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DEPARTMENT OF CLINICAL MEDICINE - MEDICAL GENETICS

Clinical and genetic investigations of patients with myotonia congenita in Northern Norway

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CONTENTS

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List of papers

I. **Sun C, Tranebjærg L, Torbergsen T, Holmgren G, and Van Ghelue M.**
Spectrum of *CLCN1* mutations in patients with myotonia congenita in Northern Scandinavia.
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II. **Warnstedt M, Sun C, Poser B, Escriva MJ, Tranebjaerg L, Torbergsen T, Van Ghelue M, and Fahlke C.**
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J Neurosci 2002;22(17):7462-70.

III. **Sun C, Henriksen OA, and Tranebjærg L.**
Proximal Myotonic Myopathy: clinical and molecular investigation of a Norwegian family with PROMM.
Clin Genet 1999;56:457-61.

IV. **Sun C, Van Ghelue M, Tranebjærg L, Thyssen F, Nilssen Ø, and Torbergsen T.**
Myotonia congenita and myotonic dystrophy in the same family: coexistence of a *CLCN1* mutation and expansion in the *CNBP* (*ZNF9*) gene.
Clin Genet 2010 Dec 15. doi: 10.1111/j.1399-0004.2010.01616.x.

Abbreviations

Ca^{2+}	Calcium ion
$(CCTG)_n$	CCTG repeat tract
ClCs	Voltage-gated chloride channel family
ClC-1	Skeletal muscle voltage-gated chloride channel
Cl^-	Chloride ion
ClCh	Chloride channelopathy associated with myotonia
CLCN1	Skeletal muscle voltage-gated chloride channel gene
CNBP/ZNF9	CCHC-type zink finger, nucleic acid binding protein gene
$(CTG)_n$	CTG repeat tract
DM	Dystrophic myotonia
DM1	Myotonic dystrophy, type 1
DM2	Myotonic dystrophy, type 2
DMC	Dominant myotonia congenita
DMPK	Dystrophia myotonica-protein kinase gene
EMG	Electromyography
G_{Cl}	Cl^- conductance
HyperPP	Hyperkalemic periodic paralysis
K^+	Potassium ion
MC	Myotonia congenita
Na^+	Sodium ion
NaCh	Sodium channelopathy associated with myotonia
$Na_v1.4$	Skeletal muscle voltage-gated sodium channel
NDM	Non-dystrophic myotonia
OMIM	Online Mendelian Inheritance in Man
PAM	Potassium-aggravated myotonia
PCR	Polymerase chain reaction
PMC	Paramyotonia congenita
PROMM	Proximal myotonic myopathy

RMC	Recessive myotonia congenita (Becker's myotonia)
SCN4A	Skeletal muscle voltage-gated sodium channel α–subunit gene
SSCP	Single-stranded conformational polymorphism

INTRODUCTION

1. Myotonia

Myotonia (*myo*, muscle; *tonus*, tension) is defined as delayed relaxation of the skeletal muscle after voluntary contraction or mechanical stimulation. This delay results from increased excitability of the skeletal muscle plasma membrane.

Patients with myotonia experience muscle stiffness. They have, for example difficulties releasing a clenched fist, getting up from a chair, or opening their eyes after squinting in bright light. They may also have a stiff, awkward gait.

Myotonia can affect all muscle groups. However, the pattern of affected muscles can vary. The severity of muscle stiffness can be influenced by several other factors (Heatwole and Moxley, 2007; Mankodi, 2008), such as emotion (Thomsen, 1876; Gutmann and Phillips, 1991; Trip et al., 2009), temperature (Thomsen, 1876; Gutmann and Phillips, 1991; Trip et al., 2009), exercise (Thomsen, 1876; Colding-Jørgensen, 2005), and pregnancy (Lacomis et al., 1999; Newman et al., 1999; Rudnik-Schöneborn et al., 2006; Trip et al, 2009). In addition, myotonia can be found in combination with muscle weakness (Becker, 1977; Trip et al., 2009). Although myotonia is often described as painless (Heatwole and Moxley, 2007; Miller, 2008), recent evidence indicates that painful myotonia is relatively common in non-dystrophic myotonias (Trip et al., 2009). Myotonia is not lethal. Nevertheless, severely affected individuals may be subjected to potentially hazardous situations due to their sudden inability to move away.

Upon neurological examination myotonic reactions may be observed in the patients as action myotonia after voluntary contraction, or/and percussion myotonia after mechanical stimulation (Figure 1 and 2). On electromyography (EMG) examination, characteristic spontaneous, repetitive electrical discharges called “myotonic runs” can be recorded (Figure 3). Classic myotonic discharges have a pattern of waxing and waning of both amplitude and frequency (Rüdel et al., 1985; Miller, 2008). In addition, the sound profile produced during EMG is distinct and typical to myotonia, described as a ‘diving bomb’. In very mild cases, EMG may be the only positive sign of myotonia, whereas muscle stiffness or/and clinical myotonic reactions might be completely absent. This is called ‘latent myotonia’ (Rüdel et al., 1985; Lossin and George, 2008).

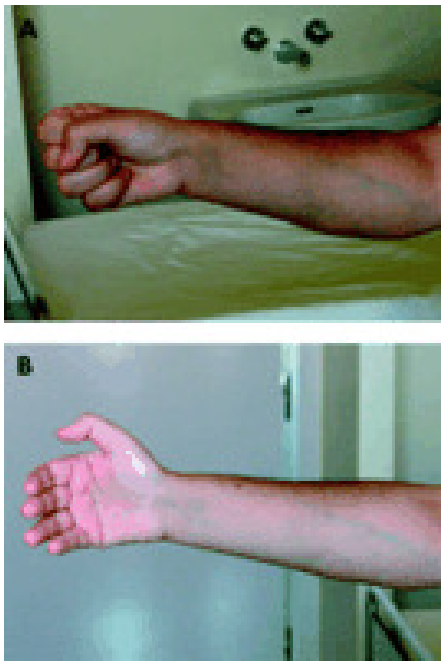

Figure 1. Action myotonia. The patient was asked to make a forceful fist for several seconds (A), and was then asked to open the hand as fast as possible (B). The ability to fully extend the fingers and release the handgrip is abnormally delayed. (Pelargonio et al., 2002; with permission from the journal: *Heart*)

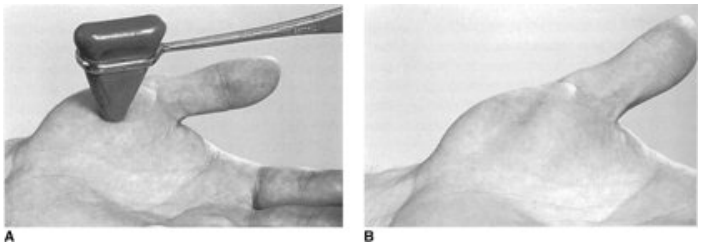

Figure 2. Percussion myotonia of the thenar muscle. A, A tap was made with a reflex hammer on the thenar eminence. **B,** The myotonic muscle became indented for a few seconds without immediate relaxation. (Miller, 2008; with permission from the journal: *Muscle Nerve*)

EMG examination does not allow a distinction between myotonic disorders. Several specialized tests have been developed, combining electrophysiological examinations with provoking factors like exercise or cooling. Some studies have shown that combinations of these specialized tests may assist in distinguishing phenotypes and accordingly direct the relevant molecular genetic investigations (Nielsen et al., 1982; McManis et al., 1986; Sander

et al., 1997; Fournier et al., 2004 and 2006; Tan et al., 2011). However, their reliability and reproducibility are not completely established.

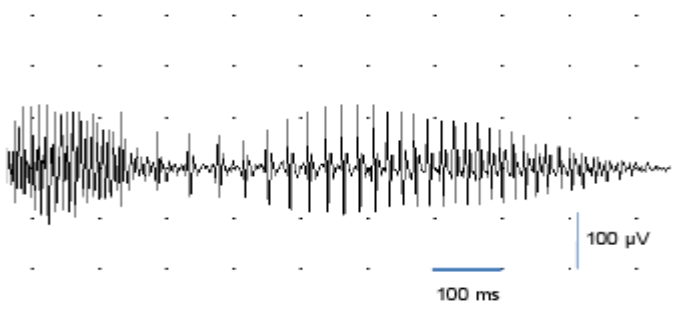

Figure 3. 'Myotonic runs' on EMG. Normal muscle responds with single action potentials upon single stimuli. However, myotonic muscle often responds with a series of repetitive action potentials. Notice that both the amplitude and the frequency of the myotonic discharges increase and decrease with a waxing and waning pattern. (Provided by Dr. Erik Stålberg in Uppsala, Sweden)

Lifestyle modification and avoidance of precipitating factors (such as cold exposure) are important in the management of myotonia. Symptomatic treatment of muscle stiffness is currently the standard therapy. Agents that block sodium channels are the most commonly used (Ceccarelli et al., 1992; Jackson et al., 1994; Savitha et al., 2006; Lyons et al., 2010; Logigian et al., 2010). The carbonic anhydrase inhibitor acetazolamide is also frequently applied (Trudell et al., 1987; Ferriby et al., 2006). However, lack of good randomized controlled studies makes it difficult to evaluate their effect as well as their safety (Trip et al., 2006). There are currently no drugs available which act directly on the muscle chloride channel. However, new therapies are under development (Wheeler et al., 2007; Cleland and Grigg, 2008; Matthews et al., 2010).

2. Pathological mechanisms underlying myotonia

Physiologically, several pathomechanisms can cause hyperexcitability of the skeletal muscle plasma membrane. Three abnormalities have been reported in patients with myotonia: 1) Reduced muscle chloride (Cl^-) conductance (G_{Cl}) observed in myotonia congenita (MC) and dystrophic myotonia (DM). 2) Alterations of sodium (Na^+) currents in sodium channelopathy associated with myotonia (NaCh) and DM. 3) Abnormal potassium (K^+) conductance observed in cultured cells derived from patients with DM (Rüdel et al., 1985).

2.1 Action potential

When a nerve impulse reaches a muscle cell at the neuromuscular junction, it causes a change in the electric potential across the plasma membrane (called action potential). Skeletal muscle cells rapidly convert this electrical signal into a rise in cytosolic calcium (Ca^{2+}) concentration, which then initiates contraction in each muscle cell (Figure 4). One nerve stimulus induces one action potential leading to one muscle contraction.

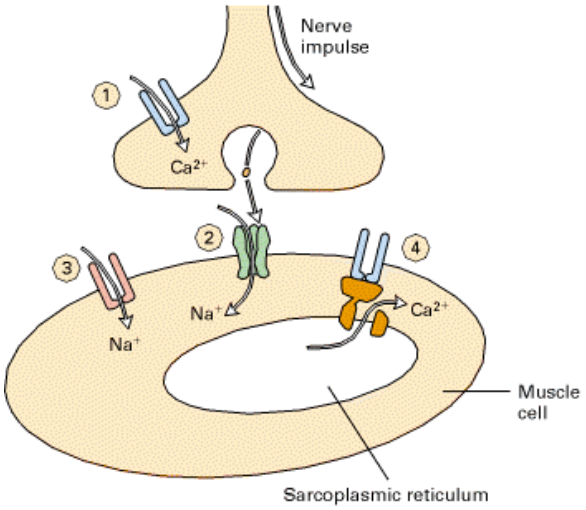

Figure 4. Sequential activation of gated ion channels at a neuromuscular junction. When a nerve impulse reaches the terminus of a presynaptic motor neuron, voltage-gated Ca^{2+} channels open (step 1) and acetylcholine is released. This, in turn, triggers the opening of the ligand-gated nicotinic receptor channels in the muscle plasma membrane (step 2), and Na^{+} ions flow into the muscle cell. The influx of Na^{+} produces a localized membrane depolarization. Voltage-gated Na^{+} channels open (step 3) and allow more Na^{+} influx, which further depolarizes the membrane. When the amplitude of the depolarization reaches the threshold (approximately -50 mV), a regenerative potential change known as an action potential is crested, which sweep down the length of the entire plasma membrane. When the depolarization reaches T- tubules, it activates voltage-gated Ca^{2+}-release channels (step 4), and Ca^{2+} ions release from the sarcoplasmic reticulum (SR) into the cytosol. The rise in cytosolic Ca^{2+} concentration causes muscle contraction. ("Molecular Cell Biology". 4th edition. Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, and Darnell J. New York: W. H. Freeman; 2000; with permission)

Action potentials are mediated by the voltage-gated ion channels embedded in the muscle plasma membrane (the sarcolemma). The two primary currents, voltage-gated Na^+ and K^+ current, are the ionic basis of the action potential (Figure 5). They control depolarization and repolarization phases of the potential change, respectively. A nerve impulse causes an opening of voltage-gated Na^+ channels (Na^+ ion influx) followed by the activation of voltage-gated K^+ channels (K^+ ion efflux). Following an action potential, there is an absolute refractory period during which no second action potential can be triggered irrespective of the stimulus strength. This is because the Na^+ channels have yet to recover from inactivation to the closed conformation. The absolute refractory period is followed by a relative refractory period during which an action potential can only be fired if the stimulus strength is increased.

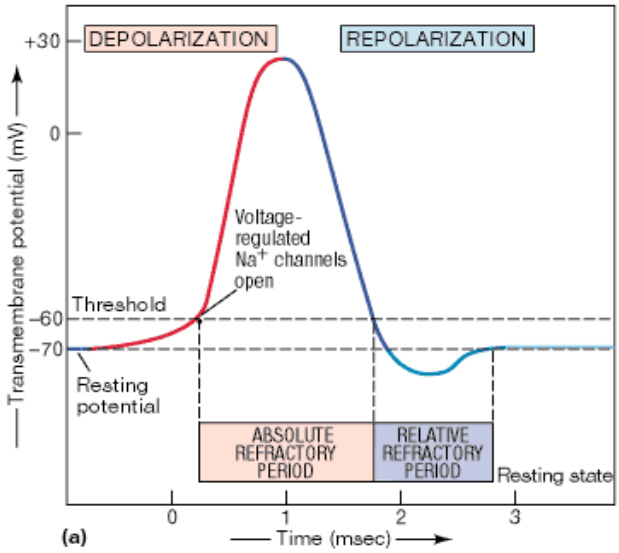

Figure 5. The action potential. An action potential is a rapid, transient reversal in membrane potential mediated by voltage-gated ion channels found in the plasma membrane ("Psychology: An introduction". 11th edition. Charles G. Morris, Albert A. Maristo. Prentice-Hall, Inc.; 1995-2002; with permission)

2.2 Increased excitability of the muscle and myotonia

The threshold potential at which the inward Na^+ current exactly balances the outward K^+ current is a critical potential, as any increase in Na^+ influx or reduction in K^+ efflux evokes subsequent depolarization. The summation of the after-depolarization may be sufficient to initiate a self-sustaining action potential. Whereas normal muscles respond with single action

potential upon single stimuli, myotonic muscles often respond with runs of action potentials, causing extended muscle contractions. In addition, any larger sustained Na^+ current could produce a prolonged depolarization, which turns the muscle fiber electrically unexcitable. This may cause muscle weakness or paralysis (Cannon et al., 1993; Colding-Jørgensen, 2005).

