

ONLINE MUTATION REPORT

RYR1 mutations in UK central core disease patients: more than just the C-terminal transmembrane region of the RYR1 gene

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The congenital myopathies are a group of disorders that are difficult to distinguish. Diagnosis is frequently dependent on the result of histological examination of muscle biopsy specimens in conjunction with clinical diagnosis. There is a significant degree of overlap in the clinical features of different congenital myopathies: identification of genetic determinants may therefore aid in establishing a firm diagnosis.

Central core disease (CCD) is a rare congenital myopathy. Histologically, using oxidative enzyme stains, it is identified by the abundance of central cores, characterised by localised areas of mitochondrial depletion and sarcomere disorganization exclusively in type 1 skeletal muscle fibres, and extending throughout their length. Cores are often central and unique, but may be eccentric or multiple within one fibre. Affected patients may present with congenital muscle hypotonia, pronounced proximal weakness, delayed motor development, and slightly elevated creatine kinase (CK) levels. In addition, skeletal anomalies such as congenital hip displacement and scoliosis are frequent. Later in life muscle strength may improve, but in rare cases progressive muscle weakness is observed. Respiratory insufficiency is rare.^{1,2} Overall, the disorder demonstrates significant phenotypic variability; in a study of 13 cases, as many as 40% of patients with histological signs of disease were clinically asymptomatic.³

CCD is predominantly reported as an autosomal dominant trait. However, there is evidence that inheritance may be autosomal recessive in some families,^{4,5} and sporadic cases have been documented.^{6,7} It is recognised as the primary disorder associated and allelic with the pharmacogenetic disorder malignant hyperthermia (MH).⁸ However, characteristic muscle cores have been reported in association with several other myopathies, namely multi-mini core disease (MmD), nemaline myopathy, and hypertrophic cardiomyopathy. All have additional definitive characteristics in their own right, although in some cases the phenotypic boundaries are difficult to assert.

MH also shows autosomal dominant inheritance, and is genetically heterogeneous with six susceptibility loci identified to date.^{9,10} MH reactions are triggered in susceptible individuals following exposure to certain anaesthetic agents. Crises reflect a disturbance of skeletal muscle calcium homeostasis. Uncontrolled sarcoplasmic reticulum calcium release involving the ryanodine receptor (RYR1) results in sustained muscle contraction, elevated temperature, and metabolic acidosis, and may be fatal if not treated.^{11,12} Clinically, MH status may be assessed by exposure in the laboratory of a patient's muscle biopsy specimen to incremental doses of specific trigger agents (caffeine, halothane) and measuring the *in vitro* contracture response using the contracture test (IVCT).^{13,14} Alternatively, in families where MH status has been confirmed by a positive IVCT in the

Key points

- Central core disease (CCD) is predominantly an autosomal dominant neuromuscular condition exhibiting variable penetrance, which also has been described in association with the pharmacogenetic disorder malignant hyperthermia (MH).
- In many cases of CCD, mutations in RYR1 have been detected, resulting in defective calcium handling of the skeletal muscle ryanodine receptor.
- CCD and MH status have been characterised in 21 investigated families, with RYR1 mutations in 57% of cases. Results suggest that regions other than the C-terminus of the RYR1 gene have important roles in CCD pathogenesis, and that certain RYR1 mutations may be specific to CCD presenting with or without predisposition to MH.
- These data may aid in the diagnosis of CCD/MH in the future.

family index case, identification of an RYR1 mutation proven causative of MH may be used for diagnosis.¹⁵

CCD patients may or may not be susceptible to MH, with phenotypic variability with respect to MH status sometimes observed within the same pedigree.^{16,17} One explanation is the independent segregation of genetic determinants of CCD and MH within a family. Alternatively, variability could reflect patient age at the time of muscle biopsy and/or the mildly progressive nature of CCD documented in some individuals.¹⁸ Reports that histological examination of muscle specimen cross sections (rather than longitudinal sections) does not enable differentiation between CCD and MmD, indicates that the method of specimen analysis is also important for the accurate characterisation of these disorders.¹⁹ All CCD patients should be considered at risk for MH unless diagnosed as MH normal on muscle biopsy testing. However, in some individuals, a positive muscle biopsy test result may be a consequence of the underlying muscle pathology rather than MH susceptibility (MHS) itself. Patients with other muscular disorders unrelated to MH, and who are considered an anaesthetic risk, have been known to test positive with the IVCT.²⁰

