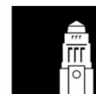

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Genetic epidemiology of malignant hyperthermia in the United Kingdom

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Abbreviated title: Genetics of malignant hyperthermia

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Abstract

Background:

Gaps in our understanding of genetic susceptibility to malignant hyperthermia limit the application and interpretation of genetic diagnosis of the condition. Our aim was to reduce the knowledge gaps by defining the prevalence and role of variants in the three genes implicated in malignant hyperthermia susceptibility in the largest comprehensively phenotyped malignant hyperthermia cohort worldwide.

Methods:

We initially included one individual from each positive family tested in the UK MH Unit since 1971 to detect variants in the *RYR1*, *CACNA1S*, or *STAC3*. Screening for genetic variants has been ongoing since 1991 and has involved a range of techniques, most recently next generation sequencing. We assessed the pathogenicity of variants using standard guidelines, including with family segregation studies. The prevalence of recurrent variants of unknown significance was compared to the prevalence reported in a large database of sequence variants in low risk populations.

Results:

We have confirmed malignant hyperthermia susceptibility in 795 independent families, for 722 of which we have a DNA sample. Potentially pathogenic variants were found in 555 families, with 25 *RYR1* and one *CACNA1S* previously unclassified recurrent variants significantly over-represented ($P < 1 \times 10^{-7}$) in our cohort compared with the ExAC database. There was genotype-phenotype discordance in 86 of 328 families suitable for segregation analysis. We estimate non-*RYR1/CACNA1S/STAC3* susceptibility occurs in 14-23% of malignant hyperthermia families.

Conclusions:

Our data provide the best estimates to date of the role of variants in *RYR1*, *CACNA1S* and *STAC3* in susceptibility to malignant hyperthermia in a predominantly white European population.

Keywords

Malignant hyperthermia; genetics, diagnosis: *RYR1:CACNA1S: STAC3*

Malignant hyperthermia (MH) is a potentially fatal reaction that occurs in genetically susceptible individuals exposed to inhalation anaesthetics or succinylcholine[1]. There has been considerable progress in elucidating the genetic basis of MH susceptibility over the past 30 years[2]. The *RYR1* gene that encodes the skeletal muscle sarcoplasmic reticulum calcium release channel was the first gene linked to MH susceptibility[3, 4] and is involved in 34-86% of cases reported[5-12]. *RYR1* is a large gene and many variants have been associated with MH susceptibility although only a minority of these have been demonstrated to be pathogenic[2]. The second gene with variants pathogenic for MH susceptibility is *CACNA1S*[13, 14], which encodes the main subunit of the skeletal muscle T-tubule voltage sensor. *STAC3* encodes a protein involved in trafficking the voltage sensor into the correct T-tubular location and subsequently a direct role in excitation-contraction coupling[15, 16]: homozygous inheritance of the *STAC3* variant p.Trp284Ser leads to a congenital myopathy associated with MH susceptibility[17]. Such findings have enabled limited application of genetic diagnoses[18] but further expansion has been constrained by the difficulty in establishing a pathogenic role for rare missense variants[19] and evidence that a simple genetic model may not apply in at least a significant minority of cases[20]. Our aim in this paper was to define the prevalence of individual variants in *RYR1*, *CACNA1S* and *STAC3* in the largest cohort of phenotypically characterized MH susceptible individuals to date and to assess their likely pathogenicity. We also present data on the proportion of families where there is evidence for more than one genetic variant contributing to MH susceptibility and the proportion where variants in *RYR1*, *CACNA1S* and *STAC3* have been excluded.

Methods

Patients

We included index cases or, where the index case could not be tested, their nearest relative from families where MH susceptibility had been confirmed following a clinical reaction suggestive of MH. We excluded cases referred where there had been no adverse anaesthetic event, such as those patients referred with a history of exertional heat illness, exertional or recurrent rhabdomyolysis, or a congenital myopathy. MH susceptibility was confirmed by *in vitro* contracture testing (IVCT) or finding of a functionally characterized genetic variant pathogenic for MH susceptibility. The criteria used for diagnosis of MH susceptibility were those of the European MH Group applicable at the time of diagnosis[21, 22, 18]. Patients tested prior to 1984 were considered susceptible if their muscle biopsy samples developed a contracture of 0.2 g or more upon exposure to 2% halothane. DNA samples were collected, stored and processed according to protocols approved by Leeds (East) Research Ethics Committee or its predecessors: Leeds Teaching Hospitals NHS Trust Clinical Research (Ethics) Committee (East) and Leeds Health Authority / St James's and Seacroft University Hospitals Clinical Research (Ethics) Committee. All patients contributing DNA samples gave written informed consent.

Detection of Genetic variants

This was as described in Merritt and colleagues[19]. In brief, we began screening MH susceptible families for *RYR1* variants following publication of the first *RYR1* variant implicated in MH susceptibility [23]. As further *RYR1* variants were reported we undertook a systematic search for all published variants principally using amplification refractory mutation system or restriction digest assays. As technology developed we used Sanger sequencing of mutation "hot-spots" and then the whole coding region of *RYR1* and

CACNA1S [5]. Most recently, next generation sequencing (NGS) technology with targeted exon capture has been used[24] to sequence the coding sequences of *RYR1, CACNA1S and STAC3*. We defined a potentially pathogenic variant as one with a minor allele frequency (MAF) < 0.001 in each of the ethnic cohorts of the ExAC browser database (http://exac.broadinstitute.org). This is the highest prevalence value that we consider compatible for a heterozygous single gene disorder with the clinical incidence and penetrance of MH. We also included STAC3 variants with MAF < 0.01 inherited in the homozygous state.

Family studies

When potentially pathogenic variants are identified in a family, a segregation study of the variant is undertaken for those individuals who have been phenotyped by the IVCT. Again, depending on when the study was done and the nature of the variant this was either using an amplification refractory mutation system test, a restriction digest assay or direct sequencing. When we encountered a case of discordance between familial variant and the IVCT, we reviewed the IVCT records (phenotype) and calculated the probability that the IVCT responses represented an abnormal response [25]. We also verified the genotype using Sanger sequencing where a DNA sample was available and, again when feasible, used deep resequencing of *RYR1* and *CACNA1S* to look for alternative disease-associated variants in cases of affected non-carriers.

