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Review Article

European Malignant Hyperthermia Group guidelines for investigation of malignant hyperthermia susceptibility

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Abstract

It is 30 yr since the *British Journal of Anaesthesia* published the first consensus protocol for the laboratory diagnosis of malignant hyperthermia susceptibility from the European Malignant Hyperthermia Group. This has subsequently been used in more than 10 000 individuals worldwide to inform use of anaesthetic drugs in these patients with increased risk of developing malignant hyperthermia during general anaesthesia, representing an early and successful example of stratified medicine. In 2001, our group also published a guideline for the use of DNA-based screening of malignant hyperthermia susceptibility. We now present an updated and complete guideline for the diagnostic pathway for patients potentially at increased risk of developing malignant hyperthermia. We introduce the new guideline with a narrative commentary that describes its development, the changes to previously published protocols and guidelines, and new sections, including recommendations for patient referral criteria and clinical interpretation of laboratory findings.

Key words: *CACNA1S*; excitation–contraction coupling; malignant hyperthermia; malignant hyperthermia susceptibility, diagnosis; *RYR1*; skeletal muscle

The European Malignant Hyperthermia Group (EMHG) was formed in 1983[1] with the principal objective of standardizing the laboratory diagnosis of malignant hyperthermia (MH) susceptibility. At that time, pharmacological challenge tests carried out on excised skeletal muscle biopsies were being used for this purpose by several groups in North America, Europe, South Africa, and Australia. These tests were based on the findings that MH muscle samples were more sensitive to the contracture-inducing properties of caffeine, or caffeine with halothane,[2] or halothane alone[3] than normal muscle, but with such interlaboratory variation that comparisons of results were impossible. One particular area of contention was the combined caffeine–halothane test, which had proponents in North America but not in Europe. In 1984, the EMHG published its protocol for an *in vitro* contracture test (IVCT),[4] and this has formed the bedrock of clinical diagnosis and phenotyping for research in Europe throughout the past

† Contributors to the guidelines from member laboratories of the European Malignant Hyperthermia Group are listed in Appendix 3.

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30 yr. The protocol has been the subject of annual review by the EMHG and, since the launch of the EMHG website (www.emhg.org), the latest version of the protocol has been available online. Member laboratories of the EMHG participate in a quality assurance-programme, with a principal focus on compliance with the protocol.

Although the protocol was developed through an informal consensus approach, it was based on the collective experience of the EMHG founding members, which in 1984 already encompassed investigation by muscle biopsy of more than 1500 individuals at risk of MH susceptibility either through a history of a suspected MH reaction or a family history. In 1984, there was no obvious means of validating the test because there were insufficient data on the outcome of the test in low-risk individuals and no consensus on the clinical criteria for assigning 'true-positive' status without recourse to results of the IVCT. After the publication of a clinical grading scale[5] and accumulation of results of the IVCT in low-risk individuals, an evaluation of the EMHG became feasible.[6] This confirmed that the IVCT was not specific (point estimate for specificity of 94%) because some low-risk individuals had positive IVCT responses. It is possible that some of these false-positive results arose from the use of atrophied muscle, because muscle sampled during the course of ipsilateral joint arthroplasty was included. The reported point estimate for the sensitivity of the IVCT was 99%. However, examination of the single patient (patient 4)[6] who reduced the sensitivity from 100% reveals that the authors misapplied the Clinical Grading Scale,[5] which specifically excludes rigidity on emergence of general anaesthesia as an indicator of MH. The Clinical Grading Scale score for this patient should have been a maximum of 35, therefore excluding this patient from the category of 'almost certain' MH used to determine the sensitivity of the test. It seems likely that this was an instance of iatrogenic hyperthermia and possible febrile convulsion in an infant who had received atropine before surgery and overly enthusiastic warming during surgery.

The rigour of the IVCT is also evident from its use to phenotype members of MH families in molecular genetic studies. The IVCT, and its North American equivalent, enabled the linkage analyses that identified *RYR1* as the major locus implicated in MH.[7 8] During the 1990s, results from the IVCT suggested, to the scepticism of many geneticists at the time, that *RYR1* was not involved in all MH patients[9–11] and that more than one genetic factor was likely to be implicated in some families.[12 13] The robustness of the IVCT has been borne out by its use to identify a second locus (*CACNA1S*)[11 14] and provide further evidence for the involvement of interacting gene products.[15 16] The complexity of the genetics of MH, which would not have been realized without the IVCT, has been confirmed further by early results of next-generation sequencing in MH families.[17 18] Although the EMHG appreciated the complexity of the genetics of MH, through collaborative working of its multidisciplinary membership it developed guidelines for the use of molecular genetic techniques in order to reduce the need for a muscle biopsy in selected patients, while recognizing that MH susceptibility could be excluded for clinical purposes only with the use of the IVCT. These guidelines were published in 2001[19] and subsequently evaluated.[20]

