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Malignant hyperthermia related DNA analysis (RYR1 gene) in Belgian families

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Abstract : This retrospective study summarizes the results of *DNA testing (Ryanodine receptor 1 (RYR1) gene* sequencing) in a cohort of 34 Belgian families with proven Malignant Hyperthermia susceptibility. Eighteen different *RYR1* sequence variants were found in 25 families. The most prevalent variant was p.Gly341Arg, detected in 7 families. Ten of the 18 variants are considered to be pathogenic, the remainder being 'variants of uncertain significance'. Complete phenotype and genotype concordance was obtained in 15 families ; in 7 discordance was found ; in 3 insufficient data did not allow to conclude. Discordance could be attributed to either limits in specificity of the in vitro contracture test, wrong blood sampling, or the possibility of more than one *RYR1* variant in a particular family.
Even though this study reflects the known important diverseness of *RYR1* variants, genetic testing can secure a diagnosis of Malignant Hyperthermia susceptibility in almost 50% of the Belgian families with known Malignant Hyperthermia.

Keywords : malignant hyperthermia ; RYR1 ; sequence variant ; phenotypet ; genotype.

Introduction

Malignant Hyperthermia (MH) is a rare but potentially life-threatening complication of general anesthesia, elicited by exposure to inhalational anesthetics and/or succinylcholine. It is clinically and biochemically expressed as hypercapnia, hyperthermia, generalized rigidity, rhabdomyolysis, mixed acidosis, arrhythmias and possibly death (1).

The underlying pathophysiology is characterized by abnormalities in excitation-contraction coupling and loss of control of myoplasmic calcium levels during muscle activation. This is the result of defects in genes coding for the proteins involved in calcium release from the sarcoplasmic reticulum (1, 2).

MH shows an autosomal dominant inheritance pattern. Diagnosis of MH in a proband puts relatives, and certainly first-degree relatives, at a higher risk for also developing MH upon anesthesia. The risk for each affected individual to transmit the mutant allele to the offspring is 50%.

Because MH is both life-threatening and preventable, it is of paramount importance to accurately diagnose susceptibility to MH and thus effectively manage the MH patient during procedures requiring general anesthesia. Until recently, the detection of MH susceptibility relied on a muscle biopsy and in vitro contracture testing (IVCT) in which muscle tissue is exposed to the triggering agents (3). An increased dose-response to the substances tested confirms the clinical diagnosis. This is however an invasive test with some morbidity, and a less than 100% sensitivity and specificity (4).

In an attempt to avoid this invasive test, molecular genetic techniques have been explored as a means of screening for MH susceptibility (1, 2, 5). In 1990, the primary gene for MH was identified as the *RYR1* gene, coding for the calcium-release channel of the sarcoplasmic reticulum. *RYR1* is

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located on chromosome locus 19q13.1 and is one of the largest human genes comprising 106 exons which encode a protein of 5,038 amino acids (6).

In contrast to the pig model for MH, in which one single mutation was found to be responsible for all cases of porcine stress syndrome (the porcine equivalent of human MH), the situation in humans is more complex with both chromosomal and allelic heterogeneity. To date, about 400 *RYR1* variants, clustering in three so-called 'hot spot' regions, have been shown to cause MH susceptibility (1). The majority of these variant are missense changes, e.g. single amino acid substitutions that alter the structure of the *RYR1* channel, causing it to open more easily in response to the triggering drugs.

In order to ascertain that a missense variant is pathogenic for MH susceptibility, functional assays are necessary to determine if a variant alters calcium release or not. Most often this is realized by expressing the mutated gene in a cell model and measuring calcium release (7). However, this is a time-consuming approach which has not been performed for all identified *RYR1* variants. To date, only 48 variants are considered to be pathogenic and when found in a particular family are classified as 'diagnostic'. The vast majority of variants remain 'variants of uncertain significance'(1).

Over the last 25 years, the MH-Laboratory at the University of Antwerp has functioned as the Belgian national reference lab for this disorder. Its database contains clinical, in vitro contracture test results, and histological data of over 500 patients. This report reviews the genetic data relative to the *RYR1* receptor obtained in this population, as well as the observed genotype and phenotype relationship.

Methods

We undertook a comprehensive analysis of our existing database with the aim to update and review the data. This was realized in several steps.

Step 1 : Update of the current database with respect to demographic, clinical, in vitro contracture testing, and histological data.

