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Genetic Risk for Malignant Hyperthermia in Non-Anesthesia-Induced Myopathies

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Abstract

Malignant hyperthermia (MH) is a pharmacogenetic, autosomal dominantly inherited disorder of skeletal muscle triggered by volatile anesthetics and infrequently by extreme exertion and heat exposure. MH has variable penetrance with an incidence ranging from 1 in 5,000 to 1 in 50,000–100,000 anesthesias. Mutations in the ryanodine receptor gene, *RYR1*, are found in 50–70% of cases. W*e* hypothesized that a portion of patients with drug-induced muscle diseases, unrelated to anesthesia, such as severe statin myopathy, have underlying genetic liability that may include *RYR1* gene mutations. DNA samples were collected from 885 patients in 4 groups: severe statin myopathy (n=197), mild statin myopathy (n=163), statin-tolerant controls (n=133), and non-drug-induced myopathies of unknown etiology characterized by exercise-induced muscle pain and weakness (n=392). Samples were screened for 105 mutations and variants in 26 genes associated with 7 categories of muscle disease including 34 mutations and variants in the *RYR1* gene. Disease-causing mutations or variants in *RYR1* were present in 3 severe statin myopathy cases, 1 mild statin myopathy case, 8 patients with non-drug-induced myopathy, and none in controls. These results suggest that disease-causing mutations and certain variants in the *RYR1* gene may contribute to underlying genetic risk for non-anesthesia-induced myopathies and should be

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included in genetic susceptibility screening in patients with severe statin myopathy and in patients with non-statin-induced myopathies of unknown etiology.

Keywords

Malignant hyperthermia; Statin myopathy; *RYR1* gene mutations; Metabolic muscle disease; Coexisting genetic risk

Introduction

Malignant hyperthermia (MH; MIM #145600) is a pharmacogenetic disorder of skeletal muscle that is triggered by volatile anesthetics, such as succinylcholine, and, in rare cases, by vigorous exercise and heat exposure. The disorder is caused by a disturbance in calcium homeostasis. Diagnostic features include an unexplained elevation in end-tidal carbon dioxide (ETCO2) concentration, muscle rigidity, tachycardia, acidosis, hyperthermia, and hyperkalemia [1]. MH is inherited as an autosomal dominant trait with variable penetrance. The incidence has been reported to range from 1 in 5,000 to 1 in 50,000–100,000 anesthesias, however, the prevalence of causative genetic abnormalities may be as high as 1 in 2,000 to 1 in 3,000 [1, 2].

Ryanodine receptor proteins (RyR) are homotetrameric intracellular calcium release channels that are located in the endoplasmic and sarcoplasmic reticulum membranes [3]. The primary RyR protein of skeletal muscle, RyR1, is encoded by a gene (*RYR1*; MIM #180901), on chromosome 19q13.1 [3].The gene spans 158kb, contains 106 exons, and transcribes to an RNA molecule that is 15 kb long. The *CACNA1S* gene (MIM #114208), encoding the calcium channel, voltage-dependent, L type, α1 subunit of the voltage-gated dihydropyridine receptor (DHPR), is located on chromosome 1q32 [4, 5]. A number of studies have reported that mutations in the *RYR1* gene are found in or associated with 50–70% of MH cases and mutations in the *CACNA1S* gene (MIM#601887) are found in 1% of cases [4, 6, 7]. Four additional loci are believed to be causally associated with MHS cases when *RYR1* and *CACNA1S* gene mutations are not found. These include the *SCN4A* gene (MIM#154275) on chromosome 17q.2-q24 encoding the sodium channel voltage-gated, type IV alpha subunit; the *CACNL2A* gene (MIM#154276) on chromosome 7q21-q22; on chromosome 3q13.1 (MIM#600467); and on chromosome 5p (MIM#601888) [5, 8–10]. In addition, the calsequestrin-1 (*CASQ1*) gene (MIM #114250) on chromosome 1q21 is a moderate affinity, high-capacity calcium-binding protein in the sarcoplasmic reticulum terminal cisternae of skeletal muscle. It functions not only as a calcium-binding protein but also as a luminal regulator of ryanodine receptor-mediated calcium release [11] and has recently been identified as a candidate gene for MH [12]. There is clinical evidence for a synergistic effect between genetic variants in the genes encoding proteins vital to skeletal muscle calcium regulation (*CASQ1*, *RYR1*, and *CACNA1S*) in a case that began with exercise-induced rhabdomyolysis, followed by a clinical episode of MH with exposure to anesthesia, and then a positive CHCT [13]. In spite of good progress in uncovering mutations causative for MH, there remain a sizeable number of cases in which the genetic etiology is unknown.

