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A Multi-dimensional Analysis of Genotype - Phenotype Discordance in Malignant Hyperthermia Susceptibility

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A short running title: Genotype - Phenotype Discordance in MH susceptibility

ABSTRACT

Background: Malignant hyperthermia (MH) susceptibility is an inherited condition, diagnosed either by the presence of a pathogenic genetic variant or by *in vitro* caffeine-halothane contracture testing. Through a multi-dimensional approach, we describe the implications of discordance between genetic and *in vitro* test results in a patient with a family history of possible MH.

Methods: The patient, whose brother had a possible MH reaction, underwent the caffeine-halothane contracture test (CHCT) according to the North-American MH Group protocol. Screening of the complete *RYR1* and *CACNA1S* transcripts was done by Sanger sequencing. Additional functional analyses included skinned myofibre calcium-induced calcium release sensitivity, calcium signaling assays in cultured myotubes and *in silico* evaluation of the effect of any genetic variants on their chemical environment.

Results: The patient's CHCT result was negative but she carried an *RYR1* variant c.1209C>G, p.Ile403Met, that is listed as pathogenic by the European Malignant Hyperthermia Group. Functional tests indicated a gain-of-function effect with a weak impact, and the variant was predicted to affect the folding stability of the 3D structure of the RyR1 protein. If American College of Medical Genetics and Genomics/Association of Molecular Pathology guidelines were used, this variant would be characterized as a variant of uncertain significance.

Conclusions: Available data do not confirm or exclude an increased risk of MH for this patient. Further research is needed to correlate RyR1 functional assays, including the current gold standard testing for MH susceptibility, with clinical phenotypes. The pathogenicity of genetic variants associated with MH susceptibility should be re-evaluated.

Key words: ACMG/AMP guidelines, functional testing, malignant hyperthermia; phenotype-genotype discordance; *RYR1* variants.

INTRODUCTION

Malignant hyperthermia (MH) is a rare pharmacogenetic disorder of skeletal muscle triggered in susceptible individuals by volatile anaesthetics and/or succinylcholine. It manifests as a potentially lethal hypermetabolic crisis associated with a rapid and uncontrolled increase in myoplasmic Ca^{2+} concentration in skeletal muscle cells, which can lead to sustained cellular hypermetabolism, muscle contracture and rhabdomyolysis.[1]

MH has variable presentation and its clinical signs are non-specific. The Clinical Grading Scale (CGS) is a tool used to assess the likelihood that a suspected clinical episode is actually MH, with scores assigned based on the clinical findings ranking from almost never (rank 1) to almost certain (rank 6), but the tool's sensitivity may be low especially when rank is low.[2-3]

The diagnosis of MH susceptibility is determined by the caffeine-halothane contracture test (CHCT) in North America[4] and the *in vitro* contracture test (IVCT)[5] in Europe and elsewhere, except for Japan, where it is based on detecting an enhancement of calcium-induced calcium release (CICR) rates from the sarcoplasmic reticulum in permeabilized muscle fibres.[6-7] CHCT and IVCT have a sensitivity of 97% and 100% (point estimates in CGS rank 6 cases), and a specificity of 78% and 94% (point estimates in low-risk control patients), respectively.[5,8-9] The diagnostic thresholds of the Japanese CICR test to diagnose susceptibility to MH are determined by statistical comparison to CICR values measured in low-risk subjects, but the test's sensitivity and specificity have not been formally assessed.[10] The diagnosis of MH susceptibility can also be made by identifying a pathogenic variant in one of the MH-related genes, *RYR1, CACNA1S or STAC3*.[5, 11-12] Molecular genetic testing for MH, however, has a low sensitivity as up to 50% of MH families do not carry potentially pathogenic genetic variants in any of the major genes. [11-12] At present only 2 *CACNA1S* variants and 48 out of more than 200 MH-associated *RYR1* variants

are accepted as MH diagnostic mutations by the European Malignant Hyperthermia Group (EMHG).[11] Therefore, negative genetic test results should be followed by muscle biopsy and contracture test for ultimate diagnosis of MH susceptibility.

