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Investigating the genetic susceptibility to exertional heat illness

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Abstract

Background: We aimed to identify rare (minor allele frequency ≤1%), potentially pathogenic non-synonymous variants in a well-characterised cohort with a clinical history of exertional heat illness (EHI) or exertional rhabdomyolysis (ER). The genetic link between malignant hyperthermia (MH) and EHI was investigated due to their phenotypic overlap.

Methods: The coding regions of 38 genes relating to skeletal muscle calcium homeostasis or exercise intolerance were sequenced in 64 patients (mostly military personnel) with a history of EHI, or ER and who were phenotyped using skeletal muscle *in vitro* contracture tests. We assessed the pathogenicity of variants using prevalence data, *in silico* analysis, phenotype and segregation evidence and by review of the literature.

Results: We found 51 non-polymorphic, potentially pathogenic variants in 20 genes in 38 patients. Our data indicate that *RYR1* p.T3711M (previously shown to be likely pathogenic for MH susceptibility) and *RYR1* p.I3253T are likely pathogenic for EHI. *PYGM* p.A193S was found in 3 EHI patients, which is significantly greater than the control prevalence (P = 0.000025). We report the second case of EHI in which a missense variant at *CACNA1S* p.R498 has been found. Combinations of rare variants in the same or different genes are implicated in EHI.

Conclusion: We confirm a role of *RYR1* in the heritability of EHI as well as ER but highlight the likely genetic heterogeneity of these complex conditions. We propose defects, or combinations of defects, in skeletal muscle calcium homeostasis, oxidative metabolism and membrane excitability are associated with EHI.

Keywords: Exertional heat illness, genetics; rhabdomyolysis; skeletal muscle, calcium signalling, membrane potential, oxidative phosphorylation; *RYR1*; *CACNA1S; PYGM*

Introduction

Exertional heat illness (EHI) is characterised by an inability to thermoregulate during physical activity.[1] It is a cause of sudden death in young athletes [2] and an important occupational hazard, particularly in the military.[3] There is consensus that there is a genetic predisposition to EHI [4] but limited data to confirm the involvement of specific genes or even the underlying genetic model. Clinical features can include hyperthermia, nausea, tachycardia, metabolic and respiratory acidosis, muscle cramps, rhabdomyolysis, elevated serum creatine kinase (CK), cerebral dysfunction, seizures, multi-organ failure, disseminated intravascular coagulation and death.[4] Rhabdomyolysis is a frequent manifestation of EHI but can also develop in the absence of hyperthermia, when it is termed exertional rhabdomyolysis (ER).[5] On exposure to sufficient heat stress anyone can develop EHI and there are well known "environmental" factors [3] that lower heat tolerance, principally by impairing heat dissipation. In 1991, we reported evidence for an inherited skeletal muscle abnormality in two EHI cases with no predisposing environmental factors:[6] we used *in vitro* pharmacological challenge tests to identify the skeletal muscle abnormality in the index cases and first degree relatives. These *in vitro* contracture tests (IVCTs) have high sensitivity to detect malignant hyperthermia (MH) susceptibility,[7] a form of heat illness exclusively seen during general anaesthesia, but also give non-specific abnormal responses in some other muscle disorders.[8-10] Over the past 15 years the Institute of Naval Medicine (INM) has conducted a standardized heat tolerance test (HTT) on UK military personnel who have experienced EHI resulting in hospital admission.[11] The HTT is able to identify individuals who fail to thermoregulate despite unimpaired heat dissipation, i.e., they generate excessive heat during exercise. Such individuals are referred for IVCT to assess their risk of developing MH and/or having an underlying skeletal muscle abnormality to explain their predisposition to EHI. It is these individuals, with unexplained and often recurrent cases of EHI, that present the greatest likelihood of harbouring a genetic predisposition to the condition.

Because of the similarities between MH and EHI in clinical and IVCT phenotypes, strategies for identifying genetic variants associated with EHI have borrowed from knowledge of the genetics of MH susceptibility. To date, three genes, *RYR1*, *CACNA1S* and *STAC3,* have been implicated in MH susceptibility. These genes encode two skeletal muscle Ca^{2+} channels (RyR1 and $Ca_v1.1$) and Stac3, which regulates the RyR1-$Ca_v1.1$ complex.[7,8] We estimated that heterozygous *RYR1* variants were implicated in ~75% of MH cases, heterozygous *CACNA1S* variants in ~2%, a homozygous *STAC3* variant in a small number of cases of African or Middle Eastern descent, while the remainder involve other genes, or non-coding variants, yet to be identified.[12] Our study reinforced earlier data [13-15] that the genetics of

MH may be best explained by a threshold oligogenic model rather than the originally presumed Mendelian autosomal dominant model. More than 200 heterozygous non-synonymous *RYR1* variants have been identified in MH families, but only 50 have been functionally characterised (www.emhg.org). There are two EHI cases in which *RYR1* variants proven to be pathogenic in MH susceptibility have been found.[16,17] However, our previous report of *RYR1* and *CACNA1S* sequencing in EHI [18] did not produce definitive confirmation for a role of either of these genes in EHI. On the other hand, *RYR1* variants have been reported in around 30% of individuals presenting with rhabdomyolysis, including ER.[5]

In this study we investigate genetic variants of a large cohort of EHI and ER patients whose clinical, HTT and IVCT phenotypes have been determined. We use a next generation sequencing approach using a custom panel of 38 genes including *RYR1*, *CACNA1S*, genes encoding additional subunits of the $Ca_V1.1$ complex and other genes that either encode further proteins involved in skeletal muscle excitation-contraction (E-C) coupling and Ca^{2+} regulation, or because they have been implicated in conditions associated with exercise intolerance.

Materials and Methods

Patients and Samples

Our cohort comprised patients referred to the UK Malignant Hyperthermia Investigation Unit in Leeds for genetic testing and IVCTs after presenting clinically with EHI or ER. Research ethics approval was granted by Leeds (East) Research Ethics Committee or its predecessors (10/H1306/70) and patients provided written consent.

The clinical details were obtained from general practitioner referral information, copies of hospital records when available, reports from the INM (for military personnel), witness accounts and a patient history. The criteria for military personnel to be referred to the INM (and therefore included in this study depending on HTT assessment) were: 1) clinical symptoms or signs suggestive of EHI (Supplementary Methods) requiring admission to hospital because of central nervous system disturbance or biochemical evidence of organ damage (including rhabdomyolysis); 2) more than one episode of suspected EHI, irrespective of the severity. Patients whose clinical features were limited to myalgia, cramps, or fatigue, with no evidence of hyperthermia and who had biochemical evidence of rhabdomyolysis (creatine kinase, CK, > 1,200 $IU.L^{-1}$, which is > 5x the upper limit of normal provided for all samples) were categorised as cases of ER. All other presentations were categorised as EHI. Non-military cases met the same clinical criteria, as did military personnel who were referred

for IVCT prior to implementation of the HTT by the INM. The protocol and interpretation of the INM HTT has been described by House et al.[11] Patients who failed the HTT on more than one occasion were referred for IVCT as were patients who passed the HTT but had rhabdomyolysis as the sole or major presenting feature. IVCTs were conducted according to the standardized European Malignant Hyperthermia Group (EMHG) protocol.[7] In brief, the EMHG IVCT protocol describes tests in which freshly excised skeletal muscle biopsies are maintained in a tissue bath under physiological conditions and exposed to increasing concentrations of halothane (inhalation anaesthetic) or caffeine. Abnormal responses are defined by an increase in resting muscle tension (a contracture) of $\geq$ 0.2 g at $\leq$ 2% halothane or $\leq$ 2 mM caffeine. The EMHG laboratory classification defines MH susceptibility according to abnormal responses to both halothane and caffeine (MHShc) or to just halothane (MHSh) or caffeine (MHSc). Patients whose muscle responds normally to both halothane and caffeine are MH negative (MHN). Relatives of patients tested MH susceptible by IVCT are subsequently offered IVCT. Where available, samples from family members of MH susceptible EHI/ER patients were sequenced alongside those of the index patients, to determine whether any genetic variants co-segregated with IVCT phenotypes in their families.

Targeted next-generation sequencing

DNA was extracted from peripheral whole blood samples using a salt precipitation method described previously.[18] DNA concentrations were measured using the Quant-iT™ dsDNA High-Sensitivity Assay kit (Invitrogen™), according to manufacturer's instructions and fluorescence was measured using a FLUOstar Galaxy microreader (BMG LABTECH) (excitation/ emission at 480/530 nm). All gDNA samples were diluted in Tris-EDTA (10 mM Tris-HCl, 1 mM disodium EDTA, pH 8.0) to a working concentration of 5 ng/µl for HaloPlex™ target enrichment.

HaloPlex™ SureDesign was used to create a custom oligonucleotide probe library to target the exons of the 38 genes (Supplementary table 1). HaloPlex™ target enrichment (Agilent Technologies, Santa Clara, CA, USA) was performed according to the manufacturer's protocol. The system relies on a combination of restriction digestion, customised probe hybridisation, magnetic bead capture, and PCR amplification to create a targeted HaloPlex™ library for downstream sequencing applications. Molar concentrations of enriched target DNA (175-625 bp) were calculated and equimolar amounts of each HaloPlex™ library (100 fmol) were combined into a single indexed pool for sequencing. The enriched HaloPlex™ libraries were sequenced using Illumina's MiSeq® platform, producing 150-bp paired-end reads.