The caffeine halothane contracture test (CHCT), requiring an open muscle biopsy, is the most sensitive means of confirming a clinical MH episode. The test measures skeletal muscle contracture response following exposure to halothane or caffeine in a temperature-controlled bathing solution to which caffeine or halothane is added in a dose-controlled manner. Interpretation of the test results vary. The European MH Group assigns MH susceptibility phenotypes as MH sensitive (MHS) when an abnormal response occurs with

both halothane and caffeine and MH equivocal (MHE) when there is an abnormal response to only one of the stimulants. The CHCT may have reduced specificity, sensitivity and predictive value in patients with coexisting neuromuscular diseases and enzymopathies. An evidence-based approach was used to score disorders that may be associated with MH [11]. Beyond the well-known association of MHS with central core disease, minicore disease, and King-Denborough syndrome, weak evidence has been presented for susceptibility to MH-like episodes with the following disorders: osteogenesis imperfecta, arthrogryposis, carnitine palmitoyltransferase (CPT) II deficiency, myophosphorylase deficiency (McArdle disease), myoadenylate deaminase deficiency and hyperCKemia [14]. Recently, patients with chronic unexplained hyperCKemia who were MHS or MHE were found to have a number of known and novel mutations and variants in *RYR1* [15].

The suspicion that MH susceptibility could be unmasked by an adverse reaction to statin therapy was proposed by Guis, et al. [16] who demonstrated positive CHCT results in a number of patients with statin myopathy. These abnormal findings clearly indicated an alteration of calcium homeostasis during statin therapy. Molecular studies for mutations in the *RYR1* or *CACNA1S* gene or any other genes involved in muscle disease were not included in their studies. In another study, the effect of statins was examined on the expression of genes implicated in the regulation of calcium and membrane repair, lipid homeostasis, remodeling of myocytes and mitochondrial function [17]. Statins were found to impact genes involved in the regulation of calcium and membrane repair but not those involved in myocyte remodeling or mitochondrial function. Most recently, a study was performed to determine if statins modified the contracture response in isolated muscle bundles in MHS versus MH-normal (MHN) pigs [18]. Both atorvastatin and simvastatin induced significant muscle contractures in muscle bundles from MHS pigs but not in bundles from MHN pigs. It was inferred that a preexisting impairment of calcium homeostasis must be necessary for this outcome and that there was a greater vulnerability of muscle cells toward statins in MHS patients.

In the present study, we hypothesized that a portion of patients with statin-induced myopathy and idiopathic myopathies have underlying genetic abnormalities that may include *RYR1* gene mutations. To test this hypothesis, a study was undertaken to broadly evaluate disease-causing mutations and variants in genes associated with muscle disease by testing several high risk groups. The need for a more comprehensive analysis of disease gene mutations in these patient groups will be determined from the outcome of this study.

Subjects

A total of 885 subjects were evaluated through analysis of genomic DNA derived from peripheral blood or skeletal muscle specimens. Subjects were derived from multiple collaborating centers across the U.S. and Canada. Subjects provided informed consent approved by the NYS Department of Health and the University at Buffalo Health Sciences Institutional Review Board.

Statin Exposure Groups

One hundred ninety-seven subjects with severe statin-induced myopathy participated (majority Caucasians; 66 females, 131 males; age range: 28–80 yrs; mean age: 59 yrs). Severe statin myopathy is defined as the onset of incapacitating muscle pain and/or weakness often accompanied by rhabdomyolysis or abnormal elevations of plasma creatine kinase (CK) and directly related to the use of statin therapy with no pre-therapy symptoms of muscle disease. Many of these individuals also had persistent symptoms post-therapy lasting from weeks to years. The most affected cases actually suffered progressive disease post-therapy and never recovered. An additional 163 subjects (majority Caucasians; 87

females, 76 males; age range: 45–80 yrs; mean age: 62 yrs) had symptoms of mild statin myopathy defined as muscle aches and pains clearly attributable to the initiation of statin therapy that were not incapacitating and were reversible with the cessation of therapy. A statin-tolerant control group consisted of 122 Caucasians, 5 African Americans, 4 Asians, and 2 Native Americans (74 females, 59 males; age range: 26–78; mean age: 50 yrs) who had been on statin therapy for a minimum of 6 months with the majority taking statins for years with no muscle side effects. The control group is questioned annually regarding their continued statin tolerance. Each patient having taken a statin, with or without adverse reactions (ADRs), completed a detailed questionnaire regarding the dose and type of statin that was the first to result in myopathic ADRs or, for the controls, the statin and dose that they were taking at the time of participation in the study. Each participant with ADRs provided specific start and end dates of statin therapy as well as the date symptoms began, to the best of their recollection. They described the symptoms that were clearly attributed to statin therapy, whether symptoms ended with cessation or changes in therapy, and, in some cases, estimated how long symptoms lasted post-therapy. Patients were contacted by phone for clarification when questions arose about their information. While approximately one-third of individuals were self-referred, most data gathering for questionnaires was performed by healthcare professionals at collaborating institutions in the U.S. and Canada. A quantifiable scoring system for information derived from the questionnaires is under development taking into account pre-existing conditions, medications, and other factors of possible influence on statin tolerance.