The diagnosis of MH susceptibility (phenotype) in the proband was based on clinical data available in the patients' anesthetic file and confirmed by IVCT with caffeine and halothane according to the European Malignant Hyperthermia Group's (EMHG) protocol (3). This test requires a sample of skeletal muscle tissue obtained by open biopsy, which is exposed in vitro to incremental doses of the testing agents caffeine and halothane, and the contracture response is measured. The test is considered positive (MHS) (susceptible) if a sustained contracture of at least 2 mN is obtained at caffeine concentrations of 2 mM or less, and/or halothane concentrations of 2 Vol% or less. Non-susceptible individuals (MHN) do not react at the threshold concentrations of either agent. In a minority of patients a significant contracture is obtained with one test substance only, and they are classified accordingly, e.g. MHSh if reacting to halothane only and MHSc when reacting to caffeine only. These different degrees in IVCT response are considered to reflect the phenotypical variability. The sensitivity and specificity of this in vitro diagnostic procedure have been reported to be respectively 99% and 94% (4).

Step 2 : Update of the pedigree for all families involved. The pedigrees contain information on the probands, the first degree relatives, and all individuals with either known IVCT or genetic data.

Step 3 : Update of the genotype in all studied families. For the genotype, only the *RYR1* gene was analyzed. Over time different labs were involved in the testing : Diagnostic lab of the Department of Medical Genetics at the Antwerp University Hospital, 'Laboratoire de Biochimie Génétique et Moléculaire, CHU de Grenoble' and the "Team Genoomdiagnostiek" of the Radboud Universitair Medisch Centrum Nijmegen.

Until 2008, mutation analysis was focused on the three known 'hot spot' regions with sequence analysis of 16 *RYR1*-exons only. This "targeted" – or "part of the exon" – sequencing was an established approach a decade ago and was then considered a rapid way to detect known and novel variants on the basis of an upfront selection of three *RYR1*-regions of interest (8). Even though this is a cost-effective way, the assay does not detect potential disease-causing variants in the other 90 exons of the gene which are left unsequenced.

Over the last 10 years, when new technologies became available and sequencing cheaper, the entire *RYR1* gene (all 106 exons) was sequenced. In our families the analysis was always initiated with the proband, and when a causal or pathogenic variant was identified, other relatives at risk were screened for the presence of this particular variant. The pathogenicity was assessed according to the international guidelines (9), and the existing genetic database of the EMHG (https://www.emhg.org/diagnostic-mutations). Informed consent was obtained from all participating families and the study was approved by the institutional review board. The databank is registered with the Data Protection Authority.

Step 4 : The final step was the charting of the correlation of the genotypic data with the phenotype. In each family we assessed if the sequence variant (genotype) was cosegregating with the phenotype (MHS) in a particular family (concordance), or not (discordance). By correlating the phenotypic and genotypic data obtained, we analyzed to what extent genetic counseling based on *RYR1* mutation analysis is feasible. The demonstration of reliable genotype-phenotype correlation is vital in prognostication and family counseling, as predicting outcome for an affected individual relying on genotyping alone is risky. Indeed, not all base-pair changes are 'pathogenic', and may or may not affect amino-acid sequence or function in the final protein. Certainly in families with a 'variant of uncertain significance', genotype-phenotype analysis is a prerequisite for genetic counseling.

In those families in which phenotype-genotype correlation was incomplete, we examined the reasons for the discordance.

Results

Over a period of 25 years, we have collected 70 Belgian families with an IVCT confirmed diagnosis of MH susceptibility. Thirty-four of these families were sufficiently explored by both muscle biopsy and genetic screening for a *RYR1* variant to allow analysis of phenotype and genotype correlation, and were therefore included in this survey.

Variants : In 25 of these 34 families a *RYR1* sequence variant was found. These comprised 18 different variants (Table 1). Ten of these are considered pathogenic by the EMHG, 8 are of 'uncertain significance'. Sixteen are missense variants ; one variant is predicted to result in deletion of one amino-acid, one variant is located at the boundary of an exon and intron. The most frequent variant is p.Gly341Arg which was observed in 7 families. In two families the co-existence of three missense variants in the same allele was found (p.1571Ile>Val, p.3366Arg>His, p.39333Tyr>Cys). The pathogenicity of the three individual variants as such remains uncertain (10).

