BJA

British Journal of Anaesthesia, xxx (xxx): xxx (xxxx)

doi: 10.1016/j.bja.2024.11.043
Advance Access Publication Date: xxx
Laboratory Investigation

LABORATORY INVESTIGATION

Pathogenicity assessment of seven *RYR1* variants in patients with confirmed susceptibility to malignant hyperthermia in the Netherlands

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Abstract

Background: Malignant hyperthermia (MH) susceptibility is associated with variants in *RYR1*, the gene encoding the skeletal muscle ryanodine receptor-1 (RyR1), in 70–75% of patients. Functional characterisation demonstrating an increased sensitivity to RyR1 agonists is necessary among other criteria for inclusion in the European Malignant Hyperthermia Group list of MH susceptibility diagnostic variants.

Methods: Seven variants in the *RYR1* gene, p.Glu342Lys, p.Leu2288Ser, p.Phe2340Leu, p.Arg2676Trp, p.Val3324Ala, p.Phe4076Leu, and p.Trp5020Cys, identified in MH-susceptible individuals were introduced into the cDNA for the human *RYR1* gene. These variants were tested in cultured human embryonic kidney HEK293 cells for their effect on calcium release in response to the RyR1 agonist 4-chloro-*m*-cresol. Calcium release of each variant was compared with wild-type and benign and pathogenic controls. Each variant was subjected to curation using the European Malignant Hyperthermia Group scoring matrix and ClinGen *RYR1* Variant Curation Expert Panel guidelines.

Results: Six of seven *RYR1* variants (p.Glu342Lys, p.Leu2288Ser, p.Phe2340Leu, p.Arg2676Trp, p.Val3324Ala, p.Phe4076Leu) showed hypersensitivity to 4-chloro-*m*-cresol compared with wild-type. The p.Trp5020Cys variant did not release calcium in response to 4-chloro-*m*-cresol. All variants had minor allele frequencies <0.1%. Rare exome variant ensemble learner scores of p.Glu342Lys, p.Leu2288Ser, p.Phe4076Leu, and p.Trp5020Cys were >0.85, supporting pathogenicity.

Conclusions: The variants p.Glu342Lys, p.Leu2288Ser p.Phe2340Leu, and p.Arg2676Trp are pathogenic or likely pathogenic for MH and can be used for presymptomatic testing for MH susceptibility. As current knowledge on the p.Val3324Ala, p.Phe4076Leu, and p.Trp5020Cys variants remains insufficient, they are still classified as variants of uncertain significance.

Keywords: adverse events; calcium; malignant hyperthermia; next-generation sequencing; presymptomatic diagnosis; ryanodine receptor-1

Received: 15 June 2024; **Accepted:** 2 November 2024

Editor's key points

- Variants in *RYR1*, the gene encoding the skeletal muscle ryanodine receptor-1 (RyR1), are associated with malignant hyperthermia (MH) susceptibility in ~75% of patients.
- Functional characterisation of seven variants in the *RYR1* gene demonstrated six with increased sensitivity to the ryanodine receptor-1 agonist 4-chloro-*m*-cresol in calcium release assays *in vitro*.
- Four of the *RYR1* variants tested were reclassified as pathogenic or likely pathogenic for MH, and can be used for presymptomatic diagnosis of MH susceptibility.
- The other three should still be considered variants of uncertain significance as the available evidence is insufficient for reclassification.

Malignant hyperthermia (MH) is a clinical syndrome caused by a disorder of calcium signalling in skeletal muscle cells. Exposure of MH-susceptible (MHS) individuals to triggering agents can result in a life-threatening hypermetabolic reaction characterised by hypercapnia, tachycardia, hyperthermia, rhabdomyolysis, renal failure, hyperkalaemia-induced cardiac arrhythmias, and death.[1,2] Given the potential severity of an MH reaction,[3] accurate and efficient presymptomatic diagnosis of MH susceptibility is of great importance to prevent life-threatening adverse anaesthetic events. Currently, MH susceptibility diagnostics rely on muscle biopsy for the *in vitro* contracture test (IVCT), and on genotyping for identification of pathogenic or likely pathogenic variants conferring predisposition to MH. The IVCT has a sensitivity of 100% and specificity of 94% and is the gold standard for presymptomatic diagnostic testing for MH susceptibility.[4,5]