Unknown Myopathy Group

A group of 392 patients (135 females, 257 males; age range: 2–80 yrs; mean age: 32 yrs) was referred to the Robert Guthrie Biochemical & Molecular Genetics Laboratory primarily for the EIP; http://www.rgbmgl.org/mutations.asp) which screens for the most common mutations causing CPT II (EC 2.3.1.21) deficiency (MIM#255110, 600649, 608836), myophosphorylase (EC 2.4.1.1) deficiency (GSD V; MIM#232600) and/or myoadenylate deaminase (EC 3.5.4.6) deficiency (MIM#102770). All had symptoms of muscle pain, weakness or cramps usually with exercise intolerance +/− rhabdomyolysis and myoglobinuria and diagnostic etiologies were unknown. Information about race was only available for 58% of this myopathic group. Among these, 15.8% were African-American, 1.4% were Asian, and 82% were Caucasian.

Methods

DNA Isolation

Genomic DNA was isolated from whole blood collected in EDTA or from frozen skeletal muscle tissue using Puregene DNA isolation kits (Qiagen, Germantown, MD).

SNP and Mutation Genotyping Assay

Genotyping was performed using Goldengate Genotyping with VeraCode technology (Illumina, Inc., San Diego, CA). A complete description of the methodology is described at http://www.uthscsa.edu/csb/CSBPDFfiles/genomics-GoldenGateGenotypingOverview.pdf. Briefly, a total of 384 variants were multiplexed in a single well of a standard microplate and 96 DNA samples were analyzed per plate. The pass rates for genotype designs in the GoldenGate system is 95% and the call rates of a designed panel are >98%. Each locus was assayed with an average 15- to 28-fold redundancy to reduce signal-to-noise ratio. Another strong contributor to data quality was outlier removal in the averaging of raw intensity data.

Genomic DNA samples (250 ng) underwent activation to enable binding to paramagnetic particles. The hybridization step combined assay oligonucleotides, hybridization buffer, and

the paramagnetic particles together with the activated DNA. The reagents, produced by Illumina, consisted of three oligonucleotides designed for each of the 384 SNP or mutation loci. Two were specific for each allele. The third was locus-specific and hybridized several bases downstream from the SNP site. All 3 oligos contained regions of genomic complementarity and universal PCR sites. The locus-specific oligonucleotide also contained a unique `address sequence' that targeted a particular VeraCode Bead type. The technology allows up to 384 SNPs to be interrogated simultaneously in this manner. No amplification bias is introduced into the assay because hybridization of the DNA sample to the paramagnetic beads occurs before any amplification steps. After several wash steps, extension of the appropriate allele-specific oligonucleotide takes place along with ligation of the extended product to the locus-specific oligonucleotide. These steps join information about the genotype present at the SNP site to the address sequence on the locus-specific oligonucleotide. The resultant ligation products serve as the PCR templates with universal primers. Following downstream processing, the single-stranded, dye-labeled PCR products are hybridized to their complement bead type through their unique address sequences. Hybridization of the GoldenGate Assay product onto the VeraCode beads separate the assay products for individual SNP genotype readout. The BeadXpress reader is used for code identification and fluorescent signal detection. During the scanning process, a laser beam penetrates the digitally inscribed VeraCode microbead causing a unique code image identification allowing for rapid and highly specific detection. Data generated from the BeadXpress reader are analyzed using the Illumina BeadStudio software for automated genotype clustering and calling. The GoldenGate Assay includes 48 assay controls, allowing for a high level of confidence and the ability to troubleshoot errors such as PCR and hybridization failures.