Phenotype-genotype correlation

Of the 25 families with both phenotype and genotype information, 15 showed complete concordance e.g. every individual with an abnormal IVCT carried the mutation while MHN patients did not (Fig. 1), while in 7 families there was discordance (Fig. 2 and 3 as an example). In three

Table 1

Listing of the different *RYR1*-variants found, as well as a number of characteristics of the variants : change of amino-acid, number of exon, the number of families in which a particular mutation was found, the sequencing method used, as well as the estimated pathogenicity (9)

Amino-acid change	Exon	Number of families	Screening method	Classification of sequence variants ACMG (9)
p.Cys35Arg	2	1	TS	pathogenic
p.Gly341Arg	11	7	TS/FGS	pathogenic
c.1122 + 5G>A*	11	1	FGS	uncertain significance
p.Arg614Cys	17	2	TS/FGS	pathogenic
p. Arg614Leu	17	2	TS	pathogenic
p.Val1436Met	30	1	FGS	uncertain significance
p.Ile1571Val	33	2	FGS	uncertain significance
p.Arg3366His	67	2	FGS	no information on 'triplet'
p.Tyr3933Cys	86	2	FGS	
p.Val2168Met	39	1	TS	pathogenic
p.Thr2206Arg	40	1	FGS	pathogenic
p.Asn2342Ser	44	1	FGS	uncertain significance
p.Gln2348del	44	1	FGS	pathogenic
p.Arg2435His	45	1	TS	pathogenic
p.Met4640Val	91	1	FGS	uncertain significance
p.Leu4838Val	101	1	FGS	pathogenic
p.Val4849Ile	101	2	FGS	pathogenic
p.Phel4857Leu	101	1	FGS	uncertain significance

Pathogenic : variant considered to be diagnostic in the EMHG databank (48 in total) (www.emhg.org/diagnostic-mutations). Uncertain significance : variant of unknown significance, and therefore not (yet) considered diagnostic for MH. No information : variant not listed in the EMHG's genetic database. On grey background : variants appear as a triplet (p.Ile1571Val, p.Arg3366His, p.Tyr3933Cys) in 2 non-related families. p.Val4849Ile was found in fam 3 as an isolated variant, and in combination with other variants in fam 58. ACMG : American College of Medical Genetics and Genomics. TS : targeted sequencing. FGS: full gene sequencing. * : Heterozygote donor splice-site variant with unknown functional significance.

Table 2

Consecutively lists the explored families, number of individuals involved (n), whether 16 exon or 106 exon sequencing has been performed, and which variants were found

Family	n	16 exon sequencing	variant	106 exon sequencing	Variant
1.	22	+	-	+	no variant
2	20	+	-	+	no variant
3	11	+	-	+	p.Val4849Ile
4	7	+	p.Gly341Arg	-	p.Gly341Arg
5	22	+	p.Gly341Arg	-	p.Gly341Arg
7	14	+	p.Arg614Leu	-	p.Arg614Leu
8	20	+	p.Arg614Leu	-	p.Arg614Leu
9	22	+	p.Gly341Arg	-	p.Gly341Arg
11	25	+	p.Arg2435His	-	p.Arg2435His
15	11	+	-	+	p.Glu2348del
16	2	+	-	+	p.Phe4857Leu
17	4	+	-	+	no variant
18	9	+	-	+	c.G1122+5G>A
19	4	+	p.Val2168Met	-	p.Val2168Met
20	2	+	p.Cys35Arg	+	p.Cys35Arg
23	5	+	p.Gly341Arg	-	p.Gly341Arg
29	3	+	p.Arg614Cys	-	p.Arg614Cys
32	8	+	-	-	p.Gly341Arg
35	3	-		+	p.Arg614Cys
36	15	-		+	p.Gly341Arg
41	6	-		+	no variant
42	8	-		+	no variant
46	5	-		+	p.Gly341Arg
51	6	-		+	p.Val1436Met
57	3	-		+	p.Tyr3933Cys + p.Arg3366His+p.Ile157Val
58	6	-		+	p.Tyr3933Cys+p.Arg3366His+p.Ile157Val.+p.Val4849 Ile
59	5	-		+	p.Thr2206Arg
60	1	-		+	no variant
62	4	-		+	no variant
63	4	-		+	p.Asn2342Ser
64	3	-		+	p.Leu4838Val
65	4	-		+	p.Met4640Val
66	1	-		+	no variant
68	1	-		+	no variant

In 25 of the 34 families a variant was detected. In 9 families no variant could be found, even after 106 exon sequencing. + : realized, - : not realized.

families no conclusion could be drawn because of either too small a number of individuals studied and/or insufficient data.