Sequence data were analysed using SureCall software (Agilent Technologies). Non-synonymous variants with read depth ≥10 were identified and annotated with minor allele frequency (MAF) from the Exome Aggregation Consortium (ExAC) database.[19] *In silico* pathogenicity scores were calculated using the Combined Annotation Dependent Depletion (CADD) tool v1.3.[20] Non-synonymous variants identified in EHI patients with a MAF ≤1% (in each ExAC cohort) and a C-score of ≥15 were selected for further investigation. Recurrent gaps in *RYR1* coverage (Supplementary table 2) were sequenced using an NGS long-PCR approach described previously.[18] These data were analysed using the same pipeline as the HaloPlex™ data.

Variant evaluation

Variants were evaluated using the relevant categories of evidence set out in the guidelines of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG-AMP) for the interpretation of sequence variants [21]. We did not attempt to score the pathogenicity of variants using the ACMG-AMP guideline because of the limitations of the generic guideline, especially for genetically heterogeneous conditions that may not be monogenic [22-24]. Instead we combine the evidence from clinical and laboratory (HTT and IVCT) phenotypes, segregation data, review of the literature, frequency data from our cohort and public databases (ExAC), and *in silico* analyses in a narrative overview of relevant variants. For *in silico* analysis we used Gene-Aware Variant INterpretation (GAVIN) [25]. This algorithm uses gene-specific data to determine C-score cut-off values that predict a pathogenic or benign variant and a MAF cut-off value to predict a benign variant. We classified a variant as pathogenic if the C-score was greater than the GAVIN cut-off for pathogenicity and the MAF was less than the GAVIN threshold. A variant was classified as benign if the C-score was less than the GAVIN cut-off for benign status. An uncertain classification was assigned to any other combination of C-score and MAF. For genes where gene-specific cut-offs could not be determined using GAVIN, we applied the genome-wide GAVIN thresholds of C-score <15 for benign, C-score > 15 for pathogenic and MAF > 0.00426 for benign. Thresholds for relevant genes are shown in Supplementary table 3.

For variants that were recurrent in the EHI/ER cohort and were significantly more prevalent in this cohort than in the ExAC database (see below) we used Sanger sequencing to screen for the presence of the variant in samples from 285 low risk controls from the UK population (European Collection of Authenticated Cell Cultures, Sigma-Aldrich Company Ltd, Gillingham, United Kingdom).

Statistical analyses

We compared the incidence of variants recurrent in our cohort to their prevalence in the ExAC database using a chi-squared test accepting a *P* value < 0.001 to indicate significance (Bonferroni correction for the total number of variants found, n=51). To explore the possibility that rarer variants in our genes of interest might be enriched in our EHI/ER cohort, we selected variants in each gene with a sample prevalence < 0.001 (MAF < 0.0005) in the ExAC database. We then compared the total number of patients in our cohort with the total number of samples in the ExAC database carrying such variants in each gene using a chi-squared test: a *P* value < 0.00131 was used to indicate significance (Bonferroni correction for the number of genes investigated, n = 38). *P* values are presented uncorrected throughout.

Results

Clinical, HTT and IVCT details

Of 64 patients, 59 were military personnel referred by the INM, while five were civilians referred to the Leeds MH Unit after presenting clinically with EHI. The subjects were predominately male, young and physically fit due to their military occupation. Of the 58 individuals who underwent HTT, five (3 ER cases and 2 EHI cases with rhabdomyolysis) were able to effectively thermoregulate but were referred to the MH Unit because of the severity of rhabdomyolysis during their index clinical event. A further EHI case (#1) passed the HTT after one episode of EHI but subsequently had another episode of EHI. Descriptions of the clinical reactions are summarised in Table 1, from which it can be seen that our patients presented with a spectrum of clinical features.

Table 1: Clinical features of the exertional heat illness/exertional rhabdomyolysis cohort.

ID	IVCT	EHI/ER episodes	Location	Core temp (ºC)	Conscious level[1]	CK (IU/L)	Complications	Other factors	HTT
1	MHShc	2 EHI	UK	39.9ºC	Unconscious	2,184	Clotting derangement-	-	Passed[2]
2	MHShc	2 EHI	1 UK, 1 deployed[3]	41ºC	Unconscious	-	Abnormal LFT	-	Failed
3	MHShc	1 EHI	UK	42ºC	Unconscious	-	AKI-	-	Failed
4	MHShc	1 EHI	UK	39.6 ºC	Normal	48,986	-	-	Failed
5	MHSh	1 EHI	UK	41ºC	Unconscious	35,000	-	-	N/A
6	MHSh	2 EHI	1 UK, 1 Germany	39 ºC -	Unconscious	-	-	-	Failed
7	MHSh	1 EHI	UK	40.7ºC	Unconscious	11,000	-	-	Failed
8	MHSh	1 EHI	UK	-	Altered	15,000	-	-	Failed
9	MHSh	2 EHI	Deployed[3]	-	Unconscious	-	-	-	Failed
10	MHSh	1 EHI	Deployed[3]	-	Normal	725	Hyponatraemia, AKI	chicken pox	Failed
11	MHSh	2 ER	UK	-	Normal	> 200,000	AKI	-	Passed
12	MHSh	1 EHI	UK	40.7ºC	Unconscious	>100,000	Clotting derangement, AKI	-	Failed
13	MHSh	2 EHI	UK	-	Unconscious	-	-	-	Failed
14	MHSh	1 EHI	UK	39.1ºC	Unconscious	> 50,000	AKI, myocardial ischaemia		Failed
15	MHSh	2 ER	UK	-	Normal	>10,000	-	-	N/A
16	MHSh	1 ER	UK	-	Normal	6,658	-	-	Passed
17	MHSh	2 ER	UK	36.8 ºC	Altered	27,000	-	-	Passed
18	MHSh	1 EHI	UK	-	Altered	-	-	-	Failed
19	MHSh	2 EHI	Deployed[3]	-	Altered	-	-	-	Failed
20	MHSh	1 EHI	UK	-	Altered	820	-	-	Failed
21	MHSh	2 EHI	UK	-	-	-	-	-	Failed
22	MHSh	2 EHI	UK	-	Altered	7,899	AKI	-	Failed
23	MHN	1 EHI	UK	-	Unconscious	30,700	AKI	Alcohol consumption	N/A
24	MHN	1 EHI	UK	38.6 ºC[4]	Altered	-	-	-	Failed
25	MHN	1 EHI	UK	41.5 ºC	Unconscious	-	-	-	Failed
26	MHN	1 EHI	UK	38.9ºC	Altered	15,000	-	Dehydration	Failed
27	MHN	2 EHI	UK	-	Unconscious	> 20,000	-	-	Failed
28	MHN	1 EHI	UK	> 40 ºC	Unconscious	39,718	Multi-organ failure	-	N/A
29	MHN	1 EHI	UK	39.5ºC	Unconscious	63,000	AKI	-	N/A
30	MHN	3 EHI	Deployed[3]	-	Unconscious	-	-	-	Failed
31	MHN	1 EHI	UK	42ºC	Unconscious	-	-	Alcohol consumption	Failed
32	MHN	3 EHI	Deployed[3]	-	Unconscious	-	-	Extreme heat	Failed
33	MHN	6 EHI	UK, Deployed[3]	hyperthermia[5]	Unconscious	-	-	-	Failed
34	MHN	1 EHI	UK	41.7ºC	Unconscious	5,510	AKI, altered LFT & clotting	-	Failed
35	MHN	1 EHI	UK	-	Unconscious	-	Altered LFT	-	Failed
36	MHN	1 EHI	UK	-	Normal	1,596	Hyponatraemia	Dehydration	Failed
37	MHN	1 EHI	Germany	41.6 ºC	Unconscious	-	-	Dehydration	Failed
38	MHN	3 EHI	UK	40ºC	Altered	32,000	-	-	Failed
39	MHN	1 EHI	UK	41.2 ºC	Altered	myoglobin 595 μg/L	AKI	-	Failed
40	MHN	2 EHI	UK	-	Altered	-	-	-	Failed
41	MHN	1 EHI	Deployed[3]	40ºC	Altered	-	-	Extreme heat	Failed

42	MHN	1 EHI	UK	hyperthermia[5]	Altered	60,000	-	-	Passed
43	MHN	2 EHI	Deployed[3]	hyperthermia[5]	-	-	-	Extreme heat	Failed
44	MHN	2 EHI	Deployed[3]	38.7 ºC[4]	Unconscious	-	-	-	Failed
45	MHN	1 EHI	Germany	hyperthermia[5]	Unconscious	-	rhabdomyolysis, AKI, hepatic failure	-	Failed
46	MHN	1 EHI	Deployed[3]	-	Unconscious	-	-	-	Failed
47	MHN	2 EHI	Deployed[3]	37.4 ºC[4]	Unconscious	232	-	-	Failed
48	MHN	1 EHI	UK	>40ºC	Unconscious	10,000	Seizure, AKI	-	Failed
49	MHN	1 EHI	UK	44ºC	Unconscious	>10,000	-	-	Failed
50	MHN	1 EHI	UK	-	Unconscious	-	-	-	Failed
51	MHN	2 EHI	1 UK, 1 deployed[3]	-	Altered	-	-	-	Failed
52	MHN	1 EHI	UK	-	Unconscious	3,848	AKI	-	Failed
53	MHN	1 EHI	UK	-	Altered	4,196	Compartment syndrome	-	Failed
54	MHN	1 EHI	UK	38.5ºC	Altered	1,289	-	-	Failed
55	MHN	1 EHI	UK	39ºC (Aural)	Unconscious	24,210	AKI		Passed
56	MHN	1 EHI	UK	39.6ºC	Altered	2,073	-	-	Failed
57	MHN	1 ER	UK	-	Normal	47,000	AKI	-	Failed
58	MHN	1 EHI	UK	-	Unconscious	>10,000	-		Passed
59	MHN	1 EHI	UK	40ºC	Altered	4,836	-	-	Failed
60	MHN	1 EHI	UK	-	Altered	-	-	-	Failed
61	MHN	1 EHI	UK	42ºC	Unconscious	7,000	AKI	-	Failed
62	MHN	1 EHI	Deployed[3]	-	Normal	-	-	-	Failed
63	MHN	1 EHI	UK	40ºC	Unconscious	>100,000	Seizures, multi-organ failure, DIC, cardiac arrest	-	N/A
64	N/A	1 EHI	UK	42.5ºC	Altered	-	-	Alcohol consumption	N/A

This table provides information about *in vitro* contracture test (IVCT) status (MHShc, abnormal responses to halothane and caffeine; MHSh, abnormal response to halothane only; MHN, normal responses to halothane and caffeine), core temperature, clinical features, predisposing factors and heat tolerance testing (HTT) outcome of the exertional heat illness (EHI) / exertional rhabdomyolysis (ER) cohort. Creatine kinase (CK) values have been reported, if available, as an indication of rhabdomyolysis. DIC = disseminated intravascular coagulation. LFT = liver function tests. AKI = acute kidney injury.

