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# Mutation screening of the RYR1-cDNA from peripheral B-lymphocytes in 15 Swedish malignant hyperthermia index cases

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**Background.** Malignant hyperthermia (MH), linked to the ryanodine receptor 1 gene (*RYR1*) on chromosome 19, is a potentially lethal pharmacogenetic disorder which may lead to a disturbance of intracellular calcium homeostasis when susceptible individuals are exposed to halogenated anaesthetics, suxamethonium, or both. Central core disease (CCD) is a rare dominantly inherited congenital myopathy allelic to MH-susceptibility.

**Methods.** In this study, 14 unrelated MH-susceptible probands and one CCD patient from Sweden were screened for mutations in the *RYR1*. Since the RYR1 is also expressed in B-lymphocytes, RYR1-cDNA was transcribed from total RNA extracted from white blood cells.

**Results.** We detected two known *RYR1* mutations and two previously described unclassified sequence variants. In addition, six novel sequence variants were detected. All mutations or sequence variants were verified on genomic DNA. Seven of the probands did not show any candidate mutation, although the total coding region of *RYR1* was sequenced. Segregation data in *in vitro* contracture tested family members of three probands support a causative role of three of the novel sequence variants.

**Conclusions.** Our study contributes to the genetic aetiology of MH in Sweden, but also raises questions about the involvement of genes other than *RYR1* since nearly half of the probands did not show any sequence variants in the total coding region of the *RYR1*.

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Malignant hyperthermia susceptibility (MHS; OMIM *145600) is a potentially lethal pharmacogenetic disturbance of intracellular calcium homeostasis, associated with the ryanodine receptor 1 (RYR1; OMIM *180901) in the sarcoplasmic reticulum of the skeletal muscle cell. In susceptible individuals, halogenated anaesthetics, suxamethonium, or both can induce a severe decompensation of muscle calcium homeostasis.[1] Early symptoms of an acute MH crisis include increased carbon dioxide in the expired air, possibly masseter spasm after suxamethonium administration, hyperthermia, and tachycardia, whereas late symptoms can be coagulation disturbances, generalized muscle rigidity, oliguria, and death.[1]

Outside anaesthesia, the diagnosis of MHS is traditionally made by an *in vitro* contraction test (IVCT). This investigation is highly invasive and requires a muscle biopsy from *m. quadriceps*.[2] Muscle strips in an organ bath are exposed to halothane and caffeine, respectively. The threshold concentrations of halothane and caffeine which cause an increase of at least 0.2 g above baseline force are measured according to the European Malignant Hyperthermia Group (EMHG) protocol.[2] From this result, a patient can be classified as MH-susceptible (MHS), MH-equivocal (MHE), or MH-negative (MHN).

Central core disease (CCD; OMIM *117000) is a rare dominantly inherited congenital myopathy allelic to MHS.[3] The clinical presentation of CCD is highly variable and symptoms may vary from clinically normal to severe myopathy with hypotonia, skeletal abnormalities, and scoliosis. As in MHS subjects, halogenated anaesthetics, suxamethonium, or both may trigger an MH reaction in CCD patients.[4]

The ryanodine receptor 1 is encoded by the *RYR1* gene on chromosome 19q13.1. The gene comprises 159 000 base pairs with 106 exons. The RYR1-cDNA has a length of 15 117 kb and encodes a protein monomer of 5038 amino acids.[5] The RYR1 is expressed mainly in striated muscle, but there is also a significant level of RYR1 expression in peripheral B-lymphocytes.[6–8] The exact function of RYR1 in B-lymphocytes is not fully understood.

At present, there are only preliminary data on the genetic basis of MHS in Sweden,[9] and systematic mutation screening has not been performed. The large size of the *RYR1* and the distribution of published mutations do not lend themselves to a streamlined diagnostic rationale. With the exception of Switzerland,[10] no founder mutations of significant prevalence have been reported from other populations.

## Methods

With Regional Ethics Committee approval, 14 unrelated Swedish MH probands and one Swedish CCD patient, living in Sweden, were contacted by telephone and informed of the study. All MH probands had suffered serious MH clinical reactions and thereafter been tested MHS by IVCT. The CCD patient had been IVC tested as a part of the diagnostic investigations and was also found MHS. All IVCTs were carried out at the National MH Laboratory of Lund University Hospital in southern Sweden. The contractions at the threshold concentrations of caffeine 2.0 mmol litre$^{-1}$ and halothane 0.44 mmol litre$^{-1}$ were measured *in vitro* according to the EMHG protocol.