Unlike other excitable cells, the resting membrane conductance in normal skeletal muscle is dominated by Cl^- rather than by K^+. The sarcolemma G_{Cl} contributes to approximately 70-80% of the resting membrane potential. It is essential for the electrical stability of the muscle fiber. In its defect or absence, Na^+ channels have enough time to recover from inactivation while the membrane potential is still depolarized. The most important role of the G_{Cl} in skeletal muscle might be to counteract the depolarizing effect of local K^+ accumulation in the T-tubules that accompanies excitation. It contributes significantly to the fast repolarization and largely eliminates after-depolarization.

A reduction of G_{Cl} to 50% apparently does not cause myotonia (Chen et al., 1997). Some reports concluded that a 70% or greater reduction of G_{Cl} is required to induce myotonia in muscle fibers (Barchi, 1975; Kwiecinski et al., 1988).

3. Myotonic disorders

Myotonia is a clinical feature associated with a number of neuromuscular diseases. Although the myotonic disorders share this symptom, they have different genetic causes. Myotonia represents the clinical end point resulting from dysfunctions in a number of membrane ion channels.

The myotonic disorders can be divided into two main categories: non-dystrophic (NDMs) and dystrophic myotonias (DMs) (Table 1). The NDMs are distinguished from the dystrophic types by the absence of progressive muscle weakness, dystrophic features in the muscle histology, and multisystem involvement. Both categories are clinically highly heterogeneous with a wide variety of phenotypes.

There are other diseases, such as Schwartz-Jampel syndrome (SJS) and hereditary familial episodic ataxia type 1 that can mimic clinical myotonia. However, additional clinical symptoms and the lack of characteristic EMG features distinguish these disorders from the NDMs. Other conditions, such as acid maltase deficiency, polymyositis, as well as some drugs, can occasionally produce myotonic potentials on EMG (Heatwole and Moxley, 2007;

Miller, 2008).

Table 1. Myotonic Disorders

	Mode of inheritance	Gene location	Gene symbol	Gene product (Protein)
Non-dystrophic myotonias (NDMs)				
Sodium channelopathy assiociated with myotonia (NaCh) Paramyotonia congenita (PMC) Hyperkalemic periodic paralysis (HyperPP) Potassium-aggravated myotonia (PAM)	AD	17q13	*SCN4A*	skeletal muscle voltage-gated sodium (Na^+) channel α-subunit
Chloride channelopathy associated with myotonia (ClCh) Myotonia congenita (MC) Thomsen's myotonia (DMC) Becker's myotonia (RMC)	 AD AR	7q35	*CLCN1*	skeletal muscle voltage-gated muscle chloride (Cl^-) channel
Dystrophic myotonias (DMs)				
Myotonic dystrophy type 1 (DM1) type 2 (DM2) Proximal myotonic myopathy(PROMM) Proximal dystrophic myotonia (PDM)	AD	19q13.3 3q21.3	*DMPK* *CNBP/ ZNF9*	Dystrophic myotonia protein kinase Cellular nucleic acid binding protein gene

(AD autosomal dominant; AR autosomal recessive; *SCN4A*, skeletal muscle voltage-gated sodium channel α–subunit gene; *CLCN1*, skeletal muscle voltage-gated chloride channel gene; *DMPK*, dystrophia myotonica-protein kinase gene; *CNBP/ ZNF9*, CCHC-type zink finger, nucleic acid binding protein gene)

3.1 Non-dystrophic myotonias (NDMs)

The NDMs belong to the skeletal muscle ion channelopathies. They are caused by dysfunction of the chloride or the sodium channels located in the plasma membranes of the muscle cells. They only affect the muscle system with myotonia as the main symptom. Despite a growing knowledge of the genetic etiology of these disorders, clinical and electrophysiological features remain paramount in diagnosing and differentiating these diseases. Family history, myotonia characteristics and its response to stimuli (including cold, potassium ingestion, exercise, and drug therapy), EMG features, together with the available molecular studies are the diagnostic determinants. However, marked phenotypic differences in patients with identical genetic etiology, even between members of the same family, are evident in all NDMs. This indicates that other modifying factors may exist. Overall, the prognosis is good, with no reduction in life expectancy.

3.1.1 Chloride (Cl^-) channelopathy associated with myotonia (ClCh)

Myotonia congenita (MC)

MC has a prevalence estimated around 1:100,000 worldwide (Emery, 1991). In Northern Finland the prevalence was reported to be much higher, about 7.3:100,000 (Baumann et al., 1998).

MC can be transmitted either as a dominant or as a recessive trait. The dominant type was first described in 1876 by Dr. Asmus Julius Thomas Thomsen (Thomsen, 1876) who himself had the disease (Thomsen's myotonia, DMC, OMIM 160800). A century later, Becker described the recessive form of MC (Becker, 1977) (Becker's myotonia, RMC, OMIM 255700). In the majority of the families with MC, the inheritance is thought to be recessive.

Both types of MC share two similar clinical signs, muscle stiffness (myotonia) and muscular hypertrophy. Muscle stiffness is most pronounced when a forceful movement is suddenly initiated after a period of rest, but decreases as the same movement is repeated several times ("warm-up" phenomenon). Stiffness is also more pronounced in the extremities, especially in the legs and the hands. In general, myotonia in RMC is clinically more severe and generalized than myotonia in DMC (Becker, 1977; Mailänder et al., 1996; Rayan and Hanna, 2010). The stiffness is usually progressive for a few years after its first appearance and then remains stable. Muscular hypertrophy is usually present, particularly in the calves and gluteal muscles.

In addition to the degree of myotonia, a few other differences between DMC and RMC have been described, which may allow clinical differentiation (Becker, 1977; Mankodi, 2008). DMC is characterized by early onset (from infancy to early childhood), while RMC is characterized by a later onset (after the first decade of life) and a commonly presented transient muscle weakness. The weakness lasts only seconds to minutes, and especially affects the arms and legs. In many patients, the weakness also improves during several minutes of continued exercise. Occasionally, permanent muscle weakness and wasting with atrophy of selected distal muscle groups may occur in RMC (Becker, 1977; Nagamitsu et al., 2000).

Muscle pain is not a typical feature of MC, but it is not uncommon (Fialho et al., 2007). Mildly elevated serum creatine kinase (CK) levels have been described as common (Fialho et al., 2007; Heatwole and Moxley, 2007). Muscle biopsy may show hypertrophic fibers, central nuclei, and deficiency in type 2 muscle fibers. None of these findings, however, are diagnostic.

Skeletal muscle voltage-gated chloride channel gene CLCN1

MC is the first recognized inherited chloride channelopathy. Disease causing mutations in the major muscle voltage-gated chloride channel gene, *CLCN1,* were identified in 1990s (Koch et al., 1992; George et al., 1993; Lorenz et al., 1994). The *CLCN1* gene, located on chromosome 7q35, encodes the major voltage-gated skeletal muscle Cl^- channel (ClC-1). Surprisingly, both types of MC, Thomsen and Becker, are caused by mutations in the same gene.

To date, over 150 mutations causing MC have been identified in the *CLCN1* gene (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=CLCN1). The number is continuing to increase, illustrating the high degree of allelic heterogeneity associated with this disorder. The *CLCN1* mutations are spread throughout the whole gene and show no specific locations for mutations leading to DMC or RMC. However, exon 8 appeared to harbor clusters of mutations that more commonly result in DMC (Fialho et al., 2007). Nevertheless, no clear correlation between genotype and phenotype for distinct mutations has been found. Despite the numerous mutations identified, *CLCN1* mutations were detected in about 40-100% of the clinically diagnosed MC patients reported in the literature (Meyer-Kleine et al., 1995; Zhang et al., 1996; Sangiuolo et al., 1998; Plassart-Schiess et al., 1998; de Diego et al., 1999; Papponen et al., 1999; Deymeer et al., 1999; Fialho et al., 2007; Trip et al., 2008).

Although the majority of the *CLCN1* mutations are recessively inherited, a few are transmitted dominantly (Pusch, 2002; Wu et al., 2002; Jurkat-Rott et al., 2010a). Curiously, several mutations were observed to have a variable mode of transmission: they can be associated with either RMC or DMC in different families (Pusch, 2002; Colding-Jørgensen, 2005; Dupré et al., 2009). The molecular mechanism of this complex behavior remains unclear. Variable penetrance (Koty et al., 1996; Kubisch et al., 1998), incomplete dominance (Plassart-Schiess et al., 1998), differences in allelic expression (Dunø et al., 2004), and lower clinical expressivity in females (Becker, 1977; Mailänder et al., 1996; Deymeer et al., 1999) have been suggested as possible explanations.

CLCN1 mutations have also been identified in other myotonic disorders. Heterozygosity for c.2680C>T (p.Arg894X) has been found in a patient with clinical hyperkalemic periodic paralysis (HyperPP) (Zhang et al., 1996) and dystrophic myotonia type 2 (DM2) (Suominen et al., 2008).

Skeletal muscle voltage-gated chloride channel (ClC-1)

ClC-1 is almost exclusively expressed in skeletal muscle (Steinmeyer et al., 1991). It belongs to the highly conserved voltage-gated chloride channel family (ClCs). These Cl^- channels are expressed in organisms ranging from bacteria and yeast to plants and animals. Currently, nine mammalian ClCs have been identified (Jentsch, 2008) and mutations in several of them are associated with human diseases.

The tertiary and quaternary structures of ClCs are distinct from that of the voltage-gated cation channels. However, compared with cation channels of similar importance, the relationship between structure and function of this channel family is poorly understood. The crystal structures of the ClC protein from *Escherichia coli* (EcClC) and *Salmonella typhimurium* (StClC) have been resolved (Mindell et al., 2001; Dutzler et al., 2002). The functional chloride channel exists as a homodimer lined in opposite direction with a "double-barreled" structure. Each of the subunits contains its own pore, and is made up of 18 α-helical regions (Figure 6) of variable length. The gating behavior is complex and consists of two main gating modes. The individual pores can open and close independently of one another (Duffield et al., 2003; Dutzler, 2004). This is the "fast gate". Both pores are deactivated and closed simultaneously by a much slower common gate ("slow gate") (Duffield et al., 2003; Dutzler, 2004). However, the gating mechanisms of ClCs remain poorly understood (Dutzler, 2004; Jentsch, 2008), and recent electrophysiological studies show that the ClCs seem to be functioning as ion transporters as well as ion channels (Jentsch, 2008; Robertson et al., 2010).

Functional expression studies have increased the understanding of the nature of these channels and the pathophysiology of myotonia (Pusch, 2002). Numerous expression studies of the *CLCN1* mutations have revealed several mechanisms of altered channel function leading to myotonia. Alteration in anion selectivity (Fahlke et al., 1997), inversion of the voltage dependence of gating (Fahlke et al., 1995; Zhang et al., 2000a), and shift of the voltage dependence of activation to more positive potentials (Pusch et al., 1995; Wagner et al., 1998; Zhang et al., 2000b) were described to cause myotonia. It has been proposed that *CLCN1* mutations causing DMC most probably affect the common gate, whereas mutations resulting in RMC involve one of the fast gates (Saviane et al., 1999; Duffield et al., 2003). In DMC the mutant subunit exerts a dominant-negative effect over the wild-type subunit by a shift in voltage dependence, while in RMC the mutant channel usually show total loss of function of both monomers (Pusch et al., 1995; Pusch, 2002). This may explain why mutations in the same gene can cause both dominant and recessive disorders, and why

recessively inherited myotonia is generally more severe than dominantly inherited myotonia. The presence of some normal channels in heterozygous patients with dominantly inherited myotonia may result in a residual Cl^- current. However, even mutations with electrophysiological characteristics consistent with dominant inheritance have been found in recessive pedigrees (Kubisch et al., 1998; Pusch, 2002).

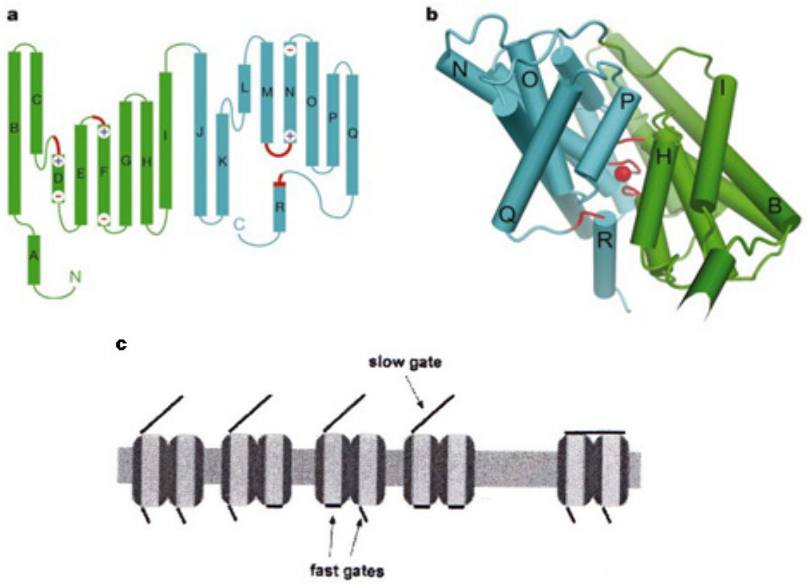

Figure 6. Schematic topology of eukaryotic members of the ClC family. a. The topology of the transmembrane domains was deduced from the known structure of the bacterial homologue EcClC. ClCs are homodimers with each monomer consists of 16 transmembrane domains (B-Q). **b.** View of dimmer interface. Cylinders represent α-helices. Cords represent loops between helices. (Dutzler, 2002; with permission from the journal: *Nature*) **c.** "double-barreled" structure of the ClCs with two pores which can gate independently (fast gating), while still be connected by a slow common gating. (Pusch, 2002; with the permission from the journal: *Human Mutation*)

Several other voltage-gated chloride channels as ClC-2 and ClC-4 are present in skeletal muscle, and with a structure similar to ClC-1. It has been shown that they can form functional channels with ClC-1 (Lorenz et al., 1996).

3.1.2 Sodium (Na^+) channelopathy associated with myotonia (NaCh)

The NaCh mainly includes three disorders, paramyotonia congenita (PMC), potassium-aggravated myotonia (PAM), and hyperkalemic periodic paralysis (HyperPP).

Clinically, these conditions frequently overlap. Myotonia in patients with PMC is precipitated by cold and is aggravated by exercise (paradoxical myotonia). The characteristic of PAM is K^+-aggravated myotonia. Attacks of muscle weakness are the hallmark of HyperPP.