1. * Conscious level described as normal, altered or unconscious. An altered conscious level includes confusion, disorientation, loss of coordination, blurred vision, drowsiness, agitation, or any combination of these.
2. Passed HTT after first episode. Second episode of EHI after return to military duties.
3. Location is described as deployed when the event occurred either during military training or operations in non-temperate climates, including Iraq, Afghanistan and Cyprus. Precise locations are not provided for each individual in order to maintain de-identification.
4. After active cooling
5. Information from patient or referring physician who was told that temperature was raised but not the value

Five patients experienced ER exclusively with no other features of EHI documented. A further 28 EHI patients presented with features consistent with rhabdomyolysis (> 1,200 $IU.L^{-1}$). A total of 22 patients had a documented maximum CK concentration > 10,000 $IU.L^{-1}$. Twenty-one cases of EHI or ER resulted in severe complications or organ damage including acute kidney injury, multi-organ failure, metabolic acidosis, disseminated intravascular coagulation, seizures and cardiac arrest. IVCT responses (Table 2) were abnormal in 35% of the EHI/ER cohort (18 EHI and 4 ER cases). Ten family members across 3 independent EHI families were sequenced alongside the 64 index patients.

Table 2: Exertional heat illness /rhabdomyolysis cohort classified by *in vitro* contracture test (IVCT) result.

IVCT	Individuals	Age at biopsy	Male/ Female	Military	Civilian	HTT
MHN	41	17-34	M	38	3	37
MHSh	18	20-34	17M/ 1F	17	1	17
MHShc	4	20-28	M	4	–	4
No IVCT	1	N/A	M	–	1	–
ALL	64	17-34	63M / 1F	59	5	58

HTT = number who had heat tolerance testing. MHShc, abnormal IVCT responses to halothane and caffeine; MHSh, abnormal IVCT response to halothane only; MHN, normal IVCT responses to halothane and caffeine

Variants identified in *RYR1* and the genes of the $Ca_v1.1$ complex

Targeted next generation sequencing revealed 15 rare and potentially pathogenic heterozygous non-synonymous variants in the genes coding for RyR1 (*RYR1*) or the $Ca_v1.1$ complex (*CACNA1S*, *CACNA2D1*, *CACNB1* and *CACNG1*) (Table 3). One was found in *CACNA1S*, 3 in *CACNA2D1* and the other 11 were in *RYR1*. Using GAVIN we classified 5 of the *RYR1* variants as variants of unknown significance (VUS) with the remainder of variants in these genes classified pathogenic (Table 3). *CACNA2D1* p.D1045A was the only variant in these genes identified more than once across the EHI/ER cohort but was not significantly more prevalent in the EHI/ER cohort compared with the ExAC cohort ($X^2 = 3.44$, $P = 0.06$). Of the 22 EHI/ER patients who demonstrated a positive IVCT (MHShc or MHSh), 6 harboured an *RYR1* variant (one patient had 3 *RYR1* variants, p.R3366H, p.T3711M, p.Y3933C) while only 3 out of 41 patients with a negative IVCT (MHN) had an *RYR1* variant ($X^2 = 4.49$, $P = 0.03$). Of these genes, only in *RYR1* did we find more than one variant with ExAC MAF < 0.0005 but we found no statistical evidence that such rarer *RYR1* variants are more prevalent in the EHI/ER cohort than the ExAC cohort ($X^2 = 0.03$, $P = 0.87$).

Table 3: Rare and potentially pathogenic non-synonymous variants identified in *RYR1* and genes of the Cav1.1 complex.

Gene	Variant ID	DNA change	Amino acid change	MAF (ExAC)	C-score	GAVIN classification	IVCT
CACNA1S	rs150590855	c.1493G>A	p.R498H	0.000238	35	Pathogenic	1 MHN
CACNA2D1	−	c.149C>T	p.T50I	N/A	24.9	Pathogenic	1 MHN
CACNA2D1	rs78086631	c.2126G>A	p.S709N	0.0027	17.75	Pathogenic	1 MHN
CACNA2D1	rs35131433	c.3134A>C	p.D1045A	0.0028	22.2	Pathogenic	1 MHN,1 MHSh
RYR1	rs199826952	c.1475G>A	p.R492H	N/A	27.6	Pathogenic	1 MHShc
RYR1	rs746904839	c.2635G>A	p.E879K	0.000008	33	Pathogenic	1 MHN
RYR1	rs780579604	c.4865G>A	p.R1622Q	0.0002	28.4	Pathogenic	1 MHSh
RYR1	rs147707463	c.8327C>T	p.S2776F	0.0007	23.7	Uncertain	1 MHSh
RYR1	rs375626634	c.9758T>C	p.I3253T	0.00004	20	Uncertain	1 MHSh
RYR1	rs200375946	c.9800C>T	p.P3267L	0.00006	23.5	Pathogenic	1 MHN
RYR1	rs137932199	c.10097G>A	p.R3366H	0.0009	23.9	Uncertain	1 MHShc
RYR1	rs143987857	c.10616G>A	p.R3539H	0.0018	26.1	Uncertain	1 MHSh
RYR1	rs375915752	c.11132C>T	p.T3711M	0.000008	24.3	Pathogenic	1 MHShc
RYR1	rs147136339	c.11798A>G	p.Y3933C	0.0009	24.1	Uncertain	1 MHSh
RYR1	rs150396398	c.13513G>C	p.D4505H	0.0061	24.3	Uncertain	1 MHN

MAF: minor allele frequency. ExAC: ExAC browser (http://exac.broadinstitute.org). GAVIN: gene-aware variant interpretation. IVCT: *in vitro* contracture test result. MHShc, abnormal IVCT responses to halothane and caffeine; MHSh, abnormal IVCT response to halothane only; MHN, normal IVCT responses to halothane and caffeine. Note added in proof: the ExAC browser website became unavailable after 31 December 2019 but the data are available at https://gnomad.broadinstitute.org . The MAF for each variant derived from the complete gnomAD dataset is provided in Supplementary table 5.

Variants identified in genes associated with an exercise intolerance phenotype

Six genes were investigated due to their previous implication in conditions featuring an exercise intolerance phenotype. These were *AMPD1*,[26-28] ACADVL,[29] ACADM,[30] *ATP2A1*,[31,32] *CLCN1*,[33] *CPT2*,[34,35] and *PYGM*.[36] Sequencing the coding region of these genes revealed 11 non-synonymous variants (Table 4) in 5 patients who tested MHSh and 8 who tested MHN in the IVCT. GAVIN annotated the three variants in *PYGM* as pathogenic, *ACADVL* p. H335Q and *ATP2A1* p.T538M as benign with the other 8 variants as VUS (Table 4). Two variants, *AMPD1* p.M343I and *ATP2A1* p.T538M, were found in the same EHI MHSh individual (patient #20- supplementary table 3), while one MHN individual who developed EHI with rhabdomyolysis carried both *CLCN1* variants (patient #63- supplementary table 4). Two variants were identified more than once across the EHI cohort. *CLCN1* p.R300Q was found in one MHSh and 3 MHN patients, while *PYGM* p.A193S was found in three (two MHSh and one MHN) patients. Comparing the incidence of these variants in our EHI/ER cohort with their prevalence in the ExAC database (European non-Finnish cohort) suggests they are over-represented in our cohort. We found *CLCN1* p.R300Q in 4 patients compared with an

ExAC MAF of 0.0067 (X^2 = 11.54, *P* = 0.0007). *PYGM* p.A193S was present in 3 patients and this has an ExAC MAF of 0.003 (X^2 = 17.76, *P* = 0.000025). However, *CLCN1* p.R300Q was found in 9 of 285 UK control samples (MAF 0.016) which was not statistically significantly different from the EHI/ER cohort (MAF 0.031, X^2 = 1.26, *P* = 0.24). The increased prevalence of *PYGM* p.A193S in the EHI/ER cohort (MAF 0.023) was confirmed in comparison to the UK control population (MAF 0.0035, X^2 = 5.823, *P* = 0.016). *ACADVL* was the only one of the genes in this category with more than one variant having ExAC MAF < 0.0005 but we found no statistical evidence that such rarer *ACADVL* variants are significantly more prevalent in the EHI/ER cohort (X^2 = 0.97, *P* = 0.32).

Table 4: Rare and potentially pathogenic non-synonymous variants identified in genes associated with exercise intolerance.

