3-mannosyltransferase ALG2
ATP	Adenosie triphosphate
bp	Base pair
CDG	Congenital disorders of glycosylation
cDNA	Complementary DNA
CGH array	Comparative genomic hybridization array
ChAT	Acetyltransferase CHAT
Chr	Chromosome
CK	Creatine kinase
CMAP	Compound muscle action potential
CNV	Copy number variation
Colq	Collagenic tail subunit of acetylcholinesterase
DNA	Deoxyribonucleic acid
Dok-7	Downstream of tyrosine kinase 7
DPAGT1	UDP-N-acetyl glucosamine-dolichyl-phosphate N-acetylglucosaminephospho transferase
EB	Elution buffer
EDTA	Ethylenediaminetetraacetic acid
EMG	Electromyography
EPP	Endplate potential
GFPT1	Glucosamine-fructose- 6-phosphate transaminase-1
H&E	Hematoxylin and eosin stain
LB	Lysogeny Broth
LG-CMS	Limb-girdle CMS
LHON	Leber hereditary optic neuropathy
Lrp4	Low-density lipoprotein receptor related protein 4
MAF	Minor allele frequency
Mb	Megabases
MELAS	Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes
MEPP	Miniature endplate potential
MERRF	Myoclonic Epilepsy with Ragged Red Fibers
MOs	Morpholino oligonucleotides
MRC	Medical Research Council
MRI	Magnetic resonance imaging
MuSK	Muscle-specific receptor tyrosine kinase
NADH-TR	Nicotinamide adenine dinucleotide diaphorase staining
NADPH	Nicotinamide adenine dinucleotide phosphate
NARP	Neuropathy, ataxia, and retinitis pigmentosa
NMJ	Neuromuscular junction
OD	Optical density

OsO4	Osmium Tetroxide
PCR	Polymerase chain reaction
Rapsyn	Receptor-associated-protein of the synapse
RNA	Ribonucleic acid
RNS	Repetitive nerve stimulation
RT-PCR	Real time polymerase chain reaction
SDH	Succinate Dehydrogenase
SDS-Page	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SFEMG	Single Fibre electromyography
SNP	Single nucleotide polymorphism
Tris.Cl	Trisamino methane
WT	Wild type

CHAPTER1. INTRODUCTION[1]

Congenital myasthenic syndromes (CMS) consist of a rare group of genetic disorders in which the safety margin of neuromuscular transmission is jeopardized. Typically, patients present with fatigable neuromuscular weakness; with ocular, bulbar and occasionally respiratory failure. Although the phenotype is similar to the more common autoimmune myasthenia gravis, the early age of onset, a positive family history, the course of disease progression and the choice of therapy are distinctly different. The precise prevalence of this condition is unknown and is likely to be underestimated. A recent UK study reported a prevalence of 9.2 per million genetically confirmed CMS children under 18 years of age (Parr et al., 2014). In Southern Brazil, the minimum prevalence was estimated at 1 in 1 million populations (Mihaylova et al., 2010).

Understanding the fundamentals of neuromuscular junction structure, development and function has been key to establishing the molecular mechanisms underlying several CMS subtypes. Generally, defects related to CMS can either target the efficiency of chemical transmission across the neuromuscular junction (*CHAT, COLQ, CHRNE, CHRNB1* and *CHRNA1*) or prevent organized formation of neuromuscular synapses (*AGRN*, *MUSK* and *DOK7*). To date, nineteen genes have been shown to cause CMS, with most patients exhibiting defects in the postsynaptic compartment. Nonetheless, the genetic cause remains elusive in almost half of clinically diagnosed CMS patients, which may either suggest that patients harbor rare private mutations in known CMS genes or that more novel genes are waiting to be unraveled. It seems likely that the introduction of next generation sequencing will help clarify the latter possibility.

In this chapter, I first discuss the clinical diagnostic strategy of congenital myasthenia and the importance of particular ancillary tools in confirming the diagnosis. I then review the salient morphological and functional characteristics of the neuromuscular junction (NMJ), illustrating how defects in particular proteins relate to distinct groups of CMS and influence the choice of treatment in patients. Finally, I outline the main objectives of the work presented in the following eight chapters.

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[1] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

1.1 Diagnostic strategy of CMS[2]

Most CMS patients present in childhood; with fatigable weakness showing varying degree of ocular, bulbar as well as respiratory involvement (Beeson et al., 2005). The pattern of muscle involvement can provide some useful diagnostic clues as to the underlying molecular diagnosis. For instance, a proximal pattern of muscle weakness is usually seen in the limb girdle CMS phenotype. The latter can be associated with a particular group of CMS genes such including *DOK7*, *COLQ*, *GFPT1*, *ALG2*, *ALG14* and *DPAGT1*. On the other hand, a more distal pattern of muscle weakness can be seen in a rare subtype of CMS, namely slow channel CMS or the recently described AGRN associated CMS with distal muscle weakness and atrophy (Nicole et al., 2014). The gold standard investigations to evaluate the integrity of neuromuscular transmission are repetitive nerve stimulation (RNS) and single fibre electromyography (SFEMG). These should preferably be completed on both proximal and distal muscles (Chaouch et al., 2012b). More details on these tools will be discussed below.

Though histochemical analysis of muscle biopsies is not specifically carried out to prove a diagnosis of CMS, this can offer valuable cues in terms of dismissing alternative neuromuscular conditions such as a myopathy or peripheral nerve disorder. Moreover, in the absence of significant structural abnormalities, particular muscle biopsy features such as type1 fiber predominance with type 2 fiber atrophy can be supportive of CMS. Other potentially relevant histological clues include tubular aggregates, which have recently been associated with the limb-girdle CMS phenotype resulting from mutations in glycosylation genes (Senderek et al., 2011, Belaya et al., 2012, Cossins et al., 2013). Nevertheless, tubular aggregates are not a specific finding to CMS and have been reported in other neuromuscular disorders such as congenital myopathies, periodic paralysis and myotonic dystrophy. Accordingly, one would often need to interpret this histological finding in the context of the clinical and electrophysiological evidence to establish accurate diagnosis (Schiaffino, 2011).

[2] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

In terms of differential diagnosis of CMS, autoimmune myasthenia gravis in children and adults and congenital myopathies in infants are common and, at times, challenging diagnostic mimics of CMS. Since some CMS patients can present late into adulthood and exhibit no positive family history, clinicians may consider a diagnosis of autoimmune myasthenia gravis, which is much more common than CMS and is also treatable. Nevertheless, the presence of ophthalmoplegia with no associated significant diplopia, contractures and scapular winging are all features of a congenital disorder, not usually seen in the acquired autoimmune form of myasthenia. Congenital myopathies have emerged as an important differential diagnosis of CMS, sometimes with significant clinical and neurophysiological overlap between the two entities. Typically, congenital myopathies are associated with fixed neuromuscular weakness with evidence of skeletal deformities in most and occasional intellectual disability. The muscle biopsy findings and more recently muscle MRI patterns of involvement have proved crucial to guide the diagnostic strategy of congenital myopathies. Lastly, although RNS and SFEMG are usually normal in congenital myopathies, some reports have shown evidence of abnormal NMJ transmission in genetically confirmed cases Wattjes et al. (2010).

1.2 Electrophysiological evaluation of neuromuscular transmission

This investigation remains an essential part of the assessment of patients with suspected abnormal neuromuscular transmission and includes repetitive nerve stimulation and single fibre EMG.

Repetitive nerve stimulation aims at measuring the amplitude (or area) of the compound muscle action potential (CMAP) of a muscle in response to the stimulation of the motor nerve being studied (Sanders, 2012). Depending on the frequency of stimulation, different parameters can be assessed:

a. Repetitive nerve stimulation at low frequency (3Hz) is usually carried out in search of decrement, which may reflect a postsynaptic defect.
b. Repetitive nerve stimulation at high frequency (20 to 50Hz) is performed to assess for increment, which would be in favour of a presynaptic defect.

CMAP amplitude is measured before and immediately after brief muscle contraction (10-20 seconds); an increment of more than 80% is suggestive of a presynaptic defect. If decrement is not present, testing for post-exercise exhaustion can be done. This relates to the development or exacerbation of decrement with sustained muscle contraction for 1 minute and after repetitive nerve stimulation at 3Hz (Sanders, 2012).

Single fibre EMG (SFEMG) is carried out using a special electrode inserted into the muscle. It is based on the principle that action potentials of muscle fibres of the same motor unit appear with consistent latencies. The action potentials from two muscle fibres belonging to the same motor unit are captured. Increased variability (jitter) of latencies across these two muscle fibres, sometimes leading to conduction block, is suggestive of impaired neuromuscular transmission. Abnormal jitter on SFEMG is reported to be highly sensitive (more than 95%) but not specific to defects of the neuromuscular junction. Indeed, abnormal jitter can occur in other neuromuscular conditions such as motor neurone disease or myositis (Sanders, 2012).

There are two other neurophysiological evaluations carried out in most patients, which have some impact in terms of interpreting some of the above mentioned NMJ investigations. Nerve conduction studies involve the measurement of conduction velocity of motor and sensory nerves whilst taking into account:

1- The amplitude of CMAP in relation to single stimulation of the motor nerve. This amplitude can be reduced in presynaptic disorders.
2- The morphology of the CMAP such as repetitive CMAP on single nerve stimulation, which can be observed in some forms of congenital myasthenia.

Of note, the motor and sensory motor conduction velocities are usually normal in disorders of the neuromuscular junction.
EMG focuses on the characteristics of the motor units (amplitude, duration, morphology, etc.). In disorders of the neuromuscular junction, some myopathic features can occasionally be observed (Sanders, 2012).

1.3 Neuromuscular junction organization[3]

In vertebrates, a motor nerve fibre divides into several axonal terminal branches, which innervate a skeletal muscle. The region of contact between the nerve terminal and the muscle fibre is termed the motor endplate. Every muscle fiber is connected to one motor nerve terminal via one neuromuscular junction apart from some of the cranial muscles, where a single muscle fibre can contain several motor endplates (Happak et al., 1997). The latter probably reflects the intricate neuronal regulation required in these muscles. Despite great heterogeneity in NMJ structure across muscle fiber types and across species, all NMJs consist of:

1- A Schwann cell process that wraps the nerve terminal without reaching the synaptic cleft.
2- A presynaptic nerve bud in which special vesicles are filled with a neurotransmitter that is released into a synaptic cleft.
3- A synaptic space that is filled with a myriad of extra cellular matrix proteins.
4- The postsynaptic membrane that contains a receptor for the neurotransmitter released from the presynaptic vesicles as well as a sarcolamma that defines and maintain the structure and function of the postsynaptic compartment (Engel, 2008).

The presynaptic nerve terminal comprises great numbers of organelles such as mitochondria, which form up to 30% of the presynaptic compartment as well as numerous synaptic vesicles. These are responsible for the uptake, concentration and storage of acetylcholine (ACh) and some usually cluster close to the active zone where the neurotransmitter is released into the synaptic space. The synaptic space itself is a complex meshwork of structural proteins and enzymes such as laminins, collagen, proteoglycan, acetylcholinesterase (AChE) and neuronal agrin. A unique feature of the neuromuscular junction is the presence of several junctional folds in the postsynaptic membrane. The sarcolemma lining these junctional folds contains nicotinic acetylcholine receptors (AChRs) as well as other proteins shown to be important for the development and maintenance of the neuromuscular synapse (Engel, 2008).

[3] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

1.4 Neuromuscular junction development and maintenance[4]

The functional properties of the NMJ are acquired through a complex but well-orchestrated process involving close interaction between the muscle fibre and the motor nerve terminal. "Pre-patterning" is a term coined to describe a critical step in the initial development of the NMJ and relates to the clustering and apposition of nascent acetylcholine receptors on the postsynaptic membrane. This process was shown to be more reliant on muscle derived postsynaptic protein such as MuSK, which can enhance the transcription of postsynaptic receptors. Interestingly, the nerve terminal seems to have little influence on this critical step of NMJ development. On the other hand, further stabilization of pre-patterned AChR clusters does require neuronal regulation through the agrin protein, which can disperse any receptor clusters, placed outside the synapse. Agrin can also regulate postsynaptic signals mediated via the AChRs. Thus, during NMJ development, the specialization of the postsynaptic compartment precedes nerve terminal differentiation (Witzemann, 2006, Sanes and Lichtman, 2001).

1.5 Transmission at the neuromuscular junction

The release of acetylcholine from presynaptic vesicles via exocytosis is activated by depolarization of the terminal motor nerve membrane. This in turn triggers entry of calcium into the presynaptic compartment. Acetylcholine is then released into the synaptic space and binds to the postsynaptic nicotinic acetylcholine receptors. The latter are ligand gated ion channels that allow entry of sodium, which in turn generates depolarization of the postsynaptic muscle membrane (endplate potential, EPP). In the absence of an action potential, presynaptic vesicles can also spontaneously release their content into the synaptic cleft and generate a small electrical response named a miniature end plate potential (MEPP). Simultaneous efflux of potassium in the postsynaptic compartment through activation of voltage gated K^+ channels permits repolarization of the membrane potential to baseline. A fundamental principal underpinning the efficacy of neuromuscular transmission is the safety margin, which

[4] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

corresponds to a threshold at which the synaptic machinery can operate without failure of transmission (Paton and Waud, 1967, Fagerlund and Eriksson, 2009).

Patch clamp study of the neuromuscular junction allows the analysis of the functional properties of the NMJ based on its electrical properties. It also provided the opportunity to examine the molecular basis of different CMS subtypes and the impact of new mutations on synaptic function. Studies on sampled motor endplates (often taken from intercostal muscles) allow the measure of the kinetic characteristics of the nicotinic AChR. This is achieved by the electrical isolation of a small number of ion channels and the record of current flowing through a single channel. This in turn determines whether the channel is an open or closed state. Variations in these different parameters offer the opportunity to refine the phenotype of CMS.

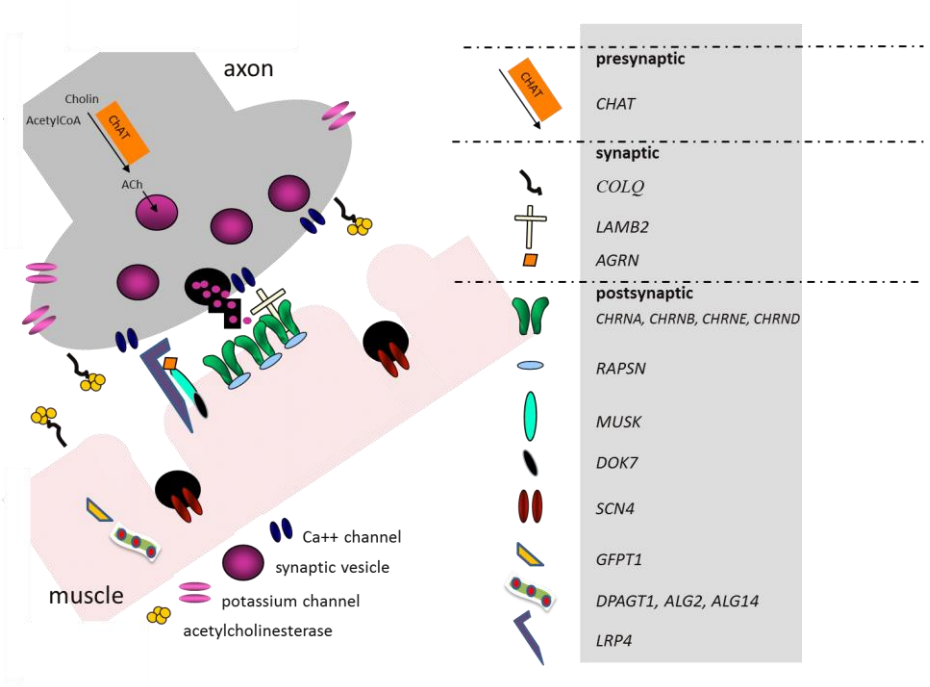


Figure 1.1. The neuromuscular junction and its three compartments. Genes known to cause CMS are classified based on their location in the NMJ. Modified from (McConville and Vincent, 2002)

To date mutations in nineteen genes have been causally associated with CMS (figure 1.1). These include the pre-synaptic acetyltransferase *CHAT* (Ohno et al., 2001), the gene *COLQ* encoding the triple stranded collagenic tail of the synaptic AChE (Ohno et al., 1998c), the genes encoding the four subunits of the nicotic postsynaptic AChR (*CHRNA1*, *CHRNB1*, *CHRND)* (Quiram et al., 1999) *and CHRNE* (Ohno et al., 1995), the *RAPSN* gene encoding the postsynaptic receptor related protein rapsyn (Ohno et al.,

2002), the postsynaptic muscle specific kinase (*MUSK*) gene (Chevessier et al., 2004), the postsynaptic sodium channel *SCN4* (Tsujino et al., 2003), the *DOK7* gene encoding the postsynaptic downstream of kinase (Dok-7) protein (Beeson et al., 2006), the *LAMB2* encoding the synaptic laminin β2 protein (Maselli et al., 2009), the *AGRN* gene encoding the synaptic agrin protein (Huze et al., 2009), the *PLEC1* gene encoding the postsynaptic protein plectin (Forrest et al., 2010), the *GFPT1*gene encoding the Glutamine-fructose-6-phosphate transaminase 1 protein (Senderek et al., 2011), the *DPAGT1* gene encoding the UDP-N-acetyl glucosamine-dolichyl-phosphate N-acetylglucosaminephospho transferase (Belaya et al., 2012), *ALG2 and ALG12* encoding N-glycosylation enzymes (Cossins et al., 2013) and *LRP4* encoding the low density lipoprotein receptor-related protein (Ohkawara et al., 2013). It is worth noting that the *CHRNG* gene, which is the foetal isoform of *CHRNE*, encodes the γ subunit of the receptor and has been associated with an antenatal form of neuromuscular disorders (Hoffmann et al., 2006).

1.6 Classification of CMS[5]

Conventionally, CMS have been categorized based on the site of molecular defect in relation to the neuromuscular junction (table1.1).

[5] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

Gene defect	Percentage of CMS patients with mutations in the gene
Presynaptic	
CHAT	**5%**
Synaptic	
COLQ	**12%**
AGRN	**<1%**
LAMB2	**<1%**
Postsynaptic	
Primary AChR deficiency ± kinetic defects	**Up to 50%**
RAPSN	**15%**
DOK7	**10%**
MUSK	**<1%**
SCN4A	**<1%**
PLEC1	**<1%**
GFPT1*	**4%**
DPAGT1, ALG2 and ALG14	**<1%**

Table 1.1. The frequency of CMS based on the Munich CMS diagnostic database. Adapted from Chaouch, A. and Lochmüller, H. (2013). Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

1.6.1 CMS caused by presynaptic defects[6]

Patients carrying *CHAT* mutations characteristically present in the neonatal period with respiratory and bulbar difficulties. Patients can experience multiple sudden apnoeic episodes over 24 hours and require intensive care support. Sometimes, specific triggers can be identified such as infection, fever, and excitement. In between episodes, patients can be remarkably well with minimal neuromuscular weakness. Several retrospective studies suggest that two subgroups of CHAT associated CMS can be identified based on the disease course. In the first, patients present in the neonatal period with respiratory distress, which improves over time in term of frequency and severity of the attacks.

[6] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

However, some patients seem to further relapse in adulthood. The second subgroup presents later, during infancy or childhood, with respiratory crises and seems to exhibit a more unpredictable course of disease from the outset. AChE inhibitors have been shown to be of benefit in patients harboring *CHAT* mutations (Schara et al., 2010, Ohno et al., 2001).

At the nicotinic presynaptic nerve terminal choline acetyltransferase (ChAT) mediates the biosynthesis of acetylcholine (Ach) from acetyl CoA and choline. Most mutations are recessive private mutations, though there are some exceptions such as the missense mutation p.I336T reported in several independent Turkish families which may suggest a founder effect (Ohno et al., 1998a). Of note, there are no anatomical footprints at the NMJ with this subtype of CMS. Mutations in the *CHAT* gene have been the only known molecular cause of presynaptic CMS until the recent report of *AGRN* mutations associated with presynaptic CMS with distal muscle weakness and atrophy.

1.6.2 CMS caused by synaptic basement membrane defects

1.6.2.1 *COLQ* mutations[6]

Patients with COLQ related CMS present usually in the neonatal period with delayed motor development, hypotonia and proximal neuromuscular weakness of the limbs. They often exhibit ptosis with ophthalmoparesis and occasionally show a delayed pupillary response to light. Patient may also develop respiratory symptoms, which necessitate ventilator support. Previous studies suggest significant clinical heterogeneity in relation to the course of the disease. Indeed, patients can either present in the neonatal period with severe neuromuscular weakness and a progressive evolution of disease; whereas others may present later in childhood with milder symptoms and slower rate of progression. In this category of CMS, AChE inhibitors are not beneficial and may worsen neuromuscular weakness. Alternative agents such as ephedrine and salbutamol have been shown to be helpful (Liewluck et al., 2011, Bestue-Cardiel et al., 2005).

The asymmetric AChE comprises one to three identical tetramers of globular subunits that mediate the rapid breakdown of acetylcholine in the synaptic space. A triple-stranded collagenic tail (ColQ) allows the anchorage of AChE to the synaptic basal lamina for its optimum function. Molecular defects in *COLQ* lead to secondary AChE deficiency, which can be observed using immunohistochemistry. No less than 30 recessive loss of-function mutations have been reported in the *COLQ* gene (Donger et al., 1998). Deficiency of AChE leads to accumulation of ACh in the synaptic space. This in turn generates prolonged motor endplate depolarization, which outlasts the refractory period of the muscle fiber, thus precipitating cationic overload and a secondary endplate myopathy.

1.6.2.2 *LAMB2* mutations[7]

There has been one case in the literature of a female patient presenting with delay in motor milestones, global limb weakness, bilateral ptosis and ophthalmoparesis. She was also noted to have macular changes and abnormal pupillary responses. Importantly, this lady had end stage kidney disease. Neurophysiological studies showed decrement in keeping with abnormal neuromuscular transmission. A trial with AChE inhibitors was of no benefit in contrast to ephedrine, which helped. The patient carried compound heterozygous mutations in the *LAMB2* gene.

Laminins are extracellular matrix glycoproteins with several isoforms and comprise three subunits (α, β and γ). Laminins are ubiquitous and represent an important component of the neuromuscular junction basement membrane. *LAMB2* gene codes for the β2 subunit, which is found in all laminins expressed in the neuromuscular junction. This subunit is thought to play part in synaptogenesis. Mutations in the laminin β2 subunit have previously been reported in association with Pierson syndrome, a fatal disease, which results in severe renal and ocular abnormalities (Maselli et al., 2009).

[7] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

1.6.2.3 *AGRN* mutations[8]

AGRN associated CMS appears to be rare and was until recently only reported in three patients. All patients exhibited generalized fatigable limb weakness with ocular involvement. The course of disease was mild in two of the patients. One of the patients developed recurrent respiratory complications necessitating ventilator support and tracheostomy. None of the patients exhibited clear improvement with conventional treatment including AChE inhibitors and ephedrine. Agrin is a glycoprotein synthesized and released from the motor nerve terminal into the synaptic cleft. Through binding to a postsynaptic transmembrane protein LRP4, agrin can activate the postsynaptic muscle specific kinase (MuSK) and trigger clustering of a number of postsynaptic proteins (Huze et al., 2009, Maselli et al., 2012). I further discuss this particular CMS subgroup in chapter 7.

1.6.3 CMS caused by postsynaptic defects

This group of CMS accounts for most of CMS patients with an established molecular diagnosis. Most postsynaptic defects appear to either directly or indirectly affect postsynaptic AChR.

1.6.3.1 *CHRNE, CHRNA1, CHRNB1, CHRND* mutations[9]

The nicotinic AChR is a pentameric structure that is expressed in two different forms. The first is a foetal form and comprises five subunits: 1α, 2 γ, 1 β and 1δ subunits. It is expressed throughout embryonic development before being gradually replaced by the adult form (ε subunit) after thirty three weeks gestation.

Defects in the adult form of AChR subunits lead to two different types of CMS categories. The first is associated with kinetic mutations, which cause either gain or

[8] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

loss of function of the receptor; the other subtype results from deficiency of the postsynaptic AChR.

Escobar syndrome is a congenital disorder characterized by arthrogryposis, pterygia, and abnormal testicular development and breathing difficulties. It was first described in association with defects in the foetal γ subunit of AChR but has since been linked with other CMS genes including other subunits of the AChR (*CHRNA1, CHRNB1*and *CHRND*) as well as *RAPSN* and *DOK7*. Patients with mutations in *CHRNG* exhibit no myasthenic symptoms after birth. There is some suggestion that homozygous mutations in the γ subunit can lead to foetal akinesia and recurrent miscarriages.

Kinetic defects in the subunits of AChR[9]

Fast channel CMS is caused by recessive loss of function mutations in any of the subunits of the adult form of AChR. Electrophysiology of the motor endplates typically shows abnormally brief channel opening events. Fast channel CMS patients usually exhibit a more severe phenotype than in AChR deficiency CMS, often associated with early respiratory crises, ptosis and ophthalmoparesis, delayed motor development and global neuromuscular fatigable weakness. AChE inhibitors can be helpful but not always. More recently salbutamol was shown to be of benefit (Webster et al., 2014, Shen et al., 2002, Palace et al., 2012).

Dominant gain of function mutations in AChR cause slow channel CMS. Physiological study typically shows prolonged channel opening events. These mutations have been reported in the transmembrane domains (M1, M2 and M4), the linker chain between M2 and M3 and the extracellular N terminal domain. Often patients display selective cervical, wrist and long finger extensor neuromuscular weakness and can occasionally develop respiratory insufficiency. Slow channel CMS patients either present in the neonatal period with significant morbidity or manifest later in adulthood showing a less aggressive disease course. Single nerve stimulation can trigger repetitive CMAP

[9] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

corresponding to over action of the receptor. The prolonged endplate potential (EPP) lasts beyond the refractory period of the muscle fiber and results in cation overload and secondary endplate myopathy. Patients do not usually show sustained response to AChE inhibitors and may in fact deteriorate on such treatment. By contrast, quinidine or fluoxetine have been shown to be beneficial and shown to abort abnormal persistent channel opening *in vitro*. It is worth noting that fluoxetine has been associated with suicidal ideation in children and should only be prescribed for adults. Furthermore, quinidine can cause long QT syndrome and close cardiac monitoring is therefore advocated.

Mutations causing AChR deficiency [10]

Mutations resulting in AChR deficiency are by far the commonest cause of CMS with most defects involving the ε subunit of the AChR. Patients typically present at birth or in the first year of life with ptosis, ophthalmoparesis, poor cry and swallowing problems; in addition to more global fatigable neuromuscular weakness. Respiratory complications and loss of ambulation are not usually seen in this CMS subgroup. The majority of these patients exhibit a relatively mild phenotype with often improvement in fatigability over time. The exquisite positive response to AChE inhibitors is a hallmark of this category of CMS.

Previous reports have shown that some mutations in the ε subunit are more common in certain populations in keeping with a possible founder effect. These include mutation *CHRNE* 1267delG in the Roma population, the *CHRNE* 1293insG mutation in North Africa, *CHRNE* 1293insG in Southern Europe and *CHRNE* 70insG in Southern Europe and Brazil (Muller et al., 2007b, Beeson et al., 2005).
Most mutations in the AChR subunits are recessive and lead to AChR deficiency on the postsynaptic membrane. The latter can be confirmed using 125α-bungarotoxin labelling, which allows the quantification and localisation of these receptors. It is worth noting that deficiency of AChR can be caused by mutations in any of the AChR subunits but

[10] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

also in other postsynaptic proteins such as rapsyn, Dok-7, MuSK and Agrin. These proteins have been linked with the maintenance and stabilization of the neuromuscular junction.

1.6.3.2 *RAPSN* mutations[11]

RAPSN mutations are a relatively common cause of CMS. By contrast to AChR deficiency CMS, patients with mutations in *RAPSN* do not exhibit ocular symptoms except for some ptosis. In addition, two groups of patients were noted based on the age of onset of disease. Indeed, patients can either present in the neonatal stage with significant fatigable neuromuscular weakness and recurrent apnoea or they can develop symptoms as adults and often exhibit a milder phenotype with better prognosis. This CMS subgroup of CMS is responsive to AChE inhibitors and 3,4-DAP.

The receptor related protein rapsyn ensures appropriate positioning and clustering of AChR at the postsynaptic membrane through interaction with MuSK and β-dystroglycan. Deficiency in rapsyn can be identified by immunostaining of the muscle endplate. The majority of patients with RAPSN CMS have been either heterozygous or homozygous for the missense mutation, N88K (Richard et al., 2003).

1.6.3.3 *DOK7* mutations[11]

Dok-7 is an essential postsynaptic downstream of kinase shown to activate MuSK, which triggers postsynaptic AChR clustering (Okada et al., 2006). Defects in Dok-7 were shown to lead to morphological abnormalities in the pre and postsynaptic compartments, simplified synapses and reduced AChR numbers. Mutations in *DOK7* were detected in patients exhibiting predominant proximal muscle weakness (limb girdle phenotype) with a lack of response to AChE inhibitors (Beeson et al., 2006). These patients tended to exhibit ptosis without ophthalmoplegia. Disease course was often progressive and disabling, resulting in respiratory failure. Occasionally, patients

[11] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

presented with vocal cord palsy and stridor in the neonatal phase. Patients displayed improvement with ephedrine and salbutamol in contrast to AChE inhibitors, which can worsen neuromuscular symptoms (Liewluck et al., 2011, Schara et al., 2010, Schara et al., 2009).

1.6.3.4 *MUSK* mutations[12]

The biological importance of MuSK was recognized prior the identification of any patients with mutations in the encoding gene. This muscle specific kinase is crucial to the development and differentiation of the neuromuscular junction through the activation of postsynaptic downstream kinases. The latter mediate the clustering of essential postsynaptic proteins and the induction of NMJ specific gene transcription, thus indirectly providing a retrograde signal to the presynaptic part. Experimental analysis of the patient motor endplate and establishing that this showed deficiency of MuSK protein was the lead to the correct genetic diagnosis in the first CMS family described with *MUSK* mutations (Chevessier et al., 2004). However, *MUSK* mutations remains a very rare cause of CMS. Despite significant variability in phenotype (Gallenmuller et al., 2014, Maggi et al., 2013, Maselli et al., 2010, Mihaylova et al., 2009, Ben Ammar et al., 2013), most patients had ocular symptoms with fatigable limb weakness. Respiratory complications were noted either in the neonatal period or later in adulthood. AChE inhibitors and 3,4-DAP were of limited benefit for treatment but salbutamol was beneficial in some (Chevessier et al., 2004, Mihaylova et al., 2009, Gallenmuller et al., 2014).

1.6.3.5 *PLEC1*mutations[12]

Plectin is ubiquitous structural protein found in most epithelia tissues and muscle. This protein is important in maintaining the stability of the cytoskeleton. Defects in the encoding gene were shown to cause Epidermolysis Bullosa Simplex. This condition is

[12] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

primarily associated with skin lesions in combination with cardiomyopathy, respiratory failure and esophageal atresia. Mutations in this gene have also been shown to cause adult onset limb girdle muscular dystrophy. Very rarely, defects in *PLEC1* can alter the morphology of the postsynaptic compartment and result in impaired transmission across the NMJ. As such, clinical symptoms can include fatigable neuromuscular weakness. Of note, all patients with PLEC1 associated CMS showed abnormal cutaneous lesions and 3,4-DAP improved the patients' neuromuscular symptoms (Forrest et al., 2010).

1.6.3.6 *SCN4* mutations[13]

This is a very rare cause of CMS and has only been described in one patient in the literature. This female patient developed global fatigable weakness in association with bulbar difficulties and frequent respiratory crises. She also exhibited learning difficulties, a rare feature in CMS. The patient required admission to intensive care for ventilator support. Repetitive nerve stimulation showed decrement and the results of patch clamp analysis were key in targeting and sequencing the correct underlying gene defect. The study indicated preserved resting membrane potential, quantal release and normal MEPP amplitude that failed to trigger action potentials (Tsujino et al., 2003). This patient was a compound heterozygote for two missense mutations in the $Na_v1.4$ channel located on the postsynaptic folds.

1.6.3.7 *GFPT1* mutations[13]

GFPT1 mutations were identified in a *DOK7* negative LG-CMS cohort showing marked proximal muscle weakness with absence of ocular signs and a positive response to AChE inhibitors. Almost all muscle biopsies analyzed showed tubular aggregates (Chevessier et al., 2005, Slater et al., 2006, Schiaffino, 2011, Senderek et al., 2011, Huh et al., 2012, Selcen et al., 2013). These are composed of packaged membranous tubule-vesicular structures mainly derived from sarcoplasmic reticulum. They are usually

[13] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

recognized by their intense NADH-TR reactivity with negative SDH reaction and their characteristic electron-microscopic appearance. GFPT1 is a cytoplasmic enzyme that catalyzes the first rate limiting step of the hexosamine biosynthesis pathway, which ultimately leads to the production of nucleotide sugar donor used as substrate for the glycosylation of proteins and lipids. Altered GFPT1 function was recently shown to affect neuromuscular junction transmission by reducing cell surface AChRs at motor endplates (Zoltowska et al., 2013). Interestingly, another study of motor endplate ultrastructure and function revealed marked variability with defects in synaptic response in some and quantal release in others. A number of patients had preserved AChR content with some poorly developed endplate regions (Selcen et al., 2013). This heterogeneous picture may reflect alternative, yet to be determined, pathomechanisms of GFPT1 associated CMS.

1.6.3.8 *DPAGT1*mutations

Defects in *DPAGT1* were discovered using exome sequencing in a subgroup of CMS patients with a limb girdle phenotype, some of whom had tubular aggregates, and no mutations in *GFPT1*(Belaya et al., 2012, Finlayson et al., 2013, Basiri et al., 2013, Selcen et al., 2014). All patients responded to AChE inhibitors, sometimes in combination with 3,4-DAP. DPAGT1 mediates the first rate-limiting step in the *N*-glycosylation pathway, which through a series of proteins, sequentially incorporates sugar moieties to the lipid dolichol. Once the core glycan is formed, it is added to the asparagine residues of nascent proteins. This step is an essential part of post-translational modification and would normally dictate the structure, stability, location and function of a particular protein. It is worth noting that the first sugar substrate used by DPAGT is the end product of the hexosamine pathway involving GFPT1. The motor endplate of a DPAGT associated CMS patient showed reduced postsynaptic folding and density of postsynaptic AChRs. Furthermore, inhibition of DPAGT using a selective enzyme inhibitor (tunicamicin) was shown to alter AChR subunit glycosylation and export to the cell surface (figure 1.2).

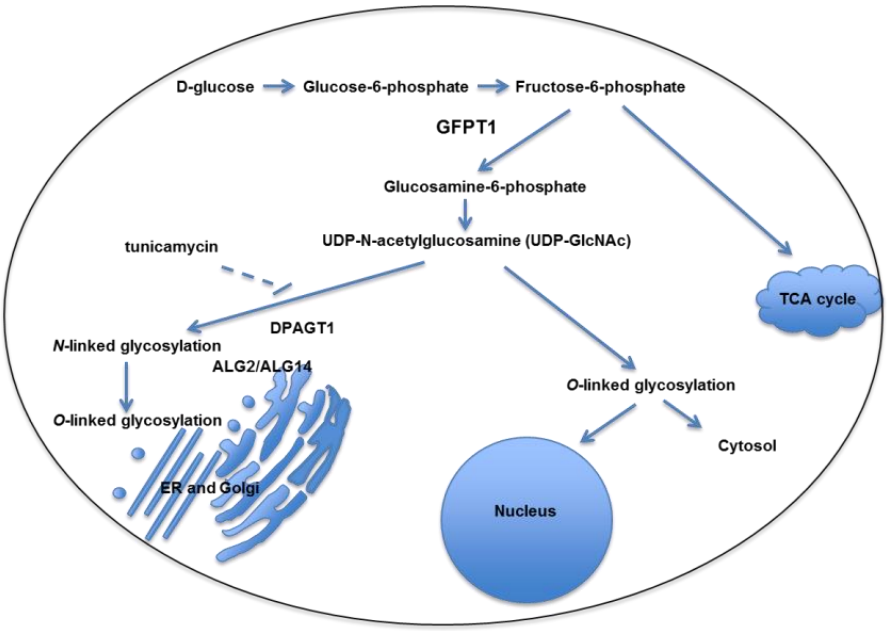


Figure 1.2. Glycosylation pathways associated with CMS. Mutations in *GFPT1*, *DPAGT1*, *ALG2* and *ALG14* have all been associated with limb girdle CMS. .

Congenital disorders of glycosylation (CDG) have also been previously reported with mutations in *DPAGT1*. These are multi system disorders, usually associated with intellectual disability, hypotonia, epilepsy and dysmorphic features, although some patients may present with the "form frustre" of this disorder and present with single organ disease. Measurement of serum transferrin levels using column anion-exchange separation followed by immune turbidimetry showed abnormal levels in two out of five CMS patients. Whether standard glycosylation assays used in most diagnostic laboratories would be sensitive to detect a change is unclear.

1.6.3.9 *ALG2* and *ALG14* mutations

Mutations in both genes were identified in three kinships, all presenting with a limb girdle CMS phenotype, minimal ocular weakness and a response to AChE inhibitors (Cossins et al., 2013, Monies et al., 2014). The disease course was static. Intellectual

disability was associated with mutations in *ALG2* with evidence of contractures proximally and distal laxity reminiscent of a collagen disorder.

Both ALG2 and ALG14 are implicated in the initial steps of *N*-glycosylation (figure 1.2) and knockdown of ALG14 expression, similar to DPAGT1, reduced AChR expression on the cell surface. Patient's tissue showed reduced expression of ALG2. The standard transferrin glycoform analysis did not detect any change. Of note, defects in *ALG2* have also been associated with congenital defects of glycosylation.

1.6.3.10 *LRP4* mutations

This protein is expressed on the postsynaptic cell membrane and can activate MuSK after forming a complex with Agrin. MuSK, in concert with Dok-7, induce Rapsyn mediated AChR anchorage and clustering on the postsynaptic membrane. More recently, Lrp4 was shown to inhibit the Wnt/beta-catenin signaling pathways and to provide a retrograde signal to the pre-synaptic compartment. Mutations in *LRP4* were found using exome sequencing in one patient who presented with ocular, bulbar and respiratory difficulties soon after birth, with mainly proximal muscle weakness and reduced mobility over time. The patient's symptoms deteriorated after receiving AChE inhibitors. Although the nerve terminal and postsynaptic regions were reported to be small in size, patch-clamping studies were normal. Mutant Lrp4 was associated with reduced Agrin mediated MuSK signaling (Ohkawara et al., 2014).

1.8 Animal models

A number of animal models including mice, flies and more recently zebrafish have focussed on the study of the NMJ, in spite of clear differences between the human phenotype and the experimental models (Muller et al., 2007b).

Transgenic mice harbouring epsilon null alleles exhibit a much more severe often fatal phenotype in contrast to the human phenotype. These mice display delayed motor development with low weight and a reduced life expectancy of less than fourteen weeks

(Missias et al., 1997). Some have hypothesized the lack of the γ subunit in adult mice may be the cause of this severe lethal phenotype. Indeed transgenic mice lacking the ε subunit but constitutively expressing the human γ subunit exhibit a phenotype reminiscent of the human disease. These mice are smaller than wild type littermates but exhibit normal development and have a normal life expectancy (Cossins et al., 2004). In keeping with AChR deficiency syndrome, these mice exhibit neuromuscular weakness, decrement on RNS with evidence of abnormal fragmented motor endplates deficient in postsynaptic AChRs.

More recently, a slow channel CMS mouse model was generated. In this AChR ε-subunit knock out (KO) model, the human AChR ε subunit containing the L221F mutation tagged with EGFP is expressed under the AChR -subunit promoter. The phenotype and neurophysiological features of these mice are reminiscent of the human disease.

In addition, a MuSK mutant mouse model sharing the same genotype as a previously reported patient with MUSK associated CMS was shown to exhibit significant heterogeneity across different muscles and across motor endplates within the same muscle (Chevessier et al., 2008).

Finally, an AGRN mouse model was recently reported and will be further discussed in chapter 7.

The short life cycle and access to a large number of zebrafish embryos has attracted much interest of this model in spite the clear differences from humans. The transparency of zebrafish embryos in association with its external embryonic development permits visualization and *in vivo* imaging of initial embryonic stages, which is often challenging in mammalian systems. Another significant advantage is that zebrafish embryos can live independent of their respiratory or cardiovascular status, which offers the opportunity to the study embryos deficient in NMJ protein. Knocking down protein expression using morpholino modified oligonucleotides into zebrafish offers the possibility of prompt and expedient examination of genes. Selective restoration of protein expression or overexpression of a particular protein can be accomplished by injecting mRNA into zebrafish embryos. Furthermore, numerous reports have exploited the zebrafish as a model for studying the neuromuscular junction with several mutant lines specifically generated with defects in NMJ proteins. These include e.g. nic-1 and sop: AChRs subunit mutations; twitch once: rapsyn mutations,

unplugged: MuSK mutation; bajan: ChAT mutations (Muller et al., 2007b, Witzemann, 2006, Chaouch et al., 2012b).

1.9 Conclusions[14]

CMS are rare inherited disorders in which the safety margin of neuromuscular transmission is compromised. In clinical practice, most CMS patients will harbour mutations in the postsynaptic proteins. Accurate diagnosis can be challenging but remains crucial to instigating early suitable treatment, which should help prevent life threatening events and improve quality of life.

Momentous progress towards refining the clinical phenotype, natural history and therapeutic strategies for CMS has been seen over the last 20 years. Nevertheless, a proportion of CMS patients remain without molecular diagnosis (figure 1.4). The recent advent of next generation sequencing has proved effective in unraveling novel CMS genes but has also exposed the complexity of the biological mechanisms of this disease and the unprecedented need for coordinated and collaborative research using the infrastructures available such as patient registries, shared genetic variant databases and through the exchange of experimental tools and animal models.

[14] Adapted from Chaouch, A. and Lochmüller, H. (2013) Congenital Myasthenic Syndromes, in Muscle Disease: Pathology and Genetics, Second edition. John Wiley & Sons, Ltd, Oxford, UK.

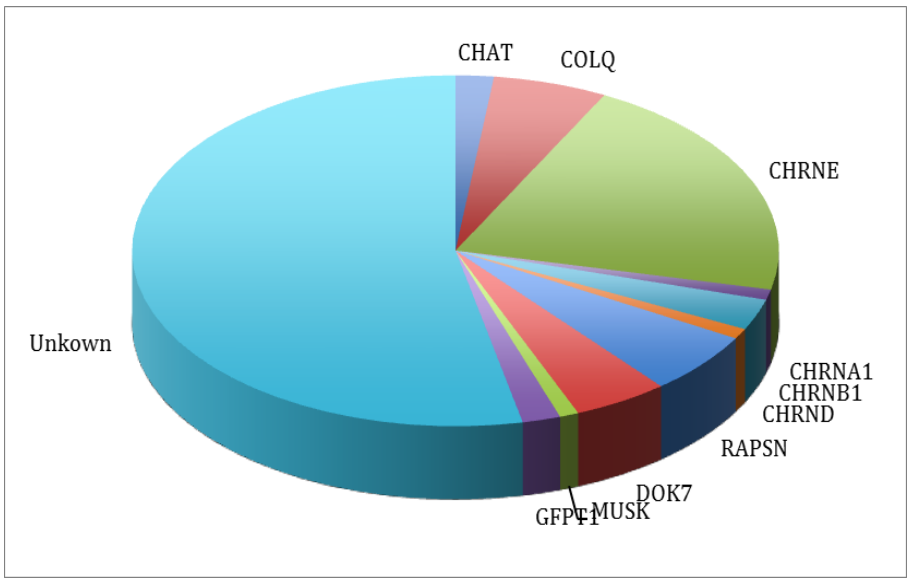


Figure 1.4. Distribution and frequency of CMS gene mutations in the Munich CMS database

1.10 Objectives

The objectives of this research project are:

Part 1:

A. To review the genotypic and phenotypic features of 680 suspected CMS patients referred to the Munich CMS diagnostic centre between 1997 and 2011 and be able to:
    - Report on the frequency and distribution of CMS gene mutations within this cohort.
    - Determine the mutation detection rate when specific clinical features are present.
    - Propose a diagnostic algorithm to improve efficiency of genetic screening of CMS patients.
    - Refine the clinical phenotype in a CMS cohort harbouring a novel CMS gene (GFPT1).
    - Review the clinical phenotype of a sizable slow-channel CMS cohort and retrospectively assess response to recommended therapy.
    - Select a cohort of genetically undiagnosed CMS patients suitable for exome sequencing aimed at novel gene discovery.

Part 2:

B. To identify the disease causing gene in a selected CMS cohort using exome sequencing and homozygosity mapping data when appropriate:
    - Test novel variants/gene in the undiagnosed CMS cohort.
    - Compile more detailed clinical data in the newly diagnosed patients and ascertain:
        - Phenotype variability.
        - Phenotype-genotype correlation.
        - Response to therapy.
    - Present *in vitro* / *in vivo* validation.

Chapter 2. Materials and Methods

2.1 Patient selection

Patients were captured primarily from the Munich CMS cohort. One family was selected from the Newcastle CMS cohort and two families were identified via the French CMS network.

2.1.1 The Munich CMS cohort

The Munich CMS diagnostic service is based in Friedrich-Baur-Institut, Ludwig Maximilians University, Germany. The two key investigators for this cohort were Professor Hanns Lochmüller and Dr Angela Abicht from 1997 until 2007. The Munich CMS service is primarily a genetic diagnostic service. Clinical evaluation of patients is based on a questionnaire that captures the salient clinical features and any relevant family history (Appendix A1). This questionnaire is subdivided into four main sections pertaining to the details of the referral centre, the demographic details of the patient, any relevant family history, consanguinity and ethnic origin. In the clinical section, data pertaining to the onset and course of disease, the presence of exacerbating or relieving factors were included. As regard the clinical examination findings, a particular attention was given to the distribution and degree of muscle weakness and the presence of additional clinical features. The results of specific investigations, when available, were checked including repetitive nerve stimulation and SFEMG reports. Levels of serum biomarkers like serum creatine kinase, AChR and MuSK antibodies and details of previous muscle biopsies were collated. The questionnaire also explored the response to conventional CMS treatments like AChE inhibitors and 3,4-DAP as well as other therapies like steroids that may point towards an underlying autoimmune myasthenic syndrome. It is worth noting that the information collected in the questionnaire was not always uploaded in full to the digital database. This service was funded primarily by research grants and referrals were from senior clinicians from within and outside Germany. A consent form was requested for each patient as per the local institutional ethical board.

The Munich database was the main source of clinical and genetic data for the work described in chapters 3, 4, 5, 6 and 7.

2.1.2 The Newcastle CMS cohort

Two affected siblings were selected from this cohort and are described in chapters 6 and 7.

In the UK, the national diagnostic and advisory service for CMS is based in Oxford. Any patient with a suspected diagnosis of CMS is referred to this unit for genetic testing and often clinical assessment. The National Commissioning Group funds this service. Newcastle in collaboration with the Oxford service offers access to a specialist CMS clinic where a total of thirty-four patients (2012) are reviewed on a regular basis. Genetic testing of this cohort is carried out in Oxford. Approximately, 50% of these patients have no molecular diagnosis. Professor Hanns Lochmuller and Professor David Beeson coordinate the Newcastle service.

The French CMS network

This is another important clinical and genetic diagnostic centre of CMS in Europe. Most referrals are from France. Two families were recruited from this cohort and the full details of these will be discussed in chapter 7.

2.2 Cross sectional analysis of the Munich CMS cohort

2.2.1 Clinical evaluation[15]

For the purpose of this study, the clinical and genetic details of all patients referred between 1997 and 2010 were reviewed. Patients were sub-classified based on whether

[15] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

they had a molecular diagnosis. In the patients harbouring pathogenic mutations, the clinical data sent by the referring clinician were then reviewed. In the undiagnosed group, a number of selection criteria were applied to exclude potential CMS mimics:

1- Patients with positive anti AChR or anti MuSK antibodies.
2- Patients with a positive response to immune-modulatory treatments including immunoglobulins and steroids.
3- Patients with evidence of large fibre peripheral neuropathy on nerve conduction studies.
4- Patients with significant myopathic or neuropathic changes on EMG.
5- Patients with markedly elevated creatine kinase suggestive of a myopathy (>1000 IU).
6- Patients with marked myopathic or neurogenic changes on muscle biopsy.
7- Patients with marked intellectual disability.

A few undiagnosed patients had no autoantibody screen completed. In this instance, only patients with a clear positive family history and an early onset of disease (age less than two years) were analysed.

2.2.2 Molecular analysis[16]

2.2.2.1 DNA samples

The Munich diagnostic Centre receives isolated human genomic DNA or EDTA samples for which genomic DNA extraction is carried out using a kit as per the manufacturer's instructions (Promega, Mannheim, Germany).

2.2.2.2 PCR amplification and Sanger sequencing

[16] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

PCR amplification and bidirectional Sanger sequencing were used to confirm mutations. The following ten CMS genes were available for sequencing at the time of this study: *CHRNA1*, *CHRND*, *CHRNB1*, *CHRNE*, *RAPSN*, *CHAT*, *COLQ*, *DOK7*, *MUSK* and *GFPT1*. The following four genes *AGRN*, *SCN4A*, *LAMB2* and *PLEC1* were not routinely sequenced in the Munich CMS Centre as these were deemed too rare or to be associated with distinct phenotypes including renal disease with *LAMB2* and skin involvement with *PLEC1*.

Primers were designed to include the exons of each gene as well as its flanking regions. The latter were most relevant with *CHRNE* (Abicht et al., 2002, Nichols et al., 1999, Ohno et al., 1999) and *RAPSN* (Ohno et al., 2003) where a 5` non coding segment was added that includes important regulation elements of the genes where previously pathogenic mutations were reported. The following transcripts were used in this study: *CHAT* (NG_011797.1; NM_020549.4); *COLQ* (NG_009032.1; NM_005677.3); *CHRNE* (NG_008029.1; NM_000080.2); *CHRNA1* (NG_008172.1; NM_001039523.2); *CHRNB1* (NG_008026.1; NM_000747.2); *CHRND* (NG_008028.1; NM_000751.1); *RAPSN* (NG_008312.1; NM_005055.3); *DOK7* (NG_013072.1; NM_173660.4); *GFPT1* (NG_029542.1; NM_001244710.1); *LRP4* (AC021573; NM_002334); *SLC18A3* (NT_017696, NM_020549.2); *CNTN1* (NG_012058.1; NM_001843.2). Of note, in genes where several transcripts exist, the longest isoform was sequenced. For *CHAT*, the M variant (NM_020549.4; NG_011797.1) was sequenced as this encodes for a longer protein due to an alternate 5` exon.

The nucleotide numbering was relative to the ATG translation initiation site with A being +1 of the corresponding cDNA sequences (www.hgvs.org/mutnomen). As previously described, the nucleotide numbering for *CHRNE* was relative to the 1st nucleotide of the 1st exon of the mature protein. The pathogenicity of novel variants was ascertained by checking segregation with the disease in the family, *in silico* prediction and screening of at least fifty controls to ensure that the variant not a common polymorphism.

As part of an exploratory gene discovery study, three candidate genes were screened in the Munich cohort: *LRP4* (MIM# 604270), *SLC18A3* (MIM# 600336) and *CNTN1* (MIM# 600016).

2.2.2.3 Genetic screening strategy[17]

Based on the family history, ethnicity and clinical phenotype, a sequential approach was undertaken to screen for mutations in this cohort. Patients were screened to varying degree for:

- Particular mutations, including the common *CHRNE* (c.1267delG; exon 12), *RAPSN* (p.N88K; exon 2, and *DOK7* (c.1124_1127dupTGCC; exon 7)
- Particular exons, including a slow channel CMS panel encoding the M2 domain of *CHRNA1, CHRNB1, CHRND* and *CHRNE* as well as the N-terminal domain of C*HRNA1*.
- One or several CMS genes.

It is important to note that the extent of gene screening evolved with the discovery of novel genes and a better grasp of phenotype genotype correlation in particular CMS categories.

