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ARTICLE

In tandem analysis of *CLCN1* and *SCN4A* greatly enhances mutation detection in families with non-dystrophic myotonia

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Non-dystrophic myotonias (NDMs) are caused by mutations in *CLCN1* or *SCN4A*. The purpose of the present study was to optimize the genetic characterization of NDM in The Netherlands by analysing *CLCN1* and *SCN4A* in tandem. All Dutch consultant neurologists and the Dutch Patient Association for Neuromuscular Diseases (Vereniging Spierziekten Nederland) were requested to refer patients with an initial diagnosis of NDM for clinical assessment and subsequent genetic analysis over a full year. Based on clinical criteria, sequencing of either *CLCN1* or *SCN4A* was performed. When previously described mutations or novel mutations were identified in the first gene under study, the second gene was not sequenced. If no mutations were detected in the first gene, the second gene was subsequently also analysed. Underlying NDM mutations were explored in 54 families. In total, 20% (8 of 40) of our probands with suspected chloride channel myotonia showed no *CLCN1* mutations but subsequent *SCN4A* screening revealed mutations in all of them. All 14 probands in whom *SCN4A* was primarily sequenced showed a mutation. In total, *CLCN1* mutations were identified in 32 families (59%) and *SCN4A* in 22 (41%), resulting in a diagnostic yield of 100%. The yield of mutation detection was 93% with three recessive and three sporadic cases not yielding a second mutation. Among these mutations, 13 in *CLCN1* and 3 in *SCN4A* were novel. In conclusion, the current results show that in tandem analysis of *CLCN1* and *SCN4A* affords high-level mutation ascertainment in families with NDM.
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Introduction

Non-dystrophic myotonias (NDMs) are a group of skeletal muscle disorders that have myotonia as their common feature, in reference to a delayed muscle relaxation after voluntary or evoked muscle contraction. Myotonic

discharges can be recorded by needle-electromyography (needle-EMG) from the skeletal muscles of these patients. Clinically, NDMs are classified as dominant and recessive myotonia congenita (dominant MC; Thomsen's disease (OMIM 160800) and recessive MC; Becker's disease (OMIM 255700)), paramyotonia congenita (PC (OMIM 168300)) and potassium-aggravated myotonias (PAMs (OMIM 608390)). As suggested by Rüdel *et al*,[1] PAM diagnosed without a potassium-loading test is referred to as a sodium-channel myotonia (SCM).

In 1971, Bryant and Morales-Aguilera showed that the membrane resistance of myotonic goat muscle fibres was considerably elevated at rest, which was found to be due to a strongly diminished sarcolemmal chloride conductance.[2] The voltage-gated chloride channel concerned was shown to be involved in dominant and recessive MC in humans.[3,4] Subsequent genetic studies demonstrated a linkage to the skeletal muscle chloride channel gene (*CLCN1* (OMIM 118425)), mapped to chromosome 7q35.[5,6] About 80 different *CLCN1* mutations have, so far, been associated with MC.[7] Meanwhile, in a second group of NDMs, impaired inactivation of voltage-gated sodium channels was observed.[8] Various researchers later independently linked PC and PAM to the skeletal muscle sodium channel gene (*SCN4A* (OMIM 603967)), genetically mapped to chromosome 17q23–25.[9–11] To date, at least 30 different missense mutations have been identified in this gene.[12]

Although many mutations have been identified in *CLCN1*, 25–60% of the MC patients who were examined lacked any identifiable *CLCN1* mutation.[13–15] Comparable studies in patients with PC or PAM have so far not been performed. Although sequencing of the entire *CLCN1* increased the yield of putative myotonia-associated mutants, this was never able to account for all patients.[16] Limitations in mutation detection methods, genetic heterogeneity and additional modifying factors were proposed to explain the discrepancy.[13–16] Plassart-Schiess *et al*[17] postulated the incomplete dominance of some mutations with variable penetrance and expressivity as another compounding factor. Additionally, patients with suspected autosomal dominant MC may show *SCN4A* mutations. The purpose of the present study was therefore to optimize the genetic characterization of patients with NDM in The Netherlands by in tandem analysis of *CLCN1* and *SCN4A*, as necessary.

