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Next Generation Sequencing of *RYR1* and *CACNA1S* in Malignant Hyperthermia and Exertional Heat Illness

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Abstract

Background—Variants in *RYR1* are associated with the majority of cases of malignant hyperthermia (MH), a form of heat illness pharmacogenetically triggered by general anesthetics, and they have also been associated with exertional heat illness. *CACNA1S* has also been implicated in MH. We applied a targeted next generation sequencing approach to identify variants in *RYR1* and *CACNA1S* in a cohort of unrelated patients diagnosed with MH susceptibility. We also provide the first comprehensive report of sequencing of these two genes in a cohort of survivors of exertional heat illness.

Methods—DNA extracted from blood was genotyped using a "long" polymerase chain reaction technique, with sequencing on the Illumina GAII® or MiSeq® platforms (Illumina Inc., San Diego, CA). Variants were assessed for pathogenicity using bioinformatic approaches. For further follow up DNA from additional family members and up to 211 MH normal and 556 MH susceptible unrelated individuals was tested.

Results—In 29 MH patients we identified three pathogenic and four novel *RYR1* variants, with a further five *RYR1* variants previously reported in association with MH. Three novel *RYR1* variants were found in the exertional heat illness cohort (n = 28) along with two more previously reported in association with MH. Two other variants were reported previously associated with

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centronuclear myopathy. We found one and three rare variants of unknown significance in *CACNA1S* in the MH and exertional heat illness cohorts respectively.

Conclusion—Targeted next generation sequencing proved efficient at identifying diagnostically useful and potentially implicated variants in *RYR1* and *CACNA1S* in MH and exertional heat illness.

Introduction

Malignant Hyperthermia (MH) is a potentially fatal adverse drug reaction, triggered during general anesthesia by volatile halogenated anesthetics and succinylcholine. The prevalence of genetic variants predisposing to MH has been estimated to be as high as 1:2,000 [1,2]. In most families MH shows an autosomal dominant pattern of inheritance. Following a suspected MH episode, the diagnosis can be confirmed using an *in vitro* contracture test (IVCT), where skeletal muscle, obtained through biopsy of the vastus muscle, is exposed to incremental concentrations of caffeine or halothane and responses recorded [3].

Physiological and biochemical studies show a severe defect in MH skeletal muscle calcium regulation [4]. The ryanodine receptor (RYR1) forms a calcium channel in the sarcoplasmic reticulum and in its active state releases Ca^{2+} from the sarcoplasmic reticulum leading to muscle contraction[5]. $Ca_v1.1$ is structurally a calcium channel located within the t-tubule membrane, but functionally it is the voltage sensor that activates RYR1. During an MH episode myoplasmic calcium concentration rises rapidly resulting in a hypermetabolic state characterised systemically by hyperthermia, acidosis, tachycardia, cardiac arrhythmias, skeletal muscle rigidity and rhabdomyolysis. Similar features are observed in exertional heat illness (EHI) suggesting possible links between MH and EHI[6].

Variants in the *RYR1* gene (Mendelian inheritance in man, MIM #180901) are thought to be responsible for about 75% of cases susceptible to MH (MHS). Less frequently MH is associated with variants in *CACNA1S* (MIM #114208), encoding the α subunit of $Ca_v1.1$. Mutations in either of these genes may disrupt signalling between $Ca_v1.1$ and RYR1 and thus confer MH susceptibility[7–9]. A significant number of families do not have a variant in a major locus identified to date.

Approximately 200 missense variants in *RYR1* have been described in association with MH[10], with 31 of them known to be functionally relevant and used diagnostically[11], while only 2 variants described in *CACNA1S* in association with MH have been functionally characterised[12,13]. Variants in *RYR1* are also known to cause distinct rare myopathies such as multiminicore disease and central core disease, with some variants associated both with MH and central core disease[14].

Because of the large size of *RYR1* screening the whole coding sequence (~15,000 bp) using Sanger sequencing is expensive and time-consuming. In a clinical setting, often only the limited number of diagnostic variants is screened. With the advent of Next Generation Sequencing (NGS) limitations of current approaches can be overcome with a potential for increased variant detection rate compared with Sanger sequencing[15]. We have assessed the feasibility of NGS using groups of MH susceptible and EHI patients previously screened for

RYR1 diagnostic variants and found to be negative, for analysis of the whole coding sequences of *RYR1* and *CACNA1S*. We applied a Long Range polymerase chain reaction (PCR) technique as an enrichment method, to amplify coding sequences, and sequenced using Illumina GAII® and MiSeq® technology (Illumina Inc., San Diego, CA).

Materials and Methods

Samples

Blood samples were obtained from patients referred to the Malignant Hyperthermia Unit, University of Leeds for genetic and functional (IVCT) tests used in the diagnosis of MH-susceptibility. The research was approved by the Leeds East Local Research Ethics Committee (Reference 10/H1306/70; Leeds, United Kingdom) and written consent was obtained from each patient. DNA was extracted from peripheral blood lymphocytes using a salting out method. Briefly, after erythrocyte lysis with a buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 1.0 mM EDTA, the leukocytes were pelleted and subjected to lysis with 2% sodium dodecyl sulphate, 25 mM EDTA solution. Next, a protein precipitation with 10 M ammonium acetate was performed. Then the proteins were pelleted and supernatant containing DNA was transferred into a new tube. DNA precipitation was carried out using isopropanol.

Fifty-seven DNA samples were chosen for sequencing the entire coding sequence of *RYR1* and *CACNA1S*, as the two main genes known to contribute to MH susceptibility. The majority of samples had already been routinely screened for the 16 most common diagnostic variants found in the United Kingdom population: DNA results from these patients have not been reported in previous publications. We sequenced DNA from 29 MH susceptible individuals, with susceptibility confirmed by IVCT, and 28 EHI individuals. All of the EHI individuals were military personnel who had had a clinical episode of heat illness during military exercises that required hospital treatment. They were referred for testing for MH susceptibility having all subsequently failed to demonstrate normal thermoregulation during a heat tolerance test carried out at the Institute of Naval Medicine (see appendix 1) on at least two separate occasions. As the sensitivity of the IVCT to detect genetic predisposition to EHI, as opposed to MH susceptibility, is unknown, we included EHI individuals in this study irrespective of their IVCT result. In the group of EHI samples, 16 were from MH normal individuals (MHN, responding to neither triggering agent used in the IVCT) and 12 from MH susceptible individuals, where MH susceptibility is defined by the IVCT.

Each of the 57 samples represented a single family. For follow up of NGS, DNA from additional family members and at least 150 MH normal and up to 556 MH susceptible genetically independent individuals, including cases of EHI, were tested. If a variant is not found in the 300 chromosomes of 150 individuals it can be concluded with 95% confidence that the prevalence of the variant in the population is < 1%, assuming the data are from a Binomial distribution. Such a variant, by definition, is not a polymorphism.

