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Translational Studies

Genetic epidemiology of malignant hyperthermia in the UK

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Abstract

Background: Gaps in our understanding of genetic susceptibility to malignant hyperthermia (MH) limit the application and interpretation of genetic diagnosis of the condition. Our aim was to define the prevalence and role of variants in the three genes implicated in MH susceptibility in the largest comprehensively phenotyped MH cohort worldwide.
Methods: We initially included one individual from each positive family tested in the UK MH Unit since 1971 to detect variants in *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 family segregation studies. The prevalence of recurrent variants of unknown significance was compared with the prevalence reported in a large database of sequence variants in low-risk populations.
Results: We have confirmed MH 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* variants previously unclassified recurrent variants significantly over-represented ($P<1\times10^{-7}$) in our cohort compared with the Exome Aggregation Consortium 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 MH families.
Conclusions: Our data provide current estimates of the role of variants in *RYR1*, *CACNA1S*, and *STAC3* in susceptibility to MH in a predominantly white European population.

Keywords: genetics; malignant hyperthermia; *RYR1*; *CACNA1S*; *STAC3*

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Editor's key points

- Malignant hyperthermia (MH) is a crucially important condition in anaesthetic practice.
- Accurate diagnosis of MH is challenging.
- This paper describes the prevalence of known gene variants associated with MH.
- This knowledge will assist prospective genetic diagnosis.

Malignant hyperthermia (MH) is a potentially fatal reaction that occurs in genetically susceptible individuals exposed to the volatile anaesthetics or succinylcholine.[1] There has been considerable progress in elucidating the genetic basis of MH susceptibility over the past 30 yr.[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 aims were to define the prevalence of individual variants in *RYR1*, *CACNA1S*, and *STAC3* in the largest cohort of phenotypically characterised 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 after 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 characterised 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.[18,21,22] Patients tested before 1984 were considered susceptible if their muscle biopsy samples developed a contracture of 0.2 g or more upon exposure to halothane 2%. 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 by Merritt and colleagues.[19] In brief, we began screening MH susceptible families for *RYR1* variants after 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 <0.001 in each of the ethnic cohorts of the Exome Aggregation Consortium (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 minor allele frequency <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 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 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 analysis 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 (accessed 18 March 2018). The C-score is derived from Combined Annotation-Dependent Depletion and scores of >15 include the 5% predicted most deleterious substitutions in the human genome.[26] Because of the uncertainty

Table 1 Non-functionally characterised *RYR1* variants present in more than one malignant hyperthermia family. ExAC, Exome Aggregation Consortium browser (http://exac.broadinstitute.org); HOM, homozygous; MAF, minor allele frequency. *MAF for the European non-Finnish cohort unless otherwise stated