For each MH proband, the Larach index was also estimated. The Larach index ranks the qualitative likelihood, from 1, almost never, to 6, almost certain, that an adverse anaesthetic event represents MH. This clinical grading scale requires the anaesthesiologist to judge whether specific clinical signs are appropriate for the patient's medical condition, anaesthetic technique, or surgical procedure.[11]

PAXgene (Qiagen, Hilden, Germany) and standard EDTA test tubes were provided to patients together with a detailed information sheet on blood sampling. All patients had two peripheral venous blood samples drawn at their primary care centres, the first in a PAXgene test tube and the second in an EDTA test tube. The blood samples were immediately sent to the genetic laboratory in Würzburg, Germany, by ordinary mail, a distance up to 2000 km and a transportation time up to 3 days.

Upon arrival in the laboratory, test tubes were stored frozen at −80°C until total RNA was extracted from the PAX tube according to the manufacturer's instructions and genomic DNA of leucocytes from the EDTA tube according to standard protocols. First-strand cDNA was synthesized from 1 μg of total RNA in a final volume of 20 μl using SuperScript™ II (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Owing to the size and nucleotide composition of the RYR1-mRNA, first-strand cDNA was synthesized using three mixes of specific primers as described previously.[12] The resulting first strands were then amplified in 500–700 bp overlapping fragments using a second set of primers, which are available from the authors on request. Sequencing was performed by BigDye 1.1 on an ABI 3130 XL.

All cDNA sequence variants leading to a change in amino acids were confirmed on genomic DNA from the same patient. A panel of 150 German and 100 Swedish DNA samples from clinically healthy subjects was used to assess the prevalence of the candidate mutations in the normal population. The MH status of these subjects was not known.

Exon 70 was found to be deleted in all cDNA samples and was therefore sequenced from genomic DNA. Likewise, exon 86 was missing in the cDNA of one MH proband and was sequenced from genomic DNA of the particular patient.

In addition, all 15 patients were screened by sequencing for the only known MH causing mutation outside RYR1, the p.Arg1086His mutation in the alpha-1 subunit gene of the dihydropyridine receptor, DHPR (CACNA1S; OMIM *114208), on chromosome 1q32.[13]

For segregation studies, the IVC-tested family members of the probands were identified from records. Genomic DNA was extracted from left over muscle tissue biopsied at the time of the IVCTs and stored at −80°C. The age of the muscle pieces varied from <1 to >10 yr. The probands' sequence variants were either confirmed or excluded by direct sequencing of the relevant exon of the *RYR1*.

## Results

Descriptive, IVCT, Larach index, and *RYR1* sequence variant data are presented in Table 1.

Eight of 15 patients (53%) in our cohort, consisting of 14 MH probands and one CCD patient, showed either a known mutation or a novel sequence variant. All sequence variants could be verified on genomic DNA and were absent from 300 German and 200 Swedish control chromosomes with two exceptions (see below).

Two MH probands had known MH causing mutations (www.emhg.org): p.Gly248Arg and p.Arg614Leu. In addition to the mutation p.Gly248Arg, the former proband showed two unreported amino acid substitutions: p.Arg1583Cys and p.Glu5034Val.

One MH proband had the mutation p.Val4849Ile which has been previously associated with MHS in the heterozygous state[14] and with congenital multi-minicore disease when homozygous.[15] However, this variant was previously found once in a healthy control.[14] Therefore, its functional impact remains to be elucidated.

**Table 1** MHS probands. MHS proband and CCD patient data. Descriptive and clinical data, the maximal contractures in the IVCT at the threshold concentrations of caffeine 2.0 mmol litre$^{-1}$ and halothane 0.44 mmol litre$^{-1}$, the Larach index, which describes the likelihood that the clinical adverse reaction is MH and *RYR1* known mutations or novel sequence variants and the exon where the mutations or sequence variants occurred. The known mutations or novel sequence variants were identified on cDNA and confirmed on genomic DNA. The observed polymorphisms are listed in the complete table available only in the Supplementary Data in the online electronic version