No mutation-positive/negative IVCT diagnosis was found, e.g. in none of the MHN patients a *RYR1* mutation was found.

Discordance was observed in 7 of the 25 families (28%), but upon analysis involved only 11 individuals out of a total of 219 patients in all families.

Post-hoc analysis of the IVCT data of these 11 discordant individuals showed:

- Four test results showed borderline contractures of ≤ 2 mN with either caffeine or halothane alone.
- Two tests were characterized by poor viability of the different muscle specimens used and borderline contracture responses.
- In one family, initial analysis showed discordance but appeared to be due to wrong blood sampling, which needed resequencing. The new results indicated concordance of pheno- and genotype.

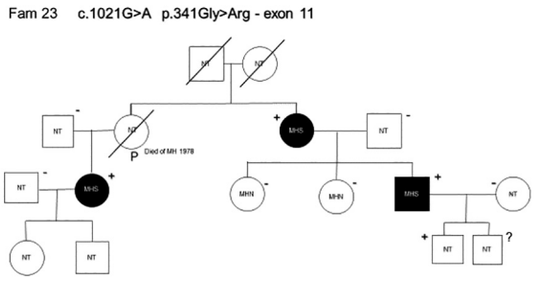


Fig. 1. — Illustrative of phenotype/genotype concordance. ○ female, □ male, / deceased, p proband, MHS : MH susceptible, MHN : not-MH susceptible. NT : not tested, + mutation present, - mutation absent, ? *RYR1* analysis not yet performed. Fifteen of 25 families showed complete phenotype-genotype concordance as the one illustrated above : all MHS patients carry the mutation, the MHN patients do not. The second son in the right hand corner indicated by ? will be offered genetic testing and not an in vitro contracture testing.

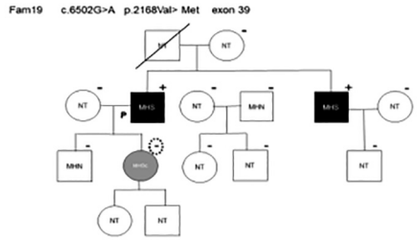


Fig. 2. — Discordance on technical grounds. The patient in gray was classified as MHSc (Malignant Hyperthermia susceptible, significant contracture to caffeine only) on the basis of an in vitro contracture testing (IVCT) performed in 1992 at the age of 3 years. The current protocol of the European Malignant Hyperthermia, as a quality control measure, requires a minimum patient age for the muscle biopsy of 10 years. In view of the absence of the mutation found in other family members it was therefore felt that this result could be a false positive IVC-diagnosis. As this lady has two children with a difficult to determine MH-status the IVCT in this patient was repeated in 2018, and found to be normal. She therefore is now considered not to be MH susceptible and neither are her children.

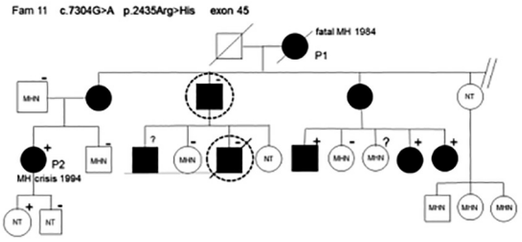


Fig. 3. — 'True' phenotype-genotype discordance: Family with 2 probandii (P1 and P2). The discordance in this family was found in both a father and his son – encircled black squares - who demonstrated convincing in vitro contracture testing results indicating MH susceptibility but did not carry the p.Arg2435His variant. As sequencing in these individuals was performed with the earlier technique of targeted 16 exon sequencing (Table 1), a new 106 exon sequencing has been initiated in order to detect a possible second sequence variant.

- Two families had both two individuals with tests of sufficient technical quality that were clearly abnormal with both halothane (4 mN at 2 Vol% Hal) and caffeine (2 mN at 2 mM caffeine) (families 8 and 11 – Fig. 3).

