THE CLASSIFICATION AND FUNCTIONAL CHARACTERIZATION OF *RYR1* SEQUENCE VARIANTS ASSOCIATED WITH MALIGNANT HYPERTHERMIA SUSCEPTIBILITY IDENTIFIED THROUGH EXOME SEQUENCING

by

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DEDICATION

To Mom,

Who took me to the library.

To Dad,

Who instilled the drive to keep returning.

To both,

Who created a passion for learning and provided me with a traveled life.

Steve

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September 15, 2014

ABSTRACT OF THE DISSERTATION

The Classification And Functional Characterization Of *RYR1* Sequence Variants Associated With Malignant Hyperthermia Susceptibility Identified Through Exome Sequencing

By

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Doctor of Nursing Science

Uniformed Services University, Bethesda, Maryland 2012

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Background: Malignant hyperthermia susceptibility (MHS) is a life-threatening, inherited disorder of muscle calcium (Ca^{2+}) metabolism, triggered by anesthetics and depolarizing muscle relaxants. An unselected cohort of 870 *ClinSeq*® participants were screened for MHS mutations using exome sequencing to identify putative pathogenic MHS variants in the *RYR1* and *CACNA1S* genes. To study the effects of *RYR1* variants on channel function, we selected benign and pathogenic variants for comparison, using an *in vitro* assay measuring Ca^{2+} release from participant-derived lymphoblasts. Our goals were to pilot a strategy for the assessment of novel variants following genomic screening of the *RYR1* and *CACNA1S* genes and to determine if incidental MHS-associated variants identified from ES showed abnormal Ca^{2+} release from lymphoblasts.

Design: Functional analysis of gene variants identified through exome sequencing.

Methods: Annotation of 870 exomes for *RYR1* and *CACNA1S* variants was performed using an algorithm that filtered results based on genotype quality, allele frequency, mutation type, and information from mutation databases. Variants were scored on a six-point pathogenicity scale. Medical histories and pedigrees were reviewed for malignant hyperthermia (MH) and related disorders. Following identification of putative pathogenic *RYR1* variants, specific variants were selected for functional testing. Pharmacologic sensitivity of the RyR receptor type-1 (RYR1[1]) in lymphoblasts derived from four *ClinSeq*® participants were tested and compared to MH-negative controls. *RYR1* c.1840C>T, which predicts p.Arg614Cys (with published functional data) was selected as a positive control. Lymphoblastoid cell lines were used to study the transient peak Ca^{2+} release induced by the ryanodine receptor agonist 4-chloro-m-cresol (4-CmC). Calcium release was averaged from eight replicates. Differences in Ca^{2+} release among cell lines were analyzed by t-test.

Results: We identified 70 *RYR1* and 53 *CACNA1S* variants among 870 exomes. Of these, 63 *RYR1* and 41 *CACNA1S* variants passed the quality and frequency metrics. We then excluded synonymous variants. This resulted in numerous variants, which had previously been described as pathogenic in mutation databases being reclassified to unknown pathogenicity as a result. Three putatively pathogenic *RYR1* variants c.1840C>T, c.5183C>T, c.11958C>G, which predict p.Arg614Cys, p.Ser1728Phe,

[1] Note that the literature typically describes this protein as RyR1. Hereinafter, we use the designation approved by the Human Gene Nomenclature Committee, RYR1 for the protein and *RYR1* for the gene.

[2]Herein after the genomic changes referred by their designated protein changes p.Arg614Cys, p.Ser1728Phe, p.Asp3986Glu, p.Arg1667Cys.

p.Asp3986Glu respectively[2], and one variant of uncertain significance c.4999C>T, which predicts p.Arg1667Cys[2], were selected for functional testing. Pharmacologic sensitivity of RYR1 in lymphoblasts derived from four ClinSeq® participants were tested and compared to *RYR1* confirmed wild type (CWT) controls. Lymphoblasts from putative pathogenic variants showed increased sensitivity of Ca^{2+} release to 4-CmC compared to normal controls. Elevated Ca^{2+} release was observed in three cell lines with distinct mutations in RYR1 (p.Arg614Cys, p.Asp3986Glu, and p.Ser1728Phe) (P <0.05) at progressive doses (from 0.4 mM to 1.0 mM) of 4-CmC. Calcium release in the cell line containing the RYR1 p.Arg1667Cys mutation was similar to CWT.

Conclusions: Exome sequencing can identify asymptomatic patients at risk for MHS, although the interpretation of exome variants can be challenging. Three *RYR1* variants identified through ES showed significantly altered Ca^{2+} release. RYR1 channel function can be successfully assayed using a biochemical assay on participant-derived lymphoblasts, without resorting to muscle biopsy for *in vitro* contraction testing.

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CHAPTER 1: INTRODUCTION AND PURPOSE

INTRODUCTION

The research contained in this dissertation focuses on genomic screening of the *RYR1* and *CACNA1S* genes and the identification and functional characterization of sequence variants in the *RYR1* gene, and their association with Malignant Hyperthermia Susceptibility (MHS), which increases the risk for a malignant hyperthermia event.

Malignant Hyperthermia (MH) is a potentially lethal, autosomal dominantly inherited disorder of skeletal muscle calcium metabolism, triggered by exposure to inhalational anesthetics or depolarizing muscle relaxants such as succinylcholine, and rarely MH can be triggered by elevated environmental temperatures and exercise (125). In addition to humans, MH is known to affect pigs, dogs, horses, giraffes, and more recently, chickens (26) (140). In humans and dogs the syndrome is inherited in an autosomal dominant pattern, while in pigs the inheritance follows an autosomal recessive pattern (131). The diagnosis of MHS in humans is by one of two criteria: (a) a positive response to an *in vitro* muscle bioassay, such as the Caffeine Halothane Contracture Test (CHCT), or (b) the presence of a causative mutation in the *RYR1* gene (from European Malignant Hyperthermia Group's panel of causative mutations) found by molecular genetic testing. Candidates for testing by the two aforementioned tests, are those with a personal history of a clinical MH episode during anesthesia and family members of MHS patients (100). Currently the CHCT, which requires a biopsy of the vastus lateralis muscle, remains the gold standard for diagnosis of MHS (158).

At least six potential genetic loci for MHS have been mapped through linkage analysis, although to date MHS-associated variants have been found in only two genes.

The *RYR1* gene on chromosome 19q13.1 encodes the ryanodine receptor isoform I protein, a Ca^{2+} release channel of the sarcoplasmic reticulum (SR). The second gene associated with MHS is *CACNA1S,* found on chromosome 1q32, which encodes the dihydropyridine receptor (DHPR), the α1-subunit of the voltage-gated Ca^{2+} channel (159).

The number of *RYR1* variants associated with MHS continues to grow and currently includes some 400 variants (180). Among these hundreds of variants are those that have been proven to be pathogenic, reputed pathogenic (where evidence of pathogenicity is present but still inconclusive), variants of unknown pathogenicity, and benign variants. Remarkably, only 34 *RYR1* variants—most of them missense mutations—out of the hundreds identified have satisfied the rigorous criteria prescribed by the European Malignant Hyperthermia Group (EMHG; http://www.emhg.org/home/) for genetic molecular diagnosis (1). Mutations in the *RYR1* gene are also associated with several congenital myopathies, specifically Central Core Disease (CCD), multi-minicore disease, congenital fiber-type disproportion, nemaline rod, and centronuclear myopathy (34; 78). For the second causal gene, *CACNA1S,* only three variants have been determined to be pathogenic for MHS (143; 178; 193).

Currently, nearly all patients with a diagnosis of MHS have received that diagnosis following a MH event affecting either him- or herself or a family member. The mortality rate of an MH event was nearly 10% (8/84) between 2007 to 2012 (95), so this method for identifying at-risk individuals is far from optimal. One potential solution to this challenge would be to use genomic screening to identify individuals at risk, before they or their family members suffer an MH event, but it has not yet been established that

DNA analysis by exome sequencing (ES) can be used to identify at-risk individuals. Beyond the identification of MHS mutations, it is currently necessary to confirm a molecular diagnosis of MHS using a functional test. More research is needed to develop less invasive approaches to confirming MHS than the current CHCT, which necessitates a biopsy. Our research proposes that individuals could be screened by ES, followed by an analysis of known MHS-associated genes, and identified putative variants could then be confirmed using a non-invasive assay to determine MHS prior to the onset of an MH episode. In this way, a predictive medicine approach can be brought to bear on this challenging and potentially lethal disorder.

STATEMENT OF PURPOSE

In this dissertation study, variant analyses of the *RYR1* and *CACNA1S* genes were performed following ES of 870 adult participants. Our first hypothesis was that ES could be used to identify putative pathogenic MHS variants from a population unselected for the disorder and that causative mutations could be filtered from ES data. A key attribute of the ClinSeq® cohort is that they were not ascertained for MHS or any congenital myopathies, nor was the presence of a personal or family history of MHS an exclusion criterion—thus they comprise a cohort that can be used to model the scenario of screening for MHS and congenital myopathies in the general population.

We proposed coupling ES-based screening to a minimally invasive assay to detect MHS. Following the identification and classification of the *RYR1* and *CACNA1S* sequence variants, we then characterized the effect of *RYR1* variants on the function of the type-1 ryanodine receptor (RYR1) channel. The functional characterization was performed by an *in vitro* Ca^{2+} release assay on lymphoblasts from research participants with a range of sequence variants from benign to pathogenic. This assay is a technique used in recent MHS functional studies to characterize *RYR1* variants (7; 33; 98; 117; 201; 220).

A functional assay is needed to confirm that a specific DNA mutation has a functional effect on Ca^{2+} release. MHS variant identification from a DNA test, coupled with a MHS-positive result from an *in vitro* Ca^{2+} release assay in lymphoblasts can feasibly be used to identify people at risk and to substitute for, or augment, the CHCT which necessitates an invasive muscle biopsy (181).

This lead to our second hypothesis that lymphoblasts derived from an individual with a pathogenic *RYR1* variant identified through ES will have increased sensitivity to the RYR1 agonist 4-chloro-m-cresol (4-CmC) as compared to MH-negative controls. The results reported from this work may provide evidence for the usefulness of ES and lymphoblasts to investigate the effects of other *RYR1* variants.

HYPOTHESES AND SPECIFIC AIMS

The overall goal of our study was to identify and functionally characterize sequence variants in the *RYR1* gene, associated with MHS that increase the risk for MH. Our *first* aim was to investigate if MH-susceptible individuals—in a cohort unselected for MHS—could be identified through ES. The ES data were derived from the ClinSeq® study (07-HG-0002), a genomic research study at the National Institutes of Health (NIH), National Human Genome Research Institute designed to be accessible to the study of all traits and the eventual acquisition of whole genome sequences (17). The aim of the first part of this investigation was to pilot a strategy to identify variants in *RYR1* and *CACNA1S* that are candidate pathogenic variants associated with MHS versus benign variants, to model the approach of population screening.

The second aim involved functionally testing selected putative pathogenic and nonpathogenic variants identified in part one—following an annotated filtering and scoring process. This work was undertaken to test the correlation of the predicted effect from the mutation to the functional properties of the RYR1 receptor. The *CACNA1S* gene was part of the analysis for MHS: although the *CACNA1S* variants identified will not be functionally analyzed, the exome analysis was nearly identical to *RYR1*. In addition, individuals with *CACNA1S* variants could bias the selection of controls as well as the phenotype analysis by,

for example, the presence of abnormalities in serum creatine kinase and potassium levels. This approach uses the recognized association of the presence or absence of pathogenic gene variants with differences in Ca^{2+} release in lymphoblasts. Lymphoblastoid cell lines were used to assay the underlying functional effects of the *RYR1* variants. This cell type has the advantage of expressing the RYR1 receptor (168). The purpose was to increase our knowledge of the underlying genetic and functional mechanisms that lead to MHS.

The following two hypotheses were tested:

Hypothesis 1

Exome sequencing can be used to identify putative pathogenic MHS variants in a population (n =870) unselected for the disorder, and pathogenic MHS variants can be filtered from ES data.

Hypothesis 2

Lymphoblasts derived from individuals with predicted pathogenic *RYR1* variants identified through ES will have increased sensitivity to the RYR1 agonist 4-CmC—as evidenced by increased RYR1 cytoplasmic Ca^{2+} release—compared to cell lines from individuals without such mutations.

To test the hypotheses, the following two specific aims (listed sequentially) were identified:

Specific Aim 1

(a) Screen the *RYR1* and *CACNA1S* genes by ES for the identification of MHS sequence variants in 870 subjects not ascertained for the phenotype of MHS; (b) annotate *RYR1* and *CACNA1S* variants using an algorithm that filters results based on mutation type,

allele frequency, information in mutation databases, and the primary literature; (c) score variants on a six-point pathogenicity scale; (d) review medical histories and pedigrees for MHS and related disorders; and (e) molecularly characterize the sequence variants identified through ES using amino acid substitution mutation analysis tools.

Specific Aim 2

Perform functional characterization of the RYR1 channels of cell lines with putative pathogenic MHS variants, variants of unknown significance and confirmed wild type (CWT i.e., the reference or non-mutated allele at each locus of the *RYR1* gene identified by ES) cell lines by a dye incorporation array; and determine the correlation of specific MHS genotypes and responses to Ca^{2+} release in lymphoblasts.

SUMMARY OF THE STUDY

The present study was designed to determine if putatively pathogenic *RYR1* variants could be identified through ES and could be confirmed to be pathogenic by the analysis of resting Ca^{2+}concentration in a proxy cell (lymphoblast). Ca^{2+} homeostasis of RYR1 channels was studied in lymphoblasts using Fura-2 (a sensitive fluorescent indicator) and the RYR1 agonist 4-CmC. We hypothesized that the putative MH mutations would shift the sensitivity of the RYR1 to a lower agonist concentration. To confirm that the reduction in 4-CmC induced Ca^{2+} release—from the lymphoblasts harboring the variants—is not due to depletion of intracellular Ca^{2+} stores, the SR Ca^{2+} content was also assessed by treating cells with thapsigargin, a potent inhibitor of Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) Ca^{2+} pumps.

Our study was designed to determine if MHS-associated pathogenic variants uncovered through ES in a population unselected for this phenotype can be identified and

confirmed to be pathogenic by demonstrating differences in Ca^{2+} release from the endoplasmic reticulum in lymphoblasts. Validation of our approach would be a powerful pilot study and rationale for the use of ES and a minimally invasive assay to identify individuals at risk for MHS. Ultimately, our work is a first step in the development of a minimally-invasive approach for the diagnosis of MHS. Our eventual goal is the ability to predict MHS solely from genomic data without the currently requisite muscle biopsies or functional assays, as having a minimally invasive assay allows us to design large scale studies to correlate mutations and function, which would then allow us to better predict function from the specific mutation alone.

CHAPTER 2: REVIEW OF THE LITERATURE

MALIGNANT HYPERTHERMIA

Clinical Description

Malignant Hyperthermia Susceptibility (MHS) is a potentially lethal, autosomal dominantly inherited disorder of skeletal muscle Ca^{2+} metabolism, most commonly caused by pathogenic variants in the gene coding for RYR1. The genetic susceptibility for MHS is estimated to be present in as many as 1 of 2,000 individuals (70). The true figure is difficult to establish, as there are no established MHS screening tests and the majority of susceptible individuals are phenotypically normal unless exposed to triggering anesthetics or depolarizing muscle relaxants or, in rare cases, by vigorous exercise, heat exposure or febrile illness (158). A malignant hyperthermia (MH) reaction (1 in 100,000 anesthetic exposures) reflects an uncontrolled increase in intracellular Ca^{2+} in skeletal muscle (20). There is no consistent clinical presentation of MH (Figure 1): affected individuals experience hypermetabolism with varying signs and symptoms, including hyperthermia, tachycardia, generalized or localized muscle rigidity, metabolic acidosis, rhabdomyolysis, or hypoxemia (166). Death results without rapid treatment. Additional life-threatening complications are not uncommon, (such as renal failure, congestive heart failure, intravascular coagulation, etc.), and the mortality rate can be as high as 19.8% when patients develop MH in a non-hospital setting (161). However, modern anesthetic monitoring, and treatment with the skeletal muscle relaxant dantrolene, results in much lower morbidity and mortality than when MHS was first recognized in the 1960s (20). A disturbing trend identified by Rosero and coworkers in 2009 is the rise in the incidence of MH episodes in the last decade (161), apparently due to the use of MH-triggering anesthetics in the growing numbers of ambulatory surgical

centers (22). These statistics emphasize the need for a readily accessible, non-invasive test for MHS with high sensitivity to more accurately identify those with the condition.

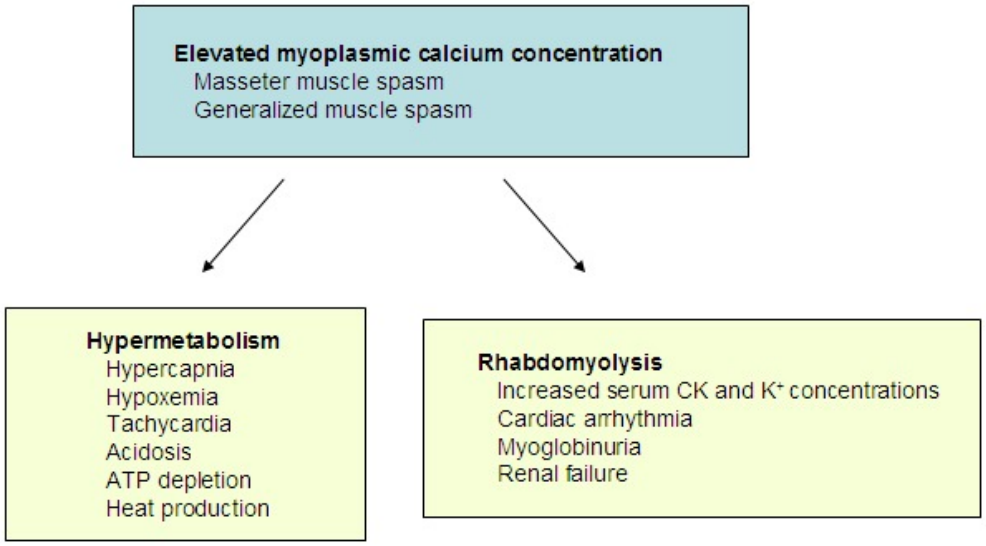

Figure 1. Clinical features of malignant hyperthermia susceptibility. Published in GeneReviews®, Rosenberg H, Sambuughin N, Dirksen R. Malignant Hyperthermia Susceptibility. Seattle (WA): University of Washington, Seattle, 2003. Available from: http://www.ncbi.nlm.nih.gov/books/NBK1146/. Adapted from Muscle & Nerve, 23, Jurkat-Rott K, McCarthy T, Lehmann-Horn F. Genetics and pathogenesis of malignant hyperthermia, 4-17. Copyright John Wiley & Sons, Inc. (2000), with permission from Elsevier.

Diagnosis of the Disorder

The diagnosis of MHS is typically based upon a personal or family history of a clinical MHS episode during anesthesia, or upon a positive response to the Caffeine Halothane Contracture Test (CHCT), or by molecular genetic testing. The CHCT is an *in vitro* muscle bioassay, which determines the contractile properties of the skeletal muscle when exposed to both caffeine and halothane (i.e., RYR1 receptor agonists). The test is invasive, costly ($6,000 to $10,000 dollars) (23), and—because of the potentially life-threatening danger of a false-negative diagnosis—is designed to err on the side of a false-

positive diagnosis. Additionally, the CHCT must be performed on freshly excised muscle (2.0 g of quadriceps muscle) following a muscle biopsy, which is available at only five MHS diagnostic centers in North America (157); patients therefore may have to travel a great distance to have the test performed. Finally, it is no small matter that a test to assess risk from anesthesia, needs anesthesia, irrespective if a trigger free general or peripheral nerve blockade with or without sedation is used, as anesthesia and complete monitoring is necessary for the excision of muscle bundles from the vastus lateralis muscle (24).

DNA analysis of the *RYR1* gene is a more recently established clinical diagnostic test for MH susceptibility (158). A DNA test is indicated for patients with a positive contracture test, or in those with an unequivocal contracture test result (i.e., positive for one but not both of the test's two parameters) to determine the presence of a mutation (100). However, for others, the DNA test needs careful patient selection because of the limited sensitivity. Genetic testing is also suggested for children or adults with a clinical history of suspicion for MH but who are unable or unwilling to undergo an invasive muscle biopsy and for the identification of MHS status in family members of MHS individuals (100). These recommendations apply only to North America, as in Europe the EMHG does not perform molecular genetic testing unless the affected individual or his/her relative has first had a positive contracture test (197). The EMHG has designated 34 accepted pathogenic variants and approximately 30% of patients with known MHS have one of these pathogenic variants (66; 153; 157). Only 34 *RYR1* variants, out of the hundreds identified, have passed the rigorous mutation criteria required by the EMHG for genetic molecular detection (126; 159). When one of the 34 pathogenic variants is

identified, the patient is considered to be MH-susceptible (1). However, because it has a high false-negative rate for MH-susceptibility, a negative genetic test result does not rule out MHS. A CHCT is still needed to determine MHS-negative status (159).

To date, muscle biopsy remains the gold standard for diagnosis of MHS. DNA analysis by panel testing offers an alternative to the CHCT and would be preferred, because it is less expensive (about $800 dollars) (101) and less invasive, requiring only a peripheral blood sample. The DNA test is 25-30% sensitive and highly specific (for example those with a positive test for one of the 34 diagnostic or causative mutations are almost certain of being at risk) at 95% for MH susceptibility (157). Currently, DNA analysis alone is not considered sensitive enough to diagnose susceptibility to MH and more research is needed before it can be used in clinical practice.

Genetic Research on Malignant Hyperthermia Susceptibility

Progress on a more sensitive genetic test for MHS has proven difficult. The molecular genetics of MHS is complex: the genes are large (154 kb gene and 15 kb cDNA length for *RYR1*), making Sanger sequencing complex and time-consuming, and the disorder has a high degree of allelic heterogeneity and locus heterogeneity (84). MHS is pleiotropic, including manifestations that may be mimicked by several clinical situations (159). Absent an anesthetic exposure, there are rarely any phenotypic signs of the disorder. Thus the development of a more sensitive DNA test for MHS is contingent on the establishment of a thorough assemblage of MHS causative variants.

Despite the difficulties, genetic testing is playing an increasingly important role in MHS diagnostics, due to advances in MHS genetic research. Among patients with demonstrated MHS, 60-86% have *RYR1* mutations and ~1% have *CACNA1S* mutations

(147). There are a few rare disorders that also include MHS as part of a more complex phenotype. The rare disorders will be discussed briefly, followed by a thorough discussion of the more common variants.

At least six potential genetic loci (MHS1 to MHS6) for MHS have been proposed (159), these include the *RYR1* and *CACNA1S* genes, and provide evidence of genetic heterogeneity in MHS. Three of the loci (designated MHS3, MHS4, and MHS6) were mapped in the late nineteen nineties but their genes have yet to be identified and the candidate loci have been found in only a few pedigrees each (21; 81). The MHS2 locus was once proposed as a candidate gene (123; 137), with the DNA mutation in the sodium channel alpha-subunit (*SCN4A* gene) associated with myotonia and abnormal contracture tests for MH-susceptibility in four individuals from one extended pedigree (199). However the earlier MHS2 findings of susceptibility to MH could not be confirmed in further studies (71; 184). A list of the six MHS loci and their chromosomal locations is provided in Table 1.

More recently, Horstick and coworkers in 2013 reported a missense variant in the *STAC3* gene mapped to chromosome 12q13-14 was reported to be a likely cause of the debilitating, autosomal recessive Native American myopathy (NAM) (68). The mutation is thought to be responsible for defective excitation–contraction (EC) coupling, as the skeletal muscle-specific T-tubule protein (STAC3) is a necessary component of the EC coupling machinery (129). The congenital myopathy is characterized by a constellation of clinical features (e.g., weakness, joint contractures, dysmorphic facial features, and short stature) and MHS (68). In contrast to the secondary loci (MHS2, MHS3, MHS4, and MHS6) being described in only a few pedigrees, and to the recent finding of a single

variant in *STAC3* to be a cause of MHS, two loci have a well-known role in MHS - the *RYR1* gene on chromosome 19q13.1, encoding the Ca^{2+} release channel of the sarcoplasmic reticulum, and the *CACNA1S* gene on chromosome 1q32, encoding the α1-subunit of the voltage-gated Ca^{2+} channel known as the dihydropyridine receptor (DHPR) (97).

Table 1. MH susceptibility loci, their gene names, chromosomal locations and presumed percent association with MHS

Locus	Chromosomal Location	Gene Name	Presumed Percent Association with MHS
MHS_1	19q13.1	RYR_1	60-87%
MHS_2	17q11.2-q24	SCN_4A	Unknown
MHS_3	7q21-q22	$CACNL_2A$	Unknown
MHS_4	3q13.1	Unidentified	Unknown
MHS_5	1q32	$CACNA_1S$	1%
MHS_6	5p	Unidentified	Unknown

In addition to MHS, the *RYR1* gene is also linked to congenital myopathies, specifically, CCD and multi-minicore disease. When the entire coding sequence of *RYR1* is examined in families with MHS, the *RYR1* pathogenic variant detection rate is 60-86% (51; 70; 120; 163). However, the MHS-causative variant detection rate for DNA testing is lower when applied to individuals and any of their relatives who have not been pre-screened with the CHCT test (21). Kraeva and coworkers studied a highly selected population likely to MHS-associated mutations based on the presence of one of three required criteria (a) a positive CHCT in individuals with a family history of MHS, or (b) a positive score from a clinical grading scale (>35) in individuals who experienced an MH episode, or (c) hyperCKemia (i.e., high serum creatine kinase level) and a family history of MHS; they found a mutation detection rate as high as 86% for at least one

RYR1 mutation, and a detection rate, or diagnostic sensitivity of genetic testing, of 42% for known casual mutations (89).

The number of identified MHS *RYR1* variants continues to grow and is currently at approximately 400 variants (180). The *RYR1* gene is 159,000 nucleotides long and contains 106 exons that are spliced into a 15 kb *RYR1* mRNA transcript (163), making it difficult to sequence with Sanger technology. For the second most common causal gene, *CACNA1S*, only three MHS-associated genetic variants (p.Arg1086His or p.Arg1086Ser, p.Arg174Trp, and p.Thr1354Ser) have been identified (11; 30; 120; 178).

Early studies of the *RYR1* gene suggested that there were three genetic variant hot spots in *RYR1* in patients with MHS, designated MHS1, MHS2, and MHS3 (116). Many laboratories screened only these *RYR1* coding regions (158). However, over time, pathogenic variants causing RYR1-related disorders have been found throughout the *RYR1* gene (151). MacLennan and Zvaritch (2011) plotted the rate of accumulation of the mutations associated with MHS or core myopathy on the 5,038 amino acid *RYR1* open reading frame. Their plots showed that, in addition to variant hotspots, there where other regions with a lower density of mutations and that the mutations are spread throughout most of the *RYR1* gene. The only exception was the seemingly mutation-free interval between amino acids 4,338 and 4,558 (113). A diagram of the hot spot is illustrated in Figure 2.

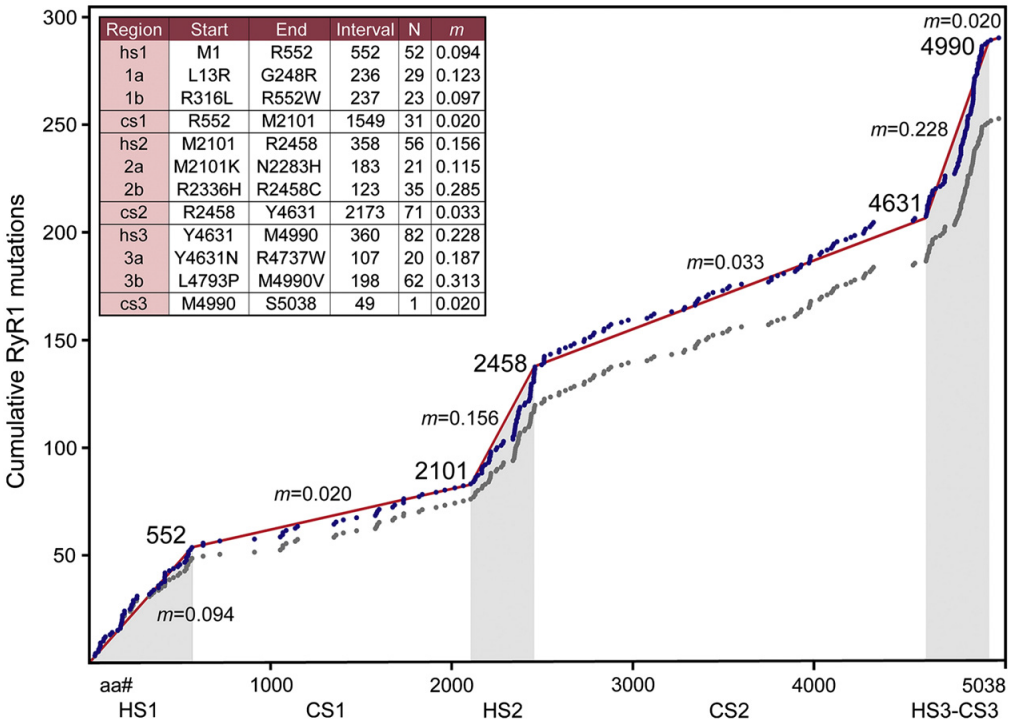

Figure 2. Diagram of hot spot regions.

A plot of the accumulation of MH-and core myopathy-associated mutations in *RYR1* against amino acids 1 to 5,038 in the human RYR1 protein. A total of 294 RYR1 amino acids in which at least one mutation has been documented are plotted cumulatively as blue dots relative to their position in the amino acid sequence. In cases where redundant substitutions have occurred, they are assigned accumulation values equal to the redundancy at this site. In a second plot, using gray dots, we have assigned a value of only one to all mutated amino acid sites, regardless of redundancy at the site. The two plots have the same overall shape, but the rate of accumulation is lower in the gray plot. The density of *RYR1* mutations in relation to their location within the protein are presented graphically in the blue plot by the slopes (m) of linearized segments (red lines) representing average values across each of the different segments of the plot. Six red lines subdivide the polypeptide chain into six major intervals designated HS1, CS1, HS2, CS2, HS3, and CS3, where HS stands for hot spot and CS for cooler spot, each with a different slope. The slope of each interval was determined as the ratio of the number of mutations within the interval (N) divided by the length of the interval (Interval) in amino acid residues. HS1, HS2, and HS3 are regions with steep slopes, their "Start" and "End" coordinates being the points of intersection of the red lines, indicated in the graph and in the inserted table. Within each of the HS sequences there are at least two subsequences, designated "a" and "b", in which the slopes differ from the average, some being much steeper. The intervals of these subsections are delineated in the inset table by the first and the last mutated amino acid residue within the interval. Published in Biochimica Biophysica Acta, 1813, Maclennan DH, Zvaritch E. Mechanistic models for muscle diseases and disorders originating in the sarcoplasmic reticulum, 948–964. Copyright Elsevier (2011), with permission from Elsevier.

Research Biases in Malignant Hyperthermia Susceptibility

Researchers studying MHS have conventionally chosen populations most likely to harbor genetic changes on the basis of being MHS positive by contracture testing, by the clinical grading scale, or by using biomarkers such as hyperCKemia, making it difficult to determine the incidence, penetrance and expressivity of the disorder. However, an improved understanding of the prevalence and significance of *RYR1* variants will require genetic studies of MHS individuals who are not well defined and MHS-negative individuals by CHCT (156). The study of a population not ascertained for MHS, or a particular myopathy, may shed light on the complexities of MHS.

Molecular genetic research into MHS has led to a greater understanding of the clinical presentation of the disorder. Sequencing of the entire coding region of *RYR1* is leading to the detection of numerous coding variants (70; 86; 163; 165). The significance of these variants will need to be studied through functional testing to determine if they are causative MH mutations, rather than benign variants. However, to date, functional testing on MHS variants has been carried out on only a small percentage of *RYR1* variants (113). Studies such as the ClinSeq® study where phenotype information is accessible, and that uses new sequencing technologies—such as exome and genome sequencing—should advance our understanding of the disease by evaluating patients with full gene sequence data but without ascertainment for the MHS phenotype. The use of technologies like ES may also lead to the discovery of other putative pathologic variants (besides the *RYR1* variants already functionally classified) that may account for cases of MHS and provide us with a full understanding of normal genomic variation.

Epidemiology

MH is a medical emergency known to clinicians in US military and civilian operating suites. In the US, it is estimated that the incidence of clinical MH reactions ranges from 1:5,000 to 1:50,000–100,000 anesthetic exposures (158). However, these reported figures underestimate the prevalence of MH susceptibility. Estimates are that the prevalence of the MHS trait may be as high as 1 in 2,000 (70; 120).

The discrepancy between the incidence of, and susceptibility to, MH is likely due to many factors: (a) not all volatile anesthetic agents or depolarizing muscle relaxants trigger a reaction in people who have MHS, (b) many individuals with MHS have an MH event after undergoing several anesthetics without an event, and (c) mild or aborted MH reactions are difficult to define and diagnose (66)—all evidence of the variable expressivity and incomplete penetrance of the MHS phenotype, which complicates our understanding of the disorder. MHS is a classic example of a gene-environment interaction: the manifestation of an MH event is dependent upon having (a) a pathogenic variant in one of the primary genes, (b) exposure to a triggering agent, and (c) additional, as yet uncharacterized genetic and environmental risk factors. All of these factors conspire to complicate estimates of the prevalence of the trait (151). For these reasons, the true penetrance and expressivity of MHS remains unknown.

Since discovery of the condition, the MH mortality rate has been reduced from 80% in the 1970s to less than 10% in the 1990s (20). A major factor in the decrease in mortality is the increased use of anesthetics that do not trigger the MH syndrome. Other significant influences were the introduction of protocols that involve the routine monitoring of signs and symptoms during anesthesia, the immediate cessation of the

offending anesthetic agent, the initiation of MH clinical care procedures, and the administration of dantrolene—a potent antidote to anesthetic-induced MH—if there is a rapid change in any of the patient parameters (14; 96).

Although current MH mortality rates are about 10%, the rate of complications from acute MH episodes is much higher. Larach and coworkers found a 34.8% morbidity rate after review of case reports to the North American Malignant Hyperthermia Registry (NAMHR, http://www.mhaus.org) between 1987 and 2006. The serious complications that were most frequently reported included a change in consciousness level, cardiac dysfunction, pulmonary edema, renal dysfunction, disseminated intravascular coagulation, and hepatic dysfunction (96).

Concern is growing as recent surveys on the occurrence of MH in North American clinical facilities show a disturbing trend in the last decade—a rise in MH-associated morbidity and mortality to 35% and 14%, respectively (96; 161). This has been attributed to the use of MH-triggering anesthetics in ambulatory surgical centers (22). A recent study showed that patients who develop MH in an ambulatory surgical center have a higher mortality rate than those in a hospital setting, associated with lesser clinical and physical resources in the former setting (161). These statistics stress the need for a better understanding of the prevalence and ability to predict MHS (156).

Malignant Hyperthermia and the Military

This project has broad public health ramifications, as advances in our understanding of MHS will benefit both civilian and military communities. Addressing this issue is critical for the military, which needs the flexibility to use any available anesthetics. Field medical stations, and other remote hospitals, should not use limited operative and perioperative resources on a

conceivably avoidable problem like MH. An in-depth understanding of the prevalence and significance of *RYR1* mutations will require studies of individuals who are not just MHS positive, but also many others who are not as well characterized. Therefore, studies that feature functional characterization of MHS genetic variants following genetic/genomic analysis of a population unselected for the disorder are particularly important to our understanding of how human genetics relates to the clinical manifestations of the disease and can model population screening to determine the propensity for MHS.

It is also imperative to increase our understanding of the many debilitating and prevalent clinical conditions mimicking or presenting with some of the symptoms of MH (e.g., rhabdomyolysis, exertional heat illness, chronic unexplained increased creatine kinase levels, statin intolerance, etc.) whether or not they occur in relation to anesthesia. Many of these conditions have been shown to be associated with a positive CHCT and, in rare cases, with known mutations causative for MHS (29; 74; 124; 125; 200). Genomic studies coupled with functional testing of MHS variants may ultimately lead to the development of better tools to identify atypical MHS phenotypes.

Pathophysiology

In the early 1960s, Hall and coworkers reported the first case of an anesthetic agent inducing MH in a pig (60). Researchers recognized that specific breeds and lineages of pigs were sensitive to certain types of anesthetics, similar to humans (41; 62; 133; 211). Clinical reports of MH occurring in humans as well as dogs were also well documented in the literature (28; 73; 175). By the late 1960s to early 1970s, an association was suspected between the well-recognized porcine stress syndrome (PSS) in pigs—triggered by a variety of non-pharmacologic stressors (unrecognized at the time as a stress-induced

MH attack)—and anesthetic-induced MH in humans (63; 76; 211). The association was eventually confirmed (61; 132). Breeders selecting for pigs with large body size and lean muscle mass had unintentionally also selected for a mutation that causes MHS (113). The finding of an MH syndrome in pigs was a boon for researchers, as it provided a useful animal model (76; 203). The signs and symptoms of MH in pigs and humans are virtually indistinguishable, so that newly developed anesthetics that do not trigger an MH reaction in pigs are considered safe in humans (97).

In pigs with a homozygous mutant allele for MH susceptibility, exposure to the anesthetic halothane led to higher Ca^{2+}concentration when compared to controls, and higher concentrations of Ca^{2+} were associated with contractures of the muscle fibers (69). Studies performed on muscle biopsies demonstrated that animals affected by PSS showed increased rates of Ca^{2+} release from the SR (50; 119; 130; 135). After the RYR1 Ca^{2+} channel was identified in 1988 (92), successive studies in pigs confirmed that the RYR channel was defective in PSS (46; 83; 118; 136). In 1992, MacLennan and Philips proposed that the mechanism for PSS was the excessive release of Ca^{2+} from the SR (112). Continued research involving various approaches confirmed the same underlying mechanisms causative for MH – a pathologically high and uncontrolled release of Ca^{2+} from the SR during exposure to triggering agents (158).

The cause of the abnormal regulation and release of Ca^{2+} in affected muscle lies in the defective RYR1 Ca^{2+} release channel in the skeletal muscle membrane, which is paired to the L-type, dihydropyridine-sensitive, voltage dependent Ca^{2+} channel, the dihydropyridine receptor (DHPR). They are referred to as ryanodine and dihydropyridine receptors as they are opened and blocked by the experimental drug ryanodine and the

clinically important class of drugs called dihydropyridines, respectively (162). DHPR is located in transverse tubule membranes and functions as a voltage sensor. Located in the SR membrane, RYR1 is not voltage-dependent, but linked to the DHPR (80).

Calcium transport from the sarcoplasmic reticulum into the sarcoplasm is a fundamental component of normal muscle contraction (also known as the excitation-contraction process) and is mediated by RYR1, which is expressed in skeletal muscle. When a muscle fiber is excited via the nerve endplate and generates an action potential, the signal (i.e., the coordinated movement of ions) is propagated along the sarcolemmal membrane into the transverse tubules (i.e., an inward-folding of the of the skeletal muscle membrane that allow action potentials on the surface membrane to spread rapidly into the depths of the fiber), the voltage change is sensed by DHPR, and the channel opens. The conformational change of the open DHPR is transmitted directly by mechanical means to RYR1, which responds by opening. The resultant Ca^{2+}release, and increase in cytoplasmic Ca^{2+}, initiates muscle contraction. This is the fundamental EC coupling needed for normal skeletal muscle function (Figure 3). A sarcoplasmic reticulum Ca^{2+}ATPase (SERCA) pump sends Ca^{2+} back into the SR, initiating the relaxation state. Abnormalities in these two receptors are thought to be responsible for most of the functional changes seen in Ca^{2+}dysregulation in MH. Since before the identification in 2013 of the rare NAM disorder caused by mutation in *STAC3* gene, decades of research had only linked RYR1 and DHPR to dysfunctional changes in MH, and disease-causing mutations had only been identified in the genes for these two receptors (158).

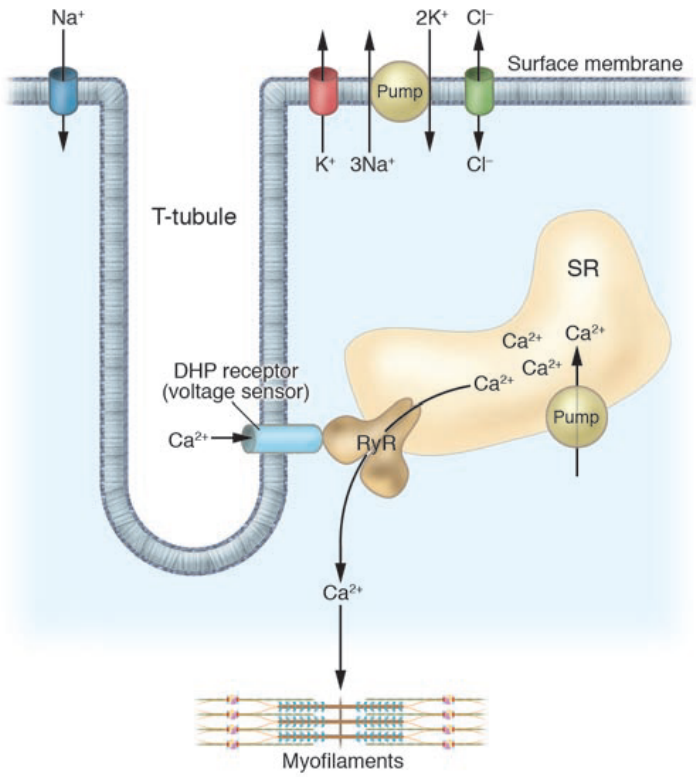

Figure 3. Excitation-contraction coupling of skeletal muscle.
Depolarization of the T-tubule membrane activates Cav1.1, triggering SR Ca^{2+} release through RYR1 and leading to sarcomere contraction. Published in the Journal of Clinical Investigation, 115, Jurkat-Rott K, Lehmann-Horn F. Muscle channelopathies and critical points in functional and genetic studies, 2000-2009. Copyright American Society for Clinical Investigation (2005), with permission from the Copyright Clearance Center.