| Age at MH crisis | Gender | Surgery | Caffeine (g) | Halothane (g) | Larach index | Novel sequence variant or known mutation and exon found on cDNA and verified on genomic DNA |
|---|---|---|---|---|---|---|
| *MHS probands* | | | | | | |
| Patient 1: 46 yr | Male | Osteosynthesis of tibial fracture | 4.4 | 2.2 | 5 | No |
| Patient 2: 29 yr | Female | Caesarean section | 6.2 | 7.55 | 6 | p.Glu1058Lys in exon 24, not previously reported sequence variant |
| Patient 3: 40 yr | Female | Thyroidectomy | 2.9 | 4.25 | 6 | No |
| Patient 4: 39 yr | Male | Nasal septoplasty | 2.4 | 4.1 | 4 | p.Arg1679His in exon 34, not previously reported sequence variant, but found once in 150 German control individuals |
| Patient 5: 29 yr | Female | Caesarean section | 0.5 | 2.3 | 5 | p.Val4849Ile in exon 101, previously reported mutation, but debate about its significance |
| Patient 6: 29 yr | Female | Laparotomy | 2.8 | 1.8 | 3 | p.His382Asn in exon 12, not previously reported sequence variant |
| Patient 7: 7 yr | Female | Knee arthroscopy | 3.9 | 6.2 | 6 | No |
| Patient 8: 9 yr | Male | Osteosynthesis of ulnar fracture | 0.6 | 1.1 | 6 | p.Gly248Arg in exon 9, known mutation, p.Arg1583Cys in exon 33, not previously reported sequence variant, p.Glu5034Val in exon 106, not previously reported sequence variant |
| Patient 9: 26 yr | Male | Colectomy | 5.25 | 3.4 | 5 | No |
| Patient 10: 44 yr | Male | Laparotomy | 0.35 | 2.4 | 6 | No |
| Patient 11: 7 yr | Male | Placement of tympanostomy tubes | 0.8 | 0.8 | 4 | No |
| Patient 12: 41 yr | Male | Parotid surgery | 0.4 | 0.6 | 4 | No |
| Patient 13: 5 yr | Male | Tendon reconstruction of the lower limb | 0.5 | 1.15 | 5 | p.Lys1393Arg in exon 29, not previously reported sequence variant, but found once in 100 Swedish control individuals |
| Patient 14: 55 yr | Male | Open cholecystectomy | 2.85 | 7.4 | 4 | p.Arg614Leu in exon 17, known mutation |
| *CCD patient* | | | | | | |
| Patient 15 | Male | Elective screening | 7.2 | 7.3 | — | p.Arg2508Gly in exon 47, previously reported sequence variant, but no proof of functional significance |

In four additional MH probands, novel amino acid substitutions were found: p.Glu1058Lys, p.Arg1679His, p.His382Asn and p.Lys1393Arg. The substitution p.Arg1679His was found in one German control sample and the substitution p.Lys1393Arg was found in one Swedish control sample.

The CCD patient had the sequence variant p.Arg2508Gly. This variant has been found once before in a CCD patient from Japan.[16]

Our segregation data were limited by the number of family members IVC tested. The available segregation data are shown in Figure 1.

The variant p.Arg1679His (Fig. 1A) was carried by two MHS individuals and by two MHE individuals in the pedigree, but absent from two MHN individuals. However, a third MHN individual (1374, child of the MHE individual 1327) also carried the sequence variant.

The variant p.His382Asn (Fig. 1B) perfectly co-segregated with MHS status in the small nuclear family available.

Seven IVC-tested relatives of Patient 8 carrying the variants p.Arg1583Cys and p.Glu5034Val together with the known mutation p.Gly248Arg were available for a segregation study. The variant p.Arg1583Cys and the mutation p.Gly248Arg are co-inherited in *cis* on a familiar haplotype (black bars in Fig. 1C). In contrast, the variant p.Glu5034Val was introduced on a different haplotype (hatched bars) by the unrelated MHN spouse (1045) and segregated in two MHS (949, 984) and two MHN individuals (985, 1732). It can therefore be excluded as being causative for the MHS disposition in this family. No conclusion can be drawn from the observed segregation for the variant p.Arg1583Cys.

Segregation analysis for the variants p.Glu1058Lys, p.Arg2508Gly, and p.Lys1393Arg was not possible because of the limited IVC testing in these families.