Discussion

The aim of the present study is to summarize the results of *RYR1* sequencing in a cohort of 34 Belgian families with proven MH susceptibility. Eighteen different variants were detected in 25 families. Ten of these are considered pathogenic, 8 are variants of 'uncertain significance'. The most frequent mutation is p.Gly341Arg, found in 7 different families. The majority of the other variants occur in one family only.

The prevalence of *RYR1* variants reported over the world varies considerably. An early study in a cohort of 50 Italian MH susceptible subjects reported a mutation detection rate of 86% (11). Screening for *RYR1* variants in 30 individuals of the North American population showed a detection rate of around 70% (12).

Other studies however indicate a much lower prevalence. One of the most comprehensive assessments of *RYR1* mutation prevalence in a sample of over 500 unrelated European MH susceptible individuals recorded a mutation rate of 30% of families (13). In 2004, a prevalence of 52% was reported in 200 German, Swiss, Dutch, and Irish families tested (5). The yield of one or more variants obtained with entire coding sequencing of *RYR1* and *CACNA1S* in Australia was reported to be 37% (14).

This report – like many others from European as well as North-American groups (1,2,5,11,12,14,19) – shows a high number of different variants, the vast majority of which are in fact found in only a few, or even single families. The relative prevalence of the individual variants varies somewhat from country to country but even the more prevalent variants (in Switzerland the Val2168Met variant is the most frequent, in the UK the Gly2434Arg variant, and Gly341Arg in this study) make up no more than 25% of the total number.

The known 'reduced penetrance' of the MH trait complicates the matter even further (15,16). Indeed, the prevalence of the genetic variants known to be associated with MH susceptibility in large genomic databases is 1:2000-1:3000. As this prevalence is far greater than the reported incidence of clinical MH crises (estimated at 1:50.000 triggering anesthesias) this is felt to reflect the

Table 3
Phenotype-genotype concordance/discordance

Amino-acid change	Exon	Nr of families	Pathogenic ±	Concordance phenotype-genotype
p.Cys35Arg	2	1	+	fam 20 concordance
p.341Gly>**A**rg	11	7	+	fam 4 concordance
			+	fam 5 discordance , 1 individual MHSh - no variant
			+	fam 9 discordance, 1 individual MHSh - no variant
			+	fam 23 concordance
			+	fam 32 concordance
			+	fam 36 discordance, 1 individual MHS - no variant
			+	fam 46 concordance
c.1122 + 5G>A	11	1	-	fam 18 insufficient data
p.614Arg>Cys	17	2	+	fam 29 concordance
			+	fam 35 discordance, 1 individual MHS - no variant
p. Arg614Leu	17	2	+	fam 7 discordance, 1 individual MHS, 1 individual MHSc - no variant
			+	fam 8 discordance, 2 individuals MHS - no variant
p.Val1436Met	30	1	-	fam 51 concordance
p.Ile1571Val p.Arg3366His p.Tyr3933Cys	33 67 86	2*	-	fam 57 concordance * fam 58 concordance
p.Val2168Met	39	1	+	fam 19 discordance, 1 individual MHSc - no variant concordance after repeat IVCT
p.Thr2206Arg	40	1	+	fam 59 concordance
p.Asn2342Ser	42	1	-	fam 63 insufficient data
p.Glu2348del	44	1	+	fam 15 concordance
p.2435Arg>His	45	1	+	fam 11 discordance, 2 individuals MHS – no variant
p.4849Val>Ile	101	2 *	+	family 57 concordance *
			+	family 3 concordance
p.Leu4838Val	101	1	+	family 64 concordance
p.Met4640Val	91	1	-	family 65 insufficient data
p.Phel4857Leu	101	1	-	family 16 concordance
				18 different variants in 25 families 15 families show concordance of phenotype and genotype, 7 show phenotype/ genotype discordance, 3 families insufficient data

"Pathogenic" refers to variants considered to be pathogenic or 'diagnostic' as listed in the database of the EMHG (www.emhg.org/diagnostic-mutations).
* the p.4849Val>Ile mutation is present in family 57 and 58.

incomplete penetrance of the MH trait. A recent article reported that the likelihood to develop MH on exposure to triggers was only 0.25 among *RYR1* mutation carriers (15). This reduced expression is in part due to the anesthetic technique (triggering of an MH crisis is both dose-and time-dependent, and the use of concomitant drugs can mitigate the symptoms), and in part due to the specific *RYR1* mutations found (and possibly modifying effects of other genetic variants), which have a differential effect on calcium release from the sarcoplasmic reticulum.