Research has revealed the pathophysiologic changes that characterize MH. In MH, the RYR1 Ca^{2+}-release channels are sensitive to lower concentrations of intracellular Ca^{2+} and remain in an open state, which increases the Ca^{2+} concentration within the cytoplasm, resulting in a sustained muscle contraction and a surge in oxygen consumption, anaerobic metabolism, and depletion in ATP. The low ATP level causes muscle rigidity, since the presence of ATP is needed for muscle relaxation. Hyperkalemia and rhabdomyolysis often follow due to the secondary degradation of the

muscle membrane integrity (158). If not quickly treated, the resultant muscle cell death leads to progressive and sometimes lethal complications (such as ventricular fibrillation and renal failure).

The Genetics of Malignant Hyperthermia Susceptibility

Point mutations in the *RYR1* gene, as well as mutations in the *CACNA1S* gene, cause human malignant hyperthermia (i.e., defective EC coupling). In 1991 Fujii and coworkers reported that the c.1843C>T mutation, which predicts p.Arg615Cys in porcine RYR1 caused PSS in five of the major breeds of lean, heavily muscled pigs (48). However, there was still no direct evidence that demonstrated the mutation completely explained the RYR1-mediated Ca^{2+} release. It remained possible that the MHS phenotype is the result of alterations in both the RYR1 receptor and other SR protein components. In 1994, Treves and coworkers addressed this issue by studying the cytosolic Ca^{2+} increase in transfected COS-7 (eukaryotic) cells expressing either wild type or mutated recombinant *RYR1* in response to 4-CmC, an RYR1 activator capable of eliciting *in vitro* Ca^{2+} release. Their results provided evidence that the porcine c.1843C>T, p.Arg615Cys change in the primary structure of the RYR1 is sufficient to alter intracellular Ca^{2+} homoeostasis in eukaryotic cells (196), and that the mutation (orthologous to c.1840C>T, p.Arg614Cys in humans) is the sole cause of PSS in pigs (140). The c.1840C>T, p.Arg614Cys mutation was one of the first of many *RYR1* mutations identified in humans (53). The majority of *RYR1* MH mutations show gain-of-function effects: they increase RYR1 sensitivity to caffeine and other chemical activators, as shown by functional tests of ryanodine receptors expressed in heterogeneous cell types (192).

The pathogenic effects of *RYR1* mutations are not limited to MHS. Mutations in *RYR1* have been found to cause several other inherited muscle disorders such as CCD, some forms of multi-minicore disease, and centronuclear myopathy (195). For example, single-base substitutions in *RYR1* can result in MHS only, CCD only, or MH with a variable portion of the individuals exhibiting clinical symptoms of CCD. However, most individuals with a pathogenic (i.e., disease causing) variant in *RYR1* gene are susceptible to MH, although they may or may not have CCD or one of the other muscle disorders (158). The fact that some of the disorders share phenotypic effects, and others in the same gene do not, demonstrates the overall complexity of the channel.

THE RYANODINE RECEPTOR CALCIUM CHANNEL

The ryanodine receptors belong to a family of intracellular Ca^{2+} release channels that are responsible for the release of Ca^{2+} from the endoplasmic reticulum and sarcoplasmic reticulum. RYRs form assemblies with four identical subunits (i.e., homotetramers), with each subunit composed of ~5,000 amino acids. With a total molecular mass of ~2.3 MDa, they are the largest and most structurally complex ion channels (198). Three mammalian paralogs that exhibit subtype-specific tissue expression and are each encoded by distinct genes have been identified. They are classified according to the order and the tissue where they were first identified. Type 1 RYR is the predominant isoform expressed in skeletal muscle and was first to be detected (185; 217). Type 2 RYR is the subtype in cardiac muscle (128; 139). Type 3 RYR is expressed throughout the central nervous system, and was originally purified from the brain (59). However, the tissue classification is not as straightforward as the categorization infers, as there is some overlap in tissue expression and each isoform is found in many different cell types:

RYR1 is also present at low levels in cardiac Purkinje cells, smooth muscle, human B-cells, and murine dendritic cells (194); RYR2 is also present in the cerebellum (139), and RYR3 is also present in mammalian striated muscle at relatively low levels (194). This suggests that mutations in *RYR1* not only affect muscle and neuron (excitable) cells, but also the immune system and other tissues (195). Of the three isoforms, RYR1 has been the most thoroughly studied due to its high expression levels in a number of tissues and the ease of purifying it from skeletal muscle (94).

In humans RYRs are encoded by three different genes: (a) the gene encoding RYR1 is located on chromosome 19q13.2 and contains 106 exons (142): (b) the gene encoding RYR2 is located on chromosome 1q43 and contains 102 exons (139), and (c) the RYR3 gene with 103 exons is on chromosome 15q13.3-14 (176). The three mammalian RYR isoforms are 65% identical in protein sequence, thus their three dimensional structures are thought to be nearly the same (59).

The structure of RYR family members has been studied extensively (59; 128; 139; 185; 217). Their structural analysis is a challenge, however, due to their size and complexity (94). An atomic construction of an intact receptor by x-ray crystallography does not yet exist (198). However, several studies have reached high resolutions by cryo-electron microscope and agree on the overall structure of the receptor (145; 146; 171-174). The basic structure has been described as a mushroom, with a large tetrameric cap representing 80% of the volume located in the cytoplasm, and a stalk composed of four thick columns with 20% of the volume crossing the membrane into the SR/ER lumen (198). A diagram of the structure of an RYR Ca^{2+}release channel is illustrated in Figure 4.

Within the EC coupling pathway, the RYR1 Ca^{2+} release channel is modulated by the T-tubule and DHPR receptor, and by several endogenous substances and diverse polypeptides such as adenosine triphosphate, $Ca^{2+,}$ calmodulin, calsequestrin, carnosine, sorcin, homer, protein kinase A, magnesium, and the 12 kD FK506 binding protein (FKBP12) (194). These substances are thought to influence the activity of the channel, and to regulate intracellular Ca^{2+} during contraction and relaxation. If mutated, they could be responsible for increasing Ca^{2+}cycling and cause MH. However, at present, no identified mutations in genes that encode for any of these proteins have conclusively been associated with MHS. As a result of the multiple coupling proteins and polypeptide interactions, and because of the involvement of RYRs in many physiological processes and their role as action sites for a range of toxins, pesticides, and drugs, they have become an interesting target for physiologic and pharmacological investigations (111).

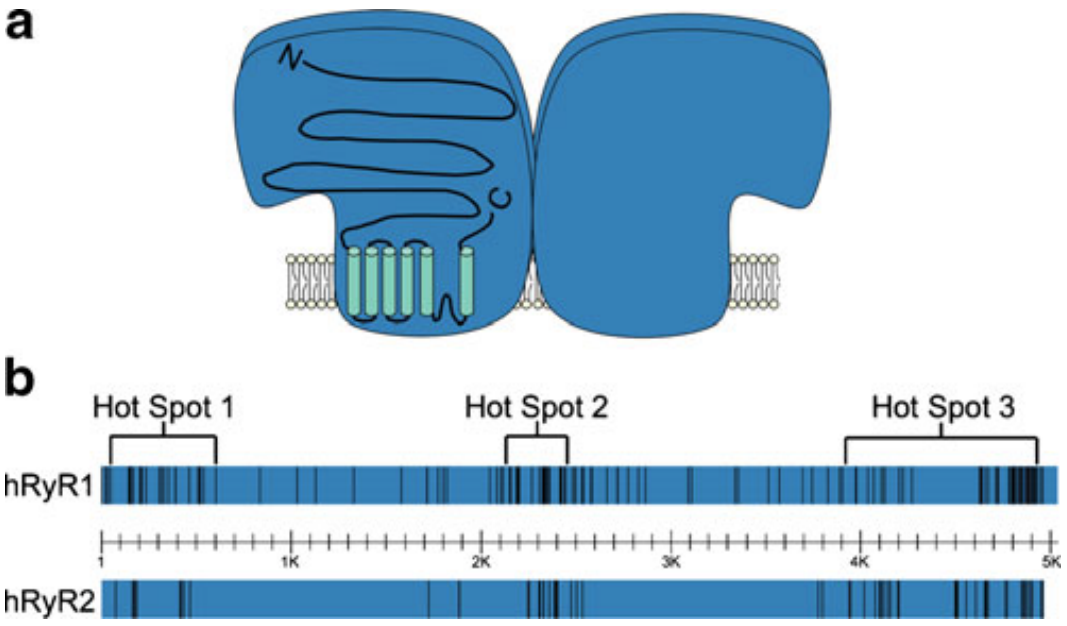

Figure 4. The structure of the RYR Ca^{2+} release channels and the location of *RYR1* and *RYR2* hot spot regions.

Disease-related mutations in human *RYR1* and *RYR2* are clustered in three mutational "hot spots." a The tetrameric structure of RyR Ca^{2+} release channels with the membrane topology superimposed on one of the subunits. b The distribution of over 200 MH and CCD-causing mutations in human *RYR1* and over 70 catecholaminergic polymorphic ventricular tachycardia-associated mutations in human *RYR2*. Published in European Journal of Physiology, 460, Betzenhauser MJ, Marks AR. Ryanodine receptor channelopathies, 467–480. Copyright Springer-Verlag (2010), with permission from Springer-Verlag.

There are many compounds that activate or inhibit RYR Ca^{2+} function and are used to study channel function or in functional assays to classify *RYR1* variants. The physiological and chemical activators of RYR1 channels include Ca^{2+}, calmodulin, adenine nucleotides (ATP and ADP), ryanodine (in low, submicromolar concentrations), caffeine, 4-CmC, and halothane; these agonists enable the channel to open, releasing Ca^{2+} ions from the SR and initiating muscle contraction. RYR1 channel inhibitors that decrease the open probability of the channel include Ca^{2+} (in millimolar concentrations), ryanodine (in micromolar concentrations), ruthenium red, dantrolene, and local anesthetics such as procaine and tetracaine (194).

The Functional Effects of *RYR1* Mutations

Functional studies of *RYR1* variants have revealed that MHS is caused by mutant RYR1 channels, and that most MH-causing mutations disturb normal Ca^{2+} intracellular regulation by shifting the sensitivity to pharmacological activation to lower agonist concentrations. Others may also cause an increase in the resting Ca^{2+} concentration, in addition to increasing the activation threshold (39; 55; 106). Although MHS and CCD are caused by different *RYR1* mutations in the majority of cases, there is some genotype-phenotype correlation—such as a hypersensitive channel in the case of MHS, or weak

muscles in the case of CCD—and in some cases both phenotypes together (79). *RYR1* mutations localized to the N-terminal and central regions of the protein (also referred to as domains 1 and 2) give rise primarily to MHS, while dominant *RYR1* mutations clustered in the hydrophobic C terminal region (domain 3) of the protein are mostly associated with CCD (194). Multi-minicore-linked mutations are distributed along the entire coding sequence (195).

RYR1 mutations cause four distinct types of channel defects (Figure 5): (a) changes in *RYR1* specific to MHS cause the channels to become hypersensitive to membrane depolarization and pharmacological stimuli; (b) *RYR1* mutations that cause CCD result in leaky channels, leading to depletion of Ca^{2+} from the SR; (c) mutations also linked to CCD that cause EC uncoupling, uncouple the channel from the DHPR voltage sensor causing inefficient activation of RYR1 and abnormal levels of SR Ca^{2+} release and muscle contraction; and (d) mutations associated with autosomal recessive multi-minicore myopathy result in protein Ca^{2+} channel instability and a decrease in mutant RYR1 channel expression on SR membranes (195). Despite the distinct channel defects resulting in different phenotypes, the majority of individuals with *RYR1* disorders (e.g., MHS, CCD, multi-minicore, or centronuclear myopathy) are susceptible to MH (158).

What differentiates the first two potential mechanisms (i.e., hypersensitive vs. leaky), correlated with MHS and CCD, respectively, is the rate of Ca^{2+} leak from the SR into the cytoplasm (66). *RYR1* mutations that cause a low leak rate lower the threshold for triggering MH, but do not deplete Ca^{2+} stores enough to cause a CCD myopathy. However, *RYR1* mutations causing a high leak rate may sufficiently deplete Ca^{2+} stores in

the SR to produce a deficiency of Ca^{2+} during normal muscle contraction, producing the chronic muscle weakness seen in CCD and, at the same time, lowering the sensitivity of this channel to RYR1 agonists—triggering MH. A single mechanism with a variable rate could account for two seemingly unrelated phenotypes in the same patient. The third mechanism, EC uncoupling, accounts for the characteristic CCD phenotype of muscle weakness. Allele silencing is used to explain the link to the MH phenotype in a few cases of multi-minicore myopathy (66),

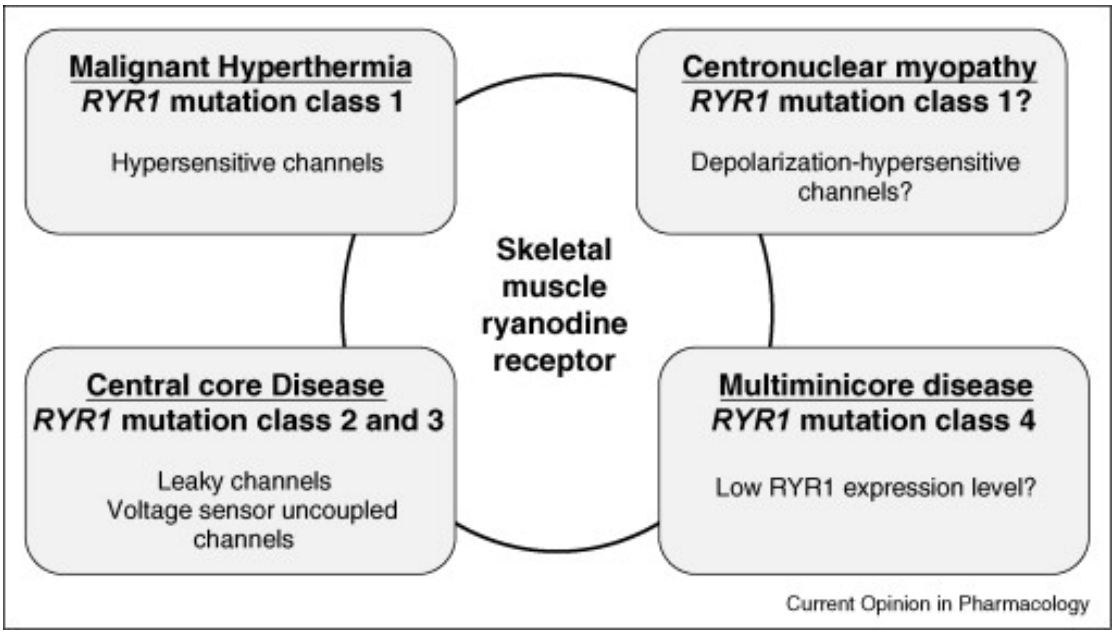

Figure 5. Classification of RYR1 channelopathies on the basis of the functional effect of mutations.
Published in Current Opinion in Pharmacology, 8, Treves S, Jungbluth H, Muntoni F, Zorzato F. Congenital muscle disorders with cores: the ryanodine receptor calcium channel paradigm, 319-326. Copyright Elsevier (2008), with permission from Elsevier.

Experiments have not been able to fully clarify the effects of *RYR1* variants on intracellular Ca^{2+} levels, nor explain the phenotypic differences between MHS and CCD

and the variations in the properties of different aspects of the RYR1 channels. Therefore, the effects of specific *RYR1* variants, previously uncharacterized by functional analysis, cannot be inferred. Functional testing and phenotype data are essential.

The Functional Assay of *RYR1* Mutations

To verify the pathogenicity of variants discovered through genetic analysis, and to understand pathophysiological mechanisms of *RYR1* variants, a number of functional *in vitro* assays have been used. These include patch clamp experiments (36; 202), intracellular Ca^{2+} measurements in human muscle cells or myotubes generated from muscle biopsy tissue (205-207), lymphoblasts expressing endogenous mutations from MHS individuals (55; 167; 191), and various heterologous non-muscle expression systems like HEK293 (human embryonic kidney cells), COS-7 (immortalized fibroblast like cells from monkey kidney tissue), and 1B5 dyspedic myotubes (multinucleated myotubes from primary fibroblast cell lines) transfected with mutant *RYR1* complementary DNA (cDNA) (32; 116; 138; 196; 208; 213). In COS-7 or HEK293 systems, the host cells are transfected with mutant cDNA from rabbit *RYR1* by site-directed mutagenesis. Mice with "knock-in" mutations are the most recent system for functional characterization of *RYR1* variants (8; 13; 43; 108; 212). All of the aforementioned systems meet the EMHG's criteria for use in functional testing for mutations in the *RYR1* gene (126). The EMHG specifies that only *RYR1* variants that have been functionally characterized for the effect of Ca^{2+} release can be adopted for use in a predictive genetic test for MHS (1).

Calcium release has been monitored and quantified in these assays by using calcium-specific florescent markers like Fura-2 and Fluo-4 (58), RYR binding assays

(148; 213), and by protein release (87). Three of the commonly used RYR agonists are caffeine, halothane, and 4-CmC. 4-CmC is preferred as an RYR1 channel agonist, as it is a more potent and specific RYR1 activator than halothane or caffeine, as it opens RYR channels at much lower agonist concentrations (micromolar versus millimolar) (64; 65; 218). In addition, 4-CmC is preferentially used in functional RYR1 assays with intact muscle, as caffeine has been shown to activate other nonspecific channels in smooth muscle cells and RYR3 is expressed in muscle cells at low levels. Therefore Ca^{2+} release results measured by the addition of caffeine might not represent RYR1 channel function but a combined Ca^{2+} release from several channels (154). Thapsigargin is another RYR1 activator often used to quantify the total stores of Ca^{2+} prior to measurement, or to block the reuptake of Ca^{2+} into SR stores (155).

A comprehensive review of a decade of MH research, by Stowell and coworkers in 2008, listed 18 studies from 1997 to 2007 that functionally characterized 34 *RYR1* mutations using one of six experimental systems: lymphoblasts (5/18), HEK293 cells or human myotubes (9/18), knock-in-mice (2/18), IB5 myotubes (1/18), and SR vesicles with mutated peptides (1/18). All but four of the 18 studies used a fluorescence or fluorimetry assay with Fura-2, and the agonists caffeine, halothane, or 4-CmC separately or in combination. The remainder used a ryanodine binding assay or the florescent indicator Fluo-4. After analysis in the various studies, 29 of the 34 variants assayed (85%) met the criteria of the EMHG for diagnostic classification (179).

The Merits and Disadvantages of Alternative Approaches

Each of the above-listed systems have their comparative advantages. The pig animal model is useful to study MH but is limited due to the fact that only one *RYR1* mutation is

involved and the recessive inheritance pattern differs from the dominant pattern in humans. Also, large animal research is expensive. A relatively new approach in MH research is the use of transgenic mice. Creating a specific *RYR1* mutation in a murine model through the use of the "knock-in" approach has provided strong evidence for functional defects of *RYR1* mutations *in vivo*. Yang and coworkers provided validation of a mouse model for studying MH by using mice with the *Ryr1* (p.Arg163Cys) mutation (212). Both heterozygous and homozygous animals had fulminant malignant hyperthermia after exposure to 1.25–1.75% halothane and myotubes from these mice had increased sensitivity to 4-CmC (212). This model enables investigators to study the long term *in vivo* effects of *RYR1* mutations on skeletal muscle. The disadvantages of this method include the needed technical effort, expense, and time involved in creating knock-in mice (for mutations that have not been made). It can also be difficult to interpret such studies, since the phenotypes of some knock-in mice have been different from human MH (180). Special lab facilities, and personnel are needed and it can take years to create and breed transgenic mice, therefore the use of transgenic mice is limited to well-funded and resourced labs.

A commonly used method to analyze the functional effects of variants that cause *RYR1* disorders is the use of rabbit *RYR1* cDNA (versus recombinant human *RYR1*) cloned and expressed in HEK-293 or COS-7 cells. This method is especially useful if human tissue samples are not available. However, the cloning of the *RYR1* cDNA can be technically difficult due to its size (~15,000 bp) and with the cell growth for needed for cloning and transfection (180).

The 1B5 myotube system is dyspedic mouse (1B5) cells differentiated into

multinucleated myotubes. The system is similar to COS-7 or HEK 293 cell systems in that it uses non-human cDNA (i.e., rabbit in the case of COS-7 and HEK 293). However, 1B5 cells are considered more physiologically relevant than the simpler COS-7 or HEK 293 cell systems, as they express all the components of skeletal muscle, such as triadic proteins, including DHPR, FKBP12, binding protein, calsequestrin, and triadin (45). They are also thought to provide a more sensitive measure of Ca^{2+} release than COS-7 or HEK 293 cells (158). A drawback to the use of 1B5 cells is that it takes the mixing of species further than COS-7 and HEK 293 cells, by using human *RYR1* mutations engineered into a non-human cDNA and expressing them in mouse myotubes. However, the amino acid sequences of human, rabbit and mouse RYR1 are similar, but not identical (164). Thus, the use of mutant forms of recombinant human *RYR1* (e.g., human myotubes or lymphoblasts) may provide a more genotypically related system to study RYR1 function. Other limitations of the myotube system are that myotubes can only be studied over a limited time period, they can over express wild type and mutant proteins—a problem when studying gain-of-function mutations like MHS—and they do not exactly reflect EC-coupling but rather an earlier developmental state of skeletal muscle (109).

Cultured myotubes (primary myoblasts), established from skeletal muscle biopsy samples of MHS individuals, is a relatively simple method that has been used commonly in functional studies. The advantages are that (a) it has most of the muscle cell components and thus closely matches conditions in vivo, (b) it does not need cDNA cloning, and (c) it is easier and faster than most non-muscle expression systems. The system's two main drawbacks are that it needs surgical excision of skeletal muscle tissue excised from an individual, and the myoblasts have limited life (206). This precludes its

use for testing or research involving large numbers of MHS individuals. Although it maybe recognized as an advantage that myotubes contain all the EC coupling machinery, thus the most representative in vivo, it may also make abnormal functional tests more difficult to interpret as it is possible that another EC coupling protein, for example the DHPR, is also involved in the abnormal response.

After the initial discovery by Sei and coworkers that human B-cells express the skeletal muscle isoform of RYR1 (168), and the subsequent complementary research by Girard and coworkers that showed MH mutant RYR1 channels in lymphoblasts cause a phenotypic response of increased Ca^{2+} consistent with that observed in skeletal muscle (55), the lymphoblast model has provided a straightforward functional assay that has been in wide use for RYR1 investigation. In contrast to other assays, lymphoblasts can be derived from a peripheral blood sample, and the assay can generally be carried out after approximately one month in culture. A sample can be readily obtained without surgery or anesthesia, which is likely to increases participation in MH genetic research and screening. The immortalized cells can be grown in large numbers, and are considered stable in culture and in storage and in re-culture cycles (freeze-thaw), for repeated usage in assays over long periods. Perhaps the two greatest advantages of lymphoblasts are that they are representative of the genotype of the individual, and they express *RYR1* but few of the other muscle components such as the DHPR; thus any altered Ca^{2+} release in response to specific RYR1 agonists in these cells will most likely be due to altered RYR1 function and not some other altered protein (154). In addition to providing a means to functionally test *RYR1* variants, this system can also be used to eliminate *RYR1* as a causative factor in MHS individuals who do not show linkage to

RYR1 (158).

SUMMARY OF THE REVIEW OF LITERATURE

From a clinical perspective, a major barrier to the management of MHS is the development of a simple, minimally-invasive diagnostic test that is sensitive, specific, and practical. It is important to replace the muscle biopsy test with a less invasive test to reduce costs, provide greater access to testing, and eliminate the need for surgical sampling. This becomes critical when many genetic variants predisposing to MHS are identified and need to be functionally characterized. Exome sequencing of the *RYR1* gene, coupled with a novel system to better identify and inventory pathogenic variants—and the use of a minimally invasive assay—will shorten the detection time for MHS genetic defects. Our pilot study is intended to evaluate the potential for large-scale studies to correlate mutation with function, which would then allow us to better predict function from mutation alone. These goals lead us to the test the hypothesis that ES can be used to identify putative pathogenic MHS variants in a population unselected for the disorder. This was coupled to functional analysis using lymphoblasts derived from individuals with predicted pathogenic *RYR1* variants identified through ES. We set out to measure sensitivity to the RYR receptor agonist 4-CmC in predicted pathogenic vs. wild type RYR1.

CHAPTER 3: MATERIALS AND METHODS

RESEARCH DESIGN AND METHODOLOGY: PARTS I AND II

We took the following steps to accomplish the aims of Part I and Part II of the study following ES of volunteers:

I. Selection of *RYR1* and *CACNA1S* variants through quality and frequency filters, then evaluation and scoring of variant pathogenicity, followed by an examination of the conservation of the amino acid residues for the *RYR1* variants selected for functional analysis, and literature and database analysis;

II. The EBV-immortalization of B-lymphocytes and culture of lymphoblastoid cell lines derived from participants carrying the sequence variants; and

III. Calcium release measurements in response to the RYR1 agonist 4-CmC in lymphoblasts.

RESEARCH DESIGN PART I: VARIANT SCREENING AND SELECTION

Data from Part I of this study were generated from the intramural NIH ClinSeq® project (07-HG-0002). The ClinSeq® project is a longitudinal pilot study on the technical, medical, and genetic counseling issues associated with large-scale medical sequencing and its application to personalized medicine (17). The participants in ClinSeq®, approximately 1,000 to date, were evaluated for personal health status and family history upon enrollment. The project was designed to identify novel genetic variants that influence clinical phenotypes, and to overcome the lack of phenotypic information often found in large-scale sequencing projects.

ClinSeq® is uniquely designed for collaboration and iterative testing: one of the aims of the project was to build an open, shared resource for clinical research in genomic

medicine; the project has the ability to recruit family members of volunteers; and the project maintains identified samples, which permits the return of volunteers for further testing (17). ClinSeq® methodology, and its use of ES technology in the discovery of causative variants, is particularly appropriate for research into MHS, as one of the aims of the project is to develop approaches to predictive medicine by giving researchers access to a cohort with deep genomic characterization, and the ability to perform iterative clinical research to validate the pathogenicity of the detected genomic variants — particularly for diseases where early identification may lead to interventions that could lower the risk of developing the disease (16; 18).

The ClinSeq® study was reviewed and approved by the Institutional Review Boards (IRBs) of the National Human Genome Research Institute and Suburban Hospital (Bethesda, MD) and all participants provided informed consent for all molecular characterizations of their samples, return of results, recontact for possible follow up studies, publication of results, and deposit of sequence data into databases. Participants were 45 to 65 years of age at enrollment with a median age of 57 years. These volunteers were unselected for MHS because they were ascertained for a spectrum of coronary artery disease, which is not associated with MHS. We did not use ES data from the entire ClinSeq® cohort of 1,000 for this dissertation research; ES data were available on only 870 participants when this work was first initiated in 2012. The subsample was 89% Caucasian, 96.3% not of Hispanic or Latino background, and 49.7% female. Family history, race, ethnicity, current medical status, and clinical data were collected at enrollment, although a personal or family history of MHS was not specifically solicited. Race and ethnicity were determined by self-report on an intake questionnaire. First-

degree relatives of another participant were excluded but one dyad of participants were first cousins and one dyad were first cousins once removed. During their initial visit, participants underwent an electrocardiogram, echocardiogram, and computed tomography scan for coronary $Ca^{2+,}$ clinical chemistries, and blood sample collection for genomic analysis. Sequence variants deemed clinically relevant were validated in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory and the results returned to the participant.

Sequence data were generated at the NIH Intramural Sequencing Center (NISC). Exome sequencing of ClinSeq® participants was performed on a massively parallel sequencing platform (Illumina, Inc., San Diego, CA) (75). The sequencing method used solution-hybridization exome capture (SureSelect 38 and SureSelect50, Agilent Technologies, Santa Clara, CA or TruSeq Illumina Inc., San Diego, CA). The sequencing with a mix of primary sequence runs — some older ones were 75 bp — and 101 base-pair (paired-end reads) was performed with the GAIIx sequencer (Illumina, Inc., San Diego, CA). One or two 101 base-pair, paired-end flow-cell lanes were sufficient to generate greater than 85 percent coverage of the targeted exome with high-quality variant detection (187). Filters were applied with the VarSifter Next-Gen variation analysis software (188). DNA isolation, library preparation, capture, sequencing, and alignment and base calling were performed as described (75). Clinical findings were analyzed and correlated with the sequence data.

METHODOLOGY PART I: METHODS OF VARIANT SCREENING AND SELECTION

Variant Screening

Our first aim was to identify pathogenic variants in *RYR1* and *CACNA1S* in a population not ascertained for MHS, as a pilot for the use of exome data for predictive medicine. New sequencing technologies, including ES, have made sequencing of the human exome (exons of known genes) feasible. Because of the very low marginal cost of sequencing any particular gene, this provides the opportunity to detect mutations in MHS genes in a large number of unselected research participants. Using this approach, we can improve our understanding of the mutational spectra of the *RYR1* and *CACNA1S* genes, and estimate their prevalence.

However, assigning pathogenicity to *RYR1* and *CACNA1S* variants is challenging for several reasons. First is the issue of locus heterogeneity. With several mapped loci without identified genes, some *RYR1* and *CACNA1S* variants may have been erroneously determined to be pathogenic when there was a causative variant in another (untested) gene. The methods of scoring the variants can vary between the different Locus Specific Databases (LSDBs), making the use of the databases for analysis problematic (75; 134). Finally, *RYR1* and *CACNA1S* genes are large with 106 and 44 exons, respectively, making mutation screening challenging. Thus, some *RYR1* and *CACNA1S* variants previously determined to be pathogenic may be benign, as has been shown for other genes (37; 40; 82). For this reason, we reanalyzed the evidence for every variant that passed frequency and quality filters by evaluating the literature on pathogenicity, as per current recommendations (110).

We enhanced the pathogenicity assessment by (a) the use of ES in an unselected cohort, (b) a variant filtering system modified for this disorder, (c) the robust phenotypic data in the ClinSeq® cohort, and (d) the ability to perform functional testing of the scored

variants. The filtering and evaluation of *RYR1* and *CACNA1S* gene variants and the details that led to the selection of the *RYR1* variants for further functional testing in this study are described below.

Following ES of 870 participants, and filtering the *RYR1* and *CACNA1S* variants for quality and frequency, we analyzed nonsynonymous, frameshift, nonsense, and splice-site variants using methods similar to those published by Johnston and coworkers (75), using an adaptation of published criteria (34) for cancer susceptibility genes (illustrated in Figure 6). We referred to the cDNA variants and their predicted protein change by their protein designations in Table 2 below. The *RYR1* and *CACNA1S* nucleotide numbering is based on transcript variant NM_000540.2 and NM_000069.2, respectively, according to the standards of the Human Genome Variation Society (HGVS, http://www.hgvs.org/) (67). Variants were filtered for quality and frequency using four criteria: (a) for genotype quality with most probable genotype (MPG) (187) score >10, (b) by MPG/read count ratio >0.5, (c) for minor allele frequency (MAF) in the ClinSeq® cohort and (d) for MAF in the National Heart, Lung, and Blood Institute's (NHLBI) Exome Variant Server (EVS, downloaded February 2, 2012).

We used MPG as a quality filter criteria, and only accepted genotype assignments with an MPG prediction score of 10 or greater, because MPG was found to be a best balance of sensitivity and accuracy (187). The MPG algorithm is built on a Bayesian model of prior probabilities that simulates sampling from one or two chromosomes with sequencing error and then calculates the likelihood of each possible genotype given the observed sequence data. An MPG score of 10 is a log-scaled value indicating that the theoretical probability of the call being incorrect is a $1/e^{10}$ or 1/22,026 (4); or seen differently, a score of 10 generally indicates a depth of coverage in the 10x to 20x range and to an expected accuracy of roughly

99.9% (49).

We also used the quality filter MPG/coverage ratio as a method for filtering out poor quality (i.e., false or incorrect) variants as many noticed that false positive calls in ES data almost always had MPG/coverage ratio less than 0.5, before Wei and coworkers quantified its utility for filtering bad variants (209). The NIH/ National Human Genome Research Institute, Genome Technology Branch's staff scientist, N. Hansen (personal communication, February 4, 2015), stated that an MPG/coverage ratio of less than 0.5 for a given coverage is a bad sequencing call because—in normal diploid sampling from two chromosomes—the Bayesian score calculated by the genotype caller program bam2mpg has a fairly tight distribution around its expected value and increases linearly with depth of coverage; so a score of less than 0.5 is an indication that the sequencing reads may not be derived from a true, heterozygous location in the genome.

We viewed the EVS data set as a random sample because, similar to ClinSeq®, the cohort was not selected for MHS status and thus representative of the general population with regard to the frequency of MHS. We excluded variants with an MAF of >0.5% in ClinSeq® and >0.005 in EVS from further pathogenicity assessment as any variant at this frequency was unlikely to cause MHS. We sacrificed sensitivity or clinical validity (i.e., the proportion of actual positives) during screening by the use of stringent quality and frequency thresholds, in order to minimize false-positives, yet ascertain variants in the two genes with a high positive predictive value.

We then categorized the *RYR1* and *CACNA1S* variants that survived this filtering into pathogenic groups based on mutation type (e.g., missense, nonsense, frameshift, or splice) and information from various locus-specific variant databases, as described in this section in

the paragraphs that follow and in Appendix A. To assign a pathogenicity score, we examined the locus-specific databases for references to the variants found in our dataset; if a database entry was found, we reviewed and assessed the cited publications citing information and assigned each variant to a pathogenicity class (class 0 to 5) according to the criteria in Appendix A. Our criteria for determining pathogenicity, formulated specifically by us for this study, are similar to those used previously in studies of other disorders (5; 15; 56; 77; 189). Our criteria were similar to those used for the established MHS mutation panels (Appendix A). To locate references to our variants, we used information from the Human Gene Mutation Database (http://www.hgmd.org; HGMD -Professional 2012.2 from BIOBASE) (177), and one of the Locus Specific Databases (LSDB) referred to as the Leiden Open Variation Database (http://www.lovd.nl/3.0/home, LOVD, v.2.0, Build 34) (47); and Universal Protein Resource (2002-2012 UniProt Consortium, http://www.uniprot.org/) (12), as well as citation trackers: Google Scholar 2012; Scopus, 2012 Elsevier B.V.; and Web of Science, 2012 Thomson Scientific.

We designated *RYR1* and *CACNA1S* variants with low genotype quality (i.e., variants that did not meet our quality threshold) as class 0, and *RYR1* and *CACNA1S* variants with a MAF greater than our ClinSeq® and EVS frequency thresholds as class 1, almost certainly benign. This classification is based on the assumption that given an MHS population frequency of 1/2,000, no single causative variant could have a frequency above these thresholds and be pathogenic because that would imply that the prevalence of MHS is much higher than 1/2,000. The population frequency is based on the estimated prevalence of the MHS trait as described previously in Chapter 2, epidemiology section. The remainder we scored on a pathogenicity scale of class 2-5. Class 2 variants were

likely benign, class 3 of uncertain significance, class 4 likely pathogenic, and class 5 pathogenic. We assigned all novel (i.e., newly discovered distinct gene alteration) *RYR1* and *CACNA1S* missense variants or in-frame insertion/deletion variants not listed in HGMD or in the other LSDBs as class 3 (uncertain significance). We designated novel missense variants as class 3 as there is insufficient information to whether the change has a damaging affect on the function of the protein or protein complexes, and current predictive mutation algorithms (i.e., *in silico* evaluation) for classification of missense variants are unreliable and can produce erroneous conclusions regarding pathogenicity (91; 149; 190). However, for other *RYR1* and *CACNA1S* mutation types such as novel nonsense, frameshift, and splice-site variants, the characteristics of the variant and a family history of MHS were considered. When an *RYR1* and *CACNA1S* variant was reported as pathogenic in HGMD and/or an LSDB, we reviewed the pertinent citations to determine whether the variant should be assigned to classes 2 to 5. *RYR1* and *CACNA1S* variants listed in but *not* reported as pathogenic by HGMD and/or an LSDB were defined as variants of uncertain significance (VUS) and assigned as class 3 or 2 based on primary studies.

We designated *RYR1* variants as class 2 (likely benign) if they were on EMHG's list (http://www.emhg.org/home/) of 156 nonpathogenic variants (1), or if an *RYR1* and *CACNA1S* variant had been published two times or more as benign, or had some evidence to support as likely benign or had multiple forms of evidence against pathogenicity. Evidence for likely benign status included equivalent frequency in cases and controls, and/or normal functional data. We also categorized *RYR1* and *CACNA1S* missense, and nonsense, frameshift, and splice variants with a single publication of pathogenic, but with multiple

forms of evidence against as class 2, likely benign.

We designated *RYR1* and *CACNA1S* missense variants as class 3 (uncertain significance) if they were reported a single time in the published literature as pathogenic without supporting evidence, or if two or more publications concluded that they were pathogenic but there were an equal number of publications or greater against pathogenicity. We also designated *RYR1* and *CACNA1S* nonsense, frameshift, or splice-site variants as class 3 (uncertain significance) if they were reported in a single publication as pathogenic with single publication providing against, or if two or more publications as pathogenic with two or more publications concluding that there was evidence against pathogenicity. The reasoning for these assignments is further described in the Supplemental Methods text in Appendix G

RYR1 and *CACNA1S* missense variants were designated class 4 (likely pathogenic) if there were two or more publications as pathogenic with single evidence against, or a single publication as pathogenic with supporting evidence (i.e., characterization of the mutation at the genetic level, segregation in at least two pedigrees, absent in 100 or greater control samples, functional characterization in an EMHG-approved test system) but the published study did not meet all the qualifying criteria set by the EMHG that a genetic mutation should fulfill prior to its use in predictive genetic testing (126). We similarly designated *RYR1* and *CACNA1S* nonsense, frameshift, or splice-site variants as class 4 (likely pathogenic) if there were two or more publications as pathogenic with single evidence against or a single publication as pathogenic without supporting evidence.

We categorized an *RYR1* variant found on the EMHG list (http://www.emhg.org/home/) of 34 approved causative mutations (1) as a class 5 (pathogenic) regardless of mutation type or personal or family history of ClinSeq® proband.

RYR1 and *CACNA1S* missense variants were also designated class 5 (pathogenic) if there were two or more published reports as pathogenic and no evidence against. *RYR1* and *CACNA1S* nonsense, frameshift, and splice-site variants were designated class 5 (pathogenic) if there was a single publication as pathogenic and no evidence against (Appendix A).

We reviewed the medical histories of the probands and their pedigrees for diagnoses or symptoms of MHS and related disorders. We learned retrospectively that one participant self-referred to the study because of a clinical diagnosis of MHS (subsequently found to have *RYR1* c.11958C>G, p.Asp3986Glu). We returned clinically relevant results to all participants with class 5 variants predicted to cause MHS for management following lab confirmation in our CLIA-certified laboratory. We provided medical and genetic counseling to the participants when returning test results, and referred them for consideration for caffeine halothane contracture testing, enrollment in the North American Malignant Hyperthermia Registry (NAMHR, http://www.mhaus.org) of the Malignant Hyperthermia Association of the United States (MHAUS, http://www.mhaus.org/), and registration with the Medical Alert Foundation (http://www.medicalert.org) for medical alert identification through MHAUS (2; 3).

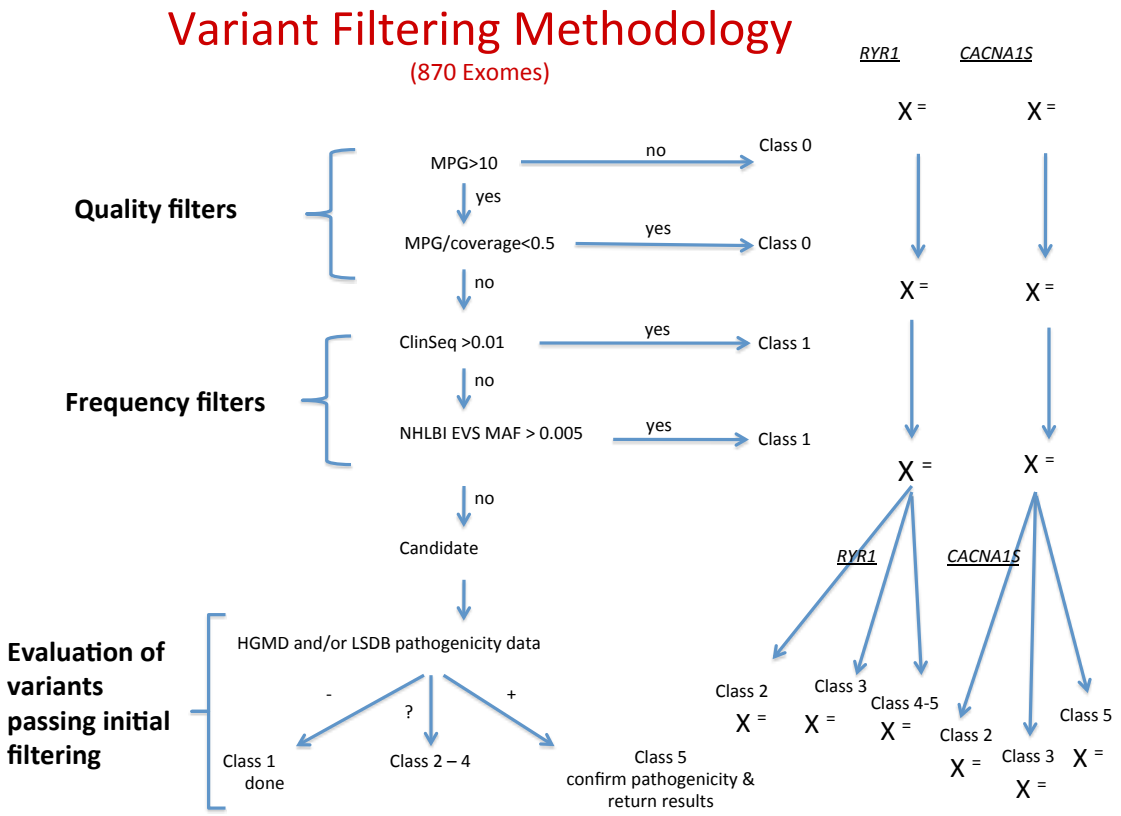

Figure 6. Quality, frequency filter algorithm.