Abbreviations: CCD, central core disease; CK, creatine kinase; MH(S), malignant hyperthermia (susceptibility); MmD, multi mini-core disease; IVCT, *in vitro* contracture test; EC, excitation contraction

Table 1 Clinical details of probands investigated

Family (LMH)	Age in years at biopsy	Gender	History	Histology	Clinical features of neuromuscular disorders	CK IU/l	IVCT data§				MH status
							At time of MH biopsy	H thr %	H contr g	C thr mM	
31	37	F	CCD referral	CCD	No cramps. Numbness and weakness in limbs	70	2.0B**	2.8	<8**	0	MHS**
32	24	F	Relatives had MH reactions. Postoperative CK 18000, temp. 39.9°C, myoglobinuria	Cores present.	Some cramps in legs	1000	0.5	0.6	–	–	MHS
33	24	M	Poor muscle development, congenital dislocating hips (CDH) Twice, muscle rigidity after suxamethonium, once with cyanosis and temp. 43°C	CCD with dystrophic changes	Scoliosis. No cramps	1419	0.5	4.3	1	0.4	MHS
34 (1P)	11	F	Known case of CCD	CCD	Scoliosis. Weak legs	35	0.5	1.1	0.5	1.55	MHS
34 (2P)	32, 37	F	Known case of CCD.	CCD and nemaline rods	Weak legs	44	2	0.2	2	0.2	MHS
36	28	F	CCD referral	CCD	Limb weakness. Scoliosis	131	>2	0.05	2	0.75	MHE
37	25	F	After halothane and suxamethonium, MMS with abdominal rigidity. ETCO ₂ off scale, temp. 40.8°C, CK (5 days postoperatively) 11856	Core myopathy	No cramps	913	0.5	3.1	0.5	2.9	MHS
38	14	F	MMS following thiopentone, atropine and suxamethonium	CCD	Scoliosis since childhood	202	0.5	2.4	0.5	1.4	MHS
39	11	M	?CCD. After nitrous oxide/halothane, stiff all over. ?MH, rectal temp. 38.1°C, CK next day 9970	Relative's muscle: cylindrical spirals	Spinal and limb problems. Cramps	120	2.0*	0.3	32*	0	MHE*
63	41	F	Muscle rigidity, high CK, hyperthermia with halothane	Core myopathy	Cramps in legs and shoulders	1828	0.5	3.25	1.5	0.45	MHS
64	33	M	CCD referral	CCD	Cramps in all limbs	492	>2	0	4	0	MHN
65	35	F	MMS after suxamethonium <24hrs stiffness	Core myopathy	No cramps	422	0.5	6.1	1.5	1.5	MHS
66	55	M	After sevoflurane, increased ETCO ₂ , HR 145, temp. 38.4°C	Core myopathy	No cramps	953	1	1	2	0.2	MHS
67	27	F	After isoflurane, increased ETCO ₂ and HR, temp. 37.3°C, CK 56	Core myopathy	No cramps	41	2	0.5	1.5	0.5	MHS
68	50	F	CCD referral	CCD	No cramps. Weakness in legs	244	>2	0	3	0	MHN
69	15	F	Muscle disease since childhood, ?limb girdle dystrophy. After sevoflurane, ETCO ₂ 6.6, HR 165, temp. 38.5°C, CK920	Features of CCD	No cramps. Weakness arms and legs; some wasting. Scoliosis	1301	0.5	4.55	1	1.45	MHS
70	10	F	Known case of CCD/MmD. Poor muscle bulk.	CCD	No cramps. Weak limbs	44	>2	0	4	0	MHN
71	38	M	After halothane, temp. 39°C, CK 779	Core myopathy	No cramps	83	>2	0	4	0	MHN
72	29	M	After isoflurane, HR 120, ETCO ₂ 10.2, temp. 39.5°C, muscle rigidity, CK 48000, myoglobinuria., muscle pain	Core myopathy	Weakness in legs	–	1	2.1	1	1.45	MHS
73 (1P)	62	F	Family history of CCD ³²	Ice crystal artefacts	No cramps	29	2	0.75	1	1.3	MHS
73 (2P)	19	M	Grandmother CCD. MMS after fractured arm age 11	Myopathic changes	No cramps	1324	1	2.7	1.5	1.05	MHS