The features that distinguish NaCh from ClCh are the fluctuations in the degree of muscle stiffness, and a substantial cold-dependence of the symptoms in the sodium channelopathy. Still, the present clinical features of NaCh may be similar to DMC. Trip and colleagues (2009) reported that 20% of patients with an initial clinical suspicion of DMC were genetically confirmed to have myotonia associated with Na^+ channel.

Skeletal muscle voltage-gated sodium channel α-subunit gene SCN4A

HyperPP, PMC, and PAM are allelic variants of sodium channelopathy. They are inherited as autosomal dominant traits, and are associated with mutations in the *SCN4A* gene located on chromosome 17q23 (George et al., 1990 and 1991). This gene encodes the α-subunit of the voltage-gated skeletal muscle Na^+ channel ($Na_v1.4$). To date, around 60 mutations have been described in the *SCN4A* gene that cause myotonia (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=SCN4A). A certain genotype and phenotype correlation exists (Jurkat-Rott et al., 2010b). However, as in Cl^- channel myotonia, phenotypic variation has been observed in association with the same *SCN4A* genotype (Matthews et al., 2010).

Skeletal muscle voltage-gated sodium channel ($Na_v1.4$)

The $Na_v1.4$ contains a larger α-subunit and a smaller β-subunit. The α-subunit forms a single ion-conducting pore. The pore contains four domains (I-IV). Each domain comprises six transmembrane segments (S1-S6) with interlinking loops (Jurkat-Rott et al., 2010b; Matthews et al., 2010) (Figure 7). The fourth segment (S4) of each domain functions as a voltage sensor. Upon the change in the membrane potential, the Nav1.4 undergoes conformational changes between three states - open, inactivated, and closed state. The inactivation conformation prevents further generation of action potential, while the close conformation prepares for channel activation upon a new action potential. All three states are voltage- as well as time-dependent (Jurkat-Rott et al., 2002). However, the exact mechanisms of voltage sensing and the conformational changes are still under investigation (Jurkat-Rott et al., 2010b). *SCN4A* mutations leading to NaCh are thought to be mainly 'gain-of-function' defects impairing channel inactivation or enhancing channel activation (Celesia, 2001; Jurkat-Rott et al., 2010b).

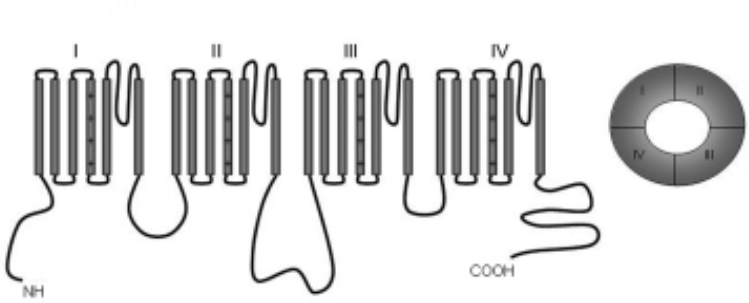

Figure 7. Diagramatic outline of the α-subunit of the skeletal muscle voltage-gated sodium channel $Na_v1.4$. It contains four domains (I-IV) which form a singe pore. Each domain consists of six transmembrane segments. (Matthews et al., 2010; with permission from the journal: *Brain*)

3.2 Dystrophic myotonias (DMs)

Myotonic dystrophy (DM) is the most common form of adult muscular dystrophy. Currently, it is subdivided into type 1 (DM1, OMIM 160900) and type 2 (DM2, OMIM 602668) according to their underlying genetic defects. DM1, also called Steinert's disease, is the "classic" type of myotonic dystrophy, first described in 1909 (Steinert, 1909) with a prevalence of 2.1-14.2/100.000 worldwide (Harper, 2001). The prevalence of DM2 is unknown, though it is thought to be equally prevalent as DM1 (Udd et al., 2003 and 2006). However, recent evidence pointed to a higher prevalence of DM2 than expected (Suominen et al., 2011).

DMs belong to nucleotide repeat expansion disorders. Both types of DM are autosomal dominantly inherited, and share similar clinical manifestations. They are multisystemic disorders characterized by myotonia, muscle weakness and wasting, muscle pain, and involvement of other organs and systems including the eye, heart, endocrine, gastrointestinal, and the central nervous system (CNS) (Harper, 2001). DMs shows marked clinical variability, similar to NDMs.

3.2.1 DM1

The muscle involvement in the classic DM1 is highly characteristic. The core features are facial and distal muscle weakness and atrophy. The facial expression in DM1 patients is characterized by frontal baldness, bilateral ptosis, atrophic temporalis muscles, and a long, lean face (Figure 8). The finger-flexor weakness is prominent and distinct. Later in the

course of the disease, wasting of the sternocleidomastoid and proximal weakness might develop. Muscle biopsies often show type I fiber atrophy, increased number of centrally localized nuclei, and ringed fibers.

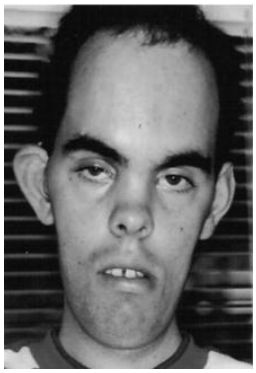

Figure 8. Characteristic facial expression of a patient with DM1. The patient showed weakness and atrophy of the facial muscles, wasting of the muscle temporalis and ptosis. (Zühlke et al., 2007; with permission from *American Journal of Medical Genetics*)

As mentioned earlier, several other organ systems are also affected in the classic DM1. Posterior iridescent/capsular cataracts are characteristic, and can be detected by slit-lamp examination. Cardiac involvement, mainly represented by conduction abnormalities is common. Rarely, overt clinical manifestations of cardiomyopathy ("myotonic" heart disease) are present in patients with DM1 (Pelargonio et al., 2002). Endocrine disturbances with abnormal glucose-insulin metabolism, testicular failure, and abnormal growth hormone regulation are common (Harper, 2001; Turner and Hilton-Jones, 2010). CNS affection, cognitive and behavioral abnormalities (Delaporte, 1998; Turner and Hilton-Jones, 2010), and gastrointestinal tract involvements, such as irritable-bowel-like symptoms are common (Harper, 2001; Turner and Hilton-Jones, 2010). The life expectancy of most DM1 patients is greatly reduced due to respiratory disease, cardiovascular disease, and sudden death presumably caused by cardiac arrhythmias (Mathieu et al., 1999; Harper, 2001; Turner and Hilton-Jones, 2010).

Ten to 15% of DM1 patients have the most severe congenital form with numerous developmental abnormalities. They have generalized muscle weakness/hypotonia at birth, respiratory insufficiency, craniofacial defects, mental retardation, and a high mortality rate.

Myotonia is absent in affected infants, and they do not have cataracts (Harper, 2001; Turner and Hilton-Jones, 2010).

DM1 is subject to anticipation, elucidating earlier onset and/or more severe symptoms in affected individuals belonging to successive generations of the same family (Harper, 2001; Turner and Hilton-Jones, 2010).

Dystrophia myotonica-protein kinase gene DMPK

The underlying genetic defect in DM1 is an expansion of a CTG trinucleotide repeat motif in the 3'-untranslated region (3' UTR) of the dystrophia myotonica-protein kinase gene, *DMPK,* located on chromosome 19q13.3 (Fu et al., 1992; Mahadevan et al., 1992; Brook et al., 1992).

There is a general correlation between the degree of expansion and the severity of the disease (Harper, 2001; Turner and Hilton-Jones, 2010). The number of repeats ranges from 5 to 37 in normal individuals. Minimal symptoms are present in people with 50 to 90 repeats (premutation), and severely affected patients have larger expansions with as many as 2000 repeats in the congenital forms (Harper, 2001; Turner and Hilton-Jones, 2010). Currently, the golden standard for diagnosis of DM1 is the identification of an abnormal $(CTG)_n$ expansion in the *DMPK* gene.

Dystrophic myotonia protein kinase

The *DMPK* gene encodes a member of the serine-threonine kinase family that interacts with members of the Rho family of small GTPases. Several alternatively spliced isoforms of the dystrophic myotonia protein kinase (DMPK) have been described (Oude Ophuis et al., 2009). It was found that in animal model DMPK was pronouncedly expressed in both the skeletal and smooth muscle as well as neural tissues that are commonly affected in DM1 (Oude Ophuis et al., 2009). However, the exact function of DMPK is currently unknown. It was suggested that this protein may regulate the cytoskeleton reorganization and intracellular trafficking process (Kaliman and Llagostera, 2008).

3.2.2 DM2

As early as the late 1970s, some neurologists identified patients who did not present with the typical features of classical DM. However, it was not until the genetic cause behind the classical DM was identified, that a new disease entity was discovered. In 1994, Thornton

et al. and Ricker et al. independently reported atypical DM patients without $(CTG)_n$ expansion in the *DMPK* gene. Since then, many similar cases with different degrees of severity have been described worldwide. They were named proximal myotonic myopathy (PROMM) (Thornton et al., 1994; Ricker et al., 1994), proximal myotonic dystrophy (PDM) (Udd et al., 1997), and DM2 (Ranum et al., 1998; Day et al., 1999). These disorders were shown to have a common genetic etiology (Udd et al., 2003) and are now collectively termed myotonic dystrophy type 2 (DM2).

Currently, our knowledge and understanding of the natural history of DM2 and its full spectrum of clinical manifestations remain incomplete. DM2 patients present with features resembling of DM1, including muscle weakness and wasting, myotonia, cataract and a whole range of manifestations of variable degree in other organ systems. DM2 differs principally from DM1 by its late onset, predominant weakness and atrophy of proximal muscles at onset, absence of a congenital form, doubtful anticipation, and its milder system symptoms with a more favorable long-term prognosis (Day et al., 2003; Schara and Schoser, 2006; Turner and Hilton-Jones, 2010) (Table 2). The onset of DM2 is typically in the third decade. The muscle weakness at onset typically affects the proximal muscle groups, neck and elbow flexor and especially hip flexors (Schara and Schoser, 2006; Turner and Hilton-Jones, 2010). Facial weakness is less common. Some features, such as myalgia, hyperhidrosis and hyperinsulinaemia, are reported to be more common in DM2 than in DM1 patients (Schara and Schoser, 2006; Turner and Hilton-Jones, 2010). Although no reports on DM2 cases gave clear evidence of a congenital form, anticipation has been described in few DM2 families (Schneider et al., 2000; Day et al., 2003). Most DM2 patients do not experience severe disabilities in their life. Respiratory failure usually does not occur (Meola and Moxley, 2004). Sudden cardiac arrest seems uncommon, but has been reported occasionally in younger patients (Moxley et al., 2002).

CCHC-type zinc finger, nucleic acid binding protein gene CNBP

DM2 is associated with a CCTG tetra-nucleotide expansion located in intron 1 of the CCHC-type zinc finger, nucleic acid binding protein gene, *CNBP,* previously called *ZNF9,* located on chromosome 3q21.3 (Liquori et al., 2001). Thus, similar to DM1, the repeat motif is located in an untranslated region of its respective gene (Table 2). Although DM2 is generally a milder disease than DM1, the DM2 $(CCTG)_n$ expansions can be much larger than DM1 $(CTG)_n$ expansions with alleles ranging in size from ~75 to 11,000 CCTG repeats (mean ~5000 CCTGs) (Udd et al., 2003) (Table 2). The smallest pathogenic repeat size has not been

determined. There does not seem to be any correlation between the size of expansion and the disease severity, nor with the age of onset (Udd et al., 2003). Moreover, there is no report that there exists a pool of premutation alleles in the DM2 population. However, Bachinski and coworkers (2009) recently suggested that uninterrupted $(CCTG)_{22\text{-}23}$ allele may represent such reservoir for DM2 disease mutations.

Table 2. DM1 and DM2

	DM1	**DM2**
Inheritance	AD	AD
Gene	*DMPK*, 19q13.3	*CNBP* (*ZNF9*), 3q21.3
Mutation	CTG repeat expansion	CCTG repeat expansion
Localization of mutation	3'-UTR	Intron 1
Number of expanded repeats	37-5000	75-11 000
Gene product	Dystrophic myotonia protein kinase	Cellular nucleic acid binding protein
Possible function	Signal pathway?	Transcriptional / Translation regulation?
Muscle weakness/atrophy at onset	Facial and distal involvement	Predominant proximal involvement, milder
Other system manifestation	+, usually shortened lifespan	+, but in milder degree
Anticipation	+	±
Congenital form	+	–

(Modified from Finsterer, 2002)

Cellular nucleic acid binding protein

Cellular nucleic acid binding protein (CNBP) is abundantly expressed in human muscle fibers (Massa et al., 2010). As for DMPK, the exact functional role of this protein is currently unclear. CNBP is a conserved single-stranded nucleic acid binding protein with a wide range of targets. Recent data proposed that this protein probably acts as a transcriptional and/or translational regulator (Gerbasi and Link, 2007; Schneider-Gold and Timchenko, 2010), operating as a nucleic acid chaperone (Calcaterra et al., 2010).

3.2.3 Pathomechanisms

The underlying molecular mechanism by which the expanded repeat tract produces the complex DM1 phenotype is poorly understood. The key question is how does a mutation

in the non-coding region of a gene exert a multisystem effect at the cellular level? Three possible mechanisms have been suggested based on molecular investigations: *haploinsufficiency* caused by the respective disease gene, *chromatin structure alteration* causing abnormal expression of neighboring genes, and *RNA processing* related pathogenesis. The identification of DM2 as the first tetranucleotide expansion disease threw more insight into the molecular pathomechanisms of DMs. The overlapping symptoms suggested that the underlying pathomechanisms may be common for both disorders. The function of CNBP appears unrelated to DMPK or any of the proteins encoded in the DM1 region of chromosome 19. Similarly, the other genes in the DM2 region bear no obvious relationship to genes at the DM1 locus. It is now generally agreed that both DMs are mainly RNA-mediated diseases caused by a dominant negative effect on the expression of other genes or their products. (Ranum and Day, 2002; Osborne and Thornton, 2006; Wheeler and Thornton, 2007) (Figure 9).

