CHAPTER ONE

INTRODUCTION

Malignant hyperthermia (MH) is an autosomal dominant pharmacogenetic disorder (Deufel *et al.*, 1992) and this potentially fatal disorder is one of the leading causes of death due to anaesthesia. An MH episode is triggered when MH susceptible individuals are exposed to certain inhalation anaesthetic agents and depolarising skeletal muscle relaxants (MacLennan, 1992).

The clinical symptoms of MH are highly variable and may even present after anaesthesia has been discontinued - generally within the first 48 hour period (Donnelly, 1994). Detection of MH susceptible individuals prior to receiving anaesthesia is important. The *in vitro* contracture test (IVCT) and the caffeine halothane contracture test (CHCT) are currently used worldwide to determine the MH status of individuals (Kalow *et al.*, 1970). The IVCT refers to the European (European Malignant Hyperpyrexia Group, 1984) and the CHCT to the North American (Larach, 1989) protocol utilised for diagnostic purposes. Both the IVCT and CHCT are highly invasive, expensive and time consuming (Hopkins *et al.*, 1994). The European IVCT protocol is 99% sensitive and 93% specific (Ørding *et al.*, 1997). The sensitivity and specificity of the CHCT is 97% and 78%, respectively (Allen *et al.*, 1998). However, false-negative diagnoses have been reported for both these protocols (Isaacs and Badenhorst, 1993; Wedel and Nelson, 1994). This type of erroneous diagnoses holds a grave risk for the MHS individual and highlights the need for a sensitive and non-invasive MH diagnostic test.

Mutations in the Ca^{2+}-release channel genes of skeletal muscle result in the cytoplasm of the cell being flooded with Ca^{2+}. The elevated cytoplasmic Ca^{2+} concentration triggers a cascade of metabolic reactions, culminating in the MH phenotype (MacLennan and Phillips, 1992). Skeletal muscle Ca^{2+}-release channels are encoded by the skeletal muscle ryanodine receptor gene (RYR1), located on human chromosome 19q13.1 (MacKenzie *et al.*, 1990). To date, twenty-three mutations in the RYR1 gene have been reported to co-segregate with the MH phenotype (Brandt *et al.*, 1999). These mutations are discussed in detail in chapter two where a review of the MH literature is presented. Clinical symptoms, diagnosis and the treatment of MH are discussed along with the pathophysiology and underlying genetic basis of MH.

It was envisaged that the specific mutation segregating in some MH families would be identified in this study leading to a molecular diagnostic service being available for individuals within particular MH families. One of the aims was that a mutation screening service for these nine reported mutations would be established and that this study might also contribute to evidence of possible interaction between mutations and polymorphisms which might modify, or contribute towards, the MH phenotype. The detailed aims of this study are presented in paragraph 2.7 (page 53) of chapter two.

Seventeen MH families, four South African and thirteen North American, were included in this study. Selected individuals in these families were screened for the presence of nine of the missense mutations (Cys35Arg, Arg163Cys, Gly248Arg, Gly341Arg, Ile403Met, Tyr522Ser, Arg614Cys, Gly2435Arg and Arg2436His) reported in the RYR1 gene. The families, pedigrees of the families and clinical information of probands are presented in chapter three. The materials and methods utilised for the detection of the investigated MH missense mutations are also presented in this chapter.

The results of this study are presented and discussed in chapter four. In this chapter each of the nine missense mutations are discussed individually, and a synopsis of the mutation detection results are presented in Table 4.3 (page 128). The conclusions drawn from the results generated in this study are presented in chapter five which also includes future perspectives on this ongoing research programme.

CHAPTER TWO

THE AETIOLOGY AND PATHOGENESIS OF MALIGNANT HYPERTHERMIA

Malignant hyperthermia (MH) is a hypermetabolic disorder of skeletal muscle that is inherited as an autosomal dominant trait (Gronert, 1980). This disorder was first described in 1960 by Denborough and Lovell who reported the case of a 21-year-old student who was reluctant to undergo general anaesthesia because of the death of his close relatives due to ether anaesthesia.

The estimated incidence of MH in the North American population is 1 in 40 000 among adults (Kaus and Rockoff, 1994). The incidence of MHS is higher in children, between 1 in 10 000 and 1 in 15 000, indicating reduced penetrance with increased age (Ball and Johnson, 1993; Kaus and Rockoff, 1994).

It has been estimated that between 1 in 1000 and 1 in 11 000 patients that undergo general anaesthesia in Japan develop MH (Moochhala *et al.*, 1994). In contrast Ball and Johnson (1993) reported a lower frequency for individuals receiving anaesthesia in the United Kingdom, between 1 in 50 000 and 1 in 75 000. In the Danish population Ørding *et al.* (1985) determined that fulminant MH occurs in 1 in 250 000 anaesthetic cases. These authors also reported an MH prevalence of 1 in 16 000 for all types of anaesthetics, and 1 in 4200 anaesthetics when a combination of potent inhalation agents and succinylcholine is used (Ørding *et al.*, 1985).

2.1 Clinical aspects of malignant hyperthermia

Clinically MH is highly variable and in most cases symptoms occur during anaesthesia. However, cases have been reported where the symptoms occurred after termination of the initial anaesthesia, when the patient was in the recovery room (Donnelly, 1994; Carr *et al.*, 1995; Souliere *et al.*, 1986). Ryan and Tedeschi (1997) reported a case of sudden unexplained death of a 23-year-old male with a family history of MH. This male, who was in excellent physical condition, died during moderate exercise and had an elevated temperature of 41°C two hours post-mortem.

Tachycardia is often the first sign of an acute MH episode, but is generally mistaken for

"light anaesthesia" which results in the administration of more anaesthesia (Donnelly, 1994). This may lead to arrhythmia such as ventricular fibrillation and unstable blood pressure. Cases have been reported where skeletal muscles become severely rigid (Hackl *et al.*, 1990). Sustained contracture of the masseter muscle then results in difficulty when intubating patients.

Although fever is a distinctive clinical feature of malignant hyperthermia during most fulminant MH episodes (Sessler, 1986; Hackl *et al.,* 1990), cases have been reported where no abnormal elevation of body temperature was recorded (Muiños *et al.*, 1995; Fierobe *et al.*, 1998, Harwood and Nelson, 1998). The skeletal muscle alone is the source of heat production (Sessler, 1986). During the acute phase of an MH episode an increase in body temperature of approximately 0.5°C every five minutes might result in a core body temperature as high as 46°C (Nelson, 1989).

Other clinical symptoms of an MH episode include tachypnoea, which is secondary to the increased carbon dioxide (CO_2) production, increased end-tidal CO_2, hypercarbia, hyperkalaemia and an increase in the following biochemical parameters: creatine phosphokinase (CPK), lactate dehydrogenase (LDH), myoglobin, and carbon dioxide partial pressure (pCO_2). A reduction in pH results in severe metabolic and respiratory acidosis. There are also coagulation abnormalities, imbalances in magnesium, calcium, phosphate and potassium levels. The skin presents with erythematous flush, peripheral mottling and cyanosis, secondary to extensive vasoconstriction and accelerated oxygen consumption by the muscles. (Allen, 1994; Donnelly, 1994; MacLennan, 1992).

Without immediate treatment, MH episodes are generally fatal. The patient might die within minutes from ventricular fibrillation, within hours from pulmonary oedema or coagulopathy, or within days from neurological damage or obstructive renal failure due to myoglobinuria (MacLennan, 1992; MacLennan and Phillips, 1992).

Due to the highly variable clinical presentation and the lack of a precise clinical definition of MH it is extremely difficult to diagnose an MH episode via clinical criteria alone. Larach *et al.* (1994) created a clinical grading scale that could be applied to two different situations. Firstly to estimate the likelihood that the adverse anaesthetic events are clinical MH, and secondly to estimate an individual's likelihood of MH susceptibility when the individual has a family history of MH susceptibility with or without any adverse personal anaesthetic events (Larach *et al.*, 1994).

The clinical grading scale used to predict MH susceptibility is listed in Table 2.1A. Scoring rules were stipulated and should be followed when using this grading system. These rules are divided into two sections: the MH indicators and the MH susceptibility indicators. Several physiological processes are involved in MH and these processes manifest as various MH indicators during anaesthesia. The list of clinical MH indicators must be reviewed and if any of these indicators are present the applicable points for each indicator should be added. However, the double-counting rule, discussed below, must be observed as it applies to multiple indicators from a single process. If none of the indicators are observed in a patient the score is zero.

Table 2.1: Clinical grading scale to predict malignant hyperthermia

2.1A Clinical indicators used to determine the malignant hyperthermia raw score

Processes and indicators	Points
Process I: *Rigidity*	
Generalised muscular rigidity (in the absence of shivering due to hypothermia, or during or immediately following emergence from inhalation general anaesthesia) ...	15
Masseter spasm shortly following succinylcholine administration	15
Process II: *Muscle Breakdown*	
Elevated creatine kinase >20 000 IU after anaesthetic with succinylcholine	15
Elevated creatine kinase >10 000 IU after anaesthetic without succinylcholine	15
Cola coloured urine in peri-operative period	10
Myoglobin in urine >60 $\mu g.l^{-1}$	5
Myoglobin in serum >170 $\mu g.l^{-1}$	5
Blood/plasma/serum K^+ >6 $mEq.l^{-1}$ (in the absence of renal failure)	3
Process III: *Respiratory Acidosis*	
$P_{ET}CO_2$ >55 mmHg with appropriately controlled ventilation	15
Arterial P_aCO_2 >60 mmHg with appropriately controlled ventilation	15
$P_{ET}CO_2$ >60 mmHg with spontaneous ventilation	15
Arterial P_aCO_2 >65 mmHg with spontaneous ventilation	15
Inappropriate hypercarbia (in anaesthesiologist's judgement)	15
Inappropriate tachypnea	10
Process IV: *Temperature Increase*	
Inappropriately rapid increase in temperature (in anaesthesiologist's judgement)	15
Inappropriately increased temperature >38.8°C in the peri-operative period (in anaesthesiologist's judgement)	10
Process V: *Cardiac Involvement*	
Inappropriate sinus tachycardia	3
Ventricular tachycardia or ventricular fibrillation	3
Process VI: *Family History (used to determine MH susceptibility only)*	
Positive MH family history in relative of first degree[1)]	15
Positive MH family history in relative not of the first degree[1)]	5
Other indicators that are not part of a single process[2)]	
Arterial base excess more negative than –8 $mEq.l^{-1}$	10
Arterial pH <7.25	10
Rapid reversal of MH signs of metabolic and/or respiratory acidosis with *intra venous* (IV) dantrolene	5
Positive MH family history together with another indicator from the patient's own anaesthetic experience other then elevated resting serum creatine kinase[1)]	10
Resting elevated serum creatine kinase [1)] (in patient with a family history of MH)	10

continued ...

Table 2.1, continued ...

2.1B: Interpreting the raw score: MH rank and qualitative likelihood		
Raw Score Range	**MH Rank**	**Description of likelihood**
0	1	Almost never
3-9	2	Unlikely
10-19	3	Somewhat less than likely
20-34	4	Somewhat greater than likely
35-49	5	Very likely
50+	6	Almost certain

As listed by Larach *et al.* (1994) and Olckers (1997). 1) Indicators should be used only for determining MH susceptibility. 2) These indicators should be added without regard to double counting (see text).

The double-counting rule applies when more than one indicator, representing a single process, is observed. In such a case only the indicator with the highest score is counted. This rule prevents double counting when the clinical process has more than one clinical manifestation. An exception to the double counting rule is the score of the final category "other indicators". These points should be added to the total score without regard to the double counting rule. The MH susceptibility indicators cannot be used to score an MH event and can only be applied to MH susceptibility. Scores are added and the raw scores are interpreted using the guidelines listed in Table 2.1B (Larach *et al.*, 1994). An "MH rank" is thus determined, ranging from 1 (almost never) to 6 (almost certain).

2.2 Diagnosis of malignant hyperthermia

Detection of MH susceptible (MHS) individuals before anaesthesia is critical. Kalow *et al.* (1970) demonstrated that the skeletal muscle strips from MHS patients showed enhanced sensitivity to caffeine and halothane. In 1972, Ellis *et al.* reported that MHS muscle specimens developed an abnormal contracture when exposed to halothane alone. These observations lead to the development of a diagnostic test for MH. A test based on contracture studies is, to date, the most widely used diagnostic procedure to determine MH susceptibility (Larach *et al.*, 1992). It involves measurement of contractions in the presence of caffeine and halothane, with the muscle fibres from MHS individuals exhibiting enhanced contracture when exposed to either of these substances (Rosenberg, 1989).

General guidelines have been suggested by Kaus and Rockoff (1994) to select individuals that should be considered for testing, and include the following:

1. Patients with a questionable MH reaction or isolated signs related to MH during or following general anaesthesia with trigger agents.
2. Patients with a suspected myopathy.
3. Close relatives of an MHS patient.

- One parent of the proband should be tested.
- The second parent should be tested whenever the first parent tested negative for MH.
- All the siblings and close relatives of an MHS individual are at risk and should, therefore, be tested for MH.

Two diagnostic protocols, the North American caffeine halothane contracture test (Larach, 1989) and European *in vitro* contracture test protocols (European Malignant Hyperpyrexia Group, 1984), are currently used for diagnostic purposes. These protocols differ in the procedure and interpretation of the results (MacLennan and Phillips, 1992) and are discussed below.

2.2.1 ***European in vitro contracture test (IVCT) protocol***

The European protocol was utilised to diagnose MHS individuals in South Africa. In an attempt to establish a standardised IVCT protocol the European Malignant Hyperpyrexia Group proposed certain criteria (European Malignant Hyperpyrexia Group, 1984). Two muscle strips obtained from the region of the quadriceps by way of biopsy, which included the motor point, are exposed to increasing concentrations of caffeine and halothane. The results of both caffeine and halothane are reported as the concentration of either caffeine or halothane at which a 0.2 g contracture was recorded (European Malignant Hyperpyrexia Group, 1984).

A positive IVCT (MHS) is defined as a 0.2 g contracture at a caffeine concentration of 2 mM or less, and a halothane concentration of 2% or less. The European protocol requires a positive reaction to both caffeine and halothane for an MHS diagnosis. Individuals that test positive for either caffeine or halothane, but not both, are classified as MH equivocal (MHE) and further subdivided as MHEc (positive only for caffeine) or MHEh (positive only for halothane), respectively. These individuals are, however, clinically treated as MHS (European Malignant Hyperpyrexia Group, 1984; Fletcher *et al.*, 1999).

The sensitivity and specificity of the IVCT was thought to be 100% and 93%, respectively (Ørding *et al.*, 1997). Initially Ørding *et al.* (1991) evaluated 119 anaesthetic events in 371 patients diagnosed via the IVCT and found that none of the individuals classified as MH normal (MHN) developed any signs of MH after receiving triggering agents. This implied that no false-negative diagnoses were made and that the sensitivity of the IVCT was

indeed 100%. However, cases of false-negative diagnoses have been reported (Isaacs and Badenhorst, 1993; Wedel and Nelson, 1994). Subsequently, the sensitivity and specificity of the IVCT was determined to be 99% and 93.6% respectively in a multi-centre study, including 22 centres (Ørding *et al.*, 1997; Islander and Twetman, 1999). The fact that false-negative diagnoses have been observed is alarming as the threshold levels of the IVCT have been set specifically to avoid false-negative diagnoses (Hopkins *et al.*, 1997). False-negative diagnoses can have fatal subsequences as these patients are erroneously considered to be MHN and are therefore exposed to triggering agents during surgical procedures.

2.2.2 ***North American caffeine halothane contracture test (CHCT) protocol***

Individuals from North American families were diagnosed utilising the North American protocol. In 1989, the North American malignant hyperthermia group compiled the standardised North American protocol for the caffeine halothane contracture test (CHCT) (Larach, 1989; Melton *et al.*, 1989). According to the protocol the preferred site for the muscle biopsy is the vastus group. Larach (1989) also suggested the rectus abdominus or other muscle groups as biopsy sites under special circumstances.

An individual is classified as MHS, for clinical purposes, when one of the viable muscle strips examined demonstrated an abnormal contracture response to either caffeine or halothane or both. A positive halothane result was defined as a contracture greater than 0.2 g - 0.7 g, after exposure to 3% halothane for 10 minutes. Each centre determined its own threshold after reviewing approximately 30 control subjects. The caffeine contracture was considered positive for MHS when the contracture was greater than 0.2 g in the presence of 2 mM caffeine (Larach, 1989).

