

Malignant Hyperthermia in the Post-Genomics Era

New Perspectives on an Old Concept

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ABSTRACT

This article reviews advancements in the genetics of malignant hyperthermia, new technologies and approaches for its diagnosis, and the existing limitations of genetic testing for malignant hyperthermia. It also reviews the various *RYR1*-related disorders and phenotypes, such as myopathies, exertional rhabdomyolysis, and bleeding disorders, and examines the connection between these disorders and malignant hyperthermia. (**ANESTHESIOLOGY** 2018; 128:168-80)

MALIGNANT hyperthermia (MH) is a pharmacogenetic disorder of skeletal muscle triggered by volatile anesthetics or succinylcholine. It manifests as a potentially lethal hypermetabolic crisis associated with a rapid and uncontrolled increase in myoplasmic Ca^{2+} in skeletal muscle cells.^{1,2} Advances in anesthesia monitoring and the discovery of the therapeutic efficacy of dantrolene have reduced the mortality and morbidity of MH substantially.³ However, over the past decade, studies have reported evidence that deaths associated with MH still occur, despite treatment.⁴⁻⁶

In parallel, our knowledge of the molecular and genetic etiology of MH has been advanced over the last three decades. Three genes, *RYR1*,^{7,8} *CACNA1S*,⁹⁻¹¹ and *STAC3*,¹² have been associated with MH susceptibility and the severe dysregulation of skeletal muscle Ca^{2+} homeostasis that results in the clinical features of an MH reaction under anesthesia. A recent report of a bleeding disorder associated with an *RYR1* variant implicated in MH susceptibility¹³ expands the range of clinical defects, already including myopathies^{14,15} and exertional rhabdomyolysis,¹⁶⁻¹⁹ that may be present in MH-susceptible individuals. These findings emphasize that the phenotypes associated with genetic defects predisposing to MH are not confined to reactions to volatile anesthetics and imply a common or overlapping pathophysiology of these disorders.

In this review, we summarize the latest evidence on the genetics of MH susceptibility, and its connection to non-anesthesia-related disorders. We review the guidelines for genetic diagnosis of MH susceptibility and discuss the limitations of current genetic screening. We also discuss the nonanesthetic phenotypes associated with *RYR1*-related disorders.

MH, a Pharmacogenetic Disorder

MH susceptibility is commonly stated to be a monogenic disorder with locus and allelic heterogeneity. The prevalence of the genetic trait has been estimated to be between 1:2,000 and 1:3,000.²⁰ Interestingly, the combined prevalence in the ExAC Browser database (<http://exac.broadinstitute.org/gene/ENSG00000196218>; accessed April 2, 2017) of functionally characterized genetic variants that have been associated with MH is 1:2,750. These prevalence rates are considerably greater than the reported incidence of clinical MH episodes,²¹ and the discrepancy is interesting to consider. Monnier *et al.*,²⁰ suggested that the penetrance of the MH genetic trait was incomplete, which indeed was the genetic model proposed by Denborough *et al.*²² when they reported the first case. Incomplete penetrance of a genetic trait implies that the genetic defect either requires additional factors for the phenotype to occur or other factors can prevent the occurrence of the phenotype. It is recognized that the lack of penetrance of the clinical MH phenotype can be for nongenetic reasons, because some people who develop MH are known to have had previous exposure to triggering anesthetics with no apparent problem. Unlike the Australian family reported by Denborough *et al.*,²² however, there are relatively few MH families where the number of clinical episodes is sufficient to draw any inference about the mode of inheritance. In most families, evidence of a dominant pattern of inheritance is derived from results of laboratory testing using pharmacologic challenge of excised skeletal muscle strips in an *in vitro* contracture test. These tests are known as the caffeine-halothane contracture test in North America and the *in vitro* contracture test in Europe. However, because these tests are invasive and costly, family studies are limited in some

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countries, whereas in others the testing strategy assumes an autosomal dominant pattern of inheritance with an inevitable bias in the resultant family structure.

Lack of penetrance of a genetic trait may also arise for genetic reasons with defects in more than one gene operating together to produce a phenotype or indeed opposing each other to modify or even obscure a phenotype. There is reproducible evidence for the presence of more than one genetic factor influencing the MH susceptibility phenotype,²³ leading Carpenter *et al.*²⁴ to propose a threshold genetic model for MH susceptibility. Such a model, in which the relatively weak pathogenic effect of the more prevalent MH-associated variants is subject to modifying effects of other genetic variants, provides a compelling explanation for the apparent discrepancy between the genetic prevalence and clinical incidence of MH.

RYR1, encoding the ryanodine receptor–Ca²⁺ release channel of skeletal muscle sarcoplasmic reticulum (RyR1), has been established as the major gene implicated in MH. Since the report⁷ of the first human MH-associated *RYR1* variant, hundreds of MH probands and thousands of members of their families have been screened for *RYR1* variants, and MH-associated variants have been found in more than half of the MH families studied from different populations.^{8,25,26} A small number of MH-susceptible families carry a variant in the second MH gene, *CACNA1S*, encoding the α -1S subunit of the T-tubular voltage-gated Ca²⁺ channel Ca_v1.1, also known as the dihydropyridine receptor.^{9–11,27} The α -1S subunit is important for the voltage sensing and Ca²⁺ conduction of the dihydropyridine receptor.

However, up to 50% of MH probands, who survived an MH event and whose MH susceptibility status was confirmed by a positive *in vitro* contracture test, do not carry any *RYR1* or *CACNA1S* variants, and the genetic basis of their MH susceptibility remains unresolved.^{21,27,28} Four additional MH loci have been implicated by linkage analysis in several European and North American families, but no MH-associated gene has been confirmed within those loci yet.²⁹ Recently, a homozygous *STAC3* mutation has been linked to Native American myopathy associated with MH susceptibility in one Native American family.¹² Normal functioning of Stac3 protein, encoded by *STAC3*, is thought to be required for effective colocalization of dihydropyridine receptors and RyR1s.