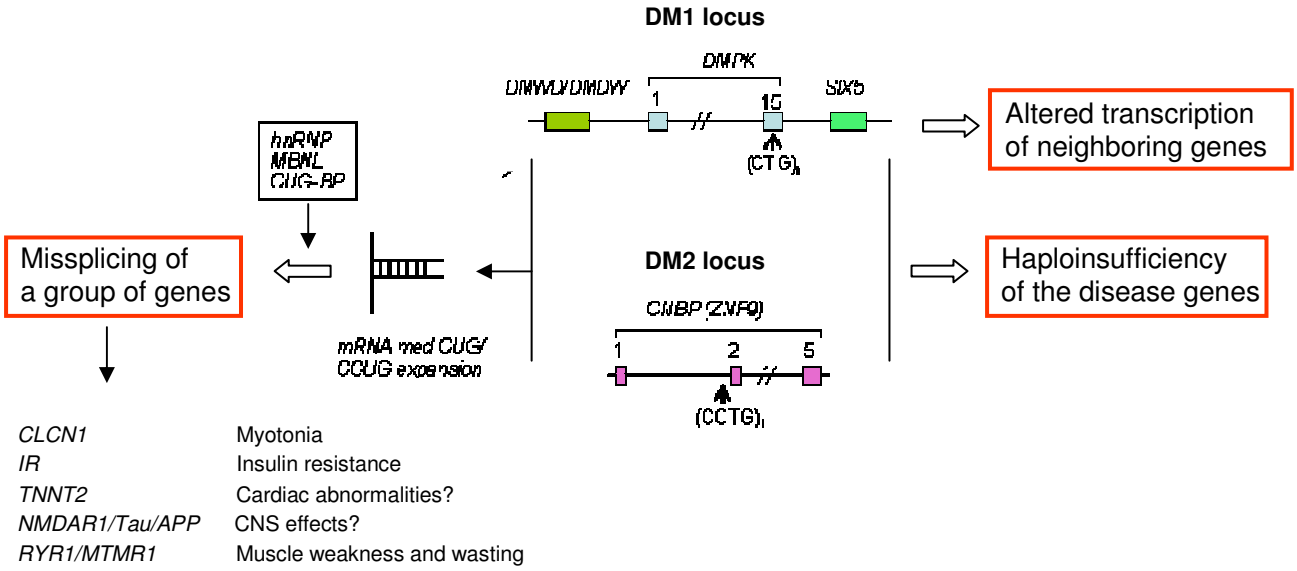

Figure 9. Molecular pathogenesis of DM1 and DM2. Three models have been suggested to be involved in DM pathogenesis. The main mechanism is the abnormal RNA processing which leads to aberrant splicing of a group of genes, such as *CLCN1* and *IR*. Reduced expressions of the gene products of the respective genes of DM1 and DM2 are also implicated. Furthermore, altered expression of the neighboring gene(s) of *DMPK* may also be involved in some of the clinical presentations in DM1. hnRNP, human nuclear ribonucleoprotein; MBNL, muscleblind-like protein; CUG-BP, CUG binding protein; *IR*, the Insulin receptor gene; *TNNT2*, the cardiac Troponin T gene; *NMDAR1*, the Nmethyl-D-aspartate receptor 1 gene; *Tau*, microtubule-associated protein gene; *APP*, the amyloid precursor protein gene; *RYR1*, the ryanodine receptor gene; *MTMR1*, the myotubularin-related protein 1 gene.

The toxic gain-of-function at the RNA level of the *DMPK* and *CNBP* encoded RNA was suggested based on the observation that both DM1 and DM2 cells exhibit multiple nuclear foci if mutant RNA is present. Both genes are transcribed into aberrant RNAs containing expanded CUG or CCUG repeat tracts. The aberrant RNAs accumulate in the nuclei foci, sequester and alter the regulation or localization of RNA-binding proteins such as muscleblind-like protein (MBNL), and CUG binding protein (CUG-BP), which in turn lead to aberrant splicing of a group of other genes (Figure 9). Some of the potential pre-mRNA targets known are: *CLCN1* (Charlet-B et al., 2002; Mankodi et al., 2002; Lueck et al., 2007; Wheeler et al., 2007; Botta et al., 2008), *IR* (Insulin receptor) (Savkur et al., 2001 and 2004; Botta et al., 2008), and *TNNT2* (cardiac troponin T) (Philips et al., 1998; Botta et al., 2008).

However, RNA processing defects alone cannot explain the entire disease pathology of DMs (Figure 9). The phenotypic difference between DM1 and DM2, especially the lack of developmental abnormalities in DM2 as there were no convincing congenital cases reported, suggested that other cellular and molecular pathways may also be involved. *Dmpk* deficient mice display a late-onset myopathy (Reddy et al., 1996), atrioventricular conduction abnormalities (Berul et al., 1999), and impaired glucose-insulin metabolism (Llagostera et al., 2007). Haploinsufficiency of *Znf9* in mice mimicked phenotypes resembling DM including myotonia, heart arrhythmia, and muscle histopathology (Chen et al., 2007). CTG repeat expansions were shown to reduce expression of the neighboring gene *SIX5* (Thornton et al., 1997), and mice deficient in *Six5* develop cataract (Klesert et al., 2000; Sarkar et al., 2000). These data suggested that all three models may contribute to the DM phenotypic variability. However, it is unclear if these three processes are independent or interact. The exact mechanism underlying DM is still unknown.

3.2.4 Other types of DM

There is still reason to believe that there are multiple genetic causes underlying the growing number of families with variable myotonic dystrophy-like symptoms with or without additional features. Several families have been reported with similar DM clinical features, but showing no linkage to the DM1 or DM2 locus (Udd et al., 2003; Meola et al., 2004). Finding additional genes involved in the non-classical forms of DM will greatly enhance our understanding of the pathological mechanisms leading to the complex DM phenotype.

4. Summary

As illustrated above myotonia is a clinical feature seen in several disorders which show extensive overlapping phenotypes. Accordingly, it may be challenging to reach the most likely clinical diagnosis. Below, an algorithm is presented suggesting a possible strategy to find the most reliable clinical diagnosis for a patient presenting with myotonia, and to subsequently direct further molecular diagnosis.

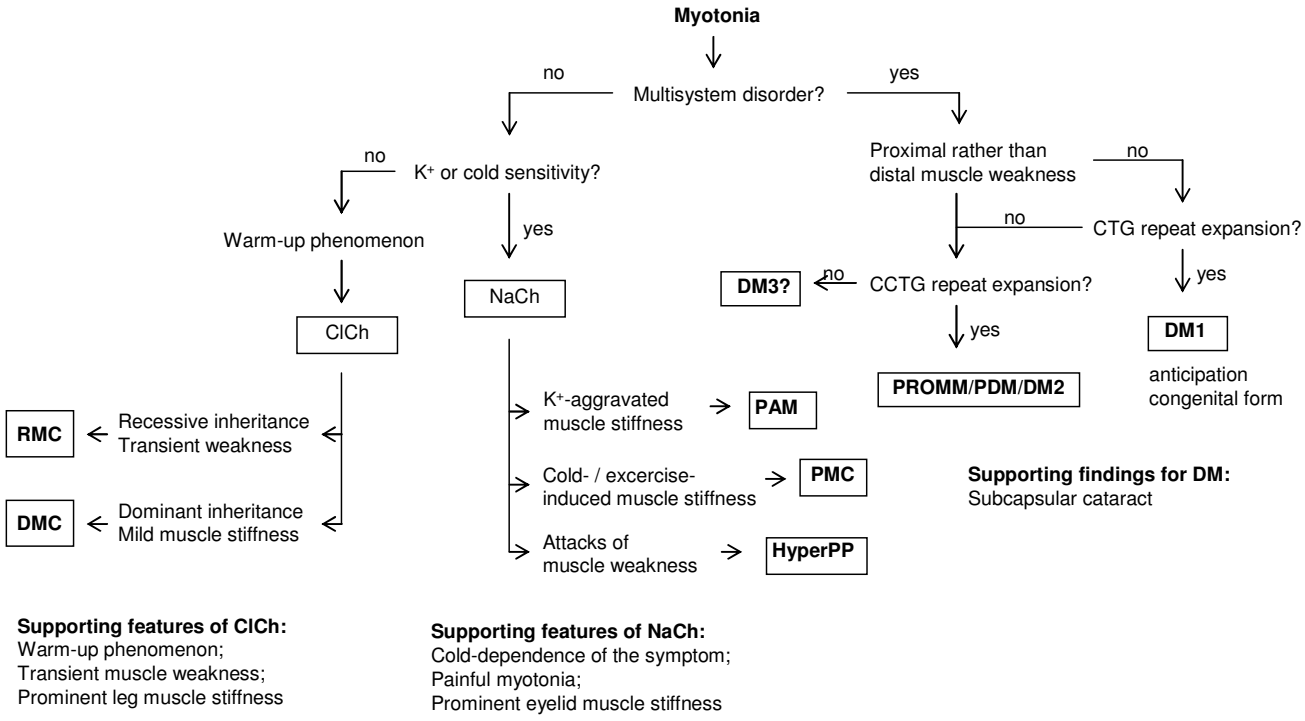

Figure 10. Clinical diagnosis of myotonic disorders. The clinical evaluation of myotonia should be supplied with EMG examination. The provocative factors of muscle stiffness and features of myotonic runs on EMG may further direct to sodium or chloride channel myotonia. The existence of symptoms from system(s) other than the muscle frequently differentiates the DMs from NDMs. Predominant proximal muscle weakness at the onset together with mild course of the multisystem features could indicate DM2 rather than DM1.

AIMS OF THE STUDY

In Northern Norway, with a population of ~465 000, at least 15 families have been clinically diagnosed with MC (Appendix I). The main goal of this project was to assess the disease etiology of MC in this population, to improve the diagnostic strategy and genetic counseling for patients with myotonia, as well as to improve the biological understanding of the myotonia and myotonic diseases.

GOALS:

1) To determine the prevalence of myotonia congenita (MC) and to study the spectrum of *CLCN1* gene mutations in the MC population of Northern Norway.

2) To develop efficient PCR-based methods for molecular diagnosis for MC causing *CLCN1* mutations.

3) To determine the carrier frequency of *CLCN1* mutations in Northern Norway

4) To increase our understanding of the relationship between mutations in the *CLCN1* gene (genotype) and the clinical presentation and mode of inheritance (the phenotype).

SUBGOALS:

1) To study the functional implications of a new *CLCN1* mutation c.991G>A (p.Ala331Thr) on ClC-1 in an *in vitro* system in order to improve our understanding of the chloride channel protein and, hence, the molecular pathogenesis of MC.

2) To investigate the influence of *CLCN1* mutations on the DM2 phenotype.

STRATEGY

Clinical investigations

Eighteen families with clinical diagnosed MC were included in this study (Appendix I): fifteen families from Northern Norway and three families from Northern Sweden. A thorough neurological examination and EMG evaluation of the Norwegian patients as well as their first-degree relatives (sibs, children, and parents) were performed by one neurologist (Dr. T. Torbergsen) with more than thirty years experience in diagnosing myotonia. Grip myotonia and percussion myotonia were examined. 'Warm-up' phenomenon and cold sensitivity were tested. The facial expression as well as muscle hypertrophy or atrophy were registered. The muscle strength and reflexes were assessed. Organ involvements other than the muscle system were investigated. Slit-lamp examination, electrocardiography (ECG), eccocardiomyography, muscle biopsy, and cerebral computer tomography (CT) scan were performed when indicated. Blood samples were investigated for the Creatine Kinase (CK), Gamma-glutamyl Transpeptidase (γ-GT), Aspartate Aminotransferase (ASAT), Alanine Aminotransferase (ALAT), and Lactate Dehydrogenase (LD). Blood glucose, Thyroid-Stimulating Hormone (TSH), free Thyroxine (FT4), Follicle-Stimulating Hormone (FSH), Luteinizing Hormone (LH), and testosterone level were tested when indicated.

EMG was performed by concentric needle electrode using Keypoint EMG equipment (Medtronic, Denmark). Four muscles were examined: the extensor digitorum communis, the dorsal interosseous I, the vastus lateralis, and the anterior tibial muscles. Myotonic discharges were recorded, and the motor unit potentials were analyzed for amplitude, durations and early recruitment.

The clinical and EMG myotonia were further classified in four categories (+/-, +, ++, and +++) depending on the degree of affection (suspected, mild, moderate, and pronounced). Presence of myotonic runs on EMG was the criteria for being classified as affected.

The clinical information regarding the Swedish patients was provided by the local physicians.

Informed consent was obtained and the study was approved by the Regional Committee for Medical and Health Research Ethics.

Molecular investigations

The methods for molecular investigation of family material are outlined in Figure 11. Briefly, genomic DNA was extracted from peripheral blood cells. All 23 exons of the *CLCN1* gene were amplified from DNA from proband(s) of each family. PCR products were then screened for mutations by single-stranded conformational polymorphism (SSCP) at three different conditions. Abnormal SSCP conformers were reamplified and subsequently sequenced. For exons showing normal SSCP patterns, PCR products were directly sequenced. Once nucleotide changes were identified, restriction enzyme digestion strategies or amplification refractory mutation system (ARMS) were designed and used to screen other family members in the respective family and normal controls.

For probands with no or only one single *CLCN1* mutation, Southern blot analysis was performed to check for large deletions, insertions and rearrangements in the *CLCN1* gene that could have been missed by PCR-based methods. If Southern blot analysis showed normal results, haplotype analysis and/or linkage analysis of the DNA samples were performed, to check for linkage of the myotonia to the *CLCN1* gene. In the families where the disorder was excluded to be co segregating with markers in the *CLCN1* locus, molecular analysis of *SCN4A*, *DMPK* and *CNBP* gene were performed.

The pathomechanism of a novel *CLCN1* mutation was subsequently studied by *in vitro* functional expression performed in embryonic kidney cells (HEK) and tsA201 cells (a simian virus 40 (SV40) T-antigen-expressing derivative of the embryonic kidney cell line HEK-293). The kinetics for the mutant Cl^- channel(s) was studied.

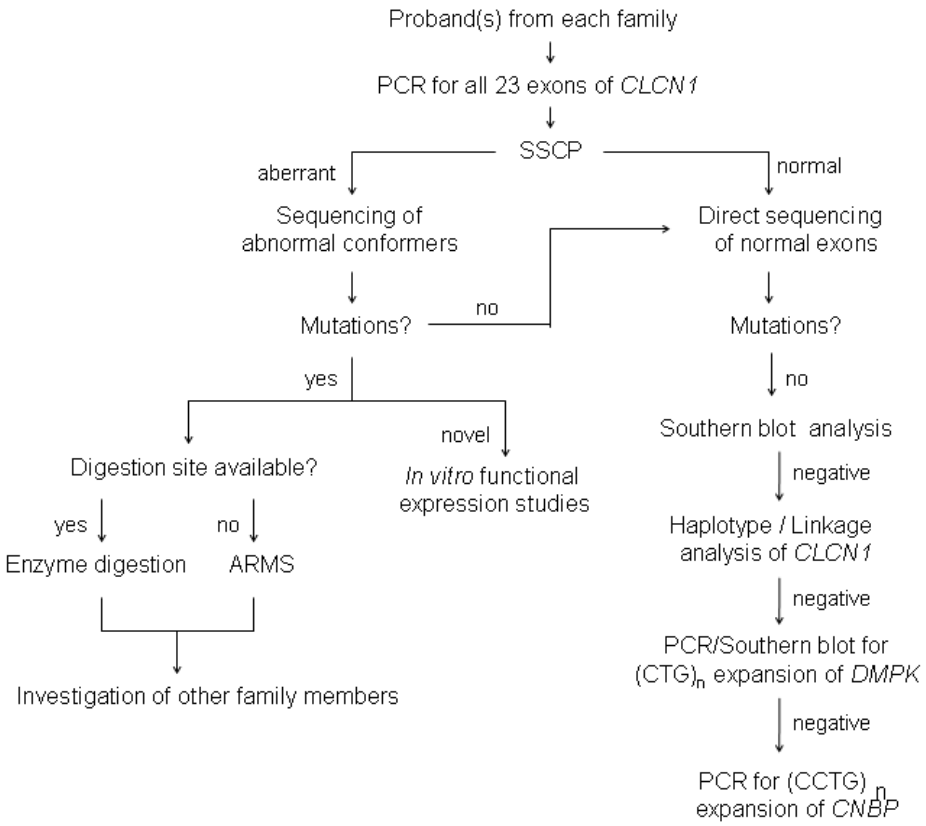

Figure 11. Flowchart of the investigation of MC families.

