Research Article

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Pharmacogenomics

RYR1 and *CACNA1S* genetic variants identified with statin-associated muscle symptoms

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Aim: To examine the genetic differences between subjects with statin-associated muscle symptoms and statin-tolerant controls. **Materials & methods:** Next-generation sequencing was used to characterize the exomes of 76 subjects with severe statin-associated muscle symptoms and 50 statin-tolerant controls. **Results:** 12 probably pathogenic variants were found within the *RYR1* and *CACNA1S* genes in 16% of cases with severe statin-induced myopathy representing a fourfold increase over variants found in statin-tolerant controls. Subjects with probably pathogenic *RYR1* or *CACNA1S* variants had plasma CK 5X to more than 400X the upper limit of normal in addition to having muscle symptoms. **Conclusions:** Genetic variants within the *RYR1* and *CACNA1S* genes are likely to be a major contributor to the susceptibility to statin-associated muscle symptoms.

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Statin-associated muscle symptoms (SAMS) occur in 10 [1] to 25% [2] of patients taking statins. Severe muscle symptoms induced by statin therapy occur in a small percentage of patients 0.1–0.5% [3]. Although genetic association studies have identified some possibly associated gene loci [4–7], these have not been reproducible in additional studies with independent patient cohorts [8,9]. The *SLC1OB1* variant, in particular, has only been found to be associated with SAMS in patients with high dose simvastatin and not consistently associated with different statins or lower doses of simvastatin [10,11].

A more reasonable explanation for susceptibility to SAMS is the presence of rare pathogenic variants in genes important for skeletal muscle structure and function. In support of this, an increased incidence of pathogenic variants in the *CPT2* and *PYGM* genes causing metabolic myopathies has been reported in patients with SAMS [12]. There are a number of genes associated with metabolic myopathies triggered by various factors such as extreme exercise, fasting, extremes in temperature, flu and exposure to volatile anesthetics [13]. In addition, many of the genes causing congenital myopathies, myofibrillar myopathies and muscular dystrophies have overlapping phenotypes with metabolic myopathies [14–16]. We propose that statins act as an additional trigger inducing muscle symptoms and a subset of patients with severe SAMS have causative genetic variants within genes associated with malignant hyperthermia susceptibility (MHS) and congenital myopathy.

A patient with statin myopathy has been previously reported to have a variant in *RYR1* known to be causative for MHS (Vladutiu 2011). Transgenic mice expressing *RYR1* with a known MH associated variant were found to be more susceptible to adverse responses to simvastatin [17]. Genetic defects causative of MHS have only been found in

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RYR1, the α-1-subunit of the dihydropyridine-sensitive L-type voltage-dependent calcium channel (*CACNA1S*) [18] and *STAC3* [19]. In addition to MHS, both *RYR1* and *CACNA1S* have been associated with other myopathic conditions. Defects in the *RYR1* gene are one of the most common causes of muscle disease [20] with a suspected prevalence of *RYR1* gene causative variants in the general population of 1 in 2–3000 [21,22]. *RYR1* variants have been classically associated with autosomal dominant forms of central core disease (CCD; MIM 117000) and MHS (MIM 145600) [23]. Further study and more extensive sequence analyses of the *RYR1* gene has revealed the existence of autosomal recessive *RYR1* variants associated with muscle disease as well [24–26]. A greater variability in phenotypes has been found including multi/minicore myopathies (MIM 255320 and 602771), King–Denborough syndrome, limb girdle myopathy, core-rod myopathy, bent spine myopathy, exertional [27], statin-induced [28] myopathies and asymptomatic CK elevation [29]. More recently, variants in the *RYR1* and *CACNA1S* genes have been associated with exertional heat stroke (EHS) and a positive response to the *in vitro* contracture test [30,31]. EHS shares certain characteristics with MHS in that both are hypermetabolic states with elevated body temperature triggered by environmental stressors and resulting in increased cytoplasmic calcium; the phenotypes vary in that EHS results in neurological dysfunction leading to coma in severe cases while MHS is usually limited to myopathy.

In this study, we have sequenced the exomes of 76 cases with severe SAMS with abnormally elevated plasma creatine kinase (CK) and 50 statin-tolerant controls. We have analyzed the resultant data using a disease model in which rare pathogenic variants, possibly within multiple genes, are causative of SAMS. In this report, we describe variants in the *RYR1* and *CACNA1S* genes. 12 probably pathogenic variants were detected in these genes in the SAMS cases and two were found in statin-tolerant controls. Additional probably pathogenic variants were also found in other muscle disease-associated genes in many of the SAMS cases with *RYR1* or *CACNA1S* variants.

Methods

Study subjects

A total of 748 subjects (392 males) with a history of statin therapy were enrolled in a retrospective case–control study from five medical centers across the USA and Canada between 2004 and 2013 and representing 20 states and provinces in the USA and Canada. Collaborating institutions included the Johns Hopkins Myositis Center, MD, USA; Cedars-Sinai Medical Center, CA, USA; the Medical College of Wisconsin, WI, USA; McMaster University Medical Center; and the University of Oklahoma College of Medicine, Tulsa, OK, USA. A standardized classification of SAMS integrates all muscle-related symptoms (e.g., pain, weakness or cramps) as 'muscle symptoms', which are then placed in subgroups depending on the presence or absence of elevated plasma CK. Pain and weakness in typical SAMS are usually symmetrical and proximal, and generally affect large muscle groups. The myalgias and weakness typically occur within 4–6 weeks after starting statin therapy, however, they may occur after many years of treatment [32]. The SAMS group was comprised of 634 individuals (340 males) and a statin-tolerant control group of 114 individuals (52 males) who continued to take statins for at least 12 months without muscle symptoms. Individuals were classified as having SAMS based on their responses to a questionnaire outlining the statin type and dosage that first led to myopathic symptoms, the date of onset of symptoms, the extent of muscle symptoms during therapy and the duration of symptoms post-cessation of statin therapy. Additional information was collected pertaining to personal or family history of heart disease, muscle disease and other co-existing medical conditions. Statin-tolerant controls completed a follow-up questionnaire 6 and 12 months post initial enrollment to confirm that statin tolerance was maintained. 26 subjects of Caucasian, Euro-American descent (19 males, mean age 57 years) of 634 individuals in the SAMS group were selected for whole exome sequencing (WES) using specific selection criteria. All had severe muscle pain and/or weakness attributed solely to statin therapy; plasma creatine kinase (CK) levels >5-times the upper limit of normal (ULN); and all were <65 years of age when muscle symptoms began. More than 70% had prolonged symptoms post therapy for at least 6 months. An additional 50 subjects (30 males); 81% Caucasian; 4% Asian; 15% African–American (mean age: 62 years) with severe SAMS were chosen for whole genome sequencing (WGS). These were selected primarily based on severity of muscle symptoms and a plasma CK level >5X ULN. One subject did not have muscle symptoms but had a CK level of 12X ULN and was included. DNA from 50 subjects (20 males, mean age: 63 years, 100% Caucasian, Euro-American) from the statin-tolerant control group were also submitted to WES.