Filtering criteria used for coding-variant interpretation. Variants were filtered on genotype quality, coverage and allele frequencies. Variants that were removed by quality filters based on most probable genotype scores and coverage were classified as 0 and variants that were removed by minor allele frequency filters, from data present in ClinSeq and in the National Heart, Lung, and Blood Institute, exome sequencing project, were classified as Class 1, with the remaining Classes 2-5 from data present in the Human Gene Mutation Database (HGMD) and locus specific databases (LSDBs) when available. MPG =most probable genotype. MAF =minor allele frequency. NHLBI EVS =The National Heart, Lung, and Blood Institute, exome sequencing project.

Variant Selection

Following the filtering, evaluation and categorization of *RYR1* and *CACNA1S* gene variants from 870 individuals between 45 and 65 years of age enrolled in ClinSeq®, we

selected a subset of the *RYR1* mutations made up of three class 5s (pathogenic), and one class 3 (uncertain significance), for testing. We experimentally assessed the functional consequences of these variants for RYR1 channel function in lymphoblasts obtained from ClinSeq® volunteers heterozygous for these mutations—as compared to an *RYR1* confirmed wild type (CWT) control group of lymphoblasts from three ClinSeq® volunteers. (The selection of matched controls is described in Methodology Part II). Calcium dysregulation was studied in four discrete lymphoblastoid cell lines carrying one of the four *RYR1* predicted missense variants identified in part 1, namely: (a) c.5183C>T, p.Ser1728Phe, (b) c.11958C>G, p.Asp3986Glu, (c) c.4999C>T, p.Arg1667Cys, and (d) c.1840C>T, p.Arg614Cys[3]. The four lymphoblastoid cell lines derived from ClinSeq® participants with these variants were chosen as the experimental group for functional analysis by several criteria:

(a) their putative pathogenicity is reported in HGMD and/ or in LSDB and these assessments are robustly supported by primary literature;

(b) they are of the type of genetic variant (i.e., nonsynonymous substitutions), and inheritance pattern (i.e., predominately autosomal dominant), which are predicted to cause a gain of function in RYR1 and thus predispose to MHS (150);

(c) they are rare, found at a minor allele frequency (MAF) <0.5% in NHLBI's Exome Sequencing Project (ESP), or in the ClinSeq® study at a MAF <1%;

[3] Herein the genomic changes c.5183C>T, c.11958C>G, c.4999C>T, c.1840C>T referred by their designated protein change p.Ser1728Phe, p.Asp3986Glu, p.Arg1667Cys and p.Arg614Cys.

(d) their pathogenicity scores, identified through ES and variant analysis, are scores of 5 (pathogenic), or categorized as a class 3 (variant of uncertain significance); and

(f) they have not been functionally characterized in peer reviewed research, except for one variant (p.Arg614 Cys)—used in our study as a *positive control*—that has been functionally characterized in lymphocytes from pigs carrying the c.1843C>T, p.Arg615Cys (117) and in HEK-293 cells and primarily human myotubes (25; 192; 213), however not from humans in lymphoblasts.

The four selected variants and their pathogenicity scores, nucleotide changes, predicted protein changes, associated disease state, allele counts, and source listings are provided in Table 2 below.

Table 2. *RYR1* variant table

***RYR1* Variants in Transcript NM_000540.2 Identified in 870 ClinSeq® Exomes**					
Nucleotide Change	**Predicted Protein Change**	**Associated Disease State**	**ClinSeq® Allele Count**	**NHLBI EVS Allele Count**	**ClinSeq® Pathogenicity Score**
***RYR1* Variants Listed in HGMD**					
c.1840C>T	p.Arg614Cys	Malignant Hyperthermia	1/1,740	NF	5^
c.5183C>T	p.Ser1728Phe	Malignant hyperthermia	1/1,740	1/10,757	5
c.11958C>G	p.Asp3986Glu	Malignant hyperthermia	1/1,740	NF	5
***RYR1* Variants Not in HGMD, Listed in Other LSDB Sources**					
c.4999C>T	p.Arg1667Cys	-?/?	4/1,740	21/10,727	3

Pathogenicity scores were determined as described in the Methods. Abbreviations used: NHLBI EVS, National Heart, Lung, and Blood Institute Exome Variant Server. NF, Not Found. HGMD, Human Gene Mutation Database (Professional 2012.2 from BIOBASE). LSDB, Locus Specific Database.

-?/? –These symbols are used in the LOVD variant pathogenicity rating, as reported on Leiden Muscular Dystrophy webpages. The first symbol indicates the conclusion of the publication cited in that publication entry. The second symbol indicates the conclusion of the database curator. Reported/Concluded; '+' indicating the variant is

pathogenic, '+?' probably pathogenic, '-' no known pathogenicity, '-?' probably no pathogenicity, '?' effect unknown. ^ on the European Malignant Hyperthermia Group's list of 34 diagnostic (causative) mutations (as of 08/2012).

The RYR1, c.5183C>T, p.Ser1728Phe Variant

The p.Ser1728Phe missense variant was listed in HGMD with references to two UK studies both by the same research team as pathogenic (31; 150). The same variant was found in the present study in one 47-year-old female ClinSeq® volunteer of Irish/British ancestry (1/1,740 alleles) without a personal or family history of MHS. The p.Ser1728Phe variant was first reported by Robinson and coworkers in three independent families from a variant and co-segregation analysis of UK patients with MH (151). In a subsequent genotype-phenotype correlation study, the p.Ser1728Phe variant was found in seven individuals and two families—six with *in vitro* contraction test (IVCT) data—but it was associated with a significantly weaker IVCT phenotype in comparison with the known pathogenic p.Gly2434Arg positive control (p.Gly2434Arg is on the EMHG panel of 34 causative MH mutations), suggesting a lesser effect on channel function as compared to their positive control (31). Extended pedigrees were investigated to assess concordance between MH susceptibility—by diagnostic IVCT—and RYR1 phenotype, and were absent in 200 controls. (The IVCT is the European protocol test for MHS and equivalent to the North American, Caffeine Halothane Contracture Test or CHCT) Since the rare p.Ser1728Phe variant (1/10,757 alleles in the NHLBI EVS) was reported multiple times as pathogenic, with no evidence against, it was scored as a class 5, pathogenic variant.

The RYR1, c.11958C>G, p.Asp3986Glu Variant

The p.Asp3986Glu variant was listed in HGMD with references to the same two UK studies cited above (31; 150). The variant was seen in five MH patients associated with significantly more severe static caffeine contractures (i.e., in place muscle contractions, versus dynamic) and greater Creatine Kinase (CK) concentrations than the p.Gly2434Arg positive control or other variants. It was also identified in one 45 year-old male ClinSeq® volunteer of Irish/German Ancestry (1/1,740 alleles) with a history of MH. The ClinSeq® volunteer, who was later determined to have been self-referred to the study (i.e., we did not know before hand that the participant had self referred for MHS, thus discovery of the MHS-related RYR1 variant was *a priori*) because of a clinical diagnosis of MHS from the Uniform Services University of the Health Sciences (USUHS) MH Muscle Biopsy Testing Center, had a history of multiple fulminant MH (anesthetic and nonanesthetic awake) episodes, as well as symptoms of myopathy, myotonia (dysphagia), proximal muscle weakness, a positive CHCT for MHS, and an elevated serum CK value 1,271 U/L and Lactate Dehydrogenase (LDH) level of 238 U/L (*RYR1* Participant Description Table, Appendix D).

In addition, the participant had a family history of myotonia and positive IVCTs for MHS in three siblings. This rare variant from *RYR1* MHS mutation hotspot region III was not found in over 6,500 human exomes in NHLBI EVS. It has been reported multiple times as pathogenic with no evidence against, so this variant was scored as a Class 5, pathogenic variant.

The RYR1, c.4999C>T, p.Arg1667Cys Variant

The p.Arg1667Cys missense variant in exon 34 was not listed in HGMD, but was referenced in LOVD with a citation to two studies (70; 89). LOVD listed the variant pathogenicity for both studies as "effect unknown" by both the publication's authors and site curators (70; 89). One study sequenced the entire *RYR1* coding region from genomic DNA of unrelated Japanese patients diagnosed with MHS by the calcium-induced calcium release (CICR) test—the Japanese equivalent of the North American CHCT or European diagnostic IVCT for MHS. The test participants were recruited after experiencing an MH episode, for having a relative with MHS, or for an increase in CK. The p.Arg1667Cys variant was present in five patients: two were positive for MH episodes, and two others had positive CICRs diagnostic for MHS. However, since the variant was also seen in two control samples, the researchers presumed the variant to be benign (70). No segregation data were included in the study.

In another study a representative cohort of 36 unrelated Canadians positive for the CHCT, or an MHS event, were screened for *RYR1* mutations, and selected regions of *CACNA1S* (89). After analyzing the correlation of the CHCT results to *RYR1* genotypes within MH families, the p.Arg1667Cys variant was found in one individual with a positive CHCT for MHS, but the absence of CHCT data for additional family members precluded segregation analysis (89). The p.Arg1667Cys variant was detected in four Caucasian (non-Hispanic) female ClinSeq® participants (4/870), all without family/personal histories and clinical findings suggestive of MHS or myopathy. The variant exists within the NHLBI ESP (21/10,727 alleles) at an allele frequency of 0.2 percent and a population frequency (assuming all are heterozygous) of 0.4 percent.

Based on the data presented, and the relatively low NHLBI EVS and ClinSeq® population frequency (<1%), this variant was categorized as a Class 3, of uncertain significance.

The RYR1, c.1840C>T, p.Arg614Cys Variant

The p.Arg614Cys variant was found in one participant, and listed in HGMD as pathogenic based on three publications, and was reported 37 times in LOVD (31; 53; 200). All of the submitting authors of these entries concluded that it was pathogenic. The p.Arg614Cys variant is one of the 34 RYR1 variants on the EMHG list of pathogenic variants, and is also included in the 2002 North American MH Working Group consensus list of 17 causative mutations (170). The variant is not in the NHLBI EVS database. Based on the data presented, we categorized this variant as class 5, pathogenic and designated the cell line with this mutation as a positive control for functional testing. It is interesting to note that the 62 year-old female ClinSeq® participant with this variant had no family or personal history of MHS. The participant characteristics for variant p.Arg614Cys and the previous described RYR1 variants are defined in Table 3 below.

Table 3. *RYR1* participant description table

Characteristics of ClinSeq® Volunteers with *RYR1* Variants					
Nucleotide Change; Predicted Protein Change	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
c.1840C>T:p.Arg614Cys	C/N/59/F	No proximal muscle weakness in family, no complications from anesthesia in any family member.	194^	194	Surgeries: (1) nasal turbinates, (2) left thigh mass removal, and (3) cholecystectomy 58y. Scarlet fever (5y)
c.4999C>T:p.Arg1667Cys	C/N/54/F	Negative	63	179	Surgeries: thyroidectomy 46y, total abdominal hysterectomy 45y. Thyroid cancer 46y. Hypothyroidism on THR
c.5183C>T;p.Ser1728Phe	C/N/47/F	Negative	97	149	Negative
c.11958C>G;p.Asp3986Glu	C/N/45/M	Proband with history of fulminant MH events and (+) CHCT; 3 siblings with myotonia	1,271	238	MHS, (+) CHCT Myopathy - myotonia with dysphagia and proximal muscle weakness; 3 fulminant MH events: 1) after surgery 1972, 2) after exercise in 2004 and 3) 2011 with rhabdomyolysis. Current medications: Dantrolene sodium, 100mg, daily in AM, by mouth; Morphine control release, 15mg, 2 times a day, by mouth

Participant information for *RYR1* variants identified in the 870 ClinSeq® subsample. Medical histories were reviewed for surgeries/ procedures and clinical conditions indicative of myopathy or that elevate serum creatine kinase (CK). Participants were evaluated for: any myopathy, MHS, and/or exertional heat illness; history of elevated CK; hypothyroidism and/or elevated thyroid stimulating hormone (TSH) > 4.00mcIU/mL; (TSH normal range: 0.40–4.00 mcIU/mL) hypokalemia <3.3 mmol/L; hyperuricemia > 8.6 mg/dL (uric acid normal range: 3.7-8.6 mg/dL); statin & nicotinic acid use; self-report alcohol use (≥3 bottles of beer, glasses of wine, or shots of distilled spirits per day); autoimmune conditions: rheumatoid arthritis, psoriasis, lupus, Sjogren syndrome, scleroderma; or renal disease. The race and ethnicity of the participants were determined by self-report.

Age, age at enrollment. Abbreviations used: CHCT, caffeine halothane contracture test. CK, creatine kinase, range: Female = 38-252 U/L; Male = 52-386 U/L. Ethnicity: N, not Hispanic or Latino; HL, Hispanic or Latino. LDH, lactate dehydrogenase, range = 113-226 U/L. MHS, malignant hyperthermia susceptible. Nucleotide change, NM_000540.2. Race: C, Caucasian, A, Asian, AA, African American, 0, other, "—" not reported. TRH, thyroid replacement hormone. Symbols used: "*" on Statin. " ^ "elevated serum TSH level.

RESEARCH DESIGN PART II: FUNCTIONAL TESTING

The proposed second aim of the project is an experimental *in vitro* design to quantitatively measure the RYR receptor type-1 response to the agonist 4-CmC. Our aim was to functionally characterize the RYR1 channels of four cell lines with the putative pathogenic MHS variants, and variants of uncertain significance identified following exome sequencing—and our annotated filtering and scoring process—by dye incorporation array, to determine their responses to Ca^{2+} release as compared to WT controls. The experimental and control groups were comprised of lymphoblasts generated from human B-lymphocytes obtained from the ClinSeq® Study. The ClinSeq® lymphoblastoid cell lines included both the MHS-positive and WT controls.

The culture of lymphoblasts from an affected individual to determine the significance of an *RYR1* DNA change is an accepted functional testing method by the EMHG (126; 152). In contrast to other assays, lymphoblasts can be derived from a peripheral blood sample, they can be grown in large numbers, the assay can generally be carried out after approximately one month in culture and the lymphoblasts are representative of the genotype of the individual. By quantitatively measuring Ca^{2+} release from the storage organelle in these cells, after exposure to RYR1-agonists—such as 4-CmC or caffeine—it is feasible to determine whether the *RYR1* variant elicits enhanced Ca^{2+} flux, as found in skeletal muscle cells of MH-susceptible individuals (55; 169; 191). The chemical 4-CmC specifically activates the RYR1 channel and can be used to determine whether or not an individual has MHS (65).

We used the Ca^{2+} flux/lymphoblast assay to functionally test experimental and control groups of lymphoblasts from four suspected MHS participants and three CWT

controls. The RYR receptor type-1 responses to 4-CmC from four MHS participants with distinct RYR1 variants, and three RYR1 CWT controls, were measured as the intracellular release of Ca^{2+}.

METHODOLOGY PART II: MATERIALS AND METHODS OF FUNCTIONAL TESTING

The functional testing method we used for the *RYR1* variants and WT controls was similar to the two methods used most recently by Perry and coworkers (141) and explained in text (93). The pharmacological sensitivity of the RYR receptor type-1 in lymphoblasts derived from four volunteers heterozygous for the RYR1 variants (p.Arg614Cys, p.Ser1728Phe, p.Asp3986Glu, and p.Arg1667Cys) were tested and compared to WT controls. We loaded the cells with a fluorescent Ca^{2+} indicator, Fura-2, and exposed to varying doses of 4-CmC (i.e., 0.47 mM, 0.81 mM) to measure the effect on Fura Ca^{2+} emission ratios. Changes in intracellular Ca^{2+} were measured and 4-CmC response curves recorded. To confirm that the increase in 4-CmC induced Ca^{2+} release—from lymphoblasts harboring the *RYR1* variants—are due to depletion of intracellular Ca^{2+} stores, we assessed endoplasmic reticulum Ca^{2+} content by treating cells with a maximal concentration of thapsigargin, a potent inhibitor of Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) pumps**.** Measurements were averaged from eight replicates for each cell line.

The procedures for both the functional testing of the *RYR1* variants, and the acquisition of WT control samples, exist as an addendum to a standing, approved protocol at the USUHS, Bethesda, Maryland. The protocol title, "*Is lymphocyte release of adenosine a metabolic marker of human susceptibility to malignant hyperthermia*?" is

approved by the USUHS IRB. This protocol is under an intramural grant (No: 80EO-A1) from USUHS.

Protocol for Acquisition of Presumed Positive MHS Group and WT Controls

For this investigation we used the existing lymphoblasts from the NIH ClinSeq® Study for the positive MHS and CWT control samples. We collected the samples as described in the ClinSeq® protocol (07-HG-002). Briefly, upon enrollment for the ClinSeq® study, blood samples are collected for basic clinical chemistry and hematologic analyses, for purifying DNA and RNA for genomic analysis, for establishing lymphoblastoid cell lines, and for long-term storage. We do not use patient names to identify samples; we assign them a ClinSeq® code. After collection and preparation, two tubes of volunteer blood samples per participant are transported to the Tissue Culture Facility at Georgetown University, Washington D.C., where the participant's B-lymphocytes are EBV-immortalized and maintained in liquid nitrogen.

For this study, after a request from us, the lymphoblasts were removed from storage and returned to the NIH—or USUHS research lab—in suspension as growing cultures. We cultured all the lymphoblastoid cell lines (experimental and control cell lines) in the identical medium solution and maintained in identical incubator conditions—listed above. At the time of testing, we confirmed the identity of the cell lines by PCR and Sanger sequencing of the variants identified by ES.

Selection of Confirmed Wild Type Controls

We matched controls for similarity on gender, race/ethnicity and age. In order to prevent the selection of controls with an undiagnosed myopathy, we selected for the absence of any personal or family history of MHS, myopathy, and exertional heat illness—by review

of the family health histories ascertained during participant enrollment—or any clinical signs or history of elevated serum myopathy markers, such as elevated serum CK, lactate dehydrogenase, $Ca^{2+,}$ or potassium level (52). We also excluded individuals if they had elevated serum markers known to elevate CK, or were on medications or had health conditions that are suspected causes of elevated muscle enzymes, such as hypothyroidism, low or elevated thyroid stimulating hormone, hypokalemia, hyperuricemia, hypo or hyperglycemia, renal disease, lipid lowering agents (e.g., statins, nicotinic acid, fibrates), and alcohol use of greater than or equal to three bottles, glasses, or shots per day (115). In addition, we selected controls for the absence of an autoimmune condition or connective tissue disease (e.g., rheumatoid arthritis, psoriasis, lupus, Sjogren syndrome, scleroderma) on their family pedigree. We collected participant and family history information, blood collection, and clinical test results at initial enrollment. The characteristics of the WT controls selected from the ClinSeq® 870 subsample, and the lab reference ranges, are shown in Table 4 below.

Table 4. Characteristics of ClinSeq® CWT controls

Characteristics of ClinSeq® Genotypic Wildtype Controls					
Control Number	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
Control 1 (#0057)	C/N/52/F	Negative	78	162	Surgeries: Total abdominal hysterectomy 51y (secondary to suspicion of ovarian cancer - none found), no medications
Control 2 (#0122)	C/N/52/F	Negative	66	135	Osteoarthritis in 40s, Obese, Temporomandibular joint disorder, Hypertension on thiazide diuretic
Control 3 (#0324)	C/N/52/F	Negative	91	153	Negative

Information for CWT controls selected from the ClinSeq® 870 subsample. Medical histories were reviewed for surgeries/ procedures and clinical conditions indicative of myopathy or that elevate serum creatine kinase (CK). Participants were evaluated for: any myopathy, malignant hyperthermia susceptibility (MHS), and/or exertional heat illness; history of elevated CK; hypothyroidism and/or elevated thyroid stimulating hormone (TSH) > 4.00 mcIU/mL; (TSH normal range: 0.40 – 4.00 mcIU/mL) hypokalemia <3.3 mmol/L; hyperuricemia > 8.6 mg/dL (uric acid normal range: 3.7-8.6 mg/dL); statin & nicotinic Acid use; self-report alcohol use (≥3 bottles of beer, glasses of wine, or shots of distilled spirits per day); autoimmune conditions: rheumatoid arthritis, psoriasis, lupus, Sjogren syndrome, scleroderma; and renal disease. The participant's race and ethnicity were determined by self-report.

Age =age at enrollment. Abbreviations used: CK range: Female = 38-252 U/L. Ethnicity: N = Not Hispanic or Latino; HL = Hispanic or Latino. LDH, lactate dehydrogenase, range = 113-226 U/L. Race: C = Caucasian, A = Asian, AA = African American, 0 =other, "—" = Not reported. TRH, thyroid replacement hormone. Symbols used: "*" on Statin. " ^ "elevated serum TSH level.

Materials and Test Solutions

Table 5. Source of materials and concentrations used in the experiments

Test Solutions	Source	Concentrations/ dilutions/ used
Bovine Serum (albumin)	Sigma Aldrich	0.1%
Dimethyl Sulfoxide	Sigma Aldrich	5 μM
EGTA	Fluka	1 mM
Fetal Bovine Serum	GIBCO Invitrogen	10%
Fura-2/AM	GIBCO Invitrogen	5 μM
Gentamicin Reagent	GIBCO Invitrogen	10 mg/ml
HBSS 1X	GIBCO Invitrogen	1X
Krebs Buffer Ca^{2+} free	Made in house	1 mM
L-glutamine	Sigma Aldrich	200 mM
RPMI 1640	GIBCO Invitrogen	87.9%
Thapsigargin	Sigma Aldrich	800 nM
4-chloro-*m*-cresol	Sigma Aldrich	400-2000 μM

The compound 4-CmC is a chlorocresol (Table 5), a phenol derivative, first used as an *in vitro* activator for RYR1-mediated Ca^{2+} release in 1993 (218), has been used pharmacologically to distinguish those who have MHS from those who do not in muscle samples and lymphoblasts (7; 55; 65; 117; 201; 220). When *RYR1* mutations are associated with the MHS phenotype, the sensitivity of the receptor to 4-CmC is increased—due in part to chronically elevated resting Ca^{2+} in MH-susceptible muscle (107). The compound is used as preservative in numerous pharmaceutical products such as succinylcholine, insulin, various heparin formulas, and human growth hormone (9).

In lymphoblasts, 4-CmC is preferred as an activator of RYR1 receptor over halothane or caffeine, as it is a more potent and specific drug: for example, (a) it opens the channel at strikingly lower concentrations (i.e., micromolar versus millimolar concentrations) and with a 10-25 times higher potency relative to caffeine (44): and (b) RYR1-mediated Ca^{2+} signals can be pharmacologically distinguished from other

intracellular sources in lymphoblasts, such as the IP3-sensitive stores and the mitochondrial stores (117). It activates RYR1 with an EC50 (i.e., the concentration of agonist that provokes a response halfway between the baseline and maximum response) between ~50-200 μM (218). The amino acids in the RYR1 protein needed for 4-CmC activation have been pinpointed to a region between amino acid residues 4007-4180 (44).

Cell Culture of Lymphoblasts

We performed experiments on Epstein-Barr virus (EBV)–transformed human lymphoblast cell lines generated at the National Institutes of Health as described in this section. Clinical center phlebotomy staff collected peripheral whole blood in sterile, Acid Citrate Dextrose yellow-top vacutainers and sent the samples, on the same day of collection, via courier to the Tissue Culture Shared Resource Center (TCSR, Lombardi Cancer Center, Georgetown University, Washington, DC) for B-lymphocyte transformation and biorepository of the cell lines. The TCSR staff isolated the mononuclear cells from anti-coagulated, peripheral whole blood. Lymphoblasts were established (i.e., transformed)—from 16 x 100 mm x 8.5 mL (tube size) blood samples collected in sterile anti-coagulated tubes and kept at room temperature and submitted within 72 hours after collection—by exposing the cells to EBV inoculation in the presence of cyclosporine A using standard procedures (19). A typical yield from a volume of 20 ml of whole blood provides approximately 3–6 $x10^6$ of fresh lymphocytes. The lymphoblasts were grown to a density of 2–3 $x10^5$ cells/ml at TCSR, harvested when the culture reached 10^7 cells in total (five to six passages), and stored in vapor–phase liquid nitrogen at –150 °C. In addition to the transformed samples, whole blood (~5 ml) was frozen and cryopreserved, after the addition of dimethyl sulfoxide, in the event that

the first, immediate transformation was unsuccessful. All frozen vials of lymphoblastoid stock preparations following cryopreservation were tested each time for viability and sterility.

The lymphoblasts were delivered for experimentation in T_{25} flasks containing cells in mid-log growth phase. We transferred the lymphoblasts and propagated them in T_{75} flasks containing growth medium on the day of delivery from the TCSR. We maintained the cells at a concentration of between 4×10^5 and 1×10^6 cells/ml and expanded as needed. We counted cells by the hemacytometer method (182). Lymphoblasts were cryopreserved for future use, and maintained at −80 °C or lower, if we could not perform testing on a cell line within 30 days. In order to minimize passages to avoid selection of oligoclonal or monoclonal cultures, we expanded the lymphoblasts as quickly as possible. As a procedural check, we submitted three cell line samples (two variant, one control) mid-way through testing (11/29/2013), as pellets of cells in growth medium and in phosphate buffered saline, to Idexx-Radil BioResearch lab (Columbia, MD) to be screened for mycoplasma bacterial contamination; all samples were negative by two distinct highly-sensitive PCR assays (detecting as little as 1-10 organisms) for 45 species of mycoplasma known to infect cells (IDEXX BioResearch Case #24680-2013).

We cultured lymphoblast cell lines in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% heat inactivated, fetal bovine serum (FBS/ Gibco Products, Invitrogen corporation, Grand Island, NY), 200 mM of L-glutamine, and 10 mg/ml of gentamicin reagent solution (Gibco Products, Invitrogen corporation). In addition, we cultured all cell lines, both MHS putative positive and CWT controls, from the same FBS batch (Lot: #1233730).

We grew cells in vented T_{75} tissue flasks (on their side) with ~30 mls of medium, and maintained them at a density of between 200,000 to 1,000,000 cells/mL. The cultures were fed every two to three days, and split when the cell count reached 2.0-x10^6 viable cells/mL. We did not maintain the cell lines in more than 30 days in continuous culture, but initiated them from frozen seed stocks (NIH/NHGRI Lab, Bethesda, MD or from TCSR, Lombardi Cancer Center) or refroze them if experiments went on for greater than 30 days. All cells in this study were cultured in the same incubation conditions at 37 °C in a humidified (80-90%), CO_2 incubator (Thermo Scientific, HeraCell 150i) at 5% carbon dioxide, and 95% oxygen (filtered air, class 100 HEPA filter). The incubator pressure was maintained at 10 mm HG by a two-stage regulator.

To validate the identity of the patient-derived lymphoblastoid cell lines in the advent of cell line misidentification, and to exclude cell line cross-contamination, we identified two unique variants from the individual's exome and genotyped their *RYR1* variant using Sanger sequencing. We then designed primers for PCR amplification for the three SNPs with the online tool (http://bioinfo.ut.ee/primer3/) Primer3 (Broad Institute, Cambridge MA). The oligonucleotide primers were obtained commercially from Invitrogen|Life Technologies (Grand Island, NY). Jennifer Johnston, PhD, of the National Human Genome Research Institute performed the PCR identification.

Intracellular Calcium Measurements

We measured changes in the intracellular Ca^{2+} concentration directly in lymphoblasts, from the ClinSeq® volunteers with the variants under study, by measuring the fluorescence intensity of the Ca^{2+} indicator on a population of Fura-2/AM loaded cells at a final concentration of 5 μM as defined in (218). We first performed the lymphoblast

Ca^{2+} release assay by loading the lymphoblasts with 5.0 µm of the ratiometric fluorescent dye Fura-2/Am dye for 30 minutes at 37 °C in dark conditions and washing in a Ca^{2+}/Mg^{2+} free medium. Our experiments were performed on an average cell population of (1.0×10^6 cells/mL, final volume 2.0 mL) in a thermostated QuantaMaster® 40 spectrofluorometer (Photon Technology International, Inc. Birmingham, NJ) equipped with a magnetic stirrer. We then measured the time course of RYR1 agonist 4-CmC-induced intracellular Ca^{2+} release by dual wavelength fluorescence spectrophotometer (340/380 nm excitation, 510 nm emission).

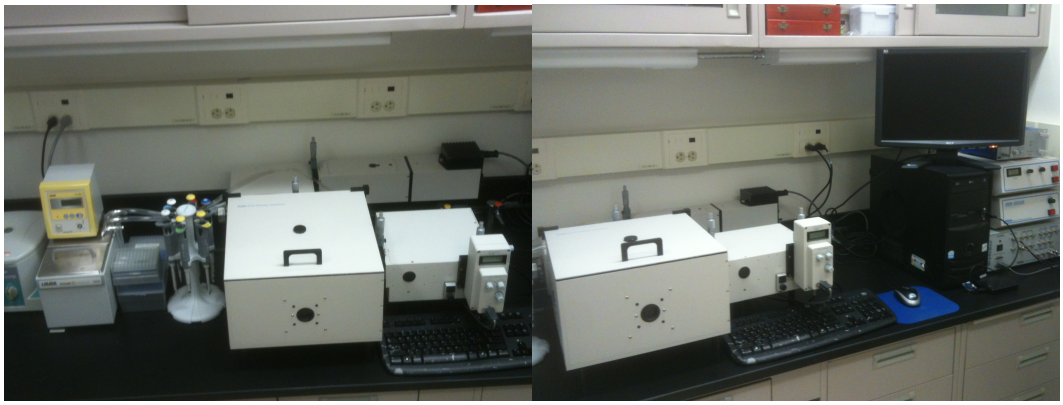

Figure 7. The QuantaMaster® 40 spectrofluorometer at the Uniformed Services University of the Health Sciences.

The spectrofluorometer (Figure 7) uses a dual wavelength light source to excite the cells (λ= 340/380 nm) and measures fluorescent emissions of Ca^{2+} ions with Fura-2 at 510 nm. From the calculated ratio, the level of intracellular Ca^{2+} can be estimated, from dissociation constants (Kd) derived from calibration curves (186). The ratioing technique from fluorescence intensities, produced by excitation at two wavelengths as compared to single wavelength, provides a number of advantages in Ca^{2+} ion measurement: (a) the ratio of intensities at these two wavelengths depends on the Ca^{2+} concentration, but is independent of the Fura-2 indicator concentration; (b) a linear response is achieved; (c)

the sensitivity (i.e., the minimal detectable quantity of compound of interest) of the measurement is increased; and (d) factors such as uneven dye distribution and photo bleaching are minimized because they should affect both measurements to the same extent (72; 88). Fura-2 fluorescence can be measured for about an hour without significant loss of fluorescence from dye leakage or photo bleaching. We reduced other unfavorable variables not uncommon to fluorescence investigations by use of a fluorescence spectrophotometer with software designed to address quenching, inner filter effect, stray light, and excitation and emission instabilities (144).

Fura-2 Dye Loading and Cell Preparation

The Fura-2 Ca^{2+} protocol used for this study was similar to the method used most recently by Perry (141) and explained in text (93). We transferred lymphoblast cultures in the T_{75} flask to 50 ml conical tubes, centrifuged them at 500g for 2 minutes, suctioned the growth medium from the flask, and then added Hanks Balanced Salt Solution (HBSS, Ca^{2+}, Mg^{2+}, Gibco Products, Invitrogen corporation) containing 0.1 g lyophilized, Bovine Serum Albumin (BSA, Sigma-Aldrich) to the remaining pellet. We next washed the cells twice (30 ml, 10 ml) with HBSS (Ca^{2+}, Mg^{2+}), re-suspended them in 10 ml HBSS/ 0.1% BSA, and then stained them with Fura-2AM dye (Invitrogen, Lot: #983913) in a final concentration of 5 μM in dimethyl sulfoxide; we wrapped the 50 ml conical tubes containing the cells in aluminum foil to protect them from light, and then incubated them for 30 minutes at 37 °C in a dark water bath. We used dimethyl sulfoxide as a solvent to solubilize 4-CmC and thapsigargin, as earlier intracellular Ca^{2+} release experiments by McKinney and coworkers with B lymphoblastoid cell lines showed dimethyl sulfoxide had no effect on Ca^{2+} release (117).

Following 30-minutes of Fura-2 dye incorporation, we stopped dye loading by centrifuging the cells, then removed the supernatant and adding fresh solution of 10 ml HBSS/ 0.1% BSA; we repeated this wash procedure twice. We maintained the cells at room temperature (20 to 23 °C, average 21 °C) in the dark for 15 minutes in 10 ml HBSS/0.1% BSA until use. At approximately 15 minutes post washout, cells were suspended at a density of 1.0 x10^6 cells/mL, final volume 2.0 mL in a 3 ml cuvette, and within 60 seconds prior to each Fura-2 florescence measurement, cells were washed with calcium-free Krebs buffer in the presence of 0.5 mM ethylene glycol tetra-acetic acid (EGTA). Emission measurements were started at a minimum of 20 minutes following loading (average 25 minutes) to allow sufficient time for all the Fura-2 in the cells to de-esterify.

We removed each replicate (i.e., 3 ml cuvette, 1 ml total volume of cells), prior to being placed into the spectrofluorometer, from dark storage (at 20 to 23 °C, average 21 °C), pipetted 1 ml aliquots, and then re-spun them at 5000 RPM for 30 seconds. We suctioned off the 10 ml HBSS/ 0.1% BSA buffer and then re-suspended the cells at a density of 2 x10-6 /ml in a 3 ml quartz cuvette with 1 ml of Ca^{2+}-free Krebs buffer. Next we added an additional 1 mL of Ca^{2+}-free Krebs buffer to the cuvette immediately before placing the 1 mL cells in suspension. We maintained the buffer at 37 °C during the procedure, and pre-warmed the cuvettes with magnetic stirrer magnets at 37 °C. Our last step, prior to the first measurement of baseline, was to place the cells —in final volume of 2 mL —in the spectrofluorometer set at 37 °C.

Spectrofluoroscopy Measurements

We added pharmacologic agents directly to a population of cells in the cuvette, and

recorded fluorescence emissions (340 nm, 380 nm, and ratio) at approximately every 0.5 seconds using the dual excitation wavelength fluorescence spectrometer. We then added 4-CmC to the cell suspension in the measuring cuvette at concentrations from 400 to 2000 μM (200 μM increments from 400 μM to 1,000 μM and then by 500 μM to 2000 μM). We selected the specific 4-CmC doses and range in order to generate dose-response curves for variant assessment, and to compare our experimental results with RYR1 functional studies in lymphoblasts, as earlier studies used these 4-CmC doses most frequently (7; 117; 169; 201). We measured the response to thapsigargin at a concentration of 800 nm, as this dose was shown by Anderson and coworkers (7) to be a good indicator of maximal Ca^{2+} release from endoplasmic reticulum stores.

We took fluorescent measurements over a gradual progression in 4-CMC doses to Ca^{2+} mobilization responses for each sample cell line. Then we calculated Base Level (BL) responses by measuring from time 0 to the first 50 seconds of Ca^{2+} emissions — prior to assessing Ca^{2+} release by adding the agonist 4-CmC or the SERCA inhibitor, thapsigargin. The Area Under the Curve (AUC) and Peak area Under the Curve (PUC) were measured over a 250 second interval: at the time of 4-CmC dosing at 50 seconds out to 300 seconds. We also recorded peak X and Y 4-CmC measurements during the 50 to 300 second interval. The peak X is the value of the transient at its maximal deflection from baseline. Peak Y is the “Time to Maximum Peak". It can be a point higher than the immediate left and right neighboring points. We chose the 300-second limit for each measurement, and 50 to 300 interval to measure the AUC and peak values for three principal reasons: (1) peak X and Y measurements were nearly always reached prior to 300 seconds; (2) the cells often became unstable after 300 seconds, and (3) an average of

50 seconds was necessary to establish baseline averages. These limits were used in similar lymphoblast assays (117; 141). To assess the size of the intracellular Ca^{2+} stores, we obtained the AUC and the peak X and PUC after the addition of 800 nM thapsigargin, which represents the total amount of Ca^{2+} present in the rapidly releasable intracellular Ca^{2+} stores.

STATISTICAL ANALYSIS

We calculated initial BL, and Fura-2, AUC, PUC, peak X, peak Y and peak minus baseline (P-B) emission ratios by using the math application on the spectrometer's PTI software Package®, for the AUC calculation by the trapezoid rule (i.e., connecting a straight line between every set of adjacent points defining the curve, and summing the areas beneath these areas). We then recorded the measurement for each replicate in a text document (.txt) on the spectrometer's computer before importing into Microsoft® Excel® for Mac 2011 (version 14.4.1) document (.xls). Later we analyzed the BL, AUC, PUC peak X, and P-B emission ratio responses to Fura-2 and thapsigargin with the statistical program SPSS (IBM SPSS Statistics, version 21). We used GraphPad Prism® (version Prism 6 for Mac OS X) software (GraphPad Software, Inc., San Diego, CA) to generate dose-response curves.

We used the paired sample t-test for the comparisons of means. For experiments with more than two samples, we used the analysis of variance (ANOVA) to test for differences within and between experimental groups examining the independent variable of dose of 4-CmC on the dependent variables of Fura-2 and thapsigargin Ca^{2+} and emissions on four measurements AUC, PUC, BL, and P-B. We considered the means statistically significant when the *P* value was <0.05. Our results were expressed as mean

value of *n* results, where *n* is the number of measurements. If they were significantly different, Tukey's multiple comparison tests was used for post-hoc analysis. We fitted the P-B dose–response data by nonlinear regression to a variable slope sigmoid curve of all data points using GraphPad Prism® software. We then calculated the effective concentration of 4-CmC at 50% (EC50) from these log-transformed curves, and then used the ANOVA test to assess for significance of EC50 values. We considered *P* values of <0.05 significant. Others in previous MHS functional studies have used these measures and analyses to quantity the differences in MHS and MH-negative lymphoblastoid cell lines Ca^{2+} responses to RYR1 agonists (33; 201).

CHAPTER 4: RESULTS

MOLECULAR CHARACTERIZATION OF *RYR1* AND *CACNA1S* SEQUENCE VARIANTS

One of our aims was to pilot a method to identify putative pathogenic MHS variants in a population unselected for the disorder from a sizeable set of exome-sequence (ES) data. At the start this pilot study there were approximately 900 ClinSeq® participants enrolled, but only 870 had ES data available for analysis. We analyzed these 870 ClinSeq® participants for the two genes known to predispose to MHS. The sequencing coverage (defined as the number of coding base-pairs with quality calls/ total number of targeted base-pairs) of the coding exons for the two genes was 83% (*RYR1*) and 93% (*CACNA1S*). A summary is provided in Figure 8 as a boxplot with the minimum, maximum, mean, and median coverage for the *RYR1* and *CACNA1S* genes.

Sequence coverage is dependent on many factors including DNA quality, capture efficiency, GC content, repeat elements, and bioinformatics issues such as alignment and base calling. Our average depth of coverage in the target region (i.e., the location of the genome region containing the two genes) for each sample was 89x. Since the coverage was not 100%, there exists an inherent risk of false-negatives. As a result, it is possible that ES may have missed some variants due to inefficient capture of certain exons in the two genes.

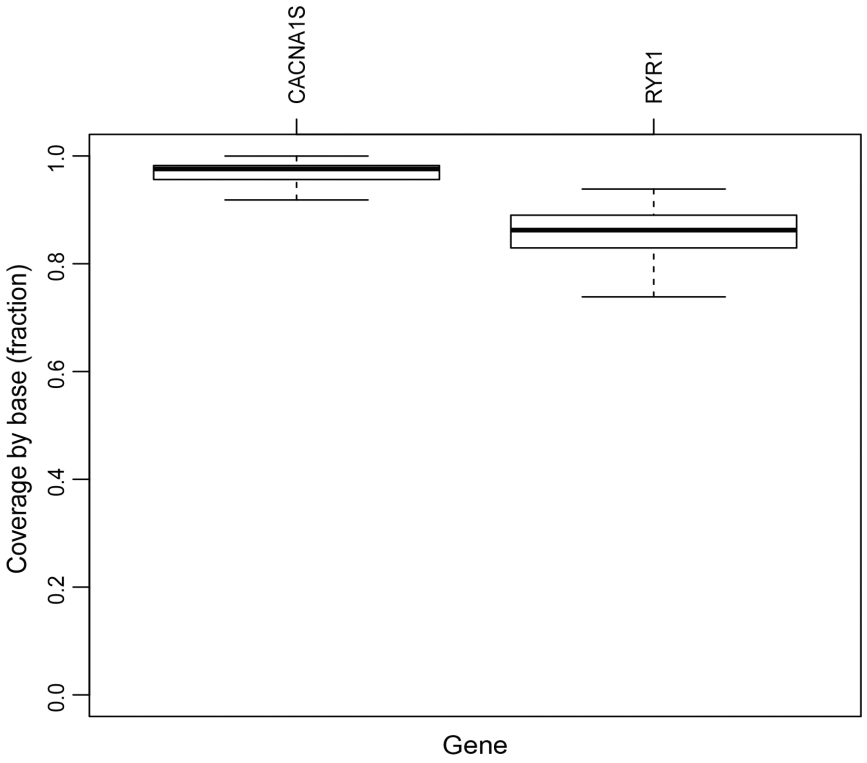

Figure 8: Base coverage for *RYR1* and *CACNA1S* and genes.
Box and whisker plots showing base coverage for the *RYR1* and *CACNA1S* genes for a subsample of 870 probands. The bottom and top lines of the box represent the 1st and 3rd quartile, respectively; the midline represents the median value. The bottom and top whiskers represent the lowest and highest values within 1.5 times the interquartile range. For the *CACNA1S* gene the minimum was 0.8803, the first quartile was 0.9053, the media was 0.9302, the mean was 0.9302, the third quartile was 0.9551 and maximum was 0.9801. For *RYR1* the minimum was 0.7425, the first quartile was 0.7839, the median is 0.8253, mean was 0.8253, the third quartile was 0.8667 and maximum was 0.9080. Outliers were excluded. Values on the y-axis are represented as a fraction of the total coding exonic bases for each gene.

From the 870 exomes, we identified 123 distinct variants nonsynonymous, frameshift, nonsense, or splice-site variants, 70 in *RYR1* and 53 in *CACNA1S*. These variants were identified in one to 419 participants, each. From the 123 variants, we removed one *RYR1* and one *CACNA1S* variant based on marginal MPG scores indicating low quality—confirmed by manual review of sequence reads. We also removed one

CACNA1S variant due to a low MPG coverage (or MPG/read count ratio >0.5)—leaving 121 variants (i.e., 69 *RYR1* and 51 *CACNA1S*). References to twenty of the 69 *RYR1* variants (29%) and seven of the 51 *CACNA1S* variants (14%) were found online in the HGMD and LSDB (*RYR1* Variant Table, Appendix B and *CACNA1S* Variant Table, Appendix C).