SNP and Mutation Inclusion

Among 384 genetic variants included, 271 were muscle disease-causing mutations or associated variants in 30 genes with overlapping symptoms. These included genes involved in disorders of energy metabolism (fatty acid oxidation disorders, glycogen storage diseases, mitochondrial disorders), selected muscular dystrophies, a regulator of muscle mass, and malignant hyperthermia. Any mutations or variants in genes that were present in patients positive for *RYR1* gene mutations and variants are reported here. These include Very Long-Chain Acyl-CoA Dehydrogenase (EC1.3.99.13; *VLCAD*; MIM#609575), myostatin (*GDF8*, MIM#601788), and myoadenylate deaminase (EC 3.5.4.6; *AMPD1*, MIM#102770). Only 18 variants on the chip failed with a call rate below 90%; 95% of the variants were successfully genotyped. Thirty-four mutations and variants in the *RYR1* gene were evaluated on the chip (Table 1).

Validation of Microarray Analysis

Positive findings were validated systematically by direct sequencing using the Sanger Method (Roswell Park Cancer Institute, Microarray Center, Buffalo, NY).

Statistical Analysis

Statistical analysis of the data employed chi-square analysis with the nominal statistical significance level set at $P \leq 0.05$.

Results and Discussion

Severe Statin Myopathy Group

Patient 1—A 47-year old Caucasian female with severe statin myopathy and symptoms of severe pain, stiffness, cramps, rhabdomyolysis with myoglobinuria and elevated plasma CK.

The patient also had persistent pain and weakness post-statin therapy. A blood sample originally had been sent to the laboratory for the EIP; the results of the profile were negative. With microarray analysis, she was found to have a proven causative mutation for MH in the *RYR1* gene (R614C; Table 1; Table 2). She was the only one of 197 severe statin myopathy cases found to have one of 34 mutations (18 proven disease-causing) and variants screened in the *RYR1* gene.

The R614C mutation, found initially to be causative for porcine MH, was ultimately linked to human MH [19] and is considered to be one of the most common causative mutations for MH in populations primarily originating from Western Europe [20]. Since this patient has been found to have this mutation she must be considered at risk for MH.

Patient 2—A 66-year old Caucasian male presented with severe statin myopathy. His symptoms included severe muscle pain with cramps and elevated serum CK. A muscle biopsy had originally been sent to the laboratory for biochemical analysis of the Mitochondrial Myopathy Profile (http://www.rgbmgl.org/profiles.asp) and CPTII activity, both of which were negative. By microarray analysis, he was found to have a variant, R3772W, in the *RYR1* gene. The R3772W variant was reported as novel and associated with MHS in 36 unrelated MHS patients [21]; it has not as yet been functionally characterized. The R3772 residue is highly conserved in inter-species amino acid sequence comparisons. Replacement of the positively charged arginine side chain could potentially have functional consequences. The R3772W mutation is predicted by PolyPhen2 software (http://genetics.bwh.harvard.edu/pph2/) likely to be damaging (score 0.997). Another non-synonymous change in *RYR1* at the same amino acid position (R3772Q) has been found in individuals of 3 unrelated Indian families and 2 separately reported unrelated families, one of which was from Asia [22, 23]. In the heterozygous state susceptibility to MH was present, while in the homozygous state the mutation was associated with susceptibility to MH and a complex myopathic phenotype with cores identified histologically in muscle. As evidence mounts linking both MH and myopathic features with changes at this amino acid residue, variants at R3772 should be included in risk analysis for cases with MH and myopathy.

Patient 3—An African-American male of unknown age with severe statin myopathy presented with muscle pain and persistently elevated plasma CK. Blood was sent to the laboratory for EIP analysis and the results were negative. By microarray analysis, the patient was found to have a variant, P4501L, in the *RYR1* gene. The P4501L variant was reported in non-synonymous members of a family with MH together with 3 other *RYR1* variants, however, it was also present alone in 2 MH-negative individuals in the family and is likely to be a nonsynonymous polymorphism. Although the P4501L was not found in 100 Caucasian controls, it appears to be a relatively frequent variant in African Americans [21, 24]. The P4501 residue is highly conserved across species. Replacement of proline with the much bulkier leucine side chain could be expected to have structural consequences, however, this polymorphism is listed in the dbSNP database, rs73933023, with a MAF of 0.04 adding further evidence to its relatively high frequency in the general population. Additional data must be gathered to determine if it contributes to an MHS phenotype when combined with other *RYR1* variants.