Nucleotide change	Amino acid change	No. of families	ExAC MAF*	χ^2	P-value
c.479A>G	p.Glu160Gly	2	0/66 620	92	$<1\times10^{-7}$
c.529C>T	p.Arg177Cys	10	0/64 006	443	$<1\times10^{-7}$
c.641C>T	p.Thr214Met	4	11/66 646	43.5	$<1\times10^{-7}$
c.1202G>T	p.Arg401His	2	0/66 660	92	$<1\times10^{-7}$
c.1598G>A	p.Arg533His	2	3/66 740	34.6	$<1\times10^{-7}$
c.1615T>G	p.Phe539Val	2	0/66 740	92	$<1\times10^{-7}$
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	0/65 086	135	$<1\times10^{-7}$
c.5183C>T	p.Ser1728Phe	8	0/65 086	361	$<1\times10^{-7}$
c.6612C>G	p.His2204Gln	5	0/66 430	230	$<1\times10^{-7}$
c.6961A>G	p.Ile2321Val	3	41/66520	4.66	0.031
c.7084G>A	p.Glu2362Lys	2	0/62 220	86	$<1\times10^{-7}$
c.7089C>G	p.Cys2363Trp	2	0/61 940	86	$<1\times10^{-7}$
c.7090T>G	p.Phe2364Val	2	0/61 754	85	$<1\times10^{-7}$
c.7291G>T	p. Asp2431Tyr	3	0/66 508	138	$<1\times10^{-7}$
c.7879G>A	p.Val2627Met	5	0/66 484	230	$<1\times10^{-7}$
c.8026C>T	p.Arg2676Trp	3	1/66 588	102	$<1\times10^{-7}$
c.9152G>A	p.Arg3051His	2	24/66 740	3.9	0.048
c.10252A>G	p.Asn3418Asp	2	0/31 266	43	$<1\times10^{-7}$
c.11708G>A	p.Arg3903Gln	2	2/66 740	44	$<1\times10^{-7}$
c.11315G>A	p.Arg3772Gln	7 (2 HOM)	0/14 896 (South Asian)	83	$<1\times10^{-7}$
c.11958C>G	p.Asp3986Glu	6	0/66 312	276	$<1\times10^{-7}$
c.12149C>A	p.Ser4050Tyr	2	0/66 732	92	$<1\times10^{-7}$
c.12700G>T	p.Val4234Leu	5	0/15 016	52	$<1\times10^{-7}$
c.14210G>A	p.Arg4737Gln	7	1/66 574	280	$<1\times10^{-7}$
c.14471T>C	p.Leu4824Pro	3	0/66 704	139	$<1\times10^{-7}$
c.14678G>A	p.Arg4893Gln	3	0/66 322	138	$<1\times10^{-7}$
c.14918C>T	p.Pro4973Leu	3	3/66 446	66	$<1\times10^{-7}$

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 analysis

We compared the prevalence estimates for potentially pathogenic variants in MH families *vs* the ExAC cohort using a χ^2 test (MedCalc Software, Ostend, Belgium https://www.medcalc.org/calc/comparison_of_proportions.php, accessed 18 March 2018). We then used an on-line package (http://www.danielsoper.com/statcalc/calculator.aspx?id=11, accessed 18 March 2018) that enables calculation of exact *P* values up to χ^2 values of 34 ($P=1\times10^{-8}$). 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 $<1\times10^{-7}$ to infer statistical significance.

Results

A total of 770 independent families with MH confirmed by a positive IVCT after 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 S1. Of these, 31 have been previously sufficiently characterised 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 three of the six 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 characterised[29,30] and are recognised as pathogenic variants by the European MH Group (www.emhg.org). We have previously reported p.Arg174Trp[14] and p.Thr1009Lys[24,31] in one and two families, respectively. We now report an additional family with p.Arg174Trp. Of the total 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 three of 66 558 alleles of the ExAC European non-Finnish cohort indicating that the variant is over-represented in MH families (χ^2 35.2, $P<1\times10^{-8}$) 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

Table 2 Rare *CACNA1S* variants in the UK malignant hyperthermia cohort. ENF, European non-Finnish cohort, MAF for other cohorts as stated; ExAC, Exome Aggregation Consortium browser (http://exac.broadinstitute.org); MAF, minor allele frequency. *Functionally characterised variant. †Black/African/Caribbean ethnicity. ‡MAF used for calculating χ^2

Nucleotide change	Amino acid change	No. of families (ethnicity)	ExAC MAF	χ^2	P-value	C-score
c.520C>T	p.Arg174Trp*	2 (Black†)	1/66 510 ENF; 0/10 376 African‡; 2/8630 East Asian; 1/16 500 South Asian	8.3	0.004	34
c.1426A>C	p.Thr476Pro	1 (Arabic)	0/66 738 ENF; 0/10 406 African‡	2.64	0.1	22.2
c.1849A>G	p.Thr617Ala	1 (White)	0/66 738 ENF	22.5	2×10^{-6}	22.4
c.2273C>T	p.Pro758Leu	1 (South Asian)	1/66 696 ENF; 0/16 512 South Asian‡	4.88	0.027	28.3
c.2654T>C	p.Leu885Pro	1 (South Asian)	0/65 598 ENF: 0/16 054 South Asian‡	4.81	0.028	23.5
c.2700G>T	p.Arg900Ser	1 (White)	0/66 652 ENF‡	22.5	2×10^{-6}	31
c.2726A>G	p.Asn909Ser	1 (White)	2/66 708 ENF‡	14.36	0.0002	25.7
c.2974C>G	p.His992Asp	1 (South Asian)	0/66 126 ENF: 6/16 362 South Asian‡	0.36	0.55	23.8
c.3026C>A	p.Thr1009Lys	2 (White)	3/66 558 ENF‡	35.2	$<1\times10^{-7}$	34
c.3332C>A	p.Arg1086Ser	2 (South Asian)	0/66 740 ENF: 3/16 512 South Asian‡	8.32	0.0039c	27
c.3628G>A	p.Gly1210Arg	1 (White)	56/61 616 ENF‡	0.063	0.8	27.1
c.5087C>T	p.Thr1696Met	1 (White)	2/65 668 ENF‡	14.28	0.0002	7.79

