Research Article

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Pharmacogenomics

Bayesian modeling to predict malignant hyperthermia susceptibility and pathogenicity of *RYR1*, *CACNA1S* and *STAC3* variants

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Aim: Identify variants in *RYR1*, *CACNA1S* and *STAC3*, and predict malignant hyperthermia (MH) pathogenicity using Bayesian statistics in individuals clinically treated as MH susceptible (MHS). **Materials & methods:** Whole exome sequencing including *RYR1*, *CACNA1S* and *STAC3* performed on 64 subjects with: MHS; suspected MH event or first-degree relative; and MH negative. Variant pathogenicity was estimated using *in silico* analysis, allele frequency and prior data to calculate Bayesian posterior probabilities. **Results:** Bayesian statistics predicted *CACNA1S* variant p.Thr1009Lys and *RYR1* variants p.Ser1728Phe and p.Leu4824Pro are likely pathogenic, and novel *STAC3* variant p.Met187Thr has uncertain significance. Nearly a third of MHS subjects had only benign variants. **Conclusion:** Bayesian method provides new approach to predict MH pathogenicity of genetic variants.

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Malignant hyperthermia (MH) is a potentially lethal pharmacogenetic sensitivity to volatile anesthetics and succinylcholine. Most patients have more than one anesthetic exposure before an MH event [1]; therefore, a negative personal and/or family history does not exclude MH susceptibility (MHS). Additionally, the diagnosis of MH may be challenging because the signs and symptoms are not specific. The Clinical Grading Scale (CGS) [2] defines the clinical features of 'very likely' and 'almost certain' MH cases, but the CGS score may also be high during anesthetics complicated by sepsis, thyroid storm, respiratory failure, adverse drug reactions unrelated to MHS, neurologic disease, or other conditions producing hyperthermia, muscle rigidity and/or hypercarbia [3]. Muscle bioassay [4–6] has been used for identification of MHS, using strips of excised muscle for an *in vitro* bioassay – known as the caffeine-halothane contracture test (CHCT) in North America and the *in vitro* contracture test in Europe.

More than 25 years ago, researchers identified *RYR1* as the gene most frequently associated with MHS [7,8]. Early genetic studies were conducted in nonmyopathic subjects with family history of clinical MH, including many individuals who had undergone muscle contracture testing. Depending on study selection criteria and testing methods, *RYR1* variants occur in as few as 23% or as many as 86% of MHS North American or European individuals [9–12]. Variants are much less common in the *CACNA1S* gene [13–15] that encodes the α1 subunit of the skeletal muscle voltage-gated calcium channel (interacting with RYR1 in the process of excitation–contraction coupling). Variants in the *STAC3* gene (encoding Stac3) were recently found in patients with congenital myopathy associated with MHS [16,17]. The Stac3 protein has an essential role in skeletal muscle excitation–contraction coupling [18].

Future Medicine

Many patients with suspected MH events never undergo muscle contracture testing since the assay is costly, invasive and often inconvenient (requiring travel to a testing facility). Clinical genetic testing for MHS has been available since 2005, and the European Malignant Hyperthermia Group (EMHG) provides an updated list of diagnostic MH mutations (currently 48 *RYR1* and 2 *CACNA1S* variants) [19]. An individual carrying one of these variants should be considered MHS (at increased risk of developing MH during anesthesia), and family members may be screened by genetic testing. An individual who does not carry a familial variant cannot be regarded as MH negative (MHN) since other pathogenic variants could be involved, and the genetics of MH are not fully known. Numerous other variants in *RYR1* have been reported but are not functionally characterized and thus are not considered to be diagnostic for MHS. Muscle contracture testing, supplemented by recombinant *in vitro* expression in HEK 293 cells or myotubes of *RYR1* knockout mice demonstrating functional change, remains a critical part of the classification of the variants based on EMHG guidelines [20].

Next-generation sequencing (NGS) for MH was described in 2012 [21], and whole exome sequencing has been performed on the known MH-associated genes, as well as genes involved in excitation–contraction coupling and skeletal muscle calcium homeostasis [22–24]. This approach is less costly and time consuming than Sanger sequencing, and it allows for identification of novel genes and/or variants [24]. The population frequency of an allele can be estimated by searching public databases containing sequencing data from >67,000 human exomes. Since most *RYR1* variants are rare, having frequencies <0.001, a low frequency cannot be used alone as a predictor of pathogenicity [25].

In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) published a joint consensus recommendation for interpreting sequence variants observed in patients with suspected inherited disorders [26]. This included weighted criteria for classifying variants as pathogenic or benign, and proposed rules for combining those criteria into five categories (pathogenic, likely pathogenic, variant of uncertain significance [VUS], likely benign and benign). Tavtigian *et al*. described Bayesian modeling using the ACMGG/AMP criteria to produce a quantitative probability of pathogenicity [27]. Bayesian reasoning uses a prior probability and modifies that based on conditional factors, resulting in a posterior probability. This methodology allows for integration of data from multiple sources to make predictions about the significance of rare genetic variants.

The aims of this study were to: identify novel variants (not previously associated with MH) by sequencing of *RYR1, CACNA1S and STAC3*; and to assess pathogenicity of identified variants (both novel and previously described in association with MH) using application of Bayesian statistics [27] to integrate data from the current and prior studies.

Materials & methods

Subjects

The study design was a case–control genetic risk assessment study. Study subjects living in the continental USA were recruited from the North American Malignant Hyperthermia Registry (NAMHR; Supplementary Material). Registrants are primarily of European descent, with <10% reporting mixed race with Native American, Asian or African ancestry. Once identified from the registry, study candidates were contacted by mail as many as three-times to request their participation. The subject population included three groups: MHS individuals with abnormal CHCT results in the NAMHR; subjects with suspected MH episodes, abnormalities on clinical genetic testing of *RYR1* or rhabdomyolysis, and their first-degree relatives with similar history who had joined the NAMHR without CHCT results; and MHN subjects with normal CHCT results in the NAMHR. Initially, only subjects meeting criteria for Group 1 were contacted to participate in the study; however, the response rate was low. Group 2 was included to expand the study and evaluate individuals with a clinical suspicion of MH or those who might have care altered based on personal/family history. Subjects were included from families with a previously reported MH variant. Blood samples for DNA analysis were collected, stored and processed according to protocols approved by the University of Pittsburgh Medical Center and Cincinnati Children's Hospital Medical Center Institutional Review Boards. All subjects gave written informed consent.