Mild Statin Myopathy Group

Patient 4—A 52-year old African American male with mild statin myopathy presented with muscle weakness. He was found to be homozygous for an *RYR1* variant, A1352G, first reported by Levano, et.al [21]. This patient was the only one of 163 mild statin myopathy patients with this variant, whether heterozygous or homozygous, and it was not found in any of 133 statin-tolerant controls. The A1352G variant has previously been shown to be

associated with MHS in a single Caucasian family and was not found in 100 controls [21]. It is polymorphic in the African American population with a frequency estimated at 2.7% [25]. This variant is in the public SNP database (Single Nucleotide Polymorphism, National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD) and has a PolyPhen2 score of 0.005 (benign), therefore, it is unlikely to be pathogenic. To our knowledge, this patient and patient #8 from the Unknown Myopathy Group, are the first homozygotes reported for the A1352G variant. The pathologic significance of this finding, if any, is unclear. Functional studies of the A1352G variant would be needed to confirm the possibility of protein instability with an impact on phenotype in the homozygous state. Patient 4 was also heterozygous for a P65L variant in the *VLCAD* gene which results in exon 3 skipping altering exon splicing but does not reduce VLCAD enzyme activity [26]. This variant was unlikely to contribute to the myopathic phenotype in this patient. The P65L variant was present at a low frequency in individuals from all of the test and control groups (Table 2).

Homozygosity for *RYR1* mutations or variants associated with human MH is reported rarely. Homozygosity for R3772Q reported by Carpenter, et al. [22], resulted in a complex myopathic presentation in addition to susceptibility to MH. Others have reported the homozygous state of *RYR1* variants in association with recessive central core disease [27] and multiminicore disease [23, 28].

Unknown Myopathy Group—Eight of 392 patients (2%) with undiagnosed myopathies had mutations or variants in the *RYR1* gene.

Patient 5—A 37-year old Caucasian male presented with muscle pain, cramps, rhabdomyolysis, myoglobinuria and elevated serum CK. His muscle biopsy was histologically normal. The biopsy was sent to our laboratory for the Myoglobinuria Profile(http://www.rgbmgl.org/profiles.asp) and the EIP, both of which were negative. Microarray analysis revealed the R614C mutation in *RYR1* causative for MH. This patient was the only one of 392 undiagnosed myopathic patients with one of the 18 disease-causing mutations screened in *RYR1;* the mutation was not found in 133 controls. The symptoms in this patient were similar to those of the severe statin myopathy case (Patient 1) in whom we also found the R614C mutation. R614C is one of the 6 most common mutations in the RYR1 gene causing MH [29]. Functional defects for all 6 mutations were found in skeletal muscle myotubes, including reduced sensitivity to calcium and magnesium inhibition and inability of ryanodine receptor isoform-1 to be fully inactivated with calcium concentrations adequate in normal myotubes [29]. Abnormalities in skeletal muscle calcium regulation are characteristics common to MH and to exertional rhabdomyolysis [30]. The finding of the R614C mutation in 2 very similar cases of rhabdomyolysis with myoglobinuria among patients diagnosed with severe, non-anesthesia-induced myopathy is intriguing. The clinical features of these cases are similar to exertional rhabdomyolysis cases reported in the literature who were found to be MHS by CHCT [13, 31]. The main difference is that our patients were screened for causative mutations for various triggerable muscle diseases based on their symptoms and a molecular diagnosis of MH was made. There were no contributing episodes of MH or CHCT testing performed in these cases. The reported cases of exertional rhabdomyolysis were classified as MHS by contracture testing, in some cases following an MH episode, however, a causal relationship cannot be proven by CHCT testing alone since its specificity is only 78% [32]. In one of the studies, 10 of 12 patients with exertional rhabdomyolysis were positive for CHCT but mutations in *RYR1* were only found in 3 of these [31]. If we had found genetic variants in the *RYR1* gene in our patients that had not been fully characterized functionally as causative for MH, then additional testing, such as CHCT, would have been necessary to further determine their risk for MH. Because we

found known causative mutations in *RYR1* without additional MH testing, we can inform the patients that they are MHS.

Patient 6—A 4-year old Caucasian male presented with hypotonia and muscle weakness. The patient's DNA had been sent to the laboratory for analysis of the common mutations in the *PYGM* gene which proved to be negative. Further analysis showed the presence of the P4501L mutation in *RYR1* also found in patient 3. The significance of this finding in association with the patient's phenotype is unknown at this time and, in isolation, is likely minimal. This finding does not preclude the possibility that additional variants may be present in *RYR1* that were not included in the screening and that coexist with P4501L as indicated in reports of certain MH families [21]. No other mutations or variants were found among the additional genes evaluated.

Patient 7—A 33-year old African American male presented with fasting-induced muscle pain, cramps, stiffness and fatigue with rhabdomyolysis and elevated serum CK. He had an abnormal EMG, exercise intolerance and was diabetic. This patient also had the P4501L variant in *RYR1* found in patients 3 and 6. No other mutations or variants were found in this patient's DNA. Because this variant was not found in any of the controls and is associated discordantly with MHS, additional data should be gathered to determine its frequency, alone or with others variants, in myopathic individuals and in those with MHS compared with normal controls.