Long PCR

The samples were processed in four batches. The coding sequence of *RYR1* and *CACNA1S* was amplified in 23 and 9 PCR products respectively (tables 1 and 2). Amplification was carried out in 20 μl reaction volumes containing 20 ng genomic DNA template, using SequalPrep Long PCR Kits with deoxyribonucleotide triphosphates (Invitrogen, Carlsbad, CA). Reactions were optimized according to the manufacturer's recommendations and contained 1x reaction buffer, 2% dimethyl sulfoxide, 0.5x SequalPrep EnhancerA, 1.8 U SequalPrep Long Polymerase and 250 nM each of the forward and reverse primers. The thermal cycling program was: 2 min at 94 °C for denaturation, followed by 35 cycles of 10 s at 94 °C, 30 s at 60 °C and 6 min 30 s at 68 °C. The final extension step was 5 min at 72 °C.

Amplicon size was verified on 0.7% agarose gels and concentrations were measured using QuantIT PicoGreen BR Kits (Invitrogen, Eugene, OR). For each patient, the PCR products were pooled in an equimolar ratio. The pooled PCR products were sheared using an ultrasonicator (Covaris S2, KBioscience, Hertfordshire, United Kingdom) to produce fragments of approximately 200 bp. Subsequently, sheared samples were purified with MiniElute Purification Kits (Qiagen GmbH, Hilden, Germany) and the quality checked on an Agilent 2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany). The SPRIworks Fragment Library System I (Beckman-Coulter, Brea, CA) was used for tagged library preparation. Ten samples can be processed in parallel. During the process unique indexed adaptors are ligated to each pooled sample. In the next step, individual libraries were subjected to enrichment. Phusion High-Fidelity Master Mix (ThermoFisherScientific, Waltham, MA) and 3′PTO modified primers (Fisher Scientific UK Ltd., Loughborough, United Kingdom) were used to amplify the libraries:

PTO-F

5′AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC* T-3′;

PTO-R

5′CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCG ATC*T-3′.

PCR was carried out in 50 μl volumes containing 5 μl of library DNA, 25 μl of Phusion HF Master Mix and 500 nM of each primer. The thermal cycling program was: 98 °C for 30 s, then 12 cycles of 98 °C for 10 s, 65 °C for 30 s and 72 °C for 30 s, and the final extension was 72 °C for 5 min. Enriched libraries were then purified with Agencourt®AMPure®XP (Beckman Coulter) according to the manufacturer's protocol. Purified libraries are pooled in equimolar ratios and loaded on one lane of a flow cell for cluster generation. Libraries were subsequently sequenced on an Illumina GAII® or MiSeq®.

Output files from the sequencing platform were sorted according to barcode tags using Illuminator Data Extractor (preprocessing software) and sequence analysis was performed using Illuminator software[16], both of which are freely available on the University of Leeds website*. This software filters out base calls with a Phred (Q) score < 25. Reads were aligned against GeneBank genomic and coding reference sequences. For *RYR1* the genomic

reference sequence was NC_000019.9 (38923137-39079419) and transcript NM_000540.2. For *CACNA1S* the genomic reference sequence was NC_0000001.10 (201008331-201081807) and transcript BC133671.1.

Testing of missing fragments, variants in family segregation studies and independent samples

Sanger sequencing was used routinely for *RYR1* exon 91, since amplification of Long PCR fragments failed and for exon 102 in all but one case for the same reason, despite protracted attempts to optimise primers. The primers used for Sanger sequencing to provide full coverage of these exons are provided in appendix 2. The primers for Sanger sequencing of other exons are available from the authors on request.

When a variant was identified in an MH patient or an EHI patient who tested MH susceptible by IVCT, there was a possibility of testing further family members to look for cosegregation of variant and disease phenotype. Sanger sequencing was used to test *RYR1* and *CACNA1S* variants, identified through NGS, on additional family members (primers not supplied).

PCR based restriction fragment length polymorphism and amplification-refractory mutation system assays were designed for specific variants and used to screen unrelated MH susceptible and MH normal DNA samples. c.641C>T was screened by PCR and restriction fragment length polymorphism (F5′ ACC CTT GGC CTG AAA ATA CC, R5′ TGA AGT CAA GGG TTC AGC TC and *BccI*). c.9676G>C was also screened by PCR and restriction fragment length polymorphism (F5′ GCA CTG CAG CCT GAG TAA CA, R5′ CCC CCG AAC CAT AAA CTC TG and *BsrBI*). c.11958C>G was tested using an assay combining PCR products from exon 87 (the mutation site) and exon 24 to differentiate products after digestion with *EcoNI* (Exon 87 F5′ GTG ATC CCT GAT CCC TTC TC, R5′ GAA GCA GGT GGA TGG AGA C; Exon 24 F5′ GAC AAG GGT CAG CAG TCA GG, R5′ GGG TCA GAG TTG GGG TAG GA). c.12028G>A in exon 88 was tested by an amplification-refractory mutation system assay (Mutation specific primers: F′ CTA GGA CTC AAG CCA GAT CA, R′ GAT GGG GTT GAG GAT TAG GG and exon 44 control primers: F′ GGG AGG TCT CTG ATG GTG, R′ CGG GAG ACT CAC TGC TCG).

Prediction of variant pathogenicity

To predict the possible functional relevance of all new variants, the impact of the amino acid substitution on the structure and function of the protein was estimated using the PolyPhen-2 (Polymorphism Phenotyping v2)[17] and Combined Annotation–Dependent Depletion[18] bioinformatics tools. The PolyPhen-2 program assigns the variant a score between 0 and 1.0: the higher the score, the more likely is the variant to be pathogenic. It should be noted that no bioinformatics tool has been validated for *RYR1* or *CACNA1S*. Our previous work suggests that a PolyPhen-2 score of >0.95 is sensitive but not specific for pathogenic *RYR1* variants[15]. The Combined Annotation–Dependent Depletion is a recently published method that combines information from multiple sources into a single C-score. The C-score provides

*http://dna.leeds.ac.uk/illuminator/ (last accessed October 3, 2014).

a better correlation with pathogenicity than any other *in silico* prediction program or combinations of these[18] but has not been validated for, or even applied in the literature to, *RYR1* and *CACNA1S* variants.

Results

Quality of sequencing

For all samples the sequence quality reached the quality threshold and read depth was above the commonly applied minimum for diagnostic use of 50 by a considerable margin in the great majority of fragments. The mean read depth by exon was 403 (see table 3 for read depth for each exon). Despite extensive efforts to optimise primers, sequence quality was insufficient to reach the base-calling threshold in all samples for *RYR1* exon 91 and all but one sample for *RYR1* exon 102. Sanger sequencing was used for these exons and other exons that failed the quality threshold in individual samples. From the first batch of samples *RYR1* fragments 4 and 15 (table 1) failed in three samples and two samples respectively. For subsequent batches of samples the primers for these fragments were redesigned with good results (table 3). Otherwise *RYR1* fragments 13 and 16 (table 1) and *CACNA1S* fragments 1 and 3 (table 2) failed in one patient each, presumably as a result of pipetting errors.