Seven patients of 15 (47%) in our cohort did not show any known mutation or candidate mutation in the entire RYR1-cDNA sequence synthesized from total RNA isolated from B-lymphocytes.

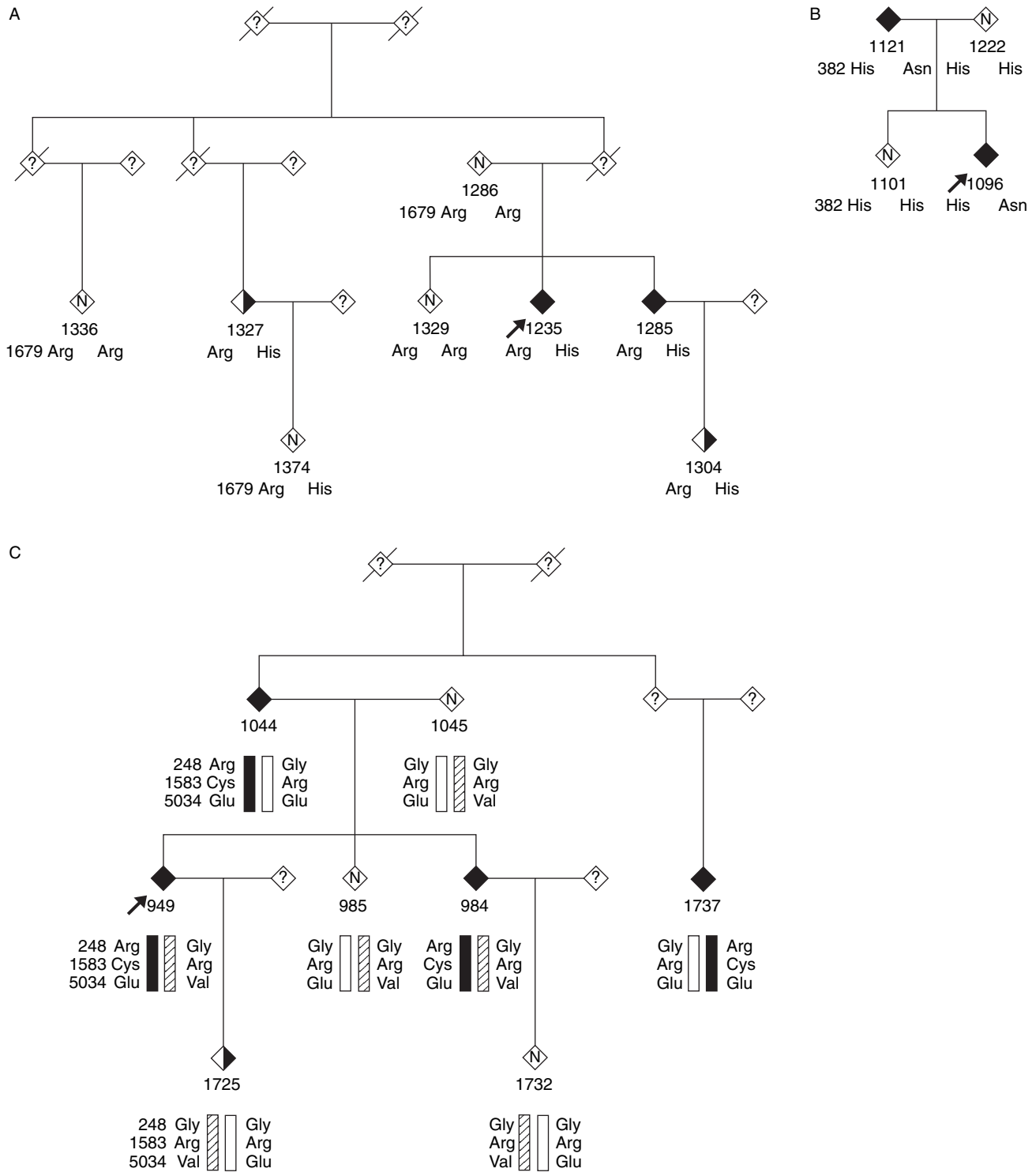


**Fig 1** Segregation of novel RYR1 variants. Segregation data in families of the probands carrying the novel variants p.Arg1679His, p.His382Asn, p.Arg1538Cys, and p.Glu5024Val. Filled symbols denote MHS, half-filled symbols MHE status. The letter N denotes MHN results, whereas the question marks indicate that IVCT has not been performed. (A) Segregation of variant p.Arg1679His. (B) Segregation of variant p.His382Asn. (C) Segregation of the mutation p.Gly248Arg and of the variants p.Arg1538Cys and p.Glu5024Val.