Patients and methods

Proband selection

The current investigation comprised a cross-sectional, nationwide study. In March 2005, consultant neurologists across The Netherlands as well as the Vereniging Spierziekten Nederland (VSN), our national Patient Association for Neuromuscular Diseases, were requested to report patients with a clinical diagnosis of NDM to our research group over a full year. All patients were subsequently contacted and those who responded positively were invited to the neurology outpatient clinic of the Radboud University Nijmegen Medical Centre for the proposed clinical assessment, needle-EMG and collection of blood samples for genetic analysis. Inclusion criteria were age over 18 years, a clinical diagnosis of NDM according to established clinical criteria (Table 1),[18] and myotonic discharges upon needle-EMG examination.

Exclusion criteria included a clinical or genetic diagnosis of types 1 or 2 myotonic dystrophy, a clinical or genetic diagnosis of primary periodic paralysis and unwillingness or inability to reduce or stop drug therapy for myotonia for the duration of the study. This latter criterion was added to optimize the clinical and electrophysiological evaluations of the myotonia. The study was approved by the Medical Ethics Committee of the Radboud University Nijmegen Medical Centre and all patients gave their written informed consent prior to their participation.

Table 1 Clinical criteria for non-dystrophic myotonias[18]

Chloride Channelopathies
Dominant myotonia congenita:
Autosomal dominant inheritance
Age at onset from birth to early childhood
Myotonia, particularly after rest
Muscle function improves with continuing exercise (warm-up)
Myotonia fluctuates only slightly during lifetime, without progression
Recessive myotonia congenita:
Autosomal recessive inheritance
Onset usually in the first decade of life
Myotonia, particularly after rest
Muscle function improves with continuing exercise (warm-up)
Often transient weakness after rest, improving with continuing exercise (warm-up)
Several years of progression, after which the condition stabilizes
Sodium Channelopathies
Paramyotonia congenita:
Autosomal dominant inheritance
Onset from birth
Muscle function worsens with continuing exercise (paradoxical myotonia)
Paradoxical myotonia is especially common when muscles are exercised in low temperatures
Muscle weakness may occur when muscles are exercised in low temperatures
Potassium-aggravated myotonia:
Autosomal dominant inheritance
Myotonia fluctuans: myotonia, which may fluctuate from day-to-day, is provoked by long periods of exercise (delayed-onset myotonia)
Myotonia permanens: persistent generalized myotonia, particularly in neck and shoulder muscles
Acetazolamide responsive myotonia congenita: myotonia fluctuates and, in addition, exercise induces muscle pain

Preliminaries and procedure

CLCN1 and *SCN4A* were sequenced in tandem following our specially designed study strategy. The decision to sequence first *CLCN1* or *SCN4A* was based on established clinical criteria, which were independently verified for each patient by two authors (JT and GD). Disagreement was resolved by discussion. When previously described mutations were identified in the analysis of the first gene, we did not proceed with sequencing the second gene. In case of a suspected recessive myotonia congenita in which we detected one mutation, we did not proceed with sequencing *SCN4A*. In case of novel mutations, their status and inheritance patterns were determined by clinical evaluations of first-degree relatives and by direct sequence analyses of their DNA. In addition, all novel missense mutations were screened in a control panel consisting of the DNA from 50 healthy Dutch individuals (100 chromosomes). In case no mutations were found in the first gene or novel mutations were not confirmed in first-degree relatives, we subsequently sequenced the second gene.

Mutation analysis

For each patient two 10-ml blood samples were collected in EDTA tubes. Genomic DNA was isolated from peripheral blood by the method of Miller *et al*[19] at the Leiden University Medical Centre and subsequently screened for mutations by direct sequence analysis of *CLCN1* and/or *SCN4A*. PCR analysis, purification of the PCR products (Millipore Multiscreen HTS PCR plates), sequencing (Big Dye Terminator Cycle Sequencer kit from Perkin-Elmer) and the final analysis (ABI3730) were performed as described previously with some minor modifications, indicated between brackets.[20] The primer sets used for amplification of *CLCN1* were made according to Lorenz *et al*[21] and primer sets designed for amplification of *SCN4A* can be found at http://www.lumc.nl/4080/DNA/SCN4A.html.

