Susceptibility to Malignant Hyperthermia

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Een wetenschappelijke proeve op het gebied
van de Medische Wetenschappen

Proefschrift ter verkrijging van de graad van doctor aan
de Radboud Universiteit Nijmegen op gezag van de
Rector Magnificus prof. dr. C.W.P.M. Blom, volgens besluit
van het College van Decanen in het openbaar te verdedigen
op donderdag 11 november 2004 des voormiddags
om 10.30 uur precies

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geboren op 3 november 1963 te Zundert

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Eerste druk september 2004, 700 exemplaren
ISBN: 90-9018522-4

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Chapter 1

GENERAL INTRODUCTION

Historical aspects

Malignant hyperthermia (MH) has been identified internationally since 1960 as a clinical pharmacogenetic entity with an autosomal dominant mode of inheritance. Denborough and Lovell reported in a letter to the editor of *the Lancet* on a well known case of a 21 years old student with a family history of unexplained deaths during anaesthesia [1]. At least ten family members had suffered an adverse reaction to anaesthesia in which ether was used. In this case the anaesthesia was performed with halothane, "a relatively new volatile anaesthetic"; the young man barely survived.

Clinical events of perioperatively occurring hyperthermia or hyperpyrexia have been observed and recorded, and even associated with a familial relationship; but, unfortunately, never published before 1960 [2]. MH has undoubtedly been a cause of anaesthetic mortality and morbidity since the introduction of diethyl ether and chloroform into regular clinical practise in the mid-nineteenth century.

Early reports on perioperatively elevated body temperature came from several surgeons during the first decades of the 20th century. General recommendations such as to provide good air-circulation during hot weather, not to cover the patient completely, restrict duration of the operation (and the general anaesthesia) and monitor the body temperature, were the result of the anaesthesia meetings at that time. A specific syndrome, composed of hyperthermia, tachycardia and tachypnoea during anaesthesia, was described for the first time by Burford in 1940 [3]. He supposed that the syndrome could be provoked by the administration of ether.

In the years after 1960 other cases were reported with the interesting observation that some patients developed muscular rigidity. This typical adverse reaction to anaesthesia became known as "*malignant hyperthermia*", because in those days 70-80% of the patients died shortly after a steep and rapid rise in body temperature.

Two groups, one working in Melbourne, Australia and the other in Johannesburg, South-Africa, independently concluded that the clinical signs of an MH reaction could be converted into an underlying muscle disease [4,5]. Denborough from Melbourne proved that succinylcholine and halothane had induced severe rhabdomyolysis in a patient, whose serum creatine kinase (CK) activity rose to 20.500 IU/l, 24 hours after anaesthesia. And in the same year, 1970, Isaacs from Johannesburg observed abnormally high serum CK activities in relatives of patients

with an MH reaction. Studies of isolated muscle specimens in vitro showed that muscle fibres from MH patients and normal individuals differ in their limits of induced tension, when exposed to incremental doses of caffeine and halothane [6,7]. It was not until 1984 that a well defined, uniform diagnostic in vitro test became available. The European MH Group was set up, followed shortly by the formation of the North American MH Group, setting of laboratory standards led to two essentially similar protocols for the caffeine and halothane in vitro contracture test (IVCT) [8,9].

In 1968 it became clear that certain inbred strains of pig were also susceptible to MH. Pigs, given anaesthesia with halothane for experiments, developed convulsions, fever and finally died [10]. It has been known that pigs could respond to stress with muscle rigidity and high fever. The stress-induced death in pigs (porcine stress syndrome) had world-wide economic consequences because acute stress prior to slaughter, resulted in a dramatic devaluation in the quality of pork. This animal model was to the researchers' advantage leading to a better understanding of MH and has since been serving as a favourable research tool [11]. Clarification of the underlying pathogenesis of MH was made possible by physiological and biochemical studies and genetic linkage analysis. The latter made a DNA-based diagnosis of susceptibility to MH possible under specific circumstances [12].

Clinical presentation of MH

The term malignant hyperthermia referred originally to a syndrome with a mortality rate of 70-80%, where pyrexia was the most impressive clinical feature. This classical fulminant presentation of hypermetabolism is nowadays rarely seen. There is no symptom or sign that is unique to MH, and MH is not one entity [13].

MH is a spectrum of disorders, ranging from a life-threatening crisis to none or an atypical reaction to anaesthesia. The diagnosis depends on the combination of clinical signs and laboratory abnormalities during or after trigger anaesthesia *(table 1)*, with reference to the timed sequence of events, concomitant drug administration and environmental factors. The clinical signs fall into two distinct categories, metabolic and muscle in origin. The patients showing variants of MH can be grouped into five types of clinical presentation:

1 Fulminant MH.
2 Moderate or mild MH.
3 Masseter muscle rigidity.
4 Unexplained anaesthetic death or cardiac arrest.
5 Atypical presentation.

Ad 1 Fulminant MH

Fulminant or classical MH is the anaesthetist's nightmare. It is a life-threatening crisis, characterized by elements of hypermetabolism in association with respiratory and metabolic acidosis and signs of increased permeability of skeletal muscle cells. Early signs are tachycardia and arrhythmias, in combination with tachypnoea and hypercapnia. Generalised muscle rigidity can be seen, especially masseter muscle spasm following the administration of succinylcholine. When

table 1 *Drugs used in anaesthesia according to their potential for triggering MH in susceptible patients[13].*

Contraindicated, trigger agents	**Safe agents**
Volatile anaesthetics	Amide/ester local anaesthetics
– Ether	Benzodiazepines
– Halothane	Barbiturates
– Enflurane	Propofol
– Isoflurane	Etomidate
– Desflurane	Ketamine
– Sevoflurane	Opioids
Depolarising muscle relaxants	Non-depolarising muscle relaxants
– Succinylcholine	– Pancuronium
	– Vecuronium
	– Rocuronium
	– Atracurium
	– Mivacurium
	(Nor) Adrenaline
	Digoxin
	N2O
	Xenon

metabolic demands outstrip muscle blood supply, the ischemic picture deteriorates with desaturation and increasing body temperature; up to 1°C every 5 minutes.

Laboratory abnormalities include: hyperkalemia, increased creatine kinase activity (CK peaks at 12–24 hours after the 'metabolic storm'), myoglobinuria, combined respiratory and metabolic acidosis, usually with an arterial base excess more negative than –8 mEq/l.

Late signs are features of renal failure, disseminated intravascular coagulopathy and cerebral oedema, often causing death. Fulminant MH requires very active treatment (see further: clinical management of MH).

Ad 2 Moderate or mild MH

This group is characterised by a less rapid development of signs and does not appear life-threatening. A variety of metabolic changes occur and muscle anomalies are mild or absent. The treatment required includes the withdrawal of trigger agents and often a single dose of dantrolene will be sufficient to normalize physiological values. Nevertheless substantial muscle breakdown (manifest by significant increase in CK) can cause oedematous and tender muscles for several days, or sometimes even weeks afterwards in moderate MH.

The classification into moderate or mild MH is arbitrary.

Ad 3 Masseter muscle rigidity

Masseter muscle rigidity (MMR) is probably the most common manifestation of MH. It is a condition where a patient's mouth can barely be opened despite great effort. MMR is a subjective sign and difficult to define.

table 2 *Probability of MH susceptibilty in the different types of clinical presentation, derived from diagnostic investigation and classification of 436 probands using IVCT in Leeds, UK. MMR: masseter muscle rigidity.*

Type of clinical presentation		nr. of patients	incidence of MHS (%)
1	Fulminant	41	96
2.1	Moderate	56	88
2.2	Mild	77	14
3.1	MMR & muscle involvement	83	76
3.2	MMR & hypermetabolism	46	56
3.3	MMR alone	59	28
4	Unexplained anaesthetic death or cardiac arrest	11	66
5	Atypical presentation	63	7

The reduced mouth opening and increased jaw muscle tension for around 90 seconds is an agonist effect of succinylcholine. This "myotonic" reaction peaks as fasciculations stop [14]. It may occur as a normal, but exaggerated response to succinylcholine, especially in children. Isolated MMR has been reported in 0.5 to 1% of cases after induction with volatile anaesthetics and an intubating dose of succinylcholine, in which none of the patients developed MH in spite of continuation of anaesthesia with the volatile agents [15].

In modern anaesthetic practise it is considered that MMR should be seen as a warning sign of MH, preceding other signs by a considerable period of time. The incidence of MH susceptibility (MHS) for patients showing MMR with evidence of muscle involvement (gross increase in serum CK and myoglobinuria) or with signs of hypermetabolism, is considerably higher than for patients with MMR alone *(table 2)* [13, 16].

Ad 4 Unexplained anaesthetic death or cardiac arrest

This group contains a modest amount of probands, often with historical or badly documented anaesthetic incidents of cardiac arrest and/or unexplained death.

The incidence of MH susceptibility among family members is 66% *(table 2)* [16]. Further information sometimes reveals a myopathy (i.e. Duchenne muscular dystrophy) associated with cardiac arrhythmia and cardiac arrest in the recovery room after an uneventful anaesthetic with halothane [13]. If the cause cannot be turned up, close relatives should be tested for MH susceptibility.

Ad 5 Atypical presentation

The most widely atypical presentation of MH is postoperative fever. It has been a common indication for IVCT, but no longer requires screening for MH susceptibility, despite dramatic case descriptions of patients being packed in ice et cetera. Referrals for unexpected postoperative renal failure or voiding of brown urine produced by myoglobin, however, do require diagnostic investigation. These signs are highly indicative of myopathies or MH [13].

Clinical grading scale

Out of the desire to create a clinical definition for MH, a group of international MH experts developed a standardised clinical case definition that determines the qualitative likelihood that an adverse anaesthetic reaction represents true MH, using the Delphi method [17]. The 'clinical grading scale to predict MH susceptibility' assigns points for abnormal signs and laboratory findings observed during the adverse reaction. These points are summed to produce a raw score *(table 3)*. The raw score is converted into an MH rank (D1 to D6) which reflects the likelihood that the reaction represents MH.

The MH clinical grading scale is recommended for use as an aid to the objective definition of MH but it has important limitations. The MH likelihood may be underestimated if indicators are missing because the anaesthesia provider failed to use appropriate monitoring or failed to obtain key laboratory data (e.g. CK). Lack of knowledge concerning the family's medical history is another reason for underestimation. Thus the calculated rank should be viewed as the lower boundry of MH likelihood and if important clinical information is missing, one should consider not to use the MH clinical grading scale.

Clinical management of MH

Early recognition and speedy intervention is vital for the successful treatment of MH. A clear intervention protocol should be available and operating room teams should practise the emergency MH protocol at training sessions.
Dantrolene rapidly reverses an MH episode [18]. Dantrolene is lifesaving; no general anaesthesia with trigger agents should be given without ready access to 36 vials of dantrolene; this recommended quantity is theoretically sufficient to treat an average patient with a total dose of 10 mg/kg.

Treatment of an MH reaction

Stop all triggering agents immediately, inform the surgeon and call for help. Continue anaesthesia with safe agents if surgery cannot be stopped *(table 1)*. Hyperventilate with 100% O_2, use high fresh gas flow to obtain a normal $PaCO_2$, replace tubing and CO_2 absorbent after the patient is stabilized (replacing respiratory tubing and/or anaesthesia machine during an MH emergency can easily lead to extra complications). Administer dantrolene 2.5 mg/kg intravenously, and repeat every five minutes in doses of 1–2 mg/kg up to a total of 10–20 mg/kg until all signs are normalised. Aids to facilitate the dissolution of dantrolene will require the full time efforts of two to three people!

Prompt dantrolene injection is the cornerstone of effective MH therapy and should have absolute priority next to withholding triggering agents [18].

Symptomatic treatment should be focussed on treatment of hyperkalemia and acidosis since these may cause dysrhythmias and cardiac arrest during MH. The heart muscle is not directly involved in MH pathology. If treatment of hyperkalemia plus acidosis does not treat dysrhythmias adequately, lignocaine or ß-receptor antagonists can be given. Calcium channel blocking drugs should be avoided since they can cause an increase in hyperkalemia and myocardial depression in the presence of dantrolene [19].

table 3 *MH clinical grading scale; clinical indicators for use in determining an MH raw score, which can be translated to an MH rank (D1–D6) and a qualitative likelihood that an adverse anaesthetic reaction represents MH [17].*

Process		Indicator	Points
I	Rigidity	Generalized muscular rigidity	15
		Masseter musclerigidity following succinylcholine	15
II	Muscle breakdown	CK activity > 20000 iu/l following succinylcholine	15
		CK activity > 10000 iu/l without succinylcholine	15
		Brown colored urine	10
		Myoglobin in urine > 60 µg/l	5
		Myoglobin in serum > 170 µg/l	5
		K^+ in serum > 6 mmol/l	3
III	Respiratory acidosis	$EtCO_2$ > 7.5 kPa during controlled ventilation	15
		$EtCO_2$ > 8.0 kPa during spontaneous ventilation	15
		$PaCO_2$ > 8.0 kPa during controlled ventilation	15
		$PaCO_2$ > 8.5 kPa during spontaneous ventilation	15
		Inappropriate hypercapnia	15
		Inappropriate tachypnoea	10
IV	Temperature increase	Inappropriate rapid increase	15
		Inappropriate increased temperature > 38.8°C in the perioperative period	10
V	Cardiac involvement	Inappropriate sinus tachycardia	3
		Ventricular tachycardia or ventricular fibrillation	3
VI	Family history	Determined MHS in relatives of first degree	15
		Determined MHS in relatives not of first degree	5
Various indicators		Arterial base excess < –8 mEq/L	10
		Arterial pH < 7.25	10
		Rapid metabolic reversal with dantrolene	5
		History of adverse anaesthetic reaction **and** determined MHS in relatives	10
		Resting elevated CK **and** determined MHS in relatives	10

Per process (I–VI): count only the indicator with the highest score; 'various indicators' should be added without regard to 'double counting'.

Raw score range	MH rank	Description of likelihood
0	D1	Almost never
3–9	D2	Unlikely
10–19	D3	Somewhat less than likely
20–34	D4	Somewhat greater than likely
35–49	D5	Very likely
50^+	D6	Almost certain

Fluid replacement and volume expansion are neccesary to compensate for fluid loss in damaged muscle. Urinary output must be kept high in order to prevent renal failure caused by myoglobin precipitation in the tubules.

table 4 *Guidelines for the treatment of an MH emergency. The different phases of MH treatment resembles a three staged rocket.*

I Immediate actions

1 Stop all triggering anaesthetic agents, inform the surgeon and call for help. If possible stop surgery, continue with safe agents if surgery cannot be stopped.
2 Hyperventilate with 100% O_2, use high flow of fresh gas to obtain a normal $PaCO_2$.
3 Administer dantrolene 2.5 mg/kg intravenously, and repeat every 5–10 minutes until all signs normalize, up to a total dose of 10 mg/kg.

II Supportive actions

4 Give (iced) saline or Ringer solution intravenously.
5 Correct any acidosis, give $NaHCO_3$ 2 mEq/kg; follow arterial bloodgases.
6 Check bloodgases, CK, electrolytes and glucose.
7 Treat hyperkalaemia with insulin and glucose.
8 Monitor temperature; start surface cooling above 38.5°C. Stop cooling at 38°C.
9 Place urinary catheter to monitor urinary output (and colour), maintain output >1 ml/kg/h with i.v. infusions, mannitol and furosemide.
10 Treat cardiac arrhythmias if persistent using lignocaine or ß-receptor antagonists.

III Post emergency actions

11 Transfer to ICU when stable, observe for 24–48 hours; monitor for recrudence and late complications.
12 Administer iv. dantrolene 1–2 mg/kg every 6 hours or continuous infusion 0.25–0.5 mg/kg/h, according to clinical and metabolic signs.
13 If the MH reaction is complicated with coagulopathy and/or high intracranial pressure that do not respond to 'standard' MH treatment, specific treatment should be given.
14 Obstructive tubular renal failure caused by myoglobinuria usually recovers following a period of dialysis.
15 After the immediate crisis, warn the patient and their families of the implications of MH and the necessity of confirming the clinical diagnosis by IVCT.
16 Counsel; refer the proband (or close relatives when the index case deceased) to an MH investigation unit (http://www.emhg.org).

A core temperature above 38.5°C should be treated promptly by infusion of ice cold fluids and surface cooling (using heat exchange or cooling blankets and ventilators). Surface cooling with ice alone is not recommended; bladder and gastric lavage provides only a small cooling effect, is time consuming, inconvenient and should therefore be avoided. Cooling should be stopped at 38°C to avoid hypothermia. Guidelines for the treatment of an MH reaction are summarized in *table 4*.

Time is of the essence and morbidity is correlated with the duration of symptoms. After a timely administration of dantrolene, following early diagnosis, little or no supportive therapy is necessary. The need for supportive therapy is directly, perhaps exponentially, proportional to the delay in diagnosis and the institution of treatment. In the treatment of MH, it must be emphasized that although we have the knowledge and the ability to diagnose and treat an MH reaction, deaths still occur.

Dantrolene

Dantrolene for intravenous administration is distributed as Dantrium™ by Procter & Gamble. The 70 ml glass bottle contains 20 mg dantrolene powder (lyophylized dantrolene sodium: 1-{[5-(p-paraphenyl)furfurylidence]amino}hydantoine), 3.0 g mannitol (which improves the solubility) and sodium hydroxide to raise the pH to 9.5 when the recommended volume of 60 ml of sterile water is added to dissolve the powder into a clear yellow/orange solution[18]. Dantrolene is highly lipid soluble, but extremely poorly soluble in water. Precious time can be saved by warming the sterile water (or store it in a operating room warming cabinet). Dantrolene solubility increases linearly with increasing temperature of the diluent between the recommended "room temperature" of 18-20°C to 40°C (maximum!); time to achieve a clear solution are respectively 180 and 30 seconds when shaking the vials thoroughly[20].

The primary pharmacological action of dantrolene is the relaxation of skeletal muscle. Administration of 2.4 mg/kg body weight dantrolene results in 75% depression of muscle twitch response. The elimination half-life of dantrolene is 12 hours; it is partly metabolized in the liver. Dantrolene and its metabolites are excreted in both the urine and the bile. Residual dantrolene concentrations in the blood are high enough to give patients a feeling of weakness for up to 48 hours after the initial dose (2-2.5 mg/kg)[21].

The relaxant action of dantrolene is unusual and still incompletely defined. Dantrolene causes skeletal muscle relaxation by either direct or indirect interaction with the ryanodine receptor, the primary calcium release channel in the sarcoplasmic reticulum membrane, involved in excitation-contraction coupling. Mainly due to inhibition of the ryanodine receptor via specific binding sites, the continued calcium release is supposed to be terminated[18, 22]. The myoplasmic free calcium will return to resting concentrations, permitting relaxation of the muscle, reversion to a normal metabolic state and termination of the MH syndrome.

Epidemiology of MH

The incidence of MH is estimated to be 1 in 10.000 to 1 in 15.000 general anaesthetics. This generally agreed, but rather high incidence has been calculated in an early extensive survey of MH published in 1970[23]. True incidence of MH is unknown because it depends on several factors: the definition of clinical MH, the age and sex of the population studied, the anaesthetic technique and drugs used for anaesthesia, and the kind of surgery and proportion of emergencies in the population studied. Over the past 25 years, the incidence of fulminant MH is approximately 1 in 225.000 anaesthetics and 1 in 65.000 general anaesthetics when trigger agents are used[24-26]. Mild forms of MH have an incidence of 1 in 4500 anaesthetics with trigger agents[25].

An MH reaction can occur regardless of human race, gender or age. However the majority of dramatic fulminant MH episodes happen to young fit male adolescents[27]. MH susceptible probands are most commonly seen in the 10-30 age group, in a male:female ratio of 2:1.

The predominance of occurrence of an MH reaction during emergency anaesthesia and anaesthetics for minor surgical procedures (ear nose throat (ENT), eye and minor orthopaedic surgery) can be explained due to the fact that in these cases

trigger agents are frequently used. This explanation is more plausible than the association of respectively ENT, eye and musculoskeletal abnormalities with MH. In recent years the incidence of clinical MH has decreased because less trigger agents are used. Total intravenous and regional anaesthesia is performed with 'safe agents' that do not elicit MH *(table 1)*. Nowadays the combination of succinylcholine and a volatile anaesthetic, which seem to potentiate each other in triggering MH, is rarely being used. This contributed greatly to the decreasing incidence of MH-reactions.

Pathophysiology of MH

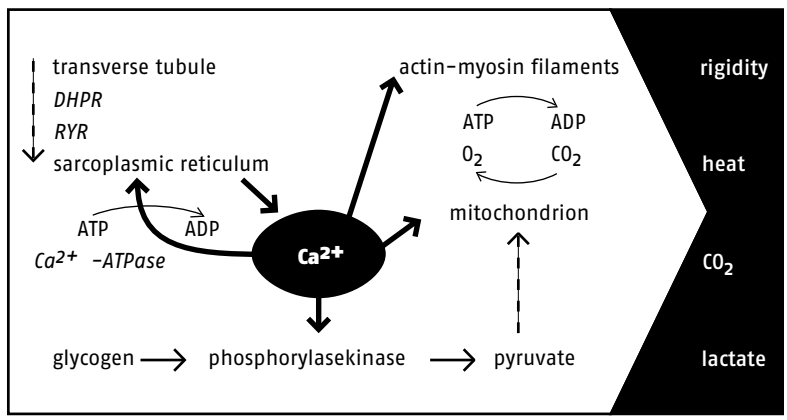
Clarification of the pathogenesis of MH was made possible by physiological and biochemical studies and genetic linkage analysis. In 1960 the MH syndrome, characterized by high fever and muscle contractures was distinguished as a pharmaco-induced syndrome in humans that could lead to death. The recognition of a corresponding condition in swine, leading to stress-induced deaths, was responsible for the fast progression in the elucidation of the pathophysiology of MH[28].

The most marked sign of acute MH is skeletal muscular rigidity. The rigidity appears not to be a regular form of muscle contraction but rather a pronounced contracture. Increase in heart rate, end tidal CO_2 and body temperature reflect the accelerated rate of energy metabolism. This increase in metabolism represents an attempt to produce ATP at a rate sufficient to counteract the rate of ATP hydrolysis by actin-myosin ATPase of the contractile apparatus in skeletal muscle fibres. Skeletal muscle accounts for approximately 40% of the body mass. Once triggered into a hypermetabolic state, a 'whole body relative ischemia' develops in which excessive metabolic demands exceed the capacity of the body to provide substrate and O_2 *(figure 1)*. Oxygen saturation and PaO_2 decrease and lactate concentration increases due to glycolysis. If the MH crisis is not actively treated, the syndrome becomes irreversible. When the ATP concentration reaches one-half of the resting concentration, breakdown of the mitochondrial membrane occur and the integrity of the muscle cell membrane alters (rhabdomyolysis); as ATP is essential for the activity of the enzymes normally involved in the repair and maintenance of cell membranes. Mg^{2+}, Ca^{2+}, K^+, inorganic phosphate and larger molecules and proteines such as myoglobin move into the extracellular space and plasma. The progressive efflux of potassium ions from the sarcoplasm, together with generalized hypoxia, lactate acidosis and elevated concentrations of circulating catecholamines provoke arrhythmias and impaired cardiac function. Hyperkalaemia causes ventricular fibrillation, cardiac arrest and ultimately, death.

Rhabdomyolysis may cause visible pigmenturia as a result of the presence of myoglobin in urine. A major threat of myoglobinuria is acute renal failure due to renal ischemia and tubular injury.

The crucial role in the pathogenesis of MH is elevated myoplasmic Ca^{2+}. Ca^{2+} regulates contraction, relaxation and energy metabolism in skeletal muscle cells. It is now clear, from 50 years research of the structure and function of subcellular components involved in excitation-contraction coupling and skeletal muscle function, that drugs (e.g. caffeine) can act on the activation site of the sarcoplasmic reticulum Ca^{2+} release channel by increasing the affinity for Ca^{2+}.

figure 1 *A proposed mechanism for induction of MH*
The release of Ca^{2+} is the end result of a cascade of events from depolarisation of nerve, muscle, transverse tubular membranes, and sarcoplasmic reticulum. Abnormalities in the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum cause continued presence of Ca^{2+} within the cell. Sustained muscle contracture accounts for all of the symptoms of MH: rigidity, and the generation of heat, CO_2, and lactate by the sustained glycolytic and aerobic metabolism.



In this way the sensitivity for Ca^{2+} release is increased [29-31].

When skeletal muscle fibres are stimulated at the motor end plate, the action potentials travel along the surface membrane and enter the transverse T-tubule system. The depolarisation signal is transmitted at specialised junctions to the terminal cisternae of the sarcoplasmic reticulum (SR). Ca^{2+} is released from the SR and the muscle contracts in response to this Ca^{2+} through the established interaction of actin, myosin, and troponin *(figure 2)*.

The junction between the T-tubule system and the SR consists of two receptor complexes: a dihydropyridine receptor (DHPR) complex in the T-tubule membrane and the ryanodine receptor (RYR) in the SR membrane *(figure 3)*. The coupling between these two calcium channels is not fully elucidated, although several structural characteristics and functional mechanisms are known [29-30]. The DHPR is a slow (L-type) voltage-sensitive calcium channel, comprised of five subunits ($\alpha 1$, $\alpha 2$, β, γ and δ). The $\alpha 1$-subunit consists of six transmembrane loops, forming four ion selective pores. The RYR acts as a single Ca^{2+} release channel when it is activated. The relatively small hydrophobic part of the RYR forms a transmembranetic SR baseplate; the huge hydrophilic cytoplasmic domain bridges the gap between the SR and the T-tubule. This cytoplasmic region, corresponding to the often called 'foot structure' of the RYR, contains binding sites for various activating ligands like calcium, ATP, calmoduline, caffeine, ryanodine and inositol trisphosphate (IP_3), and for inactivating or modulating ligands like dantrolene and calcium, magnesium or ryanodine in high concentrations (>100µM).

figure 2 *Muscle contraction is regulated by cytoplasmic Ca^{2+} concentrations. In a normal relaxation–contraction cycle, Ca^{2+} is pumped into the sarcoplasmic reticulum by a Ca^{2+}–ATPase to initiate relaxation, stored within the lumen, and released through a Ca^{2+} release channel to initiate contraction.*

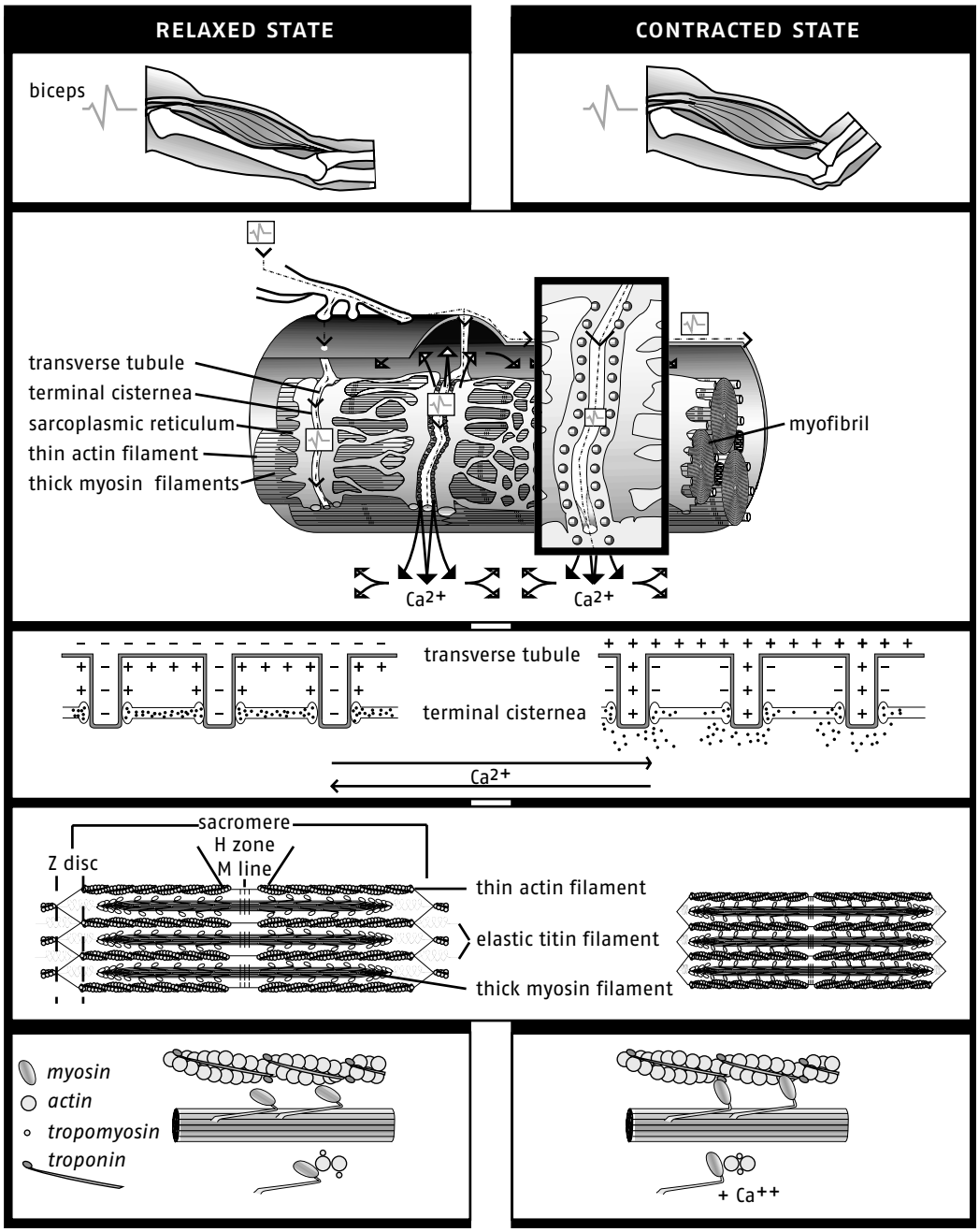
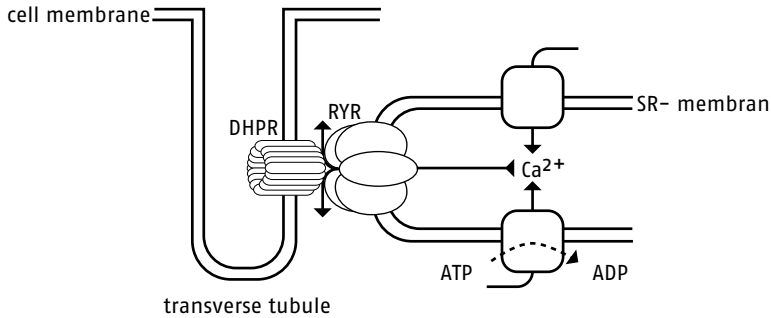


figure 3 *Proposed arrangement of the protein complexes in the transverse (T) tubule and sarcoplasmic reticulum (SR-) membranes.*
One tetrad of four dihydropyridine receptor complexes (DHPR) is physically apposed to every other ryanodine receptor (RYR), the sarcoplasmic reticulum Ca^{2+} release channel.