Table 1 Continued

Family (LMH)	Age in years at biopsy	Gender	History	Histology	Clinical features of neuromuscular disorders	CK IU/l	IVCT data§				
						At time of MH biopsy	H thr %	H contr g	C thr mM	C contr g	MH status
74	10	F	Known case of CCD	CCD	Proximal muscle weakness	52	>2	0	3	0.1	MHN
75	41	F	After desflurane, ET _{CO} 2 10.5, increased a HR, temp. 39.8°C, CK 1621	Type 2b atrophy	No cramps	74	1	0.7	3	0	MHE

*Proband disabled and unable to be tested. Patient screened was next nearest relative.

**IVCT conducted before introduction of standardised EMHG diagnostic protocol.

§ IVCT data. Pre-test twitch of muscle specimens, indicative of specimen viability, were all above 1.0 g for each patient unless highlighted in bold.

H thr,; halothane IVCT threshold = % halothane where 0.2 g contracture recorded; C thr, caffeine IVCT threshold = mM concentration of caffeine where 0.2 g contracture recorded; H contr, halothane IVCT contracture = contracture in g following application of 2% halothane; C contr, caffeine IVCT contracture = contracture in g following application of 2 mM caffeine.

LMH, Leeds malignant hyperthermia unit; * CCD, central core disease; MH, malignant hyperthermia; MmD, multi-mini core disease; P, proband/index case; HR, heart rate; ET_{CO}, end tidal carbon dioxide test; MMS, muscle masseter spasm; MHS, susceptible to MH following IVCT; MHN, MH normal following IVCT; MHE, MH equivocal following IVCT, clinically considered at risk, indicates positive reaction to one of trigger agents only; B, bolus, rather than incremental administration of 2% halothane.

CCD appears to be genetically heterogeneous. However, defects in the RYR1 gene, which encodes a skeletal muscle calcium release channel, are predominant in the majority of cases.^{7 9 21 22} Nevertheless, skeletal muscle cores characteristic of CCD have been reported in association with several other myopathies, and may implicate other loci as potential disease susceptibility candidates, such as:

- mutations in SEPNI on chromosome 1, reported in association with MmD²³
- defects in MYH7 on chromosome 14, reported in patients with hypertrophic cardiomyopathy²⁴
- mutations in ACTA1 on chromosome 1 and TNNT1 on chromosome 19, in cases of nemaline myopathy.^{25 26}

Thus, the apparent genetic heterogeneity may mirror the observed clinical heterogeneity and overlap reported in these disorders.

The RYR1 gene locates to chromosome 19q13.1, encompassing 158 Kb of genomic DNA, and has 106 exons, two of which may be alternatively spliced. The cDNA sequence is ~15 Kb. At least 44 mutations have been reported in the gene in association with CCD, the majority of these being missense changes^{6 7 22 27-30} (unpublished data from the European Malignant Hyperthermia Group). Of the mutations identified, 33 locate to the C-terminal region (exons 90–106) which forms the calcium channel pore of the protein. As a result, the RYR1 C-terminus has been identified as a CCD mutation hotspot.