Gene sequencing

Whole exome sequencing by NGS was performed by Prevention Genetics, WI, USA. Results are reported here only for *RYR1, CACNA1S* and *STAC3* genes. For this NGS panel, the full coding regions plus approximately ten base pairs of noncoding DNA flanking each exon were sequenced for each gene. Sequencing was accomplished by capturing

specific regions with an optimized solution-based hybridization method, followed by massively parallel sequencing of the captured DNA fragments. Additional Sanger sequencing was performed for any regions not captured or those having an insufficient number of sequence reads. All pathogenic, undocumented and questionable variant results were confirmed by Sanger sequencing. All sequencing data are available on request.

Initial pathogenicity classification of previously identified variants

Identified gene variants were referenced against the EMHG registry of diagnostic MH variants. The 'previously reported pathogenicity' classification of non-EMHG variants was based on data in the National Center for Biotechnology Information (NCBI) ClinVar website (www.ncbi.nlm.nih.gov/clinvar/docs/linking) [28] and reported by Prevention Genetics. Variants were classified as pathogenic, likely pathogenic, VUS, likely benign, or benign as presented in the consensus statement of the ACMG/AMP [26].

In silico analysis of nucleotide/amino acid substitutions

PolyPhen-2 [29] (individual and batch modes; http://genetics.bwh.harvard.edu/pph2) was used to predict the possible impact of amino acid substitutions on protein structure and function. PolyPhen-2 produces a qualitative prediction of probably damaging, possibly damaging, or benign, along with a numerical score ranging from 0.0 (benign) to 1.0 (damaging). The HumDiv and HumVar scores and the sensitivity and specificity estimate for the scores were recorded. The Genomic Evolutionary Rate Profiling (GERP++) score, the Combined Annotation-Dependent Depletion (CADD) [30] score, the Grantham distance and the Genome Aggregation Database (gnomAD; gnomAD.broadinstitute.org) [31] data were obtained from SeattleSeq Annotation 150 (http://snp.gs.washington.edu/SeattleSeqAnnotation150/) using the exact location of the nucleotide substitution in the individual chromosomes of interest (1, 12 and 19). These positions were obtained from the NCBI ClinVar [28] website (www.ncbi.nlm.nih.gov/clinvar/docs/linking), if the variant had been previously reported and included in ClinVar. In other cases, the location was determined using nearby previously reported variants and verifying the location on the chromosomal map for NCBI Assembly Genome Reference Consortium human build patch 38 (GRCh 38).

Classification of gene variants by Bayesian posterior probability method

Variants were initially classified based on their posterior probabilities from the Bayesian calculator described by Tavtigian *et al.* [27] (available in the Supplementary Material of that article). Assignment of evidence strength was done using the ACMG/AMP criteria presented by Richards *et al.* [26]. Briefly, pathogenic criteria were weighted as strong (PS1-4), moderate (PM1-6) or supporting (PP1-5), and benign criteria were weighted as standalone (BA1), strong (BS1-4) or supporting (BP1-7). Critical changes were made to the Tavtigian *et al.* calculator in the Bayesian prior probability and the exponential progression coefficient. The Bayesian prior probability was set to 0.02, instead of 0.10, based on the number of nonintronic variants in *RYR1* that are pathogenic and the total number of nonintronic variants present in a European-based sample (gnomAD database). The exponential progression coefficient was set to 1.9 instead of 2.0. This yields a posterior probability of 0.996 for *RYR1* p.Arg614Cys (a known EMHG pathogenic variant) given two strong (PS3, PS4), one medium (PM2) and two supporting (PP1, PP3) criteria with a Bayesian prior probability of 0.02. All EMHG pathogenic variants met criteria to achieve a posterior probability of 0.991 or greater.

A threshold for the maximum frequency of the variant allele was set at 2.0×10^{-4} (the frequency of the *RYR1* p.Arg614Cys minor allele frequency [MAF] in Europeans from the gnomAD database). Two variant frequencies were determined, including: the MAF for the European non-Finnish (EnF) cohort reported in version 2.1.1 of gnomAD; and the highest frequency observed in any subpopulation in gnomAD 2.1.1. If the variant was reported in a subpopulation but not in the EnF population, the frequency was calculated as less than one/number of EnF exomes in the dataset. If the variant was not listed in gnomAD, the value from the National Heart, Lung, and Blood Institute Exome Sequencing Project, as reported by Seattle Seq Annotation 150, was used as the denominator. If there was a numeric value for the number of times the major allele was observed in the sample but there was no value for the minor allele, the MAF was reported as one/number of observations of the major allele. For example, the *RYR1* p.Gln4837Glu major allele (C) was reported 13,006-times and there was no value for the minor allele (G); therefore, the MAF was reported as 7.69×10^{-5}. If the variant frequency in the EnF subpopulation was above threshold or the frequency in any subpopulation listed in gnomAD was more than double the threshold (true for *RYR1* p.Gly893Ser and *RYR1* p.Val4842Met), a BS1 was assigned, otherwise, a PM2 was assigned. If the allele frequency was reported as 'unknown', the entry in Supplementary Table 4 was unknown (e.g., *RYR1* p.Ala4295Val).

An abnormal CHCT was not used as the sole evidence for pathogenicity of a specific variant, as multiple variants may be present in individuals with abnormal CHCT. Assessing the impact of an abnormal CHCT on the probability that a given variant in *RYR1*, *CACNA1S* or *STAC3* is pathogenic is not straightforward. Taken alone, an abnormal CHCT has almost no impact on the conditional probability that a given variant is pathogenic. Adding sequencing information can significantly alter the conditional probability, provided that certain conditions are satisfied, including:

- A strongly positive CHCT (caffeine contracture >0.6 g; halothane contracture >1.1 g).
- Identification of all variants in *RYR1*, *CACNA1S* and *STAC3* by sequencing.
- No 'known' pathogenic variants are present in any of the three genes above.
- All but one of the variants in those three genes are known to be benign or meet strong criteria, such as allele frequency, for a benign variant.

The mean probability that the remaining VUS in *RYR1*, *CACNA1S* or *STAC3* is pathogenic is 0.755 (95% CI = 0.688–0.823; Supplementary Material). This is a Bayesian posterior, as it is a conditional probability. A variant can be classified as pathogenic without specific functional testing (PS3), based on evidence gathered from multiple families and full gene sequencing of *RYR1*, *CACNA1S* and *STAC3* as shown in Supplementary Material.