In 2012, the executive committee of the EMHG instigated a major review of the IVCT protocol and, in the course of this review, decided that a complete guideline for the diagnosis of MH susceptibility would be useful, including updated advice on the use genetic screening. The guideline has been drafted by a writing committee, with two rounds of consultation with representatives from each member laboratory of the EMHG. The final version of the guideline (Appendix 1) was agreed by the EMHG in May 2014. We will now discuss the rationale for elements of the guideline that were not included in either the IVCT protocol or the previous genetic diagnosis guideline, and the major changes to these previous documents.

Patient referral criteria

This is a new addition to previous guidelines. For possible malignant hyperthermia reactions during general anaesthesia, we considered using the score provided by the Clinical Grading Scale.[5] This can provide an estimate of the likelihood of a malignant hyperthermia reaction, but the decision required in the diagnostic setting is whether MH can be excluded or not, for which the Clinical Grading Scale is generally not helpful. When MH cannot be excluded (for example, by identification of a more likely explanation for the signs, inconsistency of the signs with MH, or both), referral is indicated.

The referral criteria also reflect our current understanding of events that, in themselves, are not malignant hyperthermia reactions (a progressive life-threatening hyperthermic reaction during general anaesthesia), but are possible manifestations of the underlying genetic defect(s) leading to malignant hyperthermia susceptibility. Many such manifestations have been proposed over the years, such as sudden infant death syndrome,[21] but most have not stood up to robust analysis.[22] There is now strong evidence that some patients susceptible to MH are at increased risk of developing rhabdomyolysis secondary to heavy exercise[23] but also to several other known causes of rhabdomyolysis.[24] There are, of course, other causes of recurrent rhabdomyolysis, and investigation for these by a neurologist should be done in conjunction with an MH referral centre. This will enable sequencing of *RYR1* and *CACNA1S* at the same time as genetic screening of other genes associated with rhabdomyolysis, such as *CPT2*, *PYGM*, *ACADM*, *AMPD1*, and *VLCAD*. If a muscle biopsy is indicated in the course of neurological assessment, this should be done in an MH centre so that samples for IVCT and for histological examination can be obtained and processed appropriately.

Persistently raised serum creatine kinase concentration was first documented in association with MH susceptibility before the principle of the IVCT was established[25] and was proposed as a diagnostic test. The utility of creatine kinase concentration is, however, limited because of a lack of sensitivity and specificity.[26] In patients with so-called idiopathic hyperCKaemia, where full neurological evaluation has excluded other causes, investigation of MH susceptibility may be warranted.[27] As with patients with rhabdomyolysis, the neurologist should liaise with an MH testing centre, where any muscle biopsy should be carried out.

There is considerable overlap between the clinical features of exertional heat illness and MH. Indeed, if diagnostic classification of heat illness, specifically the diagnosis of heat stroke, did not require a clinical assessment of mental function and level of consciousness, a separate International Classification of Disease code for MH might not have emerged. Clearly, the definition of MH as a condition occurring during general anaesthesia makes any such assessment impossible. While there appears to be sufficient evidence to support a relationship between genetic predisposition to exertional heat illness and MH susceptibility,[28–30] the evidence to define that relationship is less tangible.[31] A major difference with implications for diagnostic strategy is that MH arises exclusively in individuals with a genetic predisposition, whereas that seems likely in perhaps a minority of patients with exertional heat illness. There are indeed well-recognized non-genetic

factors that predispose to exertional heat illness,[32] and we recommend that these are excluded before further investigation, because the purpose of further tests is to identify individuals (and their relatives) at risk of recurrent episodes, rather than to confirm the clinical diagnosis of heat illness *per se*.

The *in vitro* contracture test

Only very minor adjustments have been made to the technical conduct of the IVCT compared with the last published version[6] because the validation study revealed a clinically useful and robust test.[6] One of these amendments is to stipulate an increased minimal length of muscle specimen for use in the tests, because muscle length has emerged as the major determinant of specimen viability (EMHG, unpublished data). The EMHG quality-assurance programme aims to guarantee control of the test bath constituents and conditions, including concentration measurements by a reference laboratory.

We have added a recommendation concerning the minimum age and weight of children undergoing the procedure. Before formation of the EMHG, there were anecdotal reports of normal contracture responses in young children (less than 3 yr of age) considered, on clinical grounds, to have had an MH reaction. The study that evaluated the sensitivity and specificity of the IVCT included children as young as 4 yr of age[6] and found no evidence of reduced sensitivity of the IVCT in children of 4 yr of age or older. However, most of the participating centres did not contribute data from children younger than 10 yr of age and are not minded to change their policy without more data on high-risk and low-risk children. There are also concerns among some members of the EMHG that there may be unacceptable cosmetic and functional consequences of the muscle biopsy in children below a certain body weight (e.g. 30 kg) because of the need to obtain samples of the required length.