The mechanism, for excitation-contraction coupling linked Ca^{2+} release by skeletal muscle which is widely accepted, is the mechanical or direct coupling hypothesis. The direct coupling hypothesis suggests that the DHPR is the voltage sensor which transduces a conformational change in the foot structure of the RYR, thereby regulating the opening of the calcium efflux channel, allowing the release of Ca^{2+} into the cytoplasm. The DHPR is directly linked to RYR in skeletal muscle for the purpose of controlling SR Ca^{2+} release and contrary to its function in other tissue, it does not act as a channel for Ca^{2+} uptake by skeletal muscle.

In summary, excitation-contraction coupling is believed to be mainly a mechanical mechanism, modulated by various cytoplasmic accessory proteins (calmodulin, FKBP12) and integral membrane proteins (triadin, junctin), influencing the activity of the RYR[30] *(figure 4)*.

Genetics of MH

Alterations in the kinetics of calcium release appeared to play a crucial role in MH. This fact led investigators to study the skeletal muscle sarcoplasmic reticulum (SR), since the SR had been known to be a site of calcium storage and release[29]. The calcium efflux of the SR is controlled by the ryanodine receptor (RYR), named after the neutral plant alkaloid and muscle relaxant ryanodine. Ryanodine binds with high affinity to the calcium release channel of skeletal muscle and is able to enhance calcium release from the SR[32]. RYR has three isoforms, the RYR1 is believed to be the principal form in adult skeletal muscle, the RYR2 in cardiac and the RYR3 in non-muscle[33].

Molecular cloning of the RYR cDNA from human skeletal muscle allowed the detailed structural analysis of one of the largest known proteins within the cell (2.200 kDa corresponding to 5.000 amino acids encoded by 106 exons)[34,35]. It appeared from investigation on the Ca^{2+} release channel from MH susceptible and normal pigs that structural alterations in the release channel (RYR1) were involved in porcine MH[36]. Detailed DNA sequence analysis of the RYR1 cDNA from these normal and MHS pig skeletal muscle revealed a C to T point mutation at position

1840, resulting in the substitution of cysteine for arginine in position 614[37]. Dito linkage has been established in studies of inheritance in human families, suggesting 1840C>T as a candidate mutation causing MH in patients where the disease maps to the RYR1 locus on chromosome 19q13.1[38,39]. However this 1840C>T ("pig") mutation was found to co-segregate with MHS in only 3-6% of MH-susceptible families[40,41]. Attempts to identify other mutations in the human RYR1 gene potentially causative for MH susceptibility have therefore been undertaken *(table 5)*[42-66]. Until now 22 point mutations in the RYR1 cDNA have been found to correlate with the MHS phenotype, and have been shown to directly alter RYR1 caffeine or halothane sensitivity at research on functional characterization. By this, these 22 causative mutations meet the criteria set up by the EMHG for predictive genetic testing *(guidelines + appendix, chapter 4)*[12]. The majority of the causative mutations appear to be clustered in two hotspots between amino acid residues 35-614 and 2163-2458; a small number of mutations shows linkage to central core disease (CCD; vide infra) in addition to linkage to MH-susceptibility *(figure 5)*. In over 50% of the European MH families linkage has been established

figure 4 *Diagrammatic representation of the major components of the excitation-contraction pathway of skeletal muscle.*
The dihydropyridine (DHP) receptor acts as a voltage sensor for the ryanodine receptor (RYR). The activity of the Ca^{2+} release channel (RYR) is influenced by cytoplasmic, integral membrane and intraluminal accessory proteins.

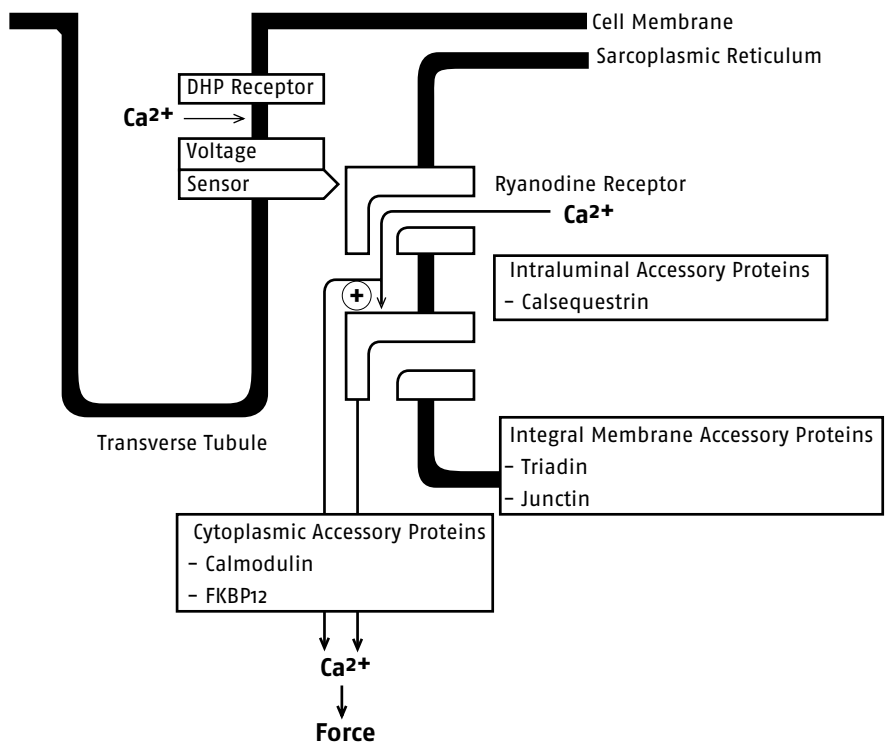
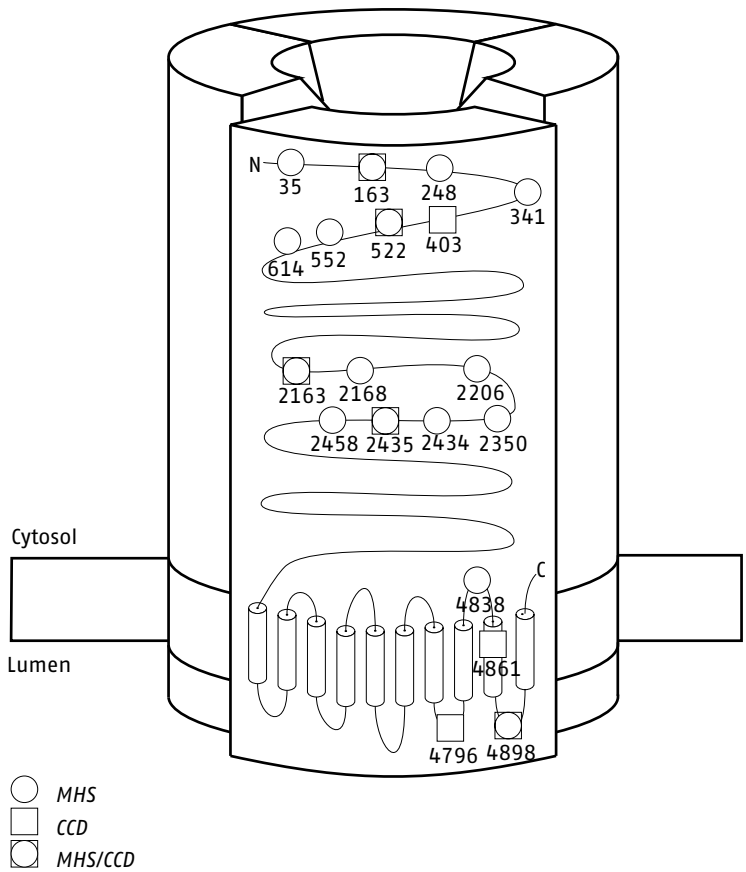


figure 5 *Diagrammatic representation of the ryanodine receptor.*
The large cytosolic part of RYR bridges the gap between the DHPR in the T-tubule and the sarcoplasmic reticulum. RYR1 mutations potentially causative for MH-susceptibility (MHS) and/or central core disease (CCD) have been presented as numbers of the human RYR1, representing the positions of the replaced amino acids. At position 614, 2163 and 2458 two mutations have been described.



to the RYR1 gene [30]. In a considerable number of families linkage to chromosome 19 could not be confirmed, pointing to genetic heterogeneity. Alternative loci containing the genes encoding the DHPR subunits, for one, have been identified on chromosomes 1, 3, 5, 7 and 17 [67-71].

MH: relationship to other diseases and syndromes

A number of muscle disorders have been associated with MH due to anaesthetic complications in the past [72, 73]. However, in most cases the muscle-related anaesthetic complications did not reflect "typical" MH. More important, with the exception of central core disease, non of the muscle disorders has been genetically linked to MH.

Neuromuscular diseases

In neuromuscular diseases (NMD), the causes of anaesthetic complications depend on whether the neuromuscular transmission or the excitability of the membrane or the function of intracellular organelles is disturbed.
Muscle relaxants and potent inhalational anaesthetics, given to patients with NMD during general anaesthesia, exert strong effects on their muscles and can provoke complications like masseter muscle rigidity or generalized muscle spasms, rhabdomyolysis, elevated core temperature, tachycardia or cardiac arrest [74]. Moreover, postanaesthetic complications have been described: weakness or prolonged paralysis of skeletal muscle leading to respiratory failure or apnoea.

Most NMD are associated with specific phenotypic features and a specific genetic mutation, whereas the clinical presentation of MH can be quite variable and the molecular basis for MH susceptibility is heterogenetic.
NMD which are at risk for adverse anaesthetic reactions are mainly progressive muscular dystrophies (Duchenne-type, Becker-type, limb girdle- and facio-scapulohumeral dystrophy), congenital myopathies (Fukuyama-type, centronuclear- and nemaline myopathy), metabolic myopathies (McArdle's disease, Brody's disease, myoadenylate deaminase deficiency), myotonias (Curschmann-Steinert-type, Thomsen, Schwartz-Jampel syndrome and myotonia fluctuance), periodic paralysis (hyper- or hypokalemic periodic paralysis), myastenia gravis, and a group of neurogenic disorders (poliomyelitis, spinal muscle atrophy, polyneuropathy).

The halothane-caffeine in vitro contracture test (IVCT) is generally accepted as the "gold standard" test for the diagnosis of MH susceptibility *(chapter 2)*. The results of in vitro contracture testing in patients with various NMD are misleading [75,76]. Although the IVCT result can be positive in patients with NMD, the inconsistent minor IVCT contractures reflect the diseased muscle rather than MH [76-78]. In fact positive results (MH-susceptible or MH-equivocal) in patients with NMD could explain the specificity of 93.6% [79]. In vitro sensitivity to the test drugs will not automatically correspond with an increased in vivo sensitivity. Nevertheless, from a practical point of view and/or just to be safe, a patient with a NMD that is tested MHS or MHE should never get anaesthesia with trigger agents. In all probands that have had a "serious" adverse anaesthetic event, a combination of clinical, morphological, genetic and contracture studies should be carried out. These studies, together with a detailed family study, will make it possible to identify the etiology.

The King syndrome or King-Denborough syndrome is often mentioned to be associated with MH [80,81]. However this association is uncertain because the King syndrome is said to be a recessive trait and patients have typical clinical features [82]. The only clearly associated muscle disorder is central core disease.

Central core disease

Central core disease (CCD) is a rare, nonprogressive myopathy, which is characterized by muscle hypotonia, proximal symmetrical muscle weakness, and CK elevation. Diagnosis is made on the basis of the typical histological central cores of type 1 skeletal muscle fibers [83]. The inheritance of CCD is autosomal dominant with variable penetrance. Within families the clinical signs may vary from severe to absent, the latter individuals having only the MH trait [84]. This association led investigators to

table 5 *Mutations that underly MH susceptibility (MHS) and/or central core disease (CCD) in the genes encoding the ryanodine receptor (RYR1). *) = 22 mutations that have been shown to directly alter RYR1 caffeine or halothane sensitivity, by which they meet the criteria set up by the EMHG for predictive genetic testing.*

Exon	Nucleotide change	Amino acid substitution	Phenotype	Reference
2	103 T > G	C35R *)	MHS	42
2	130 C > T	R44C	MHS	43
6	487 C > T	R163C *)	MHS/CCD	44
6	496 G > A	D166N	MHS	45
9	742 G>A	R248G *)	MHS	46
11	982 C > T	A328T	MHS	47
11	1021 G >A	G341R *)	MHS	44
12	1201 C > T	R401C	MHS	48
12	1202 G > A	R401H	MHS	45
12	1209 C > G	I403M *)	CCD (MH?)	44
14	1565 A > C	Y522S *)	MHS/CCD	44
15	1597 C > T	R533C	MHS	43
15	1654 C >T	R552W *)	MHS	44
17	1840 C > T	R614C *)	MHS	40
17	1841 G > T	R614L *)	MHS	44
39	6349 G > C	V2117L	MHS	43
39	6377 G > A	R2126Q	MHS	unpub.
39	6387 C > G	D2129E	MHS	49
39	6487 C > T	R2163C *)	MHS	44
39	6488 G > A	R2163H *)	MHS/CCD	44
39	6488 G > C	R2163P	MHS	50
39	6502 G > A	V2168M *)	MHS	44
40	6617 C > T	T2206M *)	MHS	44
40	6617 C > G	T2206R	MHS	51
40	6640 G > A	V2214I	MHS	52
44	7038 GAGdel	E2347del	MHS	52
44	7048 G > A	A2350T *)	MHS	52
44	7062 C > T	R2355C	MHS	53
44	7099 G > A	A2367T	MHS	52
45	7282 G > A	A2428T	MHS	45
45	7291 G > A	D2431N	MHS	52
45	7300 G > A	G2434R *)	MHS	44
45	7304 G > A	R2435H *)	MHS/CCD	54
45	7304 G > T	R2435L	MHS/CCD	55
46	7354 C >T	R2452W	MHS	56
46	7358 T > A	I2453T	MHS/CCD	57
46	7360 C > T	R2454C	MHS	51
46	7361 G > A	R2454H	MHS	55
46	7372 C> T	R2458C *)	MHS	44
46	7373 G > A	R2458H *)	MHS	44
71	10579 C > T	P3527S	rec CCD	58

91	12640 del 9	RQF4214-4216 del	CCD	59
95	13909 A>G	T4637A	CCD + rods	60
95	13910 C>T	T4637I	CCD + rods	unpub.
95	13913 G>A	G4638D	CCD	unpub.
95	13938 del 6	LS4647-4648 del	CCD	59
95	13952 A>C	His4651Pro	CCD	unpub.
96	14002 C>T	P4668S	MHS	61
100	14378 T>C	L4793P	CCD	59
100	14387 A>G	Y4796C *)	CCD + rods	62
100	14431 G>A	A4811T	CCD	unpub.
100	14473 C>T	R4825C	CCD	59
100	14477 C>T	T4826I	MHS	63
101	14558 C>T	T4853I	CCD	unpub.
101	14578 del TTC	F4860 del	CCD	59
101	14512 C>G	L4838V *)	MHS	61
101	14578 T>A	F4860I	CCD	unpub.
101	14581 C>T	R4861C	CCD	unpub.
101	14582 G>A	R4861H *)	CCD	59; 64
101	14588 del 18	4863-4969 del	CCD	65
101	14591 A>G	Y4864C	CCD	unpub.
102	14671 G>C	G4891R	CCD	64
102	14677 C>T	R4893W	CCD	59; 64
102	14678 G>A	R4893G	CCD	unpub.
102	14693 T>C	I4898T *)	MHS/CCD	66
102	14695 G>A	G4899R	CCD	64
102	14696 G>A	G4899E	CCD	59
102	14717 C>T	A4906V	CCD	64
102	14740 A>G	R4914G	CCD	59
102	14741 G>C	R4914T	CCD	unpub.
102	14749 T>C	F4917L	CCD	unpub.
102	14762 T>C	F4921S	CCD	unpub.
103	14818 G>A	A4940T	CCD	unpub.

establish linkage between CCD and RYR1 [85]. Mutations in RYR1 lead to poorly regulated Ca^{2+} release into the skeletal muscle cell; spontaneous Ca^{2+} release from the sarcoplasmic reticulum in CCD or massive Ca^{2+} release triggered by succinylcholine and/or potent inhalational anaesthetics in MH and CCD.

Heat stroke, exercise-induced or recurrent rhabdomyolysis

Heat stroke and terms such as 'exertional heat stroke', 'heat exhaustion', 'exercise-induced rhabdomyolysis' or 'recurrent rhabdomyolysis' describe situations in which the core temperature rises to perilous levels (≥40.6°C) and/or patients with recurrent attacks of rhabdomyolysis. Heat stress, physical activity and/or depletion of salt and water are important factors that cause clinical features representing different degrees of severity on a continuum of disordered thermoregulation [86]. The clinical

picture of heat stroke bears many similarities to MH. For this reason, patients with exertional heat stroke were investigated for susceptibility to MH using the caffeine–halothane IVCT [87–90]. Although approximately 30–50% of the heat stroke patients fulfilled the laboratory diagnostic criteria for susceptibility to MH, there were no known personal or family history of anaesthetic problems. Nevertheless, the high incidence of abnormal IVCT responses suggests that skeletal muscle abnormalities could be responsible for exertional heat stroke. Recurrent rhabdomyolysis proves to be the manifestation of a skeletal muscle abnormality in which a disturbance in calcium homeostasis is the main pathogenetic factor [91].

By the identification of 5 mutations in the ryanodine receptor gene (RYR1) in patients with exercise-induced rhabdomyolysis, there is claimed to be evidence that exercise-induced rhabdomyolysis is associated with susceptibiliy to MH [92, 93]. Partly due to this overlap, it is recommended that susceptibility to MH is excluded in patients who have had episodes of exercise-induced rhabdomyolysis, using the IVCT and genetic mutation screening [92, 94].

Neuroleptic malignant syndrome

The neuroleptic malignant syndrome (NMS) is a potentially fatal complication of the use of neuroleptic drugs (phenothiazines, butyrophenones and thioxanthines) [95, 96]. Three major clinical signs indicate the presence of NMS: rigidity, hyperthermia and rhabdomyolysis with elevated CK. NMS, most often, develops over a period of 24–72 hours after oral neuroleptics. Estimations of the frequency of NMS range from 0.07% to 2.20% [97, 98]. Because of the similarity of some of the clinical features, and because dantrolene has been used as a successful therapeutic agent in both syndromes, NMS has been associated with MH. The most plausible theory to explain NMS is a neuroleptic-induced alteration of central neuroregulatory mechanisms (dopamine depletion or blockade in particular). Another hypothesis could be that NMS is an abnormal reaction of predisposed skeletal muscle to neuroleptics. Although abnormal results (MH equivocal) have been found in the standard-IVCT, the muscular activity in NMS is assumed to be secondary to the central nervous system abnormality [99]. Since no patient with NMS has been reported to be MH susceptible according to the EMHG protocol, NMS and MH are not thought to be (closely) related.

Diagnosis of MH

Diagnosing MH susceptibility in patients who had survived MH reactions or in members of a family with MH is of critical importance because of the potentially disastrous consequences of an MH crisis in the future. Numerous invasive and noninvasive tests to determine the susceptibility of a person to MH have been described over the years, but few have generally been accepted as valid [13, 100].

Tests on blood

Determination of creatine kinase activity (CK) is an unreliable test for predicting susceptibility to MH unless the patient belongs to a family in which increased CK levels have been shown to correlate with in vitro determination of susceptibility to MH [101, 102]. Likewise, no correlation could be demonstrated between cholinesterase abnormalities and MH [103].

Studies on blood cells: erythrocyte tests [104] and platelet tests [105, 106], did not seem useful for diagnostic purpose. B-lymphocytes offer an interesting cellular model because expression of RYR1 has been demonstrated in B-cell lines [107, 108]. Intracellular calcium homeostasis in B-lymphocytes from MHS patients differed significantly from normal controls upon exposure to halothane [109, 110].

Electrophysiological tests

Motor unit counting [111], the tourniquet test in which twitch height was recorded before, during and after ischaemia [112], measurements of relaxation rates of the elicited twitch response [113] and investigation of the EMG recruitment pattern of the hand muscles after halothane and succinylcholine [114], all proved to be of little use for the diagnosis of MH-susceptibility due to a considerable degree of overlap between MHS and normal individuals.

Tests on muscle

Biochemical muscle tests as indicators of susceptibility to MH are considered to be limited. ATP depletion [115], increased glycolytic activity [116], increased ratio of phosphorylase [117], increased activity of adenylate cyclase and cyclic AMP [118] and reduced or absent adenylate deaminase [119], are parameters of increased sympathetic activity, but not recommended as diagnostic tools because of the lack of accuracy and specificity.

Histological differences between MHE, MHS and MHN biopsies are reported [120-122]. Abnormalities include muscle fibre hypertrophy or atrophy, internal nuclei, cores, signs of necrosis and regeneration, and hypercontracted sarcomeres. However, histology alone does not make it possible to diagnose susceptibility to MH [123, 124].

Exposure of single, skinned muscle fibres to calcium, caffeine and halothane are described as a diagnostic test [125]. These tests still need further evaluation of diagnostic sensitivity and specificity.

Measurements of intracellular calcium concentrations seems suitable for diagnosing MH susceptibility. At first, calcium sensitive microelectrodes were used on muscle biopsies [126], later on the methods changed to spectrofluorophotometric determination of free cytosolic calcium concentrations in cultured human skeletal muscle cells *(chapter 3)* [127, 128].

The in vitro contracture test using halothane and caffeine on muscle samples taken at open biopsy has been the best approach for testing for MH susceptibility since its introduction in 1970 *(chapter 2)*.

Molecular genetic tests

The complexity of the genetics of MH limits the use of molecular genetic techniques for the diagnosis of MH susceptibility [129, 130]. The European MH group have agreed on guidelines for the diagnostic use of genetic findings *(chapter 4)*.

In vivo tests

Nuclear magnetic resonance spectroscopy (NMR) using ^{31}P has been applied to measure the energy metabolism in the forearm flexor muscles [131]. Results in normal and MHS individuals showed significant overlap, that made the value of in vivo ^{31}P NMR for the diagnosis of MH risk doubtful.

table 6 *Diagnosing susceptibility to MH.*

Proband or index patient

1 Collect clinical information
2 MH clinical grading scale
3 Clinical examination
4 Creatine kinase measurement
5 Standard caffeine-halothane IVCT
6 Histological examination
7 Genetic testing for 22 mutations

Optional tests and research:

- Sevoflurane IVCT
- Ryanodine contracture test
- 4-chloro-m-cresol contracture test
- *in-vivo* metabolic test
- Test on cultured skeletal muscle cells
- Screening for novel RYR1 mutations
- Genetic segregation analysis

Member from an MH family

1 Collect information about the family
2 Family history **with** a causative mutation
3 DNA test for the MH mutation
4 Standard caffeine-halothane IVCT when the mutation is not detected
5 Family **with no** causative mutations
6 Standard caffeine-halothane IVCT

Optional tests and research:

- Sevoflurane IVCT
- Ryanodine contracture test
- 4-chloro-m-cresol contracture test
- *in-vivo* metabolic test
- Test on cultured skeletal muscle cells

Microcalorimetric studies in MH susceptible individuals showed no significant differences compared to normal individuals in heat production upon exposure to halothane [132]. Analogue to this method a metabolic test has been designed in which intramuscular injection of caffeine and halothane increases local pCO_2 in individuals susceptible to MH but not in controls [133]. The latter might become a minimally invasive alternative test for the detection of susceptibility to MH [134].

Diagnostic procedure

Based on present day knowledge and according to the European IVCT protocol and the EMHG guidelines, probands are selected for screening in accordance with *table 6*. If the proband has died then the nearest appropriate relatives are screened. The lowest age limit for the muscle biopsy is 12–14 years as IVCT results have been inconclusive below this age. Besides, the amount of muscle removed (± 1 gram), represents a significant amount in children. Once MH susceptibility is confirmed screening is offered to each family member. Preferably those individuals that are related as close as possible to the proband should be screened at first.

Aim and Outline of the Thesis

The aim of this thesis is to study the diagnostic procedures for MH susceptibility, with special emphasis upon refining the biological diagnostic test and improving protocols and guidelines for investigation of susceptibility to MH. Diagnosing MH susceptibility will improve the safety of anaesthesia in the future for MH susceptible patients and their families, because anaesthetists can provide safe anaesthesia when they know their patient is susceptible to MH. MH is a detectable and treatable condition of which no one ought to die.

The introduction contains an historical review, a description of the clinical presentation, the treatment of MH and its epidemiology, followed by the current pathophysiological and genetic insights into MH; the diagnostic tests for MH that have been described over the years, are summarized.

The in vitro contracture test (IVCT) forms the basis of investigation of MH susceptibility. Since 1984 the IVCT is performed using the European MH Group protocol *(chapter 2)*. The IVCT has an important disadvantage: it is an invasive test. An alternative, less invasive test for MH susceptibility is offered by using cultured skeletal muscle cells, obtained by needle biopsy *(chapter 3)*. Critical evaluation of the procedure for the diagnosis of MH susceptibility in four European MH centres can be used to refine family screening and to set up a system for quality assurance of all 21 European MH investigation centres *(chapter 4)*.
Chapter 5 is a review of the investigation of a family following fulminant malignant hyperthermia.

In the general discussion the items of chapter 1, will be substantiated with present-day critics *(chapter 6)*.

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Chapter 2

IN VITRO CONTRACTURE TEST

Introduction

Numerous diagnostic tests to determine the susceptibility to MH have been described over the years, but the in vitro contracture test (IVCT) has generally been accepted as "the golden standard"[1]. The IVCT determines the sensitivity of freshly obtained skeletal muscle specimens to caffeine or halothane applied to carboxygenated Krebs-Ringer solution at 37°C.