In 70–75% of patients, MH susceptibility is associated with variants in *RYR1*, which encodes the skeletal muscle ryanodine receptor-1 (RyR1).[6,7] Other MH-associated genes are *CACNA1S*, encoding the α1 subunit of the dihydropyridine receptor,[8,9] and *STAC3*, encoding the SH3 and cysteine-rich domain 3 protein in skeletal muscle cells.[10] Testing for MH susceptibility by genotyping is minimally invasive and therefore beneficial. However, to be useful for MH susceptibility diagnosis, genetic variants need to fulfil a set of criteria according to the European Malignant Hyperthermia Group (EMHG) scoring matrix for classification of genetic variants in MH susceptibility (https://www.emhg.org/genetic-scoring-matrix) or the ClinGen Variant Curation Expert Panel (VCEP) recommendations for *RYR1* pathogenicity classification.[11] Among other criteria, functional characterisation demonstrating an increased sensitivity to RyR1 agonists is pivotal for inclusion of variants in the EMHG list of MH susceptibility diagnostic variants.[12]

We report the functional characterisation of seven *RYR1* variants identified in a Dutch MH cohort. We assessed intracellular calcium release using human embryonic kidney (HEK) 293 cells, summarised the currently available bioinformatic data for pathogenicity prediction, and performed segregation analyses. By increasing the number of *RYR1* variants classified as pathogenic for MH, this study contributes to enabling MH susceptibility diagnosis by genotyping.

Methods

Regulatory authority approvals

All IVCTs and genetic investigations were carried out after obtaining informed consent as part of the usual diagnostic routine at the MH investigation unit at Canisius Wilhelmina Hospital. Functional studies were approved by the Massey University Genetic Technology Committee (GMO 05/MU/01 and GMO 00/MU/60), acting as the Institutional Biological Safety Committee for the Environmental Protection Authority, Wellington, New Zealand.

Selection of *RYR1* variants included in this study

All seven *RYR1* variants included in this study were identified in patients referred to the Dutch MH Investigation Unit with confirmation of MH susceptibility by IVCT in the index patient or, in the case of a paediatric patient, in a first-degree relative (Table 1).[4,5] Variants were identified using whole-exome, targeted, partial, or full *RYR1* sequencing. Where variants were identified using targeted or partial *RYR1* sequencing, the full *RYR1* gene in the index patient was sequenced at a later stage to exclude other *RYR1* variants that might have contributed to the MH predisposition. All variants and a summary of the relevant clinical history are given in Table 1. Variants c.38T>G, p.Leu13Arg and c.14477C>T, p.Thr4826Ile were used as pathogenic controls. Variants c.6178G>T, p.Gly2060Cys and wild-type (WT) RyR1 were used as nonpathogenic controls.[13,14]

Cloning of mutant *RYR1* constructs

The full-length human WT *RYR1* cDNA had previously been cloned into the pcDNA™5/FRT/TO expression vector (Life Technologies, Carlsbad, CA, USA) to facilitate preparation of cell lines with stable expression of the *RYR1* variants.[14] The variants were introduced into WT *RYR1* cDNA constructs using site-directed mutagenesis according to the QuikChange™ method (Stratagene, San Francisco, CA, USA; primers in Supplementary Table S1). The resulting *RYR1* mutant constructs were sequenced using Sanger sequencing (ABI3730 Genetic Analyzer with BigDye™ Terminator version 3.1 chemistry; Massey Genome Service, Palmerston North, New Zealand) to confirm the presence of the variants and to exclude any polymerase chain reaction (PCR)-induced changes.

Cell culture and transfection

Flp-In™ T-REx™ 293 cell lines (Life Technologies) were maintained in high-glucose Dulbecco's Eagle's medium (Sigma, Darmstadt, Germany) supplemented with zeocin and foetal calf serum (final concentration 10%; Sigma) at 37°C, 5% CO_2 in a humidified atmosphere. Cells were grown to ~80% confluence, the media was changed to Dulbecco's Eagle's medium supplemented with foetal calf serum 10% and blasticidin just before transfection with FuGENE® HD (Promega, Madison, WI,

Table 1 Ryanodine receptor-1 (*RYR1*) variants included in this study. IVCT, *in vitro* contracture test; MAF, minor allele frequency; MH, malignant hyperthermia; MHN, malignant hyperthermia normal; MHS, malignant hyperthermia-susceptible; NA, not applicable; REVEL, Rare Exome Variant Ensemble Learner. *Probands with a personal or family history of an MH event are awarded 0.5 points, and probands with a personal or family history of a positive (MHS) IVCT are awarded an additional 0.5 points.[11] †The Swedish patient carried a second variant in *RYR1*; also, MHS was not confirmed, and therefore these results are not included in this table. ‡The index patient in the Dutch family and one of his siblings carry both the p.Arg2676Trp variant and the p.Arg614Cys variant. Six other MHS family members do not carry additional variants in *RYR1*.