We used the sequencing data available from this study and existing class assignments of known variants to identify variants that met the four conditions above. The method described by Tavtigian *et al.* [27] for testing the internal validity of the ACMG-AMP combining criteria did not apply, without modification, to our data and to the study of MH in general. The variants found in the present study were scored using the Bayesian approach with the following modification. Novel variants meeting all of the four conditions above were assigned PS3 with a probability of 0.755. The posterior probability for a variant meeting the four criteria above was computed by adding (1 - 0.755) multiplied by the posterior without PS3 and 0.755 multiplied by the posterior including PS3.

$$\begin{aligned} &p\left(\text{path}|\text{abnormal CHCT meeting the 4 conditions}\right) = \\ &a \times p\left(\text{path}|\text{PS3 PM *PP*}\right) + (1-a) \times p\left(\text{path}|\text{PS3 PM* PP*}\right) \end{aligned} \quad \text{(Eq. 1)}$$

where α = 0.755 and the * indicates all of the evidence of strong (other than PS3), moderate or supporting that applies to the variant.

Results

Genotyping results

Complete sequencing of *RYR1*, *CACNA1S* and *STAC3* by NGS was performed for 64 subjects (57 proven or suspected MHS subjects or first-degree relatives and 7 MHN control subjects; Figure 1). Many previously reported variants (Table 1) were identified, including 8 EMHG pathogenic variants in *RYR1* [19] found in 15 subjects and 7 variants in *RYR1* or *CACNA1S* [9,11,12,14,22,24,32–43] classified as likely pathogenic. One of these likely pathogenic variants for MH is pathogenic for Central Core Disease [36,38–41]. Many other previously reported variants [6,9,11,32,34,38,43–48] were identified, and had been classified as VUS or benign/likely benign (Table 1). Additional benign genetic variants (not listed) were found in *RYR1* and *CACNA1S* from every subject and in *STAC3* from 25 subjects. The *CACNA1S* variant p.Pro1839Ser [43] was initially reported as likely pathogenic by Prevention Genetics; however, we changed the initial classification to VUS based on the Bayesian posterior probability from previously reported data. Fourteen novel variants were found in 12 subjects, including 12 *RYR1* variants (1 duplication), a *CACNA1S* variant and a *STAC3* variant (Table 2).

All genetic variants were missense mutations, except for the single duplication. An intronic variant in *RYR1* was found in one subject (Supplementary Table 1); this person also had a recognized pathogenic variant and a VUS in *RYR1*.

Clinical details of subjects with abnormal CHCT

A total of 38 subjects had abnormal CHCT results (performed between 1970 and 2015; Supplementary Table 1). Four of these CHCT results were not quantitatively comparable to the others, as they involved nonstandard methods [5]. Genotyping revealed pathogenic *RYR1* variants in 12 of the 38 subjects (32%), and likely pathogenic

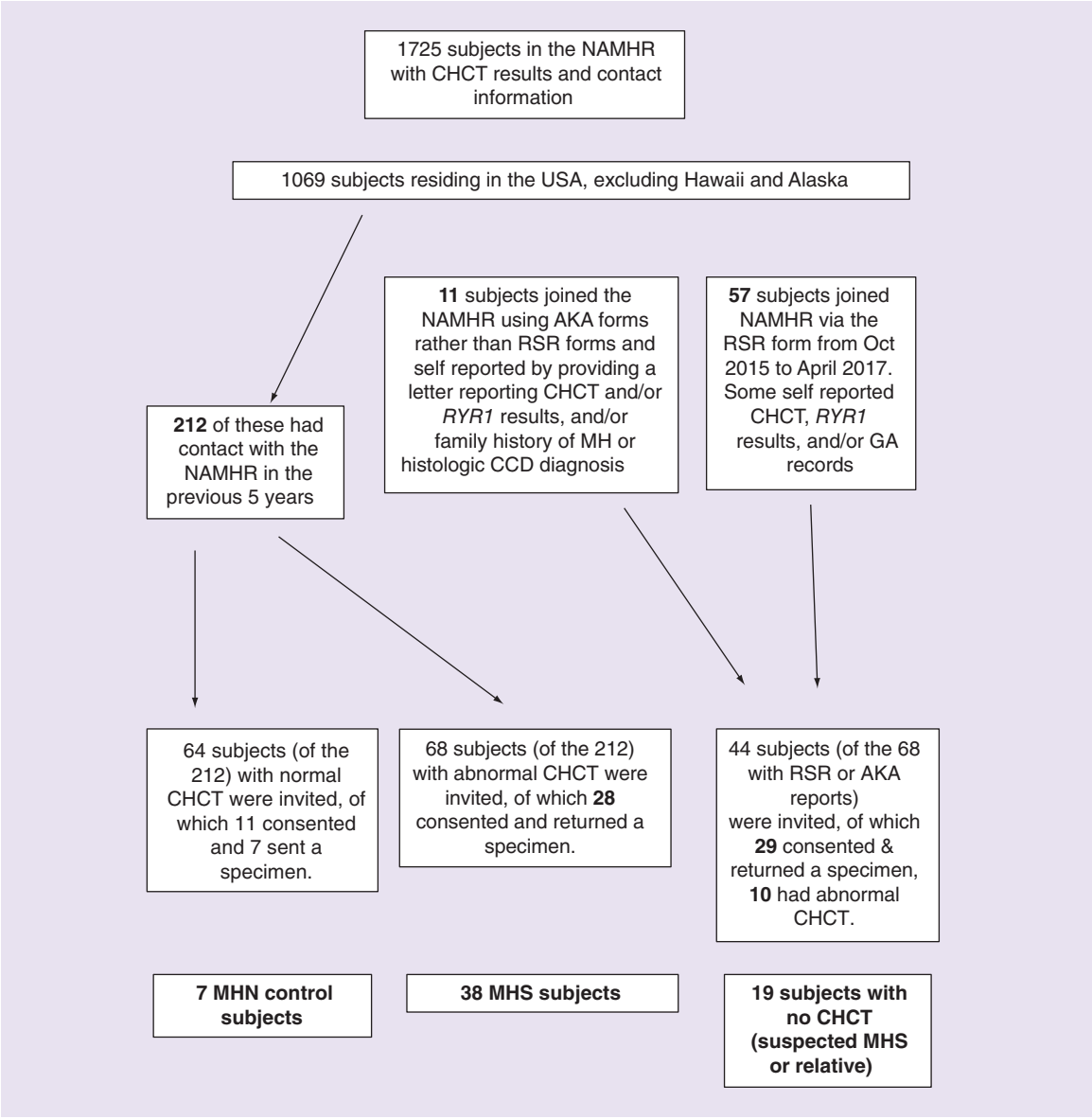

Figure 1. Details of subject recruitment from the North American Malignant Hyperthermia Registry.
AKA: Also known as; CCD: Central core disease; CHCT: Caffeine halothane contracture test; GA: General anesthesia; MH: Malignant hyperthermia; MHN: MH negative; MHS: MH susceptible; NAMHR: North American Malignant Hyperthermia Registry; RSR: Registry subject report.

variants in *RYR1* or *CACNA1S* in 7 additional subjects; therefore, 50% of subjects with CHCT results diagnostic of MHS had at least likely pathogenic previously described variants.