Patient 8—A 60-year old African-American male had symptoms of muscle pain, cramps, and weakness together with rhabdomyolysis and myoglobinuria. He also had an abnormal neurologic examination. The patient was originally referred for a Mitochondrial Myopathy Profile for the biochemical assessment of respiratory chain enzymes and the results were negative; an EIP analysis was also negative. The patient was the second participant in the study to be homozygous for the A1352G variant. The likelihood of 2 unrelated myopathic individuals to be homozygous for this variant in the same relatively small study would appear to be low considering the rarity of reports of homozygous *RYR1* variants, however, the potential impact on the respective patient phenotypes remains elusive and may be minimal. A second variant was found in Patient 8 in the *GDF8* gene encoding myostatin, a regulator of skeletal muscle size [33]. The variant, K153R, has a frequency in Caucasians of 3.3% [34]. The allele frequencies for this variant are significantly different between Caucasians and African Americans with the minor allele present at a 3 to 4-fold greater frequency in African Americans than in Caucasians [34]. Defects in *GDF8* are associated with hypertrophy of muscle [34]. The K153R variant is one of several nonsynonymous variants in *GDF8* that occurs in a highly conserved section of the coding region and is inherited as an incomplete dominant trait [34]. The allelic variants provide markers for examining associations between inter-individual variation in muscle mass and differences in loss of muscle mass with aging but have no effect on strength training in either Caucasians or African Americans [25]. The K153R variant has been associated with reduced muscle strength in women [35] and more recently with increased clinical severity of McArdle disease in women [36]. It is not clear at this time what role, if any, this variant may play in the myopathic phenotype described in this patient.

Patient 9—A 28-year old African-American male presented with muscle pain, cramps, weakness, rhabdomyolysis with myoglobinuria and elevated serum CK. He also experienced exercise intolerance. He was originally referred for mutation analysis of the *CPT2* gene which was negative. He was found to be a carrier for the A1352G variant in the *RYR1* gene as well as another variant, S1342G, located in close proximity to A1352G. S1342G is relatively common in African Americans (frequency of 4%; SNP database) and not highly

conserved among species [25]. We found both the A1352G and S1342G variants co-existing in 2 additional African American patients (Patients 11 and 12) and one Caucasian (Patient 10). It is possible that these variants may exist in linkage disequilibrium. Sambuughin, et al. [25] reported the coexistence of these variants in 2 of 6 African Americans with exertional rhabdomyolysis and MHS. Patient 9 also had the K153R variant in the *GDF8* gene as well as the harmless P65L polymorphism in the *VLCAD* gene and the common cosegregating mutations Q12X and P48L in the *AMPD1* gene. Carrier status for the Q12X and P48L mutations in *AMPD1* are very common occurring in 20% of the general population [37]. Carrier status alone does not contribute to a myopathic phenotype whereas homozygosity for these mutations does lead to myoadenylate deaminase deficiency which can result in a myopathic phenotype.

Patient 10—A 34-year old Caucasian female presented with muscle pain, weakness, fatigue and normal plasma CK. Her muscle biopsy was originally referred to the laboratory for a Mitochondrial Myopathy Profile. Significant partial reductions in enzymes of Complexes II-III and IV of the respiratory chain were found. The microarray screening for muscle disease mutations and variants detected the A1352G and S1342G variants in the *RYR1* gene.

Patient 11—A 41-year old African American male presented with muscle pain, cramps, fatigue, rhabdomyolysis and exercise intolerance. He was reported to have MH but no details were provided. The patient was originally referred for the EIP which was negative. By microarray analysis, he was found to have the A1352G and S1342G variants in *RYR1*, the K153R variant in *GDF8*, and a variant, E534K, in the *VLCAD* gene of unknown pathogenicity but found in 6% of general population (SNP database) with potentially low risk for causing disease.

Patient 12—A 45-year old African American male presented with muscle pain, cramps, stiffness, myoglobinuria, renal failure and elevated plasma CK. He also had elevated liver transaminase and aldolase. This patient was referred for *CPT2* and *PYGM* gene mutation analyses which were negative. This patient also was a carrier for the combined A1352G and S1342G variants in *RYR1* as well as the K153R variant in *GDF8*. He had no additional mutations or variants among genes analyzed. The combination of A1352G and S1342G in 4 patients in this study, 3 of whom were African American, suggests possible linkage disequilibrium between the variants with unknown phenotypic effects. Interestingly, all patients with the *GDF8* variant (Patients 8, 9, 11, 12) in this study also had the *RYR1* A1352G variant either alone or with S1342G. While all of these variants are relatively common in African Americans, there is no information on their frequencies in combination among healthy or myopathic African Americans or in other races. A role for these variants, if any, in genetic risk for statin myopathy and exercise-induced rhabdomyolysis in African Americans remains to be determined in larger test and control groups.