DNA sequencing

Genomic DNA was purified from blood or saliva specimens with Puregene or OraGene protocols, respectively. Whole exome sequencing was performed on 26 of the samples with the Illumina HiSeq instrument and Nimblegen

V2 exome enrichment kits in Dr Deborah Nickerson's laboratory at the Northwest Genome Center in Seattle, WA, USA. Sequence data was processed to generate the VCF file as described by Kim *et al.* [33]. WGS was carried out on 50 of the SAMS cases by the New York Genome Center (NYGC; NY, USA) with the Illumina HiSeq. WES was run on 50 statin-tolerant controls with the Trueseq exome capture kit (Illumina). Sequencing was performed using the Illumina HiSeq. Each sample was run in eight lanes.

Data processing

This includes sequence alignment (for WGS and WES control sequence data only, WES case data were obtained as BAM files from the Northwest Genome Center and used directly for variant calling, see below), sorting alignment by genomic coordinates and marked PCR duplicates, followed by variant calling. All of these were performed using the high performance computing cluster hosted at the Center for Computational Research, University at Buffalo. To align the raw fastq sequences to the reference assembly, we aligned the fastq files generated for each lane from the Illumina platform against the human_g1k_v37_decoy.fasta (download from **https://software.broadinstitute.org/gatk/download/bundle**) using BWA-MEM and provided read group (ID, Library, Sample, etc.) information that was extracted from sequence file names. The resulting bam files are sorted by genomic coordinates and subsequently merged to obtain one bam file per sample using Picard (v.1.131), with PCR duplicates marked during this step. For variant calling, we used an in-house developed variant calling pipeline utilizing modules from the Genome Analysis Tool Kit (GATK, v3.6, Broad Institute) with parameters according to their best practice documentation. Briefly, the bam files are subjected to base quality recalibration, joint genotyping using 'HaplotypeCaller' and 'GenotypeGVCFs.' Raw variants are filtered using the 'Variant Quality Score Recalibration' tool and output as a multisample VCF file for each of the sequencing cohort (WGS, WES case or WES control).

Coverage analysis of the sequencing runs showed in all cases that exon 91 had relatively low coverage (Supplementary Figure 1), which is consistent with no data being presented for this region in the ExAC database. The greatest overall coverage was obtained with whole exome sequencing of the statin-tolerant control group ensuring that there was no bias toward identifying more genetic variants in the case group than in the control group.

Variant annotation

VCF files from above are used as input for variant annotation using Annovar (1 February, 2016 version) [34]. We annotated variants for population frequencies (ExAC, 1000 Genome Project Phase III, ESP6500), pathogenic predictions (SIFT, PolyPhen2, CADD, REVEL), functional consequences, disease associations (ClinVar, GWAS catalog) – among others.

Candidate variants selection

Annotated VCF files were used as input to populate the Genomic Data Warehouse (GDW) developed at the Center for Computational Research, University at Buffalo. It is a database tool aimed at facilitating genomic variant data storage as well as fast and efficient retrieval for variants satisfying user defined filtering criteria. To search for candidate variants, three steps are needed, in other words, select dataset, set/select filters and select attributes/field names to return. Specifically, we used the following filtering criteria to obtain our list of candidate disease associated variants: RefGene IDs: NM_000540, NM_000069 and the cases and controls were also screened for a list of 114 genes associated with myopathy compiled by Abath Neto [16]. The list was also limited to minor allele frequency (MAF) in ExAC All populations: <0.02. For attributes/field names to return, we included chromosome, chromosomal position, reference allele, alternate allele, variant type, GATK VQSR score, dbSNP ID, gene, exon, cDNA change, amino acid change, REVEL score, dbscSNV_ADA score, dbscSNV_RF score, MAF in the ExAC All populations database, ClinVar clinical significance and sample ID.

Variants were categorized into five classes of pathogenicity based on the type of variant, frequency in the general population from the ExAC database and reports in the literature and the HGMD, ClinVar and Leiden Muscular Dystrophy databases. As previously reported [35], variant effect prediction algorithms do not correctly predict the effect of all *RYR1* and *CACNA1S* variants, both pathogenic and benign. This is shown in Supplementary Table 1 with the 44 established *RYR1* and *CACNA1S* pathogenic variants that have been functionally demonstrated to cause MHS (emhg.org). More than half of the 44 known MHS causing mutations are predicted to be tolerated by the SIFT algorithm. Consensus prediction algorithms integrating several different individual algorithms have been found to be more accurate [36,37]. We have used REVEL, which integrates prediction scores from 13 different

individual algorithm tools [38] and accurately predicts all of the EMHG mutations to be pathogenic. The REVEL score does, however, incorrectly predict one of the benign common *CACNA1S* variants, G258D, examined by Schiemann and Stoell [35] as pathogenic (Supplementary Table 2) and also incorrectly predicts the pathogenicity of a number of the variants identified in this study that are too frequent in the population to be pathogenic, such as *RYR1* K1393R, R1679H, R3539H, H3647Q and D4505H (Table 1) and *CACNA1S* G258D, S606N and R683C (Table 2). While the REVEL score cannot be relied upon to be completely accurate, it is the most accurate prediction approach currently available.