After application of Bayesian methods, 11 of these 38 subjects had only benign/likely benign variants in *RYR1*, *CACNA1S* and/or *STAC3*, or no potentially pathogenic variants identified. Muscle contracture testing (between 1984 and 2015) was performed according to the standard method for 8 of these 11 subjects and produced maximal contractures in the presence of 3% halothane of 0, 1.05, 1.1, 1.2, 2.2, 2.58, 3 and 3.94 gm. The diagnostic cutoff for the assay is 0.7 gm.

Twenty six of these 38 subjects reported a personal experience of MH during general anesthesia. The CGS scores of these MH events ranged from 10 to 70 for subjects with pathogenic or likely pathogenic variants in *RYR1* or *CACNA1S*. In the six subjects with only VUSs based on previous reports, the highest CGS score was 53. Of the ten

Table 1. Previously reported *RYR1* and *CACNA1S* variants found in subjects from North American Malignant Hyperthermia Registry with or without caffeine-halothane contracture test results.

Variant found			Previously reported pathogenicity	Bayesian posterior probability	Predicted pathogencity
Protein	AA change	CHCT			
RYR1	p.Gly341Arg	Abn	EMHG Path	0.996	Path
RYR1	p.Arg614Cys	Abn	EMHG Path [38]†	0.996	Path
RYR1	p.Val2168Met	NA	EMHG Path	0.996	Path
RYR1	p.Thr2206Met	Abn	EMHG Path [38]†	0.996	Path
RYR1	p.Arg2336His	Abn	EMHG Path	0.996	Path
RYR1	p.Gly2434Arg	Abn	EMHG Path [38]†	0.996	Path
RYR1	p.Arg2435His	Abn	EMHG Path	0.996	Path
RYR1	p.Arg2508His	Abn §	EMHG Path	0.996	Path
CACNA1S	p.Thr1009Lys	Abn	Likely Path [22,24]	0.978	Likely Path
RYR1	p.Ser1728Phe	Abn	Likely Path [9,14]	0.979	Likely Path
RYR1	p.Arg2163Pro	Abn	Likely Path [32,37]	0.925	Likely Path
RYR1	p.Val2354Met	Abn	Likely Path [38]†	0.788	VUS
RYR1	p.Leu4824Pro	Abn	Likely Path [12]† [33]	0.926	Likely Path
RYR1	p.Val4842Met	Abn	Likely Path [14,34,35]	0.150	VUS
RYR1	p.Ala4940Thr	Abn	Likely Path [41]† [36,39,40]	0.683	VUS
RYR1	p.Arg830Trp	Abn	VUS [47]	0.195	VUS
RYR1	p.Arg2248His	Abn	VUS [38]‡	0.267	VUS
RYR1	p.Val2280Ile	NA	VUS [14]	0.195	VUS
RYR1	p.Ile2358Leu	Abn	VUS [38]‡	0.267	VUS
RYR1	p.Pro2366Arg	NA	VUS	0.195	VUS
RYR1	p.Asp2730Gly	Abn	VUS [48]	0.380	VUS
RYR1	p.Arg3539His	Abn	VUS [14,34]	<0.001	Benign
RYR1	p.Thr3711Arg	NA	VUS [38]†	0.624	VUS
RYR1	p.Ala4185Thr	Abn	VUS [11,14]	0.057	Likely benign
RYR1	p.Ala4295Val	Abn	VUS [6,14,44,45]	0.002	Benign
RYR1	p.Gln4837Glu	Abn	VUS [38]‡	0.649	VUS
CACNA1S	p.Pro1839Ser	Abn	VUS [43]	<0.001	Benign
RYR1	p.Ala697Ala	Normal	Benign/likely benign	ND	ND
RYR1	p.Gly893Ser	Abn §	Benign/likely benign [45,46]	0.001	Benign
RYR1	p.Pro1423Pro	Normal	Benign/likely benign	ND	ND
RYR1	p.Arg1667Cys	Normal	Benign/likely benign [14]	<0.001	Benign
RYR1	p.Ala4293Ala	Normal	Benign/likely benign	ND	ND
RYR1	p.Gly4835Gly	Abn	Benign/likely benign	0.001	Benign

† The subject with this variant was previously reported in the referenced publication.
‡ The subject with this variant may have been previously reported in the referenced publication.
§ The CHCT results were not reported due to abnormal prestandardization results, and CHCT data were not used for assigning criteria for these variants.
AA: Amino acid; Abn: Abnormal; CHCT: Caffeine-halothane contracture test; EMHG: European Malignant Hyperthermia Group; NA: Not available; ND: Not determined; Path: Pathogenic; VUS: Variant of uncertain significance.

subjects with only benign or likely benign genetic variants by previous reports, six individuals reported suspected MH events with CGS scores between 0 and 15.

Eleven subjects underwent muscle contracture testing only because of family history of MH. Pathogenic or likely pathogenic *RYR1* variants were found in seven of these subjects and two VUSs in *RYR1* were found in one other subject.

Seven subjects reported a fatal MH event in a first- or second-degree relative, and five had either a pathogenic or likely pathogenic *RYR1* variant (p.Gly341Arg, p.Thr2206Met, p.Arg2435His or p.Leu4824Pro) or the VUS p.Gln4837Glu. The remaining two subjects had only benign variants in *RYR1*, *CACNA1S* and *STAC3*.

Table 2. Novel variants (not previously associated with malignant hyperthermia) in *RYR1*, *CACNA1S* and *STAC3* found in subjects from North American Malignant Hyperthermia Registry with or without caffeine-halothane contracture test results including results of Bayesian modeling to predict malignant hyperthermia pathogenicity.