Gene	Variant ID	DNA change	Amino Acid change	MAF (ExAc)	C-score	GAVIN classification	IVCT
ACADVL	rs113994167	c.848T>C	p.V283A	0.0014	24.6	Uncertain	1 MHSh
ACADVL	rs753624994	c.1005C>A	p.H335Q	0.00002	17.9	Benign	1 MHN
ACADVL	rs139425622	c.1567G>A	p.G523R	0.0004	22.6	Uncertain	1 MHN
AMPD1	rs139512772	c.202C>T	p.R68C	0.00005	29.1	Uncertain	1 MHN
AMPD1	rs61752478	c.1029G>T	p.M343I	0.0031	29.2	Uncertain	1 MHSh
ATP2A1	rs763211121	c.1613C>T	p.T538M	0.00002	27.1	Benign	1 MHSh
CLCN1	rs118066140	c.899G>A	p.R300Q	0.0067	32	Uncertain	1 MHSh/ 3 MHN
CLCN1	rs140205115	c.1842G>C	p.K614N	0.0014	24.4	Uncertain	1 MHN
PYGM	rs116987552	c.148C>T	p.R50*	0.0014	35	Pathogenic	1 MHSh
PYGM	rs77656150	c.577G>T	p.A193S	0.003	25.6	Pathogenic	2 MHSh/ 1 MHN
PYGM	rs146919445	c.1279C>T	p.R427W	0.00007	33	Pathogenic	1 MHN

MAF: minor allele frequency. ExAC: ExAC browser (http://exac.broadinstitute.org). GAVIN: gene-aware variant interpretation. IVCT: *in vitro* contracture test result. MHShc, abnormal IVCT responses to halothane and caffeine; MHSh, abnormal IVCT response to halothane only; MHN, normal IVCT responses to halothane and caffeine. Note added in proof: the ExAC browser website became unavailable after 31 December 2019 but the data are available at https://gnomad.broadinstitute.org . The MAF for each variant derived from the complete gnomAD dataset is provided in Supplementary table 6.

Variants identified in other genes associated with calcium homeostasis

Twenty-five non-synonymous heterozygous variants were identified in other genes associated with E-C coupling and calcium homeostasis (Table 5). These were found in 10 individuals tested MHS by the IVCT and 18 tested MHN. Two variants, *ASPH* p.V84G and p.A85P, were found in the same individual in *cis* with an upstream insert that is predicted to result in termination of the protein and so the missense variants would not be expressed (Table 5). These, and the other two variants found in *ASPH*, are annotated as benign by GAVIN; of the variants found in other genes in this category, 17 are annotated pathogenic and four VUS (Table 5). Two variants, *HOMER1*, p.P142L and *HRC* p.R88C were each found in two EHI/ER

patients. The prevalence of *HRC* p.R88C was not significantly different in the EHI/ER cohort compared to ExAC. Although the prevalence of *HOMER1* p.P142L was significantly greater than in the ExAC cohort, we found the variant in 2 of 285 UK control samples, indicating that it was not significantly more prevalent in the EHI/ER cohort ($X^2 = 2.7$, $P = 0.1$). We found more than one very rare variant (ExAC MAF < 0.0005) in three genes within this category (*ASPH*, *SCN4A*, *SLC8A3*) but such rare variants were not significantly more prevalent in our cohort compared with the ExAC cohort. Indeed, there were 4 very rare variants in *SCN4A* but with more than 1900 such variants in the ExAC cohort the X^2 value for the comparison was 1.75 ($P = 0.19$).

Table 5: Rare and potentially pathogenic non-synonymous variants identified in other genes associated with calcium homeostasis.

Gene	Variant ID	DNA change	Amino Acid change	MAF (ExAc)	C-score	GAVIN classification	IVCT
ASPH	rs527506012	c.251T>G[1]	p.V84G	0.0009	21.1	Benign	1 MHN
ASPH	N/A	c.253G>C[1]	p.A85P	N/A	21.1	Benign	1 MHN
ASPH	N/A	c.263A>C	p.K88T	N/A	20.9	Benign	1 MHN
ASPH	rs147012895	c.1189C>T[2]	p.R397C	0.00045	22.4	Benign	1 MHN
CALR	N/A	c.733C>G	p.P245A	N/A	23	Pathogenic	1 MHN
CASQ1	rs140253806	c.130G>A	p.D44N	0.002	29.3	Pathogenic	1 MHN
CASQ1	rs770893881	c.557T>A	p.F186Y	0.000008	28.6	Pathogenic	1 MHN
CHERP	rs763728092	c.1112C>T	p.P371L	0.0001	20.3	Pathogenic	1 MHN
HOMER1	rs200295734	c.425C>T	p.P142L	0.0003	22.9	Pathogenic	2 MHN
HRC	rs200176524	c.1189C>T	p.R397Ter	0.0002	34	Pathogenic	1 MHN
HRC	rs148966785	c.262C>T	p.R88C	0.0062	20.2	Pathogenic	1 MHSh/1 MHN
SCN4A	rs768087254	c.1773C>A	p.N591K	0.000008	29.1	Pathogenic	1 MHN
SCN4A	rs113462659	c.2188G>A	p.V730M	0.0006	30	Uncertain	1 MHSh
SCN4A	rs377277110	c.2995G>A	p.V999M	0.00007	25.5	Pathogenic	1 MHSh
SCN4A	rs776355318	c.4303G>C	p.D1435H	0.00002	25	Pathogenic	1 MHN
SCN4A	rs749841448	c.5233C>T	p.R1745C	0.00002	24.6	Pathogenic	1 MHSh
SLC8A3	rs144289733	c. 2383G>A	p.V795M	0.0004	33	Pathogenic	1 MHN
SLC8A3	rs141396102	c.1064G>T	p.R355L	0.0004	32	Pathogenic	1 MHSh
SLC8A3	rs376525495	c.1204C>T	p.P402S	0.000077	23.6	Pathogenic	1 MHN
SRL	rs374884498	c.1192C>T	p.R398C	0.00006	27.4	Pathogenic	1 MHN
STIM1	rs146873551	c.1511C>T	p.T504M	0.0009	21.3	Uncertain	1 MHSh
STIM1	rs35637264	c.1838C>A	p.S613Y	0.0008	27.4	Uncertain	1 MHSh
SYPL2	rs199821906	c.194G>A	p.R65H	0.0003	23.2	Pathogenic	1 MHSh
TRPM6	rs150874152	c.511G>A	p.G171R	0.0026	34	Uncertain	1 MHN
TRPM6	N/A	c.3263T>G	p.M1088R	N/A	23.6	Pathogenic	1 MHShc

1. These two variants were found in the same individual in *cis* with an upstream insert NC_000008.10: g.62580820_62580821ins; *ASPH* c.252_253insTTCTGGGA with predicted consequence p.Val84Serfs: the two missense variants would, therefore not be expressed.
2. This variant is only expressed in the full transcript (isoform 1) - the function of isoform 1 in skeletal muscle is uncertain. This variant is not expressed in isoform 4 (junctin), which interacts with calsequestrin 1, triadin and the ryanodine receptor.

MAF: minor allele frequency. ExAC: ExAC browser (http://exac.broadinstitute.org). GAVIN: gene-aware variant interpretation. IVCT: *in vitro* contracture test result. MHShc, abnormal IVCT responses to halothane and caffeine; MHSh, abnormal IVCT response to halothane only; MHN, normal IVCT responses to halothane and caffeine. Note added in proof: the ExAC browser website became

unavailable after 31 December 2019 but the data are available at https://gnomad.broadinstitute.org . The MAF for each variant derived from the complete gnomAD dataset is provided in Supplementary table 7.

Family studies

The index cases for the three families where relatives were available for IVCT and sequencing were #7 (EHI with rhabdomyolysis), #15 (ER) and #21 (non-military EHI) (Table 1). None of the relatives presented a history suggestive of MH under anaesthesia, EHI or ER. EHI patient #7 tested MHSh on IVCT and was found to carry 3 rare variants (*RYR1* p.R3539H, *ACADVL* p.V283A and *SCN4A* p.R1745C – tables 3, 4, 5 and supplementary table 4). *RYR1* p.R3539H and *ACADVL* p.V283A (annotated as VUS by GAVIN) segregated with positive IVCT phenotypes whereas *SCN4A* p.R1745C (annotated pathogenic) did not (Fig 1a). We found no variants meeting our criteria in ER patient #15, although we note that both parents had abnormal IVCT responses (Fig 1b). *SCN4A* p.V730M (annotated VUS) was identified in EHI patient #21 and his MHSh father (I-1) but was absent from his IVCT negative brother (II-2) (Fig 1c). Interestingly, both parents in this family also demonstrated abnormal IVCT responses: the mother (I-2) did not harbour any rare (MAF ≤0.01) variants in the genes investigated that could account for her MHSh IVCT response.

Phenotype-genotype associations

In patients with a history of ER only we found 4 variants in 3 patients (patients #11, #17 and #57, Supplementary table 4), with none of the variants being in *RYR1*. Of the 18 EHI patients in whom the peak creatine kinase was > 10,000 IU/L, 13 carried a total of 25 variants, with four harbouring at least one *RYR1* variant. Of the remaining EHI patients (n=41), 25 carried a total of 34 variants, of which 5 were in *RYR1*. There were 25 variants in 22 MHS individuals and 34 variants in 42 MHN individuals but the difference is largely explained by the presence of more *RYR1* variants in the MHShc/MHSh individuals (see above).