SUMMARY OF PAPERS

Paper I. Spectrum of *CLCN1* mutations in patients with myotonia congenita in Northern Scandinavia.

In order to explore the spectrum of *CLCN1* mutations in Northern Scandinavia the entire *CLCN1* gene was investigated in 15 Northern Norwegian families and 3 Northern Swedish families clinically diagnosed with myotonia congenita (MC). The prevalence of MC in Northern Norway was estimated to be as high as about 9:100 000. Eight different *CLCN1* mutations (c.180+3A>T (c.IVS1+3A>T), c.979G>A (p.Val327Ile), c.920T>C (p.Phe307Ser), c.991G>A (p.Ala331Thr), c.1238T>G (p.Phe413Cys), c.1592C>A (p.Ala531Val), c.2284+5C>T, and c.2680C>T (p.Arg894X)) and three polymorphisms (c.261C>T (p.Thr87Thr), c.2154T>C (p.Asp718Asp), and c.2180C>T (p.Pro727Leu)) were identified. The p.Phe413Cys, p.Ala531Val, and p.Arg894X substitution clearly predominated in our patient population. The p.Ala531Val has been detected only in the populations of Northern Norway and Northern Finland whereas the p.Arg894X causing mutation is one of the most common *CLCN1* mutations found in the MC population worldwide. The allele frequencies of c.1238T>G (p.Phe413Cys), c.1592C>A (p.Ala531Val), and c.2680C>T (p.Arg894X) were <0.005%, 0.3% and 0.89%, respectively. Heterozygous carriers of c.2680C>T (p.Arg894X) in our study are rarely affected by myotonia, in contrast to other reports where c.2680C>T (p.Arg894X) has been shown to follow either dominant or recessive inheritance in different families. As previously reported there was no obvious genotype - phenotype correlation. This may partially be due to the fact that the majority of our patients were compound heterozygous with various combinations of different mutations, the c.1592C>A (p.Ala531Val) / c.2680C>T (p.Arg894X) being the most frequent. Moreover, three *CLCN1* mutations were present in two probands. Despite the sequencing of the entire *CLCN1* gene in 19 probands from 18 unrelated MC families, bi-allelic mutations were detected in 15 probands and mono-allelic mutations in two probands. No *CLCN1* mutations were found in the remaining two probands.

Paper II. The myotonia congenita mutation A331T confers a novel hyperpolarization-activated gate to the muscle chloride channel ClC-1.

Mutations in the *CLCN1* gene might cause both autosomal dominant and autosomal recessive form of myotonia congenita (MC). Numerous *CLCN1* mutations have been identified scattered over the whole length of the gene. In this study, the functional implication of a novel disease-causing *CLCN1* mutation c.991G>A (p.Ala331Thr) was investigated using a heterologous expression system. The p.Ala331Thr causing mutation was introduced into a plasmid vector containing the full-length wild-type (WT) hClC-1 cDNA by site-directed mutagenesis. The mutant and WT hClC-1 channel proteins were expressed in human embryonic kidney (HEK) 293 and tsA201 cells both in homodimeric and heterodimeric forms. Whole-cell patch-clamp recordings were then performed under different conditions. The current responses, the gating properties, and the voltage dependence of the studied channel were analyzed. The p.Ala331Thr mutant channel in homodimeric form had a novel effect on ClC-1 gating. It is the first mutation reported to produce a slow hyperpolarization-induced gating step in ClC-1 without obvious alterations of the typical deactivation gating of this isoform. This slow gating reduces the open probability of mutant channels, explaining the myotonic phenotype in muscle fibers expressing the mutant channels. In contrast, heterodimeric channels consisting of WT and the mutant ClC-1 subunits do not exhibit this particular gating, suggesting that the function of this novel gate requires the mutation in both subunits.

Paper III. Proximal myotonic myopathy: clinical and molecular investigation of a Norwegian family with PROMM.

In 1994, proximal myotonic myopathy (PROMM) was established as a new entity with clinical features similar but much milder than myotonic dystrophy (DM1) and without the $(CTG)_n$ repeat tract expansion in the *DMPK* gene. Yet, its full clinical spectrum was uncertain. A new gene locus, termed DM2, on chromosome 3q was identified in 1998. We described the clinical characteristics of the first Norwegian PROMM family. The clinical features were inherited as an autosomal dominant trait. The affected family members had muscle weakness, myotonia, cataract, and normal $(CTG)_n$ repeat size in the *DMPK* gene. The patients had prominent proximal muscle weakness and wasting. The onset of muscle weakness was around the third decade rather than fifth to seventh decade as previously reported. The male patients in the family had also frontal balding. The female proband had ptosis, and her facial, jaw and swallowing muscles were also affected. Myalgia was a prominent feature in all affected family members. In addition, central nervous system involvement such as hypersomnia, previously rarely described in PROMM, was also present. The p.Arg894X causing *CLCN1* mutation was found in heterozygous form present in affected as well as unaffected family members. Haplotype analysis seemed to exclude the new DM2 locus as the disease locus in this family.

Paper IV. Myotonia congenita and myotonic dystrophy in the same family: coexistence of a *CLCN1* mutation and expansion in the *CNBP* (*ZNF9*) gene.

Myotonia is a clinical feature shared by several neuromuscular disorders. They are mainly classified into non-dystrophic and dystrophic myotonias. All myotonic disorders are clinically highly variable, and it is not always straightforward to distinguish these disorders from each other. The mechanisms underlying this clinical heterogeneity are unclear. We investigated a unique Norwegian family that was previously diagnosed with myotonic congenita (MC) and myotonic dystrophy (DM1) in different branches of the family. In one branch, all female patients had myotonia as the main symptom with onset during pregnancy and not disappearing after delivery. However, one of their uncles had bilateral cataracts, heart manifestation and other system involvement in addition to mild myotonia. Detailed clinical evaluation and EMG examination were performed. All known genes associated with myotonia were investigated. It turned out that this family had DM2 as a pathological $(CCTG)_n$ expansion in the *CNBP* (previously known as *ZNF9*) was identified in all affected family members but in none of the healthy individuals. Our study confirmed that DM2 was associated with clinical variability, even within the same family. Furthermore, our study indicated that some modifying factors, for example, a hormonal influence may exist that contribute to the high phenotypic variations in DM2. A p.Phe413Cys causing *CLCN1* mutation was also identified in this family. This mutation was found in 6 out of 13 affected family members, and we found more severe clinical affection in patients harboring both the DM2 expansion and p.Phe413Cys variation. Hence, p.Phe413Cys was likely to exaggerate the clinical presentation of myotonia in DM2.

DISCUSSION

Allelic spectrum and prevalence of myotonia congenita (MC) in Northern Norway

More than 150 disease associated *CLCN1* mutations have been identified (http://www.hgmd.cf.ac.uk/ac/gene.php?gene=CLCN1). In several MC populations of different origin a high number of different *CLCN1* mutations have been identified (Meyer-Kleine et al., 1995; Fialho et al., 2007; Trip et al., 2008).

In our study on MC in Northern Norway, we found eight different *CLCN1* mutations (paper I), in contrast to the Finnish MC population (Papponen et al., 1999) where only three *CLCN1* mutations were found (c.1238T>G (p.Phe413Cys), c.1592C>A (p.Ala531Val), and c.2680C>T (p.Arg894X)). However, these three mutations clearly predominated in MC patients from Northern-Norway/Sweden, constituting 26 of the total 33 mutant *CLCN1* alleles detected (paper I). The p.Ala531Val causing mutation has been detected exclusively in the Northern Fennoscandian population, while the p.Phe413Cys and p.Arg894X causing mutations are the most common *CLCN1* mutation detected worldwide.

We found a prevalence of about 9:100,000 of MC in Northern Norway (paper I), approximately ten fold higher than what has been reported in other populations (Emery, 1991). This prevalence is probably still an underestimate because very mild cases may remain unrecognized and, hence, never diagnosed. The high prevalence found in our study is comparable to that in the population of Northern Finland (Baumann et al., 1998). The genetic epidemiological observations from other diseases in the Finnish population (de la Chapelle, 1993) have shown that enrichment of various disease-causing mutations, through founder effects and genetic drift, in the relatively isolated, immobile populations has resulted in the accumulation of certain rare diseases. This may be the cause for the high prevalence of MC in the Northern Finnish population. The population of Northern Finland has an interesting migration history, as reflected in the molecular and genealogical studies of the autosomal recessive disorder Aspartylglucosaminuria (AGU) (Tollersrud et al., 1994). The Norwegian AGU families originated mostly from Finnish immigrants from the Tornio valley area (North-West Finland at the border between Northern Finland and Sweden) who migrated in the period between 1700 and 1900. They settled mainly in the Western part of Northern Norway, in the counties of Troms and Finnmark (Tollersrud et al., 1994). Most of the MC cases reported in Northern Finland show similar geographic locations as those of the ancestors of Norwegian AGU patients (Baumann et al., 1998; Papponen et al., 1999; Tollersrud et al., 1994). Hence, it is not unlikely that MC causing mutations, c.1592C>A (p.Ala531Val) in

particular, may share a similar migration history with that of the AGU_{Fin} mutation. Combination of founder events and genetic drift resulting in high carrier frequencies in specific sub-populations may explain the high prevalence of MC in Northern Norway. However, thorough haplotype analysis with validated SNP markers is required to verify the possibility of founder events. Founder investigations in other regions of Scandinavia or in the European Caucasian population are largely lacking at this time.

Genotype / phenotype relationships in MC

The phenotype of genetic disease such as MC can be considered in several ways: as the mode of inheritance, as the clinical features, and as the assessment of mutant channel function. A detailed analysis of clinical features was beyond the scope of this work. Therefore, only phenotypic aspects related to results from needle EMG examination and mode of inheritance are discussed here. The diagnostic methods and criteria are crucial in MC classification. Many of the investigations, including the Finnish (Papponen et al., 1999), the British (Fialho et al., 2007), and the Dutch (Trip et al., 2008) studies, define a clinical diagnosis of MC when a patient has muscle stiffness and/or clinical myotonia confirmed by needle EMG examination. How the asymptomatic individuals with electrical myotonia were classified - as healthy or affected was not clearly outlined. EMG has been proven to be the most reliable diagnostic tool in the clinical assessment of myotonia. Therefore, we used positive electrical myotonia on EMG as the only criterion for defining affected status, regardless of the existence of muscle stiffness and/or clinical myotonia. This would not only include those patients who remain unnoticed of their very mild symptoms, but also patients with electrical myotonia as the only sign at the initial investigation, who are likely to develop symptoms later on.

Several aspects of MC complicate the analysis of inheritance pattern. First, the pedigree can be misinterpreted due to pseudodominance as discussed later. Secondly, incomplete dominance with variable penetrance or expressivity may further confuse the picture (Plassart-Schiess et al., 1998). Lastly, most of the earlier studies of mutation analysis in the *CLCN1* gene were based on a PCR-SSCP strategy with a low mutation detection yield (Meyer-Kleine et al., 1995; Zhang et al., 1996; Sangiuolo et al., 1998). This may to some extent explain why some apparently ‘dominant’ mutations have later been shown to be ‘recessive’. Recent studies have shown that *CLCN1* mutations may coincidentally be detected in DM2 patients (papers III and IV; Mastaglia et al., 1998; Lamont et al., 2004; Suominen et

al., 2008) who may clinically resemble DMC. In these patients, *CLCN1* mutations might contribute to the intra-familial phenotypic variability.

In this work we have identified eight different *CLCN1* mutations (c.180+3A>T (IVS1+3A>T), c.979G>A (p.Val327Ile), c.920T>C (p.Phe307Ser), c.991G>A (p.Ala331Thr), c.1238T>G (p.Phe413Cys), c.1592C>A (p.Ala531Val), c.2284+5C>T, and c.2680C>T (p.Arg894X)) (paper I). No apparent cluster of mutations causing DMC or RMC was evident in our study (paper I). Fialho and co-workers (2007) reported that exon 8 seemed to harbor mutations mostly involved in DMC. Exon 8 encodes the H and I helix, the H – I interlink and part of the I – J interlink (Figure 6). The helices H, I, P, and Q form the interface of the channel dimer (Duffield et al., 2003), and *CLCN1* mutations causing DMC were thought to exert their dominant negative effects by abolishing dimer formation with the WT subunit. However, several other mutations located in exon 8 were found to cause RMC (Pusch, 2002). The p.Phe307Ser causing mutation (paper I, corrigendum) was previously reported to cause both RMC and DMC (Kubisch et al., 1998; Colding-Jørgensen et al., 2003). Although phenylalanine at position 307 is not highly conserved within the ClCs, a change from a nonpolar, hydrophobic phenylalanine to a polar amino acid, serine, might affect the channel function as this residue is situated in the I helix. We assumed the p.Phe307Ser was causing RMC as it was detected in a proband who harbored three *CLCN1* mutations (Figure 1c, paper I), heterozygous p.Phe307Ser causing mutation in combination with homozygosity for another putative 'recessive' splicing mutation c.2284+5C>T (Figure 1c, paper I). The c.2284+5C>T variant was absent in 100 control chromosomes. However in retrospect, the phenotypic effect of p.Phe307Ser has to be reconsidered. The fact that thymine (T) was found in +5 position in other *CLCN1* exon/intron junctions makes c.2284+5C>T doubtful as a disease causing mutation. Furthermore, recent data available from Ensemble (http://www.ensembl.org/index.html) show that the c.2284+5C>T variant has an allele frequency of 0.056. This excludes c.2284+5C>T as a disease causing mutation. Thus, it is likely that the p.Phy307Ser substitution causes DMC (S03, Appendix) rather than RMC. Consequently, the novel p.Ala331Thr substitution is also causing DMC (N05, Appendix), not RMC, since it was found in compound heterozygosity with the benign c.2284+5C>T variant (N05, paper I). A previously reported G to A substitution at nucleotide position c.979 (potential p.Val327Ile substitution), at the boundary of exon 8 (Lorenz et al., 1994) was also detected. This mutation affects a consensus splice site of the *CLCN1* gene and it was presumed to lead to myotonia by acting as a donor splice-site mutation (Lorenz et al., 1994). Using the splice site prediction program, NNSPLICE 0.9 (http://www.fruitfly.org/seq_tools/splice.html), c.979G>A was indeed predicted to disrupt the

donor splice site. Hence, it is likely that this mutation is disease causing and involved in RMC. Another putative splicing mutation c.180+3A>T was predicted not to interfere with splicing, according to the NNSPLICE 0.9 program. However, cDNA analysis is needed to determine whether these mutations disrupt normal splicing of the *CLCN1* gene transcript.