AA change	CHCT result	PolyPhen-2 score; HumDiv & HumVar (Sens; Spec)	GERP	GD	CADD score	gnomAD 2.1 variant Freq (EnF)	Weighted Bayesian posterior probabilities		
							Evidence path or benign [§]	Posterior probability	Prediction
RYR1									
p.Val164Phe[†]	NA	1.0 (0.00; 1.00); 0.997 (0.27; 0.98)	4.260	50	11.79	$<7.74 \times 10^{-6}$	PP3, PM1, PM2 BP5	0.344	VUS
p.Arg274Cys[†]	Abn	1.00 (0.00; 1.00); 0.997 (0.47; 0.96)	4.630	180	13.47	$<7.80 \times 10^{-6}$	PP3, PM2 BP5	0.094	Likely benign
p.His460Arg	Abn	1.00 (0.00; 1.00) 0.996 (0.36; 0.97)	4.700	29	18.24	7.69×10^{-5} [‡]	PP3, PM2, PS3[¶]	0.649	VUS
p.His865Arg	NA	0.999 (0.14; 0.99); 0.986 (0.54; 0.94)	4.000	29	15.83	$<7.75 \times 10^{-6}$	PM2, PP3	0.195	VUS
p.Glu1424Lys	Norm	0.669 (0.86; 0.91); 0.099 (0.91; 0.68)	5.120	56	16.68	$<7.75 \times 10^{-6}$	PM2 BP4	0.042	Likely benign
p.Met1493Arg[†]	NA	0.999 (0.14; 0.99); 0.995 (0.45; 0.96)	4.400	91	11.4	7.69×10^{-5} [‡]	PM2, PP3 BP5	0.094	Likely benign
p.Glu1642Lys	NA	1.00 (0.00; 1.00); 0.979 (0.57; 0.94)	5.000	56	22	7.8×10^{-5} [‡]	PM2, PP3	0.195	VUS
p.Ser3171Ser[†]	Abn [#]	Unknown	-1.23	0	11.76	7.69×10^{-5} [‡]	PM2, BP7, BP6, BP5, BP4	0.003	Likely benign
p.Arg3629Trp[†]	Abn	1.00 (0.00; 1.00); 0.936 (0.66; 0.91)	3.760	101	14.21	8.79×10^{-6}	PP3, PM2, PS3[¶]	0.683	VUS
p.Glu3853Lys[†]	Abn	1.00 (0.00; 1.00); 0.981 (0.57; 0.94)	4.380	56	15.81	5.43×10^{-5}	PP3, PM2, PS3[¶]	0.683	VUS
p.Thr4823Met[†]	NA	1.00 (0.00; 1.00); 0.999 (0.09; 0.99)	4.570	81	14.93	7.75×10^{-6}	PP5, PP3, PM2 BP5	0.195	VUS
CACNA1S									
p.Ser606Asn[†]	Abn	0.929 (0.81; 0.94); 0.858 (0.72; 0.89)	3.63	46	17.42	1.30×10^{-2}	PP3 BP5, BS1	<0.001	Benign
STAC3									
p.Met187Thr	Abn	0.932 (0.80; 0.94); 0.775 (0.76; 0.86)	5	81	24.1	$<8.79 \times 10^{-5}$	PM2, PP3, PS3[¶]	0.683	VUS

Also see Supplementary Table 4 for additional details on variant frequency in subpopulations. A '<' symbol indicates that this variant was not identified in the EnF population, and the minor allele frequency following the '<' symbol is an upper limit.

The sensitivity and specificity are measures of the prediction confidence for the naive Bayesian probabilities of a variant being damaging generated by the PolyPhen-2 algorithm. The FPR equals 1 – specificity. For the HumDiv probabilities, if the FPR is <0.05 the variant is probably damaging; if 0.05 < FPR < 0.10 the variant is possibly damaging; otherwise it is likely benign/benign. The corresponding critical FPR values for the HumVar probabilities are 0.10 and 0.20. Low sensitivity values are associated with greater confidence in the Bayesian probability generated by the PolyPhen-2 algorithm. The sensitivity may exceed specificity for some variants predicted to be benign.

[†]Two variants found in this patient; see Supplementary Material for more details.

[‡]No data available in gnomAD, thus the frequency was based on genomeESP as described in Methods section.

[§]Criteria were categorized as pathogenic (strong [PS], moderate [PM] or supporting [PP]) or benign (strong [BS] or supporting [BP]).

[¶]PS3 used in calculation of weighted Bayesian posterior probability as described by the equation in Methods.

[#]The CHCT results were not reported due to abnormal prestandardization results, and CHCT data were not used for assigning criteria for these variants.

AA: Amino acid; Abn: Abnormal; BS: Benign-strong evidence; BP: Benign-supporting evidence; CADD: Combined Annotation-Dependent Depletion; CHCT: Caffeine-halothane contracture test; EnF: European non-Finnish population; ESP: Exome Sequencing Project; Freq: Frequency; GD: Grantham distance; GERP: Genomic Evolutionary Rate Profiling; gnomAD: Genome Aggregation Database; NA: Not applicable or not available; Norm: Normal; Path: Pathogenic; PM: Pathogenic-moderate evidence; PP: Pathogenic-supporting evidence; PS: Pathogenic-strong evidence; Sens: Sensitivity; Spec: Specificity; VUS: Variant of uncertain significance.

Clinical details of subjects with no CHCT results

No muscle contracture testing had been performed on 19 subjects (Supplementary Table 2), although the majority (15/19) of these individuals had reported MH events (CGS scores of 3–73) between 1986 and 2017. Pathogenic variants recognized by the EMHG were found in three of these subjects (CGS scores 73, 33 and 15). Four subjects had not experienced MH events, but they joined the NAMHR due to family history. Two of these subjects had a first-degree relative with a history of repeated episodes of rhabdomyolysis without exposure to anesthetics, and they had *RYR1* variants identified by clinical genetic testing (Supplementary Material). The other two subjects had strong family histories of MH, and only benign variants were found in these individuals.

Clinical details of MHN subjects

Seven subjects, serving as MHN controls, had a record of previous normal CHCT between 1990 and 2016 (Supplementary Table 3). Six of these individuals had experienced suspected MH events (1979–2014, CGS scores 10–58), and the remaining subject had an MHS sibling. In the subject with CGS score of 10, the *RYR1* variant p.Glu1424Lys (predicted to be likely benign by Bayesian methodology) was identified. All other variants were benign or likely benign variants in *RYR1*.