Variant prevalence in MH families and the general population

We defined the prevalence in the UK MH population as the number of independent families carrying a variant divided by the number of independent MH families in whom genetic analyses has been undertaken. For an estimate of the population prevalence of each variant

we used data presented in the ExAC browser (http://exac.broadinstitute.org) for the European non-Finnish cohort, unless our cases were from a non-white ethnic background in which case the appropriate ExAC population was used.

In silico assessment of pathogenicity of variants

For each variant we obtained the C-score from http://cadd.gs.washington.edu (last accessed 18.03.2018). The C-score is derived from Combined Annotation–Dependent Depletion (CADD) and scores of >15 include the 5% predicted most deleterious substitutions in the human genome[26]. Because of the uncertainty of the validity of using *in silico* tools for prediction of pathogenicity of *RYR1* variants[27] we simply report the values rather than using them to infer likelihood of pathogenicity.

Statistical analyses

We compared the prevalence estimates for potentially pathogenic variants in MH families versus the ExAC cohort using a chi-square test (MedCalc® statistical software https://www.medcalc.org/calc/comparison_of_proportions.php, last accessed 18.03.2018). We then used an on-line package (http://www.danielsoper.com/statcalc/calculator.aspx?id=11, last accessed 18.03.2018) that enables calculation of exact *P* values up to chi-squared values of 34 ($P = 1 \times 10^{-8}$). As we had selected our genes of interest in a non-random way from ~ 20,000 genes in the genome and because we made comparisons for multiple variants we used a *P* value $< 1 \times 10^{-7}$ to infer statistical significance.

Results

A total of 770 independent families with MH confirmed by a positive IVCT following a clinical episode consistent with MH susceptibility were identified. DNA samples were

available from at least one member of 697 families. Pathogenic *RYR1* variants have been identified by NGS in the probands of a further 25 families since the introduction of NGS as a primary diagnostic test[18].

Variants in the *RYR1* gene

One hundred and forty-seven different potentially pathogenic variants were found in at least one independent MH family and these are listed in supplementary table 1. Of these, 31 have been previously sufficiently characterized to be used in prospective diagnosis (www.emhg.org). A further 29 of the 147 potentially pathogenic variants were found in more than one family. These are presented in Table 1, along with the population prevalence in the ExAC browser. The difference in prevalence between the UK MH cohort and the relevant ExAC browser cohort was statistically significant for 25 of these 29 variants (Table 1). All of these variants were found in the heterozygous state except p.Arg3772Gln which we have previously reported in 3 of the 6 families listed in Table 1 [28]. In total, 546 of 722 families carry at least one pathogenic, likely pathogenic or potentially pathogenic *RYR1* variant.

Variants in the *CACNA1S* gene

Two *CACNA1S* variants, p.Arg174Trp. and p.Arg1086His, have been functionally characterized [29, 30] and are recognized as pathogenic variants by the European MH Group (www.emhg.org). We have previously reported p.Arg174Trp [14] and p.Thr1009Lys [31, 24] in one and two families respectively. We now report an additional family with p.Arg174Trp. Of the total of 11 potentially pathogenic *CACNA1S* variants (Table 2) there were only two found in more than one family, p.Thr1009Lys and p.Arg1086Ser. For p.Thr1009Lys this prevalence compares with 3 of 66,558 alleles of the ExAC European non-Finnish cohort

indicating that the variant is over-represented in MH families (chi-sq 35.2, $P < 1 \times 10^{-8}$) and meets our criteria for classifying it as likely pathogenic.

The p.Arg1086Ser variant has previously been reported in association with MH [32] and involves substitution of the same amino acid as the functionally characterized p.Arg1086His variant. The p.Arg1086Ser variant was not found in the European non-Finnish ExAC cohort but our two families were both of South Asian origin. Comparison of the prevalence of this variant in our cohort with the ExAC South Asian cohort (3 out of 16,512 alleles) did not reach our criteria for classifying this variant as likely pathogenic (chi-sq 8.315, $P = 0.0039$). In fact, several of our potentially pathogenic *CACNA1S* variants were found in patients with a non-white ethnic background. Both patients with the p.Arg174Trp variant were black/African/Caribbean, although this variant was not found in any of 10,376 alleles in the ExAC African cohort. In addition to p.Arg1086Ser, three further variants were found only in patients of South Asian origin. Two of these, p.Pro758Leu and p.Leu885Pro were found in the same patient, while p.His992Asp was also present in a single patient. The prevalence for each of these variants in the ExAC South Asian cohort was < 1 in 1,000 and so these variants remain potentially pathogenic.

One of the *CACNA1S* variants meeting our criteria for being potentially pathogenic in MH, p.Arg900Ser, has previously been reported in association with hypokalaemic periodic paralysis [33]. This and another *CACNA1S* variant, p.Gly1210Arg, were found in a patient who we have previously reported [34] with a history of hypokalaemic periodic paralysis and MH, and the *RYR1* c.7025A>G, p.Asn2342Ser variant. Four other families with potentially pathogenic *CACNA1S* variants also had a potentially pathogenic *RYR1* variant. There were 7 families with pathogenic or potentially pathogenic *CACNA1S* variants only.

Variants in the *STAC3* gene

We found the previously reported p.Trp284Ser variant in one proband who was homozygous for this variant. This patient was originally from the Middle East. No novel potentially pathogenic variants were found in *STAC3*.

Families where variants in the coding regions of *RYR1*, *CACNA1S* and *STAC3* were not found

We found potentially pathogenic variants in 555 of 722 families. Of the remaining 167 families, *RYR1*, *CACNA1S* and *STAC3* were sequenced with NGS in 103 families, with the sequence of regions of low quality reads being confirmed by Sanger sequencing.

Segregation analyses

Segregation between genotype and IVCT phenotype was assessed in 328 families with an *RYR1* variant and 4 families with *CACNA1S* variants. The median (range) number of MH susceptible and MH normal members included per family was 2 (1 -16) and 3 (1 - 27) respectively. In families carrying a pathogenic *RYR1* variant there were 72 out of 280 families with at least one example of genotype-phenotype discordance (Table 3). In families carrying a likely pathogenic *RYR1* variant there were 14 out of 48 families with at least one example of genotype-phenotype discordance (Table 4).