RYR1—Nineteen *RYR1* variants, 4 of which are novel (p. 2248R>C, p.3226E>Q, p. 4010E>K, p.4230M>R), were found in 20 MH patients (table 4) and 11, 3 of which are novel (p.492R>H, p.4282L>V, p.4331A>T), in 13 EHI patients (table 5). Three polymorphic changes were found in the *RYR1* coding sequence, based on frequencies supplied for all populations on the Exome Variant Server† (table 6), and a further two variants have been reported not to segregate with the MH phenotype (p.1787P>L[19] and p. 3253I>T[20]). All variants were seen as heterozygotes. *RYR1* variants described in this paper are illustrated in figure 1 and their PolyPhen-2 scores and C-scores given in table 6.

Previously reported *RYR1* variants: We found three variants (p.163L>R[14,21], p. 552R>W[22], p.614R>C[23]), previously reported in MH patients, that have functionally characterised effects consistent with a pathogenic role in malignant hyperthermia. Four further variants previously described in the literature in MH patients (p.177R>C, p. 1342S>G, p.1571I>V, p.3986D>E)[14,19,24–26] are yet to be functionally characterised. p. 177R>C affects a well-conserved amino acid, segregates with the MHS phenotype and was not found in 100 control chromosomes[14,19,24]. We found *RYR1* c.11958C>G p.3986D>E in a further 3 of 541 independent MH susceptible individuals and none of 177 MH negative controls. Of the family members tested in all four families, disease phenotype and variant status were concordant but only in a small total number of meioses. p.3986D>E has a PolyPhen-2 score of 0.999 and C-score of 12.60.

The variant c.641C>T; p.214T>M was found in a patient who had a clinically suspected MH reaction during anesthesia but who did not have an IVCT. Two brothers of the proband did have an IVCT, however, and the p.214T>M variant was found in one brother who tested

†http://evs.gs.washington.edu/EVS/ (date last accessed October 12, 2014).

MH susceptible by IVCT but not the other brother who tested MH normal, which is consistent with the variant being associated with MH susceptibility. The PolyPhen-2 score for p.214T>M is 1.000 and the C-score 14.48. Furthermore, p.214T>M was found in three of 556 MH susceptible samples and all members of the three families show concordance of disease and/or IVCT phenotype with variant status. All of 177 MH negative samples tested lacked the substitution.

Variant p.4295A>V was described by Jeong *et al*.[27] as a compound mutation (with p. 2435R>H) in an MH family with histological minicores and elevated serum creatine kinase: some individuals developed a late onset myopathy. In our study this variant appears in an EHI patient who tested MH normal by IVCT.

RYR1 c.14168G>A; p.4723R>H was found in a patient who had a clinically suspected MH reaction confirmed by IVCT. Despite annotation through the 1000 Genomes Project[28], the variant did not appear on the Exome Variant Server (table 6). However the variant was found in the proband's son who was MH negative by IVCT. No further work was carried out on this variant.

RYR1 c.4178A>G p.1393K>R was found in a patient with a clinically suspected MH reaction and detected in 2 further MH families through screening 556 unrelated MH susceptible samples. Subsequent testing of family members revealed individuals discordant for variant status and disease phenotype in both families, which is perhaps not surprising considering the minor allele frequency reported in the Exome Variant Server, and suggesting that this variant is unrelated to MH status.

Novel *RYR1* variants: The four new variants found in MH patients were located in exons 41, 65, 88, and 91. The c.9676G>C; p.3226E>Q *RYR1* variant was found in a MH proband whose mother was tested MH susceptible and also carried the variant. The brother and maternal cousin of the proband did not carry the variant and were tested MH negative by the IVCT. The PolyPhen-2 score was 0.997 and the C-score 14.93. This substitution was not seen in 211 MH negative or 530 independent MH susceptible samples. There were no family members to conduct further studies on p.2248R>C (PolyPhen-2 1.000, C-score 15.92) or p. 4230T>G (PolyPhen-2 0.932, C-score 12.83).

Variant p.4010E>K was found in a MH susceptible woman who had four other *RYR1* variants (table 4): p.1571I>V, p.2060G>C, p.3366R>H and p.3933Y>C. Her son was the clinical proband and was too ill for an IVCT: he was negative for all variants except p. 4010E>K. The Polyphen-2 score for this variant is 0.07 which suggests that it is unlikely to be damaging, although the C-score is 15.14. Two hundred MH negative controls and 422 MH susceptible individuals tested negative for the variant. The father of the proband was MH negative by IVCT and negative for all variants.

One of three new *RYR1* variants found in EHI patients is c.1475G>A; p.492R>H, which occurs in an EHI patient found to be MH susceptible by IVCT. At the same amino acid position there is another variant described in the 1000 Genomes Project[28], c.1474C>T; p. 492R>C. There were no family members available for segregation analysis. The PolyPhen-2

result for the new variant is 0.986 while the C-score is only 7.26. The other new variants identified in EHI patients, p.4282L>V and p.4331A>T, occur in EHI patients found to be MHN by IVCT and so no further family members have been tested by IVCT. The PolyPhen-2 scores and C-scores (table 6) suggest that they are benign.

CACNA1S—Eight of nine missense variants found in the *CACNA1S* coding sequence were previously described[7,8]. All of these are listed in the 1000 Genomes Project (table 7). In our patients four polymorphic changes were found in the *CACNA1S* coding sequence, based on frequencies supplied for all populations on the Exome Variant Server, and a single additional low frequency variant was seen, which had been reported as polymorphic elsewhere (table 7). Consistent with polymorphic status, c.206C>G; p.69A>G and c. 1373T>A; p.458L>H were homozygous in some of the sequenced samples. A variant with a low minor allele frequency (p.683R>C; minor allele frequency 0.00976) was found in an EHI patient who had tested MH negative by IVCT (table 5). The relevance of this variant to EHI remains to be established.

In the MH group we found *CACNA1S* variant c.3026C>A, A**C**G/A**A**G; p.1009T>K in one individual (tables 4 and 7): this variant was previously found by exome sequencing in another family from the United Kingdom[15]. In the 1000 Genomes Project there is an annotated variant changing threonine at position 1,009 to methionine (c.3026C>T; A**C**G/A**T**G). The Exome Variant Server provides a minor allele frequency of 0.000154 for the T>M substitution. Both substitutions are predicted to give probably damaging amino acid changes with PolyPhen-2 scores of 1.0 and high C-scores (table 7). Segregation studies showed the new variant not to segregate with the MH IVCT phenotype in the family from the current study. Although three MHS individuals carried the variant and six MHN individuals did not carry the variant, there were two MHN individuals who carried the variant. This is a similar situation to the family with the same variant reported by Kim *et al*[15], where there was a LOD (logarithm of odds) score of 2.12 but one MHN individual carried the p.1009T>K variant.