Bayesian statistics

Predictions for the seven novel variants (not previously associated with MH) from subjects with abnormal CHCT included four VUSs and three benign or likely benign (Table 2; Supplementary Table 4). All VUSs (*RYR1* p.His460Arg, p.Arg3629Trp, and p.Glu3853Lys, and *STAC3* p.Met187Thr) had posterior probabilities of 0.649. This is the maximum Bayesian posterior probability for a novel variant, achieved with *in silico* evidence of a damaging mutation, MAF below threshold and PS3 per Equation 1. The PS3 criterion was assigned for these variants given the abnormal CHCT and the lack of other variants on full gene sequencing of the three genes in that subject. Novel variants from patients with no CHCT results were VUSs or likely benign variants with posterior probabilities from 0.094 to 0.344.

Using Bayesian statistics to combine the case details from this report with previous reports, we were able to predict pathogenicity for the previously identified variants (Tables 1 & 3; Supplementary Table 4). Modeling results predicted likely pathogenicity for the *CACNA1S* variant p.Thr1009Lys and the *RYR1* variants p.Ser1728Phe and p.Leu4824Pro with posterior probabilities of 0.978, 0.979 and 0.926, respectively. The *CACNA1S* variant p.Pro1839Ser had been reported as VUS [43], but application of Bayesian methods produced posterior probability of pathogenicity of <0.001 (consistent with benign status).

Case details for subjects with STAC3 & CACNA1S variants

The variable penetrance of MH and wide range of symptoms and signs in MHS patients with *RYR1* variants have been recognized [38,49–51]. Less information is available from MHS patients with other gene variants; therefore, we include the case details of these subjects with *STAC3* variant p.Met187Thr and *CACNA1S* variants p.Pro1839Ser and p.Thr1009Lys (with *RYR1* variant p.Arg274Cys in the same subject; Supplementary Material).

Discussion

In this study, we examined three genes, *RYR1*, *CACNA1S* and *STAC3*, believed to harbor 75% or more of MH-causative variants [14]. Since pathogenic variants in *STAC3* were found relatively recently, few published studies have described complete sequencing of these three genes in the same population sample. Miller *et al.* recently published a study from the UK in which these three genes were sequenced from 722 MH families [14]. Our study evaluated subjects living in the USA, identified additional novel variants and case details for select variants, and provided Bayesian statistics to predict pathogenicity. Our results did confirm previous reports, revealing many recognized pathogenic variants and many VUSs in *RYR1* [10,11,48,52,53].

Bayesian modeling of the ACMG/AMP variant classification guidelines was proposed by Tavtigian *et al.* in 2018, and we applied that framework to variants associated with MH. The original article presented a probability threshold of 99% for pathogenicity, indicating >99% certainty that a variant is disease causing. The posterior probability cutoff for pathogenic was thus 0.99. The ACMG/AMP guidelines are less clear, as they defined 'likely pathogenic' to mean >90% certainty that a variant causes disease, and the threshold for 'pathogenic' is higher (but not clearly defined). We maintained the threshold for pathogenic at 0.99, but an argument could be made for a lower threshold. The threshold should likely be modified based on the effective balance of risk and benefit

Table 3. Results of Bayesian modeling to predict malignant hyperthermia pathogenicity of previously reported likely pathogenic variants and selected variant of uncertain significance including *in silico* evidence and pathogenic or benign criteria assigned.

AA change	CHCT result	PolyPhen-2 score; HumDiv & HumVar (Sens; Spec)	GERP	GD	CADD score	gnomAD 2.1 variant Freq (EnF)	Weighted Bayesian posterior probabilities		
							Evidence Path or benign [§]	Posterior probability	Prediction
RYR1									
p.Arg830Trp[†]	Abn	0.999 (0.14; 0.99); 0.960 (0.63; 0.92)	1.890	101	15.5	1.32×10^{-4}	PP3, PM2	0.195	VUS
p.Ser1728Phe	Abn	0.962 (0.78; 0.95); 0.756 (0.77; 0.86)	3.770	155	12.37	$<9.15 \times 10^{-6}$	PP5, PP3, PM2, PS4, PS3[¶]	0.979	Likely path
p.Arg2163Pro[#]	Abn	1.00 (0.00; 1.00); 0.999 (0.09; 0.99)	4.630	103	15.88	7.69×10^{-5}[‡]	PP5, PP3, PM2, PM5, PS3[¶]	0.925	Likely path
p.Arg2248His	Abn	1.00 (0.00; 1.00); 0.963 (0.62; 0.92)	4.910	29	17.8	8.82×10^{-6}	PP3, PM2, PS3[¶] BP5	0.267	VUS
p.Val2280Ile	NA	0.952 (0.79; 0.95); 0.486 (0.83; 0.81)	5.040	29	15.23	3.54×10^{-5}	PP3, PM2	0.195	VUS
p.Val2354Met	Abn	0.995 (0.68; 0.97); 0.973 (0.60; 0.93)	3.990	21	17.91	7.65×10^{-5}[‡]	PP3, PP5, PM2, PS3[¶]	0.788	VUS
p.Ile2358Leu	NA	0.004 (0.97; 0.59); 0.0.014 (0.95; 0.53)	3.990	5	14.9	8.96×10^{-6}	PM2, PP3, PS3[¶] BP5	0.267	VUS
p.Pro2366Arg	NA	0.920 (0.81; 0.94); 0.346 (0.78; 0.85)	3.990	103	16.81	7.69×10^{-5}[‡]	PP3, PM2, BP5	0.195	VUS
p.Asp2730Gly	Abn	0.002 (0.99; 0.30); 0.005 (0.97; 0.44)	3.180	94	14.85	7.69×10^{-5}[‡]	PM2, PS3[¶] BP4	0.380	VUS
p.Thr3711Arg[†]	NA	1.00 (0.00; 1.00); 0.997 (0.27; 0.98)	5.330	71	15.02	$<9.00 \times 10^{-6}$	PP3, PM2, PS3[¶]	0.624	VUS
p.Leu4824Pro	Abn	0.994 (0.69; 0.97); 0.947 (0.65; 0.92)	4.570	98	13.94	7.65×10^{-5}[‡]	PP5, PP3, PM2, PS4	0.926	Likely path
p.Gln4837Glu	Abn	0.985 (0.74; 0.96); 0.981 (0.57; 0.94)	4.570	29	15.51	7.69×10^{-5}[‡]	PP3, PM2, PS3[¶]	0.649	VUS
p.Val4842Met	Abn	1.00 (0.00; 1.00); 0.999 (0.09; 0.99)	4.570	21	15.17	2.32×10^{-5}	PP3, PM2, PS3[¶], BS1	0.150	VUS
p.Ala4940Thr	Abn	1.00 (0.00; 1.00); 0.990 (0.51; 0.95)	4.670	58	12.65	1.49×10^{-5}	PP3, PM2, PS3[¶]	0.683	VUS
CACNA1S									
p.Thr1009Lys	Abn	1.00 (0.00; 1.00); 1.00 (0.00; 1.00)	5.170	78	33	2.64×10^{-5}	PP5, PP3, PM2, PS3[¶], PS4, BP5	0.978	Likely path

Also see Supplementary Table 4 for additional details on variant frequency in subpopulations. A '<' symbol indicates that this variant was not identified in the EnF population, and the minor allele frequency following the '<' symbol is an upper limit.