[17] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

2.2.2.4 Linkage Analysis[18]

Forty-seven linkage studies were completed by Professor Angela Hubner at the Technical University Dresden, Germany. The index cases were from multiplex families with more than one affected individual, sometimes in conjunction with a family history of consanguinity. The microsatellite marker set included the gene loci of the following genes were screened: *CHRNA1*, *CHRNB1*, *CHRNE*, *CHRND*, *CHAT*, *COLQ*, *RAPSN*, *DOK7*, *MUSK*, *SCN4A*, *AGRN* and *GFPT1*. It is worth noting that although *SCN4A* and *AGRN* were not routinely sequenced, the loci of both genes were included in the haplotype analysis after 2009.

2.3 Clinical characterization of a slow channel CMS cohort[19]

2.3.1 Patient data

Patients were selected based on the mutations they harboured which were either known or predicted to cause slow channel CMS. A total of fifteen patients were recruited via the Munich CMS database where genetic diagnosis was carried out. These patients had been referred between 1997 and 2012. A questionnaire was designed to collate data on the clinical phenotype, disease progression, results of investigations and response to treatment (Appendix A2). The latter focused on the therapeutic strategy for each patient. Treatment choice, dose escalation and response including tolerability were documented. The clinician involved in the care of each patient completed each questionnaire, which was circulated twice to ensure that all data was accurate and up to date.

[18] Adapted from Abicht et al 2012 (URL:http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)
[19] Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

2.3.2 Genetic analysis

This was carried in The Munich diagnostic centre. Genomic DNA extraction was carried out as above. A slow channel CMS panel was used in some of the patients. This included the M2 domain of the AChR subunits and the N terminal part of the α subunit. In others, the entire sequence of *CHRNA1*, *CHRNB1*, *CHRND* and *CHRNE* genes was analysed (Abicht et al., 1999). To establish pathogenicity of novel variants, one hundred controls were screened by direct sequencing.

2.4 Clinical characterization of GFPT1 CMS cohort[20]

For this study, nine families were recruited from the Munich CMS cohort. These patients had been referred for genetic testing over the last fifteen years. Two families (LGM7 and 8) were recruited through the Oxford CMS National Service and were previously published (Palace et al., 2007). Family LGM13 and LGM3 were reported in (Rodolico et al., 2002) and (Sieb et al., 1996) respectively.

All the pedigrees in this report were previously reported in (Senderek et al., 2011) except for LGM17, which is novel. The details of mutations were discussed in this earlier publication and will not be re-discussed in this study. It is worth noting that three of the pedigrees (LGM4, 15 and 16) discussed in (Senderek et al., 2011) did not carry mutations in *GFPT1* gene and were not included in this study.

All pedigrees were compatible with autosomal recessive inheritance; all parents of the probands were reported to be healthy. Families LGM1, LGM4, LGM10 and LGM11 were consanguineous. The patients from families LGM1, LGM3, LGM5, LGM12, LGM13 and LGM17 are siblings. The age at examination varied between seven and sixty-three years.

To define the genotype phenotype correlation in GFPT1associated CMS, an additional two patients previously published in (Palace et al., 2007) with a limb girdle phenotype and tubular aggregates were screened for mutations in *GFPT1* and did not harbour any.

[20] Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

Furthermore, a total of fifty-seven undiagnosed CMS patients exhibiting variable clinical phenotype with no predominant proximal involvement and no evidence of tubular aggregates as well a cohort of four patients with evidence of tubular aggregates on muscle biopsy without evidence of myasthenic symptoms or impaired neuromuscular transmission were screened and did not carry any mutations in *GFPT1*.

Details of clinical history and examination findings were collected for each patient. A particular attention was focused on the results of serum creatine kinase levels (CK) and titres of AChR antibodies. Neurophysiological testing was carried out as per standard protocols. Given the overlap of this category of CMS with limb girdle muscular dystrophy, the EMG reports, nerve conduction studies, repetitive nerve stimulation and SFEMG were carefully reviewed. Muscle biopsies were performed by open or needle techniques and were analysed by Dr Anders Oldfors (Goteborgs University, Sweden). Following fixation in 2.5% glutaraldehyde and OsO_4, muscle biopsies were embedded in resin before being analysed by electron microscopy. Ultrathin sections were contrasted with uranyl acetate and lead citrate. Out of the eighteen muscle biopsies available for analysis, tubular aggregates were identified in thirteen samples.

2.5 Gene discovery using exome sequencing

2.5.1 Patient selection

I searched both the Newcastle and Munich databases for suitable families in whom existing CMS genes were excluded by conventional methods. Newcastle had a cohort of thirty-one potential patients, with fourteen having a confirmed molecular diagnosis. This distribution was mirrored in Munich, where only 299 of the 680 patients with suspected CMS had a clear genetic defect.

The following inclusion criteria were used to capture this undiagnosed CMS cohort:

1- Ready access to both clinical data and genomic DNA from patients and their relatives.

2- A clinical phenotype strongly suggestive of CMS with objective evidence of neuromuscular transmission defect (abnormal neurophysiology and/or positive response to cholinesterase inhibitors).

3- More than one affected individual in each family.

4- Exclusion of all known CMS genes by conventional methods.

2.5.2 DNA samples

Genomic DNA was isolated from venous blood as per manufacturers' recommendations, using the MagAttact DNA blood MiniM48 kit (Qiagen 951336) for the Newcastle patients and the Wizard Genomic DNA purification kit (Promega, Manheim, Germany) for patients selected from the Munich database. For patients recruited through the French CMS network, standard phenol-chloroform procedures were used. Sufficient amount (at least 10µg) of high quality genomic DNA (OD 260/280 from 1.8-2.0) was obtained from each selected individual.

2.5.3 DNA library preparation and whole-exome sequencing

DNA library preparation and whole exome sequencing were outsourced to EuroFins MWG Operon (Germany) and IntegraGen (France). Sequencing was achieved using Agilent SureSelect capture and Illumina HiSeq2000 (2x100bp reads) sequencing platforms (Appendix A3).

Raw sequencing data were aligned to the human reference genome (hg19), sorted and converted to a BAM file using bwa. The BAM file was indexed and variants called using SAMtools (version 0.1.16) (Li and Durbin, 2009). The alignments were optimised for indel calling using dindel (version 1.0.12) (Albers et al., 2011). Variants called were annotated using ANNOVAR (figure 2.1) (Wang et al., 2010b).

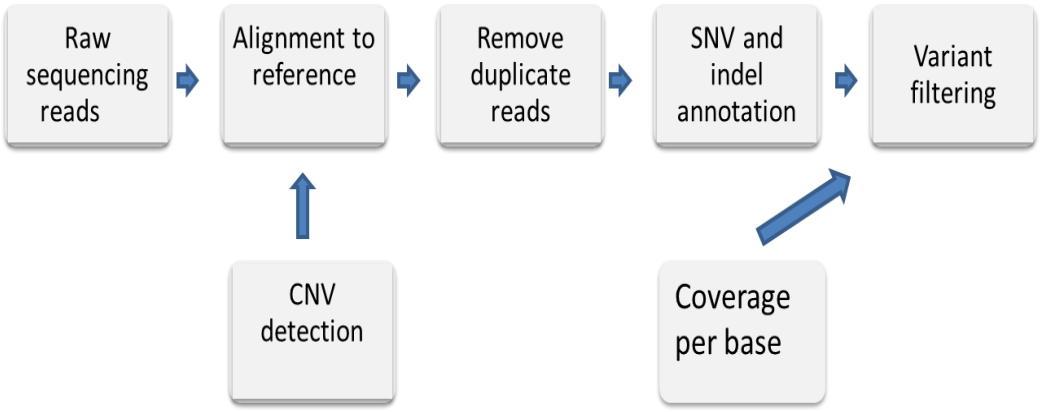


Figure 2.1. Workflow used to process raw sequencing data before selecting good quality variants

2.5.4 Filtering pipeline applied to prioritize called variants

The filtering algorithm was applied to exclude non-coding frequent variants calls (MAF > 1%) found in public databases (dbSNP [http://www.ncbi.nlm.nih.gov/SNP/], the Exome Variant Server [http://evs.gs.washington.edu/EVS/], the 1000 Genomes Project [http://www.1000genomes.org]) and an in house list of common variants from unrelated controls (figure 2.2). Given that the family history was highly suggestive of autosomal recessive inheritance (healthy parents, two affected children and parental consanguinity in half the pedigrees), and that most CMS are recessive in inheritance, the recessive model was applied to the search for genes containing homozygous, or at least two heterozygous changes, shared between siblings. Variants were visualized and evaluated using the UCSC Genome Browser (Kent et al., 2002).

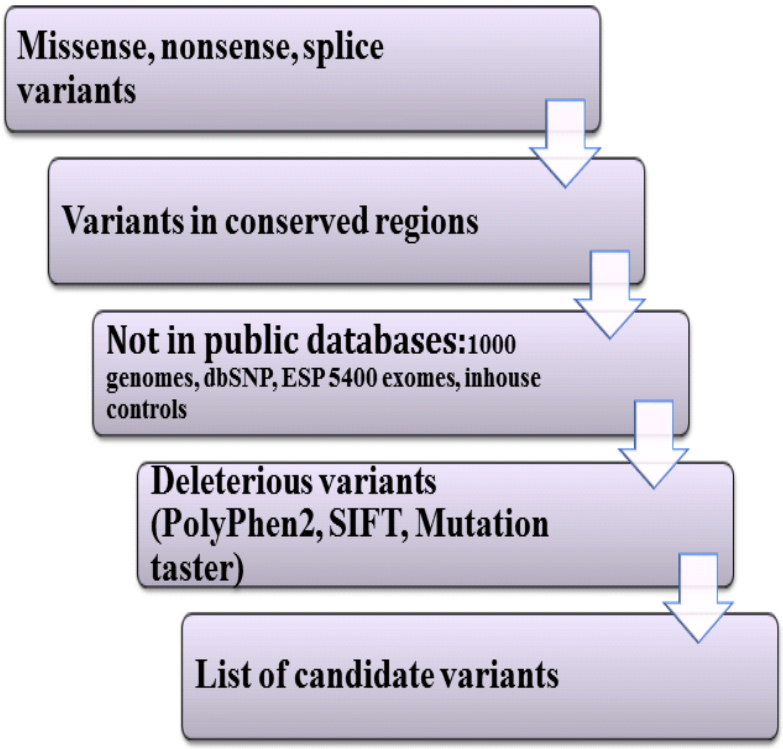


Figure 2.2. Filtering pipeline for called good quality variants

2.5.5 Copy number variants analysis using exome sequencing[21]

This analysis was carried out by Dr Helen Griffin at the Institute of Genetic Medicine, Newcastle University. Copy number variant (CNV) analysis of Patients 1 and 2 was carried out using the ExomeDepth software (Plagnol et al., 2012). The read depth per exon was compared with a pool of reference exomes from the same capture targets and the same sequence machine run. Any CNV present in the test sample was detected as a deviation from the 1:1 ratio depth (0.5 = hemizygous deletion; 0.0 = complete deletion; 1.5 = duplication of one exonic copy). The CNV predictions from each sibling were combined and compared with an in-house control list (191 exomes). A set of "common" CNVs from forty controls was used to exclude the predicted CNV overlapped by at least 50% (Conrad et al., 2010). CNVs were later confirmed by conventional CGH array.

[21] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

2.5.6 Verification of called variants

Sanger sequencing was used to verify putative disease causing variants and their segregation with the disease. Primer sequences were designed using the Primer-3 Plus programme (http://www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi). All primer sequences used are available on request. Sanger sequencing was performed using bi-directional fluorescent sequencing on an ABI 3730 XL 96 capillary sequencer, with BigDye Version 3.1 chemistry (Life Technologies). Sequences were assembled and aligned against a reference sequence downloaded from GenBank using Chromas (Technelysium Pty Ltd) or Mutation Surveyor (SoftGenetics) programmes.

2.5.6.1 PCR amplification

Moltaq polymerase (Molzym) was used to amplify DNA templates via PCR. The following was made up to 50µl with dH2O to carry out PCR

- DNA template 1µl (variable concentration)
- Forward primer 1 µl (50ng/ µl)
- Reverse primer 1 µl (50ng/ µl)
- 10mM dNTP mix 2 µl
- 10x PCR buffer 5 µl
- PCR enhancer 4 µl
- Moltaq polymerase 1 µl

Following gently mixing and spinning in the centrifugation, PCR reactions were incubated in a thermal cycler (Senso Quest, Labcycler) using the parameters in table 2.1

Step number and name	**Temperature**	**Time**	**Cycle repeat**
1) Initialisation	94°C	2 minutes	
2) Denaturation	94°C	15 seconds	
3) Annealing	58°C	30 seconds	
4) Elongation	72°C	2 minutes	Repeat steps (2-4) 40 times
5) Final elongation	72°C	7 minutes	
6) Termination	4°C	∞	

Table 2.1. Programme used for PCR reactions

2.5.6.1 Gel Electrophoresis

A 1% agarose gel was used for most PCR reactions. 1.5g agarose was heated with 150ml TAE-buffer (Tris base, acetic acid and Ethylenediaminetetraacetic acid (EDTA)). 1µl/ml of safe view nucleic acid stain (NBS biological) was mixed into the molten gel. After polymerization, the gel was placed on an electrophoresis gel-chambre and submerged with TAE buffer. The PCR products were mixed with 1µl of loading dye (dH2O, 15% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol) and loaded onto the gel. Depending on the size of the PCR product, 1Kbp or 100bp ladder (NBS biological) was used as a molecular weight size marker. Electrophoresis was performed for a minimum of 30 minutes at 100V. Gels were visualised under UV light and images were captured on a GelDoc-It 310 Imaging system (UVP). QiaexIIGel extraction Kit from Qiagen (Qiagen, Valencia, CA) was used for the elution of PCR products from the agarose gel.

2.5.7 CGH array analysis[22][23]

This study was carried by Dr Angela Abicht in Munich at the MGZ diagnostic laboratory, Germany. CGH array was carried out to analyse and confirm copy number variants. CytoChip ISCA 180K V1.0 oligonucleotide array (BlueGnome) was used according to the manufacturer´s instructions (BlueGnome). *Rsa*I and *Alu*I digested DNA products (patient´s DNA as well as reference DNA) from peripheral blood were labelled by random priming using either Cy3-dUTP or Cy5-dUTP and hybridized at 65°C for 24h. Scanning and image acquisition was done on an Agilent microarray scanner. Data analysis was performed using BlueFuse Multi software (BlueGnome).

[22] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

[23] Adapted from Chaouch et al (URL:http://iospress.metapress.com/content/l36500736v3r4132)

2.6 Functional studies of the *AGRN* variants

2.6.1 Patients data[24]

In total five patients from three kinships were recruited for this study. CMS-EN was chosen from the Munich congenital myasthenic syndromes diagnostic database whilst families 2 and 3 were recruited via the French congenital myasthenic syndromes network. Detailed information regarding the clinical phenotype, disease progression, investigations and treatment were collected for each patient. EMG, SFEMG and repetitive nerve stimulation (3 Hz) were carried out in selected muscles as per standardized protocols (Bauche et al., 2013). Patients 1 and 2 were investigated on a Keypoint machine (Medtronic) by Dr Sissel Loseth and Dr Torberg Torbergsen from Tromso University (Norway). Patients 3, 4 and 5 were assessed by Dr Emmanuel Fournier at Inserm (Paris), using a Viking IV machine (Nicolet). Motor conduction velocity studies were performed in the median, ulnar, tibial and peroneal nerves. Sensory neurographic studies were completed in the median, ulnar, radial and in the sural nerves. The amplitude of the CMAP was recorded at rest and following exercise. Pathological decrement was considered when the amplitude and surface of the CMAP was decreased by 5% and an increment when this was increased by 20%. A possible presynaptic origin was considered for an increment higher than 60% (Oh et al., 2005). SFEMG was carried out on patients 1 and 2 by Dr Sissel Loseth and Dr Torberg Torbergsen (Tromson University, Norway) and Professor Erik Stalberg (Uppsala University, Sweden) using concentric needle. Criteria for abnormal jitter were 55 μsec as the limit value. Appropriate consent for research was obtained from each patient.

2.6.2 Muscle biopsy[24]

Muscle biopsies were acquired for Patient 2 at the age of thirty-three years *(vastus lateralis*) at the Tromso hospital (Norway) and reviewed by Professor Sigurd Lindal. This biopsy was also exported to Newcastle for review by Dr Louise Anderson for immune-staining of proteins associated with inherited myopathies. The other two

[24] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

biopsied were obtained via the French CMS network and the hitochemical analysis was carried out by Dr Romero at the Institut de Myologie, CHU Pitié-Salpêtrière, Paris. Deltoid biopsies were sampled from patient 3 at the age of thirty-six years, patient 4 at the age of eighteen years. Patient 5 had two deltoid biopsies at the age of thirty-one and thirty-four years. All biopsies were analysed using standard histochemical protocols on transversal cryostat sections and included staining for hematoxylin and eosin, Gomori trichrome, ATPase and oxidative enzymes.

Electron microscopy was carried out on deltoid muscles biopsies containing NMJ-rich areas. These were collected from patients 3 and 5(Bauche et al., 2013). Fluorescent staining of NMJs on teased myofibers was carried out to ascertain the impact of the novel *AGRN* mutations on particular proteins. The following antibodies were utilized on transverse muscle sections shown to contain motor endplates. Antibody for agrin was kindly provided by Professor Markus Rüegg from the University of Basel, Switzerland. LRP4 antibodies were supplied from (Ab1 antibody, Sigma-Aldrich), C4 (LAMB2) and SV2 antibodies were purchased from The Developmental Studies Hybridoma Bank. Stained sectioned were visualized using epifluorescent and confocal microscopy (Carl Zeiss LSM510 and Axiophot).

2.6.3 Generation of wild-type and mutated agrin recombinant proteins[25]

A construct coding for a mouse full length agrin cloned into the episomal expression vector pCEP-Pu was used (figure 2.3). This was kindly provided by Prof Markus Rüegg (University of Basel, Switzerland) (Kohfeldt et al., 1997). This construct encodes for the full-length NtA isoform with the 4- and 8-amino-acid inserts at the A/y and B/z sites corresponding to the neural isoform of mouse agrin with the signal peptide BM40 in the N-terminus and a His (6) tag in the C-terminus. The BM40 peptide allows for the secretion of the recombinant protein from the cells. Site-directed mutagenesis was performed by Mutagenex Inc (Piscataway) to introduce the missense mutation found in kinship 1 (c.226G>A, p.G76S) and 2 (c.314A>T, p.N105I).

[25] Adapted from Nicole et al 2014 (URL:http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

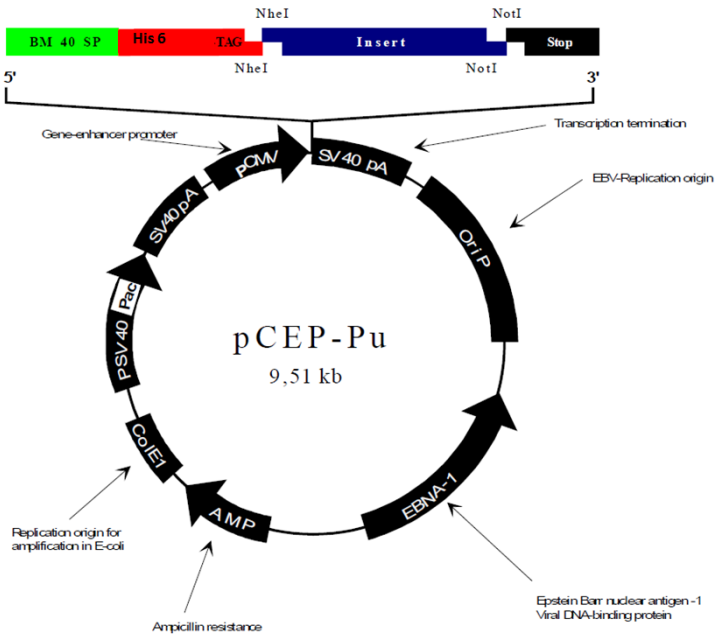


Figure 2.3. Modified pCEP Pu expression vector containing full length mouse agrin. Kindly provided by Professor Markus Ruegg, Switzerland. Modified from ((Kohfeldt *et al.*, 1997),

2.6.3.1 Bacterial "heat shock" transformation with XL1-Blue chemically competent cells (Homemade)

2 µl of plasmid DNA (up to 50ng) were gently mixed in with 50µl thawed competent cells and incubated on ice for 10 minutes. Transformation efficiency was tested using 1µl of pUC18/19 plasmid at 0.01ng/µl in positive control. Cells were heat shocked at 42˚C for 1 minute and returned to ice for 2 minutes. The cells were suspended in 150µl of LB medium then incubate at 37˚C for 1 hour. 100µl of the test transformations were spread onto LB plates containing appropriate antibiotic and incubated overnight at 37˚C.

2.6.3.2 Plasmid DNA extraction and purification

A single colony was picked from 100µg/ml Ampicllin LB agar plate (10g Bacto-tryptone, 5g yeast extract, 10g NaCl, 15g agar in 1litre dH_2O, sterilised by autoclaving) and incubated in 5ml LB media (10g Bacto-tryptone, 5g yeast extract, 10g NaCl, in 1litre dH_2O). 50µg/ml Ampicillin was added to the mixture, which was incubated

overnight at 37^{o}C in a shaking incubator (300rpm). The cells were harvested by centrifugation at 5400g for 10minutes. Plasmid DNA extraction and purification were completed as per the directions of Quiagen plasmid purification kit manual (QIAprep Spin Miniprep Kit; 27104). The final product was eluted in 20µl of EB buffer (10mM Tris.Cl, pH8.5) or water. The quality of the plasmid DNA was verified by gel electrophoresis (1µl product used) and the concentration was estimated on a NanoDrop (ND-8000, 8-Sample Spectrophotometer). The optical density of the DNA sample was measured at 260/280 nm. A ratio of OD260 to OD280 of 1.8 or more was consistent with pure DNA.

2.6.4 *In vitro* AChR clustering assay[26]

The C2C12 mouse myoblast cell line was used. Cells were cultured to form myotubes as previously described (Huze et al., 2009). The clustering of AChR experiment was carried out by Yasmin Issop at the Institute of Genetic Medicine, Newcastle. HEK293 EBNA cells were transiently transfected with wild type or mutant agrin constructs. Following five days of incubation, the culture media containing the secreted neural agrin was incubated with myotubes in Opti-MEM reduced serum medium for twenty-two hours at 37°C. Myotubes were fixed with 4% paraformaldehyde (PFA) for thirty minutes then stained for AChR by incubation in 1mM BTX conjugated to Alexa Fluor 594 (Life Technologies^{TM}) for one hour. Plates were then mounted in Vectashield mounting medium with DAPI (Vector Laboratories) and observed with a 40X objective lens under an Olympus IX70 inverted microscope (Olympus Europa) linked to a CDD camera (Princeton Cool SNAP Fx). The efficiency of AChR clustering was assessed by determining the number of AChR clusters in ten random fields and expressed in relation to the number of nuclei seen in the same field. This experiment was repeated on two occasions.

[26] Adapted from Nicole et al 2014 (URL:http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

2.7 Functional studies of *SLC25A1* variant

2.7.1 Patient data[27]

Appropriate consent for research was obtained from each patient and any family member involved in this study. This novel homozygous variant was identified in CMD-NO family. The latter was identified via the Newcastle MRC Neuromuscular Centre, Institute of Genetic Medicine. These two affected siblings were born to two healthy consanguineous parents (first cousins) and were originally from Durham in the North of England. Based on the positive family history suggestive of recessive inheritance and the clinical phenotype, a diagnosis of CMS was suspected. The elder brother was the index case and displayed from the age of five years, fatigable limb weakness. Nerve conduction studies and EMG studies revealed no evidence of a large fibre neuropathy or significant myopathy. Repetitive nerve stimulation showed no evidence of decrement. SFEMG on the other hand showed clear abnormal jitter with block in several muscles compatible with defective transmission across the neuromuscular junction. The younger sister was also affected and presented with fluctuating neuromuscular weakness of all four limbs, more marked in the legs. She also displayed moderate learning difficulties and attended a special need college.

2.7.2 Neurophysiological and morphological study of the patient's motor endplates[27]

To assess the integrity of the neuromuscular junction, the affected brother underwent repetitive nerve stimulation and SFEMG at the Oxford CMS Centre led by Professor Beeson's group. Both investigations were performed on two separate occasions (age twenty and age twenty-one years). Repetitive nerve stimulation at 3Hz was used on *Abductor digiti minimi* (ADM), anconeus and *extensor digitorum communis* (EDC) muscles to check for decrement. The same muscles were also assessed for facilitation following 20 seconds of sustained maximum muscle contraction.

[27] Adapted from Chaouch et al (URL:http://iospress.metapress.com/content/l36500736v3r4132)

The morphology and functional properties of the motor endplates were assessed in the patient's muscle (*anconeus*). This work was also carried out in Oxford by Professor Beeson's group. The number of postsynaptic AChR was calculated as previously described (Vincent et al., 1981). The *anconeus* muscle bundles were dissected stained with 125I-a-BuTX, before labelling for AChE. The number of endplates in a given field was established in relation to 125I-a-BuTX binding to generate the number of receptors per endplate. 35 NMJs were tested to determine MEPPs and quantal content with between 1 and 62 MEPPS per NMJ.

2.7.3 Homozygosity mapping[28]

A recessive pattern of inheritance was suspected based on the pedigree of two healthy parents and two affected children. This analysis was carried out by Professor Senderek in Aachen, Germany. In order to restrict the chromosomal location of the disease causing mutation in this family, genome wide genotyping of SNPs was performed using Illumina 300K chip (Illumina, San Diego, Ca). This test was implemented to identify regions of homozygosity shared by both affected sibling, which may contain a homozygous mutation segregating on the same ancestral haplotype.

2.7.4 Verification of *SLC25A1* variant

PCR amplification and Sanger sequencing were used to verify and screen for variants in the *SLC25A1* gene using primer sequences described in table 2.2.

[28] Adapted from Chaouch et al (URL:http://iospress.metapress.com/content/l36500736v3r4132)

SLC25A1	**Forward primer 5´-3´**	**Reverse primer 5´-3´**	**Product size**
exon 1	ACAGTGCACCTTGACCCACT	CAGCTGCGTCTTCACGTACTC	850bp
exon 2	GGCCCCAGCCTATGTTCC	CTAAGGCCGCGGTACAGG	386bp
exon 3 & 4	CCCACCGAGTACGTGAAGAC	GGCTGCCTTTAGCATAGACTC	683bp
exon 5 & 6	CTTGGCTGCACTGCTTCCT	CTGCAATAGCTCCGAAGACC	700bp
exon 7 & 8	AGGTGTGTCTGCATCACAGG	GGAGAGGAAGGCAGGTCAG	583bp
exon 9	TGCCTCTCACTTCAGTATCTGG	ATGCATAGATGGGTCCATACAG	795bp

Table 2.2. Forward and reverse primer sequences for the 9 exons of *SLC25A1* gene

2.7.5 SLC25A1 constructs and expression in bacteria[29]

This work was carried by Professor Luigi Palmieri's group in Bari University, Italy. The coding sequence of the yeast orthologue citrate carrier CIC (CTP1, NM_001178639.1) was amplified from *S. cerevisiae* genomic DNA via PCR and the mutations were introduced by site-directed mutagenesis as described in QuikChange® Site-Directed Mutagenesis Kit from Stratagene. Ctp1p and its mutated form (p.R241Q, p.R276H and p.G117D) were overexpressed as inclusion bodies in the cytosol of E. coli strain BL21 (DE3). Inclusion bodies were purified on a sucrose density gradient and washed at 4 °C, first with Tris-EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.0), then twice with a buffer containing Triton X-114 (3 %, w/v), 1 mM EDTA, 20 mM Na_2SO_4 and 10 mM PIPES pH 7.0, and finally with the Tris-EDTA buffer. The proteins were solubilized in 1.7 % sarkosyl (w/v). Eventual small residues were removed by centrifugation (20800 × g for 10 min at 4 °C).

2.7.6 Transport assay of recombinant mutant SLC25A1 proteins[29]

This work was carried by Professor Luigi Palmieri's group in Bari University, Italy. The recombinant proteins solubilized in sarkosyl were reconstituted by cyclic removal of detergent as described (Fiermonte et al., 1993) with some modification. The reconstitution mixture consisted of phospholipids in the form of sonicated liposomes (10 mg), protein solution (25 µl, about 9µg), 10 % Triton X-114 (70 µl), 10 mM citrate

[29] Adapted from Chaouch et al (URL:http://iospress.metapress.com/content/l36500736v3r4132)

(35µl), cardiolipin (0.7 mg), 10 mM PIPES/NaOH pH 7.0 and water (final volume 700 µl). The mixture was recycled 13-fold through an Amberlite column pre-equilibrated with 10 mM PIPES-NaOH (pH 7.0) and 50mM NaCl. All operations were performed at 4 °C except the passages through Amberlite, which were carried out at room temperature. Approximately 20% of WT or mutated CIC proteins were reconstituted. External citrate was removed from proteoliposomes on Sephadex G-75 columns pre-equilibrated with 50 mM NaCl and 10 mM PIPES at pH 7.0 (buffer A). Transport at 25 °C was started by adding 0.1 mM [^{14}C] Citrate (from PerkinElmer, Inc) to proteoliposomes and terminated by addition of 20 mM pyridoxal 5′ phosphate and 20 mM of bathophenanthroline. In controls, inhibitors were added with the labelled substrate. The external substrate was removed by Sephadex G-75 columns pre-equilibrated with buffer A, and the entrapped radioactivity was counted. The experimental values were corrected by subtracting control values and the initial transport rate was calculated from the time-course of substrate uptake by proteoliposomes (Palmieri et al., 1995).

2.7.7 Study of mitochondrial membrane potential in fibroblasts[30]

This work was carried by Professor Luigi Palmieri's group in Bari University, Italy. Fibroblasts from the affected sibling harbouring the *SLC25A1* variant c.740G>A; (p.R247Q) and control fibroblasts were grown in DMEM (high glucose). Membrane potential was measured by fluorescence microscopy using the tetramethylrhodamine methyl ester (TMRM) probe (Scaduto and Grotyohann, 1999), while mitochondrial morphology was investigated using MitoTracker® Red CMXRos. MitoTracker® Red CMXRos and tetramethylrhodamine methyl ester (TMRM) were purchased from Life Technologies. Fluorescent images of treated cells were acquired and fluorescence intensities were analyzed, as previously described (Lasorsa et al., 2004), using a Zeiss Axiovert 200 microscope equipped with a Photometrics Cascade 512B CCD camera (Roper Scientific) and the MetaFluor software (Universal Imaging).

2.7.8 Western blotting[31]

[30] Adapted from Chaouch et al (URL:http://iospress.metapress.com/content/l36500736v3r4132)

This work was carried by Professor Luigi Palmieri's group in Bari University, Italy. Immunoblotting analysis was performed using 100 x 10^3 cells (patient and control fibroblast). The cells were rinsed with ice-cold PBS and lysed using RIPA buffer. Total proteins were heated at 100 °C for 5 min, separated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was then incubated at room temperature with anti-SLC25A1 polyclonal antibodies raised in rabbit against recombinant rat SLC25A1 carrier or mouse monoclonal antibodies against the β subunit of human F1-ATPase (BD Biosciences). Antigen–antibody complexes were detected using anti-rabbit or mouse IgG-coupled horseradish peroxidase (Pierce, Thermo Scientific), in combination with the ECL system (Merck Millipore).

2.7.9 Multiple sequence alignment and *in silico* evolutionary pressure analysis[31]

This work was carried by Professor Luigi Palmieri's group in Bari University, Italy. A multiple sequence alignment of citrate carrier (CIC) orthologues from various species and other mitochondrial carriers was obtained using ClustalW(Persson, 2000). The functional or structural relevance of the mutated residues of the mitochondrial citrate carrier protein was estimated according to a scoring system based on the evolutionary pressure acting on each amino acid as described previously (Pierri et al., 2014).

2.7.10 SLC25A1 knockdown in zebrafish embryos[31]

This work was carried out by Dan Cox from the muscle group, Newcastle University. A BLAST search of zebrafish transcripts with the human SLC25A1 protein sequence as input revealed the presence of three homologues in the zebrafish genome: two on chromosome 10: *SLC25A1*a (NM_200607), *SLC25A1b* (XM_003199276.2) and one on chromosome 8 (*SLC25A1c*). Only *SLC25A1a* and *SLC25A1b* from chromosome 10 are expressed in zebrafish.

[31] Reproduced from Chaouch et al (URL:http://iospress.metapress.com/content/l36500736v3r4132)

2.7.11 Zebrafish Husbandry and Observation[32]

This work was carried out by Dan Cox from the muscle group, Newcastle University. The Golden strain (*slc24a5*$^{b1/+}$) of zebrafish was used in this study (Zebrafish International Resource Centre (ZIRC), Oregon). Zebrafish embryos were raised at 28.5°C and staged hours or days post-fertilization (hpf or dpf) according to standard procedures. Video recordings of embryos and larvae were taken with a Leica dissection stereomicroscope equipped with a Chameleon digital camera (model CMLN-13S2M, Chameleon). Touch-evoked swimming response of embryos was elicited by touching the head or the tail of the embryos with a fine pipette tip. Light microscope images of injected and non-injected embryos were taken with a digital camera (model LEICA DFC 420C) mounted on a Leica dissection stereomicroscope. Procedures involving experimentation on animal subjects were done in accordance with the local institutional guide.

2.7.12 Antisense Morpholino Oligonucleotide Knockdown[32]

This work was carried out by Dan Cox from the muscle group, Newcastle University. Antisense morpholino oligonucleotides (MOs) were purchased from Gene Tools LLC (Pilomath, OR). The MOs were designed with the mRNA sequence of the zebrafish *SLC251A* orthologues and genomic sequences available from public databases (*SLC25A1A* GeneID: 393579, accession number: NM_200607.1) and (*SLC25A1B* GeneID: 795332, accession number: XM_003199276.2) and the corresponding genomic DNA sequence obtained from the zebrafish chromosome 10 assembly.

Two MOs were generated, one per gene orthologue. Both MOs are splice-blocking and directed against the splice donor site of exon 3. The following two morpholinos were used: (MO1: zSLC25A1a 5'-AGATGAATCTTTCTTACCGTACTGC-3' and MO2: zSLC25A1b 5'-GCGGGTTAGGAAACACTCACCTCAC-3'). Both MOs are expected to interfere with the splicing process and lead to a disruption of the protein open reading frame and premature translation termination after twenty-five missense amino acids.

[32] Adapted from Chaouch et al (URL:http://iospress.metapress.com/content/l36500736v3r4132)

The Gene Tools standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') targeting a human beta-haemoglobin gene was used as a negative control for the effects of MO injection. We also used the Gene Tools anti-p53 MO (5'GCGCCATTGCT-TTGCAAGAATTG-3') to suppress any non-specific apoptotic effects that can be induced by some MOs. MOs were suspended in 1x Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5 mM HEPES; pH 7.6) with phenol red as an injection indicator. Embryos were injected with both morpholinos separately to determine the optimal concentration to produce the best range of phenotypes and then with both MOs simultaneously. 5ng SLC25A1a MO and 2.5ng SLC25A1b produced the best range of phenotypes. Four independent MO injection experiments were performed for each MO and at least 500 injected embryos were evaluated in total for each MO.

2.7.13 RNA Isolation and RT-PCR[33]

This work was carried out by Dan Cox from the muscle group, Newcastle University. RNA from approximately thirty zebrafish embryos was isolated with Trizol reagent (Invitrogen, Life Technologies) following the manufacturer's instructions. Reverse transcription was performed with 1 or 2 µg total RNA as template with the Superscript III First-Strand Synthesis System (Invitrogen, Life Technologies) and MolTaq DNA polymerase (Molzym). Regions of interest were amplified with compatible primers.

2.7.14 Immunofluorescence Staining[33]

This work was carried out by Dan Cox from the muscle group, Newcastle University. Whole-mount immunofluorescence staining and imaging of zebrafish embryos was conducted as previously described. In brief, NMJ were visualized with Alexa Fluor® 594 conjugated alpha-bungarotoxin (Invitrogen, Life Technologies, 1 mg/ml) and a mouse monoclonal anti-SV2 (synaptic vesicle protein 2) antibody (Developmental Studies Hybridoma Bank, Iowa City, IA; dilution 1:200). Alexa Fluor® 594 conjugated phalloidin (Sigma-Aldrich) was used at a concentration of 5 µg/ml to label actin.

[33] Adapted from Chaouch et al (URL:http://iospress.metapress.com/content/l36500736v3r4132)

Chapter 3. Cross Sectional Study of the Munich CMS Cohort

3.1 Introduction and Aims

Genetic screening for CMS has been available in five major centres across the world: Oxford, Paris, Munich, California and Rochester. Until recently, these diagnostic laboratories have relied on traditional Sanger sequencing and linkage studies when appropriate, to establish a molecular diagnosis. In all five centres, CMS associated with postsynaptic defects (table 3.1), and in particular low expressing alleles of the subunits of AChR were most frequent (Chaouch et al., 2012a).

Although several studies had been published on genetically characterized individual CMS subgroups, there was no detailed cross sectional analysis of such a large CMS cohort like the Munich database.

In this chapter, I report on the results of a collaborative study I carried out with the Munich CMS diagnostic centre. Dr Angela Abicht and my supervisor Professor Hanns Lochmüller led this project.

I have contributed to the analysis of the clinical data, phenotype genotype correlation and refining the proposed CMS diagnostic algorithm. I was also a contributor to the manuscript detailing this collaborative work: Abicht et al: (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

	Index cases				
	Mayo	**Munich**	**French network**	**Oxford**	**Total**
Pre-synaptic defects					
Choline acetyltransferase deficiency	17	15	4	8	44
Lambert–Eaton syndrome like	1				1
Other presynaptic defects	1				1
Synaptic basal lamina-associated defects					
Endplate AChE deficiency (COLQ)	43	38	19	20	120
Postsynaptic defects					
Primary kinetic abnormality of AChR	53	11	10	39	113
AChR deficiency	109	147	40	138	434
AChR mutations with unclear effects		1	19	1	21
Rapsyn deficiency	48	43	28	71	190
b2-laminin deficiency	1				1
MuSK deficiency		1	3		4
Na channel myasthenia	1		1		2
Agrin deficiency			1		1
Plectin deficiency	2				2
DOK7 deficiency	31	31	28	67	157
GFPT1	2	12		4	18

Table 3.1. Total numbers of index cases with mutations in known CMS genes. Data from four major CMS diagnostic centres in Europe and North America are presented. Reproduced from Chaouch et al 2012 (URL: http://www.sciencedirect.com/science/article/pii/S0960896611014210#)

3.2 Patient data[34]

In this study, I focused on patients with a high clinical suspicion of CMS. In total, 680 patients were selected. Referrals were primarily from Europe (87%), with additional clusters noted in the Middle East, North Africa and South America (figure 3.1). It is worth noting that 17% of patients were from South East Europe and were mainly of Roma ancestry. There was no record of the gender distribution of patients in this database.

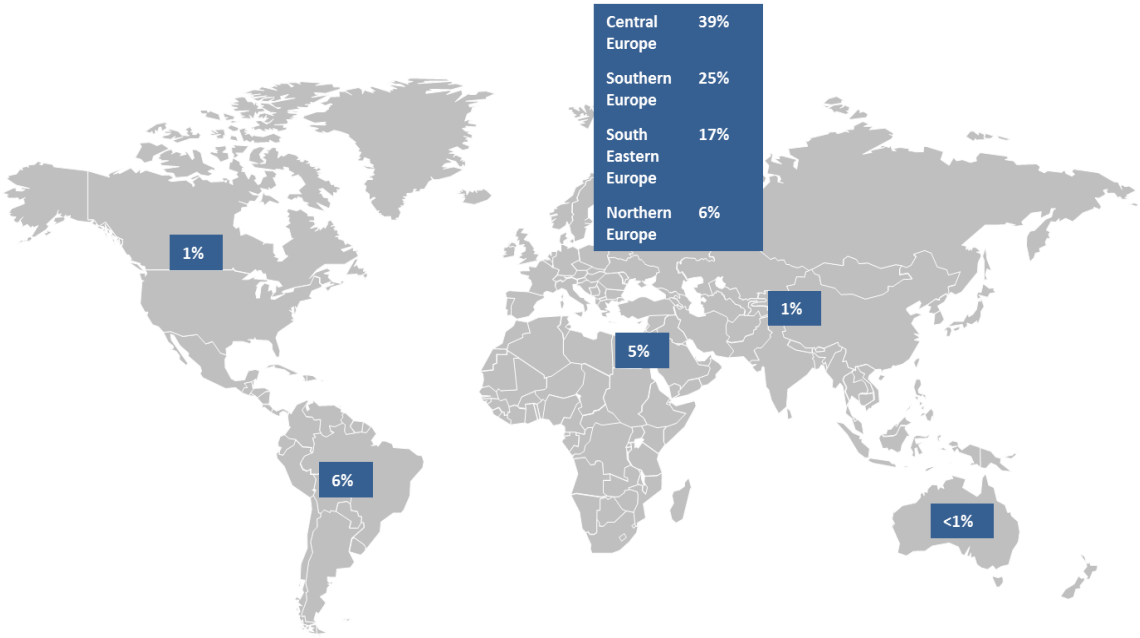


Figure 3.1: Geographical distribution of patients referred to the Munich CMS diagnostic database.

3.2.1 Clinical phenotype[34]

The largest proportion of patients presented at birth (42%). A significant proportion developed symptoms in the first two years of life (29%) or later between 3 and 10 years of age (15%) (figure 3.2). Fifty-seven patients (8%) were reported to have antenatal

[34] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

complications such as reduced fetal movements. Of note, details of age at onset of disease were only available in 511 patients out of the total cohort of 680 patients.

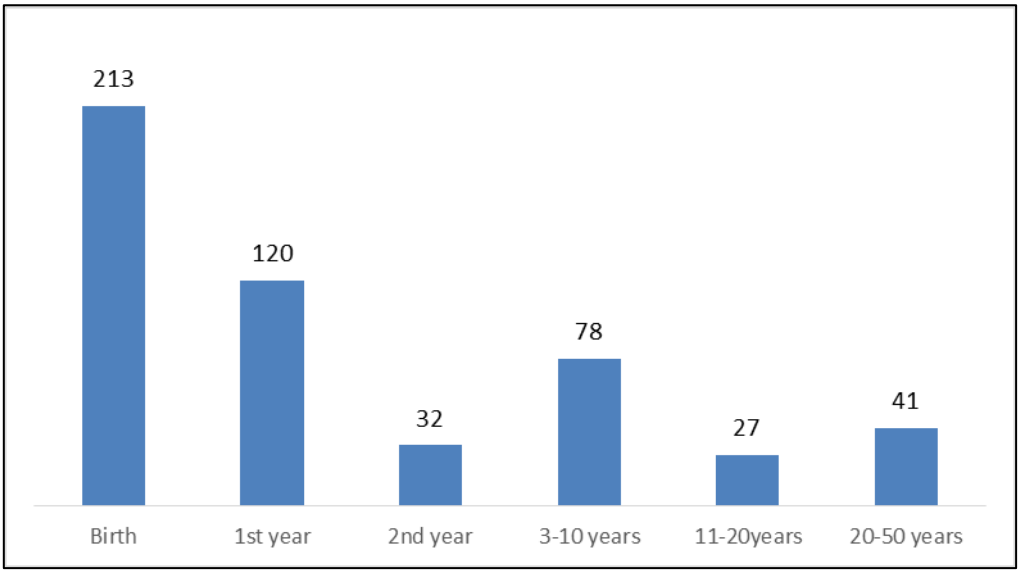


Figure 3.2: Distribution of patient numbers based on age at onset of disease

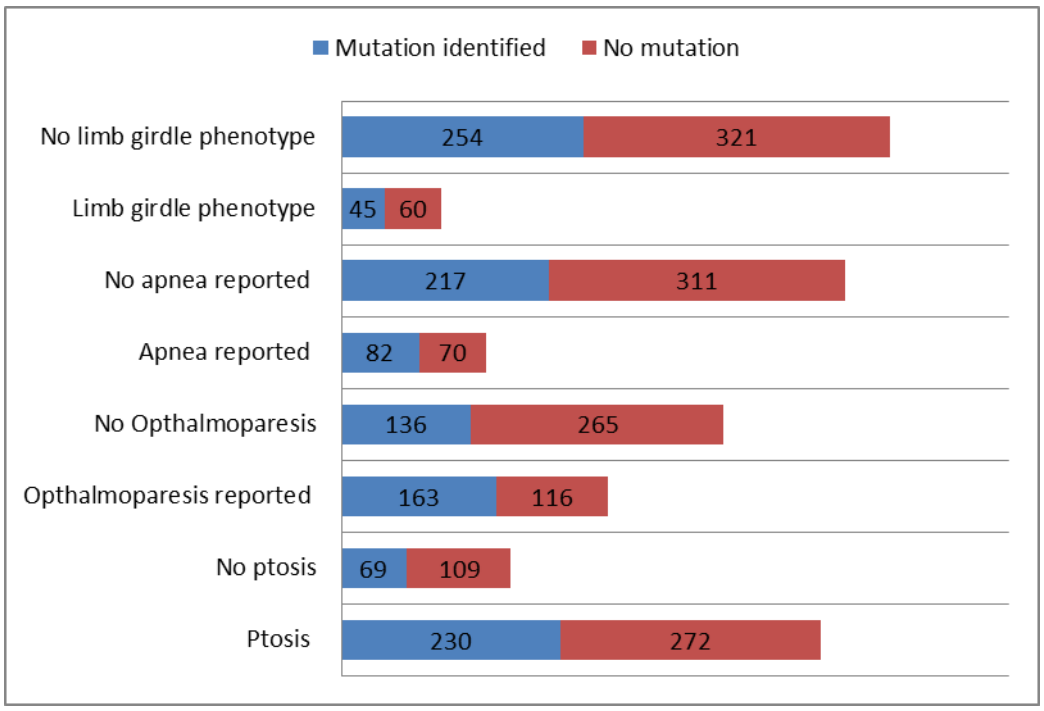


Figure 3.3: Pattern of muscle involvement in both genetically diagnosed and undiagnosed patients.

Most patients presented in childhood with generalized fatigable limb weakness, ptosis and evidence of decrement on neurophysiology. Some patients exhibited additional clinical features such as delayed motor milestones, marked bulbar difficulties, respiratory insufficiency, sometimes requiring ventilator support, congenital contractures of the fingers, wrists and elbows and high arched palate. Response to treatment was variable and not necessarily sustained over time.

In terms of pattern of muscle involvement, ptosis was by far the commonest clinical sign reported in 502 patients (figure 3.3). Ophthalmoparesis was reported in 279 patients, apnoeic episodes in 152 and a limb girdle pattern of muscle weakness in 105. Mutations were detected in 58% of patients presenting with ophthalmoparesis, 56% of patients with isolated ptosis, 54% of patients presenting with apnoea and 43% of patients with a limb girdle phenotype. 275 patients presented with combined ptosis and opthalmoparesis with 55% genetically diagnosed, a figure similar to the subgroups of patients exhibiting either clinical sign in isolation.

3.2.2 Ancillary investigations[35]

Four main criteria were used to exclude potential mimics of CMS and to provide supportive evidence of impaired neuromuscular transmission. These included negativity for anti-AChR and anti-Musk antibodies, normal or mildly elevated creatine kinase (less than 5 times normal range), and absence of large fibre neuropathy or significant myopathic changes on neurophysiology and muscle biopsy. In the muscle biopsy, the presence of tubular aggregates and type 2 fibre atrophy were specifically sought. These structures have recognized as an important pathological hallmark of a particular CMS subgroup associated with glycosylation gene defects. Type 2 fibre atrophy was reported in a number of CMS subtypes regardless of the underlying molecular diagnosis.

[35] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

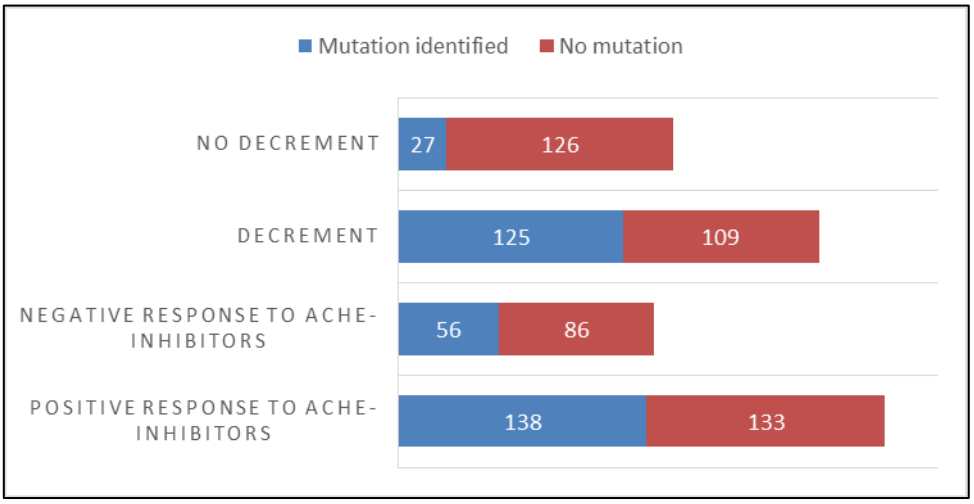


Figure 3.4: Decrement and response to AChE inhibitors in both genetically diagnosed and undiagnosed patients.

Almost two third of the patients in this cohort (387) had detailed neurophysiological studies, including repetitive nerve stimulation. 234 had evidence of decrement in keeping with impaired neuromuscular junction transmission (figure 3.4). 53% of patients shown to have decrements were genetically diagnosed in contrast to 18% with no decrement. By contrast, fewer patients had a muscle biopsy (197 patients), which was reportedly normal in most, or showing non-specific abnormalities such as type 2 fibre atrophy or mild myopathic changes. It is worth noting that the questionnaire did not allow for detailed description of the muscle biopsy findings, in particular it was often unclear how detailed was the study and whether specific stains such NADH or EM analysis were carried out. Both of these are usually required to ascertain the presence of tubular aggregates.

3.2.3 Treatment response[36]

413 patients (60%) received a course of AChE inhibitors (figure 3.4). This treatment was beneficial in 271 patients (65%) and was unhelpful in 142 patients. 51% of patient with a positive response to AChE inhibitors were genetically confirmed in contrast to 39% with negative response.

[36] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3 Genetic data[37]

3.3.1 Inheritance pattern

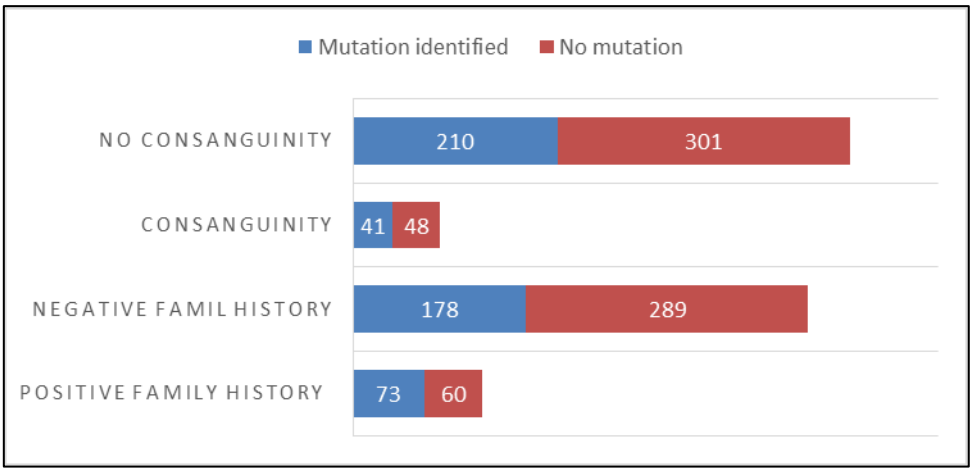


Figure 3.5 Consanguinity or a positive family history, regardless of inheritance pattern

133 patients were reported to have a positive family history with most showing a recessive pattern of transmission (89 patients). A dominant inheritance pattern was only reported in 27 patients. Consanguinity was reported in 94 index cases, accounting for 14% of the total cohort (figure 3.5).