Discussion

We have used NGS technology to sequence the coding regions of the *RYR1* and *CACNA1S* genes in 29 MH patients and 28 EHI patients. By limiting our analyses to two genes we were able to simultaneously sequence samples from multiple patients while achieving very high read depths (table 3) in all *CACNA1S* exons and all but two *RYR1* exons. Combined with the high base-calling quality of the sequencing platforms used, this provides a high level of accuracy of sequencing in an efficient manner (once the primers were optimized, preparation of the enriched libraries for sequencing took 2 weeks). We had to resort to Sanger sequencing for *RYR1* exons 91 and 102, where the quality of NGS did not meet our quality thresholds, presumably because of the high proportion of guanine and cytosine bases in these regions.

Testing for diagnostic variants by amplification-refractory mutation system and PCR-restriction fragment length polymorphism methods is time consuming and usually a staged procedure, taking place over a considerable period of time. Necessarily the most prevalent

variants are likely to be assayed first. An NGS diagnostic approach is more efficient with individuals fully sequenced for all variants at one time, with both the diagnostic variants and the wealth of uncharacterized variants, many of which may still prove to have functional relevance, detected. An NGS approach using long-range PCR has been employed successfully for diagnostic detection of *BRCA1* and *BRCA2* mutations in cases of familial breast cancer[29,30]. A real clinical and possible ethical problem with NGS approaches to diagnosis is deciding what information is given to referring clinicians and patients concerning variants of unknown or uncertain significance. Guidelines for reporting variants have been produced by professional bodies in Europe‡ (joint British/Dutch guidelines) and the United States[31]. The joint British/Dutch guidelines are especially useful as they provide a consensus view on the evidence required for inclusion in each class of variant. This includes the necessity to demonstrate the functional effects of missense variants before the variant can be considered to be pathologic, reiterating the position of the European MH Group with respect to the use of DNA findings in MH diagnosis[32]. The guidelines also caution against reliance on *in silico* predictions of pathogenicity in the diagnostic context and we would agree with this based on our previous analyses of *RYR1* and *CACNA1S* in control populations[15]. We propose to limit the use of PolyPhen-2 and C-scores as part of the process to prioritize variants for functional studies.

In the MH cohort we detected three variants that meet the European MH Group criteria for diagnostic use in assigning high risk of MH susceptibility. DNA from the individuals carrying these variants had not been sequenced prior to this study and screening for a full panel of diagnostic mutations was not complete in all cases. One of the variants detected was yet to be screened in the affected individual and another had been missed on routine screening, highlighting the sensitivity of NGS sequencing compared to conventional screening, including Sanger sequencing in this context[15,29]. Of the remaining 26 MH patients we detected variants of possible clinical relevance in 10 patients. In the 28 EHI patients we detected variants of possible clinical relevance in 7 individuals, 2 of whom had abnormal IVCT and 5 who had normal IVCT responses. In the MH cohort there was only one *CACNA1S* of possible relevance, the remainder being in *RYR1*, while 3 out of 7 of the variants of possible relevance in the EHI cohort were in *CACNA1S*.

This study represents the first report of sequencing the complete coding sequence of *RYR1* and *CACNA1S* in a cohort of survivors of EHI who have been physiologically characterized using heat tolerance testing and investigated by IVCT. Other than for several common variants, the functional and clinical relevance of the variants found in our EHI cohort is unclear. Uncommon variants were found in *RYR1* and *CACNA1S* in EHI patients who tested either positive or negative in the IVCT. However the prevalence of susceptibility to EHI is unknown, making interpretation based on variant frequency difficult. This is compounded by the absence of family segregation studies and indeed uncertainty regarding the genetic model operating in heritable predisposition to EHI. It is more than 20 yr since we first reported familial IVCT abnormalities in survivors of EHI[33] but the sensitivity and specificity of the IVCT for identifying skeletal muscle abnormalities associated with EHI is

‡http://www.acgs.uk.com/media/774853/evaluation_and_reporting_of_sequence_variants_bpgs_june_2013_-_finalpdf.pdf (date last accessed October 12, 2014).

unknown. Rather than trying to define crude IVCT cut-offs for this purpose (which has been clinically useful in MH susceptibility) it may be more fruitful to utilize the extensive quantitative information that can be derived from these tests, as we have done when analyzing the effects of different *RYR1* mutations in MH susceptible patients [34]. As all of the variants that were found in both MH and EHI cohorts were polymorphisms or of uncertain significance, our findings are not helpful in advancing understanding of the relationship between the two conditions.

Variants in the present study were detected throughout *RYR1*. Due to the size of *RYR1*, attention historically focused on three hotspot regions where the first diagnostic variants were found [34]. Sequencing of the entire gene is revealing variants throughout the gene. The RYR1 protein has many direct, diverse protein and subunit interactions. Whilst particular regions and residues will be conserved and functionally important, the expanding range of variants is not surprising. To date, the assumption has been made that primary mutations causing MH are likely to be missense, coding variants. However, the expression of messenger RNA in heterozygous MH susceptible individuals has been explored by Grievink and Stowell [35] and their findings suggest allele specific differences in *RYR1* expression may also contribute to phenotype. The possibility that noncoding variants are involved has not been explored.

Although MH shows an autosomal dominant pattern of inheritance, in some MH families discordant individuals have been identified, where IVCT confirmed phenotype is discordant with genotype [36–38], which suggests complexities in the genetics of the disease[11]. One explanation for discordancy in IVCT tested MH susceptible individuals is that there are some as yet undiscovered variants in *RYR1* or *CACNA1S* or other genes, which contribute to MH phenotype, while modifier loci have been implicated[39,40]: NGS may help to resolve discordancy in some families.

The current strategy for MH is to regard individuals carrying a familial diagnostic variant as susceptible. Diagnosis is conservative and these individuals would not require an IVCT. However individuals who do not carry a familial diagnostic variant cannot be regarded as unaffected, due to our incomplete knowledge of the genetics of MH, and these individuals undergo the IVCT. Of course this strategy is only appropriate for families where a proband has been screened for mutations and a familial diagnostic variant already identified. Incomplete knowledge concerning the genetics of MH means that a sensitive test based on identification of particular diagnostic variants is some way away. The last decade has seen many groups sequencing and cataloguing variants relevant to MH, some with information relating to diagnosis, focusing on *RYR1* and *CACNA1S*. Recently NGS methods have been employed to sequence genes involved in excitation-contraction coupling[20] or the whole exome[15,41]. To expand the range of diagnostic variants parallel efforts are required to identify further variants in *RYR1*, *CACNA1S* and other loci, and to obtain functional information on the nature of the variants.

A number of samples sequenced for *RYR1* and *CACNA1S* in the present study carried several variants, particularly those of higher frequency. Minor allele frequencies from public databases, or inferred from our own screening, were combined with other information for

interpretation of the likely nature of variants (see appendix 3 for an example). The description of variants as ‘polymorphic’ was not necessarily consistent across possible sources. Although the prevalence of MH is difficult to assess, primary causative missense mutations are highly unlikely to have frequencies greater than 0.001. However, the notion of common polymorphisms, or even rare variants, modifying the effects of mutations, such as the predicted damaging change *CACNA1S* c.3026C>A, cannot be ruled out in some instances[42,43]. *RYR1* variants are discussed here in the context of MH. However *RYR1* mutations are also known to be responsible for rare, congenital myopathies, which show both dominant and recessive modes of inheritance[44] such as autosomal recessive central core disease[45,46]. NGS will undoubtedly reveal variants of interest to a number of disorders.