We analysed RYR1 in 21 families in whom CCD and MH status had been histologically and clinically characterised, and identified 12 mutations in 12 families (one mutation was recurrent and was detected in two families, and two mutations were detected in one family). On screening a panel of unrelated individuals susceptible to MH, absence of 9/12 of the identified mutations implied that these were likely to be specific to the CCD and/or the asymptomatic core myopathy observed in the families. Seven of the 12 mutations characterised were novel, four of which were detected in the C-terminus of RYR1. Our data support the hypothesis that the RYR1 gene is a major genetic locus for CCD. Results also suggest that regions other than the C-terminus of RYR1 have important roles in CCD pathogenesis, and that certain RYR1 mutations may be specific to CCD patients with or without MH. Finally, the role of other candidate susceptibility loci should not be excluded from study of the aetiology of CCD.

METHODS

Clinical features and IVCT results for probands are given in table 1. For the purposes of discussion relevant to this report, we refer to patients as having CCD when histological and clinical signs of the disease have been recorded. Those cases with only histological signs are referred to as patients with core myopathy. All patients were referred to the Leeds MH Unit because of the risk of MH susceptibility (previous known/suspected MH reaction or case of CCD), and MH status was therefore assessed by the contracture test in the laboratory using a specimen of patient skeletal muscle. An MHS phenotype is diagnosed by a contracture of 0.2g or above at a threshold dose of halothane (2%), and separately to caffeine (2 mM).¹³

Genomic DNA was extracted from whole blood samples using a salting out method.³¹ Haplotypes were constructed for families of suitable size (LMH31,32, 33, 34, 36, 37, 38, 39, 65, 75, table 1) using markers to the RYR1 region (D19S208 - [0.9cM]- D19S224 - [2.3cM]- D19S421-[0.3cM]- D19S422 - [6.8cM]- D19S219 - [0.4cM]- CTG-DM - [0.1cM]- D19S112). Where samples were available, the family proband and a clinically normal relative were selected for mutation detection, which was performed using specific or mutation scanning assays. Detection of 21 known mutations was carried out using PCR and restriction digest assays. These involved: Thr2206Met,³³ Arg2454Cys,³⁴ Arg2454His,³⁵ Thr4637Ala,³⁶ Tyr4796Cys,⁶ Ile4898Thr,³⁷ and 15 mutations: Cys35Arg, Arg163Cys, Gly248Arg, Gly341Arg, Ile403Met, Tyr522Ser, Arg552Trp, Arg614Cys, Arg614Leu, Arg2163Cys, Arg2163His, Gly2434Arg, Arg2435His, Arg2458Cys, and Arg2458His.¹⁵ Further screening was undertaken by fluorescent SSCP (F-SSCP) analysis (exons 6, 11, 17, 39–46) and cycle sequencing using an ABI 377 (exons 91, 95–106). Primers for F-SSCP and cycle sequencing were designed to the flanking intronic sequence to ensure full exon coverage. For F-SSCP, test samples were analysed in comparison with wild type and mutation positive control samples where available, on 1X MDE gels (Flowgen; Ashby de la Zouch, UK) run at 18°C using 1X TBE buffer. Any shifts detected were sequenced to characterise the nature of the change.

For novel mutations identified, a mutation specific assay was designed (table 2) in order to perform segregation analysis within mutation positive families. A panel of 200 unrelated MHS and 100 normal control samples were also screened, to assess the prevalence of the mutation in the UK MH population, and to establish whether the variant detected was likely to be a neutral

Table 2 Primer sequences and conditions for detection of novel mutations

Mutation*		Primer sequence (5'-3')		Annealing temp. °C	Product size bp
479A>G	ARMS	AAGCAGAGGTCTGAAGCGG*	CAGACCTGGGAGCAGGAG	58	113
7036G>A	RFLP (gain of NlaIII site with mutation)	GGACCTGTGTACCCCTGGAGT	TTGCCTATTGAGGAGGGGGC	60	121
7043A>G	ARMS	CCAGGCGAGAGCGTGGCTGA*	CGGGTGCCTATTGAGGAG	55	227
14440C>T	ARMS	CTTCTTTTGTCTGCCGTT*	TTATCCCTTCACCACCCACT	55	162
14471T>C	ARMS	CATGGGGGTCAAGACACC*	TCAGTCCCTGTGGCTCTACC	56	222
14814C>G	RFLP (loss of TaqI site with mutation)	GTCGGGCACTGACTTGTT	AAGTACCCCACTGCTGGCTA	58	355
14817C>A	ARMS	CCCCAGGTCTGATCATCTAA*	CGGCCTGGAGGTACCATTAT	55	162