RyR, dihydropyridine receptor, and Stac3 protein are all essential components of the skeletal muscle excitation–contraction coupling complex.³⁰ The underlying mechanism of MH is disruption of excitation–contraction coupling resulting in abnormally enhanced Ca²⁺ release from the sarcoplasmic reticulum *via* RyR1 in response to either endogenous (*e.g.*, voltage) or exogenous (*e.g.*, halogenated anesthetics) stimuli.

Variants in *RYR1* associated with MH susceptibility are heterozygous missense changes that are shown

to impact the RyR1 channel function as gain-of-function mutations, making mutant RyR1 channels more sensitive to activation. Functional analysis of the MH-associated *CACNA1S* variants showed that their effect on excitation–contraction coupling was similar to that shown for *RYR1* mutations, *i.e.*, expression of the mutant α -1S in dysgenic myotubes (lacking α -1S) resulted in an enhanced sensitivity of RyR1 to stimuli compared with the effect of wild-type α -1S.³¹ It was suggested that α -1S functions as a negative allosteric modulator of RyR1 activation, and the *CACNA1S* mutations result in suppression of this negative modulatory effect.^{2,31,32} Currently, more than 200 *RYR1* variants are found in association with MH, but only 35 *RYR1* variants and 2 *CACNA1S* variants are recognized as being sufficiently functionally characterized (<http://www.emhg.org>; accessed April 8, 2017) to be used in diagnostic genetic testing for MH. Although these variants are frequently referred to as causative mutations, we will refer to them as pathogenic variants because the functional studies demonstrate an effect that is qualitatively consistent with our understanding of MH but, in the context of a possible threshold genetic model, do not prove that the effect would translate into a clinical MH reaction. An individual carrying one of the MH pathogenic variants is considered MH susceptible, *i.e.*, at increased risk of developing MH under anesthesia. When a familial pathogenic variant is identified, genetic testing can be extended to family members, and all members of the family carrying the variant should be considered MH susceptible.³³ However, MH susceptibility cannot be ruled out for individuals who do not carry the familial variant because of the possibility of more than one pathogenic variant being present in the same family,^{20,23,34} and they should be offered contracture testing to confirm their MH negative status.³⁴

New Genetic Technology and MH

Next-generation sequencing enables fast and cost-efficient sequencing of all protein coding regions—exons—in the human genome. The 1000 Genomes Project (2008 to 2015)³⁵ has created the largest public catalogue of human genetic variation through analysis of whole-genome sequencing data of thousands of individuals from multiple populations.

Prevalence of *RYR1* and *CACNA1S* Variants in the General Population. Analysis of next-generation sequencing variation databases for MH genes by two recent studies has corroborated previous observations of high levels of allelic heterogeneity within *RYR1* and *CACNA1S* compared to other genes of the genome. Based on the NHLBI Exome Sequencing Project data set, which includes variation data from 6,500 exomes (<http://evs.gs.washington.edu/EVS/>; accessed April 8, 2017), it was estimated that *CACNA1S* and *RYR1* are more genetically diverse than the average gene in either African American or in European-American populations,

i.e., both these genes have a high level of natural variation.³⁶ Comparably high levels of variation in both *RYR1* and *CACNA1S* were detected by a second study where the authors evaluated exome sequencing data from the ClinSeq data set on a cohort of 870 volunteers not selected for MH susceptibility.³⁷ Furthermore, using *RYR1* and *CACNA1S* variation data for this unselected cohort, the authors assessed the prevalence of MH susceptibility in the general population. Based on an allele frequency of less than 1%, genotype–phenotype data, and the primary literature, they found that only 19% of the *RYR1* variants identified in this cohort unselected for MH were probably benign, whereas 6% were pathogenic, and 75% were variants of unknown significance. Of the pathogenic *RYR1* variants, three have been previously reported in association with MH: such a high prevalence of MH-associated *RYR1* variants has not been replicated in the much larger sample presented in the ExAC Browser. For *CACNA1S*, 20% of the variants were defined as benign, and 80% were defined as variants of unknown significance.

These studies showed that a large fraction of the *RYR1* variants found in databases were rare, with a frequency of 0.00001 or less. It is noteworthy that an abundance of rare variants is a feature common to other genetic disorders. The exome sequencing of thousands of patients with monogenic disorders has revealed that about 80% of the identified variants are unique, seen only in one proband, and 96% of the variants were found three times or less.³⁸ Similar to variation data for *RYR1* and *CACNA1S*, variants of unknown significance comprised about 70% of the variants identified in Mendelian genes.

Search for MH-associated Novel Genes and Variants Using Next Generation Sequencing. When applied to studies of rare monogenic or oligogenic diseases, whole exome sequencing allows unbiased, not based on any *a priori* hypothesis, screening of the coding sequence of all of a patient's genes. On the other hand, whole exome sequencing generates a large number of variants in multiple genes whose relevance to a specific phenotype is often difficult to ascertain. At present, the use of next-generation sequencing-based targeted sequencing of a restricted panel of genes associated with a disease phenotype seems to be a more practical approach. Targeted sequencing has a higher coverage (up to 99%) and accuracy and therefore higher sensitivity than whole exome sequencing. Targeted sequencing panels can be also supplemented with Sanger sequencing for regions poorly covered by next-generation sequencing. This gives an added level of coverage and reduces the potential for false-negative results.