The major change in the protocol is in the laboratory diagnostic classification. In all previous versions of the protocol, patients were classified, on the basis of the results of their IVCT responses, into one of the following three groups: MHS (MH susceptible); MHN (MH normal); or MHE (MH equivocal). Although the original criteria for categorizing results were modified in 1985,[33] the principle was the same, such that patients with abnormal responses to both halothane and caffeine were classified as MHS, patients with normal responses to both halothane and caffeine were classified as MHN, and patients with an abnormal response to either caffeine or halothane but not both were classified as MHE. While this system undoubtedly proved beneficial for some types of collaborative research projects among EMHG members and for comparing results from different diagnostic centres, it has its limitations.

The first limitation arose from the use of the term 'equivocal'. While this indeed reflected the uncertainty of the founder EMHG members as to the clinical significance of the MHE laboratory classification, there has been a consensus since 1985[33] that patients with an MHE laboratory classification should be considered clinically at risk of developing MH under anaesthesia. The appropriateness of this approach has been borne out by a study that evaluated the sensitivity and specificity of the protocol[6] and the results of genetic analyses. However, use of the 'equivocal' label outside of its laboratory context has the potential to confuse patients and clinicians unfamiliar with its derivation. The changes in the protocol are to address this problem such that the primary designation of any patient with an abnormal response to either or both caffeine and halothane tests is MHS. A suffix is then added to indicate an abnormal response to halothane (h) or caffeine (c), so that there are now four laboratory diagnostic groups: MHS_{hc} (formerly MHS); MHS_h (formerly MHE); MHS_c (formerly MHE); and MHN (unchanged).

The second limitation of the old (and indeed new) laboratory classification system is that it is crude, not using a wealth of data that can be derived from the contracture studies. This was best appreciated at the outset of molecular genetic investigations of MH susceptibility, because accurate phenotyping is essential for robust genetics. The EMHG adopted, although never published, a recommendation that the minimal data set for the IVCT phenotype should be the threshold concentration (concentration producing a contracture of 2 mN or 0.2 g force) of halothane and caffeine, along with the tension produced at 2% halothane and at 2 mM caffeine. Even more sophisticated approaches have been proposed,[34 35] and the genetic basis for the observed phenotypic variability has been published.[36] We did, therefore, consider introducing more sophistication into the reporting of IVCT results but pragmatically decided that the primary purpose of the test was to provide a categorical diagnosis for clinical purposes with known sensitivity and specificity.

Clinical interpretation of *in vitro* contracture test results

This is another addition to previous publications. It is a reminder to the MH specialist that they must interpret laboratory findings in the light of the totality of clinical and investigative data availability for the benefit of non-specialists. It is the latter group of clinicians who provide the great majority of medical care to MH patients. The most fundamental role of MH diagnostics is to be conservative in applying the diagnosis of not susceptible to MH because false-negative diagnosis is most likely to have disastrous consequences. The evidence is that a laboratory diagnosis of MHN by IVCT provides a high degree of security if carried out in an EMHG-accredited laboratory that conducts the tests according to this protocol and where the specimens fulfil the viability criteria.[6] While the offspring of a patient tested MHN cannot inherit MH susceptibility from that parent, it is still possible that they may be susceptible by transmission from the other untested parent or through a *de novo* genetic mutation; the probability of either of these events is less than the population prevalence of MH susceptibility.

Molecular genetic detection of susceptibility to malignant hyperthermia

The original genetic guideline published in 2001 described the potential diagnostic use of screening for 15 mutations in the *RYR1* gene shown to produce functional changes compatible with a pathogenic role in appropriate model cell sytems.[19] The guideline also described the possibility of using genetic markers that showed highly statistically significant segregation with the MH trait in individual families. A key message of those guidelines was the need to confirm MHN status using the IVCT in individuals who did not carry the familial *RYR1* mutation or genetic marker. This was because of reports of discordance between *RYR1* genotype and the IVCT phenotype in a number of families across Europe. Evidence for a biological basis for these observations, rather than them being a consequence of test failings, has accumulated since then.[15 16 20 36 37]

Recent evidence has also confirmed the foresight of the previous guidelines in requiring functional analysis of missense variants before their adoption for diagnostic use. Kim and

colleagues[18] reported the prevalence of rare *RYR1* variants in a control sample to be 6%. It is interesting that generic guidelines for interpretation of findings of variants in other genes adopt a similar approach.[38] These generic guidelines also propose using segregation analyses[39] to indicate likely pathogenicity, but in our experience it is rare to generate sufficient statistical power to enable this, even when combining data from several families carrying the same variant.