Larach *et al.* (1992) suggested that the proposed diagnostic guidelines had to be modified to ensure greater specificity. These authors also suggested that a uniform method for analysing the *in vivo* adverse response to anaesthetics of the patient be developed to define the sensitivity of the North American CHCT. The sensitivity of the CHCT should be 100%, to eliminate false-negative results. Allen *et al.* (1998) reported that an adjustment to the CHCT cut off values would ensure a higher sensitivity of the CHCT. The new cut off values, yielding the highest test sensitivity were ≥ 0.5 g for 3% halothane, and ≥ 0.3 g for 2 mM caffeine (Allen *et al.*, 1998). Subsequent to these adjustments the sensitivity and specificity were calculated to be 97% and 78% respectively (Allen *et al.*, 1998; Islander

and Twetman, 1999). The North American MH registry recommended that a new cut off value of ≥ 0.5 g for 3% halothane be used for clinical purposes due to the improved sensitivity and therefore a reduced number of false-negative diagnoses – despite the fact that it necessitates the introduction of false-positive diagnoses (Fletcher *et al.*, 1999). However, for genetic studies a more stringent halothane cut off value of 0.7 g was suggested in order to reduce the false-positive diagnoses thus making the CHCT and IVCT results more comparable with each other (Fletcher *et al.*, 1999).

2.2.3 ***Non-invasive procedures for diagnosing malignant hyperthermia***

The IVCT is an invasive, expensive and time consuming procedure (Hopkins *et al.*, 1994). Furthermore, as discussed earlier in paragraphs 2.2.1 and 2.2.2, neither the European nor the North American protocols are 100% sensitive. The IVCT is also not suitable for routine diagnostic screening of small children, a group that is at high risk due to the elevated incidence of MH in children (Glauber *et al.*, 1997; Hogan, 1998).

A non-invasive or less invasive test is needed for the diagnosis of MHS. Many diagnostic tests, other than the IVCT, have been investigated over the years, some of which are listed in Table 2.2. These tests can be arranged into five categories: blood, electrophysiological, biochemical muscle, muscle histology and contractures. However, both muscle histology and contracture tests are invasive, requiring a muscle biopsy.

Many, but not all, MHS individuals had increased CK activity. Moreover, an increased CK activity is a non-specific indicator of muscle injury that may be due to factors other than MH (Ørding, 1988). The other blood tests listed in Table 2.2 were also unsuccessful in their diagnostic value for MH, mainly due to the lack of specificity. The most promise was displayed by the procedure in which the calcium concentration in lymphocytes was measured. The calcium concentration was significantly different in the MHS and MHN groups with very little overlap. However, further investigation of this test is required to evaluate its MH diagnosing potential (Ørding, 1988).

The electrophysiological tests proved not suitable for the diagnosis of MH, as there was no discernible distinction between the normal and the MH group investigated. Furthermore, the tests were less specific than the currently utilised contracture test (Ørding, 1988). Analysis of the biochemical muscle tests demonstrated that none of these could replace the muscle contracture test.

Table 2.2: Non-invasive diagnostic procedures investigated for the diagnosis of malignant hyperthermia

Diagnostic procedures
Blood tests
Serum tests
Creatine kinase (CK)[1, 2]
Cholinesterase [2]
Erythrocyte tests
Osmotic fragility [2]
Chemiluminescence for quantitating auto-oxidation [2]
Spin labelled red cell technique [3]
Platelet tests
Platelet aggregation [2]
Platelet nucleotide depletion [2, 4]
White cell tests
Human leukocyte antigen (HLA) type [2]
Calcium concentration in lymphocytes [2]
Electrophysiological tests
Motor unit counting [2]
Tourniquet-twitch test [2, 5]
Relaxation rates of the elicited twitch response [2]
Recruitment pattern after halothane and suxamethonium [2]
Biochemical muscle tests
Adenosine triphosphate (ATP) depletion [2, 6]
Glycolytic metabolites [2]
Myophosphorylase ratio [2]
Adenylate kinase deficiency [2, 7]
Adenylate cyclase activity and cyclic adenosine monophosphate (AMP) [2]
Adenylate deaminase deficiency [2]
Low weight proteins [2]
Calcium uptake by sarcoplasmic reticulum [2, 8]
Intracellular ionised calcium concentration [2, 9, 10]
Heat production [2]
Nuclear magnetic resonance scanning [2, 11, 12]

[1] Paasuke and Brownell (1986), [2] Ørding (1988), [3] Ohnishi *et al.* (1988), [4] Lee *et al.* (1985), [5] Britt *et al.* (1986), [6] Britt *et al.* (1976), [7] Cerrie *et al.* (1981), [8] Nagarajan *et al.* (1987), [9] Lopez *et al.* (1985), [10] Klip *et al.* (1987), [11] Olgin *et al.* (1988), [12] Olgin *et al.* (1991).

It seems that the IVCT and the CHCT are presently the only reliable methods for diagnosing MH. Hartung *et al.*, (1996) and Gilly *et al.*, (1997) have proposed the use of the ryanodine test protocol and the use of 4-Chloro-M-Cresol in conjunction with the European IVCT in order to define the MHE individuals more accurately on a phenotypical level. It appears as though the development of a DNA based molecular diagnostic protocol would be the only non-invasive procedure to diagnose MH 100% accurately. However, the development of a molecular diagnostic protocol presents with its own unique problems since the genetics of MH is complex due to the genetic heterogeneity that exists. The genetic aspects of MH are discussed in paragraph 2.6.

2.3 Clinical management of malignant hyperthermia

Early diagnosis and treatment of an MH episode is essential to reduce mortality. It is, therefore, important that all the necessary supplies be at hand to treat a fulminant MH episode. An MH cart with the necessary medication and supplies is therefore recommended for all operating rooms and emergency surgery centres. The supplies that should be kept in the cart are listed in Table 2.3. It is further recommended that a written protocol for the treatment of an MH episode be posted in operating rooms and surgery centres (Donnely, 1994; Golinski, 1995).

Table 2.3: Medication and medical supplies for a malignant hyperthermia cart

Medication	
36 vials	20 mg, IV dantrolene sodium
4 bottles	500 ml, sterile water for injections
6 ampoules	50 ml, 8.4% sodium bicarbonate
10 vials	12.5 g, 25% mannitol
4 ampoules	100 mg furosemide
2 ampoules	50 ml, 50% dextrose
1 vial	10 ml, 100 $U.ml^{-1}$ regular insulin (refrigerated)
3 vials	10 ml, 1000 $U.ml^{-1}$ heparin
6 bags	1000 ml, IV 0.9% sodium chloride (refrigerated)
Supplies	
Written protocol for the treatment of a malignant hyperthermia episode	
Cooling blankets	
Ice machine	
50 ml syringes	
Nasogastric tubes	
Plastic bags for ice	
Blood pump	
Hemodynamic monitoring equipment	
Foley catheters	
IV additive pin devices to ease mixing of water with dantrolene	
Extra anaesthesia breathing circuits	
Tubes for pertinent laboratory tests	

As listed by Donnelly (1994) and Golinski (1995).

In the event of an acute MH episode the following protocol should be followed:

1. Exposure to trigger agents, listed in Table 2.4, should be discontinued. If the surgery cannot be terminated the procedure should be continued with the use of safe agents (listed in Table 2.4).
2. The patient should be hyperventilated with 100% oxygen at high flow rates. This reduces the risk of hypoxia and helps prevent hypercarbia and respiratory acidosis. If at all possible the anaesthesia circuit and CO_2 absorbent should be changed.
3. Dantrolene should be administrated intravenously (initial *intra venous* dose of 2 to

4 $mg.kg^{-1}$ with a limit of 10 $mg.kg^{-1}$) as soon as possible. Administration of dantrolene should be continued until MH symptoms have disappeared and clinical signs have normalised.

4. During an acute MH episode the metabolic acidosis should be corrected with an alkalinising agent such as 1 to 2 $mEq.kg^{-1}$ *intra venous* sodium bicarbonate. *Intra venous* dextrose and regular insulin can be administered if hyperkalemia persists. This facilitates the redistribution of potassium from extracellular to intracellular compartments by both sodium bicarbonate and insulin.
5. Cooling the patient as soon as possible treats pyrexia. Surface cooling can be achieved with ice packs and cooling blankets. Iced saline solutions can be administered intravenously and surgical wounds, stomach, bladder and rectum can also be lavaged with chilled saline. As soon as the patient's temperature reaches 38°C cooling should be discontinued.
6. Further treatment may be needed and is determined by monitoring arterial and venous blood gasses, central venous pressure, urine output, temperature, $P_{ET}CO_2$, potassium, calcium, LDH, CPK, urine myoglobin and clotting studies.
7. It is not uncommon that ventricular arrhythmias, e.g. ventricular fibrillation, persist even after acidosis and hyperkalemia have been corrected. An appropriate anti-arrhythmic agent such as lidocaine or procainamide can be administered without any adverse effect. However, calcium channel blockers (verapamil) should not be used with dantrolene since they may result in myocardial depression (Kaus and Rockoff, 1994).
8. The patient should be transferred to the intensive care unit (ICU) for at least 24 hours of observation. CPK, electrolytes and clotting profiles should be monitored until normal along with the electrocardiogram, blood pressure, blood gasses, temperature and urine output.

This treatment protocol was described by Halsall and Ellis (1993), Donnely (1994), Kaus and Rockoff (1994), Golinski (1995) and Mauritz *et al.* (1997).

Table 2.4: Safe agents and trigger agents for malignant hyperthermia

Safe agents	Trigger agents
Barbiturates thiopental methohexital **Anaesthetic IV agents** propofol etomidate ketamine	**Inhalational agents** halothane[a)] enflurane isoflurane desflurane sevoflurane methoxyflurane[b)] cyclopropane[b)]

continued ...

Table 2.4, continued ...

Safe agents	Trigger agents
Inhalational agents	**Inhalational agents (continued ...)**
nitrous oxide	ether[b)]
Benzodizepines	**Neuromuscular blocking agents**
diazepam	Succinylcholine / suxamethonium
midazolam	decamethonium
lorazepam	gallamine
Opioids	d-Tubocurarine
morphine	**Local anaesthetics**
meperidine	lidocaine
hydromorphone	bupivacaine
fentanyl	mepivacaine
sufentanil	**Other**
alfentanil	belladonna alkaloids (atropine)
Neuromuscular blocking agents	phenothiazines
pancuronium	meperidine
atracurium	cardiac glycosides
vecuronium	corticosteroids
doxacurium	
pipecuronium	
mivacurium	
Local anaesthetics	
some local anaesthetics	

As listed by Ahmad *et al.* (1985), Sims (1992), Donnely (1994), Kaus and Rockoff (1994), Belmont (1995), Ducart *et al.* (1995), Ben Abraham *et al.* (1997), Michalek-Sauberer (1997), Allen and Brubaker (1998) and Garrido *et al.* (1999). a) Inhalational agent most often associated with MH. b) No longer in use clinically.

A few major complications should be considered subsequent to an acute MH episode. Recurrence of an MH reaction within hours of the first episode is possible, as well as temperature fluctuation for several days. Coagulation abnormalities such as disseminated intravascular coagulation has been reported and may be as a result of the release of thromboplastins. Myoglobinuria usually occurs within 4 to 8 hours of an MH episode and may result in renal failure if not treated. It is recommend that the bladder is catheterised and a high alkalinised urine output is maintained until myoglobinuria clears. Muscle weakness due to dantrolene and or muscle destruction may be severe and patients are at risk of respiratory compromise due to muscle weakness. It is also recommended that dantrolene be continued for 48 to 72 hours as it is the most important treatment for an acute MH episode and treats the underlying cause (Halsall and Ellis, 1993; Donnely, 1994; Kaus and Rockoff, 1994).

2.4 Disorders associated with malignant hyperthermia

MH has been associated with a number of other disorders which can be divided into three categories: (1) disorders that appear to be almost certainly associated with MH, (2) disorders which are possibly associated with MH and (3) disorders where the association appears coincidental (Brownell, 1988). It is unknown whether MH susceptibility is additional to the disorder from which the individual suffers, or if the symptoms are due to the reaction of abnormal muscle to the anaesthetic agents and, therefore, not indicative of an MH reaction (Kaus and Rockoff, 1994).

One disorder that is associated with MH is central core disease (CCD), a non-progressive or slowly progressive myopathy (Allen, 1994). Denborough *et al.* (1973) described the first association between these two disorders, after treatment of a patient with both MH and CCD. Since then the locus for both CCD and MH has been localised to chromosome 19q13.1 and the same gene, the skeletal muscle ryanodine receptor (RYR1) gene, identified to be involved in both these disorders. CCD and MH are actually allelic variants as mutations within the same gene are responsible for both these phenotypically distinct disorders (Zhang *et al.*, 1993; Quane *et al.*, 1993; Quane *et al.*, 1994b).

Masseter muscle spasm (MMS) or jaw rigidity has been described as the most common clinical symptom associated with the early stages of an MH reaction (Christian *et al.*, 1989). MMS may result in a difficulty to intubate, leading to complications in airway management (Sims, 1992; Vita *et al.*, 1995). Patients who received succinylcholine and developed MMS, (Sims, 1992) although not all of them are MHS, will suffer a fulminant MH episode. It is estimated that 50% of these patients may be at risk for MH or another undiagnosed myopathy (Christian *et al.*, 1989; Sims, 1992). Allen (1993) stated that MMS alone is not indicative of MHS. However, caution should be taken when it occurs during anaesthetic and the patient should be monitored carefully for other clinical signs indicative of MH (Allen and Rosenberg, 1990; Allen, 1993). The only pre-symptomatic indicator of MMS is the Gly1306Ala mutation identified by Vita *et al.* (1995) in the adult muscle sodium channel α sub-unit (SCN4A) gene. This mutation is associated with succinylcholine-induced MMS, whole body rigidity, and abnormal IVCT results (Vita *et al.*, 1995).

There are various other myopathic conditions (listed in Table 2.5) where an association with MH has been reported but the exact relationship between these disorders remains unclear. Duchenne muscular dystrophy (DMD) is one of the disorders that seem to be

associated with MH (Brownell *et al.*, 1983). Kaus and Rockoff (1994) reported that 50% of DMD patients have positive IVCT results. However, the positive IVCT results may be due to the abnormalities in the DMD muscle and not because of MHS. Therefore, DMD patients should be considered to be at risk for anaesthetic complications and should be carefully monitored. Although many cases of MH and DMD have been reported, it is important to state that not all DMD patients are MH susceptible, and that the association between these two disorders has not yet been elucidated (Kaus and Rockoff, 1994).

Isaacs and Gericke (1990) reported two patients in whom MH is thought to be responsible for the congenital abnormalities observed. However, these authors suggested that the association of MH and congenital abnormalities might be coincidental. In a study performed by Gericke and Isaacs (1990) 25 children with birth defects were investigated via the IVCT. Fifteen of these children were diagnosed as MH positive. Muscle strips of five of these MHS children responded to both halothane and caffeine, with the remainder only responding to halothane. It is therefore possible that an association exists between MH and certain congenital abnormalities including myotonia congenita. However, the IVCT is not 100% specific (as discussed in paragraph 2.2.1) and false-positive MH diagnoses are possible. There is, however, an anaesthetic risk for patients with congenital abnormalities which cannot be ignored (Gericke and Isaacs 1990).

There are a number of other disorders associated with MH in which the association appears to be coincidental. These disorders are also listed in Table 2.5 and include disorders such as sudden infant death syndrome, neuroleptic syndrome, Burkett's lymphoma and arthrogryposis. Baines *et al.* (1986), Brownell (1988) and Adnet *et al.* (1989) all discounted the possibility that an association may exist between these disorders.

The relationship between osteogenesis imperfecta (OI) and MH is not clear. Rampton *et al.* (1984), Ryan *et al.* (1989) and Porsborg *et al.* (1996) reported individuals with OI in whom MH reactions were observed. IVCT was performed for one individual with OI and the result was negative (Porsborg *et al.*, 1996), and none of the other individuals with OI were investigated via the IVCT (Rampton *et al.*, 1984; Ryan *et al.*, 1989). Porsborg *et al.* (1996) and Ryan *et al.* (1989) suggested that an underlying metabolic anomaly is responsible for the anaesthetic complications observed among patients with OI.