Discussion

We report 51 rare and potentially pathogenic variants in genes involved in skeletal muscle calcium regulation, membrane excitability or metabolism in a cohort of 64 EHI/ER patients. The high proportion of our patients (22 of 64) who had an abnormal IVCT adds weight to a role for skeletal muscle in the aetiology of EHI and ER. The increased prevalence of *RYR1* variants in those who had an abnormal IVCT compared with those who did not suggests that at least some of the *RYR1* variants are likely to result in a gain of function of the RyR1 protein, which is the prevailing explanation for abnormal IVCT responses to the RyR1 agonists halothane and

caffeine. The finding of potentially pathogenic RYR1 variants in 27% (95% CI 13-48%) of EHI/ER patients with an abnormal IVCT contrasts with 76% (95% CI 72-79%) of MH susceptible patients with a potentially pathogenic RYR1 variant [12]. Our data for two *RYR1* variants associated with an abnormal IVCT, in combination with other data, indicate that these variants are likely to be pathogenic for various phenotypes including EHI and ER. The other principal findings of this study are the novel evidence for the involvement of heterozygous variants in genes encoding enzymes with a role in maintaining oxidative phosphorylation during exercise (in which homozygous or compound heterozygous variants are associated with metabolic diseases) and in genes associated with the regulation of skeletal muscle membrane excitability.

Evaluation of variants in EHI and ER needs to be cogniscant that there are cases without a strong heritable basis [3]. Our diagnostic pathway is designed to identify those with a greater chance of genetic susceptibility to EHI/ER. We had anticipated that patients with higher CK, those with ER and those who had abnormal IVCT responses might be most likely to have a genetic basis for their clinical event and therefore be more likely to carry variants of interest. Other than a significantly greater number of *RYR1* variants in patients with abnormal IVCT, this was not the case. We suggest that the clinical screening and HTT alone provide an effective mechanism for identifying a high likelihood of genetic predisposition for EHI.

Previous reports have provided stronger evidence for a role of *RYR1* variants in ER rather than EHI.[5,37] However, we found no *RYR1* variants in 5 ER patients with the 11 *RYR1* variants found in 9 EHI patients. We note that Sambuughin and colleagues only found one *RYR1* variant in 7 ER patients investigated by whole exome sequencing.[38] Of the *RYR1* variants found in EHI patients, p.I3253T (found in patient #10) has been previously reported in combination with a nonsense *RYR1* variant in *trans* in a patient with a congenital core myopathy [39] and in a case of EHI from the French military.[17] We previously found this variant in a further patient referred for MH testing because of persistent muscle pains and idiopathic hyperCKaemia (unpublished): this patient was MHShc on IVCT. Although annotated as a VUS by GAVIN (see below), we conclude that *RYR1* p.I3253T is likely to be pathogenic for EHI, myalgia with hyperCKaemia and, in the hemizygous state, congenital myopathy. There is at least a possibility that it also increases the risk of developing MH under anaesthesia.

We have recently reported *RYR1* p.T3711M (annotated pathogenic by GAVIN) in two independent MH families and concluded the variant was likely pathogenic variant for MH.[12] We have also demonstrated a positive IVCT in a patient found to carry *RYR1* p.T3711M during investigation for a potential metabolic myopathy. EHI patient #4 who carried this variant

developed a CK of almost 50,000 $IU.L^{-1}$. In addition to *RYR1* p.T3711M EHI patient #4 carried two other heterozygous *RYR1* variants (p.R3366H and p.Y3933C, both annotated VUS by GAVIN). A combination of p.R3366H and Y3933C has been previously identified in *cis* in four unrelated MH families.[40] *RYR1* p.R3366H and p.Y3933C have also been reported together in a patient who experienced anaesthetic-induced cardiac arrest and rhabdomyolysis.[41] The p.R3366H/p.Y3933C haplotype was also reported in combination with *RYR1* p.R391P in a patient with limb-girdle muscular dystrophy and cardiomyopathy.[42]

RYR1 p.E879K (annotated pathogenic by GAVIN), which we found in a non-military case of EHI with rhabdomyolysis (patient #63) who tested MHN by IVCT, has been reported [43] in combination with another *RYR1* missense variant in a patient with congenital myopathy. We also found two variants in the *CLCN1* gene in this individual, both annotated as VUS by GAVIN. The *CLCN1* variant p.K614N has been implicated in a compound heterozygous combination with a splice-site donor variant in the same gene in a patient with recessive non-dystrophic myotonia.[44] The other *CLCN1* variant, p.R300Q, was present in the original family described with Thomsen's myotonia congenita but the variant did not segregate with the condition and was reported not to affect the function of the ion channel.[45]

RYR1 p.S2776F (annotated VUS by GAVIN) was found in MHSh EHI patient #18. This variant has been found in association with King-Denborough syndrome [46] and MH.[12] *RYR1* p.R3539H (annotated VUS by GAVIN), which segregated with the IVCT phenotype in the family of EHI patient #7 (along with *ACADVL* p.V283A) has previously been reported in *trans* with another *RYR1* missense variant in a patient with congenital myopathy.[43]

The only variant we found in *CACNA1S*, p.R498H (annotated pathogenic by GAVIN), has not been reported in association with MH and was found in patient EHI #52 who had a normal IVCT response. Interestingly, Sambuughin and colleagues reported a variant affecting the same amino acid position (*CACNA1S* p.R498L) in an individual with a history of EHI.[38] *CACNA1S*, p.R498H and p.R498L have a combined MAF in low risk populations of 0.00016 suggesting that the discovery of two variants at this amino acid position in a total of 71 patients with EHI/ER is unlikely to be due to chance. EHI patient #52 was also found to carry *SCN4A* p.N591K (annotated pathogenic by GAVIN and see supplementary table 4). *SCN4A* encodes the principal pore-forming subunit of $Na_v1.4$, the skeletal muscle voltage-gated sodium channel and has been implicated in various disorders on the myotonia/periodic paralyses spectrum. *CACNA1S* is also implicated in hypokalaemic periodic paralysis and sustained muscle rigidity after succinylcholine [12,47,48]. We speculate that the combination of amino acid substitutions in two proteins that are involved in skeletal muscle membrane depolarization and repolarization

may be relevant to the aetiology of EHI in this case. The skeletal muscle voltage-gated chloride channel (CLC-1 encoded by *CLCN1*) is another protein involved in skeletal muscle membrane potential regulation and which, as mentioned earlier, is associated with myotonias. In addition to patient #52, 3 other patients (ER #17, EHI #46, EHI #56) had combinations of variants in two genes encoding subunits of $Ca_v1.1$, $Na_v1.4$ or CLC-1 (supplementary Table 4).

Variants were found in three genes that encode enzymes involved in maintaining oxidative phosphorylation during exercise. *PYGM* p.A193S (annotated pathogenic by GAVIN) was found in three of our EHI patients and this incidence was significantly greater than the prevalence of this variant in the ExAC database. A variant affecting the adjacent amino acid (p.R194W) has been implicated in a compound heterozygous form of McArdle's disease,[49] a rare condition characterised by the absence of myophosphorylase.[36] Two of the patients harbouring *PYGM* p.A193S had abnormal IVCT responses (one had an additional variant, *STIM1* p.S613Y, annotated VUS by GAVIN) whereas the third patient had a normal IVCT. MHSh EHI patient #6 was found to carry PYGM p.R50*, which in the homozygous or compound heterozygous state is the most frequent mutation associated with McArdle's disease: it is annotated pathogenic by GAVIN.[50]

The MHSh EHI patient carrying *AMPD1* p.M343I (annotated VUS by GAVIN) also carried the *AMPD1* p.Q45* and p.P77L haplotype, a combination of common variants reported in myoadenylate deficiency although it should be noted that the clinical relevance of myoadenylate deficiency is debated.[28] This individual also harboured a p.T538M variant in *ATP2A1* (annotated benign by GAVIN). Three variants were identified in the *ACADVL* gene, which encodes a mitochondrial enzyme, very long-chain acyl-CoA dehydrogenase (VLCAD).[51] *ACADV*L p.V283A (annotated VUS by GAVIN), which cosegregated with the IVCT phenotype alongside *RYR1* p.R3539H (Fig 1a), has been previously associated in the homozygous form with VLCAD deficiency in a number of unrelated families.[52,53]

We have found more than one rare and potentially pathogenic variant in 16 of our EHI/ER cohort. We have described several specific combinations of variants in either the same gene or different genes to suggest that genetic susceptibility to EHI is oligogenic. Sambuughin drew a similar conclusion in their study of 7 patients with ER.[38] Indeed, in the two families where we were able to undertake IVCT in both parents of the index case both parents in each family had an abnormal IVCT. However, the size of our cohort precludes any stronger conclusion in this respect.

Study limitations

We can only speculate on the possible relevance of the variants that we found in 11 other genes. While our patients represent a relatively large and consistently phenotyped group of EHI patients, the cohort does not generate the statistical power to draw direct further inference. *CASQ1*, for example, encodes calsequestrin 1, the principal Ca^{2+}-binding protein within the skeletal muscle sarcoplasmic reticulum. Calsequestrin 1 interacts with RyR1 and other components of the E-C coupling complex, such as junctin and triadin. We found two variants in *CASQ1,* p.D44N and p.F186Y, both annotated pathogenic by GAVIN. The potential importance of calsequestrin 1 to heat and exercise tolerance is indicated by the susceptibility to exertional heat stress (and MH under anaesthesia) of *CASQ1* knockout mice.[54,55]

Limited understanding of the heritability of EHI contributes to the difficulty of making progress in this area. Without such knowledge we chose arbitrary MAF cut-offs to select variants of possible interest and we also report pathogenicity predicted by GAVIN. For 30 of the 51 identified variants, the GAVIN classification supports a pathogenic role but it may have led to an underestimate of the pathogenicity of other variants found in our cohort. GAVIN was developed using the ClinVar database but ClinVar does not distinguish between discreet phenotypes associated with a single gene, such that a variant may be benign for one phenotype but pathogenic for another. Furthermore, the prevalence of variants associated with each phenotype can differ markedly. The impact of this problem is compounded when a phenotype is sub-clinical until affected individuals are exposed to a sporadic environmental trigger, such as with MH, EHI and ER. The limitations of GAVIN may be appreciated by considering that three of the 28 confirmed pathogenic (functionally characterized) *RYR1* variants found in the UK MH population [12] are classified benign by GAVIN.