Results

Study cohort

The recruitment procedure yielded a total of 113 probands, 23 of whom did not respond to initial contacts while 10 eventually refused participation without specifying their reasons. Nine were unable to participate due to transportation problems and three were unable to visit the hospital because of serious co-morbidity. Another 14 probands were excluded based on the following exclusion criteria: primary periodic paralysis ($n=7$), unwillingness/inability to reduce and stop drug therapy for myotonia (n = 5) and no clinical diagnosis of NDM with absence of myotonic discharges by needle-EMG ($n=2$). Accordingly, 54 probands with a clinically and electrophysiologically supported diagnosis of NDM took part in this study. We, moreover, clinically and genetically examined 18 (20%) affected and 74 (80%) unaffected first-degree relatives of those probands for whom novel mutations or indistinct inheritance patterns were established. Table 2 shows the basic demographics for all 54 probands, and their 92 first-degree relatives.

Table 2 Basic characteristics of all eligible probands and their first-degree relatives (affected and unaffected) included in the *CLCN1* and *SCN4A* sequencing procedure

	Probands (n = 54)	*First-degree affected family members (n = 18)*	*First-degree unaffected family members (n = 74)*
Sex, n (%)			
Female	25 (46%)	7 (39%)	40 (54%)
Male	29 (54%)	11 (61%)	34 (46%)
Mean age years (SD)	43.1 (12.4)	49.9 (13.3)	54.8 (16.1)
Age years, (range)	19–71	26–76	18–87

Preliminaries and procedure data

For an overview of the results, see the flowchart depicted in Figure 1. In short, *CLCN1* was sequenced in 40 probands. For the remaining 14 probands, we first sequenced *SCN4A*. Mutations in *CLCN1* were identified in 32 probands. Subsequently, the remaining eight showed a mutation in *SCN4A*. For all 14 probands in whom *SCN4A* was the first to be sequenced, a *SCN4A* mutation was found. DNA analysis thus identified 32 (59%) probands with *CLCN1* mutations and 22 (41%) probands with *SCN4A* mutations, reflecting a 100% gene detection yield. However, the mutation detection yield, including homozygous recessives, was 93% (78 of 84) as in three recessive and three sporadic probands (worst case scenario) no second mutation was found.

Mutation analysis data

CLCN1 *mutations* In 32 probands, 21 different *CLCN1* mutations were identified, encompassing 9 missense, 5 splice site, 4 nonsense and 3 frameshift mutations (Table 3a). More than half (55%) of the mutations were detected in three exons (exons 3, 8 and 11). The remaining mutations were scattered across the entire chloride channel (Figure 2). Most of the 32 probands were compound heterozygote ($n=17$; Table 3b) and seven probands were homozygote, of whom four are known to result from consanguineous marriages. In eight probands, only one mutation was detected of whom two showed an autosomal dominant inheritance (families 37 and 51; Table 3b), three were sporadic (families 10, 16 and 38; Table 3b) and for the other three, autosomal recessive inheritance seemed plausible (families 9, 27 and 41; Table 3b). Overall, the F413C missense mutation was the most frequently observed mutation ($n=8$; see Table 3a).