Families with more than one variant

We have identified 27 of 293 families where sequencing of the entire coding regions of the three genes has been done in which more than one potentially pathogenic variant in *RYR1*, *CACNA1S* or *STAC3* has been identified. The number of families with 2, 3, or 4 such variants was 21, 5, and 1 respectively.

Discussion

In this paper we provide the best estimate to date of the prevalence and distribution of genetic variants in MH susceptible families in a principally European Caucasian population. *RYR1* variants were found in 546 of 722 independent families corresponding to an estimate of 76% (95% CI 72-79%). We have confirmed the extent of allelic heterogeneity within *RYR1* and demonstrated that the majority of *RYR1* variants in our population are private to individual families. However, just 28 variants are implicated in > 50% of our MH families, with one variant, p.Gly2434Arg, found in almost 16% of MH families. Interestingly, in 434 families we reported in 2006 we identified 52 *RYR1* variants of which ~ 50% were private to individual families [5]. Other than our cohort, the largest evaluation of the prevalence of *RYR1* variants associated with MH susceptibility included 120 families from the United States [11] of which 62 (52%, 95% CI 43-61%) had *RYR1* variants. A total of 96 Australian patients have been included in two reports from the group of Gillies [8, 12] and 33 of these were found to carry *RYR1* variants (34%, 95% CI 25-44%). Estimates of the prevalence of *RYR1* variants in other populations included smaller numbers of patients: Japan, 33 out of 58 patients (57%, 95% CI 43-70%) [6]; Italy, 31 out of 43 patients (72%, 95% CI 56-85%) [7]; Canada, 31 out of 36 patients (86%, 95% CI 70-95%) [10]; Sweden, 7 out of 14 patients (50%, 95% CI 23-77%) [9]. *RYR1* prevalence estimates lower than ours are likely to be at least partially attributable to either incomplete screening of the *RYR1* gene or reliance

on Sanger sequencing which is not as sensitive as NGS for variant detection [31]. For the Canadian cohort, Kraeva and colleagues [10] selected MH susceptible patients with the clearest clinical episodes and strongest caffeine-halothane contracture test responses and this may explain their point estimate of 86% prevalence of *RYR1* variants, although their 95% CI fully encompasses our 95% CI. The use of different *in vitro* diagnostic test protocols between North America (caffeine-halothane contracture test), Japan (skinned fibre test) and Europe (IVCT) could also affect *RYR1* prevalance estimates. It is only after accounting for all of the technical issues that an assessment could be made of true population differences in *RYR1* variant prevalence in MH susceptible patients from different countries.

The EMHG published its first guideline for classification of high-risk genetic variants in MH susceptibility in 2001 [35] which was based on contemporary standards of molecular genetic diagnoses. Key to confirming pathogenicity of missense variants, the type of variant most frequently associated with MH susceptibility, was the demonstration of a functional effect of the variant consistent with the known pathophysiology of the condition. The technical difficulty and cost of the necessary experiments has limited the number of variants found in MH patients that have been functionally characterized [19].

Current generic guidelines [36] for the diagnostic classification of genetic variants incorporate 5 classes: benign, likely benign, variant of unknown significance, likely pathogenic and pathogenic: variants within the last two categories are usually considered suitable for prospective diagnostic testing. Within the current European MH Group guidelines [18], there are broadly two categories of functional tests: *ex vivo* experiments on cells cultured from MH susceptible patients and *in vitro* studies where the variant has

been genetically engineered into homologous or heterologous expression systems. There is debate about the use of *ex vivo* cells for genetic variant characterization because of the potential for genetic background effects [18, 19] and it perhaps would be appropriate to classify variants characterized in this way as likely pathogenic.

The American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guideline [36] includes an algorithm to determine the classification of individual variants and we have attempted to apply this to the variants we have found. Using this algorithm, recurrent variants that have been functionally characterized using genetic engineering and homologous or heterologous expression systems are classified as pathogenic but none of our other variants could be classified beyond a variant of unknown significance (potentially pathogenic) despite many being found in multiple MH families but rarely in the control population.

Through comparing the prevalence of recurrent *RYR1* variants in our population with that of a relevant low-risk (for MH susceptibility) population presented within the ExAC browser dataset, we have provided compelling statistical evidence that a further 25 *RYR1* variants are likely to be pathogenic. We suggest that these are suitable to be used in prospective DNA diagnosis of MH susceptibility within the framework for diagnostic testing recommended by the European MH Group [18]. This framework enables the presence of a likely pathogenic or pathogenic variant to be used to confirm high-risk status but requires the absence of a familial variant to be confirmed by IVCT in order for a sufficiently low-risk status to be assigned such that the patient may safely receive MH triggering anaesthetics. Addition of these 25 *RYR1* variants to the diagnostic panel would enable a further 97 UK MH families to benefit from prospective DNA diagnosis.

Pathogenic or potentially pathogenic *CACNA1S* variants were found in 12 (1.7%, 95% CI 0.9-2.9%) UK MH families but 5 of these families also carried a potentially pathogenic *RYR1* variant. Our previous review of *RYR1* variants [5] highlighted that the distribution of variants was spread widely across the gene, rather than in three "hot-spots" previously described. We now report a similar situation in *CACNA1S* with variants that are at least potentially pathogenic occurring between amino acid positions 174 and 1696 (Table 2). Furthermore, the variants affect a variety of functional sites within the $Ca_v1.1$ protein [37]. The p.Arg174 amino acid is one of the positively charged residues of the S4 segment of domain I: the S4 segments are thought to be the voltage sensors of the protein. Mutations of the arginine residues of the S4 segments of domains III (p.Arg900) [35, 38] and IV (p.Arg1239) [39, 40] cause hypokalaemic periodic paralysis while the p.Arg1242Gly variant (domain IV S4) is associated with normokalaemic periodic paralysis [41]. The p.Asn909Ser found in our cohort also affects the S4 segment of domain III.

The amino acid p.Arg1086 is located in the cytoplasmic loop between domains III and IV and this loop has been shown to influence RyR1 channel gating [42]. One of our new variants to be associated with MH susceptibility, p.Pro758Leu, is located in the domain I-II cytoplasmic loop in a region thought to be critical for excitation-contraction coupling. Three of our potentially pathogenic *CACNA1S* variants, p.Tyr617Ala, p.His992Asp and p.Thr1009Lys, may affect the $Ca_v1.1$ channel pore regions of domains II, III and III respectively. A potential mechanism for pathogenicity of our other *CACNA1S* variants is less clear.