Conclusion

Our data confirm a role of variants in *RYR1* in the heritability of EHI as well as ER but highlight the likely genetic heterogeneity of these complex conditions. We have found that a high proportion of patients who have experienced EHI and who demonstrate impaired heat tolerance have skeletal muscle abnormalities. We propose defects, or combinations of defects, in three skeletal muscle pathways to be associated with EHI and ER: calcium homeostasis, oxidative metabolism and membrane excitability. These findings may form the basis of a pre-recruitment genetic screening test where heat stress is an occupational hazard, such as the military. Functional characterisation of genetic variants identified in this EHI/ER cohort is required to confirm their pathogenicity.

Competing interests:

The authors declare no conflicts of interest.

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Contribution of authors:

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Conduct of experiments and data collection: LG, DM, , PKG, CD, CH, DRdS, PMH

Data analysis & interpretation: all authors

Drafting of manuscript: LG

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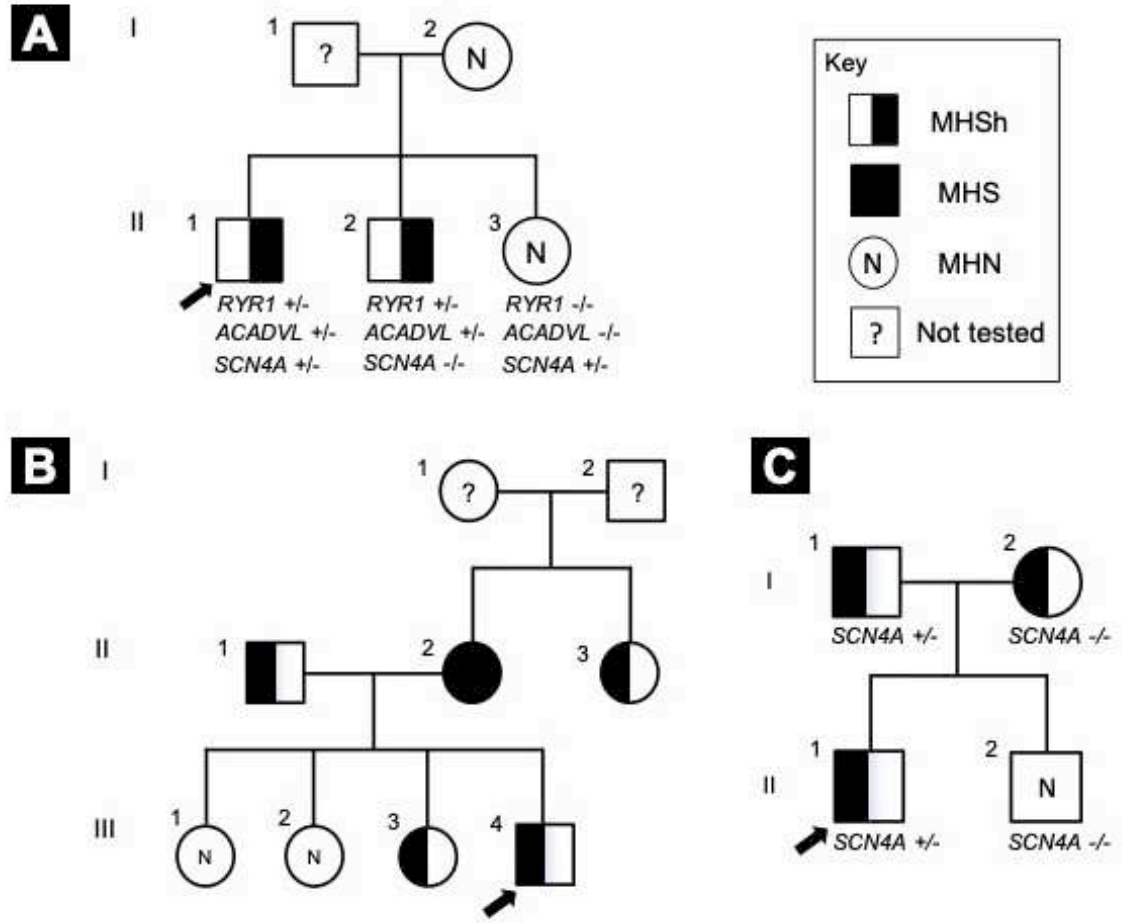
Figure Legend

Figure 1: Pedigrees representing the co-segregation of *in vitro* contracture test phenotypes and potentially pathogenic genotypes. Genotypes have been displayed below the individual symbols and the index has been indicated by an arrow. A) Family 1 pedigree representing variant *RYR1* p.R3539H, *ACADVL* p.V283A and *SCN4A* p.R1745C. B) Family 2 pedigree where no potentially pathogenic variants were identified. C) Family 3 pedigree representing variant *SCN4A* p.V730M. MHShc, abnormal *in vitro* contracture test responses to halothane and caffeine; MHSh, abnormal *in vitro* contracture test response to halothane only; MHN, normal *in vitro* contracture test responses to halothane and caffeine

Figure 1


A
I
1
?
2
N
II
1
2
3
N
RYR1 +/-
ACADVL +/-
SCN4A +/-
RYR1 +/-
ACADVL +/-
SCN4A -/-
RYR1 -/-
ACADVL -/-
SCN4A +/-
Key
MHSh
MHS
MHN
Not tested
B
I
1
?
2
?
II
1
2
3
III
1
N
2
N
3
4
C
I
1
2
SCN4A +/-
SCN4A -/-
II
1
2
N
SCN4A +/-
SCN4A -/-

Supplementary Methods

Symptoms and signs of exertional heat illness.

The symptoms and signs of heat illness are thirst, nausea or vomiting, diarrhoea, myalgia, cramps, fatigue, headache, visual disturbance, hyperventilation (which can lead to paraesthesia, tetany), agitation, anxiety, lack of judgement, hysteria, dizziness, staggering or loss of coordination, confusion, collapse or loss of consciousness.

Supplementary table 1: Target genes selected for next-generation sequencing, related to calcium homeostasis and energy metabolism.

Gene	Protein product	Exons	Coverage	Function
ACADM	Acyl-CoA dehydrogenase, medium chain	16	99.68%	Involved in fatty acid oxidation within the mitochondria
ACADVL	acyl-CoA dehydrogenase, very long chain	7	100.00%	Involved in fatty acid oxidation within the mitochondria.
AMPD1	adenosine monophosphate deaminase 1	16	100.00%	Involved in energy production within skeletal muscle (conversion of AMP to IMP).
ASPH	Aspartate beta-hydroxylase	37	99.99%	Role in calcium homeostasis and regulates calcium release from sarcoplasmic reticulum (SR).
ATP2A1	ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1	25	100.00%	SERCA pump for transport of calcium from the cytosol to the SR lumen. Contributes to calcium sequestration involved in muscular excitation/ contraction.
ANXA6	Annexin 6	29	99.86%	Ca^{2+}-binding protein which regulates release of Ca^{2+} from intracellular stores.
CACNA1S	calcium channel, voltage-dependent, L type, alpha 1S subunit	44	99.88%	alpha 1S subunit of dihydropyrdine receptor ($Ca_v1.1$ complex), a voltage-sensitive calcium channel in skeletal muscle. This subunit contains the voltage sensor.
CACNA2D1	calcium channel, voltage-dependent, alpha 2/delta subunit 1	44	99.87%	alpha 2/delta subunit of dihydropyrdine receptor ($Ca_v1.1$ complex). This subunit regulates calcium current density and kinetics of channel.
CACNB1	calcium channel, voltage-dependent, beta 1 subunit	16	99.91%	beta subunit of $Ca_v1.1$ complex, which modulates G protein inhibition, increasing peak calcium current, and shifting the voltage

				dependence of activation and inactivation.
CACNG1	calcium channel, voltage-dependent, gamma subunit 1	4	100.00%	gamma subunit of complex, plays a role in excitation-contraction coupling.
CALM1	Calmodulin 1	6	100.00%	Member of the EF-hand calcium-binding protein family.
CALR	calreticulin	9	100.00%	Calcium-binding chaperone involved in calcium homeostasis.
CASQ1	Calsequestrin 1	12	100.00%	calcium-binding protein acting as an internal calcium store in muscles.
CHERP	Calcium homeostasis endoplasmic reticulum protein	18	100.00%	Involved in calcium homeostasis, growth and proliferation.
CLCN1	chloride channel, voltage-sensitive 1	25	100.00%	Chloride channel regulates the electric excitability of the skeletal muscle membrane. Mutations involved in myopathies.
CPT2	carnitine palmitoyltransferase 2	5	94.91%	Nuclear protein which is transported to the mitochondrial inner membrane and involved in fatty acid oxidation.
FKBP1A	FK506 binding protein 1A	7	99.01%	Coordinates multi-protein RYR1 complex formation.
HOMER1	Homer homolog 1	9	99.29%	Scaffold protein that bind to and regulates RyR1.
HRC	Histidine rich calcium binding protein	6	100.00%	Luminal SR protein, binds to the cytoplasmic domain of triadin.
JPH1	Junctophilin 1	6	99.79%	Forms junctional membrane complexes (JMCs) linking the plasma membrane with the SR vital for RyR1-mediated calcium release.
JPH2	Junctophilin 2	7	99.84%	Forms junctional membrane complexes (JMCs), which is central to RyR1-mediated calcium release.
JSRP1	Junctional Protein 45			