*Mutations were screened for using ARMS (Amplification Refractory Mutation System, *mutation specific primer)/RFLP (Restriction Fragment Length Polymorphism) assays. For ARMS assays mutation specific primers were multiplexed with a second set of primers to serve as positive control for each PCR. All assays were performed using a known wild type and mutation positive control sample. Bases in bold within primer sequences denote mutation specific primer (3' end) and incorporated mismatch to improve primer specificity.

polymorphism or pathogenic mutation. The site of the mutation in the RYR1 protein was also examined to determine whether it had been conserved among species and was therefore likely to be of functional significance (table 3).

RESULTS

Linkage analysis

Family size restricted linkage analysis to 10/21 families. Nine of these families showed haplotypes compatible with linkage

of CCD/MH to RYR1. An RYR1 mutation, which cosegregated with the chromosome 19 haplotype, was identified in six of the nine families (LMH31, LMH33, LMH34, LMH36, LMH65, LMH75, table 3). In the three families where a mutation was not identified, a common chromosome 19 haplotype cosegregated with the MH positive / CCD positive phenotype in two families (LMH37 and LMH38), in contrast to LMH39 where the haplotype segregated with core myopathy observed rather than MH. In family LMH32, a single recombinant core

Table 3 Summary of mutation results

Family (LMH)	Status	Mutation status				Segregation of mutation		
		Exon	Nucleotide change	Amino acid change	+Conserved RYR1 site?	Method of detection	Concordant family CCD	Concordant family MH
31	MH/CCD	95	13913G>A‡	Gly4638Asp	Yes*	Sequencing	Yes	No
32	MH/core myopathy	-	-	-	-	-	-	-
33	MH/CCD	6	479A>G	Glu160Gly	Yes	SSCP, sequencing	Yes	Yes
34 (1P)	MH/CCD	95	13913G>A‡	Gly4638Asp	Yes*	Sequencing	Yes	No
34 (2P)	MH/CCD	95	13913G>A‡	Gly4638Asp	Yes*	Sequencing	Yes	No
36	MH/CCD	103	14814C>G	Ile4938Met	Yes	Sequencing	No	Yes
37	MH/core myopathy	-	-	-	-	-	-	-
38	MH/CCD	-	-	-	-	-	-	-
39	MH/CCD	-	-	-	-	-	-	-
63	MH/core myopathy	44, 103	7036G>A, 14817C>A	Val2346Met, Asp4939Glu	Yes	SSCP, sequencing	Yes, Yes	Yes, Yes
64	CCD	-	-	-	-	-	-	-
65	MH/core myopathy	44	7043A>G	Glu2348Gly	Yes	SSCP, sequencing	No	Yes
66	MH/core myopathy	17	1840C>T†	Arg614Cys	Yes	RFLP	No	Yes
67	MH/core myopathy	-	-	-	-	-	-	-
68	CCD	100	14440C>T	Leu4814Phe	Yes	Sequencing	Yes	No
69	MH/CCD	-	-	-	-	-	-	-
70	CCD	-	-	-	-	-	-	-
71	Core myopathy	-	-	-	-	-	-	-
72	MH/core myopathy	100	14471T>C	Leu4824Pro	Yes	Sequencing	Yes	Yes
73 (1P)	MH/CCD	46	7354C>T§	Arg2452Trp	Yes	SSCP, sequencing	No	Yes
73 (2P)	MH/CCD	46	7354C>T§	Arg2452Trp	Yes	SSCP, sequencing	No	Yes
74	CCD	101	14582G>A¶	Arg4861His	Yes	Sequencing	Yes	No
75	MH/core myopathy	46	7373G>A**	Arg2458His	Yes	SSCP	No	Yes

Mutation status †, ‡, §, ¶, and ** reported previously; -, no mutation detected.

*Conserved in all species apart from bull frog; †³⁹; ‡²²; §⁴⁰; ¶^{7 22 30}; **³³.

