The European Malignant Hyperthermia Group guideline\textsuperscript{a} 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 under controlled laboratory conditions. These tests, when carried out according to the following protocol are collectively referred to as the \textit{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\textsuperscript{b}.

\textbf{A: Patient referral criteria}

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

1. Family history of malignant hyperthermia.
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 and/or death. Initial signs should be evident during anaesthesia or within 60 minutes 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 of unknown cause (idiopathic hyperCKaemia) where no cause has been identified following neurological work-up.
6. Exertional heat stroke requiring hospital admission, where known predisposing factors have been excluded.
7. Myopathy and detection of an uncharacterised, rare, potentially pathogenic \textit{RYR1} variant.

\textbf{B: \textit{In vitro} contracture test (IVCT)}

1. The minimum patient age for the muscle biopsy is 4 years but laboratories should not test children younger than 10 years 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.

\textsuperscript{a} The 2015 guidelines were first published as part of the following article and are reproduced with permission (Copyright\textsuperscript{c} 2015 the British Journal of Anaesthesia): \textit{European Malignant Hyperthermia Group guidelines for investigation of malignant hyperthermia susceptibility} P. M. Hopkins; H. Ruffert; M. M. Snoeck; T. Girard; K. P. E. Glahn; F. R. Ellis; C. R. Muller; A. Urwyler, on behalf of The European Malignant Hyperthermia Group; British Journal of Anaesthesia 2015; doi: 10.1093/bja/aev225 (http://bja.oxfordjournals.org/content/early/2015/07/18/bja.aev225.long).

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 with a composition of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>118.1 mmol L⁻¹</td>
</tr>
<tr>
<td>KCl</td>
<td>3.4 mmol L⁻¹</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0.8 mmol L⁻¹</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>1.2 mmol L⁻¹</td>
</tr>
<tr>
<td>Glucose</td>
<td>11.1 mmol L⁻¹</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>25.0 mmol L⁻¹</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>2.5 mmol L⁻¹</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>

Freshly made or pharmaceutically stable Krebs-Ringer solution should be used. The ion concentration should be as stated with a maximal 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 employed. 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 (l₀) 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 (l₀) and is recorded. The muscle is left at optimal length (l₀) to stabilise 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. Following 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 L⁻¹. Each successive concentration of caffeine should be administered as soon as the maximum 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 which produces a sustained increase of at least 2 mN (0.2 g) in baseline force from the lowest force reached. In addition, the maximum contracture achieved at 2 mmol L⁻¹ caffeine 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 L⁻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 and/or the tissue bath concentration should be measured regularly using gas chromatography (see below). The specimen should be exposed to each halothane concentration for at least 3 min or until maximum contracture is reached. The result of this test will be reported as the threshold concentration which is the lowest concentration of halothane which produces a contracture of at least 2 mN (0.2 g) measure 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 approximately 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, i.e. 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 3 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 0.11 mmol L⁻¹ halothane (0.5 %) for 3 min and the stretch process is repeated. The procedure is repeated for 0.22 and 0.44 mmol L⁻¹ halothane (1 and 2 %). 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 L⁻¹ is also recorded.

14. Laboratory diagnostic classification

• MHSₙc: a caffeine threshold (as defined earlier) at a caffeine concentration of 2.0 mmol L⁻¹ or less in at least one caffeine test, and a halothane threshold concentration at 0.44 mmol L⁻¹ or less in at least one halothane test.

• MHSₙ: a halothane threshold concentration at 0.44 mmol L⁻¹ or less in at least one halothane test and a caffeine threshold at a caffeine concentration of 3 mmol L⁻¹ or more in all caffeine tests

• MHSₕ: a caffeine threshold at a caffeine concentration of 2.0 mmol L⁻¹ or less and a halothane threshold concentration above 0.44 mmol L⁻¹ in all halothane tests.