[37] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3.2 Mutations analysis[38]

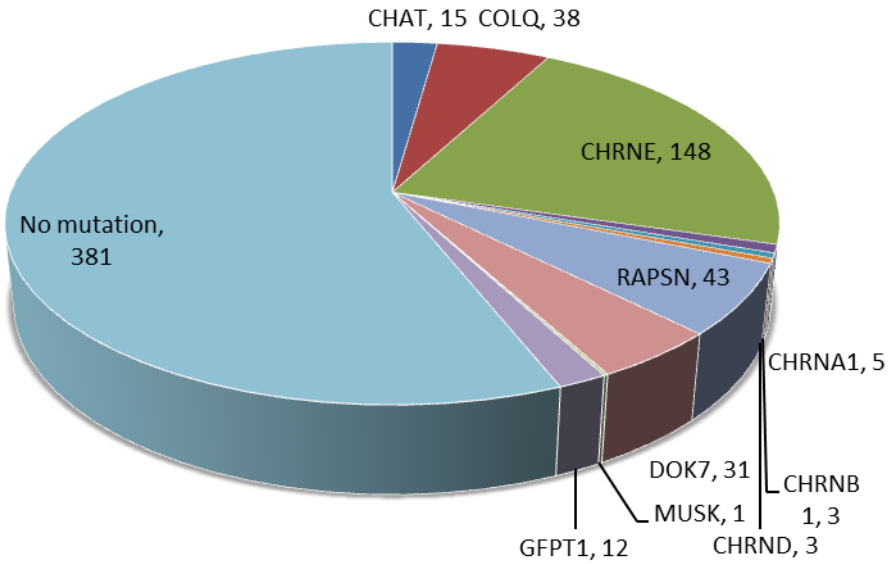


Figure 3.6: Mutation distribution in the Munich CMS database

A molecular diagnosis was confirmed in 299 out of 680 index cases (44%). Patients were screened for mutations in the following known CMS genes: *CHRNA1*, *CHRNB1*, *CHRNE*, *CHRND*, *CHAT*, *COLQ*, *RAPSN*, *DOK7*, *MUSK*, and *GFPT1* (figure 3.6) Mutations in the subunits of AChR were by far the commonest, followed by mutations in *RAPSN*, *COLQ*, *DOK7* and *CHAT* (figure 3.7).

Mutations in *CHRNE*, accounted for 49% of all mutations identified in the diagnosed cohort of 299 patients. Additionally, *RAPSN*, *COLQ* and *DOK7* mutations occurred relatively frequently accounting for 14%, 13% and 10% of all mutations respectively (figure 3.7).

[38] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

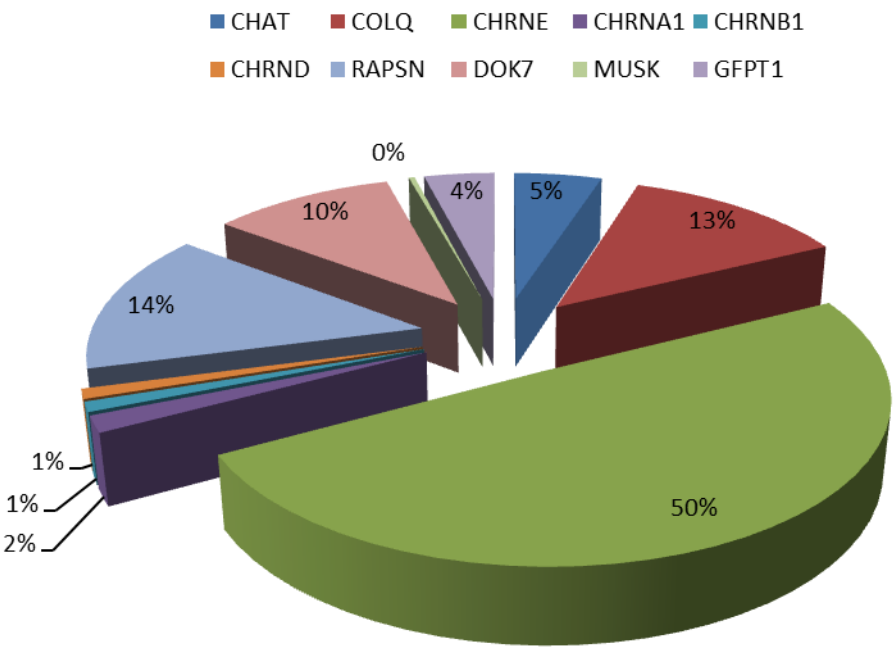


Figure 3.7: Mutation distributions across known CMS genes in the Munich database.

Most mutations were missense exonic changes. For novel variants, pathogenicity was assessed by checking segregation of the variant with the disease phenotype, *in silico* predictions and determination of variant frequency in control samples (at least 50). The majority of variants were private mutations arising in independent kinships, although occasionally, these occurred in several unrelated families, suggesting a possible common founder effect (table 3.2).

	Gene	Mutation	Patients	Ethnic origin
Recurrent mutations	*CHRNE*	c.70insG	16	Spain, Portugal, Hungary, Brazil, Chile
	CHAT	c.T1007C; p.Ile336Thr	6	Turkey
	COLQ	c.1082delC, p.Pro361Leufs∗65	5	Turkey, Italy, Georgia, Czech
	COLQ	c.1289A>C; p.Tyr430Ser	4	Spain

Table 3.2: Private mutations shared between more than three independent kinships. Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

Furthermore, known common mutations were identified in the *CHRNE*, *DOK7* and *RAPSN* genes (table 3.3). These often correlated with particular geographical or ethnic origins: *CHRNE*; c.1267delG7 in the Roma population, *CHRNE*; c.1293insG29 in North Africa, Southern Europe and Brazil, *DOK7*; c.1124_1127dupTGCC and *RAPSN*; c.264C>A (p.N88K) in Europe. Of note, 89 out of 148 patients (60%) with mutations in the *CHRNE* gene carried the common Roma mutation (c.1267delG7).

Gene	Total number of patients with gene defect	Common Mutation	Patients with mutation	Ethnic origin
CHRNE	148	c.1267delG[7]	89	South East Europe, Roma
CHRNE	148	c.1293insG[29]	14	North Africa, southern Europe, Brazil and Russia
DOK7	31	c.1124_1127dupTGCC[19]	25	Europe, Brazil
RAPSN	43	c.264C>A (p.Asn88Lys)[10, 35]	39	Europe, Canada

Table 3.3: Common mutations identified in *CHRNE, DOK7* and *RAPSN* genes. Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3.3 Common mutations identified in the Munich database

3.3.3.1 *CHRNE, CHRNA1, CHRNB1, CHRND*[39]

Mutations in the different subunits of AChR were most frequent (figure 3.7). These were identified in 159 patients out of 299 diagnosed patients and accounted for 54% of all patients. Most mutations were recessive low expressing alleles, predicted to result in deficiency or loss of the different subunits of AChR. Mutations were identified across the length of the genes.

Remarkably, mutations in the epsilon subunit (*CHRNE*) represented 49% of all patients and were detected in 148 out of 299 patients. In accordance with the geographical bias of this cohort, the common Roma mutation *CHRNE;* c.1267delG was the predominant

[39] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

variant, occurring either homozygously or heterozygously in 89 patients. The remaining 59 patients harbored other known founder mutations, seen in North Africa, Brazil and Southern Europe, or private mutations.

Occasionally, mutations known to cause kinetic defects in the different subunits of AChR were identified. These were either recessive, fast channel mutations or dominant mutations causing slow channel syndrome. In five patients, fast channel mutations were identified in *CHRNE* (p. P121L, p.A411P and p.N436del). Only two mutations in *CHRNE* (p.V259L, and p.L269F) were associated with slow channel CMS. Mutations in the other subunits of AChR were less frequent, with five patients harbouring mutations in *CHRNA1*, three patients with mutations in *CHRNB1* and three patients with mutations in *CHRND*. All mutations identified in *CHRNA1* (alpha subunit of AChR) were autosomal dominant mutations associated with a classical slow channel CMS phenotype. Mutations in *CHRNB1* and *CHRND* were either recessive low expressing mutations or kinetic defects predicted or known to cause fast channel CMS. Some of the defects were dominant mutations predicted to result in slow channel syndrome. Clearly, the exact pathomechanism of these rare mutations is dependent on further detailed functional studies.

3.3.3.2 *RAPSN*[40]

RAPSN mutations accounted for 14% of all identified patients (figure 3.7). A total of 369 patients were screened for *RAPSN* and 43 patients were found to be mutation positive. This corresponds to a mutation detection rate of 11%. Invariably, exon 2 was initially sequenced as this included the common mutation (p.N88K) which was detected in 38 out of the 43 patients either in a homozygous (18) or compound heterozygous state (20). In effect, 90% of the patients harboured this mutation in at least one allele. In one patient the p.N88K mutation was found on one allele but no second mutation was detected. Furthermore, a 4.5kb heterozygous deletion was detected in another patient spanning exon 7 and 8. None of the patients were homozygous for a null allele and the distribution of mutations across *RAPSN* is shown in figure 3.8.

[40] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

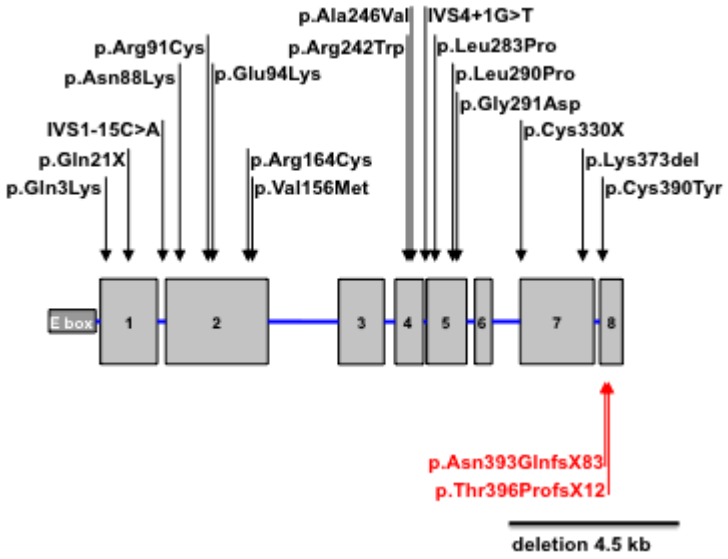


Figure 3.8: Mutations in *RAPSN* identified in patients of this study. Frame shift, nonsense, and splice-site mutations are listed below the gene diagram. Reproduced from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3.3.3 *COLQ*[41]

COLQ mutations accounted for 13% of all mutations (figure 3.7). Often the same mutation occurred independently in different kinships which may suggest a distant shared haplotype in these families or a mutational hotspot in the *COLQ* gene. A total of 126 patients were screened and 38 patients were mutation positive. This corresponded to a mutation detection rate of 30%. Out of the 17 mutations identified, only 6 were missense mutations which were almost exclusively located in the C terminal domain of the colq protein. The remaining mutations, which spread across the length of the gene were either frameshift, nonsense or splice site mutations (figure 3.9). By contrast to *RAPSN*, 20 patients were homozygous for nonsense alleles predicted to result in complete loss of protein.

[41] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

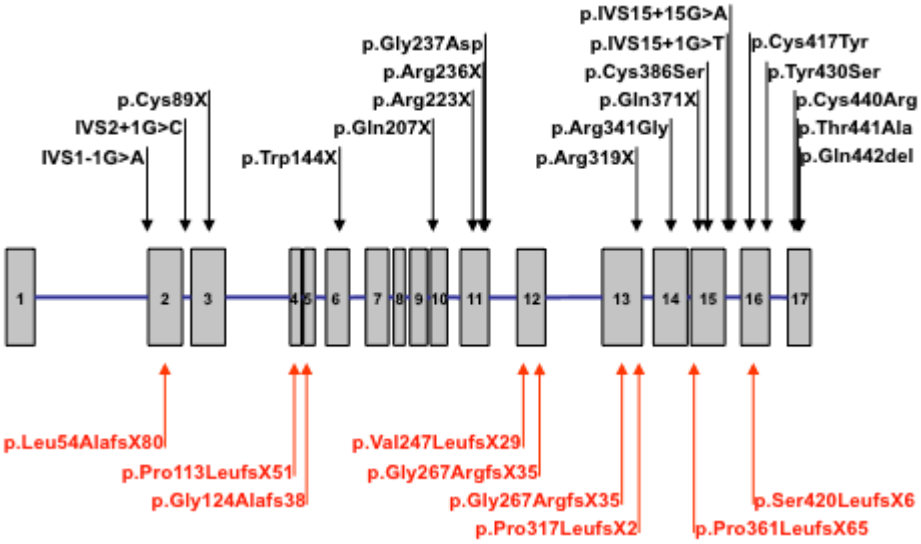


Figure 3.9: Mutations in *COLQ* identified in patients of this study. Frameshift, nonsense, and splice-site mutations are listed below the gene diagram. Reproduced from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3.3.4 *DOK7*[42]

Mutations in *DOK7* accounted for 10% of all mutations (figure 3.7). Of the 373 patients screened, 31 patients were *DOK7* mutation positive. This corresponded to a mutation detection rate of 8%. The common mutation c.1124_1127dupTGCC was present on one or both alleles in 25 out of 31 patients. Only 5 patients were compound heterozygous for defects other than the common *DOK7* mutation. It is worth noting that a second mutation was not detected in 7 patients who were heterozygous carriers for the frameshift mutation c.1124_1127dupTGCC despite the full sequencing of the coding regions (exons) of the *DOK7* gene.

[42] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

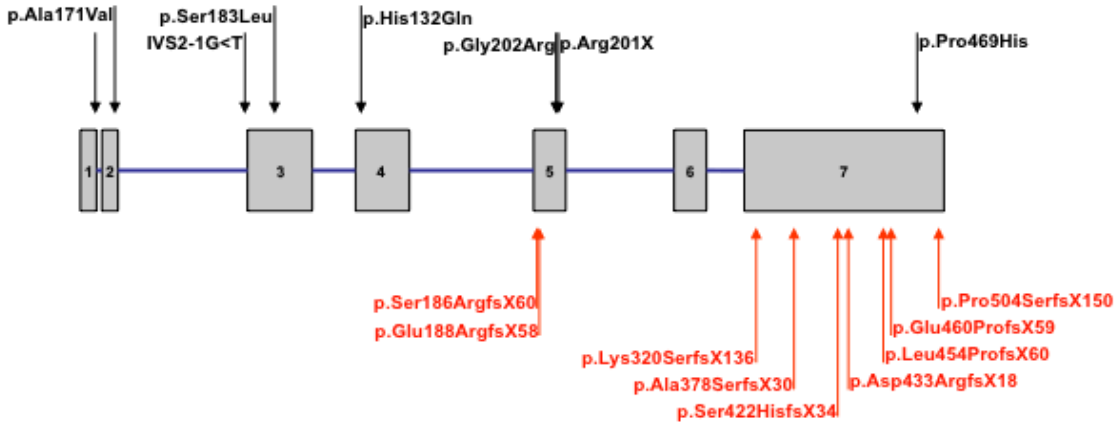


Figure 3.10: Mutations in *DOK7* identified in patients of this study. Frameshift, nonsense, and splice-site mutations are listed below the gene diagram. Reproduced from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3.3.5 *CHAT*[43]

Mutations in *CHAT* accounted for 5% of all patients. A total of 82 patients were screened and 15 patients were mutation positive. This corresponds to a mutation detection rate of 18%. 13 missense and 1 frameshift mutation were identified. These were dispersed throughout the gene. None of the patients were homozygous for nonsense or frameshift mutations. A recurrent mutation was identified in several independent families from Turkey which may point towards a founder effect (table 3.2).

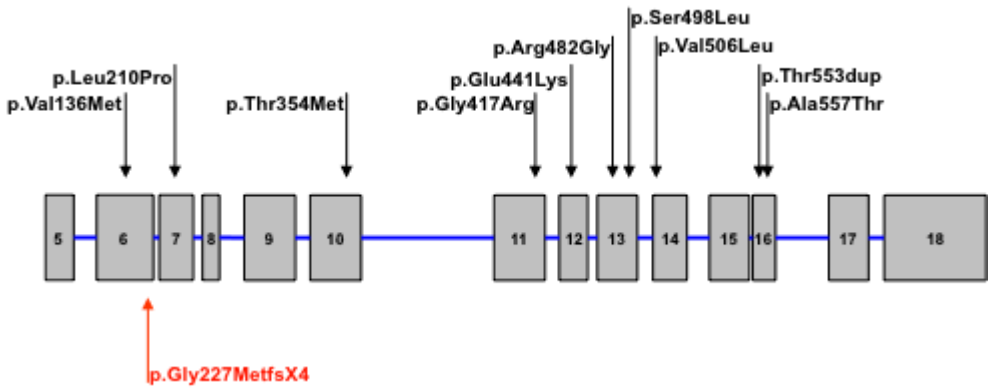


Figure 3.11. Mutations in *CHAT* identified in patients of this study. Frameshift, nonsense, and splice-site mutations are listed below the gene diagram. Reproduced from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

[43] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3.3.6 *GFPT1*[44]

This gene had only recently been discovered using positional cloning (Senderek et al., 2011). *GFPT1* was screened for mutations in 39 patients and 12 patients were mutation positive. *GFPT1* accounted for 4% of all mutations found. A detailed description of the clinical phenotype of this cohort will be presented in chapter 5.

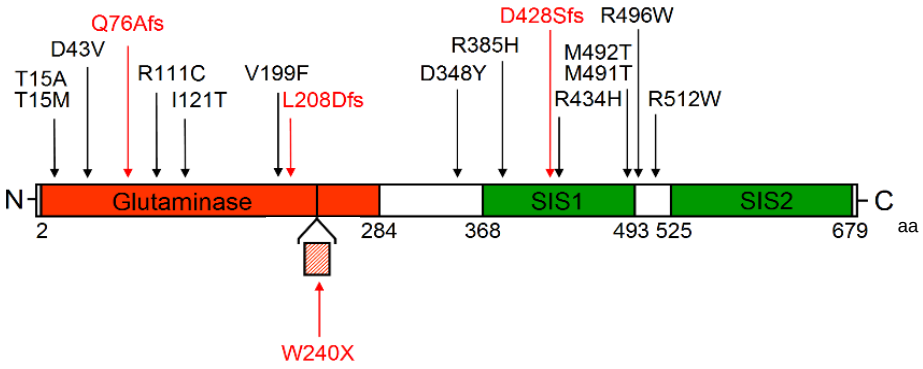


Figure 3.12. Mutations in *GFPT1* identified in patients of this study. Frameshift, nonsense, and splice-site mutations are listed below the gene diagram. Reproduced from Senderek et al 2011 (URL: http://www.sciencedirect.com/science/article/pii/S0002929711000097).

3.3.3.7 *MUSK*[44]

MUSK was screened for mutations in 24 patients and only one had a clearly pathogenic homozygous mutation, *MUSK;* p.P344R which had been previously identified in a consanguineous Sudanese kinship (Mihaylova et al., 2009). Since this study was completed another Turkish family has been shown to be compound heterozygous for a missense mutation (p.R38E) and a heterozygous deletion (Gallenmuller et al., 2014). Nevertheless, *MUSK* mutations remain rare and accounted for less than 1% of all patients.

[44] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3.3.8 Unknown sequence variants

A number of novel sequence variants were identified that had not been reported as polymorphisms or pathogenic mutations. The significance of these defects was unclear (data not shown).

3.3.4 Mutation detection[45]

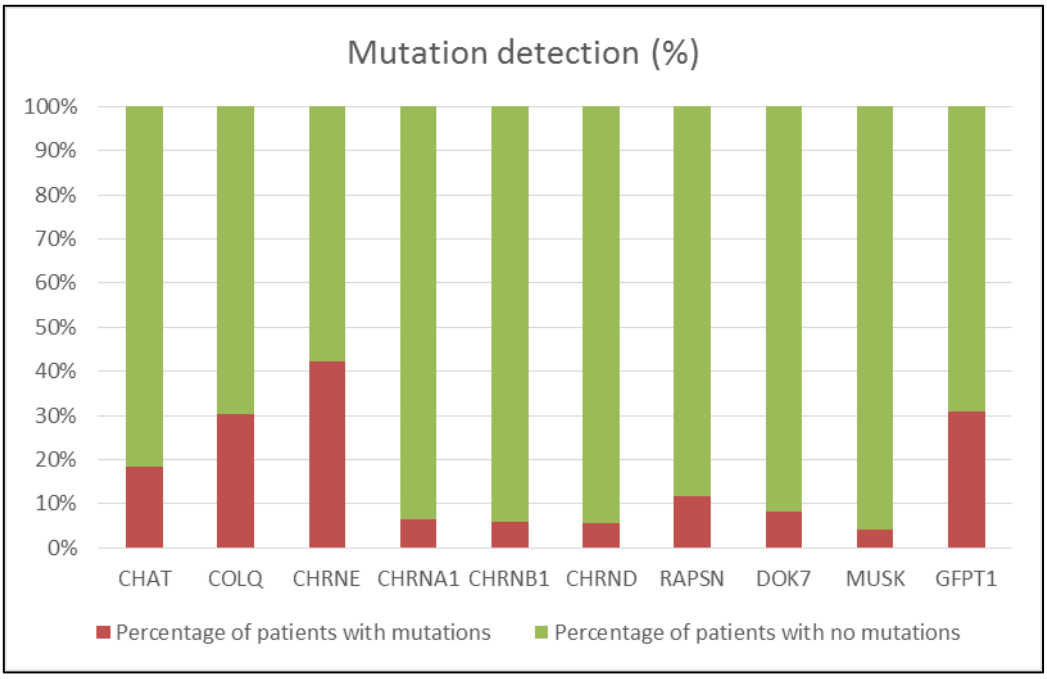


Figure 3.13: Mutation detection rate across all genes analysed.

The mutation detection rate (number of patients with mutations divided by the total tested) is given in figure 3.13 and table 3.4. This was highest for *CHRNE* (42%), followed by *GFPT1* (31%), *COLQ* (30%) and *CHAT* (18%). This figure should be interpreted in the context of a logistically restrained strategy based on our understanding of genotype phenotype in different CMS categories, common mutations related to either a founder effect in particular populations or specific hot spots. This is in part reflected in the high mutation detection rate in *CHRNE*, which probably related to the ethnic bias

[45] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

with 39% of patients referred from South East Europe, many of whom were of Roma ancestry.

Gene	Number of index patients with mutations	Number of patients analysed	Mutation detection in % of analyzed patients
CHAT	15	82	18%
COLQ	38	126	30%
CHRNE	148	350	42%
CHRNA1	5	77	6%
CHRNB1	3	50	6%
CHRND	3	54	6%
RAPSN	43	369	11%
DOK7	31	373	8%
MUSK	1	24	4%
GFPT1	12	39	31%
All genes	299		

Table 3.4: Mutation detection based on gene tested. Total of number of patients screened and found to be mutation positive for each of the 10 CMS genes screened in the Munich CMS cohort. Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.3.5 Targeted CMS gene sequencing (gene panels)[46]

Three sets of gene panels were applied as part of the diagnostic strategy at the Munich centre (table 3.5). These were primarily designed to include common CMS gene mutations. Single gene panels for *CHRNE* and *CHAT* were associated with the highest mutation detection rate at 88%, followed by the *COLQ* single gene panel (76%). Two-gene panels (*CHRNE*/*RAPSN*) and three-gene panels (*CHRNE*, *RAPSN* and *DOK7*) had much lower detection rates.

		Mutation detection: Number of patients with mutation/ Number of tested patients	**Mutation positive (%)**
Single gene	*CHRNE* only	120/136	88%
	RAPSN only	30/63	48%
	DOK7 only	12/45	26%
	COLQ only	19/25	76%
	CHAT only	7/8	88%
2 genes	*CHRNE/RAPSN*	15/33	45%
3 genes	*CHRNE/RAPSN/DOK7*	8/46	17%

Table 3.5: Mutation detection rate in different gene panels tested. Reproduced from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

The *CHRNE* gene was entirely sequenced in 156 patients whilst targeted sequencing of the *CHRNE*; c.1267delG was carried out in 241 patients. For the slow channel panel, the focus was on specific exons coding for the M2 functional domain and the binding

[46] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

site of α subunit in the N terminal domain. For *RAPSN*, the entire gene was screened in 156 patients and targeted screening (c.264C>A; p.N88K) undertaken in 213 patients. For *DOK7*, the entire gene was sequenced in seventy-one patients whilst targeted sequencing of exon 7 was done in 302 patients.

3.3.6 Haplotype analysis in informative families

Haplotype analysis was carried out in informative families with more than one affected individual. Regardless of the phenotype, fifteen out of forty-seven patients (32%) initially screened with haplotype analysis were subsequently shown to have pathogenic mutations in known CMS genes. This figure is lower than without haplotype analysis. One possible explanation is that these families may harbour mutations outside the regions frequently Sanger sequenced in Munich or that the patients share the same phenotype of CMS but have an alternative neuromuscular condition (phenocopy).

3.4 Genotype phenotype correlation[47]

Ideally, one would explore several parameters simultaneously to assess genotype phenotype correlation. However, the choice of these factors in this study was often limited by the lack or variable quality of the clinical and investigation reports. Therefore, analysis focussed on six specific features: 1) presence or absence of decrement, 2) positive or negative response to AChE inhibitors, 3) presence or absence of ptosis, 4) presence or absence of ophthalmoparesis, 5) presence or absence of apnoeic episodes, 6) ethnic origin and in particular, presence or absence of Roma ancestry, 7) presence or absence of consanguinity and lastly 8) presence or absence of a positive family history (table 3.6 and figure 3.14)

Once a particular parameter was selected, patients were categorised based on the presence or absence of this feature and calculated the total number of mutation positive or negative patients. A Chi Square test was used to assess whether there was any difference between these two unpaired groups.

[47] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

The presence or absence of consanguinity and isolated ptosis had no impact on the mutation detection rate. By contrast, all other parameters were associated a significant difference as regard the mutation detection rate. This was most notable in patients of Roma descent, patients with evidence of decrement on repetitive nerve simulation and clinical evidence of ophthalmoparesis. Other subgroups, namely, patients presenting before the age of two years, patients developing apnoeic episodes and patients responding to AChE inhibitors also exhibited a clear difference in mutation detection.

The positive predictive value of ethnic origin in our cohort is probably related to the prevalent Roma founder mutation (*CHRNE,* 1267delG), primarily found in South East Europe.

	Mutation identified	No mutation detected	Total
Positive family history	73 (55%)	60	133
Negative family history	178 (31%)	289	467
			P 0.0005
Consanguinity	41 (46%)	48	89
No consanguinity	210 (41%)	301	511
			P 0.38 not significant
Geographical origin: South-East Europe/Roma	106 (57%)	79	185
Other countries	193 (39%)	302	495
			P 1.9E-05
Age at onset (0,1,2 yrs)	151 (41%)	214	365
Age at onset (3yrs and >3yrs)	45 (31%)	101	146
			P 0.026
Positive response to AChE-Inhibitors	138 (51%)	133	271
Negative response to AChE-Inhibitors	56 (39%)	86	142
			P 0.026
Decrement	125 (53%)	109	234
No decrement	27 (18%)	126	153
			P 0
Apnea reported	82 (54 %)	70	152
No apnea reported	217 (41%)	311	528
			P 0.0049
Ptosis reported	230 (46 %)	272	502
Ptosis not reported	69 (39%)	109	178
			P 1.03 not significant
Opthalmoparesis reported	163 (58 %)	116	279
No Opthalmoparesis	136 (34%)	265	401
			P 0
Ptosis with Opthalmoparesis	152 (55 %)	123	275
No Ptosis with Opthalmoparesis	147 (36%)	258	405
			P1E-06
Limb girdle phenotype	45 (43%)	60	105
No limb girdle phenotype	254 (44%)	321	575
			P 0.8 not significant
Predominant ocular symptoms	1 (4%)	23	24
No predominant ocular smptoms	298 (44%)	358	656
			P6.3E-05

Table 3.6: Mutation detection based on distinct phenotypic features. P values calculated using Chi Square test. Modified from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

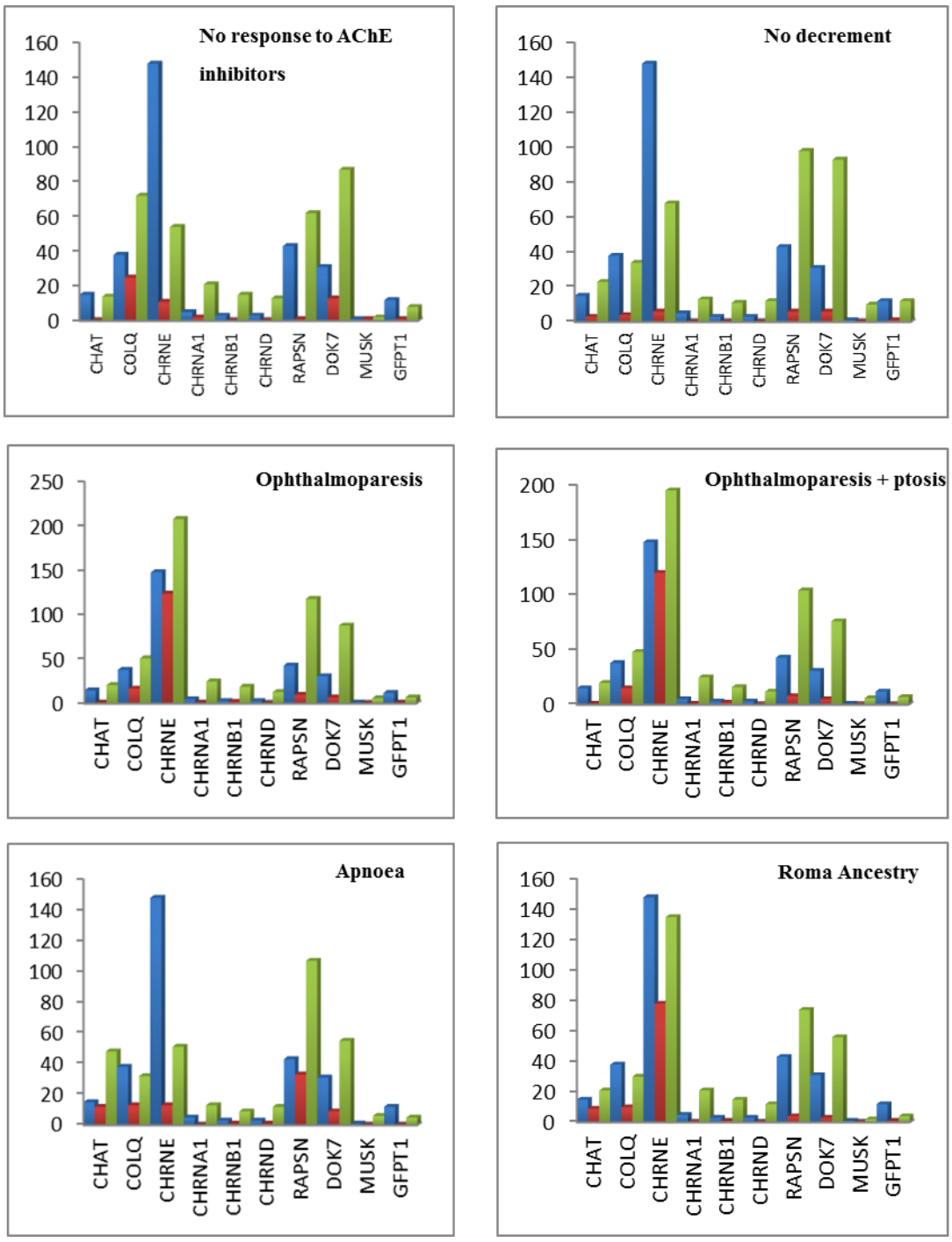


Figure 3.14: Predictive value of particular phenotypic features. The number of patients with a particular phenotype tested for a particular gene (blue) were compared to the mutation positive patients with the same phenotype (red) and in turn this was compared to the total number of mutation positive patients in the total cohort regardless of the phenotype (green). Reproduced from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

3.5 Discussion[48]

This observational cross sectional study was important in terms of determining the distribution and frequency of mutations, ascertaining whether phenotype and genotype can be correlated in a large CMS cohort and to help design a better diagnostic strategy. This seems even more relevant in the context of a rising number of novel CMS genes, and in particular in a disorder like CMS where particular molecular defects require particular therapeutic approaches (Schara and Lochmuller, 2008).

In this study, stringent selection criteria were used to include patients with a clinical diagnosis highly suggestive of CMS and exclude potential phenocopies such as congenital myopathies and autoimmune myasthenia (see chapter 1). Only fourteen known CMS genes were known when this work was carried out and only ten genes were routinely screened for mutations. With a candidate gene approach, disease causing mutations were successfully established in 299 out 680 patients, representing 44% of the total cohort. It is worth noting that this figure is lower than that described in other cohorts. In the UK, a figure of 60% (Palace and Beeson, 2008) has been reported and more recently up to 70-80% of patients have a molecular diagnosis. The discrepancy in mutation detection rates between the Munich and Oxford centres is remarkable and may be due to a number of factors:

1- Some of the undiagnosed patients may have autoimmune myasthenia gravis. Although antibody screening for anti AChR and anti-MuSK antibodies was routinely checked in the undiagnosed patients, a number of these patients may carry low titres of antibodies that would only be detected in specialist centres like the Oxford National laboratory *(Leite et al., 2008).*

2- Some of the undiagnosed patients may have an alternative genetic neuromuscular disorder.

   a. Variations in clinical protocols and expertise in performing repetitive nerve stimulation and SFEMG may result in misinterpretation of results.

   b. The use of a questionnaire to collect data, instead of formal assessment of patients, has its own limitations.

   c. The clinical criteria suggestive of CMS such as the presence of fatigue, jitter on SFEMG and even a positive response to AChE inhibitors are not

[48] Adapted from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract)

specific and may be found in other disorders such as congenital myopathies and mitochondrial disorders.

3- The Munich CMS database may be more ethnically heterogeneous than the Oxford CMS cohort. Indeed only 39% of all referrals to the Munich Centre were from central Europe. This ethnic heterogeneity may be associated with a wider range of mutations, which are not necessarily excluded with the current strategy or may be due to novel genes awaiting to be unraveled.

Regarding the relative mutation frequency across the different CMS genes, the Munich cohort (299 diagnosed patients) showed similar trends as reported in the Mayo clinic cohort, which contained 295 genetically confirmed cases (Engel et al., 2010, Chaouch et al., 2012a) and in the UK cohort which contained 189 mutation-positive patients (Beeson et al., 2005, Beeson et al., 2008). Indeed, postsynaptic defects and in particular defects in the subunits of AChR accounted for most mutations in the Munich cohort (54%). This was mirrored in the Mayo CMS cohort and the UK cohorts. Most of the mutations were recessive missense mutations predicted to result in AChR deficiency at the motor endplate.

A founder effect in different populations was associated with a number of common mutations in shared distant haplotypes. The common Roma mutation (*CHRNE*; c.1267delG) was most prevalent. This mutation was found either homozygously or heterozygously in eighty-nine patients (30% of the diagnosed cohort). It is worth noting that other common mutations were also detected: the *CHRNE*; c.1293insG29 in North Africa, Southern Europe and Brazil as well as the *DOK7*; c.1124_1127dupTGCC and the *RAPSN*; c.264C>A mutations in Europe and North America. These mutations were found in 14 out of 148 *CHRNE* positive patients, 25 out of 31 *DOK7* positive patients and 39 out of 43 *RAPSN* positive patients.

The mutation distribution, should the 89 patients harbouring the Roma founder mutation be removed, were closer to reports from other large CMS cohorts. A proportional increase in the frequency of mutations in other CMS genes was also noted: 5 to 7% in *CHAT* gene, 10 to 15% in *DOK7*, 12 to 17% in *COLQ* gene and 15 to 20% in *RAPSN* gene. In the Mayo clinic, *CHAT* accounted for 5% of the diagnosed cohort whilst

DOK7, *COLQ* and *RASPN* mutations were detected in 8%, 15% and 15% of the 295 genetically confirmed patients (Engel et al., 2010):

At the time of this study, *GFPT1* was a newly discovered gene which was shown to be associated with a limb girdle CMS phenotype with tubular aggregates (Senderek et al., 2011, Guergueltcheva et al., 2011). Based on this phenotypic picture, a total of thirty-nine independent individuals were selected for *GFPT1* mutation screening and twelve were mutation positive.

In general, exclusion criteria have been relaxed in the Munich diagnostic centre due to concerns of dismissing atypical or novel CMS phenotypes. Although patients were referred by experienced clinicians, these were not assessed by CMS experts and therefore the accuracy of the described phenotype will depend on the quality of the data received on a particular patient.

Furthermore, it is well recognized that the CMS phenotype can be difficult to distinguish from other neuromuscular entities. This has been a particular issue with congenital myopathies in which, occasionally, patients can present with neuromuscular symptoms since birth, with ptosis, fluctuations, decrement and even some response to treatment (Alseth et al., 2011, Munot et al., 2010, Srour et al., 2010, Robb et al., 2011). Such patients would be very difficult to detect unless other ancillary tools are included such as detailed muscle biopsy analysis and muscle MRI.

As described earlier, the screening strategy relied on PCR amplification and Sanger sequencing of exonic regions of ten known CMS genes. However, this strategy is inherently limited by its inability to detect large scale deletions as well as intronic defects that may be crucial in the regulation of a gene or its splicing. Moreover, sequencing was often targeted to recurrent mutations or hot spots like the Roma founder mutation in *CHRNE*, exon 2 in *RAPSN* or exon 7 in *DOK7*. Thus, it is probable that some undiagnosed CMS patients who had a robust clinical diagnosis would carry novel CMS genes as yet to be unraveled.

As part of a novel candidate gene search, three functional genes were screened in this cohort: *LRP4*, which encodes for a transmembrane receptor that binds agrin and forms a complex with MuSk; *SLC18A3*, which encodes a transmembrane vesicular transporter of acetylecholine and *CNTN1*, which encodes neuronal membrane protein that may function as an adhesion molecule. No mutation positive patients were identified in any

of these genes. It is worth noting that since this work was completed, *LRP4* mutations were shown to be disease causing in one kinship (Ohkawara et al., 2014). Therefore, it is plausible that some of the Munich centre patients may still harbour mutations in this gene since the phenotype reported had no characteristic features.

In the second part of this study, we ascertained whether particular clinical features or ancillary investigation provided more robust evidence to support a diagnosis of CMS and when possible, determine which parameter had the best predictive value. A number of clinical cues were shown to be more prevalent in certain CMS subgroups (see chapter 1). For instance, the presence of ophthalmoplegia is well documented in patients with mutations of AChR subunits, while apnoeic episodes have typically been associated with *CHAT* and *RAPSN* mutations (Beeson et al., 2005, Engel, 2012). On the other hand, the limb girdle phenotype was shown to be associated with *DOK 7* mutation (Palace et al., 2007) and more recently with several glycosylation gene defects (Belaya et al., 2012, Cossins et al., 2013, Senderek et al., 2011).

Particular neurophysiological and histochemical findings such as evidence of repetitive CMAPs in patients with COLQ related CMS and slow channel syndrome, the presence of tubular aggregates in muscle biopsies of patients with defects in *GFPT1* and other *N*-linked glycosylation genes have certainly helped delineate and refine the clinical phenotype of the patients and sometimes facilitated molecular diagnosis. The response to AChE inhibitors was also a useful tool to stratify patients and can correlate with particular gene defects with *DOK7* and *COLQ* related CMS being typically associated with a detrimental response to AChE inhibitors. Of note, the role of muscle MRI in refining the phenotype of CMS patients remains to be determined.

The correlation between the presence of a particular phenotypic feature and mutation detection rates was also assessed. Consanguinity and isolated ptosis had no impact on the mutation detection rate in the Munich cohort. By contrast, presence of ophthalmoparesis, apnoeic episodes, age at onset before two years, evidence of decrement, response to AChE inhibitors and ethnicity (Roma ancestry) were associated with a significant difference in the mutation detection rate.

In the Munich cohort, a total of 152 patients were reported to have apnoeic episodes and 82 of these were diagnosed. Most defects were detected in *RAPSN*, *CHAT* then *COLQ* genes. Although, apnoeic episodes are well recognized in patients with *CHAT* and *RAPSN* related CMS, mutations in *COLQ* have not necessarily been known to be an

important cause of this clinical phenotype. In this cohort, sudden respiratory crises were documented in twelve out of thirty-eight patients (31%) with *COLQ* mutations. Therefore, in the presence apnoea, one may need to consider screening for *COLQ* mutations if *RAPSN* and *CHAT* sequencing be negative. *CHAT* mutations accounted for 5% of all mutations in the Munich cohort. The mutation detection rate for *CHAT* was 18% ranking second only to *CHRNE* (42%). Two out of the fifteen patients with *CHAT* positive mutations presented at birth but did not develop apnoeic episodes at the time of referral and would have been missed if testing had solely relied on the presence of this feature to proceed with genetic testing.

The distinct proximal pattern of muscle weakness and atrophy with minimal ocular involvement seen in limb girdle CMS has attracted great interest over the years. This phenotype can be associated with *DOK7* mutations, when patients show a negative response to AChE inhibitors or alternatively glycosylation genes (*GFPT1*, *DPAGT1*, *ALG2* and *ALG14*) when AChE inhibitors are beneficial to patients. It is worth noting that *GFPT1* was the only known glycosylation gene when this study was completed and this may well influence the mutation detection rate in this subgroup. A total of 105 patients were referred with a limb girdle phenotype, forty-five of these were mutation positive. In this study, a limb girdle phenotype on its own was not sufficient to influence the mutation detection rate. However, if one combines this particular pattern of muscle weakness with a positive response to AChE inhibitors and ptosis, the predictive value improved. In a total of thirty patients with this constellation of features, twelve were mutation positive. Two patients were *DOK7* carrier and ten had mutations in *GFPT1* gene.

Furthermore, if one focusses on the patients with limb girdle phenotype in whom a muscle biopsy was available, fifty-nine individuals (56%) were identified. A total of eleven patients were shown to have tubular aggregates and seven of these harboured mutations in *GFPT1*. Nevertheless, one should interpret these figures with caution given the relatively large number of patients who did not have a muscle biopsy but also in terms of the technical difficulties in recognizing tubular aggregates in muscle biopsies and their variable distribution in different muscles groups and over time in the same patient (see chapter 5).

This study correlated with previous reports in terms of the importance of neurophysiological studies to explore whether there is impaired neuromuscular transmission (decrement or abnormal jitter) but also in terms of the usefulness of a trial

with AChE inhibitors, if deemed safe, in pointing towards a neuromuscular defect and potentially particular candidate genes, e.g. negative response in *COLQ* and *DOK7* patients.

In this study, haplotype analysis was not suitable in most kinship because of their small size, often including single affected individuals with no positive family history. This method uses microsatellite DNA markers and has been shown to be a cost effective screening method to determine the molecular diagnosis of neuromuscular disorders including CMS (von der Hagen et al., 2006). This indirect genetic strategy allows the restriction of candidate gene by excluding potential candidate genes for subsequent Sanger sequencing. Nevertheless, the application of this approach can be restricted by the small size of informative families, the inability to cope with de novo mutations and recombination events near to or within the candidate gene locus. A total of forty-seven index cases were analysed using this strategy. All had other affected first-degree relatives with some being derived from consanguineous parents. An average of three genes had to be screened before reaching the correct genetic diagnosis. Overall, a molecular diagnosis was successfully established in 32% of this subgroup.

In this cohort, more than 80% of all mutations were located in five CMS genes: *CHRNE*, *RAPSN*, *COLQ*, *DOK7* and *CHAT*. The probability of identifying the genetic defect was highest in patients with only one gene sequenced. This was most notable with screening for *CHRNE* and in particular with the Roma founder mutation c.1267delG. Clearly, this trend may vary in other CMS cohorts with different ethnic distribution with other genes in which there no clear hotspots. A smaller number of patients were first screened for *CHAT* and *COLQ* mutations. However, the mutation detection rate in these patients was substantial, 88% and 78% respectively. The distinct phenotypic clues associated with these two genes, namely the apnoeic episodes in CHAT CMS and a negative response to AChE inhibitors in COLQ patients, may have been useful pointers to the correct molecular diagnosis.

Based on the above observations, one can distinguish four important factors that can determine the likelihood of establishing a molecular diagnosis (figure 3.14):

1) Ethnicity.
2) A positive family history.
3) Presence of apnoeic episodes.

4) Response to AChE inhibitors.

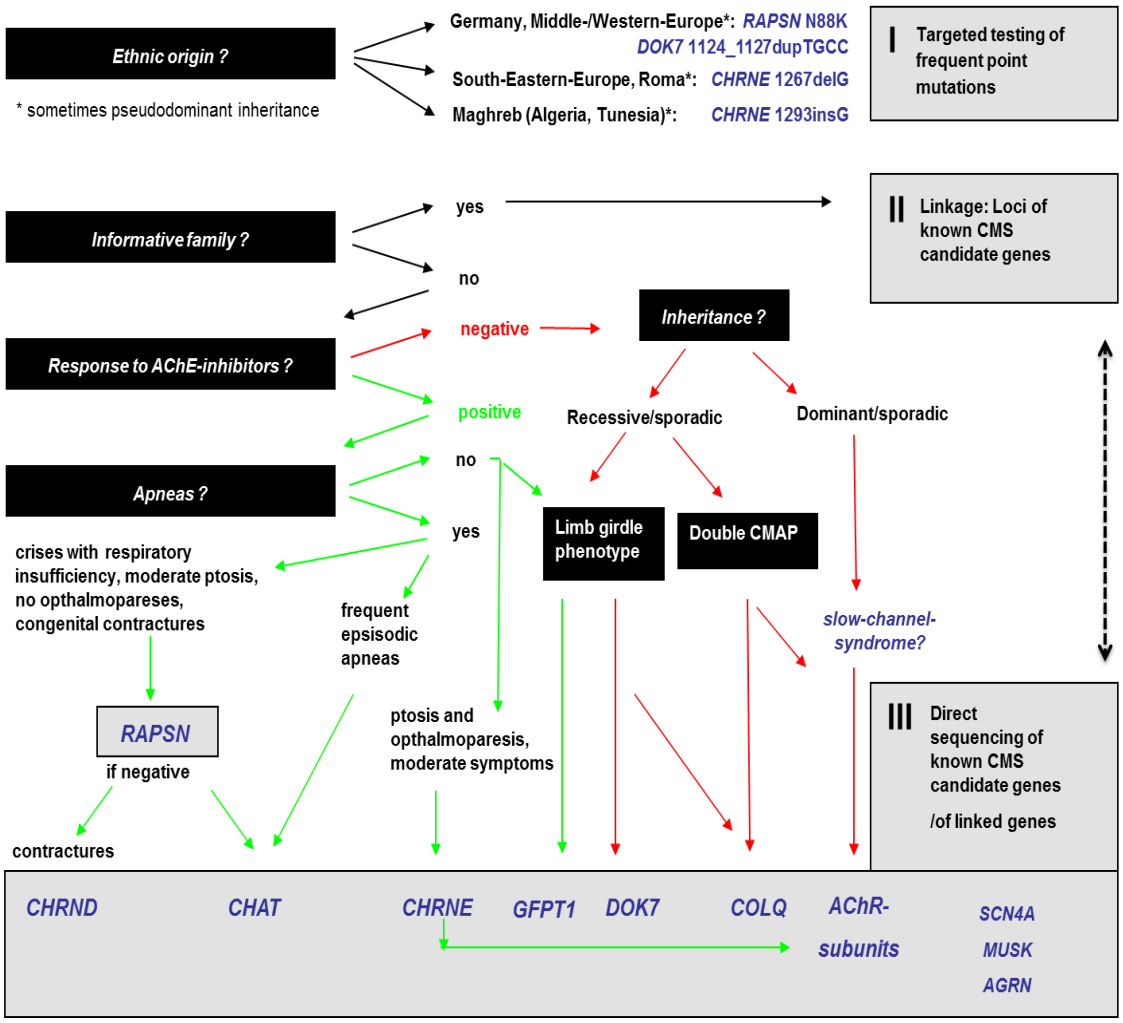


Figure 3.15: Algorithm for genetic testing. Entry points according to available clinical and electrophysiological data are given as black squares. Reproduced from Abicht et al 2012 (URL: http://onlinelibrary.wiley.com/doi/10.1002/humu.22130/abstract).

The recent discovery of more CMS genes using next generation sequencing will undoubtedly influence the diagnostic strategy for CMS (figure 3.15). In clinical practice, most CMS patients will present soon after birth or in the neonatal period with hypotonia and fatigable weakness. The differential diagnosis of such symptoms is wide and one would need to search for additional clinical clues to determine the precise genetic aetiology. Such features may include recurrent apnoeas, which can be seen in several subtypes of CMS including defects in CHAT, RAPSN, COLQ, DOK7 and fast channel CMS. In this instance, the presence of finger contractures and minimal ocular symptoms, may suggest RAPSN CMS, proximal weakness may suggest COLQ and DOK7 mutations and evidence of minimal neuromuscular weakness between episodes may suggest CHAT mutations. In the presence of apnoeas, an important step to help clarify the cause is a trial with AChE inhibitors: This treatment should be administered in a safe setting where patients can be monitored for potential deterioration in their neuromuscular symptoms. Patients with RAPSN, CHAT mutations and fast channel CMS tend to respond favourably to AChE inhibitors in contrast to DOK7 and COLQ associated CMS patients, who exhibit no sustained response to this treatment and may even deteriorate.

Although ptosis is a common symptom in CMS, it is rather non-specific. On the other hand, ophthalmoparesis can be more suggestive of a particular subgroup of CMS including mutations in the subunits of AChRs, COLQ, MUSK and some AGRN mutations. In this instance, response to AChE inhibitors can be extremely helpful to narrow the differential diagnosis since mutations in AChR, except for slow channel mutations, respond to this treatment whilst CMS associated with COLQ, MUSK and AGRN mutations do not.

Most CMS patients present with global fatigable weakness, however, some may show distinct patterns of muscle involvement. Proximal weakness with minimal ocular symptoms may be in keeping with defects in DOK7 and other glycosylation genes (GFPT1, DPAGT1, ALG2 and ALG14). COLQ CMS patients can also present with proximal involvement but ophthalmoplegia is usually present. A positive response to AChE inhibitors and presence of tubular aggregates on muscle biopsy may point towards mutations in glycosylation genes rather than DOK7-mediated CMS. Another distinguishing pattern of muscle involvement includes distal muscle weakness that can be consistent with either slow channel or AGRN mutations. The latter may also be associated with evidence of increment on repetitive nerve stimulation. Intellectual disability is a rare feature in CMS but mutations in ALG2 were recently shown to be

associated with this phenotype. Finally, other clues such as a dominant mode of inheritance and particular ethnic origins have been reported in association with slow channel CMS and particular common mutations respectively.

Chapter 4. Clinical characterization of a slow channel CMS cohort

4.1 Introduction and aims[49]

Mutations in the subunits of the AChR are responsible for the majority of CMS cases and can result in deficiency of AChR subunits or alter the kinetic characteristics of the ligand gated ion channel. (Engel and Sine, 2005, Hantai et al., 2004, Muller et al., 2007b).

Slow channel CMS is a rare CMS category and the only known autosomal dominant form. Mutations are associated with sustained channel opening and destabilized channel closure with higher receptor affinity for acetylcholine (ACh) (Engel and Sine, 2005). These aberrant properties compromise efficient and reliable transmission across the neuromuscular synapse. Characteristically, patients present with global fatigable neuromuscular weakness with a predilection for the cervical and extensor forearm muscles. Single nerve stimulation can trigger a repetitive CMAP response. Furthermore, an endplate myopathy can be a distinctive morphological footprint. The latter is thought to be secondary to sustained postsynaptic activation and excess calcium influx (Engel et al., 1982).

To date 20 mutations have been reported to cause slow channel CMS (Engel et al., 1996b, Croxen et al., 1997, Sine et al., 1995, Milone et al., 1997, Shen et al., 2006, Gomez et al., 2002, Croxen et al., 2002, Ohno et al., 1995, Vohra et al., 2004, Vohra et al., 2006). These have been identified in the α, β, δ and ε subunits of the AChR. All mutations have primarily been located in the transmembrane domains of the different subunits: M1, M2 and more recently M4 domains and a few were located in the extracellular domain of M1 as well as the N terminal part near the binding site of the receptor."