Reports of discordance between genetic testing results and the MH susceptibility diagnosis determined by the muscle contracture tests[13-15] have brought attention to the complexity of MH diagnosis. Here we describe a comprehensive approach to delineate the pathophysiological and clinical implications of genotype-phenotype discordancy in an individual with a family history of possible MH, utilizing the American College of Medical Genetics and Genomics (ACMG) and the Association of Molecular Pathology (AMP) guidelines for classifying genetic variants[16] as well as muscle physiology and structural analysis tools.

METHODS

STUDY SUBJECTS, DIAGNOSTIC WORKUP AND EXPERIMENTAL METHODS

An Italian-Canadian family first came to our MH centre's (MHIU in Toronto) attention in 1990 (Fig 1). The index case (II.1), then a 6-year old boy, had an adverse reaction during surgery for tonsillectomy consisting of masseter muscle rigidity after inhalational induction of general anaesthesia with halothane followed by succinylcholine. Neither hypercarbia nor hyperthermia was reported, and the episode resolved without the administration of dantrolene. The serum creatine kinase increased to 5000 IU/L on the second day after surgery. The proband's mother (I.2) suffered from frequent muscle cramps and had a history of postoperative fever after tonsillectomy. Because of the association between masseter muscle rigidity and MH susceptibility, the proband's mother underwent CHCT in 1990 (the proband being too young) and was diagnosed as MH negative (MHN: 2 mM caffeine - 0 g [normal response < 0.2 g]; 3% halothane - 0.1 g [normal response < 0.7 g]). Histopathology results showed no structural or histochemical abnormalities. She died of colon cancer in 2016.

The proband's father (I.1) allegedly had no personal or family history of MH. Unfortunately, neither the proband nor his father agreed to CHCT or genetic testing.

More recently, the proband's sister (II.2) was referred to us for MH susceptibility workup: a 34 years old female with history of mild asthma triggered by cold weather and exercise, otherwise healthy and with no previous general anaesthesia. She consented to undergo muscle biopsy and CHCT.

With approval of our Research Ethics Board, we obtained informed consent from the proband's sister for our MH research protocols, which include comprehensive genetic testing and studying

intracellular Ca^{2+} dynamics in skeletal muscle. A piece of her left gracilis muscle was harvested under spinal anaesthesia and transported to our lab. CHCT was done according to the North American MH Group protocol.[4] Concomitantly, we performed the CICR test in saponin-treated (skinned) single muscle fibres from the same specimen according to the Japanese protocol.[6-7] A fresh sample from the excess muscle was used for muscle pathology analysis.

To explore Ca^{2+} signaling abnormalities at the cellular level, another fresh specimen was sent on ice via overnight courier to the Rios lab for calcium signaling assays in cultured myotubes as described in Figueroa and colleagues,[17] specifically, for measurements of resting cytosolic [Ca^{2+}], frequency of spontaneous cytosolic calcium events, cytosolic calcium waves, and cell-wide calcium spikes after electrical stimulation. Measurements in myotubes obtained from the patient's myoblasts were compared with data from cultured myotubes derived from other patients who underwent CHCT at MHIU previously.

We mapped the variant on the 3D structures of rabbit RyR1, obtained from either cryo-electron microscopy[18] (PDB ID 5T15) and from more detailed crystallographic studies of the RyR1 N-terminal disease hot spot[19-20] We directly compared crystal structures of wild-type and I404M rabbit RyR1 disease hot spots (residues 1-536, PDB ID 2XOA and 4I2S). Structural representations, including superpositions and an analysis of the chemical environment of the variant, were generated using Pymol[21] and Chimera.[22]

Genetic analysis was done as described in Kraeva and colleagues.[23]

RESULTS

Good viability of each muscle specimen (six in total, three for each drug) used in the CHCT was ascertained by a pre-test twitch equal or greater to 4 g. CHCT testing demonstrated no contracture response to 2 mM caffeine, and the greatest contracture response to 3% halothane was 0.1 g (diagnostic threshold ≥0.7 g).

The patient's muscle histopathology showed no structural or histochemical abnormalities.

The CICR test in skinned fibres detected accelerated CICR rates from the sarcoplasmic reticulum (Fig 2A), which is considered as positive for MH susceptibility in Japan, in 5 out of 6 single segments of fast-twitch muscle fibres.