ORAI1	ORAI calcium release-activated calcium modulator 1	2	99.07%	Mediates Ca^{2+} influx following depletion of intracellular Ca^{2+} stores, detected by STIM1.
PVALB	Parvalbumin	19	100.00%	High affinity Ca^{2+}-binding protein involved in muscle relaxation.
PYGM	phosphorylase, glycogen, muscle	20	99.44%	Involved in glycogenolysis. Mutations associated with glycogen storage disease of muscle.
RYR1	ryanodine receptor 1 (skeletal)	106	99.86%	Calcium release channel in the sarcoplasmic reticulum that triggers muscle contraction following depolarisation of T-tubules.
S100A1	S100 calcium binding protein A1	3	100.00%	Ca^{2+}-binding protein associated with cardiomyopathies.
SCN4A	sodium channel, voltage-gated, type IV, alpha subunit	24	99.96%	Voltage-gated sodium channels that generate and propagate action potentials in skeletal muscle.
SEPN1	Selenoprotein N, 1	14	98.22%	Protects against oxidative stress and binds Ca^{2+}, associated with multi-minicore disease and congenital muscular dystrophy.
SLC8A3	solute carrier family 8 (sodium/calcium exchanger), member 3	11	100.00%	Rapidly transports Ca^{2+} during excitation-contraction coupling (Ca^{2+} homeostasis).
SRL	sarcalumenin	8	100.00%	May be involved in the regulation of calcium transport.
STAC3	SH3 and cysteine rich domain 3	12	100.00%	Skeletal muscle-specific T-tubule protein is required for myotubule formation and myogenic differentiation.
STIM1	stromal interaction molecule 1	27	99.46%	Mediates Ca^{2+} influx after depletion of intracellular Ca^{2+} stores by gating of store-operated Ca^{2+} entry (SOCE) channels.

SYPL2	synaptophysin-like 2	6	99.95%	Involved in communication between the T-tubular and junctional sarcoplasmic reticulum (SR) membranes.
TRDN	Triadin	48	99.48%	May be involved in anchoring calsequestrin to the junctional SR and allowing its coupling with RYR1.
TRPC1	Transient receptor potential cation channel, C, 1	13	99.08%	Forms a non-selective channel permeable to Ca^{2+} and other cations. Activated by intracellular Ca^{2+} store depletion.
TRPC3	Transient receptor potential cation channel, C, 3	14	100.00%	Forms a non-selective channel permeable to calcium and other cations. Activated by intracellular calcium store depletion.
TRPM6	transient receptor potential cation channel, subfamily M, member 6	45	99.55%	Essential ion channel that is crucial for magnesium homeostasis.

Supplementary table 2: Primer sequences for *RYR1* long-range PCR amplicons.

Primer ID	Exons	Size	Sequence
RYR1_FR4_F	Ex 12 - 16	5606bp	5'-CCAGTGCCTAGAACAGAGCC-3'
RYR1_FR4_R			5'-GCAACAGGAACTTGTAGGGC-3'
RYR1_FR9_F	Ex 35 – 38	3762bp	5'-GACCTGGGTGGATCTTAAGGAG-3'
RYR1_FR9_R			5'-CTATCTACCCCTCCCTTGCATCT-3'
RYR1_FR11_F	Ex 43 – 49	4036bp	5'-GTCTCTGACTGAGCCCCTTCT-3'
RYR1_FR11_R			5'-GAGATTTCTACGGGGACGCT-3'
RYR1_FR12_F	Ex 50 – 58	4004bp	5'-GCATCCATATGCCCATTTACTC-3'
RYR1_FR12_R			5'-TAGGTGAGTCTGGTCTGCAGAA-3'
RYR1_FR14_F	Ex 64 – 67	4937bp	5'-GAGGAAGTACCCCTCACTTTCA-3'
RYR1_FR14_R			5'-GAAACCAGGAGGAAGAGTCAGA-3'
RYR1_FR17_F	Ex 85 – 89	5703bp	5'-TGCTTTCTGGCATACAATAGGA-3'
RYR1_FR17_R			5'-CGGTTCTCATCTGTGTTAATGC-3'
RYR1_FR22_F	Ex 97 – 101	5034bp	5'-GACAGCTCTGATCCCTCTGG-3'
RYR1_FR22_R			5'-ATGCATCAGCTTGCCAAACT-3'

Supplementary table 3. Gene-specific criteria for classification of pathogenicity using GAVIN

Gene	Benign if MAF >	Benign if CADD <	Pathogenic if CADD >
ACADVL	0.000906	19.37	22.86
AMPD1	0.693	6.47	30
ASPH	0.000313	30	40.3
ATP2A1	0	29.2	36.2
CACNA1S	0.002735	21.12	26.97
CACNA2D1	0.00426	15	15
CALR	0.00426	15	15
CASQ1	0.00426	15	15
CHERP	0.00426	15	15
CLCN1	0.00589	18.18	25.24
HOMER1	0.00426	15	15
HRC	0.00426	15	15
PYGM	0.01088	19.87	24.59
RYR1	0.0006548	19.58	22.24
SCN4A	0.0001446	16.2	23.46
SLC8A3	0.00426	15	15
SRL	0.00426	15	15
STIM1	0.0000453	15	15
SYPL2	0.00426	15	15
TRPM6	0.000062	15	15

Supplementary table 4: Non-polymorphic (MAF ExAC ≤1%) and potentially pathogenic (C-score ≥15) variants identified EHI patients across the coding regions of all twenty-two genes.

EHI ID	Variant ID	Gene	cDNA	Amino acid	MAF ExAC	C-score
1	rs77656150	*PYGM*	c.577G>T	p.A193S	0.002	25.6
2	N/A	*TRPM6*	c.3263T>G	p.M1088R	N/A	23.6
3	rs199826952	*RYR1*	c.1475G>A	p.R492H	N/A	27.6
4	rs147136339	*RYR1*	c.11798A>G	p.Y3933C	0.009	24.1
4	rs137932199	*RYR1*	c.10097G>A	p.R3366H	0.0009	23.9
4	rs375915752	*RYR1*	c.11132C>T	p.T3711M	0.000008	24.3
5	rs780579604	*RYR1*	c.4865G>A	p.R1622Q	0.0002	28.4
5	rs141396102	*SLC8A3*	c.1064G>T	p.R355L	0.0004	32
5	rs146873551	*STIM1*	c.1511C>T	p.T504M	0.0009	21.3
6	rs116987552	*PYGM*	c.148C>T	p.R50*	0.0014	35
7	rs113994167	*ACADVL*	c.848T>C	p.V283A	0.0014	26
7	rs143987857	*RYR1*	c.10616G>A	p.R3539H	0.0018	26.1
7	rs749841448	*SCN4A*	c.5233C>T	p.R1745C	0.00002	24.6
10	rs375626634	*RYR1*	c.9758T>C	p.I3253T	0.00004	20
10	rs377277110	*SCN4A*	c.2995G>A	p.V999M	0.00007	25.5
13	rs148966785	*HRC*	c.262C>T	p.R88C	0.0062	20.2
14	rs77656150	*PYGM*	c.577G>T	p.A193S	0.002	25.6
14	rs35637264	*STIM1*	c.1838C>A	p.S613Y	0.0008	27.4
17	rs35131433	*CACNA2D1*	c.3134A>C	p.D1045A	0.0028	22.2
17	rs118066140	*CLCN1*	c.899G>A	p.R300Q	0.0046	32
19	rs147707463	*RYR1*	c.8327C>T	p.S2776F	0.0007	23.7
19	rs199821906	*SYPL2*	c.194G>A	p.R65H	0.0003	23.2
20	rs61752478	*AMPD1*	c.1029G>T	p.M343I	0.0031	29.2
20	rs763211121	*ATP2A1*	c.1613C>T	p.T538M	0.00002	27.1
21	rs113462659	*SCN4A*	c.2188G>A	p.V730M	0.0006	30
24	rs139512772	*AMPD1*	c.202C>T	p.R68C	0.00005	29.1
24	N/A	*CALR*	c.733C>G	p.P245A	N/A	23
26	rs140253806	*CASQ1*	c.130G>A	p.D44N	0.002	29.3
27	rs147012895	*ASPH*	c.1189C>T	p.R397C	0.00045	22.4
28	rs200176524	*HRC*	c.1189C>T	p.R397Ter	0.0002	34
29	rs200295734	*HOMER1*	c.425C>T	p.P142L	0.0003	22.9
31	rs527506012	*ASPH*	c.251T>G	p.V84G	0.0009	21.1
31	N/A	*ASPH*	c.253G>C	p.A85P	N/A	21.1
33	rs77656150	*PYGM*	c.577G>T	p.A193S	0.002	25.6
34	rs376525495	*SLC8A3*	c.1204C>T	p.P402S	0.000077	23.6

35	rs148966785	*HRC*	c.262C>T	p.R88C	0.0062	20.2
37	rs200295734	*HOMER1*	c.425C>T	p.P142L	0.0003	22.9
39	rs200375946	*RYR1*	c.9800C>T	p.P3267L	0.00006	23.5
43	rs35131433	*CACNA2D1*	c.3134A>C	p.D1045A	0.0028	22.2
44	rs770893881	*CASQ1*	c.557T>A	p.F186Y	0.000008	28.6
45	rs763728092	*CHERP*	c.1112C>T	p.P371L	0.0001	20.3
46	N/A	*ASPH*	c.263A>C	p.K88T	N/A	20.9
46	rs118066140	*CLCN1*	c.899G>A	p.R300Q	0.0046	32
46	rs776355318	*SCN4A*	c.4303G>C	p.D1435H	0.00002	25
49	rs78086631	*CACNA2D1*	c.2126G>A	p.S709N	0.0027	17.75
51	rs753624994	*ACADVL*	c.1005C>A	p.H335Q	0.00002	22.9
52	rs150590855	*CACNA1S*	c.1493G>A	p.R498H	0.000238	35
52	rs768087254	*SCN4A*	c.1773C>A	p.N591K	0.000008	29.1
53	rs146919445	*PYGM*	c.1279C>T	p.R427W	0.00007	33
56	N/A	*CACNA2D1*	c.149C>T	p.T50I	N/A	24.9
56	rs118066140	*CLCN1*	c.899G>A	p.R300Q	0.0046	32
58	rs139425622	*ACADVL*	c.1567G>A	p.G523R	0.004	26.4
58	rs374884498	*SRL*	c.1192C>T	p.R398C	0.00006	27.4
59	rs150396398	*RYR1*	c.13513G>C	p.D4505H	0.0061	24.3
59	rs150874152	*TRPM6*	c.511G>A	p.G171R	0.0026	34
61	rs144289733	*SLC8A3*	c. 2383G>A	p.V795M	0.0004	33
63	rs140205115	*CLCN1*	c.1842G>C	p.K614N	0.0014	24.4
63	rs118066140	*CLCN1*	c.899G>A	p.R300Q	0.0046	32
63	rs746904839	*RYR1*	c.2635G>A	p.E879K	0.000008	33

Supplementary table 5: Rare and potentially pathogenic non-synonymous variants identified in *RYR1* and genes of the Cav1.1 complex (updated version of table 3 using gnomAD minor allele frequency).