+ RYR1 cDNA sequences for Human (P21817, gi: 19857096), Pig (P16960, gi: 1173335), Rabbit (P11716, gi: 134134), C. elegans (D45899, gi: 1871446), and Bull frog (D21070, gi: 1856972) were aligned using CLUSTAL W (1.81) to assess whether the mutations detected arose at evolutionary conserved sites in the RYR1 gene; conserved in all species apart from bull frog; CCD, central core disease; MH, malignant hyperthermia; LMH, Leeds Malignant Hyperthermia Unit.

myopathy / MHS individual was observed for the high risk susceptibility haplotype. Further investigations are in progress to characterise the putative disease susceptibility locus in this family.

RYR1 mutation analysis

Of 20 RYR1 mutations screened, a known, functional MH mutation—LMH66 with 1840C>T—was found in one family. Histological analysis for this patient showed core myopathy, and the patient was also IVCT MHS (tables 1 and 3).

F-SSCP of 11 candidate exons, representing mutation hotspot regions 1 and 2 of RYR1, detected 10 shifts. All shifts were sequenced; five missense mutations, three novel (table 3), and five neutral polymorphisms were identified (data not shown). Of the three novel mutations: 479A>G was detected in family LMH33 (CCD/MHS); 7043A>G in family LMH65 (core myopathy / MHS); and 7036G>A in family LMH63 (core myopathy / MHS). Of the two previously reported mutations: 7373G>A was detected in LMH75 (family with evidence of non-specific type-2b atrophy / CCD/MHS); and 7354C>T was detected in LMH73 (family history of CCD/MHS).

Sequencing of 13 C-terminal exons, representing mutation hotspot region 3 of RYR1, identified six missense mutations: four novel and two previously reported (table 3); and also four neutral polymorphisms (data not shown). The four novel mutations were: 14440C>T in family LMH68 (CCD/MHN); 14471T>C in family LMH72 (core myopathy / MHS); 14814C>G in family LMH36 (CCD/MHS); and 14817C>A in family LMH63 (core myopathy / MHS). Of the two previously reported mutations: 13913G>A was detected in families LMH31 (CCD/MHS) and LMH34 (CCD/MHS); and 14582G>A was detected in family LMH74 (CCD/MHN).

Prevalence of mutations in the UK population

Mutation specific assays were designed for all novel mutations detected (table 2). The prevalence within the UK MHS population (sample of 200 subjects) and a UK normal control population (sample of 100 subjects) was assessed. Of the mutations identified within the sample, 1840C>T, 7373G>A, and 14471T>C were detected in the unrelated screened MHS population. There was no evidence in this population of CCD in 1840C>T:6 families, 7373G>A:6 families, or 14471T>C:3 families. Mutation 13913G>A was recurrent in two CCD/MHS families—LMH31 and LMH34. Remaining mutations detected occurred in isolated cases only, and were not detected in the unrelated MHS population screened, nor in the 100 normal controls. All novel mutations identified occurred at evolutionary conserved sites, indicating a site of functional significance within the protein (table 3).

Segregation analysis was conducted in all mutation positive families where DNA samples were available (table 3). In 3/12 mutation positive families, each mutation detected was concordant with both the CCD and MHS phenotypes: mutations 479A>G, 7036G>A, 14471T>C, and 14817C>A, where 7036G>A and 14817C>A were detected in one family. In 5/12 families, the mutation was concordant with MH status only (1840C>T, 7043A>G, 7373G>A, 7354C>T, 14814C>G). In 4/12 families, the mutation detected was concordant with CCD status only (13913G>A detected in two families, 14440C>T, 14582G>A). These data imply that mutations in RYR1 may be specific to CCD and/or MHS, and that further variants may be responsible for the CCD pathology and MH susceptibility within some families.