Because of its distinct molecular mechanism, slow channel CMS patients have required alternative treatments and indeed do not usually benefit from AChE inhibitors (Schara and Lochmuller, 2008). Based on previous published evidence, long acting open

[49] Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

channel blockers like fluoxetine and quinidine were prescribed in such patients with some benefits (Harper et al., 2003, Harper and Engel, 1998b).

It is worth noting that fast channel CMS is also caused by kinetic mutations in the AChR subunits (Palace et al., 2012). This entity however is autosomal recessive in inheritance and mutations lead to brief channel opening events and occasionally diminished affinity to its substrate. There are no anatomical footprints with these mutations (Engel and Sine, 2005).

The main objectives of this study were to refine the clinical phenotype of a sizeable cohort of slow channel CMS patients and determine the efficacy of recommended therapies. This cohort was identified via the Munich CMS diagnostic database. For details of patient selection, clinical assessments and mutation analysis please see Chapter 2 (Methods). Briefly, I generated and circulated a questionnaire to all the referring clinicians to capture relevant information. The results of this study were published in Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9).

4.2 Patient data

4.2.1 Clinical phenotype[50]

Fifteen slow-channel CMS patients from families were identified in the Munich CMS centre. Patients referred from1997 to 2010 (table 4.1and figure 4.1). Nine female and six male patients, of varied ethnic backgrounds, were monitored over a period extending from 5 to 20 years. Patient 3 deceased in 2009 subsequent to a car accident and patient 14 was deceased in 2011 following complications from a lower respiratory tract infection. Patients 3, 4 and 10 were previously published (Colomer et al., 2006, Mihaylova et al., 2010).

The patients presented either in early infancy or later as adults. The majority of patients demonstrated selective neuromuscular weakness of the upper limbs and neck muscles.

[50] Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

Patients 7, 8, 10, 13 and 15 showed prominent wrist and finger extensor muscle weakness.

More than half of the patients exhibited facial, ocular and bulbar involvement. Striking masseter muscle atrophy was noted in patients 12, 13 and 14, who were from the same pedigree.

Patients 10 and 11, developed neonatal respiratory complications necessitating ventilator support and have remained on this treatment since.

Most patients' neuromuscular symptoms fluctuated from days to weeks with a clear progressive trend over time except for patient 9 who reported improvement in her muscle weakness later in life, without administration of any medication.

Interestingly, 3 of the 9 female patients (patients 8, 9 and 13) developed their symptoms subsequent to giving birth and patients 2 and 11 described temporary exacerbation of neuromuscular weakness in relation to menstruation.

	Age/Gender /ethnicity	Mutation	Onset										Disease course		
			Age	Symptoms	Signs								Static	Progre-ssive	Fluctu-ating
					FVC	Ocular muscles	Cervical muscles	Prox UL	Dist UL	Prox LL	Dist LL	Fatigue	Other features			
1	44/F/African	CHRNA1 G153S	25y	Gen limb weak, occasional dyspnoea	69%	-	+++	++	+	++	+	+	Scap wing	+	-	+
2	46/F/Swedish	CHRND S268Y	6y	Neck and gen limb weak and thyrotoxicosis age 13	103%	-	+	++	+++	++	++	+	-	-	+	+
3	31/M/ Portuguese Lebanese	CHRNB1 V266M	Birth	Ocular, bulbar and proximal limb weak	ND	+	+	+	++	-	-	+	Bulbar weak, scap wing, scoliosis, hand atrophy	-	+	+
4	16/ F/ Spanish Italian	CHRNA1 G153S	1y	Ocular, neck and proximal limb weak	ND	-	+	+	+	+	-	+	-	-	+	+
5	19/F/German	CHRNE V259L	2y	Ocular and prox limb weak	79%	+	+	-	+	+	-	+		+	-	+
6	15/ F/ German	CHRNE V259L	Birth	Ocular, prox limb weak and recurrent chest infections	81%	+	+	++	++	+	+	+	Facial diplegia	-	+	+
7	44/M/ German	CHRNE V259L	20y	Gen limb weak	ND	-	-	+	+	-	+	+	Left hemiparesis after stroke	+	-	-
8	47/ F/Serbian	CHRNB1 T265S	38y	Ocular and gen limb weak	110%	+	+	++	++	+	+	+		-	+	-

Table 4.1. (page 84-85). Clinical features of the slow channel CMS cohort. F; female, M; male, ND; not done, y; year, Gen; generalised, FVC; forced vital capacity, Prox; proximal, Dist; distal, scap; scapular, wing; winging, UL; upper limbs, L; lower limbs, +; mild, ++; moderate, +++; severe. Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

	Age/Gender/ ethnicity	Mutation	Onset										Disease course		
			Age	Symptoms	Signs								Static	Prog-ressive	Fluctu-ating
					FVC	Ocular muscles	Cervical muscles	Prox UL	Dist UL	Prox LL	Dist LL	Fatigue	Other features			
9	73/F/Serbian	CHRNB1 T265S	26y	Gen upper limb weak	ND	-	+	+	+	-	-	+		-	-	+
10	20/ M/Spanish	CHRNE L269F	Birth	Hypotonia, failure to thrive, ocular and gen limb weak	ND	+	++	+	++	-	-	+	Facial weak, gen muscle atrophy scoliosis	-	+	+
11	18/ F/Spanish	CHRNE L269F	Birth	Hypotonia, failure to thrive, ocular, facial, gen limb weak	35%	+	++	++	+	++	+	+	Facial weak, gen muscle atrophy scoliosis	-	+	+
12	48/M/Ashkenazi Jewish	CHRNA1 G153S	43y	Chewing difficulties, involuntary mouth opening, gen fatigue	ND	+	++	-	+	-	+	+	Facial weak, masseter atrophy high arched palate	-	+	+
13	74/F/Ashkenazi Jewish	*	28y	Chewing & speech diff, gen limb weak	ND	+	+	-	++	+	++	-	Facial weak, UL atrophy, masseter atrophy	-	+	+
14	70/M/Ashkenazi Jewish	*	15y	Hand weak, diff standing on tiptoes, chewing diff	ND	-	+	-	+	-	+	+	Masseter atrophy	-	+	-
15	19/M/Ashkenazi Jewish	*	15y	Hand weak	ND	-	+	+	++	-	-	-	Facial weak, high arched palate, micrognathia	+	-	-

4.2.2 Ancillary investigations[51]

In terms of investigations, most patients had normal creatinine kinase levels and undetectable AChR antibodies titres. Two patients were noted to have moderately elevated creatine kinase levels. Repetitive nerve stimulation at 3Hz showed decrement in all the 9 patients tested. Notably, 7 had demonstrated repetitive CMAPs on single nerve stimulation. Unspecific myopathic features were reported in all 5 muscle biopsies carried out as part of the initial assessment of these patients (table 4.2).

Patient/Age/Gender	CK	AChR Abs	Nerve stimulation	Muscle biopsy
1/44y/F	↑	-	Decrement, double CMAPS	NS
2/46y/F	-	-	Decrement, double CMAPS	ND
3/31y/M	-	-	Decrement, double CMAPS	NS
4/16y/ F	-	-	Decrement, double CMAPS	NS
5/19y/F	-	-	Decrement	NS
6/15y/ F	-	-	Decrement	ND
7/44y /M	ND	ND	ND	ND
8/47y/ F	↑	-	Decrement, double CMAPS	NS
9/73y/F	ND	-	ND	ND
10/20y/ M	-	-	Decrement, double CMAPS	NS
11/18y/ F	-	ND	ND	ND
12/48/M	-	ND	Decrement, double CMAPS	ND
13/74/F	ND	ND	ND	ND
14/70/M	ND	ND	ND	ND
15/19/M	ND	ND	ND	ND

Table 4.2. Results of ancillary investigations in slow channel CMS cohort. M; male, F; female, ↑; elevated, - ; normal, ND; not done, NS; nonspecific, CMAP; compound muscle action potential, Abs; antibodies. Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

[51] Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

4.2.3 Mutation analysis[52]

Ten patients from four families exhibited autosomal dominant inheritance while five cases were sporadic. Genetic testing was carried out in the Munich diagnostic centre. Patients were screened using a slow channel CMS panel that included exons encoding the M2 domain of the ε, α, β, δ subunits of the receptor. In the α subunit, exons encoding the N terminal region were also sequenced. In total, six missense mutations in the α, β, δ and ε subunits were detected: αG153S, βT265S, εL269F, εV259L and δS268Y. Mutations βT265S and δS268Ywere not previously reported. Most mutations were located in the channel pore M2 functional domain (figure 4.1).

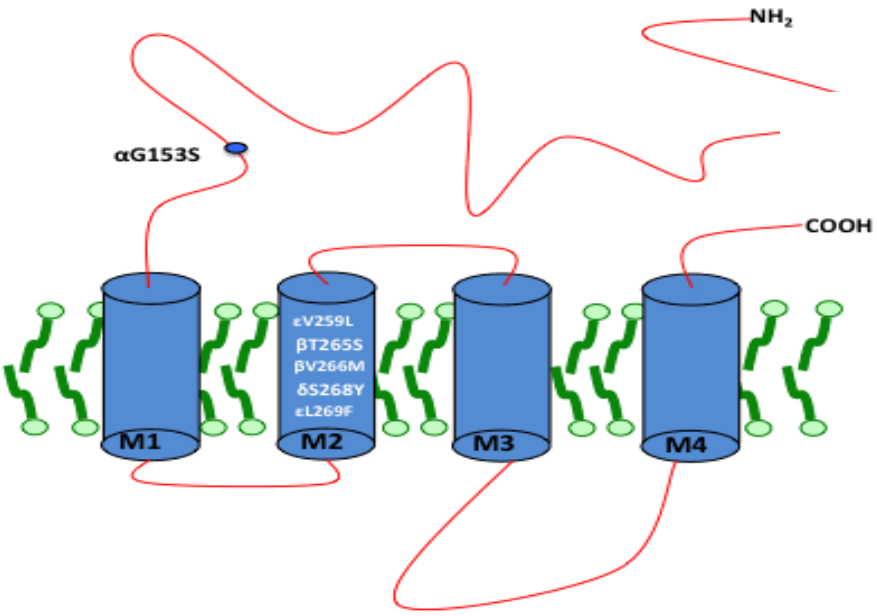


Figure 4.1. Slow channel mutations in relation to the functional domains of the AChR.

4.2.4 Treatment regimens

Choice of therapy was dependent on whether molecular diagnosis was established (table 4.3). In the eight patients with no initial molecular diagnosis available, AChE inhibitors were prescribed with no reported clinical benefit. 3,4-DAP was initiated as substitute in two patients and prednisolone was added as an adjunct to AChE inhibitors in a further

[52] Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

two patients. Though well tolerated, prednisolone, 3,4-DAP and AChE inhibitors were ineffective and were later stopped.

Patient/Age/Gender	1st line therapy (max dose)	Response to therapy
1/44y/F	Age 30y. Pyridostigmine 60mg tds	No benefit, worsening fatigue
2/46y/F	Age 38y. Pyridostigmine 120mg tds	No benefit
3/31y/M	Age 20y. Pyridostigmine 60mg tds	Initial benefit then relapse
4/16y/ F	Age 5y. Pyridostigmine 20mg tds	No benefit, side effects (headache)
5/19y/F	Age 17y. Quinidine 150mg tds	Clear benefit
6/15y/ F	Age 13y. Quinidine 150mg tds	Clear benefit, mild side effects (vertigo and tachycardia)
7/44y /M	Never treated	
8/47y/ F	Age 39y. Pyridostigmine 60mg qds	No benefit
9/73y/F	Never treated	
10/20y/ M	Age 1y. Pyridostigmine 60mg qds	No benefit
11/18y/ F	Age 1y. Pyridostigmine 60mg qds	No benefit
12/48/M	Pyridostigmine 180 mg/d	No benefit
13/74/F	Age 70. Fluoxetine 40mg/d	Clear benefit with no side effects
14/70/M	Age 67. Fluoxetine 40mg/d	Clear benefit with no side effects
15/19/M	Never treated	

Table 4.3. Summary of therapies prescribed. M; male, F; female, y; year, bd; twice a day, tds; three times a day, qds; four times a day Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

Patient/Age/Gender	2nd line therapy (max dose)	Response to therapy	3rd line therapy (max dose)	Response to therapy
1/44y/F	Age 30y. 3,4-DAP 15mg x 6/d	Initial benefit, then relapse	Age 40y. Fluoxetine. 40mg/d	Clear benefit but significant side effects (serotoninergic crisis 3 months after starting treatment)
2/46y/F	Quinidine 200mg bd	No benefit	Age 43y. Fluoxetine 20mg od	No benefit and significant side effects (lethargy and hypotension)
3/31y/M	Age 23y. Pyridostigmine with prednisolone 20mg/d	No benefit, symptoms worse	Age 25y. Fluoxetine 20mg/d	Some benefit but unable to tolerate higher dose (40mg/d)
4/16y/ F	Age 6. 3, 4 DAP 25mg/day	No benefit	Age 13y. Fluoxetine 60mg/d	Clear benefit with no side effects
5/19y/F				
6/15y/ F				
7/44y /M				
8/47y/ F	Age 40y. Pyridostigmine with prednisone 60mg alt days	No benefit	Age 43y. Fluoxetine 80mg/d	Clear benefit with no side effects
9/73y/F				
10/20y/ M	Age 15y. Fluoxetine 60mg/d	Clear benefit but relapsed after 2 years of therapy. Remains on fluoxetine but reliant on NIV		
11/18y/ F	Age 12y. Fluoxetine 60mg/d	Clear benefit		
12/48/M	Age43. Quinidine 400 mg/d	No benefit	Age43. Fluoxetine (60mg/d)	Clear benefit with no side effects
13/74/F				
14/70/M				
15/19/M				

Table 4.3. (Cont) Summary of therapies prescribed. Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

Subsequent to establishing a molecular diagnosis, fluoxetine was prescribed in patients 1, 3, 4, 8, 10, 11, 13, 14 and quinidine was introduced in patients 2, 5, 6 and 12. Notably, three patients received no treatment. In patients 9 and 15 neuromuscular weakness was deemed too mild to warrant therapy. Patient 7 was still undergoing assessments to decide on optimum management (table 4.3). Patients 5 and 6 reported definite amelioration in their neuromuscular weakness after receiving quinidine. On the other hand, patients 2 and 12 showed no benefit and were later switched to fluoxetine. It is worth noting that quinidine was well tolerated in all patients.

Fluoxetine was prescribed in a total of ten patients but had to be withdrawn in patients 1, 2 and 3 due to side effects. The latter comprised life threatening serotonergic crisis, symptomatic hypotension that failed to improve by reducing treatment dose. In patients in whom fluoxetine was tolerated, all reported clear benefit in their neuromuscular weakness. Sustained improvement with fluoxetine was reported up to six years after instigating treatment although one patient (patient 10) developed significant respiratory insufficiency two years after instigation of fluoxetine. Only one patient (patient 1) had ephedrine as 4th line therapy and showed clear benefit though he reported excess lethargy and malaise.[53]

5.3 Discussion[53]

The findings from this study, which describes a sizeable cohort of patients, were comparable to previous reports of slow channel CMS cohorts as regard the preferential involvement of cervical and upper limb muscle groups with clear inter familial and intra familial variability (Beeson et al., 2005, Engel and Sine, 2005). Craniofacial weakness was a common feature and the degree of ophthalmoparesis was less severe than what is normally seen in AChR deficiency CMS. The age at onset of disease was also distinctly variable with some patients presenting in infancy while others manifesting often mild symptoms late in life. I was interested to note that 50% of the female patients reported exacerbation of their myasthenic symptoms in relation to hormonal changes and pregnancy.

[53] Adapted from Chaouch et al 2012 (URL:http://link.springer.com/article/10.1007%2Fs00415-011-6204-9)

Repetitive nerve stimulation consistently revealed decrement suggestive of impaired NMJ transmission. Furthermore, repetitive CMAPs were detected in several patients, which can reflect aberrant sustained stimulation of the postsynaptic membrane. It is important to emphasize that repetitive CMAP are not pathognomonic of slow channel CMS and can be found in other conditions like COLQ CMS (or treatment with AChE inhibitors). Nonetheless, neurophysiological studies were helpful in supporting the diagnosis of CMS.

Predictably, unravelling the exact genetic diagnosis had substantial effect on choice of treatment and clinical response. Out of the six dominant missense mutations identified, five had previously been published and one was novel. *CHRNB1* T265S is the novel mutation and was shown to modify a conserved residue at the pore forming M2 domain thus delaying channel opening events on patch clamp study (Hoellen, unpublished). The mother and daughter from the Serbian kinship (patients 8 and 9) were heterozygotes for this mutation. They both developed adult onset neuromuscular weakness, which was comparatively mild and improved with fluoxetine in the daughter. The mother never wished to be treated given her mild symptoms.

Mutation αG153S is the commonest slow channel mutation in the UK. It is located near the extracellular AChR binding domain and was reported to impede the disconnection of ACh from its binding site (Sine et al., 1995). In this cohort, this mutation was also the most frequent and occurred in patients of different ethnic origin. There were clear differences with respect to age at disease onset and response to fluoxetine in patients sharing mutation αG153S. Remarkably, all individuals from the Ashkenazi Jewish family demonstrated the common feature of atrophied masseter muscles. This clinical feature may well be a clinical sign specific to this family, as it was not observed in other cases.

The other two known mutations identified in this cohort are mutations βV266M and εL269F. These are positioned in the M2 domain and have been shown to prolong channel opening events (Engel et al., 1996b). In contrast to βV266M, mutation εL269F also enhanced the affinity of the receptor for ACh. In this study, both mutations were associated with a relatively disabling phenotype with neonatal onset neuromuscular weakness and a good response to fluoxetine.

Mutation εV259L was reported in a French family with a slow channel phenotype. This mutation is also positioned in the M2 domain (Outteryck et al., 2009) and was shown to augment AChR permeability to calcium. In this study, this mutation was detected in the same kinship from Germany in which the father and both daughters were affected. There was evidence of intra familial phenotypic variability with both daughters presenting at much earlier stage than their father and demonstrated moderate fatigable weakness. Both daughters had a beneficial response to quinidine.

Mutation in the δ subunit (S268F) was formerly shown to prolong channel opening events (Gomez et al., 2002). In this study, a mutation (δS268Y) at the same position was detected in patient 2, but the resulting amino acid is altered and the exact functional consequence is unclear. Although no functional work was carried out to assess the kinetic properties of this mutation, this patient had a positive family history compatible with dominant transmission, and showed selective distal extensor forearm and neck muscle weakness. Of note, this patient was unable to tolerate several therapies and reported no clear benefit with any.

Overall, the site and kinetic properties (when available) of the slow channel CMS mutations did not correlate with the clinical phenotype in any of the fifteen patients assessed in this study.

This study also underlined the prolonged time interval in reaching the precise molecular diagnosis, which predictably prevented prompt and appropriate treatment in several patients. In some patients particular features such as a dominant pattern of inheritance, selective distal extensor forearm and neck muscle weakness, a clear negative response to AChE inhibitors are useful clues that could guide clinicians towards the correct diagnosis.

Choice, titration and escalation of treatment were explored in the second part of this study. It is worth noting that AChE inhibitors, usually contra indicated in slow channel CMS, were introduced as first line therapy in most patients. This highlights the importance of a detailed neurological examination to depict any preferential muscle involvement and neurophysiology at the time of presentation. In this cohort, fluoxetine was the most frequently used agent once genetic diagnosis was confirmed. Although

fluoxetine had a favourable outcome in several patients, often harbouring different mutations; its effectiveness was restricted by substantial side effects. Furthermore, fluoxetine was associated with clear improvement in one patient which was not sustained after three years. It would be helpful to prospectively monitor this cohort to ascertain whether this is a common feature with fluoxetine usage or whether this in fact relates to a more severe relapsing phenotype in that particular patient.

Fluoxetine is a long acting open channel blocker that curtails the periods of channel opening in a concentration dependent fashion (Engel, 2007, Schara and Lochmuller, 2008). This agent has been endorsed for the treatment of slow channel CMS based upon a prospective study on two patients who were heterozygotes for slow channel mutations εT264P and εL269F respectively. This open label study ran over three years, during which patients were assessed both clinically and neuro-physiologically (Harper et al., 2003). Patients were prescribed doses of fluoxetine ranging from 80 to 120mg per day and exhibited clear improvement in their neuromuscular weakness. However, in one patient escalation of his fluoxetine dose above 80mg per day was limited by side effects including sleep fragmentation and excess sedation as well as loss of appetite. An additional concern with fluoxetine, especially in children, is the development of suicidal ideation (Whittington et al., 2005). This was indeed noted in a child with slow-channel CMS who developed mood disturbance and ideas of self-harm subsequent to the introduction of fluoxetine (Kinali et al., 2008).

Quinidine was used either as first line or second line treatment in a small group of patients in this study. This medication was associated with a favourable outcome and good side effects profile with most patients tolerating this treatment, which correlated with better concordance. Quinidine is also a long acting open channel blocker recommended in slow-channel CMS. Similar to fluoxetine, it shortens the duration of channel opening events in a concentration dependent manner (Engel, 2007, Schara and Lochmuller, 2008). This treatment was shown to improve significantly both functional and electrophysiological measurements at a dose of 1-2.5μg/mL (Fukudome et al., 1998, Harper and Engel, 1998a). In this prospective open label therapeutic trial, a total of six patients were recruited and evaluated over 8 to 14 months. All patients carried known slow channel mutations: βV266M, αN217K, αG153S, αV249F, εL269F and εT264P (Harper and Engel, 1998a).

Four patients were able to tolerate treatment titration and reported clear benefit. In two patients, a hypersensitivity reaction and deranged liver tests were reported. Both complications resolved once quinidine was withdrawn. Moreover, one patient deteriorated while on treatment and necessitated ventilator support. Of note this patient exhibited borderline low respiratory function at the time of recruitment.

In conclusion, the present study provides a useful overview of the clinical phenotype and prescribed treatments of a sizeable slow channel CMS group as well as highlights potential treatment complications in clinical practice. Nonetheless, this study is limited by its retrospective observational nature, which did not permit for any standardization of assessment tools or randomization of patients to different treatment arms, including a placebo group. Accordingly, a collaborative multicentre approach would be key in recruiting a sufficient number of subjects to establish a prospective randomized controlled study or a head to head trial that would provide a better appreciation of optimum therapy for this rare CMS subtype.

Chapter 5 Clinical Characterisation of A GFPT1 Related CMS Cohort

5.1 Introduction and Aims[54]

The limb girdle CMS (LG-CMS) phenotype is a distinct clinical entity attracting much attention since the mid-1960s (McQuillen, 1966). Perhaps, this is in part due to the phenotypic overlap with limb girdle muscular dystrophies, which in contrast to CMS, are not treatable. LG-CMS is associated with marked muscle wasting and weakness of the shoulder and hip girdle muscles with minimal ocular, bulbar and facial involvement. Patients are noted to exhibit variable response to AChE inhibitors, with some showing clear benefit while other exhibiting clear deterioration in neuromuscular symptoms. In addition, several patients reported fluctuation in their symptoms over days and weeks instead of the typical diurnal variation seen in myasthenia gravis. At an ultra-structural level, abnormalities of varying extent, in the pre and postsynaptic compartment were detected in all patients (Slater et al., 2006).

The significant discovery that *DOK7* mutations are disease causing in some of these patients allowed a better clinical stratification of LG-CMS patients and the realisation that some cues, such as a negative response to AChE inhibitors, presence of ptosis and a progressive, often disabling course, were an integral part of the *DOK7* phenotype (Beeson et al., 2006, Palace et al., 2007, Muller et al., 2007a, Anderson et al., 2008). Furthermore, some distinct ultra-structural features were recognized with *DOK7* mutations (Beeson et al., 2006), namely, the presence of small motor endplates with preserved expression and localisation of crucial endplate proteins as well as intact kinetic properties of AChR. This discovery also underscored the functional importance of the Dok7 and MuSK signalling pathway in NMJ development (Beeson et al., 2006, Bergamin et al., 2010, Yamanashi et al., 2008).

[54] Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z) and Chaouch et al (URL: http://jnnp.bmj.com/content/83/3/e1.79)

More recently, *GFPT1* mutations have been shown to be another important cause of LG-CMS in a cohort of 22 patients (Senderek et al., 2011). Using genome wide linkage in a large Libyan consanguineous family, a locus was mapped on chromosome 2p13.3 and subsequent Sanger sequencing of this region revealed mutations in *GFPT1* in this family and several other kinships (figure 5.1). GFPT1 (glutamine-fructose-6-phosphate transaminase 1) is the first and rate-limiting step of the hexosamine pathway that leads to the biosynthesis of UDP-N-acetylglucosamine. The latter is an essential substrate for protein glycosylation (Haltiwanger and Lowe, 2004).

In this section, I focus on the clinical phenotype and genotype phenotype correlation of a sizable GFPT1 associated CMS cohort. My contribution was focussed on analysing the clinical data and refining the phenotype genotype correlation. I have also contributed to the draft of the manuscript (Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z) and presented the data at the Annual British Association National meeting in 2012 (Chaouch et al (URL: http://jnnp.bmj.com/content/83/3/e1.79).

5.2 Patient data[55]

A total of fifteen kinships were included in this study with several derived from the original cohort described by (Senderek et al., 2011). From the Senderek cohort, a new genetically confirmed family was incorporated into this analysis: LGM 17 and three families: LGM4, LGM15 and LGM 16 were excluded as they did not carry *GFPT1* mutations. In total, 17 males and 7 females with a mean age at report of 31 years were studied. Most patients were Caucasian referred primarily from Europe. Additional families from Iran, Libya, Senegal and Turkey were also included (figure 5.1).

[55] Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

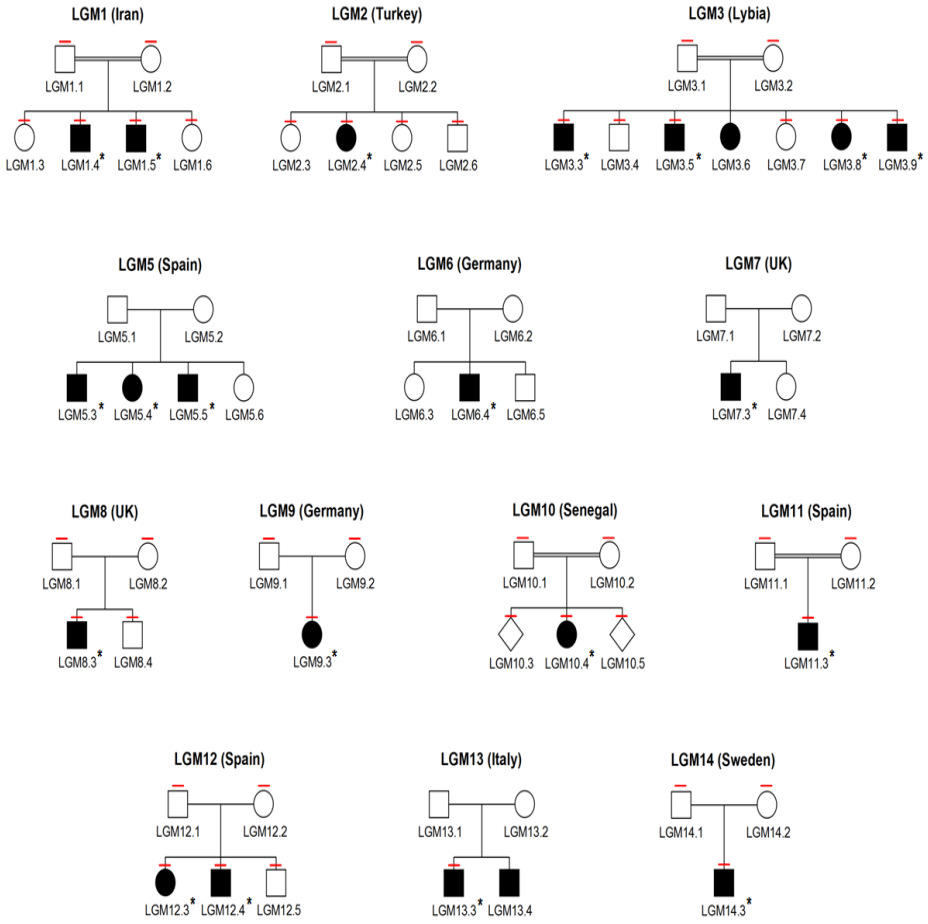


Figure 5.1 Pedigrees shown to carry *GFPT1* mutations. Please note that LGM17 is a new family with *GFPT1* mutations not reported in the original cohort. Adapted from Senderek et al 2011 (URL: http://www.sciencedirect.com/science/article/pii/S0002929711000097)

All patients reported normal motor milestones except patients LGM10.4, LGM12.3, LGM17.3 and LGM17.4 (table 5.1). 21 out of 24 patients developed neuromuscular weakness before or at age 10 years with a range 1- 40 years across the cohort. Patients invariably presented with proximal muscle weakness, reporting difficulties climbing stairs, lifting the arms above the head and lifting heavy objects. Patients LGM5.3, LGM5.4 and LGM3 displayed upper limb weakness at presentation (Sieb et al., 1996) while patient LGM7.3 and LGM8.3 reportedly had equal weakness of the shoulder and hip girdle muscles but had first noted difficulties in the hip muscle before progressing to the shoulders six years later. Five patients suffered several falls prior to presentation. Most patients reported clear fluctuation in their neuromuscular weakness except patients LGM5.3, LGM5.4, LGM7.3, LGM11.3, LGM13.3 and LGM13.4, who had a fixed pattern of weakness, reminiscent of limb girdle muscular dystrophies. Patients LGM1, LGM2.4, LGM3 family and LGM8.2 reported certain triggers to their weakness such as hot weather and infective episodes. The course of disease was slowly progressive in most, often preceded by a static phase in the first 10-20 years. Twenty-one out of the twenty-four patients remained ambulant. Neurological assessment revealed minimal ocular involvement with only one patient (LGM7.3) noted to exhibit mild bilateral ptosis. None of the patients showed ophthalmoparesis. Facial weakness was rare with only five patients being affected: LGM1.4, LGM1.5, LGM5.4, LGM7.3 and LGM8.3 as was neck flexion weakness noted in patients LGM7.3, LGM12.3, LGM12.4 and LGM5.4 (table 5.1).

None of the patients were reported to have bulbar difficulties. In the limbs, shoulder and hip girdle muscle involvement was a consistent finding in all patients. Distal muscle weakness, affecting long finger flexors, extensors as well as foot extensors muscles, was observed in the following patients: LGM1, LGM5.3, LGM5.5, LGM7.3, LGM8.3 and LGM12.3. Patient LGM7.3 suffered a more severe phenotype, losing ambulation at 4 years and exhibiting more generalised muscle weakness. He also developed respiratory insufficiency but has yet to require respiratory support.[56]

Additional features were noted in some patients: Patients LGM17.3 and LGM17.4 were both reported to exhibit intellectual disability. Patient LGM7.3 developed early strabismus and was later diagnosed with retinitis pigmentosa at the age of 5 years. Both

[56] Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

LGM1.4 and LGM1.5 were reported as visually impaired and had an established diagnosis of macular degeneration. In this cohort, no other patients were reported to have visual impairment or retinal disease, although formal retinal assessments were not routinely carried out[57].

[57] Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

Kinship/gender	Mutation	Age at onset / age at report	Symptom at onset / motor milestones	Facial / bulbar / respiratory muscle weakness / other	Limb girdle weakness / fluctuations / course	Response to AChE inhibitors
LGM1.4/m	p. D348Y	6y/31y	Muscle weakness, fatigue, worse in summer/normal	Yes/no/no/no/distal limb	Yes/yes/improving after age 20	Positive but side effects
LGM1.5/m	p. D348Y	6y/26y	Muscle weakness, fatigue, worse in summer/normal	Yes/no/no/no/distal limb	Yes/yes/improving after age 20	Positive but side effects
LGM2.4/f	p. W240X	6y/26y	Muscle weakness, fatigue and pain/normal	None	Yes/yes/worsening	Positive
LGM3.3, LGM3.5, LGM3.6, LGM3.8, LGM3.9/2m, 3f	p. R111C	6y/23-35y	Muscle weakness, fatigue/normal	None	Yes, shoulder>pelvic girdle/ND/ND	Partially positive
LGM5.3/m	p. M492T; c. *22C>A	14y/55y	Weakness in the upper limbs/normal	No/no/no/distal limb	Yes/no/slight worsening	Positive
LGM5.4/f	p. M492T; c. *22C>A	40sy/54y	Weakness in shoulder girdle/normal	No/no/no/neck muscles	Yes/no/slight worsening	
LGM5.5/m	p. M492T; c. *22C>A	10y/50y	Muscle weakness, falls/normal	No/no/no/distal limb	Yes/yes/slight worsening	Positive, +3,4 DAP
LGM6.4/m	p. D43V; p. I121T	5y/16y	Muscle weakness/normal	None	Yes/yes/slight worsening	Positive
LGM7.3/m	P.R385H; p. R434H	8y/23y	Fatigue on walk/slight delay	Yes/no/slight/slight neck and distal limb weakness	Yes, shoulder and pelvic girdle/no/worsening	Positive +3,4 DAP
LGM8.3/m	p. T15M; p. R496W	6y/37y	Repeated falls/normal	Yes/no/no/ distal limb	Yes, shoulder and pelvic girdle/yes/ improving after age 20	Positive +3,4 DAP
LGM9.3/f	p. V199F; c. *22>A	13y/26y	Fatigue/normal	None	Yes/yes/stable	Positive

Table 5.1 Clinical features of patients with *GFPT1* mutations. m; male, f; female, y; year, ND; not detected, AChE ; acetylcholinesterase. Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

Kinship/gender	Mutation	Age at onset / age at report	Symptom at onset / motor milestones	Facial / bulbar / respiratory muscle weakness / other	Limb girdle weakness / fluctuations / course	Response to ache inhibitors
LGM10.4/f	p. R512W	1y/7y	Muscle weakness, falls/normal	None	Yes/yes/worsening	Positive +3,4 DAP
LGM11.3/m	p. M491T	7y/19y	Muscle weakness/normal	None	Yes/no/stable	Positive +3,4 DAP
LGM12.3/f	c.1278_1281dup; c.*22C>A	1y/37y	Muscle weakness, falls/slight delay	No/no/no/neck and distal muscles	Yes/yes/worsening	Positive +3,4 DAP
LGM12.4/m	c.1278_1281dup; c.*22C>A	10y/39y	Muscle weakness /normal	No/no/no/neck muscles	Yes/yes/worsening	Not known
LGM13.3/m	c. T15A; c.621_622del	10y/55y	Muscle weakness/normal	None	Yes/no/ worsening	Positive
LGM13.4/m	DNA not available	7yr/36y	Muscle weakness/normal	None	Yes/no/ND	Positive
LGM14.3/m	p.222_223ins; p.R111C	10y/40y	Muscle weakness/normal	None	Yes/yes/worsening	Positive +3,4 DAP
LGM17.3/f	p. M491T; c.714_715ins	8y/9y	Frequent falls, fatigability, when, walking/delayed	Learning difficulties	Yes/yes/worsening	Positive at first, but effect lost after 3 years despite adding 3,4 DAP
LGM17.4/m	p. M491T; c.714_715ins	7y/13y	Difficulties in running, fatigability when walking/ normal	Learning difficulties	Yes/yes/worsening	Positive at first, but effect lost after 3 years despite adding 3,4 DAP

Table 5.1(cont.) Clinical features of patients with *GFPT1* mutations. Adapted from Guergueltcheva et al 2011 (URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

5.3 Ancillary investigations[58]

Before screening for any mutations in CMS genes, patients were checked for anti-AChR antibodies to exclude autoimmune myasthenia gravis. Three main investigations were reviewed in this cohort: creatine kinase (CK) levels, neurophysiological studies and muscle biopsies (table 5.3).

Kinship	CK	Decrement/muscle sampled	Myopathic EMG	Tubular aggregates on muscle biopsy
LGM1.4	X1.5	Yes/proximal	ND	Yes
LGM1.5	X1.5	Yes/deltoid	Yes	ND
LGM2.4	No	Yes/distal	Yes	Yes
LGM3.3, LGM3.5, LGM3.6, LGM3.8, LGM3.9	No	Yes/trapezius	Yes	Yes, biopsy performed in one of the siblings
LGM5.3	X1.5	Yes/deltoid	Yes	TAs
LGM5.4	No	Yes/deltoid	ND	ND
LGM5.5	X1.5	Yes/deltoid	Yes	TAs
LGM6.4	X2-8	Yes	ND	Yes, on re-revision
LGM7.3	X2	Yes/anconeus	ND	Yes
LGM8.3	X5-10	Yes	ND	Yes
LGM9.3	No	Yes/trapezius	Yes	Yes, on revision
LGM10.4	No	Yes/trapezius	Yes	Suggestive
LGM11.3	No	No/distal	Yes	One inclusion
LGM12.3	No	Yes/proximal	Yes	ND
LGM12.4	No	Yes/deltoid	Yes	Yes
LGM13.3	Minimal	Yes/trapezius	Yes	Yes
LGM13.4	Minimal	Yes/proximal	Yes	Yes
LGM14.3	X12	Yes/proximal and distal	Yes	Yes
LGM17.3	Minimal	No/distal	ND/ND	No
LGM17.4	Minimal	ND	ND/ND	No

Table 5.3: Results of ancillary investigations in GFPT1 CMS cohort. ck; creatine kinase, ND; not done, EMG; electromyography, TA; tubular aggregates. Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z).

[58] Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

Creatine kinase levels were available in 12 patients and ranged from normal to 12 times the upper limit of normal. Indeed, three patients, LGM6.4, LGM8.3 and LGM14.3, were reported to have levels between 8 and 12 times the normal range.

Neurophysiological reports were available in 19 patients. None had evidence of a large fibre neuropathy on nerve conduction studies. EMG reports were accessible in 13 patients. These revealed mild myopathic features with no spontaneous activity. Repetitive nerve stimulation reports were available in 19 patients. These showed decrement in all patients when the proximal muscles were sampled. In patients LGM1.4, LGM1.5, LGM5.4, LGM10.4, LGM11.3 and LGM17.3, no decrement was detected in the distal muscles. On the other hand, abnormal jitter (when SFEMG was done) was observed in all patients. One patient showed double CMAP on single nerve stimulation, typically seen in *COLQ* and slow channel CMS. This finding was detected on two separate occasions and reproducible on and off AChE inhibitors (table 5.3).

A total of 18 muscle biopsies were available. These were from, *biceps brachii*, *vastus lateralis*, *biceps brachii*, *deltoid*, and *quadriceps* muscles. The site of the muscle biopsies was not documented in three patients. Most muscle biopsies showed non-specific or mild myopathic changes. Three patients showed type 1 fibre predominance. The muscle biopsy findings (NADH and EM appearances) of LGM3 and 4 families were previously published (Rodolico et al., 2002, Sieb et al., 1996). Tubular aggregates (TAs) were confirmed in 13 biopsies (figure 5.2, table 5.3 and 5.4). These were visualized using NADH staining in 7 patients and EM in 5 patients. In patients LGM5.3, LGM5.5 and LGM12.4, several small subsarcolemmal aggregates were noted on NADH staining but were subsequently confirmed to be tubular aggregates. These would have been overlooked should a second evaluation with EM have not been carried out. In two patients, NADH staining was inconclusive and no further EM study was performed. Morphological review of the motor endplates was available in only one patient and showed simplified postsynaptic membranes with seemingly preserved presynaptic architecture.

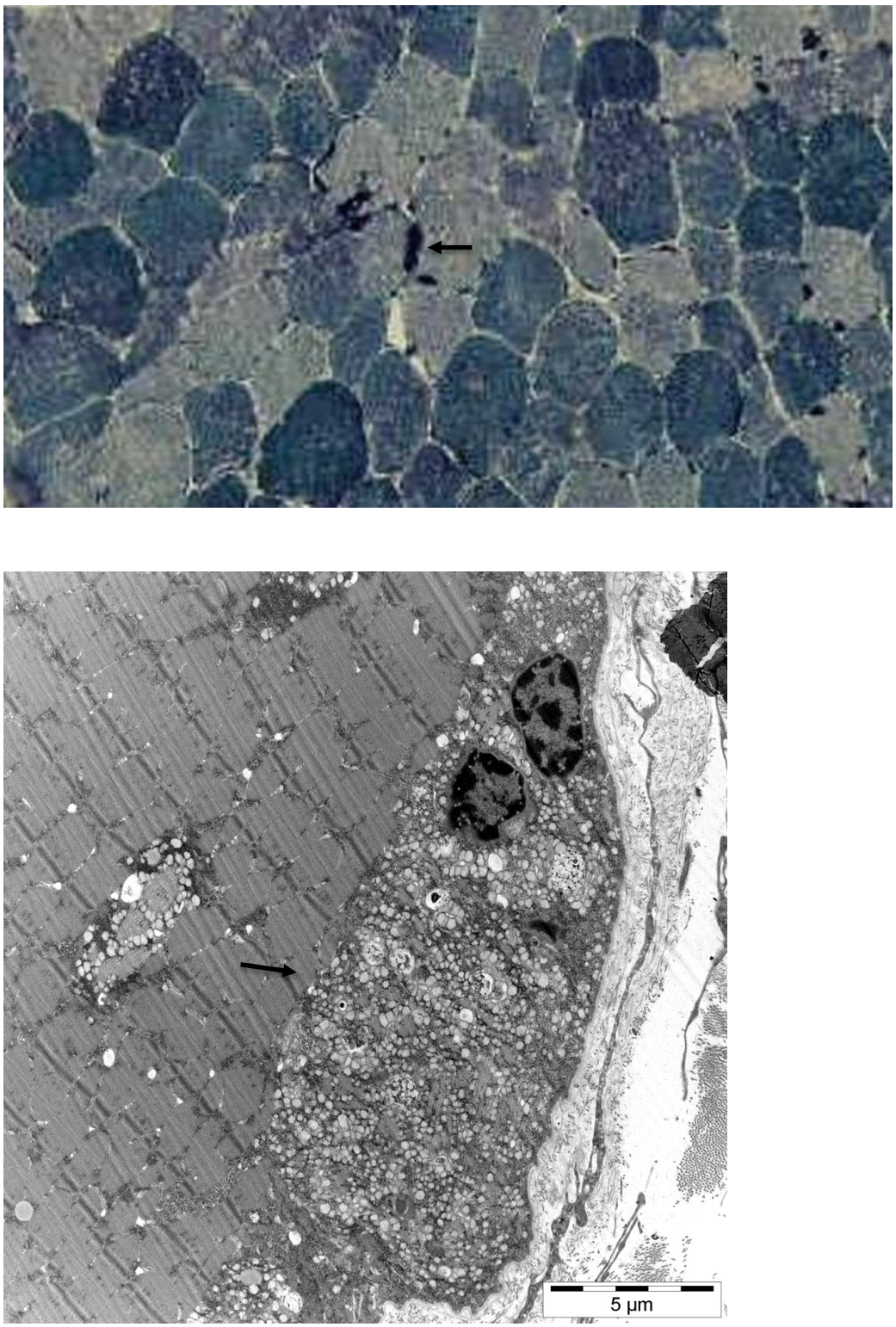


Figure 5.2: Example of tubular aggregates. (arrow). Top image: NADH staining of tubular aggregates in muscle biopsies of *GFPT1* patients. Bottom image: Electron microscopy appearance of tubular aggregates beneath the sarcolemma. Reproduced from Chaouch and Lochmuller 2013, 86 -94, © John Wiley & Sons, Ltd

Patient	Muscle sampled	NADH and EM findings	Other findings
LGM1.4	Biceps brachii	Yes, NADH staining	Fibre size variation, type 1 fibre predominance, round or angular fibres
LGM2.4	Vastus lateralis	Yes, TAs exclusively in type 2 fibres	Chronic myopathic changes
LGM3	Biceps brachii	Yes, small subsarcolemmal aggregates (NADH), TAs in EM	-
LGM5.3	Deltoid	Yes, subsarcolemmal aggregates (NADH), TAs in EM	Mild myopathic changes, type 1 fibre predominance and ragged red-like fibres
LGM5.5	Deltoid	Yes, subsarcolemmal aggregates (NADH), TAs in EM	Unspecific myopathic changes, type 1 fibre predominance, ragged red-like fibres
LGM6.4	Unknown	Yes, NADH staining	Unspecific myopathic changes
LGM7.3	Unknown	Yes	Muscle atrophy, multiple internal nuclei, vacuoles, features consistent with denervation
LGM8.3	Unknown	Yes	-
LGM9.3	Biceps brachii	Yes	-
LGM10.4	Deltoid	Unclear suggestive of TAs (NADH)	Uneven oxidative staining, accumulation of mitochondria
LGM11.3	Deltoid	Probably no, one fibre with a possible inclusion (NADH)	-
LGM12.3	Deltoid	NADH and EM not done	Unspecific myopathic changes
LGM12.4	Biceps brachii	Yes, enhancement and subsarcolemmal aggregates (NADH), tas in EM	Unspecific myopathic changes
LGM13.3	Vastus lateralis	TAs in EM	-
LGM13.4	Vastus lateralis	Yes	-
LGM14.3	Deltoid	Yes, frequent fibres with TAs, TAs in EM	Increased fibre size variability. Frequent fibres with internalized nuclei and autophagic vacuoles
LGM17.3	Quadriceps	No	Fibre size variability, oxidative enzyme staining showed type 2 predominance with occasional core-like areas and subtle uneven staining
LGM17.4	Quadriceps	No	Fibre size variability, oxidative enzyme staining was pale and inconclusive

Table 5.3. Muscle biopsy findings in GFPT1 cohort. TAs; tubular aggregates. Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

5.4 Treatment response[59]

All patients received a trial of AChE inhibitors except patients LGM5.4 and LGM12. (table 5.1). AChE inhibitors were used as monotherapy in thirteen patients and in combination with 3,4-DAP in nine patients: LGM 5.5, LGM7.3, LGM8.3, LGM10.4, LGM11.3, LGM12.3, LGM14.3, LGM17.3 and LGM17.4. The introduction of 3,4-DAP was helpful in all patients except two (LGM17.3 and LGM17.4). Treatment was beneficial in 20 out of the 22 treated patients. Patients received a dose of AChE inhibitors ranging from 80 to 540 mg. In patients LGM1.4 and LGM1.5, dose titration was limited by side effects. Indeed, although AChE inhibitors led to significant improvement at a dosage of 2.8mg/kg/day in the older brother and 2.4mg/kg/day in the younger brother, treatment was withdrawn due to increased muscle twitching as well as mood disturbance. Patients LGM17.3 and LGM17.4 showed a clear but transient benefit from AChE inhibitors with evidence of relapse after three years (table 5.1).

5.5 Discussion[59]

This clinical study was primarily based on the cohort first described in the seminal paper (Senderek et al., 2011), reporting novel mutations in *GFPT1* in a sizeable cohort of patients with a LG-CMS phenotype. Three case series have since described additional patients with both known and novel *GFPT1* mutations (Huh et al., 2012, Selcen et al., 2013, Maselli et al., 2013). Furthermore, three other glycosylation genes have been implicated with a similar LG-CMS phenotype: *DPAGT1*, *ALG2* and *ALG14* (Belaya et al., 2012, Cossins et al., 2013).

For this study, a total of 15 independent kinships were recruited from different geographical origins including Europe, North Africa and Sub-Saharan Africa, Asia and the Middle East with no particular clustering or founder effect reported.

I was interested to note the relatively early age at onset of most patients, presenting in their first decade of life, but also the delay in diagnosing CMS in such patients, with a mean time to diagnosis of 21.5 years. This clearly may have implications in terms of

[59] Adapted from Guergueltcheva et al 2011(URL: http://link.springer.com/article/10.1007%2Fs00415-011-6262-z)

instigating early therapy and potentially reducing long-term disability. Fluctuation in neuromuscular weakness was present in three quarters of the patients and the course of disease was relatively mild with most patients retaining ambulation 20 years after presentation. In this cohort only one patient was reported to have lost ambulation at the age of 4, and went on to develop early respiratory insufficiency.

In this study, all patients exhibited a distinctive and relatively homogenous phenotype, with a predominant proximal pattern of muscle weakness with minimal ocular involvement, a positive response to AChE inhibitors and evidence of tubular aggregates on muscle biopsy. This constellation of clinical and histological features has been a consistent finding in other newly reported GFPT1 patients (Huh et al., 2012, Selcen et al., 2013, Maselli et al., 2013). These features were also key in the identification of 12 mutation positive patients out of 39 screened in the Munich cohort, corresponding to one of the highest mutation detection rate (31%) in the Munich database. Interestingly, sequencing of another cohort of 52 undiagnosed CMS patients, with variable phenotype and no tubular aggregates, did not yield any mutation positive patients, which reinforces the hypothesis that there is a strong genotype phenotype correlation with *GFPT1* mutations. I was interested to note evidence of retinitis pigmentosa in LGM1and macular degeneration in LGM7.3. Since no additional GFPT1 patients have been reported with these clinical features, it seems reasonable to speculate that such findings are either incidental or a very rare association with *GFPT1* mutations.

In terms of ancillary tests, the levels of creatine kinase were moderately elevated in a few patients (LGM 6.4, LGM 8.3 and LGM 14.3). However these did not correlate with the extent and severity of muscle disease nor did they correspond to evidence of significant myopathic or dystrophic changes on muscle biopsy. Nonetheless, a myopathic EMG was reported in more than half of our cohort which can certainly mislead clinicians to consider a primary myopathy as a diagnosis; as was the case in several of our patients initially diagnosed with limb girdle muscular dystrophy.
The presence of decrement was key in ascertaining that patients had a neuromuscular junction disorder. In this study, decrement was always present when proximal muscles were assessed. However, repetitive nerve stimulation of the distal muscles showed no decrement in a quarter of all patients. By contrast, abnormal jitter was detected in all patients tested. This highlights the importance of extensive sampling, of both proximal

and distal muscles and SFEMG in confirming any defect related to the neuromuscular junction.

Furthermore, a clear genotype histo-pathological correlation was noted in most patients since all muscle biopsies, except two, contained tubular aggregates either on NADPH or EM analysis. The two patients with no TAs on muscle biopsy were some of the youngest in this cohort and may therefore develop these tubular structures later in life since TAs form in an age dependent fashion in inbred male mice (Agbulut et al., 2000). *GFPT1* mutations seem to be directly linked to defects in NMJ function, but the formation of tubular aggregates is not predictive of NMJ dysfunction since screening of patients with muscular weakness and tubular aggregates without evidence of impaired NMJ transmission did not detect mutations in *GFPT1*.
Indeed, tubular aggregates are a nonspecific histo-pathological finding associated with a variety of muscle conditions including alcohol and drug induced myopathies, inherited myopathies, inflammatory myositis, periodic paralysis, dyskalaemia, myotonia congenita (Funk et al., 2013, Engel et al., 1970, Pavlovicova et al., 2003).

TAs have previously been shown to represent re-organization of the sarcoplasmic reticulum and manifest as densely packed tubular membrane structures of variable shape and size (Pavlovicova et al., 2003, Chevessier et al., 2005). A number of proteins have been consistently present in TAs, including SAR-1 thought to reflect the involvement of the endoplasmic reticulum in the formation of these tubular structures and proteins participating in calcium homeostasis (Chevessier et al., 2005). The recent discovery of mutations in the main Ca2+ sensor on the endoplasmic reticulum (Stromal interaction molecule 1 (STIM1)), in association with a dominant congenital myopathy with tubular aggregates (Bohm et al., 2013), seems to reinforce the idea that both the ER and calcium may be important players in TAs formation.

Another important cause of LG-CMS includes mutations in *DOK7* (table 5.5), which accounted for 10% of all mutations in the Munich database (see chapter 3). These mutations were shown to account for half of all patients exhibiting an LG-CMS phenotype (Palace et al., 2007). Other rare causes of proximal fatigable limb weakness include *RAPSN* and *COLQ* mutations, which accounted for 15% and 12% of all mutations in the Munich database and should be considered in the differential diagnosis (Mihaylova et al., 2008, Muller et al., 2003).