Table 2.5: Disorders associated with malignant hyperthermia

Disorders	Reference
Probable association	
Central core disease (CCD)	Denborough *et al.*, 1973; Loke and MacLennan, 1998
Possible association	
Duchenne muscular dystrophy (DMD)	Brownell *et al.*, 1983.
King-Denborough syndrome	King and Denborough, 1973
Becker's muscular dystrophy	Gericke and Isaacs, 1990; Isaacs and Gericke, 1990
Myotonia congenita	Gericke and Isaacs, 1990; Isaacs and Gericke, 1990
Schwartz-Jampel syndrome	Seay and Ziter, 1987
Myoadenylate deaminase deficiency	Brownell, 1988
Sarcoplasmic reticulum adenosine triphosphate deficiency syndrome	Brownell, 1988
Mitochondrial myopathy	Ohtani *et al.*, 1985
Smith-Lemli-Opitz syndrome	Petersen and Crouch, 1995; Haji-Michael and Hatch, 1996; Peterson and Crouch, 1996
Masseter muscle spasm (MMS)	Christian *et al.*, 1989; Allen and Rosenberg, 1990
Coincidental association	
Sudden infant death syndrome	Brownell, 1988
Burkett's lymphoma	Brownell, 1988
Neuroleptic malignant syndrome	Adnet *et al.*, 1989
Osteogenesis imperfecta (OI)	Rampton *et al.*, 1984; Ryan *et al.*, 1989; Porsborg *et al.*, 1996
Arthrogryposis	Baines *et al.*, 1986
Hernia	Kaus and Rockoff, 1994
Kyphoscoliosis	Kaus and Rockoff, 1994
Strabismus	Kaus and Rockoff, 1994
Ketoacidotic diabetic coma	Wappler *et al.*, 1996

2.5 Pathophysiology and biochemistry of malignant hyperthermia

The primary abnormality of MH is located within skeletal muscle (Gallant *et al.*, 1979). Cytoplasmic calcium ions (Ca^{2+}) regulate the contraction, relaxation and energy metabolism of the muscle cell. The Ca^{2+} concentration in skeletal muscle is regulated by the sarcoplasmic reticulum (SR) and other ion-pumps, -channels and -exchangers located within the muscle cell membrane (MacLennan, 1992; MacLennan and Phillips, 1992). The SR is the main storage site for Ca^{2+} in skeletal muscle cells. Within the SR the Ca^{2+} associate with calsequestrin, a SR luminal calcium binding protein, and is released through a Ca^{2+}-release channel into the cytosol. Here calcium binds to troponin and skeletal muscle contraction is initiated (MacLennan, 1992).

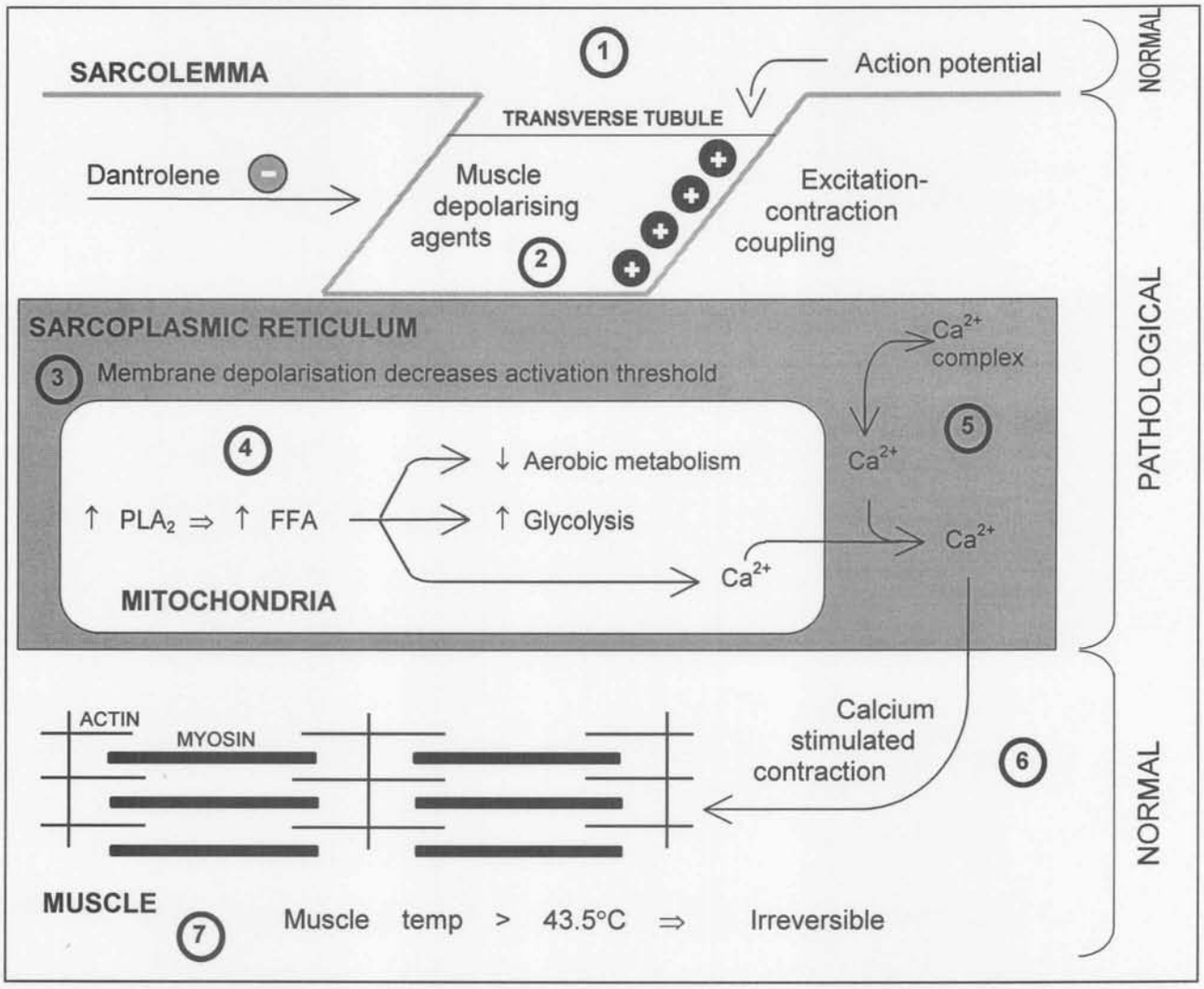
The skeletal muscle ryanodine receptor (RYR1) gene located on human chromosome 19q13.1 (MacKenzie *et al.*, 1990), as discussed in paragraph 2.6.1, encodes the Ca^{2+}-release channel of skeletal muscle. Abnormalities in the Ca^{2+}-release channels located in the SR result in these channels opening for longer periods of time, thus flooding the cytoplasm of the cell with Ca^{2+} (Fill *et al.*, 1990; MacLennan, 1992). The excess Ca^{2+} cause sustained muscle contraction, resulting in rigidity and increased glycolytic and aerobic metabolism that generate lactic acid and heat. The generated heat results in the elevation of the body temperature, which is the symptom most often associated with MH (MacLennan, 1992; MacLennan and Phillips, 1992; Ball and Johnson, 1993).

A schematic representation of the mechanism involved in muscle contraction is presented in Figure 2.1. In muscle the sarcolemma is depolarised by an action potential from a neuromuscular junction. The nerve impulse is transferred to the SR via excitation-contraction coupling (EC-coupling) after the signal travelled along the cell membrane and down the transverse tubule ①. Excitation-contraction coupling is the process which involves depolarisation and contraction initiation of skeletal muscle. The major components responsible for EC-coupling in skeletal muscle are the transverse tubule (T-tubule) and the sarcoplasmic reticulum. T-tubule are formed by folds within the sarcolemma that has the ability to conduct the muscle contraction signal through which muscle contraction is triggered. The SR is an intracellular Ca^{2+} sequestering and releasing membrane system, which contains the Ca^{2+} transporting adenosine triphosphatase (ATPase) and Ca^{2+}-release channel. The SR is activated via a voltage-gated Ca^{2+}-channel, the dihydropyridine receptor, located in the T-tubule membrane.

After depolarisation of the T-tubule the charge movement within the dihydropyridine sensitive L-type Ca^{2+}-channel (DHPR) activates the RYR1 causing it to open (Sessler, 1986; Miller, 1992). Ca^{2+} is released from the lumen of the SR into the myoplasm, resulting in muscle contraction. Although the precise mechanism involved in the interaction of the DHPR and the RYR1 is not yet fully understood it is most likely that protein-protein interaction between the DHPR and the RYR1 results in calcium release (Marty *et al.*, 1994; Meissner and Lu, 1995; Greenberg, 1997; Wingertzahn and Ochs, 1998). Upon activation of the DHPR, a conformational change results in direct physical interaction between the DHPR and the RYR (Marty *et al.*, 1994; Wingertzahn and Ochs, 1998).

The EC-coupling process is enhanced by trigger agents such as halothane, caffeine and succinylcholine ②, but inhibited by dantrolene. In MHS individuals the activation threshold is sufficiently lower to allow a small depolarisation signal by the triggering agents ③ (listed in Table 2.4) that stimulates calcium release. Normal muscle cells are depolarised at -54 mV for contraction, while in MHS muscle the contraction occurs at -86 mV (Sessler, 1986).

Figure 2.1: A schematic representation of the mechanism involved in muscle contraction



Adapted from Olckers (1997).

The phospholipase A_2 (PLA_2) activity is elevated in the mitochondria ④, due to unknown factors, increasing the concentration of mitochondrial free fatty acids (FFA). Mitochondrial calcium is, therefore, released into the SR as a result of the decreased aerobic metabolism and compensatory increase in glycolysis ⑤. SR calcium release is increased and re-uptake inhibited in the presence of FFA. An increase in the calcium concentration allows for the binding of calcium to troponin C ⑥. The myosin binding sites are uncovered and cross–links formed between actin and myosin result in contraction. Eventually when

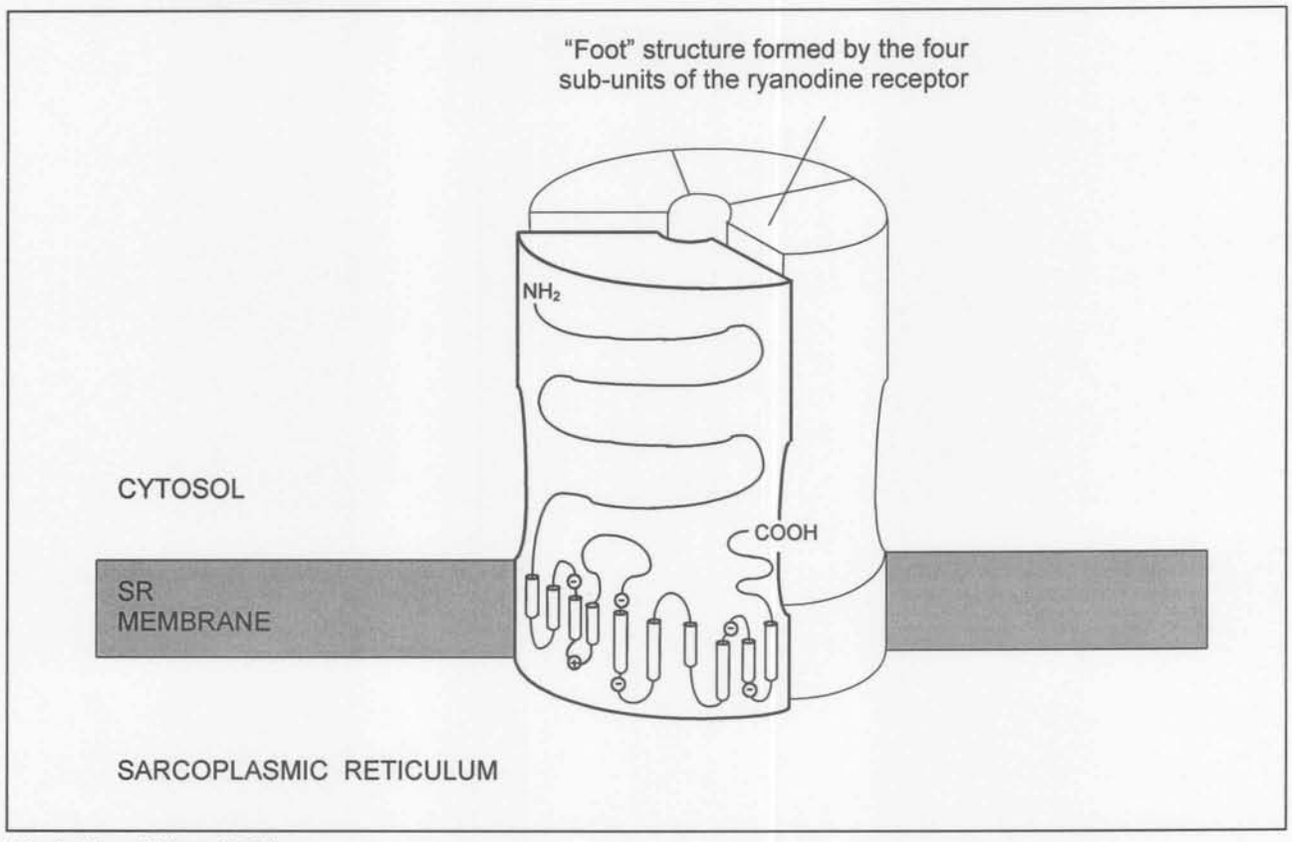
the muscle temperature rises above 43.5°C ⑦, calcium is no longer required for the interaction between actin and myosin and the muscle contraction becomes irreversible (Sessler, 1986).

2.5.1 ***The skeletal muscle ryanodine receptor***

Ryanodine receptors (RYRs) are intracellular Ca^{2+}-channels involved in the releasing of Ca^{2+} from the SR of various tissues. A plant alkaloid, ryanodine, has been essential in the identification of the SR Ca^{2+}-release channels, due to the ability of ryanodine to bind these channels (Pessah *et al.*, 1985). The SR Ca^{2+}-release channels are commonly known as ryanodine receptors. Three ryanodine receptor isoforms, RYR1, RYR2 and RYR3, encoded by three different genes have been identified in mammals (Sorrentino and Volpe, 1993). Isoforms RYR2 and RYR3 are expressed in cardiac muscle and the brain respectively with the RYR1 isoform being expressed predominately in fast- and slow-twitch skeletal muscle but also in the cerebrum, hippocampus, cerebellum heart and esophagus (McPherson and Campbell, 1993; Sorrentino and Volpe, 1993; Coronado *et al.*, 1994; Mickelson and Louis, 1996).

The skeletal muscle ryanodine receptor is a large ion channel composed of four identical polypeptide sub-units (Lai *et al.*, 1989). Wagenknecht *et al.* (1989) demonstrated the three dimensional structure of the ryanodine receptor and the tetrameric structure of the functional channel was shown by electron microscopy (Wagenknecht *et al.*, 1989). Two regions of the ryanodine receptor, the region associated with the junctional face membrane of the terminal cisternae of the SR, and the region associated with the T-tubule, can be distinguished (Wagenknecht *et al.*, 1989). The large N-terminal region of the receptor protein molecule protrudes into the cytoplasm, and can be observed under an electron microscope. It appears as electro-dense square-like structures, referred to as "feet", protruding from the junctional SR membrane (Wagenknecht and Radermacher, 1995). A central cavity, 1-2 nm in diameter, is observed in the centre of the "foot"-like structure (Figure 2.2). This is postulated to be the ion pore formed by the four membrane spanning sub-units (Lai *et al.*, 1988). The "foot"-like structure extends across the gap between the junctional SR membrane and the surface membrane transverse tubule, also known as the triad junction (Sorrentino and Volpe, 1993).

Figure 2.2: Representation of the skeletal muscle ryanodine receptor protein structure



Adapted from Olckers (1997).

Only a relatively small section of the large ryanodine receptor (RYR) molecule is intra-lumenal. The C-terminal of the molecule is contained within the transmembrane segment which anchors the receptor to the membrane of the SR. The receptor protein has ten transmembrane domains located on the intralumenal side of the membrane and which are involved in the control of the gating properties of the Ca^{2+}-release channel (Zorzato *et al.*, 1990). The C-terminal region of the ryanodine receptor forms the channel region (Hawkes *et al.*, 1992).