The p.Phe413Cys causing mutation showed the most consistent results in our studies. None of the individuals heterozygous for the p.Phe413Cys substitution was affected by myotonia (paper I), which is in accordance with the fact that it has been reported only as an RMC causing mutation in the literature.

The p.Arg894X causing sequence variant has been reported in several studies to give a dual mode of inheritance in MC (Meyer-Kleine et al., 1995; Zhang et al., 1996; Plassart-Schiess et al., 1998; Sangiuolo et al., 1998; Papponen et al., 1999). However, among the MC families reported here, the heterozygous carriers of this mutation were generally not affected with MC, except in three individuals, I:2 and II:1 in family N10 (Figure 1b, paper I) and II:5 in family N04 (Figure 1d, paper I). The latter two patients had 'latent' myotonia. In addition, heterozygosity for the p.Arg894X causing mutation was also identified in IV:3 in the PROMM family (paper III). This individual carried no CCTG expansion, but reported symptoms of muscle stiffness without clinical and electrical myotonia demonstrated (paper III). Due to its high allele frequency (0.87%), the c.2680C>T (p.Arg894X) mutation is unlikely to cause DMC in our population. However, it might contribute to the relatively high prevalence of MC. Larger population studies are needed to investigate this hypothesis.

The p.Ala531Val causing mutation seemed to be involved in both RMC and DMC in the MC cohort presented here (paper I). None of the heterozygous carriers of this mutation were analyzed by sequencing the whole *CLCN1* gene. Thus, myotonia might be caused by a second unidentified mutation, or, alternatively, by heterozygote manifestation in some of the heterozygous carriers. This is consistent with the findings from MC families in Finland (Papponen et al., 1999).

The clinical outcome of MC, such as age of onset, muscular hypertrophy, site or severity of myotonia, myalgia, muscle weakness and disease progression cannot be predicted based on the mutant genotype alone. A high fraction of our verified MC patients was compound heterozygous (9 of the 15 probands) (paper I). This was observed even in families with apparently dominant segregation of myotonia and is consistent with several other studies in which a direct sequencing strategy has been applied (Papponen et al., 1999; Trip et al., 2008). Frequent occurrence of compound heterozygosity and the pronounced allelic heterogeneity contribute to the difficulties in determining the genotype-phenotype relationship

at the clinical level. In our study, MC patients, who were compound heterozygous, experienced more severe muscle stiffness and showed more pronounced clinical and electrical myotonia than heterozygous family members. This is in accordance with early studies (Mailänder et al., 1996; Plassart-Schiess et al., 1998; Colding-Jørgensen, 2005).

A few reports have described the clinical features related to the different genotypes (Papponen et al., 1999; Fialho et al., 2007; Dupré et al., 2009). Papponen et al. (1999) reported that the p.Ala531Val substitution in a homozygous form or in combination with either the p.Phe413Cys or the p.Arg894X substitution was associated with the most severe muscular hypertrophy and myotonia. Patients homozygous for the p.Arg894X causing mutation rarely experience tongue myotonia, while those homozygous for the p.Ala531Val causing mutation had no myotonia during swallowing. However, these observations are limited and clinical studies in large cohorts are necessary to validate these and other possible genotype correlations.

In vitro systems as tools to understand molecular pathogenesis in MC

Functional studies of the disease-causing *CLCN1* mutations have enhanced our understanding of MC, especially the functional implications of different genotypes underlying MC. For example, the p.Arg894X mutant channel conducted reduced Cl^- currents with otherwise WT characteristics (Meyer-Kleine et al., 1995). When co-expressed with the WT *CLCN1*, the p.Arg894X mutant channel subunit showed only a weak dominant negative effect. The Cl^- conductance produced by the heterologous channel is close to the border between a “recessive” and “dominant” mutation. (Meyer-Kleine et al., 1995). This may explain why this mutation could be associated with both DMC and RMC.

In a follow up study (paper II), we investigated the functional implication of the novel p.Ala331Thr variant identified in our mutation analysis study (paper I). The Alanine codon at position 331 is evolutionary conserved in ClC-0, ClC-1 and ClC-2. It is localized in the I-J interlink (Dutzler et al. 2002; paper I) (Figure 6). The mutation causes replacement of a small hydrophobic side chain (Ala) with a polar one (Thr). How this affects the structure of the I-J interlink remains to be elucidated. The whole-cell current recording of the mutant channel, by patch clamp technique, was apparently normal (paper II). However, when tested under more physiological conditions, there was a clearly rightward shift of the activation curve of the mutant channels as compared with the WT channel under identical conditions (paper II). The alterations caused by the p.Ala331Thr mutant channel are distinct from any

functional effects previously described for other mutations showing the rightward shift of open probability. The mutant channel exhibited a novel slow activation gating step. However, this gating process was absent in heterodimeric channels when the p.Ala331Thr was co-expressed with WT *CLCN1* (paper II). The patient with the p.Ala331Thr also carried a putative splice site mutation c.2284+5C>T on the other allele. The electrophysiological features exhibited by the mutant channels were in agreement with a recessive inheritance. However, in retrospect as discussed above, c.2284+5C>T was shown to be a polymorphism, which leaves the p.Ala331Thr variant to cause DMC rather than RMC. This is in contrast to the conclusion based upon the functional expression study (paper II). In addition to myotonia, the patient had spastic paraparesis. To which extent, if at all, this novel gating property of the p.Ala331Thr mutant channel might contribute to this clinical feature is currently unknown.

With regard to our findings, *in vitro* electrophysiological studies of other ClC-1 variants have also given controversial results, or results that have been difficult to interpret. Even mutations with electrophysiological characteristics consistent with a dominant inheritance pattern have been found in recessive pedigrees (Pusch et al., 1995; Wollnik et al., 1997; Kubisch et al., 1998; Aromataris et al., 2001; Pusch, 2002). Several mutations involved in RMC showed little or no evidence of abnormal channel function in heterologous expression systems (Wollnik et al., 1997; Pusch et al., 2002; Simpson et al., 2004). Discrepant results concerning the same mutation in similar types of expression systems have also been reported. Wagner and coworkers (1996) could not detect expression of the p.Tyr150Cys substitution in *Xenopus* oocytes, while Wollnik et al. (1997) reported normal channel function in the same expression system. The endogenous expression in the different cells or cell lines has been thought to be negligible. However, heterologous expression systems do not necessarily reproduce the true 'physiological' conductance of the channel as mechanisms present in the intact skeletal muscle may not be faithfully reproduced. Papponen and colleagues (2008) demonstrated that the p.Phe413Cys and p.Ala531Val substitutions, but not the p.Arg894X substitution showed defects in trafficking of the channel protein from the endoplasmic reticulum (ER) to the Golgi apparatus. This may explain the minimal shift in G_{Cl} upon functional expression of the p.Phe413Cys mutant channel *in vitro* (Zhang et al. 2000b). Thus, one should be aware that decay or impaired intracellular transport of mutant proteins may, in some cases, preclude the interpretation of the results from *in vitro* functional studies.

Challenges in classification of MC

The marked phenotypic heterogeneity of myotonia in our MC population is in accordance with what has previously been reported. In the patient material presented here clinical differentiation between Becker's and Thomsen's myotonia in single patients seems to be difficult despite the earlier described clinical distinction between DMC and RMC (Becker, 1977; Heatwole and Moxley, 2007; Mankodi, 2008). Age of onset and site of onset (upper or lower limb) of myotonia showed variations and considerable overlap with respect to the classical clinical criteria for Thomsen's and Becker's myotonia. Muscle hypertrophy of lower extremities is usually equally present in both types. The peculiar transient muscle weakness, described in RMC, was rarely observed in our patient material, despite the fact that the majority of our families most likely had recessively inherited myotonia. Thus, the clinical classification as either Thomsen's or Becker's disease in our MC population was entirely based on the inheritance patterns in each family. This is in accordance with conclusions based on a study of a Dutch cohort of patients with NDMs (Trip et al., 2009). In that study no statistically significant differences were observed between clinical phenotypes of RMC and DMC. Transient muscle weakness was described as a unique feature that could distinguish ClCh from NaCh. However, it could not distinguish RMC from DMC in the Dutch cohort. Fialho and co-worker (2007) studied a large British cohort of MC patients. No significant differences were observed with regard to age of onset or site of onset between RMC and DMC. However, they found that transient muscle weakness and muscle hypertrophy occurred more frequently in RMC than in DMC.

In the majority of the fifteen Norwegian MC families myotonia was inherited in an autosomal recessive fashion (paper I), which is consistent with that of the Finnish MC families (Baumann et al, 1998; Papponen et al., 1999). Five families showed autosomal dominant inheritance. Of these, two families (family N11 and N15, paper I; paper III) were later proven to have DM2. Two other families (N01 and N04, paper I) showed pseudodominant inheritance, as several spouses introduced mutations into the family, in similarity to one Finnish MC family (Papponen et al, 1999). In the last family (N06, paper I) no *CLCN1* mutation could be identified in the proband. In a Dutch population of 32 genetically confirmed MC patients, only two of them were diagnosed with DMC (Trip et al., 2009), whereas Fialho and coworkers (2007) reported an unexpectedly high proportion of DMC in a large British MC population. Dissimilar mode of inheritance among geographically separated MC populations may reflect different spectrums of *CLCN1* mutations. It should be noted that after the genetic bases of myotonia were discovered, several clinical aspects were

reconsidered and many of the original DMC families turned out to have NaCh (Koch et al, 1989; Wagner et al., 1998; Jurkat-Rott et al., 2010a). Thus, some DMC cases in which mutations in *CLCN1* remained to be identified may actually have NaCh.

Family pedigrees have to be interpreted with caution. The pedigree pattern can easily be misinterpreted since variable penetrance has been described for several dominantly inherited *CLCN1* mutations (Colding-Jørgensen, 2005). Some patients may have very mild symptoms, and 'latent myotonia' is not uncommon among MC patients. In addition, our studies showed that the inheritance pattern of myotonia in some pedigrees may be obscure (paper I). Examples are given by the pedigrees N01 (paper I) presenting with apparently dominant segregation of myotonia. However, molecular analysis revealed different combinations of two recessively inherited *CLCN1* mutations with four independent mutant alleles segregating with myotonia in the family. Two of the mutant alleles were introduced by married-in individuals as a consequence of high carrier frequency of some mutations (paper I).

These data indicate that the earlier classification of MC into dominant Thomsen and recessive Becker myotonia is to date less valuable and may sometimes be misleading. One should not simply rely on the inheritance pattern in the family when assessing risk for transmission of MC. In general, the combination of clinical, genetic and functional studies provides a more profound basis for a more accurate genetic counseling.

The impact of *CLCN1* mutation on the DM2 phenotype

CLCN1 mutations have also been identified in DM2 patients (Mastaglia et al., 1998; Lamont et al., 2004; Suominen et al., 2008). Suominen and colleagues (2008) reported that about 5% of the German and 3% of the Finnish DM2 patients carry the most frequent p.Arg894X causing *CLCN1* mutation, whereas, the carrier frequency in the normal German and Finnish population was 1% and 1.3%, respectively. In addition, 2% of the Finnish DM2 population carries the p.Phe413Cys causing mutation, compared to 0.7% in the normal population. Until now, no *CLCN1* mutations have been reported in DM1 patients. The frequencies of *CLCN1* mutations within the DM2 population are not expected to be different from the normal population. The disproportional frequency of *CLCN1* mutations within the DM2 patients might simply reflect a selection bias towards the diagnosis of DM2 patients at the severe end of the phenotypic spectrum, as suggested by Souminen et al. (2008).

The first identified Norwegian PROMM family harbored the p.Arg894X causing mutation in heterozygous form in all affected family members. Haplotype analysis seemed to exclude the DM2 locus as the disease locus, since the only healthy son (IV:I, paper III) as well as his affected brother (IV:3, paper III) inherited the same haplotype from their affected mother (III:2, paper III). However, after the genetic cause of DM2 was found, the pathological $(CCTG)_n$ expansion was subsequently identified both in III:2 and IV:2, while both IV:1 and IV:3 had normal DM2 alleles (data not shown). Thus, heterozygosity for the p.Arg894X causing mutation may contribute to the myotonic phenotype of IV:3 in this family. Alternatively, another unidentified *CLCN1* mutation may exist that may account for the difference in phenotypic expression in IV:1 and IV:3.

In the genetically verified DM2 family (family N15, paper I; paper IV), a coincident heterozygous p.Phe413Cys causing *CLCN1* mutation was identified in some of the family members. The affected family members with both the *CNBP* expansion and the p.Phe413Cys substitution exhibited more severe myotonia both subjectively, clinically and electrically compared to patients harboring the *CNBP* expansion alone. Further clinical investigations are needed to establish if *CLCN1* mutations act as modifying factors in manifestations of DM2.

Like MC, DM2 is probably underdiagnosed in the general population because of the mild character in some patients. Furthermore, DM2 patients with very mild symptoms may present with a clinical picture that is very difficult to distinguish from NDMs such as MC (paper IV). To date, the prevalence of misdiagnosed DM2 in the MC, especially the DMC, population is unknown. Thus, the clinically diagnosed MC patients heterozygous for doubtful *CLCN1* mutations, or patients in whom *CLCN1* mutations remain undetected, should be reconsidered carefully for DM2 diagnostics. This is important since the extramuscular manifestations of DM2 have important implications for prognosis and management.

Modifying factors of myotonia

Several lines of evidence indicate the existence of additional modifying factors which may account for the broad clinical manifestations and severity of myotonia (Thomsen 1876; Becker, 1977; Deymeer et al, 1999; Simpson et al., 2004; Colding-Jørgensen, 2005). The intrafamilial phenotypic variation seen in patients sharing the same genotype (paper I) is in agreement with these previous observations.

Lower clinical expressivity in females has been well documented (Becker, 1977; Mailänder et al., 1996; Baumann et al., 1998; Deymeer et al., 1999). In one branch of our

DM2 family (paper IV) all affected females developed myotonia during pregnancies, and the myotonia never disappeared after delivery. This is in contrast to the previous observations (Lacomis et al., 1999; Newman et al., 1999; Rudnik-Schöneborn et al., 2006). Dupré and co-workers (2009) reported significant improvement of myotonia after menopause. These observations indicate that hormonal factors may influence the clinical manifestations of myotonia. Recently, both testosterone and progesterone were shown to rapidly and reversibly inhibit WT ClC-1 channels expressed in the *Xenopus* oocytes (Fialho et al., 2008).