Several reports have highlighted a number of distinct characteristics that can allow physicians to distinguish the DOK7 and glycosylation defect linked CMS (Beeson et al., 2006, Muller et al., 2007a, Palace et al., 2007, Selcen et al., 2008, Huh et al., 2012, Selcen et al., 2013, Cossins et al., 2013, Monies et al., 2014, Maselli et al., 2013, Guergueltcheva et al., 2011). Indeed, *DOK7* mutation positive patients were shown to present earlier, usually in the first 2 years, and often have clear ptosis with facial and bulbar weakness. In addition, DOK7 associated CMS seems to result in a more aggressive disease course with several patients reported to lose ambulation and require ventilator support over time (Eymard et al., 2013). The negative response to AChE inhibitors is significant and can be utilized as an objective tool to distinguish DOK7 from GFPT1 linked CMS. The former is usually treated with ephedrine and salbutamol (Schara et al., 2009, Lashley et al., 2010). Lastly, tubular aggregates have never been reported in association with *DOK 7*, *RAPSN* or *COLQ* mutations and should raise the clinical suspicion of glycosylation defects.

	GFPT1	**DOK7**
Onset	1st decade (birth – forties)	2nd year of life (birth – late 20s)
Ocular involvement	No	Ptosis with no ophthalmoparesis
Facial muscles	Rarely affected	Affected
Bulbar muscles	Not affected	Affected
Respiratory problems	No	Can be affected
Course of disease	Progressive in half but all retained ambulation	Progressive with loss of ambulation in some
CK	Can be elevated	Can be elevated
Tubular aggregates	Present	None
Endplate pathology	Unspecific changes	Small simplified endplates
Treatment	Pyridostigmine	Ephedrine, salbutamol

Table 5.4: Comparison of GFPT1 CMS with DOK7 CMS phenotype. CK; creatine kinase. Reproduced from Chaouch and Lochmuller 2013, 86 -94, © John Wiley & Sons, Ltd.

Since the publication of our report, Selcen et al., 2013 described 11 patients with LG-CMS with *GFPT1* mutations and provided valuable details in terms of the homogenous

clinical phenotype, the ultra-structural appearance at the motor endplates and kinetic abnormalities. Remarkably, one patient was heterozygous for a null allele and a splice site mutation disrupting the muscle specific exon. This patient exhibited the most severe and disabling phenotype described so far in association with *GFPT1* mutations. In addition to reduced foetal movements in the antenatal period, the patient was arthrogrypotic and suffered apnoeic episodes soon after birth, with marked bulbar and limb weakness. The patient was non-ambulant and showed little response to AChE inhibitors. In this particular patient, glycoprotein expression was absent in muscle tissue.

In addition to tubular aggregates, rimmed vacuoles in three out of nine muscle biopsies were observed. At an ultra-structural level, abnormalities within the muscle fibres were also observed including apoptotic nuclei and autophagic vacuoles. The motor endplates were often poorly developed with synaptic contact of small and varying morphology ranging from single to grape-like appearance. The density of AChR was comparable to controls in all but one study. Patch clamp analysis showed a presynaptic defect in some, with reduced quantal release, and postsynaptic changes in others with impaired response to acetylcholine. This study underscored the significant ultra-structural variability at the NMJ in spite of a clinically homogeneous phenotype.

GFPT1 is ubiquitously expressed and mediates the biosynthesis of N-acetylglucosamine (UDP-GlcNAc), which is a substrate for N-linked and O-linked protein glycosylation (Wellen and Thompson, 2012, Haeuptle and Hennet, 2009). Morphological studies of the motor endplate of patient LGM14.3 showed non-specific abnormalities and the ultra-structural data were unable to define the primary defect underlying impaired neuromuscular transmission. Mutations in *GFPT1* have since been shown to affect neuromuscular junction transmission by reducing cell surface muscle AChRs at motor endplates (Zoltowska et al., 2013).

Importantly, three glycosylation genes, *DPAGT1*, *ALG2* and *ALG14*, have since been identified in patients with LG-CMS with minimal ocular and facial involvement and a beneficial response to AChE inhibitors (Belaya et al., 2012, Selcen et al., 2014, Cossins et al., 2013).

All *DPAGT1* patients had LG-CMS with tubular aggregates, abnormal jitter with block, and a good response to AChE inhibitors. The latter was sometimes prescribed in

conjunction with 3,4-DAP and salbutamol. Ultra structural studies revealed reduced postsynaptic folding and AChR density. Pharmacological inhibition of DPAGT1 was shown to reduce cell- surface AChR expression *in vitro* (Belaya et al., 2012, Finlayson et al., 2013).

ALG14 mutations were described in an affected sib pair presenting in infancy with predominant proximal weakness, contractures, evidence of abnormal jitter and block and a good response to AChE inhibitors. No Tubular aggregates were detected (Cossins et al., 2013).

ALG2 mutations were reported in 2 consanguineous kinships of Saudi Arabian and Italian descent respectively (Cossins et al., 2013). Both had an LG-CMS phenotype with abnormal jitter, minimal facial, bulbar and ocular weakness and evidence of tubular aggregates in one family. Remarkably in the Saudi family, patients were also noted to have proximal contractures with distal laxity, reminiscent of an extra cellular matrix disorder (Bushby et al., 2014). They were non ambulant and had learning difficulties. The patient's biopsy showed myopathic changes but no tubular aggregates. ALG2 expression levels in patients' muscle were significantly reduced compared to controls and knockdown of ALG14 expression impaired AChR expression on the cell surface (Cossins et al., 2013).

There are several reports in the literature that have described patients with LG-CMS with no tubular aggregates (Palace et al., 2007, Rodolico et al., 2002, Chevessier et al., 2005, Slater et al., 2006, Azulay et al., 1994, Dobkin and Verity, 1978, Furui et al., 1997, Zephir et al., 2001). It seems reasonable to consider screening these patients for *DPAG1*, *ALG2*, *ALG14* and *GFPT1*as absence of TAs does not exclude an underlying glycosylation defect.

In conclusion, defects in *GFPT1* and *N*-linked glycosylation genes have reinforced the paradigm in which ubiquitous proteins can selectively alter the structure and function of the neuromuscular junction. Despite the relatively homogeneous clinical phenotype, we noted striking ultra-structural variability of motor endplates, encompassing both the pre and postsynaptic compartments. *In vitro* work so far has shown the important role of impaired AChR glycosylation in causing NMJ dysfunction. Nevertheless, the preserved postsynaptic AChR density in several patients suggests that other mechanisms are at

play and require further study. Furthermore, evidence of intellectual disability and retinal disease in occasional patients may suggest that a deeper phenotyping of patients is required to look for other organ involvement which may be subclinical. Glycosylation is a crucial posttranslational modification step that dictates the structure; location, stability and function of proteins and one may speculate that several aspects of the NMJ may be deranged at any one particular time and patient.

Chapter 6. Gene Discovery Using Exome Sequencing

6.1 Introduction and aims

Gene identification is an essential step to the understanding of disease biology, which could point towards potential drug targets, ultimately resulting in better care for patients. Conventional gene discovery has relied on either candidate gene approaches, which require a comprehensive understanding of the biology of the disease or positional cloning, based on linkage analysis that defines a candidate region likely to include the disease gene. The main caveat to this approach has been the need for large families to increase statistical power, and failure of linkage studies to cope with phenocopies and incomplete penetrance.

Most CMS genes have been identified using position independent strategies, which requires a deep understanding of the disease pathophysiology and biology (Sine et al., 1995, Engel et al., 1996a). Only two genes (*GFPT1* and *CHRNE*) were discovered by linkage studies in large consanguineous families (Senderek et al., 2011, Ohno et al., 1998b). More recently exome sequencing has allowed four additional disease causing genes to be unravelled (figure 6.1) (Belaya et al., 2012, Cossins et al., 2013).

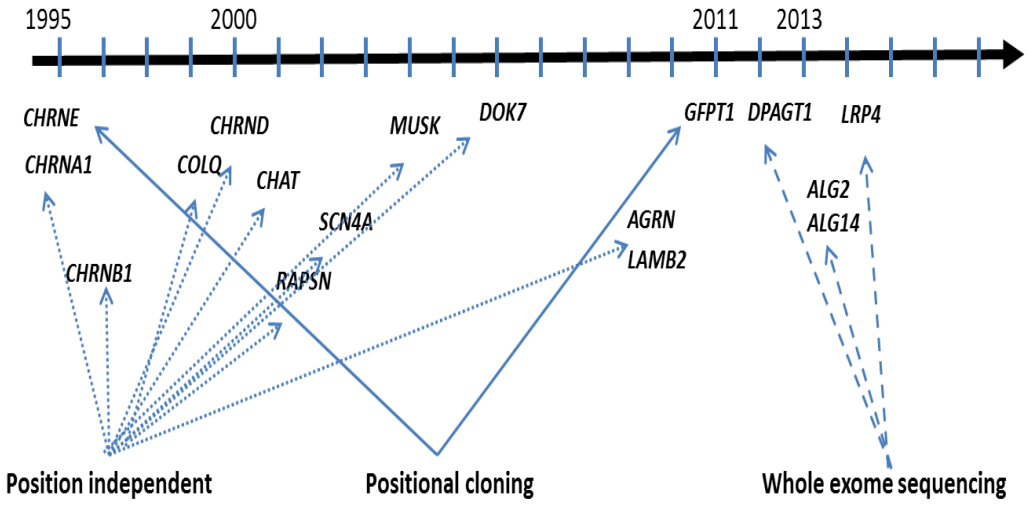


Figure 6.1. Strategies applied for CMS gene discovery.

Until recently, only half of all inherited Mendelian disorders were associated with a gene defect and in particular, with mutations found in the coding regions of the genome (exome) (Chen et al., 2010). Thus, whole exome sequencing has been proposed as an alternative screening strategy for disease gene identification; with clear advantages over conventional sequencing, being able to sequence all genes in one run and only requiring a few patients.

Ng and colleagues were able to show in a proof of concept study that whole exome sequencing combined with a filtering methodology can accurately determine the molecular basis of a known disease gene (Ng et al., 2009). This approach relied on targeted exome capture with oligonucleotide probes. Target sequences were amplified in a massively parallel fashion and unwanted sequences were discarded (Mamanova et al., 2010). Predictably, a colossal number of sequencing data were generated through this process, which proved to be an inherent challenge to this technique. The same authors were first to describe a stepwise filtering approach to analyse sequencing variants whereby genes containing non synonymous changes, splice site mutations or coding indels (small nucleotide deletions and insertions) were prioritized, while common variants that were presumed to be non-pathogenic were excluded. Variants predicted to be non-deleterious were also removed during this process (Kuhlenbaumer et al., 2011).

Depending on the number of individuals available for exome screening, different approaches have been applied by different groups to isolate disease-causing variants in a candidate gene. Although possible, disease gene discovery based on a single affected individual remains very difficult (Pierce et al., 2010). Alternatively, the "intersecting" filtering method aiming at detecting mutations shared between several affected individuals has been more effective. Indeed, Ng and colleagues applied this strategy to identify *DHODH* and *MLL2* as the disease causing genes in Miller and Kabuki syndromes respectively (Ng et al., 2010a, Ng et al., 2010b).

Another important group of patients where a linkage approach has not been possible includes sporadic cases in whom *de novo* mutations are presumed to be the culprit. Recently, exome sequencing on several "trios" composed of both parents and an affected child successfully isolated a convincing candidate gene (Vissers et al., 2010).

Lastly, family based filtering strategies have been shown to be successful in disease gene identification. These focus on shared homozygous or heterozygous genomic regions between related individuals. This approach has also been combined with linkage or homozygosity mapping in consanguineous families with positive results (Vermeer et al., 2010, Rehman et al., 2010, Wang et al., 2010a).

In this chapter, the clinical details of a cohort of undiagnosed CMS patients selected for exome sequencing are reviewed along with the results of prioritized variants identified through exome sequencing. I searched the Munich and Newcastle CMS cohorts for suitable patients for novel gene discovery and ensured that known CMS genes corresponding to the phenotype were excluded. I designed a strategy to analyse the data and carried out all the sequencing of candidate variants, segregation analysis and screening of variants in undiagnosed patients. I also identified independent kinships that were later shown to share the same genotype. The bioinformatics work was carried out by Dr Helen Griffin and Dr Steven Laval at the Institute of Genetic medicine, Newcastle University.

6.2 Patient selection

The first step for patient selection was to focus on families in whom more than one individual was affected. The rationale for this was to limit data analysis to chromosomal regions shared between two or more siblings. In consanguineous families, the focus was on shared homozygous segments.

Both the Munich and Newcastle CMS cohorts were searched for suitable patients. In the Munich undiagnosed cohort, seventy-two kinships were reported to have more than one affected individual and twenty-five of these were consanguineous. In Newcastle, one consanguineous family with two affected siblings was captured out of a cohort of fourteen kinships. An important criterion in this selection process was access to both clinical and genetic data of patients.

The next step was to evaluate the robustness of the clinical diagnosis of congenital myasthenic syndrome in this subset of patients, based on the reported clinical phenotype and in conjunction with the results of their ancillary investigations. Certain clinical

features such as neonatal onset disease, ocular involvement, apnoeic crises in the neonatal period, a response to AChE inhibitors and evidence of decrement were sought. Table 6.1 shows the clinical details of the six sibpairs selected for exome sequencing.

Patient	Age at onset (yrs)	Motor delay	Pattern of muscle involvement						Other features	Fatigability	Decrement	CK	Muscle biopsy	Positive response to AChE inhibitors
			Ocular	Bulbar	Limbs			Respi-ratory						
					Global weak	Prox > Dist	Dist > Prox							
CMS-OV1	Birth	+	Ptosis	+	+	+	-	+	Pseudoesterase deficiency	+	NA	NA	NA	+
CMS-OV2	Birth	-	Ptosis	+	+	+	-	-	High arched palate	+	-	N	N	+
CMS-MA1	Birth	-	Ptosis	+	+	-	-	-		Unclear	+	N	NA	-
CMS-MA2	Birth	-	Ptosis	+	+	-	-	-		Unclear	+	N	NA	-
CMS-GH1	1	+	-	+	+	-	-	+	Hypercalciuria	-	NA	N	NA	+
CMS-GH2	Birth	+	-	+	+	-	-	-	Hypercalciuria and premature puberty, scoliosis	-	+	N	NA	+

Table 6.1. Clinical details of the six families selected for exome sequencing. Yrs; years, Prox; proximal, Dist; distal, NA; not available, N; normal, CK; creatine kinase, - absent, +; present.

Patient	Age at onset years	Motor delay	Pattern of muscle involvement						Other features	Fatigability	Decrement	CK	Muscle biopsy	Positive response to AChE inhibitors
			Ocular	Bulbar	Limbs			Respi-ratory						
					Global weak	Prox > Dist	Dist > Prox							
CMS-NO1	1	+	Ptosis	+	+	-	-	-	Obsessive compulsive disorder, autonomic dysfunction	+	No, increased jitter	N	Possible transmitter release defect on patch clamp	-, (on 3,4-DAP)
CMS-NO2	1	+	Diplopia	-	+	-	-	+	Moderate Learning difficulties	+	NA	N	NA	Equivocal
CMS-SC1	3-6	+	Ptosis	+	+	-	-	-	Muscle atrophy	-	-	↑	Neurogenic, normal staining of AChE	+
CMS-SC 2	3-6	+	Ptosis	+	+	-	-	-	Muscle atrophy	-	-	↑	Neurogenic, normal staining of AChE	+
CMS-EN1	14-15	-	-	-	+	-	+	-	Distal limb atrophy	-	Yes with facilitation	N	Myopathic	NA
CMS-EN2	17	-	-	-	+	-	+	-	Distal limb atrophy	-	Yes with facilitation	N	Myopathic	NA

Table 6.1. (cont.).

The final step was to ensure that most known CMS genes were excluded either via direct sequencing or linkage based on the reports available in the Munich diagnostic centre, Dresden and Oxford. Table 6.2 provides details of the genes excluded in individual kinship. Three of the families were noted to be consanguineous.

By process of elimination, a total of 12 affected individuals from 6 unrelated kinships were deemed suitable for exome sequencing. Of these, one consanguineous family was from Newcastle (CMS-NO) and 5 from the Munich CMS database (figure 6.2)

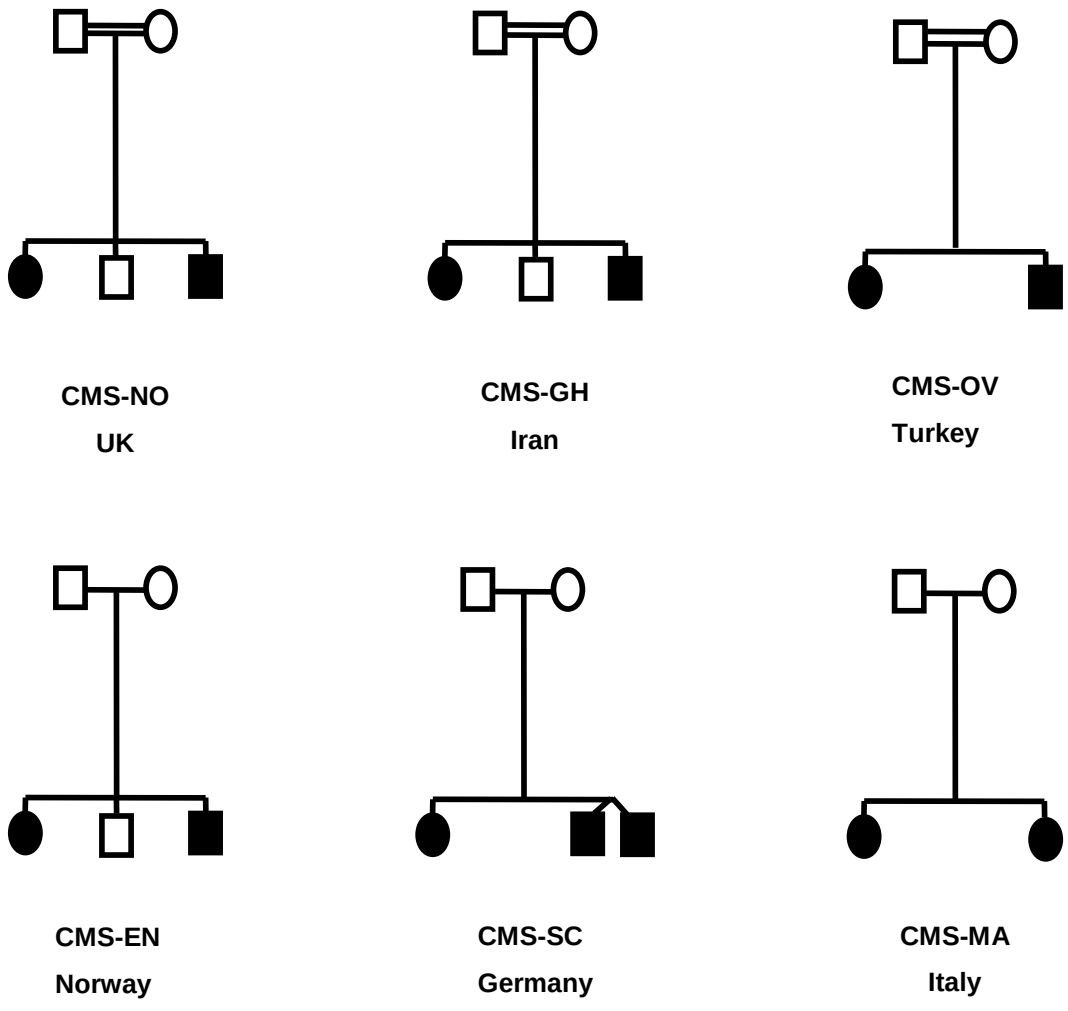


Figure 6.2. Pedigrees of the six undiagnosed families selected for exome sequencing, Details of the patients' geographical origin also included

Kinship	Ethnicity	Consanguinity	Presumed inheritance	Genes excluded		Other features
				Direct sequencing	**Linkage**	
CMS-NO	United Kingdom	+ (1st cousins)	Recessive	*CHRNA1, CHRNB1, CHRND, CHRNE, RAPSN, CHAT, DOK7, COLQ, MD1*	*COLQ, CHRNA1, CHRNB1, CHRNE, CHAT, RAPSN, MUSK, SCN4A, CNTN1, DOK7, GFPT1*	CMS-NO2 has microdeletion on X chromosome
CMS-OV	Turkey	+ (1st cousins)	Recessive	*CHAT, COLQ, CHRNE, CHRNA1, CHRNB1, CHRND, DOK7, RAPSN*	*CHRND, CHRNE, SCN4A, MUSK, CNTN1, AGRN, GFPT1*	Paternal grandparents also first cousins and father may be symptomatic (ptosis and decrement but no limb weakness). Father also has pseudoesterase deficiency
CMS-EN	Norway	No	Recessive	*CHRNE, CHRNB1, COLQ, RAPSN, DOK7, CNTN1*	All excluded except *CHRNE, CHRNB1* and *COLQ*	
CMS-SC	Germany	No	Recessive but in database father affected!	*CHRNE, CHRNA1, CHRNB1, CHRND, DOK7*	*COLQ, CHAT, RAPSN, SCN4A, DOK7, GFPT1*	3 affected siblings (monozygotic twins)
CMS-GH	Iran	+ (2^{nd} cousins)	Recessive	*CHAT a*nd *COLQ*	*ACHE, CHRNA1, CHRNB1, CHRND, CHRNE, RAPSN, MUSK, DOK7 CNTN1, SCN4A, AGRN, GFPT1*	
CMS-MA	Italy	No	Recessive	*CHRNE, COLQ, DOK7, MUSK*	*ACHE, COLQ, CHRNA1, CHRNB1, CHRND, CHRNE, CHAT, RAPSN, CNTN1*	

Table 6.2. CMS genes excluded by Sanger sequencing or linkage in the undiagnosed CMS cohort selected for exome sequencing.

6.3 Patient data

Patients were of diverse ethnic origins including the UK, Germany, Norway, Italy, Turkey and Iran. As often seen with rare genetic disorders, the pedigrees were relatively small with only two affected offspring. In CMS-SC, three offspring were reported to be affected but two of these were identical twins. Only one of the twins was selected for exome sequencing.

Age of onset of disease ranged from birth to 17 years and motor development was delayed in four patients (table 6.1). In CMS-NO, the affected sister exhibited moderate intellectual disability. Patients presented with global weakness, sometimes associated with fatigability. CMS-EN was noted for selective distal muscle atrophy and weakness in both upper and lower limbs. Ptosis and bulbar difficulties were common clinical features in all individuals. The twelve selected patients were ambulant and none required ventilator support. Patients either had evidence of decrement on repetitive nerve stimulation or a positive response to AChE inhibitors in keeping with impaired neuromuscular transmission. Creatine kinase levels were within the normal range in all families except CMS-SC. Muscle biopsy reports were available in three of the families and showed unspecific changes in all with no tubular aggregates. In addition to exhibiting myasthenic symptoms, some families were noted to have other medical conditions. CMS-OV had a diagnosis of pseudoesterase deficiency typically associated with an abnormal reaction to anaesthetic agents without evidence of neuromuscular weakness. In CMS-GH, there was a history of hypercalciuria.

In this cohort, Sanger sequencing was used to screen for mutations in known CMS gens that could not be excluded by haplotype analysis. It is worth noting that particular genes like *AGRN*, *LAMB2* and *SCNA4* were not routinely sequenced. Furthermore, haplotype analysis included microsatellite loci for *AGRN* after 2009 and *GFPT1* after 2011. Therefore the extent of CMS gene screening varied between patients and expanded with the discovery of new CMS disease causing genes.

In CMS-NO, CGH array was carried out given the presence of moderate learning difficulties in the affected sister. This revealed a large hemizygous deletion in the X

chromosome. More details on this are discussed in chapter 8. In CMS-OV family, consanguinity was reported in two generations (paternal grandparents related) and the father himself may have been symptomatic with both pseudoesterase deficiency and fatigable ptosis. It was subsequently confirmed that the father had evidence of decrement in keeping with NMJ disorder.

6.4 Exome sequencing data

The coverage of the coding regions using BWA aligner was comparable across all 12 individuals with most bases being covered at least 1-fold. In this study, the filter was set at 5-fold coverage for homozygous variants and 10-fold for heterozygous variants (figure 6.3).

6.4.1. Coverage of exome data

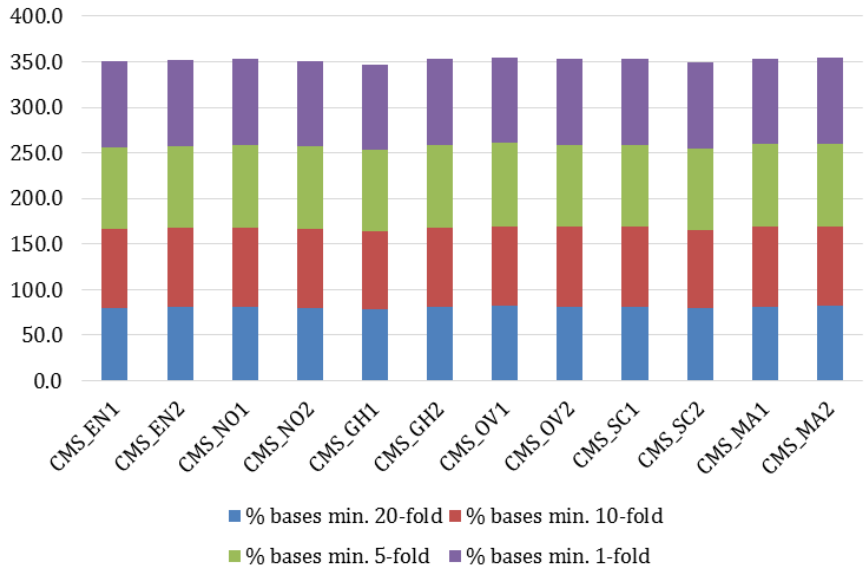


Figure 6.3. Coverage of consensus coding sequence (CCDS) bases across different individuals.

6.4.2 Coverage of known CMS genes

Although patients had already been screen for known CMS genes, it was important to ensure that these were also excluded by exome sequencing. In the first instance, the coverage of CMS genes was assessed. Inconsistent coverage was noted in some of the CMS genes, notably *AGRN* and *CHRNE* (figure 6.4 A and B). In *AGRN*, only one patient out of the cohort of 12 individuals had partial sequencing of exon 1.

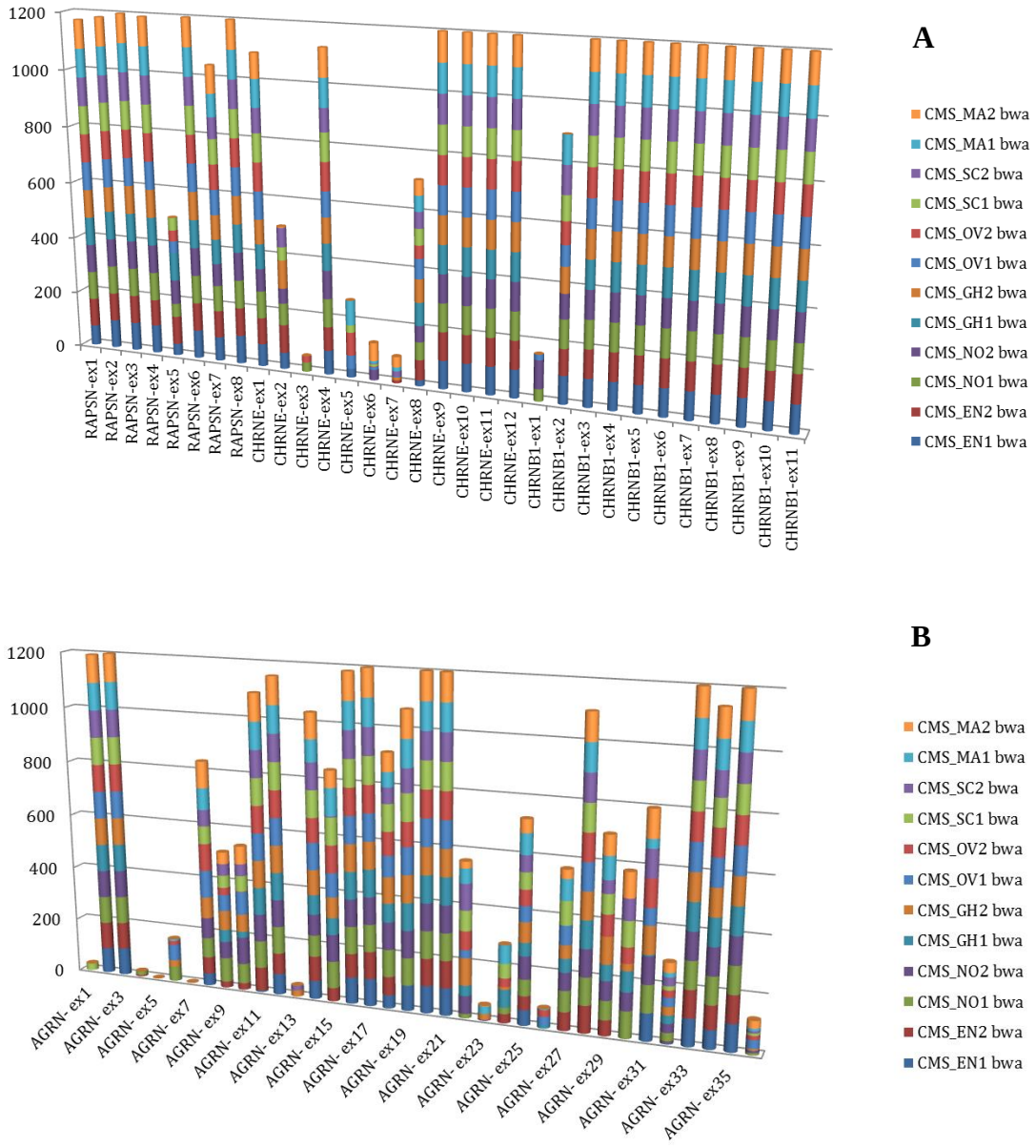


Figure 6.4. Coverage of *AGRN*, *RAPSN*, *CHRNB1* and *CHRNE* genes.

6.4.3 Sensitivity and specificity of called variants

The sensitivity and specificity of the SNP predictions were evaluated based on a panel of SNPs with HapMap frequencies (table 6.3). Sensitivity was variable across different families and between siblings. The specificity of SNP predictions appeared to be more consistent across the group.

Patient	Sequence Aligner	Estimated Sensitivity[+]	Estimated Specificity[+]
CMS_EN1	BWA	0.692302	0.998699
CMS_EN2		0.695829	0.999262
CMS_NO1		0.695191	0.998673
CMS_NO2		0.700991	0.998695
CMS_GH1		0.673925	0.997389
CMS_GH2		0.694720	0.997655
CMS_OV1		0.670149	0.997918
CMS_OV2		0.672444	0.997803
CMS_SC1		0.709786	0.998827
CMS_SC2		0.687046	0.998651
CMS_MA1		0.698994	0.997885
CMS_MA2		0.695727	0.998178

Table 6.3. Sensitivity and Specificity of SNP predictions

6.4.4 Prediction of variants

Good quality variants were selected for being 62Mb targets +/- 500bp and detected on both DNA strands with a minimum depth of coverage of 5-fold. Common variants (MAF>0.01) found in dbSNP135 or 1000 Genomes (Feb 2012 download) and variants identified in an in-house list of 60 unrelated controls were removed. For consanguineous families, the filtering was limited to homozygous variants, whilst for non-consanguineous families, both homozygous and compound heterozygous were prioritized. Table 6.6 shows the total number of variants generated for each patient and the final number of variant prioritized. It is worth noting that no variants were prioritized in CMS-MA and CMS-SC (table 6.4).

Patient	Aligner	Variant Type	Total predicted (*)	On Target (a)	dbSNP135 & 1000 Genomes (b)	Rare/Novel Variants (c)	Shared Rare/Novel (d)	Number of Genes					
								Comp Het	Comp Het - Protein Altering	Comp Het- Predicted disease causing (e)	Hom	Hom- Protein Altering	Hom - Predicted disease causing (e)
CMS_EN1	BWA	**SNV**	5,091,951	66,632	56,691	7,930	1,621	168	26	26	22	2	3
CMS_EN2			5,065,829	66,736	57,038	7,722							
CMS_EN1		**Indel**	28,180	6,515	3,698	573	161						
CMS_EN2			25,860	6,546	3,717	562							
CMS_SC1		**SNV**	5,526,108	67,958	57,319	8,643	1,790	223	35	30	16	0	0
CMS_SC2			4,956,825	67,320	56,447	8,996							
CMS_SC1		**Indel**	28,131	6,649	3,812	506	141						
CMS_SC2			27,303	6,418	3,673	530							
CMS_MA1		**SNV**	5,096,255	71,276	58,227	10,998	2,552	351	54	45	11	0	0
CMS_MA2			5,269,379	73,219	58,032	13,111							
CMS_MA1		**Indel**	25,540	6,458	3,779	520	136						
CMS_MA2			25,026	6,367	3,703	520							

Table 6.4 a. Number of Variant Predictions: non-consanguineous families. SNV; single nucleotide variant, indel; small insertion or deletion, Hom; homozygous, Het; heterozygous.

Patient	Aligner	Variant type	Total predicted (*)	On Target (a)	dbSNP135 & 1000 Genomes (b)	Rare/Novel Variants (c)	Shared Rare/Novel (d)	Number of Genes					
								Comp Het	Comp Het - Protein Altering	Comp Het- Predicted disease causing (e)	Hom	Hom- Protein Altering	Hom - Predicted disease causing (e)
CMS_NO1	BWA	**SNV**	5,045,879	66,418	23,242	92	25	-	-	-	28	4	4
CMS_NO2			4,729,958	66,565	23,399	92							
CMS_NO1		**Indel**	27,396	6,495	1,561	49	3						
CMS_NO2			27,695	6,367	1,558	57							
CMS_GH1		**SNV**	4,613,050	65,928	22,286	109	15	-	-	-	20	1	1
CMS_GH2			5,270,469	70,653	22,738	85							
CMS_GH1		**Indel**	23,037	6,114	1,458	48	5						
CMS_GH2			25,955	6,493	1,516	46							
CMS_OV1		**SNV**	5,204,509	67,558	25,301	219	77	-	-	-	99	16	10
CMS_OV2			5,469,740	66,847	25,222	228							
CMS_OV1		**Indel**	25,918	6,429	1,739	79	22						
CMS_OV2			30,129	6,533	1,751	88							

Table 6.4 b. (Cont.) Number of Variant Predictions: consanguineous families

6.5 Homozygosis mapping

Genome wide SNP genotyping identified several large homozygous blocks (table 6.5) in the three consanguineous families (CMS-NO, CMS-OV and CMS-GH).

Consanguineous kinship	Chr.	Position start (bp)	Position end (bp)	Size (Mb)	SNP start	SNP end
CMS-NO	5	2725775	10699490	7.97	rs1039161	rs2930049
CMS-NO	13	74746182	94173688	19.43	rs7990716	rs9301891
CMS-NO	16	31860504	48879322	17.02	rs11150644	rs4785552
CMS-NO	22	17255335	27855335	10.62	rs16984825	rs2342945
CMS-OV	4	31307699	37604570	6.3	rs9985891	rs2973280
CMS-OV	4	124286915	143220613	18.93	rs12512633	rs10002445
CMS-OV	6	91393320	100642650	9.25	rs1923064	rs7738443
CMS-OV	10	13328248	78713115	65.38	rs500800	rs10824473
CMS-OV	11	44550047	66081211	21.53	rs2863104	rs10791867
CMS-GH	**3**	87834410	93588564	5.75	rs2046796	rs9683303
CMS-GH	**5**	113149886	119795765	6.65	rs10055433	rs6883068
CMS-GH	**9**	101168637	110238361	9.07	rs10986125	rs17725250
CMS-GH	**16**	31814299	48556573	16.74	rs8060086	rs9933187

Table 6.5. Blocks of homozygosity in consanguineous families. Bp; base pair, chr; chromosome.

6.6 Prioritized variants in known CMS genes

Non synonymous variants that were mapped on target and seen on both strands at a coverage of 5-fold or more were prioritized based on their MAF <0.01 and *in silico* predictions. Variants in known CMS genes were reviewed first. Variants in *AGRN* were identified in two kinships: CMS-EN and CMS-GH.

6.6.1 CMS-EN kinship

This was a non-consanguineous family from Norway. A novel homozygous variant (*AGRN*; c.G226A:p.G76S) was identified. This variant was predicted to be disease causing by different *in silic*o prediction tools. It is worth noting that the coverage of this variant was limited on the reverse strand and may have been missed should a higher level of depth of coverage was applied to the filtering pipeline (figure 6.5).

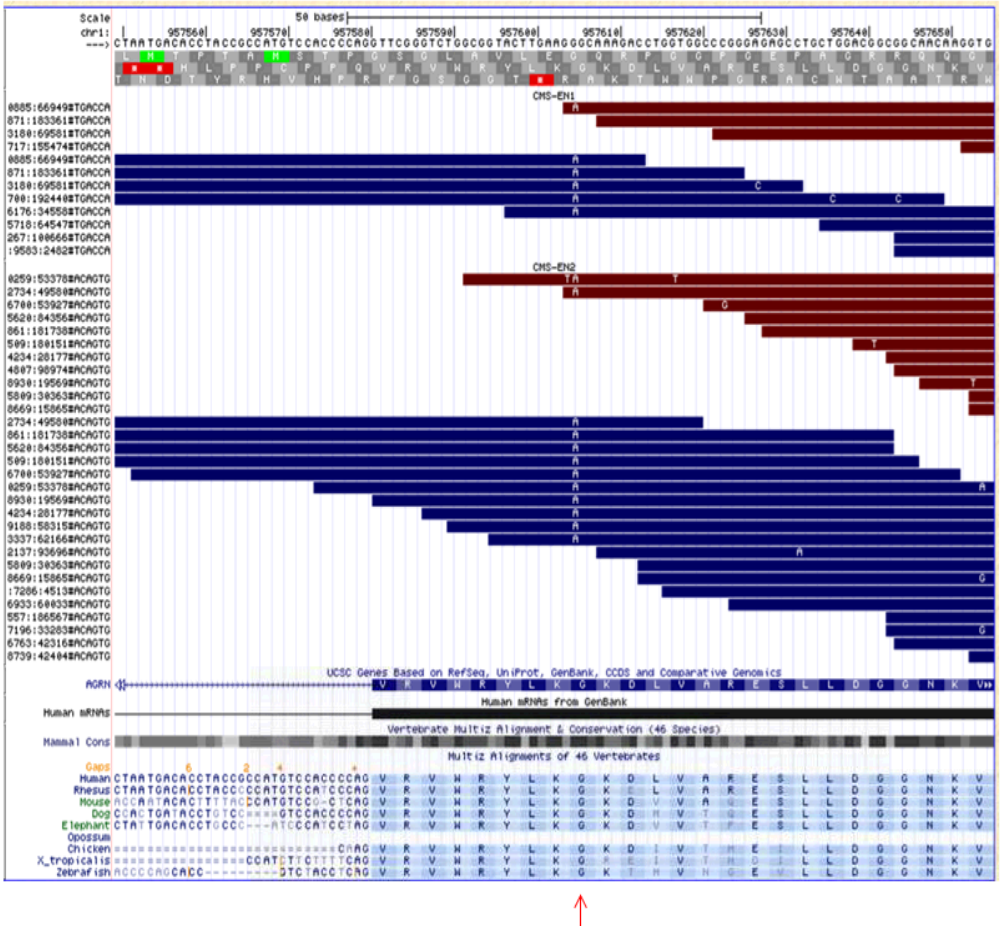


Figure 6.5 *AGRN* variant in CMS-EN kinship (visualized using UCSC browser, accessed February 2012)

6.6.2 CMS-GH kinship

This was a consanguineous Iranian family. Compound heterozygous variant in *AGRN* were prioritized (c.A3404G; p.Q1135R) and (c.G5687A; p.S1896N). The former was previously reported in dbSNP with a low frequency (MAF 0.005 in 1000 genomes) and was predicted to be benign. The second variant was novel but also predicted to be non-deleterious.

6.6.3 Prioritized variants in non CMS genes in CMS-NO kinship

This was a consanguineous family from England. Only one novel homozygous variant in the *SLC25A1* gene (c.G740A; p.R247Q) was identified and predicted to be disease causing. The gene *SLC25A1* encodes a citrate transporter that has recently been linked to human disease. The final list of filtered variants in this family is available on request. Of note, a change in *CLTL1* was shortlisted. This gene encodes a clathrin related protein involved in synaptic vesicle cycle. The prioritized variant was not predicted to alter protein structure or cause disease. In addition, it was found in both dbSNP and exome variant databases at a MAF 0.07%. Accordingly, it was presumed that this variant was a benign polymorphism.

6.6.4 Other variants in CMS-OV kinship

In CMS-OV, a novel frameshift homozygous variant (*BCHE*; c.436_437insG:p.F146fs), predicted to be damaging was found. Mutations in this gene have been associated with pseudo-cholinesterase deficiency. In a homozygous state, mutations in this gene cause respiratory complication after administration of particular muscle relaxants during anaesthesia. This particular family was known to have this disorder although this was never confirmed genetically. In the absence of triggers, the patient is asymptomatic.

In CMS-MA, CMS-SC and CMS-OV families, the current filtering pipeline did not allow the identification of candidate variants causing CMS.

6.7 Confirmation of prioritized variants

6.7.1 *AGRN* variant in CMS-EN kinship

Sanger sequencing in both affected siblings confirmed this variant. The first step to ascertain pathogenicity of this variant was to check its segregation with the disease phenotype. The mother was found to be heterozygous for the mutant allele whilst the father and healthy brother were homozygous for the wild type allele (figure 6.6).

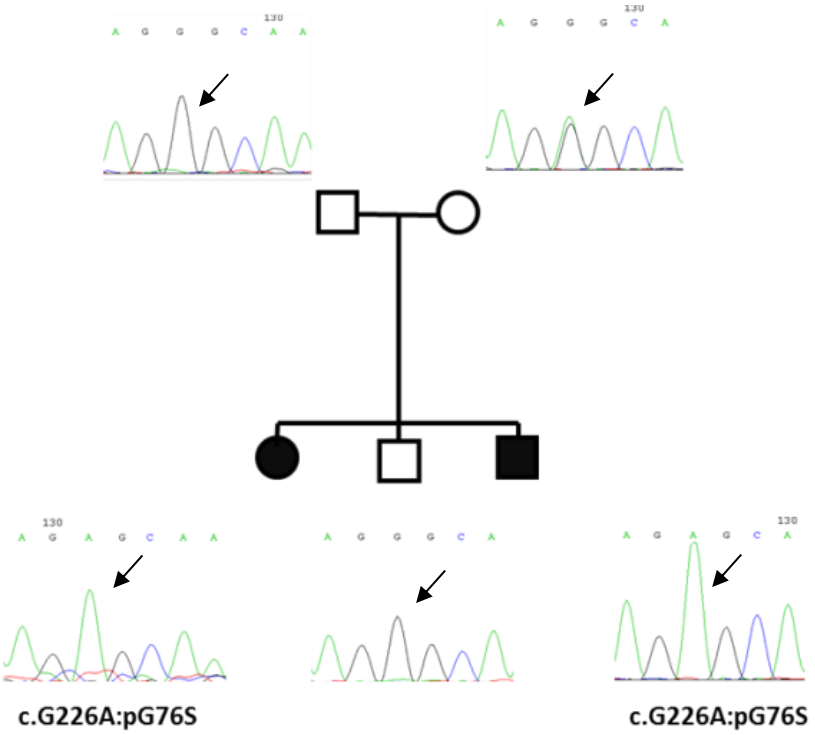


Figure 6.6. Segregation of the *AGRN* variant in CMS-EN.

The seemingly homozygous state of the father for the wild type allele could not account for the homozygous state for the mutant allele in both off springs. One possible explanation was that the father harbours a deletion or rearrangement spanning exon 2 of the *AGRN* gene (position of our mutation). This would prevent PCR amplification of the mutant allele and result in "an apparent" homozygous state in the father and both affected siblings. It is worth noting that PCR amplification and sequencing were repeated using a fresh DNA sample from the father to confirm sample identity and paternity of the father was confirmed based on previous haplotype results (not shown).

To look for evidence of chromosomal deletions or rearrangements involving the *AGRN* gene, CGH array analysis was carried out looking for copy number variations. This confirmed evidence of a 461 kb heterozygous deletion on chr1:746,649-1,207,683 (figure 6.7). The deleted region included *AGRN*. Using a read depth approach comparing the number of reads mapping to a particular region between a test and a set of reference samples, it was possible to detect this copy number variants using the exome data in combination with a statistical mode

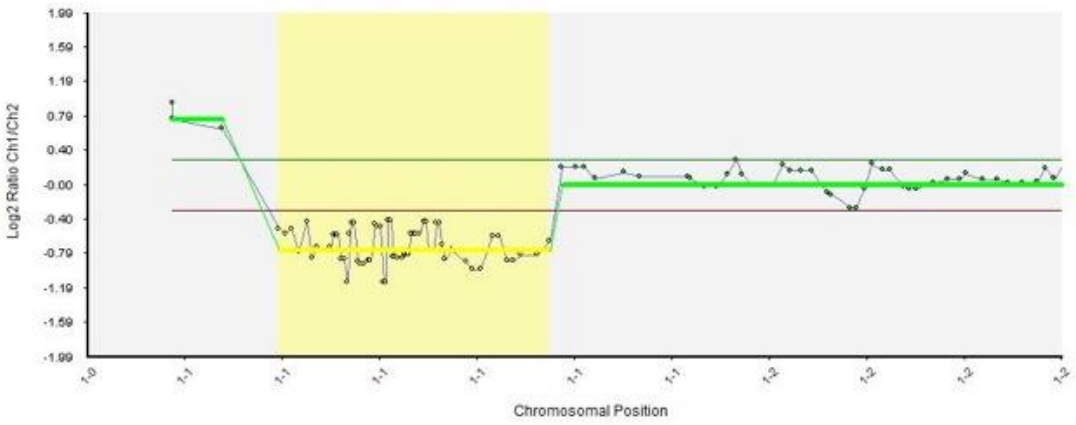


Figure 6.7. CGH array in CMS-EN kinship. Evidence of loss of chromosomal material on chr1.

6.7.2 *AGRN* variants in CMS-GH kinship

Sanger sequencing in both affected siblings confirmed this variant. However, the variant did not segregate with the disease since the asymptomatic mother and both affect siblings with compound heterozygous for the same *AGRN* variants (data not shown). Bearing in mind that these variants were predicted to be benign, it seems unlikely that these are pathogenic in this family.

Figure 6.6: Segregation analysis of *AGRN* variant in CMS-EN family.

6.7.3 *SLC25A1* variant in CMS-NO kinship

Sanger sequencing in both affected siblings confirmed this novel homozygous variant. Segregation analysis of this variant showed that both parents were heterozygous for the mutant allele whilst the brother was homozygous for the wild type allele (figure 6.7).

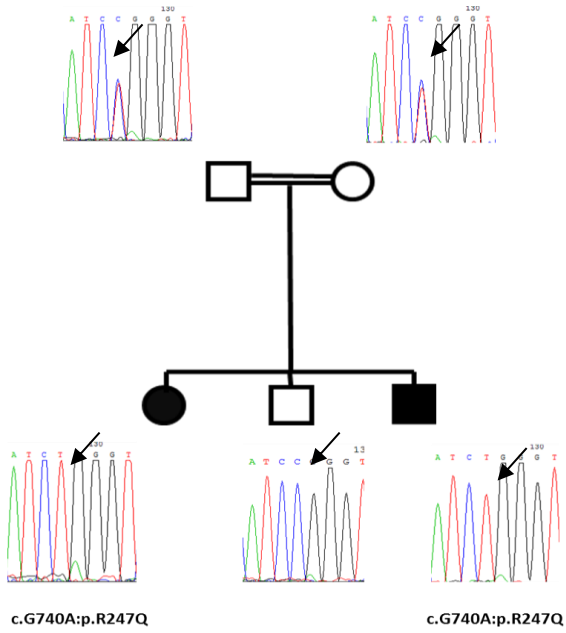


Figure 6.8. Segregation of *SLC25A1* variant in CMS-NO family.

6.8 Discussion

In this study, six pairs of affected siblings were selected from the Munich and Newcastle CMS cohorts. Three of these were from consanguineous families. The choice of these families relied on a high clinical suspicion of CMS and an extensive screening for known CMS genes using conventional methods. Whenever possible, patients with unusual phenotypic features, potentially caused by novel molecular mechanisms, were prioritized. For instance, CMS-EN exhibited marked pre-synaptic defects and distal muscle atrophy and weakness both very unusual in CMS. In the CMS-NO family, the affected siblings exhibited some intellectual disability and psychiatric tendencies.

In this study, disease causing variants, related to the CMS phenotype, were successfully identified in two out of the six families. One of the variant was in a known CMS gene (*AGRN*) that was not excluded in this family either by Sanger sequencing or linkage. The latter had been carried out before the discovery that *AGRN* mutations cause CMS. Although the *AGRN* variant was detected in one of the siblings during the initial filtering, this had been excluded in the second sibling due to poor coverage. This variant was therefore not prioritized during data intersection aimed at collating shared candidate variants between the two siblings. Reducing the filter setting for a read depth of 10 to 5-fold allowed the resolution of this issue.

The other important finding in CMS-EN, was the presence of a large hemizygous deletion on chromosome 1, which contained *AGRN* and resulted in loss of heterozygosity in this non-consanguineous family. The detection of copy number variants using exome sequencing is an evolving field, which is intrinsically linked to optimum coverage and is likely to be superseded by whole genome sequencing.

Based on the phenotypic features of this family, an additional three patients have been identified with *AGRN* mutations. The details of these and the functional work carried out to prove pathogenicity of the *AGRN* variant are described in chapter 7.

The second candidate variant identified was in the CMS-NO family. This was the only novel homozygous variant predicted to be disease causing. It also segregated with the

phenotype. This gene has recently been linked to human disease in 13 kinships. One of the patients was shown to have impaired neuromuscular transmission. The full details of these patients and the functional work carried are presented in chapter 8.

The variant identified in *BCHE* gene in the CMS-OV family accounts for the pseudoesterase deficiency but not for the myasthenic syndrome. Therefore, exome sequencing was only able to solve two out of the six families selected.

One inherent limitation to exome sequencing is failure to detect intronic mutations and regulatory elements of the genes. These undiagnosed families may have harboured mutations in the non-coding regions. An alternative possibility is that these families harboured copy number variants missed by exome sequencing. Families have since been screened for copy number variants suing CGH array and none was found. Although whole-genome sequencing will in part overcome this challenge, the true limitation seems to relate to the lack of understanding of the biology of these genomic entities and how they interact with each other.

Regardless of the platform applied, the first step of exome sequencing consists of DNA library preparation. This is designed to generate DNA fragments that can be chemically enriched to allow the capture of the coding regions of the genome. These are then amplified and sequenced using different signals to determine each nucleotide (Cooper and Shendure, 2011). In this project, the platform used was for paired-end sequencing, which has the advantage of generating forward and reverse strands for each template fragment. This in turn should improve the quality of the sequencing and provide information on the position of the read in relation to the reference sequence, thus facilitating the mapping process. Nonetheless, one can expect some errors related to suboptimal DNA library preparation and sequencing bias.

The quality of exome sequencing is intrinsically linked to the coverage of the coding regions and the accuracy (sensitivity and specificity) of the variants called (Metzker, 2010). The current bioinformatics tools available, although very effective, are not perfect. It is recognized that the quality of alignment and mapping of the sequence reads have a direct impact on the coverage as well as sensitivity and specificity of the called variants. If variants are not covered they cannot be found and if they are poorly

covered they cannot be accurately annotated (Flicek and Birney, 2009, Li and Homer, 2010).

In this project, the filtering pipeline initial objective was to shortlist good quality variants i.e. variants positioned within or near exonic regions, with at least 5-fold coverage and present on both forward and reverse strands. The next step was aimed at prioritizing rare disease causing variants using established public variant databases and *in silico* prediction tools. These provide a score based on the physical properties and conservation across species of residues.

The use of public databases to remove common polymorphisms can be limited by the lack of accurate frequency data which are essential for optimum restriction of variants to a manageable number. Setting the minimum allele frequency to less than 1% seems an arbitrary choice that should probably be adjusted based on the prevalence of the disease and the penetrance of phenotype. In this study, the CMS-GH siblings were compound heterozygous for 2 variants in *AGRN* but only one of the variants was prioritized in the rare variant list because the MAF of the other change in 1000 genomes database was exactly 0.01.