Unfortunately, the costs and technical difficulties associated with conducting rigorous functional analyses are rate limiting. Although more than 180 *RYR1* variants have been associated with MH susceptibility, only 33 have been shown to have functional effects consistent with MH pathogenicity (www.emhg.org). Two variants in *CACNA1S* have also been shown to be functionally consistent with pathogenicity.[40 41] The inclusion of the use of *ex vivo* preparations for functional analysis is controversial (Appendix 2), even with the safeguard that consistent results need to be obtained using preparations derived from at least two unrelated individuals. We consider that the balance of risk from misdiagnosis (false-positive diagnosis of MH susceptibility) is not sufficient to outweigh the benefit of avoiding muscle biopsy in these families, so long as the muscle biopsy and IVCT are required to confirm that an individual is not susceptible. If functional characterization is done using the more rigorous genetic manipulation of heterologous[42] or homologous[43] expression systems, we have removed the need for the variant to have been described in more than one family (Appendix 2), in line with generic guidelines.[38] This recognizes that many variants are so far private to individual MH families. We also recognize that reports of functional analyses of further *RYR1* variants may not necessarily continue to be easy to publish, and we have therefore removed the need for functional analyses using previously described methods to be published before accepting the relevant variant for diagnostic use. Instead, reports of functional analyses will be peer reviewed by members of the EMHG with the relevant expertise.

Advances in genetic technology have prompted a change in our recommendations for the diagnostic pathway of patients referred for investigation of MH susceptibility (Fig. 1). Our previous genetic testing guideline[19] recommended muscle biopsy and IVCT as the primary investigation for the index case, with subsequent mutation screening when the IVCT confirmed MH susceptibility. We now consider DNA screening to be a viable alternative primary diagnostic approach to the IVCT.[45] The more than doubling in number of functionally characterized variants associated with MH susceptibility has increased the proportion of MH families able to benefit from DNA diagnostics from ~25% in 2001 to more than 40% now (data extrapolated from Carpenter and colleagues).[36] The potential expense in screening for these additional variants has been more than compensated by the advances in genetic technology.

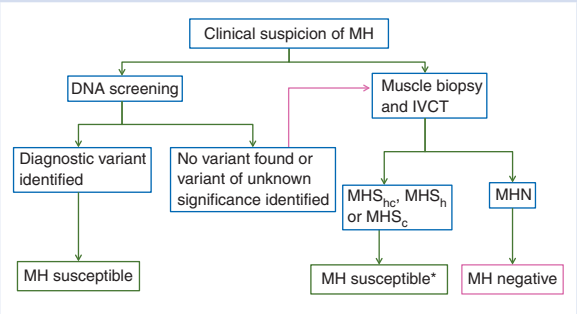

Fig 1 Diagnostic pathway for investigation of MH susceptibility. The decision to pursue either DNA screening or muscle biopsy and IVCT in the first instance will be made on a patient-by-patient basis by the MH diagnostic centre in consultation with the patient and their health-care funder. Factors that may be taken into consideration will include the availability of the respective tests (including turnaround time), the urgency of the test (for example, if the patient is awaiting surgery), the prior probability of a positive diagnosis, and the costs of the tests in the relevant laboratory. The prior probability of a positive diagnosis will be estimated by the clinical director of the MH centre based on clinical judgement, published data,[5 44] or a combination of these. *These patients should be invited to take part in research studies of the genetic basis of malignant hyperthermia. IVCT, *in vitro* contracture test; MH, malignant hyperthermia; MHN, *in vitro* contracture test laboratory classification applied when all contracture tests are negative; MHS$_{hc}$, MHS$_h$, and MHS$_c$, IVCT laboratory classifications applied when contracture responses to both halothane and caffeine are abnormal, response to halothane alone is abnormal, or response to caffeine alone is abnormal, respectively.

Conclusion

The EMHG has led the way in the standardization and quality assurance of diagnostic testing for MH susceptibility throughout the past 30 yr. This new guideline updates and adds to previous publications as an important contribution to the safety of patients requiring general anaesthesia.

Authors' contributions

Conception, planning, and drafting of the manuscript: P.M.H., H.R., M.M.S., T.G., K.P.E.G., F.R.E., C.R.M., A.U.
Approval of the final manuscript: P.M.H., H.R., M.M.S., T.G., K.P.E.G., F.R.E., C.R.M., A.U.
Reviewed and provided suggestions on drafts of the guidelines: additional listed members of the European Malignant Hyperthermia Group†.