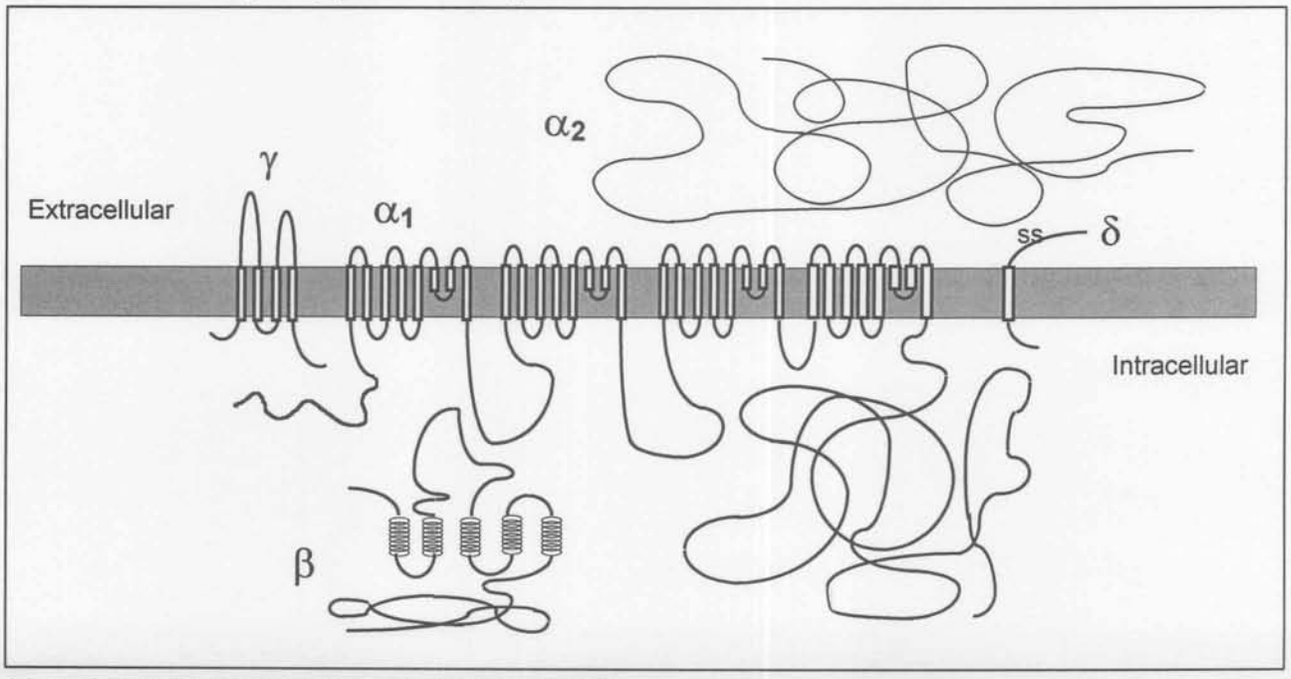
The large cytoplasmic domain of the RYR has a delicate structure with various cavities and grooves. The functional significance of this type of assembly is unclear, but it was suggested that its design allows it to withstand the stresses of muscle contraction while still allowing diffusion of ions through the transmembrane domain (Wagenknecht and Radermacher, 1995). In MH the gating regulation, or the gating mechanism of the ryanodine receptor is affected, resulting in the Ca^{2+}-release channel remaining open for an increased period of time (Hawkes *et al.*, 1992).

2.5.2 *The dihydropyridine receptor*

The dihydropyrodine (DHP) receptor is an L-type Ca^{2+}-channel and can be defined as a high voltage activated channel which is sensitive to 1,4-dihydropyridine compounds (Catterall, 1988; Tsien *et al.*, 1991). These DHP receptors are located in the T-tubule membrane of the skeletal muscle and are the essential components involved in EC-coupling. The DHPR is both a voltage sensor for triggering intracellular Ca^{2+}-release and a slow-voltage-activated Ca^{2+}-channel (Tanabe *et al.*, 1988; Miller, 1992; Cannon, 1996). However, these two processes are independent as Ca^{2+} entry into the cytoplasm through the dihydropyridine receptor (DHPR) is not required for EC-coupling and the DHPR is involved in EC-coupling by acting as a voltage sensor (McPherson and Campbell, 1993; Cannon, 1996).

The DHPR is a heteropentamer protein complex composed of five different sub-units, α_1, α_2, β, δ, and γ, (Miller, 1992; McPherson and Campbell, 1993). The α_1 and α_2 sub-units are large high molecular weight sub-units while the β, δ, and γ sub-units are smaller in size. There is a strong association of α_1 with β and γ indicating direct interaction between these sub-units, as illustrated in Figure 2.3 (Catterall, 1988; Catterall and Striessnig, 1992). In skeletal muscle four genes encode the five different sub-units of the DHPR (Perez-Reyes and Schneider, 1995).

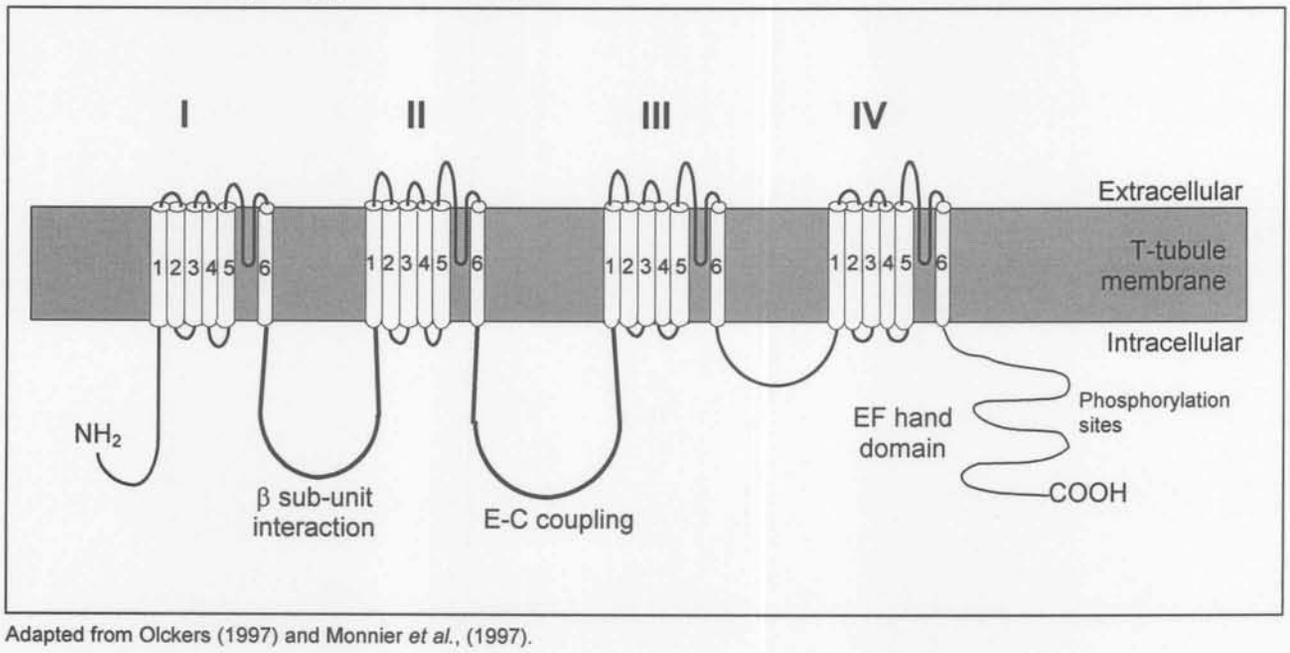
Figure 2.3: Interaction between the sub-units of the skeletal muscle dihydropyridine receptor



Adapted from Olckers (1997) and Catterall and Striessnig (1992).

The α_1 sub-unit is a large 212 kDa protein of the skeletal DHPR and has four homologous repeat units, each comprised of six α-helix transmembrane segments, illustrated in Figure 2.4 and labelled S1-S6. In the fourth α-helix segment (S4) every third or fourth amino acid of each repeat is a positively charged arginine or lysine. These segments are, therefore, thought to form part of the voltage sensing mechanism of the channel (Catterall, 1988; Tsien *et al.*, 1991; Catterall and Striessnig, 1992; Miller, 1992). The cytoplasmic segments include the amino- and the carboxyl-terminal regions as well as the intercellular loops linking repeats I and II, repeats II and III and repeats III and IV. The intracellular loop linking repeat units II and III has been identified as the region involved in EC-coupling in skeletal muscle (Tanabe *et al.*, 1990; Lu *et al.*, 1994). The I-II loop region of the α_1 sub-unit is thought to interact with the β sub-unit (Meissner and Lu, 1995; Perez-Reyes and Schneider, 1995). The region between the C-terminal and the IV repeat unit of the α_1 sub-unit is known as the EF hand domain and is implicated in calcium dependent inactivation and the binding of Ca^{2+} (Tsien *et al.*, 1991; Perez-Reyes and Schneider, 1995). The EF hand is a domain which has a consensus sequence that is folded into a helix-loop-helix pattern with hydrophilic side chains which bind to calcium ions (Baimbridge *et al.*, 1992). The α_1 sub-unit also forms the voltage-gated Ca^{2+} selective pore (Cannon, 1996) and is encoded by the α_1 skeletal muscle dihydropyridine receptor (CACNL1A3) gene on chromosome 1q31-32 (Monnier *et al.*, 1997).

Figure 2.4: Schematic representation of the α_1 sub-unit of the skeletal muscle dihydropyridine receptor



Adapted from Olckers (1997) and Monnier *et al.*, (1997).

The β sub-unit is a 55 kDa protein (Catterall and Striessnig, 1992; Miller, 1992) with a secondary structure which indicates that this protein contains several α-helixes. This is a cytoplasmic protein as the helixes contain many charged amino acid residues and is, therefore, not embedded in the membrane. The gene encoding the β sub-unit of the DHPR is located on chromosome 17q11.2-q22 (Iles *et al.*, 1993). Interaction of the β sub-unit with the cytoplasmic domain of the α_1 sub-unit may be via phosphorylation, modulating the function of α_1 (Powers *et al.*, 1992). The amino (N) terminal region of the β sub-unit is capable of interaction with the α_1 sub-unit and this is thought to control the kinetic effect of the β sub-unit (Perez-Reyes and Schneider, 1995).

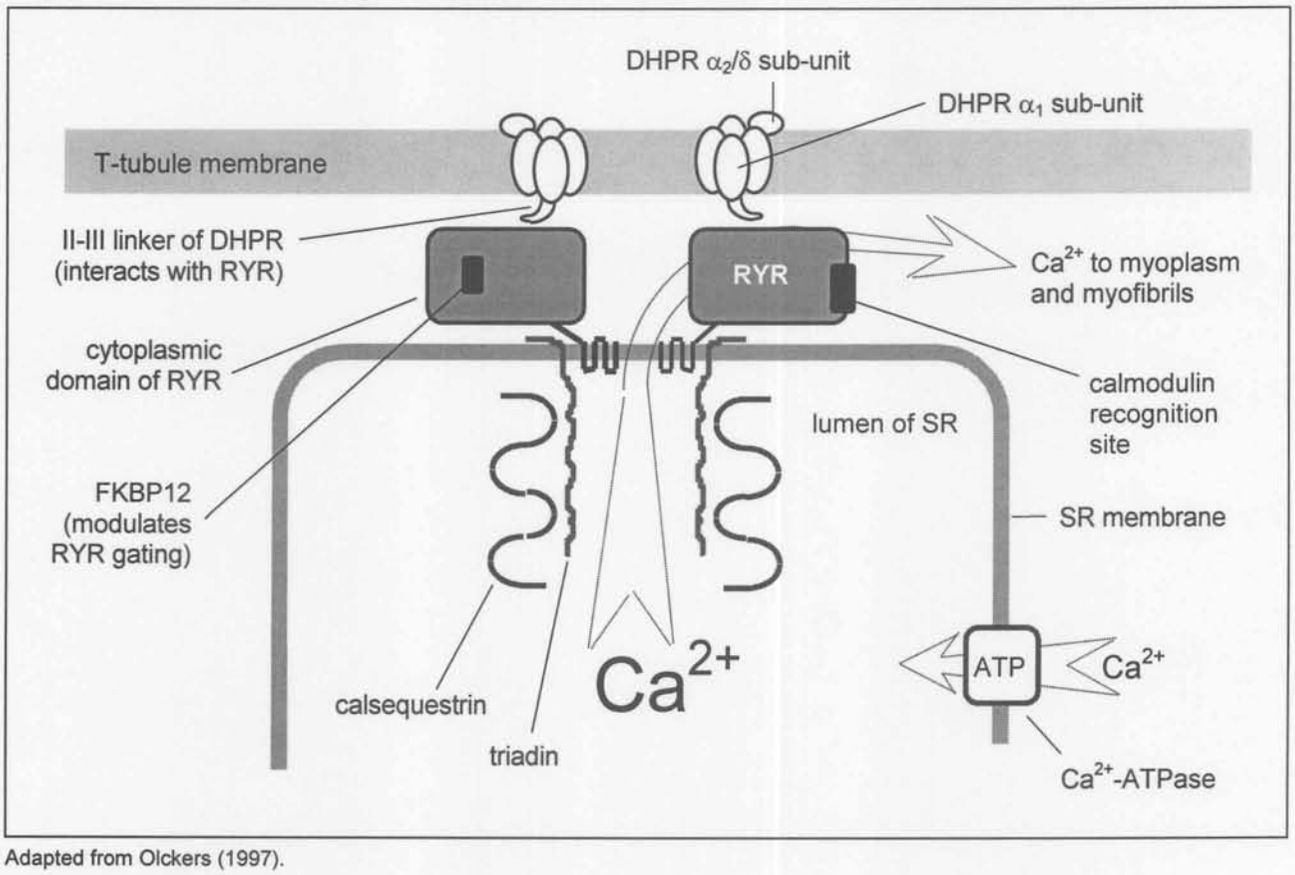
The α_2 sub-unit is a large (142 kDa) glycoprotein that is bound to the 22-28 kDa δ peptide through disulphide bonds (Catterall and Striessing, 1992; Miller, 1992). Both the α_2 and δ sub-units are encoded by the same gene located on the proximal long arm of chromosome 7q (Miller, 1992; Iles *et al.*, 1994). A single precursor protein, α_2/δ propeptide, is proteolytically cleaved near the carboxyl terminal to form the δ and α_2 sub-units, but the α_2 and δ sub-units remain covalently linked through a disulphide bond. The suggested secondary structure of the δ sub-unit contains a transmembrane segment that anchors the α_2/δ complex to the membrane. The functional co-expression of the α_2/δ and α_1 in the presence of the β sub-unit appears to enhance the expression of the α_1 sub-unit and increase the voltage-sensitivity of the channel activation and inactivation (Miller, 1992; Iles *et al.*, 1994; Perez-Reyes and Schneider, 1995). The α_2/δ complex may also play a role in determining the spatial distribution of Ca^{2+}-channels and may be involved in the insertion of the α_1 sub-unit into the membrane (Iles *et al.*, 1994; Perez-Reyes and Schneider, 1995).

The 32 kDa γ protein has been purified from skeletal muscle (Jay *et al.*, 1990; Miller, 1992). The protein is encoded by the gene on chromosome 17q24 (Iles *et al.*, 1993), and contains four possible membrane spanning regions that separate the intracellular amino (NH_2) and the carboxyl (COOH) terminal (Jay *et al.*, 1990). Although this protein is almost exclusively expressed in the skeletal muscle, the role of the γ sub-unit in the DHPR complex has not yet been determined (Jay *et al.*, 1990; Miller, 1992; Perez-Reyes and Schneider, 1995; Greenberg, 1997). Therefore, the γ sub-unit may play a role in the assembly, modulation or structure of the Ca^{2+}-channels, as well as being involved in EC-coupling (Jay *et al.*, 1990; Perez-Reyes and Schneider, 1995).

2.5.3 *Interaction of the skeletal muscle ryanodine receptor with other proteins involved in skeletal muscle contraction*

The RYR protein interacts with at least five other triadic proteins, as presented in Figure 2.5. The proteins are the DHPR α_1 sub-unit, FK-506 binding protein (FKBP12), triadin, calsequestrin and calmodulin. These triadic proteins seem to contribute to the function and regulation of the Ca^{2+}-release mechanism (Coronado *et al.*, 1994; Pessah *et al.*, 1996).

Figure 2.5: Major components involved in skeletal muscle contraction



Adapted from Olckers (1997).