In cultured myotubes derived from the patient's myoblasts, mean myoplasmic calcium concentration at rest (Fig. 2B) was 76% higher than in myotubes derived from MHN individuals ($p < 0.001$), whereas the spontaneous calcium release activity of the sarcoplasmic reticulum did not differ from that of MHN myotubes (Fig. 2C).

Sequencing of the whole *RYR1* and *CACNA1S* transcripts obtained from the patient's muscle tissue revealed an *RYR1* variant, c.1209C>G p.Ile403Met, currently included among the 48 MH diagnostic mutations (www.emhg.org). The presence of the variant was confirmed using genomic DNA isolated from a separate blood sample.

The variant c.1209C>G (p.Ile403Met) is rare with a minor allele frequency (MAF) of 0.000008 (dbSNP entry rs118192116), located in the N-terminal mutational hot spot region of the *RYR1*; thus, the criterion PM1 is met. However, *in silico* analyses yielded ambiguous results: SIFT and PolyPhen predict the variant to be deleterious (SIFT score of 0.02; PolyPhen score of 0.952), while REVEL (score of 0.618) and CADD (score of 16) predictions are inconclusive.

The availability of cryo-EM and crystal structures of the rabbit RyR1 protein allow mapping of the p.Ile403Met variant on the 3D structures. A comparative analysis of several disease-associated *RYR1* variants, including those considered pathogenic by the EMHG, with cryo-EM and crystal structures of the related rabbit RyR1 showed that many variants cluster at domain-domain interfaces[24] and have the potential to change the relative domain orientations, thus affecting channel opening allosterically[19,20,25]. Another subset of sequence variants, known to affect function, affects residues buried within individual domains, where they are involved in hydrophobic packing. Such changes can affect the folding stability. The p.Ile403Met variant affects a residue within the N-terminal solenoid (NSol) and through interaction with other hydrophobic residues is involved in packing within this domain (Figure 3).

DISCUSSION

Across our long practice at the Malignant Hyperthermia Investigation Unit at Toronto General Hospital, we report here our first discordant case of negative CHCT phenotype with positive MH genotype. Despite her CHCT results and because of the potential consequences of a false negative MH susceptibility diagnosis, our patient has been assigned a diagnosis of MH susceptibility based on the presence of the MH diagnostic p.Ile403Met variant in the *RYR1* gene.[5] We have analyzed possible reasons for the discordance using the CHCT/IVCT sensitivity study reports, bioinformatics, muscle physiology and crystallography tools.

The CHCT/IVCT muscle contracture test is the gold standard for MH susceptibility diagnosis.[1] For almost 50 years, negative CHCT/IVCT results have been used to reliably rule out MH susceptibility. A large study in New Zealand, found no evidence of malignant hyperthermia episodes in 479 anaesthetic records from 280 patients who had tested negative on the IVCT.[26] However, with an estimated sensitivity of 97%,[8] it is possible that the CHCT produced a false-negative result in our patient. False-negative CHCT results as well as cases of discordance between the negative IVCT results and the positive *RYR1* genotype have been reported by MH research groups around the world.[13-15] Discordance between CHCT/IVCT phenotype and *RYR1* genotype hindered the demonstration of linkage between MH and *RYR1* in several pedigrees, despite the presence of familial *RYR1* variants fulfilling the criteria for pathogenicity.[14] Thus Robinson and colleagues,[15] found 2.6% IVCT negative tests in carriers of pathogenic *RYR1* variants among 196 European MH families. In a more recent study[27] aimed to estimate the prevalence of genetic variants implicated in MH in the United Kingdom, out of 280 families carrying at least one of the

pathogenic *RYR1* variants, there were 16 families (5.7%) that included individuals with positive genotype and negative IVCT phenotype.

In 2015 the American College of Medical Genetics and Genomics (ACMG) and the Association of Molecular Pathology (AMP) published revised guidelines[16] to define criteria or evidence types in order to classify genetic variants as benign, likely benign, likely pathogenic, and pathogenic. If there is insufficient evidence to reach pathogenic/likely pathogenic or benign/likely benign classification, the variant is to be regarded as a variant of uncertain significance (VUS). Publication of the ACMG/AMP guidelines prompted reassessment of individual variants, and led to reclassification of up to 15% of variants in the ClinVar database.[28] These criteria can also be applied in order to classify incidentally found variants.