Targeted sequencing of panels of genes implicated in excitation–contraction coupling, alongside whole exome sequencing, holds great promise for finding a genetic cause of MH in cases where no *RYR1* and *CACNA1S* variants were found. Four recently published studies used next-generation sequencing-based whole exome sequencing and targeted sequencing of panels of genes with a potential involvement in excitation–contraction coupling, skeletal muscle calcium homeostasis, or

immune response, as well as targeted *RYR1* and *CACNA1S* gene sequencing to search for MH-associated variants and novel MH genes in cohorts of unrelated MH-susceptible patients.^{34,36,39,40} However, these first studies using next-generation sequencing for identification of MH-associated variants did not result in the discovery of novel candidate genes. They confirmed the findings of previous studies, where Sanger sequencing was used for MH variant screening, namely, that variants in *RYR1* and, to a lesser degree in *CACNA1S*, are associated with MH in the majority of MH cases. Known MH pathogenic variants comprised about 30% of the identified variants, and the remaining variants were variants of unknown significance. These studies also found that up to 50% of MH-susceptible individuals do not carry potentially pathogenic variants in either MH gene, corroborating previous evidence.

Rare variants in several additional genes (*CACNB1*, *CASQ1*, *SERCA1*, *CASQ2*, and *KCNA1*) encoding proteins involved in calcium homeostasis in skeletal muscle have been identified using next-generation sequencing⁴⁰ and Sanger sequencing.^{41,42} However, these variants will remain variants of unknown significance until functional assays are developed to validate their role in MH susceptibility.

Current Approaches to Characterization of Potentially Pathogenic *RYR1* and *CACNA1S* Variants. To increase the sensitivity and specificity of genetic testing for MH and expand the number of pathogenic variants that can be used in clinical genetic testing, all MH-associated variants have to be validated at the genetic level, as well as functionally.³³ Advances in our current knowledge about genetic variation within MH genes have prompted changes in the approach to genetic characterization of the variants. Two approaches, genetic and functional characterization, are used in combination to assess the pathogenic effect of novel variants.

Genetic Characterization. Today, instead of genotyping hundreds of control individuals to exclude common variants or neutral polymorphisms, estimation of the variant allele population frequency can be done by searching publicly available variation databases containing data from sequencing more than 67,000 of human exomes (*e.g.*, the Short Genetic Variations database [http://www.ncbi.nlm.nih.gov/SNP; accessed April 8, 2017], the 1000 Genomes project [http://browser.1000genomes.org; accessed April 8, 2017], the NHLBI Grand Opportunity Exome Sequencing Project [https://esp.gs.washington.edu/drupal/; accessed April 8, 2017], the exome variant server [http://evs.gs.washington.edu/EVS/; accessed April 8, 2017], and the Exome Aggregation Consortium Browser [http://exac.broadinstitute.org; accessed April 8, 2017]). Pathogenic variants are likely to have a minor allele frequency not higher than 0.001.³⁴ However, because the majority of identified variants of unknown significance in *RYR1* are rare and have frequencies lower than 0.001, a low frequency of a variant cannot serve as a predictor of its pathogenicity.

The effect of a variant on protein function and/or stability can be assessed using bioinformatics prediction software

tools, such as SIFT,⁴³ PolyPhen-2,⁴⁴ and CADD.⁴⁵ These prediction tools use protein sequence information and annotations to protein functional domains to compute predictions with relatively low false-positive and false-negative error rates.⁴⁶ However, such predictions should be taken with caution. Different prediction tools use different prediction algorithms and different input data sets (disease-associated mutation sets and neutral variation sets identified in the same protein and available from variation databases, such as the Human Gene Mutation database, Online Mendelian Inheritance in Man database, and the Short Genetic Variations database), and their predictions might be discordant. In addition, the bioinformatics tools are based on imperfect algorithms and on imperfect databases.⁴⁷ A recent study compared the predicted and actual consequences of missense mutations and found that half of the *de novo* or low-frequency missense mutations found by genome sequencing and inferred as deleterious correspond to nearly neutral variants that have little impact on the clinical phenotype of individual cases.⁴⁸ Similarly, a significant proportion of *RYR1* sequence variants in the human gene mutation database classified as “disease-causing mutations” was found to be benign, probably benign, or as being of unknown pathogenicity.³⁷ The sensitivity of commonly used bioinformatics prediction tools for *RYR1* and *CACNA1S* has been estimated⁴⁶ at 84 to 100% with specificity of 25 to 83%. Therefore, other approaches such as segregation analysis and functional studies are necessary to accurately differentiate clinically relevant variants from neutral variants.

Another challenge in genetic characterization of a variant of unknown significance is the small size of the families. It is not always possible to perform a meaningful analysis of segregation of the variant with the disease phenotype (susceptibility to malignant hyperthermia, MHS) and to generate sufficient statistical power even when combining data from several families carrying the same variant.^{33,34}

Functional Characterization. Functional characterization of candidate *RYR1* variants remains a key component of their validation. MH-associated *RYR1* variants are dominant gain-of-function variants. They render the RyR1 channels hypersensitive to depolarization and pharmacologic agonists or lead to greater depolarization-induced Ca^{2+} influx into the muscle cell.^{2,32} The effect of each MH candidate variant on RyR1 function should be assayed in one of the recombinant *in vitro* expression systems, HEK293 cells, or myotubes of the dyspedic/dysgenic mouse (*RYR1/CACNA1S* knock-out).^{49,50} These systems use expression of a rabbit or human *RYR1* cDNA construct with incorporated variants and measure the properties of expressed channels. The advantage of *in vitro* systems is the defined cDNA and the standardized genetic background of the recipient cell line. In view of the large number of private familial variants found to date, the revised European Malignant Hyperthermia Group guidelines have removed the need for mandatory description of the variant in more than one family, if functional characterization is

done using the more rigorous genetic manipulation of heterologous or homologous expression systems.³³

Systems using *ex vivo* expression utilize tissues from MHS patients with characterized *RYR1* variants such as myotubes, microsomal sarcoplasmic reticulum preparations from muscle biopsies, or lymphoblasts.³³ Assays of RyR1 function in *ex vivo* systems are controversial, because they assume that the identified gene variant is the only variant present, when this may not be the case. The compromise presented in the European Malignant Hyperthermia Group guideline is the stipulation that *ex vivo* analyses should be done on samples from at least two unrelated patients with the same variant to reduce the likelihood of confounding genetic factors.