cDNA change	Aa change	dbSNP ID	MH events[11] (frequency)	MH case points[11, *]	MHS families (frequency)	MHS individuals (frequency)	MHN individuals (frequency)	gnomAD4.1.0 MAF[15]	REVEL score[16]
Variants studied in the present study									
c.1024G>A	p.Glu342Lys	rs2145361853	4	6.5	1†	9	0	NA	0.927
c.6863T>C	p.Leu2288Ser	NA	4	3	2	3	0	0.00000677	0.888
c.7018T>C	p.Phe2340Leu	rs1969983957	1	1	1	2	0	NA	0.789
c.8026C>T	p.Arg2676Trp	rs193922826	3‡	4.5	2	8‡	0	0.00000372	0.601
c.9971T>C	p.Val3324Ala	rs141515745	1	1.5	1	2	0	0.0000124	0.555
c.12226T>C	p.Phe4076Leu	rs1298885611	0	0	1	1	0	0.00000438	0.888
c.15060G>T	p.Trp5020Cys	NA	1	2	1	3	0	NA	0.892
Controls									
Wild-type (WT)	NA	NA	NA	NA	NA	NA	NA	NA	NA
pcDNA™5/FRT/TO (negative control)	NA	NA	NA	NA	NA	NA	NA	NA	NA
c.38T>G (pathogenic control)	p.Leu13Arg	rs193922744	NA	NA	NA	NA	NA	NA	0.828
c.6178G>T (benign control)	p.Gly2060Cys	rs35364374	NA	NA	NA	NA	NA	0.0667	0.172
c.14477C>T (pathogenic control)	p.Thr4826Ile	rs121918595	NA	NA	NA	NA	NA	0.00000137	0.977

USA), and full-length human *RYR1* cDNA constructs were added in an 8:2 (μl:μg) ratio of FuGENE HD to plasmid DNA. The cells were subsequently grown into stable cell lines in the above media using hygromycin as the selectable marker. Once stable cell lines were established, cells were seeded into T25 flasks for immunoblotting, chamber microscope slides for immunofluorescence, or 96-well plates for calcium release essays 72 h after seeding. To induce expression of RyR1, tetracycline was added 24 h before further processing. Presence of the *RYR1* variant was confirmed by Sanger sequencing of genomic DNA isolated from the stable lines.

Immunoblotting

Protein was extracted from Flp-In™ T-REx™ 293 cells using cell lysis buffer (Tris-HCl 0.1 M, pH 7.8; Triton X-100 0.5%) supplemented with 1× cOmplete™ Mini EDTA-free protease inhibitor (Roche, Basel, Switzerland). Protein extracts (~200 μg) were resolved by sodium dodecylsufate−polyacrylamide gel electrophoresis (7.5% polyacrylamide) and transferred to a polyvinylidene fluoride (PVDF) membrane. The 34C antibody (Sigma) was used for detection of RyR1, and anti-α-tubulin antibody (Sigma) was used to detect α-tubulin followed by horseradish peroxidase-conjugated anti-mouse secondary IgG antibody (Sigma).

Immunofluorescence

Cells were fixed with 2% paraformaldehyde in phosphate-buffered saline (PBS), followed by permeabilisation with 0.1% Triton X-100 in PBS. After blocking in 0.5% bovine serum albumin in PBS, protein was detected using primary antibodies as follows: 34C (Sigma) for RyR1 and anti-protein disulfide isomerase (Sigma) as an endoplasmic reticulum marker. Proteins were visualised using the ZOE fluorescence imaging system (Bio-Rad, Hercules, CA, USA) using secondary antibodies: fluorescein isothiocyanate-labelled anti-mouse immunoglobulin G (Jackson ImmunoResearch, West Grove, PA, USA) for RyR1 and tetramethylrhodamine isothiocyanate-labelled anti-rabbit immunoglobulin G (Jackson ImmunoResearch) for protein disulfide isomerase. 4′,6-diamidino-2-phenylindole was also used for nuclei staining.

Measurement of calcium release

Flp-In™ T-REx™ 293 cells were washed once with a balanced salt solution (BSS) buffer (NaCl 140 mM, KCl 2.8 mM, $MgCl_2$ 1 mM, 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid [HEPES] 10 mM, $CaCl_2$ 2 mM, and glucose 10 mM; pH 7.3). Subsequently, cells were loaded with 4 μM fura-2-AM (Invitrogen, Carlsbad, CA, USA) and Pluronic™ F-127 0.01% (Sigma) in BSS buffer and incubated for 1 h at 37°C in the dark.