Outside of *in silico* predictions, two criteria hold for all of the EMHG established mutations; they are absolutely conserved in sequence comparisons across species (Supplementary Figures 2 & 3) and they have a very low frequency in the general population. The most frequent of the mutations, T2206M and R2355W, were only present in the ExAC database with a MAF = 0.00003. Based on this consideration, we have set <0.0001 MAF in the ExAC database as the lowest frequency to be considered as a potentially pathogenic mutation. Class 5, pathogenic variants (mutations), have <0.0001 MAF, have been reported more than once in disease cases and have not been identified in unaffected individuals, are nonsense or splicing variants or nonsynonymous variants with REVEL scores >0.5 and absolutely conserved in interspecies amino acid comparisons. Class 4, probably pathogenic variants, have <0.0001 MAF, are nonsense or splicing variants or nonsynonymous variants with REVEL scores >0.5, affect amino acid residues that are absolutely conserved in interspecies amino acid comparisons and have not been previously reported. Class 3, uncertain pathogenicity, have REVEL scores >0.5, <0.0001 MAF, introduce nonconservative amino acid substitutions into residues that are not absolutely conserved in interspecies comparisons, but have only conservative, structurally similar substitutions in other species. Class 2, likely benign variants, have <0.001 MAF and are nonsynonymous variants that may have failed to meet all of the criteria of strong algorithmic predictions, sequence conservation or complete segregation with disease cases. Class 1, benign variants (polymorphisms), include synonymous variants and nonsynonymous variants with >0.001 MAF.

All class 3, 4 or 5 genetic variants identified by exome or genome sequencing were verified by bidirectional Sanger sequencing. Sequencing was performed with the BigDye Terminator v3.1 cycle sequencing kit (ABI) and an ABI 3500 Genetic Analyzer. Oligonucleotide primer sequences used for PCR amplification and sequencing are available upon request.

Results

In the examination of the *RYR1* and *CACNA1S* genes in 76 genomic DNA samples from subjects with severe SAMS, 66 rare variants (<0.02 MAF in the ExAC database) were found compared with 22 in the control group of 50 statin-tolerant subjects (Tables 1 & 2). Categorization of these variants into five classes of pathogenicity resulted in one class 5, pathogenic variant, nine class 4, probably pathogenic variants and two class 3, variants of uncertain pathogenicity in the SAMS cases. In the statin-tolerant controls, there was one class 4 and one class 3 variant. Three of the variants in *RYR1* have been previously reported as causative of congenital myopathy (G893S [39,40], R2676W [41]) or MHS (E2404K, R2676W [42]). By our classification scheme, R2676W, was ranked class 5, E2404K was ranked class 3 and G893S was categorized as class 2, probably benign.

Case 2236: a 56-year-old Caucasian male with severe SAMS presented with muscle pain, weakness and rhabdomyolysis, plasma CK of 8000 IU/dl and was diagnosed with inflammatory myositis (Table 3). The subject had coronary artery disease and a history of a previous heart attack. He had been taking simvastatin (80 mgs) at the time muscle symptoms began. After stopping statin therapy, his symptoms persisted for 25 months. The subject was found to have a probably pathogenic variant, R2676W, in the *RYR1* gene which has been previously reported in a family study as a novel variant associated with MHS and the presence of multiminicores in muscle biopsies from MHS family members [41]. In that study, the R2676W variant as well as an additional variant, T2787S, on the same allele segregated completely with disease in 19 family members studied by genetic testing. R2676W has also been reported in an unrelated MHS patient along with three additional *RYR1* variants, A1352G, T2787S and P4501L [42]. Arg2676 is absolutely conserved in interspecies sequence comparisons (Supplementary Figure 2) and the R2676W variant is extremely rare, 0.000008 MAF in the ExAC database. Thr2787 is also absolutely conserved but the variant T2787S is too frequent in the general population (0.003 MAF) to be considered pathogenic. The co-occurrence of this variant along with the R2676W in unrelated MHS patients by both Guis *et al.* [41] and Levano *et al.* [42] is suggestive of a possible role of the T2787S influencing the effect of the R2676W variant; however, Thr2787 is located 25 Å from Arg2676 and would not have an obvious effect. The T2787S variant was not detected in the sample containing R2676W in this study.

Table 1. *RYR1* variants <0.02 minor allele frequency.