Declaration of interest

P.M.H. is a Trustee and Director of the *British Journal of Anaesthesia*. All other authors: none declared.

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Appendix 1: European Malignant Hyperthermia Group guideline for the investigation of malignant hyperthermia susceptibility

Investigation of malignant hyperthermia (MH) susceptibility initially involves clinical evaluation of a patient's risk based on their anaesthetic and medical history, and relevant family history. Further investigation is indicated when increased risk of susceptibility to MH cannot be excluded. The highest sensitivity for detecting susceptibility to MH is provided by pharmacological challenge tests carried out on freshly excised skeletal muscle in controlled laboratory conditions. These tests, when carried out according to the following protocol, are collectively referred to as the *in vitro* contracture test, or IVCT. The IVCT is recommended for individuals considered to be at increased risk of MH either as a first-line test or when DNA analyses have failed to confirm the high-risk status. DNA analyses are less invasive than the IVCT but not as sensitive. They have a major role in family screening and, with recent improvements in cost-effectiveness of genotyping, can play a role in the primary investigation of index cases.[45]

A. Patient referral criteria

The following are the most common reasons for referral for investigation of MH susceptibility.

1. Family history of MH.
2. Adverse reaction to general anaesthesia where a trigger agent has been used, involving any combination of signs of increased metabolism (unexplained increase in carbon dioxide production, tachycardia, temperature increase), muscle rigidity, rhabdomyolysis, disseminated intravascular coagulation or death, or both. Initial signs should be evident during anaesthesia or within 60 min of discontinuation of anaesthesia.
3. Family history of unexplained perioperative death.
4. Postoperative rhabdomyolysis after clinical exclusion of other myopathies.
5. Exertional rhabdomyolysis, recurrent rhabdomyolysis, or persistently raised serum creatine kinase concentration where no cause has been identified after neurological work-up (idiopathic hyperCKaemia).
6. Exertional heat stroke requiring hospital admission, where known predisposing factors have been excluded.
7. Myopathy and detection of an uncharacterized, rare, potentially pathogenic *RYR1* variant.

B. *In vitro* contracture test

1. The minimum patient age for the muscle biopsy is 4 yr, but laboratories should not test children younger than 10 yr of age without relevant control data. Laboratories may also set minimum body weight limits.
2. The biopsy should be performed on the quadriceps muscle (either *vastus medialis* or *vastus lateralis*), using local (avoiding local anaesthetic infiltration of muscle tissue), regional, or trigger-free general anaesthetic techniques.
3. The muscle samples can be dissected *in vivo* or removed as a block for dissection in the laboratory within 15 min.
4. The excised muscle should be placed immediately in precarboxygenated Krebs–Ringer solution of the following composition (in millimoles per litre): NaCl, 118.1; KCl, 3.4; $MgSO_4$, 0.8; KH_2PO_4, 1.2; glucose, 11.1; $NaHCO_3$, 25.0; and $CaCl_2$, 2.5 (pH 7.4). Freshly made or pharmaceutically stable Krebs–Ringer solution should be used. The ion concentration should be as

stated with a maximum deviation of (10%), and its pH should be in the range 7.35–7.45 at 37°C.