None of the 15 patients showed the MH causative mutation p.Arg1086His in the alpha-1 subunit of the CACNA1S gene.

## Discussion

The aim of the present study was a systematic screening of the total coding region of the *RYR1* gene in a cohort of 15

MHS index patients from Sweden. For logistic reasons, we designed a simple and practical protocol for the collection of blood samples drawn at the patients' local primary care centres. The protocol included two venous peripheral blood samples of 5 ml each, the first in a PAXgene test tube for mRNA extraction and the second in an EDTA test tube for genomic DNA preparation. Since PAXgene test tubes are not normally available at primary care centres, they were sent to the patients before sampling.

With the RNA stabilizer used in the PAXgene tubes, we managed to extract sufficient RYR1-mRNA from all samples after 2–3 days of transportation at ambient temperature over a distance up to 2000 km. RYR1-cDNA was synthesized from the RYR1-mRNA fraction of B-lymphocytes for direct sequencing, and sequence variants were confirmed on genomic DNA isolated from leucocytes.

In a previous study of 27 Swedish MHS probands, only exons 17, 39, 40, 45, and 46 were screened on genomic DNA. These exons harbour 14 published MH-related mutations (www.emhg.org) which together cover about 45% of all cases in a German MHS-population (C.R.M., unpublished data). In contrast, only five mutations (19%) were identified among the 27 Swedish probands.[9] This suggested that the mutation spectra differ between the Nordic and the Central European MHS populations.

Upon screening of the entire coding region of the *RYR1* from cDNA derived from B-lymphocytes, 10 amino acid substitutions were identified in eight of 15 patients raising the detection rate to 53%. Patient 5 (p.Val4849Ile), Patient 8 (p.Gly248Arg), and Patient 14 (p.Arg614Leu) carried mutations which have been reported before (www.emhg.org). The latter two have been extensively tested by segregation, functional, or both studies[17–19] and are considered causative of MH by the EMHG criteria.[14 19] The p.Val4849Ile substitution is more puzzling: as a homozygous genotype, it has been reported to cause a severe congenital form of multi-minicore myopathy.[15] However, Monnier and colleagues[14] found this as a heterozygous genotype in MHS probands but also once among 100 healthy control subjects. Pharmacological activation of immortalized B-lymphocytes carrying this mutation homozygously showed a small but significant effect on resting calcium concentration but did not alter the amount of calcium released after RYR1 activation.[20] The mutation carrier in our study does not have any clinical signs of myopathy or a diagnosis of CCD, but she suffered a severe MH reaction during anaesthesia with inhalation anaesthetics. Thus, the functional relevance of this amino acid substitution remains to be elucidated. The only CCD patient in our study; Patient 15 carried the substitution p.Arg2508Gly which has been found once before in a Japanese CCD patient, but its significance as a causative mutation has not been elucidated.[16]

Six of the 10 amino acid substitutions observed have not been reported to date. Interestingly, Patient 8 carried one known mutation (p.Gly248Arg) and two novel substitutions: p.Arg1583Cys and p.Glu5034Val. Patient 2 (p.Glu1058Lys), Patient 4 (p.Arg1679His), Patient 6 (p.His382Asn), and Patient 13 (p.Lys1393Arg) all carried a novel amino acid substitution each.

The evidence for disease causality of the variants presented in this study must be considered incomplete and circumstantial.

(i) All substitutions affect amino acids which are highly or perfectly conserved in the RYR proteins from several vertebrates (Fig. 2). Conservation of protein domains over long evolutionary distances is generally thought to indicate functional relevance. Substitution of an amino acid within such a domain is, therefore, regarded to compromise the function of the protein with the potential sequela of disease.

(ii) All variants were absent from 300 German and 200 Swedish control chromosomes with two exceptions: the substitution p.Arg1679His was found in one German control and the substitution p.Lys1393Arg in one Swedish control. Since the MH status of the control subjects was not known, these individuals might have an as yet undiagnosed MH predisposition. Alternatively, the substitutions of Arg1679 by histidine and Lys1393 by arginine may be 'neutral' polymorphisms, although they both occurred in highly conserved domains (Fig. 2).