DISCUSSION

We screened 21 individuals of UK origin with core myopathy or CCD, diagnosed on the basis of histological findings with or without clinical presentation and assessed for MHS by the

IVCT. We identified RYR1 mutations in 12 families. Only one mutation was recurrent in two families. In one family two mutations were detected. This analysis gives an overall mutation detection rate of 57%. The mutations identified can be classified into four distinct groups:

- those that cosegregate with the CCD and MHS phenotype (change at nucleotide 479)
- those that cosegregate with histologically diagnosed core myopathy and MHS phenotype (changes at nucleotides 7036, 14471, 14817)
- those that cosegregate with CCD only (changes at nucleotides 13913, 14440, 14582)
- those that appear specific to the MHS phenotype (changes at nucleotides 1840, 7043, 7354, 7373, 14814).

Therefore, in line with previous reports, mutations in RYR1 specifically associated with CCD appear to cluster in C-terminal exons of the gene (exons 95, 100, 101, in our patient sample). However, it is evident that mutations in N-terminal regions—exon 6—may also be associated with a CCD/MHS phenotype. Conversely, mutations in the C-terminus may give rise to MHS in the absence of CCD—for example, exon 103 and 14814C>G in LMH36.

CCD is the only myopathy that has been consistently reported in association with MH. It exhibits extensive histological and clinical variability within and between families. Histological examination shows cores in type 1 muscle fibres and type 1 fibre predominance.¹⁹ There are also reports of worsening clinical status from one generation to another.⁷ Patients with MH may also present with cores, despite clinically being asymptomatic of CCD. The boundaries of CCD diagnosis with respect to the extent of myopathic changes on histological examination are unclear. At least three CCD families within the cohort studied also had members who were either:

- clinically affected with CCD and MH positive
- or positive for MH, with core myopathy on histology but without clinical CCD
- or MH positive with no clear myopathic changes
- or MH negative and CCD positive.

The variation in phenotype observed within the same family could reflect incomplete penetrance for CCD which has been described in association with some RYR1 mutations,³⁸ the mildly progressive nature of CCD reported in some individuals,³² or the independent segregation of MH and CCD within some families. Because of the observed phenotypic overlap, we investigated patients presenting with CCD and asymptomatic patients showing core myopathy following histological examination.

Patients with CCD are at high risk of MHS, and in almost all cases are diagnosed MHS positive by the IVCT—for example 7/11 (64%) in our study. Within the entire cohort of 23 probands (from 21 families), 18 gave a positive response on IVCT (78%). Five cases were clearly IVCT MH normal (table 1). Of the 18 MH positive IVCT cases, investigation of further family members showed CCD positive and MH normal individuals to be present in two families (LMH31, LMH34). In seven families, CCD/core myopathy negative MHS individuals were also identified (LMH32, LMH36, LMH65, LMH66, LMH67, LMH73, LMH75). This substantiates the concept that CCD / core myopathy and MH may exist independently within families. In characterisation of pathogenic mutations, some variants clearly predispose to both MH and CCD, others being disease specific. Candidates include specific mutations within RYR1, or at sites in alternative loci. We identified one family (LMH63) presenting with MHS and

core myopathy with two mutations in RYR1. One mutation may be specific for the core myopathy observed because of its location to the C-terminus (14817C>A), the other for predisposition to MHS (7036G>A). Neither mutation was detected on screening a panel of normal control samples, and both occur at evolutionary conserved sites within the RYR1 protein, evidence which supports the concept that both mutations may have some functional consequence. A second family, LMH32, did not display haplotypes compatible with linkage to the RYR1 region, confirming the possible role of alternative susceptibility loci in CCD / core myopathy aetiology. Work is ongoing for the analysis of this family.

Of the 12 mutations identified, five are known. In contrast to other studies, none of the cases represented sporadic events,^{7,22} but were reported as follows:

- mutation 7354C>T in association with MH in a New Zealand family³⁹
- mutation 13913G>A in a sporadic case of CCD in an Australian family²²
- mutation 14582G>A in
 - two Australian CCD families (one sporadic case and one with a family history of CCD²²)
 - three French CCD families (one sporadic case, two with a family history of CCD⁷)
 - seven families of mixed European origin, all of which had a family history of CCD.³⁰

The remaining two mutations, 1840C>T and 7373G>A, did not show concordance with the core myopathy phenotype observed in the families, although 1840C>T and mutation 7372C>T, which alters the same amino acid residue as 7373G>A, have both been previously described in association with MH and asymptomatic cores.³⁶ Our findings imply either that additional genetic determinants may predispose to CCD/asymptomatic cores in these cases, or that penetrance is incomplete. Overall, the spectrum of mutations reported for CCD is diverse, the majority being private to individual families. To date, the 14582G>A mutation is the most commonly reported CCD mutation.