Chr	Position (hg19)	cDNA change	aa change	Exon	Class	REVEL	Conserved	dbscSNV_ADA	dbscSNV_RF	ExAC ALL	Sample ID
***RYR1* variants in statin myopathy cases**											
19	38937141	c.C661T	p.L221F	exon8	4	0.726	Yes	None	None	0	2155
19	38948227	c.C1882T	p.R628C	exon17	4	0.745	Yes	None	None	2.47E-05	2048
19	38948268	c.C1923G	p.T641T	exon17	1	None	Yes	0.5625	0.336	0.0009	2063
19	38949859	c.G2241A	p.L747L	exon19	1	None	Yes	None	None	0.0014	1443, 2157
19	38954162	c.G2677A	p.G893S	exon21	2	0.837	Yes	None	None	0.0006	2157
19	38956779	c.C2919T	p.H973H	exon24	1	None	Yes	None	None	0.0021	2168
19	38958397	c.G3326A	p.R1109K	exon25	1	0.088	Yes	None	None	0.0017	1443, 2157
19	38960067	c.A3679G	p.I1227V	exon27	2	0.364	Yes	None	None	8.26E-06	2170
19	38964109	c.T3858C	p.L1286L	exon28	1	None	No	None	None	0.0149	2048, 2074, 2157
19	38964317	c.G4066A	p.A1356T	exon28	2	0.157	No	None	None	0	2063
19	38964322	c.C4071T	p.P1357P	exon28	1	None	Yes	None	None	0.0006	2074
19	38966001	c.C4204T	p.P1402S	exon29	4	0.563	Yes	None	None	0	2198
19	38968484	c.G4428C	p.G1476G	exon30	1	None	Yes	None	None	0	2257
19	38973933	c.A4711G	p.I1571V	exon33	1	0.56	Yes	None	None	0.0013	2168
19	38973941	c.G4719A	p.P1573P	exon33	1	None	Yes	None	None	0.003	1443, 2157
19	38974154	c.C4932T	p.N1644N	exon33	4	None	No	0.9923	0.898	0	2196
19	38976770	c.C5475A	p.H1825Q	exon34	2	0.285	No	None	None	0	2162
19	38979903	c.G5634C	p.E1878D	exon35	1	0.061	No	None	None	0.0014	2074
19	38985101	c.C6384T	p.Y2128Y	exon39	1	None	Yes	None	None	0.0032	2074
19	38985215	c.C6498T	p.L2166L	exon39	1	None	Yes	None	None	0.0021	2157
19	38987117	c.G6732A	p.R2244R	exon41	1	None	Yes	None	None	0.001	2063
19	38990456	c.C7209T	p.R2403R	exon44	1	None	No	None	None	0.0154	2162
19	38990457	c.G7210A	p.E2404K	exon44	3	0.676	No	None	None	2.85E-05	2168
19	38990632	c.C7299T	p.L2433L	exon45	1	None	Yes	None	None	6.61E-05	2077
19	38991600	c.C7584T	p.P2528P	exon47	1	None	Yes	None	None	0.0142	2078
19	38993269	c.G7737A	p.V2579V	exon48	1	None	No	None	None	0.0029	2257
19	38993279	c.C7747T	p.L2583L	exon48	1	None	Yes	None	None	3.35E-05	2059
19	38994925	c.C7992T	p.F2664F	exon50	1	None	No	None	None	4.13E-05	2189
19	38994959	c.C8026T	p.R2676W	exon50	5	0.601	Yes	None	None	8.25E-06	2236
19	38996014	c.G8376A	p.R2792R	exon53	1	None	Yes	None	None	0.0048	2152
19	38998362	c.G8827A	p.D2943N	exon58	2	0.725	Yes	None	None	0.0009	2074
19	39001154	c.T8949C	p.S2983S	exon59	1	None	Yes	None	None	0.0012	2074
19	39001380	c.C9081T	p.S3027S	exon60	1	None	Yes	None	None	4.94E-05	2214
19	39003103	c.A9452G	p.Q3151R	exon63	4	0.684	Yes	None	None	0	2189
19	39009954	c.G10119A	p.V3373V	exon67	1	None	Yes	None	None	0.0007	1406
19	39016132	c.G10616A	p.R3539H	exon71	1	0.877	Yes	None	None	0.0018	2264
19	39018329	c.C10729T	p.R3577W	exon73	2	0.344	No	None	None	0.0001	2261
19	39018347	c.G10747C	p.E3583Q	exon73	1	0.32	No	None	None	0.0149	2216, 2242
19	39019242	c.C10941G	p.H3647Q	exon75	2	0.509	Yes	None	None	0.001	2074
19	39026667	c.G11547A	p.Q3849Q	exon82	1	None	Yes	None	None	0.0168	2145, 2221, 2223
19	39057597	c.C13484T	p.P4495L	exon92	2	0.244	No	None	None	5.18E-05	2024
19	39057598	c.G13485A	p.P4495P	exon92	1	None	No	None	None	0.0001	2157
19	39061267	c.T13680C	p.F4560F	exon94	1	None	Yes	None	None	0.0007	2261
19	39070762	c.G14505A	p.G4835G	exon100	1	None	Yes	None	None	0.0034	1443
***RYR1* variants in statin-tolerant controls**											
19	38943526	c.G1312A	p.E438K	exon13	3	0.73	No	None	None	1.15E-05	1017
19	38948268	c.C1923G	p.T641T	exon17	1	None	Yes	0.5625	0.336	0.0009	1167
19	38948886	c.C2121A	p.G707G	exon18	1	None	Yes	None	None	0.002	1410

Table 2. *CACNA1S* variants <0.02 minor allele frequency.

Chr	Position (hg19)	cDNA change	aa change	Exon	Class	REVEL	Conserved	dbscSNV_ADA	dbscSNV_RF	ExAC ALL	Sample ID
***CACNA1S* variants in statin myopathy cases**											
1	201079385	c.G165A	p.T55T	exon2	1	None	Yes	None	None	0.0003	2157
1	201079298	c.C252T	p.L84L	exon2	1	None	No	None	None	0.0017	2157
1	201058428	c.C858T	p.Y286Y	exon6	1	None	Yes	None	None	0.0061	2078, 2184, 2257
1	201054624	c.C1090T	p.R364W	exon8	3	0.554	No	None	None	1.65E-05	2214
1	201047078	c.G1548A	p.S516S	exon11	1	None	No	None	None	0.0094	2155, 2179, 2239
1	201044689	c.G1882A	p.G628S	exon13	4	0.742	Yes	None	None	8.24E-06	2142
1	201043702	c.C1995T	p.A665A	exon14	1	None	Yes	None	None	0.0072	2155, 2179, 2239
1	201043650	c.C2047T	p.R683C	exon14	1	0.6	Yes	None	None	0.0031	2185
1	201042742	c.2089_2091del	p.E697del	exon15	4	None	Yes	None	None	0	2157
1	201038705	c.C2385T	p.I795I	exon18	1	None	No	None	None	0	2339
1	201038636	c.G2454A	p.A818A	exon18	1	None	No	None	None	0.0018	2194
1	201038610	c.T2480C	p.M827T	exon18	1	0.316	No	None	None	0.0042	2239
1	201035429	c.C2673T	p.S891S	exon21	1	None	No	None	None	0.0121	2257
1	201035035	c.C2784T	p.I928I	exon22	1	None	No	None	None	0.0017	2179
1	201029939	c.A3261G	p.Q1087Q	exon26	1	None	Yes	None	None	0.0088	2066, 2143, 2181
1	201028425	c.C3417G	p.H1139Q	exon27	4	0.804	Yes	None	None	0	1394
1	201020172	c.A4053G	p.T1351T	exon33	1	None	Yes	None	None	0.0014	2179
1	201017811	c.G4340A	p.R1447Q	exon36	4	0.502	Yes	0.9847	0.868	8.28E-05	2024
1	201012542	c.G4915C	p.E1639Q	exon40	2	0.429	No	None	None	0	2155
1	201012503	c.C4954T	p.R1652C	exon40	2	0.366	No	None	None	0.0003	2078
1	201012449	c.T5008A	p.Y1670N	exon40	2	0.234	No	None	None	0.0002	2189
1	201009404	c.T5325C	p.N1775N	exon43	1	None	No	None	None	4.18E-05	2024
***CACNA1S* variants in statin-tolerant controls**											
1	201058513	c.G773A	p.G258D	exon6	1	0.784	Yes	None	None	0.0074	1010, 1088, 1121
1	201046058	c.G1817A	p.S606N	exon12	1	0.521	Yes	None	None	0.0088	1167
1	201043637	c.C2060A	p.S687Y	exon14	2	0.435	No	None	None	0	1072
1	201029939	c.A3261G	p.Q1087Q	exon26	1	None	Yes	None	None	0.0088	1113
1	201013506	c.G4747A	p.E1583K	exon39	2	0.681	Yes	None	None	0.0002	1237
1	201009011	c.G5570A	p.S1857N	exon44	1	0.339	No	None	None	0.0019	1113, 1147