We applied the 2015 ACMG/AMP guidelines to reassess the prior classification of "pathogenic-diagnostic for MH" for the *RYR1* variant c.1209C>G (p.Ile403Met) using our own as well as publicly available clinical, genetic and functional data. We are specifically applying the criteria for the gene-disease dyad of *RYR1* genotype and malignant hyperthermia susceptibility phenotype, which is often inherited in an autosomal dominant pattern and associated with heterozygous variants in the gene. The myopathy phenotype associated with *RYR1* variants, which is inherited in either an autosomal dominant or recessive pattern, was disregarded here as the biopsied patient did not exhibit any myopathic symptoms nor had any histopathological abnormalities.

The first striking finding was that this *RYR1* variant has never been reported in association with the clinical MH phenotype, nor been shown to co-segregate with the IVCT/CHCT phenotype. It has an OMIM entry (117000) for central core disease; the most common congenital myopathy associated with *RYR1*; it was identified in a single Italian family - in two siblings with core myopathy, whose parents were clinically asymptomatic. There was no family history of MH and

no family member had undergone IVCT.[29] Thus, there are no cases to count for comparison between the variant's prevalence in MH individuals and its prevalence in controls, nor for its co-segregation with the disease. Without such data, we cannot exclude the possibility of this variant being just a private benign variant.

Furthermore, although the variant is rare and is located in the hot spot region of the *RYR1*, *in silico* analyses yielded inconclusive results. Thus, the criterion for supporting variant pathogenicity based on multiple lines of computational evidence is not met.

Functional studies of the rabbit p.Ile404Met mutant RyR1 protein, orthologous to the human p.Ile403Met mutant, expressed in HEK-293 cells, showed an increased sensitivity to caffeine and to halothane when analyzed by cellular Ca^{2+} photometry[30] indicating a gain-of-function pathogenic change. In a different set of experiments, the same authors[31] found no increase in resting Ca^{2+} concentration compared with wild type RyR1, which might reflect the ability of HEK-293 cells to compensate for a weak pathogenic calcium leak from the sarcoplasmic reticulum. It is important to mention that the cellular assay system[30-31] mimics a homozygous occurrence of the p.Ile404Met variant, while all three known variant-carriers are heterozygous, thus any potential effect of the variant will be expected to be even milder *in vivo*. In another study, the impact of the p.Ile404Met variant on release channel function was characterized, along with several other CCD-associated RyR1 variants, by means of its incorporation into a rabbit RyR1 cDNA and expression in myotubes derived from RyR1-knockout (dyspedic) mice.[32] Unlike the other mutants assessed in that study, the p.Ile404Met change had essentially no effect on resting Ca^{2+} levels or sarcoplasmic reticulum Ca^{2+} depletion, and showed only a very small shift in voltage sensitivity. In contrast, we detected a marked increase of cytosolic calcium at rest in myotubes derived from the patient's myoblasts and enhanced CICR rates from the sarcoplasmic reticulum in her skinned muscle fibres, thereby

pinpointing a gain of the RyR1 function. Although, due to the undefined genetic background of our *ex-vivo* experiments, we cannot conclude that the observed effects are directly caused by dysfunctional p.Ile403Met mutant RyR1 channels, our results are in line with the previous *in vitro* characterization of this variant[30-31] as damaging to the RyR1 function.

Additionally, in a cryo-EM and crystal structure study that compared several disease-associated variants in the N-terminal disease hot spot, the rabbit p.Ile404Met RyR1 mutant, orthologous to human p.Ile403Met, was found to cause a small decrease in thermal stability[19], in agreement with its involvement in hydrophobic packing. Structural perturbations were limited to local changes, resulting in reorientations and dual conformations of side chains in the vicinity. In contrast to other variants, the p.Ile404Met substitution did not cause any relative reorientations of the domains, suggesting the functional impact would be milder than other variants (Fig 3A). Of note, the domain-domain packing is also affected by crystal contacts, and minor changes that affect the stability of the domain-domain interactions may thus not be observed. We postulate that, at elevated temperatures and in full-length RyR1, the variant may affect the nearby Glu481 residue, which is directly involved in an interaction with a neighbouring domain (Figure 3B). Taking together, however, the above studies do not seem to be unequivocally supportive of a damaging effect of the p.Ile403Met variant on RyR1 function.