(iii) The available segregation data are too limited to formally prove causality for any of the novel sequence variants found. However, co-segregation could be observed in a small nuclear family for p.His382Asn (Fig. 1B) and for the two linked variants p.Gly248Arg and p.Arg1583Cys (Fig. 1C). In this family, the segregation study allowed an exclusion of the third variant p.Glu5034Val as the causative mutation for MHS. In the third pedigree studied, the variant p.Arg1679His occurred in all MHS and MHE family members but also in one MHN individual (Fig. 1A). Interestingly, the substitution was also found in one of 150 German, but not in 100 Swedish controls. Thus, p.Arg1679His could also be a neutral variant.

The discordance between IVCT results and mutation data has been assessed in a large segregation study of MH causative mutations in 196 European pedigrees and was found to be 10%.[21] Discordance was observed in both directions: MHS individuals who did not carry their family's mutation and MHN individuals who did. It is not known, however, whether these data reflect the false-positive and false-negative error rates of IVCT or have other reasons.

Few studies have screened the total *RYR1* coding region: in Japan, a mutation was found in 57%,[22] in a North American study in 70%,[19] in a British cohort in 70%,[5] and in an Italian cohort in 86%[23] of the patients screened. In comparison, the detection rate of 53% in our study must be considered low.

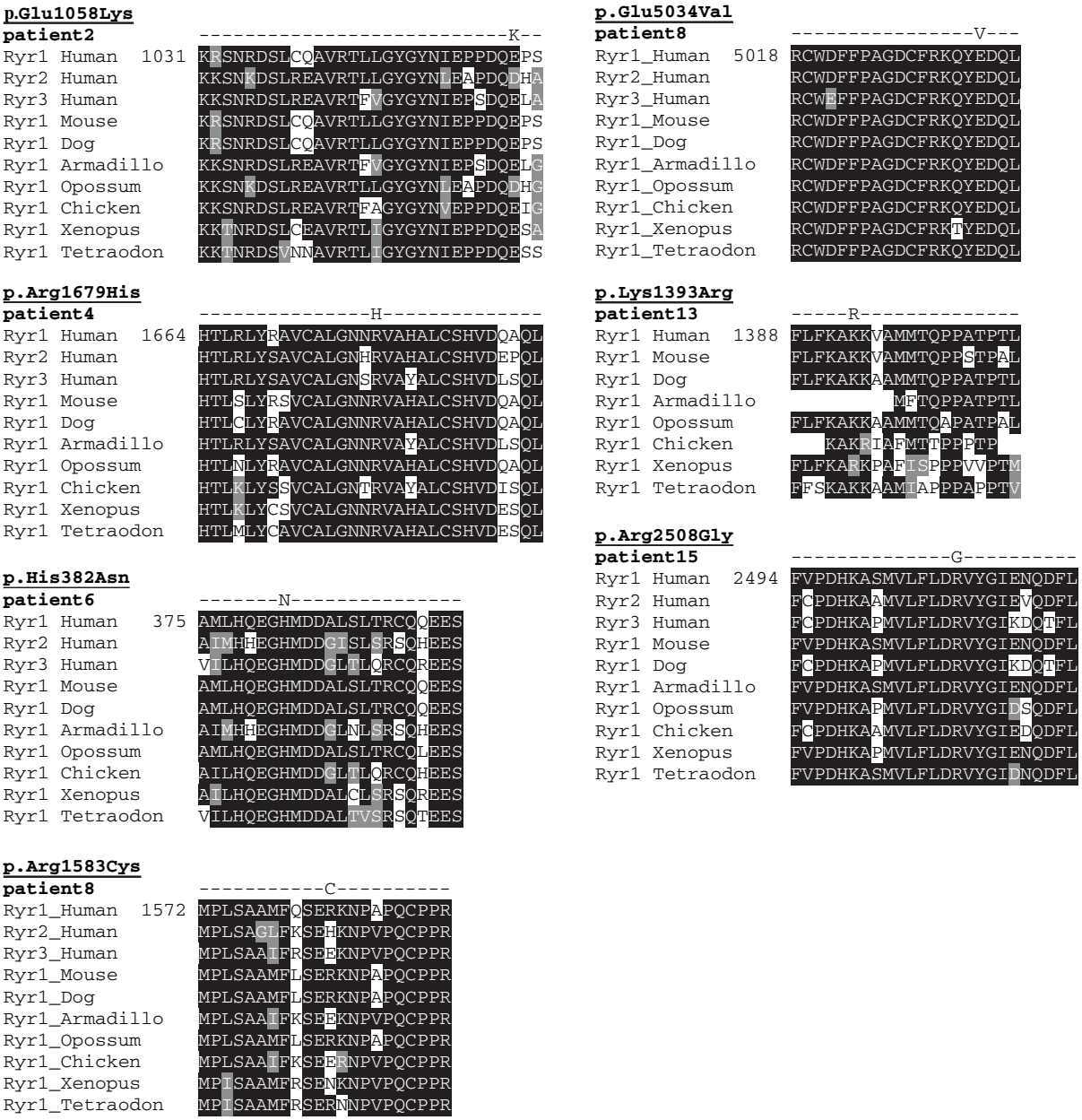


**Fig 2** Amino acid conservation of RYR1 variants detected in Swedish MH probands. For each of the novel sequence variants found in the MH probands and the CCD patient, the relevant cutout from the amino acid sequence of RYR1 is aligned to the RYR sequences from other species. Amino acids are denoted by the single-letter code. Shading of letters is used to indicate the identity (in black) or functional similarity (in grey) of amino acids. The first line of dashes refers to the sequence of the human RYR1 protein and the patient's amino acid variant is indicated by a letter at the respective position. The second line contains the reference (normal) sequence of human RYR1 followed by the other two human RYR proteins (the cardiac isoform RYR2 and the ubiquitous RYR3). To illustrate the evolutionary conservation of the RYR protein domains, the corresponding sequences from other vertebrates are given: mouse and dog as higher mammals, armadillo as a primitive mammal, and opossum as a marsupial. The other vertebrate classes are represented by chicken (birds), *Xenopus* (amphibia) and *Tetraodon* (fish). A high degree of amino acid conservation between species is regarded as a strong indication of functional relevance of the respective protein domain. An amino acid variant which falls into a highly conserved domain and introduces a significant change in physico-chemical properties is more likely to be disease-causing than a substitution in a less conserved region, with lower alteration of chemical properties, or both. All variants observed fall into strongly conserved protein domains, lead to significant changes in amino acid properties, or both.

