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A multi-dimensional analysis of genotype–phenotype discordance in malignant hyperthermia susceptibility

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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 using Sanger sequencing. Additional functional analyses included skinned myofibre calcium-induced calcium release sensitivity, calcium signalling 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. Based on American College of Medical Genetics and Genomics/Association of Molecular Pathology guidelines, this variant would be characterised 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.

Keywords: ACMG/AMP guidelines; functional testing; malignant hyperthermia; phenotype–genotype discordance; *RYR1* variants

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Editor's key points

- Malignant hyperthermia is diagnosed either by the presence of a pathogenic genetic variant or by *in vitro* caffeine–halothane contracture testing.
- The implications of discordance between genetic and *in vitro* test results were evaluated in a patient with a family history of possible malignant hyperthermia using multiple analytic approaches.
- The patient had a negative caffeine–halothane contracture test but carried the *RYR1* variant c.1209C>G, p.Ile403Met, which is considered pathogenic for malignant hyperthermia.
- The discordance in phenotype–genotype testing in this patient makes diagnosis inconclusive, which emphasises the importance of carefully evaluating the pathogenicity of genetic variants associated with malignant hyperthermia susceptibility.

Malignant hyperthermia (MH) is a rare pharmacogenetic disorder of skeletal muscle triggered in susceptible individuals by volatile anaesthetics, succinylcholine, or both. 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 nonspecific. 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 its 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 enhancement of calcium-induced calcium release (CICR) rates from the sarcoplasmic reticulum in permeabilised 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 with CICR values measured in low-risk subjects, but test 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 two *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 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, using the American College of Medical Genetics and Genomics (ACMG) and the Association of Molecular Pathology (AMP) guidelines for classifying genetic variants,[16] and muscle physiology and structural analysis tools.

Methods

Study subjects, diagnostic workup, and experimental methods

An Italian–Canadian family first came to the Malignant Hyperthermia Investigation Unit (MHIU) in Toronto in 1990 (Fig 1). The index case (II.1), then a 6-yr-old boy, had an adverse reaction during tonsillectomy consisting of masseter muscle rigidity after inhalation induction of general anaesthesia with halothane followed by succinylcholine. Neither hypercarbia nor hyperthermia was reported, and the episode resolved without administration of dantrolene. The serum creatine kinase increased to 5000 IU L^{-1} 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]; halothane 3% 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-yr-old female with a history of mild asthma triggered by cold weather and exercise, but

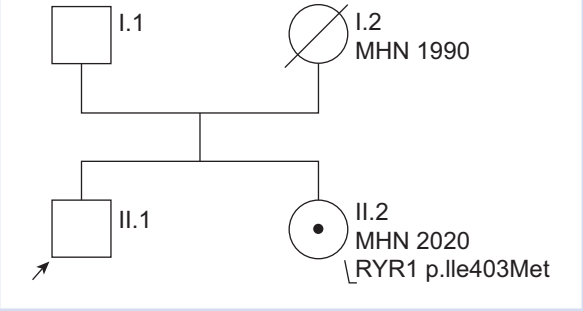

Fig 1. Pedigree diagram. Generations and birth order are identified by Roman and Arabic numerals, respectively; squares and circles represent males and females, respectively; diagonal strikethrough indicates a deceased person; MHN and MHS indicate negative and positive contracture test results, respectively;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