On the whole, combining all the evidential criteria (i.e. population data, functional data, computational prediction data, the criteria developed by the ACMG/AMP[16] and lack of segregation data) to determine the classification of the p.Ile403Met variant, we conclude that at present there is not enough evidence to classify the p.Ile403Met variant as either pathogenic or likely pathogenic, or to classify it as benign. Thus, on the basis of available data the p.Ile403Met variant should be downgraded to a variant of uncertain significance (VUS). We are aware that the

ACMG/AMP criteria are being specifically adapted for use in *RYR1*-related MH susceptibility with a more relevant classification system by a Variant Curation Expert Panel of the ClinGen consortium. Unless that expert panel identifies substantial data for pathogenicity, we expect that this variant would be downgraded to a VUS by them as well.

It is hard to ascertain based on the clinical, diagnostic and functional data whether our patient actually has an increased risk of developing MH on exposure to triggering anaesthetics. Her brother developed masseter muscle spasm following the administration of halothane and succinylcholine. Although this sign is associated with an increased risk of MH susceptibility, on its own it does not represent a hypermetabolic reaction characteristic of MH *per se*. Indeed, the proband did not develop features of cellular hypermetabolism; his peak postoperative CK was 5000 IU/L. Using the MH CGS score, the proband's adverse anesthetic reaction would be rank 3 or "somewhat less than likely." Among the differential diagnoses of masseter muscle spasm are neuromuscular disorders other than MH, although at the time of the reaction the boy was apparently asymptomatic.

There are no agreed criteria for the magnitude of RyR1 gain of function defects in different experimental models that is required to render a carrier clinically susceptible to MH. To date, any gain of function that is shown to be significantly different from the wild type response is considered to indicate pathogenicity. While there are agreed criteria for abnormal CHCT/IVCT responses, we have only a limited understanding of the correlation between CHCT/IVCT responses and the severity of the MH reaction, or indeed if an MH reaction is possible in patients with CHCT/IVCT responses just above the threshold. The CICR and myotube data from this study confirm the findings of Tong and colleagues[30] that the p.Ile403Met variant does confer a mild gain of function

defect but the normal CHCT responses suggest that the gain of function may not be sufficient to trigger a clinical MH episode. An alternative explanation remains, however, that they point to a false negative CHCT and, until shown otherwise, it is safer from the clinical point of view to manage our patient as MH-susceptible.

Our findings underscore the importance of further research to correlate all RyR1 functional assays, including the current gold standard testing for MH susceptibility, with clinical phenotypes. They also highlight the value of re-evaluating the pathogenicity of genetic variants associated with MH susceptibility using the 2015 ACMG/AMP guidelines.[16]

Authors' contributions

CAIM: Study design, CICR data acquisition & analysis, writing and revising the draft.

NK: Study design, genetic data acquisition & interpretation, writing and revising the draft

EZ: Study design, CHCT data acquisition & data analysis, writing and revising the draft.

LF: Ca index data acquisition & analysis, writing and revising the draft.

ER: Ca index data interpretation writing and revising the draft.

LB: Genetic data interpretation, writing and revising the draft.

FVP: Crystallography data acquisition & interpretation writing and revising the draft.

PMH: Study design, data interpretation, writing and revising the draft.

SR: Study design, data analysis & interpretation, writing and revising the draft.

All authors approved the final manuscript

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FIGURE LEGENDS

FIGURE 1. Pedigree diagram. Generations and birth order are identified by Roman and Arabic numerals, respectively; squares and circles represent males and females, respectively; diagonal strikethrough indicates a deceased person; MHN and MHS indicate negative and positive contracture test results, respectively; proband and carrier of *RYR1* variant are identified by an arrow and a centralized dot, respectively; open symbols indicate unknown disease status; crossed symbol indicates a deceased person.

