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Malignant Hyperthermia in the Post-Genomics Era: New Perspectives on an Old Concept

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Brief Summary Statement (32 words).

This review summarizes evidence on the genetics of MH, its complexity and development of new genetic techniques. It also discusses the connection of MH and RYR1 related disorders to other morbid phenotypes.

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Summary of the key points of the article (63 words)

This article reviews the advancements in genetic understanding of malignant hyperthermia. It summarizes the new technologies and approaches for diagnosis of MH, and discusses the existing limitations due to the complexity of the genetics of MH. It discusses 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.

Introduction

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 definitively 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 susceptibility[13] 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 non-anesthetic phenotypes associated with RYR1-related disorders.

A- 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 1:2000 and 1:3000.[20] Interestingly, the combined prevalence in the ExAC Browser database (http://exac.broadinstitute.org/gene/ENSG00000196218 accessed 4.02.2017) of functionally characterized genetic variants that have been associated with MH is 1:2750. 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 and colleagues when they reported the first case[22]. 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 non-genetic reasons, as 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, 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 pharmacological challenge of excised skeletal muscle strips in an in vitro contracture test. These tests are known as the caffeine-halothane contracture test (CHCT) in North America and the in vitro contracture test (IVCT) in Europe. However, because these tests are invasive and costly, family studies are limited in some countries, while 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 to propose a threshold genetic model for MH susceptibility[24]. 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 – Ca^{2+}release channel of skeletal muscle sarcoplasmic reticulum (RyR1), has been established as the major gene implicated in MH. Since the report[7] 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 alpha-1S subunit of the T-tubular voltage-gated Ca^{2+} channel $Ca_v1.1$, also known as the dihydropyridine receptor (DHPR).[9-11,27] The alpha-1S is important for the voltage sensing and Ca^{2+} conduction of the DHPR.

Yet, 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 is thought to be required for effective co-location of DHPRs and RyR1s.

RyR, DHPR and STAC3 are all essential components of the skeletal muscle excitation-contraction coupling complex.[30] The underlying mechanism of MH is disruption of EC-coupling resulting in abnormally enhanced Ca^{2+} 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 EC coupling was similar to that shown for RYR1 mutations, i.e. expression of the mutant alpha-1S in dysgenic myotubes (lacking alpha-1S) resulted in an enhanced sensitivity of RyR1 to stimuli compared with the effect of wild-type alpha-1S.[31] It was suggested that alpha-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 (www.emhg.org) 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 as 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.[33]

B- New genetic technology and MH

Next-generation sequencing (NGS) enables fast and cost-efficient sequencing of all protein coding regions - exons - in the human genome. The 1000 Genomes Project (2008-2015)[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 NGS 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 ESP dataset, that includes variation data from 6500 exomes (http://evs.gs.washington.edu/EVS/), 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 dataset 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 (VUS). 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% as VUS.

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, VUS 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 (WES) 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, WES 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 NGS-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 WES. Targeted sequencing panels can be also supplemented with Sanger sequencing for regions poorly covered by NGS. 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 WES, 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 NGS-based WES and targeted sequencing of panels of genes with a potential involvement in EC 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 NGS 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 VUS. 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, KCNA1) encoding proteins involved in calcium homeostasis in skeletal muscle have been identified using NGS[40] 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., dbSNP (http://www.ncbi.nlm.nih.gov/SNP), 1000 Genomes (http://browser.1000genomes.org), ESP_Cohort_Populations ESP6500 (https://esp.gs.washington.edu/drupal/), exome variant server (http://evs.gs.washington.edu/EVS/), ExAC Browser (http://exac.broadinstitute.org/gene/)]. Pathogenic variants are likely to have a minor allele frequency not higher than 0.001.[34] However, since the majority of identified VUS 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 datasets (disease-associated mutation sets and neutral variation sets identified in the same protein and available from variation databases OMIM, HGMD, NCBI dbSNP and UniProtKBd), and their predictions might be discordant. Besides, 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 (HGMD) classified as ‘‘disease-causing mutations’’ was found to be benign, probably benign, or as being of unknown pathogenicity (VUS).[37] The sensitivity of commonly used bioinformatics prediction tools for RYR1 and CACNA1S has been estimated[46] at 84% -100% with specificity of 25% -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 VUS 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 (MHS phenotype) 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 pharmacological 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 a large number of private familial variants found to date, the revised EMHG 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 (EMHG) 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 Y522S[51] and R163C[52] and a mouse model of CCD carrying an equivalent of the human uncoupling CCD mutation I4898T[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 and colleagues have expressed EGFP-RyR1(RyR1 N-terminally tagged with Enhanced Green Fluorescent Protein) constructs 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.[55] 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 CCD mutant, Ile4897Thr, resulted in a reduction of Ca^{2+} release

compared to non-expressing 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 EMHG 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.