Gene	Variant ID	DNA change	Amino acid change	MAF (gnomAD)	C-score	GAVIN classification	IVCT
CACNA1S	rs150590855	c.1493G>A	p.R498H	0.000279	35	Pathogenic	1 MHN
CACNA2D1	rs1477267428	c.149C>T	p.T50I	0.000008	24.9	Pathogenic	1 MHN
CACNA2D1	rs78086631	c.2126G>A	p.S709N	0.00253	17.75	Pathogenic	1 MHN
CACNA2D1	rs35131433	c.3134A>C	p.D1045A	0.003	22.2	Pathogenic	1 MHN,1 MHSh
RYR1	rs199826952	c.1475G>A	p.R492H	0.000014	27.6	Pathogenic	1 MHShc
RYR1	rs746904839	c.2635G>A	p.E879K	0.000016	33	Pathogenic	1 MHN
RYR1	rs780579604	c.4865G>A	p.R1622Q	0.0002	28.4	Pathogenic	1 MHSh
RYR1	rs147707463	c.8327C>T	p.S2776F	0.0007	23.7	Uncertain	1 MHSh
RYR1	rs375626634	c.9758T>C	p.I3253T	0.000036	20	Uncertain	1 MHSh
RYR1	rs200375946	c.9800C>T	p.P3267L	0.00005	23.5	Pathogenic	1 MHN
RYR1	rs137932199	c.10097G>A	p.R3366H	0.0014	23.9	Uncertain	1 MHShc
RYR1	rs143987857	c.10616G>A	p.R3539H	0.003	26.1	Uncertain	1 MHSh
RYR1	rs375915752	c.11132C>T	p.T3711M	0.000004	24.3	Pathogenic	1 MHShc
RYR1	rs147136339	c.11798A>G	p.Y3933C	0.001	24.1	Uncertain	1 MHSh
RYR1	rs150396398	c.13513G>C	p.D4505H	0.005	24.3	Uncertain	1 MHN

MAF: minor allele frequency. gnomAD: gnomAD browser (https://gnomad.broadinstitute.org). GAVIN: gene-aware variant interpretation. IVCT: *in vitro* contracture test result. MHShc, abnormal IVCT responses to halothane and caffeine; MHSh, abnormal IVCT response to halothane only; MHN, normal IVCT responses to halothane and caffeine.

Supplementary table 6: Rare and potentially pathogenic non-synonymous variants identified in genes associated with exercise intolerance (updated version of table 4 using gnomAD minor allele frequency).

Gene	Variant ID	DNA change	Amino Acid change	MAF (gnomAD)	C-score	GAVIN classification	IVCT
ACADVL	rs113994167	c.848T>C	p.V283A	0.0022	24.6	Uncertain	1 MHSh
ACADVL	rs753624994	c.1005C>A	p.H335Q	0.00013	17.9	Benign	1 MHN
ACADVL	rs139425622	c.1567G>A	p.G523R	0.00042	22.6	Uncertain	1 MHN
AMPD1	rs139512772	c.202C>T	p.R68C	0.00005	29.1	Uncertain	1 MHN
AMPD1	rs61752478	c.1029G>T	p.M343I	0.0032	29.2	Uncertain	1 MHSh
ATP2A1	rs763211121	c.1613C>T	p.T538M	0.000035	27.1	Benign	1 MHSh
CLCN1	rs118066140	c.899G>A	p.R300Q	0.0078	32	Uncertain	1 MHSh/ 3 MHN
CLCN1	rs140205115	c.1842G>C	p.K614N	0.0028	24.4	Uncertain	1 MHN
PYGM	rs116987552	c.148C>T	p.R50*	0.0015	35	Pathogenic	1 MHSh
PYGM	rs77656150	c.577G>T	p.A193S	0.0032	25.6	Pathogenic	2 MHSh/ 1 MHN
PYGM	rs146919445	c.1279C>T	p.R427W	0.000034	33	Pathogenic	1 MHN

MAF: minor allele frequency. gnomAD: gnomAD browser (https://gnomad.broadinstitute.org). GAVIN: gene-aware variant interpretation. IVCT: *in vitro* contracture test result. MHShc, abnormal IVCT responses to halothane and caffeine; MHSh, abnormal IVCT response to halothane only; MHN, normal IVCT responses to halothane and caffeine.

Supplementary table 7: Rare and potentially pathogenic non-synonymous variants identified in other genes associated with calcium homeostasis (updated version of table 5 using gnomAD minor allele frequency).

Gene	Variant ID	DNA change	Amino Acid change	MAF (gnomAD)	C-score	GAVIN classification	IVCT
ASPH	rs527506012	c.251T>G[1]	p.V84G	0.0007	21.1	Benign	1 MHN
ASPH	N/A	c.253G>C[1]	p.A85P	N/A	21.1	Benign	1 MHN
ASPH	N/A	c.263A>C	p.K88T	N/A	20.9	Benign	1 MHN
ASPH	rs147012895	c.1189C>T[2]	p.R397C	0.00024	22.4	Benign	1 MHN
CALR	rs1239263833	c.733C>G	p.P245A	0.000004	23	Pathogenic	1 MHN
CASQ1	rs140253806	c.130G>A	p.D44N	0.00195	29.3	Pathogenic	1 MHN
CASQ1	rs770893881	c.557T>A	p.F186Y	0.000008	28.6	Pathogenic	1 MHN
CHERP	rs763728092	c.1112C>T	p.P371L	0.000027	20.3	Pathogenic	1 MHN
HOMER1	rs200295734	c.425C>T	p.P142L	0.00033	22.9	Pathogenic	2 MHN
HRC	rs200176524	c.1189C>T	p.R397Ter	0.00018	34	Pathogenic	1 MHN
HRC	rs148966785	c.262C>T	p.R88C	0.0062	20.2	Pathogenic	1 MHSh/1 MHN
SCN4A	rs768087254	c.1773C>A	p.N591K	0.000011	29.1	Pathogenic	1 MHN
SCN4A	rs113462659	c.2188G>A	p.V730M	0.0011	30	Uncertain	1 MHSh
SCN4A	rs377277110	c.2995G>A	p.V999M	0.00011	25.5	Pathogenic	1 MHSh
SCN4A	rs776355318	c.4303G>C	p.D1435H	0.000026	25	Pathogenic	1 MHN
SCN4A	rs749841448	c.5233C>T	p.R1745C	0.000042	24.6	Pathogenic	1 MHSh
SLC8A3	rs144289733	c. 2383G>A	p.V795M	0.001	33	Pathogenic	1 MHN
SLC8A3	rs141396102	c.1064G>T	p.R355L	0.0012	32	Pathogenic	1 MHSh
SLC8A3	rs376525495	c.1204C>T	p.P402S	0.000017	23.6	Pathogenic	1 MHN
SRL	rs374884498	c.1192C>T	p.R398C	0.00005	27.4	Pathogenic	1 MHN
STIM1	rs146873551	c.1511C>T	p.T504M	0.00083	21.3	Uncertain	1 MHSh
STIM1	rs35637264	c.1838C>A	p.S613Y	0.001	27.4	Uncertain	1 MHSh
SYPL2	rs199821906	c.194G>A	p.R65H	0.00034	23.2	Pathogenic	1 MHSh
TRPM6	rs150874152	c.511G>A	p.G171R	0.0041	34	Uncertain	1 MHN
TRPM6	N/A	c.3263T>G	p.M1088R	N/A	23.6	Pathogenic	1 MHShc

1. These two variants were found in the same individual in *cis* with an upstream insert NC_000008.10: g.62580820_62580821ins; *ASPH* c.252_253insTTCTGGGA with predicted consequence p.Val84Serfs: the two missense variants would, therefore not be expressed.
2. This variant is only expressed in the full transcript (isoform 1) - the function of isoform 1 in skeletal muscle is uncertain. This variant is not expressed in isoform 4 (junctin), which interacts with calsequestrin 1, triadin and the ryanodine receptor.

MAF: minor allele frequency. gnomAD: gnomAD browser (https://gnomad.broadinstitute.org). GAVIN: gene-aware variant interpretation. IVCT: *in vitro* contracture test result. MHShc, abnormal IVCT responses to halothane and caffeine; MHSh, abnormal IVCT response to halothane only; MHN, normal IVCT responses to halothane and caffeine.