Other possible modifying candidates could be the additional Cl^- channels expressed in the muscle with similar structure, especially ClC-2 and ClC-4. The ClC-2 was shown to have the ability to form heteromultimeric channels with ClC-1 (Lorenz et al., 1996). They may partially compensate for the absence of functional ClC-1 in myotonia congenita. The *CLCN4* gene is located on the X chromosome. One might speculate about its role in reduced clinical expressivity of myotonia in females.

Knowledge on the possible involvement of environmental modifying factors for myotonia is lacking. However, myotonic episodes might be triggered by factors such as stress and mental discomfort. This indicates that the emotional state of a patient might play a role in manifestation of myotonia.

Future challenges

The crystal structure of prokaryote ClCs has been solved (Dutzler, 2002) and its 3-dimensional structure has facilitated the understanding of the functional implications of the different *CLCN1* mutations on channel function. The ClC 3D structure may provide further aid in understanding the genotype - phenotype relationship in MC. The explanations for variable expressivity and incomplete penetrance are not understood. The mechanism underlying the warm-up phenomenon is still unclear. The intriguing inheritance of MC needs to be clarified. Solving these questions will have a great impact on genetic counseling.

Few studies have been designed in order to investigate the genotype – phenotype relationship in MC at the clinical level. Standardization of the definition of affected status of myotonia as well as that of assessment of symptoms, clinical signs and EMG measures are important for future investigations. To genetically exclude DM2 in MC cases with dominant inheritance and doubtful *CLCN1* mutation is elemental. In this respect, challenges also exist in promoting improved awareness of MC and DM among health care providers.

The knowledge about the molecular mechanisms underlying DM has greatly

increased during the past decade. However, many questions concerning the pathophysiology still remain unanswered. The comprehensive mechanisms underlying the whole spectrum of DM manifestation as well as the possible connection between the different pathways need to be elucidated. Future research is also required to establish if there exist other DM loci.

Therapy for DM and myotonia is currently supportive aiming at ameliorating symptoms. Randomized control clinical trials on antimyotonic drugs are underway (http://clinicaltrials.gov). However, accurate outcome measures that reliably assess the myotonic responses quantitatively need to be established. Our understanding of DM pathologies has advanced the possibility for more direct treatments aiming at the underlying genetic defects. As of to date, reports based on early stage gene therapy approaches are few, but promising (Rogers et al., 2002; Wheeler et al., 2007; Cleland and Grigg, 2008; Matthews et al., 2010).

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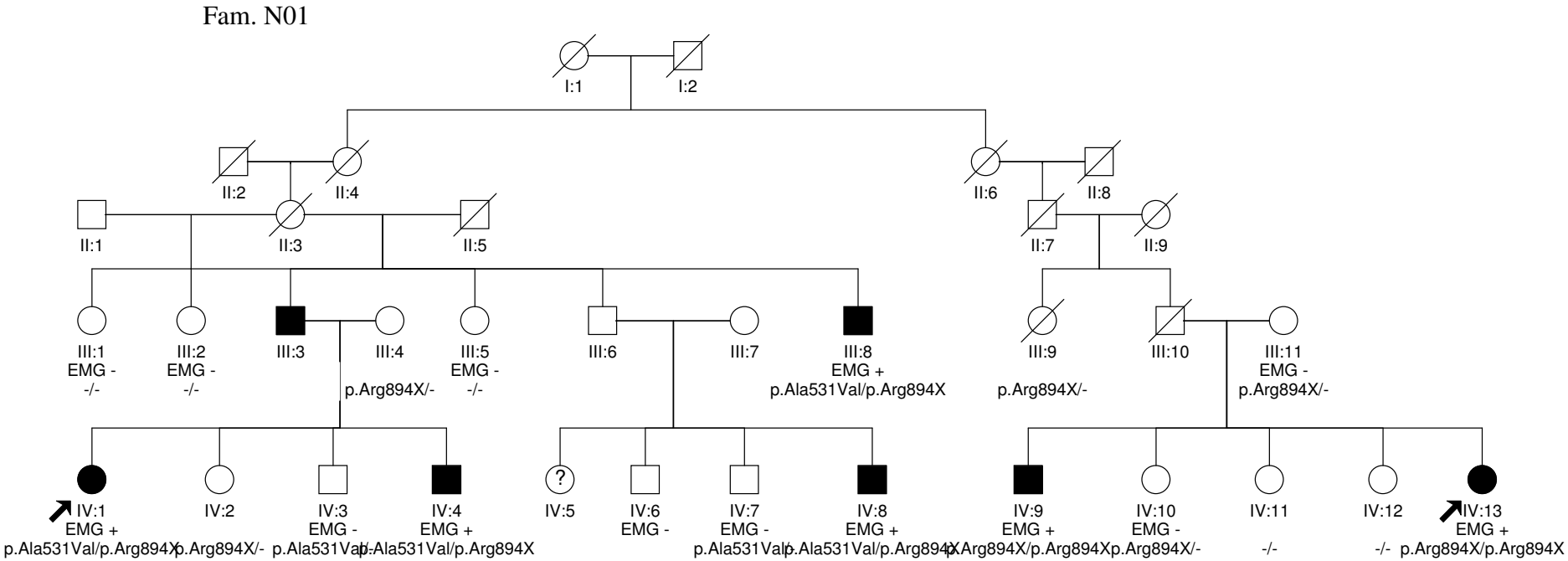
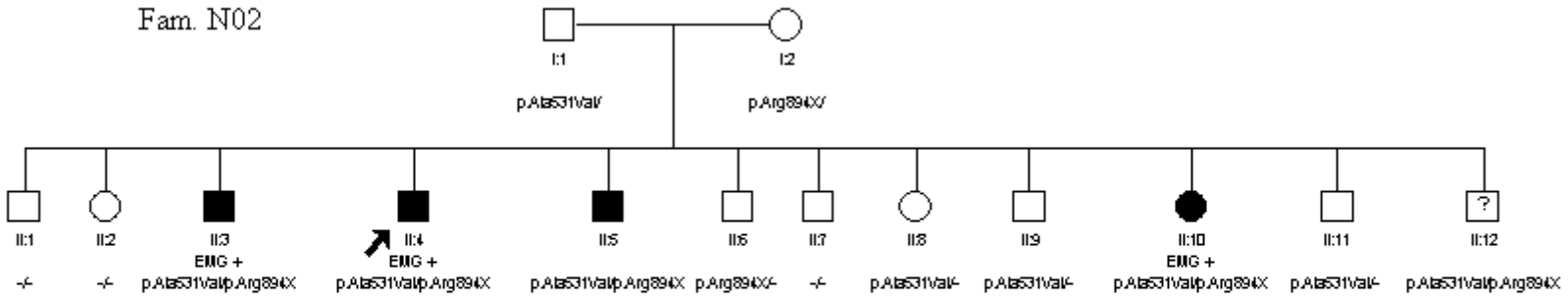
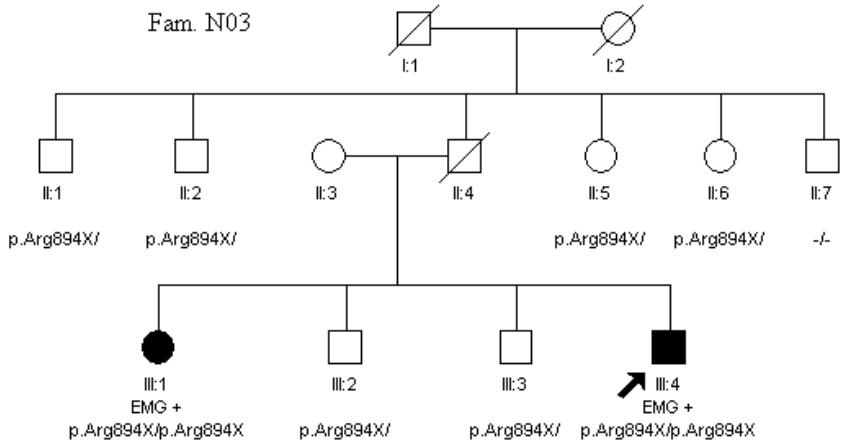
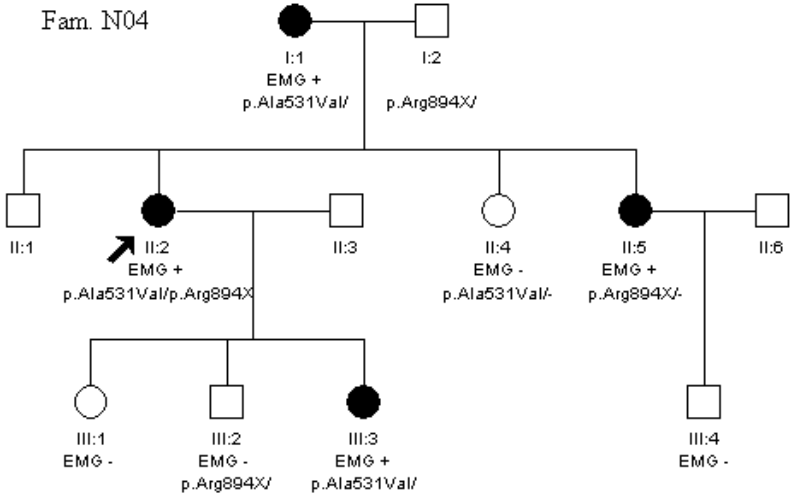
Appendix I

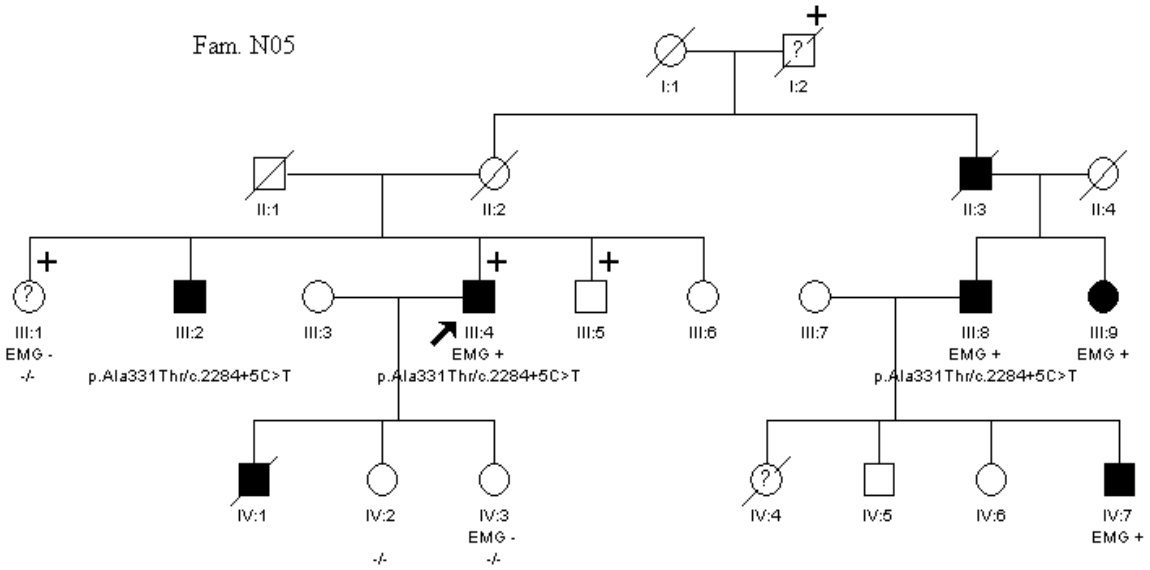
Pedigrees of the fifteen Northern-Norwegian MC families (N01-15) and three Swedish MC families (S01-03) studied.



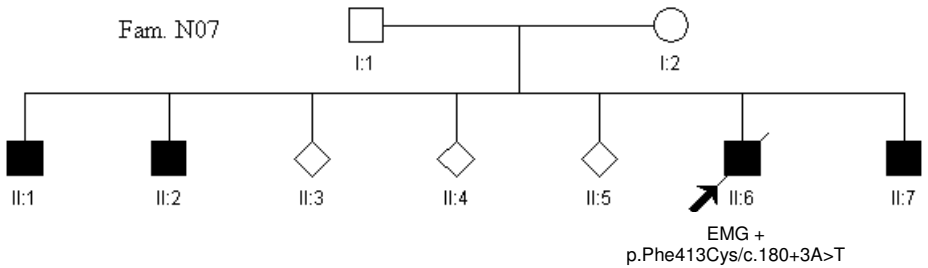

Fam. N02
I:1
p.Ala531Val/
I:2
p.Arg894X/
II:1
-/-
II:2
-/-
II:3
EMG +
p.Ala531Val/p.Arg894X
II:4
EMG +
p.Ala531Val/p.Arg894X
II:5
p.Ala531Val/p.Arg894X
II:6
p.Arg894X/-
II:7
-/-
II:8
p.Ala531Val/-
II:9
p.Ala531Val/-
II:10
EMG +
p.Ala531Val/p.Arg894X
II:11
p.Ala531Val/-
II:12
?
p.Ala531Val/p.Arg894X

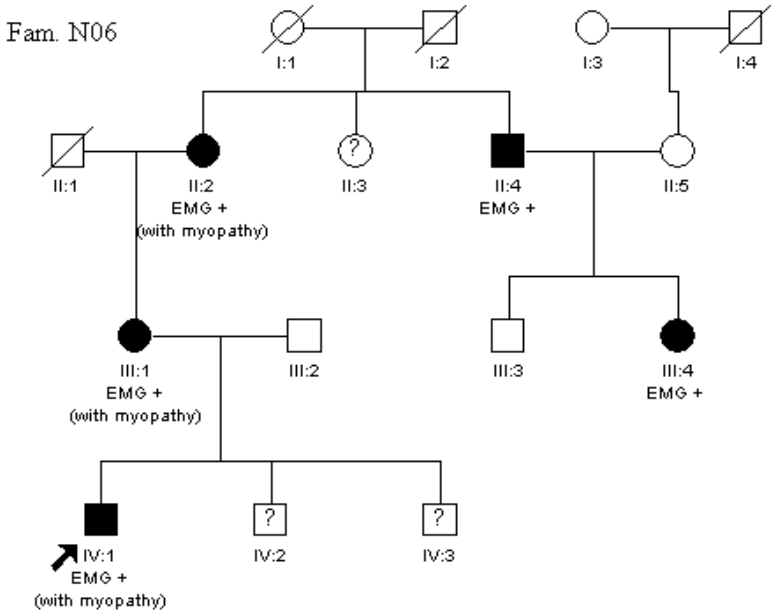

Fam. N03
I:1
I:2
II:1
p.Arg894X/
II:2
p.Arg894X/
II:3
II:4
II:5
p.Arg894X/
II:6
p.Arg894X/
II:7
-/-
III:1
EMG +
p.Arg894X/p.Arg894X
III:2
p.Arg894X/
III:3
p.Arg894X/
III:4
EMG +
p.Arg894X/p.Arg894X