Cryo-electron 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 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.[56-58] They showed that RyR1 channel opening coincides with subtle changes in the cytoplasmic domain that affect interfaces between individual RyR1 subunits. Mapping the MH/CCD 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] Since 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 6 membrane-spanning helices (S1–S6) of each RyR1subunit and allowed mapping of the majority of CCD-associated variants to the pore forming domain.[64,65] (See supplementary figure)

Ramachadran and colleagues[65] by using homology modeling and high resolution cryo-EM data, succeeded in identification a novel interface between the pore-lining helix (amino acids 4912-4948) and a S4-S5 linker helix (amino acids 4830-4841) 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. They 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 mutations within S4-S5 linker on RyR1 gating. They further expressed recombinant wild-type and mutant RyR1 channels carrying the same mutations in HEK-293 cells and used single channel experiments to characterize the mutant channels. The effect of each of the mutations on channel gating (activating for some and deactivating for other mutations) 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

WES 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 WES was offered. Such variants are called incidental findings. The American College of Medical Genetics and Genomics (ACMG) 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 ACMG 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 WES for non-MH indications will be labelled as potentially susceptible to MH when only a small minority will be at risk.

C- RYR1-related disorders

Besides MH, variants in RYR1 have been previously associated with several other skeletal muscle conditions and congenital myopathies, namely, central core disease (CCD), multiminicore disease (MmD), congenital myopathy with central or internalized nuclei and congenital fiber-type disproportion (CFTD).[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 (CCD) and recessive (MmD, CNM, CFTD) 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 WES with clinical and histopathological investigations to reach an accurate diagnosis for several patients with congenital myopathies. Different sets of recessive RYR1 variants were found in 4 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]

NGS 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, WES allowed identification of a de novo RYR1 variant in a patient who was originally diagnosed with limb girdle muscular dystrophy (LGMD) on the basis of clinical and histological presentations: the histological features were in fact myopathic rather than dystrophic,

emphasizing the importance of establishing a genetic diagnosis in order 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 pyridostigimine: 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 CCD, 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 WES to expand the phenotypes associated with recessive RYR1 variants to include arthrogryposis multiplex congenital fetal akinesia,[85,86] and lethal multiple pterygium syndrome (LMPS, OMIM #253290).[87] LMPS is a fatal disorder associated with prenatal growth failure with pterygium present in multiple areas, akinesia, and severe arthrogryposis. LMPS has been associated with variants in genes encoding components of the neuromuscular junction. Identification of RYR1 variants in fetuses affected by LMPS, together with variants in genes encoding proteins at the neuromuscular junction (CHRNA1, CHRND, CHRNG and RAPSN), might indicate that LMPS is caused by defects in the excitation-contraction (EC) coupling mechanism.[87] Interestingly, LMPS in association with MH has been described before.[88]

WES 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 (HOKPP1).[90,91] The authors hypothesize that the p.Gln1265His variant results in disruption of coupling between DHPR 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.[89] The authors hypothesized that since this variant showed dominant inheritance, it likely had a dominant negative effect on RyR1 tetramer formation and function.

WES 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 non-consanguineous pedigree, respectively.[92] While 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 histopathological 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 clinico-pathological conditions reflects the distinct effects of different RYR1 variants on skeletal muscle Ca^{2+} homeostasis and EC coupling.[95] Functional studies showed that different CCD variants exhibited varying degrees of EC 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 SR 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 RYR1variants 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, histopathological, in vitro contracture testing, MRI and genetic findings.[3]

RYR1 in non-skeletal muscle cells

Lopez and colleagues reported that some MH susceptible patients, carrying specific gain-of-function RYR1 variants, give a history of mild bleeding abnormalities.[13] 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 of prolonged bleeding in MH patients carrying RYR1 variants are confirmed, their animal studies offer a pathological mechanism and indicate a potential therapeutic use of dantrolene for such cases.

D- MH, a metabolic disorder

Since RyR1 plays an essential role in maintenance of Ca^{2+} homeostasis and in EC 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 (ROS) 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 neurological 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]

Exertional rhabdomyolysis (ER) often presents with severe muscle pain and is diagnosed by elevated serum CK 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; skeletal muscle breakdown may cause hyperkalemia, myoglobinuria and acute kidney injury. On the

basis of abnormal IVCT 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 ER[74] supporting a notion of a common pathological 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 CHCT/IVCT 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.R614C, the analog of the p.R615C porcine stress syndrome mutation,[20,113-115] and p.C35R,[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.

Second question: Are EHI/ER patients at risk of MH?

A notion that EHI patients may be predisposed to MH mostly stems from the reports of positive IVCT/CHCT contracture testing results, i.e. MHS diagnoses in a substantial number of EHI patients.[18,34,98,105,108,119-121]

It is important to emphasize that the CHCT/IVCT tests have been validated only for patients with a suspected anesthetic-induced MH reaction, and the sensitivity and specificity of CHCT/IVCT testing in patients with EHI/ER are unknown. Patients with certain myopathies, such as muscular dystrophies and muscle channelopathies, may have abnormal CHCT/IVCT 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 (VUS).[16,34,110] The relevance of these VUS to either MH susceptibility or EHI/ER remains unclear, especially since some rare, potentially deleterious VUS have been identified in EHI patients who have had normal IVCT responses, while others are found in EHI patients with abnormal IVCT 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, 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 NGS seems to be an especially appropriate approach. Currently some laboratories offer screening of panels of genes using NGS technologies for patients with myopathy/rhabdomyolysis, among those in North America are Baylor Miraca Genetics Laboratories, Baylor College of Medicine (http://bmgl.com/) and Fulgent Therapeutics LLC (https://fulgentgenetics.com/).