The sensitivity and specificity are measures of the prediction confidence for the naive Bayesian probabilities of a variant being damaging generated by the PolyPhen-2 algorithm. The FPR equals 1 – specificity. For the HumDiv probabilities, if the FPR is <0.05 the variant is probably damaging; if 0.05 < FPR < 0.10 the variant is possibly damaging; otherwise it is likely benign/benign. The corresponding critical FPR values for the HumVar probabilities are 0.10 and 0.20. Low sensitivity values are associated with greater confidence in the Bayesian probability generated by the PolyPhen-2 algorithm. The sensitivity may exceed specificity for some variants predicted to be benign.

[†]Two variants found in this patient; see Supplementary Material for more details.

[‡]No data available in gnomAD, thus the frequency was based on genomeESP as described in Methods section.

[§]Criteria were categorized as pathogenic (strong [PS], moderate [PM], or supporting [PP]) or benign (strong [BS] or supporting [BP]).

[¶]PS3 used in calculation of weighted Bayesian posterior probability as described by the equation in Methods.

[#]c.6488 G>A = p.Arg2163His is a known EMHG pathogenic variant; therefore, c.6488 G>C = Arg2163Pro is expected to be pathogenic.

BS: Benign-strong evidence; BP: Benign-supporting evidence; EMHG: European Malignant Hyperthermia Group; ESP: Exome Sequencing Project; FPR: False positive rate; AA: Amino acid; Abn: Abnormal; CADD: Combined Annotation-Dependent Depletion; CHCT: Caffeine-halothane contracture test; EnF: European non-Finnish population; Freq: Frequency; GD: Grantham distance; GERP: Genomic Evolutionary Rate Profiling; gnomAD: Genome Aggregation Database; NA: Not available; Path: Pathogenic; PM: Pathogenic-moderate evidence; PP: Pathogenic-supporting evidence; PS: pathogenic-strong evidence; Sens: Sensitivity; Spec: Specificity; VUS: Variant of uncertain significance.

to patients for the disease of interest. The 0.99 threshold may be appropriate for diseases such as cancer when prophylactic surgery may be chosen in the presence of certain pathogenic variants. However, a lower threshold may be more appropriate for MH due to the risks associated with underclassification of a variant as pathogenic. Another recent study described a quantitative approach to variant classification in hypertrophic cardiomyopathy, and the authors chose an even lower pathogenic threshold of 0.95 [54].

We have introduced new considerations for assessing the pathogenicity of genetic variants in MH beyond the standard application of the ACMG/AMP criteria. Data from sequencing and the *ex vivo* CHCT were applied to the computation of probability that a variant is pathogenic. While not elevating any previously identified variants to pathogenic, this modification did increase the posterior probability from the range of 0.15–0.20 to 0.6 or greater. The higher probability of pathogenicity suggests that a variant merits more attention.

Importantly, we were able to combine new and previously published case data to predict that three variants, *CACNA1S* p.Thr1009Lys and *RYR1* p.Ser1728Phe and p.Leu4824Pro, are likely pathogenic for MH. The posterior probability for two of these variants exceeded 0.975 after applying data from other studies. Miller *et al.* [14] provided evidence for assignment of a PS4 criteria for the two *RYR1* variants showing that the frequency is significantly greater in the MHS population than the general population ($p < 1 \times 10^{-7}$).

The *RYR1* variant, p.Thr3711Arg, was found in a subject with a known EMHG pathogenic variant (p.Arg614Cys) and no CHCT, but a very high CGS score of 73. The same variant was found by Brandom *et al.* [38] as a single variant with a strongly positive CHCT in a second individual. These two subjects with the p.Thr3711Arg variant were siblings (as the current study and the 2013 Brandom *et al.* study shared a few subjects); therefore, it is possible that some other shared genetic element contributed to their MH risk. The PS3 criterion was assigned to this variant with an $\alpha = 0.677$ because there was sequence data for *RYR1* only from the sibling with the positive CHCT.

The *CACNA1S* variant Thr1009Lys was described in a family by Kim *et al.* [24], and the pedigree analysis showed MHS transitions to MH equivocal and even MHN over three to four generations (suggesting the variant is subject to epigenetic or other modification in successive generations). Additional data will be required to determine if this is a consistent pattern for this variant or restricted to the reported family.

About 10% of the subjects in this report had multiple variants that might be responsible for the MH phenotype. Similarly, Miller *et al.* [14] reported that 9.2% (27/293) families, in which full 3-gene sequencing was done, carried two or more potentially pathogenic *RYR1* variants. These findings demonstrate the need for complete sequencing of *RYR1*, and preferably all three of these genes, in an MH proband, as opposed to targeted sequencing restricted to specific loci. Relatives who could be identified as MHS by genetic testing might conclude that they are not at increased risk of MH if only one exon of *RYR1* was to be examined for a familial mutation [38,55]. If full sequencing of the genes known to be associated with MH is performed for the first degree relatives of probands, then those with one of the two variants found in the proband will be identified. This reinforces the importance of collecting broad genetic data from families. Unfortunately when relatives have healthcare provided by different systems, personal data cannot be shared without the initiative of the proband. All MH probands and their relatives should be encouraged to send their medical records with their genetic reports to a research registry such as the North American Malignant Hyperthermia Registry.