Data filtering may also have been limited by the degree of ethnic diversity within public databases. In this study, two out of the four undiagnosed families were from Turkey and Iran. This issue may be overcome with the use of ethnically matched public databases. Prioritization of variants is also reliant on the accuracy of the prediction software used. In this study, a very large number of variants were prioritized as disease causing, which suggests that the current prediction tools are over estimating the impact of these changes on protein function. One way to tackle this issue has been introduction of additional parameters like gene ontology data and phenotypic data from animal models that would provide some additional information on gene function and phenotypic characteristics in other species.

Finally, it is likely that some of the limitations of this strategy are secondary to the biology of Mendelian diseases. Incomplete penetrance is one example that would impact on the accuracy and interpretation of data derived from public databases. Although, stringent criteria were used to ensure that patients exhibited clinical features

of CMS, some of these undiagnosed patients may have an alternative genetic diagnosis. Indeed, CMS can overlap with other neuromuscular conditions such congenital myopathies. In this study, screening of 275 known neuromuscular genes did not detect any disease causing variants (Kaplan, 2011). A deeper phenotyping of patients to determine more specific clinical features and biomarkers seem crucial to distinguish phenocopies. In the next two chapters, the clinical features and functional work carried out in relation to the novel *AGRN* and *SLC25A1* variants are presented.

Chapter 7. AGRN related CMS with distal muscle weakness and wasting

7.1 Introduction and aims[60]

In this chapter, I discuss the CMS-EN family in whom candidate disease causing variants in *AGRN* were found. As covered in chapter 6, this Norwegian non-consanguineous family exhibited marked distal muscle wasting and weakness with decrement and marked increment on repetitive nerve stimulation, in keeping with a presynaptic defect. Based on the distinct phenotype of this family, I was able to identify a second French family via the French CMS network. This family was previously reported by Professor Bruno Eymard during the ENMC CMS workshop (Chaouch et al., 2012a). Subsequently, a third independent kinship from Turkey was shown to share the same phenotype and to carry mutations in *AGRN*.

Neuronal agrin is an extracellular matrix proteoglycan, which is key in organizing and maintaining the neuromuscular junction. McMahan was the first to speculate that agrin may have such properties and postulated that this glycoprotein may be implicated in the stabilization and maintenance of the nascent postsynaptic receptors, transiently formed on the muscle membrane. McMahan's agrin hypothesis was later shown to be essentially accurate (Bezakova and Ruegg, 2003, McMahan, 1990, Singhal and Martin, 2011).

Several agrin isoforms are generated through alternative splicing. The neuronal form is synthesized in the presynaptic compartment and released into the synaptic cleft. In the basal lamina, agrin attaches to a low-density lipoprotein (LRP4) via its C terminal domain. LRP4 then binds and activates MuSK, a muscle specific kinase. The latter mediates the concentration and anchorage of the nascent, aneurally formed AChRs, to the postsynaptic membrane. MuSK also allows the clustering of other postsynaptic proteins including new AChRs (Kim et al., 2008).

[60] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

In the C terminal of agrin, an additional binding site was identified. This attaches to α-dystroglycan via the LG2 domain. The N terminal was shown, *in vitro*, to bind laminins. It is unclear whether these basement membrane proteins interconnection are primarily aimed at maintaining structural stability or whether other functions are at play (Stetefeld et al., 2001, Gesemann et al., 1998, Yamada et al., 1996, Burgess et al., 2000).

It is worth noting that the biological properties of agrin were studies with great interest prior to the discovery of any patients with mutations in the *AGRN* gene. Before the publication of this series, only three patients had been reported in the literature with mutations in *AGRN* in association with congenital myasthenic syndrome (Huze et al., 2009, Maselli et al., 2012). The clinical phenotype of these patients was typical of CMS with evidence of generalized neuromuscular weakness, fatigability and ptosis. One of the patients had a severe phenotype with recurrent respiratory crises in childhood needing tracheostomy, whilst the other two cases had fairly mild disease. Response to AChE inhibitors and ephedrine was variable. Patients harboured missense mutations in the C terminal of agrin except for the patient with the more severe phenotype. It is unclear if the discrepancy in phenotypic expression is related to the null allele (p.Q353X) in the more severe case.

Moreover, different patterns of muscle involvement often correlate with different clinical entities, for instance proximal symmetrical weakness in myopathies. Distal muscle weakness is unusual feature in congenital myasthenia as most patients present with global and sometimes predominantly proximal weakness, as seen in LG-CMS. Distal muscle weakness is rare and has only been reported in slow channel syndrome (see chapter 4). Distal neuromuscular weakness is also typically seen in neuropathies and in some rare inherited myopathies. More than twenty distal myopathy subtypes have been reported so far, many of which remain genetically undiagnosed (Udd, 2012). Distal myopathies have been categorized based on the following criteria (Mastaglia et al., 2005):

1-Age at onset.
2-Distribution of muscle weakness.
3- Cardiac and respiratory involvement.

4- Muscle biopsy features.

5-Family history.

This chapter will discuss the results of the exome sequencing carried out in family CMS-EN in which compound heterozygous mutations in the *AGRN* gene were identified. I also describe two additional kinships that harboured mutations in *AGRN* and shared strikingly similar clinical and electrophysiological features.

7.2 Results

7.2.1 Patient data[61]

In this chapter, the two affected siblings (CMS-EN1 and CMS-EN2) will be referred to as patient 1 and patient 2 respectively. In this Norwegian kinship two of the three siblings were affected with no parental consanguinity. The two affected siblings share a similar phenotype, were the product of a normal pregnancy and birth, and reported normal motor milestone and no autonomic symptoms.

The elder affected sister, who is now 45 years old had normal psychomotor development. She presented at the age of 15 years with simultaneous bilateral distal lower limb atrophy and weakness and recurrent falls. There was no suggestion of swallowing difficulties, visual or sphincter disturbance. Her disease course was slowly progressive. Three years after her presentation, this patient developed wasting and weakness of both distal upper limbs and later noted weakness of her shoulder and hip girdle muscles.

Clinical assessment at the age of 43 years showed no evidence of cranio-bulbar weakness. Shoulder abduction was marginally weak. The Biceps, triceps, forearm flexors and extensors were preserved. In the hands, she had a split pattern of muscle atrophy with marked wasting of the thenar and dorsal interossei muscles whilst the hypothenar muscles including *abductor digiti minimi* were preserved. There was global

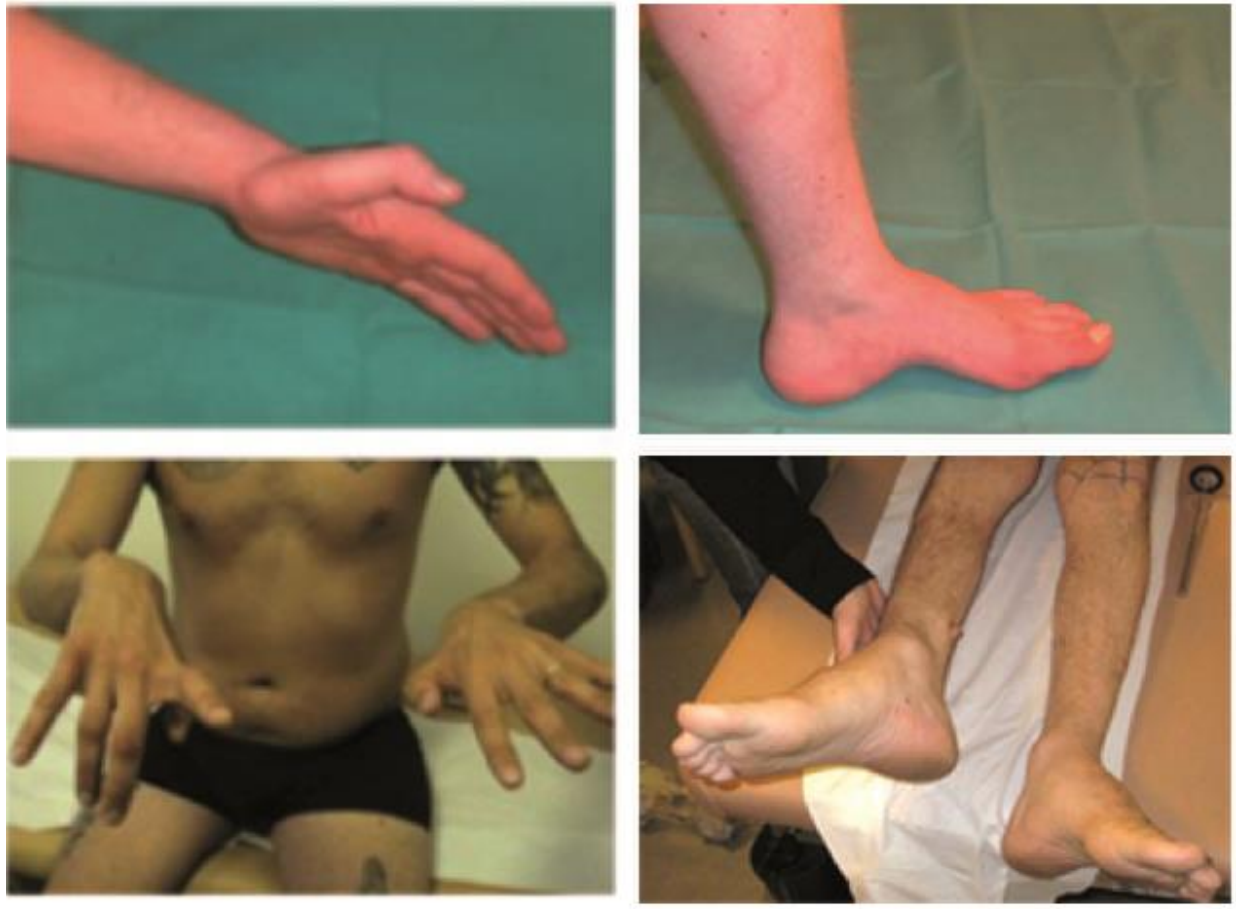
[61] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

and symmetrical weakness of all the small muscles of both hands. In the legs, she had a non-pyramidal pattern of muscle weakness, more marked distally with weakness of toe extension and foot eversion. She also showed some mild hip flexion weakness with some suggestion of a waddling gait. Foot dorsiflexion and knee extension were affected to a lesser degree. This patient reported no benefit from AChE inhibitors.

Patient 2 is the younger affected brother of Patient 1. He presented at the age of 15 years with difficulties running and recurrent falls. He later reported difficulties with both hands with marked muscle wasting. There was no history of visual disturbance, swallowing difficulties or sphincter disturbance. He also had no symptoms suggestive of nocturnal hyperpnoea. Although, he reported some progression in symptoms, there was no evidence of any significant change in the last ten years.

Neurological evaluation revealed no evidence of cranio-bulbar weakness. Neck flexion was normal. In the upper limbs, the salient findings were confined to both hands. He had marked wasting of the thenar and dorsal interossei muscles with sparing of his hypothenar eminence. Power was paradoxically preserved in the small muscle of both hands except for thumb abduction, which was weak. In the lower limbs, foot dorsiflexion and plantarflexion and to a lesser extent knee extension were affected. The patient was unable to walk on his heels and toes. Similar to his sister, the patient reported no benefit from AChE inhibitors and later declined alternative treatments given the lack of progression in his disease[62].

[62] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)



P2

P3

Figure 7.1. Distal limb involvement, Patient 2 (top) and patient 3 (bottom). Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

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To exclude potential mimics of CMS, a number of investigations was carried out in both siblings. An autoimmune screen including anti-AChR and anti-MuSK antibodies and creatine kinase levels were checked and were shown to be negative or normal. Cardiac evaluation, which comprised a baseline ECG and echocardiography were satisfactory. In addition, a muscle biopsy from *vastus lateralis* was performed at the age of 33 years. This biopsy transferred to Newcastle for further immune staining in 2002 and was used for the ultrastructural analysis described below. Histological study of this muscle revealed chronic myopathic features with type 2 fibre atrophy and evidence of clusters of atrophic muscle fibres, which encircled a number of hypertrophic muscles fibres. Immunostaining for dystrophin, sarcoglycan, laminin $\alpha 2$, telethonin, caveolin 3, emerin, lamin A/C, calpain and dysferlin was reported as normal. Both the basement membrane and sarcolemma morphology appeared intact.

To assess the extent of muscle involvement, a muscle MRI of the lower limbs was carried out in Patient 2 at the age of 42 years. Three muscles were preferentially affected with evidence of atrophy and fat infiltration of semitendinosus bilaterally (figure. 7.2B). There was asymmetrical and bilateral fat infiltration in *medial gastrocnemius*, more prominent on the right. The right *medial soleus* muscle was also involved with evidence of central muscle atrophy and fat infiltration.[63]

[63] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

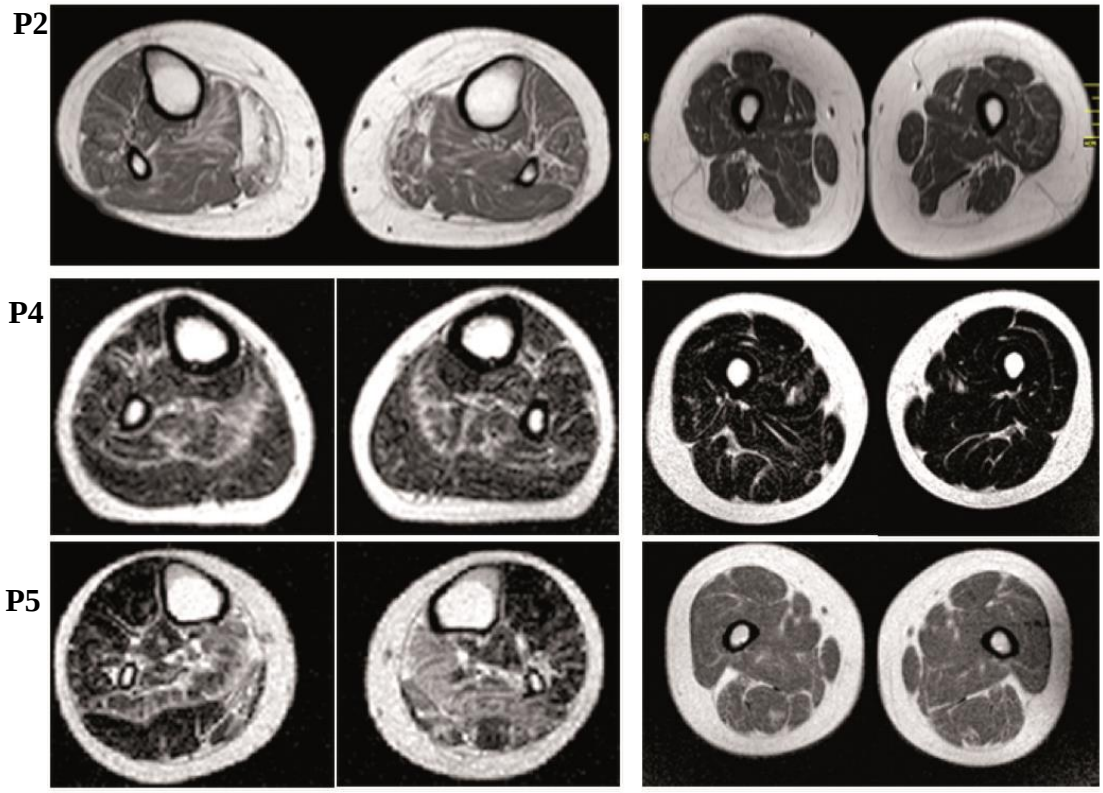

Figure 7.2: Muscle MRI of the lower limbs of Patients 2, 4 and 5. P2: Evidence of soleus, medial gastrocnemius (left) and semitendinosus atrophy (right). P4 and 5: evidence of Gastrocnemius atrophy bilaterally. The thigh muscles were spared in Patients 4 and 5. Reproduced from Nicole et al 2014 (URL:http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full).

The second kinship (Patients 3 and 4) was first reported in 2012 during the CMS workshop in Naarden, 2012. Based on a strikingly similar phenotype consisting of distal muscle weakness and wasting and presynaptic features on repetitive nerve stimulation, I was able to liaise with the French CMS network and check if *AGRN* mutations were screened in this family and indeed they were. The full details of their mutation status will be discussed below.

Similar to the CMS-EN kinship, this was a non-consanguineous family in which two out of the five siblings were affected. Both parents were healthy. Patient 3 was a male patient who was the product of normal pregnancy and delivery and displayed normal developmental milestones. He was 36-year-old when the diagnosis was confirmed. During early infancy, around two years of age, he developed recurrent apnoeic episodes, which spontaneously resolved through his childhood. From the age of seven years, he noted fatigable lower limb weakness and distal wasting which were slowly progressive and subsequently warranted the introduction of foot splints. The patient reported extension of his symptoms to both upper limbs at the age of nineteen years with clear bilateral thinning and weakness of both hands and forearms. In addition, he later developed intermittent stridor, suggestive of upper airway obstruction, as well as intermittent dyspnoea that were often triggered by physical activity. The patient did not report any swallowing difficulties, visual disturbance or autonomic features.

The course of his illness was slowly progressive with a stepwise decline often preceded by a relapse necessitating medical attention. This patient required hospital admission at the age of fourteen, nineteen, thirty and thirty two years. At the age of 30 years, his neuromuscular exacerbation was associated with marked respiratory insufficiency for which he was admitted to intensive care for ventilator support. The patient has been dependent on non-invasive ventilation ever since. It is worth noting that in between these episodes, the patient experienced marked diurnal variations in his symptoms similar to what is seen in myasthenia gravis.[64]

[64] Adapted from Nicole et al 2014 (URL:http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

His latest marked neuromuscular relapse was at the age of thirty two years. This was associated with marked reduction in his exercise tolerance and loss of hand function. This patient was able to walk two hundred metres with a stick before needing to stop[65].

Neurological assessment carried out at the age of thirty three showed normal cranial nerves. In particular, he had no ptosis, ophthalmoparesis or bulbar difficulties. Neck flexion was also normal. He had mild scapular wining but no evidence of any spinal deformities or contractures. His forced vital capacity was marginally low in sitting at 58% of predicted value.

In the limbs, he had distinct distal muscle wasting and weakness (figure. 7.1). In the arms, similar to the first kinship described, there was evidence of bilateral multifocal involvement with preferential weakness of the extensors of the wrists, thumbs and fingers. The right arm was more affected than the left and the third and fourth fingers were most affected (MRC grade 1/5). The forearm flexors were also involved with striking weakness of the pincer movement of the thumb and index finger, reminiscent of an anterior interosseus syndrome. Elbow flexion and extension were minimally affected as were shoulder abduction and adduction with an MRC grading ranging from 4+ to 5/5. Bilateral and symmetrical scapular winging was present but mild. The patient was able to elevate both arms at ninety degrees for more than two minutes. In the lower limbs, he had marked wasting distally. Proximal power was normal. Knee flexion and extension were minimally weak (MRC grade 4/5) with marked ankle dorsiflexion and plantarflexion involvement (MRC grade 1/5). The patient was unable to heel or toe walk.[65]

AChE inhibitors were initially introduced at the age of twenty four with little benefit. A similar negative response was reported with 3,4-DAP, which was later instigated at the age of twenty nine. By contrast, ephedrine correlated with a clear instantaneous benefit with marked increase in his exercise tolerance (increasing from 50 to 200 metres) along with improvement in his ability to carry out activities of daily living. The patient was able to mobilise with no aids or foot splints although objectively, distal muscle power did not dramatically improve. Despite improvement in his forced vital capacity from 59

[65] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

to 78%, he remained dependent on nocturnal non-invasive ventilation, which reflects residual diaphragmatic weakness. This improvement was maintained for three years after instigating this treatment. Ephedrine was titrated from 45mg up 135mg with no reported side effects.

In terms of investigations, baseline cardiac assessment including ECG and transthoracic echocardiography were normal. Screening for anti-AChR and anti-MuSK antibodies were negative and creatine kinase levels were within the normal range. A deltoid muscle biopsy was performed at the age of thirty six years. Histochemical analysis of this biopsy showed some variation in fibre size with type 2 fibre atrophy and type 1 fibre predominance. It is worth noting that this muscle was minimally affected clinically (figure 7.3).[66]

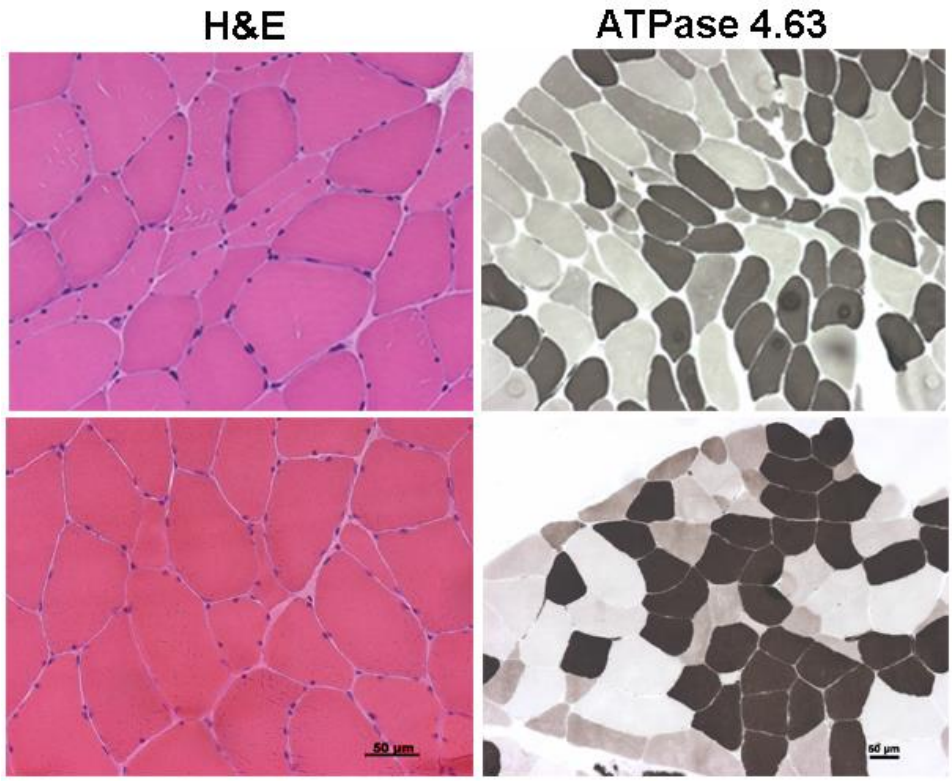

Figure 7.3. Histochemical analysis of muscle biopsy of P3 (top) and P5 (bottom). H&E staining shows occasional fibre size variation and fibre type grouping. Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

[66] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

Patient 4 was twenty seven years old and was the second affected offspring in the French kinship and first presented to the French CMS network at the age of twenty-three years. She was the product of normal pregnancy and birth and exhibited normal psychomotor development. Notably, she was noted to have stridor in the neonatal period, which was self-limiting. She subsequently sought medical attention at the age of seven years with fluctuating exercise related dyspnoea. By the age of eleven years, the patient had suffered a number of falls, which were attributed to worsening bilateral foot weakness. She started complaining of difficulties with hand function at the age of sixteen years and struggled to blow up her cheeks or whistle from the age of eighteen years. Fluctuations in her symptoms were reported since the onset of her illness and often evolved over days and weakness, instead of the diurnal variations typically seen in myasthenia gravis. Particular triggers like hot weather were noted[67].

Clinical examination at the age of twenty three years revealed no evidence of ptosis or ophthalmoparesis. She had mild eye closure and mouth closure weakness. She was unable to whistle. She had some bulbar difficulties but respiratory function was preserved with a forced vital capacity in sitting of 98% of predicted value. In the upper limbs, she had clear atrophy of both *thenar* eminences and first *dorsal interossei* muscles. Proximal power was normal. Extension of the fingers and forearm flexors were weak (MRC grade 3/5). Similar to her brother, the patient was unable to perform pincer movement of her thumb and index finger. Finger extensors and pinch grip (thumb-index finger) were weak (MRC grade 3). In the legs, she had marked distal and symmetric muscle wasting of both anterior and posterior compartments. Proximal power, knee flexion and extension were normal. Similar to her affected sibling, she had bilateral foot drop with marked weakness of ankle dorsiflexion, plantarflexion and eversion (MRC grade 1/5). Toe extension was also weak (MRC grade 2/5).[67]

Akin to her affected brother, the patient reported no benefit from AChE inhibitors or 3,4-DAP. By contrast, ephedrine was associated with a favourable response with marked improvement in her exercise tolerance and activities of daily living. This agent was commenced at the age of twenty-four years at a dose of 45mg daily.

[67] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

Baseline ECG was normal. Autoantibody screen for anti AChR and and MuSK was negative. A muscle biopsy of the deltoid was carried out at the age of eighteen and was reported as normal. Of note, this muscle was not affected clinically. Muscle MRI (figure 7.2) showed selective muscle atrophy with fat infiltration of the *soleus*, the inner portion of *tibialis anterior* and *extensor digitorum* muscles. Correlating with the clinical evaluation, there was no suggestion of more proximal muscle involvement including pelvic girdle, quadriceps and hamstring muscles[68].

Patient 5 was a 34 year old male who was identified via the French CMS network. He was the only affected offspring in a consanguineous Turkish kinship. The patient was the product of normal pregnancy and birth and reported normal motor milestones. There was no history of early respiratory difficulties. Disease onset was at the age of five years when the patient noted myalgia and weakness in relation to exercise. The course of his disease was slowly progressive with clear rapid deterioration over the past three years and increasing difficulties walking and climbing stairs. From a baseline exercise tolerance of 5 kilometers he can now barely walk 100 meters. Fatigability has become a more prominent feature over the recent past, often associated with exertional dyspnea. The patient never required ventilator support nor did he report any visual, bulbar or autonomic symptoms.

Neurological assessment at the age of thirty one years showed some limitation of eye movements but no ptosis. Cranial nerves were otherwise normal. Neck flexion and extension were intact. Upper limb examination revealed minimal wasting of the hands with moderate weakness of finger extension (MRC grade 3+/5). The remaining muscle groups were normal in the upper limbs. In the legs, he had moderate and more diffuse muscle atrophy of the posterior more than the anterior compartments. Proximal power was marginally weak with hip flexion scoring 4+/5 and knee extension 4/5 (MRC grade). The patient displayed some difficulties standing from a sitting position. In the foot, ankle dorsiflexion was weak (MRC grade 3+/5) as were ankle plantarflexion and eversion 3-4/5.[68]

[68] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

A similar battery of screening tests was carried out in this patient, comprising ECG, creatine kinase levels and autoantibody screening which were normal or negative. Sequential muscle biopsies of the deltoid were carried out at the age of thirty one and thirty four years. These revealed some atrophied fibres with fibre type grouping (figure 7.3). Muscle involvement was more marked in the posterior compartment of both forelegs on muscle MRI. There was bilateral *soleus* and *gastrocnemius* atrophy with fat infiltration sparing of the medial right *gastrocnemius muscle* (figure 7.2). This patient was recently commenced on a 6 mg daily dose of salbutamol with moderate improvement in his neuromuscular weakness and in particular in his exercise tolerance and ability to climb stairs[69].

7.2.2 Neurophysiological studies[69]

Nerve conduction studies showed no evidence of large fibre neuropathy with preserved conduction velocities and distal latencies. EMG in patient 1 showed myopathic features with short moderately polyphasic motor units and fibrillation potentials with positive sharp waves in several muscle groups, most noticeable in *abductor pollicis brevis* muscle (figure 7.4A). In contrast to the *deltoid* and *vastus lateralis* muscles that seemed to exhibit the least abnormalities.

[69] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

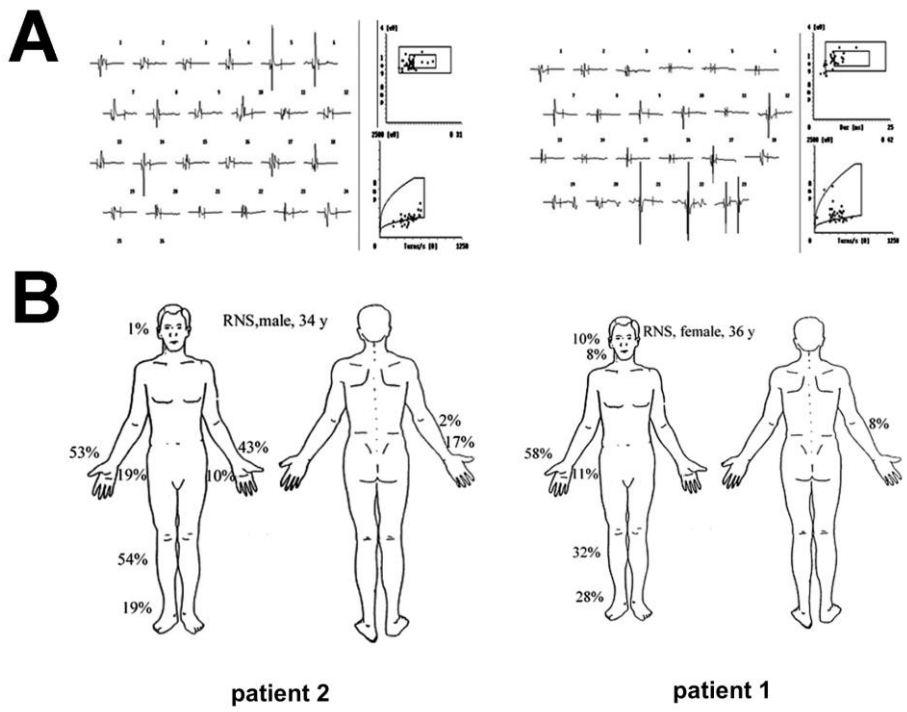

Figure 7.4. A. EMG findings in P1. Polyphasic short complexes reminiscent of myopathy. B. Percentage of decrement induced by repetitive nerve stimulation. Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

Repetitive nerve stimulation at 3 Hz demonstrated decrement in both upper and lower limbs, more marked in the distal muscles (table 7.1, figures 7.4 and 7.5). Marginal decrement was detected in *abductor digiti minimi* and no decrement was seen in the facial muscles. Notably, baseline amplitude of CMAP was reduced in some muscle groups (figure 7.5).

In the majority of muscles, increment was detected following ten seconds of sustained muscle contraction. This was most noticeable in the *tibialis anterior* muscle. In some muscles like *abductor pollicis brevis*, sustained muscle contraction for ten seconds was associated with decrement, which was later followed by significant increment (+ 58%) after twenty seconds muscle activation. The decrement did not alter with the intravenous administration of edrophonium, a short acting AChE inhibitor. Of note, progressive reduction in the amplitude of trains of motor responses was seen in several muscles including *tibialis anterior*[70].

[70] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

SFEMG showed variable and patchy changes across individual muscles (data not shown), which did not necessarily correlate with the results of repetitive nerve stimulation. Whilst SFEMG was normal in *extensor digitorum,* marked jitter with block was detected in the *tibialis anterior* muscle. Furthermore, repetitive nerve stimulation in the same hand (with normal SFEMG) displayed clear decrement in *abductor pollicis brevis*. A similar pattern of variable and patchy muscle and NMJ involvement was also noted in patient 2 (table 7.1, figures 7.4 and 7.5)[71].

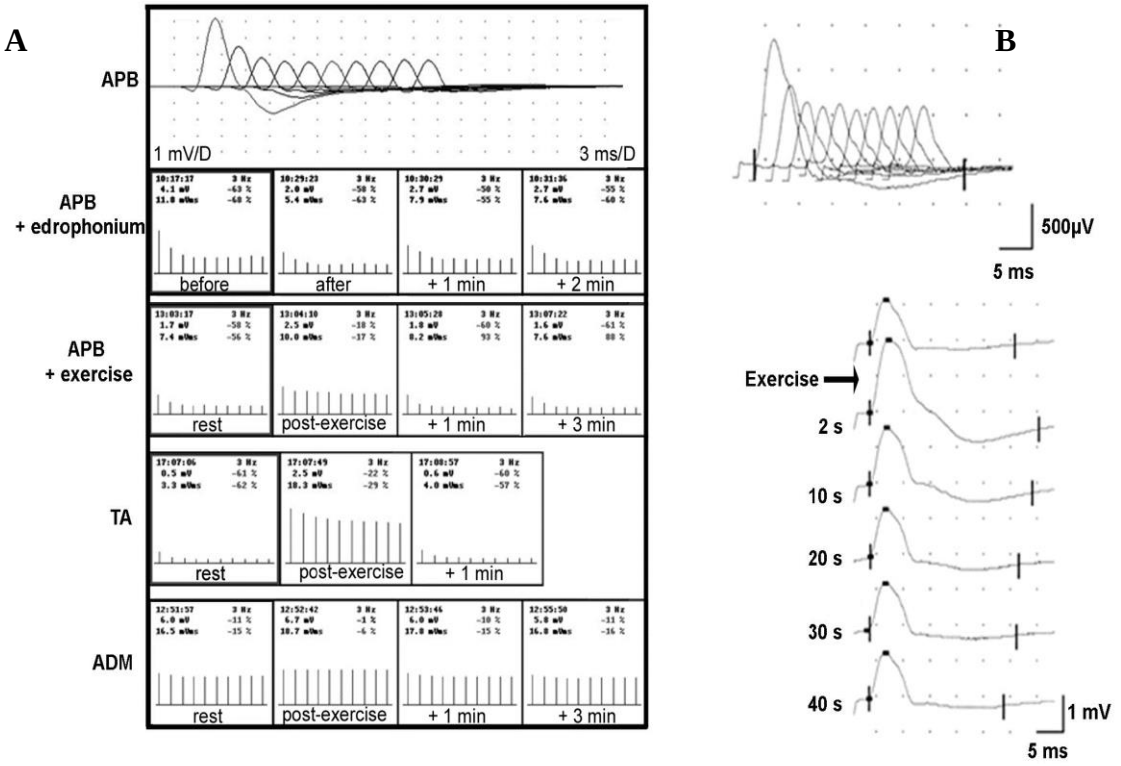

Figure 7.5 (A) Repetitive nerve stimulation (3 Hz) in P1 showed marked decrement in several muscles with no improvement after edrophonium. There is also increment after 20 s sustained muscle contraction in APB (68%) and TA (500%). Decrement was also seen without increment in ADM. (B) P3. Decrement with post-exercise facilitation in TA. APB; abductor policis brevis, TA; tibialis anterior, ADM; abductor digiti minimi. Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

[71] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

Patient	Anconeus (3Hz)	TA (mV) at rest	TA (3Hz)	TA (post-exercise)	EDB (mV) at rest	EDB (3Hz)	EDB (post-exercise)
1	-8%	0.9	-33%	133%	1.6	-27%	23%
2	-16%	0.3	-67%	267%	2.8	-20%	25%
3	-29%	1.3	-55%	87%	0.9	-72%	126%
4	-10%	0.1	-53%	285%	4.7	-22%	32%
5	-9%	1.5	-22%	67%	1.8	-33%	47%

Table 7.1 Repetitive nerve stimulation. Amplitude of compound muscle action potential in one proximal and two distal muscles in relation to repetitive nerve stimulation and brief exercise in P1, 2, 3, 4, 5. TA; tibialis anterior, EDB ; extensor digitorum brevis. Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

Neurophysiological studies in patients 3, 4 and 5 recruited through the French CMS network also showed similar results. Nerve conduction studies showed no evidence of large fibre neuropathy with persevered motor conduction velocities and distal latencies. EMG study of all three patients revealed myopathic features with no spontaneous activity. These myopathic changes were most noticeable in the distal muscles. Decrement on repetitive nerve stimulation was detected in several muscle groups. Similar to patients 1 and 2, such finding was more marked in distal muscles (figure 7.5 and table 7.1). Furthermore, sustained muscle contraction was associated with significant increment (285% *tibialis anterior*) consistent with a presynaptic defect.[72]

7.2.3 Morphological studies of the NMJ[72]

Muscles biopsies from patients 3 and 5 were selected for this study. The *deltoid* muscle was sampled in both patients as this was one of the least affected muscles in which NMJ structure was less likely to be affected secondary to muscle atrophy.

In both patients' biopsies, pre and postsynaptic variations were detected using fluorescent immunostaining on whole-mount preparations (figure 7.6A). In total, 33 NMJs from patient 3 and 43 NMJs from patient 5 were analysed. None of the synapses retained their normal architecture. There was evidence of disjointed and occasionally dispersed postsynaptic grooves along with irregular AChR staining reflected by the postsynaptic streaky pattern of staining. This unusual pattern of staining was more prominent in patient 3. Neurofilament staining was completely (full NMJ) or partially (some postsynaptic gutters only) absent in six out of eight NMJs in patient 3 and 8 out of twenty two NMJs in patient 5. Some NMJs displayed extensive branching of terminal axons with nodal, terminal or *en passant* sprouting that innervated individual groups of AChR. S100 immunostaining revealed hypertrophic or phantom-like terminal Schwann cells apposing synaptic gutters. This pattern has occasionally been reported during active denervation re-innervation processes (figure 7.6B).

[72] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

Ultrastructural analysis of three NMJs sampled from patient 5 exhibited synaptic folds with no associated apposing nerve terminal or Schwann cell (figure 7.6C). This feature can be seen in neurogenic pathology. Terminal nerves appeared morphologically intact but contained a higher number of mitochondria, synaptic vesicles, and active zones compared to control. The secondary junctional folds were poorly formed. It is worth noting that the motor endplates analysed were sampled from a proximal minimally affected muscle. Therefore the marked abnormalities seen are likely to represent a primary NMJ defect as opposed to secondary changes due to muscle pathology[73].

[73] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

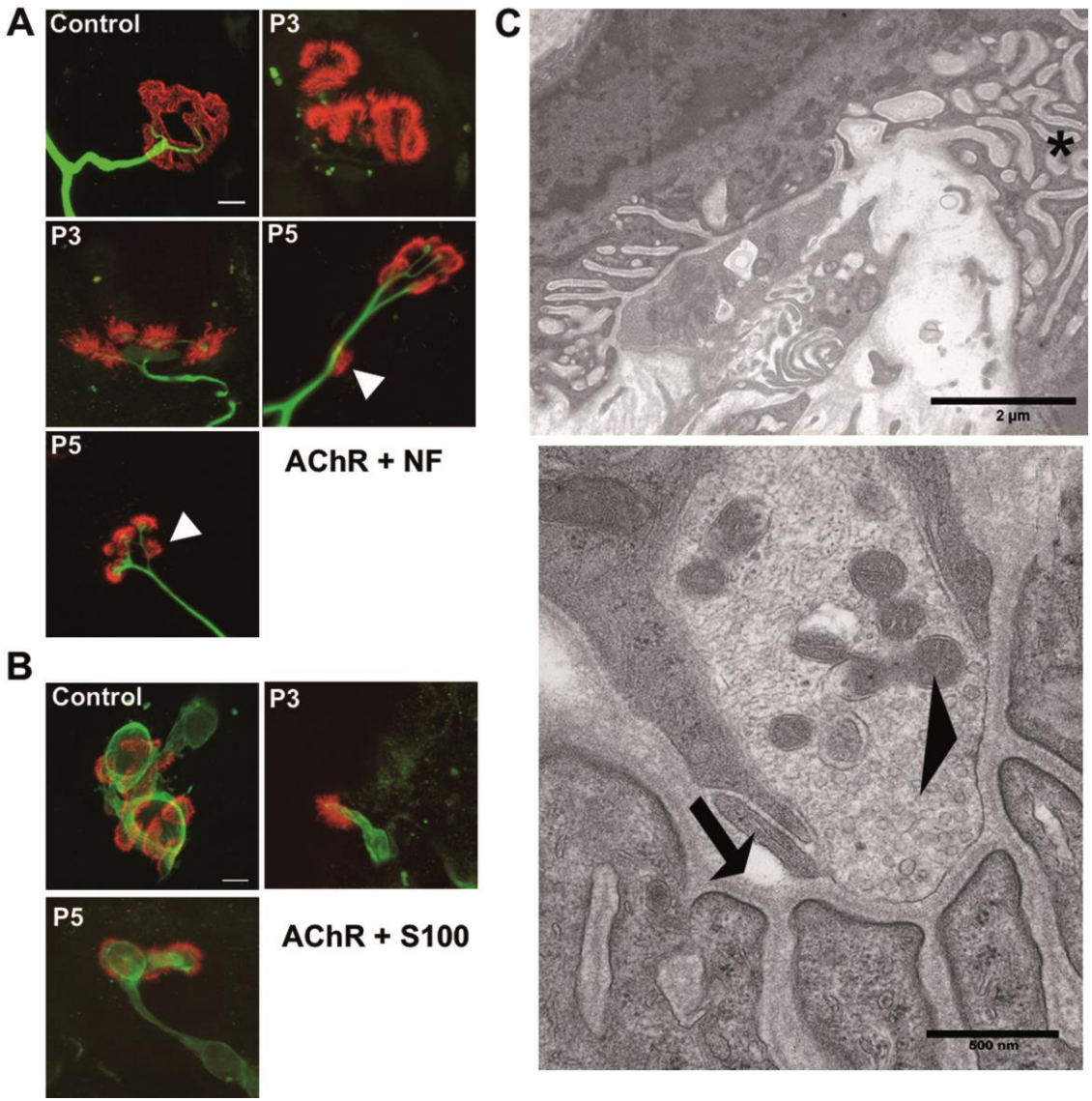

Figure 7.6 (A) Immunofluorescence analysis of NMJ on teased muscle fibres from control subject, P3 and P5. Staining of postsynaptic AChR with α-bungarotoxin (red) showed a well-organized postsynaptic element innervated by one axonal branch (NF168 stain, green) with one terminal arborization in the control sample. In P3 and P5, marked disorganization of postsynaptic AChR was seen with fragmented postsynaptic structures and each AChR cluster was covered either by a thin axonal branch or no axon at all. Terminal and en passant axonal sprouting (arrow) was also visible. An unusual streaky pattern of postsynaptic AChR staining was seen in P3 and to a lesser extent in P5. Scale bar = 10 mm. (B) Schwann cell staining (S100, green) showed terminal Schwann cells with cytoplasmic extensions (P5) or no terminal Schwann cells (P3) in some NMJs. Scale bar = 10 mm. (C) Electron microscopy of two NMJs in P5 showed denervation of postsynaptic structures which neither nerve terminal or terminal Schwann cells (top, asterisk), or incomplete apposition between the postsynaptic element and nerve terminal (bottom, arrow). When present, nerve terminals were filled with synaptic vesicles and mitochondria, and were covered by terminal Schwann cells. Well-defined synaptic basement membrane and active zones facing the crest of the postsynaptic folds (arrowhead) were observed in some terminal nerves at higher magnification. Scale bar = 2 mm (top) and 500nm (bottom). NF; neurofilament. Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

7.2.4 Confirmation of exome variants [74]

As shown in the exome chapter, patient 1 and 2 had been extensively screened for known CMS genes using both Sanger sequencing and linkage studies.
This was a non-consanguineous family in which both parents were healthy and had two affected offspring. Based on this pedigree and the predominance of recessive mutations in association with CMS, I used a recessive model in this filtering analysis searching for both compound heterozygous or homozygous variants in the same gene.

On target good quality variants were categorized based on their genomic location, frequency in public databases and *in silico* predictions of pathogenicity. A total of three candidate variants were prioritized with one located in the known CMS causing gene *AGRN*. Of note, none of the short listed variants were associated with a known neuromuscular disorder and in particular with any myopathies or neuropathies characterized by marked distal muscle weakness (phenocopies). Surprisingly, we identified a seemingly novel homozygous mutation *AGRN*; c.226G>A; p.G76S in exon 2. This variant results in the substitution of a conserved hydrophobic glycine residue to a polar serine in the N-terminal domain.

Segregation analysis of this variant using Sanger sequencing showed that the mother was heterozygous for the pathogenic allele whereas the father and the healthy brother were homozygous for the wild-type allele c.266G. This seemingly wild type homozygous state of the father could not account for the homozygous state of both offspring for the mutant allele c.226A. I was able to exclude non-paternity based on previous haplotype reports and exome data. An ExomeDepth analysis looking for copy number variants successfully detected a ~0.48 Mb hemizygous deletion on chromosome 1, shared between both affected siblings, which contained a total of 236 exons, including the entire *AGRN* gene (table 7.2). To confirm this finding, a standard CGH was carried out on patient 1 and confirmed the presence of a 461 kb hemizygous deletion on chromosome 1 comprising twenty-two genes (1p36.33, position 0,746,649-1,207,683 Mb).

[74] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

For the French family, a "trio approach" was carried in which both parents and one affected offspring (patient 3) underwent exome sequencing. The filtering strategy in this family focussed on genes where either homozygous or more than two heterozygous defects were transmitted from each parent. Thereafter, rare or novel exonic and splice site variant predicted to be deleterious were prioritized. A total of eighteen variants were identified. Similar to patient 1 and 2, disease causing variants were identified in *AGRN* and none of the other variants were positioned in genes associated with known neuromuscular disorders where distal weakness and atrophy are a feature. Patient 3 was shown to be heterozygous for a missense mutation (c.314A>T; p.N105I) in exon 2 on the maternal allele and a frameshift mutation resulting from one nucleotide duplication (c.1362dupC; p.S455Qfs*8) in exon 7 on the paternal allele. Sanger sequencing of both patents and offspring confirmed the variants and revealed appropriate segregation of these two *AGRN* variants with the disease (figure 7.7A).

In summary, in the Norwegian family, both affected siblings were compound heterozygous for a missense mutation (p.G76S) and a large hemizygous deletion containing *AGRN*. This would imply that the only agrin protein expressed would contain the N terminal mutation. Similarly, in the French family, both affected siblings were compound heterozygous for a missense mutation in the N terminal and a frameshift mutation in *AGRN*, which would suggest that the phenotype is dictated by the N terminal mutation in *AGRN*.[75]

Based on the striking clinical phenotype of the first two families, patient 5 was selected via the French CMS network. He was the only affected offspring from a Turkish consanguineous family. Using Sanger sequencing, two homozygous missense variants were identified in *AGRN*. The first was positioned in exon 12 (c.2234C>T; p.A745V) and predicted to be a benign polymorphism by *in silico* software (Mutation Taster). The second defect was a substitution found in exon 32 (c.5611G>A; p.G1871R) in the LG3 domain of agrin. This variant segregated with the disease in the family and involved a well conserved residue of the C terminal of agrin. The substitution of Glycine to

[75] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

Arginine in position 1871 was predicted to be deleterious and most likely to be the disease causing mutation in this patient (figure. 7.7B). [76]

[76] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

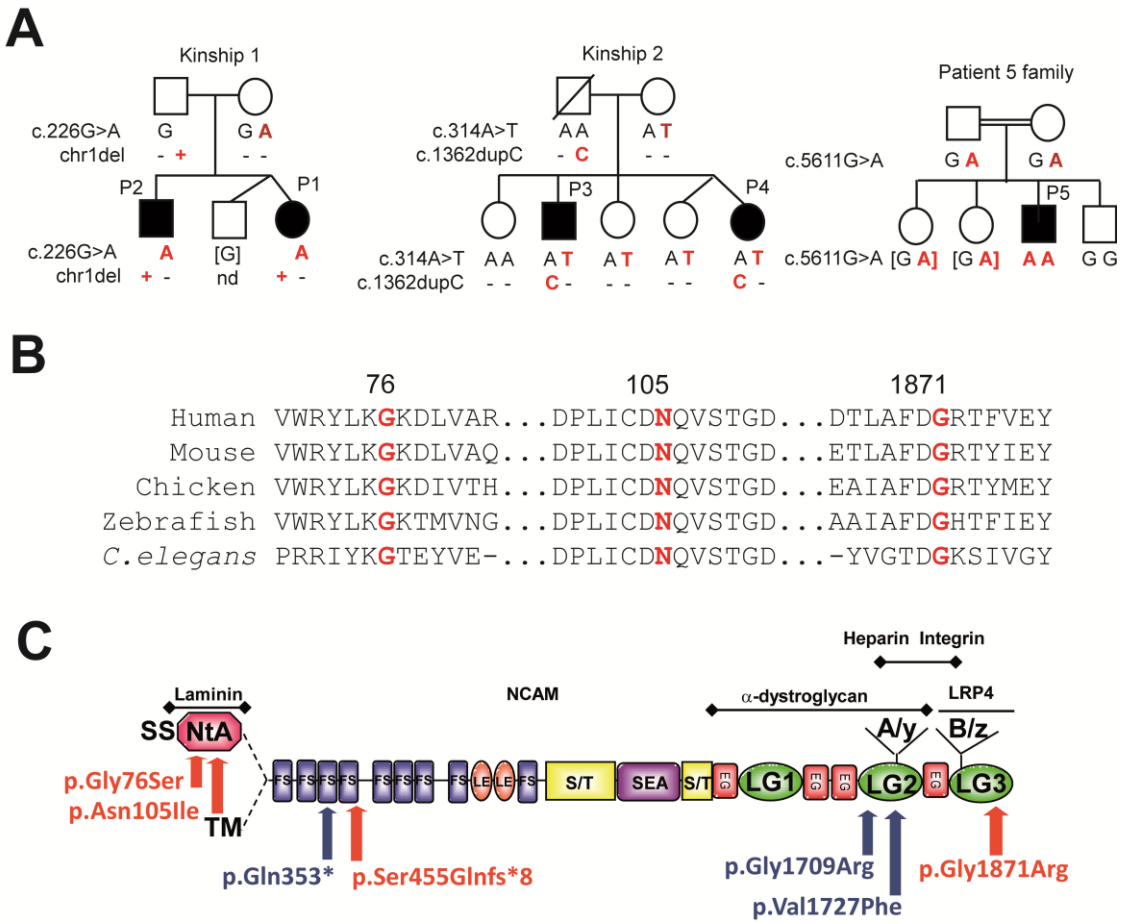

Figure 7.7 (A) *AGRN* mutations (families 1, 2 and Patient 5). These segregated with the disease in each kinship. (B) All three mutated residues were highly conserved. (C) Schematic representation of agrin and its important functional domains and binding partners. The position of the missense mutations linked to congenital myasthenic syndrome with distal muscle weakness and atrophy position of the novel mutations identified in this cohort (red) and previously reported mutations in AGRN related CMS patients (blue). SS: signal sequence; TM: transmembrane segment, FS: follistatin-like domain, LE: laminin EGF-like domain, S/T: serine/threonine-rich region; SEA: sperm protein, enterokinase and agrin domain; EG: epidermal growth factor domain; LG: laminin globular domain. Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

7.2.5 Functional effect of the mutations on agrin function[77]

Mutations p.G76S and p.N105I (figure 7.7C) are the first N terminal agrin mutations reported in relation to human disease. The precise role of this functional domain within the NMJ is unclear. The normal staining of agrin in the NMJ of patient 3 and 5 suggests that these mutations did not significantly alter protein expression and deposition in the synaptic basement membrane (figure 7.8A). Furthermore, immunostaining of the binding partners of agrin (laminin β2 and LRP4) did not display any abnormalities when compared to control (figure 7.9).

AGRN mutations described so far have been shown to alter the ability of agrin to cluster postsynaptic AChRs (Huze et al., 2009, Maselli et al., 2012). Therefore the *in vitro* ability of the two NtA mutant agrins (p.G76S and p.N105I) to aggregate AChRs in C2C12 myotubes was tested. C2C12 myotubes were treated with conditioned media from 293 EBNA cells transiently transfected with the wild type or the NtA mutant agrin constructs. The number of AChR clusters was then calculated. Both NtA agrin mutant-containing media showed reduction in the number of AChR clusters compared to wild type, suggesting that these mutations can, *in vitro*, impair clustering properties of agrin and result in loss of function (figure 7. 5B and C).