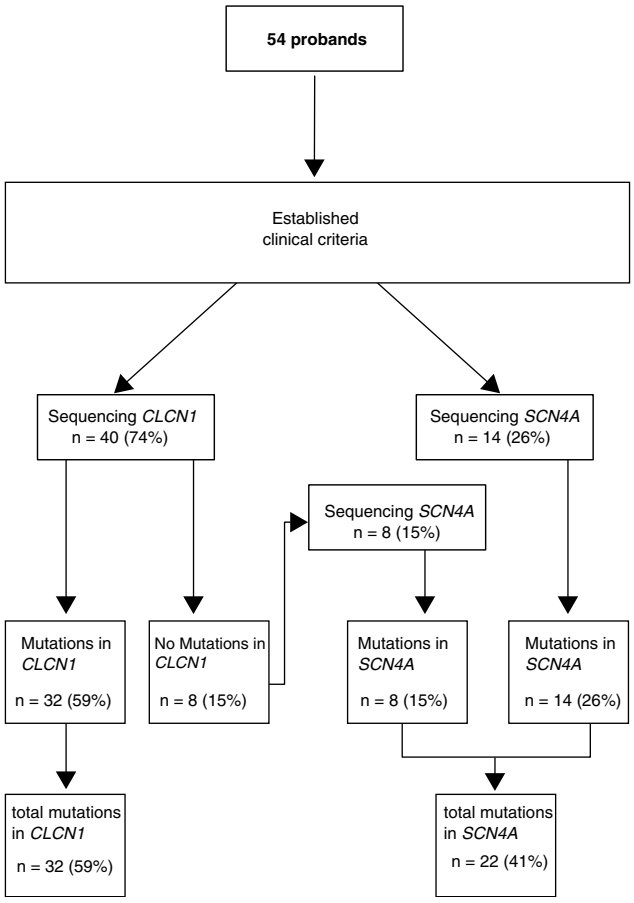

Figure 1 Flowchart depicting the results of the in tandem analysis of *CLCN1* and *SCN4A*. Flowchart presenting the numbers of probands (*n*, %) in whom the direct sequence analysis of *CLCN1* or *SCN4A* was performed. The flowchart also presents the results. Note that based on established clinical criteria in 40 probands *CLCN1* was sequenced first and that in 8 of these probands no mutation was identified. Also note that in 22 probands *SCN4A* was sequenced, 14 based on the mentioned clinical criteria and 8 based on the negative sequencing result of *CLCN1*. In total, *CLCN1* mutations were identified in 32 families and *SCN4A* in 22.

In total, 13 of the 21 different mutations in *CLCN1* were newly identified mutations (62%) and comprised two nonsense, four splice site, two frameshift and five missense mutations. The novel mutations 302−1G>A ($n=4$), S264fsX ($n=4$), M646I ($n=3$), 1167−10T>C ($n=3$), 302−2A>C (n = 2) and A129T (n = 2) were detected more than once in our families and none of the novel missense mutations were detected in the 100 control chromosomes.

Novel *CLCN1* mutations

Y137X and C819X (recessive) Two novel nonsense mutations were identified. The Y137X mutation was detected in a recessive pedigree (family 26). The C819X was identified as the only mutation in two affected sibs of family 41. The healthy mother appeared to be the carrier of this mutation.

K195fsX (recessive) This novel frameshift mutation was identified in two patients of family 1 alongside the I479fsX mutation. A healthy sister, son and daughter appeared to be carriers of the K195fsX mutation.

S264fsX (recessive) This mutation was detected in two patients of family 6 alongside the novel missense mutation G305E. The father was carrier of the S264fsX mutation and the mother and an unaffected brother were heterozygous for the G305E mutation. Furthermore, the S264fsX mutation was the only recessive mutation in the sporadic probands of families 10 and 16. Two unaffected children and three unaffected siblings of family 10 and the mother of proband 16 were carriers of this mutation. Lastly, S264fsX was also identified in family 43 alongside the novel splice-site mutation 1065−2A>C.

302−1G>A (recessive) This novel splice-site mutation was identified in a compound heterozygous state in all three affected sibs of families 2, 4 and 5 and in the recessive pedigree of family 11.

302−2A>C (recessive) This second novel splice-site mutation was observed in two families: homozygous in the proband of family 7, issue of a consanguineous marriage and compound heterozygous in a sporadic patient with a recessive mode of inheritance (family 54) alongside the newly identified missense mutation M646I.

1065−2A>C (recessive) The third novel splice-site mutation was identified in the proband of family 43 alongside the novel frameshift mutation S264fsX. The mother and an unaffected sister were carriers of the novel splice-site mutation and the father and two siblings were carriers of the novel frameshift mutation.

1167−10C>T (recessive) All affected sibs ($n=5$) of the recessive pedigrees 4 and 5 showed this novel splice-site mutation together with the novel 302−1G>A mutation. Unaffected first-degree relatives in both families appeared to be carriers of one of the novel mutations. This mutation was also identified in a compound heterozygous state in a single patient of family 14. First-degree unaffected relatives carried one of the two mutations.

A129T (dominant) This missense mutation emerged in two families (37 and 38), ie one sporadic and one with a presumably dominant inheritance pattern. This could not be confirmed by DNA analysis because the affected father was deceased and the proband did not have children. *SCN4A* was also analysed retrospectively in both families and showed no mutations.