The functional consequences of missense mutations are difficult to predict. Certain RYR1 mutations have been more fully characterised using a model system in the laboratory by analysis of either recombinant RYR1 channels expressed in a heterologous system,^{6,37,41–43} or myotubes / lymphoblastoid cells isolated and cultured from mutation carriers.^{30,44,45} Differences in calcium flux between mutant and wild type RYR1 channels / cell preparations have been demonstrated. Few mutations can be distinguished on the basis of their relative sensitivity to specific calcium release trigger agents.^{41,42} However, there is some evidence from analysis of recombinant RYR1 channels in the laboratory,³⁷ and from RYR1 genotype/IVCT phenotype correlation studies,^{33,46} that certain CCD/MH RYR1 mutations are associated with more severe phenotypes compared with RYR1 mutations reported principally in association with MH.

In skeletal muscle excitation contraction (EC) coupling, the dihydropyridine receptor (DHPR) and the ryanodine receptor (RyR) are linked mechanically. The DHPR undergoes a voltage / calcium induced conformational change, which in turn triggers RyR calcium release and ultimately muscle contraction. One proposed functional consequence of RYR1 mutations associated with CCD is to increase the permeability of the RYR1 channel to calcium; another mechanism may be functional uncoupling of SR calcium release from sarcolemmal depolarisation. Muscle weakness is thought to arise because of the reduction in available sarcolemmal calcium for the normal process of muscle contraction.

Alternatively, persistent calcium leak from the sarcolemma could predispose muscle damage and therefore weakness.⁴⁶ Seven CCD mutations (G4890R, I4897T, I4898T, G4898E, G4898R, A4905V, and R4913G) have so far been found to give rise to an EC uncoupled SR calcium release channel.^{36,47} Locating to exon 102 of the gene, and the region contributing to the RYR1 channel pore (residues 4861 to 4918⁴⁸), other mutations in this area may have the same effect. One mutation identified in this study, Arg4861His; 14582G>A, locates to this interval, with mutations Arg4939Glu, 14817C>A, and Ile4938Met (14814C>G being close to the boundary of the pore encoding region). Data from analysis of 14582G>A mutant lymphoblastoid cell lines support a pathological mechanism of channel leakiness.³⁰ Only two other mutations identified in the study, 1840C>T and 7373G>A, have been characterised in an independent system in the laboratory and shown to alter the normal functioning of the RYR1 channel.^{41,42}

In summary, we have identified RYR1 mutations in 57% of the families investigated. Three mutations were specific to the CCD phenotype (13913G>A, 14440C>T, 14582G>A). Four mutations cosegregated with the CCD and MHS phenotype in three families (mutation 479A>G in a family with CCD/MH, and mutations 14471C>T, 7036G>A/14817C>A in two MHS families presenting with core myopathy). In five families, the mutation detected cosegregated with the MHS phenotype only, showing discordance with CCD and/or core myopathy in at least one individual (1840C>T, 7043A>G, 7354C>T, 7373G>A, 14814C>G). This indicates that specific RYR1 mutations predispose to MHS or CCD, and that others may predispose to both CCD and/or core myopathy in addition to MH. Mutations which segregated specifically with CCD status were detected in the C-terminal region of the RYR1 gene only. For the purposes of molecular characterisation of CCD patients, with a wide spectrum of RYR1 mutations now reported in individual families, the C-terminal region of the gene remains the primary site for initial investigation and mutation screening. It is, however, important to recognise that mutations in other regions of the RYR1 gene may also occur, and that the risk to MHS is significant and should be investigated initially by the IVCT in the family index case.

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