5. The muscle should be transported to the laboratory in Krebs–Ringer solution at ambient temperature. In the laboratory, it should be kept at room temperature and carboxygenated.
6. The time from biopsy to completion of the tests should not exceed 5 h.
7. The tests should be performed at 37°C in a tissue bath perfused either intermittently or continuously with Krebs–Ringer solution and carboxygenated continuously. At least four tests should be performed, each one using a fresh specimen. These include two static caffeine tests (see 11 below) and two halothane tests. The halothane test could consist of either one static (see 12 below) and one dynamic test (see 13 below) or two static tests. Each laboratory should be consistent in the method used. Separate tissue baths should be used for different agents.
8. **Muscle specimen dimensions.** Muscle specimens suitable for *in vitro* investigation should measure 20–25 mm in length between ties, with a thickness of 2–3 mm. For measurement of length, see 8 below. The weight of the specimens should be 100–200 mg. The specimens are blotted and weighed after the test, between sutures.
9. **Determination of specimen length and predrug force.** The static tests (see 11 and 12 below) are performed at optimal length (l_0) which is determined 5 min after suspension of the specimen in the tissue bath by slowly stretching the muscle to force of 2 mN (0.2 g). The length between sutures is measured (initial length). Leave the muscle for another 4 min at initial length, then commence electrical stimulation (see 10 below) and stretch the muscle slowly until optimal twitch results are obtained (usually corresponding to 2–3 g or to 120–150% of initial length). This new length is considered to be the optimal length (l_0) and is recorded. The muscle is left at optimal length (l_0) to stabilize for at least 15 min and until baseline force does not vary more than 2.0 mN (0.2 g) within a 10 min period. Drugs may then be added. The baseline force immediately before addition of drug is recorded as the predrug force.
10. **Electrical stimulation.** To demonstrate viability, the muscle specimen should be electrically stimulated (field stimulation) with a 1–2 ms supramaximal stimulus at a frequency of 0.2 Hz. After suspension of the muscle in the tissue bath and with the muscle at optimal length, current or voltage is slowly increased until twitch height does not increase any more (initial stimulus intensity). For the supramaximal stimulation, the current or voltage is increased to 120% of initial stimulus intensity.
11. **The static cumulative caffeine test and measurement of the caffeine threshold.** The concentrations of caffeine (as free base, analytical grade) in the tissue bath should be increased stepwise as follows: 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 32 mmol litre^{-1}. Each successive concentration of caffeine should be administered as soon as the maximal contracture plateau induced by the previous concentration of caffeine has been reached, or after exposure of the muscle to the caffeine concentration for 3 min if no contracture occurs. The muscle is not washed with fresh Krebs–Ringer solution between successive concentrations of caffeine. Caffeine should be added to the tissue bath either as a bolus by injection or, with low-volume (<5 ml) baths, in the Krebs–Ringer perfusate. A rapid change of caffeine concentration must be achieved. The result of this test will be reported as the threshold concentration, which is the lowest concentration of caffeine that produces a sustained increase of at least 2 mN (0.2 g) in baseline force from the lowest force reached. In addition, the maximal contracture achieved at a caffeine concentration of 2 mmol litre^{-1} should be reported. Please note that the lowest force is not necessarily the same as the predrug force.
12. **The static halothane test and measurement of static halothane threshold.** The halothane threshold is obtained using the halothane concentrations 0.11, 0.22, 0.44, and an optional concentration of 0.66 mmol litre^{-1} as equivalent to 0.5, 1.0, 2.0, and 3.0 Vol%, respectively, from a serviced and calibrated vaporizer. It is recommended that the halothane concentration in the gas phase should be measured close to the inlet port of the tissue bath or the tissue bath concentration should be measured regularly using gas chromatography, or both (see under Quality Control, below). The specimen should be exposed to each halothane concentration for at least 3 min or until maximal contracture is reached. The result of this test will be reported as the threshold concentration, which is the lowest concentration of halothane that produces a contracture of at least 2 mN (0.2 g) measured as an increase in baseline force from the lowest force reached. The measurement of halothane should also be reported. For determination of halothane concentration, see 14 below. The flow rate of gas should be set to maintain the correct halothane concentration in the tissue bath. The gas flow into the tissue bath should be controlled using a low-flow rotameter or similar device, situated close to the inlet port of the tissue bath. The time to reach equilibration of the halothane concentration in the bath should be determined in order to ensure that the muscle sample is exposed to the test drug for the required period. The equilibration time will depend on bath volume, gas flow rate, rate of perfusion, and the dynamics of the tissue bath.
13. **The dynamic halothane test and measurement of dynamic halothane threshold.** This test requires a motor to enable stretching and relaxation cycles of the muscle specimen at predefined constant rates. Initially, the muscle is stretched at a constant rate of 4 mm min^{-1} to achieve a force of ~30 mN (3 g) and held at this new length for 1 min. The stretching process is then reversed for 1.5 min. The movement of the transducer from the end of the 1 min rest period to the low force is measured accurately using a vernier scale. This measurement is then used to achieve all subsequent length–tension curves; the muscle is stretched and shortened 6 mm in each cycle. The muscle is allowed to rest for 3 min. The process is then repeated to obtain three control curves with 1 min rest at high force and 3 min rest at low force. At the end of the descent of the third control curve, the muscle is exposed to halothane at 0.11 mmol litre^{-1} (0.5%) for 3 min, and the stretch process is repeated. The procedure is repeated for halothane concentrations of 0.22 and 0.44 mmol litre^{-1} (1 and 2%, respectively). The force is measured at the end of the 1 min rest after stretching, and the dynamic halothane threshold is the lowest concentration increasing force 2 mN (0.2 g). The contracture at 0.44 mmol litre^{-1} is also recorded.
14. **Laboratory diagnostic classification**
    - MHS_{hc}: a caffeine threshold (as defined earlier) at a caffeine concentration of 2.0 mmol litre^{-1} or less in at least one caffeine test, and a halothane threshold concentration at 0.44 mmol litre^{-1} or less in at least one halothane test.
    - MHS_h: a halothane threshold concentration at 0.44 mmol litre^{-1} or less in at least one halothane test and a caffeine threshold at a caffeine concentration of 3 mmol litre^{-1} or more in all caffeine tests.