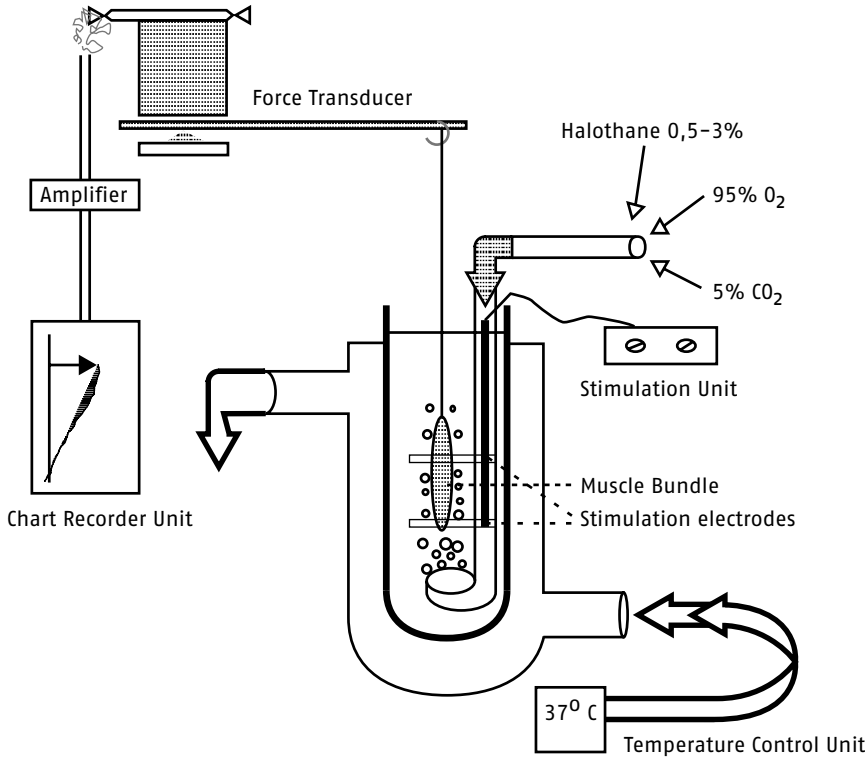
Kalow introduced the use of caffeine for in vitro diagnosis of MH susceptibility in 1970, and Ellis introduced halothane as a test drug, the following year[2, 3]. The European MH investigation units, forming the European Malignant Hyperthermia Group (EMHG), agreed upon a standardised protocol in 1984[4]. The IVCT protocol prescribes the way the muscle should be handled after biopsy and in vitro and how the muscle should be exposed to the test agents. Furthermore, the protocol contains the diagnostic criteria. Since 1984 the European IVCT protocol had been revised several times; the current protocol has been published in 1997[5] (appended at the end of this chapter). The North American MH Group (NAMHG) agreed upon a testing protocol in 1989[6]. Both protocols differ in certain points, but correlation between the testresults is quite good: 84-100%[7, 8].

IVC testing primarily aims at determining the clinical risk of MH reactions. In the attempt to identify as many patients at risk as possible, the diagnostic criteria are established in a way so that sensitivity approaches 100%. This pursuit of minimising the chance of false-negative results, is inextricably bound up with loss of specificity. Taking in account that the IVCT is a biologic test with many variables, the test shows a high sensitivity: 99% for EMHG and 92-97% for NAMHG, and a satisfactory specificity: 93.6% for EMHG and 53-78% for NAMHG[5, 9].

Basic principle

Immediately after excision, the muscle specimen of the vastus group of the thigh is placed in a (precarboxygenated) physiological Krebs-Ringer solution and transported to the IVCT-laboratory. Muscle bundles are tied on both ends and attached vertically in a tissue bath. The bath is filled with Krebs-Ringer solution at 37°C and bubbled with 5% CO_2 in oxygen. The lower end of the muscle bundle is fixed, the other end is hanging on an isometric force transducer *(figure 1)*. The muscle bundles are stretched to optimal length (preload) and viability of the muscle is ensured by stimulation at a supramaximal voltage (1 ms duration, 0.2 Hz). Before addition of the test drug, the muscle bundles are equilibrated for a stable resting tension.

figure 1 *The test set-up for in vitro contracture testing.*
Muscle bundles are stimulated at 37°C in Krebs-Ringer solution. Isometric contractions are recorded after the application of caffeine or halothane.



According to the EMHG test protocol, the cumulative static caffeine contracture test and the cumulative static halothane contracture test are performed in duplo. A fresh muscle bundle is used for each test. The "old" dynamic halothane contracture test and the "modern" ryanodine and 4-chloro-m-cresol contracture tests are optional tests.

In the cumulative static caffeine contracture test, the caffeine is added cumulatively to the tissue bath at 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 32 mmol/l *(figure 2)*. A positive reaction is dependent on a contracture force of at least 2 mN at maximally 2.0 mmol/l caffeine (EMHG threshold concentration).
In the cumulative static halothane contracture test, the halothane is added cumulatively to the tissue bath at 0.11, 0.22, 0.44 and 0.66 mmol/l (consistent with 0.5, 1.0, 2.0 and 3.0% v/v) *(figure 3)*. A positive reaction is dependent on a contracture force of at least 2 mN at maximally 0.44 mmol/l halothane (EMHG threshold concentration).

Three categories result by each test:

- MHS (MH-susceptible): contractures ≥ 2 mN under or at the threshold concentrations of caffeine and halothane.
- MHN (MH-normal or not susceptible): normal reactions to both agents.
- MHE (MH-equivocal): only one type of test is abnormal.
  - MHEh: contractures ≥ 2 mN under or at the threshold concentrations of halothane only.
  - MHEc: contractures ≥ 2 mN under or at the threshold concentrations of caffeine only.

Contracture test results may be falsely positive due to coincidental changes in skeletal muscle resulting from neuromuscular diseases[10, 11], non specific muscle damage secondary to metabolic factors or drug exposure[12], and technical laboratory errors[13, 14].
The MHE category was introduced to define a group with a possible false positive or borderline result. For clinical purposes MHE individuals are considered as MH susceptible.

More specific activators of the skeletal muscle ryanodine receptor have been used in the IVCT instead of caffeine: ryanodine and 4-chloro-m-cresol *(figure 4)*[15, 16]. Ryanodine is a plant alkaloid that was used formerly as an insecticide. Because of its great affinity for the sarcoplasmic reticulum calcium release channel, pharmacologists named this calcium release channel: 'ryanodine receptor' after it. 4-chloro-m-cresol is a preservative that is commonly present in some pharmaceutical preparations.

Multicentre evaluation of in vitro contracture testing with bolus administration of either ryanodine (1 μmol/l) or 4-chloro-m-cresol (75 μmol/l) for diagnosis of MH susceptibility, confirmed that both agents can usefully discriminate between MHS and MHN[17, 18]. Ryanodine IVCT and 4-chloro-m-cresol IVCT might be useful to improve the reliability of diagnosis of MH susceptibility[19]. However, both tests cannot replace the standard caffeine and halothane diagnostic tests. In order to do that it would be necessary to determine sensitivity and specificity by the method of Ørding and colleagues[5]. Ryanodine IVCT and 4-chloro-m-cresol IVCT remain optional tests; only for research purposes they can be applied as additional tests with a view to assign MHE individuals to MH-susceptible or MH-normal.

figure 2 *Example of the static caffeine in vitro contracture test.*
Upper trace MH-normal (MHN); lower trace MH-susceptible (MHS). The threshold caffeine concentration is reached when the increase in resting (baseline) tension surpasses 2 mN.

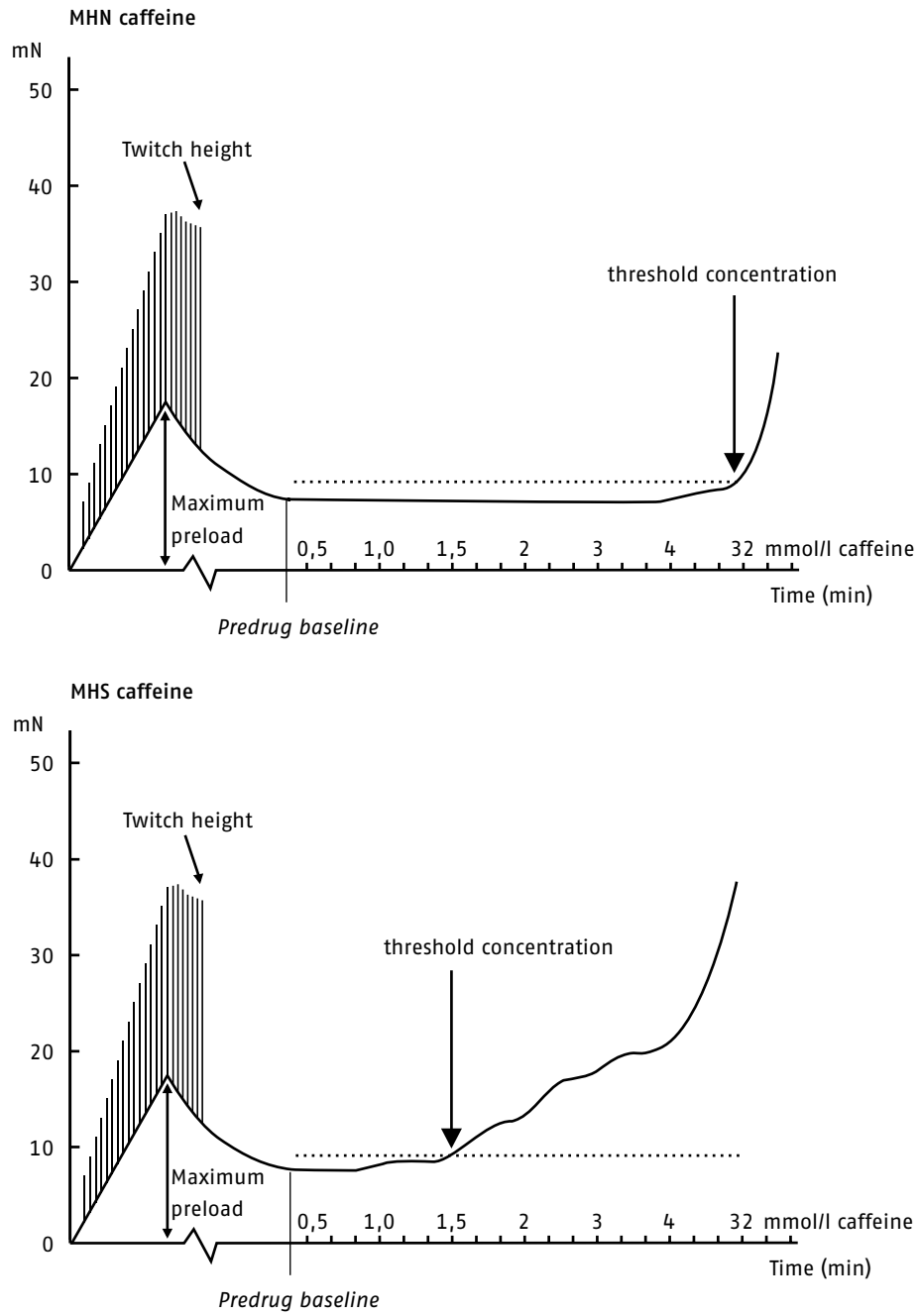


figure 3 *Example of the static halothane in vitro contracture test*
Upper trace MH-normal (MHN); lower trace MH-susceptible (MHS). The threshold halothane concentration is reached when the increase in resting (baseline) tension surpasses 2 mN.

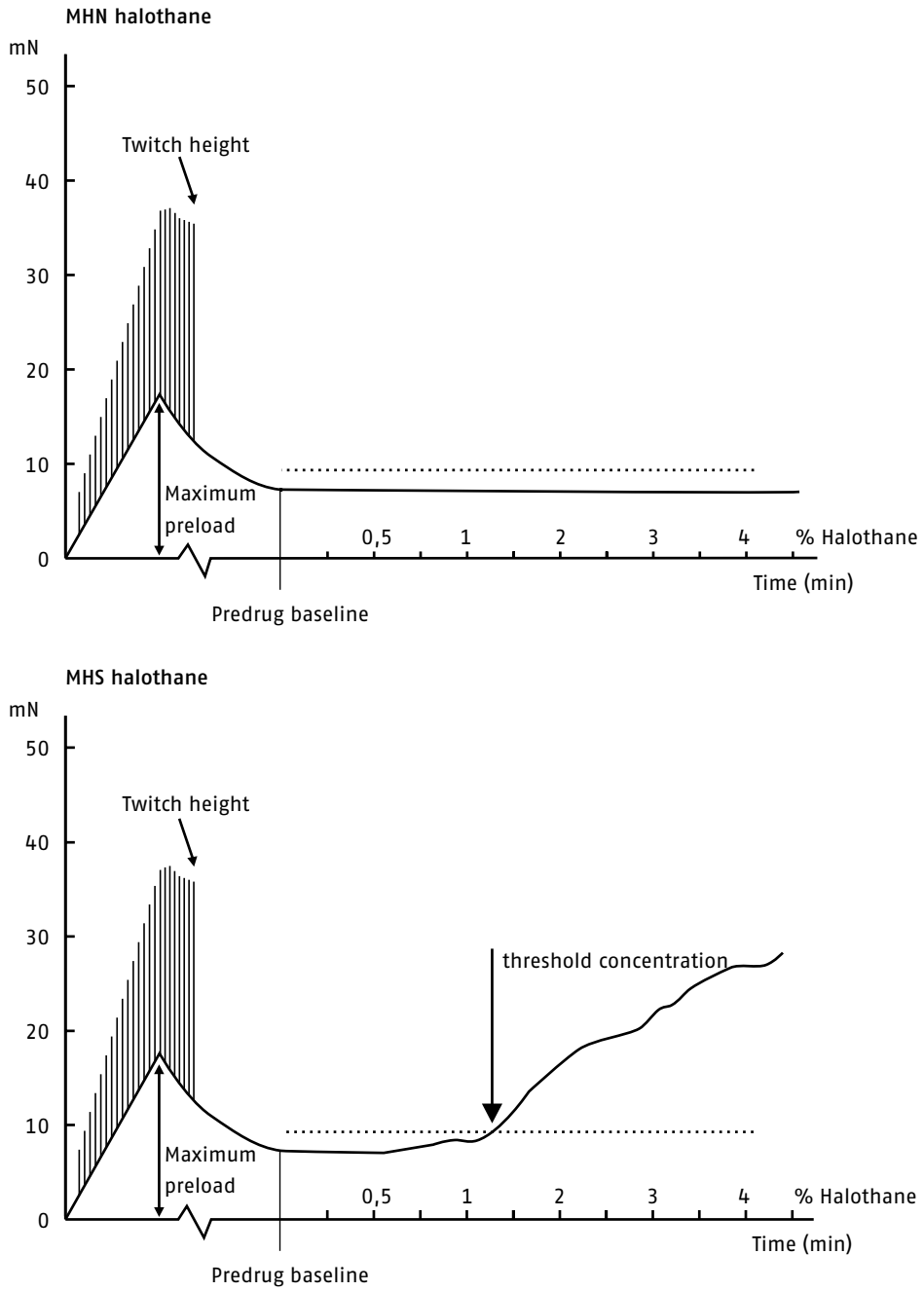
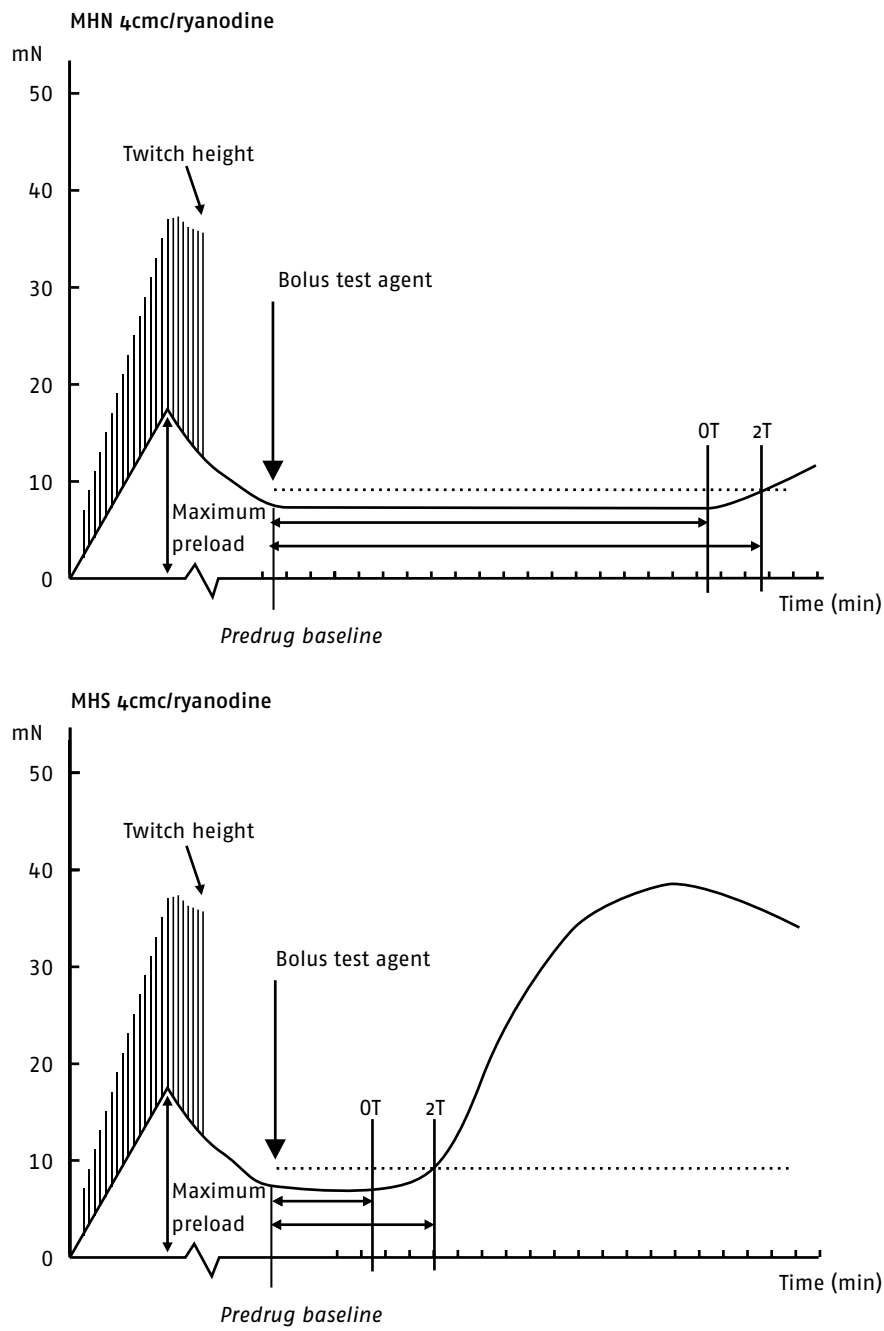


figure 4 *Example of the ryanodine / 4-chloro-m-cresol in vitro contracture test*
In this optional IVCT either ryanodine (1 µmol/l) or 4-chloro-m-cresol (75 µmol/l) is added to the tissue bath. Upper trace MH-normal (MHN); lower trace MH-susceptible (MHS). Onset time (0T) = the time following administration until the contracture increases; 2T = the time to achieve a contracture level of 2 mN.



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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

Acta Anaesthesiologica Scandinavica 1997; 41: 955-966

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Abstract

Determination of sensitivity and specificity of the in vitro contracture test (IVCT) for malignant hyperthermia (MH) susceptibility using the European MH Group (EMHG) protocol has been performed in some laboratories but only on a small sample from the combined EMHG. Thus the purpose of the present study was to determine combined EMHG sensitivity and specificity of the test.

Methods: Results of IVCT of patients with previous fulminant MH and normal, low-risk subjects (controls) were collected from 22 centres of the EMHG. IVCT was performed according to the EMHG protocol. Patients were included in the study if the clinical crisis had a score of at least 50 points with the Clinical Grading Scale. Low-risk subjects were included provided they did not belong to a family with known MH susceptibility, they had not at previous anaesthetics developed any signs of MH, and they did not suffer from any neuromuscular disease. For inclusion of both MH patients and low-risk subjects, at least 1 muscle bundle in the IVCT should have twitches of 10 mN (1 g) or more. For evaluation of individual tests, only muscle bundles with twitch heights of 10 mN (1 g) or more were used.

Results: A total of 1502 probands had undergone IVCT because of a previous anaesthesia with symptoms and signs suggestive of MH. Of these, 119 had clinical scores of 50 and above. From these 119 MH suspected patients and from 202 low-risk subjects IVCT data were collected. Subsequently 14 MH suspected patients were excluded from further analysis for the following reasons: In 3 patients, the suspected MH episode could be fully explained by diseases other than MH; in 11 MHS patients, IVCT was incomplete (n = 1), data were lost (n = 3), or none of the muscle bundles fulfilled twitch criteria (n = 7). Of the remaining 105 MH suspected patients, 89 were MHS, 10 MHEh, 5 MHEc, and one MHN. Thus we observed a diagnostic sensitivity of the IVCT of 99.0% if the MHE group is considered susceptible (95% confidence interval 94.8%–100.0%). Of the 202 low-risk subjects, 3 were MHS, 5 MHEh, 5 MHEc, and 189 MHN. This gives a specificity of the IVCT of 93.6% (95% confidence interval 89.2%–96.5%).

Conclusion: The IVCT for diagnosis of MH susceptibility in Europe has a high sensitivity and a satisfactory specificity.

Introduction

Malignant hyperthermia (MH) is a pharmacogenetic disease of skeletal muscle which is mainly of concern during and following anaesthesia. Diagnosis of susceptibility to MH may be established using an in vitro contracture test (IVCT) with halothane and caffeine. Two international protocols for the performance of such tests have been published, one by the European MH Group (EMHG)[1,2] and the other by the North American MH Group (NAMHG)[3]. The two protocols are similar in many ways, both including a halothane and a caffeine test. They differ in some details, and apparently these minor differences are enough to

account for some variations in the results of the tests[4,5].
The ability of a test to discriminate between those who have a disease and those who are disease-free is measured by the sensitivity and specificity of the test[6]. Sensitivity and specificity are inherent characteristics of the test and in principle independent of the prevalence of the disease tested for. The sensitivity measures the proportion of those with the disease who are correctly identified by the test and the specificity measures the proportion of those without the disease who are correctly called disease-free by the test[6]. In both Europe and North America the threshold between a normal and an abnormal test result, for reasons of safety, has been deliberately chosen so as to secure a high sensitivity of the test, well knowing that such a step will sacrifice specificity.

In a preliminary evaluation of the test results from a few centres in Europe, the sensitivity and specificity of the test was found to be 100% and 93%, respectively[7]. Similar figures have been observed in some individual European centres[4,8]. Apparent false negative results of the IVCT have been reported[9,10] although it must be questioned if the cases described are truly false negatives. Other formal studies have not found evidence of false negative test results[11-13]. One of the problems encountered when determining sensitivity of the IVCT is that the symptoms and signs of MH are non-specific. Thus other diseases and conditions may mimic the clinical presentation of MH[14]. Another problem is that individuals with the MH phenotype may be anaesthetised with triggering agents without developing clinical MH[15,16]. Recently, a Clinical Grading Scale was developed by an international panel of MH experts for assessment of the likelihood that any adverse clinical event could be considered to be MH[17]. This grading scale cannot be specific for MH but allows a comparison of the severity of the observed clinical episode and therefore could help to categorise patients when it is impractical to describe in detail the individual case histories.

The European protocol for the IVCT has been published[1,2], is used widely and has been upgraded regularly. Thus, viability criteria and drug concentrations have been specified. Also, a common database format for recording results has been agreed upon. In the protocol four tests are required, two halothane and two caffeine tests. The caffeine tests are static tests performed at optimal length of the fibre bundle. Concerning the halothane tests, at least one is a static test, whereas the second may be either a static or a dynamic test in which the length of the fibre bundle is cyclically changed[18]. Few European centres use the dynamic test at present, and it is not settled if this test has any advantages compared to the static test[19,20]. However, more patients in Europe are tested with the dynamic halothane test than without it because it is used by the Leeds MH centre which is by far the largest MH centre in Europe.

A patient is considered susceptible to MH (MHS) if at least one halothane test result and one caffeine test result are abnormal[2]. If the results of all 4 tests are normal, the patient is considered non-susceptible (MHN). In the case with abnormal test results to either caffeine or halothane but not to both agents, the result is categorised as MHE (equivocal) and for reasons of safety most of these patients are clinically treated as MH susceptible, whereas in genetic investigations they are assigned unknown disease status.

The present study was initiated to establish combined sensitivity and specificity of the test on data from many different European centres applying the viability criteria which are now part of the testing protocol on individual muscle bundles. For the clinical safety of patients it is essential to secure a high sensitivity of the IVCT but for research it is important to estimate the specificity. Such data have hitherto not been available on a large scale for data obtained with the protocol of the European MH Group.

Materials and Methods

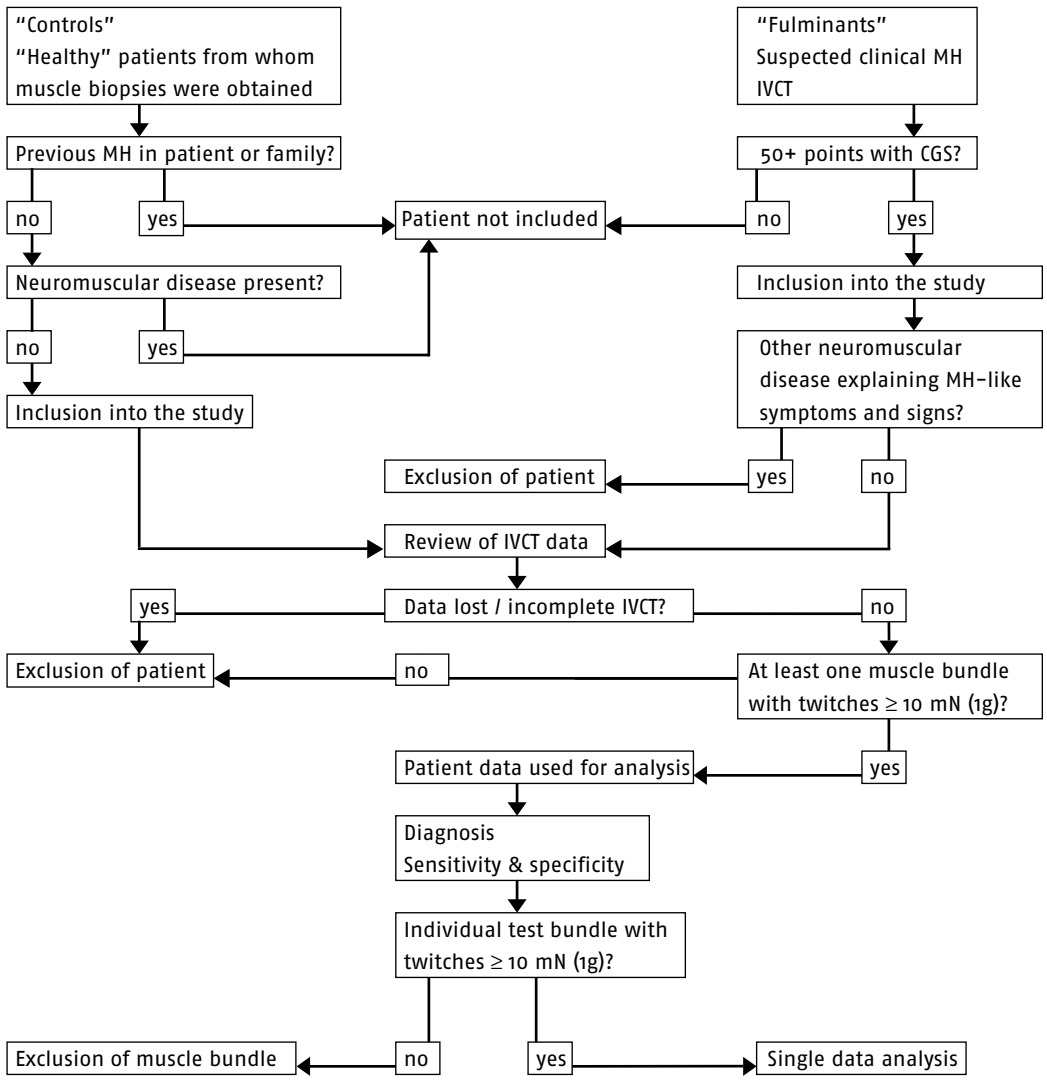
All centres performing IVCT in Europe were asked to forward results of IVCT in patients considered to have survived fulminant MH, and in normal, low-risk subjects (controls), using the database format of the EMHG for reporting results. Patients were assigned a diagnosis according to the result of the IVCT: MHS (MH-susceptible) if the result of at least one halothane test and one caffeine test were abnormal; MHE (MH-equivocal) if either the halothane or the caffeine test result was abnormal; and MHN (MH-negative) if both halothane and caffeine test results were normal. Abnormal results of IVCT were those with increases in muscle bundle force of at least 2 mN (0.2 g) at halothane 0.44 mmol/l or less and at caffeine 2 mmol/l or less. Contracture thresholds at higher concentrations than these were considered normal.