[77] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

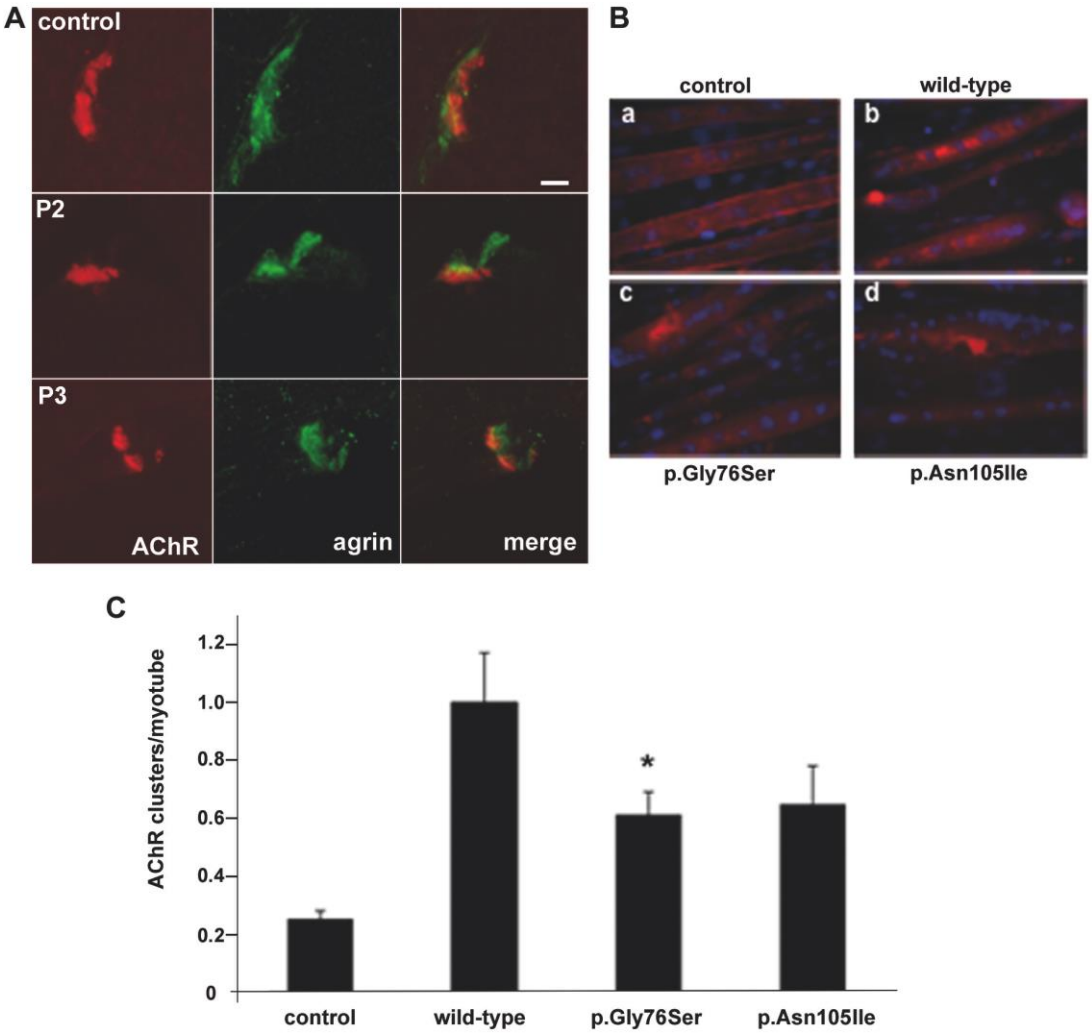

Figure 7.8 (A) Impact of mutation on agrin expression and clustering ability. Immunostaining performed on transversal muscle sections of P2 and 3 showed no reduction in agrin (green) at the NMJ (stained red with bungarotoxin. Scale bar = 5 mm. (B) C2C12 myotubes labelled with Alexa Fluor 594-conjugated - bungarotoxin and DAPI for nuclei (blue). Conditioned medium containing wild-type agrin (wild-type, b) induced numerous clusters of AChR when compared to the medium conditioned by untransfected cells (control, a). AChR clustering was reduced when C2C12 myotubes were incubated with conditioned media containing the p.Gly76Ser (c) and the p.Asn105Ile (d) NtA mutant agrins. (C) Estimation of the numbers of AChR clusters formed by myotube (expressed as the proportion of the AChR cluster number/myotube in response to conditioned culture medium containing wild-type agrin) showed reduced AChR clustering in response to conditioned media containing the p.Gly76Ser and p.Asn105Ile mutant agrins. This difference was statistically significant for the p.Gly76Ser mutation (*P50.02, 2 test) as compared to the wild-type but not for the p.Asn105Ile mutation (P40.05,2 test). AChR clustering in response to mutant agrins was nevertheless still higher than conditioned medium from untransfected cells (control). Each bar graph epresents the mean SEM of AChR clusters per myotube. Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

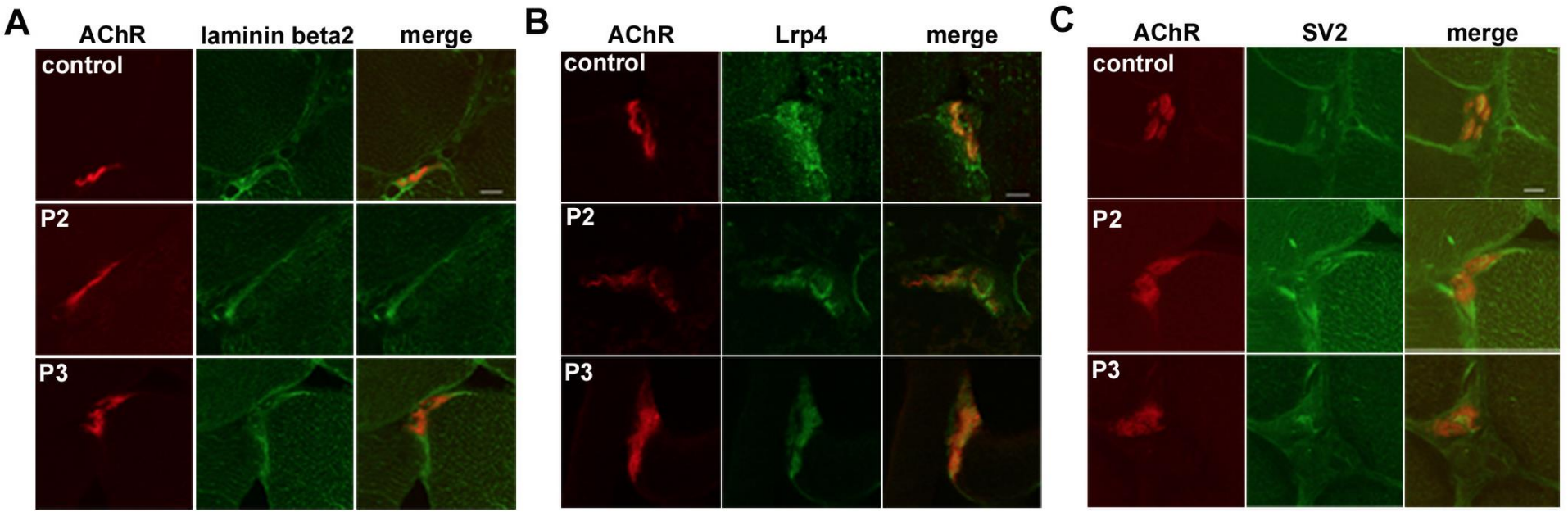

Figure 7.9 (A) laminin beta 2, lrp4, SV2 immuno-staining. This was performed on transversal muscle sections of P 2 and 3 and showed normal labelling. Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

7.3 Discussion[78]

Congenital myasthenic syndromes are a heterogeneous group of genetic disorders associated with defects in transmission between the nerve terminal and the muscle fibre. To date, nineteen genes have been associated with CMS, including *AGRN*, which seems distinct in its involvement with all three compartments of the NMJ. Indeed, this protein is synthesized and released from the presynaptic nerve terminal into the basement membrane and is thought to have a significant role in the maintenance postsynaptic structures (Hantai et al., 2013, Ohkawara et al., 2013).

Agrin associated CMS is very rare and has been reported in two unrelated kinships (Huze et al., 2009, Maselli et al., 2012). The first report was of a 42-year-old female, who presented with ptosis and early motor difficulties, being unable to run with her peers (Huze et al., 2009). Clinically, she had bilateral ptosis with global limb weakness and muscle atrophy of the trunk. She had no ophthalmoplegia, bulbar difficulties or respiratory insufficiency. EMG showed mild myopathic features. Repetitive nerve stimulation showed clear decrement with no facilitation post exercise or supra maximal stimulation. Her brother who was also affected, presented with a similar mild phenotype with clear decrement on neurophysiology. The patients were homozygous for the G1907R in the LG2 domain of agrin. A second kinship with *AGRN* was subsequently described (Maselli et al., 2012). In this family, the patient presented with ophthalmoparesis, facial and generalised limb weakness with clear fluctuations. He developed chronic respiratory insufficiency. Electrophysiological studies revealed clear presynaptic defects; although conventional repetitive stimulation showed decrement usually suggestive of postsynaptic defects in the absence of reduced CMAP amplitude or increment. This patient was compound heterozygote for a nonsense mutation (Q353X) and a missense mutation (V1727F) in in the LG2 domain of agrin.

In contrast to the relatively severe and heterogeneous phenotype described previously, the three kinships described in this chapter show a milder and more consistent phenotype (table 8.2) associated with:

- Onset in the lower limbs with progression to the upper limbs evolving over years.

[78] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

- Differential muscle weakness being more marked in the legs relative to the arms.
- Distal neuromuscular weakness and atrophy.
- Absence of ptosis, ophthalmoparesis or swallowing difficulties.
- Evidence of asymmetric distal and proximal muscle atrophy in the legs on MRI.
- A negative response to AChE inhibitors
- A positive response to ephedrine and salbutamol.
- Evidence of decrement in several muscles, sometimes associated with jitter but not always.
- Evidence of increment, sometimes marked, in several muscles.

However, it is worth noting that some of the patients in this cohort exhibited a more severe phenotype as seen in patient 3 who presented in the neonatal period with respiratory involvement transiently needing respiratory support or the presence of marked fatigability. Furthermore, marked facial weakness was reported in patient 4 but was not a feature in the remaining patients.

It is remarkable that most of the patients described in this cohort were initially diagnosed with a distal form of myopathy. In addition to the overlapping phenotype, these patients exhibited myopathic features on EMG and to a lesser degree on muscle biopsy as well as atrophic muscles on MRI, which may, in a clinical setting, point towards such a diagnosis.

Distal myopathies are a diverse group of inherited disorders where molecular diagnosis can be a challenge and is often guided by the pattern of inheritance, histo-pathological changes, muscle MRI appearance and evidence of cardio respiratory disease. There are least three subtypes of distal myopathies that overlap with the phenotype described in this cohort.[79]

1-Laing myopathy, which results from defects in *MYH7* gene. It is typically associated with distal muscle weakness and wasting, preferentially involving the anterior compartment. By contrast to this cohort, Laing myopathy is a dominant form of distal myopathy that can be associated with a cardiomyopathy (Laing et al., 1995).

[79] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

2- Distal nebulin myopathy, which is allelic with Rod myopathy, is a recessive disorder that also results in distal anterior compartment muscle wasting and weakness. The muscle biopsy is often unremarkable but EM study can show Z line streaming (Wallgren-Pettersson et al., 2007).

3- Distal myopathy with mutations in *DNM2* is a dominant disorder typically involves the posterior compartment of the lower limbs but almost half of the patients will exhibit ophthalmoparesis and have markedly abnormal muscle biopsies showing central nuclei and core like lesions (Bohm et al., 2012).

Distal muscle weakness and wasting have been reported in one subtype of CMS, namely slow channel syndrome. This form is associated with dominant kinetic mutations in the subunits of AChR and characteristically affects the long finger flexors of the forearms. By contrast to this cohort, slow channel CMS does not present with distal leg wasting and weakness and patients will often have a more global pattern of muscle involvement with cervical and ocular muscle weakness (Chaouch et al., 2011). Single nerve stimulation can show repetitive CMAP and muscle biopsy may reveal an endplate myopathy. Slow channel CMS patients benefit from fluoxetine and quinidine but not AChE inhibitors. It is worth noting that none of the variants prioritized using exome sequencing was located in known CMS or distal myopathy genes. Additionally, screening of twenty patients with undiagnosed distal myopathy and 12 patients with suspected CMS and marked distal decrement was negative for mutations in the N terminal domain of agrin. This portion of the *AGRN* gene was sequenced as this seemed to dictate the phenotype in two out of the three families described in this chapter. In this cohort, fatigability and respiratory failure in infancy were useful cues to make the correct diagnosis of CMS.[80]

The neurophysiological findings in all three kinships have emerged as important indicators to the correct diagnosis. Interestingly, despite finding decrement in some muscles (*tibialis anterior*), SFEMG did not detect abnormal jitter in the same muscle group. This is in contrast to what is typically seen in other forms of CMS and

[80] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

autoimmune myasthenia gravis. The clear presynaptic features of low amplitude motor units at rest and marked facilitation (up to 500%) seen in several muscles was striking and reminiscent of Lambert Eaton syndrome caused by an aberrant immune response targeting pre-synaptic (P/Q) voltage gated calcium channel receptors.

Inherited pre-synaptic defects in neuromuscular transmission are very rare in CMS. These have been reported in the context of episodic ataxia caused by *CACNA1* mutations. The phenotype of these patients is distinct with fluctuating cerebellar signs, triggered by exercise and stress. Patients respond well to acetazolamide. The neurophysiological presynaptic features are probably the result of great abundance of Cav1.2 receptors at the neuromuscular junction. An additional two cases have been reported in the literature that remains genetically undiagnosed. These exhibited ophthalmoplegia with marked global limb weakness with respiratory compromise in one patient. None had cerebellar features (Milone et al., 2006)[80].

Patient/Age/Gender	Age at onset	Symptoms/ Course	Fluctuation: Daily/Long term exacerbation/ Other	Signs: Ocular/Face/ Bulbar/ Cervical	Signs					Response to treatment	CK	EMG pattern
					Signs	Distal	Fatigue	Res-piratory	Other features			
1/45y/Female	15y	Weakness in feet, trips, muscle wasting, hand 3 years later, proximal weaknessweakness and wasting. Few years later, waddling gait	No	-/-/-/-	UL+; LL+	UL+; LL++	-	-	Atrophy thenar and inter-ossei muscles	Pyrodostigmine -	N	Myopathic changes with fibrillation potentials and positive sharp waves (distal); Decrement (distal); Post exercise increment in some muscle groups
2/43y/Male	15y	Weakness in feet, trips, difficulties running, hand wasting and weakness few years later	No	-/-/-/-	UL-, LL -	UL+, LL++		-	Atrophy of thenar and inter-ossei muscles.	Pyrodostigmine -	N	Myopathic changes with fibrillation potentials and positive sharp waves (distal); Decrement (distal); Post exercise increment in some muscle groups
3/32y/Male	2y	Episodic apnea; Weakness in feet (7y) and hands (23y); Respiratory failure (30 y); Progressive worsening	-/ + / -	-/-/-/-	UL-; LL- (except knee flexor)	UL+++; LL+++	+	++ NIV	Atrophy of thenar and inter-ossei muscles, legs	AChE inhibitors -; 3,4 DAP -; Ephedrine +70% (proximal muscles)	N	Myopathic pattern (distal); Decrement, post exercise increment (distal)
4/23y/Female	Birth	Isolated stridor at birth; Exercise fatigability (7y); Stumble due to feet weakness (11y);Distal upper limb weakness and atrophy (16y); Progressive worsening	-/-/hot atmosphere worsening	-/+/mild swallowing/-	UL-; LL- (except right knee flexor)	UL++; LL++	+	-	Atrophy of thenar and inter-ossei muscles, legs	AChE inhibitors -; 3,4 DAP -; Ephedrine +70% (proximal muscles)	N	Myopathic pattern (distal); Decrement, post exercise increment (distal)
5/25y/Male	5y	Exercise fatigability (5y); Distal lower limb weakness worsening since the age of 20y; Successive periods of rapid worsening without regression (step pattern)	-/ + / -	Mild questionable/-/-/-	UL-; LL-	UL+; LL++	+	-	No hand atrophy but lower limbs atrophy	Salbutamol + 40% (distal muscles)	N	Myopathic pattern with reduced motor unit potential (distal); Decrement, post-exercise increment (distal)

Table 7.2: Clinical details of all five patients. N ; normal values, UL; upper limbs ; LL ; lower limbs ; NIV; non-invasive ventilation, - ; absent, + ; mild, ++ ; moderate, +++ ; severe. Reproduced from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

All patients harboured at least one missense mutation in *AGRN,* which suggests some correlation between genotype and a phenotype of distal muscle weakness and atrophy. Importantly, this is the first report of N terminal mutations of agrin causing disease since two of the three missense mutations were positioned in this domain. Of note, all previously reported cases harboured mutations in the C terminal domain (LG2) of agrin.[81]

In mice, alternative splicing of agrin at the N terminal generates two discrete isoforms. These seem to differ in terms of length of the initial peptide sequence, which can comprise either 49 short (SN) or 150 long N-terminal (LN) amino acids attached to a 1,900 peptide chain of shared amino acid sequence. Alternative splicing at the N terminal is pivotal for agrin localization, tissue distribution and functional properties. The LN isoform localizes to the basement membrane of the neuromuscular junction as well as other tissues apart from the brain where the alternative splice isoform "SN" is expressed as a transmembrane protein. In the neuromuscular junction, the N terminal domain contains a signal peptide that allows the secretion of agrin into the basement membrane (Kammerer et al., 1999, Burgess et al., 2000). [82]

X ray crystallography of the chicken NtA-portion of agrin (figure 7.10) revealed a β-barrel fold with a high content of charged amino acids, which is bordered on either side by α-helices (Stetefeld et al., 2001).
None of the mutations identified in this cohort involved residues shown to directly bind to the laminin γ1 subunit. Although the mutations reported here did not directly alter the charge of the amino acid, the residues involved exhibit some distinctive features. Mutation G76S implicated a highly conserved Glycine residue, which has the unique property of containing hydrogen at its side chain that provides conformational flexibility to glycine and allows it to reside in portions of the protein where other residue would not tolerate. The second mutation involved a highly conserved Asparagine residue, which is a polar amino acid which has the tendency to lie on the surface of proteins close to aqueous elements. Its substitution with the aliphatic hydrophobic Isoleucine, which is thought to have less conformational flexibility because of its non-hydrogen substituents, may well be significant. Therefore, it seems plausible that the novel

[81] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

N terminal mutations would influence the conformational properties of agrin and can either alter its binding ability to laminin γ1 or result in protein misfolding which would ultimately diminish the content of functional agrin in the neuromuscular junction. [82]

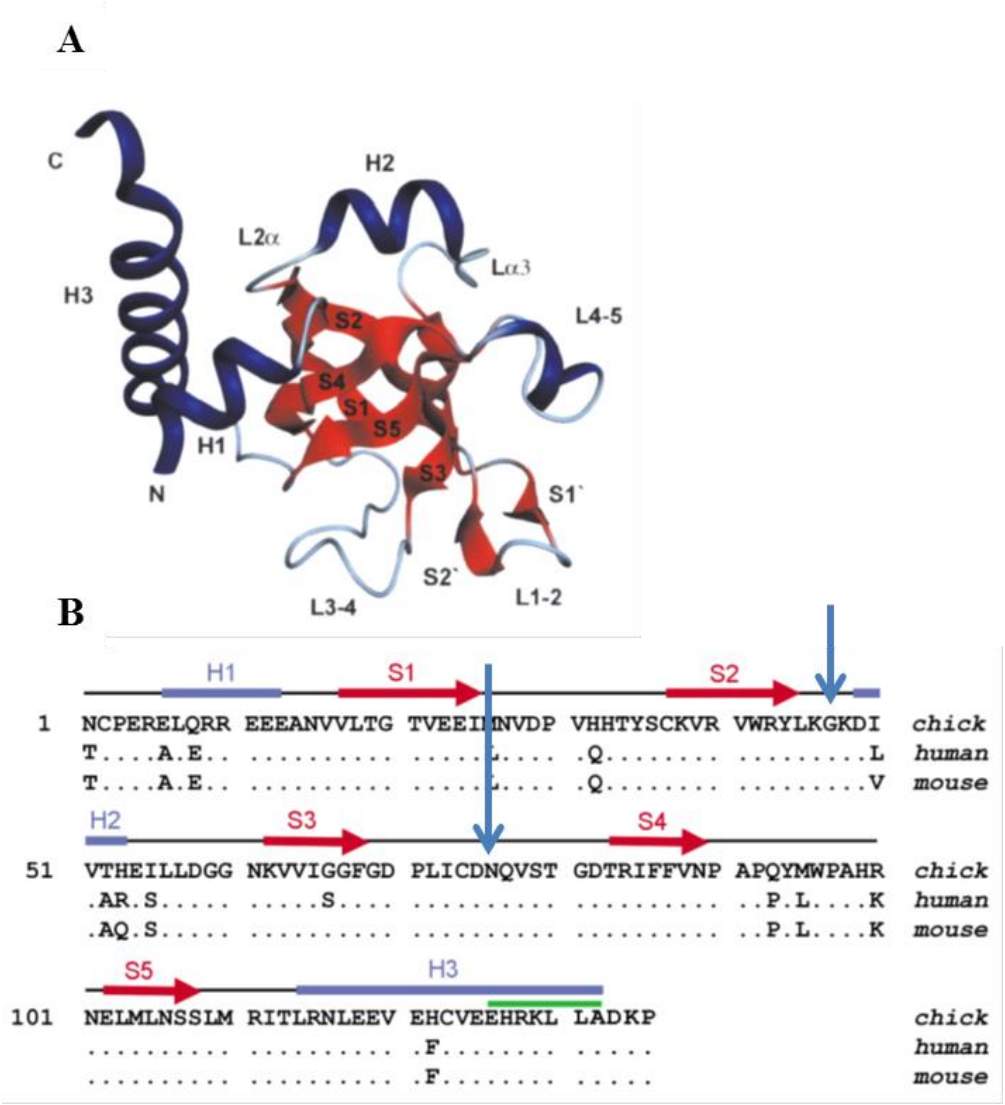

Figure: 7.10 **A**, Schematic diagram of the NtA structure of agrin. β-strands are represented in red and numbered from S1 to S5. α-helices (H1–H3) and the 310-helix in loop L4–5 are in blue. The loops joining strands S1 and S2 (L1–2), strands S3 and S4 (L3–4), and strands S4 and S5 (L4–5) are oriented to the identical surface of the protein. **B**, Alignment of known NtA sequences. Sequence numbers correspond to the mature protein. Helix H3 comprises a splice insert, residues Glu 126–Ala 132 (demarcated by the green bar), whose function is yet unknown. Blue arrows show the two residues mutated (G76S and N105I) in our cohort. Adapted from Stetefeld et al, (2001). Nature Structural Biology. 8, 705 - 709 (2001) doi:10.1038/90422

[82] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

As regard the latter, the results of agrin immunostaining in the patient tissue suggest otherwise. Indeed, there was no reduction in agrin labeling in muscle biopsies. Nevertheless, a more quantitative approach such as western blotting may be more accurate in this setting. Lastly, the AChR binding assay showed altered clustering of the receptors with both N terminal mutants of agrin, more marked with the G76S mutation. This suggests that these novel N terminal mutations may result in loss-of-function effect, similar to previous reports related to mutations in the C terminal domain of agrin (Huze et al., 2009, Maselli et al., 2012). Nevertheless, this consequence appears to be subtle and it is likely that other functions of agrin are compromised by defects in the N terminal domain. Clearly, specific *in vitro* experimental work focused on the N terminal binding properties of the mutant agrin are required to make this study more comprehensive.[83]

The phenotype of all three kinships was characterized by marked distal muscle atrophy, which was confirmed on muscle MRI. Nevertheless, the muscle biopsies available in patients 2 (*vastus lateralis*), 3, 4 and 5, did not show sufficient myopathic changes to account for such muscle wasting. One can therefore speculate that defective neuromuscular junction was the primary drive of this phenomenon, caused in this instance by mutations in agrin. One may also suggest that this length dependent pattern of neuromuscular weakness with evidence of fibre type grouping in the least affected deltoid muscles is reminiscent of a neurogenic process. Morphology of NMJs in the clinically not affected deltoid muscle of two unrelated patients supports active denervation re-innervation events with partial denervation of synaptic gutters, nerve sprouting, and Schwann cell extension. Therefore mutations in agrin may be related to abnormal motor nerve terminals and one can speculate that the primary pathomechanism is pre synaptic neuronal dysfunction.

In addition to widespread denervation-re-innervation events, a remarkable though nonspecific streaky pattern of post-synaptic AChRs was detected. Based on the EM findings in patient 5, this streaky pattern may have resulted from secondary synaptic folds that were not covered by terminal motor axons or Schwann cells as observed in patient 5. This in turn suggests aberrant presynaptic differentiation which correlates

[83] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

with the significant increment observed in several muscles following high frequency stimulation or sustained muscle contraction.[84]

Bearing in mind that several alternatively spliced AGRIN isoforms are synthesized by muscle and nerve and that all patients exhibited marked distal muscle atrophy, one may need to consider the impact of these novel mutations in muscle AGRIN. Muscle AGRIN transcripts lack inserts at the C terminal which markedly reduces the AChR clustering properties of these isoforms. Muscle AGRIN N-terminal sequence is homolougous to the neuronal one. Therefore, muscle AGRIN is expected to carry the N terminal mutations as would the neuronal isoform. Muscle AGRIN, which is expressed on the muscle cell surface, was shown not to be essential for synapse differentiation (Burgess et al., 1999, Fallon and Gelfman, 1989). It has also been shown that muscle AGRIN can bind more avidly to α dystroglycan than its neuronal counterpart (Yamada et al., 1996, Sugiyama et al., 1994). α dystroglycan is a heavily glycosylated protein that has AGRIN and laminin as binding partners in the extracellular matrix and the trans-membraneous β dystroglycan. Both α and β dystroglycans in association with several other proteins form the dystrophin glycoprotein complex, which is believed to be a key structure underlying the pathogenesis of muscular dystrophies (Leite et al., 2008). This complex is thought to confer mechanical stability to the muscle fibre by linking the extracellular matrix to the muscle fibre basement membrane (Lapidos et al., 2004). Defects in muscle AGRIN may therefore destabilise this complex and result in muscle abnormalities. It is worth noting that the CK levels were normal in all patients with AGRIN associated CMS with distal weakness and atrophy, by contrast to the high levels found in α dystroglycan mediated muscular dystrophies".

Time-induced deletion has showed that whole agrin is required for the postsynaptic maintenance in adult mice, and that the postsynaptic alterations progressively lead to presynaptic changes (Samuel et al., 2012). The missense mutations identified here may also point towards domains critical for presynaptic function of agrin.

Agrin knockout mice exhibit early respiratory failure and limb weakness and die within days regardless of the position of the mutations introduced in *AGRN* (Burgess et al.,

[84] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

2000, Gautam et al., 1996). Recently, a mouse model homozygous for a point mutation (p.F1061S) in the SEA domain (agrinnmf387) was shown to be a viable model that appears to accurately replicate human disease showing variable and patchy pre and post synaptic changes with muscle atrophy. The mutation causes partial loss of protein function with abnormal posttranslational glycosylation and diminished protein cleavage and retention within the cell (Bogdanik and Burgess, 2011). This animal model may shed light into some of the molecular mechanisms accounting for the distinct phenotype in these patients.

It has been shown that the disruption of the interaction between laminin β2 and the $Ca_v2.1$ leads to fragmentation of the presynaptic active zones parallel to what is observed in Lambert-Eaton myasthenic syndrome (Nishimune et al., 2004). Through this interaction, the laminins may be critical to the organization of the presynaptic machinery within the active zone to maintain its configuration in relation to the corresponding postsynaptic structures in the muscle fibre. One may therefore postulate that this trans-synaptic platform necessitates agrin and involves several of its functional domains including the N terminal portion. Through its N terminal binding partners (laminins), agrin may partake in the specialization of the presynaptic compartment within the active zone and ensure their alignment with postsynaptic specializations. In the patients harbouring mutations in the N terminal domain, laminin β2 immunostaining was comparable to control. However, no motor endplates were available for EM analysis to corroborate this hypothesis.

In patient 5 who harboured a homozygous mutation in the LG3 domain of agrin, EM analysis was available and showed preserved arrangement of the active zone with normal content of synaptic vesicles within the motor nerve ends. Given the marked streaky pattern of nerve sprouting shared between these patients, one may need to explore the direct influence of mutant agrin on the development and differentiation of the motor nerve which were shown to be implicated *in vitro* studies (Campagna et al., 1997, Wu et al., 2012). Lrp4 was recently shown to be a crucial retrograde signal that modulates presynaptic growth and function (Yumoto et al., 2012, Wu et al., 2012). Through this mechanism, one may be able to explain some of the features seen in patient 5. Furthermore, given the similarities in phenotype between patients, one may

hypothesize that agrin with lrp4 is an important retrograde signal for pre synaptic differentiation[85].

In this study, three CMS families with a distinct distal myopathy phenotype and presynaptic features on neurophysiology are reported. All three families harboured novel mutations in *AGRN* with two families sharing a strikingly similar genotype since both were compound heterozygous for a missense mutation in the N terminal domain of agrin and a null allele. Although, the morphological changes seen at the level of the neuromuscular junction hint to both pre and post synaptic derangement, there seems to be a peculiar pathology in the motor nerve terminal that clearly requires further characterization[85].

[85] Adapted from Nicole et al 2014 (URL: http://brain.oxfordjournals.org/content/early/2014/06/20/brain.awu160.full)

Chapter 8. Mutations in SLC25A1 Cause Impaired Neuromuscular Transmission

8.1 Introduction and aims[86]

Whole exome sequencing was performed in an affected sib pair from an English consanguineous family (CMS-NO). These patients had remained genetically undiagnosed for more than 23 years, having had a suspected clinical diagnosis of congenital myasthenia with a reported response to pharmacotherapy. In this chapter, I present the diagnostic strategy and the evidence collected to support the pathogenicity of the novel *SLC25A1* homozygous variants.

A sizable fraction of CMS patients are without genetic diagnosis (Chaouch et al., 2012a). One may conjecture that some of these genetically undetermined patients may be carriers of extremely rare private mutations in a gene selectively expressed in the NMJ. On the other hand, affected individuals may harbour mutations in genes encoding proteins abundantly expressed in different tissues which may manifest mainly as an NMJ defect but may also be associated with a much more diverse disease spectrum. The recently discovered glycosylation defects in relation to LG-CMS are a good paradigm for this. These defects were until recently primarily linked to congenital glycosylation disorders (Belaya et al., 2012, Cossins et al., 2013). Other examples include mutations in *PLEC1* and *LAMB2* genes in which mutations can lead to a CMS phenotype combined with cutaneous and renal disease respectively (Forrest et al., 2010, Maselli et al., 2009).

Whilst mitochondrial gene defects can result in a multitudinous of neurological disorders such as myopathies and neuropathies, they have not been explicitly implicated in defects of the NMJ (McFarland et al., 2010, Ly and Verstreken, 2006). SLC25A1 is a mitochondrial citrate carrier thought to play a pivotal role in fatty acid and sterol biosynthesis, gluconeogenesis and glycolysis (Kaplan et al., 1993). More recently, it has been proposed that it may also play a part in the maintenance of chromosome

[86] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

integrity and in the regulation of autophagy (Morciano et al., 2009, Catalina-Rodriguez et al., 2012).

In mitochondria, SLC25A1 mediates the exchange of mitochondrial citrate/isocitrate with cytosolic malate. ATP-citrate lyase cleaves the exported citrate into acetyl-coenzyme-A and oxaloacetate (Kaplan et al., 1993, Moraes and Reithmeier, 2012). Acetyl-coenzyme A, is the main substrate for lipid and sterol biosynthesis in the cell whilst oxaloacetate is usually reduced to malate and recycled back into the mitochondria for further exchange with mitochondrial citrate. Citrate can also indirectly influence the rate of glycolysis in the cell by inhibiting cytosolic phosphofructokinase 1. Citrate and malate are both involved in modulating NADPH cellular content (Sun et al., 2010, Moraes and Reithmeier, 2012).

The recent identification of several patients with mutations in this mitochondrial carrier has underscored the critical biological importance of this protein in humans (Edvardson et al., 2013, Nota et al., 2013). The first report was of a patient presenting with a neurodevelopmental disorder manifesting as profound psychomotor retardation, hypotonia, apnoeic crises, poor feeding, epilepsy, postnatal microcephaly, sensori-neural deafness, agenesis of the corpus callosum (ACC) and hypoplastic optic nerves. He had raised urinary hydroxyglutaric acid levels and Krebs metabolites (Edvardson et al., 2013). Subsequently, a series of 12 individuals with mutations in *SLC25A1* were described. These patients were from twelve independent kinships and presented with a homogenous, often lethal, clinical phenotype and recessive *SLC25A1* mutations (Nota et al., 2013). Eight of these patients died in the neonatal period whilst the others survived until early infancy (range 1month - 5years 1month). All displayed delayed psychomotor milestones, hypotonia and seizures (Nota et al., 2013). A large peak of 2 hydroxyglutaric acid was identified in the 13 patients previously reported with *SLC25A1* mutations with no variants identified in L2 or D2 hydroxyglutaric acid gene which implies that this metabolic disturbance is a consequence of mutations in *SLC25A1*.[87]

[87] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

8.2 Results

8.2.1 Clinical phenotype[88]

This was a consanguineous English family (first cousins) with two affected siblings and one healthy offspring. The parents were reportedly both healthy. The index case was a 33-year-old man who was the product of normal pregnancy and birth but displayed speech and motor developmental delay.Early in his childhood, he experienced intermittent, exercise induced leg weakness that improved with rest. He had no ocular, bulbar or respiratory involvement. He had mild intellectual disability but was able to attend mainstream schooling and attain full time employment in a passport office. His neuromuscular symptoms have remained stable over the intervening years. Most recently, a neuropsychiatric picture has emerged in this patient with obsessive compulsive tendencies. Clinical examination revealed mild bilateral ptosis but no ophthalmoplegia. He was dysarthric with fatigable speech. Muscle tone and power (MRC grade 4) were normal at rest with inducible fatigability on exertion. He had bilateral pes cavus as well as calf hypertrophy bilaterally. Reflexes were reduced at rest with clear potentiation with exercise (table 8.1).

His sibling, who is now 19 year old, is the product of normal pregnancy and birth. She exhibited more marked delayed psychomotor milestones, compared to her brother. She sustained multiple falls in early infancy with clear fatigable weakness in all four limbs. She had no bulbar or respiratory symptoms but she complained of intermittent double vision. The course of her illness remained static. Neurological assessment showed mild facial weakness with no ophthalmoplegia or ptosis. She had mild neck flexion weakness (MRC4) with fatigable arm and leg weakness that did not impede her ability to mobilise. Tendon reflexes were preserved. She has moderate learning disability and presently attends a special needs college (table 8.1).

In the presence of intellectual disability and evidence of jitter with block on SFEMG, the differential diagnosis included a congenital myasthenic syndrome or a mitochondrial disorder. Mitochondrial disorders have been associated with abnormal jitter in some

[88] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

patients in the absence of a large fibre neuropathy or significant myopathy (Girlanda et al., 1999).

MRI brain together with MR spectroscopy were normal. Blood and CSF lactate levels were within the normal range. Genetic screening for MELAS, MERRF, LHON, and NARP was negative. Free and total carnitine levels were normal. Acetylcholine receptor antibodies were negative. While the index case had normal karyotype, we identified a 30.4 Mb deletion Xq25 to Xq28, position 122,440,897-154,886,079 using CGH array in the affected sister. The parents have not yet been tested for this deletion. Histo-chemical analysis of an earlier muscle biopsy was reported as normal with no abnormalities in respiratory enzyme chain activity (cytochrome oxidase, succinate dehydrogenase and NADH). There was also no evidence of multiple mitochondrial DNA deletions on muscle analysis. Of note, EM analysis reportedly showed increased number and size of mitochondria in the same muscle. Unfortunately, there was no record of the site of the muscle sampled nor was the full biopsy report available.

Neurophysiological studies were carried out in the index case at aged 20 and 21 years. At age 20, repetitive nerve stimulation of ADM and *anconeus* at 3 Hz stimulation showed no decrement of CMAP and no facilitation after maximum voluntary muscle contraction for 20 seconds. SFEMG on EDC showed evidence of increased jitter in 52% of fibre pairs (25% were normal); 20% showed block, from twenty-five pairs tested. Jitter MCD (mean consecutive difference) from non-blocking pairs was 78.5 +/- 44.4 (microsec +/- ISD). Subsequent repetitive nerve stimulation (3Hz) of ADM and *anconeus*, at age 21 years showed no decrement. SFEMG on EDC showed 81% of potential pairs had increased jitter or jitter with blocking (19% were normal, 62% were increased, 19% were blocking (n=16 pairs; mean MCD was 71.6% +/- 27.8). It is worth noting that nerve conduction studies showed no evidence of a large fibre neuropathy and EMG showed no significant myopathy. The findings of SFEMG were not assessed in relation to the treatment prescribed.[89]

A basic evaluation of endplate morphology in the earlier biopsy revealed increased size of motor endplates. The density of post synaptic AChR was found to be normal on repeat muscle biopsy (*anconeus* muscle). Microelectrode studies showed preserved pre and post synaptic function with normal MEPP amplitude and quantal content.

[89] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

The index case was initially treated with pyridostigmine with no clear benefit. He was later instigated on 3,4-DAP with a favourable response. His sister received pyridostigmine since early childhood, which she continues to this date. It is unclear whether she has any benefit from this treatment[90].

[90] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

8.2.2 Confirmation of exome variant[91]

Since these two affected siblings were born to healthy consanguineous parents suggesting recessive Mendelian inheritance, the following CMS genes were excluded by targeted linkage analysis: *COLQ, CHRNA1, CHRNB1, CHRNE, CHAT, RAPSN, MUSK, SCN4A, DOK7* and *GFPT1*. Sanger sequencing was then carried out to further exclude *CHRNA1, CHRNB1, CHRND, CHRNE, RAPSN, CHAT, DOK7* and *COLQ*. Four large homozygous segments were delineated using homozygosity mapping. These were shared between the two siblings and located on chromosome 5 (7.97Mb); chromosome 13 (19.43Mb); chromosome 16 (17.02Mb) and chromosome 22 (10.62Mb). Given the significant size of these homozygous regions, exome sequencing was carried out to restrict the number of candidate variants.

Whole exome sequencing generated an average of 8.73 Gb of mappable sequences per patient and 53.92% of reads mapped to target. 87% of targeted variants had a 10-fold coverage, which was used for variant calling in our filtering pipeline. Filtering of variants shared by both siblings by restricting the search to coding, rare (frequency <1%), predicted to be deleterious changes, allowed the shortlisting of one homozygous missense mutation in the *SLC25A1* gene (exon7: c.740G>A; p.R247Q, GenBank: NM_005984). This variant was located in a large homozygous block on chromosome 22 and led to the substitution of a highly conserved positively charged arginine residue to a neutral glutamine residue. Of note, the same mutation was identified by the Oxford Group using whole genome sequencing on the older sibling. A second variant in *CLTL1* (c.A719G; p.N240S), involved in synaptic vesicle cycle was also identified. However, this variant was predicted to be non deleterious and was subsequently identified in other public databases, which suggests that it was likely to be a benign polymorphism.

The *SLC25A1* variant (c.740G>A; p.R247Q) segregated with the disease in the family. Both parents were heterozygous for the mutant allele whilst the unaffected brother was homozygous for the wild type allele. Screening of 58 patients from an extended CMS cohort (Munich, Newcastle and Oxford) did not identify any other candidate disease causing variants in *SLC25A1*. Exome sequencing of a further 100 undiagnosed

[91] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

neuromuscular patients including CMS cases did not detect any other *SCL25A1* variants.

Shortly after the results of exome sequencing became available, a patient with compound heterozygous mutations in *SLC25A1* gene was reported in the literature (Edvardson et al., 2013). This patient was compound heterozygous for two *SLC25A1* variants, Chr.22:19163734 C->T resulting in (p.R282H) and Chr.22:19165292 C->T resulting in (p.G130D). This patient exhibited some clinical features reminiscent of abnormal neuromuscular transmission including hypotonia, bulbar and respiratory difficulties. The authors of the report were therefore contacted to check if the patient had evidence of decrement or abnormal jitter. SFEMG at the age of 2 years revealed markedly increased jitter and blocking in the muscles studies. This patient is now aged 3 years and was the only affected offspring of a non-consanguineous Ashkenazi Jewish family. She presented in the neonatal period with poor suck, hypotonia, recurrent apnoea and apathy. Due to her recurrent apnoeic crises and persistent respiratory insufficiency, this patient had a tracheostomy inserted and was commenced on non-invasive ventilation. She exhibited marked psychomotor developmental delay with diminished spontaneous movements. She also suffered several generalised tonic clonic seizures. After establishing that the patient had impaired neuromuscular transmission based on SFEMG results, she was given a trial of AChE inhibitors, 3,4-DAP and ephedrine with little benefit.

In terms of her investigation results, her MRI brain showed corpus callosum agenesis and optic atrophy. Analysis of urinary organic acids revealed a large peak of 2-hydroxyglutaric acid and Krebs cycle intermediates. Mitochondrial function was assessed in cultured skin fibroblasts and revealed reduced growth in glucose free medium, elevated ROS biosynthesis and decreased mitochondrial membrane potential. ATP content was normal in mitochondria[92].

The kinetic properties of mutations (p.R282H) and (p.G130D) were studied using mutant recombinant protein into liposomes. This revealed complete loss of transport function with the former variant and a minor residual activity with the latter (table 8.1).

[92] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

	Case 1	Case 2	Case 3
Gender/age/ethnicity	Male/33y/English	Female/19y/English	Female/18m/Israeli
Mutation	Rg247Q	R247Q	G130D; R282H
Progression	Stable with some long term fluctuations	Stable	Progressive
Age at onset	Less than 2 years	Less than 2 years	10 days
Symptoms at onset	Fluctuating leg weakness	Fluctuating arm and leg weakness	Poor suck, hypotonia, apneoas
Psychomotor delay/intellectual disability	No but developed obsessive compulsive disorder in adulthood	Yes	Yes
Ocular involvement	Ptosis	Ptosis	Optic nerve hypoplasia
Bulbar weakness	No	No	Yes
Apneic crises	No	No	Yes
Epilepsy	No	No	Yes
Agenesis of the corpus callosum	No	No	Yes
Hearing loss	No	No	Yes
Elevated urinary organic acids	No	No	Yes
Abnormal jitter with block	Yes	Not done	Yes
Positive response to treatment	Yes	Equivocal	No

Table 8.1: Clinical features of patients with mutations in the *SLC25A1* gene. Reproduced from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

8.2.3 Biochemical properties of the *SLC25A1* variants[93]

SLC25A1 contains eight exons, which encode the 311 amino acid mitochondrial citrate carrier protein. The percentage of identical amino acids between the yeast and the mammalian orthologous mitochondrial carriers is usually comparable to that observed across the different members of the mitochondrial carrier family (25-30% of identical amino acids (Palmieri et al., 2006). Remarkably, the yeast Ctp1p shares the 37% of sequence identity (52% of sequence similarity) with the human citrate transporter coded by *SLC25A1*. Nonetheless, *S. cerevisae* was shown to be useful for assessing the functional impact of pathogenic mutations in human mitochondrial transporters which have often proved problematic as regard their over-expression in bacteria (Fontanesi et al., 2004, Edvardson et al., 2013).

The amino acid arginine 247 is highly conserved in *SLC25A1* of *fungi* and *metazoan as well as* in numerous other mitochondrial carriers (figure 8.1A, 8.1B). This residue (arginine 247) is part of the transmembrane helix 5 and correlates with the second basic amino acid of the sequence motif [D/E]GxxxxAr[R/K]G. This motif is characteristic of members of the mitochondrial carrier family (Palmieri, 2013).

Using the crystallized structure of the ADP/ATP carrier (Pebay-Peyroula et al., 2003), one can extrapolate that residue R247 is implicated in ionic exchanges with the acidic residues of the second portion of the MCF sequence motif [D/E]Gx5Ar[K/R]G located at the start of even transmembrane helices (figure 8.1c). Two other amino acids of the citrate carrier positioned at the same height on transmembrane helix 1 (Q52) and 3 (K149) share this same property. During substrate transport cycle, alternate closing and opening of the carrier on the matrix side is believed to be facilitated via these intra-repeat interactions (Palmieri and Pierri, 2010).

[93] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

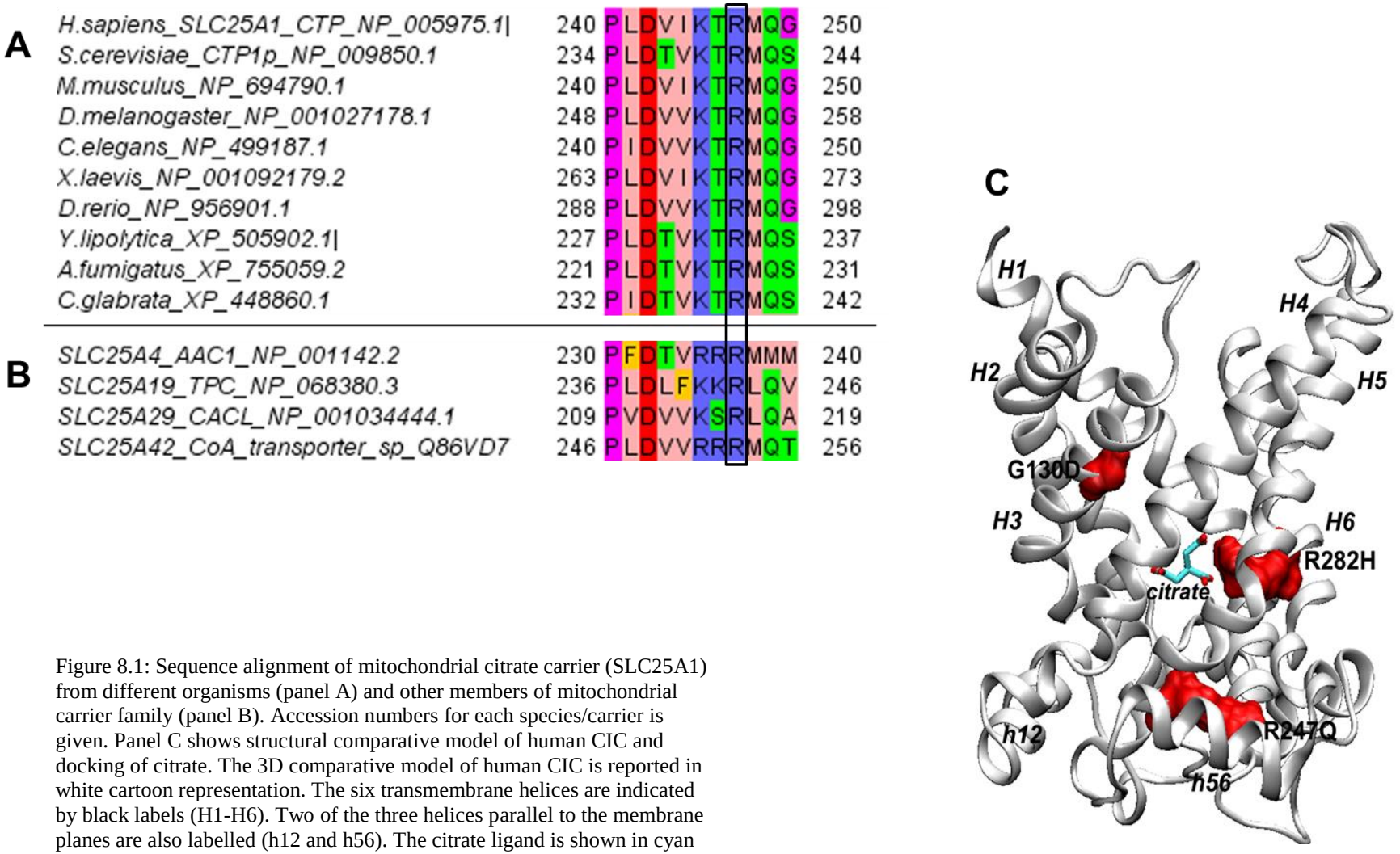

Figure 8.1: Sequence alignment of mitochondrial citrate carrier (SLC25A1) from different organisms (panel A) and other members of mitochondrial carrier family (panel B). Accession numbers for each species/carrier is given. Panel C shows structural comparative model of human CIC and docking of citrate. The 3D comparative model of human CIC is reported in white cartoon representation. The six transmembrane helices are indicated by black labels (H1-H6). Two of the three helices parallel to the membrane planes are also labelled (h12 and h56). The citrate ligand is shown in cyan licorice. The pathogenic mutations R247Q (this work) G130D, R282H (Edvardson et al., 2013) are displayed in red surf representation. Reproduced from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

Kinetic studies of the R241Q Ctp1p mutant were carried out after reconstitution of purified recombinant protein into liposomes. The substitution of R241 with glutamine, which correlates with the substitution of residue R247 in the human mitochondrial transporter, was associated with significant reduction in transporter activity when compared to WT Ctp1p (figure 8.2). It is worth noting in spite of this reduction, some residual activity of the transporter was detected. Furthermore, this reduction was partially corrected by incorporating more SLC25A1 protein (approximately 30%) as shown in figure 8.3. This Western Blotting analysis was performed using cultured skin fibroblasts of the index case and control cells. A small (not significant $p>0.05$) reduction in membrane potential of mitochondria was detected in the patient cultured fibroblasts when compared to control (data not shown). On the other hand, mitochondrial content and network morphology showed no abnormalities.

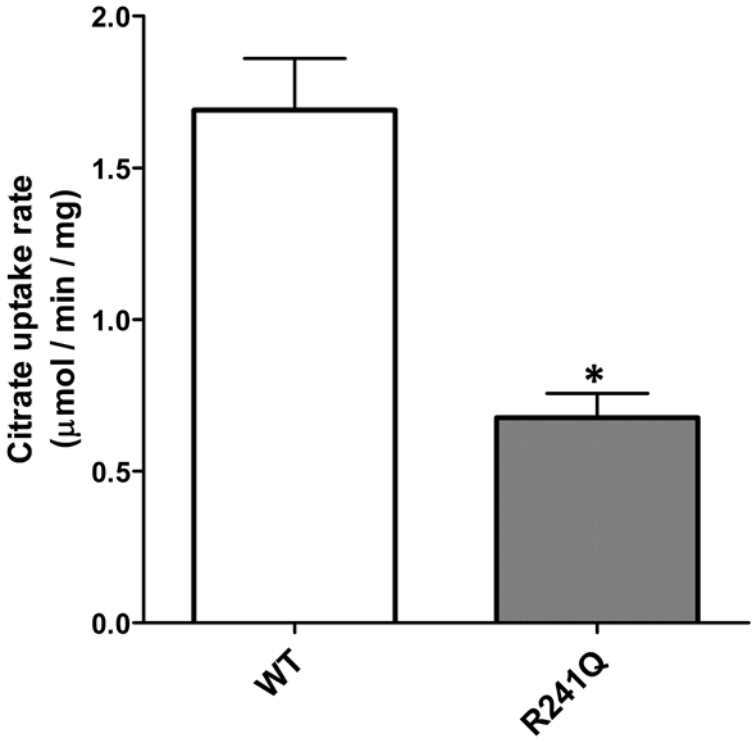

Figure 8.2: Functional characterization of the wild-type (WT) and the p.R241Q Ctp1 p. The uptake rate of (14C) citrate was measured by adding 0.1 mM of (14C) citrate to proteoliposomes reconstituted with purified WT or with the mutated Ctp1 protein. The proteoliposomes were preloaded internally with 10 mM of citrate. The means and SDs from five independent experiments are shown (*$p<0.01$, two-tailed unpaired Student's t-test). Reproducedfrom Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

Recently, *in silico* evaluation the evolutionary limitation of individual amino acid residues in the human mitochondrial carriers was shown to successfully predict the impact of this residue in conserving the function of the mitochondrial transporter (Pierri et al., 2014). When reviewing the 216 residues formerly scored for the mitochondrial carrier SLC25A1 (Pierri et al., 2014), residue R247 lies lower than a median threshold of 4.68 (i.e. in the bottom 50% of all scores). Accordingly, this scoring method suggests that the R247 residue of SLC25A1 bears less functional impact compared to other mutations in SLC25A1 residues. This would be in keeping with the higher protein activity seen in the context of a milder clinical phenotype of the two affected siblings and the normal ranges of urinary 2-hydroxyglutarate (table 8.2).

It is worth noting that all previously reported disease causing mutations in *SLC25A1* scored above the median threshold of 4.68 and were indeed present in patients with a much more severe often lethal phenotype. This includes the previously studied mutations G130D and R282H identified in patient 3 (table 8.2, figure 8.4).