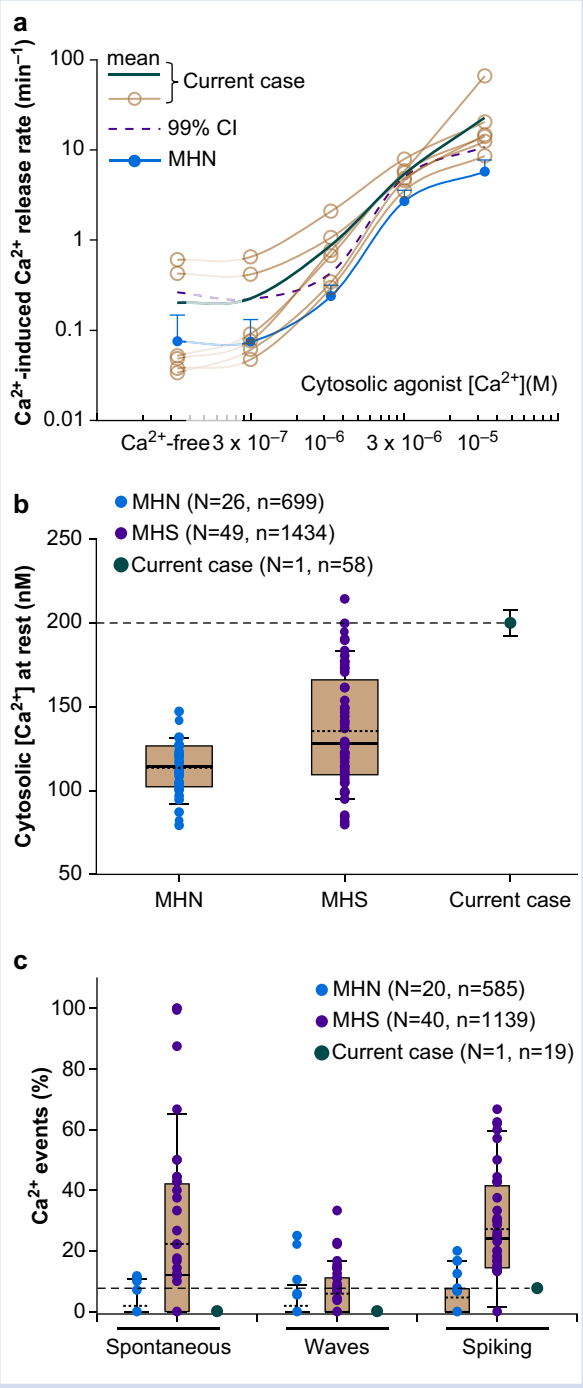

Fig 2. (a) The patient's calcium-induced calcium release from the sarcoplasmic reticulum was accelerated as compared with that of MHN patients. Solid circles and error bars represent mean (standard deviation) (N=28). Empty circles and grey lines are individual assays from the patient's skinned fibres (n=6). Distribution of resting calcium concentration (b) and calcium signalling (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 (standard error of the mean). Individual patient's values are average measurements from 20 to 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. Spontaneous 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. CI, confidence interval; MHIU, Malignant Hyperthermia Investigation Unit; MHN, malignant hyperthermia normal; MHS, malignant hyperthermia susceptible.

otherwise healthy and with no previous general anaesthesia. She consented to undergo muscle biopsy and CHCT.

With the 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 laboratory. CHCT was performed 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+} signalling abnormalities at the cellular level, another fresh specimen was sent on ice via overnight courier to the Rios laboratory for calcium signalling assays in cultured myotubes as described by 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 cryogenic electron microscopy (cryo-EM)[18] (PDB ID 5T15) or 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] Any identified variants were then classified according to ACMG and AMG guidelines.

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 to or greater than 4 g. CHCT testing showed no contracture response to 2 mM caffeine, and the greatest contracture response to halothane 3% 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 showed accelerated CICR rates from the sarcoplasmic reticulum (Fig 2a), which is considered as positive for MH susceptibility in Japan, in five out of six 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 *RYR1*. 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), whereas Rare Exome Variant Ensemble Learner; score of 0.618 (REVEL) and Combined Annotation Dependent Depletion; score of 16 (CADD) 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 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 (Fig 3).