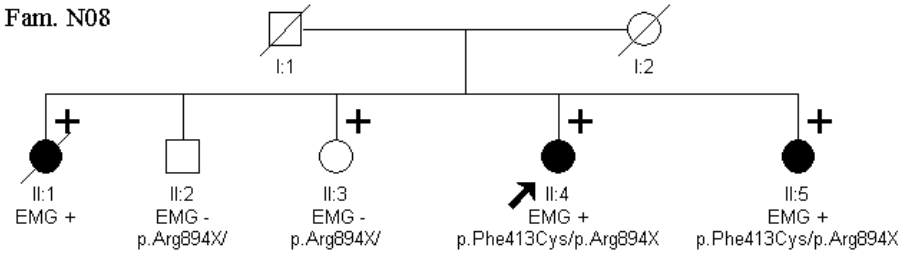

Fam. N04
I:1
EMG +
p.Ala531Val/
I:2
p.Arg894X/
II:1
II:2
EMG +
p.Ala531Val/p.Arg894X
II:3
II:4
EMG -
p.Ala531Val/-
II:5
EMG +
p.Arg894X/-
II:6
III:1
EMG -
III:2
EMG -
p.Arg894X/
III:3
EMG +
p.Ala531Val/
III:4
EMG -



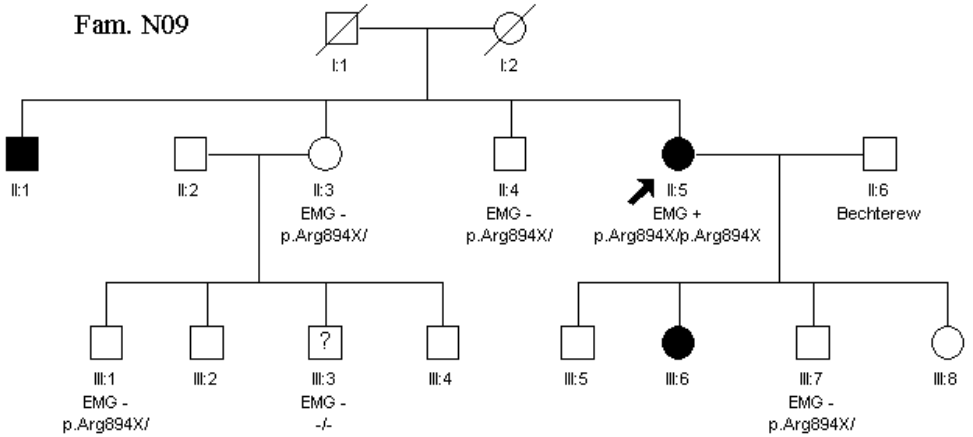
(+ Members with spastic paralysis)

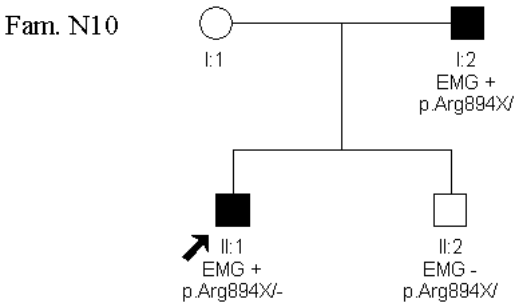


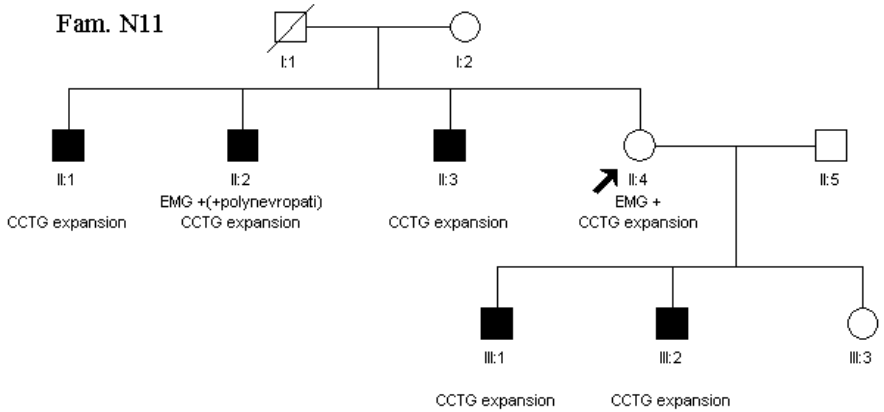


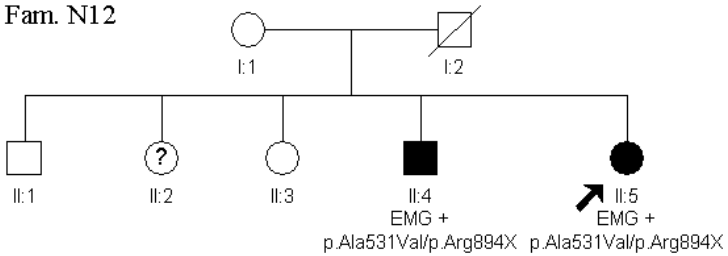


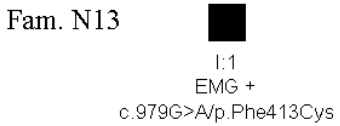
(+ Members with mental retardation)

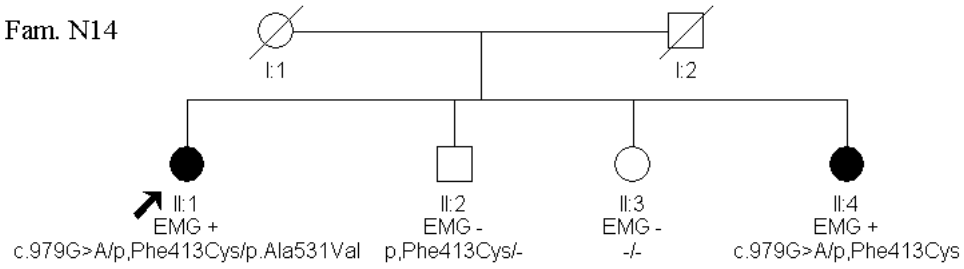


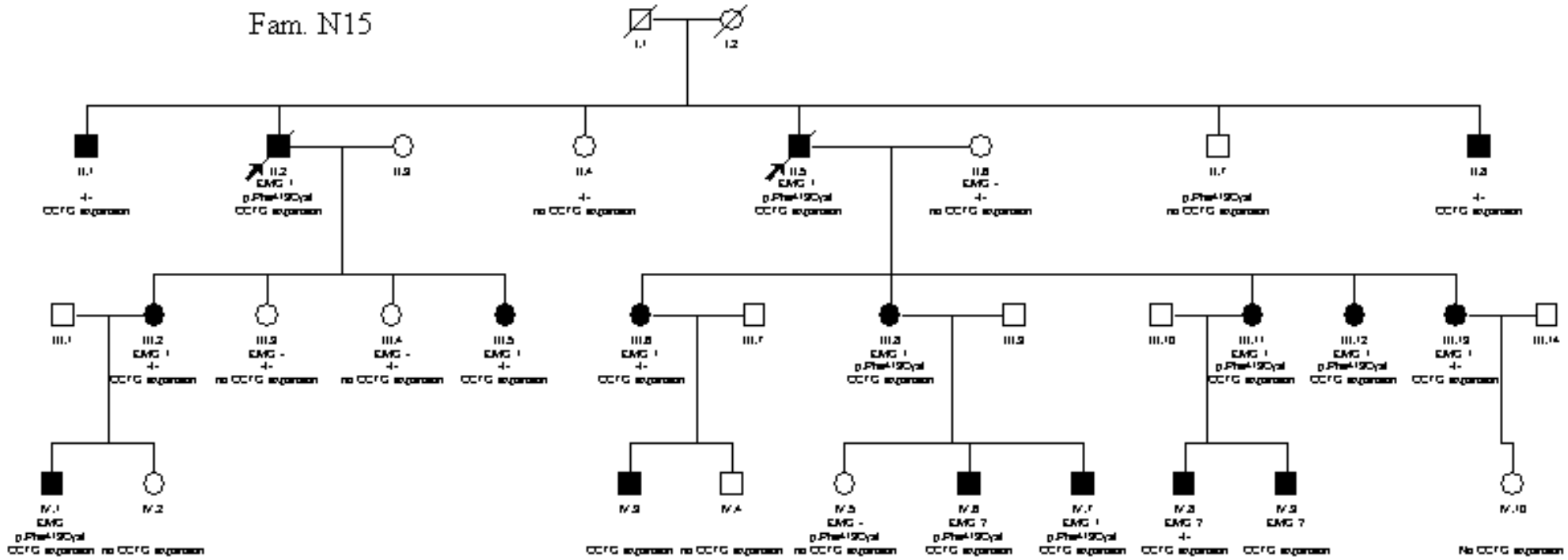



Fam. N11
I:1
I:2
II:1
CCTG expansion
II:2
EMG +(+polynevropati)
CCTG expansion
II:3
CCTG expansion
II:4
EMG +
CCTG expansion
II:5
III:1
CCTG expansion
III:2
CCTG expansion
III:3

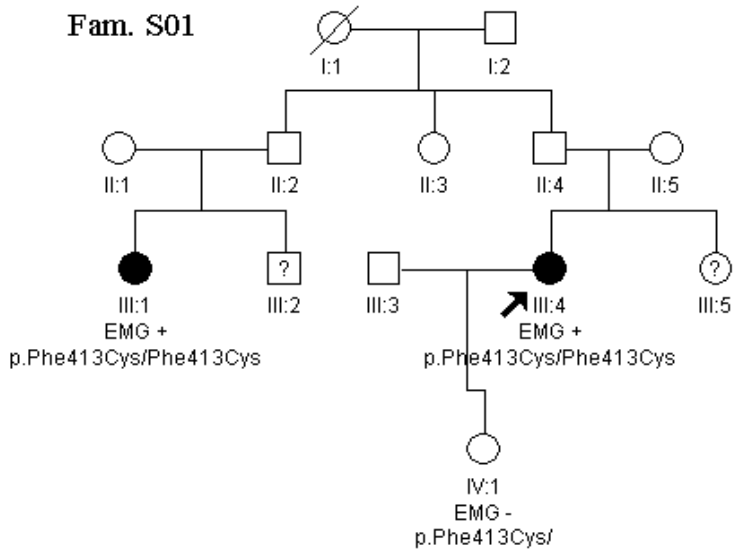

Fam. N12
I:1
I:2
II:1
II:2
II:3
II:4
EMG +
p.Ala531Val/p.Arg894X
II:5
EMG +
p.Ala531Val/p.Arg894X


Fam. N13
I:1
EMG +
c.979G>A/p.Phe413Cys

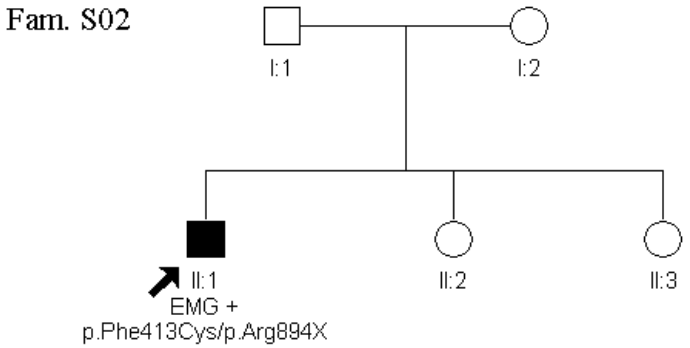

Fam. N14
I:1
I:2
II:1
EMG +
c.979G>A/p,Phe413Cys/p.Ala531Val
II:2
EMG -
p,Phe413Cys/-
II:3
EMG -
-/-
II:4
EMG +
c.979G>A/p,Phe413Cys


Fam. N15
I.1
I.2
II.1
+/-
CCTG expansion
II.2
EMG +
p.Phe413Cys
CCTG expansion
II.3
II.4
+/-
no CCTG expansion
II.5
EMG +
p.Phe413Cys
CCTG expansion
II.6
EMG -
+/-
no CCTG expansion
II.7
p.Phe413Cys
no CCTG expansion
II.8
+/-
CCTG expansion
III.1
III.2
EMG +
+/-
CCTG expansion
III.3
EMG -
+/-
no CCTG expansion
III.4
EMG -
+/-
no CCTG expansion
III.5
EMG +
+/-
CCTG expansion
III.6
EMG +
+/-
CCTG expansion
III.7
III.8
EMG +
p.Phe413Cys
CCTG expansion
III.9
III.10
III.11
EMG +
p.Phe413Cys
CCTG expansion
III.12
EMG +
p.Phe413Cys
CCTG expansion
III.13
EMG +
+/-
CCTG expansion
III.14
IV.1
EMG
p.Phe413Cys
CCTG expansion
IV.2
no CCTG expansion
IV.3
CCTG expansion
IV.4
no CCTG expansion
IV.5
EMG -
p.Phe413Cys
no CCTG expansion
IV.6
EMG ?
p.Phe413Cys
CCTG expansion
IV.7
EMG +
p.Phe413Cys
CCTG expansion
IV.8
EMG ?
+/-
CCTG expansion
IV.9
EMG ?
CCTG expansion
IV.10
No CCTG expansion

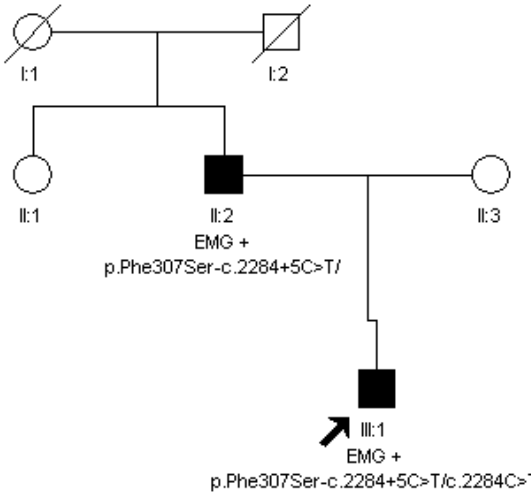
Fam. S01



Fam. S02



Fam. S03



Appendix II

Allele frequencies of selected mutations in the Fennoscandian populations.

Mutations	Number of alleles studied in the normal population				Number of alleles carrying the mutation				Allele frequency (%)
	Norwegian	Finnish	Swedish	Total	Norwegian	Finnish	Swedish	Total	
p.Ala331Thr	88			88	0				0
p.Phe413Cys	92	94	100	286	0	0	0	0	0
p.Ala531Val	188	82	86	356	0	0	1	1	0.3
p.Arg894X	194	162	100	456	2	1	1	4	0.87

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