A limitation of the study is that "non-*RYR1"* variants such as reported to occur in *CACNA1S* on chrom1 were not systematically looked for in our families. This gene was not screened in view of the considerable cost involved in the screening of a cohort of 34 families, and the known relative rarity of these variants (less than 1 %) (1).

When considering the feasibility of genetic counseling in these families an important distinction has to be made between the families with *RYR1* variants of known or proven pathogenicity, and the *RYR1* variants of unknown causality. Ten of the 25 families in which a variant was found proved to carry a pathogenic variant or 'diagnostic mutation'. In these families further genetic counselling is possible in the following sense : if an individual carries one of the MH pathogenic variants, then he or she is clearly at high risk for MH when exposed to volatile anesthetics and does not need to undergo muscle biopsy and IVCT. However, in individuals

who do not carry this mutation, MH susceptibility cannot be ruled out on the basis of genetic testing because of the possibility of more than one pathogenic *RYR1* mutation being present in a given family as reported by several groups (17, 18, 19). Therefore, the European Malignant Hyperthermia Group still advises these patients to undergo IVC testing to confirm non-susceptibility to MH (3).

This point of view is supported in this series, as 7 of the 19 families with a confirmed pathogenic mutation show one or maximum two individuals which have an abnormal IVC test indicative of MH-susceptibility, but do not carry the family variant. In all but two families, the discordance concerned only one individual, and as the contracture responses were borderline (≤ 2 mN at either 2Vol% Hal or 2 mM caffeine), the discordance may be attributable to the fact that the test carries with it a small inherent risk of false positive phenotyping (4). Indeed from a clinical point of view, borderline results usually are considered abnormal in order to be on the safe side of decision making. This kind of caution however may result in false positive phenotyping in a few individuals.

In two families (family 8 and 11) there is more convincing evidence of discordance in the sense that two of the family members have good quality IVC tests with more than borderline contractures at both 2 mM caffeine and 2 Vol% halothane, but do not carry the familial *RYR1* mutation. In these two families a second mutation in *RYR1* or a different gene in EC coupling could be involved. Indeed multiple *RYR1* variants and even polygenic variants have been reported to contribute to MH in a single family (17, 18, 19).

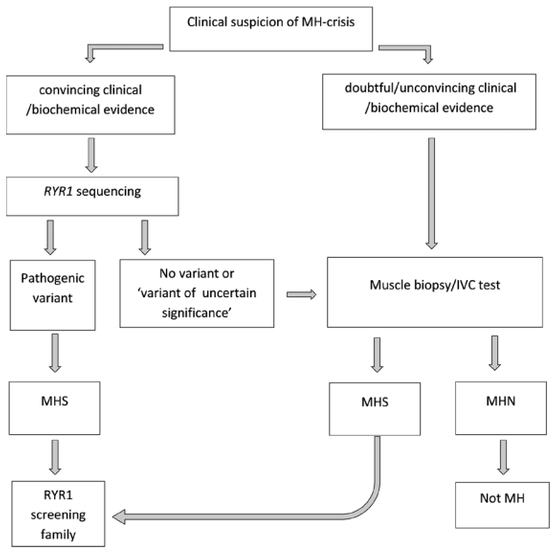
In one third (8/25) of the families, variants of unknown significance were found for which there is insufficient genetic and/or experimental evidence (yet) that they are pathogenic for MH. About 300 different of these variants have been reported in the literature, most of them in only a few families and some even in only one family worldwide. For these 'variants of uncertain significance' it is recommended that even with the availability of several bioinformatics tools to predict pathogenicity (20), the results hereof should only be used in combination with other available data such as IVCT results obtained in key individuals within that family, and segregation analysis (21).

In three of our six families with a 'variant of uncertain significance', segregation and phenotype-genotype analysis does indeed allow to offer genetic counseling as phenotyping completely correlates with the presence or absence of the variant found. In three other families, on post hoc analysis, the number of family members that could be included or information that had been obtained was too limited to be able to offer genetic counseling.

This study points to the obvious need for functional studies of more variants than the current 48 'pathogenic mutations' in the European MH database (www.EMHG.org). Likewise, central reporting of phenotype/genotype concordance and discordance in a database (as planned to be set up within the EMHG) will allow to convert a number of 'variants of unknown significance' into either pathogenic variants or clearly non-pathogenic variants. The newest genetic mutation-detection techniques allow massive parallel screening of a multitude of genes and hence have the potential to identify other DNA changes that account for the 50% of MH cases in patients not harbouring neither *RYR1* nor *DHPR* mutations.