This subject also has a nonsynonymous variant, G178R (REVEL = 0.741, MAF = 0.0002), in the *ISPD* gene which is predicted to affect splicing. *ISPD* variants have been associated with congenital muscular dystrophy-dystroglycanopathy with brain and eye anomalies type A7 [43,44].

Case 2155: a 34-year-old Caucasian male with severe statin myopathy presented with muscle pain and a plasma CK of 7500 IU/dl. A history of heart disease was absent in this subject. The subject was taking simvastatin (20 mg) when symptoms began and persisted for 3 weeks post therapy. The subject had a probably pathogenic variant, L221F, in the *RYR1* gene. The L221F variant is located within the N-terminal disease hotspot of *RYR1*, has been previously unreported and is not present in the ExAC database. There are several disease causative nonsynonymous variants located close to Leu221 including T214M [31,45], G215E [46,47], V218I [23,47], R220C [48], M226K [23] and D227V [49]. The Leu221 residue is absolutely conserved in inter-species comparisons and the L221F substitution

Table 3. Clinical features of individuals with probably pathogenic variants in *RYR1* and *CACNA1S*.

ID	Sex/Race	Gene	Variant	Sx Onset (y)	Skeletal muscle symptoms			CK (IU/l)	Statin (mg)	Pers Sx (mos)	Cardiac symptoms			Other features				
Pain	Weakness	Inflam				CAD	Ht Attack	Fam Hx Dis	Hypothy	Hypertens	Diabetes	Obesity	Other					
Test Subjects																		
2236	M/Cauc	*RYR1*	R2676W	56	Y	Y	Y	8000	ATOR (80)	25	Y	Y	N	N	N	N	N	Rhabdo
2155	M/Cauc	*RYR1*	L221F	34	Y	N	N	7500	SIM (20)	0.75	N	N	N	N	N	N	N	
2048	M/Cauc	*RYR1*	R628C	77	N	Y	N	3000	ATOR (10)	>3	N	N	N	N	N	Y	N	Fam Hx MD
2189	M/Cauc	*RYR1*	Q3151R	37	Y	N	N	35,000	ATOR (NA)	24	N	N	N	N	N	N	N	Elev liv enz
2198	F/Cauc	*RYR1*	P1402S	60	Y	N	Y	1074	SIM (10)	0	N	N	Y	Y	N	N	N	
2196	M/Trindad	*RYR1*	N1644N	56	Y	Y	N	1600	ROSUV (25)	NA	Y	Y	Y	Y	Y	Y	N	
1394	M/Cauc	*CACNA1S*	H1139Q	60	Y	N	Y	1000	ATOR (20)	0	N	N	N	N	Y	Y	Y	Smoker
2142	M/Cauc	*CACNA1S*	G628S	63	Y	Y	N	78,000	ROSUV (20)	NA	N	N	N	N	N	N	N	Rhabdo/ Myogl
2024	M/Cauc	*CACNA1S*	R1447Q	80	N	N	N	2200	SIM (40)	Ongoing	N	N	N	N	Y	N	N	Liv dis
2157	F/AA	*CACNA1S*	E697del	61	Y	Y	N	23,000	SIM (40)	3	N	N	N	N	N	N	N	Rhabdo
2214	F/First Nation	*CACNA1S*	R364W	60	N	Y	N	5590	ATOR (10)	>12	N	N	Y	N	Y	Y	Y	Smoker
2168	M/Mauritius	*RYR1*	E2404K	46	Y	Y	N	1300	ATOR (NA)	4+	Y	Y	N	N	Y	N	N	Liv dis
Control subjects				**Age entering study**														
1007	M/Cauc	*RYR1*	R1035W	49	N	N	N	67	LOVA (40)	N	N	N	N	N	N	N	N	
1017	M/Cauc	*RYR1*	E438K	49	N	N	N	145	ATOR (10)	N	N	N	N	N	N	N	N	

Table 3 describes 12 subjects with severe statin myopathy and class 3–5 variants in the *RYR1* or *CACNA1S* genes; 2 of 50 statin-tolerant controls also had class 3 or 4 variants. The age of onset of symptoms in the myopathic group ranged from 34 to 80 years. All myopathic subjects had plasma CK >5X ULN.

AA: African–American; ATOR: Atorvastatin; CAD: Coronary artery disease; Cauc: Caucasian; CK: Creatine kinase; Dis: Disease; Elev: Elevated; Enz: Enzymes; Exerc Intol: Exercise intolerance; F: Female; Fam: Family; Ht: Heart; Hx: History; Hypertens: Hypertension; Hypothy: Hypothyroidism; Inflam: Inflammation; Liv: Liver; M: Male; MD: Myotonic dystrophy; mos: Months; Myogl: Myoglobinuria; N: No; NA: Not available; Pers: Persistent; Rhabdo: Rhabdomyolysis; ROSUV: Rosuvastatin; SIM: Simvastatin; Sx: Symptoms; y: Years; Y: Yes.

is predicted to be damaging by REVEL. This is within a region of RyR1 with high-resolution crystal structure information [47]. Modeling based on this structure indicates that Leu221 is located at the interface between N-terminal domains A and B and the L221F substitution is predicted to destabilize this interface (Supplementary Data).