Our single case of homozygous presentation of the p.Trp284Ser *STAC3* variant was in a patient from the Middle East. It is interesting that this variant is present in 0.12% of the African ExAC population, suggesting that it did not originate in the Native American population from which the congenital myopathy derived its name. There are no reported cases of MH associated with the presence of this variant in the heterozygous state and indeed the prevalence of the variant in the African population makes this unlikely.

Of 722 families, we have excluded *RYR1*, *CACNA1S* and *STAC3* variants in 103 families using NGS. No variants in these genes have been found in a further 64 families but the genetic analyses of these families have not been so extensive as to conclude that variants in *RYR1*, *CACNA1S* and *STAC3* are not present. We can therefore provide a range of estimates for non-*RYR1/CACNA1S/STAC3* MH susceptibility of between 14% (95% CI 11.5-17%) and 23% (95% CI 20-26%). As with other groups [43] we have used exome sequencing to search for variants in other genes [31] but the analytical approach to distinguish potentially pathogenic from benign variants is challenging [2].

We first reported discordance within a family between a functionally relevant *RYR1* variant and the IVCT phenotype 20 years ago [44]. Similar findings have been reported across European laboratories [20]. Since the introduction of predictive testing for familial variants, high risk status indicated by the presence of a familial variant has not required to be confirmed with a subsequent IVCT, which is not the case for low risk status in the absence of a familial variant. There has therefore been an inevitable bias in the type of discordance recorded over the past 15 years, with only a susceptible phenotype in the absence of a familial variant detected. The occurrence of discordance appears to be distributed equally among the various *RYR1* variants, with the number of discordant cases reflecting the

number of families harbouring the variant where segregation analyses have been done. The possible exception to this is *RYR1* p.Arg2435His where we found no cases of genotype-phenotype discordance in 10 families where segregation analyses had been conducted. It is interesting to note that this variant was found to be associated with one of the "strongest" IVCT phenotypes [45]. In that paper we proposed a threshold genetic model for MH susceptibility to explain genotype-phenotype discordance. If this is the case, the extent of genotype-phenotype discordance that we now present could suggest that very few *RYR1* variants are sufficiently penetrant to consistently cause MH susceptibility in the absence of other genetic risk factors. Such a situation would be consistent with the high combined prevalence of known pathogenic *RYR1* variants [2].

An alternative explanation for genotype-phenotype discordance is errors in either genotyping or phenotyping. The genotypes of all our discordant cases involving pathogenic variants have been confirmed under strict diagnostic laboratory quality control procedures. The IVCT responses of discordant cases have been evaluated using a predictive model [25] to minimize the likelihood of misdiagnosis. We also routinely send a sample of the muscle biopsy for histological and histochemical analyses to exclude muscle pathology as a cause of a false positive IVCT [46-48]. The number of false positive IVCT results in a cohort of 202 subjects at low risk for MH susceptibility was reported by the European MH Group to be 13 (6.43%) [49]. Out of 656 patients tested negative for a familial mutation, we found 79 (12.04%) to have a positive IVCT phenotype (Tables 3 and 4). The difference in these proportions is 5.61% (95% CI 0.78-9.39%, P = 0.024), which further argues against phenotyping error as an explanation for genotype-phenotype discordance. Our hypothesis that genotype-phenotype discordance is a result of the presence of more than one genetic risk factor for MH susceptibility is supported by our finding of more than one potentially

pathogenic variant in 9.2% (95% CI 6.2-13.1%) of the 293 families in which *RYR1*, *CACNA1S* and *STAC3* had been fully sequenced.

In conclusion, we have described the most comprehensive genetic analysis of MH susceptibility to date. All of the families included have a relevant anaesthetic history and the diagnosis has been confirmed by internationally accepted diagnostic tests. Our data confirm the importance of variants in *RYR1* and the high proportion of these that are private to single families. We propose that 25 recurrent *RYR1* variants can be used for prospective genetic diagnosis of high risk status for MH susceptibility. The prevalence of potentially pathogenic variants in *CACNA1S* is slightly higher than previous estimates and our data suggest that their role in non-white populations may be even more important. We present further evidence that not all cases of MH are explained by genetic variants in *RYR1*, *CACNA1S* or *STAC3* and that combinations of potentially pathogenic variants in these genes are present in a significant minority of MH families.

Contribution of authors

Conception and design of the study: PMH, M-AS
Conduct of experiments and data collection: DMM, CD, LG, SJH, KR, SS, RLR, JGB, PKG, PMH
Data analysis & interpretation: all authors
Drafting of manuscript: PMH
All authors reviewed drafts of the manuscript and approved the final version

Declaration of Interests

PMH is an Editorial Board Member of BJA. He is also Chair of the European Malignant Hyperthermia Group

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Table 1. Non-functionally characterized *RYR1* variants present in more than one MH family