- MHS_c: a caffeine threshold at a caffeine concentration of 2.0 mmol litre^{-1} or less and a halothane threshold concentration above 0.44 mmol litre^{-1} in all halothane tests.
- MHN: a caffeine threshold at a caffeine concentration of 3 mmol litre^{-1} or more in all caffeine tests and a halothane threshold concentration above 0.44 mmol litre^{-1} in all halothane tests.

15. **Quality control.** Viability in any specimen used should be demonstrated by twitches ≥10 mN (1 g) at the beginning of a test and, for the caffeine test, a response to 32 mmol litre^{-1} ≥50 mN (5 g) at the end. The concentrations of halothane and caffeine in the tissue bath should be checked at least every 6 months. The samples should be obtained directly from the tissue bath in the same dynamic conditions as when testing. Samples for determination of halothane concentrations should be obtained immediately after the gas flow has been stopped to avoid sampling from the gas phase. Halothane concentrations can be measured using gas chromatography or high performance liquid chromatography and caffeine using ultraviolet spectroscopy. Halothane 0.11 and 0.44 mmol litre^{-1} and caffeine 0.5 and 2 mmol litre^{-1} should be checked. Accepted maximal deviation from the desired concentrations are (10%). Lambda halothane (air/Krebs–Ringer solution) is taken to be 0.72 at 37°C. The vaporizer should be serviced and calibrated in accordance with the manufacturer's recommendations.
16. **Control biopsies.** Prospective MH units should test 30 control muscle samples according to this protocol before commencing their diagnostic programme. All MH units are asked to investigate further control samples when feasible. For control samples, the following groups of patients are considered suitable: healthy volunteers; patients having amputations for localized disease (not systemic or vascular disease); patients with varicose veins; brain-dead patients within the first 24 h; and patients with fractures within the first 24 h. Control biopsies should be conducted within the ethical framework of the local institutional review board or ethics committee.
17. **Optional tests.** Tests with other drugs may be performed on an optional basis. Results of optional tests are not used for diagnosis. However, to allow for comparison of results between centres it is recommended that optional tests are performed in a uniform way, agreed upon by the European Malignant Hyperthermia Group (EMHG) Board of Directors. At present, protocols exist for tests with ryanodine, sevoflurane, and 4-chloro-*m*-cresol. These protocols may be accessed through the EMHG homepage (www.emhg.org).
18. **Protocol review.** The EMHG protocol for investigation of MH susceptibility by IVCT is reviewed annually.

C. Clinical interpretation of *in vitro* contracture test results

Clinical advice provided by the diagnostic laboratory director remains the responsibility of the individual physician. All available information should be taken into account, including clinical evaluation and IVCT results. Muscle histopathology, serum biochemistry, and molecular genetic analysis may provide additional information. However, in general all patients with any subtype of MHS IVCT classification should be considered at risk of developing malignant hyperthermia under anaesthesia. Laboratory MHN diagnosis is good evidence that the patient is not at increased risk of developing malignant hyperthermia. An MHN-tested individual cannot transmit MH risk to their offspring.

D. Molecular genetic detection of susceptibility to malignant hyperthermia

Although an MH episode must be considered a multifactorial sequence of events, the genetic basis for MH susceptibility is largely attributable to mutations in the *RYR1* gene. Despite several linkage and screening studies, mutations associated with MHS have been found only in *RYR1* and, more rarely, in *CACNA1S*, the gene for the skeletal muscle L-type voltage-dependent Ca^{2+} channel ($Ca_v1.1$ or DHPR).

The great majority of mutations reported in *RYR1* result in the replacement of an individual amino acid. With the currently available algorithms, it is challenging to predict the functional consequence of a given amino acid substitution within a large tetrameric protein complex, such as the skeletal muscle ryanodine receptor, RYR1. We include, as Appendix 2, an updated guideline for the interpretation of *RYR1* sequence variants in order to classify them as MH associated or not. The same principles should be applied to variants in *CACNA1S* and other genes implicated in the future. A list of proven MH-associated *RYR1* mutations is available on the EMHG website (www.emhg.org).