In summary, we have reported NGS using long range PCR of *RYR1* and *CACNA1S*, the only two loci with variants with functional evidence supporting their role in MH. NGS is a fast and efficient diagnostic tool and a significant improvement on the current screening of diagnostic genetic variants. Additionally, complete coding sequence information will rapidly expand our knowledge of variants associated with MH and related disorders. However, functional studies will then be required to define the importance of these variants to the condition in question.

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Appendix 1. Summary of the Heat Tolerance Assessment Undertaken at the Institute of Naval Medicine, Alverstoke, United Kingdom

For the British military there are published guidelines[47], which require all military personnel who have suffered a significant episode of heat illness or required urgent admission to hospital with heat illness to be subsequently referred and assessed by the Institute of Naval Medicine. A function of the assessment is to establish if thermoregulation is affected by an underlying metabolic, biochemical or physiological disorder.

Exertional Heat Illness encompasses a spectrum of disorders deriving from the combined stresses of exertion and thermoregulation[48]. Such disorders may include heat related dehydration, stroke, cramps, exhaustion, injury, stress, syncope, rhabdomyolysis, acute renal failure and hyponatremia[49,50]. Individuals across the whole spectrum may be assessed. The assessment usually takes place a minimum of 6 calendar weeks following the original episode, or following full biochemical recovery. Individuals also undergo a limited guided and supervised return to physical activity prior to the assessment.

The heat tolerance assessment is conducted in an environmentally controlled climatic chamber (dry bulb temperature 34 °C (±0.25 °C), 40% (± 2%) relative humidity, Wet Bulb Globe Temperature (WBGT) index 27 °C, and wind speed 7 $km \cdot h^{-1}$). Patients first undergo a full medical examination, including cardiac screening and 12 lead electrocardiogram. Anthropometric measurements, height weight, estimation of body surface area, lean mass and fat mass are taken. This is followed by a maximal oxygen uptake assessment (VO_2 max) with electrocardogram telemetry. This exercise test is conducted wearing shorts and a T-shirt on a treadmill in the climatic conditions described. Following this assessment patients are allowed an hours rest in a cool room to allow body temperature to return to normal. They are also allowed to drink ad libitum.

Prior to undergoing the full heat tolerance assessment, patients are weighed nude, they self insert a rectal thermistor to a depth of 10 cm, are instrumented with further skin thermistors for the measurement of skin temperature[51], electrocardiogram telemetry and heart rate monitors. They then dress in combat trousers, T-shirt and combat jacket. They reenter the environmental chamber and are asked to march on the treadmill, carrying a weighted rucksack at a speed and gradient that is adjusted until they are working at 60% of their VO_2 max. Steady state VO_2 measurements are taken at regular intervals throughout the assessment. The duration of the assessment is a minimum of 60 min but may proceed to a maximum of 90 min. After 30 min of marching with the load, subjects remove the rucksack and jacket and then continue walking. Patients who are able to rapidly attenuate the rate of rise of their deep body temperatures and demonstrate the ability to achieve plateau in their core temperature are considered to be able to demonstrate physiological thermoregulation and the attainment of thermal equilibrium. Those who cannot do so, or those whose deep body temperatures rise rapidly towards the safety threshold of 40 °C are considered heat intolerant. Other measures such as sweat rate, which can be shown for body surface area, VO_2 consumption, heart rate, rating of perceived exertion are made and occasionally blood sampling for biochemical analysis is considered if clinically indicated.

Patients who have an uneventful heat tolerance assessment are passed as fit to return to fitness and exercise training and are eventually returned to full military activities. Those who are thermally intolerant of exercise in the proscribed conditions remain in a protected medical category. They are retested at intervals of no less than 6 weeks. Persistent heat intolerance under these conditions, especially when this is combined with evidence of exertional rhabdomyolysis or elevations of muscle markers after the assessment and the absence of other medical conditions may be referred to The Malignant Hyperthermia Investigation Unit at the University of Leeds, United Kingdom.

Appendix 2. Primers for Sanger Sequencing

RYR1 Exon 91

Fragment 1

F5′ GCT GAC GGC GCC CTA TCC TGT

R5′ GCG CCG CCG CAG GCT GCG GTA A

Fragment 2

F5′ GCC GGG CCC TGC GAG GCC TCA

R5′ GGT GGG GTC GGG CAT GCC TGC C

Fragment 3

F5′ GCT CTG GGC AGC AGT GAC

R5′ ATC CCC CAT CTT TCC AAA AC

RYR1 Exon 102

F5′: AAT GTC GAA TGA ATG CGT GA

R5′: CTG GGC CTG CAT TCT TAG C

Appendix 3. Interpretation of the Results of a Malignant Hyperthermia Sample in which Five Missense Variants in RYR1 Were Found

In one MH sample, five missense variants in the gene coding for RYR1 were found (p.1571I>V, p.2060G>C, p.3366R>H, p.3933Y>C, p.4010E>K). Histopathology showed nonspecific myopathic changes in the skeletal muscle of our patient, but serum creatine kinase concentration was normal. The variants p.1571I>V and p.3933Y>C in *cis* were first described in one family with four missense changes by Tammaro *et al*.[26], while we found p.1571I>V in one United Kingdom family as a single variant (unpublished sequence data, 2013, Hopkins PM, Leeds, United Kingdom). The Italian family members carrying these two variants were MH negative but the patient who inherited an additional variant from her father (p.3903R>Q) was diagnosed by IVCT as MH susceptible. The MH susceptible phenotype seemed to segregate with the p.3903R>Q variant but there were discordant members in this family who were MH negative while carrying the p.3903R>Q variant,

which may suggest that more than one variant is necessary to develop MH susceptibility[26]. Duarte *et al*.[45] also reported p.3366R>H and p.3933Y>C inherited in *cis* in a patient with a family history of serious adverse reactions to anesthetics. These variants were considered pathogenic as they were not detected in 150 control samples and involve with phylogenetically conserved residues. Since the *RYR1* variant p.2060G>C is polymorphic[23,14] and the novel variant p.4010E>K shows a very low PolyPhen-2 score of 0.07, it could be suggested that a compound effect of the three other variants may contribute to the susceptibility of our patient. However a limited family study does not support this suggestion since p.4010E>K was the only variant clearly segregating with disease in this particular family. It may well be that variants in other genes contribute to, or are wholly responsible for, MH susceptibility in these cases.