Calcium release was measured by the change in fluorescence in response to the RyR1 agonist 4-chloro-*m*-cresol (4-CmC) using an Olympus (Tokyo, Japan) IX81 microscope linked to a CoolLED pE-340^{fura} light source, Photometrics (Tucson, AZ, USA) prime 95B camera, and CellSens imaging software (version 3.1; Olympus). The concentrations of 4-CmC used were 0, 10, 40, 100, 200, 300, 400, and 500 μM. For each assay, calcium release was normalised and calculated as a percentage of maximum calcium release. Maximum calcium release was defined as release by 500 μM 4-CmC, and was confirmed by calcium release essays in WT cells in response to increasing concentrations of thapsigargin (maximum 1000 nM) and ionomycin (1.0 μM). The 4-CmC concentrations were lower than in previous similar studies performed by our laboratory. The assays of the present study were performed using new equipment and imaging software. According to the thapsigargin and ionomycin maximum calcium release essays described above, the microscope and imaging software set-up has a lower threshold to be saturated for maximum calcium release, which explains the lower 4-CmC concentrations used in the present study.

Segregation analysis

The number of MH families and the number of MHS individuals harbouring each variant is reported, including the variant segregation, with the IVCT results. To increase the value of the segregation analysis, other EMHG laboratories were contacted and asked whether they had any IVCT or clinical data on the variants included in the present study.

Bioinformatic evidence

For each *RYR1* variant, minor allele frequencies (MAFs) from the Genome Aggregation Database (gnomAD, dataset v4.0.0 [https://gnomad.broadinstitute.org/], accessed December 13, 2023) are reported.[15] Further bioinformatic evidence is included using Rare Exome Variant Ensemble Learner (REVEL) scores. A REVEL score of 0.5 or lower is evidence against pathogenicity, and a score of 0.85 or higher supports pathogenicity. A REVEL score of 0.5−0.85 neither refutes nor supports pathogenicity.[16]

Variant classification

Finally, after reviewing all available evidence on the pathogenicity of the *RYR1* variants included in this study, variants were classified according to the ClinGen *RYR1* VCEP recommendations[11] and the EMHG scoring matrix (https://www.emhg.org/genetic-scoring-matrix). Variants were classified as pathogenic, likely pathogenic, variant of uncertain significance, likely benign, or benign.

Statistical analysis

The statistical analysis plan was approved by the authors before accessing the data. The number of MH families, number of MHS individuals, and IVCT results are reported using descriptive statistics.

Calcium release was normalised for each assay as a percentage of the total calcium released by 500 μM 4-CmC. Sigmoidal curves were fitted using GraphPad Prism (version 9.5.0; GraphPad Software Inc., Boston, MA, USA) to calculate the 50% effective concentration (EC_{50}). EC_{50} values are reported as mean and standard error of the mean (SEM). EC_{50} values of the *RYR1* mutant constructs were compared with WT using a two-tailed unpaired Student's *t*-test followed by Bonferroni correction for multiple testing. As the EC_{50} values of nine variants were compared with WT, *P*-values of <0.006 (0.05/9=0.006) were considered significant.

No power calculation was conducted; the sample sizes used were based on similar cell models in previous studies.[13]

Results

Expression of RyR1 was confirmed for all controls and for all study variants as shown in Supplementary Figure S1.

Immunofluorescence microscopy was used to confirm co-localisation of the RyR1 protein with endoplasmic reticulum probes (data not shown).

Calcium release

Six of the seven variants (p.Glu342Lys, p.Leu2288Ser, p.Phe2340Leu, p.Arg2676Trp, p.Val3324Ala, p.Phe4076Leu) included released calcium in response to 4-CmC. The selected controls (WT, benign, and pathogenic controls) released calcium as well. The p.Trp5020Cys variant and the pcDNA™5/FRT/TO vector did not release any calcium in response to 500 μM 4-CmC, which was the highest 4-CmC concentration used.

The 4-CmC EC_{50} values of variants p.Glu342Lys, p.Leu2288Ser, p.Phe2340Leu, p.Arg2676Trp, p.Val3324Ala, and p.Phe4076Leu, and of the pathogenic control variants p.Leu13Arg and p.Thr4826Ile, were all significantly lower compared with WT, indicating hypersensitivity to 4-CmC. The EC_{50} of the benign control Gly2060Cys was not statistically different from WT. Results of the calcium release assays are summarised in Figure 1 and Table 2.