We then used frequency filters, and two data sources, to further exclude 16 *RYR1* and *CACNA1S* variants that were too common to be plausible causes of autosomal dominant MHS, basing this on an estimate of population frequency of MHS of 1/2,000 (70; 120). Based on the NHLBI EVS data set, we set the minor allele frequency threshold at 0.5%, as this was approximately 10-fold higher than the higher end of the MHS prevalence estimate. We also filtered against our own ClinSeq® data, for which the frequency was set to 1%, because it includes about 1/10 as many exomes as the EVS data set and therefore chance variation could inadvertently exclude a variant. Following this frequency analysis, sixteen of the remaining 121 variants (six *RYR1* and 10 *CACNA1S*) were excluded because they were too common (Figure 9, and *RYR1* Variant Table, Appendix B and *CACNA1S* Variant Table, Appendix C).

The remaining 104 variants (63 in *RYR1* and 41 in *CACNA1S*) were considered rare variants. Seventeen of the 63 (or 27%) *RYR1* rare variants were listed in the HGMD as "disease-causing" for MHS, central core disease, multi-minicore disease, atypical periodic paralysis, or a congenital myopathy (*RYR1* Variant Table, Appendix B). Three of the 63 *RYR1* variants were not present in HGMD but were listed in the LSDB as pathogenic. One of the 41 *CACNA1S* variants (p.Thr1354Ser) was listed in HGMD as pathogenic for MHS, and one (p.Arg498His) was listed in the LSDB as pathogenic but

without any supporting evidence. Of the 20 *RYR1* variants (present in HGMD or LSDBs, and with a mean allele frequency <1%), only four met our criteria for class 5 pathogenicity (*RYR1* Variant Table, Appendix B); the remaining 16 were scored as class 3 (variants of uncertain significance) or class 2 (likely benign). There were no class 4 variants. After excluding synonymous variants, we identified 65 *RYR1* missense mutations, one nonsense, two that affected splicing, and one non-frameshift indel. In *CACNA1S*, 48 missense, one frameshift deletion, one splicing, and one non-frameshift indel were identified.

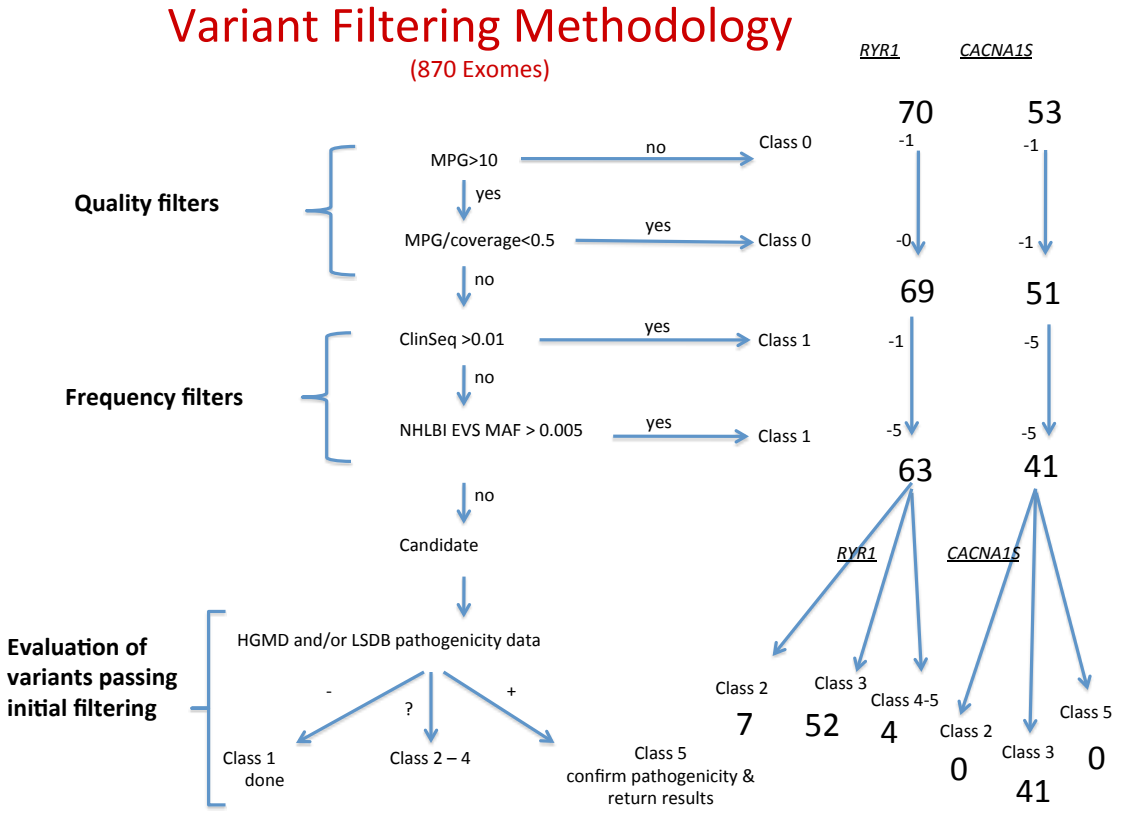

Figure 9. Quality/ frequency filter algorithm.
Filtering criteria used for coding-variant interpretation. Variants were filtered on genotype quality, coverage, and allele frequencies. Prior to filtering, 123 distinct variants were identified—70 in *RYR1* and 53 in *CACNA1S*—from 870 exomes. One

RYR1 and one *CACNA1S* variant were removed due to marginal MPG scores. One CACNA1S variant was removed due to a low MPG coverage (or MPG/read count ratio >0.5)—leaving 121 variants (69 *RYR1* and 51 *CACNA1S*). Frequency filters, and two data sources, were next used to further exclude 16 *RYR1* and *CACNA1S* variants that were too common to be plausible causes of autosomal dominant MHS, based on an estimate of population frequency of MHS of 1/2,000. Variants removed by quality filters were classified as 0 and frequency filters as Class 1. The remaining 104 variants (63 in *RYR1* and 41 in *CACNA1S*) were assessed for pathogenicity (Class 2-5) based on data present in the Human Gene Mutation Database (HGMD) and locus-specific databases (LSDBs). MPG =most probable genotype. MAF = minor allele frequency. NHLBI EVS = The National Heart, Lung, and Blood Institute, exome variant server.

Characterization of Class 1-3 Variants

Of the 16 rare *RYR1* variants (eight found in both HGMD & the LSDBs, three in LSDBs only) we identified in ClinSeq®, ten were assigned to class 3, and six to class 2, based on the criteria in the pathogenicity table. The reasoning for these assignments is described in the Supplemental Methods text in Appendix G. We reviewed the family, personal, medical and surgical histories of all participants with *RYR1* and *CACNA1S* variants; all but two were negative for MHS (*RYR1* Participant Description Table, Appendix D and *CACNA1S* Participant Description Table, Appendix E).

One participant was found to have a novel *RYR1* missense variant p.Arg3498Gly and a three-generation family history of MHS with an *in vitro* contracture test diagnostic for MHS (160). To assess the potential pathogenicity of this variant, we performed a segregation analysis of the variant in the family. The variant did not segregate with the MHS phenotype (Family Pedigree, Appendix F). We ruled out misdiagnosis, after acquiring muscle biopsy and caffeine halothane contracture test results for seven of the family members from The North American Malignant Hyperthermia Registry. Next, a candidate linkage analysis of the *RYR1* locus was next done for us (data not shown) in

consultation with Dr. Jennifer Johnston. Genotyping and manual haplotyping showed that a *RYR1* haplotype cosegregated with the phenotype, but this haplotype was in *trans* to p.Arg3498Gly. We concluded that p.Arg3498Gly was not pathogenic and hypothesized that this family most likely had MHS attributable to an undetected *RYR1* variant in *trans* to p.Arg3498Gly in the proband. We next evaluated the ES coverage of *RYR1* in this proband and found that it was 91.9%. We used Sanger sequencing to evaluate exons with poor ES read-depth but found no mutations. We concluded that the exome sequencing of *RYR1* generated both a false-negative and a false-positive result in that the p.Arg3498Gly is not pathogenic and the participant likely has a mutation in *RYR1* not captured by ES or Sanger sequencing.

One of the 41 *CACNA1S* rare variants, p.Arg498His, identified in one exome, was listed in LOVD as pathogenic (it was not listed in HGMD). However, the pathogenicity of this entry was not supported by the primary literature, nor did LOVD provide details of the associated phenotype. We contacted the LOVD curators and learned that the variant had been recategorized as 'unknown pathogenicity', although the database itself had not been updated. We therefore categorized it as a variant of uncertain significance (score 3).

The *CACNA1S* variant p.Thr1354Ser was identified in 9/870 ClinSeq® exomes (1/1,740 alleles, minor allele frequency 0.5%) and in the EVS with an allele count of 48/12,958 (minor allele frequency 0.4%). HGMD listed this variant as pathogenic, citing a publication showing segregation of p.Thr1354Ser in one family, its absence in 282 controls, and functional data demonstrating abnormal Ca^{2+} flux (143). However, we categorized the p.Thr1354Ser variant as a class 3, of uncertain significance and concluded that this was more likely a benign variant in linkage disequilibrium with the

(undetected) true pathogenic variant in the family described by Pirone and coworkers based on the frequency of the variant, which was approximately 20 times higher than the frequency of MHS attributed to all loci and all mutations (0.74–1% p.Thr1354Ser heterozygotes) (143). Although there are good functional data implicating this variant in MHS (143), we believe that the population genetic data mandate that it be scored class 3, of uncertain significance. Our findings, supported by the Exome Variant Server *CACNA1S* allele frequencies, suggest that other previously implicated MHS variants may also be benign.

Characterization of *RYR1* Class 5 Variants

We identified four class 5 *RYR1* variants in 870 exomes. Three of these variants (c.1840C>T, p.Arg614Cys, c.5183C>T, p.Ser1728Phe , c.1840C>T, p.Asp3986Glu) were described earlier in the methods section. Here we describe an *RYR1* nonsense mutation (a premature stop codon that results in truncation of the resulting protein) that the literature indicates is strongly predictive of an autosomal recessive *RYR1*-related myopathy as well as the second novel missense variant c.2122G>A, p.Asp708Asn that co-occurred with c.6721C>T, p.Arg2241X.

The class 5 *RYR1* pathogenic variant, p.Arg2241X, was detected in two participants. It was described as pathogenic in HGMD, based on a single patient with congenital myopathy, episodes of generalized, atypical normokalemic paralysis, multi-minicore disease with external ophthalmoplegia, and episodes of atypical periodic paralysis (215). The molecular data in this published report were complex. The patient had, in addition to p.Arg2241X, p.Asp708Asn in *cis* and p.Arg2939Lys in *trans* to p.Arg2241X, an apparent nonsense-mediated messenger RNA decay of the p.Arg2241X-

bearing allele. In another study of 37 patients with dominant or recessive *RYR1*-related myopathies, the p.Arg2241X variant was described in three patients with recessive myopathies and ophthalmoparesis (85). In two siblings, seven and five years old, the p.Arg2241X variant co-occurred with the previously described putatively pathogenic variant p.Arg109Trp (214; 216), and in a third patient the p.Arg2241X variant co-occurred with two missense variants, the putatively pathogenic p.Arg2939Lys (214) and p.Asp708Asn. (These three variants are likely from the same patient reported in two case series by this same group) (214; 215). The *RYR1* variant p.Arg2241X was also categorized as a pathogenic recessive mutation in a patient with a congenital myopathy and muscle biopsy finding of an *RYR1*-related myopathy from a study of 71 families with *RYR1* mutations (86). The patient had two additional recessive pathogenic variants, p.Asp708Asn and p.Met485Val, and a synonymous variant of uncertain significance c.11547G>A (p(=)). The p.Arg2241X variant was not detected in the EVS. We categorized p.Arg2241X as class 5, since it was described in affected patients and of the category of variants (nonsense) strongly predictive of an autosomal recessive RYR1-related myopathy.

Summary of Part I Results

We reduced the number of *RYR1* and *CACNA1S* variants detected through sequencing using our quality and frequency filters to 70 *RYR1* and 53 *CACNA1S* variants among 870 exomes. *RYR1* variants predicted to be pathogenic for MHS were found in three participants without medical or family histories of MHS. We reclassified numerous variants, previously described as pathogenic in mutation databases, to be of uncertain pathogenicity. Through ES, we have discovered missense and nonsense variants in the

exons of *RYR1* and *CACNAS1*. A few have been implicated as being causative for Malignant Hyperthermia. A major contribution of the ES approach we have piloted here is a method to categorize putative or benign *RYR1* and *CACNA1S* variants. As has been demonstrated for many other genes, it is apparent that some previous MHS studies that classified *RYR1* and *CACNA1S* variants without complete data may have led to their misclassification as pathogenic or benign. However, until such time as it becomes possible to implement accurate, non-invasive testing on all at MH-susceptible individuals for the final validation of MHS variants, it remains essential to further functionally characterize the variants identified through ES.

FUNCTIONAL CHARACTERIZATION OF *RYR1* SEQUENCE VARIANTS

The second part of the results of the current work focuses on the functional effects of *RYR1* MHS variants on Ca^{2+} homeostasis, and the feasibility of using lymphoblasts expressing RYR1 amino acid substitutions identified through exome sequencing in a population not ascertained for MHS or other neurological disorders. Following the establishment and culture of lymphoblastoid cell lines from volunteers carrying three putative pathogenic variants c.5183C>T, p.Asp3986Glu, c.5183C>T, p.Ser1728Phe , c.4999C>T, p.Arg1667Cys, a positive control cell line carrying the c.1840C>T, p.Arg614Cys[4] mutation, and three *RYR1* CWT controls, we studied the cells Ca^{2+} release. We confirmed the presence of the *RYR1* variants in the cell lines by Sanger Sequencing. Two of the cell lines harbored mutations that are located in known mutation hotspot clusters: the positive control p.Arg614Cys mutation is located in the N-terminal (Cys35-

[4] Herein the genomic changes c.5183C>T, c.11958C>G, c.4999C>T, c.1840C>T referred as p.Ser1728Phe, p.Asp3986Glu, p.Arg1667Cys, and p.Arg614Cys.

Arg614, MH region 1) and the variant p.Asp3986Glu in the C-terminal (Ile3916-Ala4942, MH region 3) pore forming region. Both these regions contain a high frequency of evolutionarily conserved sites, which suggests the positions may be of functional importance (31).

All the lymphoblastoid cell lines in our study released Ca^{2+} from intracellular stores in the presence of exogenous trigger agents (e.g., 4-CmC or thapsigargin) - as described in Materials and Methods. The intracellular Ca^{2+} increase was unlikely to be an experimental artifact as evidence by: (a) the fact that changes in the fluorescence level occurred at dual wavelengths (at 340 nm the fluorescence level increased while at 380 nm the fluorescence decreased); (b) the increase in fluorescence ratio varied among the different cells; (c) the Ca^{2+} levels returned to baseline; and (d) the Ca^{2+} stores could be depleted by treatment with the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin after the addition of 4-CmC to Fura-2-loaded lymphoblasts (Figure 10).

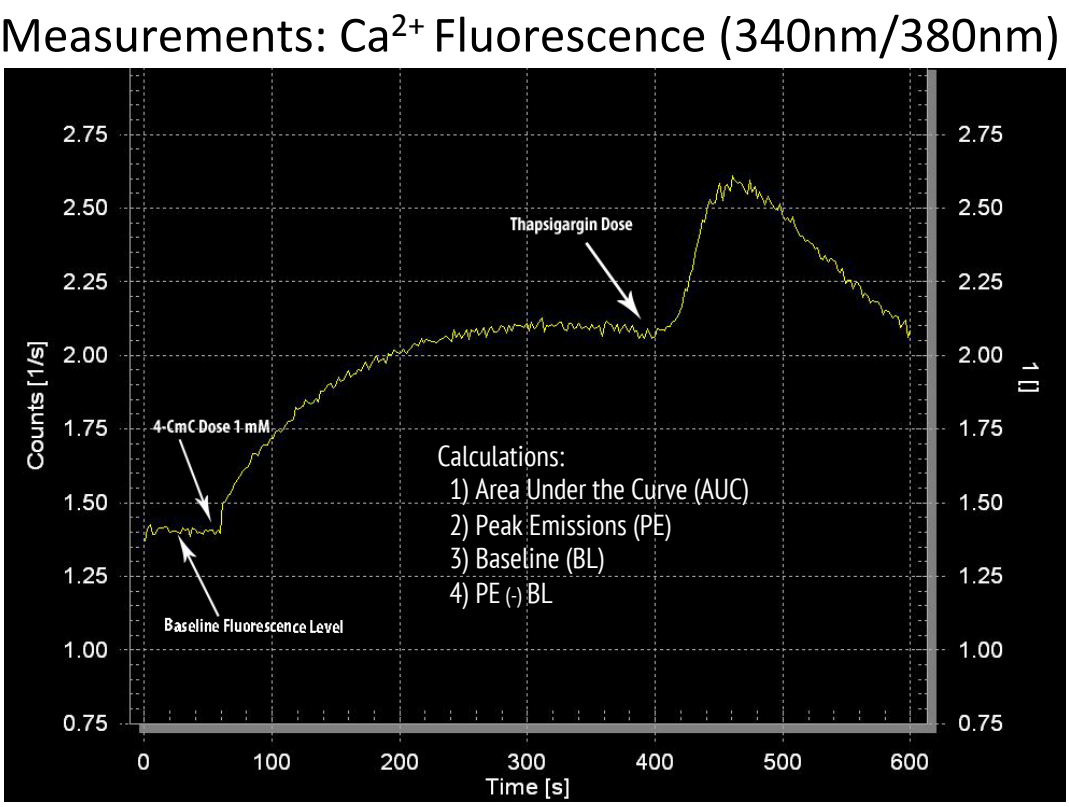

Figure 10. Fura-2 emission ratio (340 nm/ 380 nm) curve.
The picture is an example of the two RYR1 agonists (4-CmC and thapsigargin) and the Ca^{2+} fluorescence measurements for a given experiment. The picture shows thapsigargin being added after 4-CmC. The Ca^{2+} stores could be depleted by treatment with 400 nM thapsigargin after the addition of 4-CmC Fura-2-loaded lymphoblasts. The application of thapsigargin after 1 mM of 4-CmC still produced an increase in Ca^{2+} fluorescence (at 340 nm/ 380 nm) to the same absolute level as with thapsigargin alone. This is an indication that 1.0 mM of 4-CmC did not fully deplete the Ca^{2+} pool but thapsigargin after 4-CmC released all of the available Ca^{2+}.

Figures 11.1 through 11.4 show that cells harboring the RYR1 variants p.Asp3986Glu (Figure 11.1), p.Ser1728Phe (Figure 11.2), p.Arg1667Cys (Figure 11.3), and p.Arg614Cys (Figure 11.4) release Ca^{2+} from intracellular stores when placed in Ca^{2+}-free medium and exposed to the triggering agent 4-CmC. The computer tracings (multiple tracings in each) are representative of one experiment carried out on a specific

cell line on a single day. All our experiments were carried out on each cell line separately at least six times on different days.

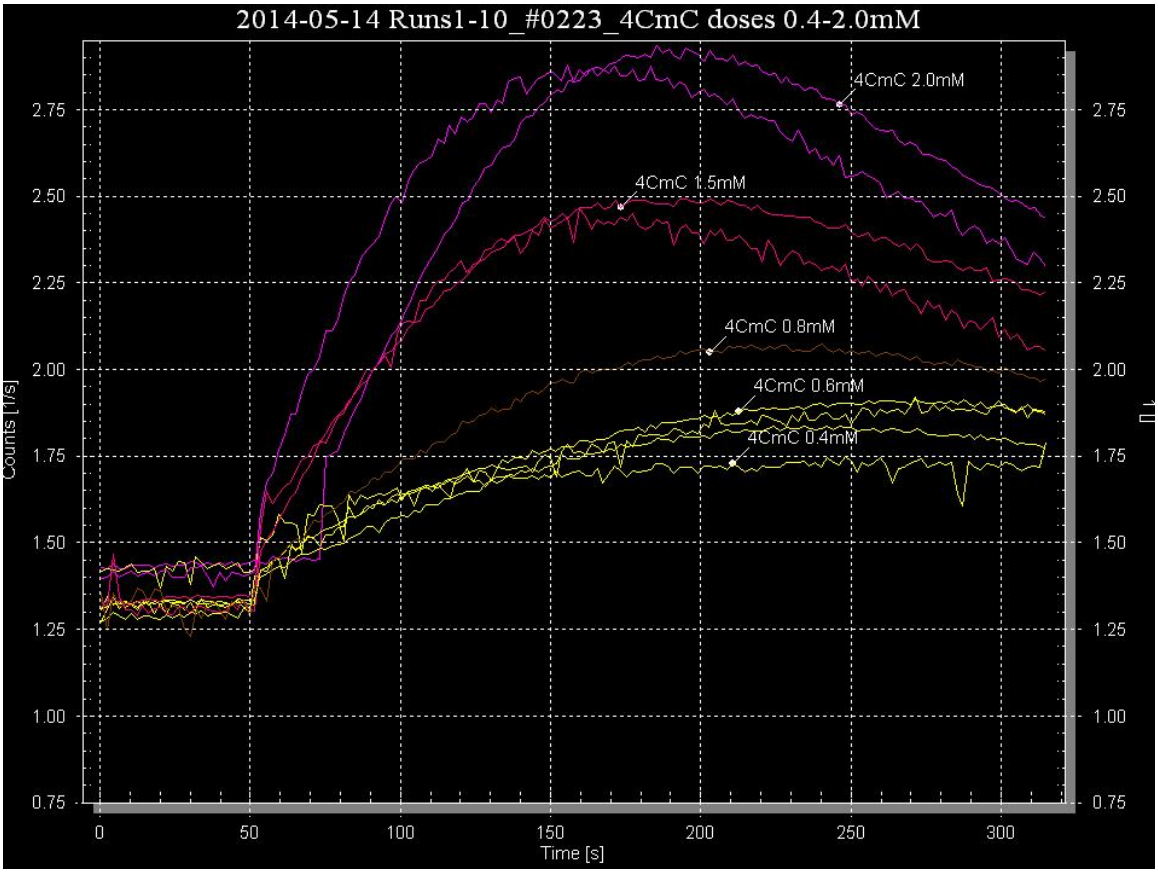

Figure 11.1. RYR1 variant p.Asp3986Glu agonist response.
Shows that addition of the RYR1 agonist to lymphoblasts harboring the RYR1 variant p.Asp3986Glu (labeled #0223 in figure above) is accompanied by a transient increase in the 340/380 nm fluorescence ratio when placed in Ca^{2+}-free medium and exposed to triggering agents. The multiple computer tracings are representative of multiple experiments (one tracing at each dose concentration of 4-CmC) carried out on the p.Asp3986Glu cell line.

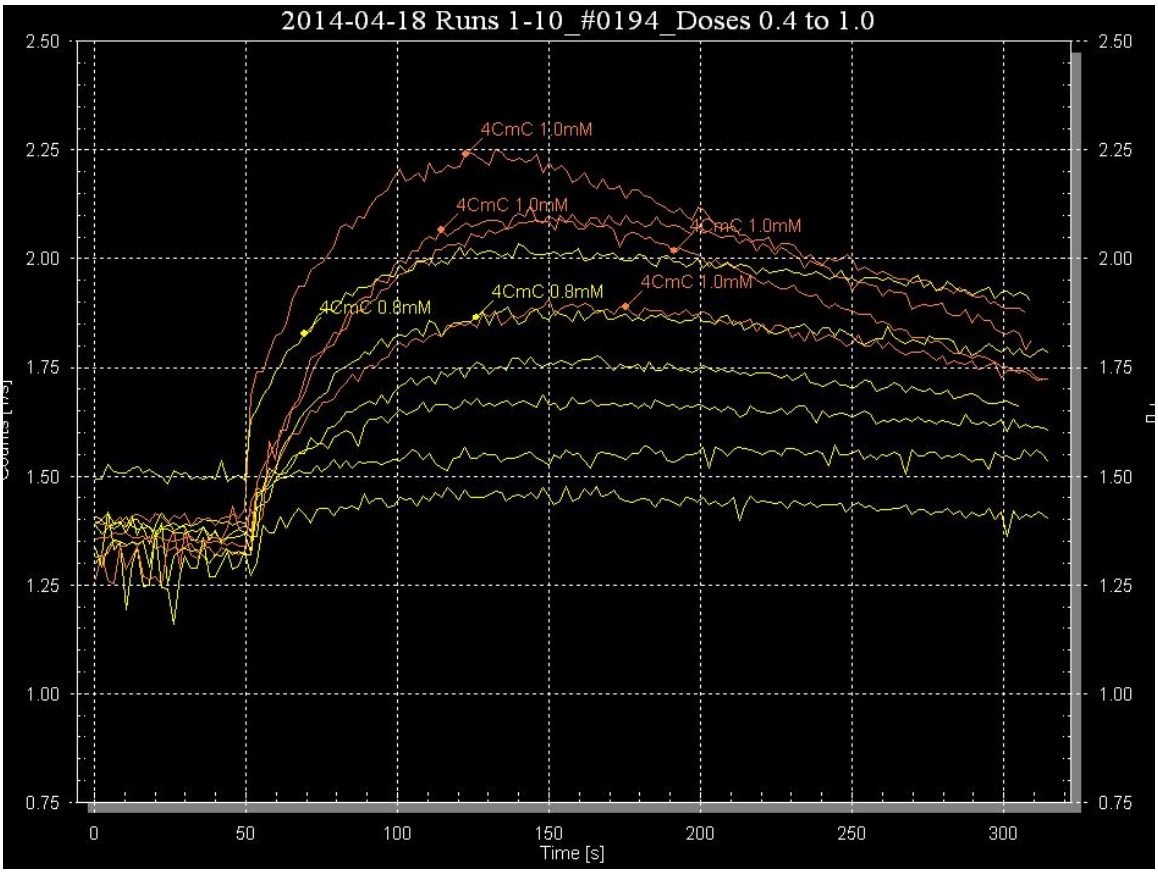

Figure 11.2. RYR1 variant p.Ser1728Phe agonist response.
Shows that cells harboring the RYR1 variant p.Ser1728Phe (labeled #0194 in above figure) release Ca^{2+} from intracellular stores when placed in Ca^{2+}-free medium and exposed to triggering agents. The multiple computer tracings are representative of multiple experiments (one tracing at each dose concentration of 4-CmC) carried out on the p.Ser1728Phe cell line.

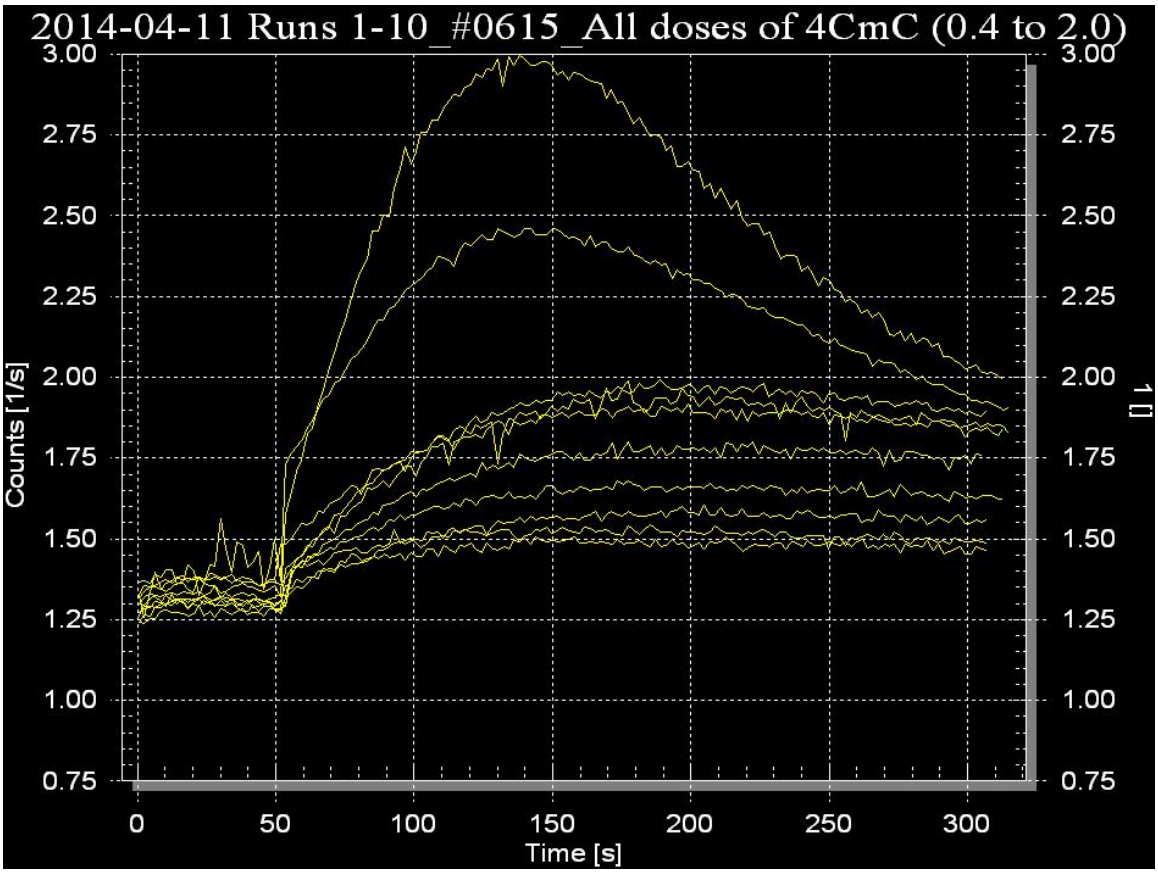

Figure 11.3. RYR1 variant p.Arg1667Cys agonist response.
Shows that cells harboring the RYR1 variant p.Arg1667Cys (labeled #0615 in above figure) release Ca^{2+} from intracellular stores when placed in Ca^{2+}-free medium and exposed to triggering agents. The multiple computer tracings are representative of multiple experiments (one tracing at each dose concentration of 4-CmC) carried out on the p.Arg1667Cys cell line.

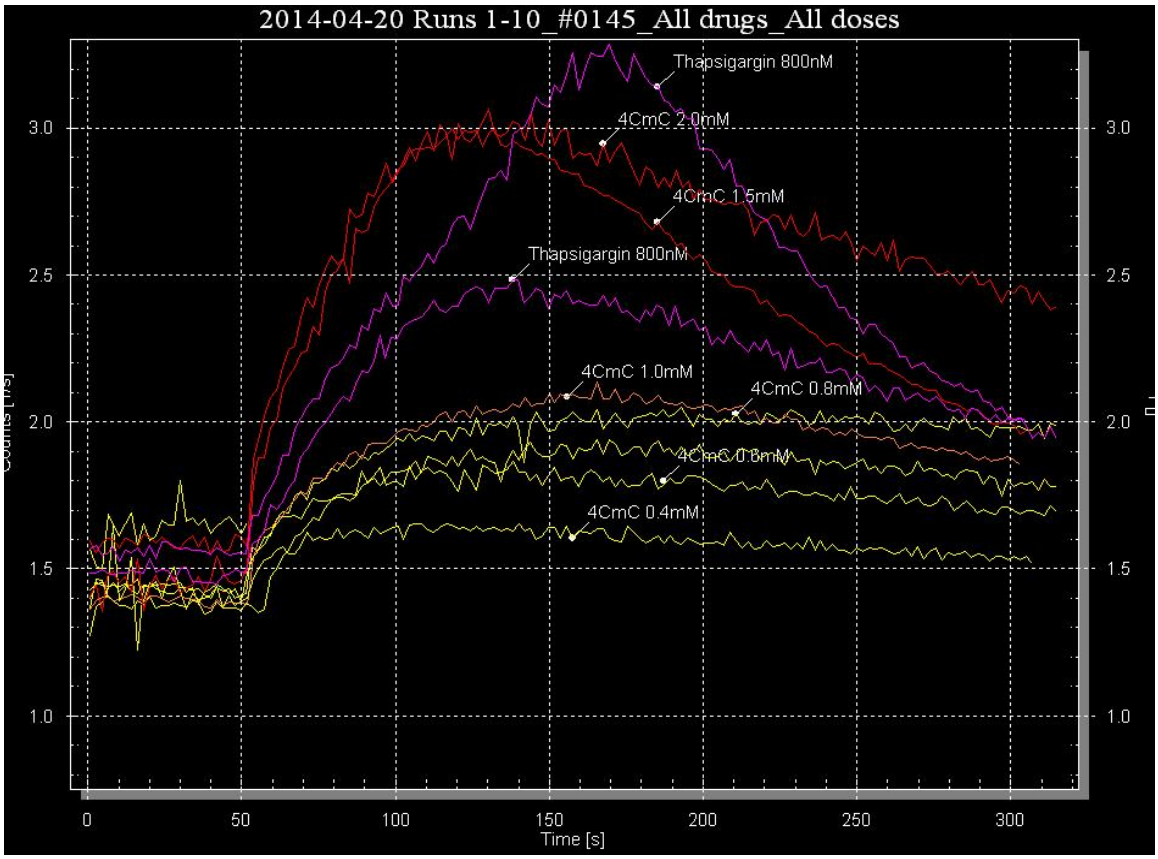

Figure 11.4. RYR1 variant p.Arg614Cys agonist response.
The tracing are a summary of data in one lymphoblastoid cell line harboring the RYR1 positive control p.Arg614Cys (labeled #0145 in above figure) release Ca^{2+} from intracellular stores when placed in Ca^{2+}-free medium and exposed to triggering agents. The multiple computer tracings are representative of multiple experiments (one tracing at each dose concentration of 4-CmC) carried out on the p.Arg614Cys cell line.

Resting Ca^{2+} Fluorescence Level in Lymphoblasts

One of the aims of our pilot study was to functionally characterize the cytoplasmic Ca^{2+} stores of the lymphoblastoid cells from individuals bearing the different RYR1 substitutions: p.Asp3986Glu, p.Ser1728Phe, P.Arg1667Cys, and p.Arg614Cys. For this purpose, we first examined the resting Ca^{2+} of lymphoblasts with putative *RYR1* variants and compared them with that observed in cells from three *RYR1* CWT control

individuals, as well as the p.Arg614Cys positive control. Following multiple experiments, we found the resting Ca^{2+} concentration in the three RYR1 variants p.Asp3986Glu, p.Ser1728Phe, p.Arg1667Cys, and positive p.Arg614Cys, in the heterozygous state had higher mean resting Ca^{2+} than that observed in the pooled (n =3) CWT controls (p <0.001 ANOVA). The results of the resting fluorescence level are displayed in Figure 12.

We first conducted a one-way between-groups analysis of variance (ANOVA) to compare resting baseline fluorescence levels between the pooled CWT control group and variants. The sample size included a total of 227 resting baseline measurements with a minimum group size of 24—each of the three CWT controls, and two variant cell lines—and a maximum of 62 measurements for variant p.Arg1667Cys (Figure 12). Post hoc comparisons using the Tukey HSD test indicated that the mean resting Ca^{2+} emissions for the MHs variants and the CWT cell lines were different at the p =0.001 level for the five groups [F (4, 74.9) = 13.66].

Next, our independent t test analyses showed that the mean resting fluorescence for variant p.Asp3986Glu (M = 1.365, SD =0.045) was significantly different (p <0.001) from the pooled CWT control group (M =1.295, SD =0.0288). The two variants p.Ser1728Phe (M =1.319, SD =0.050), p.Arg1667Cys (M =1.318, SD =0.042) and the p.Arg614Cys positive control (M =1.325, SD =0.046) differed significantly from the three CWT controls. The mean resting florescence level for p.Asp3986Glu was also significantly greater than each of the other three RYR1 variants (p.Arg614Cys p <0.007, p.Ser1728Phe p <0.001, and p.Arg1667Cys p <0.001). The resting fluorescence level of the p.Arg614Cys positive control was greater than the p.Ser1728Phe and p.Arg1667Cys

RYR1 variants but it did not differ significantly from either variant (p.Ser1728Phe, p =0.98 and p.Arg1667Cys, p =0.96).

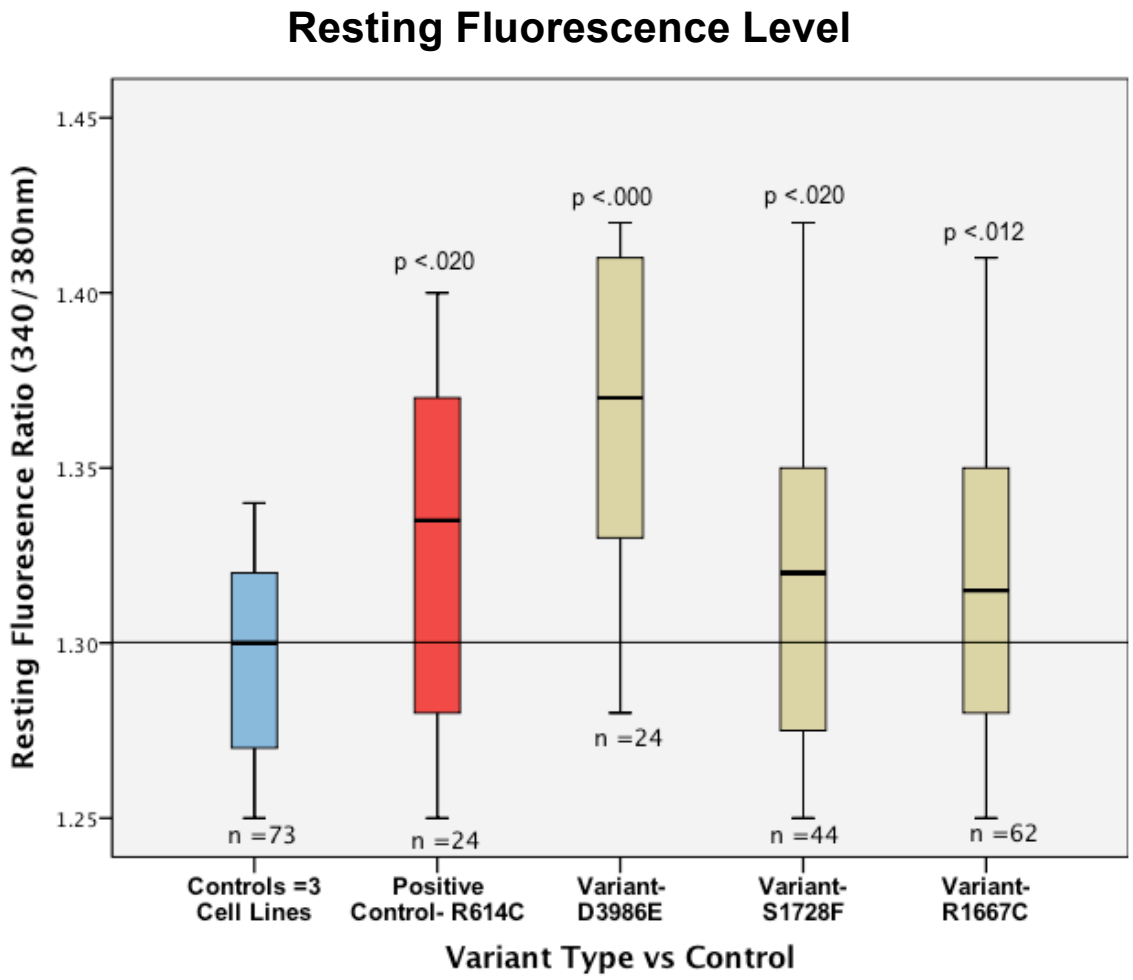

Figure 12. Mean resting Ca^{2+} florescence level (340/380 nm) of lymphoblasts. The average resting Ca^{2+} fluorescence level of lymphoblasts from the pooled CWT control individuals (n =3) and individuals` bearing different RYRI variants. Values are means (n =24-73) of the indicated number of measurements. Means are displayed in a boxplot: the length of the box is the interquartile range and includes 50 percent of the cases. The line across the inside of the box represents the median value. The whiskers protruding from the box project out to the variable's smallest and largest values. The measurements were made on populations of Fura-2 loaded lymphoblastoid cells (1-1.5**x**10^6 cells/ml) in nominally Ca^{2+}-free Krebs–Ringer solution supplemented with 0.5 mM EGTA.

Our results are consistent with studies that show *RYR1* mutations related to an MH-only phenotype (i.e., non Central Core Disease, CCD) increase resting cytosolic Ca^{2+} levels (10; 103; 206). Chronically elevated resting Ca^{2+} in MHS muscle cells is one mechanism proposed to account for the hypersensitivity of MHS RYR1 channels (179), and has been suggested to be the cause for the increased sensitivity of MHS cells to RYR1 agonists caffeine and 4-CmC (104; 106). The association of increased resting Ca^{2+} levels to hypersensitive RYR1 channels is further supported by a number of diverse functional assays that have used: (a) human MHS muscle fibers with unidentified *RYR1* variants (102), (b) muscle cells of MHS pigs expressing the RYR1 mutation p.Arg614Cys (103; 105), (c) by muscle and lymphoblasts in culture expressing MH mutations (201; 210), and (d) dyspedic myotubes using mutant RYR1 channels (107). These various functional studies found that RYR1 variants from patients with an MH phenotype had higher resting cytosolic Ca^{2+} levels than controls.

Status of the Intracellular Stores in Lymphoblasts

To assess whether the amino acid changes affected the amount of Ca^{2+} present in the intracellular stores, we next treated the lymphoblastoid cell lines—both variants and controls—with an 800 nM concentration of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor thapsigargin in the presence of Ca^{2+}-free Krebs buffer supplemented with 0.5 mM EGTA. We found that the thapsigargin-induced Ca^{2+} release measurements were all within the same range, regardless of whether the cells were from p.Arg614Cys positive or CWT control cell lines or from RYR1 variants p.Arg1667Cys, p.Ser1728Phe, p.Asp3986Glu (one-way ANOVA P =0.368). The measurements are the

fluorescence level (fluorescence ratio 340/380 nm) presented as the mean AUC for the four RYR1 variants and CWT controls. The AUC results for the thapsigargin effect are displayed in Figure 13.