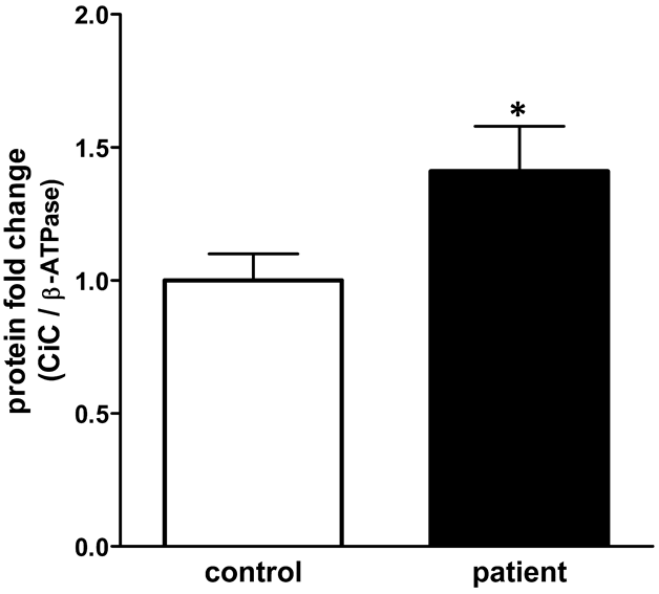

Figure 8.3: Western blot analysis of patient fibroblast cells (and control). Upon separation by SDS-PAGE and SLC25A1 (~31-kDa) and β-ATPase (~55-kDa) levels were determined by densitometric analysis. The relative ratio was calculated and the means and SDs from three independent experiments are shown (*p<0.01, two-tailed unpaired Student's t-test). Reproduced from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

Patients	Clinical phenotype	2-HG levels	Human mutation	SLC25A1 Activity (%)	Residue-specific score
Present study	mild	normal	R247Q	40 (c)	3,38
(Edvardson et al., 2013)	severe (a)	high	G130D	25(c)	5,50
			R282H	0 (c)	5,61
(Nota et al., 2013) patient 1	severe (b)	high	S193W	12.9 (d)	5,51
(Nota et al., 2013) patient 2	-	high	R282G	12.3 (d)	5,61
(Nota et al., 2013) patient 3	severe (b)	high	R282C	6.1 (d)	5,61
(Nota et al., 2013) patient 5	severe (b)	high	G167R	19.8 (d)	5,10
(Nota et al., 2013) patient 6	severe (b)	high	P45L	-	5,00
(Nota et al., 2013) patient 8	severe (b)	high	E144Q	-	5,04
(Nota et al., 2013) patient 10	severe (b)	high	M202T	-	5,33
			Y297C	-	4,89

Table 8.2: Phenotype genotype correlation. Phenotype and mutations of patients affected by SLC25A1-associated pathologies (Edvardson et al., 2013, Nota et al., 2013). A prediction of the functional relevance of each residue is also reported (residue-specific score), as calculated in (Pierri et al., 2014).(a) agenesis of the corpus callosum and optic nerve hypoplasia with marked jitter on SFEMG (b) neonatal epileptic encephalopathy and absence of developmental progress. (c) Percentage of activity of the mutated yeast orthologous protein compared to the yeast wild-type protein [(Edvardson et al., 2013) and this study]. (d) Percentage of citrate export in human mutated fibroblasts compared to human control fibroblasts (Nota et al., 2013). 2-HG: urinary 2- hydroxyglutarate. Reproduced from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132).

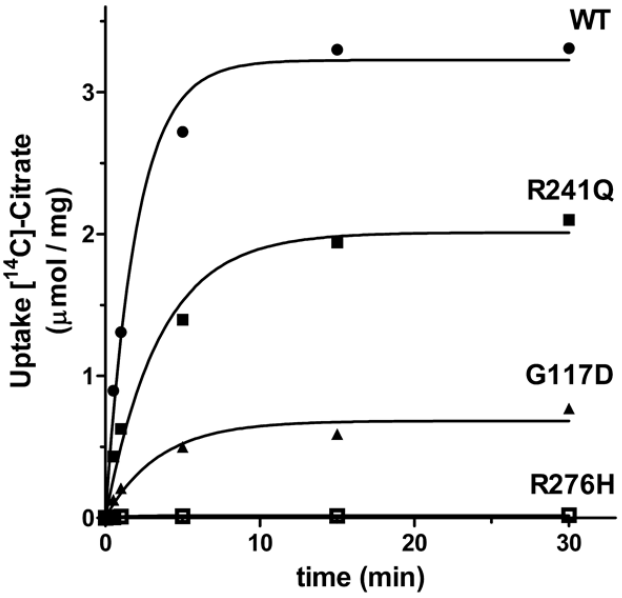

Figure 8.4: Transport assay of wild type (WT) and mutated SLC25A1 protein into liposomes. At time zero 0.1 mM (^{14}C) citrate was added to liposomes reconstituted with the recombinant wild-type or mutants and containing 10mM citrate. At the indicated times, the uptake of the labeled substrate was terminated by adding 20 mM pyridoxal 5′-phosphate and 20 mM bathophenanthroline. Similar results were obtained in four independent experiments. Reproduced from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

8.2.4 Zebrafish SLC25A1 knockdown[94]

The influence of reduced expression of SLC25A1 on the neuromuscular junction was assessed using zebrafish knockdown of this mitochondrial carrier. In zebrafish embryos, two orthologues (*SLC25A1*a and *SLC25A1b*) were shown to be expressed. Injection of both SLC25A1a and SLC25A1b antisense MOs into the embryos produced mis-spliced transcripts in addition to the wild-type transcript at 48hpf (data not shown).

Typically, knockdown of neuromuscular junction proteins impacts on the motility and swimming behaviour of injected embryos. MO-injected embryos exhibited abnormal tail morphology, swimming and touch-evoked escape responses at 48 hpf (figure 8.5). Muscle fibres appeared normal on histochemical analysis in contrast to the NMJ morphology, which was clearly disrupted (figure 8.6, 7). Synapse development was often incomplete with marked abnormalities noted in the presynaptic compartment. The

[94] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

motor nerve terminals appeared short with evidence of haphazard outgrowth in the direction of the muscle fibre (figure 8.6). In embryos injected with a standard control MO and non-injected wild type embryos, the structure and function of NMJ were indistinguishable (data not shown). There was also evidence of multi organ involvement with knockdown embryos frequently exhibiting oedema of the hindbrain, heart, yolk sac and tail (figure 8.5). Cardiac abnormalities correlated with the severity of the phenotype and were associated with diminished blood flow to the tail. Knockdown embryos displayed these characteristics in both the presence and absence of the apoptotic suppressing anti-p53 MO (data not shown).

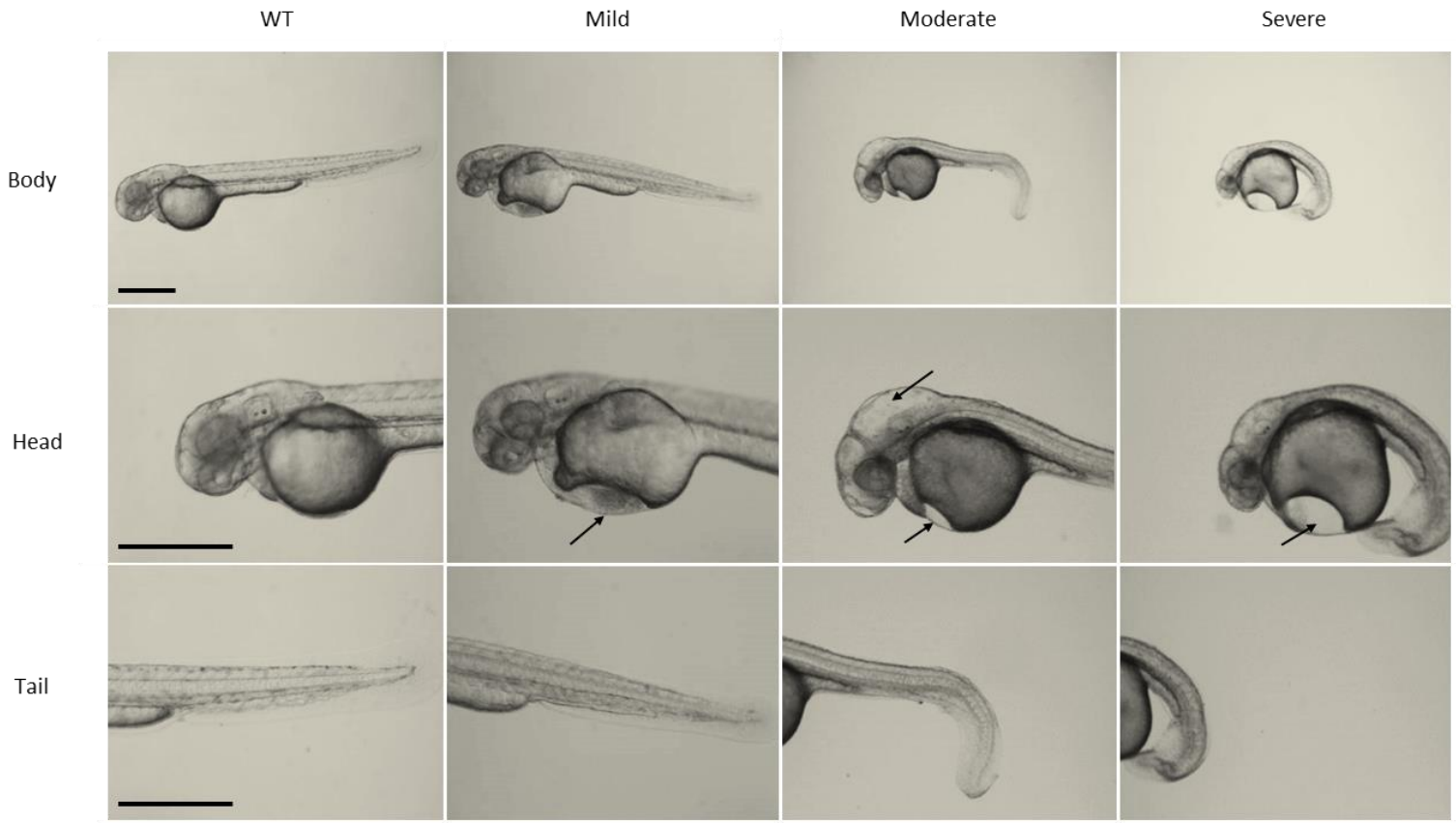

Figure 8.5: Phenotype of SLC25A1 knockdown zebrafish. Live embryos imaged at 48hpf following co-injection of SLC25A1a (5ng) and SLC25A1b (2.5ng). Injected embryos demonstrate a range of phenotypes with mild to severe morphological abnormalities. Embryos exhibit a developmental delay with curvature and shortening of the tail and also oedema of the hindbrain, heart, yolk sac and tail. Scale bars: 500 μm

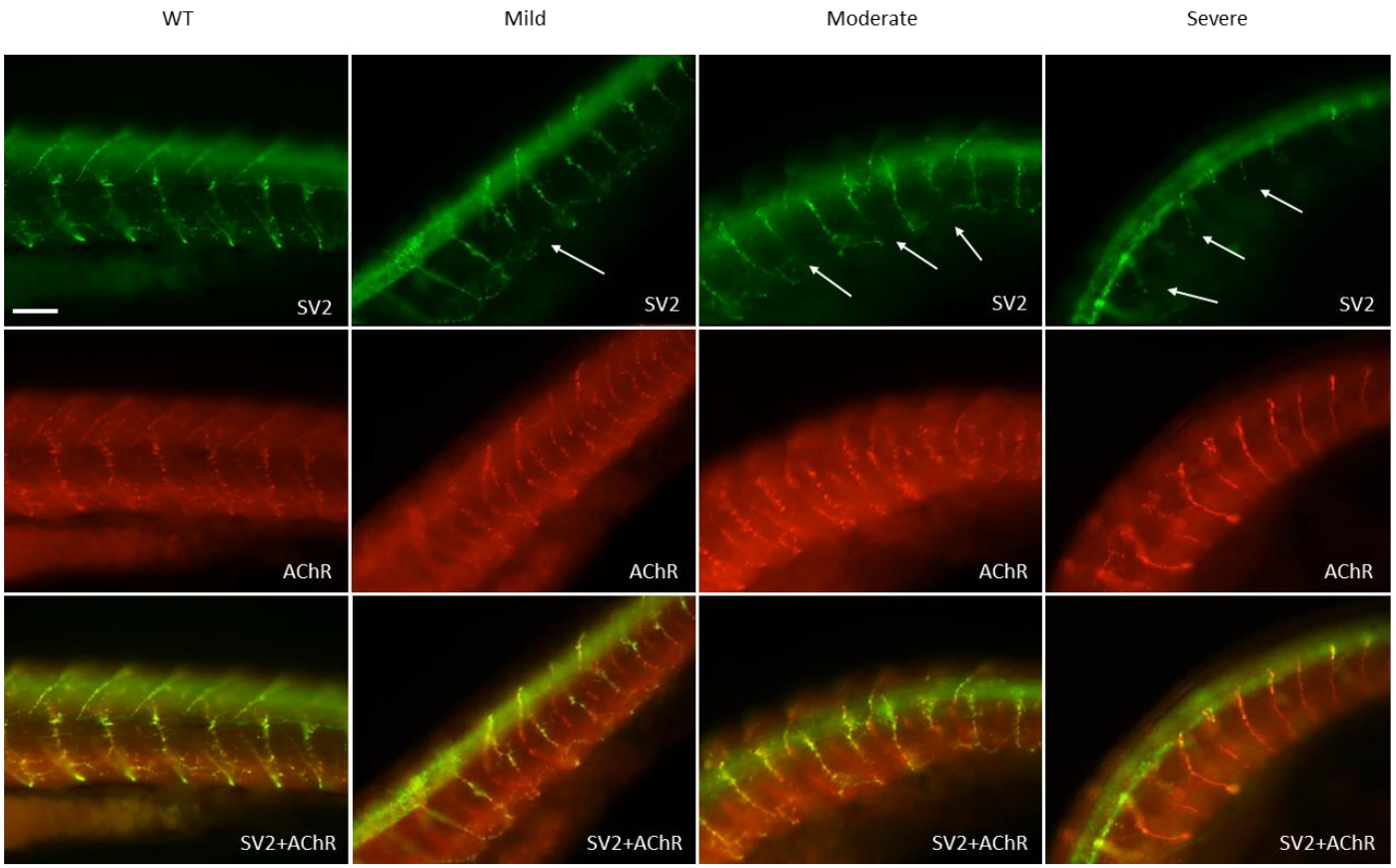

Figure 8.6: Neuromuscular junctions following injection with SLC25A1 MOs. Non-injected control Wild Type Golden embryos (left) and SLC25A1 MO injected embryos were stained for postsynaptic AChR (α-bungarotoxin, red staining) and presynaptic nerve endings (SV2 antibody, green staining). Combined SLC25A1 MO (a 5ng & b 2.5ng) injected 48hpf embryos demonstrate short motor axons and erratic outgrowth toward the muscle fibre. Scale bar: 50 μm. Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

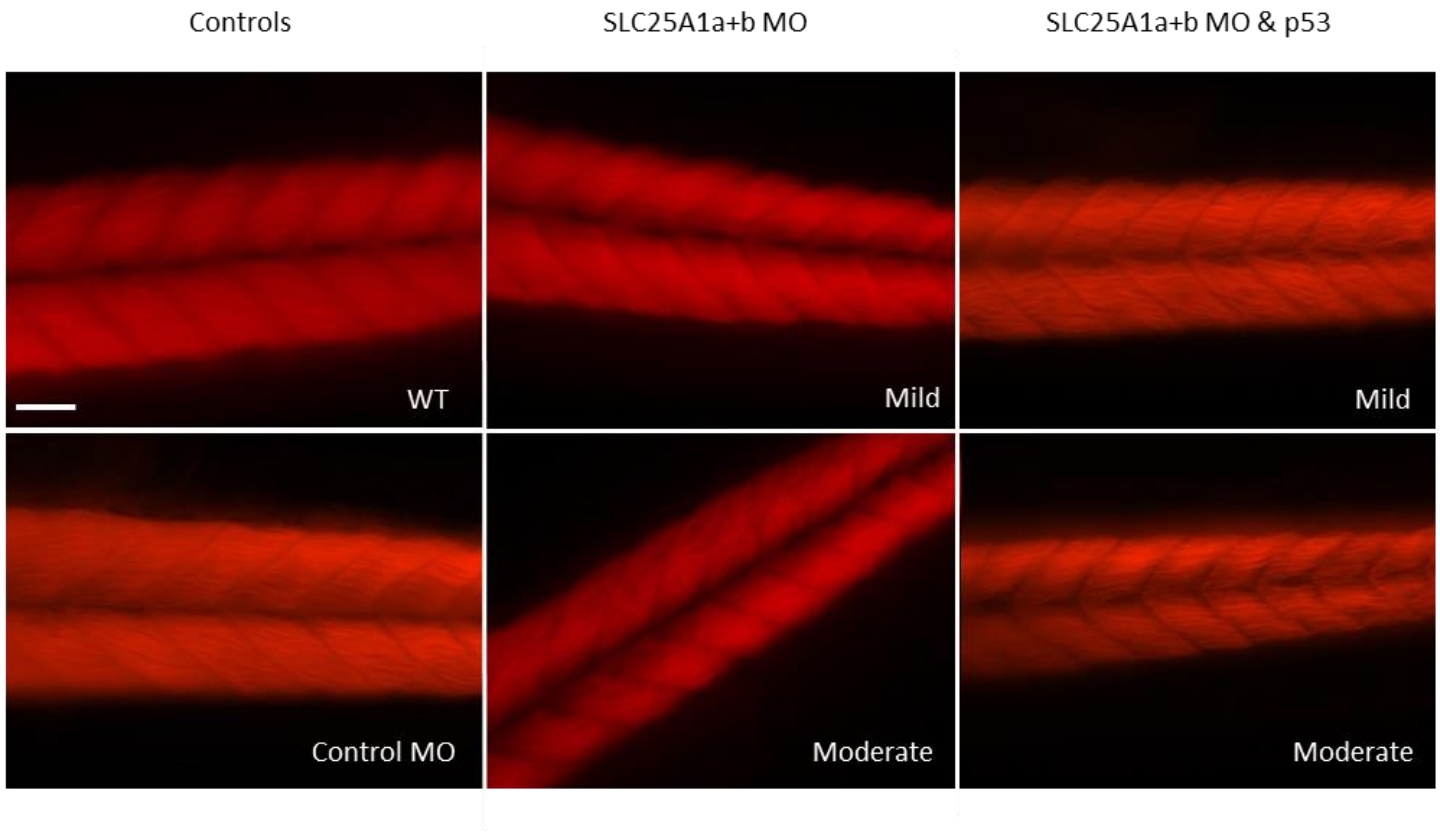

Figure 8.7: Muscle morphology following injection with SLC25A1 MOs. WT and control MO injected embryos (left) and SLC25A1 MO injected embryos (centre and right) were stained for F-actin with Alexa Fluor® 594 conjugated phalloidin (5 µg/ml). WT embryos and those injected with a standard control MO showed normal muscle morphology. Combined SLC25A1 MO with and without the addition of anti-p53 MO also demonstrate normal muscle morphology. Scale bar: 50 µm. Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132

8.3 Discussion[95]

The hallmarks of congenital myasthenic syndrome are fatigable neuromuscular weakness, neurophysiological evidence of abnormal NMJ transmission, absence of anti AChR and anti-MuSK antibodies and response to recommended treatment (Beeson et al., 2005, Hantai et al., 2013). The affected patients reported in this study exhibited all these features, which prompted the working diagnosis of CMS. SFEMG in the index case revealed that a significant proportion of myofiber pairs showed increased jitter with block, in keeping with defective neuromuscular transmission. Both patients exhibited little fluctuations or progression in their neuromuscular symptoms; with no bulbar or respiratory compromise and remain ambulant to this date. Accordingly, the disease course in this kinship seems less severe compared to other CMS categories (Chaouch et al., 2012a).

To define the molecular defect in this family, homozygosity mapping and whole exome sequencing were carried out and successfully identified a novel homozygous missense mutation in the *SLC25A1* gene. This variant was the sole novel variant which involved a highly conserved amino acid predicted to be deleterious and was shown to be expressed in both nerve and muscle tissues.

One unusual feature in this family was the presence of intellectual disability of varying severity in both siblings. The X chromosomal deletion confirmed in the female patient may have contributed to this. This 30 Mb large heterozygous deletion comprised 273 deleted genes including Fragile X Mental Retardation gene (*FMR1*). It has been shown that trinucleotide repeat expansions of this gene cause fragile-X-syndrome which is a common cause of intellectual disability in boys. Given the large size of this hemizygous deletion, a much more severe or probably lethal phenotype would be expected. In the affected female patient, a non-random X-inactivation was confirmed which may point towards a female manifestation of this syndrome triggered by selective inactivation of the intact X (Schmidt et al., 1990).

Dysfunction of SLC25A1 has recently been shown to affect brain, eye and perhaps neuromuscular development, as well as producing an idiosyncratic urinary organic acid

[95] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

profile. It is currently unclear whether this metabolic derangement is solely responsible for CMS like novel phenotype but it may be that additional factors such as oxidative stress, ROS toxicity and cellular NADPH content are also pertinent.
Establishing that one of the previously reported patients with mutations in *SLC25A1* had abnormal neuromuscular transmission, provided further supportive evidence that mutations in this gene were responsible for the disease phenotype in this family (Edvardson et al., 2013).

Of note, the two siblings described in this chapter had a relatively mild phenotype in comparison to the previous reports and exhibited no agenesis of the corpus callosum. They also exhibited a normal urinary organic acid analysis in keeping with a milder clinical phenotype.

SLC25A1 is well conserved across species and was only recently associated with disease in humans (Edvardson et al., 2013, Nota et al., 2013). This mitochondrial citrate transporter was shown to be critical in intermediary metabolism, glucose induced insulin secretion and chromatin integrity (Catalina-Rodriguez et al., 2012, Palmieri, 2013, Morciano et al., 2009, Iacobazzi et al., 2009). The chromosomal location of this gene (22q11.2) also attracted some attention as this is either translocated or amplified in some tumours and deleted in DiGeorge syndrome and possibly schizophrenia (Maynard et al., 2008, Williams et al., 2002).

In this study, the preserved protein expression in patient's skin fibroblast along with the moderately diminished carrier function and the low *in silico* prediction score suggest that the novel pathogenic mutation (p.R247Q) is associated with a less deleterious effect in comparison to all the recently described mutations in *SLC25A1*(Edvardson et al., 2013, Nota et al., 2013).[96]

Despite significant discrepancies amongst the *in silico* scores of R247 and other previously reported mutated residues like G130 (Edvardson et al., 2013), the transporter activity of the yeast orthologous protein carrying either of the two pathogenic mutations at corresponding positions only shows 15% reduction in activity, i.e. 40% and 25%, respectively (table 8.2 and figure 8.4). There are two potential explanations to this

[96] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

observation: The first relates to the yeast model system used in this experiment which may not accurately replicate the *in vivo* carrier activity of the human mitochondrial citrate transporter. Bearing in mind the marked homology between the human and yeast orthologues of *SLC25A1*, in terms of amino acid sequence and transporter activity, this hypothesis seems unlikely. It is worth noting that the p.G130D mutation is heterozygous and the other affected allele carried the p.R282H mutation (Edvardson et al., 2013) which completely abolished transport activity and would be associated with a more severe phenotype. Secondly, the *in silico* prediction score is an evolutionary based value, which relies on the strength of natural selection and correlates with the fitness of organisms, i.e. the probability of organisms to survive and generate fertile progeny (Eyre-Walker and Keightley, 2007). The presented patients achieved reproductive age and may be considered fertile even though the protein activity of the p.R247Q mutant was partially reduced. Accordingly, one expects the prediction score of p.R247Q to be much lower compared to any other tested *SLC25A1* mutations that were instead responsible for lethal phenotype with premature death in neonatal period. Correspondingly, this hypothesis may explain the close scores of the previously reported mutations G130 and R282 (Edvardson et al., 2013), as well as other previously reported pathogenic mutations (Nota et al., 2013), all of which were associated with a more severe often lethal phenotype[97].

Zebrafish has recently been shown to be a useful animal model to assess the impact of protein Knockdown on the neuromuscular junction (Muller et al., 2010, Senderek et al., 2011). Remarkably, *SLC25A1* knockdown confirmed aberrant NMJ development and function similar to that seen in the affected patients regardless of the severity of the phenotype which ranged from mild, moderate to severe. Ultra-structural study of the NMJ showed intact morphology of post synaptic muscle fibres, with marked anomalies in nerve outgrowth and synapse formation. This observation underscores the role of *SLC25A1* in development and maintenance of the NMJ. In addition to the synaptic defects, we demonstrate further alteration in brain and heart morphology and development in the more severely affected zebrafish morphants, in keeping with a more general function of mitochondrial *SLC25A1* in various tissues. It is worth noting that a previous report used an SLC25A1 knockdown zebrafish model to assess its influence on embryogenesis, the integrity of mitochondrial DNA and autophagy. However, limited

[97] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

details were available in terms of organ development and synapse formation (Catalina-Rodriguez et al., 2012)[98].

One may conjecture that the mild CMS phenotype may have facilitated the detection of an NMJ defect in these patients, while this remains undiagnosed in patients with the more disabling, often fatal disease form. Further studies are required to elucidate why the NMJ seems to be particularly sensitive to mild SLC25A1 dysfunction, while other organs such as the brain remain largely asymptomatic. Interestingly, the knockdown experiments in zebrafish showed a similar dependency of symptoms in relation to the knockdown: less severely affected zebrafish morphants showed motility defects and NMJ abnormalities, but more severely affected morphants had additional defects of the brain and heart compatible with a generalized mitochondrial disorder[100].

Mitochondria are ubiquitous organelles that mediate a host of cellular functions including energy production, cellular metabolism, calcium homeostasis and apoptosis (McFarland et al., 2010, Ly and Verstreken, 2006). The role of mitochondria in synapse development and maintenance is probably complex and remains poorly understood. How defects in a ubiquitous mitochondrial citrate carrier can manifest primarily as a NMJ defect remains to be resolved[100].

It is worth stressing that *SLC25A1* is likely to be an extremely rare cause of a pure CMS phenotype. Screening of undiagnosed CMS patients from extended cohorts did not yield other pathogenic mutations in *SLC25A*. Furthermore, only fifteen missense mutations in *SLC25A1* were identified after exome sequencing more than six thousand random individuals (Exome Variant Server; http://evs.gs.washington.edu/EVS/, accessed April 2014). Each mutation was heterozygous and present in less than 0.1% of the screened population. Based on the described *in silico* prediction scoring system, a minimum of seven mutations were predicted to significantly impair transporter function and would in effect represent an existing pool of pathogenic alleles circulating in the human population. Consequently, while the presence of homozygous or compound heterozygous mutations in *SLC25A1* is likely to be a rare event, should a patient display

[98] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

fatigable neuromuscular weakness, screening of *SLC25A1* mutations may be considered.[99]

[99] Adapted from Chaouch et al 2014 (URL:http://iospress.metapress.com/content/l36500736v3r4132)

9. Final conclusions and future directions

Congenital myasthenic syndromes are rare disorders, the phenotype of which primarily reflects abnormal transmission across the neuromuscular junction. Despite being rare, this disease entity remains at the centre of research focused on deciphering the biological relevance of synaptic proteins and unravelling how particular defects can disturb sophisticated mechanisms that ensure reliable neuromuscular transmission.

The review of the nineteen CMS subtypes based on their underlying molecular defects revealed significant heterogeneity in terms of clinical phenotype, neurophysiological findings and response to treatment. Nevertheless, there was evidence of some correlation between particular symptoms and molecular defects as seen with presence of apnoea in CHAT or RAPSN related CMS. Although decrement is a useful indicator of impaired NMJ function, it is less able to predict the underlying molecular defect. Nonetheless, certain electrical patterns can be more specific such evidence of repetitive CMAP on single nerve stimulation, seen in slow channel CMS and COLQ related CMS. In clinical setting, the majority of CMS patients carry defects in the postsynaptic proteins with mutations in the *CHRNE* gene being the commonest and the most compatible with life. The choice of pharmacotherapy is dependent on the molecular defect and it is crucial that clinicians approach CMS patients in a holistic fashion with attention to respiratory and bulbar difficulties.

9.1 Cross sectional study of the Munich CMS cohort

The cross sectional study of the Munich CMS cohort aimed at reporting on the clinical and genetic profile of a large cohort of 680 independent patients with suspected CMS. It also helped ascertain the efficacy of the chosen diagnostic strategy based upon a step-by-step Sanger based mutation screening. In total, disease causing mutations were successfully detected in 44% of patients in various known genes, endorsing the marked genetic heterogeneity observed in CMS. Except for the four previously reported founder mutations in the *CHRNE*, *DOK7* and *RAPSN* genes, the majority of molecular defects identified were private mutations in isolated kinships. In the Munich cohort, the

mutation c.1267delG in *CHRNE* in South Eastern European patients was most prevalent.
This study also revealed that in the absence of specific phenotypic clues, one may consider screening for the three common mutations in *CHRNE*, *DOK7*, and *RAPSN*.

Based on the current understanding of genotype phenotype correlations in CMS, it was possible to confirm the importance of some parameters such as ophthalmoplegia, decrement and positive family history, in predicting a higher mutation detection rate. These different cues were used to design an algorithm for genetic testing which highlighs the importance of close collaboration between health professionals including clinicians, genetic counsellors, neurophysiologists and pathologists to ensure accurate genetic diagnosis and optimum patient care. In the context of emerging next generation sequencing, this study has reinforced certain concepts of phenotype genotype correlation and facilitated the production of a reliable and cost efficient algorithm which will be crucial in terms of interpreting the large number of variants likely to be generated from next generation sequencing.

9.2 Clinical characterization of a slow channel CMS cohort

Slow-channel syndrome is a rare form of CMS and exhibits distinct clinical and genetic characteristics. This retrospective study focused on the clinical phenotype, phenotype genotype correlation and response to treatment in a sizable cohort of patients. The data revealed some heterogeneity with regard to onset of symptoms, severity of disease and mutations. Nonetheless, patients shared a number of features including the preferential involvement of the cervical and forearm extensor muscles and lack of response to conventional treatment with AChE inhibitors. Several mutations were detected, most of which were situated in a transmembrane domain or near the binding site of the receptor. Although this study endorses the observation that treatment with fluoxetine and quinidine are beneficial in most patients; occasionally, patients suffered serious adverse effects related to fluoxetine that hampered optimum escalation of the dose. The current lack of alternatives leaves patients vulnerable to potentially life threatening events related to this condition.

9.3 Clinical characterization of CMS cohort with *GFPT1* mutations

Limb girdle myasthenia is a rare and distinct phenotype characterized by fatigable proximal muscle weakness with minimal ocular and facial involvement. Until recently, *DOK7* mutations were the only known molecular defect associated with this phenotype. Mutations in *GFPT1* were recently unravelled in a cohort of undiagnosed limb girdle myasthenia using positional cloning approach. This study was able to provide a more comprehensive review of the phenotypic spectrum of this disease and highlighted differences compared to DOK7 related CMS. In general, patients with mutations in *GFPT1* exhibited a homogenous clinico-pathological phenotype with symptoms starting within the first decade, a consistent pattern of proximal limb weakness, presence of tubular aggregates on muscle biopsy and a positive response to AChE inhibitors. This correlation is likely to contribute to the better diagnosis; treatment and counselling of patients with GFPT1 related CMS.

9.4 Gene discovery in CMS using whole exome sequencing

Over the past five years, whole exome sequencing has transformed disease gene discovery. This approach relies on a highly sophisticated biotechnology for parallel sequencing and relies on a computational analysis aimed at setting filters to restrict the search to novel or rare variants, known or predicted to be pathogenic. The success rate from whole exome sequencing is unknown. In this study, less than 50% of families were successfully diagnosed. Limitations were primarily related to suboptimum coverage, evolving *in silico* prediction tools and public databases that do not always provide accurate frequency data nor necessarily reflect the genotype of smaller ethnic groups.

9.5 AGRN related CMS with distal muscle weakness and wasting

Using exome sequencing, compound heterozygous mutations in the *AGRN* gene were successfully identified in a Norwegian kinship. These mutations were associated with a strikingly homogenous phenotype with impaired neuromuscular transmission and distal

muscle weakness and atrophy. Based on this phenotype, an additional three patients from two independent kinships were identified via the French CMS network. These patients shared distal limb muscle wasting and weakness and showed no response to AChE inhibitors.

Neurophysiological studies were key in unravelling impaired neuromuscular junction. In addition to marked decrement, there was evidence, sometimes, of significant increment after sustained muscle contraction (up to 285%), in keeping with a presynaptic defect. The latter is not a common finding in CMS but is a landmark of autoimmune Lambert Eaton myasthenic syndrome. Muscle biopsy showed patchy myopathic changes, which may mislead clinicians to a diagnosis of primary myopathy. At ultra-structural level, there was evidence of synaptic remodelling with a combination of denervation and re-innervation events. Two of the novel mutations identified were situated in the agrin NtA domain and were shown to diminish acetylcholine receptors clustering. The effect of these mutations on laminin binding was not assessed but no abnormal labelling of agrin, lrp4 or laminin β2 was noted in the patients' muscle.

This study expanded the phenotypic spectrum of AGRN related CMS and revealed a remarkable correlation between genotype and phenotype. Accordingly, one can stipulate the need to investigate patients with distal myopathy for an underlying neuromuscular transmission disorder and to consider genetic testing for *AGRN* mutations.

9.6 Mutations in SLC25A1 cause impaired neuromuscular transmission

A novel homozygous variant in the *SLC25A1* gene was also identified using exome sequencing in a consanguineous English kinship. This gene encodes the mitochondrial citrate carrier. The phenotype of these patients was suggestive of CMS with fatigable weakness and ptosis. They also exhibited some intellectual disability and neuropsychiatric features. Response to conventional treatment was equivocal. Of note, a previously reported patient with different compound heterozygous missense mutations of *SLC25A1* was later shown to have abnormal neuromuscular transmission. This gene *SLC25A1* had been reported to cause a severe often lethal disease. The two patients

described in this study displayed a less severe phenotype manifesting mainly as a neuromuscular junction disorder.

To assess the pathogenicity of this novel mutation, mutant SLC25A1 expression in yeast and its transporter function were assessed *in vitro* and a knockdown zebrafish model was generated. These experiments showed normal SLC25A1 protein expression with abnormal carrier function in the patient's tissue. Additionally, knockdown of SLC25A1 expression in zebrafish replicated the human disease, exhibiting variable brain and eye abnormalities with impaired motility. The zebrafish model also demonstrated defective development of the neuromuscular junction with abnormal axonal outgrowth irrespective of the severity of the phenotype.

9.7 Future perspectives

Using traditional methods, a number of CMS patients remain with no genetic diagnosis. The introduction of high throughput sequencing technology is likely to improve the diagnostic yield in known genes, but will require a collaborative approach to help interpret the colossal number of variants generated and refine the diagnostic algorithm. The study of very rare disorders such as CMS will continue to be limited by the small number of patients available. The discovery of mutations in *GFPT1*and more recently *DPAGT1*, *ALG2* and *ALG14* genes associated with limb girdle myasthenia have established the importance of glycosylation in maintaining the integrity of the neuromuscular junction. Given the ubiquitous function of these enzymes, it would be worth ascertaining if patients have subclinical involvement of other organs. The presence of variable abnormalities in both the pre and postsynaptic compartments suggest that additional mechanisms other than impaired glycosylation of the AChR subunit are at play. The challenge here may be to successfully generate a mammalian animal model that can replicate the human disease.

The striking homogenous clinical and neurophysiological features seen in AGRN related CMS with distal muscle weakness, are likely to reflect a common molecular mechanism. In the first instance, the effect of N terminal of agrin mutations on laminin binding should be assessed followed by further *in vivo* work to understand the

mechanism of presynaptic changes seen in all patients. Given the marked distal muscle atrophy seen in these cases, the expression and function of the muscle isoform of agrin would be pertinent to assess. Finally, the effect of mutant SLC25A1 on the presynaptic compartment awaits to be elucidated. One may consider exploring the impact of the mutant protein on the bioenergy of the synapse and transmitter release from the presynaptic compartment.

Our report on the treatment of slow channel CMS has underlined the difficulties accoutred with conventional treatment. This is also true of other CMS subgroups. One would hope that future progress leads to more specific and safer therapies.

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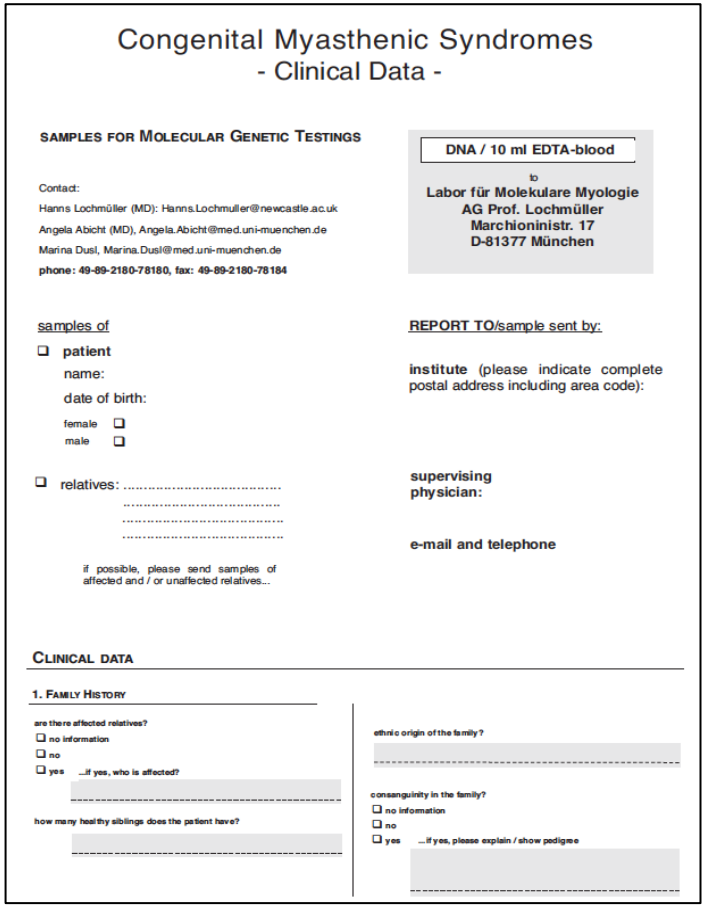
Appendix

A1. Munich CMS questionnaire



Congenital Myasthenic Syndromes
- Clinical Data -

SAMPLES FOR MOLECULAR GENETIC TESTINGS

Contact:
Hanns Lochmüller (MD): Hanns.Lochmuller@newcastle.ac.uk
Angela Abicht (MD), Angela.Abicht@med.uni-muenchen.de
Marina Dusl, Marina.Dusl@med.uni-muenchen.de
phone: 49-89-2180-78180, fax: 49-89-2180-78184

DNA / 10 ml EDTA-blood

to
Labor für Molekulare Myologie
AG Prof. Lochmüller
Marchioninistr. 17
D-81377 München

samples of

❑ **patient**
name:
date of birth:
female ❑
male ❑

❑ relatives: .....
.....
.....
.....

if possible, please send samples of affected and / or unaffected relatives...

REPORT TO/sample sent by:

institute (please indicate complete postal address including area code):

supervising physician:

e-mail and telephone

CLINICAL DATA

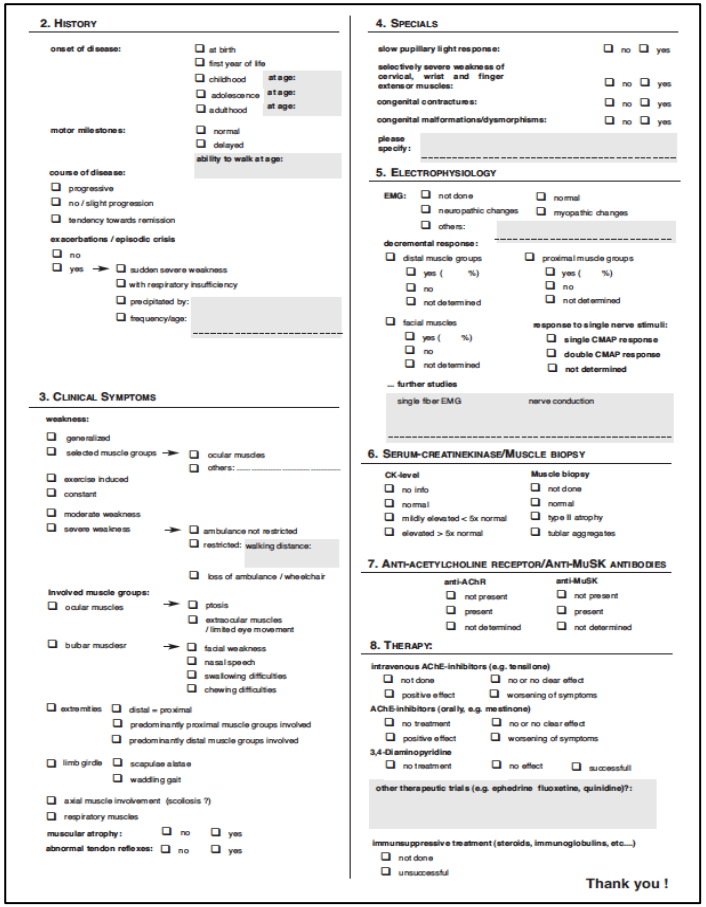
1. FAMILY HISTORY

are there affected relatives?
❑ no information
❑ no
❑ yes ...if yes, who is affected?

how many healthy siblings does the patient have?

ethnic origin of the family?

consanguinity in the family?
❑ no information
❑ no
❑ yes ...if yes, please explain / show pedigree



2. HISTORY

onset of disease:
❑ at birth
❑ first year of life
❑ childhood **at age:**
❑ adolescence **at age:**
❑ adulthood **at age:**

motor milestones:
❑ normal
❑ delayed
ability to walk at age:

course of disease:
❑ progressive
❑ no / slight progression
❑ tendency towards remission

exacerbations / episodic crisis
❑ no
❑ yes → ❑ sudden severe weakness
❑ with respiratory insufficiency
❑ precipitated by:
❑ frequency/age:

3. CLINICAL SYMPTOMS

weakness:
❑ generalized
❑ selected muscle groups → ❑ ocular muscles
❑ others:
❑ exercise induced
❑ constant
❑ moderate weakness
❑ severe weakness → ❑ ambulance not restricted
❑ restricted: walking distance:
❑ loss of ambulance / wheelchair

involved muscle groups:
❑ ocular muscles → ❑ ptosis
❑ extraocular muscles / limited eye movement
❑ bulbar muscles → ❑ facial weakness
❑ nasal speech
❑ swallowing difficulties
❑ chewing difficulties
❑ extremities ❑ distal = proximal
❑ predominantly proximal muscle groups involved
❑ predominantly distal muscle groups involved
❑ limb girdle ❑ scapulae alatae
❑ waddling gait
❑ axial muscle involvement (scoliosis ?)
❑ respiratory muscles
muscular atrophy: ❑ no ❑ yes
abnormal tendon reflexes: ❑ no ❑ yes

4. SPECIALS

slow pupillary light response: ❑ no ❑ yes
selectively severe weakness of cervical, wrist and finger extensor muscles: ❑ no ❑ yes
congenital contractures: ❑ no ❑ yes
congenital malformations/dysmorphisms: ❑ no ❑ yes
please specify:

5. ELECTROPHYSIOLOGY

EMG: ❑ not done ❑ normal
❑ neuropathic changes ❑ myopathic changes
❑ others:

decremental response:
❑ distal muscle groups ❑ proximal muscle groups
❑ yes (%) ❑ yes (%)
❑ no ❑ no
❑ not determined ❑ not determined
❑ facial muscles
❑ yes (%)
❑ no
❑ not determined

response to single nerve stimuli:
❑ **single CMAP response**
❑ **double CMAP response**
❑ **not determined**

... further studies
single fiber EMG nerve conduction

6. SERUM-CREATINEKINASE/MUSCLE BIOPSY

CK-level
❑ no info
❑ normal
❑ mildly elevated < 5x normal
❑ elevated > 5x normal

Muscle biopsy
❑ not done
❑ normal
❑ type II atrophy
❑ tubular aggregates

7. ANTI-ACETYLCHOLINE RECEPTOR/ANTI-MUSK ANTIBODIES

anti-AChR
❑ not present
❑ present
❑ not determined

anti-MuSK
❑ not present
❑ present
❑ not determined

8. THERAPY:

intravenous AChE-inhibitors (e.g. tensilone)
❑ not done ❑ no or no clear effect
❑ positive effect ❑ worsening of symptoms
AChE-inhibitors (orally, e.g. mestinone)
❑ no treatment ❑ no or no clear effect
❑ positive effect ❑ worsening of symptoms
3,4-Diaminopyridine
❑ no treatment ❑ no effect ❑ successful

other therapeutic trials (e.g. ephedrine fluoxetine, quinidine)?:

immunsuppressive treatment (steroids, immunoglobulins, etc....)
❑ not done
❑ unsuccessful

Thank you !

A2. Slow channel CMS questionnaire

Referring clinician:.....

Patient name:.....

DOB:.....

Gender:.....

Origin:.....

Mutation:.....

Please answer yes or no

Age of onset of symptoms.....

Symptoms at presentation

Ocular: yes ☐ no ☐

Bulbar: yes ☐ no ☐

Respiratory: yes ☐ no ☐

Limb weakness: yes ☐ no ☐

If yes, please specify.....

.....

.....

.....

.....

.....

.....

Was there any suggestion of fluctuation of symptoms in the history?

Yes ☐ no ☐

If yes, was it Diurnal (morning vs. Evening) ☐ day to day ☐

Week to week ☐ month to month ☐

Did symptoms worsen during menstrual cycle/ pregnancy?

Yes ☐ no ☐

If yes, please specify.....

.....

.....

Was there a history of respiratory crises?

Yes ☐ no ☐

If yes, what was the cause? .....

.....

How would you describe the course of the illness since onset?

Static: yes ☐ no ☐

Progressive: yes ☐ no ☐

Fluctuating: yes ☐ no ☐

Pregnancy/birth and development

Were there any problems during pregnancy or birth of the child/patient?

Yes ☐ no ☐

If yes, please specify.....

.....

Did the child/patient have normal developmental milestones? Yes ☐ no ☐

If no, please specify.....

Clinical examination at presentation: please answer yes or no

Cranial nerves

Ptosis: Yes ☐ no ☐ if yes, was it unilateral ☐ or bilateral ☐

Ophthalmoparesis: yes ☐ no ☐

Facial weakness: yes ☐ no ☐

Bulbar weakness: yes ☐ no ☐

Neck flexion weakness: yes ☐ no ☐

if yes, what was the power (MRC grade).....

Limbs

Inspection

Muscle atrophy yes ☐ no ☐

if yes, was it proximal ☐ distal ☐

Scapula winging yes ☐ no ☐

If yes, was this symmetrical ☐ or asymmetrical ☐

Scoliosis yes ☐ no ☐

Rigid spine yes ☐ no ☐

Contractures yes ☐ no ☐

If yes, which joints? fingers ☐ wrists ☐

Elbows ☐ ankles ☐

Other, please specify.....

Assessment of weakness

Respiratory: what was the force vital capacity (FVC)? (.....%)

Proximal power (MRC grade): Upper limbs...../5 Lower limbs...../5

Distal power (MRC grade): upper limb...../5 Lower limbs...../5

Evidence of fatigability clinically yes ☐ no ☐

If yes, please specify which muscle.....

Reflexes: normal yes ☐ no ☐ if no, please specify.....

Sensory deficit: yes ☐ no ☐ if yes, please specify.....

Autonomic features yes ☐ no ☐ if yes, please specify.....

Investigations:

CK value: .....**at what age**.....

Muscle biopsy report (which muscle and at what age)

.....

.....

.....

Tensilon test (if available)

Age when test performed..... Dose of edrophonium.....

Muscle tested.....Response (negative or positive).....

Neurophysiology results

At what age: .....

Nerve conduction studies report (if available).....

.....

EMG results (please specify which muscle).....

.....

Repeat nerve stimulation results (please specify which muscle, frequency of stimulation, percentage decrement/increment).....

.....

Single fibre EMG report (please specify which muscle).....

.....

Antibody results:

Acetylcholine receptor antibodies

Negative ☐ positive ☐

MUSK antibodies

Negative ☐ positive

Gene testing results

At what age was correct genetic diagnosis made? .....

Therapy: please provide as much information as possible

Which therapy was given first?.....

At what age?.....

What was the starting dose? .....

How was the dose titrated and over how long?.....

.....

Response to treatment (please specify)

Subjective (patient's perception).....

Objective (clinical examination, FVC, neurophysiology.....

.....

.....

Time lag before clear response noted by patient.....

Any side effects?.....if yes please specify.....

.....

.

Was treatment stopped because of side effects?.....If yes, how soon after starting therapy?.....

Was a second line treatment given?.....please specify.....

What was the starting dose?.....

How was the dose titrated and over how long?.....

.....

Response to treatment (please specify)

Subjective (patient's perception).....

Objective (examination, FVC, RNS).....

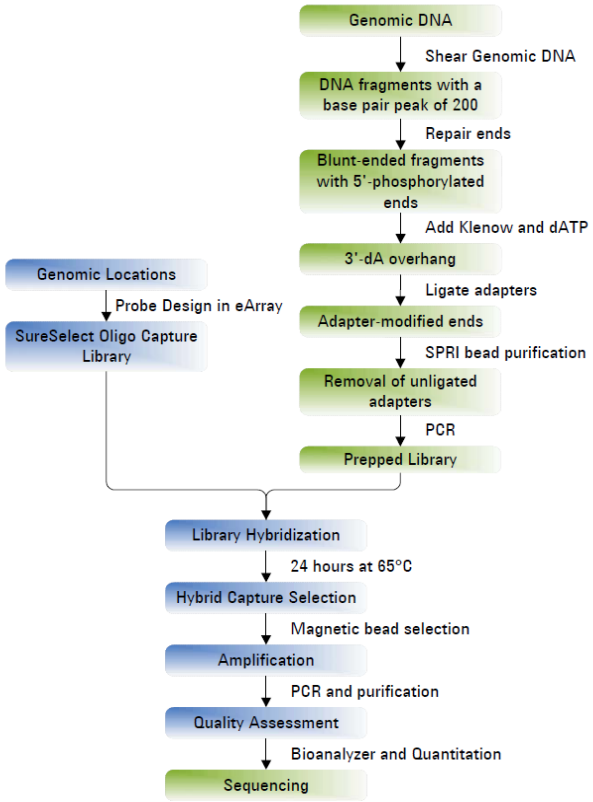
.....

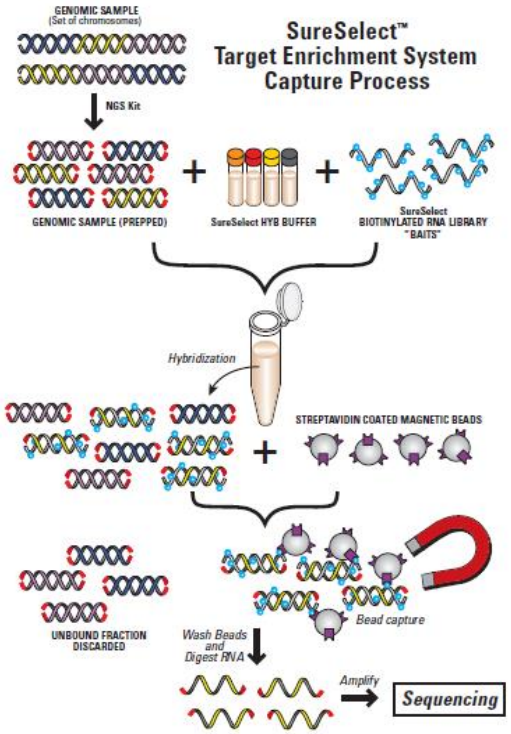
Time lag before clear response noted by patient.....

Any side effects?.....if yes please specify.....

Was treatment stopped, if yes how soon after starting therapy?.....

A3. SureSelect DNA library preparation and target enrichment capture[100]





[100] From SureSelect Human All Exon Kit Protocol (version 1.0.1 October 2009).