Segregation analysis

Data relevant for segregation analyses are presented in Table 1 and Supplementary Figure S2. p.Glu342Lys was identified in two individuals from a Dutch family who had a clinical MH reaction, whereas no DNA was available for analysis from two other individuals from the same family who died during or shortly after having MH reactions (Supplementary Fig. S2a). The same variant was identified in a Swedish patient who harboured a second *RYR1* variant of uncertain significance (p.Glu572Ala) after an anaesthetic adverse event suspected to have been MH but unconfirmed by IVCT. p.Leu2288Ser was identified in two MHS individuals from one Dutch family whereas no DNA was available for analysis from three other individuals from the same family who suffered a clinical MH reaction (Supplementary Fig. S2b). p.Leu2288Ser was also identified in a Swedish patient who suffered a clinical MH reaction and was confirmed as MHS by IVCT. p.Phe2340Leu was identified in an individual who had an MH event and in two first-degree relatives who were diagnosed as MHS by IVCT. The mother of the index patient harboured a second *RYR1* variant of uncertain significance, c.7760A>G, p.Tyr2587Cys, in trans (rs767553612, MAF 0.00001232, REVEL score 0.911) (Supplementary Fig. S2c). p.Arg2676Trp was identified in a patient who had an MH event and also harboured the pathogenic *RYR1* variant p.1840C>T, p.Arg614Cys in trans. Six of his relatives confirmed as MHS by IVCT harboured p.Arg2676Trp alone, whereas another one carried both p.Arg614Cys and p.Arg2676Trp, like the index patient (Supplementary Fig. S2d). p.Arg2676Trp was also identified in two individuals from an unrelated Australian family who both had had MH events and harboured no additional *RYR1* variant. p.Val3324Ala was identified in two MHS-confirmed relatives of an individual who died from MH (Supplementary Fig. S2e). p.Phe4076Leu was identified during diagnostic screening in a patient presenting with cramps, myalgia, and muscle weakness, who was confirmed as MHS by IVCT but had been never exposed to MH-triggering anaesthetic agents. p.Trp5020Cys was identified in an individual who had an MH event, and in three of his relatives confirmed as MHS by IVCT (Supplementary Fig. S2f). There were no phenotype-negative (malignant hyperthermia normal [MHN], i.e. diagnosed as not susceptible to MH by IVCT) individuals among any of the variant carriers included in this study.

Bioinformatic analysis

All the variants included in this study had low MAFs in the gnomAD dataset,[15] meeting neither the EMHG scoring matrix benign stand-alone criterium (MAF >0.1%) nor the ClinGen *RYR1* VCEP benign strong criterium (MAF >0.08%).[11] REVEL scores of p.Glu342Lys, p.Leu2288Ser, p.Phe4076Leu, and p.Trp5020Cys were >0.85, supporting their pathogenicity. None of the other variants analysed had REVEL scores <0.5, supportive of a benign impact. MAF and REVEL scores of all variants are summarised in Table 1.

Variant classification

The EMHG scoring matrix and the ClinGen *RYR1* VCEP Guidelines for *RYR1* Pathogenicity Assertions in MH Susceptibility were applied. The p.Glu342Lys and p.Leu2288Ser variants were classified as pathogenic according to both the EMHG scoring matrix and VCEP guidelines. The p.Phe2340Leu and p.Arg2676Trp variants were classified as pathogenic according to the EMHG guidelines and likely pathogenic according to the VCEP guidelines. Variants p.Val3324Ala, p.Phe4076Leu, and p.Trp5020Cys remain classified as variants of uncertain significance according to both guidelines. The criteria met according to the EMHG scoring matrix and VCEP guidelines are summarised in Table 3.

Discussion

Four out of seven variants studied met the criteria to be reclassified and included in the EMHG list of diagnostic variants for MH susceptibility. Six of the seven *RYR1* variants analysed demonstrated hypersensitivity to 4-CmC in calcium release assays (p.Glu342Lys, p.Leu2288Ser, p.Phe2340Leu, p.Arg2676Trp, p.Val3324Ala, p.Phe4076Leu). To the best of our knowledge, none of the variants studied have been identified in individuals with negative IVCT results (i.e. not susceptible to MH).

The hypersensitive *RYR1* variants p.Glu342Lys, p.Leu2288Ser, p.Phe2340Leu, and p.Arg2676Trp, all identified in MHS individuals referred to the Dutch MH Investigation Unit because of an MH reaction after exposure to MH-triggering agents, have also been identified in MHS individuals unrelated to the Dutch families. p.Glu342Lys was found in a Swedish patient after an anaesthetic adverse event suspected to be MH (clinical grading score 30) (Table 1), whose phenotype has not yet been confirmed by IVCT. The Swedish patient also harboured the *RYR1* variant of uncertain significance c.1715A>C, p.Glu572Ala (rs779551357, MAF 0.0001146, REVEL score 0.899), absent from the Dutch family. Variant p.Leu2288Ser was identified in another Swedish patient who developed MH triggered by anaesthesia (CGS 33), and was confirmed as MHS by IVCT. The *RYR1* variant c.7020C>G, previously identified in a combined Swiss/German MH cohort, results in the same amino acid change (p.Phe2340Leu) as variant c.7018T>C identified in an MHS individual from the