FINAL BOX SUMMARY

What we already know about this topic

- Variants in the genes for ryanodine receptor 1 and the Cav1.1 calcium channel account for most cases of malignant hyperthermia susceptibility
- Rapid targeted DNA sequencing was used to identify variants in the coding sequence of these two genes in a cohort of patients with malignant hyperthermia susceptibility or exertional heat illness

What this article tells us that is new

- Variants in the ryanodine receptor gene were identified in 13 of 29 malignant hyperthermia patients, with one variant in Cav1.1
- Targeted DNA sequencing is a potentially useful diagnostic approach to identifying genetic variants associated with malignant hyperthermia and exertional heat illness

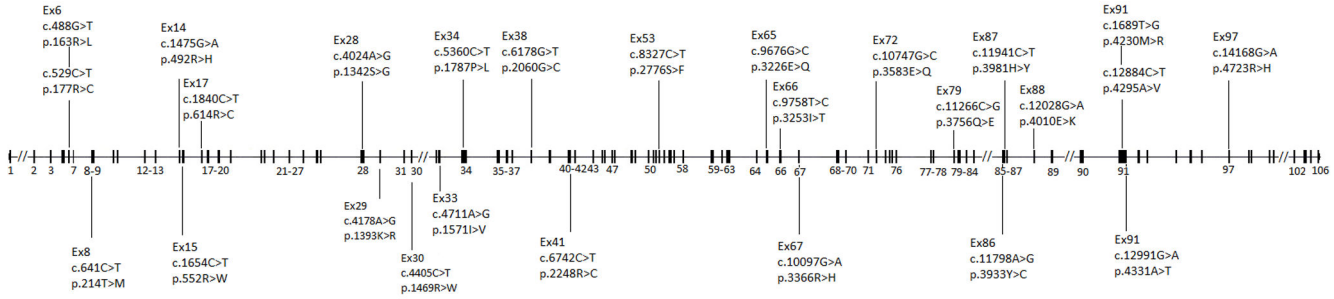

Fig. 1.

Map of the *RYR1* gene indicating the position of variants found in this study.

Table 1

Primer Sequences for 23 Amplicons Covering All *RYR1* Exons with Splice Site Intronic Fragments

	Genomic start and end of amplicon	Exons covered	Length of Amplicon (bp)	Primer sequence
Fr1	11192355	Ex 1	1285bp	5′-GAAAGCGCAGGTACCTCCTA-3′
	11193639			5′-CTCCCATATTTACGACCCCA-3′
Fr2	11198817	Ex 2 – 7	4781bp	5′-AAAAAGCAATTTAACTTGAGCAAGT-3′
	11203597			5′-GGGCAACATTAAGGGTCTGTT-3′
Fr3	11205200	Ex 8 – 11	3279bp	5′-CAACTGCAAAACCCCAAACT-3′
	11208478			5′-GGAACAGGTCGCTACAGCTC-3′
Fr4	11209942	Ex 12 – 16	5606bp	5′-CCAGTGCCTAGAACAGAGCC-3′
	11215547			5′-GCAACAGGAACTTGTAGGGC-3′
Fr5	11216101	Ex 17 – 20	4181bp	5′-TCCTTCCTTACATTGCTGATACC-3′
	11220281			5′-ACTGCGATTCTGCTCTGACAG-3′
Fr6	11222082	Ex 21 – 27	6469pb	5′-TTCTGAGAATGCTAAGCCCTC-3′
	11228550			5′-GCTGTGGTTTGCCAAAGTTAC-3′
Fr7	11231956	Ex 28 – 31	5677bp	5′-GGAGGTTTTGGAAGTTACCCC-3′
	11237632			5′-GGGCCTTAGTTTCATGGAAG-3′
Fr8	11241613	Ex 32 – 34	3576bp	5′-TGAATGAGTATGCTTGGGAGAG-3′
	11245158			5′-TTGGATGCATAAATAGAGGAGGAT-3′
Fr9	11247847	Ex 35 – 38	3762bp	5′-GACCTGGGTGGATCTTAAGGAG-3′
	11251608			5′-CTATCTACCCCTCCCTTGCATCT-3′
Fr10	11252848	Ex 39 – 42	3246bp	5′-AGATTCTTGGGCGAATAGAAAA-3′
	11256093			5′-GGAAGTTAGAGTGCGGATCC-3′
Fr11	11257937	Ex 43 – 49	4036bp	5′-GTCTCTGACTGAGCCCCTTCT-3′
	11261972			5′-GAGATTTCTACGGGGACGCT-3′
Fr12	11262753	Ex 50 – 58	4004bp	5′-GCATCCATATGCCCATTTACTC-3′
	11266756			5′-TAGGTGAGTCTGGTCTGCAGAA-3′
Fr13	11269208	Ex 59 – 63	2265bp	5′-GGTTTATCTCAAAGCCAACACA-3′
	11271472			5′-ACTGACTGCATGCTTTGCTTA-3′
Fr14	11273519	Ex 64 – 67	4937bp	5′-GAGGAAGTACCCCTCACTTTCA-3′
	11278455			5′-GAAACCAGGAGGAAGAGTCAGA-3′
Fr15	11281751	Ex 68 – 76	6296bp	5′-CAGATGACCCTAGAAACCCCAT-3′
	11288046			5′-ATTTAAAGATCCGTCTGTCTTGGG-3′
Fr16	11291290	Ex 77 – 84	5608bp	5′-TTATAAGATGGGGGTCCTCTCC-3′
	11296897			5′-GCGGAAACTAATTCAGTCAACC-3′
Fr17	11302085	Ex 85 – 89	5703bp	5′-TGCTTTCTGGCATACAATAGGA-3′
	11307787			5′-CGGTTCTCATCTGTGTTAATGC-3′
Fr18	11318013	Ex 90	3492bp	5′-GCAAATCACAAGAGCAAAGTCTA-3′
	11321504			5′-CAAGGGTTTGTGTACTGGCTC-3′

	Genomic start and end of amplicon	Exons covered	Length of Amplicon (bp)	Primer sequence
Fr19	11322259	Ex 91	3282bp	5′-ATTACAGCCATGTGCAACCA-3′
	11325540			5′-CAAGTGCAGCTCTGCTTGTC-3′
Fr20	11325725	Ex 92 – 93	1334bp	5′-CGCTTAGGGTGAGGACTCAG-3′
	11327058			5′-AGGATGGAGGTGGAAGTGTG-3′
Fr21	11329396	Ex 94 – 96	2821bp	5′-GATGGGATGAATTCTCCAGG-3′
	11332216			5′-AAACCCAGGTCCCCTCCT-3′
Fr22	11334714	Ex 97 – 101	5034bp	5′-GACAGCTCTGATCCCTCTGG-3′
	11339747			5′-ATGCATCAGCTTGCCAAACT-3′
Fr23	11343628	Ex 102 – 106	3010bp	5′-GACCATTTCTGGCTGTTGGT-3′
	11346637			5′-CTGCTCCCAGCAACCTCTAC-3′

bp = base pairs.