In order to avoid any bias in the selection of patients we applied the Clinical Grading Scale[17] to the clinical episodes of the patients investigated with IVCT because of previous symptoms and signs of MH during or following anaesthesia. Only those cases with a score of 50 or more (i.e. the patients belonging to group 6 with the most severe and well-documented reactions) were accepted for inclusion into this study as fulminant MH cases. The flowsheet for evaluation of patient data is shown in *figure 1*.

Normal low-risk subjects (controls) were defined as patients undergoing surgery who had no symptoms or signs of neuromuscular disease themselves, and who were not genetically related to any such patient. If these low-risk subjects had previously undergone anaesthesia, it was a condition that no side effects which could resemble MH had been observed.

An additional criterion should be met by both MH patients and normal low-risk subjects in order for them to be included into the study: at least one muscle bundle should have a twitch height of

figure 1 *Screening of data submitted for the study.*
Flowsheet showing how data were screened for inclusion into the study and at which levels patients or muscle bundles were excluded. Scoring with the Clinical Grading Scale (CGS) was performed in individual centres and checked for the patients with 50 points or more by the first author. See text for further explanation.



10 mN (1 gram) or more *(figure 1)*. For analysis of individual tests, only muscle bundles with twitch heights of at least 10 mN (1 g) were included because this viability criterion is the one applied in the protocol for the IVCT. Thus inclusion into the study was determined on 2 levels: a subject level and a muscle bundle level *(figure 1)*. Patients were excluded if they were found to have another neuro-

table 1 *Results of scoring the adverse anaesthetic reaction of all probands investigated for MH using the Clinical Grading Scale[17], and results of IVCT.*
The proportion of individuals in each diagnostic category varies with scoring. Diagnostic category and scoring group are significantly related (P < 0.001, Chi-square analysis).
**Three of these 4 MHN individuals were excluded from the study because other diseases fully explained the clinical episode (neuroleptic malignant syndrome in one patient, limb girdle muscle dystrophy in one, and myotonia fluctuans in one patient) – see text for further details.*

Category/diagnosis		MHS	MHEh	MHEc	MHN	Total
1	(0)	4	2	2	21	29
2	(3–9)	4	3	3	24	34
3	(10–19)	161	81	22	474	738
4	(20–34)	118	56	23	214	411
5	(35–49)	96	27	8	40	171
6	(50^+)	99	11	5	4*	119
Total		482	180	63	777	1502

muscular disease which could fully explain the MH-like symptoms and signs *(figure 1)*. For both MH patients and low-risk subjects, patients were excluded if data were lost or the IVCT was not complete. In addition, results from individual muscle bundles were excluded if the bundle did not fulfill viability criteria (i.e. a twitch height of 10 mN (1 g)) *(figure 1)*.

For statistical evaluation, the Mann-Whitney Rank Sum Test and the Chi-square test were used. Confidence intervals were calculated for binomially distributed data. $P < 0.05$ was considered significant.

Results

A total of 22 MH centres from 13 countries in Europe participated in the project. Altogether, 1502 probands had been investigated with IVCT in these centres. The 1502 suspected MH reactions were scored according to the Clinical Grading Scale[17]. The result of this scoring and the outcome of the IVCT performed in these 1502 patients are shown in *table 1*. A significant correlation was found between the category of the clinical reaction and diagnosis ($P < 0.001$).

IVCT data concerning the 119 patients scoring 50 points or more in the Clinical Grading Scale (i.e. group 6 patients with a likelihood of MH described as "almost certain") were collected. In addition, data were collected from 202 normal low-risk subjects. Results from 14 of the group 6 patients were subsequently excluded for the following reasons *(see also figure 1)*: 3 patients were found to suffer from other neuromuscular diseases; these patients were MHN and their clinical episodes are reviewed below. In 4 patients the IVCT was incomplete ($n = 1$) or data were lost ($n = 3$) and in 7 patients none of the muscle bundles for IVCT had twitch heights of at least 10 mN. These 11 patients were all MHS. Thus, test results from 105 patients with previously

table 2 *Type of anaesthetic agent triggering MH in 105 patients suspected of fulminant MH.*

Anaesthetic	N
Halothane	44
Enflurane	12
Isoflurane	45
Other agents	4
Total	105

table 3 *The use of suxamethonium, clinical signs, and treatment of MH with dantrolene in 105 patients suspected of fulminant MH.*

Sign or treatment	Yes	No	Unknown
Suxamethonium	80	23	2
Masseter muscle rigidity	55	38	12
Generalised rigidity	64	24	17
Myoglobinuria	68	11	26
Ventricular extrasystoles	37	32	36
Dantrolene	77	26	2

suspected fulminant MH and 202 low-risk subjects were evaluated in the study. The data finally included in the study originated from 20 centres for MH patients and 14 centres for low-risk subjects. Thirteen centres contributed data on both MH patients and low-risk subjects.

The case histories of the three patients who had scores of 50 or more in the Clinical Grading Scale, and who were excluded because of other diseases are summarised below to illustrate the appropriateness of the exclusion.

Patient 1: A 60 yr old male was anaesthetised for a laparotomy because of a perforated peptic ulcer. Drugs given during anaesthesia included atropine, diazepam, thiopentone, droperidol, suxamethonium, fentanyl, and isoflurane in O_2/N_2O. The anaesthesia was uneventful. The following day the patient developed delirium tremens and was treated with haloperidol. Following this drug, the temperature started to increase. Within 2 days temperature reached 42°C, the patient became rigid and myoglobinuria was observed. CK was 20,900 U/l. The patient was treated with dantrolene without any effect. Following symptomatic treatment in the ICU, the condition of the patient slowly improved during the next month. IVCT (result MHN) was made for a differential diagnosis of MH and neuroleptic malignant syndrome (NMS) and the patient is considered to have suffered from NMS.

Patient 2: A 13 yr old boy was anaesthetised for dental surgery. Following suxamethonium he developed masseter muscle rigidity, which did not impede intubation. Anaesthesia was maintained with

table 4 *Clinical details of 105 patients suspected of fulminant MH. Age: at the time of the MH crisis; duration: of anaesthesia until MH was diagnosed; temperature: the maximum temperature measured during the crisis; $EtCO_2$: maximum end-tidal tension of CO_2 during the crisis; CO_{2art}: maximum tension of CO_2 measured during the crisis in arterial blood; CK: maximum concentration of the enzyme creatine kinase measured in blood following the crisis; K^+: maximum concentration of potassium measured in blood during the crisis; score: result of application of the Clinical Grading Scale[17]; N: the number of patients in whom a given variable was measured.*

	Mean (SD)	Maximum	Minimum	N
Age (yrs)	21.3 (11.7)	65	1	105
Duration (min)	56.6 (60.9)	360	5	85
Temperature (°C)	39.9 (1.4)	44.0	37.1	100
$EtCO_2$ (kPa)	11.0 (3.6)	28.0	6.3	42
CO_{2art} (kPa)	10.7 (4.8)	32.3	5.3	70
pH_{art}	7.09 (0.15)	7.40	6.60	83
CK (U/l)	29,840 (35,503)	225,000	300	94
K^+ (mmol l^{-1})	5.6 (1.2)	8.0	3.1	71
Score	63.5 (9.4)	88	50	105

halothane. He became acidotic with an arterial pH of 7.24 and a base excess of -9. Temperature increased to 38°C. Postoperatively, he had marked myoglobinuria but no renal failure. CK was 111,740 U/l. Subsequently resting CK was found to be increased to 1163 U/l. The patient had no clinical signs of a neuromuscular disease at the time. IVCT was normal (MHN), but muscle histology was abnormal and indicating limb girdle muscle dystrophy. This latent neuromuscular disease is considered to have caused the anaesthetic problems in this patient.

Patient 3: A 16 yr old male was anaesthetised for torsion of the testis. Rapid sequence induction was performed. Following suxamethonium masseter and generalised muscle rigidity was observed. A few minutes later oxygen saturation decreased to 26% and end-tidal pCO_2 increased to 12.7 kPa in spite of ventilation with 100% oxygen and a high flow. Heart rate increased to 120 bpm and temperature to 39.6°C. Arterial blood gases 30 minutes after induction showed acidosis with pH 7.09, pCO_2 8.9 kPa, and base excess –11.7. The patient was treated with dantrolene and sodium bicarbonate and recovery was rapid. Serum potassium remained normal, CK increased to a maximum of 743 U/l. IVCT was normal. However, the patient was found to suffer from myotonia fluctuans and a mutation in the sodium channel gene on chromosome 17 was identified[21].

Details of the clinical episodes of MH in the remaining 105 patients suspected of fulminant MH are summarised in *tables 2-4*.

It is apparent that several variables were not measured or reported for all patients. Some measurements of end-tidal or arterial pCO_2 *(table 4)*

table 5 *Clinical details relating to the muscle biopsy in 105 patients suspected of MH and 202 low-risk subjects. Sex: m = male, f = female; anaesthesia for the biopsy: r = regional, g = general, u = unknown; abnormal neurological signs: p = present, a = absent, u = unknown; histopathology: n = normal, a = abnormal, np = not performed.*

	Fulminant	Control	P value
Sex, m/f/u	67/38/0	106/94/2	0.091
Age, yrs	23.7 (11.6) (4 – 66)	51.3 (17.9) (7 – 90)	< 0.001
Anaesthesia, r/g/u	62/27/16	127/58/17	0.185
Neurology, p/a/u	11/79/15	0/143/59	< 0.001
Histopathology, n/a/np	62/18/25	43/0/159	< 0.001

were done during hyperventilation or following other treatments. The reporting system did not allow details concerning this problem.

Clinical details of the MH patients and low-risk subjects relating to the muscle biopsy are shown in *table 5*. The low-risk subjects were significantly older than the patients surviving MH (Mann-Whitney test, $P < 0.001$), whereas the sex distribution was similar in the two groups of patients ($P = 0.091$). A significantly larger proportion of MH patients than controls had abnormal neurological signs and abnormal histopathology although histopathology was not performed in the majority of control patients.

Details relating to the IVCT are shown in *table 6* (halothane test) and *table 7* (caffeine test). No significant differences were found between the two groups for any variables of the IVCT with the exception of the number and sizes of contractures which were significantly more frequent and of larger size in the MH group ($p < 0.001$).

Whereas 168 of 181 viable specimens from MH patients developed a contracture of > 2 mN (0.2 g) in the halothane test, only 12 of 272 specimens from low-risk subjects did so ($P < 0.001$) *(table 6)*. In the caffeine test, 128 of 143 viable specimens from MH patients developed contractures whereas only 8 of 258 viable specimens from the low-risk subjects did so ($P < 0.001$).

From the number of muscle bundles with contractures exceeding the threshold of 2 mN (0.2 g) at halothane 0.44 mmol/l or caffeine 2 mmol/l, the false positive or false negative rate of results may be calculated for the two tests. Altogether 13 of 181 muscle bundles from MH patients did not develop contractures in the halothane test *(table 6)*, giving a false negative rate of 7.2% for individual muscle bundles. The false negative rate for muscle bundles in the caffeine test was 15/143 *(table 7)*, i.e. 10.5%. The false positive rate for the halothane test was 12/272 muscle bundles *(table 6)*, i.e. 4.4%, whereas the false positive rate for the caffeine test was 8/258 *(table 7)*, i.e. 3.1%. It must be noted that these rates are not for patients, only for specimens tested.

table 6 *Muscle bundle characteristics in the halothane test for those bundles included in the study (criterion: twitch heights of at least 10 mN).*
*Fulm = bundles from patients with a score in the Clinical Grading Scale of 50 and above (n = 105 patients). Contr = bundles from low-risk subjects (n = 202 subjects). $N_{samples}$ for twitch are the number of specimens fulfilling viability criteria (twitch height of 10 mN or above). For length and weight, $N_{samples}$ signify the number of specimens fulfilling viability criteria and for which length and weight were reported. For contracture, $N_{samples}$ signify the number of viable specimens with a contracture of at least 2 mN at halothane 0.44 mmol/l. Nindividuals signify the number of individuals from whom the $N_{samples}$ come. All patients had at least one halothane test and one caffeine test performed. * P < 0.001.*

		Halothane test				
		Median	**Mean (SD)**	**Range**	**$N_{samples}$**	**$N_{individuals}$**
Twitch (mN)	Fulm	35	43 (28)	10 – 132	181	103
	Contr	20	24 (15)	10 – 98	272	181
Length (mm)	Fulm	18.0	18.9 (5.5)	6 – 33	173	64
	Contr	17.0	19.9 (5.1)	6 – 33	231	107
Weight (mg)	Fulm	150.0	165.2 (81.8)	49 – 684	127	65
	Contr	153.5	156.7 (72.5)	22 – 340	168	98
Contracture	Fulm	17	20 (16)*	2 – 89*	168*	99*
(mN)	Contr	4	4 (3)*	2 – 12*	12*	8*

table 7 *Muscle bundle characteristics in the caffeine test for those bundles included in the study (criterion: twitch heights of at least 10 mN).*
*Fulm = bundles from patients with a score in the Clinical Grading Scale of 50 and above (n = 105 patients). Contr = bundles from low-risk subjects (n = 202 subjects). $N_{samples}$ for twitch are the number of specimens fulfilling viability criteria (twitch height of 10 mN or above). For length and weight, $N_{samples}$ signify the number of specimens fulfilling viability criteria and for which length and weight were reported. For contracture, $N_{samples}$ signify the number of viable specimens with a contracture of at least 2 mN at caffeine 2 mmol/l. $N_{individuals}$ signify the number of individuals from whom the $N_{samples}$ come. All patients had at least one halothane test and one caffeine test performed. * P < 0.001.*

		Caffeine test				
		Median	**Mean (SD)**	**Range**	**$N_{samples}$**	**$N_{individuals}$**
Twitch (mN)	Fulm	27	38 (27)	10 –146	143	92
	Contr	20	25 (17)	10 –99	258	179
Length (mm)	Fulm	18.0	18.2 (5.2)	10 – 32	113	65
	Contr	18.0	19.5 (5.9)	5 – 35	172	141
Weight (mg)	Fulm	167.5	169.5 (74.8)	47 – 550	112	65
	Contr	160.0	155.1 (74.0)	27 – 350	166	130
Contracture	Fulm	11	14 (13)*	2 – 62	128*	94*
(mN)	Contr	2.8	3 (1)*	2 – 5	8*	8*

table 8 *Diagnostic outcome of the IVCT in 105 patients suspected of fulminant MH and 202 normal low-risk subjects.*
For calculation of the diagnostic sensitivity and specificity of the IVCT, patients with an MHE diagnosis are considered susceptible to MH, as most would be clinically for reasons of safety. For sensitivity and specificity 95% confidence limits are given in brackets. If, for calculation of specificity, the MHS diagnosis is exclusively used, specificity would increase to 98.4%.

	MHS	MHEh	MHEc	MHN	N	Sensitivity	Specificity
Fulm	89	10	5	1	105	99.0% (94.8-100.0%)	-
Contr	3	5	5	189	202	-	93.6% (89.2-96.5%)

Diagnoses resulting from the IVCT are shown in *table 8*. For reasons of safety, the MHE group is considered to be at risk of MH. The sensitivity and specificity of the IVCT are 99.0 % (104/105) (95% confidence interval 94.8%-100.0%) and 93.6% (189/202) (95% confidence interval 89.2%-96.5%), respectively. If the MHE group is omitted from the MH group, the specificity increases to 98.4% (189/192). However, we have agreed the MHE groups are under constant review.

One patient among those categorised by the Clinical Grading Scale as fulminant tested MHN in the IVCT. The MH suspected episode occurred in 1978 and is reported below.

Patient 4: This patient was a 10 month old boy requiring plastic surgery for cleft palate. The anaesthetic itself was uncomplicated and included halothane and atropine, but at recovery generalised rigidity and possibly convulsions were observed. The body temperature was 40.6°C and an arterial blood sample showed a combined metabolic and respiratory acidosis with pH 7.10 and $PaCO_2$ 8.7 kPa. No information about possible myoglobinuria, plasma increases in CK or K^+ were available. Treatment consisted of oxygen and cooling (the patient had been on a warming blanket throughout a two hour period in addition to being wrapped in blankets and completely covered by surgical drapes). Further recovery was uneventful. The patient had an IVCT performed in 1989 at the age of 12 years. He looked "peculiar" and was intellectually somewhat retarded. There was no clinical signs of neuromuscular disease except for the treated cleft palate. IVCT was normal. Histology showed fibre type one predominance and centrally located nuclei but no cores, indicating the presence of a nonspecific myopathy. Following this result the mother was investigated to rule out a false negative result of the IVCT. She was also MHN. The father has refused to undergo IVCT. Thus it is impossible to determine if this is a false negative result of the IVCT or the episode was due to over-heating with ensuing convulsions, hypoxia, hypercapnia, and acidosis in a patient with a latent myopathy.

table 9 *Effect on sensitivity and specificity of different contracture thresholds applied to the IVCT data presented in this study.*

	Threshold (mN)	MHS	MHEh	MHEc	MHN	N	Sensitivity	Specificity
Fulm	2	89	10	5	1	105	99.0%	-
	3	81	16	5	3	105	99.0%	-
	4	77	16	6	6	105	94.2%	-
	5	66	24	6	9	105	91.4%*	-
Contr	2	3	5	5	189	202	-	93.6%
	3	2	3	2	195	202	-	96.5%
	4	1	4	1	196	202	-	97.0%
	5	0	3	0	199	202	-	98.5%*
Fulm	H 5, C 3	77	15	9	4	105	96.2	-
Contr	H 5, C 3	2	1	2	197	202	-	97.5%

Values are number of patients within a diagnostic category, given a particular threshold. If nothing is mentioned the threshold applies to both halothane and caffeine data. The currently used threshold for both halothane and caffeine tests is a 2 mN contracture with 0.44 mmol/l halothane or less and 2 mmol/l caffeine or less. * denotes a significant decrease in sensitivity or increase in specificity compared to that obtained with 2 mN.

The number of data reported from individual centres were too small to calculate centre-specific variability and the number of data were not evenly distributed from the 22 participating centres. Concerning the MH patients, the 16 non-MHS responses originated from 9 different centres, which does not hint at skewness in distribution. Likewise, the 13 non-MHN responses in the control group originated from 6 different centres out of the 14 centres contributing control data.

With the present large sample of data, it is possible to investigate the effect on sensitivity and specificity of other thresholds than the current of 2 mN (0.2 g). In *table 9* calculated sensitivity and specificity are shown for thresholds of 2 - 5 mN. It is apparent that increases in the threshold value above 3 mN reduces sensitivity. This is significant for the 5 mN threshold ($P = 0.023$, Chi-square test with Yates correction). A threshold of 3 mN maintains sensitivity and increases specificity. However, this increase in specificity is not statistically significant ($P = 0.25$, Chi-square test with Yates correction). In addition data are given for different halothane and caffeine thresholds: 0.3 g for caffeine and 0.5 g for halothane, but this does not improve sensitivity and specificity.

Discussion

Safety for all patients undergoing anaesthesia is a main goal of anaesthetists. MH has been one of the severe complications of

anaesthesia with an early mortality rate of 70% [22]. In recent years, the number of deaths from MH has significantly decreased, and in some countries is now zero [4]. Performing IVCT has several purposes: The main one is to eliminate the threat of MH from all those individuals who do not have the MH phenotype so that they can be anaesthetised without any specific precautions and be given volatile anaesthetics if the anaesthetist wishes to use these agents. Because MH is an inherited disease, the number of individuals who may thus benefit from IVCT is large compared to the number of patients who have themselves developed signs of MH. Another purpose is to establish a definite diagnosis in those individuals who do have the MH phenotype and to inform the patients and their attending doctors of this disease. A research purpose is to establish a link between the clinical phenotype, the IVCT result, and the presence or absence of the mutations associated with MH. For this purpose, a systematic analysis of the clinical signs of MH present during a crisis and the results of the IVCT must be performed.

There has been no formal assessment of the Clinical Grading Scale which was developed by an international panel of MH experts by the Delphi method [17]. It represents an attempt at providing objective criteria for classification of clinical MH. For each individual process involved in MH, points are awarded according to the occurrence of specific signs, realising that several variables may be signs of the same process. Thus double-counting is avoided although not completely. Points may be given for both hypercapnia, low pH, and negative base-excess, although these parameters are related. Another problem is that since none of the symptoms or signs of MH are specific, it follows that the Clinical Grading Scale cannot be specific either. Thus severe rhabdomyolysis following cardiac arrest during anaesthesia may give rise to high scores classifying the episode as highly likely to be MH although nothing points to MH [14, 23]. The three patients with other neuromuscular diseases whom we excluded from the analysis of IVCT data represent patients with such non-specific high scores. We find the exclusion of these patients appropriate because it is well-known that patients with other neuromuscular diseases may develop signs similar to MH without having the MH phenotype [14].

The result of scoring is dependent on the quality of documentation of the case. In this study, some cases of real and severe MH, mostly occurring many years ago, were not well documented at the time of the crisis and thus did not obtain a score high enough to be included. Other cases were well documented and considered to represent real, fulminant MH in the investigation centre but were excluded because they did not obtain a score of at least 50. Such cases are considered representative of less severe MH, often due to early diagnosis and treatment. The fact that we observed a significant correlation between the score and the proportion of MHS responses seems to validate the usefulness of the Clinical Grading Scale for comparison of

groups of patients though it should not be used for diagnostic purposes for an individual.

The sensitivity of 99% observed in this study is satisfactory for a diagnostic test, the more so because the clinical adverse event in the single MHN patient scoring above 50 points may well be due to other factors than MH. This case illustrates the fact that a definite diagnosis may not always be established even with use of the invasive IVCT. The observed sensitivity of the IVCT is comparable to that obtained by the North American MH Group [24].

The specificity of the IVCT observed in the present study is also satisfactory, given the high sensitivity and also compared with the observations made by the North American MH Group [25]. However, for other purposes than patient safety, it seems wise not to include patients with the MHE response into the group of MH susceptible patients. This suggested guideline is supported by the fact that in families with known chromosome 19 mutations related to MH, MHE individuals rarely do have the mutation in question [26–28]. Thus the original statement concerning the MHE group, that this group is under permanent review, is still valid [2].

Should the critical contracture size be changed from the present 2 mN value? Based on our present rather large data sample there is no justification for change. As shown in *table 9* only a change from 2 mN to 5 mN significantly increases specificity, and this occurs at a cost of a significantly reduced sensitivity which would be unacceptable. The reason why our halothane threshold contracture is smaller than that used by the North American MH Group is that our dose–response curve has cumulative increments in concentration to 2%, whereas the NAMHG uses a single addition of 3% halothane. The single dose has previously been shown to result in larger contractures [4].

For clinical decision making it is not enough to know the sensitivity and specificity of the IVCT. What really counts is the predictive value of a positive or negative response, which depends on the prevalence of the disease (in this case MH) in the population tested [4, 6]. With a very high sensitivity of the test and a somewhat lower specificity, as presently observed for the IVCT, the predictive value of a negative test result is around 99%. The predictive value of a negative result is not much influenced by the prevalence of the disease when the specificity is as high as that found for the IVCT and it increases with decreasing disease prevalence [4, 6]. On the other hand, the predictive value of a positive test result is heavily influenced by the disease prevalence in the test population. The a priori risk of a first degree relative of a patient surviving fulminant MH having inherited the disease is 50% and in such a patient the predictive value of a positive test result is about 90% [4]. However, if instead a cousin with an a priori risk of 12.5% is investigated the predictive value of a positive result of the IVCT is reduced to approximately 50% [4]. The predictive value of a negative result in the same patient is close to 100% [4].

An abnormal result in a member of the general population who has a very low a priori risk of MH is therefore much more likely to be a false positive result than indicating MH susceptibility. These considerations are important when selecting patients for IVCT. The greatest confidence in the results is obtained in those with the highest a priori risk of susceptibility.

Although data are insufficient to prove that results vary between centres, it is obvious from *tables 6 and 7* that individual test procedures probably could be more standardised. Since data were collected for this study steps have been taken to standardise muscle length and weight as well as the size of the preload and the method to obtain this preload. These guidelines are presented in the updated current protocol.

In conclusion, the observed sensitivity of 99% and specificity of 93.6% in the IVCT which we have obtained in this joint Europan study is considered satisfactory for patient safety. For research purposes it is recommended to increase the specificity to 98% by not regarding MHE patients as susceptible.

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Contractures in skeletal muscle of malignant hyperthermia susceptible patients after in vitro exposure to sevoflurane

Acta Anaesthesiologica Scandinavica 2000; 44: 334-337

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Abstract

Sevoflurane, a potent inhalational anaesthetic agent that is structurally similar to halothane, has some favorable characteristics, but may also be able to trigger malignant hyperthermia (MH) in susceptible patients. The diagnosis of malignant hyperthermia susceptibility relies on the in vitro contracture test on skeletal muscle. The present study was undertaken to investigate whether exposure to sevoflurane of muscles of malignant hyperthermia susceptible (MHS) patients would also cause an abnormal contracture.

Methods: Muscle fascicles from three MHS patients, one malignant hyperthermia non-susceptible (MHN) patient, two control patients and one malignant hyperthermia equivocal (MHE) patient were exposed to sevoflurane instead of halothane in the in vitro contracture test, carried out according to the protocol of the European Malignant Hyperthremia Group. The muscle fascicles were surplus to diagnostic requirements. Sevoflurane concentrations in the testbath were measured using a headspace gas chromatographic technique.

Results: The kinetics of sevoflurane concentration in the testbath were similar to those of halothane. An in vitro contracture response of 2 mN or more was seen in all four MHS/MHE patients with sevoflurane and not in the three control/MHN patients. The magnitude of muscle contracture in the sevoflurane test was less than in the conventional halothane test at comparable testbath concentrations.

Conclusions: Sevoflurane can trigger an abnormal contracture in human muscle in vitro. This is indicative of malignant hyperthermia susceptibility. Exposure to sevoflurane should be avoided in patients thought to be susceptible to malignant hyperthermia.

Introduction

Sevoflurane is a volatile anaesthetic agent and is used for the induction and maintenance of general anaesthesia. Unlike other currently clinically used halogenated inhalational anaesthetics (e.g. enflurane, isoflurane and desflurane), sevoflurane does not irritate the airways, causing less coughing and mucous production during induction of anaesthesia. The blood/gas partition coefficient at 37°C of sevoflurane is low (0.63–0.69). These characteristics of sevoflurane make it a useful drug for induction of anaesthesia, especially in children, because of a rapid and smooth induction and a fast recovery.

Potent inhalational anaesthetics can trigger Malignant Hyperthermia (MH)[1,2]. Sevoflurane is not thought to be an exception. MH susceptible swine have been exposed to sevoflurane and MH-reactions did not occur as often as after halothane administration, and the MH-reactions after sevoflurane were less fulminant compared to the MH-reactions after halothane[3]. In Japan, where sevoflurane has been used since 1990, four patients have been described in whom MH developed during sevoflurane anaesthesia[4–6]. Sevoflurane may or may not have been the cause of the MH-reactions. Otsuka et al.

encountered a case of fulminant MH in a child with central core disease, which is closely related to MH [4]. Ochiai et al. described two patients with a MH-reaction but in one of these cases an other trigger-agent (isoflurane) had been administered [5]. Maeda et al. described delayed recovery from muscle weakness in a patient with a likely diagnosis of MH triggered by sevoflurane [6]. In Europe, two patients have been described in whom MH developed during sevoflurane anaesthesia [7,8]. In both cases diagnosis of susceptibility to MH was established using an in vitro contracture test (IVCT).