Clearly, RYR1-mRNA in B-lymphocytes differs from that of muscle,[24] for example, exon 70 was removed by alternative splicing in all our samples. In our protocol, this could be managed by filling the gaps with sequences obtained from genomic DNA. It could be argued that the cDNA synthesis step from a low-level transcript (as RYR1 in B-lymphocytes) might be prone to error. However, during sequencing of 15 kb of cDNA in 15 patients (about 200 000 nucleotides), we detected only two sequence variants in cDNA that could not be verified on genomic DNA

from the same patient. This demonstrates that the reverse transcriptase used (see Methods) worked with high fidelity.

With cDNA synthesis and subsequent PCR, there is always the concern of selective amplification or allele drop-out due to large heterozygous deletions. The large number of single nucleotide polymorphic variants, which were detected (see additional material in the electronic version), can be considered as a good though not perfect monitor for allele loss. Furthermore, it has been shown that allele silencing of *RYR1* by epigenetic or other mechanisms leads to severe congenital myopathies.[25 26] Thus, quantitative defects of RYR1 expression are not compatible with normal muscle function. It is therefore highly unlikely that hitherto undetected large heterozygous deletions contribute to the genetic aetiology of MH.

According to our sequencing results, in about half of the patients included in our cohort, no candidate mutation was found in the *RYR1*. This raises the question whether other genes contribute to the aetiology of MH in Swedish probands. The only MH-related mutation in the alpha-1 subunit of the CACNA1S gene, p.Arg1086His, has been ruled out, but the remainder of this large gene has not been analysed.

As a conclusion, the present study underlines the heterogeneity of the genetic background of MH. With the present evidence, the novel variants observed should be considered candidate mutations for MH and CCD, respectively. Experiments are underway to study the impact of the identified amino acid substitution on RYR1 function in order to draw final conclusions on their relevance for MH disposition.

## Supplementary material

Supplementary material is available at *British Journal of Anaesthesia* online.

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