G305E (recessive) The second novel missense mutation was identified in family 6 and occurred in all affected

Table 3a Spectrum of *CLCN1* mutations identified in selected probands residing in The Netherlands

Exon	*Nucleotide change*	*Amino-acid change*	*No. of families with mutations*	*References*
1	180+3A>T	splice-site donor	2	[22]
3	**302−1G>A**	**splice-site acceptor**	4	Present study
3	**302−2A>C**	**splice-site acceptor**	2	Present study
3	**385G>A**	**A129T**	2	Present study
3	**411C>G**	**Y137X**	1	Present study
4	501C>G	F167L	3	[13,23]
5	**585_589del**	**K195fsX**	1	Present study
7	**789delC**	**S264fsX**	4	Present study
8	854G>A	G285E	5	[24]
8	**914G>A**	**G305E**	1	Present study
10	**1065−2A>G**	**splice-site acceptor**	1	Present study
11	**1167−10T>C**	**splice-site acceptor**	3	Present study
11	1238T>G	F413C	8	[13,23]
11	**1250A>G**	**E417G**	1	Present study
13	1437_1450del	I479fsX	1	[13,14,22,25]
13	1439C>T	P480L	1	[13,26]
16	**1841A>T**	**K614M**	1	Present study
17	**1938G>A**	**M646I**	3	Present study
21	2419C>T	Q807X	1	[7,27]
21	**2457C>A**	**C819X**	1	Present study
23	2680C>T	R894X	3	[13,14,17,28,23]

Abbreviation: No., number.
Novel mutations are printed in bold.

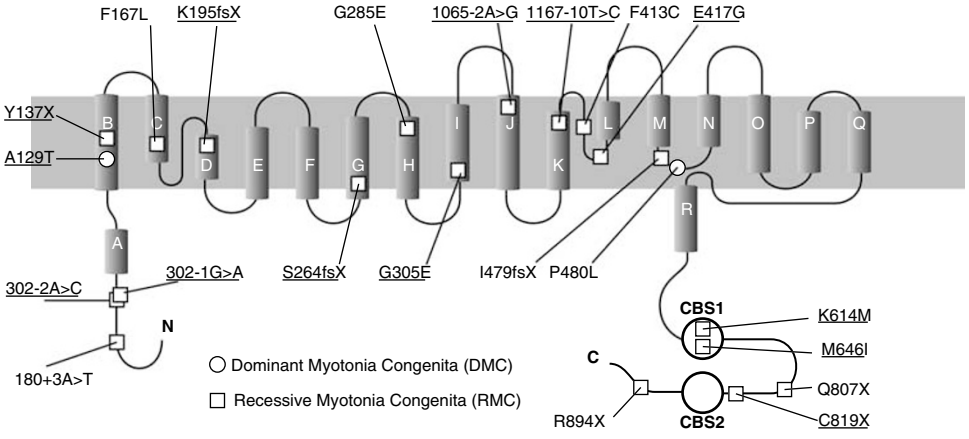

Figure 2 The sites of the mutations in the skeletal muscle chloride (ClC-1) channel as identified in the present study. Novel mutations are underlined, circles represent dominant myotonia congenita (Thomsen) and squares recessive myotonia congenita (Becker). The membrane topology of the ClC-1 channel is adapted from Dutzler *et al*.[45]

family members alongside a novel recessive frameshift mutation (S264fsX). The mutation was also identified in two unaffected siblings and the unaffected father.

E417G (recessive) This mutation was detected in the proband (E417G/G285E) of family 40. The unaffected mother was heterozygous for the E417G mutation; the father was deceased.

K614M (recessive) This fourth novel missense mutation was identified in family 42. The affected proband was compound heterozygous (K614M/180+3A>T). Both parents were deceased, while one brother was a carrier of the 180+3A>T and one sister was a carrier of the K614M mutation.