The first knock-in mouse models of MH carrying *RYR1* variants analogous to the MH pathogenic human variants Tyr522Ser⁵¹ and Arg163Cys⁵² and a mouse model of central core disease carrying an equivalent of the human uncoupling central core disease mutation Ile4898Thr^{53,54} allowed *in vitro* and *in vivo* functional studies of these mutations in fully differentiated adult muscle fibers. However, generation of mouse models for validation of each of more than 150 MH-associated variants is not realistic. To circumvent this obstacle, a promising novel approach has been developed to study the function of RyR1 mutant channels.⁵⁵ Using localized *in vivo* electroporation, Lefebvre *et al.*⁵⁵ have expressed constructs of RyR1 N-terminally tagged with enhanced green fluorescent protein carrying MH variants in fully differentiated normal mouse muscle fibers and found that the results were consistent with those obtained for MH variants in previous studies. They showed that expression of the RyR1 channels carrying MH mutations, Tyr523Ser, Arg615Cys, or Arg2163His, was associated with an increased Ca^{2+} release in response to depolarization, whereas expression of the central core disease mutant, Ile4897Thr, resulted in a reduction of Ca^{2+} release compared to nonexpressing regions of the same muscle cell. These results indicate that *in vivo* expression in adult mouse muscles might serve as a novel technique for assessment of functional properties of mutant RyR1s.

Some of the *RYR1* variants have been already functionally and genetically characterized and found to be likely or very likely pathogenic. The revised European Malignant Hyperthermia Group guidelines recommend an individual carrying a potentially MHS-associated variant to be considered as being at risk for MH until contracture testing can be performed.

Cryoelectron Microscopy and X-ray Crystallography Contribution to Functional Assessment of *RYR1* Variants.

Recent determination of crystal structures for the N-terminal domains together with the development of cryoelectron microscopy (cryo-EM) maps of the full-length RyR at nanometer resolution allowed elucidation of the three-dimensional architecture and domain organization of RyR1 and facilitated modeling interactions between its N-terminal, central, and C-terminal domains. These studies revealed how small conformational changes in the cytoplasmic domains,

induced by the binding of RyR regulators, are transmitted to the C-terminal domain regulating the channel opening.^{55–58} They showed that RyR1 channel opening coincides with subtle changes in the cytoplasmic domain that affect interfaces between individual RyR1 subunits. Mapping the MH/central core disease variant hot spots and individual disease-related variants onto the high resolution structure of RyR1 domains helped reveal mechanisms by which disease-related *RYR1* variants might disrupt RyR1 function.^{59,60} Because most of the N-terminal MH-associated variants are mapped onto the interfaces between N-terminal domains or at interfaces between subunits in the tetrameric channel, it is probable that those variants weaken the interdomain interactions, thus lowering the energetic barrier to channel opening.^{61–63}

The high resolution (near 3Å) cryo-EM images of the transmembrane region that contains the ion conducting pore revealed the presence of the six membrane-spanning helices (S1 to S6) of each RyR1 subunit and allowed mapping of the majority of central core disease-associated variants to the pore-forming domain^{64,65} (fig. 1).

Ramachandran *et al.*,⁶⁵ by using homology modeling and high resolution cryo-EM data, succeeded in identification a novel interface between the pore-lining helix (amino acids 4,912 to 4,948) and a S4–S5 linker helix (amino acids 4,830 to 4,841) and showed that this interface controls RyR gating. They built structural models for the RyR1 membrane-spanning domains based on the alignment between RyR1 and two other ion channels with known crystal structures, docked the structural models onto the cryo-map, and showed the close fit between them, indicating that their

structural model is suited to model interactions involved in RyR1 gating. Using the models of membrane-spanning domains in the open and closed state, they showed the S4–S5 linker helix interacts with the S6 helix and thus plays a role in gating. Based on this model, they computationally predicted the effect of several variants within S4–S5 linker on RyR1 gating. They further expressed in HEK-293 cells recombinant wild-type and mutant RyR1 channels carrying the same variants and used single-channel experiments to characterize the channels. The effect of each of the variants on channel gating (activating for some and deactivating for other variants) was similar to that predicted by their structural models, thus confirming the role of the S4–S5 linker helix in RyR1 gating.

Generation of structural models of the open and closed states of RyR1 facilitated comparison of computational impact predictions of C-terminal variants with the results of single-channel experiments and identification of amino acid residues in the predicted pore-lining helix and a linker helix that are important for channel gating.^{65,66}

Future studies will further refine the three-dimensional RyR1 structure and elucidate the complex molecular mechanisms involved in RyR1 channel regulation and function, thus allowing a more reliable computational prediction of the functional impact of newly discovered variants.