Statin-Tolerant Control Group—None of the *RYR1* mutations or variants was present in members of the statin-tolerant control group of 133 individuals of mixed ethnicities. Certain relatively common variants in other genes described in the test groups were found in this control group and are listed in Table 2.

The finding of *RYR1* disease-causing mutations and variants in individuals with severe and mild statin myopathy and none in the statin-tolerant control group suggests that *RYR1* mutations and selected variants should be added to the existing list of associations with risk for mild and severe statin myopathy [38]. Only 34 variants in RYR1 were analyzed in this study of which only 18 were proven to be disease-causing according to the European Malignant Hyperthermia Group (www.emhg.org). It will be important to repeat the

screening with all of the known MH-causing mutations in *RYR1* to determine if even more positive cases will be found. The finding of *RYR1* mutations and variants in the unknown myopathy group without anesthesia exposure suggests that cost-effective screening for the known disease-causing mutations in the *RYR1* gene should be considered in myopathy cases with symptoms of rhabdomyolysis and myoglobinuria.

There were several limitations to consider in this study. Our results provided identification of disease-causing mutations in the *RYR1* gene associated with myopathic disorders triggered by statins and other factors, however, we were unable to perform a comprehensive screening for all known disease-causing mutations in the *RYR1* gene and those not yet tested functionally for causation. Only 18 of the 30 EMHG listed disease-causing mutations in *RYR1* could be evaluated on this chip due to spatial constrictions. The technology limited the number of mutations that could be interrogated due to their close proximity to each other. Therefore, the prevalence of pathogenic mutations in *RYR1* and other genes studied may have been underestimated from this study. Using the Goldengate platform, generation of high quality genotype calls depends on the production of accurate cluster profiles for each genotype (homozygous major allele, homozygous minor allele, and heterozygous for both alleles). SNPs with a higher polymorphic frequency produce the most accurate clusters. SNPs with a low or rare polymorphic rate require the analysis of a greater number of samples to determine accurate profiles. Without accurate cluster profiles it is extremely difficult to determine the rare genotype calls expected in this study from random noise produced in the assay. Limitations in the microarray platform not only did not allow for mutation saturation within certain genes of interest but also did not accommodate deletion mutations for analysis. These limitations may have lowered the yield of positive outcomes. The technology clearly works most effectively in the analysis of highly polymorphic variants of low effect and not as effectively in the analysis of rare variants of high effect. The limitations of the platform necessitated sequence analysis for the validation of all positive findings. We found that all of the positive controls in the analysis were validated, however, there were false positives among the unknowns (not among *RYR1* variants), i.e., apparent homozygous individuals for mutant alleles that were actually heterozygous. It was not feasible to perform comprehensive sequence analysis for all variants in search of possible false negatives.

The data presented in this report are limited to findings for relatively small groups of several hundred patients collected from around the U.S. and Canada who were found to have mutations and variants in the *RYR1* gene together with additional genetic variants in other genes in some cases. There is the possibility that DNA samples with multiple rare variants may be more common in select populations in the country suggesting that any perceived clinical effects may be more population driven than phenotype driven. Findings for statin myopathy patients and clinical unknown cases evaluated on this microarray platform for variants or mutations in other genes, not including *RYR1* gene variants, will be reported separately and categorized by primary disease state in a similar format as presented for malignant hyperthermia.

The value of a multiplexed approach was demonstrated in detecting disease mutations and additional genetic variants that may have never been sought in statin myopathy cases or other unknown myopathy cases without the multiplex microarray platform. Most patients specifically referred to the laboratory for a particular indication proved to be negative on testing. A search for coexisting mutations and variants among many different genes associated with muscle disease is a pursuit rarely taken in traditional diagnostic laboratory studies. The significance of these findings, the potential synergy between variants, and the impact on phenotype of co-existing mutations and variants will be explored further using

different technology more amenable to inclusivity and allow for the successful interrogation of rare mutations of high impact.

The finding that mutations and variants in the *RYR1* gene were present in patients with either statin-induced myopathy or undetermined myopathies without anesthesia exposure was significant considering that none of 133 normal controls had any of the variants or mutations studied in the *RYR1* gene. This suggests that patients with severe statin myopathy as well as those with unknown myopathies characterized by elevated plasma CK, rhabdomyolysis and myoglobinuria should be screened for *RYR1* mutations and selected variants in addition to gene mutations causative for other metabolic myopathies.