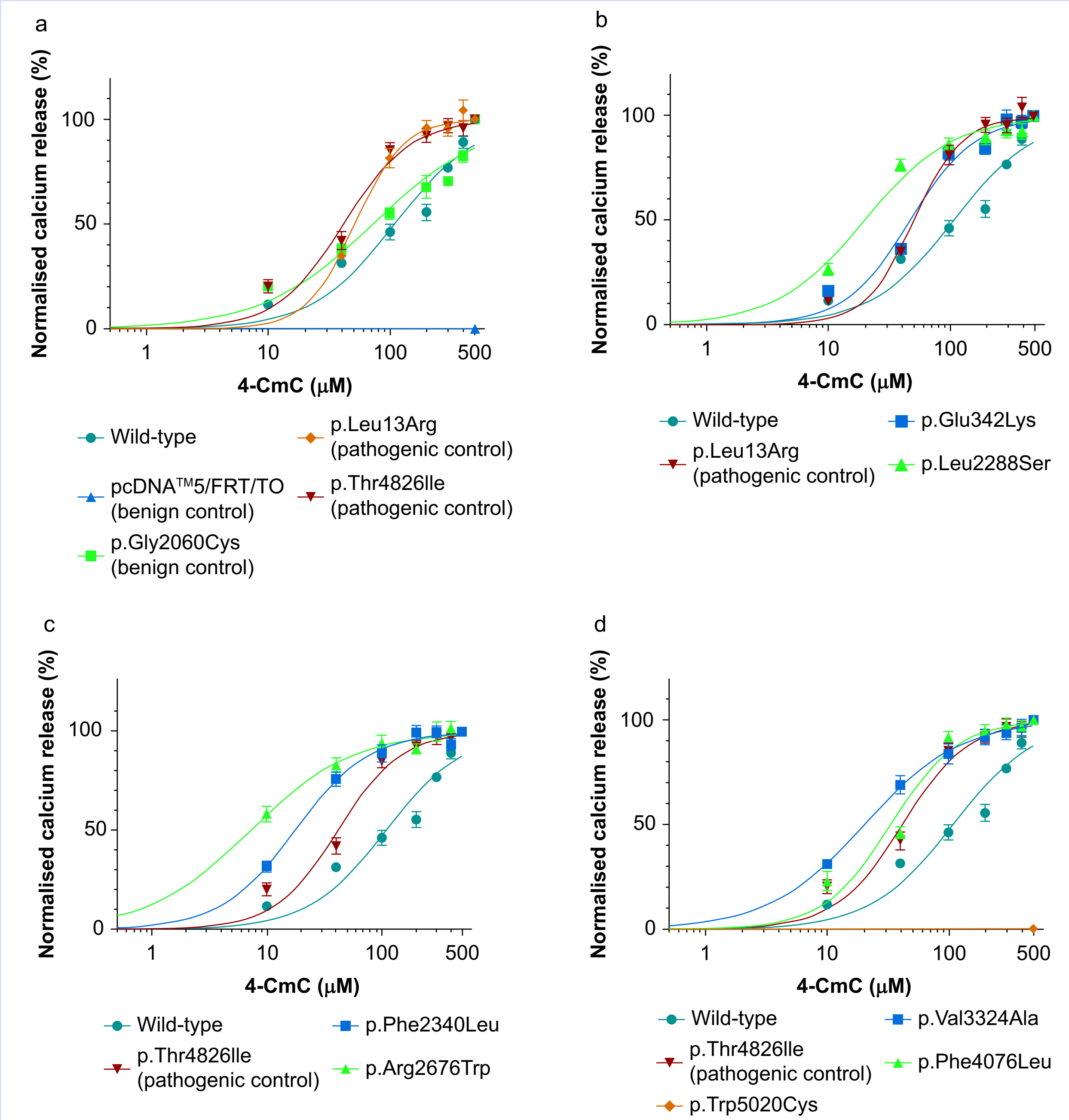

Fig 1. Normalised concentration–response curves for calcium release in response to the ryanodine receptor-1 agonist 4-chloro-m-cresol (4-CmC) in Flp-In™ T-REx™ HEK293-T cells. Response was normalised to calcium release to 500 μM 4-chloro-m-cresol. Sigmoidal curves were fitted using GraphPad Prism (version 9.5.0; GraphPad Software Inc., Boston, MA, USA). p.Leu13Arg and p.Thr4826Ile represent pathogenic controls. p.Gly2060Cys and wild-type present a benign control.