Nucleotide Change	Amino acid change	No of families	ExAC MAF*	X^2	*P* value
c.479A>G	p.Glu160Gly	2	0/66620	92	$< 1x\ 10^{-7}$
c.529C>T	p.Arg177Cys	10	0/64006	443	$< 1x\ 10^{-7}$
c.641C>T	p.Thr214Met	4	11/66646	43.5	$< 1x\ 10^{-7}$
c.1202G>T	p.Arg401His	2	0/66660	92	$< 1x\ 10^{-7}$
c.1598G>A	p.Arg533His	2	3/66740	34.6	$< 1x\ 10^{-7}$
c.1615T>G	p.Phe539Val	2	0/66740	92	$< 1x\ 10^{-7}$
c.3166G>C	p.Asp1056His	2	0/7566	10.48	0.0012
c.4763C>T	p.Pro1588Leu	2	1/9516	7.51	0.0061
c.5024T>C	p. Leu1675Pro	3	0/65086	135	$< 1x\ 10^{-7}$
c.5183C>T	p. Ser1728Phe	8	0/65086	361	$< 1x\ 10^{-7}$
c.6612C>G	p.His2204Gln	5	0/66430	230	$< 1x\ 10^{-7}$
c.6961A>G	p.Ile 2321Val	3	41/66520	4.66	0.031
c.7084G>A	p.Glu2362Lys	2	0/62220	86	$< 1x\ 10^{-7}$
c.7089C>G	p.Cys 2363Trp	2	0/61940	86	$< 1x\ 10^{-7}$
c.7090T>G	p.Phe2364Val	2	0/61754	85	$< 1x\ 10^{-7}$
c.7291G>T	p. Asp2431Tyr	3	0/66508	138	$< 1x\ 10^{-7}$
c.7879G>A	p.Val2627Met	5	0/66484	230	$< 1x\ 10^{-7}$
c.8026C>T	p. Arg2676Trp	3	1/66588	102	$< 1x\ 10^{-7}$
c.9152G>A	p.Arg3051His	2	24/66740	3.9	0.048
c.10252A>G	p.Asn3418Asp	2	0/31266	43	$< 1x\ 10^{-7}$
c.11708G>A	p.Arg3903Gln	2	2/66740	44	$< 1x\ 10^{-7}$
c.11315G>A	p. Arg3772Gln	7 (2 HOM)	0/14896 (South Asian)	83	$< 1x\ 10^{-7}$
c.11958C>G	p.Asp3986Glu	6	0/66312	276	$< 1x\ 10^{-7}$
c.12149C>A	p.Ser4050Tyr	2	0/66732	92	$< 1x\ 10^{-7}$
c.12700G>T	p.Val4234Leu	5	0/15016	52	$< 1x\ 10^{-7}$

c.14210G>A	p.Arg4737Gln	7	1/66574	280	< 1x 10^{-7}
c.14471T>C	p.Leu4824Pro	3	0/66704	139	< 1x 10^{-7}
c.14678G>A	p.Arg4893Gln	3	0/66322	138	< 1x 10^{-7}
c.14918C>T	p.Pro4973Leu	3	3/66446	66	< 1x 10^{-7}

ExAC: ExAC browser (http://exac.broadinstitute.org). HOM: homozygous. MAF: minor allele frequency. *-MAF for the European non-Finnish cohort unless otherwise stated.

Table 2. Rare *CACNA1S* variants in the UK malignant hyperthermia cohort

Nucleotide change	Amino acid change	No. of families (ethnicity)	ExAC MAF	X^2	*P* value	C-score
c.520C>T	p.Arg174Trp#	2 (Black^)	1/66510 ENF; 0/10376 African*; 2/8630 East Asian; 1/16500 South Asian	8.3	0.004	34
c.1426A>C	p.Thr476Pro	1 (Arabic)	0/66738 ENF; 0/10406 African*	2.64	0.1	22.2
c.1849A>G	p.Thr617Ala	1 (White)	0/66738 ENF	22.5	2×10^{-6}	22.4
c.2273C>T	p.Pro758Leu	1 (South Asian)	1/66696 ENF; 0/16512 South Asian*	4.88	0.027	28.3
c.2654T>C	p.Leu885Pro	1 (South Asian)	0/65598 ENF: 0/16054 South Asian*	4.81	0.028	23.5
c.2700G>T	p.Arg900Ser	1 (White)	0/66652 ENF*	22.5	2×10^{-6}	31
c.2726A>G	p.Asn909Ser	1 (White)	2/66708 ENF*	14.36	0.0002	25.7
c.2974C>G	p.His992Asp	1 (South Asian)	0/66126 ENF: 6/16362 South Asian*	0.36	0.55	23.8
c.3026C>A	p.Thr1009Lys	2 (White)	3/66558 ENF*	35.2	$< 1 \times 10^{-7}$	34
c.3332C>A	p.Arg1086Ser	2 (South Asian)	0/66740 ENF: 3/16512 South Asian*	8.32	0.0039c	27
c.3628G>A	p.Gly1210Arg	1 (White)	56/61616 ENF*	0.063	0.8	27.1
c.5087C>T	p.Thr1696Met	1 (White)	2/65668 ENF*	14.28	0.0002	7.79

ExAC: ExAC browser (http://exac.broadinstitute.org). # functionally characterized variant. ^ Black/African/Caribbean ethnicity. MAF: minor allele frequency. *- MAF used for calculating chi-square. ENF: European non-Finnish cohort, MAF for other cohorts as stated

Table 3. Segregation analyses of *RYR1* variants reported as pathogenic by the European Malignant Hyperthermia Group (www.emhg.org)

Nucleotide change	Amino acid change	No. of UK families		No. of discordant UK families		
		total	segregation	G+/P-	G-/P+	Both
c.103T>C	p.Cys35Arg	0				
c.487C>T	p.Arg163Cys	21	14	1	2	
c.488G>T	p.Arg163Leu	2	1			
c.742G>A	p.Gly248Arg	5	4	1	1[a]	
c.742G>C	p.Gly248Arg	3	2			
c.1021G>A	p.Gly341Arg	31	27	1	6[b]	2
c.1201C>T	p.Arg401Cys	2				
c.1209C>G	p.Ile403Met	0				
c.1565A>C	p.Tyr522Ser	0				
c.1589G>A	p.Arg530His	1				
c.1654C>T	p.Arg552Trp	4	4		1	
c.1840C>T	p.Arg614Cys	14	7		1	
c.1841G>T	p.Arg614Leu	1				
c.6487C>T	p.Arg2163Cys	2	1	1		
c.6488G>A	p.Arg2163His	9	8	2	3	
c.6502G>A	p.Val2168Met	8	6		3[b]	
c.6617C>G	p.Thr2206Arg	1				
c.6617C>T	p.Thr2206Met	28	24	3	4	1
c.7007G>A	p.Arg2336His	9	7		1[b]	1[a]
c.7042GAG>del	p.Gln2348del	0				
c.7048G>A	p.Ala2350Thr	7	7		2	
c.7063C>T	p.Arg2355Trp	8	6		1	
c.7124G>C	p.Gly2375Ala	0				
c.7282G>A	p.Ala2428Thr	1				
c.7300G>A	p.Gly2434Arg	118	96	6	13[b c]	2
c.7304G>A	p.Arg2435His	11	10			

c.7354C>T	p.Arg2452Trp	2	2			
c.7360C>T	p.Arg2454Cys	0				
c.7361G>A	p.Arg2454His	14	11	1	2 [b]	
c.7372C>T	p.Arg2458Cys	0				
c.7373G>A	p.Arg2458His	15	12		2 [b]	
c.7522C>T	p.Arg2508Cys	1				
c.7523G>A	p.Arg2508His	4	2		1	
c.9310G>A	p.Glu3104Lys	5	3			
c.11969G>T	p.Gly3990Val	11	7		1	
c.14387A>G	p.Tyr4796Cys	0				
c.14477C>T	p.Thr4826Ile	10	10		3	
c.14497C>T	p.His4833Tyr	0				
c.14512C>G	p.Leu4838Val	1	1			
c.14545G>A	p.Val4849Ile	8	8		3 [b]	
c.14582G>A	p.Arg4861His	0				
c.14693T>C	p.Ile4898Thr	0				