This individual also had rare (MAF <0.001), REVEL-predicted deleterious nonsynonymous variants in three additional myopathy associated genes, *CAPN3* (M666T), *PLEC* (R102L) and *MYH2* (A111V). Variants in *CAPN3* cause limb-girdle muscular dystrophy type 2A [50]. *PLEC* variants cause limb-girdle muscular dystrophy type 2Q [51]. Variants in *MYH2* cause congenital myopathy [52,53].

Case 2048: a 77-year-old Caucasian male with statin myopathy presented with muscle weakness only and a plasma CK of 3000 IU/dl. He was taking atorvastatin (10 mgs) at the time symptoms began and reported unexpected weight loss 3 months before stopping statin therapy. Muscle weakness was ongoing post-therapy for at least 3 months. The subject had diabetes and a family history of myotonic dystrophy; however, myotonic dystrophy was ruled out genetically in this individual. He was found to have a probably pathogenic variant, R628C, in the *RYR1* gene. R628C is a previously unreported very rare (MAF = 0.00002) *RYR1* variant, absolutely conserved in inter-species sequence comparisons and strongly predicted to be deleterious by *in-silico* analysis (REVEL = 0.745). Modeling of this region of RyR1 based on structures of Yuchi *et al.* [54] suggests that the R628H variant would lead to the destabilization of FKBP12 binding (Supplementary Data).

This individual also had a nonsynonymous variant in the *CRYAB* gene, G154S, REVEL = 0.507, MAF = 0.0008. Variants in *CRYAB* have been associated with myofibrillar myopathy [55,56].

Case 2189: a 37 year-old Caucasian male presented with statin-induced muscle pain and a plasma CK of 35,000 IU/dl at its highest post-exercise. He was taking atorvastatin (dose unknown) at the time of symptom onset and symptoms persisted 24 months post-therapy. He had exercise intolerance and abnormally elevated liver enzymes. For 6 years post therapy he continued to have aches in his muscles primarily in the quadriceps. The subject had a family history of heart disease without a personal history. The subject was found to have a probably pathogenic variant, Q3151R, in the *RYR1* gene and a co-existing Class 2 variant, Y1670N, in the *CACNA1S* gene. Q3151R has not been reported and is not in the ExAC database. Q3151R is located in helical domain 2 [57] and could play a role in destabilizing the helical region. Gln3151 is absolutely conserved in inter-species comparison and is predicted to be deleterious (REVEL = 0.684).

Case 2198: a 60-year-old Caucasian female presented with muscle pain and a plasma CK of 1024 IU/dl 4 days after initiating simvastatin (10 mgs) therapy; symptoms did not persist post-therapy, although the subject self-reported having inflammatory myositis. The subject had a family history of heart disease and a personal history of hypothyroidism. The subject had a probably pathogenic variant, P1402S, in the *RYR1* gene that has not been reported and is not found in the ExAC database. Pro1402 is absolutely conserved in interspecies comparisons and P1402S is predicted to be deleterious (REVEL = 0.563). Pro1402 is located in the SPRY3 domain and is not well resolved in available structural models.

This subject also had a nonsynonymous variant in *MYH7*, R1475C (REVEL = 0.659, MAF = 0.00008). Genetic variants in *MYH7* are primarily causative of hypertrophic cardiomyopathy [58], but have also been associated with Laing distal myopathy and myosin storage myopathy [59].

Case 2196: a 56-year-old male from Trinidad presented with muscle pain, cramps stiffness, muscle swelling and weakness with a plasma CK of 1600 IU/dl while taking rosuvastatin (25 mgs). He had originally taken atorvastatin 15 years earlier that made pre-existing muscle aches worse. His symptoms persisted for 10 years at which time he began rosuvastatin therapy. The subject had many pre-existing conditions including coronary artery disease, a previous heart attack, diabetes, hypertension, hypothyroidism and a family history of heart disease and muscle disease. The subject had a probably pathogenic variant, N1644N, in the *RYR1* gene. This synonymous variant was not present in the ExAC database and is predicted to cause aberrant splicing (dbscSNV_ADA score = 0.9923, dbscSNV_RF score = 0.898). It is categorized as Class 4 since it is a predicted splicing defect but has not been demonstrated experimentally.

This individual also had a nonsynonymous variant in *DYSF*, E1471K (REVEL = 0.651, MAF = 0.0003), that is predicted to affect splicing. *DYSF* variants cause two main types of muscle disease, Myoshi myopathy and limb-girdle muscular dystrophy type 2B [60,61].

Case 1394: a 60-year-old Caucasian male presented with muscle pain primarily in his arms and a plasma CK of 1000 IU/dl while taking atorvastatin (20 mgs). The subject self-reported dark colored urine that may be indicative of unconfirmed myoglobinuria. He reported having diabetes, hypertension and a family history of inflammatory

muscle disease in his father and brother. A probably pathogenic variant, H1139Q, was found in the *CACNA1S* gene. H1139Q is very rare, not present in the ExAC database and strongly predicted to be deleterious (REVEL = 0.804). This subject also had a nonsynonymous variant, R1064W (REVEL = 0.716, MAF = 0.0002) in *COL6A3*. Collagen VI-related disorders are caused by variants in *COL6A1, COL6A2* and *COL6A3* and include Bethlem myopathy and congenital Ullrich muscular dystrophy [62,63].

Case 2142: a 63-year-old Caucasian male presented with pain and weakness predominantly in his arms and calves with rhabdomyolysis and a plasma CK of 78,000 IU/dl occurring within 3 months of taking rosuvastatin (20 mgs). The subject reported no personal or family history of heart disease or muscle disease. A probably pathogenic variant, G628S, was found in the *CACNA1S* gene. G628S is rare (MAF = 8×10^{-6}), Gly628 is absolutely conserved and G628S is strongly predicted to be deleterious *in silico*.