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Table 1

RYR1 Mutations and Variants Evaluated by Microarray Analysis n=34

EMHG-1*	EMHG-2**	OTHER1***	OTHER2****
C35R	L13R	R1043C	S1342G
R163C	M226K	S3217P	A1352G
G248R	R367L	R3772W	E2880K
G341R	D544Y	I4938T	G3806R
I403M	R2336H		P4501L
Y522S	R2676W		
R614C	D2730G		
R614L			
V2168M			
T2206M			
A2350T			
G2375A			
R2435H			
R2458C			
R2458H			
T4826I			
R4861H			
I4898T			

* Mutations functionally proven to be disease-causing by the European Malignant Hyperthermia Group (EMHG)

** Variants that have met certain EMHG criteria for pathogenicity but are still being validated

*** Variants recently reported as associated with MHS but not yet validated as disease-causing

**** Variants recently reported that are likely to be neutral polymorphisms

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Table 2

Non-MH Cases with *RYR1* Gene Mutations and Variants (n=12)

Type/ID[#]	Race/Age/Gend	*RYR1* Mutations[*]	& Varian	Otsther Gene Variant	Lab Tests Completed	Muscle Symptoms
SEVERE STATIN MYOPATHY (n=197)						
1	C/47/F	R614C[*]		none	EIP(−)	Cramps, stiff, rhabdo, myogl, elev CK, persistent pain, weakness
2	C/66/M	R3772W		none	MMP(−), CPT(−)	Pain, cramps, elev CK
3	AA/?/M	P4501L		none	EIP(−)	pain, persistent elev CK
MILD STATIN MYOPATHY (n=163)						
4	AA/52/M	A1352G[#]		*VLCAD* P65L	EIP(−)	Weak
UNKNOWN MYOPATHY (n=392)						
5	C/37/M	R614C*		none	MYGL(−), EIP(−)	pain, cramps, myogl, rhabdo, elev CK normal biopsy
6	C/4/M	P4501L		none	*PYGM* (−)	hypotonia, weak
7	AA/33/M	P4501L		none	EIP(−)	diabetes, fasting-induced pain, cramps fatigue,stiff, exerc intol, rhabdo, elev CK, abnormal EMG
8	AA/60/M	A1352G[#]		*GDF8* K153R	MMP(−), EIP(−)	diabetes, pain, weak, cramps, myogl abnormal neurologic exam
9	AA/28/M	A1352G + S1342G		*VLCAD* P65L *GDF8* K153R *AMPD1* Q12X;P48L	*CPT2*(−)	pain, cramps, weak, myogl, elev CK, exer intol
10	C/34/F	A1352G + S1342G			MMP (SCR & COX def)	pain, weak, fatigue, normal CK
11	AA/41/M	A1352G + S1342G		*GDF8* K153R *VLCAD* E534K	EIP(−)	pain, cramps, fatigue, exerc intol, rhabdo, MH
12	AA/45/M	A1352G + S1342G		*GDF8* K153R	*CPT2*(−), *PYGM(−)*	pain, cramps, stiff, myogl, renal failure, elev CK, transamin, aldolase

STATIN-TOLERANT CONTROLS (n=133)[**]

No *RYR1* gene mutations or variants found

GDF8 K153R(3.8%)

VLCAD P65L (1.5%)

VLCAD E534K (1.5%)

AMPD1 all mutations (24.8%)

[**] Figures in parentheses represent % of total in statin-tolerant group; C, Caucasian; AA, African American; MMP, mitochondrial myopathy profile; CPT, carnitine palmitoyltransferase enzyme; *CPT2*, carntine palmitoyltransferase II gene; EIP, Exercise Intolerance Mutation Profile; SCR, succinate cytochrome c reductase; COX, cytochrome c oxidase; MYGL, Myoglobinuria Profile; myogl, myoglobinuria; stiff, muscle stiffness; rhabdo, rhabdomyolysis; EMG, electromyography; *PYGM*, myophosphorylase gene; transamin, elev.liver enzyme; exerc intol, exercise intolerance; Weak, muscle weakness; MH, malignant hyperthermia; *AMPD1*, myoadenylate deaminase gene; *VLCAD*, very long-chain acyl-CoA dehydrogenase gene; *GDF8*, myostatin gene.

#Patients 4 and 8 are homozygous for the A1352G variant

*disease-causing mutations in RYR1 gene