G+/P-: +ve for genotype and -ve for phenotype
G-/P+:–ve for genotype and +ve for phenotype
a: 1 family has 3 individuals –ve for genotype and +ve for phenotype
b: 1 family has 2 individuals –ve for genotype and +ve for phenotype
c: 1 family has 5 individuals –ve for genotype and +ve for phenotype

Table 4. Segregation of recurrent *RYR1* variants in the UK malignant hyperthermia cohort

Nucleotide change	Amino acid change	No. of UK families		No. of discordant UK families		
		total	segregation	G+/P-	G-/P+	Both
c.479A>G	p.Glu160Gly	2	0			
c.529C>T	p.Arg177Cys	10	8	1	2	
c.641C>T	p.Thr214Met	4	3			
c.1202G>T	p.Arg401His	2				
c.1598G>A	p.Arg533His	2				
c.1615T>G	p.Phe539Val	2				
c.3166G>C	p.Asp1056His	2	1			
c.4763C>T	p.Pro1588Leu	2				
c.5024T>C	p.Leu1675Pro	3	0			
c.5183C>T	p.Ser1728Phe	8	6			1
c.6612C>G	p.His2204Gln	5	1			
c.6961A>G	p.Ile2321Val	3	1	1[a]		
c.7025A>G	p.Asn2342Ser	8	1			
c.7084G>A	p.Glu2362Lys	2	1			
c.7089C>G	p.Cys2363Trp	2	1		1	
c.7090T>G	p.Phe2364Val	2				
c.7291G>T	p.Asp2431Tyr	3	3		1	
c.7879G>A	p.Val2627Met	5	2		1	1
c.8026C>T	p.Arg2676Trp	3	3			1
c.9152G>A	p.Arg3051His	2				
c.10252A>G	p.Asn3418Asp	2	0			
c.11708G>A	p.Arg3903Gln	2				
c.11315G>A	p.Arg3772Gln	7 (2 HOM)	2	1[b]		
c.11958C>G	p.Asp3986Glu	6	2			
c.12149C>A	p.Ser4050Tyr	2	2			
c.12700G>T	p.Val4234Leu	5	1			

c.14210G>A	p.Arg4737Gln	7	7	1[b]	2	
c.14471T>C	p.Leu4824Pro	3				
c.14678G>A	p.Arg4893Gln	3	1			
c.14918C>T	p.Pro4973Leu	3	2			

G+/P-: +ve for genotype and -ve for phenotype
G-/P+:–ve for genotype and +ve for phenotype
a: 1 family has 3 individuals –ve for genotype and +ve for phenotype
b: 1 family has 2 individuals –ve for genotype and +ve for phenotype

Supplementary table 1. Variants in the RYR1 gene found in 722 independent UK malignant hyperthermia families

Nucleotide change	Amino acid change	Functionally characterized	ExAC MAF (European non-Finnish)	No. of UK families with variant	C-score
c.38T>G	p.Leu13Arg	No	0/66712	1	29
c.131G>A	p.Arg44His	No	1/55852	1	15.23
c.178G>A	p.Asp60Asn	No	1/66550	1	24.8
c.251C>T	p.Thr84Met	No	1/66398 (East Asian 5/8648)	1 (East Asian)	26.6
c.366C>G	p.His112Gln	No	0/66560	1	27.7
c.455C>A	p.Ala152Asp	No	0/66676	1	33
c.479A>G	p.Glu160Gly	No	0/66676	2	26.9
c.487C>T	p.Arg163Cys	Yes	0/66620	21	32
c.488G>T	p.Arg163Leu	Yes	0/66620	2	29.7
c.488G>A	p.Arg163His	No	0/66620 (1/11548 Latino)	1 (Italian)	30
c.526G>A	p.Glu176Lys	No	1/64606 (1/15770 South Asian)	1 (White)	32
c.529C>T	p.Arg177Cys	No	0/64006 (1/5962 Finnish))	10 (All white British)	32
c.533A>C	p.Tyr178Ser	No	0/64006	1	26.9
c.641C>T	p.Thr214Met	No	11/66646 (1/10388 African, 1/16498 South Asian)	4 (all white British)	23.8
c.652G>A	p.Val218Ile	No	0/66628	1	27.7
c.677T>A	p.Met226Lys	No	0/66716	1	28.9
c.742G>A	p.Gly248Arg	Yes	0/66454	5	26.3
c.742G>C	p.Gly248Arg	Yes	2/66454 (1/16510 South Asian)	3 (2 white British, 1 South Asian)	26.3
gtg/gtGGAg	p.Val330ValGlu ins	No	0/66642	1	29.4
c.1021G>C	p.Gly341Arg	No	0/66668	1	29.8
c.1021G>A	p.Gly341Arg	Yes	0/66668	31	32
c.1201C>T	p.Arg401Cys	Yes	0/66660	2	32
c.1202G>T	p.Arg401His	No	0/66660	2	32
c.1459C>G	p.Leu487Val	No	0/66740	1	27.7
c.1475G>A	p.Arg492His	No	0/66740	1	10.16
c.1565A>G	p.Tyr522Cys	No	0/66740	1	26.6
c.1589G>A	p.Arg530His	Yes	3/66740 (3/11756 Latino, 1/16512 South Asian)	1 (White British)	15.9
c.1598G>A	p.Arg533His	No	3/66740 (7/10406 African), 2/11578 Latino)	2 (1 white British, 1 white German)	16.65
c.1615T>C	p.Phe539Leu	No	0/66740	1	27.8
c.1615T>G	p.Phe539Val	No	0/66740	1	27.8
c.1654C>T	p.Arg552Trp	Yes	0/66730	4	27.9