The FK-506 binding protein is a 12 kDa protein that binds to the ryanodine receptor and modulates the gating of the Ca^{2+}-release channel (Jayaraman *et al.*, 1992; Timerman *et al.*, 1993; Brillantes *et al.*, 1994). The molar ratio of FKBP12 to RYR is 4:1 in highly purified RYR preparations. This indicates that one FKBP12 molecule is associated with each subunit of the Ca^{2+}-release channel (Jayaraman *et al.*, 1992; Coronado *et al.*, 1994; Marks, 1997). Timerman *et al.* (1993) reported that the FKBP12 specifically modulates the activity of the RYR1. It appears as though the FKBP12 stabilises the closed conformation of the RYR1 and may therefore be important in modulating the gating of the Ca^{2+}-release channel (Timerman *et al.*, 1993). Studies conducted by Brillantes and co-workers (1994) also indicated that the function of FKBP12 is the stabilisation of the RYR1. Brillantes *et al.*

(1994) and Brooksbank *et al.* (1998) both indicated that a functional Ca^{2+}-release channel is dependent on the binding of FKBP12 to the RYR1. The FKBP12 impedes the opening of the Ca^{2+}-channel, but stabilises it once opened, therefore optimising Ca^{2+}-release into the cytoplasm. It also appears as though FKBP12 improves co-operation between the four RYR1 sub-units, resulting in a more stable Ca^{2+}-release channel (Brillantes *et al.*, 1994).

Triadin, a 95 kDa SR protein, appears to have a functional role in EC-coupling in skeletal muscle (Caswell *et al.*, 1991; Liu and Pessah, 1994). Caswell *et al.* (1991) proposed that the triadin protein binds to both the DHP and the ryanodine receptors and form a ternary complex at the triad junction. Analysis of the amino acid sequence and peptide mapping revealed that triadin has three domains: a single transmembrane segment located within the SR membrane that separates the protein into two domains, a cytoplasmic, and SR luminal domain. The C-terminal of the triadin protein is postulated to be on the luminal side of the SR and is the largest domain. The smaller N-terminal of the protein is thought to be the cytoplasmic domain (Coronado *et al.*, 1994; Meissner and Lu, 1995). As the cytoplasmic domain is such a short segment it might not be able to interact with the DHPR as suggested previously (Coronado *et al.*, 1994). Guo and Campell (1995) suggested that triadins interact with the RYR and calsequestrin but not with the DHPR. It has also been suggested that triadin might functionally link the RYR receptor and calsequestrin (Ikemato *et al.*, 1991).

Calsequestrin is highly acidic and the primary Ca^{2+} binding protein of the SR (Fliegel *et al.*, 1987). Binding of luminal Ca^{2+} to calsequestrin coincides with major conformational changes of the protein. This protein has no transmembrane region and resides within the lumen of the SR (Fliegel *et al.*, 1987; Coronado *et al.*, 1994). Collins *et al.* (1990) reported that a segment of the calsequestrin protein binds to the junctional face membrane of the SR. It was suggested that the activation of the Ca^{2+}-release channel by ligands evoke a signal in the junctional face membrane, which is in turn transmitted to calsequestrin. This signal results in Ca^{2+} being released from calsequestrin to increase the free Ca^{2+} concentration within the lumen of the SR (Ikemato *et al.*, 1991; Coronado *et al.*, 1994).

Calmodulin is a regulatory protein consisting of only 148 amino acid residues with a molecular weight of approximately 16–17 kDa (Landers *et al.*, 1989). This protein binds four Ca^{2+} ions and regulates various calcium dependent signal pathways (O'Neil and DeGrado, 1990). Calmodulin was indicated to bind to the skeletal muscle ryanodine receptor (Seiler *et al.*, 1984), reducing the rate of Ca^{2+}-release from the skeletal muscle

SR (Meissner and Henderson, 1987; Coronado *et al.*, 1994; Meissner, 1994). Zorzato *et al.* (1990) identified three possible calmodulin binding sites in the C-terminal region of the RYR1 sequence.

2.5.4 *The calcium adenosine triphosphatase (Ca^{2+}-ATPase) pump*

Calcium homeostasis within the muscle cell is maintained by sequestering Ca^{2+} from the cytoplasm to the SR and mitochondria. The calcium adenosine triphosphatase (Ca^{2+}-ATPase) pump located in the membrane of the SR actively transports the Ca^{2+} across the membrane (Nelson, 1989; Duthie and Arthur, 1993; McPherson and Campbell, 1993). Active removal of the Ca^{2+} from the cytosol ensures subsequent relaxation of the contracted muscle (Duthie and Arthur, 1993).

The Ca^{2+}-ATPase is a membrane bound protein with two globular cytoplasmic domains which are joined to a set of 10 transmembrane helixes by a pentahelical stalk (MacLennan *et al.*, 1985; Toyoshima *et al.*, 1993). This protein removes two calcium ions from the cytoplasm for each mole of ATP hydrolysed. Calcium binds to two sequentially high affinity sites in the Ca^{2+}-ATPase located close to the cytoplasmic face of the SR membrane. The two binding sites of the Ca^{2+}-ATPase are formed by negatively charged helixes within the transmembrane domain. Conformational changes occur once Ca^{2+} binds to the binding sites. The bound Ca^{2+} is distributed into pools where it is phosphorylated by ATP and released into the lumen of the SR reservoir. The ATPase is converted from an adenosine diphosphate (ADP)-sensitive to an ADP-insensitive form by the translocation and dissociation of the bound Ca^{2+}. The cycle is completed by the hydrolysis of the ATPase (Brandl *et al.*, 1986; Clarke *et al.*, 1989).

If the increase in Ca^{2+} concentration in the cytocol is excessive, muscle contraction will be sustained and a contracture results. Excessive cytoplasmic Ca^{2+} also leads to uncoupling which results in a decreased ATP supply and subsequent failure of the SR Ca^{2+}-ATPase (Duthie and Arthur, 1993). The high cytoplasmic Ca^{2+} concentration in the cytosol stimulates the active transport of Ca^{2+} by Ca^{2+}-ATPase resulting in an increase in ATP hydrolysis. This increase in ATP hydrolysis results in a greater demand for ATP than the rate of ATP production. Since Ca^{2+} uptake is ATP dependent the rate of Ca^{2+} uptake decreases. Consequently, the cytoplasmic calcium concentration escalates and the ATP levels decline resulting in failure of the SR Ca^{2+}-ATPase to remove the excess cytoplasmic Ca^{2+}. If the Ca^{2+}-release channel is unable to close the Ca^{2+} will continue to flow from the

SR into the cytoplasm (Steinmann, 1994). Fleischer *et al.* (1985) suggested that the inability of the SR to store the Ca^{2+} would also result in the muscle not relaxing but retaining the contraction which would then result in contracture. Nelson (1989) reported that triggering agents (listed in Table 2.4) responsible for an MH episode do not block the SR Ca^{2+} pump that actively transports the Ca^{2+} back into the SR, but increases the Ca^{2+}-release channel open time, which leads to the cytoplasmic Ca^{2+} concentrations remaining high.

2.6 Genetic aspects of malignant hyperthermia

MH is inherited as an autosomal dominant trait (Gronert, 1980) and was originally believed to be a single gene disorder (Denborough and Lovell, 1960). It is currently known that MH is not a single gene disorder, and that seven loci in the human genome have been associated with this disorder (MacLennan, 1995; Robinson *et al.*, 1997; Olckers, 1997), to date.

2.6.1 Linkage to chromosome 19

The porcine ryanodine receptor gene was localised to porcine chromosome 6p11-q21 (Harbitz *et al.*, 1990). This gene is closely linked to the glucose phosphate isomerase (GPI) linkage group which is highly conserved across species (Archibald and Imlah, 1985; Davies *et al.*, 1988). The region where the human GPI linkage group is located on chromosome 19q was syntenic to the region harbouring the RYR1 on porcine chromosome 6p (Clamp *et al.*, 1993). The human GPI linkage group that contains the GPI and RYR1 genes was localised to chromosome 19q, resulting in this region being investigated for linkage to MHS (Lusis *et al.*, 1986).

As demonstrated in porcine MH, human MHS is also controlled by a locus closely linked to the GPI gene (McCarthy *et al.*, 1990). These authors reported linkage to human chromosome 19q12-q13.2 using three Irish MHS families, displaying an autosomal dominant pattern of inheritance. In 1990 Zorzato *et al.* cloned the complementary DNA (cDNA) of the human RYR1 gene and the gene was subsequently mapped to the long arm of human chromosome 19q (MacKenzie *et al.*, 1990).

MacLennan *et al.* (1990) performed linkage studies on MH families to determine whether the MH phenotype segregated with the markers on chromosome 19q, which included

markers within the RYR1 gene. Co-segregation of the RYR1 markers with the MH phenotype was observed. This indicated linkage between the MH phenotype and the RYR1 gene, and mutations in the RYR1 gene may cause MH (MacLennan *et al.*, 1990). Twenty-three missense mutations in the RYR1 gene have subsequently been identified and are discussed in paragraph 2.6.1.2.

2.6.1.1 Skeletal muscle ryanodine receptor (RYR1) gene

The human RYR1 gene is ca. 158 kilobase pairs (kb) in size, from the 5' transcriptional initiation site to the 3' polyadenylation signal (Phillips *et al.*, 1996). The cDNA coding sequence including the stop codon is 15 117 base pair (bp) in length. Analysis of the cDNA revealed an open reading frame that codes for 5038 amino acids, resulting in a protein with a molecular weight of 563 584 kDa (Phillips *et al.*, 1996).

2.6.1.1.1 The 5' untranslated region of the human skeletal muscle ryanodine receptor (RYR1) gene

Phillips *et al.* (1996) assigned the 5'-untranslated region of the human RYR1 gene 130 bp upstream of the initiator methionine (Met) codon. This region contains 75% G and C residues, which have been observed in other sarcoplasmic reticulum protein cDNAs (MacLennan *et al.*, 1985; Zorzato *et al.*, 1990). The 5'-untranslated region of the human RYR1 gene display 72% homology to that of the 5' upstream sequence of the rabbit skeletal muscle ryanodine receptor. The rabbit transcription initiation site was assigned to a residue 131 bp upstream of the initiator codon (Takeshima *et al.*, 1989). This data supported the assignment of the human transcription initiation site to the –130 bp position (Phillips *et al.*, 1996).

In a search for the potential DNA binding domains a poorly conserved CCAAT box was identified at position –198 bp upstream from the initiator codon. This CCAAT box was located in the same position as the CCAAT box found in the rabbit RYR1 gene. However, it differs from the consensus sequence (CCAATCT) in that it had purines rather that pyrimidines downstream of the CCAAT box sequence (Lewin, 1994).

Three Sp1 sites (GC box) were identified downstream of the CCAAT box. One site (GGGCGG) in the forward direction and two (CCGCCC) in the reverse direction are indicated by sequence underlined with a dotted line in Table 2.6. These Sp1 sites are usually clustered near the initiation site of a gene (Lewin, 1994).

Phillips *et al.* (1996) also identified three copies of a muscle specific promoter element GGCTGGGG. These elements were first identified by Korczak *et al.* (1988) in the Ca^{2+}-ATPase gene of rabbits, and occur in the reverse direction as CCCCAGCC in the human RYR1 gene. Two of these elements are also present in the reverse direction in the identical position in the RYR1 gene of the rabbit (Takeshima *et al.*, 1989). No consensus TATA box was found in the promoter sequence but a cluster of five TATA box sequences were found further upstream from the initiation codon at position -1500 bp (Phillips *et al.*, 1996).

Table 2.6: Partial genomic DNA (gDNA) sequence of the human ryanodine receptor (RYR1) gene, promoter region, and exon one

Nucleotide number	DNA Sequence: Promoter region and exon 1
-480	cactatgttt ggtgcttttt aaagtcagga gatctggtgc acatccctgc tctgctgcgt
-420	gatatcttgc aagaacttcc ccactcagag cctcagtctg ccctctgtga aatgggagaa
-360	tgatggcacg ttacttaccg tggtggggag aaagcgcagg tacctcctag atactctctc
-300	tcccacccca cctccggcgg ccaacggcaa gcaaacctcc agccaagatt tggggtatgt
-240	gggcagggct ccggcgaagg ggagtggccg gggagtcctg gtccaatggg gcccgggggcg
-180	gggacttcct cccatctctg tccagcatgc gtgtactcct cgcagttcca TCTACCTCGC
-120	GGGTGCCTCT GGTGTCTCCA GAGGTCTCCG ACCCCAGCGC GCCCCCAGCC CTCCCGCCCA
-60	GCCCGCAGCC CCCTCCCTCT GTTCCCCGAC CTCAGACCCT GGGCTTCCGA CCTCGACATC
1	**ATG**GGTGACG CAGAAGGCGA AGACGAGGTC CAGTTCCTGC GGACGgtgcg tctctctggg
61	ttaggggctg ttggggctat ctcttggggc tctctgaggc tctct

cap site; Exon 1

Partial gDNA sequence was retrieved from Genbank with accession number U48449. The sequence in capital letters indicates exon 1 of the gene and the underlined sequence in capital letters is the translated region with the rest of the sequence in capital letters being the 5'-untranslated region of the gene. The boxed sequences indicate the muscle specific promoter elements and the three Sp1 sites are also indicated within the sequence (...). The initiation codon is the boldface capital letter sequence **ATG**. The CCAAT box (___) located at position -198 bp and a cap site located at position -138 bp are also indicated above.

2.6.1.1.2 The coding region of the skeletal muscle ryanodine receptor (RYR1) gene

The ATG initiator codon is located 15096 bp upstream from the termination codon (Zorzato *et al.*, 1990). The initiator methionine was located in a longer sequence of ACATC**ATG**G and closely resembles the consensus initiation sequence CCA(G)CC**ATG**G reported by Kozak (1984).

The gene contains a total of 106 exons with an average exon size of 144 bp and an average intron size of 1377 bp. The smallest exon, exon 70, is 15 bp in length and the largest exon, exon 91, is 813 bp in length. The introns range from 85 bp to 16 000 bp in size, with intron 4 being the smallest and intron 89 being the largest. Two alternatively spliced sites were identified in the RYR1 gene. These sites generate two distinct exons,

exon 70 (15 bp) and exon 83 (18 bp). Alternative splicing of gDNA leads to the insertion of a 15 bp exon (exon 70) into the cDNA after nucleotide G10437. The second alternative splice site results in the deletion of exon 83 (Zhang *et al.*, 1993; Phillips *et al.*, 1996).

The splice sites of the RYR1 gene are primarily identical to the consensus sequences for the 5' donor (GT) and the 3' acceptor (CAG) sequences. The 5' donor site of exon 57 is the only donor site that differs from the consensus sequence in that the sequence is GC instead of GT. The largest number (83%) of the 3' acceptor sites ended in CAG and the remaining 17% ended in TAG (Phillips *et al.*, 1996).

Phillips *et al.* (1996) identified 12 hydrophobic transmembrane sequences in the C-terminal half of the molecule. Takeshima *et al.* (1989) identified four transmembrane sequences in the rabbit skeletal muscle receptor gene. The transmembrane sequences are labelled M', M" and M1-M10 and are indicated in Table 2.7 as the sequence underlined by a solid line. Three potential calmodulin-binding sites identified by Zorzato *et al.* (1990) are also indicated in Table 2.7.

Table 2.7: Partial amino acid sequence of the human skeletal muscle ryanodine receptor (RYR1) gene

Amino acid sequence	Amino acid number
▼ exon 37 AERYVDKLQANQRSRYGLLIKAFSMTAAETARRTREFRSPPQEQINMLLQFKDGTDEEDCPLPEEIRQDL	2030
▼ ▼ LDFHQDLLAHCGIQLDGEEEEPEEETTLGSRLMSLLEKVRLVKKKEEKPEEERSAEESKPRSLQELVSH	2100
MVVRWAQEDFVQSPELVRAMFSLLHRQYDGLGELLRALPRAYTISPSSVEDTMSLLECLGQIRSLLIVQM	2170
▼ exon 40 ▼ GPQEENLMIQSIGNIMNNKVFYQHPNLMRALGMHETVMEVMVNVLGGGESKEIRFPKMVTSCCRFLCYFC	2240
▼ ▼ RISRQNQRSMFDHLSYLLENSGIGLGMQGSTPLDVAAASVIDNNELALALQEQDLEKVVSYLAGCGLQSC	2310
▼ PMLVAKGYPDIGWNPCGGERYLDFLRFAVFVNGESVEENANVVVRLLIRKPECFGPALRGEGGSGLLAAI	2380
▼ exon 45 ▼ EEAIRISEDPARDGPGIRRDRRREHFGEEPPEENRVHLGHAIMSFYAALIDLLGRCAPEMHLIQAGKGEA	2450
▼ LRIRAILRSLVPLEDLVGIISLPLQIPTLGKDGALVQPKMSASFVPDHKASMVLFLDRVYGIENQDFLLH	2520
▼ VLDVGFLPDMRAAASLDTATFSTTEMALAVNRYLCLAVLPLITKCAPLFAGTEHRAIMVDSMLHTVYRLS	2590
▼ ▼ exon 50 RGRSLTKAQRDVIEDCLMSLCRYIRPSMLQHLLRRLVFDVPILNEFAKMPLKLLTNHYERCWKYYCLPTG	2660
▼ WANFGVTSEEELHLTRKLFWGIFDSLAHKKYDPELYRMAMPCLCAIAGALPPDYVDASYSSKAEKKATVD	2730
▼ ▼ AEGNFDPRPVETLNVIIPEKLDSFINKFAEYTHEKWAFDKIQNNWSYGENIDEELKTHPMLRPYKTFSEK	2800
▼ exon 55 ▼ DKEIYRWPIKESLKAMIAWEWTIEKAREGEEEKTEKKKTRKISQSAQTYDPREGYNPQPPDLSAVTLSRE ▼	2870
▼ ▼ ▼ LQAMAEQLAENYHNTWGRKKKQELEAKGGGTHPLLVPYDTLTAKEKARDREKAQELLKFLQMNGYAVTRG	2940

continued ...