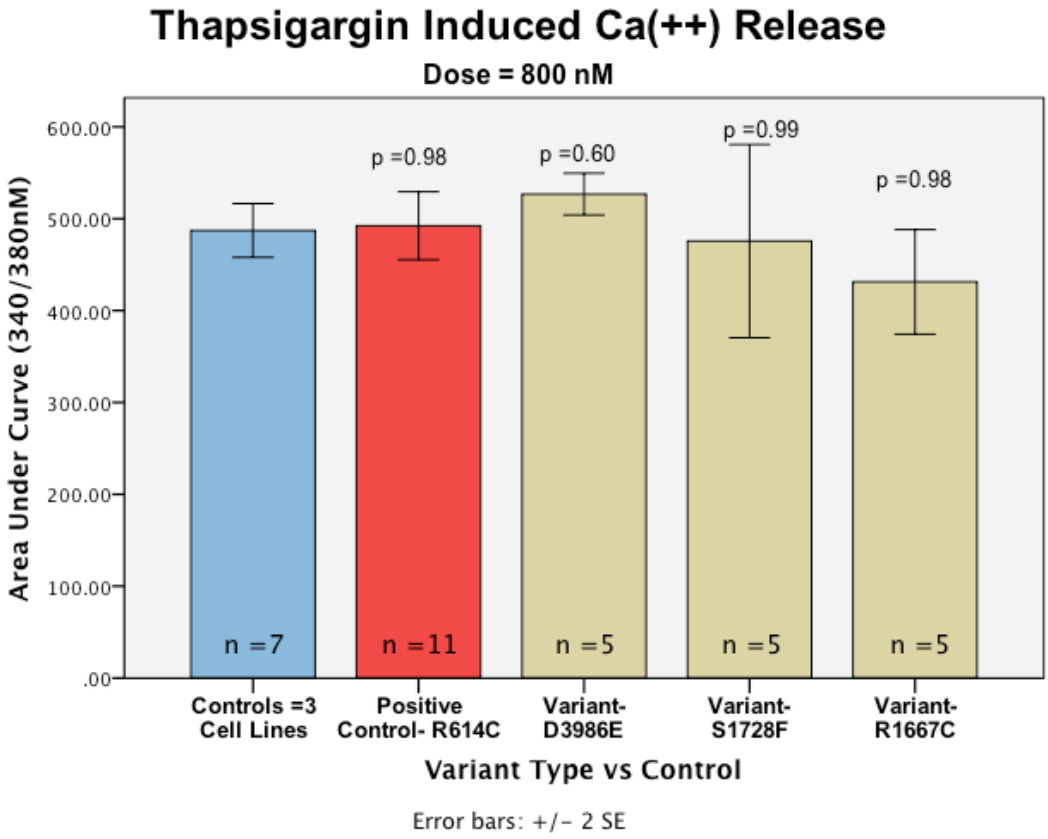

Figure 13. Mean AUC (340/380 nm) results for the thapsigargin effect.
The status of thapsigargin-sensitive Ca^{2+} stores in lymphoblastoid cells from control and MHS individuals with the putative *RYR1* mutations. No difference were observed in the thapsigargin-sensitive stores of lymphoblasts from the positive control or pooled control lymphoblast cell lines or from individuals with the p.Asp3986Glu, p.Ser1728Phe, and p.Arg1667Cys variants (one-way ANOVA, P =0.368). The measurements are the fluorescence level (fluorescence ratio 340/380 nm) presented as the mean area under the curve induced by the addition of 800 nM thapsigargin. Fluorescence measurements were performed on a population of Fura-2 loaded lymphoblastoid cells (1-1.5x10^6 cells/ml) in a 2 ml cuvette equipped with magnetic stirrer. Results represent the mean of the indicated number (n) of dedicated experiments. P values were calculated by student t test. Error bars represent the (+/- 2) standard error of the means.

Our results suggest that none of the amino acid substitutions affect the size of the

cytoplasmic Ca^{2+} stores in a way that could not be counteracted by the lymphoblasts. This is evidenced by the fact that thapsigargin causes a rapid release of Ca^{2+}—present in the intracellular pool—into the cytoplasm that then cannot be pumped back into the ER. Since very little Ca^{2+} is present in the extracellular space, the peak Ca^{2+} level recorded by fluorescence (ratio 340/380 nm) represents the total amount of Ca^{2+} present in intracellular stores (38). However, the RYR1 variants still affect Ca^{2+} release from the SR/ER because they cause an increase in the resting cytosolic Ca^{2+} levels. The outcome of no difference in AUC or peak florescence measurements—following thapsigargin treatment of MHS mutations and controls—despite an increase in resting florescence level, has been demonstrated in many human B lymphoblast studies in individuals with MHS associated mutations (7; 38; 98; 117; 201). Inversely, studies with lymphoblastoid cells harboring CCD-associated *RYR1* mutations in the C-terminal (MH region 3) and from individuals with the CCD phenotype have shown significantly *less* Ca^{2+} release then controls indicating that their stores had previously been depleted (191; 219).

Sensitivity of Lymphoblasts to Pharmacologic Activation of RyR Agonists

We next investigated whether the RYR1 amino acid changes in these lymphoblastoid cell lines alter the sensitivity of RYR1-mediated Ca^{2+} release induced by varying concentrations (0.4 mM, 0.6 mM, 0.8 mM, 1.0 mM, 1.5 mM, and 2.0 mM) of the compound 4-CmC—an RYR1 agonist shown to activate type 1 channels (65; 218). McKinney and coworkers showed that the 4-CmC assay can be a sensitive indicator of RYR1 function in lymphoblasts. In their work, they characterized the pharmacologic properties of Ca^{2+} release in two lymphoblastoid cell lines (DAKIKI and PP, EBV-

infected lymphoblastoid cell lines commercially available for research), and demonstrated that Ca^{2+} release is specific to RYR1 (despite the presence of multiple intracellular pools of Ca^{2+}, including inositol trisphosphate–sensitive stores and mitochondria), and that lymphocyte sensitivity to 4-CmC is increased in MHS (117).

Peak Dose Response (-) Baseline Fluorescence Level

A series of one-way ANOVAs were run to explore the difference between peak fluorescence response minus the baseline fluorescence level (ratio 340/380 nm) between the pooled control group and variants for four dose levels of 4-CmC (0.4 mM, 0.6 mM, 0.8 mM, 1.0 mM). Our results, following multiple experiments at four different dose levels, show that the dose response of 4-CmC induce Ca^{2+}– release in lymphoblasts from individuals bearing RYR1 substitutions p.Asp3986Glu, p.Ser1728Phe, p.Arg1667Cys and p.Arg614Cys for peak minus the baseline (P-B) fluorescence level was different at the $p<0.01$ level (ANOVA) in mean P-B fluorescence level compared to pooled CWT controls. Post hoc comparisons using Tukey HSD test indicated the mean P-B fluorescence level achieved significance for the p.Asp3986Glu and p.Ser1728Phe variant, and the p.Arg614Cys positive control at two 4-CmC dose levels (0.6 mM and 1.0 mM), compared to a pooled population of CWT controls. Mean peak fluorescence ratios (minus baseline) for the full range of 4-CmC concentrations are plotted in Figure 14.

Two RYR1 variants p.Asp3986Glu and p.Ser1728Phe, and the positive p.Arg614Cys control, showed a significantly greater response at two dose levels of 4-CmC for peak Ca^{2+} emissions minus baseline (P-B) compared to controls. The positive p.Arg614Cys control, and variant p.Ser1728Phe, showed a significantly greater response

at the 0.6 mM (p.Arg614Cys *M* =0.685, *SD* =0.114, *p* <0.03; p.Ser1728Phe, *M* =0.696, *SD* =0.123 *p* <0.01) and 1.0 mM dose (p.Arg614Cys *M* =0.450, *SD* =0.066, *p* <0.01; p.Ser1728Phe *M* =0.395, *SD* =0.128, *p* <0.01) level of 4-CmC compared to controls. Whereas, the p.Asp3986Glu variant showed greater responses achieved significance at only the 1.0 mM dose level (*M* =0.044, *SD* =0.133, *p* <0.04). The p.Arg1667Cys variant responses were greater than control cell lines but did not reach significance at any dose of 4-CmC for the P-B measurement.

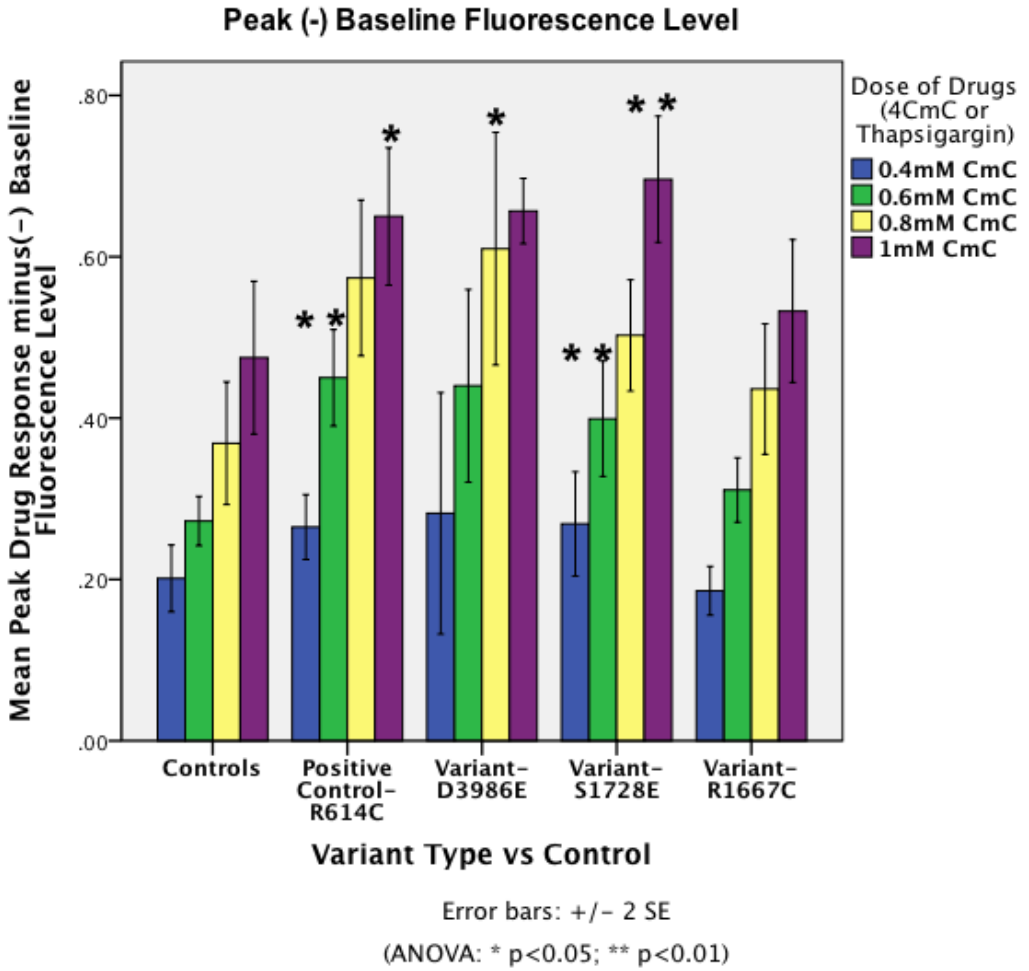

Figure 14. P-B fluorescence ratios for a range of 4-CmC concentrations.
Peak fluorescence ratios (minus baseline) for the full range of 4-CmC concentrations in Fura-2–loaded lymphoblasts (1.0-1.5 x10^6 cells) are plotted. The mean Ca^{2+}

release (P-B) was averaged from 5 to 12 replicates per dose. The dose response P-B to 4-CmC induced Ca^{2+} release of lymphoblasts from three participants with heterozygous *RYR1* variants are significantly greater at varying dose levels than the pooled (n =3) controls (ANOVA: *p<0.05, * p<0.01).

Area Under the Curve Dose Response to 4-CmC

We next examined the dose response of the lymphoblasts by measuring the AUC to increasing concentrations of 4-CmC at 0.4 mM, 0.6 mM, 0.8 mM, or 1.0 mM. The AUC is used to estimate the sensitivity of RYR1-mediated Ca^{2+} release, compared to the peak response alone, because it increases the sensitivity of the estimates. The AUC has traditionally been used in pharmacology to calculate the drug exposure over time and in skeletal muscle fiber tests to estimate caffeine dose versus contracture tension response. The AUC is preferred over the peak response, as low concentrations of RYR agonists (e.g., 4-CmC, caffeine) can cause short Ca^{2+} responses in wild type RYR with peak amplitudes as large as those reached by higher concentrations, but have been found to have a much smaller total Ca^{2+} release (213). Frequently, the only significant difference between the averaged 4-CmC Ca^{2+} measurements recorded with Fura-2 in muscle fiber is an increase of the slope of the curves (183). Thus many MHS functional and screening assays use the mean fluorescence level of the AUC Ca^{2+} release to compare responses. As further proof of usefulness, Ginz and coworkers researching a new mathematical modeling approach to resolving the differences between the MH contracture and MH genetic test results, found that if the AUC measurements are used in the analysis of the contracture response to halothane or caffeine (rather than just the peak contracture response) they improved the sensitivity and specificity to predict putative *RYR1* MHS variants to 93% and 95% (54).

Our results from a series of one-way ANOVAs, following multiple experiments in

a concentration-dependent manner of 4-CmC at 400 μM, 600 μM, 800 μM, and 1000 μM, showed that there was a difference in the overall effect between *RYR1* variant groups and CWT control group at each dose level (0.4 mM, $p < 0.01$; 0.6 mM, $p < 0.01$; 0.8 mM, $p < 0.01$, and 1.0 mM, $p < 0.01$). Tukey post hoc comparisons indicated the amount of Ca^{2+} released from the cells with RYR1 variants p.Asp3986Glu and p.Arg614Cys was significant at all 4-CmC dose levels compared to CWT controls. The RYR1 variant p.Ser1728Phe showed a significant increase in response to 4-CmC at all dose levels compared to CWT controls—except for the lowest (0.4 mM) dose. Similar to the B-P measurements, the responses to 4-CmC were greater in cell lines with the p.Arg1667ys variant than control cell lines but did not reach significance at any dose of 4-CmC for AUC measurements. The mean AUC fluorescence ratios for the full range of 4-CmC concentrations are plotted in Figure 15.

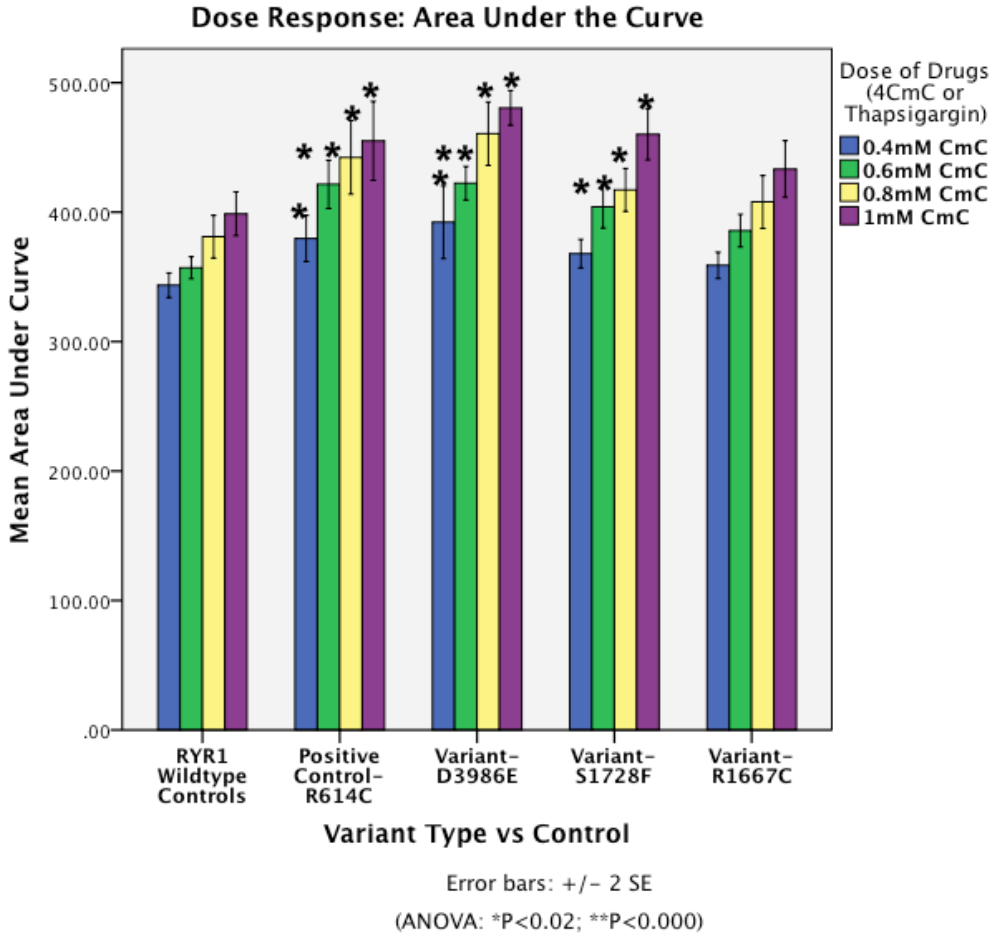

Figure 15. Mean AUC fluorescence ratios for range of 4-CmC concentrations. Lymphoblasts were stimulated with increasing concentrations of 4-CmC up to 1,000 uM, and the amount of Ca^{2+} released was calculated as AUC. The dose response to 4-CmC-induced Ca^{2+} release of lymphoblasts, following multiple experiments, from three individuals bearing *RYR1* variants are significantly greater at each dose level than control individuals (ANOVA: *p<0.02; **p<0.001).

Interestingly, the greatest difference we observed in dose effect was achieved at the 0.6 mM concentration of 4-CmC. This has been observed in other human B-lymphoblastoid experiments as well. Sei and coworkers reported that their dose–response experiments with 4-CmC on Fluo-3-loaded lymphoblastoid cells indicated a clearer separation of MHS individuals from MHN individuals at the 0.6 mM 4-CmC than

was obtained at 0.2 or 0.4 mM concentration (169). The fact that a different dye was used provides confidence to the supposition that the inter-individual differences might be an attribute of RYR or the cell type. Vukcevic and coworkers using 4-CmC concentrations from 0.2 mM to 1.2 mM also observed the greatest separation in dose response curves among MHS lymphoblasts and controls at the 0.6 mM level (201).

Model Analysis of Area Under the Curve to Dose and Variant Type

Prior to the One-way ANOVA analysis, a two-way between groups analysis of variance (two-way ANOVA) was conducted to examine the model validity of the impact of the dependent variable AUC on the two independent variables of variant type and dose level. The analysis showed a significant main effect of the drug p=0.001, and of the variant type p=0.001 (Figure 16). The effect sizes for both were large (partial eta squared 0.33 and 0.23, respectively). The F-test statistics, degrees of freedom and p values are displayed in Table 6. Post hoc comparisons (Tukey) indicated the mean AUCs for the variants p.Asp3986Glu, p.Arg614Cys, and p.Ser1728Phe variants were different ($p < .001$) than the control group. The p.Arg1667Cys variant did not differ significantly from the control group (Table 7).

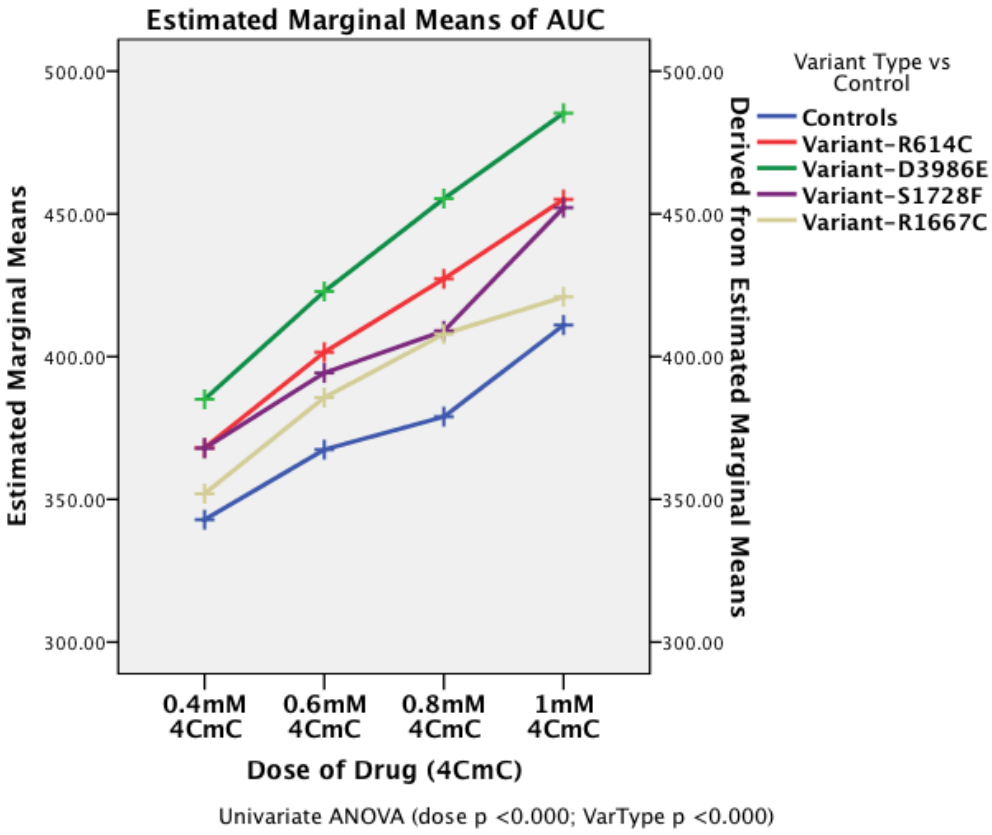

Figure 16. Two-way ANOVA for variant type and drug dose on AUC.
A two-way, between groups analysis of variance (2-way ANOVA) for variant type and drug (4-CmC) dose on AUC showed a main effect for drug F (3, 167) =26.9, p=0.001, and for variant type F (4, 167) =12.2, p=0.001.

Table 6. Tests of between-subjects effects for two-way ANOVA model analysis of AUC to dose and variant type

Tests of Between-Subjects Effects

Dependent Variable: Area Under Curve

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	196437.803[a]	19	10338.832	10.003	.000	.532
Dose	83490.334	3	27830.111	26.927	**.000**	.326
VarType	50666.677	4	12666.669	12.256	**.000**	.227
Dose * VarType	5276.914	12	439.743	.425	.952	.030

a. R Squared = .532 (Adjusted R Squared = .479)

Table 6 displays the F-test statistics, degrees of freedom and p values for the between groups analysis of variance (2-way ANOVA). Variant type and drug (4-CmC) dose on AUC showed a main effect for drug F (3, 167) =26.9, p=0.001, and for variant type F (4, 167) =12.2, p=0.001.

Table 7. Multiple comparisons for two-way ANOVA model analysis of AUC to dose and variant type

Multiple Comparisons

Dependent Variable: Area Under Curve

Tukey HSD

(I) Variant Type vs Control	(J) Variant Type vs Control	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Controls	Variant-0223	-53.3207*	11.46765	**.000**	-84.9463	-21.6951
	Variant-0145	-31.0945*	7.23249	**.000**	-51.0403	-11.1486
	Variant-0194	-32.6475*	6.57044	**.000**	-50.7675	-14.5274
	Variant-0615	-16.8804	6.18076	.054	-33.9257	.1650

Based on observed means.

The error term is Mean Square(Error) = 1033.533.

*. The mean difference is significant at the

Table 7 displays the post hoc comparisons (Tukey) for model analysis of two-way ANOVA. The mean AUCs for the p.Asp3986Glu (labeled #0223 in the table), p.Arg614Cys (#0145 above), and p.Ser1728Phe (#0194) variants were significantly different ($p < .001$) than control group. The p.Arg1667Cys variant (#0615) did not differ significantly from the control group.

CREATINE KINASE AND RESTING Ca^{2+} LEVELS

We next examined the relationship between genotype and phenotype for creatine kinase (CK) level for the three RYR1 variants, positive p.Arg614Cys, control and pooled CWT controls. Our objective was to assess if the different *RYR1* variants in our study are associated with a quantitative difference in MH phenotype (even if inferred from a quantitative surrogate marker)—specifically, the individual's serum CK level. We hypothesized that participants with the pathogenic *RYR1* variants for MHS would have higher mean CK levels than participants of the control lymphoblastoid cell lines. We hypothesized that lymphoblasts harboring pathogenic RYR1 variants for MHS would have higher mean CK levels than control lymphoblastoid cell lines. Studies have reported higher CK concentrations in individuals with MHS (27; 42; 99). Carpenter and coworkers reported an association between CK concentration and *RYR1* variants, and suggested that some *RYR1* variants are more likely to be associated with abnormal Ca^{2+} levels under physiologic conditions, versus only upon pharmacologic activation (31). However, it must be emphasized that the level of CK increase has not been found to correlate with the occurrence of MHS and, even in individuals at risk for MHS, CK levels are inadequately sensitive and nonspecific as a screening test (204).

Among the seven study participants, only one, the p.Asp3986Glu variant, had a clinically abnormal CK level (>3.3x above upper limit). For the other three participants with *RYR1*variants, and the participant with the p.Arg614Cys variant (positive control), there was a relationship between CK level and mean resting Ca^{2+} fluorescence level compared to the CWT controls (Figure 17). However, no correlation was found between:

(a) the CK level and the resting Ca^{2+} fluorescence level (CK level -63 U/L, 97 U/L, 194 U/L, 1271 U/L = to 54%, 100%, and 85% increase, compared to mean florescence-1.319, 1.320, 1.325, 1.366 = to 76%, 38%, and 3% increase); nor (b) the rank order of the CK level and mean resting fluorescence level in all cell lines. The CK levels of two the participants from whom the confirmed wild type (CWT) control lines were derived were greater than that of the participant who had the p.Arg1667Cys variant. Table 8 shows the mean resting fluorescence level and creatine kinase level by *RYR1* variant type.

To investigate the relationship between the serum CK level (drawn from peripheral blood), and the resting fluorescence level for the participants with the four *RYR1* variants and three individual CWT control participants, we conducted a zero-order correlation using a Pearson's product-moment correlation coefficient. We observed a large, significant, positive correlation between the two variables, r=.87, n =7, p <.01 (low correlation =.10-.29; medium r=.30-.49; large =.50-1.0) with high levels of CK associated with high levels of resting Ca^{2+} fluorescence in lymphocytes (Table 9)**.** We next tested the correlation without the p.Asp3986Glu variant with the abnormal CK to test if the correlation remained among the remaining six cell lines with normal levels of CK and found no significant correlation among the remaining six r=.58, n= 6, p =.111 (Table 10).

Resting Fluorescence Level and Creatine Kinase Level

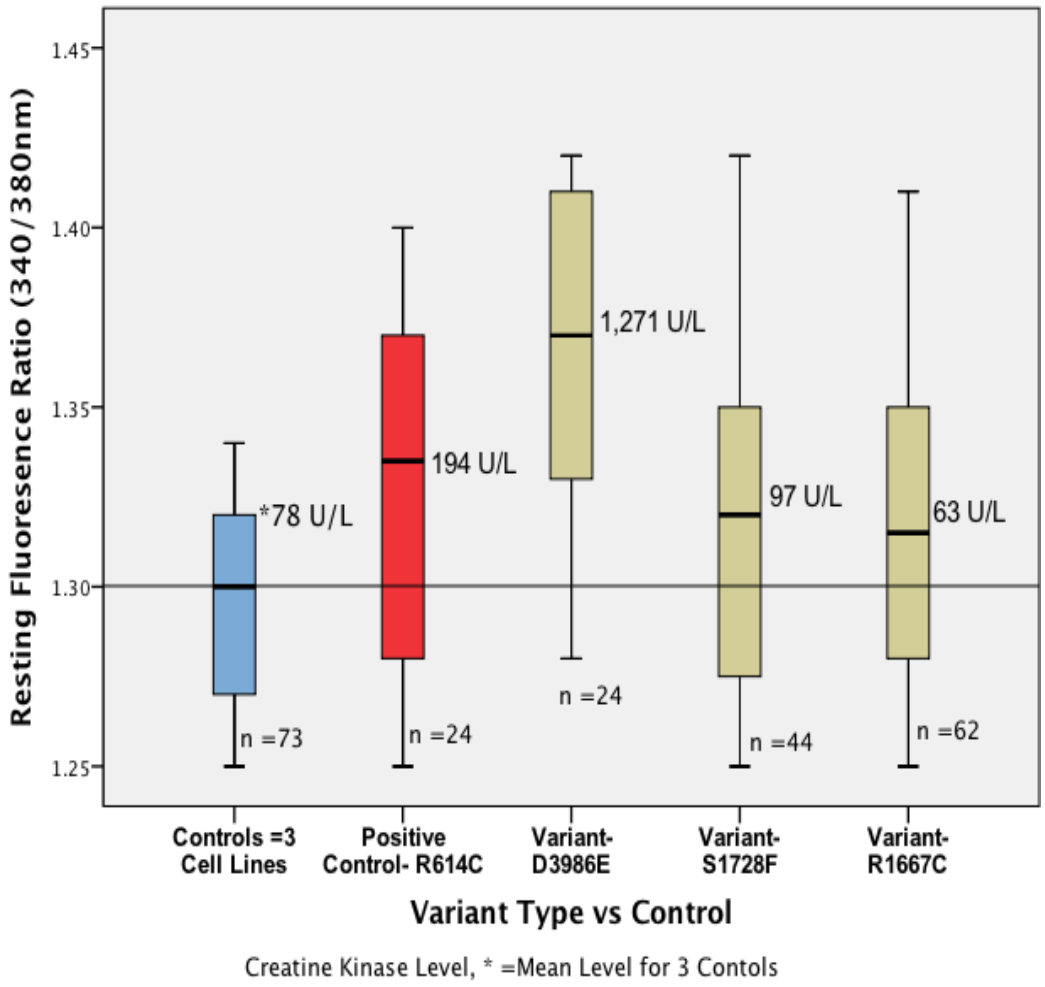

Figure 17. Creatine kinase level (CK) and mean resting Ca^{2+} fluorescence level. The average resting Ca^{2+} fluorescence levels of lymphoblastoid cell lines from the pooled CWT control individuals (n =3) and individuals bearing *RYR1* variants and their associated peripheral blood serum creatine kinase (CK) values. The CK level for CWT controls is an average for the three cell lines. Means are displayed in a boxplot. CK range: Female = 38-252 U/L; Male =52-386 U/L.

Table 8. Mean resting fluorescence level and creatine kinase level by *RYR1* variant type

Mean Resting Fluorescence Level and Serum CK Level by *RYR1* Variant Type				
Cell Line	**Serum CK Level**	**Mean Resting Fluorescence Level**	**N Resting Ca^{2+} Level**	**Gender**
Variant-p.Asp3986Glu	1,271 U/L	1.366	24	Male
Positive Control-p.Arg614Cys	194 U/L	1.325	24	Female
Variant-p.Ser1728Phe	97 U/L	1.320	44	Female
CWT Controls	78 U/L	1.292	73	All 3 Females
Variant-p.Arg1667Cys	63 U/L	1.293	62	Female

Table 8 displays the mean resting Ca^{2+} fluorescence level of lymphoblastoid cell lines from the pooled CWT control individuals (n =3) and individuals bearing *RYR1* variants as compared to their serum creatine kinase (CK) values drawn from peripheral blood. Means are displayed in the table. CK range: Female = 38-252 U/L; Male =52-386 U/L.

Table 9. Correlation of resting fluorescence level and creatine kinase level: all cell lines

Correlation of Resting Fluorescence Level and CK Level: All Cell Lines				
			Baseline Fluorescence level	Serum CK Level
Pearson Correlation	Serum CK Level	Correlation Coefficient	1	.873**
		Sig. (1-tailed)		.005
		N	7	7
	Mean Baseline Fluorescence Level	Correlation Coefficient	.873**	1
		Sig. (1-tailed)	.005	
		N	7	7

**. Correlation is significant at the 0.01 level (1-tailed).

Table 9 shows the zero order correlation using a Pearson's product-moment correlation coefficient for mean baseline fluorescence level and serum creatine kinase level (CK) among the seven B-lymphocyte cell lines. A low correlation r =.10-.29; medium r = .30-.49; large = .50-1.0. The symbol ** = correlation is significant at the p =0.01 level (1-tailed).

Table 10. Correlation of resting fluorescence level and creatine kinase level (-) D3986E variant

Correlation of Resting Fluorescence Level and CK Level: (-) D3986E variant				
			Baseline Fluorescence level	Serum CK Level
Pearson Correlation	Serum CK Level	Correlation Coefficient	1	.585
		Sig. (1-tailed)		.111
		N	7	350
	Mean Baseline Fluorescence Level	Correlation Coefficient	.585	1
		Sig. (1-tailed)	.111	
		N	6	6

*Correlation is significant at the 0.05 level (1-tailed).

Table 10 shows a zero order correlation using a Pearson's product-moment correlation coefficient for mean baseline fluorescence level and serum creatine kinase (CK) level among participants from whom the six B-lymphocyte cell lines were derived, excluding the RYR1 p.Asp3986Glu variant with the abnormal CK level. A low correlation r =.10-.29; medium r = .30-.49; large = .50-1.0.

The results from our restricted sample of four *RYR1* variants agree with Carpenter and coworkers findings that *RYR1* variants from MHS individuals are more likely to be associated with higher CK levels, as observed in Figure 17 (31). For the individuals with WT copies of *RYR1*, it is likely there is insufficient power within our data set to find a correlation, if one exists. It maybe crucial to first test individuals more than once, while controlling for excessive physical activity and possible iatrogenic muscle damage among

participants, to increase the validity of this assessment (99; 204).

SUMMARY OF PART II RESULTS

All of the *RYR1* variants we functionally characterized caused a significantly increase in the resting cytosolic Ca^{2+} levels when compared to WT controls. In addition, the sensitivity of mutated RYR1 to pharmacological activation was increased in all mutations relative to *RYR1* CWT controls, and reached significance for two RYR1 variants (p.Asp3986Glu, p.Ser1728Phe) and the p.Arg614Cys positive control. Two of the *RYR1* variants (p.Asp3986Glu, p.Ser1728Phe) and the positive p.Arg614Cys control in our study had a significantly ($P<0.001$) greater response to 4-CmC than controls at the 0.6 mM dose levels and a significantly greater response ($P<0.05$) at most dose levels. The elevated resting intracellular Ca^{2+} levels and greater dose response to the RYR1 channel agonist of the mutant RYR1 cell lines suggests altered functional status in these mutated channels and support a causal role in MHS. The results show that the RYR1 variants, with the exception of the p.Arg1667Cys variant, are associated with the MH status in these individuals. The results from the functional analyses of these *RYR1* variants identified contribute to a better understanding of *RYR1* variants identified through ES.

CHAPTER 5: DISCUSSION

We piloted a method using ES to identify rare, likely pathogenic *RYR1* variants associated with MHS in a population unselected for the disorder. We then functionally characterized three mutant RYR1 channels, one variant of unknown significance, and three confirmed wild type (CWT) controls, using EBV-transformed lymphoblastoid cell lines by dye incorporation array and examined the correlation of specific MHS genotypes and responses to Ca^{2+} release. The identification and pathogenicity classification of the *RYR1* variants from ES, followed by functional results that corresponded to the *RYR1* variant's categorization, supports our hypothesis that putative pathogenic variants can be identified through ES data.

Using ES, we identified 123 distinct variants (70 *RYR1* and 53 *CACNA1S*) among 870 participants (Figure 9, Appendix B and Appendix C). We classified these variants into six categories of pathogenicity, based on sequence quality, allele frequency, information from mutation databases, and review of primary literature and family history. Our analyses yielded a spectrum of pathogenicity scores from benign to pathogenic (Appendix A).

Of these 123 distinct variants, 21 were classified by HGMD as disease causing (Appendix B and Appendix C). All but two of the *RYR1* variants, classified as disease causing mutations in HGMD, were reclassified by us as benign, likely benign, or variant of uncertain significance, scores 1-3, respectively. We reclassified these variants based on the criteria in our Variant Pathogenicity Classification System (Appendix A), under the assumption that the variant was a variant of unknown significance, unless a critical review of the data supported a higher pathogenicity category. It is critical to recognize

that our assessment of 'benign' or 'likely benign' is limited to the specific context of using such a variant for individualized predictive medicine and that it was not our intention for it to be interpreted to mean that the variant has no role in the pathogenicity of MHS, myopathy, or other phenotypes. In addition, more than half of the *RYR1* variants (43/69, 62%) we identified were not listed by HGMD or the LSDB databases, or in biomedical literature citations. Because we screened a cohort unselected for MHS, we predicted that most of the novel variants would be benign. More than half (40/69) of the *RYR1* variants (class 1-5) were rare and not found in the Exome Variant Server. A fifth (10/51) of the *CACNA1S* variants were common variants, which we assigned to class 1 (almost certainly benign), with the remaining assigned to class 3 (Appendix C). Four individuals (three males, one female) had more than one *RYR1* variant, and two of the four participants with two *RYR1* variants had putatively benign *CACNA1S* variants as well.

The purpose of this study was to identify high penetrance variants associated with MHS from exome data of unselected individuals. As noted above, the conclusion that a variant is class 1-3 does not necessarily mean that the variant has no physiological effects. Moreover, the data did not allow us to evaluate whether interactions could have occurred between variants in a given individual (151), although this should be specifically addressed in future studies. We have deliberately set our threshold for pathogenicity high to avoid the error of wrongly diagnosing an individual as susceptible in an ascertainment mode where the prior probability that they are affected is low. However, the number of errors will diminish with time as future ES and follow-up studies generate additional data.

The filtering process for analysis of MHS variants from ES currently requires a manual method of evaluating variants to extract meaningful information. We used variant sequence quality, allele frequency, genotype-phenotype databases, family history and the primary literature to identify pathogenic variants. Unfortunately, there is no single information source that allows us to reliably ascertain if a variant is benign or pathogenic. Many sequence databases (e.g., the Exome Variant Server and The Single Nucleotide Polymorphism Database) include pathogenic, potentially pathogenic, and non-pathogenic variants, and do not include phenotype data. Further, there is no indication as to whether some individuals harbor multiple variants within a single gene, which limits our ability to evaluate the data. Our evaluation of 870 exomes using HGMD and LSDBs indicates likely significant levels of misclassification and variability in the pathogenicity determination in HGMD and the LSDBs, which is primarily attributable to the source literature.

Following the identification of RYR1 variants through ES, four *RYR1* nonsynonymous substitutions (c.5183C>T, p.Asp3986Glu, c.5183C>T, p.Ser1728Phe, c.4999C>T, p.Arg1667Cys, and c.1840C>T, p.Arg614Cys[5]) were selected for functional analysis based on their level of pathogenicity – three class 5s, pathogenic and one class 3, variant of uncertain significance. All four *RYR1* variants had been described in the MH literature as inherited in an autosomal dominant pattern in patients with MH. However, only the variant that we designated as the positive control, the p.Arg614Cys mutation on the European MHS group's mutation panel of 34 *RYR1* mutations, had been functionally characterized—although not in human B-lymphoblasts (1). The p.Arg614Cys mutation

[5] Herein the cDNA changes c.5183C>T, c.11958C>G, c.4999C>T, c.1840C>T referred as p.Ser1728Phe, p.Asp3986Glu, p.Arg1667Cys, and p.Arg614Cys, respectively.

has also been associated with the MHS/CCD phenotype and with exertional/environmental heat stroke and exercise-induced rhabdomyolysis (31; 160). Similarly, the MHS participant with the p.Asp3986Glu variant, in addition to the two life threatening MH reactions under anesthesia (e.g., masseter muscle rigidity during surgery in 1972, and a full-blown episode during surgery in 1998), had episodes of both awake MH and rhabdomyolysis. Awake MH is a term used to describe a rare subset of identified MHS individuals that develop signs of MH without anesthesia (160).

The three class 5 variants, functionally characterized in our study as showing increased sensitivity to the RYR1 agonist 4-CmC as compared to cells expressing CWT *RYR1* and suggesting the three variants would be associated with MH-susceptibility—were profiled earlier in a genotype-phenotype study. Carpenter and coworkers described c.5183C>T, p.Asp3986Glu, c.5183C>T, p.Ser1728Phe, and c.1840C>T, p.Arg614Cys, in addition to 20 other *RYR1* variants, in a study that evaluated the association of a clinical and laboratory phenotype (*in vitro* muscle contracture response and serum CK level) and *RYR1* genotype (31). Common *RYR1* variants were compared to the most prevalent *RYR1* mutation in the UK (c.7300G>A, p.Gly2434Arg), as the phenotype was previously described as relatively mild (i.e., weaker contractures in the *in vitro* test). They found that each *RYR1* variant differed in comparison to the *RYR1* positive p.Gly2434Arg control for specific quantitative contracture-test phenotypes (e.g., response time and change in tension to static caffeine, halothane and ryanodine), and serum CK concentration. The *RYR1* p.Gly2434Arg positive control was functionally characterized in SR vesicles from human MHS skeletal muscle in an earlier study by Richter and coworkers and shown to have a heightened sensitivity to 4-CmC (148).

Important parallels exist between the earlier studies and our pilot characterization. Carpenter and coworkers found that individuals with the RYR1 variant p.Asp3986Glu had significantly greater responses to caffeine, a more severe static caffeine contracture test, a greater median clinical reaction time to anesthetic, and a greater serum CK concentration than the p.Gly2434Arg positive control(31). In fact, the p.Asp3986Glu variant had a stronger response to the static caffeine contracture test and greater serum CK concentration compared to all 22 *RYR1* putative pathogenic variants profiled in the UK study. Individuals with the RYR1 p.Arg614Cys mutation also had higher CK concentrations than did the individual with the p.Gly2434Arg variant, but were not significantly different in contracture test responses. However, the p.Ser1728Phe variant was consistently associated with a significantly weaker contracture test response phenotype in comparison with p.Gly2434Arg, suggesting a lesser effect on channel function. None of the variants p.Asp3986Glu, p.Ser1728Phe, or p.Arg614Cys were associated with a statistically significantly lower CK concentrations than was the p.Gly2434Arg variant cell line and no overlap of the serum CK concentration 95% confidence interval (CI) was observed for any of the three individuals with variants compared with the 100 MH-negative controls (31).