Incidental Findings. Whole exome sequencing generates a large number of variants in multiple genes, and some of those variants might be of clinical relevance to a condition that is different from the original clinical condition for which whole exome sequencing was offered. Such variants are called

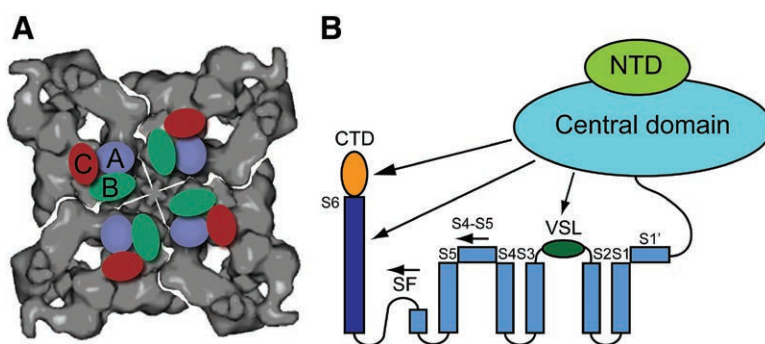


Fig. 1. (A) A schematic illustration of the N-terminal domains docked in the pseudoatomic model of the RyR1 tetramer. The RyR1 N-terminal domain (NTD), corresponding to malignant hyperthermia (MH) hot spot 1, is composed of three subdomains: A, B, and C. Interactions among the domains A, B, and C on the same RyR1 subunit together with the interactions between domains of the neighboring subunits are involved in the global conformational RyR1 transmissions that control effector-induced channel gating. The variants impair the domain–domain interactions and thus would cause the RyR1 channel dysfunction. (B) A schematic model of domain organization in a RyR1 monomer, composed of the NTD, the central domain, and the channel domain. Each domain consists of several interconnected subdomains. The channel domain consists of the six transmembrane fragments (S1 to S6), and pore helices with the selectivity filter (SF), the linker helix of S4 and S5, the voltage sensor-like domain (VSL), and the C-terminal domain (CTD). Binding Ca^{2+} to the central domain initiates a cascade of conformational transmissions via allosteric intradomain and interdomain interactions from the central domain to the NTD, to the VSL, the CTD and S6, ultimately inducing opening of the channel.⁶⁴ Together with the amino acids forming the ion channel—the pore helix and the selectivity filter (amino acids 4,894 to 4,900); the S4–S5 linker (amino acid 4,830 to 4,841); Gly4934, which serves as a “hinge” for the outward movement of the helix S6, and the CTD (amino acids 4,957 to 5,033) are all critical for RyR1 channel gating (modified with permission from Wei *et al.*).⁶⁴

incidental findings. The American College of Medical Genetics and Genomics has included MH genes *RYR1* and *CACNA1S* among the list of clinically relevant genes, whose potentially pathogenic variants should be reported as incidental findings.⁶⁷ Reporting of incidental findings, however, has created clinical and ethical challenges.^{47,67–69} The American College of Medical Genetics and Genomics guidelines on reporting the incidental findings emphasize the need for accurate assessment of clinical and research evidence supporting a variant's pathogenicity before reporting it to a patient. The guidelines also caution against excessive reliance on *in silico* predictions of pathogenicity in the diagnostic context. In light of the prevalence of rare variants in *RYR1* and *CACNA1S* and the difficulty in assessing their pathogenicity, it is likely that a significant number of patients undergoing whole exome sequencing for non-MH indications will be labeled as potentially susceptible to MH when only a small minority will be at risk.

***RYR1*-related Disorders**

In addition to MH, variants in *RYR1* have been previously associated with several other skeletal muscle conditions and congenital myopathies, namely, central core disease, multiminicore disease, congenital myopathy with central or internalized nuclei, and congenital fiber-type disproportion.^{14,15,70,71} To this list, King–Denborough syndrome,⁷² benign Samaritan congenital myopathy,⁷³ heat/exercise-induced exertional rhabdomyolysis,^{16,74} atypical periodic paralysis,⁷⁵ and statin myopathies^{76,77} were recently added.

RYR1-related congenital myopathies show both dominant (central core disease) and recessive (multiminicore disease, centronuclear myopathy, and congenital fiber-type disproportion) modes of inheritance. Moreover, some *RYR1* variants may act as dominant with regard to the MH phenotype but as recessive with regard to the congenital myopathy phenotype.^{78,79}

These myopathies present a challenge for clinical molecular diagnosis due to their strong phenotypic and genetic heterogeneity. A recent study applied an integrated strategy combining whole exome sequencing with clinical and histopathologic investigations to reach an accurate diagnosis for several patients with congenital myopathies. Different sets of recessive *RYR1* variants were found in four patients whose phenotypes ranged from a severe lethal neonatal myopathy to a mild adult-onset muscle weakness, underscoring the phenotypic variability of *RYR1*-related disorders.⁸⁰

Next-generation sequencing panel-based analysis of neonatal hypotonia in a Chinese cohort found several *RYR1* variants in this genetically heterogeneous condition, although in the majority of cases these were heterozygous changes involving variants for which a dominant pathogenic effect is not established.⁸¹

In another study, whole exome sequencing allowed identification of a *de novo* *RYR1* variant in a patient who was originally diagnosed with limb girdle muscular dystrophy on the basis of clinical and histologic presentations: the

histologic features were in fact myopathic rather than dystrophic, emphasizing the importance of establishing a genetic diagnosis to exclude an *RYR1* etiology.⁸²

The spectrum of *RYR1*-related diseases was expanded further to include a myasthenic-like component of muscle weakness with partial response to pyridostigmine: direct sequencing of the *RYR1* gene in this case revealed compound heterozygous *RYR1* variants: c.6721C>T (p.Arg2241X) nonsense variant and novel c.8888T>C (p.Leu2963Pro) missense variant.⁸³