FIGURE 2. (a) Patient's calcium-induced calcium release from the sarcoplasmic reticulum was accelerated as compared to that of MHN patients. Solid circles and error bars represent mean + SD (N = 28). Empty circles and gray lines are individual assays from the current patient's skinned fibres (n = 6). Distribution of resting calcium concentration (b) and calcium signaling (c) in myotubes derived from biopsies of MHIU patients. Blue and orange dots represent the mean resting [Ca^{2+}] MHN and MHS individuals, respectively. Solid black dots and error bars represent the index patient's mean±SEM. Individual patient's values are average measurements from 20-40 myotubes. "N" and "n" indicate the number of investigated individuals and myotubes per group, respectively. Box plots, solid black line and dotted lines show the 25th and 75th percentile, median, and mean, respectively. **S**pontaneous Ca^{2+} events, waves and spikes are more frequent in MHS than in MHN myotubes. However, note the absence of spontaneous activity/waves and infrequent Ca^{2+} spikes in myotubes from the index patient. Statistical comparison was carried out with the Mann-Whitney Rank Sum Test.

FIGURE 3. Mapping of the p.Ile404Met variant on the 3D structures of rabbit RyR1 (p.Ile403Met of human RyR1). **A.** View from the cytosolic face of full-length rabbit RyR1 (PDB ID 5T15). Different structural regions are shown in different colours, with the N-terminal solenoid (blue, Nsol) indicated for one of the four subunits. Helices are shown as cylinders. Residue I404, equivalent to human RyR1 I403, is highlighted in pink. The N-terminal domains A and B are shown in orange. B. Superposition of crystal structures of wild-type (colours, PDB ID 2XOA) and I404M (grey, PDB ID 4I2S) N-terminal disease hot spot of RyR1. The main chain is shown in coils, and the side chains in sticks. I404 (pink) makes hydrophobic interactions with M483, F478, and part of E481

in the wild type, and is thus involved in helical packing within the N-terminal solenoid. In the mutant, M404 (black) adopts two different conformations, resulting in a double conformation of M483. These changes are predicted to weaken the helical packing in the N-terminal solenoid domain. E481, which interacts with a neighbouring domain, has minimal perturbations.