Table 2.7, continued ...

Amino acid sequence	Amino acid number
▼ exon 60 LKDMELDSSSIEKRFAFGFLQQLLRWMDISQEFIAHLEAVVSSGRVEKSPHEQEIKFFAKILLPLINQYF	3010
TNHCLYFLSTPAKVLGSGGHASNKEKEMITSLFCKLAALVRHRVSLFGTDAPAVVNCLHILARSLDARTV	3080
M' MKSGPEIVKAGLRSFFESASEDIEKMVENLRLGKVSQARTQVKGVGQNLTYTTVALLPVLTTLFQHIAQH	3150
▼ exon 65 M" QFGDDVILDDVQVSCYRTLCSIYSLGTTKNTYVEKLRPALGECLARLAAAMPVAFLEPQLNEYNACSVYT	3220
TKSPRERAILGLPNSVEEMCPDIPVLERLMADIGGLAESGARYTEMPHVIEITLPMLCSYLPRWWERGPE	3290
APPSALPAGAPPPCTAVTSDHLNSLLGNILRIIVNNLGIDEASWMKRLAVFAQPIVSRARPELLQSHFIP	3360
TIGRLRKRAGKVVSEEEQLRLEAKAEAQEGELLVRDEFSVLCRDLYALYPLLIRYVDNNRAQWLTEPNPS	3430
▼ exon 70 AEELFRMVGEIFIYWSKSHNFKREEQNFVVQNEINNMSFLTADNKSKMAKAGDIQSGGSDQERTKKKRRG	3500
DRYSVQTSLIVATLKKMLPIGLNMCAPTDQDLITLAKTRYALKDTDEEVREFLHNNLHLQGKVEGSPSLR	3570
WQMALYRGVPGREEDADDPEKIVRRVQEVSAVLYYLDQTEHPYKSKKAVWHKLLSKQRRRAVVACFRMTP	3640
▼ exon 75 LYNLPTHRACNMFLESYKAAWILTEDHSFEDRMIDDLSKAGEQEEEEEEVEEKKPDPLHQLVLHFSRTAL	3710
TEKSKLDEDYLYMAYADIMAKSCHLEEGGENGEAEEEVEVSFEEKQMEKQRLLYQQARLHTRGAAEMVLQ	3780
▼ exon 80 MISACKGETGAMVSSTLKLGISILNGGNAEVQQKMLDYLKDKKEVGFFQSIQALMQTCSVLDLNAFERQN	3850
▼ exon 85 KAEGLGMVNEDGTVINRQNGEKVMADDEFTQDLFRFLQLLCEGHNNDFQNYLRTQTGNTTTINIIICTVD	3920
M1 YLLRLQESISDFYWYYSGKDVIEEQGKRNFSKAMSVAKQVFNSLTEYIQGPCTGNQQSLAHSRLWDAVVG	3990
M2 FLHVFAHMMMKLAQDSSQIELLKELLDLQKDMVVMLLSLLEGNVVNGMIARQMVDMLVESSSNVEMILKF	4060
▼ exon 90 FDMFLKLKDIVGSEAFQDYVTDPRGLISKKDFQKAMDSQKQFSGPEIQFLLSCSEADENEMINCEEFANR	4130
FQEPARDIGFNVAVLLTNLSEHVPHDPRLHNFLELAESILEYFRPYLGRIEIMGASRRIERIYFEISETN	4200
M3 RAQWEMPQVKESKRQFIFDVVNEGGEAEKMELFVSFCEDTIFEMQIAAQISEPEGEPETDEDEGAGAAEA	4270
M4 GAEGAEEGAAGLEGTAATAAAGATARVVAAAGRALRGLSYRSLRRRVRRLRRLTAREAATAVAALLWAAV	4340
TRAGAAGAGAAAGALGLLWGSLFGGGLVEGAKKVTVTELLAGMPDPTSDEVHGEQPAGPGGDADGEGASE	4410
GAGDAAEGAGDEEEAVHEAGPGGADGAVAVTDGGPFRPEGAGGLGDMGDTTPAEPPTPEGSPILKRKLGV	4480
DGVEEELPPEPEPEPEPELEPEKADAENGEKEEVPEPTPEPPKKQAPPSPPPKKEEAGGEFWGELEVQRV	4550
M5 ▼ exon 95 KFLNYLSRNFYTLRFLALFLAFAINFILLFYKVSDSPPGEDDMEGSAAGDVSGAGSGGSSGWGLGAGEEA	4620
M6 EGDEDENMVYYFLEESTGYMEPALRCLSLLHTLVAFLCIIGYNCLKVPLVIFKREKELARKLEFDGLYIT	4690
EQPEDDDVKGQWDRLVLNTPSFPSNYWDKFVKRKVLDKHGDIYGRERIAELLGMDLATLEITAHNERKPN	4760
exon 100 ▼ M7 PPPGLLTWLMSIDVKYQIWKFGVIFTDNSFLYLGWYMVMSLLGHYNNFFFAAHLLDIAMGVKTLRTILSS	4830
M8 M9 VTHNGKQLVMTVGLLAVVVYLYTVVAFNFFRKFYNKSEDEDEPDMKCDDMMTCYLFHMYVGVRAGGGIGD	4900
M10 EIEDPAGDEYELYRVVFDITFFFFVIVILLAIIQGLIIDAFGELRDQQEQVKEDMETKCFICGIGSDYFD	4970
▼ exon 105 TTPHGFETHTLEEHNLANYMFFLMYLINKDETEHTGQESYVWKMYQERCWDFFPAGDCFRKQYEDQLS	5038

Partial amino acid sequence was retrieved from Genbank with accession number J05200. The transmembrane sequences are underlined and labelled M', M" and M1-M10 (Zorzato *et al.* 1990; Phillips *et al.*, 1996). The 106 exons are indicated (▼) and numbered after every 5 exons, with the first amino acid of each exon underlined. Three potential calmodulin-binding sites are also indicated by boxed sequences.

Phillips *et al.* (1996) confirmed a number of errors in the human RYR1 cDNA published by Zorzato *et al.* (1990). A series of sequencing compressions in a GC rich region between nucleotide g4092 and g4105 were corrected by Zhang *et al.* (1993). Both the original and corrected nucleotide sequences are presented in Table 2.8. Nucleotide g4107 was corrected to c4107 and three additional nucleotides (g4093, g4094 and g4104) were identified resulting in a frameshift of the original amino acid sequence RGAP1368 to GEAQP1369 as indicated in Table 2.8. The addition of an amino acid shifted the original amino acid numbering by +1 downstream of amino acid 1369.

Table 2.8: Correction of the sequencing compressions within the RYR1 gene

Original amino acid sequence		Original nucleotide sequence		Corrected nucleotide sequence		Corrected amino acid sequence	
Amino acid number	Amino acid sequence [1]	Nucleotide number	Nucleotide sequence	Nucleotide sequence	Nucleotide number	Amino acid sequence [1]	Amino acid number
1364	Gly (G)	4090 4091 4092	g g g	g g g	4090 4091 4092	Gly (G)	1364
1365	Arg (R)	4093 4094 4095	a g a	**g** [3] **g** [4] a	4093 4094 4095	Gly (G)	1365
1366	Gly (G)	4096 4097 4098	g g c	g a g	4096 4097 4098	Gly (E)	1366
1367	Ala (A)	4099 4100 4101	g c a	g c g	4099 4100 4101	Ala (A)	1367
1368	Pro (P)	4102 4103 4104	c c **g** [2]	c a **g** [5]	4102 4103 4104	Gln (Q)	1368
1369	Ala (A)	4105 4106 4107	g c c	c c **c** [6]	4105 4106 4107	Pro (P)	1369
1370	Arg (R)	4108 4109 4110	a g g	g c c	4108 4109 4110	Ala (A)	1370
				a g g	4111 4112 4113	Arg (R)	1371

Adapted from Zhang *et al.* (1993). 1) The one letter amino acid code is indicted in brackets. 2) Corrected to **c** as indicated by 6. 3, 4 and 5) Not detected in original sequence, added to corrected sequence.

Due to one of the alternatively spliced exons, exon 70, an additional five amino acids, AGDIQ, were added to the original sequence after Lys3479 (corrected to Lys3480) (Zhang *et al.*, 1993). Therefore all the amino acids following the Lys3480 are shifted by +6 due to the addition of the one amino acid from the compression and the other five from exon 70. Phillips *et al.* (1996) also confirmed the following four sequencing corrections identified by

Gillard *et al.* (1992): Leu792 (CT**G**) to Leu792 (CT**T**), Lys2323 (AA**G**) to Asn2324 (AA**C**), Ala2839 (GCG) to Arg2840 (CGG) and Ala3379 (GCC) to Arg3380 (CGC). These sequencing corrections altered the amino acids in three instances but did not affect the amino acid numbering as the change in amino acid numbers are due to the shift in the original amino acid numbering of +1 downstream from amino acid 1369.

2.6.1.1.3 The 3'-untranslated region of the skeletal muscle ryanodine receptor (RYR1) gene

The 3'-untranslated region of the RYR1 gene that begins after the termination codon TAG, is 146 bp in length (Phillips *et al.*, 1996). Proudfoot and Brownlee (1976) reported that the AAUAAA sequence is possibly present in all eukaryotic mRNA about 20 bp away from the 3' terminal polyadenylation sequence. A consensus polyadenylation signal, AAAATAAA, is located 19 bp upstream of the polyadenylation site of the RYR1 gene, followed closely by the characteristic TG-rich sequence TCTGTCGTACG and is indicated in Table 2.9 (McLauchlan *et al.*, 1985; Zorzato *et al.*, 1990).

Table 2.9: Partial gDNA sequence of the human ryanodine receptor (RYR1) gene, exon 106 and the 3'-untranslated region

Nucleotide number	DNA Sequence: exon 106 and the 3'-untranslated region
1321	ctgaggtgga gaatcgcttg agccaggagg ctgtagtgag ctgtgattgt cgccactgca
1381	ctccagcctg ggcaacagag caacaccctg tctaaaaata tatatatata tatgtctcaa
1441	gggtttgaag atgtgaccaa tgaactcttt ctatccccaa tcctagGAGT CTTATGTCTG
1501	GAAGATGTAC CAAGAGAGAT GTTGGGATTT CTTCCCAGCT GGTGATTGTT TCCGTAAGCA
1561	GTATGAGGAC CAGCT**TAG**CT GACACACCCC CAGCTGGCCC TCCACCCCCA CCTCAAGTGC
1621	CTTATTCTCA CAGCAAGCCC CTTAGTCCCC AAGCCCCTCC CCCTAAGGCA GCTGGGGGAG
1681	AGGTGACCTA GTACtggaaa ataaa**tctgt cgtacg**cccc ccag

Exon 106

Partial gDNA sequence was retrieved from Genbank with accession number U48508. The sequence in capital letters indicates exon 106 and the underlined sequence the 3'-untranslated region of the gene. The polyadenylation signal is indicated by the sequence underlined with a double line. The termination codon is the boldface capital letter sequence **TAG**.

2.6.1.2 Reported polymorphisms and mutations within the skeletal muscle ryanodine receptor (RYR1) gene

The first mutation described within the RYR1 gene was the Arg614 missense mutation, which is analogous to the porcine Arg615 mutation (Gillard *et al.*, 1991). Since the identification of the Arg614 mutation twenty-two other mutations in the RYR1 gene have been described of which eighteen is responsible for MHS, four have been linked to both MHS and CCD, and one was observed only in a CCD family (Brandt *et al.*, 1999). Gillard *et al.* (1992) identified twenty polymorphisms in the RYR1 gene by sequence analysis.

Three additional polymorphisms were identified (Zhang *et al.*, 1993; Manning *et al.*, 1998b) and are listed with the missense mutations in Table 2.10.

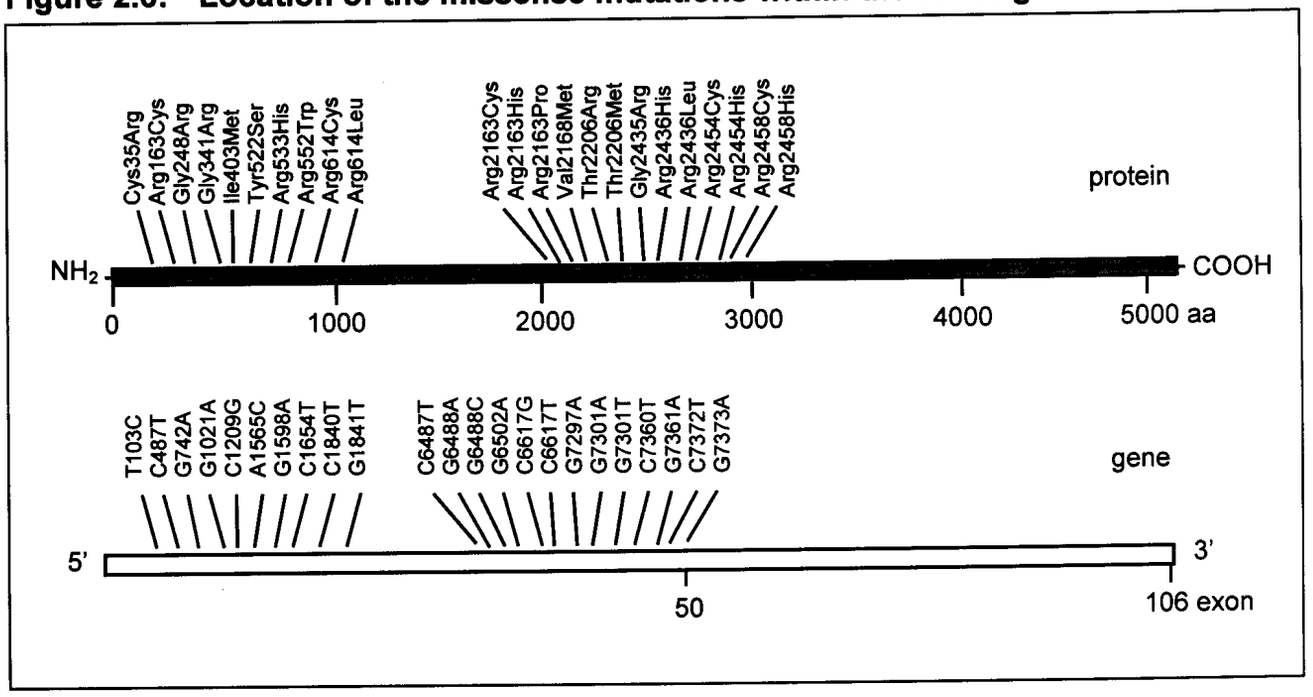
Table 2.10: Reported missense mutations and polymorphisms in the skeletal muscle ryanodine receptor gene