M646I (recessive) The M646I mutation was identified in three different families (46, 48 and 54). It was identified alongside the recessive missense mutation G285E, the nonsense mutation R894X and the novel splice-site mutation 302−2A>C, respectively. In family 48, the mutation was also detected in two affected sibs. In

Table 3b The mutational combinations in *CLCN1* as identified in the 32 probands with myotonia congentia

Family nos.	*Consequences allele 1/2*	*Inheritance*
01	**K195fsX**/I479fsX	Recessive
02	**302−1G>A**/F413C	Recessive
04 and 05	**302−1G>A/1167−10T>C**	Recessive
06	**S264fsX/G305E**	Recessive
07	**302−2A>C/302−2A>C**	Recessive
08 and 22	F167L/F413C	Recessive
09	F413C/−	Probably recessive
10 and 16	**S264fsX**/−	Sporadic
11	180+3A>T/**302−1G>A**	Recessive
14	F413C/**1167−10T>C**	Recessive
21	F413C/F413C	Recessive
25 and 44	G285E/G285E	Recessive
26	**Y137X**/F413C	Recessive
27	F167L/−	Probably recessive
29 and 32	R894X/R894X	Recessive
37 and 38	**A129T**/−	Dominant and sporadic
40	G285E/**E417G**	Recessive
41	**C819X**/−	Probably recessive
42	180+3A>T/**K614M**	Recessive
43	**S264fsX/1065−2A>G**	Recessive
46	G285E/**M646I**	Recessive
47	E807X/E807X	Recessive
48	**M646I**/R894X	Probably recessive
51	P480L/−	Dominant
54	**302−2A>C/M646I**	Recessive
56	G285E/F413C	Recessive

Abbreviation: Nos., numbers.
Novel mutations are printed in bold.

the recessive pedigree of family 54, the described mutations occurred in a single patient. In family 46, the father was a carrier of G285E, and the mother was deceased.

Clinical features of the probands with *CLCN1* mutations

All probands with *CLCN1* mutations showed obvious clinical signs of myotonia and 97% showed the warm-up phenomenon. About 15% of the probands showed transient paresis and muscle wasting, 48% only showed transient paresis and 37% showed neither transient muscle weakness nor muscle wasting. The probands with novel mutations did not show new clinical features compared with those having already known mutations.

SCN4A *mutations* In 22 probands, 11 different missense mutations were identified (Table 4). Three mutations were located in domain I, one in domain II and six in the domains III and IV of the voltage-gated sodium channel, $Na_v1.4$ (Figure 3). In 59% of the probands, a mutation was identified in exon 22, of which the G1306V mutation was the most common ($n=8$).

In total, 3 of the 11 different missense mutations were novel (27%), comprising two mutations in codon 250: L250V and L250P and one in codon 689: L689F. Each of the three novel mutations was detected once and none of the novel missense mutations were detected in the 100 control chromosomes. All probands with the novel mutations showed a phenotype mimicking Thomsen's disease. Therefore, DNA of these three probands was first sequenced for *CLCN1* mutations, but no mutations were identified.

Novel *SCN4A* mutations

L250V (dominant) This missense mutation in the proband of family 12 is most probably a *de novo* mutation. Presumably, both his parents (deceased) were unaffected while his daughter and granddaughter were both affected and showed the same L250V mutation.

L250P (dominant) This mutation was identified in the proband of family 39. The affected father and brother in this family also showed this mutation. It was not detected in an unaffected brother and sister.

L689F (dominant) This mutation was detected as a *de novo* mutation in family 36. Both parents of the proband were unaffected and did not carry the mutation. The mutation was identified in both the proband and her affected daughter.

Clinical features of the probands with *SCN4A* mutations

All probands with *SCN4A* mutations showed obvious clinical signs of myotonia, especially in the eyelid muscles. Furthermore, probands with SCM, including the three probands with novel mutations, showed the warm-up phenomenon. The phenomenon was detected in the eyelid muscles (80%) as well as in the hand flexor muscles (80%). In contrast, probands with paramyotonia congenita showed paramyotonia in the eyelid muscles (40%) as well as in the hand-flexor muscles (75%). Finally, almost all probands with paramyotonia congenita showed an increase of paramyotonia or a flaccid paresis after cooling. Probands with SCM did not react to cooling.