Case 2024: an 80-year-old Caucasian male presented with an elevated plasma CK of 2200 IU/dl without apparent muscle pain or weakness while initially taking simvastatin (40 mgs) which was changed to pravastatin (40 mg). Since the subject did not have a baseline CK measured before starting statins, it is unclear whether he had pre-existing elevations of CK, however, CK decreased after stopping statins altogether. A probably pathogenic variant, R1447Q, was found in the in the *CACNA1S* gene. R1447Q is rare (MAF = 0.00008), Arg 1447 is absolutely conserved and R1447Q is strongly predicted to be deleterious *in silico*. This subject also had a nonsynonymous variant in *ENO3*, K193N (REVEL = 0.591, MAF = 8×10^{-6}). Muscle ENO3 deficiency has been associated with exercise intolerance and rhabdomyolysis [64].

Case 2157: a 61-year-old African–American female presented with muscle aches and burning pain as well as muscle weakness and rhabdomyolysis with dark-colored urine and plasma CKs exceeding 23,000 IU/dl while taking simvastatin (40 mgs). In addition, she reportedly had hypertension, liver disease and renal failure. A probably pathogenic variant, E697del, was found in the *CACNA1S* gene with a co-existing Class 2 variant, G893S, in the *RYR1* gene. E697del is extremely rare, not present in the ExAC database and Glu697 is absolutely conserved in interspecies amino acid sequence comparisons. Glu697 is located in the region of CACNA1S linking repeats II and III. These loop regions, particularly residues within the II–III loop, have been shown to be important for excitation–contraction coupling [65,66]. While the *RYR1* variant, G893S, has been reported in patients with congenital myopathy [39,40], it has also been identified in general population samples [67]. The identification of variants in the general population does not necessarily rule out potential pathogenicity since nonmanifesting patients with known established pathogenic *RYR1* variants have been previously reported. For example, in one study [68], a patient with the known MHS causative *RYR1* variant, R614C, did not have a family history of disease and did not have adverse reactions to previous surgical anesthesia. Gly893 is absolutely conserved across species and the G893S variant is strongly predicted to be damaging by all variant effect prediction algorithms; however, we have categorized G893S as class 2, likely benign, because of the relatively high frequency (0.0006 MAF) of this variant in the ExAC database. Furthermore, although the location of Gly893 is clearly resolved in structural studies [54], the alteration to a serine residue is not obviously predicted to influence other residues. Subject 10 also had a nonsynonymous variant in *AGRN*, G719S (REVEL = 0.56, MAF = 0.0005). *AGRN* variants have been associated with myasthenia syndrome and muscle weakness [69].

Case 2214: a 60-year-old First Nation female presented with muscle weakness in the absence of muscle pain and a plasma CK of 5590 IU/dl while taking atorvastatin (10 mgs). Her symptoms persisted for more than 12 months post-therapy with CKs remaining in the range of 3500–5000 until she was given prednisone with subsequent normalization of CK. She was eventually tested for HMG-CoA reductase autoantibodies and found to be positive with unknown significance. She had hypertension, diabetes, obesity and there was a family history of heart disease. A class 3 variant was found in the *CACNA1S* gene, R364W, that is rare (MAF = 2×10^{-6}) and predicted to be deleterious (REVEL = 0.554). In interspecies amino acid comparisons only the conservative change to Lys is observed in distant species. Arg364 is in the linker region between CACNA1S repeats I and II.

Case 2168: a 46-year-old Mauritian male developed muscle pain and weakness with a plasma CK of 1300 IU/dl while taking atorvastatin (unknown dose). He had persistent symptoms post therapy for more than 4 months. The subject had a history of hypertension, a previous heart attack, coronary artery disease and liver disease of unspecified etiology; there was no family history of heart disease. A variant (E2404K) was found in the *RYR1* gene that has been previously reported once in a MHS patient [42]. E2404K is located within the central hotspot region of *RYR1*, is rare (<0.00003 MAF) and strongly predicted to be deleterious (REVEL = 0.676). Characterization of lymphoblastoid cells containing DNA from patients with the E2404K variant show increased resting calcium levels and an enhanced response to caffeine [42]. We have categorized E2404K as class 3, of uncertain pathogenicity, since Glu2404 is not

completely conserved across species. Levano *et al.* [42] mentioned that it was interesting that this residue was also Lys in the homologous *RYR2* gene, however, in our alignment of human *RYR1* and *RYR2* amino acid sequences, Glu2404 is altered to Thr in *RYR2*. Subject 12 also contained a probably pathogenic nonsynonymous variant in *DYSF*, D467E (REVEL = 0.549), that is not present in the ExAC database.

Control 1007. A 49-year-old Caucasian male had no muscle symptoms while taking lovastatin (40 mgs) and had a plasma CK of 67 IU/dl. The subject was taking lovastatin for 4 months before entering the study. He reported no personal or family history of heart disease or other related risk factors. A probably pathogenic variant, R1035W, was found in the *RYR1* gene. R1035W is rare (MAF = 0.00001), absolutely conserved and strongly predicted to be deleterious (REVEL = 0.791). At 6 weeks and 6 months follow-up, the subject continued to be asymptomatic with no change in his statin medication.

Control 1017. A 49-year-old Caucasian male had no muscle symptoms while taking atorvastatin (10 mgs). There was no personal or family history of heart disease or other cardiovascular risk factors. The subject had a plasma CK of 145 IU/dl. A probably pathogenic variant, E438K, was identified in the *RYR1* gene. This variant is located within the N-terminal disease hotspot region and is both rare and predicted to be deleterious *in silico*. We have classified E438K of uncertain pathogenicity, class 3, because Glu438 is not completely conserved across species, although only the conservative aspartate substitution is found in other species at this residue.