Conclusion

Genetic counseling appeared to be feasible in 16 out of 25 families with a *RYR1* mutation, both 'pathogenic variants' and 'variants of uncertain significance', and in 16/34 (47%) of families overall.



Flowchart of the currently employed diagnostic work-up of a clinical diagnosis of an Malignant Hyperthermia (MH)-crisis in a proband. The decision to commence by either muscle biopsy or molecular genetic analysis is based on the available clinical information: convincing evidence allows for first line RYR1 sequencing whereas a doubtful episode first requires in vitro contracture testing (IVCT) either corroborating the clinical diagnosis, or negating it. Further family counselling is based upon a combination of IVCT data and molecular genetic analysis. MHS : Malignant Hyperthermia susceptible ; MHN : Malignant Hyperthermia non-susceptible.

The following algorithm summarizes the present diagnostic approach used in our lab :

References

1. Rosenberg H., Pollock N., Schiemann A., Bulger T. and Stowell K. 2015. Malignant hyperthermia : a review. Orphanet J. Rare Dis. 28: 93-145.
2. Brandom B.W. 2006. Genetics of malignant hyperthermia. Sci World J. 6: 1722-1730.
3. Hopkins P.M., Ruffert H., Snoeck M.M., Girard T., Glahn K.P. and Ellis F.R., et al. 2015. European Malignant Hyperthermia Group guidelines for investigation of malignant hyperthermia susceptibility. Br. J. Anaesth. 115**:** 531-539.
4. Ording H., Brancadoro V., Cozzolino S., Ellis F.R., Glauber V. and Gonano E.F., et al. 1997. In vitro contracture test for diagnosis of malignant hyperthermia following the protocol of the European MH Group: results of testing patients surviving fulminant MH and unrelated low-risk subjects. The European Malignant Hyperthermia Group. Acta Anaesth. Scand. 41: 955-966.
5. Girard T., Treves S., Voronkov E., Siegemund M. and Urwyler A. 2004. Molecular Genetic Testing for Malignant Hyperthermia Susceptibility. Anesthesiology 100:1076-1080.
6. MacLennan D.H., Duff C., Zorzato F., Fujii J., Phillips M. and Korneluk R.G., et al. 1990. The ryanodine receptor gene: a candidate gene for the predisposition to malignant hyperthermia. Nature. 343: 559-561.
7. Parker R., Schiemann A.H., Langton E., Bulger T., Pollock N. and Bjorksten A. 2017. Functional characterization of C-terminal ryanodine receptor 1 variants associated with central core disease and malignant hyperthermia. J. Neuromusc. Dis. 4: 147-158.
8. Heytens L. 2007. Molecular genetic detection of susceptibility to malignant hyperthermia in Belgian families. Acta Anaesth. Belg. 58: 113-118.
9. Richards S., Aziz N., Bale S., Bick D., Das S. and Gastier-Foster J., et al. 2015. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet. Med. 17: 405-424.
10. Kraeva N., Heytens L., Jungbluth H., Treves S., Voermans N. and Kamsteeg E., et al. 2015. Compound *RYR1* heterozygosity resulting in a complex phenotype of malignant hyperthermia susceptibility and a core myopathy. Neuromuscul disord. 225: 567-576.
11. Galli L., Orrico A., Lorenzini S., Censini S., Falciani M. and Covacci A., et al. 2006. Frequency and localization of mutations in the 106 exons of the *RYR1* gene in 50 individuals with malignant hyperthermia. Hum. Mutat. 27: 830-839.
12. Sambuughin N., Holley H., Muldoon S., Brandom B.W., de Bantel A.M. and Tobin J.R., et al. 2005. Screening of the entire ryanodine receptor type 1 coding region for sequence variants associated with malignant hyperthermia susceptibility in the North American population. Anesthesiology 102**:** 515-521.
13. Robinson R.L., Anetseder M.J., Brancadoro V., Van Broekhoven C. and Carsana A., et al. 2003. Recent advances in the diagnosis of malignant hyperthermia susceptibility: How confident can we be of genetic testing? Eur. J. Human Genet. 11: 342-348.
14. Gillies R.L., Bjorksten A.R., Du Sart D. and Hockey B.M. 2015. Analysis of the entire ryanodine receptor type 1 and alpha 1 subunit of the dihydropyridine receptor (CACNA1S) coding regions for variants associated with malignant hyperthermia in Australian families. Anaesth. Intensive Care 43: 157-166.
15. Ibarra Moreno C.A., Hu S., Kraeva N., Schuster F., Johannsen S. and Rueffert H., et al. 2019. An assessment of penetrance and clinical expression of malignant hyperthermia in individuals carrying diagnostic ryanodine receptor 1 gene mutations. Anesthesiology. 131: 983-991.
16. Riazi S., Kraeva N. and Hopkins P.M. 2018. Malignant Hyperthermia in the post-genomics era: new perspectives on an old concept. Anesthesiology. 128: 168-180.
17. Rosenberg H., Davis M., James D., Pollock N. and Stowell K. 2007. Malignant hyperthermia. Orphanet J. Rare Dis. 2: 21-53.
18. Stewart S.L., Hogan K., Rosenberg H. and Fletcher J.E. 2001. Identification of the Arg1086His mutation in the alpha subunit of the voltage-dependent calcium channel (CACNA1S) in a North American family with malignant hyperthermia, Clin. Genet. 5:178-184.
19. Tammaro A., Di Martino A., Bracco A., Cozzolino S., Savoia G. and Andria B., et al. 2011. Novel missense mutations and unexpected multiple changes of *RYR1* gene in 75 malignant hyperthermia families. Clin. Genet. 79: 438-447.
20. Flanagan S.E., Patch A.M. and Ellard S. 2010. Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. Genet. Test. Mol. Biomarkers 14: 533-537.
21. Schiemann A.H. and Stowell K.M. 2016. Comparison of pathogenicity prediction tools on missense variants in *RYR1* and CACNA1S associated with malignant hyperthermia. Brit. J. Anaesth. 117: 124-128.