Table 2

Primer Sequences for Nine Amplicons Covering All *CACNA1S* Exons with Splice Site Intronic Fragments

	Genomic start and end of amplicon	Exons covered	Length of Amplicon (bp)	Primer sequence
Fr1	201081807	Ex 1–2		5′-ATCCCAGGCCTGTGTGTAAG-3′
	201078421		3406bp	5′-CAGAGAGACCAAGCTGGAGG-3′
Fr2	201063193	Ex 3–5		5′-TCAGAGAGCACAGAGGCATG-3′
	201060615		2598bp	5′-AGTAGAGCTGCCCTTCCTCC-3′
Fr3	201058618	Ex 6–10		5′-GATGTTCCCTTTGCCTTCTG-3′
	201052161		6477bp	5′-TCCAGCACTCATGGTCCATA-3′
Fr4	201047287	Ex 11–16		5′-GGAGTCAGGAGAAGGGAAGG-3′
	201041788		5519bp	5′-ATGGAGGGGTACAGGTAGGG-3′
Fr5	201039853	Ex 17–22		5′-CCCCCAGCCTATGGATATTT-3′
	201034869		5004bp	5′-TGGAAATCTGTGCATTCGAA-3′
Fr6	201031968	Ex 23–28		5′-ACTCATGTCCATCCCCTCAG-3′
	201027075		4913bp	5′-AGCCTTGCCCTCTCTGTTCT-3′
Fr7	201023879	Ex 29–32		5′-AGCTTCAGTCCTCACTCCCA-3′
	201021442		2457bp	5′-GGAGGAAAATGCAGCACTTC-3′
Fr8	201020682	Ex 33–38		5′-TTGTGACTCAGCCAGAGTGG-3′
	201015627		5075bp	5′-CCCATACCCAGTGGAACATT-3′
Fr9	201013956	Ex 39–44		5′-TAAGCCATACGAAGCGGTTC-3′
	201008352		5604bp	5′-CCAATGCAACATGGTCGTAG-3′

bp = base pairs.

Table 3

Average Read Depth for Each Exon of *RYR1* and *CACNA1S*

RYR1						*CACNA1S*	
Exon	**Average read depth**	**Exon**	**Average read depth**	**Exon**	**Average read depth**	**Exon**	**Average read depth**
1	358	48	218	95	370	1	360
2	346	49	437	96	842	2	465
3	319	50	394	97	599	3	454
4	393	51	431	98	358	4	371
5	405	52	369	99	327	5	468
6	348	53	453	100	446	6	402
7	420	54	465	101	360	7	433
8	328	55	418	102	1	8	418
9	429	56	440	103	368	9	326
10	354	57	344	104	418	10	417
11	372	58	902	105	416	11	349
12	487	59	199	106	313	12	471
13	425	60	417			13	402
14	495	61	544			14	440
15	524	62	375			15	409
16	436	63	459			16	700
17	403	64	357			17	381
18	338	65	327			18	427
19	374	66	317			19	435
20	361	67	356			20	399
21	254	68	251			21	326
22	296	69	476			22	964
23	341	70	487			23	435
24	316	71	406			24	416
25	344	72	459			25	475
26	353	73	378			26	428

RYR1						*CACNA1S*	
Exon	**Average read depth**	**Exon**	**Average read depth**	**Exon**	**Average read depth**	**Exon**	**Average read depth**
27	353	74	472			27	429
28	298	75	437			28	468
29	395	76	534			29	450
30	318	77	547			30	492
31	334	78	291			31	544
32	434	79	320			32	371
33	326	80	404			33	408
34	353	81	394			34	415
35	378	82	199			35	379
36	371	83	305			36	386
37	447	84	278			37	410
38	563	85	274			38	410
39	374	86	281			39	414
40	397	87	301			40	409
41	364	88	327			41	373
42	358	89	335			42	416
43	675	90	405			43	364
44	253	91	0			44	390
45	376	92	1128				
46	334	93	423				
47	351	94	360				

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Table 4

Variants Detected in *RYR1* and *CACNA1S* in 29 Malignant Hyperthermia Samples

Sample ID	*RYR1*	*CACNA1S*
MH1	c.1840C>T; p.614R>C	c.1373T>A; p.458L>H
MH2	c.641C>T ; p.214T>M	c.1373T>A; p.458L>H
MH3	c.9676G>C; p.3226E>Q	c.1373T>A; p.458L>H-
MH4	c.14168G>A; p.4723R>H	c.1373T>A; p.458L>H
MH5	-	-
MH6	-	c.1373T>A; p.458L>H
MH7	c.488G>T; p.163R>L	c.4615C>T; p.1539R>C
MH8	-	c.1373T>A; p.458L>H c.3026C>A; p.1009T>K-
MH9	-	-
MH10	-	c.206C>G; p.69A>G c.4615C>T; p.1539R>C
MH11	-	c.4615C>T; p.1539R>C
MH12	c.1654C>T; p.552R>W c.11266C>G; p.3756Q>E	c.4615C>T; p.1539R>C
MH13	-	c.1373T>A; p.458L>H-: c.1817G>A; p.606S>N-
MH14	c.12689T>G; p.4230M>R	c.206C>G; p.69A>G c.1373T>A; p.458L>H
MH15	-	c.206C>G; p.69A>G: c.1373T>A; p.458L>H
MH16	c.6178G>T; p.2060G>C c.8327C>T; p.2776S>F	c.1817G>A; p.606S>N
MH17	-	c.1373T>A; p.458L>H
MH18	c.529C>T; p.177R>C	c.206C>G; p.69A>G c.1373T>A; p.458L>H
MH19	c.4711A>G; p.1571I>V c.6178G>T; p.2060G>C c.10097G>A; p.3366R>H c.11798A>G; p.3933Y>C c.113964G>A; p.4010E>K	-
MH20	-	c.1373T>A; p.458L>H
MH21	c.6742C>T; p.2248R>C	-
MH22	c.6178G>T; p.2060G>C	c.206C>G; p.69A>G c.1373T>A; p.458L>H
MH23	-	c.1373T>A, p.458L>H
MH24	c.6178G>T, p.2060G>C	c.5399T>C, p.1800S>L
MH25	c.4178A>G, p.1393K>R	c.206C>G, p.69A>G c.1373T>A, p.458L>H
MH26	-	c.1373T>A, p.458L>H
MH27	c.6178G>T, p.2060G>C c.11958C>G, p.3986D>E	-
MH28	-	c.1373T>A, p.458L>H

Sample ID	*RYR1*	*CACNA1S*
MH29	c.4405C>T, p.1469R>W c.6178G>T, p.2060G>C	c.1373T>A, p.458L>H