To prove that sevoflurane alone can trigger MH in humans, we exposed muscle biopsy samples from MH susceptible patients to sevoflurane in the IVCT. The caffeine and halothane IVCT is generally accepted as the best standardized test we have available at the moment for diagnosis of MH susceptibility [9-11].

To compare the potency of inducing a contracture, sevoflurane concentrations in the testbath were measured.

Methods

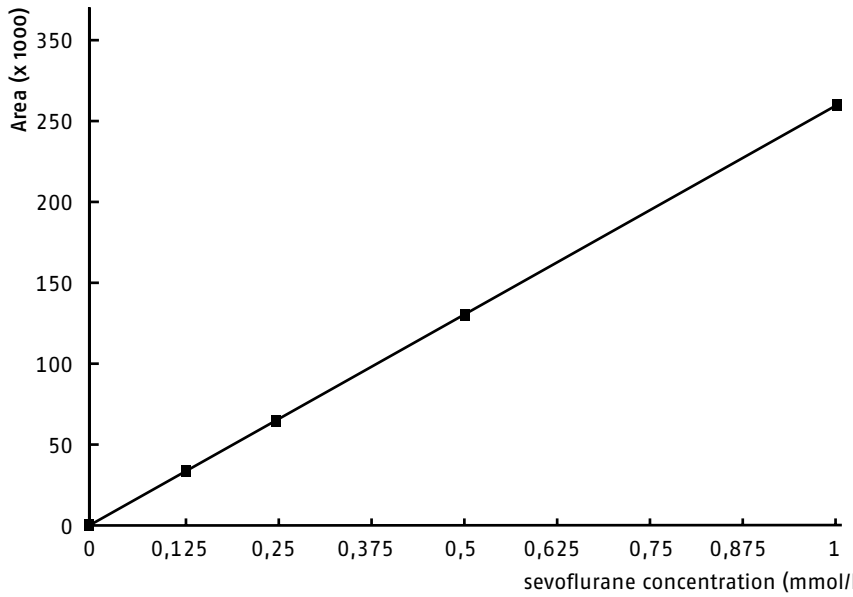
Muscle fascicles were tested from three MH susceptible (MHS) patients, one MH negative (MHN) patient, two negative tested (MHN) control patients and one patient whose muscle had an abnormal contracture response to halothane but normal response to caffeine and was therefore classified as MH equivocal (MHE) according to the protocol of the European MH Group [9,11]. The muscle fascicles for this study were surplus to diagnostic requirements or from control patients who gave written informed consent; the study was approved by the local Ethics Committee.

Immediately after excision, the muscle specimen from the quadriceps femoris muscle was placed in Krebs-Ringer solution (118.1 mmol/l NaCl; 3.4 mmol/l KCl; 2.5 mmol/l $CaCl_2$; 1.2 mmol/l KH_2PO_4; 0.8 mmol/l $MgSO_4$; 11.1 mmol/l glucose; 25.0 mmol/l $NaHCO_3$; pH: 7.4) at ambient laboratory temperature and bubbled with carbogen (5% CO_2; 95% O_2). Muscle fascicles (length 20-25 mm, weight 100-200 mg) were dissected from a larger piece of muscle and placed in a 25 ml testbath, filled with Krebs-Ringer solution at 37°C bubbled with carbogen 100 ml/min. After a 5-min rest, the muscles were stretched slowly to 150% of the initial length and stimulated directly with a 1 ms square wave single supramaximal stimulus at a frequency of 0.2 Hz (Grass S48 stimulator). The twitch responses were recorded on a Kipp & zn BD100 recorder using a Schaevitz FTD-G-10 force transducer and measured at least 10 mN before the administration of sevoflurane. After another rest of 15 minutes during which stable baseline tensions were achieved, sevoflurane was delivered incrementally at 1, 2, 3, 4, 5, 6, 7 and 8% through a calibrated vaporiser (Penlon™, Sigma Elite). Each dose being maintained for 5 min.

The threshold value is the minimal concentration of sevoflurane at which a sustained baseline contracture of 2 mN or greater occurs.

Sevoflurane concentrations in the carbogen were measured with

figure 1 *Calibration graph: gas chromatographic peak area of sevoflurane after injection of 1 ml of headspace gas, sampled out of 4 septum closed glass vials of 15 ml, filled with 1 ml Krebs-Ringer solution with 0.25, 0.5, 1.0 and 2.0 ml standard gas (sevoflurane concentration 0.5 mmol/l), resulting in concentrations of respectively 0.125, 0.25, 0.5 and 1.0 mmol/l. The standard error of the x-coefficient (260 area units/(mmol/l)) is < 1%.*



an Ohmeda 5250 RGM analyser. Sevoflurane concentrations in the liquid phase were measured using a headspace gas chromatographic technique (Chrompack, CP-9001, equipped with a flame ionisation detector)[12]. The injection port of this chromatograph was installed with a glass liner (length: 8 cm; o.d. 6 mm; i.d. 3 mm). Column: 2 m x 2 mm i.d. glass, packed with 10% SP 1200/1% H_3PO_4 on 80/100 Chromosorb W AW. The column and glass liner were stoppered with glass wool. Column temperature: 100°C (isothermal), injection port temperature: 200°C; detector temperature: 180°C. Carrier gas: N_2 30 ml/min; H_2 30 ml/min; air 250 ml/min. Injection (1 ml of headspace gas) was performed in the empty liner, in one stroke.

For external standardization, we added to 4 septum closed glass vials of 15 ml filled with 1 ml of Krebs-Ringer solution, respectively 0.25, 0.50, 1.0 and 2.0 ml of a standard gas mixture with a sevoflurane concentration of 0.5 mmol/l. The mixtures were vortexed and equilibrated for 2 min. Injection of 1 ml of the headspace gas resulted in a single peak of sevoflurane with a retention time of 0.70 min. Plotting of the peak area against the concentration resulted in a linear calibration graph *(figure 1)*. The testbath solutions were sampled using gas-tight glass syringes. One millilitre of these testbath solutions was injected

tabel 1 *In vitro contracture test results in skeletal muscle specimens of malignant hyperthermia susceptible (MHS), equivocal (MHE), non-susceptible (MHN) and control patients. Threshold: the lowest concentrations of halothane and caffeine which produce a sustained increase of at least 2 mN in baseline force from the lowest tension reached [9, 11]. The contractures were at 0.44 mmol/l halothane and 2.0 mmol/l caffeine. NR = not reached.*

	Halothane			Caffeine		
	threshold		0.44 mmol/l	threshold		2.0 mmol/l
	mmol/l	(mN)	mN	mmol/l	(mN)	mN
MHS 1	0.11	(12)	24	0.5	(5)	28
MHS 2	0.11	(5)	26	0.5	(3)	16
MHS 3	0.22	(6)	13	1.0	(2)	12
Control 1	NR		0	32	(110)	0
Control 2	NR		0	4.0	(2)	0
MHE	0.44	(2)	2	3.0	(3)	1
MHN	NR		0	32	(140)	0

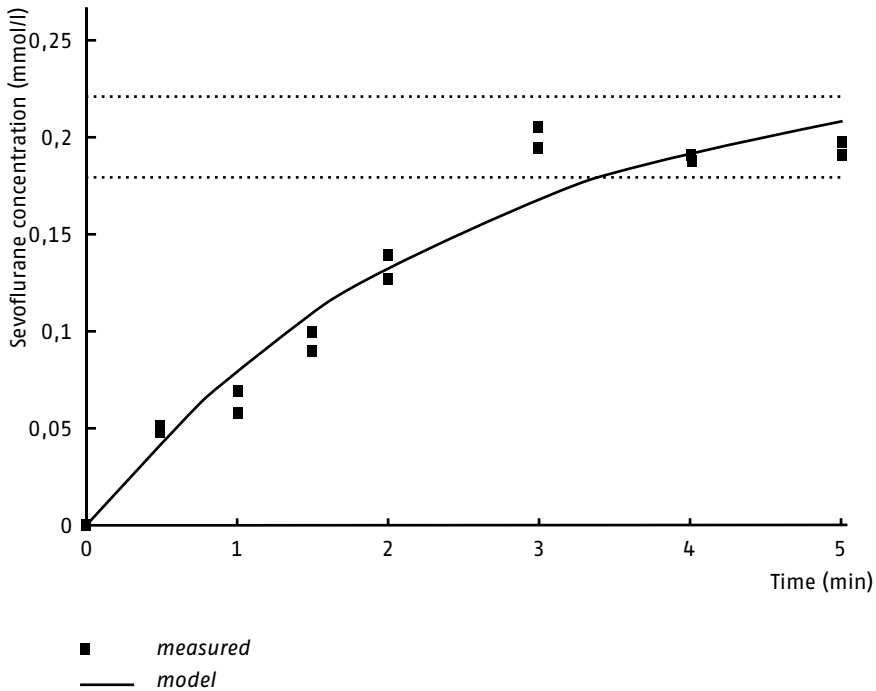
table 2 *Sevoflurane in vitro contracture test results. Contracture response in mN following cumulative administration of sevoflurane in skeletal muscle specimen (2 mN or greater is interpreted as a positive contracture response).*

	1%	2%	3%	4%	5%	6%	7%	8%
MHS 1	1	6	8	10	11	10	11	12
MHS 2	0	1	3	4	6	8	8	7
MHS 3	0	0	1	2	3	4	4	3
MHE	1	1	1	2	2	3	3	4
MHN	0	0	0	0	0	0	1	0
Control 1	0	0	0	0	0	0	0	0
Control 2	0	0	0	0	0	0	0	0

into a septum closed 15 ml glass vial and treated in the same way as described above for the calibration solutions. The concentrations of the testbath solutions were derived from the calibration graph. By using a fixed 1 ml fluid to 14 ml gas ratio in both measuring and standardization experiments, we did not have to take the Oswald lambda into account, since it is eliminated from the formulas [12].

The equilibration characteristics in the bath were investigated by measuring sevoflurane concentrations in the testbath as a function of duration of bubbling. Samples were taken after 30 s, 1, 1.5, 2, 2.5, 3, 4 and 5 min of bubbling with 2% sevoflurane in carbogen, 100 ml/min. The kinetics of sevoflurane concentrations in the bath were evaluated on mean data by non-linear regression analysis, assuming a one-exponential saturation process.

figure 2 *Equilibration of sevoflurane in the liquid phase. Sevoflurane (2% vaporiser concentration) in carbogen (100 ml/min) bubbles through a 25 ml testbath. The testbath solutions were sampled before and at 0.5, 1, 1.5, 2, 3, 4 and 5 minutes after the start of the sevoflurane perfusion. The dotted dividing lines represent the range of the target values (0.20 mmol/l ±10%). Data points indicate the means of two measurements. Equilibration time is 3 minutes. After 5 minutes 1/e time is 2.41 min, meaning the target value (90%) is always reached at 5 minutes.*



Statistical analysis were performed using exponential time fitting for the equilibration time and linear regression with a logarithmic scale, resulting in coefficients with standard errors.

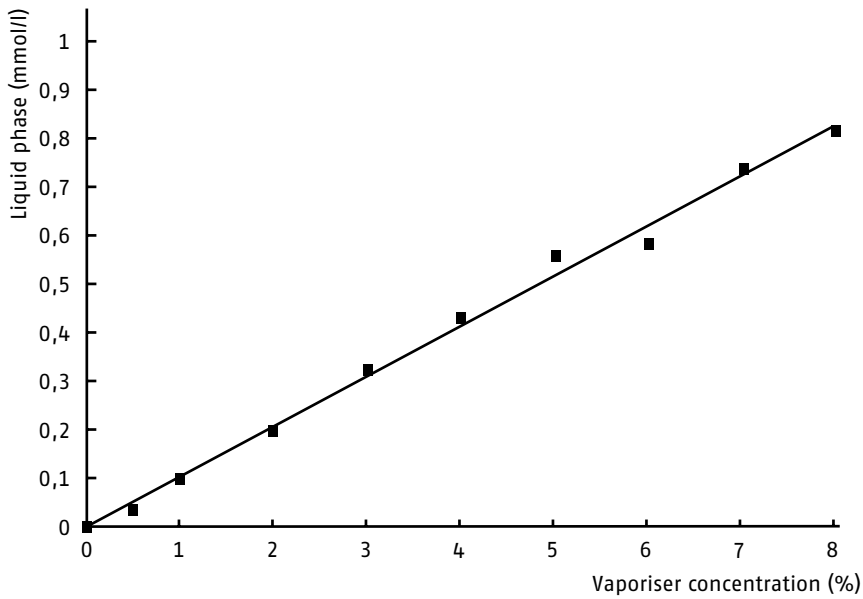
Results

The halothane and caffeine threshold values, the contractures at 0.44 mmol/l halothane and at 2 mmol/l caffeine are given in *table 1*.

The equilibration time for the 25 ml testbath, filled with Krebs-Ringer solution and bubbled with 2% sevoflurane in carbogen, is 3 min *(figure 2)*. A linear correlation was obtained between the percentage-vaporiser concentrations and the resulting concentrations of sevoflurane in the liquid phase *(figure 3)*.

The results from the sevoflurane IVCT are given in *table 2*. In patient MHS 1, a positive contracture response of ≥ 2 mN was reached at 2% sevoflurane, corresponding to 0.20 mmol/l in the liquid phase. In muscle tissue from patients MHS 2, MHS 3 and MHE, sevoflurane

figure 3 *The kinetics of sevoflurane in the liquid phase, sampled from the 25 ml testbath. Sevoflurane is delivered in carbogen 100 ml/min, incrementally at 0.5, 1, 2, 3, 4, 5, 6, 7 and 8% vaporiser concentration for 5 minutes. Data points indicate the means of 3 measurements. Deviation of a linear relation is less than 5% (standard error of the x-coefficient (0,103) is 0,0151).*



threshold values were reached at 4%, corresponding to 0.40 mmol/l. No contractures developed after exposure to sevoflurane in muscle tissue from MHN and control patients.

Discussion

Identification of patients suspected to be susceptible to MH may be accomplished on the basis of clinical history that documents the signs of hypermetabolism during or after anaesthesia [13]. To date, the caffeine and halothane IVCT is generally accepted as the best standardized test we have available for the diagnosis of MH susceptibility, at least until a less invasive DNA based diagnosis of MH susceptibility is available [10,11].

We found an equilibration time for 2% sevoflurane in the open system testbath (25 ml) of 3 min. Equilibration time for halothane, which bubbles with 2% in a 25 ml testbath, was 1.5 to 3 min during calibration studies, comparable to other centres [14]. This halothane target value of 2% in the gas phase should correspond to 0.44 mmol/l ±10% in Krebs-Ringer solution, assuming a Krebs-Ringer/gas coefficient of 0.78 [14,15]. Experimentally, it was determined that a vaporisor concentration of 2% sevoflurane resulted in a liquid concentration of 0.20 mmol/l *(figure 3)*.

The sevoflurane threshold values were 0.20 mmol/l to 0.40 mmol/l in the MHS group and 0.40 mmol/l in the MHE individual. In the EMHG protocol, the halothane threshold for susceptibility is 0.44 mmol/l [9, 11].

In terms of alveolar concentrations, 2% halothane in carbogen is clinically equivalent to 3 MAC (Minimum Alveolar Concentration). The MAC of sevoflurane, in O_2, in a 25-year-old person is 2.5% thus, 3 MAC sevoflurane is around 7% in carbogen. According to this line of reasoning, the sevoflurane threshold for susceptibility should be 0.70 mmol/l, which makes our MHS and MHE patients susceptible and the MHN patients and the controls non-susceptible in the sevoflurane IVCT. We realize that the small sample size we employed makes this comparison more clinically relevant than statistically significant.

Differences in the physico-chemical properties of inhalational anaesthetics, with special regard to solubility and volatile potency may be an explanation of the different concentrations at which contractures developed in vitro. Variations in the genetic substrate responsible for MH may be another explanation. The different responses in vitro may partially explain the variation in clinical signs and symptoms of MH. Variations in MH responses, with special attention to uneventful previous exposure to potent inhalational agents are well-known and particularly hazardous because they are unpredictable [16, 17].

In summary, sevoflurane can trigger malignant hyperthermia and MH susceptible patients, therefore, should never be exposed to it.

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The European Group protocol for investigation of malignant hyperthermia susceptibility

1 The biopsy should be performed on the quadriceps muscle (either vastus medialis or vastus lateralis), using regional (avoiding local infiltration) or general anaesthetic techniques.

2 The muscle samples can be dissected in vivo or removed as a block for dissection in the laboratory within 15 min.

3 The excised muscle should be placed immediately in precarboxygenated Krebs–Ringer solution with a composition of:

NaCl	118.1	mmol/l	KCl	3.4	mmol/l
$MgSO_4$	0.8	mmol/l	KH_2PO_4	1.2	mmol/l
Glucose	11.1	mmol/l	$NaHCO_3$	25.0	mmol/l
$CaCl_2$	2.5	mmol/l	pH	7.4	

The ion concentration of the Krebs–Ringer solution should be as stated with a maximal deviation of ±10%, and should be checked at least every month. pH should be in the range 7.35–7.45 at 37°C.

4 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.

5 The time from biopsy to completion of the tests should not exceed 5 h.

6 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 a fresh specimen. These include two static caffeine tests and two halothane tests. The halothane test could consist of either one static and one dynamic test of two static tests. Each laboratory should be consistent in the method employed. Separate tissue baths should be used for different agents.

7 Muscle specimen dimensions. Muscle specimens suitable for in vitro investigation should measure 15–25 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 specimen are blotted and weighed after the test, between sutures.

8 Determination of specimen length and predrug force. The static tests 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 9 below) and stretch the muscle slowly to 150 ± 10% of initial length. This new length is considered to be optimal length (l_0) and is recorded. Gassing of the Krebs–Ringer solution is stopped temporarily during measurement of length. The muscle is left at optimal length (l_0) 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. Then drugs may be added. The baseline force immediately before addition of drug is recorded as the predrug force.

9 Electrical stimulation. To demonstrate viability, the muscle specimen should be electrically stimulated with a 1–2 ms supramaximal stimulus at a frequency of 0.2 Hz. Following suspension of the muscle in the tissue bath and obtainment of initial 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.

10 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. 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 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 caffeine should be reported. Please note that the lowest force is not necessarily the same as the predrug force.

11 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 0.66 mmol/l as equivalent to 0.5; 1.0; 2.0 and 3.0 % v/v, respectively. The specimen should be exposed to each halothane concentration for 3 min. The measurement of halothane should also be reported. For determination of halothane concentration see 14 below. The flowrate of gas should be set to maintain the correct halothane concentration in the tissue bath. The gasflow 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 and perfusion flowrate and aerodynamics of the system.

12 The dynamic halothane test and measurement of dynamic halothane threshold. This test is dependent on a motor. Initially, the muscle is stretched at a constant rate of 4 mm/min 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 halothane (0.5 %) for 3 min and the stretch process is repeated. The procedure is repeated for 0.22 and 0.44 mmol/l 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 maximal contracture

at 0.44 mmol/l is also recorded.

13 Diagnostic criteria
- MHS: A caffeine threshold (as defined earlier) at a caffeine concentration of 2.0 mmol/l or less, and a halothane threshold concentration at 0.44 mmol/l or less.
- MHN: A caffeine threshold at a caffeine concentration of 3.0 mmol/l or more and a halothane threshold concentration above 0.44 mmol/l.
- MHE: All other results are deemed equivocal but designated MHEh if reacting to halothane only or MHEc if reacting to caffeine only.

It is envisaged that most MHE patients will be regarded clinically as MH susceptible. MHE results must be considered to be under permanent review pending the acquisition of further control and mutation data. MHE results should be treated separately in research studies.

14 Quality control
Viability in any specimen used should be demonstrated by twitches ≥ 10 mN (1 g) at the beginning of a test, or for the caffeine test a response to 32 mmol/l ≥ 50 mN (5 g) at the end.
The concentrations of halothane and caffeine in the tissue bath should be checked at least every 3 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 0.44 mmol/l and caffeine 2 and 32 mmol/l 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 at yearly intervals. It is recommended that halothane concentrations in the gas phase are monitored close to the gas inlet port to the tissue bath.
Temperature of the tissue bath should be checked.

15 Control biopsies. All MH units are asked to investigate control muscle biopsies according to this protocol. For control biopsies, the following groups of patients are considered suitable: healthy volunteers, patients having amputations for localized diseased (not systemic of vascular disease), patients with varicose veins, brain-dead patients within the first 24 hours, patients with fractures within the first 24 hours.

16 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 and 4-chloro-m-cresol. These protocols may be obtained from the group.

Protocol revision. The EMHG protocol for investigation of MH susceptibility is regularly revised, latest in May 1997, Nijmegen, The Netherlands

Chapter 3

CULTURED HUMAN SKELETAL MUSCLE CELLS

Halothane-induced calcium release in cultured human skeletal muscle cells from a family susceptible to malignant hyperthermia with an unidentified mutation in chromosome 19

Anesthesiology 2002; 97:272-274

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Abstract

Studies on Ca^{2+} homeostasis in cultured human skeletal muscle cells, obtained from fragments of biopsies of patients undergoing the in vitro contracture test (IVCT) for malignant hyperthermia (MH) susceptibility, have been performed. The purpose of the present study was to investigate the usefulness of cultured skeletal muscle cells, obtained by percutaneous needle biopsies for determining MH susceptibility.

Methods: Muscle samples from 6 MH susceptible (MHS) patients and from 4 control individuals were used to culture myotubes. The free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of myotubes was determined after exposure to halothane. $[Ca^{2+}]_i$ on-line ratio measurement and calibration were performed using Fura-2. To guarantee quality control, all halothane concentrations were determined using a head space gas chromatographic technique.

Results: There was no statistical difference in the mean resting $[Ca^{2+}]_i$ between cultured muscle cells from MHS or control individuals. The dose-dependant Ca^{2+} response in cultured muscle cells of MHS individuals is significantly different from that of control individuals after exposure to halothane; in clinically used halothane concentrations.

Conclusion: Cultured human muscle cells, obtained from needle biopsies may well be applied in diagnostic tests for MH susceptibility.

Introduction

Malignant Hyperthermia (MH) is an autosomal dominant muscle disorder characterized by a hypermetabolic crisis triggered by succinylcholine and/or volatile halogenated anesthetic agents. The underlying cause of MH is believed to be abnormal regulation of myoplasmic calcium concentration in skeletal muscle [1,2]. A single mutation in the cDNA sequence encoding the muscle ryanodine receptor (RYR1) was the first to be considered as a candidate for causing MH in pigs [3]. In human MH the genetics is more complex; at least fifteen mutations in the RYR1 gene have been reported to be potentially causative [4,5]. Beside RYR1, secondary loci containing genes encoding proteins involved in excitation-contraction coupling, such as the dihydropyridine receptor

(DHPR) appeared to be causative [2].

A well defined diagnostic test for MH became available in 1971: the in vitro caffeine and halothane contracture test (IVCT) [6,7]. The IVCT is based on the hypersensitivity of muscle strips, obtained by biopsy, to caffeine or halothane. Standardization in Europe and North America led to two essentially similar protocols for the IVCT [8,9]. Cultured human skeletal muscle cells are often used to study muscle pathology in which calcium homeostasis might be disturbed. Excitation–contraction coupling of cultured muscle cells and their excitability at stimulation is determined by their basal intracellular calcium concentration ($[Ca^{2+}]_i$) [10]. We studied the effects of halothane on the $[Ca^{2+}]_i$ transients in human myotubes made up of cultured skeletal muscle cells from MH susceptible patients and healthy controls. Since such cells can be obtained by needle biopsy, our goal is to determine if this would be a less invasive alternative of determining MH susceptibility.

Materials and Methods

Patients

Muscle biopsies were obtained from four individuals without any known muscular disorder, and from six MH susceptible (MHS) patients. All patients gave written informed consent, formulated by the Committee on Medical Ethics of the University of Nijmegen. The MHS patients were members of one single family in which a man in 1972 had died from MH. The MHS phenotypes were recognized by IVCT two years before this study *(table 1)*. Genetic analysis in this family showed linkage to a candidate locus on chromosome 19 (lod score greater than +3.0).

Human skeletal muscle cell cultures

Samples of the quadriceps femoris muscle were obtained by percutaneous needle biopsies (25–30 mg). Fragments were attached on the bottom of a 35-mm culture dish containing 1 ml proliferation medium (Dulbecco's modified Eagle medium (DMEM), 20% fetal calf serum (FCS), 4.5 mg/ml glucose and 4 mM glutamine) and cultured in a humidified CO_2 incubator (5% CO_2) at 37°C. The next day, this medium was substituded by proliferation medium containing 4% Ultroser G and 10% rat brain extract instead of FCS. After 7 to 10 days the explants were removed, and the myoblasts were plated out on glass coverslips (10x30 mm) in 35-mm dishes. Further proliferation took place in 20% FCS containing medium until confluency was reached. Differentiation to polynucleated myotubes was achieved in DMEM containing 10% horse serum for 7 days.

For cryopreservation about 10^6 myoblasts / ml DMEM containing 20% FCS and 10% dimethyl sulfoxide were stored in liquid nitrogen.

table 1 *IVCT results of muscle biopsies from six MHS individuals and averages for control individuals (n = 4). MHS: a caffeine threshold concentration at 2.0 mM or less, and a halothane threshold concentration at 0.44 mM (2.0% v/v) or less; the threshold concentration is the lowest concentration which produces a sustained increase of at least 2 mN (0.2 g) in baseline force 8.*

	Halothane		**Caffeine**	
Patient(s)	**Threshold (mM)**	**Contracture (mN) at 0.44 mM**	**Threshold (mM)**	**Contracture (mN) at 2 mM**
MHS1	0.22	8	1	10.5
MHS2	0.11	15	2	4
MHS3	0.22	12	1	7.5
MHS4	0.22	13	1	11
MHS5	0.44	4	0.5	21
MHS6	0.11	20	0.5	14
Controls	–	0.2	32	0.1

$[Ca^{2+}]_i$ on-line ratio measurement and calibration

The free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined in skeletal muscle cells using Fura-2 [11]. Myotubes were loaded with 5 µM Fura-2/acetoxymethyl ester (Fura-2/AM) and 10 µM Pluronic acid for 90 min at 37°C in physiological salt solution (PSS: containing 10 mM HEPES, 125 mM NaCl, 10 mM $NaHCO_3$, 1 mM NaH_2PO_4, 5 mM KCl, 2 mM $MgSO_4$, 1.8 mM CaCl2, and 10 mM glucose, pH 7.4). On-line ratio measurement were recorded with a Shimadzu RF-5301 spectrofluorophotometer. Fura-2 fluorescence was measured at an emission wavelength of 492 nm (bandwidth, 5 nm) and alternating excitation wavelengths of 340 and 380 nm (bandwidth, 3 nm). During the measurements the cells in the cuvette were superfused with PSS (4.0 ml/min; 37°C) without or with different concentrations of halothane (0.11, 0.22, 0.44, 1.0, 2.0, 4.0, 8.0, 12.0 mM). Halothane was solved in dimethyl sulfoxide (DMSO) and added to PSS in airtight dark bottles. All fluorescence signals were corrected for autofluorescence.

The 340/380 ratios (Fura-2) were calibrated using PSS containing 4 µM ionomycin and 10 mM Ca^{2+} (pH 7.7; R_{max}) or 4 µM ionomycin and 20 mM EGTA without external Ca^{2+} (pH 8.5; R_{min}). $[Ca^{2+}]_i$ was calculated using the equation:

$[Ca^{2+}]_i = K_d \times ß \times \{(R-R_{min}) / (R_{max}-R)\}$ K_d of Fura-2 is 224 nM [12]

Assay of halothane concentration

Halothane concentrations in PSS were measured using a head space gas chromatographic technique (Chrompack, CP-9001, equipped with a flame ionisation detector) [13].

Materials

Dulbecco's modified Eagle medium (DMEM) and Ultroser G were from Gibco BRL Life Technologies, Paisly, UK; fetal calf serum (FCS) and horse serum from Flow Laboratories, Irvine, UK. Brain extract was prepared from brains of 10 day-old Wistar albino rats as a 10% (w/v) homogenate. Fura-2/acetoxymethyl ester (Fura-2/AM), ionomycine and pluronic acid were purchased from Molecular Probes, Eugene, OR, USA. Halothane was from Tempus b.v., Oegstgeest, The Netherlands.