• MHN: a caffeine threshold at a caffeine concentration of 3 mmol L⁻¹ or more in all caffeine tests and a halothane threshold concentration above 0.44 mmol L⁻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/or for the caffeine test a response to 32 mmol L⁻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 taken directly from the tissue bath under the same dynamic conditions as when testing. Samples for determination of halothane concentrations should be taken immediately after the gas flow has been stopped to avoid sampling from the gas phase. Halothane concentrations can be measured using GLC or HPLC and caffeine using UV spectroscopy. Halothane 0.11 and 0.44 mmol L⁻¹ and caffeine 0.5 and 2 mmol L⁻¹ should be checked. Accepted maximal deviation from the desired concentrations are ±10 %. Lambda halothane (air / Krebs-Ringer) 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 of vascular disease), patients with varicose veins, brain-dead patients within the first 24 h, patients with fractures within the first 24 hours. 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 EMHG Board of Directors. At present, protocols exist for tests with ryanodine, sevoflurane and 4-chloro-m-cresol. These protocols may 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 IVCT 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 as well as 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 MH**

Although an MH episode must be considered a multifactorial sequence of events, the genetic basis for MH susceptibility is largely due 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$\alpha_{1.1}$ 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 RYR1. We include, as an Appendix, 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](http://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 MHS, i.e. at increased risk of developing MH under triggering anaesthetic conditions. In contrast, persons-at-risk who do not carry the familial mutation cannot be regarded as completely risk-free. This is due to the limited sensitivity of the tests. It is known from the study of large pedigrees that in about 5 % of cases 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 under triggering anaesthetic conditions. In the absence of a 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 be frequently 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 at increased risk for MH until further diagnostic tests, i.e., an IVCT, have been performed.

Appendix Characterisation of RYR1 sequence variants

1. Genetic characterisation.

Each variant should be fully characterised 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,
- Assessment of the prevalence of the variant in a relevant population by means of database searches, e.g., dbSNP, 1000 Genomes, exome variant server. It is anticipated that pathogenic variants will have a minor allele frequency, MAF <1%. The estimate of MAF should be based on a sample size of > 150 subjects.

2. Functional characterisation.

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 HEK293 cells. Calcium release is measured fluorimetrically in response to trigger agents. Although this is a non-muscle cell type, the advantage of the system is the defined cDNA and the standardised 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. Again, cDNA construct and genetic background are well defined and standardised. The genetic expression profile of myotubes may be closer to mature muscle. For this reason, results may not be directly comparable to the HEK system.

- Assays of RYR1 function in ex vivo tissues. Calcium measurements and ligand binding studies have been performed on tissues from MHS patients with characterised RYR1 variants:

  c Tong J, Oyamada H, Demaurex N, Grinstein S, McCarthy TV, MacLennan DH. Caffeine and Halothane sensitivity of intracellular Ca2+ release is altered by 15 calcium release channel (ryanodine receptor) mutations associated with malignant hyperthermia and/or central core disease. J Biol Chem 1997; 272:26332-9
  e Tong J, McCarthy TV, MacLennan DH. Measurement of resting cytosolic Ca2+ concentrations and Ca2+ store size in Hek-293 cells transfected with malignant hyperthermia or central core disease mutant Ca2+ release channels. J Biol Chem 1999;274:693-702
In myotubes\textsuperscript{1}, in microsomal sarcoplasmic reticulum preparations from muscle biopsies\textsuperscript{5}, and in lymphoblasts\textsuperscript{1}\textsuperscript{m}. Interpretation of altered RYR1 function was based on Ca\textsuperscript{2+} flux and resting [Ca\textsuperscript{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 taken 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 over-ride 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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\textsuperscript{1} Wehner M, Rueflert H, Koenig F, Neuhaus J, Olthoff D. Increased sensitivity to 4-chloro-m-cresol and caffeine in primary myotubes from malignant hyperthermia susceptible individuals carrying the ryanodine receptor 1 Thr2206Met (C6617T) mutation. Clin Genet 2002;62:135-46


\textsuperscript{1} Girard T, Urwyler A, Censier K, Mueller C, Zorzato F, Treves S. Genotype-phenotype comparison of the Swiss malignant hyperthermia population. Hum Mut: Mutation in Brief 2001:#449