Explanatory list of genetic terminology used :

- *RYR1* gene: protein coding gene containing the genetic information for the making of a protein called 'ryanodine receptor 1' which functions as the main calcium release channel in the sarcoplasmic reticulum in striated muscle
- Gene: DNA unit of hereditary information, consisting of two alleles (one inherited from the father, one from the mother), each on one of the homologous chromosomes
- Exon: coding part of a gene ; contains the genetic information for making a protein
- Intron: part of a gene that does not contain genetic information for the protein ; introns lie in between exons and allow the gene to make different proteins ; they are characteristic for eukaryotic genes
- Sequencing, or sequence analysis: identifies the sequence or order of nucleotides in a gene
- DNA sequence variant: the overall term for a change in the sequence code of a gene which may be a 'non-disease causing change' (polymorphism), a 'disease causing or pathogenic change' (mutation) or a change with an unknown effect (variant of uncertain significance). As the distinction is not always clear the term 'variant' is used throughout the article
- Phenotype = description of an individuals' physical characteristics (in this article it signifies the presence or absence of MH susceptibility)
- Genotype: the genetic make-up or constitution of an individual, or a section of DNA (gene) that encodes a particular protein (in this article: *RYR1*)
- Segregation analysis: aims to determine the transmission pattern of the trait within families and tests whether the presence/absence of a particular DNA variant corresponds with the presence/absence of a particular phenotype ; concordance meaning agreement, discordance meaning lack of agreement
- Missense variant: a DNA sequence variant that leads to a single amino acid substitution. In MH this will alter the

structure of the *RYR1*-channel causing it to open more easily in response to the triggering drugs

- Genetic heterogeneity: a particular phenotype (here: MH susceptibility) may be the result of several allelic variants (allelic heterogeneity ; variants in one gene) or can be the result of mutations in different genes (localized on different chromosomes ; chromosomal heterogeneity)
- Proband : the first affected family member who seeks medical attention for a genetic disorder
- Penetrance is the probability of a symptom being manifest given a certain genotype ; it refers to the proportion of people with a particular genotypic change who exhibit symptoms of that genetic disorder. When less than 100% of the individuals who carry the mutation develop the disease, the condition is said to have reduced penetrance
- *CACNA1S* gene = protein coding gene for part (alfa-1 subunit) of the dihydropyridine receptor, the second major protein involved in the calcium release process from the sarcoplasmatic reticulum.