Statistical analysis

Data are represents as mean (SD). Statistical analysis is performed using the unpaired Students t-test. Significance was set at $p < 0.05$. Curve fittings were obtained by linear regression analysis. Individual area under the curves (AUC) were determined from the curves by interpolation.

Results

Halothane concentrations in PSS were measured in the cuvette on completion of the calcium measurements. We found a substantial difference between the intended concentrations and the measured concentrations (intended→measured concentrations respectively: 0.11→0.095 (0.012) mM, 0.22→0.187 (0.029) mM, 0.44→0.33 (0.039) mM, 1.0→0.69 (0.16) mM, 2.0→1.4 (0.20) mM, 4.0→2.75 (0.42) mM, 8.0→5.2 (0.99) mM, 12.0→7.5 (1.3) mM).

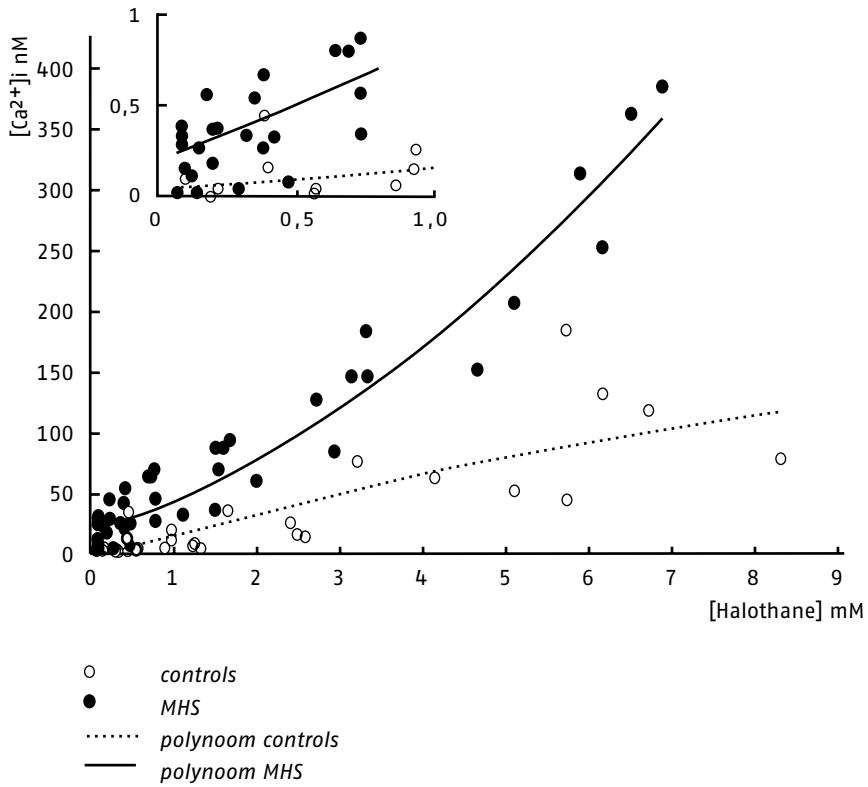
The 340/380 ratios of the Fura-2 calibration resulted in a R_{max} of 11.88 (1.03) and R_{min} of 1.59 (0.15); mean (SD) of eight experiments. The constant ß, i.e. the ratio of the fluorescence emission of the free dye and the Ca^{2+}-saturated dye measured at 380 nm, is 3.09 (0.33) n = 7. We did not find a statistical difference between the mean resting $[Ca^{2+}]_i$ in cultured muscle cells of MHS or control individuals, which were respectively 65 (20) nM; (n = 72) and 58 (17) nM; (n = 50).

Halothane produced a dose-dependent increase of $[Ca^{2+}]_i$ *(figure 1)*. In the MHS group the Ca^{2+} response is observed in the clinically used halothane concentrations (<1 mM). The Ca^{2+} response in cultured muscle cells of MHS individuals is significantly different from that of control individuals and there is no overlap beyond 0.44 mM halothane (equivalent to 2% v/v).

Discussion

Cultured human skeletal muscle cells have been very useful for studying ion homeostasis in relation to MH [14-17]. Interpretation of the results is complicated by the existence of incomplete maturation and differences related to the heterogeneous population of cells at the myotube stage. By analyzing the fluorescence signal composed of the whole monolayer of myoblasts / myotubes on glass coverslips we were able to compare the common behavior of thousands of cells instead of a small selection of cells which was done in previous studies with comparable aims [14,15,17]. This could explain why we could discriminate MHS from control muscle tissue using halothane in clinically used

figure 1 *Halothane-induced, dose-dependent increase of $[Ca^{2+}]_i$ in cultured skeletal muscle cells from 6 MHS individuals (n = 42: solid dots and line) and 4 control individuals (n = 32: open dots and dotted line). Discrimination between MHS and control is 100% beyond 0.5 mM halothane ($p < 0.05$). Inset: $[Ca^{2+}]_i$ response at clinically used halothane concentrations*



concentrations, whereas the other investigators needed much higher halothane concentrations [15, 17]. Further standardization of cell culturing could improve reproducibility of test results even more. In the IVCT a vaporizer is used to add halothane to carboxygen to solve halothane in Krebs-Ringer solution [8]. To reach supraclinical halothane concentrations, halothane was solved in DMSO before it was added to the physical salt solution. This procedure requires assay of halothane concentration in the test bath at completion of each experiment because the variations are common.

In this study we confirm that there is no difference in the resting intracellular calcium level between cultured muscle cells from MHS and control individuals [2, 14, 15].

A test based on cultured muscle cells obtained by a needle biopsy is less invasive and is easily reproducible. The time between biopsy and performance of the test is not critical because cell cultures can be expanded, frozen and thawed. In contrast IVCT requires large muscle

samples (surgically collected) and must be performed within 5 hours [8].

In conclusion: cultured human muscle cells obtained from needle biopsies may well be applied in diagnostic tests for MH susceptibility. More studies comparing data from the IVCT and cultured cells have to be undertaken to determine sensitivity (and specificity).

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Chapter 4

PROCEDURE FOR THE DIAGNOSIS OF MH SUSCEPTIBILITY IN EUROPE

Malignant hyperthermia susceptibility: diagnostic procedure in four European countries

European Journal of Anaesthesiology 2004; in press

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Abstract

The European Malignant Hyperthermia Group (EMHG) was formed in 1983 and now comprises 22 MH centres in 13 countries. However although laboratory protocols have been critically evaluated, little has been done to evaluate inter-centre standards for the clinical aspects of MH. The purpose of this study was to see how MH centres deal with the referral of patients and how this is reflected in workload and funding.

Methods: 4 MH centres (Belgium, The Netherlands, Switzerland and UK) were asked to complete a questionnaire about their last 25 consecutive consultations and their overall workload for the past 5 years.

Results: All 100 questionnaires were sent back. The main reason (81%) for consultation was a request for diagnosing MH susceptibility; resulting in screening by the in vitro contracture test in 60 cases. The variability in the number of tests performed during 1997-2001, put in perspective of the catchment area of the centre, is high between centres but constant within centres.

Conclusion: The number of tests performed appeared to be related to the way a centre is funded. This study helped to gain an insight into the need for further standardisation and quality assurance for the diagnosis of MH.

Introduction

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic disease occurring during anaesthesia in which potent inhalational anaesthetics and/or succinylcholine are used. It is inherited as an autosomal dominant characteristic.

We studied the diagnostic procedures in 4 MH investigation units. The purpose of this study was to see how MH centres deal with the referral of patients and how this is reflected in workload and funding. A well defined diagnostic in vitro test became available in 1970 [1, 2]. The in vitro contracture test (IVCT) is based on the hypersensitivity of viable muscle samples from "MH susceptible individuals", obtained by biopsy, to caffeine and halothane. At present, the IVCT, performed according to the European MH Group (EMHG) protocol, is generally accepted as the "gold standard" test for the diagnosis of MH susceptibility [3]. Molecular genetic studies brought a DNA-based diagnosis of MH susceptibility within reach. However, because MH displays a high level of heterogeneity, MH susceptibility should only be diagnosed on the basis of genetic studies according to guidelines published by the EMHG [4].

The EMHG was formed in 1983 and now comprises 22 MH centres in 13 countries. One purpose of the EMHG is to ensure comparable standards both in the diagnostic testing for MH and in how patients and their families are dealt with. Laboratory technology has been subjected to critical evaluation [5], but little has been done to evaluate

inter-centre standards for the clinical aspects of MH dealing with the clinical referrals and the criteria for refusing or accepting a proband for testing.

Methods

Representatives of the MH investigation units in Belgium, The Netherlands, Switzerland and the United Kingdom were asked to complete a two-part questionnaire. The first part contained general questions, including questions about funding and the total number of tests performed and their outcome during the last 5 years. In the second part the centres were asked to describe their last 25 consultations and to describe the basis of their decision to select patients requiring further investigation. This included questions about the type of clinical referral, patient information, outcome of the consultation and the diagnostic results when these were performed.

Results

The MH investigation units in Belgium, The Netherlands, Switzerland and the UK have been members of the EMHG since 1985, 1983, 1987 and 1983 respectively. The catchment areas of the four national MH investigation units in Antwerp (B), Nijmegen (NL), Basel (CH) and Leeds (UK) encompass 11, 16, 7, and 58 million inhabitants respectively *(table 1)*. National health care system in the UK covers all costs in Leeds, based on 230 biopsies per year. Basel has been reimbursed for all costs since July 2000 (before that Swiss health care system covered approximately 50% of IVCT costs). IVCT costs in Belgium and The Netherlands have to be paid from (shrinking) institutional budgets. The number of tests performed in latter 2 countries, is just over 1 test per 1.000.000 inhabitants per year while this amount is at least 5 times higher in both countries where all IVCT costs are covered by the healthcare system. Based on this finding it appears that adequate funding influences the decision whether IVCT will be performed to a major extent.

The majority of consultations were by telephone (85%), either by the patients themselves or more usually by anaesthetists *(table 1)*. In 81 of 100 cases the reason for the consultation was a request for diagnosing MH susceptibility. 29 cases concerned patients that have had an adverse reaction to anaesthesia. The remaining 52 cases concerned relatives of a known MH susceptible family. In total, 65 individuals were offered further investigation, 60 by IVCT and 5 by a DNA test according to the guidelines 4. Because of incomplete clinical information, 9 referrals were put on a waiting list, in expectation of further details. 7 cases were deemed not to require further investigation on the basis of clinical information alone i.e. there was a clear reason for the clinical reaction other than MH.

table 1 *Data collected by a questionnaire; reply from 4 European MH investigation units. NOT: Number of in vitro contracture tests (IVCT) performed during 1997–2001; area of catchment (AOC) in million inhabitants; the national healthcare system (NHS) is funding the IVCT separately. Data collected from 100 consultations.*

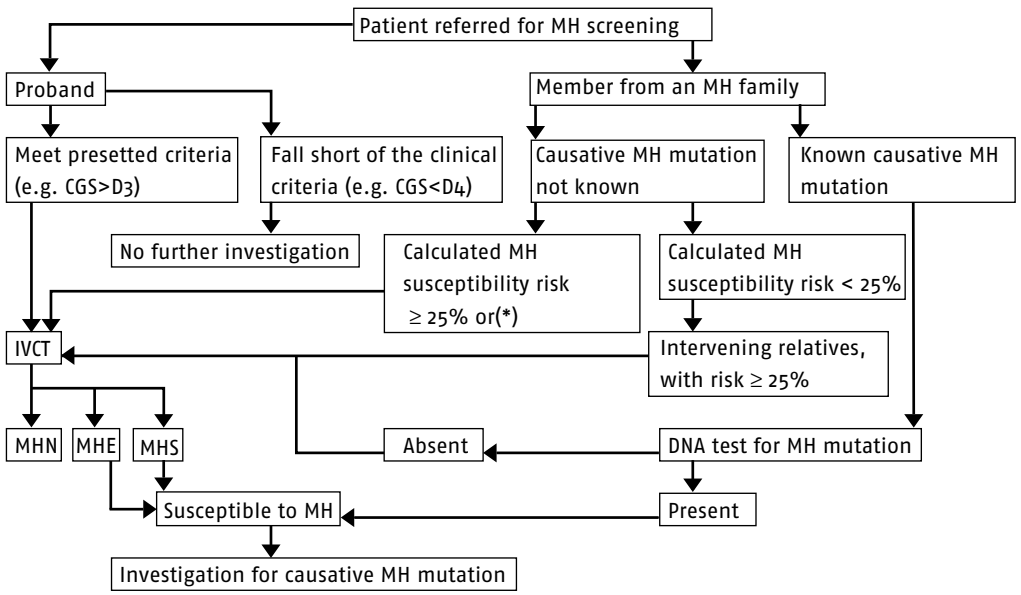
	UK	B	CH	NL	Total
NOT					
1997	257	21	30	14	322
1998	241	14	36	15	306
1999	256	15	43	10	324
2000	198	15	37	14	264
2001	202	16	73	18	309
AOC	58	11	7	16	92
NHS funding	+	–	+	–	
CONSULTATIONS					
Referred by					
Anaesthetist	9	4	6	9	28
GP	4	5	6	4	19
Other clinician	2	5	1	4	12
Self referral	4	11	12	7	34
Centre advise	6	–	–	1	7
Reason for consultation					
General info about MH	8	2	–	4	14
Anaesthesia advise	3	–	–	2	5
Request for diagnosing	14	23	25	19	81

The 4 MH investigation units select probands for screening in a similar but individual way based on experience *(figure 1)*. All 4 centres except the one from the UK use the Clinical Grading Scale (GSC) as a basis for selection. Although the GCS is objective it can be hampered by incomplete clinical information, which is a common situation [6]. For this reason the Leeds centre uses clinical categories developed from a review of 402 probands that gives an incidence rate or likelihood of MH for each category of clinical presentation but it is a subjective method [7].

Discussion

MH centres have been offering advice about MH and providing a diagnostic screening service for patients and their families. This has led to thousands of patients being assigned to be MH-susceptible (MHS/E), or MH-normal (MHN) with regard to future anaesthesia. It might be argued that the major role of an MH investigation unit was to exclude the clinical diagnosis of MH rather than confirm it, thus preventing patients and their whole families from becoming falsely labelled as MH susceptible.

Figure 1 *Suggested route for counselling.*
IVCT = in vitro contracture test [3]; MHS = malignant hyperthermia susceptible; MHN = malignant hyperthermia normal; MHE = malignant hyperthermia equivocal. (*) = when calculated MH susceptibility risk <25% but intervening relatives either deceased or refused investigation. CGS = clinical grading scale to predict MH susceptibility 6.



In recent years the "incidence" of clinical MH has tended to decrease because of increased use of regional anaesthesia and total intravenous anaesthesia, both "MH safe" techniques together with a decrease in the use of succinylcholine. As the combination of succinylcholine and inhalational anaesthetic agents produces a more profound MH reaction, the signs of MH are now often less pronounced and can occur insidiously. Mild forms of MH could therefore be missed, although conversely the increased index of suspicion of MH by anaesthetists has lead to an increase in referrals of mild, non-specific reactions. However fulminant classical MH reactions do still occur. A decrease in referrals maybe be expected, but for the time being the referral rate varies considerably from year to year, so it is not yet clear if this is a true trend (unpublished information from MH experts during the Xth International Workshop on MH, June 2003, Brunnen, Switzerland).

The present study provided an insight into the need for further standardisation and quality assurance for the diagnosis of MH. The number of referrals was to low to demonstrate inter-centre difference between IVCT indication criteria and IVCT results. Meanwhile a European quality assurance project financed by the EMHG has been started, based on an independent on-site visiting programme to all 22 European MH investigation units. Quality assurance and greater

standardisation within the EMHG will improve confidence in the diagnostic screening for MH susceptibility. It will encourage communication and collaboration within the MH research infrastructure. Finally it will improve the safety of MH susceptible patients and their families, who need to undergo anaesthesia.

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Guidelines for molecular genetic detection of susceptibility to malignant hyperthermia

British Journal of Anaesthesia 2001; 86: 283–287

Urwyler A, Deufel T, McCarthy T, West S, Anetseder M, Brancadoro V, Cozzolino S, Ellis F, Fagerlund T, Gilly H, Heytens L, Heffron J, Glahn K, Islander G, Krivosic-Horber R, Lehmann-Horn F, Lingnau W, Mortier W, Nivoche Y, Olthoff D, Sigurdsson S, Snoeck M, Tegazzin V, Tzanova I, Wappler F.

Abstract

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic disease triggered by several anaesthetic agents. The in vitro muscle contracture test (IVCT) is the standard test to establish an individual's risk of susceptibility to MH. Clinical practitioners and geneticists of the European MH Group have agreed the present guidelines for the detection of MH susceptibility using molecular genetic techniques and/or IVCT to predict the risk of MH.

Introduction

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic disease triggered by commonly used potent inhalation anaesthetics and/or succinylcholine. The in vitro muscle contracture test (IVCT) is the standard test to establish an individual's risk of susceptibility to MH [1]. The European MH Group has developed a standardized protocol for the IVCT and has initiated and fostered international collaborative molecular genetic studies to investigate the molecular basis of MH. Data from these studies demonstrate that MH displays a high level of locus heterogeneity. Thus, it is not feasible to diagnose MH susceptibility, and, more specifically, to exclude MH risk, on the basis of a simple genetic test alone. However, it is of utmost importance to avoid false MH-negative (MHN) diagnoses because of the potential risk of MH during general anaesthesia for these patients and their offspring. These general obstacles notwithstanding, there may be specific situations where genetic data provide additional diagnostic information or contribute information independent of IVCT. It is the purpose of this document to outline recommended procedures for the potential diagnostic use of such genetic findings depending on the different clinical situations that may arise.

Referrals

The usual route of entry for individuals into MH investigations follows a suspected MH crisis and referral of the patient to an MH Investigation Unit, where diagnostic procedures and genetic counselling should be performed according to *figure 1*.

In vitro muscle contracture test (IVCT)

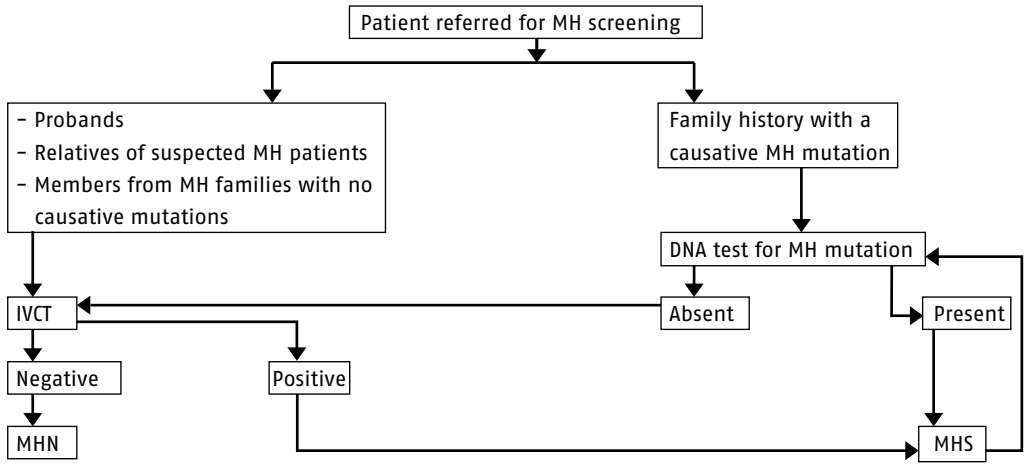
An IVCT is performed on the patient or, if the patient is too young or has not survived the anaesthetic event, his or her parents. If MH susceptibility status is confirmed by IVCT, then there is a clinical responsibility to offer the IVCT to the relatives of the index case, assuming autosomal dominant inheritance and starting with first-degree relatives.

Genetic investigations

Mutation analysis

At this stage molecular genetic testing for causative mutations in the

figure 1 *Suggested route for MH susceptibility testing. IVCT = in vitro muscle contracture test; MHN = malignant hyperthermia negative; MHS = malignant hyperthermia susceptible.*



ryanodine receptor gene (RYR1) of the index case could lead to quicker results for the rest of the kinship. An up-to-date list of mutations that have been shown to directly alter RYR1 caffeine or halothane sensitivity is shown in *table 1 and table 2*.

Genetic analysis should be performed in, or only after consultation with an MH Investigation Unit. Once a causative mutation has been detected in the proband or index patient, it can be used to test relatives who have not yet been tested by IVCT. Mutation carriers should consequently be regarded as susceptible to MH. However, family members who do not carry the mutation observed in the pedigree should still undergo IVCT investigation. The reason for such caution is the observation in several pedigrees investigated by members of the European MH Group of discordance between genetic and IVCT results, implicating a second MH susceptibility gene segregating in the kinship [2,3].

Segregation analysis

Once the MH status of the extended pedigree (e.g. 10 informative meioses) has been determined by the IVCT, it may be possible to undertake genetic segregation analysis with markers close to the known MH susceptibility loci. An up-to-date list of recommended markers and details of genetic modelling compiled by the European Malignant Hyperthermia Group, Genetics Section, is available on the internet (http://www.emhg.org).

Rarely a single pedigree may be sufficiently large to establish linkage to a candidate locus with a high probability (lod score > 3.0). In such a situation the question arises as to whether or not haplotype analysis can be used to assign MH status. Under these circumstances

table 1 *List of RYR1 mutations potentially causative for MH susceptibility (MHS) and central core disease (CCD). Residue numbering within the RYR1 nucleotide and amino acid sequence corresponds to the human RYR1 sequence according to Zorzato and colleagues[18] (accession number J05200.1), updated according to Zhang and colleagues [16] and Phillips and colleagues[19].*

Exon	Mutation position codon change	RYR1 amino acid change	Reference
2	103 T>G	Cys35→Arg	4
6	487 C>T	Arg163→Cys	5
9	742 G>A	Gly248→Arg	6
11	1021 G>A	Gly341→Arg	7
12	1209 C>G	Ile403→Met	5
14	1565 A>C	Tyr522→Ser	8
15	1654 C>T	Arg552→Trp	9
17	1840 C>T	Arg614→Cys	10
17	1841 G>T	Arg614→Leu	11
39	6487 C>T	Arg2163→Cys	12
39	6488 G>C	Arg2163→His	12
45	7300 G>A	Gly2434→Arg	13-15
45	7304 G>A	Arg2435→His	16
46	7372 C>T	Arg2458→Cys	17
46	7373 G>A	Arg2458→His	17

individuals carrying the high-risk haplotype should be regarded as susceptible to MH even without confirmation by a positive IVCT. The converse is not true, that is, identification of the low risk haplotype does not equate with MHN status and such individuals should have IVCT determination of their MH status.

In families where linkage to a candidate gene, RYR1 or another locus, is suggested but not firmly established (i.e. lod score < 3.0) haplotype analysis for predictive testing is not appropriate due to the high level of locus heterogeneity in MH. In such families, however, it is desirable to search for unknown mutations in the suggested candidate gene for research purposes.

Failure to reach a lod score of + 3.0 in a single family due to the occurrence of a single individual in whom there is recombination between the haplotype and IVCT-determined MH status will require closer scrutiny and possible reassessment of the genetic and bioassay results to attempt to resolve the basis of the discordance. For predictive diagnosis in such families the more conservative estimation, i.e. the higher risk outcome (either the MH susceptibility test result from the IVCT or the high-risk haplotype) should be the basis for the clinical decision.

table 2 *Functional characterization, phenotype and estimated incidence of the RYR1 mutations in Table 1. RYR1 channel activity have been performed by calcium photometry on myotubes and/or COS-7 or HEK cells transfected with RYR1 genes bearing the mutation[20-24].*

Mutation position codon change	Functional comparison with wild-type RYR1 (sensitivity)		Phenotype	Estimated incidence
	caffeine	halothane		
103 T>G	normal	increased	MHS	1 family
487 C>T	increased	increased	MHS/CCD	2%
742 G>A	increased	increased	MHS	1 family
1021 G>A	increased	increased	MHS	6-10%
1209 C>G	increased	increased	CCD/MHS?	1 family
1565 A>C	increased	increased	MHS/CCD	1 family
1654 C>T	increased	increased	MHS	1 family
1840 C>T	increased	increased	MHS	4-9%
1841 G>T	increased	increased	MHS	2%
6487 C>T	increased	increased	MHS	4%
6488 G>A	increased	increased	MHS/CCD	1 family
7300 G>A	increased	increased	MHS	4-10%
7304 G>A	increased	increased	MHS/CCD	1 family
7372 C>T	increased	increased	MHS	4%
7373 G>A	increased	increased	MHS	4%

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Appendix

Clinical practitioners and geneticists of the European MH Group (EMHG) have agreed on the guidelines for detection of MH susceptibility using molecular genetic techniques and/or IVCT to predict the risk of MH. The original (2001) guidelines contained 15 RYR1 mutations for genetic testing. Once one of these causative mutations has been detected in the proband, who's MH susceptibility status is confirmed by IVCT, it can be used to test relatives.

On its 22nd Annual Meeting in Brunnen, Switzerland, the EMHG has discussed criteria which any genetic mutation should fulfil prior to its use in predictive genetic testing. The following criteria concerning genetic and functional characterization have been adopted.

Genetic characterization:

- 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 mutation with the disease in at least 2 pedigrees.
- Absence of the sequence change from 100 control samples for exclusion of polymorphisms.

Functional characterization:

- Recombinant in vitro expression on a defined genetic background.
  1. The standard system 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.
  2. Alternatively, myotubes of the dyspedic mouse (RYR1-knock out) have been used as recipients for the expression of cDNA constructs. 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 mutations:
  1. in myotubes
  2. in microsomal SR preparations from muscle biopsies
  3. in lymphoblasts

  Read-out parameters were Ca^{2+} flux and resting [Ca^{2+}] or ^{3}H-ryanodine binding to SR-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 SR preparations, muscle biopsies of several patients were pooled thus eliminating individual

table 1 *As of December 2003, 7 novel RYR1 mutations have been recommended for use of predictive genetic testing within pedigrees, as these 7 causative mutations met the EMHG criteria.*

Exon	Nucleotide	Protein	Phenotype	Assay system(s)
39	6502 G>A	V2168M	MH	Ca^{2+} release in mutant lymphoblastoid cells and myotubes from several mutation carriers
40	6617 C>T	T2206M	MH	Ca^{2+} release in mutant myotubes cultured from several mutation carriers
44	7048 G>A	A2350T	MH	Mutant rabbit RYR1 expressed in HEK-293cells.
100	14387 A>G	Y4796C	MH? / CCD & nemaline rods	Mutant rabbit RYR1 expressed in HEK-293cells.
101	14512 C>G	L4838V	MH	Mutant rabbit RYR1 expressed in CHO cells.
101	14582 G>A	R4861H	CCD / MH?	Measured changes in intracellular [Ca^{2+}] of mutant lymphoblastoid cells
102	14693 T>C	I4898T	MH / CCD	Mutant rabbit RYR1 expressed in HEK-293 cells and in dyspedic myotubes

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.

Please note: candidate mutations are eligible for the list of "Mutations for genetic testing" only if results of their genetic and functional characterisation have been published in the scientific literature. An up-to-date list of mutations, recommended markers and details of genetic modelling is available on the internet: http://www.emhg.org

Chapter 5

SCREENING FOR MH SUSCEPTIBILITY

Investigation of a family following fulminant malignant hyperthermia

Journal of Clinical Neuromuscular Disease 2004; 5: 122–128

Snoeck M, Sengers R, Iles D, ter Laak H, Robinson R, Padberg G.

Abstract

Malignant hyperthermia (MH) is a pharmacogenetic neuromuscular disorder triggered by inhalational anaesthetics or succinylcholine. We studied in detail 24 relatives of a patient who died after suffering MH. All steps of the screening procedure, follow developments in testing for the diagnosis of MH susceptibility from 1984 until 2002.

Patients and methods: The screening procedure contained a general assessment and a clinical examination; CK measurement; genomic DNA isolation for linkage analysis and mutation screening; muscle samples were tested according to the in vitro contracture test protocol (IVCT) and examined histologically; cultured skeletal muscle cells were used to examine the effect of halothane on the intracellular calcium concentration.