Discussion

By sequencing *CLCN1* and *SCN4A* in tandem, we detected mutations in all our probands. The yield of mutation detection was 93%, with six cases (7%) not yielding a second mutation. This is a high percentage, especially compared to previous studies that identified *CLCN1* mutations in 40 to 75% of their MC patients, whereas analysis of *SCN4A* was not included.[13–15] Although the yield of our mutation detection was high, we failed to detect a second mutation in six probands. Possibly, deletions or other types of mutation deep in the intron

Table 4 Spectrum of *SCN4A* mutations identified in selected probands residing in The Netherlands

Family nos.	*Exon*	*Nucleotide substitution*	*Amino-acid substitution*	*No. of families with mutation*	*References*
12	**6**	**748C>G**	**L250V**	1	Present study
39	**6**	**749T>C**	**L250P**	1	Present study
3, 18	9	1333G>A	V445M	2	29
36	**13**	**2065C>T**	**L689F**	1	Present study
52	19	3472C>T	P1158S	1	30
	19	3555A>G	I1160V	1	31
15, 24	22	3917G>C	G1306A	2	10,11,22
19, 28, 30, 33, 45, 50, 53, 55	22	3917G>T	G1306V	8	10,32
13, 17, 23	22	3938C>T	T1313M	3	32,33
35	24	4343G>A	R1448H	1	34
20	24	4372G>T	V1458F	1	35

Abbreviation: No., number.
Novel mutations are printed in bold.

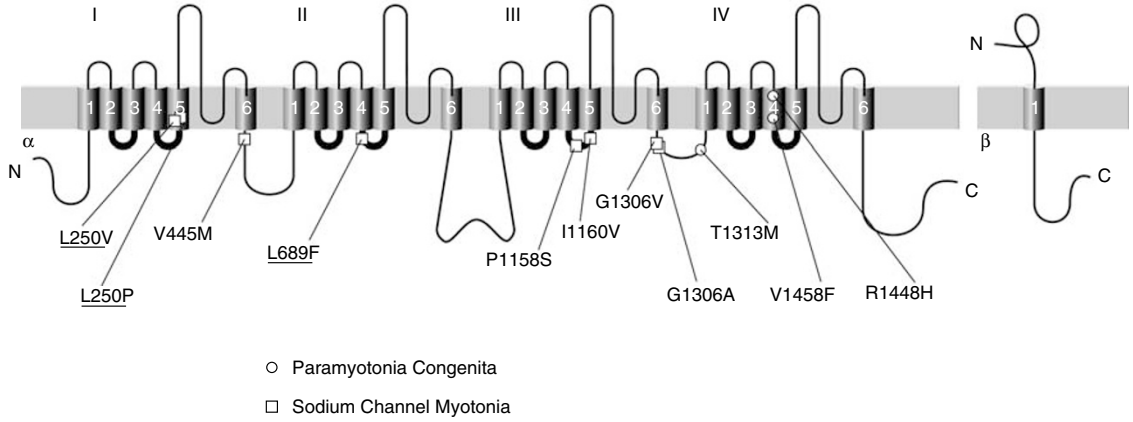

Figure 3 The sites of the mutations in the skeletal muscle sodium ($Na_v1.4$) channel as identified in the present study. Novel mutations are underlined, circles represent paramyotonia congenita and squares sodium channel myotonias. The membrane topology of the $Na_v1.4$ channel is adapted from Jurkat-Rott *et al*.[44]

or the promoter region of *CLCN1* may underlie the disease in these cases.[16]

Our strategy yielded 13 novel mutations in *CLCN1* and 3 in *SCN4A*. Although we did not perform *in vitro* studies, there are four lines of evidence affirming the suggestion that these mutations are pathogenic. First, 8 of the 13 novel mutations in *CLCN1* were splice site, frameshift or nonsense mutations, which are predicted to eliminate channel function.[27] Second, none of the novel missense mutations occurred in the 100 control chromosomes. Third, all original wild-type amino acids at the sites of the missense mutations were well conserved across chloride or sodium channels of different species and/or among human chloride or sodium channels, and fourth, all but one (A129) of the novel mutations were segregating with the disease.

Since the pathogenic status of missense mutations is less clear than the status of the other mutations, these mutations will individually be discussed by their location and conservation.