We found a similar hierarchy and separation in our study within p.Asp3986Glu, p.Arg614Cys, and p.Ser1728Phe and between the CWT controls to the basal Ca^{2+} fluorescence level, 4-CmC responses and to serum CK. In our study, p.Asp3986Glu, p.Arg614Cys, and p.Ser1728Phe followed the same rank order of highest to lowest (i.e., p.Asp3986Glu, then p.Arg614Cys, p.Ser1728Phe, and CWT controls) in relation to a surrogate phenotype marker (observed serum CK levels), and functional tests: statistical

significance for mean resting Ca^{2+} fluorescence levels and dose response to four concentrations of 4-CmC calculated as AUC (two-way ANOVA). We suggest that the association between genotype-phenotype may persist for functional characterization in the B-lymphoblasts and validates the B-lymphoblast model as an approximation of the severity level of RYR1 channel pathology *ex vivo*. It also demonstrates that genetic differences may explain some of the variability in the functional results.

A potential reason for the distinct effects on RYR1 of the agonist among the four variants we selected for analysis is the impact of the distinctive conformational states induced by each amino acid substitution, depending on the location of the amino acid within the protein. The study by Carpenter and coworkers found that genetic differences explain some of the variability in the functional *RYR1* variants responses (31), and likewise Amburgey and coworkers, in a genotype-phenotype correlation study of a cohort of unpublished and previously reported *RYR1* cases, showed that histological phenotype (i.e., microscopic findings on muscle biopsy) is at least partially determined by mutation position (6). Their analysis revealed that the diagnosis of a particular recessive *RYR1* myopathy was associated with enrichments of mutations in known functional domains of the protein (6).

Our data show that *RYR1* variants from specific hot-spot regions had greater 4-CmC responses and CK levels compared to variants outside those regions. Most *RYR1* variants are located in three clusters or hot-spots corresponding to the following regions of the RYR1 protein: N-terminal (Cys35-Arg614, MH/CCD region 1), central (Asp2129-Arg2458, MH/CCD region 2, or cytoplasmic region), and C-terminal (Ile3916-Ala4942, MH/CCD region 3) (150; 194). MHS-related *RYR1* variants are predominantly located in

regions 1 and 2 of the RYR1 protein, whereas the majority of *RYR1* variants associated with CCD occur in the hydrophobic pore-forming region 3 of the channel (35; 121; 122; 219). These regions contain a high frequency of evolutionarily conserved sites (191; 219). Altering a site within *RYR1* that is highly conserved is correlated with stronger RYR1 channel agonist responses and more severe *RYR1* phenotype (31; 57; 90)**.** Table 11 displays the characteristics of the non-synonymous recurrent *RYR1* variants in this study. However, despite the strong association, the functional effects of specific *RYR1* variants cannot be predicted based solely on their location; several mutations in the C-terminal region of *RYR1* enhance the agonist sensitivity characteristic of MHS mutations, others in the region exhibit varying degrees of impaired Ca^{2+} release, and still others show Ca^{2+} release in the absence of pharmacologic activators (90; 191).

Table 11. Characteristics of *RYR1* variants studied

Codon	Exon / Hotspot	Amino Acid Change	Conditions	Level of Conservation	Conserved in RyR Isoforms
c.1840C>T	17 / Yes	p.R614C	MH/ CCD/ EHI /Rhabdo / incr. CK	In all species	Yes
c.4999C>T	34 / No	p.R1667C	MH/ incr. CK	In some mammals	No
c.5183C>T	34 / No	p.S1728F	MH/ incr. CK	In all mammals	No
c.11958C>G	87 / Yes	p.D3986E	MH/ EHI/ Rhabdo/ incr. CK	In all species	Yes

Table 11 shows the characteristics of the non-synonymous *RYR1* variants studied in this functional assay. Hotspot refers to RYR1 terminal region 1, 2 or 3. "Condition" refers to the disease state of individuals from known studies. The "level of conservation" refers to species alignments of all available full RYR1 protein sequences from NCBI. Conserved refers to RYR paralogs RYR1 ,RYR2, and RYR3. They were described as preserved if they were present in all three RYR paralogs; otherwise the site was described as not conserved.

In terms of clinical severity, mutations within the C-terminal domain (MH/CCD hotspot 3) are more often associated with a more severe phenotype when compared to

other areas in the protein than would be expected by chance (6) and therefore, these changes are more likely associated with full penetrance of CCD (114). The RYR1 variant p.Asp3986Glu exhibited the most severe phenotype among the four mutations in terms of clinical symptoms (i.e., serum CK level) and functional outcomes (i.e., results in the assay). The participant with the p.Asp3986Glu variant was symptomatic with severe myotonia (prolonged contraction), but did not manifest the clinical features of CCD in that signs of myasthenia, hypotonia, and central cores on muscle biopsy were absent. Similarly, no Ca^{2+} store depletion was observed in the p.Asp3986Glu cell line as confirmed by Ca^{2+} release measurements from thapsigargin. A CCD-only mutation would likely show a leaky phenotype (i.e., high resting Ca^{2+} release), coupled with reduced thapsigargin-sensitive stores, and a decrease in Ca^{2+} release to an RYR1 agonist (191). The addition of 4-CmC did trigger an increase in Ca^{2+} release in the lymphoblasts with the p.Asp3986Glu variant, indicating that the RYR1 intracellular stores were not depleted; thus the mutated RYR1 channel could compensate for the leak consistent with a MHS phenotype.

Mutations in *RYR1* that occur in the N-terminal (Cys35-Arg614) and central regions (Asp2129-Arg2458) are thought to affect these two regions main functions as domain switches involved in control of the conformation of the channel. Mutations in *RYR1* region 1 (e.g., p.Arg614Cys) weaken the interdomain interaction resulting in a hypersensitive channel due to this destabilization (179). Murayama and coworkers provided the first evidence that the inter-domain interaction between regions 1 and 2 is the underlying mechanism for dysfunction of Ca^{2+} homoeostasis by using isolated SR vesicles from MHS pigs with the RYR1 c.1843C>T, p.Arg615Cys mutation (the porcine

equivalent to c.1840C>T, p.Arg614Cys) (127). McKinney and coworkers tested a porcine lymphoblast population with the c.1843C>T, p.Arg615Cys mutation and found a nearly twofold increase (normalized 4-CmC dose–response curves) in sensitivity to 4-CmC compared to porcine wild type controls and reported a similar shift is observed on human myocytes from MH patients carrying *RYR1* mutations (c.6617C>T, p.Thr2206Met, c.7358T>C, p.Ile2453Thr, c.6502G>A, p.Val2168Met) from the second hotspot region (117). We observed increases in both resting Ca^{2+} levels and a hypersensitivity response to 4-CmC that was intermediate in response to mutations in domain 3 and variants located outside hotspot regions as compared to CWT controls.

_____The *RYR1* variants p.Ser1728Phe and p.Arg1667Cys are located outside a hotspot region. Both variants lie in the dihydropyridine receptor (DHPR)-binding domains (amino acid residues 1085-1208, 1341-1402, 1635-2636, and 3495-349). They lie in regions that MacLennan and Zvaritch characterized as a mutation "cool region" with a lower density (but not lacking) of mutations (113). Amburgey and coworkers found that the DHPR-binding domains had fewer mutations than expected in their analysis. They concluded that as a result, the interactions with the RYR1-binding proteins to DHPR may not be important in the pathogenesis of the known *RYR1* myopathies (6). In our study, both the p.Ser1728Phe and p.Arg1667Cys variants showed the lowest basal Ca^{2+} level and increase in Ca^{2+} sensitivity to RYR1 agonists. The p.Ser1728Phe variant, which had a greater resting Ca^{2+} basal response compared to p.Arg1667Cys, is in close proximity to hotspot region two. The p.Ser1728Phe variant, positive in our functional analysis, has also been reported in multiple cases of MH patients with positive contracture tests and is absent in control samples in these studies (30; 151; 163) and in 1/10,757 alleles from the

Exome Variant Server. This suggests that RYR1 contains other key regions that result in an increased sensitivity to the RYR1 agonist if mutated.

The RYR1 p.Arg1667Cys substitution represents a novel finding. Our data showed lymphoblastoid cells with this mutation in the heterozygous states behaved similarly to CWT cells in terms of Ca^{2+} store content and total amount of Ca^{2+} released after pharmacological RYR1 activation; however, they also had a small, but significant increase in resting Ca^{2+} compared to CWT controls. In addition, we observed a trend toward increased sensitivity in 4-CmC responses at all dose levels. However, this was not statistically significant and had considerable overlap in the 95% CI compared to pooled CWT controls. Ibarra and coworkers showed that RYR1 p.Arg1667Cys might be causative for MHS, since it was identified in five unrelated Japanese patients diagnosed as MH-susceptible by the calcium-induced calcium release test (i.e., Japanese equivalent of the *in vitro* muscle contracture test), and Kraeva and coworkers (reported one individual with the p.Arg1667Cys variant and a positive caffeine-halothane contracture test for MHS (70; 89). However, in both studies RYR1 p.Arg1667Cys was found in control samples. In our study, the p.Arg1667Cys variant was detected in four ClinSeq® participants (4/870), and in 21/10,727 alleles, for a carrier frequency of 1/255 and at an allele frequency of 0.2% in the National Heart, Lung, and Blood Institute, Exome Sequencing Project's Exome Variant Server. Since the p.Arg1667Cys variant affects the resting Ca^{2+}, and lymphoblastoid cells do not express all of the muscle proteins that interact with RYR1 in the SR, that mutation may influence channel function in a way that could not be detected by our system. It may be possible that the p.Arg1667Cys variant manifests an intermediate response, with high resting fluorescence but normal peak Ca^{2+}

emissions. Further studies with a different functional model system or assay will be necessary to test this hypothesis. The occurrence of an *RYR1* mutation with previously uncharacterized properties highlights the issue that understanding the physiology of the entire spectrum of *RYR1* variants occurring in both MHS and WT individuals would facilitate the identification of mutations that are associated with the MHS phenotype.

CONCLUSION

We have demonstrated that it is possible to screen exomes from unselected individuals to identify variants in *RYR1* that pose potential risk of MH. We have followed this genomic ascertainment by demonstrating functional derangements in cells from the individuals harboring putatively pathogenic variants. We demonstrated increased sensitivity of the cell lines with these mutations to the RYR1 agonist 4-CmC in B-lymphoblastoid cells with the three RYR1 variants (p.Asp3986Glu, p.Ser1728Phe, and p.Arg614Cys) compared to cells expressing CWT RYR1. We conclude that the *in vitro* results suggest that the three variants would be associated with MHS. We have provided evidence that distinct *RYR1* missense variants varied in the severity of 4-CmC responses and that elevated basal Ca^{2+} level was correlated with serum CK concentration in our sample. The level of RYR1 channel agonist response was in turn observed to be associated with alterations in sites within *RYR1* that are highly conserved. We conclude that human EBV immortalized B-lymphoblastoid cells can be used as a proxy tissue to identify and study the effects of putative *RYR1* variants identified through ES, compared to immortalized B-lymphoblasts with *RYR1* CWT or benign variants.

Analyses of thousands of exomes have the potential to provide the MHS field with an exhaustive catalog of variants to determine the true prevalence, penetrance, and

expressivity of this life-threatening disorder. While the assessment of the pathogenicity of both known and novel variants remains challenging, we demonstrate that causative mutations can be identified from ES data. These data suggest that clinically relevant mutations can be identified as incidental or secondary findings in exomes sequenced for clinical care and clinical research. This should inform the debate on the return of such secondary results to research participants. Further, the application of ES technology to large and diverse cohorts has the potential to accelerate the pace of MHS gene mutation discovery. We speculate that the results of these studies will allow the development of clinical genomic screening for MHS, which should reduce the incidence of life-threatening events and increase life expectancy for those individuals who harbor pathogenic variants in these genes.

LIMITATIONS OF THE STUDY

ES has some limitations: the method is subject to have false negative results for variants such as structural variation, or copy-number variants (CNVs). Indeed, it is not 100% sensitive for single nucleotide substitutions. Although the technology has improved target coverage over the years, it does not currently reach 100%. In view of the distribution of variants and the complexity of the genome, ES remains an efficient way to identify most mutations predicted to alter the protein sequence from a DNA sample. However, to our knowledge, the only genomic variants so far associated with MH are missense variants in coding exons, so most of these limitations do not pertain, given our current knowledge of the disorder.

The published prevalence of MHS mutations varies widely from 1 in 2,000 (70; 158) to 1/10,000 (120) but the penetrance has been difficult to determine. Our study of

870 exomes, although it represents a prodigious amount of data, is still too small to estimate the prevalence of MHS. The ES of patients not ascertained for a personal or family history of MHS allows, in principle, an unbiased approach to genotype-phenotype correlation that has not been practical with previous technologies. In addition, the sample of *RYR1* variants we characterized was small and some associations are expected by chance when making multiple comparisons. It would be ideal to confirm our results by replicating our findings with a second functional analysis involving a larger cohort of *RYR1* variants. The use of CWT controls without contracture test results can be problematic; there remains a chance a mutation in another gene might result in myopathy. Our use of multiple controls reduces this likelihood, as the probability of all three CWT controls showing a similar and significantly lower basal Ca^{2+} fluorescence levels remain small. We conclude that some *RYR1* and *CACNA1S* variants may have been misclassified as pathogenic without adequate genetic (e.g., cosegregation) or functional data. It is important to stress that in addition to robust genetic analysis, there is a critical need for a robust and non-invasive functional test for MHS, which together with genetic data could allow accurate determination of the prevalence and penetrance of this trait.

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Using Exome Data to Identify Malignant Hyperthermia Susceptibility Mutations

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ABSTRACT

Background: Malignant hyperthermia susceptibility (MHS) is a life-threatening, inherited disorder of muscle calcium metabolism, triggered by anesthetics and depolarizing muscle relaxants. An unselected cohort was screened for MHS mutations using exome sequencing. The aim of this study was to pilot a strategy for the *RYR1* and *CACNA1S* genes.

Methods: Exome sequencing was performed on 870 volunteers not ascertained for MHS. Variants in *RYR1* and *CACNA1S* were annotated using an algorithm that filtered results based on mutation type, frequency, and information in mutation databases. Variants were scored on a six-point pathogenicity scale. Medical histories and pedigrees were reviewed for malignant hyperthermia and related disorders.

Results: The authors identified 70 *RYR1* and 53 *CACNA1S* variants among 870 exomes. Sixty-three *RYR1* and 41 *CACNA1S* variants passed the quality and frequency metrics but the authors excluded synonymous variants. In *RYR1*, the authors identified 65 missense mutations, one nonsense, two that affected splicing, and one non–frameshift indel. In *CACNA1S*, 48 missense, one frameshift deletion, one splicing, and one non–frameshift indel were identified. *RYR1* variants predicted to be pathogenic for MHS were found in three participants without medical or family histories of MHS. Numerous variants, previously described as pathogenic in mutation databases, were reclassified by the authors as being of unknown pathogenicity.

Conclusions: Exome sequencing can identify asymptomatic patients at risk for MHS, although the interpretation of exome variants can be challenging. The use of exome sequencing in unselected cohorts is an important tool to understand the prevalence and penetrance of MHS, a critical challenge for the field.

What We Already Know about This Topic

- Exome sequencing is likely to become more common in the movement toward personalized medicine
- A more thorough description of variants in genes associated with malignant hyperthermia may aid in interpreting the results of exome sequencing

What This Article Tells Us That Is New

- In 870 volunteers not ascertained for malignant hyperthermia susceptibility, numerous variants in *RYR1* and *CACNA1S* genes were observed, some consistent and others inconsistent with presumed pathogenicity in current databases

MALIGNANT hyperthermia susceptibility (MHS) is a rare disorder of calcium dysregulation triggered by volatile anesthetics and the depolarizing muscle relaxant succinylcholine. It is an important cause of morbidity and mortality, and in its fulminant form manifests nearly always as metabolic and/or respiratory acidosis, rhabdomyolysis and

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hyperkalemia, as well some or all of the following symptoms: tachycardia, tachypnea, arrhythmias, skeletal muscle rigidity, and lethal hyperthermia. It is inherited in a predominately autosomal dominant pattern and associated with *RYR1* or *CACNA1S* mutations, with other mapped loci. Seventy to 86% of patients with MHS have *RYR1* mutations[1–5] and 1% have *CACNA1S* mutations.[6] The prevalence and penetrance of MHS mutations are difficult to determine because the pharmacologic exposure rate is low and it is an inconsistently manifesting gene–environment interaction; that is, when a susceptible patient is exposed to a triggering agent, the probability of malignant hyperthermia (MH) is less than 100%.

Most MHS gene and variant studies have been performed on families with multiple generations affected with typical MHS. Studying these families made possible the discovery of the two implicated genes. However, these studies had ascertainment biases for those with severe reactions to the drugs. This has complicated efforts to establish the true prevalence and penetrance of MHS mutations.

In addition, assigning pathogenicity to *RYR1* and *CACNA1S* variants is challenging for several reasons. First is the issue of locus heterogeneity. With several mapped loci without identified genes, some *RYR1* and *CACNA1S* variants may have been erroneously determined to be pathogenic when there was a causative variant in another (untested) gene. In addition, *RYR1* and *CACNA1S* are large genes with 106 and 44 exons, respectively, making mutation screening challenging. Thus, some *RYR1* and *CACNA1S* variants previously determined to be pathogenic may be benign, as has been shown for other genes.[7]

New sequencing technologies, including exome sequencing (ES), have made sequencing of the human exome (exons of known genes) feasible. This provides the opportunity to detect mutations in MHS genes in a less biased manner. Using this approach, we can improve our understanding of the mutational spectra of the *RYR1* and *CACNA1S* genes, and estimate their penetrance. Our objective was to identify mutations in *RYR1* and *CACNA1S* in a population not ascertained for MHS, as a pilot for the use of exome data for predictive medicine.

Materials and Methods

To pilot the identification of MHS in an unselected population (mostly from the metropolitan areas of Washington D.C. and Baltimore in the United States), we evaluated ES data from the ClinSeq® study[8] (n = 870)—a longitudinal cohort design to study the technical, medical, and genetic counseling issues associated with medical sequencing on large scale (*i.e.*, exome or genome sequencing). The ClinSeq® study was reviewed and approved by the National Human Genome Research Institute's Institutional Review Board (Bethesda, MD) and all subjects provided informed consent to publish results and deposit sequence data in databases. Participants were 45–65 yr of age at enrollment with a median age of 57 yr. These volunteers were unselected for MHS because they were ascertained for a spectrum of coronary artery disease, which is not associated with MHS. This sample of 870 participants was 89% Caucasian, 96.3% not of Hispanic or Latino background, and 49.7% female. Details of family history, race, ethnicity, current medical status, and clinical data were collected at enrollment, although a personal or family history of MHS was not specifically solicited. Race and ethnicity were determined by self-report on an intake questionnaire. First-degree relatives of another participant were excluded but one dyad of participants were first cousins and one dyad were first cousins once removed. During their initial visit, participants underwent an electrocardiogram, echocardiogram, and computed tomography scan for coronary calcium, clinical chemistries, and blood sample collection for genomic analysis. Sequence variants deemed clinically relevant were validated in a Clinical Laboratory Improvement Amendments–certified laboratory and the results returned to the participant.

The sequence data were generated at the National Institutes of Health's Intramural Sequencing Center. The sequencing method used solution–hybridization exome capture, performed with the *SureSelect All Exon System* (Version 1.0) from Agilent Technologies (Santa Clara, CA). The sequencing of 101 base-pair (paired-end reads) was performed with the GAIIx sequencer from Illumina, Inc. (San Diego, CA). One or two 101 base-pair, paired-end flow-cell lanes were sufficient to generate more than 85% coverage of the targeted exome with high-quality variant detection.[9] Filters were applied with the VarSifter Next-Gen variation analysis software.§§[10] DNA isolation, library preparation, capture, sequencing, and alignment and base calling were performed as described.[11]

RYR1 and *CACNA1S* variants were filtered for mutation type, frequency, and information in locus-specific mutation databases (LSDBs). The complementary DNA variants and their predicted protein changes are referred to by their protein designations in the text (see variant tables, Supplemental Digital Content 1, http://links.lww.com/ALN/A976, and Supplemental Digital Content 2, http://links.lww.com/ALN/A977, which are tables of the *RYR1* and *CACNA1S* variants identified in this study, respectively). *RYR1* nucleotide numbering is based on transcript NM_000540.2, and *CACNA1S* NM_000069.2, according to the Human Genome Variation Society nomenclature.‖‖ Variants with low genotype quality were designated class 0; the remainder were scored 1–5 using an adaptation of published criteria.[12–17] Briefly, class 1 variants were definitely benign, class 2 probably benign, class 3 of uncertain pathogenicity, class 4 probably pathogenic, and class 5 definitely pathogenic.

§§ The VarSifter program website. A graphical software program designed to view massively parallel sequencing variation output. Available at: http://research.nhgri.nih.gov/software/VarSifter/index.shtml. Accessed June 30, 2013.

‖‖ The Human Genome Variation Society website. Nomenclature for the description of sequence variations. Available at: www.hgvs.org/mutnomen/. Accessed May 20, 2013.

Further evaluation of the variants was performed using the Human Gene Mutation Database (HGMD)##[18] and the LSDB, Leiden Open Variation Database (LOVD)***[19] and for potentially pathogenic variants, review of the medical literature (table 1). We elected not to use amino acid substitution mutation analysis tools because their predictive power is variable.

Medical histories of the probands and their pedigrees were reviewed for diagnoses or symptoms of MHS and related disorders. We learned retrospectively that one participant self-referred to the study because of a clinical diagnosis of MHS (subsequently found to have RYR1 p.Asp3986Glu). Clinically relevant results were returned to participants for management. For the family with a history of MH, we used standard linkage methods, typing short tandem repeat polymorphism markers and polymerase chain reaction amplification and Sanger sequencing of exons not covered by exome data.

Results

The sequencing coverage (defined as the number of coding base-pairs with quality calls/total number of targeted base-pairs) of the coding exons was 83% (*RYR1*) and 93% (*CACNA1S*) (fig. 1), and there exists an inherent risk of false negatives. Sequence coverage is dependent on many factors including DNA quality, capture efficiency, percent GC (guanine-cytosine) content, and repeat elements. Our average depth of coverage in the target region for each sample was 89x.

One *CACNA1S* variant was a false positive, recognized by its marginal most probable genotype score and confirmed by manual review of sequence reads. We identified 123 total variants, 70 in *RYR1* and 53 in *CACNA1S*, among 870 exomes. These variants were identified in 1–419 participants, each. Seventeen of the 122 variants (7 *RYR1* and 11 *CACNA1S*) were excluded because they were too common (fig. 2, and see the *RYR1* and *CACNA1S* variant tables Supplemental Digital Content 1, http://links.lww.com/ALN/A976, and Supplemental Digital Content 2, http://links.lww.com/ALN/A977). The National Heart, Lung, and Blood Institute, Exome Variant Server††† frequency threshold was set to 0.5% as this was approximately 10-fold higher than the higher end of the MHS prevalence estimate,[1] and the ClinSeq® frequency was set to 1% because it includes approximately 1/10 as many exomes as the Exome Variant Server and therefore chance variation could inadvertently exclude a variant. Our focus was on the identification of highly penetrant alleles that cause autosomal dominant MHS, though we did detect some recessive myopathy alleles.

The remaining 104 variants (63 in *RYR1* and 41 in *CACNA1S*) were considered rare variants. Seventeen of the 63 *RYR1* rare variants were listed in the HGMD as "disease-causing" for either MHS, central core disease, multiminicore disease, atypical periodic paralysis, or congenital myopathy. Three of the 63 *RYR1* variants were not present in HGMD but were listed in the LSDB as pathogenic. One of the 41 CACNA1S variants (p.Thr1354Ser) was listed in HGMD as pathogenic for MHS, and one (p.Arg498His) was listed in the LSDB as pathogenic but without any supporting evidence. Of the 20 *RYR1* variants (present in HGMD or LSDBs, and with an allele frequency <1%), only four met our criteria (table 1) for class 5 pathogenicity; the remaining 16 were scored as a 3 (variants of unknown significance) or class 2 (likely not pathogenic).

Four class 5 *RYR1* variants were identified in 870 exomes. The p.Arg614Cys variant was found in one participant and listed in HGMD as pathogenic based on three publications[20–22] and reported 37 times in LOVD. All the submitting authors of these entries had concluded that it was pathogenic. This p.Arg614Cys variant is one of the 31 RYR1 mutations on the European Malignant Hyperthermia Group‡‡‡ list of pathogenic mutations and is also included in the 2002 North American MH consensus list of 17 causative mutations.[23] We designated this mutation as class 5, pathogenic. It is interesting to note that the 62-yr-old female participant with this variant had no family or personal history of MHS, despite having surgery with general anesthesia thrice.

The second class 5 RYR1 pathogenic variant, p.Arg2241X, was detected in two participants. It was described as pathogenic in HGMD, based on a single patient with congenital myopathy, episodes of generalized, atypical normokalemic paralysis, and multiminicore disease with external ophthalmoplegia and episodes of atypical periodic paralysis.[24] The molecular data in this published report were complex. The patient had, in addition to p.Arg2241X, p.Asp708Asn in cis and p.Arg2939Lys in *trans* to p.Arg2241X with apparent nonsense-mediated messenger RNA decay of the p.Arg2241X-bearing allele. In another study of 37 patients with dominant or recessive *RYR1*-related myopathies, the p.Arg2241X variant was described in three patients with recessive myopathies and ophthalmoparesis.[25] In two siblings, 7 and 5 yr old, the p.Arg2241X variant cooccurred with the previously described putatively pathogenic variant p.Arg109Trp,[26,27] and in a third patient the p.Arg2241X variant cooccurred with two missense variants, the putatively pathogenic p.Arg2939Lys[27] and p.Asp708Asn (these three variants were likely from the same patient reported in two case series by this same group).[24,27] The RYR1 variant p.Arg2241X was also categorized as a pathogenic recessive mutation in a patient with a congenital myopathy and muscle biopsy finding of an *RYR1*-related myopathy from a study

The Human Gene Mutation Database (HGMD), Professional version 2012.2 from BIOBASE. A database of germline mutations in genes associated with human inherited disease. Available at: www.hgmd.org. Accessed May 20, 2013.

*** Leiden Open Variation Database (LOVD), v.3.0. Available at: www.lovd.nl/3.0/home. Accessed May 20, 2013.

††† The National Heart, Lung, and Blood Institute's, Exome Sequencing Project, data browser. Available at: http://evs.gs.washington.edu/EVS/. Accessed May 20, 2013.

‡‡‡ The European Malignant Hyperthermia Group website. Available at: www.emhg.org/home. Accessed May 20, 2013.

Table 1. Variant Pathogenicity Classification System

Database Literature Designation	Novel (Not Published)		Published as Pathogenic		Published as VUS	Published as Benign
Mutation Type	Missense In-frame Insertion/Deletions	Nonsense Frameshift Splice	Missense	Nonsense Frameshift Splice	Any	Any
Class 5 (pathogenic)		Similar to disease-causing mutation and consistent family history	On EMHG list of 31 approved diagnostic (causative) mutations, and/or NAMH group's mutation panel, or two or more reports as pathogenic and no evidence against	On EMHG list of 31 approved diagnostic (causative) mutations, and/or NAMH group's mutation panel, **or** single report as pathogenic with supporting evidence		
Class 4 (likely pathogenic)		Similar to disease-causing mutation and inconsistent family history	Two or more reports as pathogenic with single evidence against, **or** single report as pathogenic with supporting evidence	Two or more reports as pathogenic with single evidence against, **or** single report as pathogenic without supporting evidence		
Class 3 (uncertain)	All novel missense or in-frame insertions/deletions without supporting publications	No similar disease-causing mutation reported as pathogenic, **or** inconsistent family history	Two or more reports as pathogenic with multiple evidence against, **or** single report as pathogenic without supporting evidence	Two or more reports as pathogenic with multiple evidence against, **or** single report as pathogenic with single evidence against	Reported as VUS (no convincing evidence that they have a causative effect, no evidence to support polymorphism), or single case reported as pathogenic	Single report as benign with insufficient supporting evidence
Class 2 (likely not pathogenic)			Single report as pathogenic with multiple evidence against		Some evidence to support as polymorphism, or multiple evidence against pathogenicity	On EMHG list of 156 nonpathogenic variants, **or** multiple cases reported as benign with insufficient evidence, **or** multiple report as benign with supporting evidence
Class 1 (not pathogenic)			ClinSeq®/NHLBI ESP Minor Allele Frequency ≥*1%/0.5%*			

Variant pathogenicity classification system, criteria for assignment of pathogenicity class 1–5 for MH gene variants. *RYR1* and *CACNA1S* variants were filtered for quality and frequency, and then assigned to pathogenicity classes based on data available in the HGMD, LSDBs, and family history, as well as from the EMHG's list of diagnostic and nonpathogenic variants and the NAMH mutation panel. Variants that did not pass quality filters were defined as class 0, variants that did not pass frequency filters were defined as class 1, all other variants were assessed according to the criteria presented in the table.

EMHG = European Malignant Hyperthermia Group; HGMD = Human Gene Mutation Database; LSDBs = locus-specific databases; MH = malignant hyperthermia; NAMH = North American Malignant Hyperthermia; NHLBI ESP = National Heart, Lung, and Blood Institutes, exome sequencing project; VUS = variant of unknown significance.

of 71 families with *RYR1* mutations.[28] The patient had two additional recessive pathogenic variants, p.Asp708Asn and p.Met485Val, and a synonymous variant of unknown significance c.11547G>A (p(=)). The p.Arg2241X variant was not detected in the Exome Variant Server. We categorized p.Arg2241X as class 5 because it was described in affected patients and of the category of variants (nonsense) strongly predictive of an autosomal recessive *RYR1*-related myopathy.

The third class 5 *RYR1* variant, c.5183C>T; p.Ser1728Phe, was listed in HGMD with references to two studies as pathogenic.[5,22] We found this variant in a 47-yr-old (Irish/British ancestry) female (1/1,740 alleles) without a personal or family history of MHS. The p.Ser1728Phe variant was reported in three independent families from an analysis of the U.K. MH patients.[5] In a subsequent genotype–phenotype correlation study, the p.Ser1728Phe variant was found in seven individuals and two families—six with a weaker *in vitro* contraction test phenotype compared with the known pathogenic p.Gly2434Arg mutation, suggesting a lesser effect on channel function as compared with their control.[22] Because the rare p.Ser1728Phe variant (1/10,757 alleles in the Exome Variant Server) was reported multiple times as pathogenic, with no evidence against, it was scored as a class 5, pathogenic variant.

The fourth class 5 *RYR1* variant, c.11958C>G; p.Asp3986Glu, was listed in HGMD with references to the same two U.K. studies cited above.[5,22] The variant was seen in five MH patients with MH disease status and associated with more severe static caffeine contractures and higher creatine kinase levels than the p.Gly2434Arg control or other variants. It was also identified in one 45-yr-old (Irish/German ancestry) male, ClinSeq® volunteer (1/1,740 alleles) with a history of MH. The volunteer had a history of multiple fulminant MH events—symptoms of myopathy, myotonia (dysphagia), proximal muscle weakness, and a positive *in vitro* contracture test and a serum creatine kinase value of 1,271 U/l and lactate dehydrogenase level of 238 U/l (see participant description table, Supplemental Digital Content 3, http://links.lww.com/ALN/A978, which is a table containing the characteristics of the ClinSeq® volunteers with *RYR1* variants). In addition, he had a family history of myotonia and positive *in vitro* contracture test in three siblings. This rare RYR1 variant, which resides in MHS mutational hotspot region III, was not found in more than 6,500 human exomes in Exome Variant Server. Since it has been reported multiple times as pathogenic with no evidence against pathogenicity, we concluded this was a class 5, pathogenic mutation.

Of the 20 rare *RYR1* variants (17 in HGMD and the LSDBs, 3 in LSDBs only) that were identified in ClinSeq®—10 were assigned to class 3, and 6 to class 2, based on the criteria in the pathogenicity table. The reasoning for these assignments is described in the Supplemental Digital Content 4, http://links.lww.com/ALN/A979, Supplemental Methods, which describes the variants—of less than class 5 pathogenicity—identified in ClinSeq® and in databases. The family, personal, medical, and surgical histories of all participants with *RYR1* and *CACNA1S* variants were reviewed; all but two were negative for MHS (see participant description tables, Supplemental Digital Content 3, http://links.lww.com/ALN/A978, and Supplemental Digital Content 5, http://links.lww.com/ALN/A980, containing the characteristics of the ClinSeq® volunteers with *RYR1* and *CACNA1S* variants, respectively).

One participant was found to have a novel RYR1 missense variant p.Arg3498Gly and a three-generation family history of MHS with an *in vitro* contracture test diagnostic for MHS.[29] To assess the potential pathogenicity of this variant, we performed a segregation analysis of the variant in the family. The variant did not segregate with the MHS phenotype (see Supplemental Digital Content 6, http://links.lww.com/ALN/A981, a pedigree of the MH family). We ruled out an error in phenotyping, after acquiring muscle biopsy and caffeine halothane contracture test results for seven of the family members from The North American Malignant Hyperthermia Registry.§§§ We next performed a candidate linkage analysis of the *RYR1* locus. Genotyping and manual haplotyping showed that an *RYR1* haplotype cosegregated with the phenotype, but this haplotype was in *trans* to p.Arg3498Gly. We concluded that p.Arg3498Gly was not pathogenic and hypothesized that this family most likely had MHS attributable to an undetected *RYR1* variant in trans to p.Arg3498Gly in the proband. We next evaluated the exon coverage of *RYR1* in this proband and found that he had 91.9% sequence coverage. We performed a Sanger sequencing of exons with poor exome sequence read-depth but found no mutations. We concluded that the ES of *RYR1* generated both a false-negative and a false-positive result in that the p.Arg3498Gly is not pathogenic and the participant likely has a mutation in *RYR1* not captured by ES or Sanger sequencing.

One of the 41 CACNA1S rare variants, p.Arg498His, identified in one exome, was listed in LOVD as pathogenic (it was not listed in HGMD). However, the pathogenicity of this entry was not supported by the primary literature, nor did LOVD provide details of the *CACNA1S*-associated phenotype. We contacted the LOVD curators and learned that the variant had been recategorized as "unknown pathogenicity," although the database itself had not been updated. We therefore categorized it as a variant of uncertain significance (score 3).

The CACNA1S variant p.Thr1354Ser was identified in 9 of 870 ClinSeq® exomes (minor allele frequency 0.7%) and in the Exome Variant Server with an allele count of 48 of 12,958 (minor allele frequency 0.4%). HGMD listed this variant as pathogenic, citing a publication showing segregation of p.Thr1354Ser in one family, its absence in 282

§§§ The North American Malignant Hyperthermia Registry of the Malignant Hyperthermia Association of the United States. Available at: www.mhaus.org/registry/#.USKCe-2TxsQ. Accessed May 20, 2013.

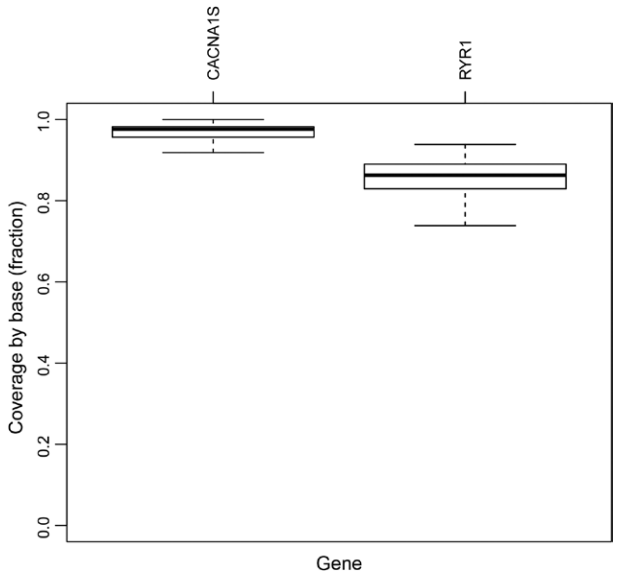

Fig. 1. Box and whisker plots showing base coverage for the *RYR1* and *CACNA1S* genes for a cohort of 870 probands. The *bottom and top lines* of the box represent the 1st and 3rd quartile, respectively; the *midline* represents the median value. The *bottom and top whiskers* represent the lowest and highest values within 1.5 times the interquartile range. Outliers have been excluded. Values on the y-axis are represented as fraction of total coding exonic bases for each gene.

controls, and functional data demonstrating abnormal Ca^{++} flux.[30] However, we concluded that this was more likely a benign variant in linkage disequilibrium with the (undetected) true pathogenic variant in the family described by Pirone *et al.*[30] Of the remaining 39 *CACNA1S* rare variants, none were present in either HGMD or the LSDBs. These variants were also assigned to class 3. None of these patients had a personal or family history of MHS (see Supplemental Digital Content 5, http://links.lww.com/ALN/A980, with the characteristics of the ClinSeq® volunteers with *CACNA1S* variants).

Discussion

Four examples of both the power and the limitations of ES for studying MHS were identified in this study. First, we detected a causative (class 5) RYR1 mutation, p.Arg614Cys, in a proband who had no clinical/phenotypic evidence of MHS and a negative family history (see participant description table, Supplemental Digital Content 3, http://links.lww.com/ALN/A978). The p.Arg614Cys variant was included in both the North American MHS and the European Malignant Hyperthermia Group causative mutation lists. We conclude that this represents a presymptomatic diagnosis of MHS in this participant, which is an example of the predictive, personalized genomic medicine in practice. We confirmed this variant in a clinical testing lab, returned it to the participant

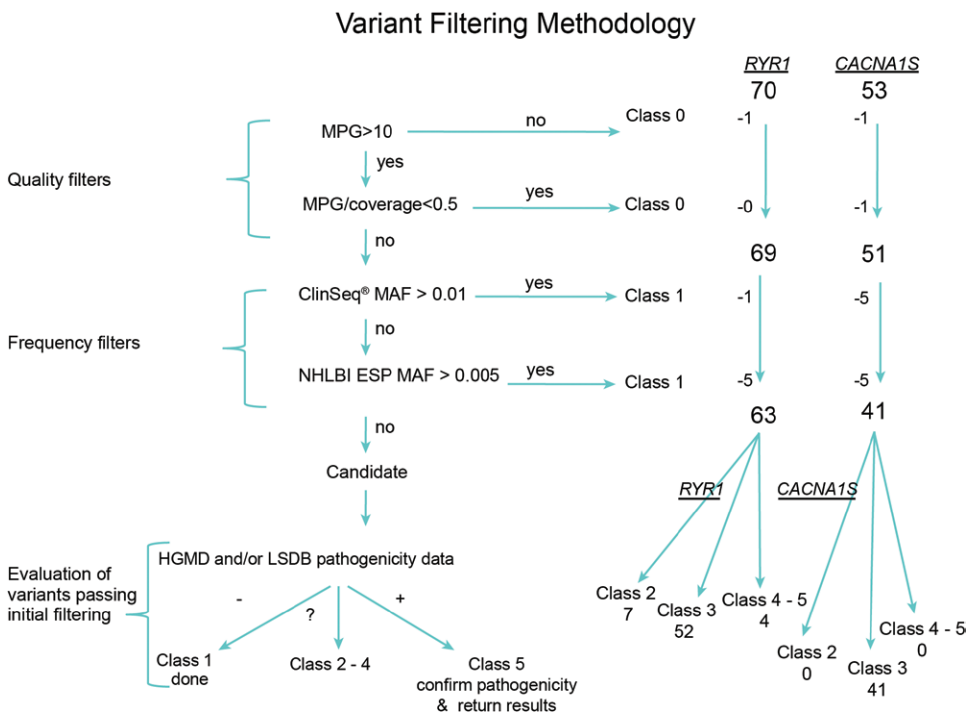

Fig. 2. Quality/frequency filter algorithm. Filtering criteria used for coding-variant interpretation. Variants were filtered on genotype quality, coverage, and allele frequencies. Variants removed by quality filters were classified as 0 and frequency filters as class 1, with the remaining assessed for pathogenicity (class 2–5) based on data present in the Human Gene Mutation Database (HGMD) and locus-specific databases (LSDBs). MAF = minor allele frequency; MPG = most probable genotype; NHLBI ESP = The National Heart, Lung, and Blood Institute, exome sequencing project.

with medical and genetic counseling, and referred her for consideration for caffeine halothane contracture test testing and enrollment in the Malignant Hyperthermia Association of the U.S. registry. Until such testing is performed on the patient or she has a reaction to a triggering agent, we cannot claim to have proven she has MHS. However, because this variant is listed in both the North American MHS and the European Malignant Hyperthermia Group causative mutation lists, we believe that it is extremely unlikely that this variant is benign solely because it was ascertained in context of this study design. Second, the p.Thr1354Ser CACNA1S variant, previously assumed to be pathogenic, was deemed likely to be class 3, that is, of uncertain pathogenicity. The frequency of this single variant was approximately 20 times higher than the frequency of MHS attributed to all loci and all mutations (0.74–1% p.Thr1354Ser heterozygotes). Although there are good functional data implicating this variant[30] in MHS, we believe that the population genetic data mandate that it should be scored class 3, of unknown pathogenicity. Our findings, supported by the Exome Variant Server *CACNA1S* allele frequencies, suggest that other previously implicated MHS variants may be benign. Caution is warranted regarding variants claimed to be causative for MHS, especially when used for predictive individualized medicine. Third, we found a novel RYR1 p.Arg3498Gly variant that was *not* pathogenic in an individual positive for MH by the caffeine halothane contracture test and a family history of MHS. The variant was rare but did not segregate with the phenotype, and this family most likely had MHS attributable to an undetected *RYR1* variant, or, less likely, a variant at another locus. We suggest that other previously reported rare *RYR1* variants without robust genetic data may

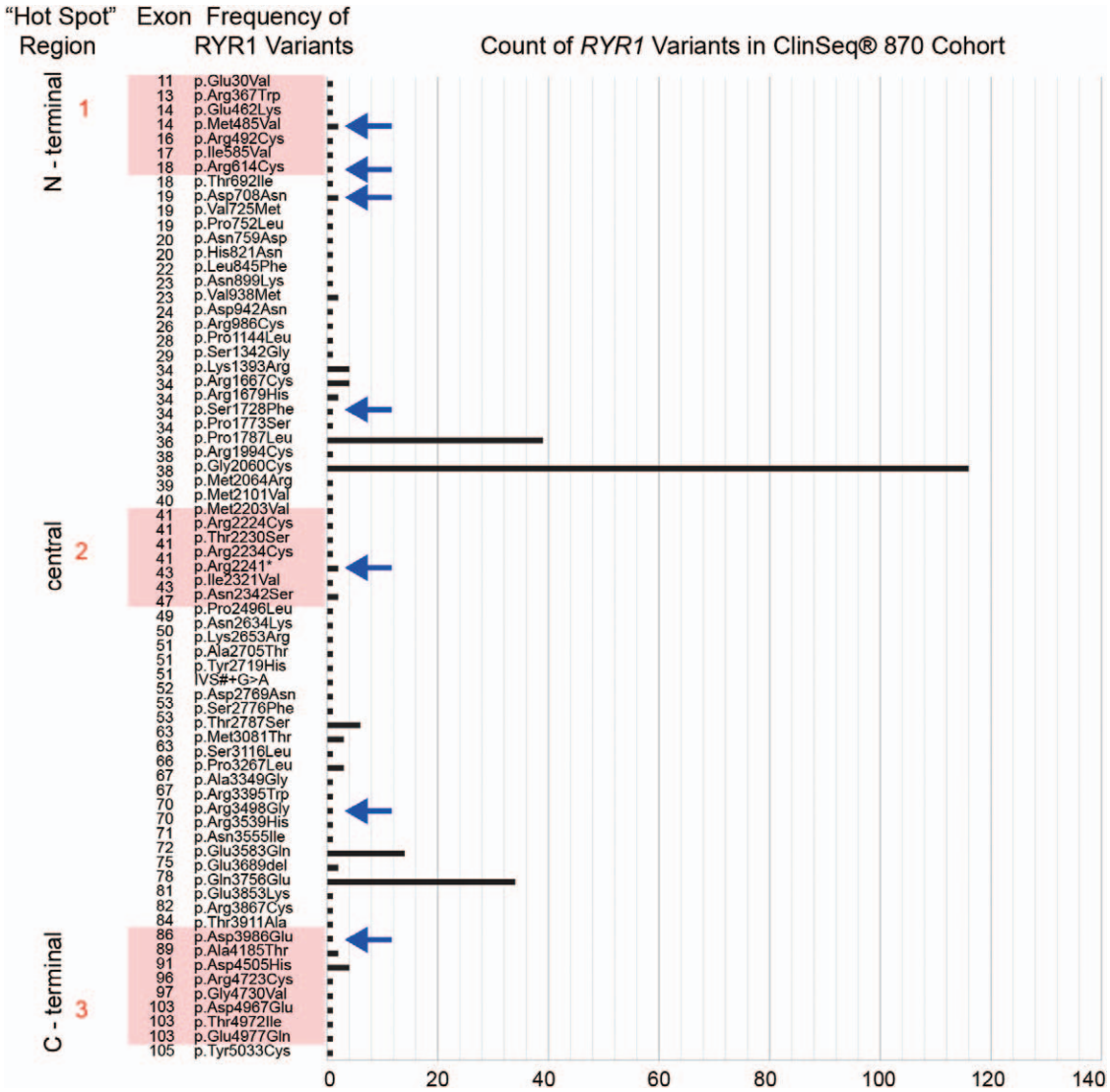

Fig. 3. Frequency histogram of *RYR1* variants. Frequency histogram of the 69 *RYR1* variants with predicted protein changes from the ClinSeq® 870 cohort. The three *RYR1* hotspot regions (Region 1/N-terminal, Region 2/central, and Region 3/C-terminal) are emphasized for purposes of orientation. *Blue arrows* in the figure point to variants referenced in the text. (ClinSeq® trademark held by National Institutes of Health, Bethesda, MD.)