Results: No correlation was found between IVCT results, serum CK or abnormal findings following histological examination, though CK elevation and the observation of cores seemed indicative for MH susceptibility in this family. Linkage analysis implicated RYR1 on chromosome 19q13.1 as the disease susceptibility locus in the family. The calcium response was found to be significantly different.

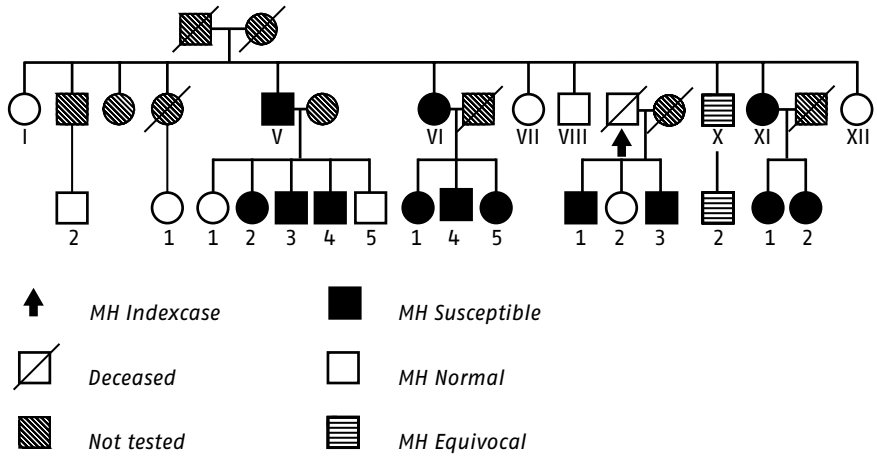
Conclusion: Following three decades of screening for MH, the gold standard for diagnosis remains the IVCT for detection of susceptibility to MH.

Introduction

A 34 years old man died in intensive care on August 21th 1973 after suffering a fulminant malignant hyperthermia crisis during general anaesthesia in the ear, nose and throat department for correction of a deviated nasal septum the previous day. The patient developed hyperkalaemia that led to recurrent ventricular fibrillation and ischemia. As a result, the patient suffered multiple organ failure that later proved fatal.

During case reconstruction, ten years later, the attendant anaesthetist reported reduced mouth opening and increased jaw tone which made intubation of the trachea difficult. The patient was anaesthetised with thiopental, meperidine, succinylcholine, nitrous

figure 1 *Pedigree.*



oxide, oxygen and halothane. Other early clinical signs were tachycardia, cyanosis and low blood pressure. In spite of supplementary clinical information, we concluded that we had been presented with a fulminant MH crisis. As a result, we felt it necessary to test the close relations of the deceased proband for susceptibility to MH.

Denborough and co-workers were the first to recognise the hereditary nature of MH susceptibility[1]. By their observations on an Australian family in which several members had died during anaesthesia, they proposed that the pattern of predisposition to MH was compatible with an autosomal dominant mode of inheritance.

At present the in vitro contracture test (IVCT) is generally accepted worldwide as the most reliable test for diagnosis. Based on two tests involving the exposure of living muscle samples to caffeine, developed by Kalow in 1970, and halothane, developed by Ellis in 1971, the IVCT is used to assess susceptibility to MH[2,3]. Standardisation of the IVCT in Europe and North America has led to two essentially similar protocols for the caffeine and halothane IVCT[4,5].

In this paper we describe a pedigree of a patient with MH. All steps of the MH-screening procedure from 1987 until 2002 follow the developments in testing for the diagnosis of MH susceptibility.

Patients and Methods

Patients

24 first and second degree relatives of the proband were screened for MH susceptibility. Family screening was initially offered to first-degree relatives with 50% chance of susceptibility, assuming autosomal dominant inheritance. In this family this included 10 siblings, 2 sons and the daughter of the proband; the parents and sister of the proband are deceased and were not tested (pedigree in *figure 1)*.

The screening procedure consisted of a general assessment, history and clinical examination of each patient by a specialist in neuromuscular diseases. Blood samples were taken for CK measurement and genetic investigations. A muscle biopsy was performed on the quadriceps muscle (either vastus medialis or vastus lateralis), using a regional anaesthetic technique (a femoral 3-in-1 nerve block). Muscle tissue was removed as a block for dissection in the laboratory; parts of the biopsy were reserved for histological examination.
The appropriate informed consent was obtained from all patients.

In vitro contracture test
The IVCT was performed according to the guidelines of the protocol of the European MH Group [4]. Briefly, fresh muscle bundles were placed in tissue baths, filled with carboxygenated Krebs-Ringer solution and stretched for optimal preload. After equilibration for a stable resting tension, caffeine or halothane were added cumulatively to the tissue bath. Two caffeine tests and two halothane tests were performed in separate baths. For each test, fresh muscle bundles were used and during testing the muscle bundles were stimulated to indicate the viability.

The results were regarded as positive if a sustained increase in resting tension (contracture) of 2 mN or greater was developed with 2 mM caffeine or less and with 0.44 mM halothane or less. In this case, the patient was classified as MH-susceptible (MHS). When both tests were negative, the patient was classified as MH-normal (MHN). All other results were designated MHE: equivocal. Both MHS and MHE patients are regarded as clinically susceptible to MH.

Histological examination
Histological examination is a part of the muscle biopsy screening procedure to exclude the existence of an underlying muscle disease that could account for any problems encountered during anaesthesia. The usual colourations and histoenzymatic reactions carried out routinely for diagnosis were performed on selected, oriented and rapidly frozen muscle specimens.

In order to compare muscle biopsy abnormalities with MH-status, each abnormal finding was scored *(table 1)*. The MA-score (morphological abnormality score) of the biopsy is the number obtained by summation of scores belonging to the defined abnormal findings in the biopsy.

Statistical analysis is performed using the unpaired Student t test; significance was set at $P < 0.05$.

Genetic investigation
MH susceptibility in the majority of white families is linked to the gene encoding the skeletal muscle ryanodine receptor (RYR1), the calcium release channel of the sarcoplasmic reticulum, on chromosome

table 1 *List of abnormal findings that may be found in the studied muscle biopsies, contributing to the Morphological Abnormality score (table 3). In cases with 3 or more abnormal fibres, the score is increased to 2.*

Morphological abnormality	incidence	score
Internal nuclei (IN)	4–5%	1
	6–10%	2
	11–20%	3
	21–40%	4
	41–80%	5
Type I fibres	<20% or >65%	1
Type IIC fibres	>5%	1
Ring fibres	1 or 2	1
Basophilic fibres	1 or 2	1
Necrotic fibres or myophagia	1 or 2	1
Tubular aggregates in fibres	1 or 2	1
Cores	1 or 2	1
Fibre splitting	1 or 2	1
AcP signalled changes	1 or 2	1
Granular fibres	1 or 2	1
Ragged red fibres	1 or 2	1
Fibres containing large fat vacuoles	1 or 2	1
Lobulated fibres	1 or 2	1
Cellular infiltration	small	1
Angular fibres	1 or 2	1
Atrophic fibres with only nuclei	1 or 2	1
Atrophic fibres	1–10	1
	>10	2
	marked	3
Hypertrophic fibres	1–10	1
	>10	2

19q13.2 [6]. Genomic DNA was isolated from EDTA whole blood according to the method described by Miller [7]. For linkage analysis, microsatellite markers D19S75, D19S191, D19S414, D19S220 and D19S412 were used to generate haplotypes. Using the LINKAGE based package, MSIM, the average and maximum two-point lod scores were computed.

Screening for all known RYR1 mutations in the hot-spot regions (106 to 2024 bp, 6188 to 7462 bp and 14313 to 15204 bp), was undertaken by direct sequencing of polymerase chain reaction (PCR) products as outlined elsewhere [8].

Skeletal muscle cell cultures

Muscle samples from 6 known MHS family members were compared to 4 unrelated control patients without any known neuromuscular disease. Samples of the quadriceps femoris muscle were obtained by

table 2 *IVCT data*

Pat. Number	Tension in mN		Threshold concentration		
	Caffeine at 2mM	Halothane at 0.44mM	Caffeine (mM)	Halothane (mM)	IVCT Status
I	0	0.5	32	–	MHN
II:2	0.5	0	4	–	MHN
IV:1	0	0.5	32	0.66	MHN
V	9	7.5	1	0.22	MHS
V:1	0	1	32	0.66	MHN
V:2	3	10	2	0.11	MHS
V:3	3	30	2	0.11	MHS
V:4	7.5	11	1	0.22	MHS
V:5	0	0	32	–	MHN
VI	10	8	1	0.44	MHS
VI:1	4	12	1.5	0.11	MHS
VI:4	3.5	32	2	0.11	MHS
VI:5	3	6	0.5	0.22	MHS
VII	1.5	1.5	3	–	MHN
VIII	0	0	32	–	MHN
IX:1	12	14	1	0.22	MHS
IX:2	0	0	32	–	MHN
IX:3	15	4	0.5	0.44	MHS
X	–	–	–	–	MHE
X:2	0.5	5	4	0.22	MHE
XI	22	15	1	0.22	MHS
XI:1	10	12	1	0.22	MHS
XI:2	28	14	1	0.11	MHS
XII	0.5	0	4	–	MHN

percutaneous needle biopsies (25–30 mg). Fragments were attached on the bottom of a culture dish containing proliferation medium and cultured at 37°C [9]. After 7 to 10 days the myoblasts were plated out on glass coverslips, proliferated further and than allowed to differentiate into polynucleated myotubes for 7 days. The free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of myotubes was determined after exposure to increasing concentrations of halothane (0.11, 0.22, 0.44, 1.0, 2.0, 4.0, 8.0, 12.0 mM); for each concentration fresh myotubes were used. $[Ca^{2+}]_i$ on-line ratio measurement and calibration were performed using a spectrofluorophotometer; the myotubes were loaded with Fura-2. To verify the halothane concentrations, samples were determined using a head space gas chromatographic technique [9].

Results

Clinical examination found no evidence of neuromuscular disorders and no incidents had been recorded during previous anaesthesia in

this population of patients.

After exposure of muscle bundles to caffeine and halothane in the IVCT, patients were classified according to the diagnostic criteria[4]. A clear negative result (MHN) was found in muscle specimens of 9 individuals. 13 Relatives were designated MHS and 2 were designated MHE because muscle specimens reacted abnormally to halothane only *(table 2)*.

CK measurement showed significant differences between MHS (n = 13) and MHN (n = 6) individuals, respectively 198 ± 189 and 68 ± 15 (mean ± SD); p = 0.03 *(table 3)*.

Histological examination showed abnormalities in all muscle specimens except in those from 2 MHN individuals *(table 3)*. The mean morphological abnormality (MA) score of the 13 MHS individuals (mean ± SD: 4.8 ± 2.3) is significantly different from that of the 9 MHN individuals (1.7 ± 1.4); p = 0.001. Under light microscopy we observed cores in muscle fibres of 6 individuals (5 MHS and 1 MHE); the cores were isolated lesions in the individual fibres. In the MHS group, no correlation could be demonstrated between IVCT results, CK, MA-score and/or the detection of cores.

In this family linkage was detected to the RYR1 candidate region on chromosome 19. The expected maximum lod score at a recombination fraction of q = 0.01 for this pedigree was +3.98 (average lod score: 2.37, SD ± 1.13) *(figure 2)*[10].

However, no mutation was detected which segregated with MH susceptibility in the family following analysis of the three hotspot-regions in the RYR1 gene containing the causative MH and CCD mutations [11].

Halothane produced a dose-dependent increase of the free cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$ of all cultured myotubes *(figure 3)*. The Ca^{2+} response in MHS individuals proved significantly different from that of control individuals with no overlap beyond 0.5 mM halothane.

Discussion

The past three decades have seen an immense increase in our understanding of MH, its clinical presentation[12], its pathophysiology[13] and its molecular basis[6]. However, screening for MH susceptibility remains a difficult problem to solve. The presentation of an MH reaction can vary enormously and there is no unique clinical sign of MH. Individuals who are susceptible for MH often have no clinical or histological evidence of a muscle disorder. Many diagnostic tests have been described but are now obsolete for MH screening because they failed to discriminate sufficiently between MHS and MHN [14]. Despite significant difference between mean values of MHN and MHS individuals after CK measurements and MA scoring in histological examination, the lack of sensitivity prevents the introduction of these procedures into clinical practise[15,16]. In selected MH-families an elevated CK increases the probability of MHS from 50% (on the basis of family history alone) to 94%[15,17]. In this family the risk of MHS increased to 92%. However, in patients with no clinical or family history of MH, CK is of no value as a screening test for MH[15,18]. The observation of cores

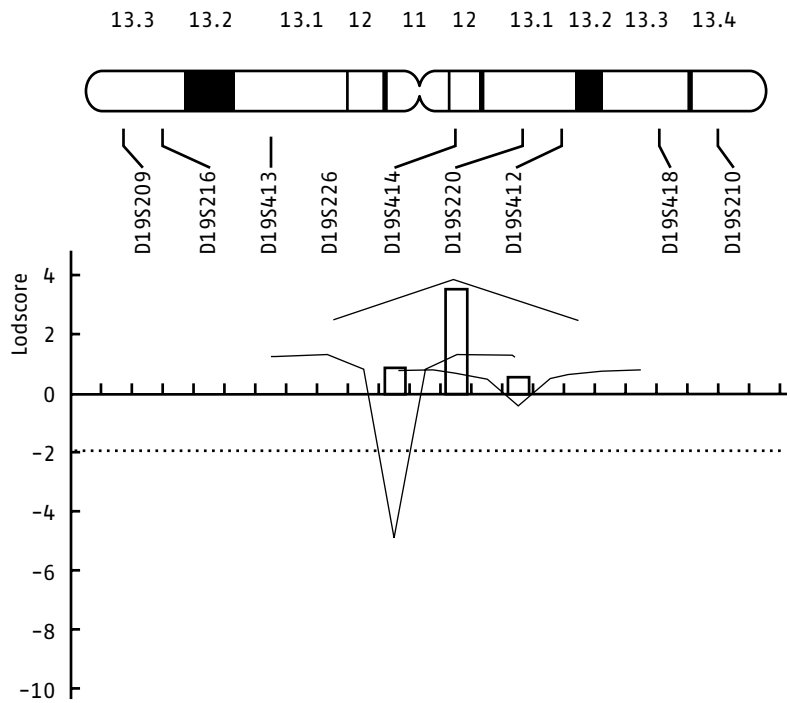
table 3 *MA-score and CK measurement results related to age, gender and IVCT result. The MA-score of the biopsy is the number obtained by summation of scores belonging to the defined abnormal findings in the biopsy. Normal CK level < 90 IU/L*

Pat. Nr	Age	IVCT result	M/F	CK IU/L	MA-score	Cores
I	68	MHN	F	41	4	-
II:2	31	MHN	M	66	0	-
IV:1	43	MHN	F	84	0	-
V	56	MHS	M	100	4	+
V:1	33	MHN	F	78	1	-
V:2	33	MHS	F	65	6	+
V:3	28	MHS	M	265	2	-
V:4	27	MHS	M	782	6	-
V:5	19	MHN	M	-	1	-
VI	58	MHS	F	128	9	+
VI:1	41	MHS	F	291	2	+
VI:4	31	MHS	M	211	5	-
VI:5	30	MHS	F	145	8	+
VII	53	MHN	F	-	2	-
VIII	52	MHN	M	75	4	-
IX:1	36	MHS	M	187	3	-
IX:2	32	MHN	F	63	1	-
IX:3	30	MHS	M	134	7	-
X	45	MHE	M	144	6	+
X:2	26	MHE	M	65	4	-
XI	50	MHS	F	61	5	-
XI:1	30	MHS	F	104	1	-
XI:2	28	MHS	F	98	5	-
XII	44	MHN	F	-	2	-
Mean MHS (n = 13)				198	4.8	
SD				189	2.3	
Mean MHN (n = 9)				68	1.7	
SD				15	1.4	
p-value				0.03	0.001	

also increased the probability of MHS in this family, though this histopathological finding, without clinical problems, has little diagnostic value in the general population.

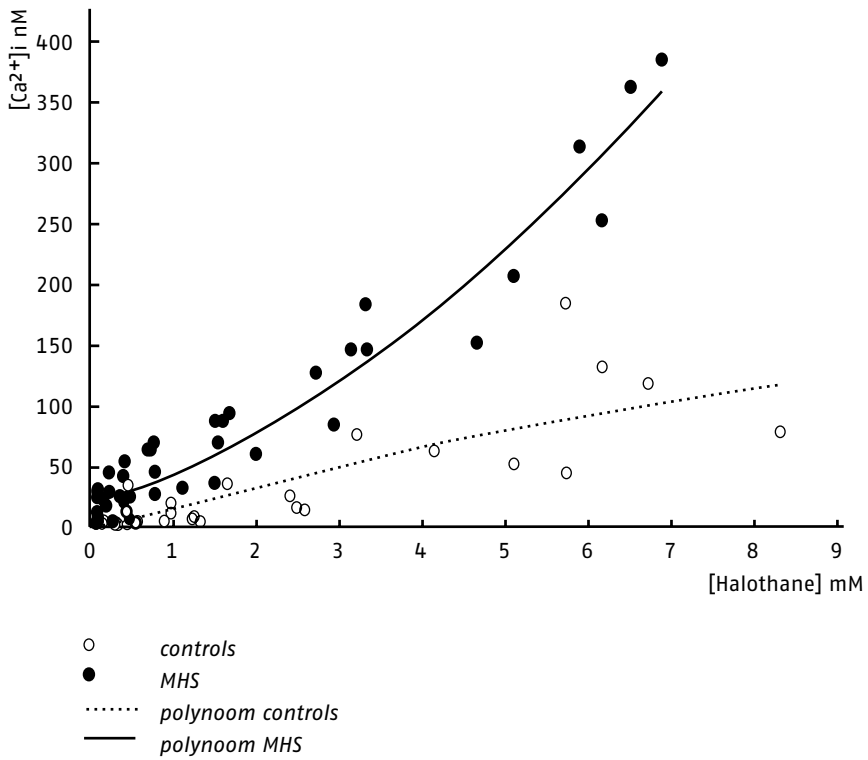
What remains for detection of susceptibility to MH in probands is the standard caffeine halothane IVCT. Validated against the clinical presentation, the sensitivity and specificity of IVCT results can best guarantee patient safety during anaesthesia [19]. Unfortunately, the IVCT has several limitations; it requires large muscle samples (surgically collected), it must be performed within 5 hours of the removal of tissue and it is a technically demanding test that requires expertise.

figure 2 *Graphical representation of two-point lod scores generated for the RYR1 region of chromosome 19q.*
Data is presented in a format generated in LODVIEW EXCEL in which each chromosomal graph represents 2 point lod score data from specific microsatellite markers spaced at ~11cM intervals along the chromosome 10. Shaded bars represent two-point lod score values at q = 0.02 for specific markers. Line distributions for each bar represent lod score values from q = 0 (peak) to q = 0.2 (termini). The y axis is graduated in ± 2 lod score intervals (+4 to -10), the stippled line denoting the -2 lod score threshold for exclusion of linkage. The physical relationship of the markers on the chromosome is shown above the genetic LODVIEW map



Indeed, the IVCT is only available in 22 MH investigation centres across Europe. An important diagnostic disadvantage of the IVCT is that it is not absolutely specific[20,21]. Research studies in which respectively cultured myotubes and an in vivo metabolic test have been used, may have the potential for development as less invasive diagnostic tests; they need to be validated with large numbers to define sensitivity and specificity[9,22]. Despite extensive screening of the RYR1 gene, we were unable to identify any known or new mutations that may underlie MHS in this family. Nevertheless, we have been able to confirm linkage of the MHS locus with chromosome 19 markers around RYR1. This means that we may still be able to apply genetic testing for MHS among other members of this family to complement IVC-testing, according to recently issued EMHG guidelines[11].

figure 3 *Halothane induced, dose-dependent increase of $[Ca^{2+}]_i$ in cultured skeletal muscle cells from 6 MHS individuals (n = 42: solid dots and line) and 4 control individuals (n = 32: open dots and dotted line). Discrimination between MHS and control is 100% beyond 0.5 mM halothane (p < 0.05)*



Perspectives

Well documented and IVCT-phenotyped MH families showing linkage between their MHS trait and RYR1, but not carrying any of the almost 30 mutations described in this gene, will encourage future inter-disciplinary research between anaesthetists, geneticists and basic scientists to solve the puzzle of this heterogeneous disorder. In the meantime, further elucidation of the mechanism of pathogenesis will continue to improve the safety of patients susceptible to MH or with related muscle disorders, who need to undergo anaesthesia.

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Chapter 6

GENERAL DISCUSSION


SUSCEPTIBLE

'Malignant hyperthermia' has been recognised as an anaesthesia-related, potentially life-threatening, genetic disorder since the mid-1960s. The combination of halothane and succinylcholine in routine general anaesthesia revealed an abnormality in the calcium release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine receptor) as the cause of MH. Malignant referred to dramatic case descriptions that had a fatal outcome in approximately 80%. Hyperthermia was the most impressive clinical feature, describing patients who became very hot and sweaty. Nowadays the term malignant hyperthermia is a misnomer. Better monitor facilities, in particular measuring end-tidal CO_2, indicate hypermetabolism well before the core temperature begins to rise. Increased awareness of MH by anaesthetists and dantrolene have contributed greatly to the drop in mortality to almost zero. Due to its major symptoms, other conditions, syndromes or disorders have been associated with MH. However, clarification of MH pathogenesis has made it possible to rule out almost all of these MH-associated conditions or myopathies (e.g. heatstroke, sudden infant death syndrome, neuroleptic malignant syndrome, myotonic disorders and muscular dystrophies).

MH presents with multiple non-specific clinical signs and laboratory findings. The latter relate to skeletal muscle hypermetabolism and ischemia. These 'clinical indicators' observed during an acute anaesthetic reaction can be scored using the MH clinical grading scale to determine the qualitative likelihood that the adverse anaesthetic event actually represents MH. The MH clinical grading scale has been recommended for use as an aid to an objective definition of MH. In actual practice it is hampered by incomplete clinical information, which is a common situation.

In recent years the "incidence" of clinical MH has tended to decrease because of increased use of regional anaesthesia and total intravenous anaesthesia (TIVA), both "MH safe" techniques, together with a decrease in the use of succinyl-choline. As the combination of succinylcholine and potent inhalational anaesthetic agents produces a more profound MH reaction, the signs of MH are now often less pronounced and can occur insidiously. Mild forms of MH could therefore be missed. However, fulminant classical MH reactions do still occur.

MH is a rare disease. The frequency of clinical MH, is therefore not easy to estimate. The highest incidence of clinical MH that has been reported is 1 in 100, where MH was defined as 'masseter muscle rigidity' in a study of children when only halothane and succinylcholine were used for anaesthesia. However, in surveys of mixed surgical populations fulminant MH has occurred in 1 in 200,000 to 250,000 anaesthetics. Several factors are responsible for this wide disparity in estimated incidence, including: the definition of MH; the anaesthetic procedure and the agents that were used; and the age and sex of the patients. The incidence of MH is generally agreed to be 1 in 10.000 to 15.000 anaesthetics.
Some questions remain unsolved despite a good understanding of MH pathogenesis: why does the incidence of clinical MH decrease with increasing age? Why is it most commonly seen in the 10-30 age group? Why are males affected more frequently than females? What is the impact of stress? Why do patients carrying a 'risk allele' not always develop MH during trigger anaesthesia? These phenomena could be explained if clinical MH is triggered only when a combination of various factors together disturb calcium regulation in the skeletal muscle cell.

Abnormalities in the calcium release channel of skeletal muscle sarcoplasmic reticulum (SR) have been implicated as the central factor in the cause of the MH syndrome. Succinylcholine and all potent inhalational anaesthetics are able to induce MH episodes by increasing the rate of Ca^{2+} release from the SR. Potent inhalational anaesthetics may interact with 'risk variants' of the SR calcium release channel (ryanodine receptor) to induce an abnormal increase in the release of Ca^{2+}. This may be further aggravated by the destabilisation of Ca^{2+}-sensitive intracellular regulatory mechanisms believed to result from exposure to potent inhalational anaesthetics.

A lot of effort has been, and is being made to design diagnostic tests for MH-susceptibility (MHS). While this research has helped our understanding of excitation-contraction coupling, no test so far developed has been sufficiently reliable or generally applicable to replace the caffeine-halothane in vitro contracture test (IVCT). The IVCT is a biological test that measures muscle contracture upon exposure to triggering (anaesthetic) agents. Actually, the IVCT could be seen as a surrogate for the clinical scenario, because abnormal muscle contractures observed in vitro reflect the alterations that occur in the patient. The IVCT, however, is invasive, time consuming and it is a technically demanding test that requires expertise.

A test based on cultured muscle cells obtained by percutaneous needle biopsy is less invasive and is easily reproducible. Halothane produces a dose-dependent increase of the intracellular calcium concentration in cultured human skeletal muscle cells. The calcium response in MHS individuals proved significant different from that of control individuals. The time between biopsy and performance of the test is not critical, because cell cultures can be expanded, frozen and thawed.
In contrast IVCT requires large muscle samples (surgically collected) and must be performed within 5 hours. More studies, comparing data from IVCT and cultured cells have to be undertaken to determine sensitivity (and specificity).
A second alternative test that could have the potential for development as a less

invasive diagnostic procedure is an in vivo metabolic test. Intramuscular injection of halothane or caffeine leads to a local hypermetabolic reaction with concomitant local increase in pCO_2 in MHS individuals that is significantly higher than in normal controls. This test will require large numbers of patients and controls to be studied in a multicentre trial to define sensitivity and specificity.

MHS was first linked to the gene encoding the skeletal muscle ryanodine receptor (RYR1) on chromosome 19q13.1 in 1990. It was thought that a genetic test for MH was within reach, but the large size and complex nature of the RYR1 gene has made identification of RYR1 mutations difficult. More than 80 individual mutations in RYR1 have been detected to date in patients with MH and the associated myopathy, central core disease (CCD). However, there is no prevalence of particular mutations in all populations, and many mutations have been found only in single families.

Linkage studies have indicated that variations in other genes located elsewhere in the human genome can also give rise to susceptibility to MH. Actually, MHS shows a high level of genetic heterogeneity, the proportion of cases not linked to RYR1 (designated as the MHS1 locus) may be as high as 50%. To date, six loci, including RYR1, have been identified as containing potential candidate MHS genes. Because of this heterogeneity it is not as yet generally feasible to diagnose or to exclude susceptibility to MH, on the basis of molecular genetic testing alone. However, there are specific situations where detection of susceptibility to MH using molecular genetic techniques can replace IVCT. Clinical practitioners and geneticists of the European MH Group (EMHG) have agreed on guidelines for DNA-based investigation for the sake of the safety of MHS patients and their families who need to undergo anaesthesia. The original (2001) guidelines contained 15 RYR1 mutations for genetic testing. Once one of these causative mutations has been detected in the proband, whose MHS status has been confirmed by IVCT, it can be used to test relatives. Mutation carriers should consequently be regarded as susceptible to MH, although confirmation of this diagnosis can only be carried out by the IVCT.
Recently, a further 7 RYR1 mutations have met the EMHG criteria for their use in predictive genetic testing. Mutations will be add to the list only after genetic and functional characterization. The criteria that will need to be met are: 1) a full description at the DNA and protein level; 2) co-segregation of the allele with MHS and/or CCD in at least 2 pedigrees; 3) absence of the sequence change in 100 control samples for exclusion of polymorphisms; and 4) the effect of the mutation on RyR1 function has been confirmed by recombinant in vitro expression or in ex vivo tissues.

In the meantime, the search for novel mutations in RYR1 goes on. Leukocytes seem to be an adequate substitute tissue for screening the RYR1 gene. Leukocytes can simply be isolated from human blood and used for extraction of RNA and reverse transcription of messenger RNA into cDNA. Since the sequence of full-length leukocyte RYR1 cDNA were shown to be identical to the sequence of human muscle RYR1 cDNA, a standard, 'noninvasive', 10 ml blood sample is sufficient to allow a rapid search for new mutations.