1. **Predictive testing based on a known familial mutation.** If an MH-associated *RYR1* mutation has been identified in the index case (i.e. a person who has a clinical history consistent with MH or who has a clearly positive IVCT result), the *RYR1* mutation can be used for predictive genetic testing of relatives. Persons at risk who are found to carry the familial mutation should be regarded as MH susceptible (i.e. at increased risk of developing MH in triggering anaesthetic conditions). In contrast, persons at risk who do not carry the familial mutation cannot be regarded as completely risk free. This is because of the limited sensitivity of the tests. It is known from the study of large pedigrees that in ~5% of patients the IVCT results and genetic data are discordant. Should such persons seek maximal safety, an IVCT should be considered.
2. ***RYR1* mutation screening as a diagnostic test.** A number of genetic testing methods are available for mutation screening of *RYR1* either as targeted analysis of the known MH-associated mutations or as screening of the entire coding regions. Irrespective of the methods applied, a clear clinical indication is a prerequisite for genetic testing [i.e. either a positive IVCT (any subtype of MHS) or a clinically suspected MH episode]. If one of the known MH-associated mutations has been identified, the person should be considered at increased risk of developing MH in triggering anaesthetic conditions. In the absence of an *RYR1* mutation, a disposition to MH cannot be excluded. The decision on the next diagnostic steps must then be based on the clinical indication. When the entire coding region of *RYR1* is being screened, as yet unclassified sequence variants will frequently be identified. The genetic laboratory is responsible for checking the available published evidence (literature and databases) and for applying prediction algorithms with the aim of eventually classifying the variant as neutral or potentially MH associated. For patient safety, individuals carrying a 'potentially MH-associated' *RYR1* variant should be regarded as at increased risk for MH until further diagnostic tests (i.e. an IVCT) have been performed.

Appendix 2: Characterization of *RYR1* sequence variants

1. **Genetic characterization.** Each variant should be fully characterized at the genetic level, including:
   - A full description at the DNA and protein level, considering aspects of evolutionary conservation and change in charge, polarity, or structure introduced by the amino acid replacement;
   - Co-segregation of the variant with the disease in the family or families affected; and
   - Assessment of the prevalence of the variant in a relevant population by means of database searches [e.g. dbSNP (http://www.ncbi.nlm.nih.gov/SNP), 1000 Genomes (http://browser.1000genomes.org), and exome variant server (http://evs.gs.washington.edu/EVS/)]. It is anticipated that pathogenic variants will have a minor allele frequency <1%. The estimate of minor allele frequency should be based on a sample size of >150 subjects.
2. **Functional characterization.** The effect of each variant on RYR1 function should be assayed by one or more of the following test systems.
   - Recombinant *in vitro* expression on a defined genetic background. The standard system, introduced by D. H. MacLennan's group, uses the expression of a rabbit *RYR1* cDNA construct (with appropriate mutations) in HEK 293 cells. Calcium release is measured fluorimetrically in response to trigger agents.[46–50] Although this is a non-muscle cell type, the advantage of the system is the defined cDNA and the standardized genetic background of the recipient cell line. This allows for direct comparison between mutations and eliminates the potential influence of mutations in other genes, which could modify RYR1 function in cells taken from patients. Alternatively, myotubes of the dyspedic mouse (RYR1 knock out) have been used as recipients for the expression of cDNA constructs.[51] Again, cDNA construct and genetic background are well defined and standardized. The genetic expression profile of myotubes may be closer to mature muscle. For this reason, results may not be directly comparable to the HEK 293 system.
   - Assays of RYR1 function in *ex vivo* tissues. Calcium measurements and ligand-binding studies have been performed on tissues from MHS patients with characterized *RYR1* variants, in myotubes,[52 53] in microsomal sarcoplasmic reticulum preparations from muscle biopsies,[54] and in lymphoblasts.[55 56] Interpretation of altered RYR1 function was based on Ca^{2+} flux and resting [Ca^{2+}] or ryanodine binding to sarcoplasmic reticulum RYR1 preparations. Myotubes and lymphoblasts were derived from individual patients and, therefore, the potential influence of other individual genetic factors cannot be excluded. For the sarcoplasmic reticulum preparations, muscle biopsies of several patients were pooled, thus eliminating individual variation. In order to avoid the interference of genetic factors other than *RYR1*, it is recommended that all assays which are based on cells obtained from patients should be performed on samples from at least two independent patients with the same mutation.
3. **Criteria for inclusion on EMHG list of diagnostic variants.** Genetic and functional characterization both must be consistent with a pathological role in MH. For variants that have been functionally characterized using any of the previously described methods (section 2 above), data can be submitted directly to the EMHG through its website. Functional data acquired using novel methods will require validation through publication in a peer-reviewed journal.

Disclaimer

These guidelines represent the views of the European Malignant Hyperthermia Group. They are based on careful consideration and interpretation of the available evidence at the time that they were agreed. They are intended principally for clinical scientists and clinicians involved in the laboratory diagnosis of malignant hyperthermia, who are encouraged to take them fully into account when exercising their diagnostic judgement. The guidelines do not override the individual responsibility for laboratory directors and diagnostic clinicians to make appropriate decisions and give the best advice according to the circumstances of individual patients. Where appropriate, decisions should be made in consultation with the patient and, where relevant, their guardian.

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