Table 5

Variants Detected in *RYR1* and *CACNA1S* in 28 Exertional Heat Illness Samples

Sample ID	IVCT Result	*RYR1*	*CACNA1S*
EHI1	MHN	c.12844C>G; p.4282L>V	-
EHI2	MHN	c.5360C>T; p.1787P>L c.6178G>T; p.2060G>C c.12884C>T; p.4295A>V	c.1373T>A; p.458L>H
EHI3	MHN	-	c.206C>G; p.69A>G c.1373T>A; p.458L>H
EHI4	MHN	c.11266C>G; p.3756Q>E	c.1373T>A; p.458L>H
EHI5	MHN	-	c.1373T>A; p.458L>H
EHI6	MHN	c.6178G>C; p.2060G>C; c.12991G>A; p.4331A>T	-
EHI7	MHN	-	c.1373T>A; p.458L>H
EHI8	MHN	c.6178G>T; p.2060G>C; c.10747G>C; p.3583E>Q	c.1373T>A; p.458L>H
EHI9	MHN	-	c.5515C>T; p.1839P>S
EHI10	MHN	-	-
EHI11	MHN	c.5360C>T; p.1787P>L c.6178G>T; p.2060G>C	c.1493G>A; p.498R>H c.4615C>T; p.1539R>C
EHI12	MHN	-	c.1373T>A; p.458L>H
EHI13	MHN	-	-
EHI14	MHN	c.6178G>T, p.2060G>C	c.4615C>T, p.1539R>C
EHI15	MHN	c.6178G>T, p.2060G>C	-
EHI16	MHN	c.6178G>T, p.2060G>C	c.4615C>T, p.1539R>C
EHI17	MHS	-	c.1373T>A; p.458L>H
EHI18	MHS	-	c.1817G>A; p.606S>N
EHI19	MHS	c.4024A>G; p.1342S>G c.11941C>T; p.3981H>Y	c.1373T>A; p.458L>H c.2047C>T; p.683R>C
EHI20	MHS	c.6178G>T; p.2060G>C c.11266C>G; p.3756Q>E	c.1373T>A; p.458L>H
EHI21	MHS	-	-
EHI22	MHS	c.4024A>G; p.1342S>G	-
EHI23	MHS	c.9758T>C; p.3253I>T	c.1817G>A; p.606S>N
EHI24	MHS	-	c.4615C>T; p.1539R>C
EHI25	MHS	-	-
EHI26	MHS	c.1475G>A; p.492R>H	c.4615C>T; p.1539R>C
EHI27	MHS	-	-
EHI28	MHS	-	c.206C>G; p.69A>G c.1373T>A; p.458L>H

IVCT = *in vitro* contracture test; MHN = not susceptible to malignant hyperthermia; MHS = susceptible to malignant hyperthermia.

Table 6

RYR1 Variant Characteristics

Exon	Nucleotide change	Amino acid change	Sample phenotype (this study)	Reported disease association or polymorphism	Variant ID	MAF (from EVS)
6	c.488G>T	p.163R>L	1 MHS	MH diagnostic	rs193922753	-
6	c.529C>T	p.177R>C	1 MHS	MH uncharacterized	rs193922757	-
8	c.641C>T	p.214T>M	1 MHS	MH uncharacterized	-	-
14	c.1475G>A	p.492R>H	1 EHI (MHS)	-	-	-
15	c.1654C>T	p.552R>W	1 MHS	MH diagnostic	rs193922770	-
17	c.1840C>T	p.614R>C	1 MHS	MH/CCD diagnostic	rs118192172	0.000077
28	c.4024A>G	p.1342S>G	2 EHI (MHS)	MH uncharacterized	rs34694816	0.04
29	c.4178A>G	p.1393K>R	1 MHS	MH uncharacterised	rs137933390	0.004
30	c.4405C>T	p.1469R>W	1MHS	CNM	rs200546266	0.000077
33	c.4711A>G	p.1571I>V	1 MHS	MH uncharacterized	rs146429605	0.000929
34	c.5360C>T	p.1787P>L	2 EHI (MHN)	-	rs34934920	0.00538
38	c.6178G>T	p.2060G>C	6 MHS 1 EHI(MHS) 7 EHI (MHN)	Polymorphism	rs35364374	0.05
41	c.6742C>T	p.2248R>**C**	1 MHS	-	-	c.6743G>A 0.000077
53	c.8327C>T	p.2776S>F	1 MHS	-	rs147707463	0.000923
65	c.9676G>C	p.3226E>Q	1 MHS	-	-	-
66	c.9758T>C	p.3253I>T	1 EHI (MHS)	-	Rs375626634	0.000077
67	c.10097G>A	p.3366R>H	1 MHS	MH/myopathy	rs137932199	0.00846
72	c.10747G>C	p.3583E>Q	1 EHI (MHS)	Polymorphism	rs55876273	0.03
79	c.11266C>G	p.3756Q>E	1 MHS 1 EHI (MHS) 1 EHI (MHN)	Polymorphism	rs4802584	0.018
86	c.11798A>G	p.3933Y>C	1 MHS	MH/myopathy	rs147136339	0.000846
87	c.11941C>T	p.3981H>Y	1 EHI (MHS)	CNM	rs148772854	0.0039
87	c.11958C>G	p.3986D>E	1 MHS	MH uncharacterized	rs193922842	-
88	c.12028G>A	p.4010E>K	1 MHS	Unknown	-	-
91	c.12689T>G	p.4230M>R	1 MHS	Unknown	-	-

Exon	Nucleotide change	Amino acid change	Sample phenotype (this study)	Reported disease association or polymorphism	Variant ID	MAF (from EVS)
91	c.12844C>G	p.4282L>V	1 EHI (MHN)	Unknown	-	-
91	c.12884C>T	p.4295A>V	1 EHI (MHN)	MH/minicores	rs193922855	-
91	c.12991G>A	p.4331A>T	1 EHI (MHN)	-	-	-
97	c.14168G>A	p.4723R>H	1 MHS	-	rs200766617	c.14167C>T 0.000077

CCD = central core disease; CNM = centronuclear myopathy; EHI = exertional heat illness; EVS = exome variant server; MAF = minor allele frequency; MH = malignant hyperthermia; MHN = not susceptible to malignant hyperthermia; MHS = susceptible to malignant hyperthermia.

Table 7

CACNA1S Variant Characteristics

Exon	Nucleotide change	Amino acid change	Sample phenotype (this study)	Reported disease association	Variant ID	MAF (from EVS)
5	c.206C>G	p.69A>G	6 MHS 1 EHI (MHS) 1 EHI (MHN)	Polymorphism	rs12406479	0.0392
10	c.1373T>A	p.458L>H	20 MHS 4 EHI (MHS) 7 EHI (MHN)	Polymorphism	rs12742169	0.274
11	c.1493G>A	p.498R>H	1 EHI (MHN)	Unknown	rs150590855	0.00084
12	c.1817G>A	p.606S>N	2 MHS 2 EHI (MHS)	Unknown	rs142356235	0.00868
14	c.2047C>T	p.683R>C	1 EHI (MHS)	Unknown	rs35708442	0.00976
24	c.3026C>A	p.1009T>K	1 MHS	Unknown	-	c.3026C>T 0.000154
38	c.4615C>T	p.1539R>C	4 MHS 2 EHI (MHS) 3 EHI (MHN)	Polymorphism	rs3850625	0.0892
44	c.5399T>C	p.1800L>S	3 MHS 4 EHI (MHS) 2 EHI (MHN)	Polymorphism	rs12139527	0.269
44	c.5515C>T	p.1839P>S	1 EHI (MHN)	Unknown	rs149547196	0.00253

EHI= exertional heat illness; EVS = exome variant server; MAF = minor allele frequency; MHN = not susceptible to malignant hyperthermia; MHS = susceptible to malignant hyperthermia.