c.1840C>T	p.Arg614Cys	Yes	9/66740 (1/11578 latino))	14 (all white)	15.96
c.1841G>T	p.Arg614Leu	Yes	0/66740	1	34
c.2050G>C	p.Gly684Arg	No	0/66090	1	29.9
c.2447C>T	p.Pro816Leu	No	4/66732 (All ENF)	1	17.96
c.2537C>T	p.Ser846Leu	No	0/66144	1	27.7
c.2654G>A	p.Arg885His	No	15/65228 (6/16386 South Asian)	1 White British	12.46
c.2924G>A	p.Arg975Gln	No	2/59490 (both ENF)	1	26
c.3095G>A	p.Arg1032His	No	8/44268 (1/12856 South Asian)	1 (white British)	16.06
c.3166G>C	p.Asp1056His	No	0/7566	2	1.802
c.3172G>A	p.Glu1058Lys	No	0/7566	1	2.566
c.3224G>A	p.Arg1075Gln	No	0/66676 (1/11578 Latino))	1 (white British)	27.2
c.3418C>T	p.Arg1140Cys	No	1/66660 (1/16512 South Asian)	1 (white British)	34
c.3527C>T	p.Tyr1176Ile	No	0/66718 (11/16510 South Asian)	1 (South Asian)	22.5
c.3667G>A	p.Glu1223Lys	No	0/66596	1	3.071
c.4024A>G	p.Ser1342Gly	No	4/7866 (415/1824 African)	1 (Black British)	9.86
c.4763C>T	p.Pro1588Leu	No	1/9516	2 (white British)	19.33
c.4999C>T	p.Arg1667Cys	No	146/65054 (62/8512 East Asian, 33/11502 Latino)	1 (White British)	15.55
c.5024T>C	p.Leu1675Pro	No	0/65086	3	23.7
c.5033A>G	p.Asn1678Ser	No	2/65086 (/11462 latino, 1/16398 South Asian)	1 (white british)	12.04
c.5183C>T	p.Ser1728Phe	No	0/65228	8	12.37
c.5186T>G	p.Met1729Arg	No	0/65326	1	25.8
c.5341T>C	p.Cys1781Arg	No	1/64684	1	11.4
c.5360C>T	p.Pro1787Leu	No	1404/63752 (662/16322 South Asian, 136/11364 Latino, 30/9304)	6	10.13
c.5440A>G	p.Met1814Val	No	0/62450	1	23.1
c.5441T>A	p.Met1814Lys	No	0/62450	1	24.8
c.6178G>T	p.Gly2060Cys	No	4577/66574 (2586/16510 South Asian, 906/11566 Latino,	7	13.64

			116/10364 African)		
c.6302T>A	p.Met2101Lys	No	20/63676 (1/11504 Latino)	1	23.4
c.6478G>A	p.Gly2160Ser	No	0/66104 (6/10300 African, 4/16506 South Asian, 1/11550 Latino))	1 (white british)	13.69
c.6487C>T	p.Arg2163Cys	Yes	0/66104	1	34
c.6488G>A	p.Arg2163His	Yes	0/66104	9	29.5
c.6502G>A	p.Val2168Met	Yes	0/66104	8	24.1
c.6599C>T	p.Ala2200Val	No	3/66414 (1/8632 East Asian, 1/11568 Latino)	1 (white british)	21
c.6612C>G	p.His2204Gln	No	0/66104	5	20.6
c.6617C>G	p.Thr2206Arg	Yes	0/66428	1	25.4
c.6617C>T	p.Thr2206Met	Yes	4/66428 (all ENF)	28	25.4
c.6670C>T	p.Arg2224Cys	No	4/66122 (17/16508 South Asian, 1/11546 Latino)	1 (white british)	34
c.6742C>T	p.Arg2248Cys	No	6/65896 (1/8594 East Asian, 1/11544 Latino)	1 (white British)	27.9
c.6838G>A	p.Val2280Ile	No	3/64894	1	24.6
c.6961A>G	p.Ile2321Val	No	41/66520 (3/10390 African, 2/11562 Latino)	3 (2 white British, 1 unrecorded)	11.9
c.7007G>A	p.Arg2336His	Yes	0/66518	9	24.9
c.7025A>G	p.Asn2342Ser	No	81/66528 (40/16512 South Asian, 5/11574 Latino, 1/10396 African)	8 (all white british)	11.42
c.7036G>A	p.Val2346Met	No	1/63418	1	27.1
c.7043A>G	p.Glu2348Gly	No	0/63508	1	24.6
c.7048G>A	p.Ala2350Thr	Yes	0/63508	7	19.17
c.7063C>T	p.Arg2355Trp	Yes	3/63724	8	34
c.7076G>A	p.Arg2359Gln	No	0/62820	1	26.3
c.7084G>A	p.Glu2362Lys	No	0/62220	2	25.3
c.7089C>G	p.Cys2363Trp	No	0/62220	2	27.4
c.7090T>G	p.Phe2364Val	No	0/62220	2	24.1
c.7123G>A	p.Gly2375Arg	No	0/58938	1	24.6
c.7282G>A	p.Ala2428Thr	Yes	0/66524	1	21.2
c.7291G>T	p.Asp2431Tyr	No	0/66508	3 (All white British)	23.7