Figure 1

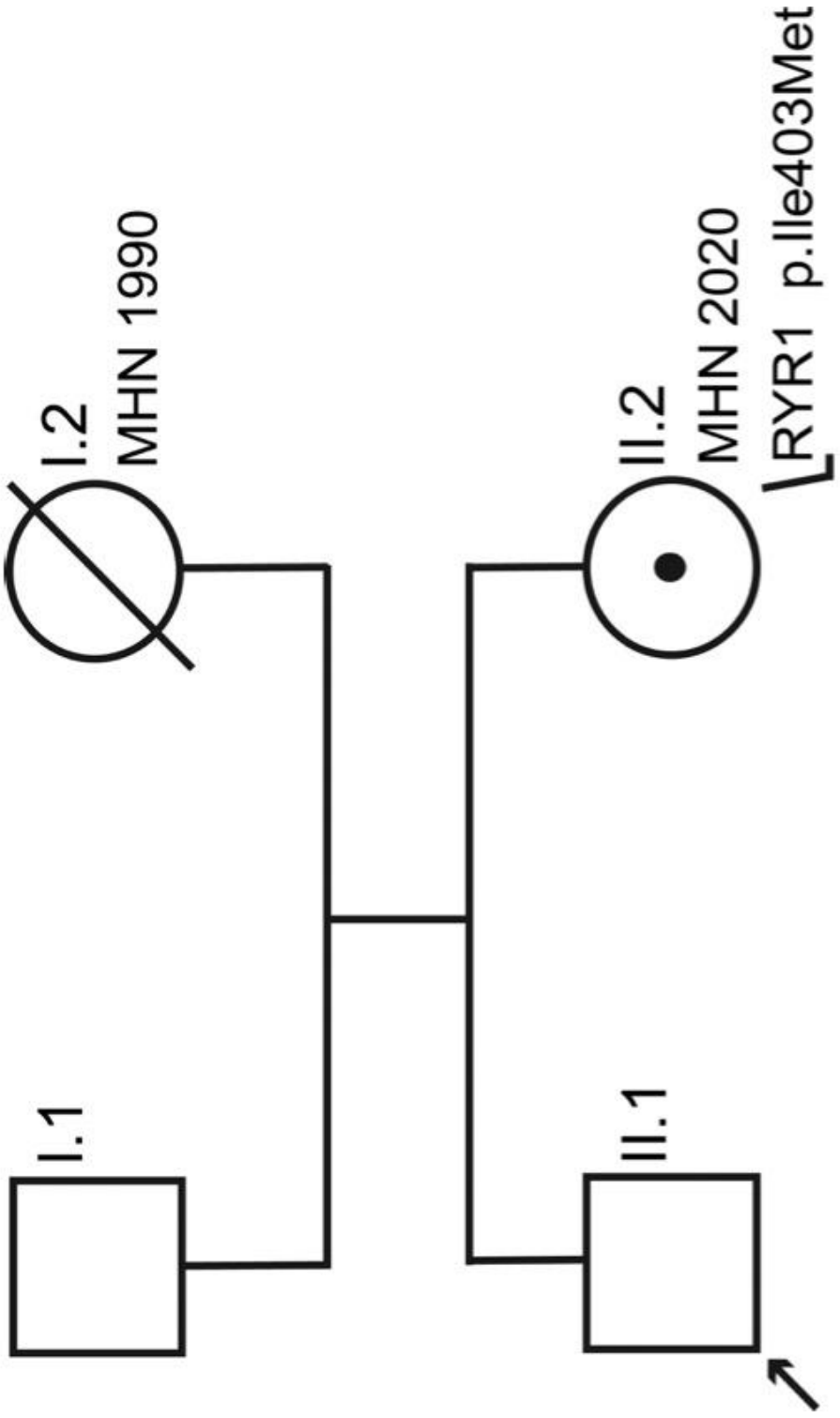

Figure 2

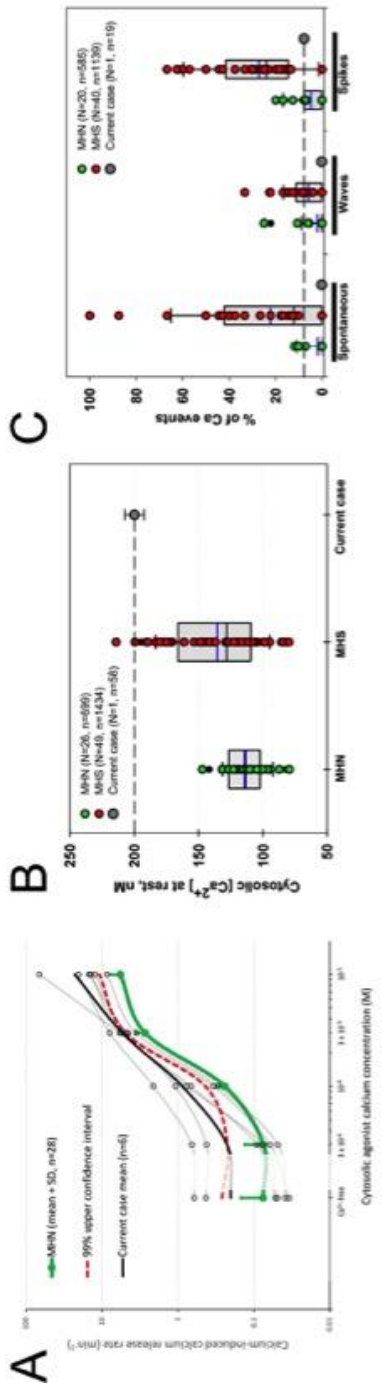
A
Calcium-induced calcium release rate (min⁻¹)
MHN (mean + SD, n=28)
99% upper confidence interval
Current case mean (n=6)
Cytosolic agonist calcium concentration (M)
B
Cytosolic [Ca2+] at rest, nM
MHN (N=26, n=699)
MHS (N=49, n=1434)
Current case (N=1, n=56)
MHN
MHS
Current case
C
% of Ca events
MHN (N=20, n=585)
MHS (N=40, n=1139)
Current case (N=1, n=19)
Spontaneous
Waves
Spikes

Figure 3