First, the A129T mutation is located in transmembrane segment B of the human ClC-1 channel. A129 is a highly conserved amino acid across ClC channels of different species and is well conserved among the human plasma membrane ClC isoforms ClC-1, -2, -Ka and -Kb. Furthermore, A129T is in the vicinity of the already established M128V and S132C mutations. Both mutations segregated with the Thomsen's phenotype.[36,37] M128V and S132C were both electrophysiologically characterized and showed a rightward shift in the current–voltage relationship, explaining their pathogenicity.[36–38]

Second, the G305E mutation is located in transmembrane segment 1 of ClC-1. G305 is a highly conserved amino acid across ClC channels of different species and is well conserved among the human ClC isoforms ClC-1, -2, -Ka and -Kb. G305E is in the vicinity of F307S, which was reported to drastically shift the voltage dependence of ClC-1 to positive potentials, preventing these channels from repolarizing muscle action potentials efficiently.[24]

Third, the E417G mutation is situated in the last codon of exon 11 and is therefore predicted to affect the splicing of this exon. However, further RNA studies are needed to explore this. Furthermore, E417, located in the linker between helix K and L of ClC-1, is highly conserved among ClC channels of different species.

Fourth, the K614M mutation, conserved across ClC channels of different species but not among human ClC isoforms, and the L646I mutation, conserved across ClC channels of different species and among the human isoforms ClC-1 and −2, are located in the β1 and β2–3 linker of the cystathionine β-synthase (CBS1) domain, respectively. Although the precise role of the CBS-domains is unknown, Estévez *et al*[39] suggested that mutations in this domain will influence the voltage-gated dependence of gating through the common gate.

The L250P/V mutations in *SCN4A* are located in the membrane-spanning segment 5 of domain I of $Na_v1.4$. Although in the vicinity of a benign polymorphism (S246L),[40] L250 is highly conserved across $Na_v1.4$ channels of different species and among the α-subunits of human sodium channels $Na_v1.1$–$Na_v1.8$. Furthermore, the mutations were retrospectively absent in 200 control chromosomes. Finally, both probands with these mutations showed a definite phenotype of NDM in the absence of other mutations in *CLCN1* or *SCN4A* and both mutations were segregating with the disease.

The third novel *SCN4A* missense mutation (L689F), located in the linker between segments 4 and 5 of domain II in $Na_v1.4$, is located at the same codon as the already established mutation L689I. This mutation was shown to cause *in vitro* effects of a hyperpolarizing shift in the voltage dependence of activation causing hyperkalemic periodic paralysis.[41] The proband with the L689F mutation in our population showed a Thomsen-like phenotype without symptoms of weakness.

To exclude benign polymorphisms, we tested 100 control chromosomes in accordance with the current best practice guidelines. Furthermore, the Leiden University Medical Centre analysed approximately 500 patients with suspected NDM worldwide during the last 5 years and none of the detected variants were identified. Others mainly performed *in vitro* studies for the confirmation of novel mutations in one of these genes.[36,42,43] In the future, such studies should also be done for the eight novel missense mutations detected in our study.

In this study, we identified 21 different *CLCN1* and 11 different *SCN4A* mutations. Meyer-Kleine *et al*[13] also found a high number of different mutations in their German-based cohort. In Scandinavian studies, three and eight different *CLCN1* mutations were detected, respectively.[16,28] In one of these studies, the A513V, F413C and R894X mutations clearly predominated.[16] Thus, our study yielded a broad spectrum of mutations underlying NDM in The Netherlands without clear evidence of a manifest founder effect. We hypothesize that these findings may be attributable to the high population density in The Netherlands, especially when compared to the low population densities in Sweden, Norway and Finland.

Our analyses revealed dominant MC to be scarce in The Netherlands, which is in sharp contrast with the initial clinical diagnoses. In 20% of the patients, the initial referral diagnosis was a dominant MC. However, only two of these patients were finally classified as dominant MC while the others proved to have SCM. All the probands with a SCM mutation showed a clinical picture of a generalized myotonia in combination with the warm-up phenomenon, mimicking Thomsen's disease.[44]

In conclusion, we have shown that in tandem analysis of *CLCN1* and *SCN4A* affords a high level of mutation ascertainment in families with NDM. With this strategy, we were able to identify 13 novel *CLCN1* and three novel *SCN4A* mutations. Moreover, it enabled us to confirm earlier suggestions that the prevalence of *SCN4A* mutations is higher than previously assumed.[18] Based on the results presented here, we feel safe in suggesting that our approach shows great diagnostic potential and may offer optimal conditions for future genotype–phenotype studies.

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