Amino acid	Amino acid change	Nucleotide substitution	Polymorphism / mutation	Reference
Cys35	Cys→Arg	tgc→cgc: T103C	Mutation	Lynch *et al.*, 1997
Leu197	None	ctg→cta	Polymorphism	Gillard *et al.*, 1992
Arg163	Arg→Cys	cgc→tgc: C487T	Causative mutation	Quane *et al.*, 1993
Gly248	Gly→Arg	ggg→agg: G742A	Causative mutation	Gillard *et al.*, 1992
Gly341	Gly→Arg	ggg→agg: G1021A	Causative mutation	Quane *et al.*, 1994a
Ala359	None	gcc→gct	Polymorphism	Gillard *et al.*, 1992
Ile403	Ile→Met	atc→atg: C1209G	Causative mutation	Quane *et al.*, 1993
Arg470	Arg→Cys	cgc→tgc	Polymorphism	Gillard *et al.*, 1992
Tyr522	Tyr→Ser	tat→tct: A1565C	Causative mutation	Quane *et al.*, 1994b
Arg533	Arg→His	cgt→cat: G1598A	Mutation	Brandt *et al.*, 1999
Arg552	Arg→Trp	cgg→tgg: C1654T	Causative mutation	Keating *et al.*, 1997
Ser555	None	tca→tcg:	Polymorphism	Gillard *et al.*, 1992
Arg614	Arg→Cys	cgc→tgc: C1840T	Causative mutation	Otsu *et al.*, 1992
Arg614	Arg→Leu	cgc→ctc: G1841T	Causative mutation	Quane *et al.*, 1997
Pro762	None	cct→ccc	Polymorphism	Zhang *et al.*, 1993
Thr980	None	aca→acg	Polymorphism	Gillard *et al.*, 1992
Asn993	None	aac→aat	Polymorphism	Zhang *et al.*, 1993
Ile1151	None	atc→att	Polymorphism	Gillard *et al.*, 1992
Pro1785	Pro→Leu	cca→cta	Polymorphism	Gillard *et al.*, 1992
Lys2012	None	aaa→aag	Polymorphism	Gillard *et al.*, 1992
Gly2059	Gly→Cys	ggc→tgc	Polymorphism	Gillard *et al.*, 1992
Arg2163	Arg→Cys	cgc→tgc: C6487T	Causative mutation	Manning *et al.*, 1998a
Arg2163	Arg→His	cgc→cac: G6488A	Causative mutation	Manning *et al.*, 1998a
Arg2163	Arg→Pro	cgc→ccc: G6488C	Mutation	Brandt *et al.*, 1999
Val2168	Val→Met	gtg→atg: G6502A	Mutation	Manning *et al.*, 1998a
Thr2206	Thr→Arg	acg→agg: C6617G	Mutation	Brandt *et al.*, 1999
Thr2206	Thr→Met	acg→atg: C6617T	Mutation	Manning *et al.*, 1998a
Pro2366	None	ccc→cct	Polymorphism	Manning *et al.*, 1998b
Gly2435	Gly→Arg	gga→aga: G7297A	Causative mutation	Keating *et al.*, 1994
Arg2436	Arg→His	cgc→cac: G7301A	Causative mutation	Zhang *et al.*, 1993
Arg2436	Arg→Leu	cgc→ctc: G7301T	Mutation	Barone *et al.*, 1999
Arg2454	Arg→Cys	cgc→tgc: C7360T	Mutation	Brandt *et al.*, 1999
Arg2454	Arg→His	cgc→cac: G7361A	Mutation	Barone *et al.*, 1999
Arg2458	Arg→Cys	cgc→tgc: C7372T	Causative mutation	Manning *et al.*, 1998b
Arg2458	Arg→His	cgc→cac: G7373A	Causative mutation	Manning *et al.*, 1998b
Ala2499	None	gcg→gca	Polymorphism	Gillard *et al.*, 1992
Val2507	None	gtg→gta	Polymorphism	Gillard *et al.*, 1992
His2620	None	cac→cat	Polymorphism	Gillard *et al.*, 1992
Arg2623	None	cgc→cgt	Polymorphism	Gillard *et al.*, 1992
Thr2658	None	acg→aca	Polymorphism	Gillard *et al.*, 1992
Ile2704	None	att→atc	Polymorphism	Gillard *et al.*, 1992
Asp2729	None	gat→gac	Polymorphism	Gillard *et al.*, 1992
Glu2778	None	gag→gaa	Polymorphism	Gillard *et al.*, 1992
Ser2862	None	agt→agc	Polymorphism	Gillard *et al.*, 1992
Pro3060	None	cca→ccg	Polymorphism	Gillard *et al.*, 1992
Leu3229	None	ctg→cta	Polymorphism	Gillard *et al.*, 1992

Adapted from Olckers (1997) and Brandt *et al.* (1999).

All twenty-three of these mutations are missense mutations resulting in the substitution of one amino acid for another due to a nucleotide substitution. Nineteen of these mutations involve an Arg amino acid: three mutations entail the substitution of a Gly to an Arg, two mutations result from the substitution of a Cys to an Arg and one is due to a Thr to an Arg substitution. Fourteen of the mutations result in an Arg being substituted for another amino acid, Cys and His in five instances, Leu in two and Trp and Pro in the others. These mutations can be divided into two distinct groups within the RYR1 gene, one group clusters between residues 35 and 614 at the N-terminal region of the RYR1 gene and the other group clustering between residues 2163 and 2458 located in the central region of the gene. Figure 2.6 illustrates the location of the mutations clustered in the two regions of the RYR1 gene.

Figure 2.6: Location of the missense mutations within the RYR1 gene



Adapted from Olckers (1997) and Zorzato *et al.* (1997).

A missense mutation is causative when it meets the following requirements as listed by Cooper and Krawczak (1995) and Hogan (1998):

1. The mutation must occur in a functional or structural region of the protein.
2. The region must be conserved across species.
3. The mutation must occur in unrelated patients and not in the normal population.
4. The missense mutation must segregate with the disease phenotype in a family.
5. The biochemical properties of an *in vitro* produced mutant protein should be identical to that of the *in vivo* mutant protein.
6. The pathological phenotype should be reversible when the mutant gene or protein is replaced with the wild-type.

Tong *et al.* (1997) conducted expression studies with mutant and wild type RYR1 recombinant DNA for fifteen of the mutations (C35R, R163C, G248R, G341R, I403M, Y522S, R552W, R614C, R614L, R2163C, R2163H, G2435R, R2436H, R2458C and R2458H) reported in the RYR1 gene. All of the mutants investigated by Tong *et al.* (1997), with the exception of C35R, were more sensitive to caffeine that the wild type RYR1. These authors concluded that the abnormal Ca^{2+}-release of the transfected cells were due to the differences in the gating properties of the mutant RYR1. These findings provide supporting evidence of the causal role of these fourteen mutations in MH as indicated in Table 2.10 (Tong *et al.*, 1997). In the following paragraphs each of the missense mutations will be discussed in greater detail.

2.6.1.2.1 The Cys35Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Lynch *et al.* (1997) reported the Cys35Arg mutation in a large MHS family that displayed linkage to the RYR1 gene. After none of the MH mutations reported at the time (Arg163Cys, Gly248Arg, Gly341Arg, Ile403Met, Tyr522Ser, Arg614Cys, Gly2435Arg and Arg2436His) were observed in this family, a T to C transition at position 103 was identified via sequencing analysis. This substitution results in the replacement of amino acid Cys35 with an Arg. This mutation is the most N-terminal mutation reported to date.

Two individuals within the extensive family described by Lynch *et al.* (1997) were diagnosed as homozygous for the Cys35Arg RYR1 mutation. The two homozygous individuals are offspring of a consanguineous marriage. Only one other homozygous MHS individual has been reported to date (Deufel *et al.,* 1995) – and is homozygous for the Arg614Cys mutation. The Cys35Arg mutation segregated perfectly with the MHS phenotype in the family described by Lynch *et al.,* (1997). These authors investigated sixty-five unrelated MHS individuals for the T103C substitution, but it was only detected in the above-mentioned family. It is, therefore, possible that this mutation is unique to this particular MHS family (Lynch *et al.*, 1997).

The mutation was not detected in 200 normal chromosomes, indicating that this substitution is not a polymorphism but a possible causative mutation. Eight RYR receptors, as listed in Table 2.11, from five species indicated that the Cys35 amino acid is conserved across these species and is therefore a functionally important amino acid (Lynch *et al.*, 1997).

Table 2.11: Alignment of amino acid sequence of the RYR receptors from five species

Species	RYR receptors	Amino acid sequence [a]
Human	RYR1	...TVLKEQLKL C LAAEGFGNRLCFLE...
Pig	RYR1	...TVLKEQLKL C LAAEGFGNRLCFLE...
Rabbit	RYR1	...TVLKEQLKL C LAAEGFGNRLCFLE...
Rabbit	RYR2	...TIHKEQQKL C LAAEGFGNRLCFLE...
Rabbit	RYR3	...TVHKEQRKF C LAAEGLGNRLCFLE...
Bullfrog	RYR α	...TIRKENLKM C MGVEGFGNRLCYLE...
Bullfrog	RYR β	...TIHKEQRKF C LAAEGLGNRLCFLE...
D. melanogaster	RYR	...TG----ERV C LAAEGFGNRHCFLE...

Adopted from Lynch *et al.* (1997). a) The isolated C in the amino acid sequence is Cys35.

2.6.1.2.2 The Arg163Cys mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Quane *et al.* (1993) identified the Arg163Cys mutation in one Italian CCD family and one Danish MH family. This nucleotide substitution was identified when the cDNA of the RYR1 gene was screened for mutations that caused CCD (Quane *et al.*, 1993). An aberrant single-stranded conformational polymorphism (SSCP) was identified, and a single base substitution, C487T, was revealed upon sequencing. It was reported that two to three percent of MHS cases investigated is caused by the Arg163 point mutation (Quane *et al.*, 1994a; Manning *et al.*, 1998a). Fagerlund *et al.* (1994) confirmed the presence of the Arg163Cys mutation in the Danish family initially investigated by Quane *et al.* (1994a).

One hundred and eighty eight normal chromosomes were examined to determine if this mutation is present in the normal population and none of these chromosomes harboured the mutation. Segregation analysis indicated that this mutation co-segregated with the MHS phenotype in the families where the mutation was identified. The amino acid Arg in position 163 is also conserved across species which indicated that it is a functionally important amino acid in the RYR protein (Quane *et al.*, 1993). Transfection studies by Censier *et al.* (1998) indicated that the Arg163Cys mutation is an MH causative mutation. This study confirmed the results of the study by Tong *et al.* (1997) which indicated that fourteen mutations in the RYR1 gene, including the Arg163Cys, are causative mutations for MH.

2.6.1.2.3 The Gly248Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Gillard *et al.* (1992) detected an G742 to A substitution when systematically sequencing the cDNA of the RYR1 gene. This point mutation results in the substitution of amino acid

Gly248 with an Arg. This substitution does result in the alteration of restriction sites. It abolishes an *Mnl I* restriction site, and also creates an *Mnl I* restriction site close to the original site (Sei *et al.*, 1998). The alteration of the restriction site could only be utilised when a modified primer was designed to eliminate the newly created restriction site. Segregation between this mutation and the MHS phenotype has, to date, only been observed in one family (Gillard *et al.*, 1992).

2.6.1.2.4 The Gly341Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

In an attempt to identify new MHS mutations in the RYR1 gene the 15.3 kb RYR1 gene was screened and the Gly341Arg mutation was identified (Quane *et al.*, 1994a). This missense mutation was detected via SSCP analysis, where aberrant conformer migration rates indicate the presence of DNA alterations. After identification of aberrant conformers the samples were sequenced, identifying the nucleotide replacement G1021 with A (Quane *et al.*, 1994a).

This mutation was first identified in three Irish, two Belgian and one French MHS patients by Quane *et al.* (1994a). The Gly341Arg mutation has subsequently been identified in seven British MHS families (Adeokun *et al.*, 1997), three Belgium families (Monieurs *et al.*, 1998) and an Italian family (Barone *et al.*, 1999).

The absence of the candidate mutation in the normal population was established by examination of 500 chromosomes with SSCP analysis. However, SSCP is not specific for any mutation and is, therefore, not the recommended detection method for the Gly341Arg mutation. This issue is discussed in more detail in chapter four. Segregation studies performed on several European MH families indicated that the mutation co-segregated with the MHS phenotype (Quane *et al.*, 1994a).

Adeokun *et al.* (1997) identified seven additional unrelated MHS individuals, via the amplification-created restriction site (ACRS) protocol (Alestrϕm *et al.*, 1995), harbouring the Gly341Arg mutation. Segregation studies in six of the seven MHS families were not possible but the presence of the mutation in these individuals supported the report by Quane *et al.* (1994a), stating that this mutation is associated with MHS. However, the Gly341Arg mutation was only observed in a single Danish MH family from 48 Danish and 41 Swedish families investigated (Fagerlund *et al.*, 1996). It was also not observed in the large North American populations investigated, which included 165 MHN and 114 MHS

individuals (Stewart *et al.*, 1998). Brandt *et al.* (1999) investigated 96 German, 4 Austrian, two Swiss, one French and one Italian family and also did not observe the Gly341Arg mutation. These findings suggested that the G1021A gene pool is restricted to Northern European populations (Stewart *et al.*, 1998).

In one British pedigree the mutation is present in only MHS individuals but does not co-segregate with the MHS phenotype of five MHS individuals (Adeokun *et al.*, 1997). Haplotype analysis indicated that the haplotype identified in the seven MHS individuals with the mutation was not present in the five MHS individuals who do not harbour the Gly341Arg mutation (Adeokun *et al.*, 1997). These authors suggested that it is highly unlikely that five of the MHS individuals were diagnosed incorrectly, even though the IVCT is not 100% sensitive (Ørding *et al.*, 1997). However, another explanation of the observed results is possible, but was not discussed by Adeokun *et al.* (1997). It is possible that these individuals have another myopathy which could result in the positive (MHS) IVCT results and would therefore explain the phenotype-genotype discordance.

Adeokun *et al.* (1997) offered several possible explanations for the phenotype-genotype discordance observed in the British pedigree. It was suggested that two genes might be involved in this kinship, thus resulting in the Gly341Arg mutation not being the causative mutation in this family but rather a polymorphism. Incorrect IVCT or mutation detection results seem unlikely since the mutation detection protocol was repeated in two other laboratories. Confirmation of results by heteroduplex analysis, a mutation-specific amplification refractory-mutation system test, and sequencing analysis of a key individual demonstrated that the initial mutation analysis was correct (Adeokun *et al.*, 1997). These authors do, however, favour the explanation that two genes might be responsible for the discordance observed in the British family. However, the IVCT results and the clinical background of the MHS individuals were not discussed in sufficient detail to conclude that other myopathies did not cause the positive IVCT results.

Phenotype-genotype discordance was also observed in an Irish family (Healy *et al.*, 1996). Two MHS individuals did not harbour the Gly341Arg mutation. However, when the IVCT thresholds were altered to be more specific the two individuals were phenotyped as MHN in which case the mutation then co-segregated with the IVCT results. Haplotype analysis also indicated that the same haplotype was present in the individuals that harboured the mutation. The two individuals not harbouring the mutation also did not have the haplotype

observed in the other MHS individuals suggesting that the two individuals were falsely diagnosed as positive (Healy *et al.*, 1996).

Monsieurs *et al.* (1998) suggested that the Gly341Arg mutation might be the cause of chronic elevated serum kinase activity. These authors identified two families having elevated resting CK activity. The Gly341Arg mutation co-segregated with the IVCT results in these two families. Moreover, all the individuals positive for the mutation had elevated CK levels. This suggested a possible relationship between CK activity and the Gly341Arg mutation. Monsieurs *et al.* (1998) also reported that the results of their study suggested that the defect in the RYR receptor might result in the loss of the integrity of the sarcolemmal membrane thus leading to increased levels of skeletal muscle proteins such as CK.

2.6.1.2.5 The Ile403Met mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Screening the RYR1 gene for possible mutations causing CCD revealed an Ile403Met substitution (Quane *et al.*, 1993). This substitution was detected via SSCP analysis of the cDNA after the aberrant conformers were identified and sequenced. A C1209G nucleotide substitution was detected which results in the loss of an *Mbo I* restriction enzyme site.

To establish whether the Ile403Met mutation was present in the normal population, 188 normal chromosomes were examined for the mutation. None of the chromosomes investigated harboured the candidate mutation. Co-segregation between the mutation and the CCD phenotype in one Italian CCD pedigree was indicated (Quane *et al.*, 1993). This family had no history of MH and had not been investigated via the IVCT. To date, the Ile403Met mutation has only been observed in this small Italian CCD pedigree (Manning *et al.*, 1998b). Examination of the amino acid sequence revealed that the Ile403 amino acid is conserved across species, and might thus be functionally important in the RYR1 protein (Quane *et al.*, 1993).