Despite the arrival of new diagnostic tests, contracture testing remains the only reliable means of ascertaining MH status. Standardization of the IVCT has led to the development of 2 essentially similar protocols in Europe and in North America. Diagnosis of MHS has been achieved successfully with the standard caffeine-halothane IVCT; but with any in vitro pharmacological test, such as the IVCT, there is always going to be some overlap in results between true positives and true negatives. In the interest of patient safety and from an anaesthesiological point of view, the major role of a clinical MH investigation unit should be to exclude the diagnosis of MH rather than to confirm it. In this way, patients and their entire families are not falsely identified as MH-susceptible. However, the inevitable consequence of the need to avoid false negative results (sensitivity), is that a considerable number of false-positive results will be made. Multi-centre evaluation showed a high diagnostic sensitivity of the IVCT in Europe: 99%, and in North America: 97%; a satisfactory specificity in Europe: 94% and a low specificity in North America: 78%.
While it may not be possible to improve sensitivity, we should aim to minimise the number of false positives. Our diagnostic procedure must be adequately specific, otherwise anaesthesia will be made unnecessarily complicated and expensive. In addition, the power of the molecular genetic studies could be increased by improving the specificity of the IVCT.

The addition of other, potentially more specific, test agents to the standard caffeine-halothane IVCT could increase its reliability. Ryanodine and 4-chloro-m-cresol have proved to be useful in improving reliability of MH diagnosis, although further studies should determine the sensitivity and specificity of both tests before inclusion into the standard IVCT protocol. Unfortunately, with the addition of more test agents, we have automatically created more MH-equivocal (MHE) categories. Since this MHE group contains a high percentage of 'false positive' test results, the specificity is likely to decrease. An important comment on the determination of sensitivity and specificity of the IVCT includes the selection of patients. Both the European and the North American MH Groups applied the MH Clinical Grading Scale to identify case subjects. Only cases ranked as "almost certain to be MH susceptible" (MH rank D6 with a score of 50 or more) were accepted for inclusion into the studies, whereas investigation with IVCT in most MH investigation centres is done from MH rank D4: "somewhat greater than likely to be MH susceptible". Latter comment mainly regard specificity. In spite of the above-mentioned problems, the standard caffeine-halothane IVCT remains the cornerstone in the diagnostic procedure for patients who had survived MH reactions. Patients and their relatives who are "labeled" MHS or MHE by the IVCT should never be placed in a "triggering" environment.
Other important parts of the procedure are a clinical examination, measurement of serum creatine kinase activities and a histological examination of the muscle biopsy, to exclude an underlying muscle disease which could account for the adverse anaesthetic event. Genetic analysis can not yet replace contracture testing, and may remain impractical due to the complexity of the genetics of MH. Further research needs to be done before an in vivo metabolic test or a test on cultured skeletal muscle cells can replace the invasive in vitro contracture test.

Summary

Malignant hyperthermia (MH), a potentially fatal adverse reaction during or after an operation was first recognised as an anaesthetic-related complication in 1960. It was not until 1985 that international researchers agreed upon a protocol for the diagnosis of MH susceptibility. MH has undergone an explosion of information since.
The aim of this thesis is to study the diagnostic procedures for MH susceptibility, with special emphasis upon refining the biological diagnostic test and developing a new test. By this and by improving protocols and guidelines for investigation of susceptibility to MH, we have gained confidence in the diagnosis "MH susceptible (MHS)".

Chapter 1 reviews the present knowledge of MH. Historical aspects of MH will be adjusted to results of pathophysiological and genetics research. In humans genetically predisposed to MH, anaesthesia with succinycholine and/or volatile anaesthetics can induce an MH reaction. Unfortunately there is no one symptom or sign that is unique to MH, and MH is not one entity. The clinical diagnosis of MH is made on the combination and the severity of abnormal signs, metabolic and muscular in origin. The type of clinical presentation sets the treatment of an MH reaction. Prompt dantrolene injection is the cornerstone of effective MH therapy, time is of the essence and morbidity is correlated with the duration of symptoms.
True incidence of MH is unknown because of the variable clinical presentation. The incidence is estimated to be 1 in 10,000 to 1 in 225,000 anaesthetics.
The crucial role in the pathogenesis of MH is elevated myoplasmic calcium. Abnormalities in the calcium release channel of skeletal muscle sarcoplasmic reticulum (the ryanodine-dihydropyridine receptor complex) have been implicated in the cause of MH. Genetic linkage analysis in human families with MH have indicated that a mutation in the ryanodine receptor gene (RYR1) could account for susceptibility to MH. The RYR1 locus is located on chromosome 19.
Clarification of MH pathogenesis has made it possible to rule out MH-associated conditions or myopathies. The only clearly associated muscle disorder is central core disease.

Chapter 2 describes the in vitro contracture test (IVCT). The IVCT determines the sensitivity of freshly obtained skeletal muscle specimens to several test agents. It has generally been accepted as "the gold standard" to determine the susceptibility to MH. European MH investigation units, forming the European Malignant Hyperthermia Group (EMHG), agreed upon a standardised protocol in 1984.
This protocol contains the diagnostic criteria.
To determine the sensitivity and specificity of the IVCT, the results of IVCT in 1502 patients with previous fulminant MH were collected from 22 centres of the EMHG. Of these 1502 probands, 119 had clinical scores of 50 and above in the Clinical Grading Scale to predict MH susceptibility such scores describing a likelihood of MH as "almost certain". By comparing IVCT results from these 119 probands with those of 202 low-risk individuals, a diagnostic sensitivity of the IVCT of 99.0% was observed if the MHE group (MH equivocal: muscle specimens only react abnormally to halothane or caffeine) is considered susceptible. Accompanying specificity was 93.6%.

Halothane, one of the test agents in the IVCT, has been replaced by sevoflurane in modern anaesthesia because of favourable characteristics. In this chapter the in vitro effects of sevoflurane are described. Sevoflurane can trigger an abnormal contracture in muscle from MH susceptible patients in vitro; the threshold concentration measures 7% or 0.70 mmol/l. This is indicative of malignant hyperthermia susceptibility. Exposure to sevoflurane should be avoided in patients thought to be susceptible to malignant hyperthermia.

Chapter 3 describes a study to investigate the usefulness of cultured skeletal muscle cells, obtained by percutaneous needle biopsies, for determining MH susceptibility. Muscle samples from 6 MHS patients and from 4 controls were used to culture myotubes. The free cytosolic calcium concentration ($[Ca^{2+}]_i$) of myotubes was determined after exposure to halothane. The dose-dependant calcium response in cultured muscle cells of the MHS individuals is significantly different from that of the control individuals after exposure to halothane. There is no overlap beyond halothane concentrations ≥ 0.5 mmol/l. Cultured human muscle cells, obtained from needle biopsies, may well be applied in alternative and less invasive diagnostic tests for MH susceptibility. More multi-centre studies, comparing data from IVCT and results from tests using cultured cells, have to be undertaken to determine sensitivity.

In chapter 4 the diagnostic procedures in four European MH centres have been evaluated to see how they deal with the referral of patients and how this is reflected in workload and funding. The 4 national MH investigation units in Belgium, the Netherlands, Switzerland and the United Kingdom select probands for screening in a similar but individual way based on experience. The number of referrals was too low to demonstrate inter-centre difference between IVCT indication criteria and IVCT results. This study provided an insight into the need for further standardisation and quality assurance for the diagnosis of MH. A European quality assurance project has been started, based on an independent on-site visiting programme to all 22 European MH investigation units. This project will improve confidence in the diagnostic screening for MH susceptibility. Ultimately it will improve the safety of MH susceptible patients and their families, who need to undergo anaesthesia.
In the second part of chapter 4, the European guidelines for the detection of MH susceptibility using molecular genetic techniques are presented. The main purpose for drawing up the present guidelines was to avoid false MH-negative (MHN) diagnoses because of the potential risk of MH during general anaesthesia for these patients and their blood relatives. At this stage molecular genetic testing for 22 causative mutations in the ryanodine receptor gene (RYR1) of the index case could lead to quicker results for the rest of the kinship. An up-to-date list of mutations that have been shown to directly alter RYR1 caffeine or halothane sensitivity is shown on the following website: http://www.emhg.org.

Chapter 5 is a survey of three decades of screening for MH in a Dutch family. 24 relatives of a patient who died in 1973 after suffering MH have been studied in detail. All steps of the screening procedure, follow developments in testing for

the diagnosis of MH susceptibility from 1984 until 2002. Linkage analysis implicated RYR1 on chromosome 19q13.1 as the disease susceptibility locus in the family. Unfortunately, following analysis of the three hotspot-regions in the RYR1 gene containing the causative MH and CCD mutations, no mutation was detected which segregated with MH susceptibility in the family.
Histological examination and CK measurements showed significant differences between MHS (n = 13) and MHN (n = 6) individuals. Despite significant difference between mean values of MHN and MHS individuals that belong to this family, the lack of sensitivity in the general population prevents the introduction of these procedures into screening for susceptibility to MH. In this family the IVCT remains the gold standard for diagnosis of susceptibility to MH.

Chapter 6 contains the general discussion. Nowadays the term malignant hyperthermia is a misnomer. Better monitoring facilities, in particular measuring end-tidal CO_2, indicate hypermetabolism well before the core temperature begins to rise. Increased awareness of MH by anaesthetists and the availability of dantrolene have contributed greatly to the drop in mortality to almost zero.
MH is a rare disease. In recent years the "incidence" of clinical MH has tended to decrease because of increased use of regional anaesthesia and total intravenous anaesthesia (TIVA), both "MH safe" techniques. However, whilst succinylcholine and potent inhalational anaesthetic agents are being used MH reactions will still occur, and as a consequence there is the need to identify individuals who are susceptible to MH. Since MH is inherited as an autosomal dominant condition, diagnosing MH susceptibility is a matter of family concern.
Whenever an "MH reaction" occurs in a patient that is exposed to triggering drugs, all available clinical signs should be analysed to predict the MH likelihood. Through such analysis, together with a personal or family history suggestive of MH, probands are selected for screening. If the proband has died then the nearest appropriate relatives should be screened. The lowest age limit for the muscle biopsy is 12–14 years as IVCT results have been inconclusive below this age. Despite the arrival of new diagnostic tests, contracture testing remains the only reliable means of ascertaining MH status. The IVCT, however, is invasive, time consuming and a technically demanding test that requires expertise. There are specific situations where detection of susceptibility to MH using molecular genetic techniques can replace the IVCT. Clinical practitioners and geneticists of the European MH Group (EMHG) have agreed on guidelines for DNA-based investigation for the sake of the safety of MHS patients and their families who need to undergo anaesthesia. Further research needs to be done before an in-vivo metabolic test or a test on cultured skeletal muscle cells can replace the invasive in-vitro contracture test, which has a sensitivity of 99% in Europe. This research will require large numbers of patients and controls to be studied in a multi-centre trial to determine sensitivity and specificity.

Samenvatting

Maligne hyperthermie (MH) een zeldzame potentieel dodelijke reactie tijdens of na een operatie, werd voor het eerst als anesthesiecomplicatie onderkend in 1960. Rond 1985 werd de methode beschreven om MH-risicodragers op te sporen waarover internationaal overeenstemming bestond. Sindsdien zijn de inzichten over MH danig gewijzigd. De doelstelling van het onderzoek in dit proefschrift is tweeledig. Enerzijds werden de diagnostische procedures van de afgelopen 25 jaar bestudeerd en geëvalueerd. Anderzijds werden de bestaande diagnostische tests verfijnd en werden nieuwe tests ontwikkeld. Dit heeft geleid tot verbetering van onderzoeksprotocollen c.q. verhoging van de betrouwbaarheid van de diagnose 'gevoelig voor maligne hyperthermie' (MHS).

Hoofdstuk 1 geeft een overzicht van de huidige kennis over MH. Historische veronderstellingen worden bijgesteld naar aanleiding van nieuw verworven pathofysiologische en genetische feiten. Een maligne-hyperthermiecrisis treedt op bij daarvoor gevoelige personen tijdens algehele anesthesie als reactie op toediening van succinylcholine en/of gehalogeneerde inhalatieanaesthetica. Helaas bestaat er geen klinisch kenmerk dat uniek is voor MH. Een MH-reactie kan zich verschillend presenteren, afhankelijk van de ernst van de metabole stoornissen en de mate van spierbeschadiging. De klinische presentatie bepaalt welke therapeutische maatregelen dienen te worden genomen om verdere schade te voorkomen. Hoeksteen van behandeling van een MH crisis is zo snel mogelijk dantroleen intraveneus toedienen.
De precieze incidentie van MH is niet te berekenen omdat er een zeer gevarieerde presentatie is. Schattingen lopen uiteen van 1 op 10.000 tot 1 op 225.000 anesthesieën.
MH berust op een defect in het ryanodine-dihydropyridine receptorcomplex, gelokaliseerd in het sarcoplasmatisch reticulum in de skeletspiercel. Onder bepaalde omstandigheden tijdens anesthesie geeft dit aanleiding tot een abnormale regulatie van de myoplasmatische calciumconcentratie. Door middel van koppelingstudies is bij enkele families een verband aangetoond tussen mutaties in het ryanodinereceptor-gen (RYR1) en gevoeligheid voor MH. Dit RYR1 is bij de mens gelokaliseerd op chromosoom 19.
Met de ontdekking van de oorzaak van gevoeligheid voor MH, maar ook doordat meer bekend is geworden over de oorzaken van neuromusculaire ziekten en syndromen met temperatuursontregeling, kunnen we MH tegenwoordig onderscheiden van andere aandoeningen. Alleen central core disease (CCD) en MH lijken nog een zelfde oorzaak te kunnen hebben met overlap in klinische kenmerken.

Hoofdstuk 2 beschrijft de in vitro contractuur test (IVCT). Deze biologische test waarbij vers gebiopteerd skeletspierweefsel wordt blootgesteld aan verschillende teststoffen geldt sinds 1984 als de gouden standaard voor diagnostiek naar maligne hyperthermie gevoeligheid. De IVCT wordt uitgevoerd volgens het protocol van de European Malignant Hyperthermia Group (EMHG), een samenwerkingsverband van alle Europese maligne hyperthermiecentra. In dit protocol zijn de criteria voor de diagnose vastgelegd.
Als Europese groep hebben wij de sensitiviteit en de specificiteit van de IVCT bepaald. Hiertoe werden 1502 patiënten onderzocht die allen een IVCT ondergingen

nadat bij hen een maligne hyperthermiereactie was opgetreden. Bij 119 van deze 1502 maligne hyperthermieprobandi kon op voorhand de diagnose "MH bijna zeker" gesteld worden omdat op grond van de 'klinische graderingsschaal voor MH' een puntenwaardering van ≥ 50 punten werd toegekend aan de ernst en aard van symptomen. De IVCT-resultaten van deze 119 van MH verdachte patiënten werden vergeleken met die van 202 controlepatiënten. Hieruit hebben wij een diagnostische sensitiviteit berekend van 99.0% waarbij we de groep patiënten die in de IVCT slechts op 1 van de 2 teststoffen positief reageerde (MHE) als 'gevoelig voor MH' hebben beschouwd. De specificiteit van de IVCT hierbij bedroeg 93.6%. Omdat halothaan, één van de teststoffen in de IVCT, in de hedendaagse anesthesiologische praktijkvoering nagenoeg geheel vervangen is door sevofluraan en omdat de MH-reacties van de laatste jaren veelal na toediening van sevofluraan zijn opgetreden, beschrijven wij in dit hoofdstuk de uitkomst van een studie naar de in-vitro effecten van sevofluraan. Spierweefsel van individuen met de diagnose MHS reageerde eveneens positief op sevofluraan. Anesthesie met gebruikmaking van sevofluraan bij deze mensen moet dus te allen tijde worden voorkomen. De verdamperconcentratie van de sevofluraan werden vergeleken met de sevofluraan-concentraties in de vloeistoffase. Hierdoor waren we in staat de sevofluraan drempelwaarde vast te stellen bij een contractuur van ≥ 2 mN: 7% of 0.70 mmol/l.

Hoofdstuk 3 beschrijft de studie waarin wij onderzocht hebben of gekweekte skeletspiercellen kunnen worden aangewend om gevoeligheid voor MH aan te tonen. Spiercellen uit de musculus quadriceps femoris, verkregen door middel van een naaldbiopsie, werden opgekweekt tot myotubes. Deze myotubes werden vervolgens blootgesteld aan halothaan, waarbij de intracellulaire calciumconcentratie ($[Ca^{2+}]_i$) werd gemeten. Vergelijking van de resultaten van myotubes gekweekt uit spierweefsel van 6 MHS individuen met die van 4 controlepersonen gaf, als reactie op toediening van halothaan, een dosis afhankelijke stijging te zien van de $[Ca^{2+}]_i$. Bij een halothaanconcentratie ≥ 0.5 mmol/l wordt er geen overlap meer waargenomen. De beschreven methode zou een alternatieve diagnostische test voor het aantonen van gevoeligheid voor MH kunnen zijn met als voordeel dat het minder invasief is dan de IVCT. Vervolgstudies en multicentre studies zijn nodig om de sensitiviteit van de test te bepalen.

Hoofdstuk 4 bevat een studie waarin de diagnostische procedures in 4 Europese MH centra zijn geëvalueerd en met elkaar zijn vergeleken.
In de 4 nationale MH diagnostiek centra in België, Nederland, Zwitserland en Groot-Brittannië worden verwijzingen van patiënten die van MH worden verdacht op een verschillende manier afgehandeld. Het aantal onderzochte verwijzingen in de studie bleek echter te klein om consequenties uit de verschillen in handelswijze te trekken. De studie heeft wel mede de aanzet gegeven tot het opzetten van een visitatieprogramma van alle Europese MH-centra. Kwaliteitscontrole van de IVCT en meer standaardisatie in de behandeling van verwijzingen komen de betrouwbaarheid in de diagnose 'gevoelig voor maligne hyperthermie' (MHS), maar met name ook in de diagnose 'niet gevoelig voor maligne hyperthermie' (MHN), in heel Europa ten goede. Dit zal de veiligheid doen toenemen indien MHS patiënten

en hun familieleden een operatie onder algehele anesthesie moeten ondergaan. Het tweede deel van dit hoofdstuk is een weergave van de richtlijnen die door de EMHG zijn uitgevaardigd voor het verrichten van MH-diagnostiek op moleculair genetisch niveau. De richtlijnen (met een update in de appendix) zijn geschreven als een dringend advies om deze te volgen omdat foutieve of onvolledige vertaling van genetische bevindingen kan leiden tot klinische problemen met nadelige gevolgen. Met name vals-negatieve resultaten van genetisch onderzoek kunnen ernstige gevolgen hebben als deze mensen onder algehele anesthesie worden gebracht met MH uitlokkende medicijnen. Diagnostiek naar gevoeligheid voor MH op DNA niveau is mogelijk maar blijft beperkt tot families waarvan de MHS-geteste individuen drager zijn van één van de 22 mutaties in het ryanodinereceptor gen (RYR1) waarvan een oorzakelijk verband is aangetoond. Een up-to-date overzicht is opvraagbaar op het internet (http://www.emhg.org).

Hoofdstuk 5 is een overzicht van 30 jaar diagnostisch onderzoek in een Nederlandse MH-familie. 24 eerste- en tweedegraads verwanten van een patiënt die in 1973 na een maligne-hyperthermiecrisis overleed zijn sinds 1984 onderzocht op gevoeligheid voor maligne hyperthermie. De diagnostische procedure van deze familie in de periode van 1984 tot 2003 evolueert met de ontwikkelingen van de MH-diagnostiek in het algemeen. In de familie bestond voldoende bewijs dat MH-gevoeligheid gekoppeld is aan het RYR1 gen op chromosoom 19. Echter, ondanks grote inspanning kon er geen van de bekende mutaties worden aangetoond in het RYR1 gen, noch kon een nieuwe mutatie worden geïdentificeerd. Histologisch onderzoek en bepaling van de creatinekinase activiteit (CK) gaf evidente verschillen tussen MHS- en MHN-individuen; doch beide onderzoeken blijken te weinig sensitief voor een betrouwbare diagnose. Concluderend blijft ook in deze familie de IVCT de 'gouden standaard' voor het aantonen van MH-gevoeligheid.

Hoofdstuk 6 bevat de general discussion. 'Maligne hyperthermie' is inmiddels een ongelukkige term. Patiënten overleven tegenwoordig nagenoeg altijd een MH-reactie indien deze snel herkend wordt en er onmiddellijk adequaat gehandeld wordt. Een serieuze MH-reactie openbaart zich namelijk doorgaans in een vroeg stadium met stijging van het CO_2 in de uitademingslucht, en is goed behandelbaar d.m.v. dantroleen. Ernstige temperatuurontregelingen met een toename boven 40 °C worden zelden of nooit meer gerapporteerd. MH is zeldzaam en een MH-reactie zal steeds minder optreden of 'subklinisch' verlopen omdat de uitlokkende medicijnen in de moderne anesthesiologische praktijkvoering langzaam maar zeker worden vervangen door medicijnen die geen MH-reactie uitlokken bij patiënten die daarvoor gevoelig zijn. Echter, zo lang er nog succinylcholine of gehalogeneerde inhalatieanaesthetica (halothaan, isofluraan, enfluraan, sevofluraan en desfluraan) gebruikt worden, blijft het noodzaak om risicodragers op te sporen. MH berust op een autosomaal dominant overervende aanleg: een mutatie in het ryanodinereceptor gen. Diagnostisch onderzoek betekent dus screening van de hele familie. Indien men bij een patiënt tijden anesthesie MH vermoedt en er geen plausibele verklaring voor de verschijnselen gevonden wordt, komt de patiënt in aanmerking voor nadere diagnostiek. Aan de hand van een analyse van

de klinische kenmerken en persoonlijke en familiaire anamnestische gegevens, wordt op voorhand het risico op vatbaarheid voor MH ingeschat om de indicatie voor de IVCT te stellen. Als de 'index case' is overleden of jonger is dan 12–14 jaar worden eerstegraads familieleden getest. De IVCT heeft het nadeel dat het een invasieve test is die alleen bij volwassenen kan worden toegepast. De test is bewerkelijk en vereist veel expertise maar wordt desondanks wereldwijd erkend als de meest betrouwbare test om MH-gevoeligheid aan te tonen. Moleculair genetisch diagnostisch onderzoek naar gevoeligheid voor MH is verantwoord onder bepaalde voorwaarden, binnen één familie. Van alternatieve tests voor de diagnostiek van MH-gevoeligheid zoals een in vivo metabole test of een test op gekweekte myotubes is de sensitiviteit nog onbekend; daar waar de sensitiviteit van de IVCT in Europa 99% bedraagt.

Curriculum vitae

Marc Snoeck werd op 3 november 1963 in Zundert geboren. In 1982 behaalde hij het diploma VWO aan de Katholieke Scholengemeenschap te Etten-Leur. Aansluitend studeerde hij Geneeskunde aan de Katholieke Universiteit te Nijmegen. Het doctoraal-examen behaalde hij in augustus 1986 en het arts-examen in mei 1989. In de periode 1989-1992 heeft hij klinische ervaring opgedaan als AGNIO. De opleiding tot anesthesioloog werd in april 1992 begonnen in het Academisch Ziekenhuis Nijmegen (opleider Prof. Dr. L.H.D.J. Booij). Sinds 1 april 1997 is hij ingeschreven in het specialistenregister. Vanaf 14 april 1997 is hij als anesthesioloog werkzaam in het Canisius-Wilhelmina Ziekenhuis in Nijmegen. Sedert 1994 maakt hij deel uit van de landelijke werkgroep maligne hyperthermie. Hij is hierbij persoonlijk verantwoordelijk voor de in vitro contractuur test en hij vertegenwoordigt Nederland in de European Malignant Hyperthermia Group. Marc Snoeck is getrouwd met Marion de Bruin

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Dankwoord/acknowledgements

Het moet ergens eind 1993, begin 1994 zijn geweest. Ik was ingedeeld op OK 15, met als supervisor dokter Gielen. Aan de muur hing een slordig A4tje dat met plakband op de tegels was geplakt: het 'protocol Maligne Hyperthermie'. Als ervaringsdeskundige, maar vooral als landelijke bekendheid op het gebied van MH vertelde Mathieu mij die dag alles over MH. Zo werd mijn belangstelling voor MH gewekt en daar ben ik hem nog steeds dankbaar voor. Beste Mathieu, jou wil ik verder bedanken dat je mij hebt geïntroduceerd in de Nijmeegse werkgroep MH en in de Europese MH groep.

De Nijmeegse werkgroep MH stond onder voorzitterschap van professor Sengers. Beste Rob, jij hebt mij vanaf het begin onder je hoede genomen. Voor mij leek het of dit kleine stukje van je werk even belangrijk was als de rest. Je was altijd makkelijk toegankelijk voor mij, terwijl ik toch een vreemde eend in de kindergeneeskundevijver ben. Jij hebt mij de inspiratie gegeven om de diagnostiek naar MH-gevoeligheid in Nederland uit te blijven voeren. Beste Rob bedankt voor al het promoten.

De in vitro contractuur test is al die jaren de rode draad geweest. De meeste 'MH-tijd' heb ik de afgelopen 10 jaar doorgebracht in het lab. In een hoekje van het anesthesiologisch laboratorium op de tweede verdieping van het oude dierenlab heb ik met hulp van Wim Kleinhans en Francien van de Pol de IVCT-opstelling opgebouwd. Francien en Wim, bedankt voor jullie onontbeerlijke ondersteuning bij het uitvoeren van de IVCT in de periode 1993-2003.

Op het anesthesiologisch laboratorium in het nieuwe centrale dieren-laboratorium kreeg de IVCT opstelling een prominente plek. Hier kon ik terugvallen op Jan van Egmond. Beste Jan, bedankt voor je hulp in het lab maar zeker ook voor je antwoorden op vele andere 'vraagjes'.

Sinds een jaar wordt de IVCT op het klinisch-chemisch laboratorium in het CWZ uitgevoerd en zijn we druk bezig om routinematig genetisch onderzoek voor alle MH-patiënten op te zetten. Yvonne, Truus, Jos, Corné en Jos ik hoop dat jullie mij met jullie deskundigheid nog lang bijstaan; alvast bedankt.

Op deze plaats is ook een bedankje op zijn plaats aan alle patiënten die zich lieten onderzoeken en daarbij tegelijkertijd mijn onderzoek dienden.

Sandra Eichelsheim en Annemiek Milder, bedankt voor al jullie perfecte secretariële regelwerk.

Ook de chirurgen die de spierbiopsieën verrichtten ben ik veel dank verschuldigd. Waren het in het Radboud de kinderchirurgen: Festen, Rieu, Severijnen en van der Staak, nu zijn het de vaatchirurgen Barendregt en Boll die de biopten zorgvuldig nemen. Beste Cees, Paul, René, Frans, Wout en Bart dank voor jullie medewerking.

Bij het fundamenteel gedeelte van mijn onderzoek ben ik welwillend bijgestaan door Arie Oosterhof, Albert Tangerman en Henk ter Laak; heren bedankt. Met jullie kennis en kunde hebben jullie ervoor gezorgd dat ik als anesthesioloog niet ben verdwaald in de biochemische, fysisch-chemische en morfologische aspecten van MH.

Misschien wel de belangrijkste motivatie voor mij vormde de vriendschap en verbondenheid die ik binnen de Europese MH groep heb mogen ervaren. Dear EMHG colleagues, thank you very much for your friendship, inspiration and the feeling of solidarity. I hope our co-operation may continue for a long time.

Beste collegae anesthesiologen in het CWZ, bedankt dat jullie mij de ruimte gaven en nog steeds geven, om het MH-werk erbij te doen. Ondanks de frequente wisselingen in de samenstelling van onze vakgroep zijn de amicale sfeer en de collegialiteit niet aangetast. Ik voel me nog steeds als een vis in het water. Willem, Frans, Wils, Henriëtte, Huub, Amar, Henry, Eric, Karel, Frans, René, Stijn, Robert en Lonneke, bedankt.

Lieve Marion, er is niemand zoals jij bent voor mij. Voor wat betreft mijn promotie dank ik je voor je relativerende opmerkingen; ondanks of juist dankzij deze bespiegelingen heb ik dit boekje toch maar mooi afgemaakt.

Rest mij tot slot nog Willem Teunissen en Ivo van Sluis te bedanken voor hun adviezen ten aanzien van de vormgeving van dit boekwerk, waarbij ik Anne, Dave, Christel en Andrew zeer erkentelijk ben voor hun "comments" op mijn engels, thanks!

Colofon

Ontwerp, fotografie en dtp Studuo, Ivo van Sluis en Willem Teunissen BNO *Nijmegen*
Drukwerk Drukkerij Benda bv *Nijmegen*