The role of RyR1 is not limited to skeletal muscle: a mouse model of central core disease, homozygous for a dominant *RYR1* variant that causes a loss of function of the RyR1 channel, exhibited embryonic developmental delay and neonatal lethality with multisystem developmental defects, including atrial septal defect: it was hypothesized that RyR1 plays an important role in early cardiac development.⁵³ In favor of this hypothesis, exome sequencing revealed two rare, potentially deleterious missense *RYR1* variants in a patient with atrioventricular septal defect who had no potentially pathogenic variants in other candidate genes.⁸⁴

RYR1 variants have been previously associated with fetal akinesia.⁷⁰ Several recent studies used whole exome sequencing to expand the phenotypes associated with recessive *RYR1* variants to include arthrogryposis multiplex congenital fetal akinesia,^{85,86} and lethal multiple pterygium syndrome.⁸⁷ Lethal multiple pterygium syndrome is a fatal disorder associated with prenatal growth failure with pterygium present in multiple areas, akinesia, and severe arthrogryposis. Lethal multiple pterygium syndrome has been associated with variants in genes encoding components of the neuromuscular junction. Identification of *RYR1* variants in fetuses affected by lethal multiple pterygium syndrome, together with variants in genes encoding proteins at the neuromuscular junction (*CHRNA1*, *CHRNA2*, *CHRNA3*, and *RAPSN*), might indicate that lethal multiple pterygium syndrome is caused by defects in the excitation–contraction coupling mechanism.⁸⁷ Interestingly, lethal multiple pterygium syndrome in association with MH has been described before.⁸⁸

Whole exome sequencing analysis revealed the first case of severe congenital myopathy with ophthalmoplegia caused by a variant in the *CACNA1S* gene,⁸⁹ pathogenic variants in which have been associated with hypokalemic periodic paralysis type 1.^{90,91} The authors hypothesize that the p.Gln1265His variant results in disruption of coupling between dihydropyridine receptor and RyR1, causing *CACNA1S*-related myopathy. Interestingly, another patient from this study with similar myopathic symptoms was found to carry an in-frame insertion in *RYR1*.⁹² The authors hypothesized that because this variant showed dominant inheritance, it likely had a dominant-negative effect on RyR1 tetramer formation and function.

Whole exome sequencing of patients presenting with severe congenital ophthalmoplegia and facial weakness in association with malignant hyperthermia revealed the presence of missense variants resulting in two homozygous *RYR1*

amino acid substitutions and two compound heterozygous *RYR1* substitutions in a consanguineous and a nonconsanguineous pedigree, respectively.⁹³ Whereas ophthalmoplegia may occur in *RYR1*-related myopathies, these children were atypical because they lacked significant muscle weakness, respiratory insufficiency, or scoliosis. The common *RYR1* variant in these cases, p.R3772Q, was previously reported to be associated with MH susceptibility in the heterozygous state and MH susceptibility with myopathy in the homozygous state.⁷⁹

Another interesting case is of congenital ptosis, scoliosis, and MH susceptibility in siblings who are homozygous for the MH-pathogenic *RYR1* variant, p.T2206M.⁹³ This variant in heterozygous carriers was previously reported in association with mild clinical and histopathologic features.⁹⁴ The last two studies emphasize the notion that *RYR1*-associated myopathies should be included in the differential diagnosis of congenital ptosis with scoliosis and of congenital ophthalmoplegia and facial weakness without scoliosis, especially because a risk of MH can be high in these patients.

This wide spectrum of clinicopathologic conditions reflects the distinct effects of different *RYR1* variants on skeletal muscle Ca^{2+} homeostasis and excitation–contraction coupling.⁹⁵ Functional studies showed that different central core disease variants exhibited varying degrees of excitation–contraction uncoupling with impaired Ca^{2+} release. Certain dominant variants displayed dual functional characteristics accounting for both MH (hypersensitivity to voltage activation and to agonists) and myopathy (reduced sarcoplasmic reticulum Ca^{2+} content and voltage-gated Ca^{2+} release) phenotypes.⁹⁶ Additionally, some recessive *RYR1* variants led to a reduction in RyR1 protein levels.^{2,32}

Complexity of functional effects of *RYR1* variants together with clinical overlap between different *RYR1*-related myopathies complicates MH susceptibility counseling in patients with *RYR1*-related myopathies. Certainly, patients with myopathies carrying MH-associated *RYR1* variants, as well as potentially pathogenic variants of unknown significance, should avoid triggering anesthetics. However, patients carrying uncoupling, loss-of-function *RYR1* variants may be considered as being at a low risk of developing MH. Counseling in *RYR1*-related myopathic patients as for MH susceptibility requires a combined approach, integrating clinical, histopathologic, *in vitro* contracture testing, magnetic resonance imaging, and genetic findings.³

***RYR1* in Nonskeletal Muscle Cells.** Lopez *et al.*¹³ reported that some MH-susceptible patients, carrying specific gain-of-function *RYR1* variants, give a history of mild bleeding abnormalities. They demonstrated that RyR1^{Y522S} mice carrying the MH gain-of-function variant had abnormalities of vascular smooth muscle cell Ca^{2+} homeostasis consistent with a bleeding phenotype.¹³ Indeed, although RyR1 is predominantly found in skeletal muscle, it is also present at lower levels in immune and smooth muscle cells. The study found that primary vascular smooth muscle cells from RyR1^{Y522S} mice

had an increased frequency of Ca^{2+} spark events and were significantly more hyperpolarized than those from wild-type mice. In contrast to skeletal muscle cells where gain-of-function *RYR1* variants led to an increased sensitivity to activating stimuli and to sustained muscle contractions, primary vascular smooth muscle cells from RyR1^{Y522S} mice showed a decreased Ca^{2+} influx through the dihydropyridine receptor and smooth muscle relaxation, causing prolonged rather than shorter bleeding times. Administration of the specific RyR1 antagonist dantrolene, which is clinically approved for the treatment of MH reactions, reversed the bleeding phenotype by decreasing spark activity in murine vascular smooth muscle cells. Thus, this study suggested that *RYR1* variants may be responsible for certain cases of mild bleeding abnormalities. If the clinical findings of Lopez *et al.*¹³ of prolonged bleeding in MH patients carrying *RYR1* variants are confirmed, their animal studies offer a pathologic mechanism and indicate a potential therapeutic use of dantrolene for such cases.