c.7291G>A	p.Asp2431Asn	No	0/66508 (1/10374 African)	1 (white british)	15.92
c.7292A>T	p.Asp2431Val	No	0/66508	1	23.4
c.7300G>A	p.Gly2434Arg	Yes	2/66466 (1/10366 African)	118	23.8
c.7304G>T	p.Arg2435Leu	No	0/66466	1	24.1
c.7304G>A	p.Arg2435His	Yes	0/66466	11	24.1
c.7307G>A	p.Cys2436His	No	0/66466	1	22.9
c.7354C>T	p.Arg2452Trp	Yes	0/65940	2	32
c.7361G>A	p.Arg2454His	Yes	1/66108	14	26.3
c.7373G>A	p.Arg2458His	Yes	0/66188 (1/8826 East Asian)	15 (all white British)	26.2
c.7373G>T	p.Arg2458Leu	No	0/66188	1	26
c.7522C>T	p.Arg2508Cys	Yes	0/66312	1	25.7
c.7523G>A	p.Arg2508His	No	0/66312	4	25.1
c.7528T>C	p.Tyr2510His	No	0/66312	1	22.3
c.7778G>A	p.Arg2593His	No	2/65618	1	24.5
c.7816T>A	p.Cys2606Ser	No	1/64776	1	23.6
c.7879G>A	p.Val2627Met	No	0/66484	5	24
c.8026C>T	p.Arg2676Trp	No	1/66568	3	28.6
c.8054C>T	p.Ser2685Phe	No	0/66402 (1/16454 South Asian)	1 (white British)	25.1
c.8198G>A	p.Gly2733Asp	No	0/66672	1	24.6
c.8327C>T	p.Ser2776Phe	No	67/59798 (4/8804 African, 1/10266 Latino, 1/14786 South Asian)	1 White British)	12.68
c.8360C>G	p.Thr2787Ser	No	39/59610 (299/9026 African, 19/10420 Latino)	1 (white British)	12.5
c.8729C>T	p.Tyr2910Met	No	0/66446	1	1.339
c.9152G>A	p.Arg3051His	No	24/66740	2	21.6
c.9268G>A	p.Ala3090Thr	No	0/66590	1	21.6
c.9310G>A	p.Glu3104Lys	Yes	1/66662 (1/16510 South Asian)	5 (all white British)	23.9
c.9353C>T	p.Ala3188Val	No	0/6030 (0/1884 African)	1 (Black British)	15.84
c.9635A>G	p.Glu3212Gly	No	7/7162 (1/528 Latino)	1 (white British)	24.7
c.9652G>A	p.Val3218Met	No	0/8134	1	25.7
c.9676G>C	p.Glu3226Gln	No	0/11588	1	21.5
c.9797T>C	p.Met3266Thr	No	0/66222	1	9.265
c.10042C>T	p.Arg3348Cys	No	9/66046	1	10.58
c.10252A>G	p.Asn3418Asp	No	0/31266	2	18.62
c.10616G>A	p.Arg3539His	No	181/64324 (7/9828 African, 11/16220 South Asian,	6	15.28

			1/11076 Latino)		
c.10747G>C	p.Glu3583Gln	No	1147/66216 (399/16498 South Asian, 71/11530 Latino, 37/10248 African)	10	10.32
c.10870C>T	p.Arg3624Trp	No	0/66562	1	28.2
c.10891G>T	p.Ala3631Ser	No	0/66604	1	24.4
c.11086G>C	p.Asp3696His	No	0/63224	1	24.7
c.11132C>T	p.Thr3711Met	No	0/65158	2	14.95
c.11266C>G	p.Gln3756Glu	No	1094/62698 (1349/10422 latino, 462/8234 East Asian, 477/14794 South Asian, 72/9228 African)	8	14.12
c.11315G>A	p.Arg3772Gln	No	0/62702 (0/14896 South Asian)	7 (all South Asian)	26.9
c.11708G>A	p.Arg3903Gln	No	2/66740	2	28.4
c.11958C>G	p.Asp3986Glu	No	0/66266	8	24.1
c.11969G>T	p.Gly3990Val	Yes	0/66266	11	24.8
c.12028G>A	p.Glu4010Lys	No	4/66682 (1/10388 African)	1 (white British)	24.5
c.12115A>T	p.Ile4039Phe	No	0/66714	1	24
c.12149C>A	p.Ser4050Tyr	No	0/66732	2	24.5
c.12383C>T	p.Ala4128Val	No	0/66458	1	16.8
c.12533G>T	p.Gly4178Val	No	0/66250	1	24.2
c.12553G>A	p.Ala4185Thr	No	28/65996 (2/10306 African, 1/11552 Latino)	1 (white british)	12.39
c.12689T>G	p.Met4230Arg	No	0/18706	1	24.4
c.12700G>C	p.Val4234Leu	No	0/13000	5	24.7
c.12884C>T	p.Ala4295Val	No	0/14	2	10.95
c.13502C>T	p.Pro4501Leu	No	41/26404 (145/5512 African, 42/9932 South Asian, 13/3270 Latino)	1 (white british)	22.6
c.13513G>C	p.Asp4505His	No	234/24010 (29/9634 South Asian, 8/4948 African)	4 (2 white British, 1 South Asian, 1 Black British)	8.184
c.13672C>T	p.Arg4558Trp	No	1/66736	1	27.1
c.14168G>A	p.Arg4723His	No	38/66678 (1/10362 African)	1 (white british)	28.6
c.14201G>A	p.Gly4734Glu	No	0/66530	1	23.5
c.14209C>T	p.Arg4737Trp	No	0/66574	1	28.8

c.14210G>A	p.Arg4737Gln	No	1/66574	7	25.4
c.14270G>A	p.Arg4757His	No	12/66574 (2/16476 South Asian, 1/10280 African)	1 (white british)	25.7
g.39070671delACA	p.Asn4806del	No	0/66738	1	38
c.14443G>A	p.Gly4815Arg	No	0/66738	1	25
c.14449A>T	p.Ile4817Phe	No	0/66738	1	24.7
c.14458G>T	p.Gly4820Trp	No	0/66728	1	25.5
c.14471T>C	p.Leu4824Pro	No	0/66710	3	24.9
c.14477C>T	p.Thr4826Ile	Yes	0/66696	9 (all white British)	25.9
c.14512C>G	p.Leu4838Val	Yes	0/66436	1	26.6
c.14545G>A	p.Val4849Ile	Yes	0/66654 (1/11546 Latino)	8	25.8
c.14581C>T	p.Arg4861Cys	No	0/66734	1	32
c.14678G>A	p.Arg4893Gln	No	0/66336	3	0.298
c.14814C>G	p.Ile4938Met	No	0/66716	1	16.4
c.14817C>A	p.Asp4939Glu	No	0/66716	1	22.9
c.14918C>T	p.Pro4973Leu	No	3/66446 (2/11500 Latino)	3	10.79

ExAC: ExAC browser (http://exac.broadinstitute.org).
MAF: minor allele frequency.