amino acid as the functionally characterised 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 (three out of 16 512 alleles) did not reach our criteria for classifying this variant as likely pathogenic (χ^2 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 less than one in 1000 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 seven 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 analysis

Segregation between genotype and IVCT phenotype was assessed in 328 families with an *RYR1* variant and four families with *CACNA1S* variants. The median (range) number of MH susceptible and MH normal members included per family was two (1–16) and three (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 two, three, or four such variants was 21, five, and one, respectively.

Discussion

This analysis provides 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% confidence interval (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 USA[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 Gillies and colleagues[8,12] and 33 of these were found to carry *RYR1* variants

Table 3 Segregation analyses of *RYR1* variants reported as pathogenic by the European Malignant Hyperthermia Group (www.emhg.org). G+/P−: +ve for genotype and −ve for phenotype. G−/P+: −ve for genotype and +ve for phenotype. *One family has three individuals −ve for genotype and +ve for phenotype. †One family has two individuals −ve for genotype and +ve for phenotype. ‡One family has five individuals −ve for genotype and +ve for phenotype

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*	
c.742G>C	p.Gly248Arg	3	2			
c.1021G>A	p.Gly341Arg	31	27	1	6†	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†	
c.6617C>G	p.Thr2206Arg	1				
c.6617C>T	p.Thr2206Met	28	24	3	4	1
c.7007G>A	p.Arg2336His	9	7		1†	1*
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†,‡	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†	
c.7372C>T	p.Arg2458Cys	0				
c.7373G>A	p.Arg2458His	15	12		2†	
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†	
c.14582G>A	p.Arg4861His	0				
c.14693T>C	p.Ile4898Thr	0				

(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]; and Sweden, seven 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* prevalence 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 characterised.[19]

Table 4 Segregation of recurrent *RYR1* variants in the UK malignant hyperthermia cohort. G+/P−: +ve for genotype and −ve for phenotype. G−/P+: −ve for genotype and +ve for phenotype. *One family has three individuals −ve for genotype and +ve for phenotype. †One family has two individuals −ve for genotype and +ve for phenotype

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*		
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†		
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†	2	
c.14471T>C	p.Leu4824Pro	3				
c.14678G>A	p.Arg4893Gln	3	1			
c.14918C>T	p.Pro4973Leu	3	2			

Current generic guidelines[36] for the diagnostic classification of genetic variants incorporate five 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 characterisation because of the potential for genetic background effects[18,19] and it perhaps would be appropriate to classify variants characterised in this way as likely pathogenic.

The American College of Medical Genetics and Genomics and the Association for Molecular Pathology 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 characterised 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 five 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 yr 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 been required to be confirmed with 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 yr, 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 minimise 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 result.[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 who 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.

Authors' contributions

Study conception/design: P.M.H., M.-A.S.
Conduct of experiments and data collection: D.M.M., C.D., L.G., S.J.H., K.R., S.S., R.L.R., J.G.B., P.K.G., P.M.H.
Data analysis and interpretation: all authors.
Writing paper: P.M.H.
Revising paper: all authors.

Declarations of interest

P.M.H. is an Editorial Board Member of *British Journal of Anaesthesia*. He is also Chair of the European Malignant Hyperthermia Group.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bja.2018.06.028.

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