MH, a Metabolic Disorder

Because RyR1 plays an essential role in maintenance of Ca^{2+} homeostasis and in excitation–contraction coupling in skeletal muscle cells,² MH-susceptible individuals carrying *RYR1* variants may have skeletal muscle metabolism abnormalities even in the absence of triggering anesthetics.^{97,98} Studies on animal models of MH^{51,99,100} have shown that MH-associated *RYR1* variants result in a significant increase in mitochondrial matrix Ca^{2+} , increased reactive oxygen species production, and lower expression of mitochondrial proteins, which in conjunction with lower myoglobin and glycogen contents and lower glucose utilization suggested a compromised bioenergetics state. Furthermore, the elevation in resting myoplasmic Ca^{2+} may lead to an enhanced oxidation of RyR1, which in turn may increase open channel probability, enhanced Ca^{2+} -induced Ca^{2+} release,¹⁰¹ thus increasing muscle sensitivity to heat and other stimuli.¹⁰² Such studies might explain the connection between MH and exertional rhabdomyolysis (ER) and/or exercise-related or exertional heat illness (EHI). The most severe form of EHI, exertional heat stroke (EHS), is characterized by a rapid increase in body temperature and neurologic impairment, with rhabdomyolysis as a common feature.^{75,103} It occurs during sustained exercise frequently in physically fit young adults and children, especially under hot or/and humid ambient conditions but it may occur in temperate climates.^{16,34,104}

ER often presents with severe muscle pain and is diagnosed by elevated serum creatine kinase levels five times higher than the upper limit of normal values.^{16,103} ER is one of the frequent signs of EHI but often does not involve a drastic increase in body temperature. EHS and MH share clinical features such as hyperthermia, muscle rigidity, tachycardia, tachypnea, elevated serum creatine kinase, and disseminated intravascular coagulation; and skeletal muscle breakdown may cause hyperkalemia, myoglobinuria, and acute kidney injury. On the basis of

abnormal *in vitro* contracture test results in survivors of EHS and their first degree relatives, it was postulated that there may be a familial skeletal muscle abnormality in some EHS patients similar to that in MH, *i.e.*, uncontrolled increase in intracellular calcium and hypermetabolism.¹⁰⁵

Dantrolene, the only drug available for treating MH, has been shown to reduce clinical symptoms in patients with ER⁷⁴ supporting a notion of a common pathologic pathway of these potentially fatal conditions.

Recurrent ER as an inherited condition has been linked to defects in the genes known to be associated with a number of neuromuscular disorders, such as metabolic myopathies and muscular dystrophies, many of which are autosomal-recessive or X-linked.^{106,107}

Recent genetic studies have suggested that *RYR1* variants may be implicated in EHI/ER.^{16,17} Moreover, identification of MH-associated *RYR1* variants in up to 30% of cases of recurrent ER^{16,98,108–110} has strengthened the possibility of a link between MH and EHI/ER. This possible link, important to MH researchers, clinicians, and especially patients with MHS and EHI, has been discussed in several publications.^{16,103,111} We have considered two questions in summarizing the clinical and genetic evidence regarding the relationship between MH and EHI/ER.

The First Question: Are MH-susceptible Patients at a Higher Risk of EHI/ER? Based on the clinical data available on MH-susceptible patients, there seems to be no strong correlation between MH susceptibility and predisposition to EHI. There are numerous MH families with at least one family member who survived an MH crisis under general anesthesia. Hundreds of individuals from these families have been diagnosed as MH susceptible by the *in vitro* contracture testing and additionally have been found to carry one of the known MH pathogenic variants.¹¹² These MH-susceptible individuals show no apparent predisposition to EHI and are reported to be clinically healthy.^{2,8} Moreover, none of the MH-susceptible individuals who are homozygous for MH pathogenic variants: p.Arg614Cys, the analog of the p.Arg615Cys porcine stress syndrome mutation,^{20,113–115} and p.Cys35Arg,¹¹⁶ nor individuals who are compound heterozygous for two MH variants have been reported to present with any clinical symptoms suggestive of EHI.^{8,20,26,115} Indeed, there have been only a few documented cases of patients with a previous personal or family history of MH who later in life experienced an EHS/EHI episode.^{103,109,117,118} There is perhaps a greater risk of MH-susceptible individuals developing ER than EHI. In addition to reported cases,¹⁷ the authors have been contacted by several patients tested MH susceptible in their units who have developed ER. Nevertheless, these observations do not support a notion of all MH-susceptible patients being at an increased risk of EHI/ER.