Discussion

In our 45 yr practice at the MHIU at Toronto General Hospital, we have phenotyped and genotyped more than 700 individuals. Here we report 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 analysed 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 yr, negative CHCT/IVCT results have been used to rule out MH susceptibility. A large study in New Zealand found no evidence of MH 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 and cases of discordance between negative IVCT results and positive *RYR1* genotype have been reported by MH research groups around the world.[13–15] Discordance between CHCT/IVCT phenotype and *RYR1* genotype hindered 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% negative IVCT 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 UK, out of 280 families carrying at least

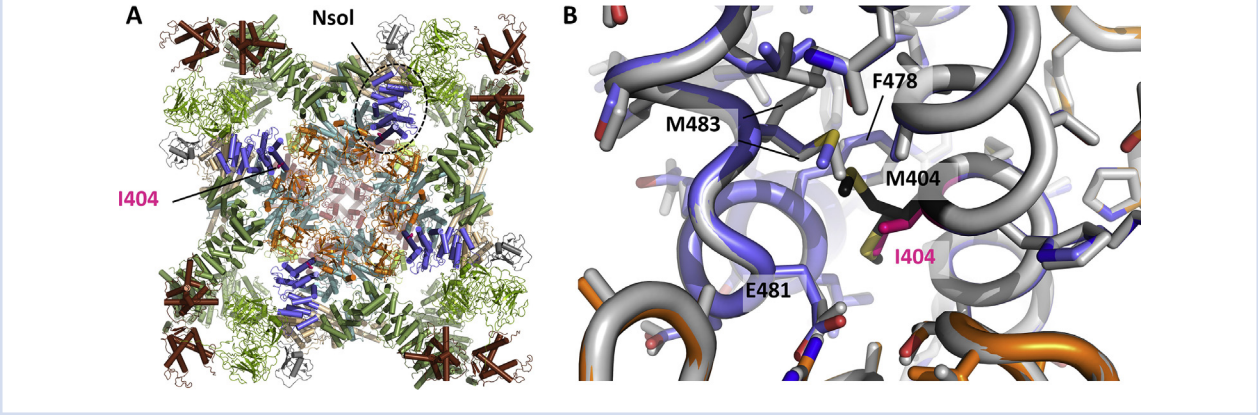

Fig 3. Mapping of the p.Ile404Met variant on the 3D structures of rabbit RyR1 (p.Ile403Met of human RyR1). (a) View from the cytosolic face of full-length rabbit RyR1 (PDB ID 5T15). Different structural regions are shown in different colours, with the N-terminal solenoid (blue, Nsol) indicated for one 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. Nsol, N-terminal solenoid.

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 ACMG and the 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 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 and publicly available clinical, genetic, and functional data. We are specifically applying the criteria for the gene-disease dyad of *RYR1* genotype and MH 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 have any histopathological abnormalities.

The first striking finding was that this *RYR1* variant has never been reported in association with the clinical MH phenotype, nor has it been shown to co-segregate with the IVCT/CHCT phenotype. It has an OMIM (Online Mendelian Inheritance in Man) 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 in our patient. 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 increased sensitivity to caffeine and to halothane when analysed 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, whereas 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 characterised, along with several other Central Core Disease (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 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 skinned muscle fibres, thereby pinpointing a gain of RyR1 function. Although, because of 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* characterisation of this variant[30,31] as damaging to RyR1 function.

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, caused 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 compared with 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 (Fig 3b). However, taken together the above studies do not unequivocally support a damaging effect of the p.Ile403Met variant on RyR1 function.

Combining all the evidence (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 VUS.

It is difficult to ascertain based on 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 after 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^{-1}. Using the MH CGS score, the proband's adverse anaesthetic 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 brother was apparently asymptomatic.

There are no agreed-upon criteria for the magnitude of RyR1 gain-of-function defects in different experimental models that are 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. Although there are 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. However, the alternative of a false negative CHCT indicates that 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 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

Study design: CAIM, NK, EZ, PMH, SR
CICR data acquisition and analysis: CAIM
Genetic data acquisition: NK
Genetic data interpretation: NK, LB
CHCT data acquisition and analysis: EZ
Ca index data acquisition and analysis: LF
Ca index data interpretation: ER
Crystallography data acquisition and interpretation: FVP
Data interpretation: PMH, SR
Data analysis: SR
Writing and revising of the draft: CAIM, NK, EZ, LF, ER, LB, FVP, PMH, SR
All authors approved the final manuscript.

Declarations of interest

PMH is an editorial board member of the *British Journal of Anaesthesia*. Otherwise, the authors declare no conflicts of interest.

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