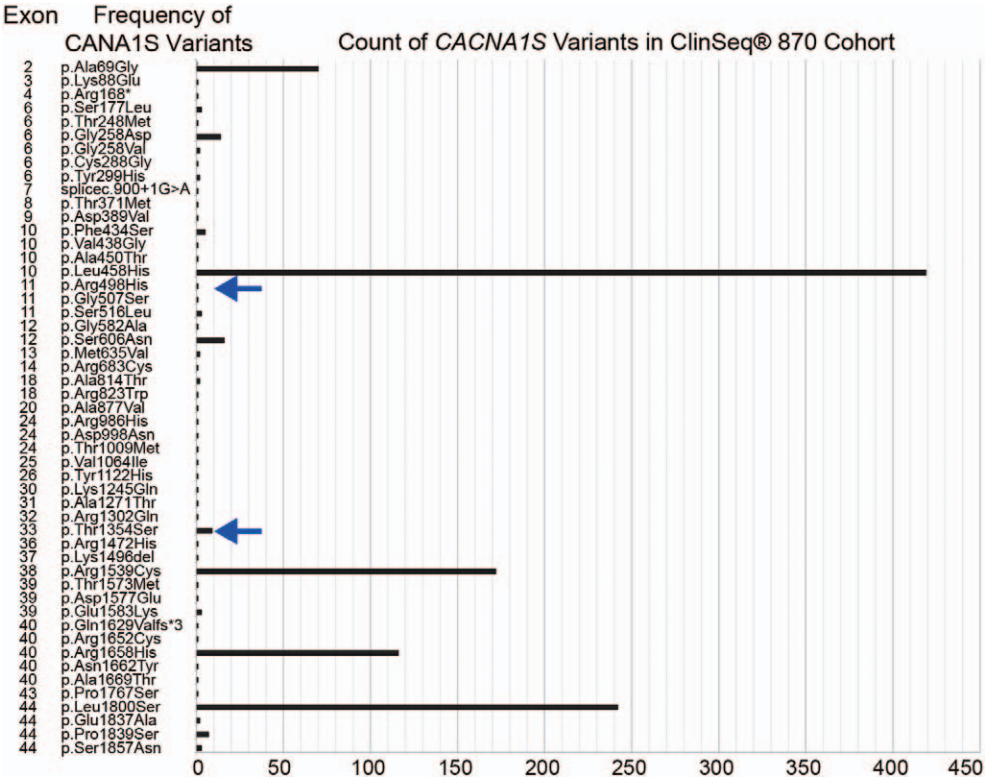

Fig. 4. Frequency histogram of *CACNA1S* variants. Frequency histogram of 51 *CACNA1S* variants with predicted protein changes from the ClinSeq® 870 cohort. *Blue arrows* in the figure point to variants referenced in the text. (ClinSeq® trademark held by National Institutes of Health, Bethesda, MD.)

have been misclassified as pathogenic. Fourth, we identified the class 5 variant, p.Arg2241X, which has been associated with phenotypes inherited in a recessive pattern, but recent publications have questioned the pathogenicity of this variant.[15,24–26,28] The risk of MHS in most recessive myopathies is uncertain, and has only been proven for central core disease.[31] This example shows that even when one can identify pathogenic variants, it can be challenging to associate them unequivocally with specific phenotypes.

Using ES, we identified 123 distinct variants (70 *RYR1* and 53 *CACNA1S*) among 870 participants (figs. 3 and 4). Our analyses yielded a spectrum of pathogenicity scores from benign to pathogenic (figs. 5 and 6). All but two of the *RYR1* variants classified as "disease causing mutations" in HGMD were reclassified by us as benign, probably benign, or variant of unknown significance, scores 1–3. We

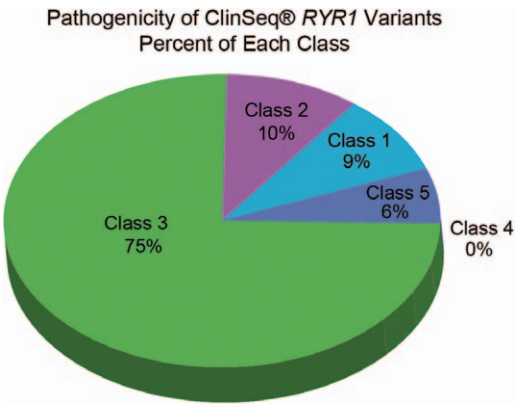

Fig. 5. Percentage of ClinSeq® *RYR1* variants, class 1–5. Proportion of 69 *RYR1* variants from the ClinSeq® 870 cohort in pathogenicity class 1 through 5. (ClinSeq® trademark held by National Institutes of Health, Bethesda, MD.)

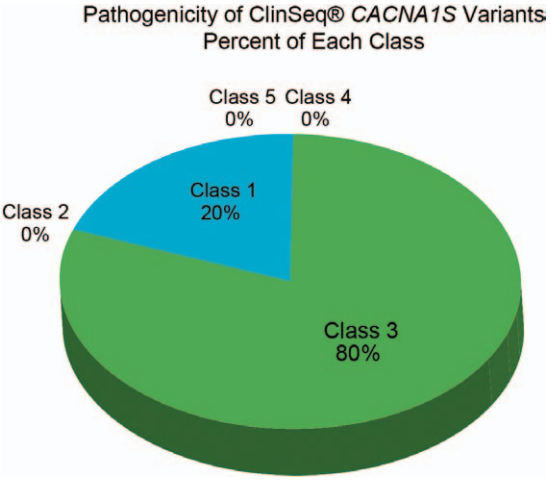

Fig. 6. Percentage of ClinSeq® *CACNA1S* variants, class 1–5. Proportion of 51 *CACNA1S* variants from the ClinSeq® 870 cohort in pathogenicity classes 1 through 5. (ClinSeq® trademark held by National Institutes of Health, Bethesda, MD.)

reclassified these variants based on the criteria in table 1, under the assumption that a variant was benign, unless a critical review of the data supported a higher pathogenicity category. It is critical to recognize that our assessment of "benign" or "probably benign" is limited to the specific context of using such a variant for individualized predictive medicine and that it is certainly not our intention for it to be interpreted to mean that the variant has no role in the pathogenicity of MHS, myopathy, or other phenotypes. In addition, more than half of the *RYR1* variants (43 of 69, 62%) we identified were not listed by HGMD or the LSDB databases, or in biomedical literature citations. Because we screened a cohort unselected for MHS, we predicted that most of the novel variants would be benign. More than half (40 of 69) of the *RYR1* variants were rare and not found in the Exome Variant Server. A fifth (10 of 51) of the *CACNA1S* variants were common polymorphisms, which we assigned to class 1 (benign), with the remaining assigned to class 3 (unknown; fig. 6). Four individuals (three males, one female) had more than one *RYR1* variant and two of the four participants with two *RYR1* variants had benign *CACNA1S* variants as well (see Supplemental Digital Content 3, http://links.lww.com/ALN/A978, and Supplemental Digital Content 5, http://links.lww.com/ALN/A980, containing the characteristics of the ClinSeq® volunteers with *RYR1* and *CACNA1S* variants).

The purpose of this study was to identify high-penetrance variants associated with MHS. As noted above, that we conclude a variant is class 1–3 does not automatically mean that the variant has no physiological effects. Moreover, the data did not allow us to evaluate whether interactions could have occurred among variants in a given individual,[5] although this should be specifically addressed in future studies. We deliberately set our threshold for pathogenicity high to avoid the error of wrongly diagnosing an individual as susceptible in an ascertainment mode where the previous probability that they are affected was low. The risk of false negatives in ES will diminish as future ES and follow-up studies generate additional data.

The filtering process for analysis of MHS variants from ES requires a manual method of evaluating variants to extract meaningful information. We used allele frequency, genotype–phenotype databases, and the primary literature to identify pathogenic variants. Unfortunately, there is at present no single information source that allows one to reliably ascertain whether a variant is benign or pathogenic. Many sequence databases (*e.g.*, the Exome Variant Server and The Single Nucleotide Polymorphism Database) include pathogenic, potentially pathogenic and nonpathogenic variants and do not include phenotype data. Furthermore, there is often no indication as to whether some individuals harbor multiple variants within a single gene, which limited our ability to evaluate these data. Our evaluation of 870 exomes using HGMD and LSDBs indicated likely significant levels of misclassification and variability in the pathogenicity determination not only in HGMD and the LSDBs, which is primarily attributable to the source literature.

Exome sequencing has some limitations: the method can miss pathogenic variants such as structural variation, or copy-number variants, in the genome—larger insertions and deletions, duplications, and inversions. Although technology has improved target coverage over the years, it will most likely never reach 100%. In view of the distribution of variants and the complexity of the genome, ES remains an efficient way to identify most mutations altering protein sequence in any single DNA sample. However, to our knowledge, to date the only genomic variants associated with MH are missense variants in coding exons, so most of these limitations do not pertain, given our current knowledge of the disorder.

The published prevalence of MHS mutations varies widely from 1 in 2,000[1,32] to 1 in 10,000[4] but the penetrance has been difficult to determine. Our study of 870 exomes, although it represents a prodigious amount of data, is still too small to estimate the prevalence of MHS. The ES of patients not ascertained for a personal or family history of MHS allows, in principle, an unbiased approach to genotype–phenotype correlation that has not been feasible with previous technologies. We conclude that some *RYR1* and *CACNA1S* variants may have been misclassified as pathogenic without adequate genetic (*e.g.*, cosegregation) or functional data. It is important to stress that in addition to robust genetic analysis, there is a critical need for a robust and noninvasive functional test for MHS, which together with genetic data could allow accurate determination of the prevalence and penetrance of this trait. Presently, ES cannot replace clinical investigations, but rather assists clinicians in determining which patients should undergo further genetic and/or functional analyses. This approach to variant identification in MHS should be extended to other cohorts undergoing ES, and may be useful as a first screening approach, before more invasive and time-consuming investigations. Analysis of thousands of exomes has the potential to provide the MHS field with an exhaustive catalog of variants to determine the true prevalence, penetrance, and expressivity of this life-threatening disorder. Although the assessment of the pathogenicity of both known and novel variants remains challenging, we demonstrate that causative mutations can be identified from ES data. These data suggest that clinically relevant mutations can be identified as incidental findings in exomes sequenced for clinical care and clinical research. This should inform the debate on the return of such secondary results to research participants. Furthermore, the application of ES technology to large and diverse cohorts has the potential to accelerate the pace of MHS gene mutation discovery. We speculate that the results of these studies will allow the development of clinical genomic screening for MHS, which should reduce the incidence of life-threatening events and increase life expectancy for those individuals who harbor pathogenic variants in these genes.

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Appendix. The NIH Intramural Sequencing Center Group (5625 Fishers Ln, Rockville, MD 20892-9400)

Center	
Director	Jim Mullikin, Ph.D.
Deputy director	Jim Thomas, Ph.D.
Sequencing group	
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Group deputy director	Alice Young, B.A.
Robotic specialist	Sean Lovett, B.Sc.
Library construction	
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Coleader	Richelle Legaspi, M.Sc.
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Leader	Michael Gregory, M.Sc.
Production technician	April Hargrove, B.Sc.
Production technician	Taccara Johnson, B.Sc.
Production technician	Nancy Riebow, B.Sc.
NextGen production	
Leader	Brian Schmidt, B.Sc.
Sequence finishing	
Leader	Jyoti Gupta, M.Sc.
Finishing technician	Betty Benjamin, B.Sc.
Finishing technician	Shelise Brooks, B.Sc.
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Staff bioinformatician	Meg Vemulapalli, M.Sc.

NIH = National Institutes of Health.

Appendix A: Variant Pathogenicity Classification System

Database Literature Designation	**Novel (Not Published)**		**Published as pathogenic**		**Published as VUS**	**Published as benign**
Mutation Type	**Missense In Frame Insertion/ Deletions**	**Nonsense Frameshift Splice**	**Missense**	**Nonsense Frameshift Splice**	**Any**	**Any**
Class 5	ClinSeq® / NHLBI EVS Mean Allele Frequency < 1%/ 0.5%					
(Pathogenic)		Similar to disease causing mutation and consistent family history	On EMHG list of 34 approved diagnostic (causative) mutations, and/or NAMH Group's mutation panel **OR** Two or more reports as pathogenic and no evidence against	On EMHG list of 34 approved diagnostic (causative) mutations, and/or NAMH Group's mutation panel **OR** Single report as pathogenic with supporting evidence		
Class 4 **(Likely pathogenic)**		Similar to disease causing mutation and inconsistent family history	Two or more reports as pathogenic with single evidence against **OR** Single report as pathogenic with supporting evidence	Two or more reports as pathogenic with single evidence against **OR** Single report as pathogenic without supporting evidence		
Class 3 **(Uncertain)**	All novel missense or in frame insertions/deletions without supporting publications	No similar disease causing mutation reported as pathogenic **OR** Inconsistent family history	Two or more reports as pathogenic with multiple evidence against **OR** Single report as pathogenic without supporting evidence	Two or more reports as pathogenic with multiple evidence against **OR** Single report as pathogenic with single evidence against	Reported as VUS (no convincing evidence they have a causative effect, no evidence to support polymorphism) **OR** single case reported as pathogenic	Single report as benign with insufficient supporting evidence

Class 2 **(Likely not pathogenic)**		Single report as pathogenic with multiple evidence against	Some evidence to support as polymorphism **OR** Multiple evidence against pathogenicity	On EMHG list of 156 nonpathogenic variants, **OR** Multiple cases reported as benign with insufficient evidence **OR** multiple report as benign with supporting evidence
Class 1 **(Not pathogenic)**	ClinSeq® / NHLBI EVS Mean Allele Frequency > 1%/ 0.5%			

Table 1. Variant Pathogenicity Classification System, Criteria for assignment of pathogenicity class 1 to 5 for MH gene variants *RYR1* and *CACNA1S* variants were filtered for quality and frequency, and then assigned to pathogenicity classes based on data available in the Human Gene Mutation Database (HGMD), locus specific databases (LSDBs) and family history, as well as from the European Malignant Hyperthermia Group's (EMHG) list of diagnostic and non-pathogenic variants and the North American Malignant Hyperthermia (NAMH) mutation panel. Variants that did not pass quality filters were defined as class 0, variants that did not pass frequency filters were defined as class 1, all other variants were assessed according to the criteria presented in the table. Additional abbreviations used: ESP, NHLBI Exome Sequencing Project. VUS, variant of uncertain significance.

Appendix B

RYR1 Variant Table

***RYR1* Variants in Transcript NM_000540.2 Identified in 870 ClinSeq® Exomes**							
Database Source	**Number of Variants Referenced**	**Total Variants**	***RYR1* Variant Pathogenicity Score**				
HGMD	20 (29% of total)						
Not in HGMD, only LSDB	6 (9% of total)		1	2	3	4	5
No listing	43 (62% of total)	69	6	7	52	0	4

Nucleotide Change	**Predicted Protein Change**	**Associated Disease State**	**ClinSeq® Allele Count**	**NHLBI EVS Allele Count**	**ClinSeq® Pathogenicity Score**
***RYR1* Variants Listed in HGMD (N=20)**					
c.1453A>G	p.Met485Val	Malignant Hyperthermia	2/1,740	1/10,757	2
c.1840C>T	p.Arg614Cys	Malignant Hyperthermia	1/1,740	NF	5^
c.2122G>A	p.Asp708Asn	Congenital Myopathy	2/1,696	1/10,753	3
c.2956C>T	p.Arg986Cys	Myopathy, congenital	1/1,530	4/10,746	3
c.4024A>G	p.Ser1342Gly	Malignant hyperthermia	1/146	386/9,608	1

***RYR1* Variants in Transcript NM_000540.2 Identified in 870 ClinSeq® Exomes**					
c.4178A>G	p.Lys1393Arg	Malignant Hyperthermia	4/1,202	47/10,703	2
c.5036G>A	p.Arg1679His	Malignant Hyperthermia	2/1,726	11/10,741	2
c.5183C>T	p.Ser1728Phe	Malignant hyperthermia	1/1,740	1/10,757	5
c.6721C>T	p.Arg2241X	Multi-minicore & Atypical Periodic Paralysis	2/1,740	NF	5
c.6961A>G	p.Ile2321Val	Malignant Hyperthermia	1/1,736	10/10,748	2^
c.7025A>G	p.Asn2342Ser	Malignant Hyperthermia	2/1,734	12/10,746	2
c.7487C>T	p.Pro2496Leu	Malignant hyperthermia	1/1,718	NF	3
c.8327C>T	p.Ser2776Phe	Possible association with Malignant Hyperthermia	1/1,724	10/10,746	2
c.8360C>G	p.Thr2787Ser	Multi-minicore Disease	6/1,716	113/10,645	1
c.9242T>C	p.Met3081Thr	Myopathy, congenital	3/1,738	23/10,735	3
c.10616G>A	p.Arg3539His	Central Core Disease	1/1,666	17/10,741	3
c.10747G>C	p.Glu3583Gln	Possible association with Progressive Axial Muscular Dystrophy	15/1,678	142/10,616	1

***RYR1* Variants in Transcript NM_000540.2 Identified in 870 ClinSeq® Exomes**					
c.10747G>C	p.Glu3853Lys	Muscular dystrophy and arthrogryposis	1/1,714	1/10,757	3
c.11958C>G	p.Asp3986Glu	Malignant hyperthermia	1/1,740	NF	5
c.13513G>C	p.Asp4505His	Possible association with Progressive Axial Myopathy with Cataracts	4/556	36/10,710	3
***RYR1* Variants Not in HGMD, Listed in Other LSDB Sources (N=6)**					
c.4999C>T	p.Arg1667Cys	-?/? (See 1)	4/1,740	21/10,727	3
c.5360C>T	p.Pro1787Leu	-?/?	40/1,704	174/10,578	1^
c.6178G>T	p.Gly2060Cys	-/?	120/1,740	548/10,210	1^
c.6301A>G	p.Met2101Val	NF	1/1,556	NF	3
c.11266C>G	p.Gln3756Glu	-?/?	34/1,728	123/10,635	1
c.12553G>A	p.Ala4185Thr	?/?	2/1,682	6/10,752	3
***RYR1* Variants Not in HGMD or LSDB (N=43)**					
c.89A>T	p.Glu30Val	N/A	1/886	4/10,730	3

RYR1 Variants in Transcript NM_000540.2 Identified in 870 ClinSeq® Exomes					
c.1099C>T	p.Arg367Trp	N/A	1/1,492	1/10,757	3
c.1384G>A	p.Glu462Lys	N/A	1/1,604	NF	3
c.1474C>T	p.Arg492Cys	N/A	1/1,740	NF	3
c.1753A>G	p.Ile585Val	N/A	1/1,740	NF	3
c.2075C>T	p.Thr692Ile	N/A	1/1,724	NF	3
c.2173G>A	p.Val725Met	N/A	1/1,716	NF	3
c.2255C>T	p.Pro752Leu	N/A	1/1,740	NF	3
c.2275A>G	p.Asn759Asp	N/A	1/1,740	2/10,756	3
c.2461C>A	p.His821Asn	N/A	1/1,740	NF	3
c.2533C>T	p.Leu845Phe	N/A	1/1,732	NF	3
c.2697C>A	p.Asn899Lys	N/A	1/1,720	NF	3
c.2812G>A	p.Val938Met	N/A	2/982	1/10,735	3

RYR1 Variants in Transcript NM_000540.2 Identified in 870 ClinSeq® Exomes					
c.2824G>A	p.Asp942Asn	N/A	1/1,040	NF	3
c.3431C>T	p.Pro1144Leu	N/A	1/1,740	NF	3
c.5317C>T	p.Pro1773Ser	N/A	1/1,740	NF	3
c.5980C>T	p.Arg1994Cys	N/A	1/1,736	1/10,757	3
c.6191T>G	p.Met2064Arg	N/A	1/1,740	1/10,757	3
c.6607A>G	p.Met2203Val	N/A	1/1,738	NF	3
c.6670C>T	p.Arg2224Cys	N/A	1/1,738	NF	3
c.6688A>T	p.Thr2230Ser	N/A	1/1,738	NF	3
c.6700C>T	p.Arg2234Cys	N/A	1/1,740	NF	3
c.7902C>A	p.Asn2634Lys	N/A	1/1,740	2/10,756	3
c.7958A>G	p.Lys2653Arg	N/A	1/1,642	NF	3
c.8113G>A	p.Ala2705Thr	N/A	1/1,740	NF	3

RYR1 Variants in Transcript NM_000540.2 Identified in 870 ClinSeq® Exomes					
c.8155T>C	p.Tyr2719His	N/A	1/1,740	NF	3
c.8231+1G>A	Splicing IVS51+G>A	N/A	1/1,740	NF	3
c.8305G>A	p.Asp2769Asn	N/A	1/1,740	NF	3
c.9347C>T	p.Ser3116Leu	N/A	1/1,738	NF	3
c.9800C>T	p.Pro3267Leu	N/A	3/1,664	NF	3
c.10046C>G	p.Ala3349Gly	N/A	1/1,700	NF	3
c.10183C>T	p.Arg3395Trp	N/A	1/978	NF	3
c.10492C>G	Arg3498Gly+	N/A	1/1,714	NF	2
c.10664A>T	p.Asn3555Ile	N/A	1/1,740	NF	3
c.11061_11063del	p.Glu3689del	N/A	2/1,056	NF*	3
c.11599C>T	p.Arg3867Cys	N/A	1/1,520	3/10,755	3
c.11731A>G	p.Thr3911Ala	N/A	1/1,740	NF	3

***RYR1* Variants in Transcript NM_000540.2 Identified in 870 ClinSeq® Exomes**					
c.14167C>T	p.Arg4723Cys	N/A	1/1,740	NF	3
c.14189G>T	p.Gly4730Val	N/A	1/1,740	NF	3
c.14901C>G	p.Asp4967Glu	N/A	1/1,740	NF	3
c.14915C>T	p.Thr4972Ile	N/A	1/1,740	NF	3
c.14929G>C	p.Glu4977Gln	N/A	1/1,740	NF	3
c.15098A>G	p.Tyr5033Cys	N/A	1/1,738	NF	3
Variant total: **Variant Type:** 1 stopgain SNV (p.Arg2241X) 1 splicing (IVS51G>A) 1 nonframeshift_substitution (p.Glu3689del) 66 Missense			=69	NF =40 (58% of total variants)	

Pathogenicity scores were determined as described in the Methods. HGMD =Human Gene Mutation Database (Professional 2012.2 from BIOBASE). LSDB =Locus Specific Database. LOVD =Leiden Open Variation Database (v.3.0). NHLBI EVS =The National Heart, Lung, and Blood Institutes, Exome Variant Server. NF =Not Found.

1 –These symbols are used in the LOVD variant pathogenicity rating, as reported on Leiden Muscular Dystrophy webpages. The first symbol indicates the conclusion of the publication cited in that publication entry. The second symbol indicates the conclusion of the database curator. Reported/Concluded; '+' indicating the variant is pathogenic, '+?' probably pathogenic, '-' no known pathogenicity, '-?' probably no pathogenicity, '?' effect unknown. **^ =** on European Malignant Hyperthermia Group's list of 34 approved diagnostic (causative) mutations, or 156 non-pathogenic variants (as of 01/2013). **+** = Proband with family history of MHS over 3 generations. * = The NHLBI exome variant server does not include any insertions or deletions.

Appendix C

CACNA1S Variant Table

***CACNA1S* Variants In Transcript NM_000069.2 Identified in 870 ClinSeq® Exomes**							
Database Source	**Number of Variants Referenced**	**Total Variants**	***CACNA1S* Variant Pathogenicity Score**				
HGMD	1 (2% of total)						
Not in LSDB, only LSDB	6 (12% of total)		1	2	3	4	5
Not listing	44 (86% of total)	51	10	--	41	--	--

Nucleotide Change	**Predicted Protein Change**	**Associated Disease State**	**ClinSeq® Allele Count**	**NHLBI EVS Allele Count**	**ClinSeq® Pathogenicity Score**
***CACNA1S* Variants Listed in HGMD (N=1)**					
c.4060A>T	p.Thr1354Ser	Malignant Hyperthermia	9/1,740	48/12,958	3
***CACNA1S* Variants Not in HGMD, Listed in Other LSDB Sources (N=6)**					
c.206C>G	p.Ala69Gly	PMID 19825159; benign	70/1,738	510/12,496	1
c.773G>A	p.Gly258Asp	PMID 19825159; benign	14/1,710	101/12,903	1
c.1373T>A	p.Leu458His	PMID 19825159; benign	518/1,732	3,572/9,434	1

***CACNA1S* Variants In Transcript NM_000069.2 Identified in 870 ClinSeq® Exomes**					
c.1493G>A	p.Arg498His	+/+ (See1)	1/1,740	NF	3
c.1817G>A	p.Ser606Asn	PMID 19825159; benign	16/1,740	113/12,893	1
c.5399T>C	p.Leu1800Ser	PMID 12636044; benign and PMID 20010423; VUS	266/1,724	3,509/9,497	1
***CACNA1S* Variants Not Listed in HGMD or LSDB (N=44)**					
c.900+1G>A	*Splice-site*	N/A	1/1,682	NF	3
c.262A>G	p.Lys88Glu	N/A	1/1,740	3/13,003	3
c.502C>T	p.Arg168*	N/A	1/1,584	NF	3
c.530C>T	p.Ser177Leu	N/A	3/1,444	7/12,999	3
c.743C>T	p.Thr248Met	N/A	1/1,652	NF	3
c.773G>T	p.Gly258Val	N/A	2/1,710	NF	3
c.862T>G	p.Cys288Gly	N/A	1/1,728	NF	3
c.895T>C	p.Tyr299His	N/A	2/1,700	91/12,915	1

CACNA1S Variants In Transcript NM_000069.2 Identified in 870 ClinSeq® Exomes					
c.1112C>T	p.Thr371Met	N/A	1/1,740	3/13,003	3
c.1166A>T	p.Asp389Val	N/A	1/1,740	4/13,002	3
c.1301T>C	p.Phe434Ser	N/A	5/1,740	8/12,998	3
c.1313T>G	p.Val438Gly	N/A	1/1,650	NF	3
c.1348G>A	p.Ala450Thr	N/A	1/1,740	1/13,005	3
c.1519G>A	p.Gly507Ser	N/A	1/1,740	NF	3
c.1547C>T	p.Ser516Leu	N/A	3/1,738	8/12,998	3
c.1745G>C	p.Gly582Ala	N/A	1/1,740	NF	3
c.1903A>G	p.Met635Val	N/A	2/1,740	4/13,002	3
c.2047C>T	p.Arg683Cys	N/A	1/1,738	127/12,879	1
c.2440G>A	p.Ala814Thr	N/A	2/656	20/12,976	3
c.2467C>T	p.Arg823Trp	N/A	1/852	NF	3
c.2630C>T	p.Ala877Val	N/A	1/1,556	1/13,005	3

CACNA1S Variants In Transcript NM_000069.2 Identified in 870 ClinSeq® Exomes					
c.2957G>A	p.Arg986His	N/A	1/1,694	4/13,002	3
c.2992G>A	p.Asp998Asn	N/A	1/1,728	17/12,989	3
c.3026C>T	p.Thr1009Met	N/A	1/1,730	2/3,004	3
c.3190G>A	p.Val1064Ile	N/A	1/1,740	2/13,004	3
c.3364T>C	p.Tyr1122His	N/A	1/1,740	NF	3
c.3733A>C	p.Lys1245Gln	N/A	1/1,736	NF	3
c.3811G>A	p.Ala1271Thr	N/A	1/1,608	93/12,913	1
c.3905G>A	p.Arg1302Gln	N/A	1/1,740	1/13,005	3
c.4415G>A	p.Arg1472His	N/A	1/1,726	NF	3
c.4483_4485del	p.Lys1496del	N/A	1/1,740	NF	3
c.4615C>T	p.Arg1539Cys	N/A	184/1,740	1,161/11,845	1
c.4718C>T	p.Thr1573Met	N/A	1/1,740	1/13,005	3

***CACNA1S* Variants In Transcript NM_000069.2 Identified in 870 ClinSeq® Exomes**					
c.4731C>G	p.Asp1577Glu	N/A	1/1,740	1/13,005	3
c.4747G>A	p.Glu1583Lys	N/A	3/1,740	4/13,002	3
c.4885_4886del	p.Gln1629Valfs*3	N/A	1/1,728	NF*	3
c.4954C>T	p.Arg1652Cys	N/A	1/1,734	5/13,001	3
c.4973G>A	p.Arg1658His	N/A	123/1,726	1,572/11,434	1
c.4984A>T	p.Asn1662Tyr	N/A	1/1,720	NF	3
c.5005G>A	p.Ala1669Thr	N/A	1/1,678	NF	3
c.5299C>T	p.Pro1767Ser	N/A	1/1,628	NF	3
c.5510A>C	p.Glu1837Ala	N/A	2/1,740	NF	3
c.5515C>T	p.Pro1839Ser	N/A	7/1,740	33/12,973	3
c.5570G>A	p.Ser1857Asn	N/A	3/1,740	33/12,973	3
Variant Type: 1 frameshift deletion 1 nonframeshift substitution			**Variant total**:	= 51	NF = 20

CACNA1S Variants In Transcript NM_000069.2 Identified in 870 ClinSeq® Exomes		
1 splicing 48 missense		

Pathogenicity scores were determined as described in the Methods. HGMD = Human Gene Mutation Database (Professional 2012.2 from BIOBASE). LSDB = Locus Specific Database. LOVD = Leiden Open Variation Database (v.3.0). NHLBI EVS = The National Heart, Lung, and Blood Institutes, Exome Variant Server. NF = Not Found. PMID = PubMed unique identifier.

1 –These symbols are used in the LOVD variant pathogenicity rating, as reported on Leiden Muscular Dystrophy webpages. The first symbol indicates the conclusion of the publication cited in that publication entry. The second symbol indicates the conclusion of the database curator. Reported/Concluded; '+' indicating the variant is pathogenic, '+?' probably pathogenic, '-' no known pathogenicity, '-?' probably no pathogenicity, '?' effect unknown. ^ = on European Malignant Hyperthermia Group's list of 30 approved diagnostic (causative) mutations, or 158 non-pathogenic variants (as of 01/2013). * = Insertion and deletion variants are not included in the NHLBI exome variant server.

Appendix D

RYR1 Participant Description Table

Characteristics of ClinSeq® Volunteers with *RYR1* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
c.89A>T;p.Glu30Val	135473	C/N/63/F	Negative	103*	166	Intermittent hypokalemia (on K+ supplements)
c.1099C>T;p.Arg367Trp	190330	C/N/58/F	Negative	76	174	Psoriasis
c.1384G>A;p.Glu462Lys	145545	A/N/57/M	Negative	214*	180	Surgeries: CABG
c.1453A>G;p.Met485Val	134364	C/N/57/M	Negative	69	146	Negative
	144294	C/N/59/F	Negative	36	147	Negative
c.1474C>T;p.Arg492Cys	177065	A/N/65/M	Negative	80*	122	Surgeries: CABG 50 y
c.1753A>G;p.Ile585Val	157045	C/N/61/F	Negative	81*	123	Surgeries: cardiac stenting 59 y, mastectomy and breast implants 30 y. Alcohol consumption: self-reports >4 glasses of wine daily
c.1840C>T:p.Arg614Cys	131685	C/N/59/F	No proximal muscle weakness in family, no complications from anesthesia in any family member.	194^	194	Surgeries: (1) nasal turbinates, (2) left thigh mass removal, and (3) cholecystectomy 58 y. Scarlet fever (5 y)
c.2075C>T:p.Thr692Ile	*117126*★	C/N/63/M	Negative	143	126	Negative
c.2122G>A:p.Asp708Asn	*181028*+	C/N/63/F	Negative	50*^	148	Negative
	115542	C/N/61/M	Negative	53	---	Colonoscopy
c.2173G>A:p.Val725Met	163600	C/N/58/M	Negative	103*	174	Cardiac catheterization 57y
c.2255C>T:p.Pro752Leu	107068	C/N/50/M	Negative	182	162	Negative
c.2275A>G:p.Asn759Asp	118968	C/N/58/M	Negative	363	188	Surgeries: prostate (TURP)

Characteristics of ClinSeq® Volunteers with *RYR1* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
						52y. Colonoscopy 57 y. Rheumatic fever 7 y, Hypothyroidism on THR
c.2461C>A;p.His821Asn	194655	C/N/63/F	Negative	29	151	Surgeries: cholecystectomy 40s; Statin intolerance
c.2533C>T:p.Leu845Phe	189882	C/N/56/M	Negative	***727****	207	Surgeries: cardiac stenting 56 y
c.2697C>A;p.Asn899Lys	*132267*+	C/N/48/M	Negative	279*	189	Negative
c.2812G>A:p.Val938Met	159946	AA/N/56/M	Negative	***487****	175	Negative
	142923	AA/N/59/F	Negative	60	124	Hypothyroidism on THR
c.2824G>A:p.Asp942Asn	132306	C/N/57/M	Negative	60	137	Negative
c.2956C>T;p.Arg986Cys	154982	C/N/58/F	Negative	48	137	Hypothyroidism on THR
c.3431C>T:p.Pro1144Leu	128062	A/N/63/M	Negative	79*	129	Surgeries: CABG 61 y, then cardiac stenting 61 y
c.4024A>G;p.Ser1342Gly	121670	O/N/55/F	Negative	196	184	Surgeries: total abdominal hysterectomy, unilateral salpingo-oophorectomy
c.4178A>G:p.Lys1393Arg	106963	C/N/55/M	Negative	37	144	Colonoscopies x2 since 40s. Gilbert disease
	115065+★	C/N/59/M	Negative	120	179	Negative
	185991	C/N/65/M	Negative	332	153	Hypothyroidism on THR Colonoscopies
	136706	C/N/52/F	Negative	77^	111	Negative
c.4999C>T:P.Arg1667Cys	112443	C/N/54/F	Negative	63	179	Surgeries: thyroidectomy 46 y, total abdominal hysterectomy 45 y. Thyroid cancer 46 y.
	193390	C/N/59/F	Negative	92	140	Hypothyroidism on THR Negative
	167185	C/N/63/F	Negative	149*	132	Ehlers-Danlos Syndrome Surgeries: umbilical hernia repair 8 y, cholecystectomy

Characteristics of ClinSeq® Volunteers with *RYR1* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
						42 y, right inguinal hernia repair and total abdominal hysterectomy 48 y, repeat bladder resuspension and right inguinal hernia repair 49 y, left inguinal hernia repair 53 y
	152415	C/N/57/F	Negative	86	199	Negative
c.5036G>A:p.Arg1679His	106945	C/N/58/M	Negative	***516****	176	Hypothyroidism on THR; Alcohol over consumption: self-reports >3 glasses of wine daily
	148840	C/N/55/M	Negative	143	187	Negative
c.5183C>T;p.Ser1728Phe	134994	C/N/47/F	Negative	97	149	Negative
c.5317C>T;p.Pro1773Ser	191186	A/N/46/F	Negative	160	175	Surgeries: total abdominal hysterectomy 43 y
c.5360C>T:p.Pro1787Leu	N =39					
c.5980C>T;p.Arg1994Cys	155247	C/N/48/M	Negative	130	152	Negative
c.6178G>T:p.Gly2060Cys	N =116					
c.6191T>G:p.Met2064Arg	195344	C/N/60/F	Negative	128	146	Surgeries: cholecystectomy.
c.6301A>Gp.Met2101Val	118954	C/N/45/F	Negative	***288***	177	Hyperuricemia (8.9 mg/dL) Colonoscopies x3 starting 35 y
c.6607A>G;p.Met2203Val	182262	C/N/56/M	Negative	248*	160	Surgeries: right radical nephrectomy 55 y
c.6670C>T;p.Arg2224Cys	151637	C/N/57/F	Negative	70	135	Negative
c.6688A>T:p.Thr2230Ser	*115065*+★	C/N/59/M	Negative	120	179	Negative
c.6700C>T:p.Arg2234Cys	116576	C/N/51/F	Negative	73	145	Surgeries: total abdominal hysterectomy 49 y Colonoscopy
c.6721C>T:p.Arg2241X	154209	C/N/58/F	Negative	58*	190	Negative

Characteristics of ClinSeq® Volunteers with *RYR1* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
	181028+	C/N/63/F	Negative	50*^	148	Negative
c.6961A>G:p.Ile2321Val	100288	C/N/58/M	Negative	108*^	167	Surgeries: cardiac stenting 48 y
c.7025A>G:p.Asn2342Ser	187842	C/N/52/M	Adopted	170	152	Negative
	106838	C/N/45/M	Negative	181	208	Negative
c.7487C>T;p.Pro2496Leu	*132267*+	C/N/48/M	Negative	279*	189	Negative
c.7902C>A;p.Asn2634Lys	135629	C/N/62/M	Negative	82	106	Negative
c.7958A>G:p.Lys2653Arg	114689	C/N/56/F	Negative	72^	140	Colonoscopy
c.8113G>A;p.Ala2705Thr	120721	C/N/63/F	Negative	82^	141	Subclinical hypothyroidism (TSH 4.16 mcIU/mL / FT4 1.6 ng/dL)
c.8155T>C:p.Tyr2719His	190433	--/HL/59/M	Negative	142	127	Negative
c.8305G>A:p.Asp2769Asn	195358	A/N/61/F	Negative	48*	161	Negative
c.8327C>T:p.Ser2776Phe	104866	C/N/58/M	Negative	106	164	Negative
c.8360C>G:p.Thr2787Ser	N =6					
c.9242T>C;p.Met3081Thr	*177243*★	AA/N/58/F	Negative	247	223	Negative
	179265★	AA/N/52/F	Negative	143	161	Negative
	110716	AA/N/64/F	Negative	143	154	Negative
c.9347C>T:p.Ser3116Leu	139926	C/N/65/F	Negative	66	151	Myositis second to autoimmune disease. Surgeries: cerebral aneurysm; repair total abdominal hysterectomy, unilateral salpingo-oophorectomy 55 y
c.9800C>T:p.Pro3267Leu	119835	C/N/61/F	Negative	102*	171	Negative
	195394	C/N/60/M	Negative	90*	145	Surgeries: cardiac stenting 59 y
	169830	C/N/53/M	Negative	119	143	Negative
c.10046C>G:p.Ala3349Gly	100818	C/N/54/F	Negative	93*	160	Negative

Characteristics of ClinSeq® Volunteers with *RYR1* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
c.10183C>T:p.Arg3395Trp	132356	C/N/50/F	Negative	107	162	Negative
c.10492C>G:p.Arg3498Gly	133237	C/N/51/M	3 Gen History of MH (MH events, (+) CHCT	151	138	MHS, (+) CHCT
c.10616G>A:p.Arg3539His	178697	C/N/61/F	Negative	85	164	Negative
c.10664A>T:p.Asn3555Ile	117201	A/N/45/M	Negative	159	129	Colonoscopy
c.10747G>C:p.Glu3583Gln	N =14					
c.11061_11063del:p.Glu3689del	108006	C/N/65/F	Negative	148*	180	Surgeries: colectomy 62 y. Colonoscopies
	162256	C/N/57/M	Negative	94*	159	Surgeries: cardiac stenting 57 y
c.11266C>G:p.Gln3756Glu	N =34					
c.10747G>C;p.Glu3853Lys	179251	C/N/60/F	Negative	149^	169	Hypothyroidism on THR (TSH 6.1 mcIU/mL / FT4 1.2 ng/dL)
c.11599C>T:p.Arg3867Cys	101329	C/N/60/M	Negative	121	189	Negative
c.11731A>G:p.Thr3911Ala	164979	C/N/47/ M	Negative	152	135	Hypothyroidism on THR
c.11958C>G;p.Asp3986Glu	194459	C/N/45/M	Proband with history of fulminant MH events and (+) CHCT; 3 siblings with myotonia	***1,271***	***238***	MHS, (+) CHCT Myopathy - myotonia with dysphagia and proximal muscle weakness; 3 fulminant MH events: 1) after surgery 1972, 2) after exercise in 2004 and 3) 2011 with rhabdomyolysis. Current medications: Dantrolene sodium, 100mg, daily in AM, by mouth; Morphine control release, 15 mg, 2 times a day, by mouth

Characteristics of ClinSeq® Volunteers with *RYR1* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
c.12553G>A:p.Ala4185Thr	104905	C/N/52/F	Negative	51	128	Negative
	140929	C/N/56/M	Negative	***702***	200	Negative
c.13513G>C:p.Asp4505His	102488	C/N/63/M	Negative	111	132	Negative
	137940	C/N/51/M	Negative	186*	160	Negative
	174073	C/N/62/M	Negative	96*	202	Surgeries: inguinal hernia repair 41 y; nephrectomy - benign tumor 40s; cardiac stenting x2 (52 y and 62 y) Colonoscopies x3
	119120	C/N/52/F	Negative	100	143	Negative
c.14167C>T:p.Arg4723Cys	100199	C/N/60/M	Negative	175	165	Negative
c.14189G>T;p.Gly4730Val	160480	C/N/53/F	Negative	54	138	Negative
c.14901C>G:p.Asp4967Glu	*142361*+★	C/N/63/M	Negative	84	131	Surgeries: appendectomy 10 y
c.14915C>T:p.Thr4972Ile	*142361*+★	"	"	"	"	"
c.14929G>C:p.Glu4977Gln	116259	C/N/52/M	Negative	208	134	Negative
c.15098A>G:p.Tyr5033Cys	157401	C/N/60/F	Negative	149	160	Negative
			Positive Family History =2			

Participant information for *RYR1* variants identified in the 870 Clinseq® cohort. Medical histories were reviewed for surgeries/ procedures and clinical conditions indicative of myopathy or that elevate serum creatine kinase (CK). Participants were evaluated for: any myopathy, MHS, and/or exertional heat illness; history of elevated CK; hypothyroidism and/or elevated thyroid stimulating hormone (TSH) > 4.00 mcIU/mL; hypokalemia <3.3 mmol/L; hyperuricemia > 8.6 mg/dL (uric acid normal range: 3.7-8.6 mg/dL); Statin and Nicotinic Acid use; self-report alcohol use (≥3 bottles/glasses/shots per days); autoimmune conditions: rheumatoid arthritis, psoriasis, lupus, Sjogen's syndrome, scleroderma; and renal disease. Participants race and ethnicity were determined by self report.