The Second Question: Are EHI/ER Patients at Risk of MH? The notion that EHI patients may be predisposed to MH mostly stems from the reports of positive *in vitro* contracture testing results, *i.e.*, MHS diagnoses in a substantial number

of EHI patients.^{18,35,98,105,108,119–121} It is important to emphasize that the *in vitro* contracture tests have been validated only for patients with a suspected anesthetic-induced MH reaction, and the sensitivity and specificity of the *in vitro* contracture testing in patients with EHI/ER are unknown. Patients with certain myopathies, such as muscular dystrophies and muscle channelopathies, may have abnormal *in vitro* contracture test results due to their persistent muscle cell abnormalities not necessarily related to MH.^{122–124}

The genetic connection between EHI/ER and MH also remains inconclusive.^{17,19,34,97,120} An increasing number of reported EHI/ER cases with MH-associated *RYR1* variants favor this connection, and undoubtedly, EHI/ER patients carrying MH-associated variants should be considered MH susceptible until demonstrated otherwise. However, in more than 70% of the EHI/ER patients, the identified *RYR1* variants are rare variants of unknown functional significance.^{16,34,110} The relevance of these variants of unknown significance to either MH susceptibility or EHI/ER remains unclear, especially considering some rare, potentially deleterious variants of unknown significance have been identified in EHI patients who have had normal *in vitro* contracture responses, whereas others are found in EHI patients with abnormal *in vitro* contracture responses. Remarkably, among the numerous EHI/ER cases with identified *RYR1* variants, there have been no reports of personal or familial history of MH; only one episode of MH in a patient who had a previous ER event has been reported so far.⁷⁴

Thus, although current studies cannot rule out a possible connection between MH and EHI/ER, the extent of the overlap between EHI/ER and MH remains unknown. Both conditions have a complex etiology and in the majority of cases probably result from the interplay between genetic and environmental factors and indeed, in some cases, possibly multiple genetic factors. Importantly, in patients with ER or EHI differential diagnoses, such as muscular dystrophies and metabolic and mitochondrial disorders, should be considered. To make an efficient and definitive diagnosis in a clinically and genetically heterogeneous condition, such as ER, targeted parallel sequencing of a panel of candidate genes using next-generation sequencing seems to be an especially appropriate approach. Currently some laboratories offer screening of panels of genes using next-generation sequencing technologies for patients with myopathy/rhabdomyolysis; some of those in North America are Baylor Miraca Genetics Laboratories, Baylor College of Medicine (USA), Prevention-Genetics (USA), and Fulgent Therapeutics LLC (USA).

The existence of a possible link between ER and disorders of muscle calcium metabolism warrant an expansion of the next-generation sequencing gene panel for myopathy/rhabdomyolysis to include the genes involved in excitation-contraction coupling, such as *RYR1* and *CACNA1S*. Indeed, several centers have already included the *RYR1* gene in their expanded panels, *e.g.*, next-generation sequencing rhabdomyolysis and metabolic myopathies panel (Greenwood

Genetic Center Diagnostic Laboratories, USA) and metabolic myopathy and rhabdomyolysis panel (Blueprint Genetics, Finland).

Summary and Implication for Anesthesiologists

With the discovery of genes associated with MH (*RYR1*, *CACNA1S*, and *STAC3*) and developments in genetic screening tools, such as next-generation sequencing, our understanding of the genetics of MH has improved dramatically. The cost effectiveness of next-generation sequencing has enabled genetic testing for MH susceptibility to be a viable first-line diagnostic test for patients suspected of having an MH reaction under anesthesia and for relatives of known MH cases in many countries.

However, initial experience with next-generation sequencing-based whole exome sequencing studies has not brought about an anticipated increase in the sensitivity and specificity of MH genetic testing. Whole exome sequencing applied to MH cohorts has revealed that up to 50% of MH-susceptible individuals do not carry potentially pathogenic variants in known MH-associated genes, corroborating previous evidence for the genetic heterogeneity of MH susceptibility. Additionally, due to the possible presence of more than one pathogenic variant in the same family, MH susceptibility cannot be ruled out for individuals who do not carry a familial variant, leaving muscle contracture testing as the only reliable diagnostic test for MH susceptibility for such patients. The specificity (true negative rate) of genetic testing is limited by the fact that of more than 200 *RYR1* variants identified in MH families, only 35 *RYR1* and 2 *CACNA1S* variants are sufficiently characterized to be regarded as pathogenic for MH. The majority of newly identified *RYR1* and *CACNA1S* variants remain to be functionally evaluated as to their role in MH. Individuals carrying variants of unknown significance in these genes should therefore be considered as being at risk of developing MH under anesthesia and should be offered contracture testing to ascertain their MH status.

The relatively low cost of whole exome sequencing, the prevalence of rare variants of unknown significance in *RYR1* and *CACNA1S*, and the designation of these variants of unknown significance as reportable incidental findings has already led to many people under investigation for other diseases or even those just curious about their genetic heritage being labeled as potentially at risk of developing MH. Such labeling is not risk-neutral for anesthesia and can also have implications for the individuals and their family with respect to eligibility for certain occupations, ease of access to insurance policies, and concerns regarding some overseas travel. We would therefore question the ethics of offering sequencing of *RYR1* and *CACNA1S* to individuals at low *a priori* risk without appropriate counseling and funded access to definitive *in vitro* contracture testing for MH susceptibility diagnosis should variants of unknown significance be found.

The phenotypic variability exposed in recent studies of *RYR1*-related disorders has taught us that abnormalities

in this gene may confer not only a potentially life-threatening reaction to anesthesia but may predispose individuals to myopathies, metabolic derangements, EHI/ER and even possibly bleeding disorders. Specifically, anesthesiologists should insist on a genetic workup for *RYR1* variants in patients with a previous history of recurrent rhabdomyolysis or those with congenital myopathies without a genetic diagnosis, before administration of triggering anesthetics. Such patients in whom a *RYR1* variant is found or indeed patients with a known *RYR1*-related myopathy should be referred to a specialized MH center for assessment of their MH risk and advice on further investigation.

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Competing Interests

The authors declare no competing interests.

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