Dutch cohort in the present study.[22] Variant p.Arg2676Trp has been reported in at least six other MHS families from the UK, Switzerland, and Australia, and in a combined French/Italian MH cohort.[7,23,24]

The index patient harbouring variant p.Arg2676Trp carried a second *RYR1* variant c.1840C>T, p.Arg614Cys, classified as pathogenic for MH according to both EMHG and VCEP criteria.[25] p.Arg614Cys has been frequently associated with MH since it became the first *RYR1* variant ever linked to MHS.[6,7,17,26] Therefore, we assume that p.Arg614Cys actively contributed to the occurrence of the MH reaction in our index patient. However, p.Arg2676Trp was identified in six MHS relatives of the index patient who did not harbour p.Arg614-Cys, and also in at least six other unrelated MH families who did not harbour a second *RYR1* variant (Supplementary Fig. S2d). Because our essays demonstrated RyR1

Table 2 EC_{50} values for 4-chloro-m-cresol-induced calcium release in Flp-In™ T-REx™ cells. EC_{50}, 50% effective concentration; NA, not applicable; SEM, standard error of the mean. *Statistically significant difference from wild-type is denoted by $P<0.006$.

Variant	Mean EC_{50} (μM 4-chloro-*m*-cresol) (SEM)	P-value
Controls		
Wild-type (n=9)	104.4 (6.0)	NA
pcDNA™5/FRT/TO (negative control) (n=5)	NA	NA
p.Gly2060Cys (benign control) (n=9)	78.0 (5.0)	0.0203
p.Leu13Arg (pathogenic control) (n=12)	39.8 (2.2)	0.0000158*
p.Thr4826Ile (n=10)	33.5 (2.7)	0.00000163*
Variants included in the present study		
p.Glu342Lys (n=8)	40.5 (3.4)	0.0000431*
p.Leu2288Ser (n=11)	19.6 (2.0)	0.000000374*
p.Phe2340Leu (n=7)	16.6 (2.3)	0.0000240*
p.Arg2676Trp (n=7)	9.0 (1.8)	0.0000181*
p.Val3324Ala (n=9)	20.2 (1.8)	0.00000161*
p.Phe4076Leu (n=9)	27.3 (3.2)	0.00000104*
p.Trp5020Cys (n=2)	NA	NA

hypersensitivity to calcium release agonist 4-CmC in cells expressing p.Arg2676Trp, and considering its bioinformatic evidence and segregation, p.Arg2676Trp can be reclassified as pathogenic according to the EMHG guidelines and as likely pathogenic according to VCEP criteria.[11]

Of the related MHS individuals who harboured p.Phe2340Leu, the mother of the index patient was also heterozygous for the variant of uncertain significance c.7760A>G, p.Tyr2587Cys, whereas an MHS sibling did not carry the second variant (Supplementary Fig. S2c). Although the REVEL score of the second variant (0.911) is predictive of a damaging effect, p.Tyr2587Cys had never before been reported in association with MH susceptibility. As shown by previous studies, REVEL scores and other *in silico* pathogenicity prediction tools are rather nonspecific, and are only considered supportive in variant pathogenicity assessments.[6,27,28] However, mutants expressing p.Phe2340Leu showed hypersensitivity to 4-CmC. In addition, three related variant carriers from the Dutch cohort and an unrelated individual from a different cohort[22] were all diagnosed as MHS by IVCT. It remains uncertain whether the second *RYR1* variant p.Tyr2587Cys found in one of the individuals contributed to the MHS phenotype, as there are no segregation data available for that variant, and the possibility of a compound heterozygous synergistic effect with regards to hypersensitivity to RyR1 agonist was not assessed in our study.

RYR1 variant p.Trp5020Cys, identified in an individual who had an MH event triggered by anaesthesia and in three of his MHS relatives (Supplementary Fig. S2f), did not release any calcium in response to 4-CmC even at high concentrations of the RyR1 agonist. Immunoblotting showed that the apparent null effect of p.Trp5020Cys was not caused by reduced expression of RyR1 constructs carrying this variant, as those were expressed at a level similar to that of other calcium-releasing variants. Immunofluorescence showing co-localisation within the endoplasmic reticulum membrane

Table 3 Summary of variant classification according to the European Malignant Hyperthermia variant scoring matrix and the Variant Curation Expert Panel recommendations. EMHG, European Malignant Hyperthermia Group; MH, malignant hyperthermia; VCEP, Variant Curation Expert Panel; VUS, variant of uncertain significance.