Age = age at enrollment. CABG = coronary artery bypass graft. CHCT = caffeine halothane contracture test. CK = creatine kinase, range: Female = 38-252 U/L; Male = 52-386 U/L. Ethnicity: N =Not Hispanic or Latino; HL =Hispanic or Latino. LDH =lactate dehydrogenase, range = 113-226 U/L. K+, potassium, range = 3.3-5.1 mmol/L. MHS = Malignant Hyperthermia Susceptible. Nucleotide change = NM_000540.2. Race: C = Caucasian, A = Asian, AA = African American, 0 = other, "—" = Not reported. THR = thyroid replacement hormone. TSH range: 0.40–4.00

mcIU/mL. Thyroxin, FT4 range = 0.9 - 1.5 ng/dL. TURP = transurethral resection of the prostate. "✚" = participant has a second *RYR1* variant. "★" = participant with *CANA1S* variant. "*" = on Statin. "∧" =elevated serum TSH level. "∨ " = low serum TSH level. "---" = sample hemolyzed.

Appendix E

CACNA1S Participant Description Table

Characteristics of ClinSeq® Volunteers with *CACNA1S* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
c.900+1G>A Splice-site	*142361*++	C/N/63/M	Negative	84	131	Surgeries: appendectomy 10 y
c.206C>G;p.Ala69Gly	N =70					
c.262A>G;p.Lys88Glu	102303	C/N/45/F	Negative	78	125	Negative
c.502C>T;p.Arg168*	197875	C/N/61/M	Negative	313	174	Colonoscopies x3
c.530C>T;p.Ser177Leu	102072	C/N/60/F	Negative	139^	149	Hypothyroidism on THR (TSH 6.31 mcIU/mL / FT4 1.7 ng/dL)
	155322	C/N/65/F	Negative	111	150	Colonoscopies x2
	198019	C/N/60/F	Negative	52	149	Surgeries: total abdominal hysterectomy, bilateral salpingo-oophorectomy 55 y Hypothyroidism on THR
c.743C>T;p.Thr248Met	146006	C/N/58/F	Negative	84*	180	Surgeries: total abdominal hysterectomy 52 y Hypothyroidism on THR
c.773G>T;p.Gly258Val	148573	C/N/61/M	Negative	153	140	Hypothyroidism on THR
	150635	C/N/45/F	Negative	48	159	Alcohol over consumption: self-reports >3 glasses of wine daily
c.773G>A;p.Gly258Asp	N =14					
c.862T>G;p.Cys288Gly	100395	C/N/60/F	Negative	79	130	Negative
c.895T>C;p.Tyr299His	*177243*+	AA/N/58/F	Negative	247	223	Negative
	179265+	AA/N/52/F	Negative	143	161	Negative
c.1112C>T;p.Thr371Met	180133	AA/N/58/F	Negative	108	161	Colonoscopy 56 y
c.1166A>T;p.Asp389Val	195059	C/N/59/F	Negative	74	147	Surgeries: unilateral salpingo-oophorectomy

Characteristics of ClinSeq® Volunteers with *CACNA1S* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
c.1301T>C;p.Phe434Ser	107143	C/N/62/M	Negative	264*	171	Negative
	122640	C/N/49/F	Negative	52	133	Duane syndrome
	115837	C/N/48/F	Negative	87*	121	Cardiac catheterizations 46 y, 48 y
	182995	C/N/53/F	Negative	63*	122	Colonoscopy 52 y
	130259	C/N/59/F	Negative	99	153	Negative
c.1313T>G;p.Val438Gly	126157	C/N/53/F	Negative	90	205	Surgeries: total abdominal hysterectomy, bilateral salpingo-oophorectomy 50 y Sarcoidosis
c.1348G>A;p.Ala450Thr	186925	C/N/54/F	Negative	65	124	Surgeries: mitral valve repair 54 y
c.1373T>A;p.Leu458His	N =419					
c.1493G>A;p.Arg498His	192035	C/N/56/M	Negative	83	174	Colonoscopies x2
c.1519G>A;p.Gly507Ser	190479	C/N/51/F	Negative	114	200	Hypothyroidism on THR
c.1547C>T;p.Ser516Leu	150738	C/N/61/F	Negative	56	164	Negative
	179902	C/N/53/M	Negative	68	188	Marfan Syndrome
	154722	C/N/60/F	Negative	113*	192	Hypothyroidism on THR (TSH 0.15 mcIU/mL / FT4 1.7 ng/dL)
c.1745G>C;p.Gly582Ala	100733	A/N/51/M	Negative	217*	182	Negative
c.1817G>A;p.Ser606Asn	N =16					
c.1903A>G;p.Met635Val	105733	C/N/61/M	Negative	70*	139	Surgeries: cardiac stenting 61 y
	184006	C/N/56/M	Negative	186	145	Negative
c.2047C>T;p.Arg683Cys	136532	AA/N/50/M	Negative	782*	145	Hyperuricemia 9.7 mg/dL
c.2440G>A;p.Ala814Thr	*117126*+	C/N/63/M	Negative	143	126	Negative
	165903	C/N/59/F	Negative	71*	177	Negative
c.2467C>T;p.Arg823Trp	107509	A/N/52/M	Negative	61*	115	Surgeries: cardiac stenting 52 y
c.2630C>T;p.Ala877Val	174649	C/N/56/M	Negative	135*	166	Surgeries: unilateral

Characteristics of ClinSeq® Volunteers with *CACNA1S* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
						nephrectomy 40s, cardiac stenting 56 y. Colonoscopy 52 y
c.2957G>A;p.Arg986His	132619	C/N/61/F	Negative	174^	177	Sjogren's syndrome types I & II. Hypothyroidism on THR (TSH 6.53 mcIU/mL / FT4 1.5 ng/dL)
c.2992G>A;p.Asp998Asn	185599	AA/N/48/F	Negative	99	114	Negative
c.3026C>T;p.Thr1009Met	131005	C/N/63/F	Negative	168	171	Negative
c.3190G>A;p.Val1064Ile	151708	C/N/55/M	Negative	396	166	Circumferential Pulmonary-Vein Ablation for Chronic Atrial Fibrillation 50s, colonoscopy
c.3364T>C;p.Tyr1122His	152465	A/N/63/M	Negative	59*	138	Negative
c.3733A>C;p.Lys1245Gln	179603	C/N/58/M	Negative	68*^	135/ 4.5	Surgeries: cardiac stenting. TSH =6.39 mcIU/mL/ FT4=1.1 ng/dL
c.3811G>A;p.Ala1271Thr	197544	AA/N/47/F	Negative	102	180	Negative
c.3905G>A;p.Arg1302Gln	142624	C/N/58/F	Negative	80*	168	Surgeries: knee replacement, total abdominal hysterectomy, bilateral salpingo-oophorectomy
c.4060A>T;p.Thr1354Ser	147218	C/N/48/M	Negative	375*^	205	TSH =4.3 mcIU/mL, FT4=1.3 ng/dL
	112126	C/N/61/F	Negative	61	143	Negative
	139980	C/N/61/M	Negative	314*	195	Negative
	117247	C/N/55/F	Negative	59*	146	Hypothyroidism on THR
	104923	C/N/63/F	Negative	100	207	Negative
	141060	C/N/61/F	Negative	93	190	Negative
	195817	C/N/62/M	Negative	132*	132	Surgeries: cardiac stent at 62 y. Colonoscopies x3

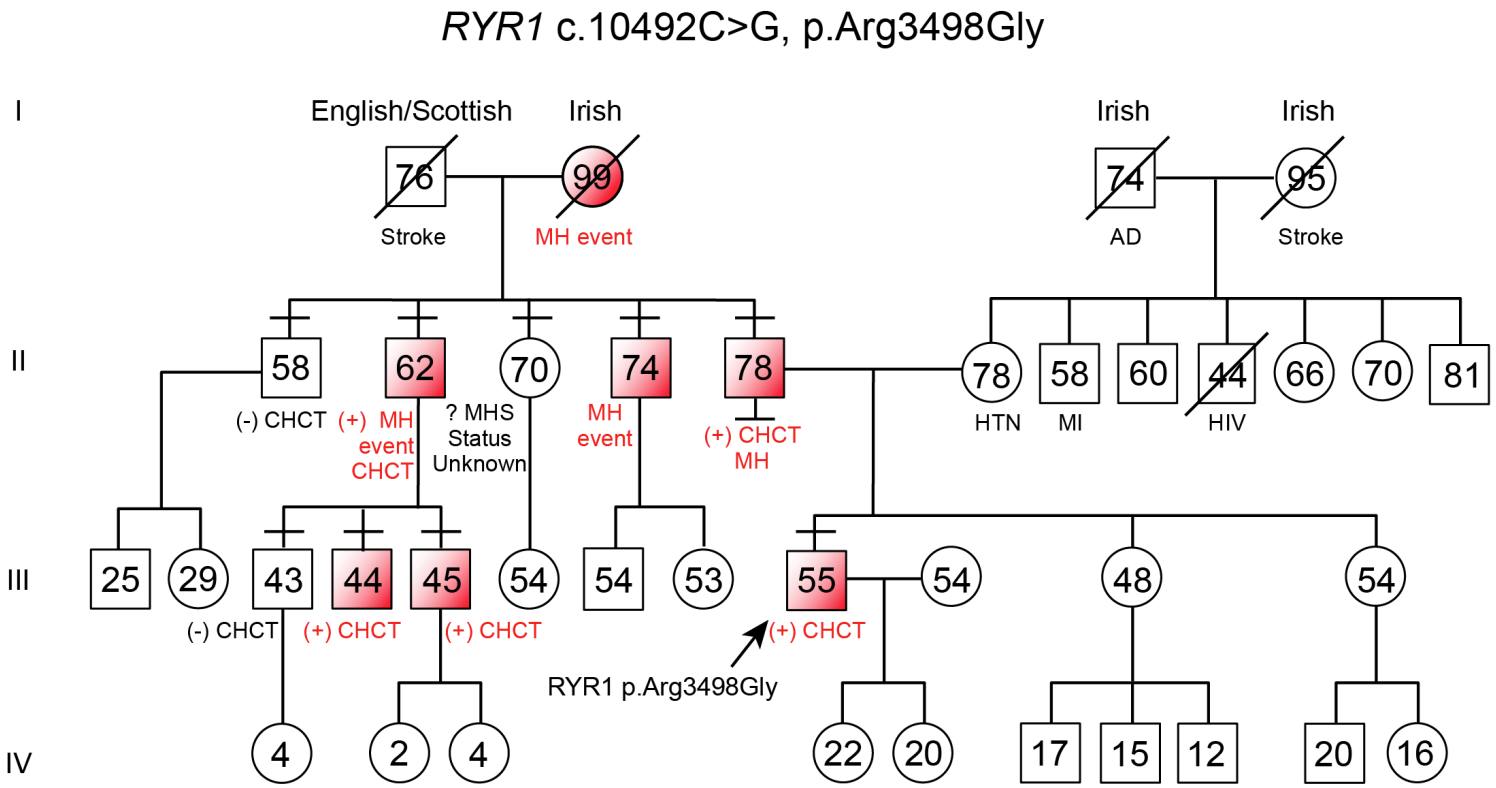
Characteristics of ClinSeq® Volunteers with *CACNA1S* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
	154231	C/N/58/M	Negative	107*	161	Negative
c.4415G>A;p.Arg1472His	159259	C/N/45/M	Negative	264	175	Negative
c.4483_4485del;p.Lys1496del	151463	A/N/57/M	Negative	136^	165	Negative
c.4615C>T;p.Arg1539Cys	N =172					
c.4718C>T;p.Thr1573Met	184042	C/N/56/F	Negative	51	158	Negative
c.4731C>G;p.Asp1577Glu	125959	C/N/59/F	Negative	168*	232	Hypothyroidism on THR (TSH 0.26 mcIU/mL/ FT4 1.7 ng/dL)
c.4747G>A;p.Glu1583Lys	166268	C/N/60/M	Negative	86	175	Negative
	179304	C/N/45/F	Negative	36	134	Surgeries: total abdominal hysterectomy 44 y
	120632	C/N/59/M	Negative	48	114	Negative
c.4885_4886del; Gln1629Valfs*3	114693	C/N/41/M	Negative	83	126	Surgeries: hip replacement 49 y
c.4954C>T;p.Arg1652Cys	101892	C/N/57/M	Negative	163^	142	TSH =7.74 mcIU/mL, FT4=1.4 ng/dL
c.4973G>A;p.Arg1658His	N =116					
c.4984A>T;p.Asn1662Tyr	152700	C/N/51/F	Negative	123*	216	Cardiac catheterization 51 y
c.5005G>A;p.Ala1669Thr	183652	C/N/59/F	Negative	77*	156	Surgeries: CABG 51 y, cardiac stents 57.
c.5299C>T;p.Pro1767Ser	169812	C/N/52/M	Negative	252^	164	TSH =6.57 mcIU/mL, FT4=1.1 ng/dL
c.5399T>C;p.Leu1800Ser	N =242					
c.5510A>C;p.Glu1837Ala	138326	C/HL/46/M	Negative	164	131	Negative
	147197	C/N/50/M	Negative	123*	134	Negative
c.5515C>T;p.Pro1839Ser	116697	C/N/64/M	Negative	112	145	Negative
	136180	C/N/59/F	Negative	270	177*	Surgeries: cardiac stent
	139001	C/N/55/F	Negative	65*	129	Negative
	114451	C/N/57/F	Negative	50	72	Hypothyroidism on THR (TSH 0.36 mcIU/mL/ FT4 1.3 ng/dL)

Characteristics of ClinSeq® Volunteers with *CACNA1S* Variants						
Nucleotide Change; Predicted Protein Change	**Subject No.**	**Race/ Ethnicity/Age/ Gender**	**Family History MHS, Heat Illness or Myopathy**	**Serum CK*^ level (U/L)**	**LDH (U/L)**	**Medical/ Surgical/ Procedures History**
	115065	C/N/59/M	Negative	120	179	Negative
	184131	C/N/54/M	Negative	84*	176	Procedures: Endoscopic balloon dilation -esophageal stricture. Gilbert's Disease. TSH =5.31 mcIU/mL, FT4=1.1 ng/dL
	119657	C/N/52/M	Negative	118	177	Negative
	118481	C/N/62/M	Negative	182*	149	Colonoscopy
c.5570G>A;p.Ser1857Asn	158592	C/N/64/M	Negative	169*	166	Cardiac catheterization
	146462	C/N/57/M	Negative	114*	161	Negative

Participant information for *CACNA1S* variants identified in the 870 Clinseq® cohort. Medical histories were reviewed for surgeries/ procedures and clinical conditions indicative of myopathy or that elevate serum creatine kinase (CK). Participants were evaluated for: any myopathy, MHS, and/or exertional heat illness; history of elevated CK; hypothyroidism and/or elevated thyroid stimulating hormone (TSH) > 4.00 mcIU/mL; hypokalemia <3.3 mmol/L; hyperuricemia > 8.6 mg/dL (uric acid normal range: 3.7-8.6 mg/dL); Statin and Nicotinic Acid use; self-report alcohol use (≥3 bottles/glasses/shots per days); autoimmune conditions: rheumatoid arthritis, psoriasis, lupus, Sjogen's syndrome, scleroderma; and renal disease. Participants race and ethnicity were determined by self-report.

Age = age at enrollment. CABG = coronary artery bypass graft. CHCT = caffeine halothane contracture test.
CK = creatine kinase, range: Female = 38-252 U/L; Male = 52-386 U/L. Ethnicity: N = Not Hispanic or Latino; HL = Hispanic or Latino. LDH = lactate dehydrogenase, range = 113-226 U/L. K+ = potassium, range = 3.3-5.1 mmol/L. MHS = Malignant Hyperthermia Susceptible. Nucleotide change = NM_000540.2. Race: C = Caucasian, A = Asian, AA = African American, 0 = other, "—" = Not reported. THR = thyroid replacement hormone. TSH range: 0.40–4.00 mcIU/mL. Thyroxin, FT4 range = 0.9 - 1.5 ng/dL. TURP = transurethral resection of the prostate. "**+**" = participant has an *RYR1* variant. "**++**" = participant with two *RYR1* variants. "⋆" = participant with another *CANA1S* variant. "*****" = on Statin. "∧" = elevated serum TSH level. "∨ " = low serum TSH level. "---" = sample hemolyzed.

Appendix F



Pedigree of Malignant Hyperthermia Susceptible Family
Pedigree of volunteer's family with novel *RYR1* variant shown not to be causative (arrow indicates ClinSeq® volunteer). MHS status is indicated below tested individuals as positive/negative (+/-) contracture test, or for those who had an MH event following the administration of anesthetics, as a near-fatal MH event. P = proband. CHCT = caffeine halothane contracture test. A&W = alive and well. HTN = hypertension. AD dx 60's =

Alzheimer's disease diagnosis in sixth decade. Stroke 90s = stroke diagnosis in ninth decade. MI = myocardial infarction. HIV = Human immunodeficiency virus.

Appendix G

Supplemental data for the publication titled "Using Exome Data to Identify Malignant Hyperthermia Susceptibility Mutations".

1. Supplemental Methods
2. Descriptions of variants identified in ClinSeq® and in databases, but determined to be of less than class 5 pathogenicity.

1. Supplemental Methods

The *RYR1* variants listed in this supplemental were annotated as described in the methods section of this paper and similar to that described by Johnston et al (13). The *RYR1* nucleotide numbering is based on transcript variant NM_000540.2, according to the standards of the Human Genome Variation Society[**]. Variants with low genotype quality were designated as class 0; the remainder were scored on a pathogenicity scale of class 1-5 using an adaptation of published criteria (1; 2; 9; 13; 14; 24; 30) and Johnston et al (13). Briefly, class 1 variants were definitely benign, class 2 probably benign, class 3 of uncertain pathogenicity, class 4 probably pathogenic, and class 5 definitely pathogenic. Further evaluation of the variants was performed using the Human Gene Mutation Database (HGMD)[††] (26) and the Locus-Specific database, Leiden Open Variation Database (LOVD)[‡‡] (7) and Universal Protein Resource[§§] as well as citation trackers (Google Scholar 2012; Scopus, 2012 Elsevier B.V.; and Web of Science, 2012 Thomson Scientific). The criteria for determining pathogenicity were similar to those used previously in studies of other phenotypes (See Table 1, Variant Pathogenicity Classification System, from original article).

[**] The Human Genome Variation Society website. Nomenclature for the description of sequence variations. Available at: www.hgvs.org/mutnomen/. Accessed May 20, 2013.
[††] The Human Gene Mutation Database (HGMD), Professional 2012.2 from BIOBASE. Available at: www.hgmd.org. Accessed May 20, 2013.
[‡‡] Leiden Open Variation Database (LOVD), v.3.0. Available at www.lovd.nl/3.0/home. Accessed May 20, 2013.
[§§] The Universal Protein Resource website, 2002-2012 UniProt Consortium. Resource for protein sequence and annotation data. Available at http://www.uniprot.org/. Accessed May 20, 2013.

2. Descriptions of 14 variants that were identified both in ClinSeq® and in databases, but determined to be of less than class 5 pathogenicity.

The *RYR1,* c.7025A>G, p.Asn2342Ser Variant

The p.Asn2342Ser variant was listed in the Human Gene Mutation Database as pathogenic based on two publications (21; 34), and in LOVD (25) with the variant pathogenicity in the cited publication as "pathogenic" by the authors (indicated with "+"), although the LOVD database curators concluded "effect unknown" (indicated with "?")[***]. Citation tracking of Malignant Hyperthermia Susceptibility (MHS) publications by author returned more listings (17; 29). The first described a 14 year old with documented MHS but who also had hypokalemic periodic paralysis (the latter not having been associated with *RYR1* mutations) (21). The second report was a screen of 23 patients with MHS, one of which had p.Asn2342Ser, although no additional genetic data or *In Vitro* Contraction Test (IVCT) results were provided. Functional data of a metabolic protein release assay supported pathogenicity (34).

In a more recent study of families with patients selected for anesthetic reactions or for chronic, unexplained elevated blood levels of creatine kinase, the p.Asn2342Ser variant was identified in one patient with moderate CK level and confirmed MHS phenotype by positive IVCT (29). The patient presented with another known RYR1 mutation p.Arg3903Gln in *trans* with p.Asn2342Ser. In the proband's family, the p.Asn2342Ser variant was carried by the patient's three sons diagnosed as MHS. The allele p.Arg3903Gln co-segregated with the MHS status in the family, whereas p.Asn2342Ser did not and was classified as a "variant allele without

[***] Variant pathogenicity, in the format Reported/Concluded; '+' indicating the variant is pathogenic, '+?' probably pathogenic, '-' no known pathogenicity, '-?' probably no pathogenicity, '?' effect unknown.

any pathological meaning". In addition, the study analyzed the skeletal muscle mRNA in two of the proband's sons and found no evidence for monoallelic silencing.

The p.Asn2342Ser variant was most recently categorized as a recessively inherited missense mutation in a patient with a congenital myopathy and muscle biopsy finding compatible with the diagnosis of an *RYR1* related myopathy from a United Kingdom (UK) study of 92 patients from 71 families with *RYR1* mutations (17). The patient was also reported to have the recessive RYR1 p.Arg3539His missense mutation (reported in ClinSeq®) in combination with a dominantly inherited mutation in the α-tropomyosin (*TPM3*) gene, (c.503G>A, p.Arg168His).The proband's daughter with the same phenotype, carried the *TPM3* mutation and the known recessive *RYR1* mutation c.10616G>A, p.Arg3539His.

This variant from a known hotspot MHS region II was identified in 12 of 10,746 alleles in the National Heart, Lung, and Blood Institute's, Exome Variant Server (EVS) ENREF 15, which (assuming all are heterozygous), would indicate a carrier rate of 1/448, and an allele frequency of 0.1%, similar to the frequency in our data set (2/1,734). We conclude that this is a Class 2 variant, probably benign.

The *RYR1*, c.10616G>A, p.Arg3539His Variant

The p.Arg3539His variant was identified in a single ClinSeq® participant. It was described as pathogenic in HGMD based on a single report of a patient with central core disease without segregation, histologic, or functional data(5). The LOVD database returned results for a single report of the p.Arg3539His variant pathogenicity with an indication of "pathogenic" (+) by the authors, and concluded as "effect unknown" (?) by the curators (23). In a study of nine families that met clinical and histological criteria for core congenital myopathies, one patient

with generalized muscle weakness and severe skeletal complications had the p.Arg3539His missense variant along with another RYR1 mutation p.Gly4935 Thr4957>AspfsX11 that affected the acceptor splice site of intron 102 and was thought to likely be the cause of the severe phenotype in the proband (23). In the previously referenced UK study of 92 patients, the p.Arg3539His variant was reported in two patients with congenital myopathies and muscle biopsy findings compatible with the diagnosis of an *RYR1*-related myopathy. The variant was classified as an *RYR1* mutation associated with an "uncertain inheritance pattern", for in one patient it was the only missense identified, and in another family it was seen with a clear recessive inheritance pattern (17). This variant was identified in 17 of 10,741 alleles in the EVS for an allele frequency of ~0.2%. We concluded that this variant was a Class 3 mutation of uncertain significance.

The *RYR1*, c.2122G>A, p.Asp708Asn Variant

The p.Asp708Asn variant was detected in two ClinSeq® participants (2/870) and detected in 1/10,753 alleles in the EVS. It was described as pathogenic for congenital myopathy in HGMD (16) and found in two additional publications on *RYR1*-associated myopathies (17; 32). It was initially reported with the p.Arg2241X nonsense variant (32) and again in two patients with *RYR1* related myopathies of moderate severity, and in a third patient with a *RYR1* myopathy and a third recessive variant p.Arg2939Lys (16). It was most recently categorized as a recessively inherited missense mutation with a high probability of pathogenicity in a patient with a congenital myopathy and muscle biopsy finding compatible with the diagnosis of an *RYR1* related myopathy from the UK study of 92 patients with *RYR1* mutations (17). In this case, the patient had two additional recessive pathogenic variants, and one variant of unknown

significance (VUS), in addition to p.Asp708Asn: the nonsense p.Arg2241X variant, the recessive missense p.Met485Val, and VUS c.11547G>A(p(=)). The evidence of pathogenicity for the variants was in part based on *in silico* analysis. The p.Asp708Asn variant has been seen twice without p.Arg2241X—in one ClinSeq® participant and once in the EVS. We categorized it as a Class 3 variant of uncertain significance.

The *RYR1*, c.4178A>G, p.Lys1393Arg and c.5036G>A, p.Arg1679His Variants

The p.Lys1393Arg and p.Arg1679His variants (here described together, as two of the largest studies reported them jointly) were described as pathogenic in HGMD and first reported as novel *RYR1* missense variants in the same publication (3). The p.Lys1393Arg and p.Arg1679His variants were found in a 5 year-old and 39 year-old Swedish male, MHS patients, respectively, known to be predisposed by prior clinical event—i.e., development of life-threatening Malignant Hyperthermic (MH) reactions during anesthesia—and positive IVCT. However, both variants were also found once in the study's 100 Swedish and 150 German control individuals. Segregation data available for the p.Arg1679His variant were inadequate to prove causality. The variant p.Arg1679His occurred in all MHS and MH-equivocal family members, based on the IVCT threshold concentrations, and in one MH-negative individual. Although the missense variants occurred in conserved domains, they were initially acknowledged as "may be 'neutral' polymorphisms" by the study's authors (3).

In their follow-up study, the authors tested the same Swedish MHS p.Lys1393Arg and p.Arg1679His variant patients—and three others—for MHS by using Epstein-Barr virus-transformed lymphoblasts cultured from the subject's lymphocytes (31). The B lymphoblastoid cell lines were used to study resting cytoplasmic calcium concentration, and the dose-dependent

calcium release induced by the ryanodine receptor agonist 4-chloro-m-cresol. The study showed that cell lines from the p.Lys1393Arg and p.Arg1679His variant patients, and other MHS individuals with distinct *RYR1* variants, responded to 4-chloro-m-cresol with statistically significant, half maximal concentration (EC50) than cells from normal individuals, and that the magnitude of the responses differed between individuals with different *RYR1* variants (31). However, the size of the study was small, and the novel diagnostic test, experimental. Also, it has not been firmly established whether the response in the *RYR1* receptor from circulating B-cells are a valid model for the MHS phenotype in skeletal myocytes.

A recent published abstract of a mother and son presenting with a severe, slowly progressive adult-onset axial myopathy and cataracts implicated the RYR1 p.Lys1393Arg variant in both. Serum creatine kinase (CK) was slightly elevated in the son, and MRI showed involvement of the lumbar, and less pronounced pelvic and posterior thigh muscles in both. The quadriceps muscle biopsy showed minor, non-specific changes in both (27). No functional studies were done.

The p.Lys1393Arg was most recently identified in three patients with congenital *RYR1* myopathies—from the UK study of *RYR1* mutations—and described as a putative dominant missense mutation with a high probability of pathogenicity (i.e., high nucleotide and amino acid conservation) (17). Two of the three p.Lys1393Arg patients also had additional synonymous variants (i.e., one patient with three *RYR1* VUS, and another patient with two VUS).

The p.Lys1393Arg variant was identified in four ClinSeq® participants (4/870), but detected in 47 of 10,703 alleles in the EVS, indicating an allele frequency of 0.4%. The p.Arg1679His was found in two ClinSeq® participants and detected in 11 of 10,741 alleles in the

in the EVS for a carrier rate of 1/318, and an allele frequency of 0.1%. Based on the equivocal data presented in the published reports, we categorized these as Class 2, probably benign variants.

The *RYR1,* c.8327C>T, p.Ser2776Phe Variant

The p.Ser2776Phe variant was found in a single ClinSeq® participant and detected in 10 of 10,746 alleles in the in the EVS for an allele frequency of 0.09%. It was described as pathogenic for MHS in HGMD and reported in two publications on *RYR1*-associated myopathies (4; 6). In the most recent study, a 16-year-old patient with suggestive features of King-Denborough syndrome—a rare syndrome characterized by the triad of dysmorphic features, a myopathy, and MHS—was found to have the heterozygous p.Ser2776Phe missense variant identified by sequencing of *RYR1* from muscle-derived complementary DNA (6). The p.Ser2776Phe variant affected a highly conserved amino acid residue and was not found in 100 control chromosomes. There was no history of clinically manifest malignant hyperthermia reactions in the family. The sequence variant was considered not in itself sufficient to cause a pathological phenotype by the authors, as the same substitution was also identified in her asymptomatic father. Subsequent Western blot analysis of protein extracted from muscle tissue showed a marked reduction in RYR1 protein levels compared to an unaffected control, suggesting the presence of unidentified allelic *RYR1* mutations not detectable on routine sequencing.

In another study of MHS patients diagnosed by the IVCT, a male Swedish patient with a history of a life threatening MH reaction during surgery, and with a high positive score in the IVCT, was found to have the p.Ser2776Phe missense variant. However, the variant was also

found in one German and in one Danish individual in controls (4). Based on the ambiguous data from the reports, the p.Ser2776Phe variant, we categorized as a Class 2, probably benign variant.

The *RYR1,* c.13513G>C, p.Asp4505His Variant

The p.Asp4505His variant was detected in four ClinSeq® participants (4/870) and detected in 36 of 10,710 alleles in the EVS for a carrier rate of 1/149 and an allele frequency of 0.3%. The variant was listed as pathogenic in HGMD based on one published report (10) and a published abstract (27). Two additional reports were found in a literature search(17; 20).

The p.Asp4505His variant was first described in a 29-year old asymptomatic male with an elevated serum CK level (407 UI/I) - slight variation in fiber size found on muscle biopsy, and MHS by IVCT results (20). However, the patient had an additional *RYR1* novel nucleotide change c.7085A>G of exon 44 predicted to cause p.Glu2362Gly. No segregation data were included in this analysis of 37 Italian subjects with elevated CK levels, but without significant weakness or other neurological symptoms (20).

A more recent study included a functional analysis of the p.Asp4505His variant and introduction into a full-length rabbit *RYR1* complementary DNA, described two children who had died from of non-anesthetic MH-like episodes—described as drug-free fatal "awake" events—triggered by either exposure to environmental heat or infection (10). A 6 year-old girl with the maternally inherited p.Asp4505His variant, and an additional RYR1 variant, p.Arg3983Cys, on another allele, showed marked increased bulk of the leg muscles and recurrent cramping with rigid gait. The girl had a previous awake episode of high fever (>105 °F) and total body rigidity at 4 years of age. There was no family history of MHS or other neuromuscular disorders (10).

In this study, the p.Asp4505His variant was *not* found in the 100 previously reported MHS individuals from North America, or in 100 controls representing the U.S. Caucasian population. In the girl's family, the novel p.Asp4505His variant was identified on a separate haplotype from the p.Arg3983Cys variant, and was present in both the patient's mother and brother. Segregation analysis within the *RYR1* gene in the second family showed association of the p.Asp4505His variant with the maternal haplotype. The p.Asp4505His variant was reported to result in approximately two-fold increase in the sensitivity to activation by caffeine. Importantly, this increase in caffeine sensitivity occurred when the p.Asp4505His variant was co-expressed with wild-type RYR1, consistent with the known autosomal dominant pattern of inheritance of MH. They reported that *allelic segregation* could be a significant pathogenic factor in individuals with MHS, and that the unusually high caffeine sensitivity when the two variants localize to the same subunit demonstrates an allele-dependent synergism of two novel *RYR1* gene variants (10).

The c.13513G>C;p.Asp4505His variant was reported in a UK study with *RYR1* mutations, together in four patients with congenital myopathies and muscle biopsy findings compatible with the diagnosis of an *RYR1* related myopathy (17). The p.Asp4505His variant was described as a putative dominant mutation in two patients with a relatively severe and a mild phenotype, and in two other patients with an apparently recessive inheritance. The two patients with recessive inheritance each had an additional missense variant (17). No information was provided if those variations were in *trans* or *cis*, and family members were not tested in the two patients with recessive inheritance.

A recent case report of single 80 year-old patient harboring the p.Asp4505His variant with severe, slowly progressive adult-onset axial myopathy, marked head-drop, respiratory impairment, cataracts, and increased fiber size and cores on muscle biopsy, expanded the spectrum of the phenotype seen with this variant (27). Based on the equivocal data presented in the multiple published reports and the low frequency in the EVS, we categorized the p.Asp4505His variant as Class 3 variant of uncertain significance.

The *RYR1,* c.4999C>T, p.Arg1667Cys Variant

The c.4999C>T,p.Arg1667Cys variant was not listed in HGMD, but referenced in LOVD with a citation to two studies (12; 18). LOVD listed the variant pathogenicity for the first study (reported/concluded) as effect unknown and effect unknown by the publication's authors and site curators, respectively (?/?) (12), and for the second as probably no pathogenicity and effect unknown (-?/?) (12;18). One study sequenced the entire *RYR1* coding region from genomic DNA of unrelated Japanese patients diagnosed as MHS by the calcium-induced calcium release test—the Japanese equivalent of the IVCT test. The tests subjects were recruited after experiencing an MH episode, or for having an MH relative, or an increase in CK. The p.Arg1667Cys variant in exon 34 was present in five patients. However, since the variant was also seen in two control samples, the researchers presumed the variant to be benign (12). No segregation data were included in the study.

In another study, a representative cohort of 36 unrelated Canadian individuals positive for the Caffeine Halothane Contracture Test (CHCT) test for MHS or with a history of an MH event, were screened for *RYR1* mutations, and selected regions of *CACNA1S* transcripts (18). After analyzing the correlation of the CHCT results to the *RYR1* genotypes within MHS families, the

p.Arg1667Cys variant was found in one individual with a positive the CHCT for MHS, but absent in two of the proband's tested relatives with negative test results. In the remaining families with variant, the absence of CHCT testing results for additional family members prevented segregation analysis (18). The p.Arg1667Cys variant was detected in four ClinSeq® participants (4/870) and in 21/10,727 alleles for a carrier frequency of 1/255 and an allele frequency of 0.2% in the EVS. Based on the data presented and the relatively low allele frequency of 0.2% we categorized this as a Class 3 variant.

The *RYR1,* c.6301A>G, p.Met2101Val Variant

The rare p.Met2101Val variant was found in a single ClinSeq® participant (1/870) but was not listed in the EVS, HGMD or LOVD, but referenced in the Universal Protein Resource database for a single publication (28). The p.Met2101Val variant was identified in one family—in members positive for MHS by IVCT test—in a study of 52 Italian families (one to 12 individuals in each family) recruited for *RYR1* DNA analysis that included at least one family member identified by increased serum CK concentrations or selected for episodes of hyperthermia associated with an anesthetic. The variant was determined to be nonpathogenic based on the results from the study's functional test—denaturing high performance liquid chromatography. The affected families were only screened for 27 *RYR1* exons from critical regions (28).

The p.Met2101Val variant was recently reported again by the same authors in a study of 75 families with patients selected for anesthetic reactions or chronic, unexplained and elevated CK levels. Fifty-four families were confirmed for the MHS phenotype by positive IVCT. The p.Met2101Val variant, located within putative hotspot MH region II, was identified in one

patient with MHS and classified as a change of "unknown pathological meaning" (29). Based on the data presented from the limited citations with incomplete confirmation, and the low allele and population frequency, we categorized this as a Class 3 variant of uncertain significance.

The *RYR1,* c.12553G>A, p.Ala4185Thr Variant

The p.Ala4185Thr variant was not listed in HGMD, but found in LOVD with a reference to one study with a reported/concluded variant pathogenicity as effect unknown (?/?) (18). In this study of 36 unrelated Canadian MHS individuals screened for *RYR1* mutations by sequencing *RYR1* transcripts, one patient carried two novel mutations, c.12553G>A, p.Ala4185Thr variant (exon 90) along with c.14524G>A, p.Val4842Met (exon 101). No information was provided if those variations were in *trans* or *cis*. The p.Ala4185Thr variant was predicted to be neutral by bioinformatic programs (i.e., SIFT, Pmut, and PolyPhen2) and was absent in 200 controls. The p.Ala4185Thr variant was detected in two ClinSeq® participants (2/870) and in 6/10,752 alleles for an allele frequency of 0.06% in the EVS. We categorized this as a Class 3 variant of uncertain significance.

The *RYR1,* c.1453A>G, p.Met485Val Variant

The p.Met485Val variant from a known hotspot MHS region I was detected in two ClinSeq® participants (2/870) and in 1/10,757 alleles in EVS. It is described as pathogenic in HGMD based on a single report without segregation, histologic, or functional data (5), in LOVD with reference to a single study(33), and a literature search found additional references (8; 15; 17). In an early study of 11 affected individuals from five families with Multi-minicore disease and external ophthalmoplegia where *RYR1* haplotyping and mutational analysis were carried out, the p.Met485Val variant was identified on the paternal allele of the asymptomatic parent in one

UK family, along with the RYR1 p.Gly2060Cys variant, and classified as a polymorphism (15). In another study of four patients from three families with core myopathies, two affected siblings carried the p.Met485Val substitution along with p.Arg109Trp in *cis*, inherited from the unaffected father (33). The two affected siblings only transcribed the mutated paternal allele in skeletal muscle, whereas the maternal allele was silent. Functional measurements showed that the mutant *RYR1* channels—carrying both substitutions—lost the ability to conduct calcium. However, the study classified the p.Met485Val variant as "probably a polymorphism" due to the variant not being a conserved residue, and p.Met485 shows as a leucine in all other species (33). In the most recent study, involving patients with *RYR1* related congenital myopathies, the p.Met485Val variant was seen in two unrelated patients. Both patients, in addition to p.Met485Val**,** had an additional nonsense and missense variant (17). We categorized this as a Class 2, probably benign variant.

The *RYR1,* c.8360C>G, p.Thr2787Ser Variant

The p.Thr2787Ser variant was seen in six ClinSeq® participants (6/870) and in 113/10,645 alleles (109 African American alleles, four European American Alleles) for an allele frequency of 1% in EVS. It is described as pathogenic in HGMD based on a single entry (11), and in LOVD for a single entry (22). We categorized this as a Class 2*,* probably benign variant, based on its high allele frequency in both our cohort and in the EVS.

The *RYR1,* c.6961 A>G, p.Ile2321Val Variant

The p.Ile2321Val variant was detected in one ClinSeq® participant (1/870) and in 10/10,748 alleles for an allele frequency of 0.1% in EVS. It is described as pathogenic in HGMD based on a single review entry (25) and in LOVD for an additional entry (19). The

HGMD entry reported the p.Ile2321Val variant in association with MHS in one family. In a study of 36 unrelated MHS patients five members of one family harbored both the p.Ile2321Val and p.Leu13Arg variants. However, the p.Ile2321Val variant was recorded as one of eight non-causative variants due to a previous description as polymorphism in the National Center Biotechnology Information, Database of Single Nucleotide Polymorphisms (19). We categorized this as a Class 2, benign polymorphism.

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