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

Rhabdomyolysis and Metabolic Myopathies Panel (the Greenwood Genetic Center Diagnostic Laboratories http://www.ggc.org/diagnostic.html) and Metabolic Myopathy and Rhabdomyolysis Panel (Blueprint Genetics http://blueprintgenetics.com).

E- 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 NGS, our understanding of the genetics of MH has improved dramatically. The cost-effectiveness of NGS 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 NGS-based WES studies has not brought about an anticipated increase in the sensitivity and specificity of MH genetic testing. WES 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 of genetic testing is limited by the fact that out 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 WES, the prevalence of rare VUS in RYR1 and CACNA1S, and the designation of these VUS 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 a VUS 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 work-up 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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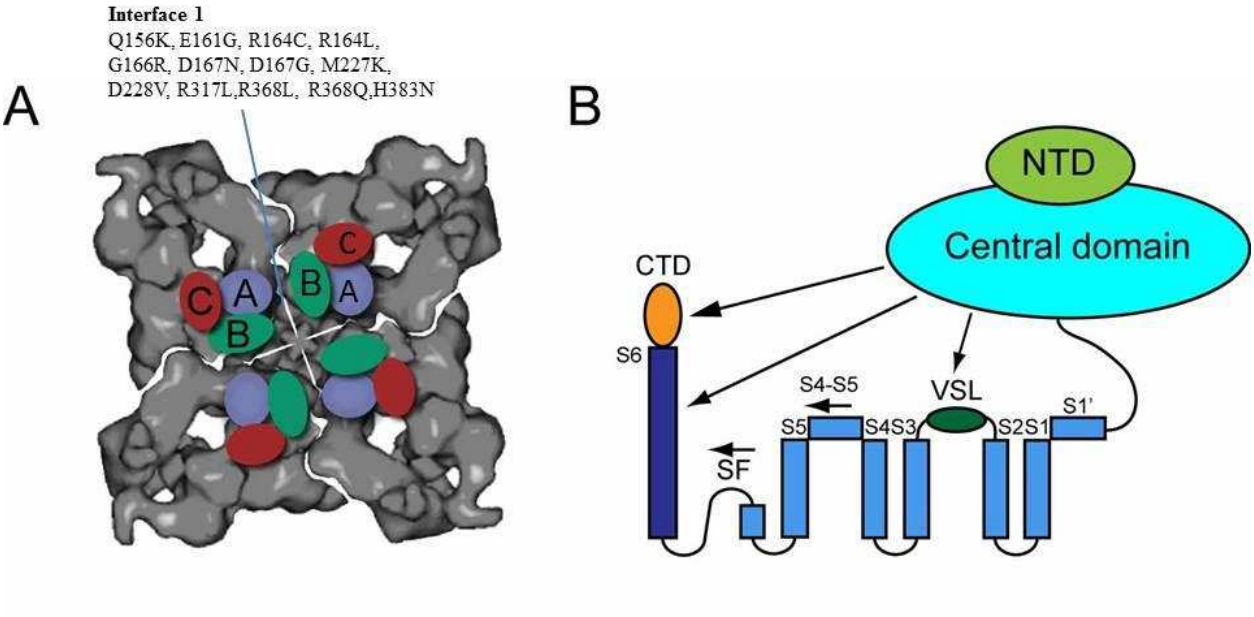
Legends for Figure-1

A. A schematic illustration of the N-terminal domains docked in the pseudo-atomic model of the RyR1 tetramer. The RyR1 N-terminal domain (NTD), corresponding to the 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. As an example, we indicated the interface between A and B domains of the neighboring RyR1 subunits (shown as a blue line) together with the variants associated with MH/CCD that map to this interface.[61,62,64]

B. A schematic model of domain organization in a RyR1 monomer, composed of the N-terminal domain (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-S6), and pore helices with the selectivity filter (SF), the linker helix of S4-S5, the voltage sensor like domain (VSL), and 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 acid 4894-4900), the S4-S5 linker (amino acid 4830-4831), Gly4934 – which serves as a "hinge" for the outward movement of the helix S6, and the CTD (amino acid 4957-5033) are all critical for RyR1 channel gating (adapted with modification from reference 64).

Figure 1


A
Interface 1
Q156K, E161G, R164C, R164L,
G166R, D167N, D167G, M227K,
D228V, R317L,R368L, R368Q,H383N
C
A
B
B
A
C
B
B
NTD
Central domain
CTD
S6
S4-S5
VSL
S1'
S5
S4S3
S2S1
SF