Variant	Previously reported in association with MH	EMHG criteria met	EMHG prediction	VCEP criteria met[11]	Odds of pathogenicity[11]	Posterior probability of pathogenicity[11]	VCEP prediction[11]
p.Glu342Lys	Yes, same family[6,17–19]	PSb, PPa, PPb, PPc	Pathogenic	PP1_strong, PM1_mod, PS3_mod, PP3_mod,	1486.8	0.994	Pathogenic
p.Leu2288Ser	No	PSb, PPa, PPb, PPc	Pathogenic	PM1_mod, PS3_mod, PS4_mod, PP1_mod, PP3_mod	1470,1	0.994	Pathogenic
p.Phe2340Leu	Yes,[6,20,21] same family and one other family[22]	PSb, PMb, PPc	Pathogenic	PM1_mod, PS3_mod, PP1_sup, PS4_sup	81.5	0.901	Likely pathogenic
p.Arg2676Trp	Yes, same family[20] and five other families[7,23,24]	PSb, PPa, PPc, PMb	Pathogenic	PP1_strong, PS3_mod, PS4_mod	345.8	0.975	Likely pathogenic
p.Val3324Ala	Yes,[6] same family	PSb, PPc	VUS	PS3_mod, PS4_sup	9.0	0.501	VUS
p.Phe4076Leu	Yes,[18,19] same family	PSb, PPb	VUS	PS3_mod, PP3_mod	18.5	0.673	VUS
p.Trp5020Cys	Yes,[6,20] same family	PPb, PPc,	VUS	PP3_mod, PS4_sup, PP1_sup	4.3	0.325	VUS

indicates the protein was expressed in the correct location. Residue 5020 resides in the cytoplasmic C-terminal domain of the RyR1 protein, forming part of the channel's pore where binding sites for the agonists calcium, ATP, and caffeine have been identified at interdomain interfaces.[29–31] The substitution of cysteine for tryptophan could have resulted in a structural alteration that made the channel insensitive to 4-CmC, as a complete RyR1 loss of function would be inconsistent with the MHS phenotype observed in p.Trp5020Cys variant carriers, none of whom had overt signs of myopathy.

An important limitation of our study is the use of Flp-In™ T-REx™ 293 cells, because these non-muscle (kidney) cell models overexpressing homozygous mutant constructs, unlike more sophisticated *in vitro* assays,[32] lack skeletal muscle regulatory proteins critical for physiological RyR1 function. However, Flp-In™ T-REx™ 293 cells are a relatively simple inexpensive system and a useful model to assess the effect of a single amino acid variant on intracellular calcium release in a standardised genetic background, validated for assessing the pathogenicity of *RYR1* variants for MH susceptibility. It would have been very useful to evaluate mutant responses to other RyR1 agonists such as calcium, ATP, cAMP, or caffeine, to assess potential functional effects overlooked by 4-CmC assays alone, as for variant p.Trp5020Cys (which resides in a crucial ligand-binding domain). Another limitation of our study is the limited data available for segregation analysis in families carrying the *RYR1* variants p.Val3324Ala and p.Phe4076Leu. Finally, the *RYR1* variant p.Phe4076Leu was identified during diagnostic screening in a patient who presented because of neuromuscular symptoms and was diagnosed as MHS by IVCT, but had no history of a hypermetabolic reaction triggered by anaesthesia.

Conclusions

RYR1 variants p.Glu342Lys, p.Leu2288Ser p.Phe2340Leu, and p.Arg2676Trp, reclassified as pathogenic or likely pathogenic for MH, can be used for presymptomatic diagnosis of MH susceptibility. Although our calcium release assays demonstrated that RyR1s expressing p.Val3324Ala and p.Phe4076Leu were hypersensitive to the RyR1 agonist 4-CmC, and that p.Trp5020Cys expression resulted in no response to 4-CmC, they should still be considered variants of uncertain significance as the available evidence is insufficient for their reclassification.

Authors' contributions

Study conception: LB, NC, MS, GS, AH, EK, RG, AB, KS
Experimental procedures: LB, AH, CY, MS, KS
Statistical analysis: LB, MS
Writing of the first draft of the manuscript: LB
Revision of the manuscript: all authors
All authors reviewed and approved the manuscript in its final form before submission.

Acknowledgements

The authors thank the Canisius Wilhelmina Hospital—Radboudumc regional junior research funds and the European Malignant Hyperthermia Group (EMHG; https://www.emhg.org/) for their financial support. Liam Hewson and Ella Eagar (graduate students at Massey University) assisted in site-directed mutagenesis. NV, MS, CY, and LB are members of the European Reference Network for rare neuromuscular diseases (EURO-NMD).

Declaration of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bja.2024.11.043.

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Handling Editor: Hugh C Hemmings Jr