Only benign or likely benign, or no potentially pathogenic variants were found in *RYR1*, *CACNA1S* or *STAC3* in about a third (11/38) of the MHS subjects in this study, as well as 10/19 subjects with MH history/MH relative but lacking CHCT results. Two possibilities may explain these findings – either the subjects were not truly MHS or other, yet unidentified, genetic loci are involved in MH. Misdiagnosis of MHS is possible for some subjects since specificity estimates for CHCT are as low as 78% [56], and specificity decreases as the force of the contractures approaches the lower limits of a positive test. In two previous studies in different geographic regions, MHS subjects with no *RYR1* variants had lower muscle contracture results than did those with *RYR1* pathogenic variants [38,53]. Seven of the 11 subjects with only benign variants in our study had strong evidence by CHCT for MHS with halothane contracture results >1 g; however, caffeine contracture results were weaker for some subjects. CHCT specificity might best be determined by the weaker of the halothane or caffeine contractures [56]. The binomial probability of >4 false positives among 38 tests, with a specificity of 80%, is 0.96; thus, half of these cases may be false positives. The CHCT results from the remaining four subjects with benign variants were not definitive, as three had nonstandard test protocols or qualitative results, and one subject was positive only on the caffeine contracture test. One individual, with both halothane and caffeine contractures greater than the median in this

small group, had a sibling with a recognized MH causative mutation. Perhaps a second locus of MHS exists in this family [57].

Other studies have also identified subjects diagnosed MHS by contracture testing that had no variants in *RYR1* [11,38]. Miller *et al.* found no pathogenic variants in *RYR1*, *CACNA1S*, or *STAC3* in 103/722 MH families (14%) and estimated non-*RYR1*/*CACNA1S*/*STAC3* MHS between 14 and 23% [14]. Whole exome and Sanger sequencing targeted to genes involved in calcium movement in skeletal muscle have been used to search for other MH genes [23,58,59]. Variants of low frequency were found in the *CACNB1*, *CASQ1*, *CASQ2*, *SERCA1*, *KCNA1* and other genes; however, a causative role in MH has not been proven yet. Kraeva *et al.* reported that variants in the *CASQ1* gene are unlikely to be a cause of MH in the North American population, despite evidence that *CASQ1* deletion is a candidate for MHS in mice [60]. Additional studies are needed to search for other genes involved in MH.

For two of the subjects with *RYR1* VUSs (a previously identified variant, p.Val2280Ile and a novel variant, p.Arg4321Leu4323dup), we reported exertional myopathy (Supplementary Material). Neither family had a history of suspected MH events or muscle contracture testing, so MHS could not be defined in these individuals. Additionally, a novel *STAC3* VUS was found in one study subject. This individual had an anesthetic-induced episode that was consistent with either MH or hyperkalemic cardiac arrest due to an occult myopathy. Because these VUSs were identified in single subjects, and relatives were not available to study, it is impossible to assess segregation of these genetic variants with MH. Nevertheless, documentation of these variants with clinical details may prove useful for future reference as new methods become available. Additional clinical reports and functional testing might lead to reclassification of these variants as pathogenic for MH. For example, *RYR1* mutations at amino acid 2508 were added to the EMHG list of MH causative variants after functional testing [61] and multiple reports of MH events in patients with the variant.

Applying the ACMG/AMP criteria to estimate the pathogenicity of genetic variants in MH is controversial. At the time of writing of this manuscript, other researchers are actively working in this area. The concepts presented here are independent of any process or product of international debates on estimating pathogenicity, except in so far as we have responded to reviewers' comments. Some reviewers may be part of these groups working on developing guidelines for interpretation of pathogenicity. As standards are introduced, some of the decisions made in our data analysis may become obsolete. We aimed to present enough details that the reader could re-analyze using different judgments regarding the contribution of elements of the criteria first published by Richards *et al.* [26].

Conclusion

We applied *in silico* analysis and Bayesian statistics to genetic variants from an MHS population in the USA, thus aggregating new and older data to assign a probability of pathogenicity to variants in *RYR1*, *CACNA1S* and *STAC3*. Three previously described variants, *CACNA1s* p.Thr1009Lys and *RYR1* p.Ser1728Phe and p.Leu4824Pro, were predicted to be likely pathogenic for MHS with very high probability. This method could help to accelerate expansion of the library of MH pathogenic variants, and it leverages the data obtained from whole gene sequencing. As nearly a third of MHS subjects had only benign variants in *RYR1*, *CACNA1S* and *STAC3*, other genes are likely involved in MH.

Future perspective

Additional studies in MHS populations will likely identify more genetic variants in *RYR1*, *CACNA1s* and *STAC3*, as well as other genes (e.g., those involved in excitation–contraction coupling and skeletal muscle calcium homeostasis). Bayesian modeling will be used to integrate data and predict pathogenicity for certain variants, therefore supporting/expediting the use of functional studies. Complete or nearly complete characterization of the library of pathogenic MH variants would allow for clinical genetic testing to replace muscle bioassays as the most widely applied diagnostic tool for identification of MHS patients. Genetic testing performed after a suspected MH event will provide information to facilitate the future care of the proband and family members.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: https://www.futuremedicine.com/doi/suppl/10.2217/pgs-2019-0055

Author contributions

S Sadhasivam contributed to the conception, design and conduct of the work. BW Brandom contributed to the conduct of the work. BW Brandom and R Henker contributed to data acquisition. J McAuliffe contributed to the analysis and interpretation of the data. All authors contributed to the drafting of the work or revising it critically for content. All authors have reviewed and approve of this manuscript for submission. All authors agree to be accountable for all aspects of the work.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Summary points

- Variants in *RYR1*, *CACNA1S* and *STAC3* genes have been implicated in malignant hyperthermia (MH) pathogenesis, although the genetics of MH are not fully characterized.
- Whole exome sequencing including *RYR1*, *CACNA1S* and *STAC3* was performed on 64 subjects in the continental USA from the North American Malignant Hyperthermia Registry including:
  - 38 individuals with previous abnormal caffeine-halothane contracture test (CHCT) results.
  - 19 individuals with no CHCT results but history of MH event or first-degree relative with MH history.
  - Seven individuals with normal CHCT.
- Pathogenicity of variants was assessed by *in silico* analysis, allele frequency and Bayesian statistics integrating prior data.
- Identified variants included 14 novel variants and many other previously reported variants (classified as pathogenic by the European Malignant Hyperthermia Group, likely pathogenic, variant of uncertain significance, likely benign, or benign).
- Bayesian statistics predicted that previously reported *RYR1* variants p.Ser1728Phe and p.Leu4824Pro and *CACNA1S* variant p.Thr1009Lys are likely pathogenic for MH with very high posterior probabilities.
- Novel (not previously associated with MH) *RYR1* variants p.His460Arg and p.Arg3629Trp, and *STAC3* variant p.Met187Thr were variants of uncertain significance with posterior probability of 0.649–0.683.
- Other genes are likely involved in MH, as only benign variants were found in nearly one out of thee of the MHS subjects in this study.
- These results may facilitate expansion of the library of genetic testing for MH so that clinical genetic testing may be improved.

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