Discussion

Of 76 subjects with clinically severe SAMS and whose genomic DNA was analyzed by WES or WGS, 12 (16%) had probably pathogenic nonsynonymous variants in either the *RYR1* gene or the *CACNA1S* gene using stringent *in silico* criteria. Since 44 variants in these genes have been functionally and genetically proven to be causative for malignant hyperthermia as determined by the European Malignant Hyperthermia Group (EMHG.org), two criteria met by all 44 EMHG variants were used as a basis in the assignment of pathogenicity to variants in this study. These included absolute sequence conservation across species and a very low frequency in the general population (MAF <0.0001). In addition, REVEL scoring, which integrates prediction scores from 13 different algorithm tools was used with a cutoff of >0.50 for pathogenicity [38]. It is possible that we have left out variants that are actually pathogenic using these strict criteria as many had REVEL scores >0.50 and/or were very rare but, considering all factors, were predicted as being Class 2 variants (e.g., D943N in the *RYR1* gene with a REVEL score of 0.725 and MAF 0.0009) (Table 1). The statin-tolerant controls carrying probably pathogenic variants should still be monitored for muscle symptoms during statin therapy over time since, by definition, 'awake state' phenotypes caused by pathogenic variants in MH-causing genes must be initiated by environmental triggers including but not limited to anesthesia, exertion, heat stroke and statin therapy [28,70]. The two positive controls may be at risk for MHS, but may also have other genetic variants or lifestyle habits that make them less susceptible to statin side effects or the dose of statin taken did not exceed a threshold for triggering muscle symptoms in these cases.

Clusters of pathogenic variants or hotspots have been identified in three regions of the *RYR1* gene: in the N-terminal region, the central region and the C-terminal region [23,71]. Additional *RYR1* pathogenic variants are increasingly being identified outside of the predominant hotspots [39]. *RYR1* pathogenic variants causative of MHS only or of both MHS and CCD are generally gain of function mutations and result in calcium leakage [72,73]. *RYR1* mutations causative of CCD without MHS are loss of function variants and primarily located in the C-terminal region of RyR1 [74,75]. *RYR1* mutations causative of MHS with mild pathology or in asymptomatic individuals are mostly located in the N-terminal and central region hotspots. *RYR1* nonsynonymous variants identified here in this group of SAMS cases were primarily located within the N-terminal region of RyR1 consistent with a disease mechanism similar to MHS.

Structural data is available for the overall RyR1 tetramer from cryo-EM experiments [57,76–78]. Some regions of RyR1 have been expressed and used to obtain higher resolution crystal structures such as the N-terminal hotspot [47,54,79,80]. Many of the nonsynonymous *RYR1* variants identified in this study were not in regions of RyR1 that have sufficient resolution to look for possible structural or functional effects. However, substitutions L221F, R628C, R2676W and Q3151R were in regions that were highly resolved.

L221F is located within the N-terminal hotspot region. The N-terminal region contains domains A, B and C [79], also referred to as β-trefoil domains 1 and 2 and part of the α-solenoid 1 [81]. Docking of high-resolution crystal structures of the N-terminal region, residues 1-559 of RyR1, with lower resolution cryo-EM structures has shown that the N-terminal region is located at the central rim of RyR1 and form a cytoplasmic vestibule [79]. Interactions between domains within the N-terminal region in the closed state appear to be disrupted by the transition to an

open state [47,79]. A disease mechanism has been proposed for pathogenic variants in the N-terminal region in which MHS pathogenic variants disrupt interactions between N-terminal domains and act as gain of function mutations that favor the transition to an open state and increased calcium release [47,79].

R628C in RyR1 falls within a region predicted by docking studies to contact FKBP12 (Supplementary Data). FKBP12 binding has been shown to promote the closed state of RyRs and reduce the occurrence of subconductance states [82]. Because of this, variants that interfere with FKBP binding have been proposed to cause RyR Ca^{++} leakage and associated disease states [54]. The precise binding site for FKBP12 is not completely understood, perhaps due to the large allosteric changes that occur within the RyR N-terminal regions [54].

Limitations of this study include aspects of its retrospective design such as the acquisition of limited or incomplete clinical details for review and summary for individual subjects. Another limitation is that because of our stringent selection of the probably pathogenic variants, we likely have left behind other important variants among the remaining 54 found with MAF <0.02 in the ExAC database. These should not be discounted in future studies.

All of the subjects with probably pathogenic *RYR1* or *CACNA1S* variants also had co-existing variants of unknown significance within these genes that are too common in the general population to be individually pathogenic. The association of disease with *RYR1* variants of predicted pathogenicity, but with frequencies too high to be considered as pathogenic have not been well studied, particularly when occurring along with other known deleterious variants. Likewise, the predicted pathogenic rare variants in other muscle disease genes (*DYSF, CAPN3, MYH2, MYH7* and others) found in subjects with variants in *RYR1* or *CACNA1S* may be partially, synergistically or completely responsible for the manifesting symptoms, however, we have no way of testing this possibility.

Conclusion

Probably pathogenic variants in the *RYR1* and *CACNA1S* genes were found to be significant potential contributors to risk for severe SAMS. Previously unreported, predicted pathogenic variants were found in the *RYR1* and *CACNA1S* genes in 16% of the severe SAMS cases examined and may explain their susceptibility to statin therapy.

All of the subjects with probably pathogenic *RYR1* or *CACNA1S* variants also have less rare co-existing variants of unknown significance within these genes as well as probably pathogenic variants within other genes associated with myopathic phenotypes. Clinicians should be mindful of the severity and longevity of muscle symptoms in patients with SAMS as well as plasma CK levels ≥4X ULN and consider in severe cases molecular testing for triggerable myopathies known to cause rhabdomyolysis.

Future Perspectives

Continued study of variants in *RYR1* and *CACNA1S* at both the clinical and experimental level will contribute to a better understanding of their contributions to disease. Further, the identification and characterization of additional genes and pathogenic variants contributing to SAMS will lead to better therapeutic strategies for patients requiring cholesterol-lowering treatment.

Summary points

- Probably pathogenic variants in the *RYR1* and *CACNA1S* genes are prominent in 16% of cases with statin-associated muscle symptoms representing a fourfold increase over statin-tolerant controls.
- Structural modeling of selected variants found in the *RYR1* gene demonstrated a range of predicted alterations associated with disease including disruption of the RYR1 receptor from the closed to open state and interference of FKBP binding causing calcium leakage.
- Subjects with *RYR1* or *CACNA1S* variants had plasma CK 5X- to more than 400X upper limit of normal as well as muscle pain and/or weakness. Several had co-existing conditions including cardiac symptoms, hypertension or diabetes.
- Genetic variants within the *RYR1* and *CACNA1S* genes are likely to be major contributors to susceptibility to statin-associated muscle symptoms.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at:
https://www.futuremedicine.com/doi/suppl/10.2217/pgs-2018-0106

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