2.6.1.2.6 The Tyr522Ser mutation in the skeletal muscle ryanodine receptor (RYR1) gene

The Tyr522Ser amino acid substitution was identified by Quane *et al.* (1994b) in a French MHS family referred for MH testing. The mutation was observed during routine SSCP analysis of the cDNA from the French MHS pedigree. Direct sequencing of the

polymerase chain reaction (PCR) product resulted in the observation of an A1565C substitution in exon 14 of the RYR1 gene. The A1565C missense mutation does not result in the alteration of a restriction site. Therefore the presence of the mutation was detected via SSCP analysis of gDNA.

The Tyr522Ser mutation was absent from the 264 normal chromosomes screened for the mutation. Co-segregation of the mutation and the MHS phenotype was observed in the French family in which it was first detected. It was suggested that the Tyr522 amino acid is important for the function of the RYR proteins as it is conserved across species (Quane *et al.*, 1994b). To date, no other MHS family has been identified with this mutation (Manning *et al.*, 1998b).

2.6.1.2.7 The Arg533His mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Brandt *et al.* (1999) listed the Arg533His mutation as one of twenty-three mutations identified within the RYR1 gene. This mutation is due to the G1598A substitution which does not result in the alteration of a restriction site. The reported detection method for the Arg533His mutation is SSCP. The Arg533His mutation has been associated with the MH phenotype. Unfortunately no other information regarding the presence of the mutation in the normal population or segregation analysis has been reported for this mutation (Brandt *et al.*, 1999).

2.6.1.2.8 The Arg552Trp mutation in the skeletal muscle ryanodine receptor (RYR1) gene

The Arg552Trp mutation was detected via SSCP analysis in a large Irish family (Keating *et al.*, 1997). Sequence analysis displayed a single nucleotide substitution, C1654T. This substitution resulted in the amino acid replacement of an Arg by a Trp at position 552. The Arg552Trp mutation abolished a *Sau 3AI* restriction site, allowing for the detection of the mutation via restriction fragment length polymorphism (RFLP) analysis.

In order to provide evidence for the causative nature of the Arg552Trp mutation 200 normal chromosomes were analysed to confirm the absence of the mutation in the normal population (Keating *et al.*, 1997). Segregation analysis in the large Irish pedigree indicated that the mutation co-segregated with the MHS phenotype in this family. Arg522 may be a functionally important amino acid as it is conserved across species (Keating *et al.*, 1997).

2.6.1.2.9 The Arg614Cys mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Fujii *et al.* (1991) identified the Arg615 point mutation in pigs subsequently identified as the causative mutation of MH in swine. Gillard *et al.* (1991) analysed the syntenic human DNA region to identify the analogous human mutation. The Arg614Cys mutation was subsequently identified, which resulted from a C to T substitution at position 1840. This nucleotide substitution caused the deletion of a *Rsa I* restriction site that could be utilised in molecular diagnosis of this mutation via RFLP analysis (Otsu *et al.*, 1992).

This mutation was demonstrated to segregate with the MHS phenotype in one of 35 Canadian MH families (Gillard *et al.*, 1991). The C1840T missense mutation also co-segregated with the MH phenotype in two North German families (Steinfath *et al.*, 1995). Monori *et al.* (1995) reported the co-segregation of the Arg614Cys mutation in a large three generation pedigree. Analyses of 78 normal chromosomes were performed and none displayed the mutation (Gillard *et al.* 1991). Hogan *et al.* (1992) also identified a family with the Arg614Cys mutation and it was subsequently estimated that this mutation is present in 3-5% of MH families (Quane *et al.*, 1994a).

Treves *et al.* (1994) studied the intracellular Ca^{2+} of transfected COS-7 cells expressing the wild type and mutant recombinant DNA. These authors found that the Arg615Cys mutation alters the Ca^{2+} homeostasis of eukaryotic cells, thus providing further evidence for the causal nature of the mutation. The Arg614Cys mutation is the human analogue of the Arg615Cys porcine mutation and contributes towards the evidence for the causative nature of the Arg614Cys mutation. The pig could also be utilised as an animal model for MH.

Phenotype-genotype discordance have been reported in five families for the Arg614Cys mutations (Olckers *et al.*, 1994; Deufel *et al.*, 1995; Serfas *et al.*, 1996; Fagerlund *et al.*, 1997; Fortunato *et al.*, 1999). Olckers *et al.* (1994) first described two individuals in a large South African family displaying discordance. One MHS individual did not harbour the mutation and the other individual, although diagnosed as MHN via the IVCT, did harbour the mutation. This family is discussed in more detail in chapter four, paragraph 4.7. This was the first report of an MHN individual harbouring the Arg614Cys mutation.

Deufel *et al.* (1995) also observed phenotype-genotype discordance in a large complex family. Three MHS individuals do not harbour the mutation, while one MHN individual

does. A homozygote for the mutation was also identified in this family. The observation of these four discordant individuals was explained via three hypotheses (Deufel *et al.*, 1995). The first hypothesis is that two independent Arg614Cys mutations in one branch of the family and a third unrelated mutation in the other branch accounts for the discordance observed together with the false-negative IVCT result in one individual. The second hypothesis is that the C to T mutation alone is the cause of the MHS phenotype in this family, with the MHN individual being erroneously diagnosed as negative with the IVCT. The third is that a single dominant mutation is responsible for all MHS phenotypes in this family, which is not linked to the RYR1 locus (Deufel *et al.*, 1995). These authors agreed that none of these hypotheses can be rejected but the first hypothesis is highly unlikely, as it would require three independent mutations and false-negative IVCT results. The second hypothesis is also in disagreement with data available in the literature, according to Deufel *et al.* (1995). The authors reported that the third is compatible with the results they observed in this particular family.

Serfas *et al.* (1996) observed discordance in a large Manitoba Mennonite family. Initially five individuals displayed discrepancies between their IVCT and the DNA-based diagnosis. However, after re-evaluation of the IVCT data it was concluded that four of the tests were inconclusive due to poor muscle quality. One individual was re-biopsied and was subsequently diagnosed as MHN in contrast to the original MHS diagnosis. Two individuals did, however, display phenotype-genotype discordance. Both the individuals were diagnosed as MHS but did not harbour the mutation (Serfas *et al.*, 1996).

Discordance between the IVCT results and the Arg614Cys mutation results were observed in one Danish and one Swedish family (Fagerlund *et al.*, 1997). In the Danish family three MHS individuals did not harbour the Arg614Cys mutation. The Arg614Cys mutation was present in five MHS individuals and absent in all seven MHN individuals and two MHEh individuals. The Swedish family consisted of only three individuals, the proband that exhibited the mutation, her husband in whom it was absent, and their daughter who was diagnosed MHS via the IVCT but did not inherit the Arg614Cys mutation (Fagerlund *et al.*, 1997).

Fortunato *et al.* (1999) observed phenotype-genotype discordance in an individual who was phenotyped as MHN via the IVCT and in whom the mutation was detected. This is a rare case of discordance and has only been observed in two other individuals (Olckers *et al.*, 1994; Deufel *et al.*, 1995).

The phenotype-genotype discordance observed for the Arg614Cys mutation is alarming, and several explanations for this anomaly exist. The following provides a synopsis:

1. There may be no linkage to chromosome 19q13.1. Haplotype analysis performed for all the families in which discordance were observed indicated that the MH-designated haplotype segregated with the Arg614Cys mutation results, but not with the IVCT results. (Olckers *et al.*, 1994; Serfas *et al.*, 1996; Fagerlund *et al.*, 1997; Fortunato *et al.*, 1999).
2. There may be more than one MH mutation segregating in the discordant family (Serfas *et al.*, 1996; Fagerlund *et al.*, 1997; Fortunato *et al.*, 1999).
3. The phenotypic assessment of the individuals may be incorrect (Olckers *et al.*, 1994; Serfas *et al.*, 1996; Fagerlund *et al.*, 1997; Fortunato *et al.*, 1999).
4. The genotypic assessment of the individuals may be incorrect (Fortunato *et al.*, 1999). However, since the discordant individuals were sequenced and the RFLP results verified, this explanation of the discordant results seem unlikely.

It was suggested by Olckers *et al.* (1994), Serfas *et al.* (1996), Fagerlund *et al.*, (1997) and Fortunato *et al.*, (1999) that the most likely explanation for the observed phenotype-genotype discordance is incorrect phenotypic assessment.

2.6.1.2.10 The Arg614Leu mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Screening of the coding sequence of the RYR1 gene for new mutations lead to the identification of a novel Arg614Leu mutation in a Danish MHS family and two unrelated MHS probands (Quane *et al.*, 1997). Direct sequencing of the aberrant SSCP fragments revealed a base substitution, G1841T, that lead to the amino acid substitution of Arg with Leu at position 614.

A hundred and forty eight chromosomes from the normal population were examined for the presence of the novel mutation. This candidate mutation was not observed in any of the analysed chromosomes. The Arg614Leu mutation and the MHS phenotype co-segregated in the Danish MHS family in which it was originally detected. Examination of the RYR1 sequences of several species indicated that the Arg614 amino acid is conserved across species and is functionally important in the ryanodine receptor (Quane *et al.*, 1997).

2.6.1.2.11 The Arg2163Cys mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Manning *et al.* (1998a) undertook mutation screening for novel mutations in the RYR1 gene by utilising the SSCP technique. Previously reported mutations were not observed in the individuals included in their screening study. Unique SSCP patterns were detected in MHS individuals from several families. Sequence analysis of the amplified fragment identified one of the aberrant SSCP patterns that was due to a C6487T alteration, which results in an Arg for a Cys amino acid substitution in position 2163 (Manning *et al.*, 1998a).

This putative causative mutation was not present in the two hundred normal chromosomes analysed via SSCP. However, SSCP is not specific enough to determine the presence or absence of a particular mutation. Therefore, even though no aberrant conformer was observed in the normal chromosomes investigated it is not conclusive evidence that the Arg2163Cys mutation is absent from the general population. The mutation segregated with the MHS phenotype in the families in which it was identified. A comparison of the amino acid sequence showed that the Arg2163 amino acid is conserved across species (Manning *et al.*, 1998a).

2.6.1.2.12 The Arg2163His mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Screening for novel mutations in the coding region of the RYR1 gene revealed a unique SSCP conformer in one Belgian MHS individual and in an Italian MHS family (Manning *et al.*, 1998a). Direct sequencing of the aberrant SSCP conformer identified a novel mutation where the G6488A substitution results in the replacement of an Arg for a His amino acid in position 2163.

To determine whether this mutation was present in the normal population 200 normal chromosomes were analysed and none of the chromosomes investigated harboured the proposed novel mutation. Segregation studies were performed in the Italian family and the candidate mutation segregated with the MHS phenotype. The Arg2163His mutation seems to be associated with CCD as histological examination of the proband in the Italian family indicated asymptomatic CCD. To meet the genetic criteria for a causative mutation the amino acid sequence of several species were compared and it was found that the Arg2163 amino acid is conserved across species (Manning *et al.*, 1998a).

2.6.1.2.13 The Arg2163Pro mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Recently Brandt *et al.* (1999) reported the Arg2163Pro missense mutation. This mutation can be detected via RFLP analysis as the G6488C substitution results in the loss of a *Bsr BI* restriction enzyme site. The mutation was reported to be associated with the MH phenotype (Brandt *et al.*, 1999). No data providing evidence of the causal nature of the Arg2163Pro mutation was available other than the fact that the Arg2163 amino acid is conserved across species (discussed in the previous two sections).

2.6.1.2.14 The Val2168Met mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Screening for novel mutations in the coding region of the RYR1 gene revealed an unique SSCP fragment pattern in an MHS individual from a family of Swiss descent (Manning *et al.*, 1998a). Analysis of this aberrant fragment identified the nucleotide replacement of a G to an A in position 6502. This mutation results in the amino acid alteration Val2168Met. After subsequent investigation this mutation was identified in three additional Swiss individuals and one German individual. The detection of the Val2168Met mutation in the additional individuals may be an indication that this mutation is one of the most common mutations in the European population or that there may be a founder effect in the Swiss and German populations.

None of the 200 normal chromosomes investigated harboured the Val2168Met mutation. Segregation analysis of the mutation in the Swiss family indicated that the mutation co-segregated with the MHS phenotype. The Val2168 amino acid is conserved across several species (Manning *et al.*, 1998a).

2.6.1.2.15 The Thr2206Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

The Thr2206Arg mutation was identified in exon 40 of a MHS patient. The migration of an aberrant SSCP conformer differed from that of the Thr2206Met mutation. Sequencing of the aberrant conformer identified a novel C to G substitution at nucleotide position 6671. The Thr2206Arg mutation was not present in 80 normal individuals analysed and segregated with the MHS phenotype in the family in which it was identified (Brandt *et al.*, 1999).

2.6.1.2.16 The Thr2206Met mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Since none of the previous reported mutations were observed in a group of MHS individuals, SSCP analysis was employed to screen for possible causative mutations in the RYR1 gene (Manning *et al.*,1998a). A possible causative mutation C6617T was identified in one Irish family and one German MHS individual. The C6617T mutation results in the Thr2206Met amino acid substitution.

Two hundred normal chromosomes were analysed and none of the chromosomes harboured the Thr2206Met mutation. The MHS phenotype co-segregated with the mutation in the Irish family. The Thr2206 amino acid is considered to be a functionally important mutation as it is conserved across species (Manning *et al.*, 1998a).

2.6.1.2.17 The Gly2435Arg mutation in the skeletal muscle ryanodine receptor (RYR1) gene

An aberrant SSCP pattern was observed in a patient investigated for possible mutations (Keating *et al.*, 1994). Direct sequencing of this segment of the RYR1 gene indicated the base substitution of G7297 to A. Translation of this codon resulted in the replacement of a Gly with an Arg at position 2435.

The presence of the mutation needed to be investigated in the normal population and for this reason 200 normal chromosomes were analysed. None of the normal chromosomes harboured the G7297A substitution (Keating *et al.*, 1994). Phillips *et al.* (1994) also investigated 256 normal chromosomes and 746 chromosomes, which included 267 individuals with a history of chronic fatigue syndrome and muscle cramping, and 106 MHS pedigrees with at least one MHS individual. The mutation was not present in any of these chromosomes. The absence of this substitution in the general population and the individuals investigated contribute to the evidence that this substitution is a possible causative mutation and not a polymorphism. In addition, this Gly2435 amino acid is conserved across species and is therefore considered to be functionally important (Keating *et al.*, 1994). This mutation is thought to account for the MH phenotype in 4% of MH families (Phillips *et al.*, 1994).

It was established that this mutation was present in one Irish, two German MHS pedigrees and four Canadian families. The mutation was shown to co-segregate with the phenotype

for MHS in each of the families with the exception of the last Caucasian family in which one MHS individual did not display the mutation (Keating *et al.*, 1994). Phillips *et al.* (1994) also identified this mutation and subsequently found it in four Canadian pedigrees investigated. They also observed phenotype-genotype discordance in four individuals from the two families. In one family two of the discordant individuals were diagnosed as MHS and one as MHN with the IVCT. The fourth discordant individual was diagnosed as MHS but did not display the Gly2435Arg mutation (Phillips *et al.*, 1994). Two possible explanations for the observed discordance are that the IVCT results are incorrect or that a possible second mutation in the RYR1 gene or even in a second gene involved in MH may be responsible (Keating *et al.*, 1994; Phillips *et al.*, 1994). Both these authors concluded that the phenotype-genotype discordance observed was due to false-positive and false-negative diagnoses via the IVCT.

2.6.1.2.18 The Arg2436His mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Zhang *et al.* (1993) detected the substitution of A to G at position 7301. This missense mutation results in the substitution of an Arg by a His. The change in nucleotide sequence abolished the *Hga I* restriction enzyme site, creating an RFLP which can be used to detect the mutation. This mutation has been associated with both CCD and MH in one Canadian family. This provides evidence that CCD and MH are allelic disorders of the same gene (Zhang *et al.*, 1993). The mutation was also absent from 126 normal chromosomes analysed. The Arg2436His mutation appears to be restricted to the Canadian family in which it was identified (Manning *et al.*, 1998a). Zhang *et al.* (1993) suggested that the Arg2436His mutation is associated with CCD rather than MH, as it is located in the central region of the RYR1 gene and not in the N-terminal region where the other MH mutations (Gly248Arg and Arg614Cys) are located. No mutations associated with MH were identified in the central region of the gene at that time. However, several mutations (Arg2163Cys, Arg2163His, Arg2163Pro, Val2168Met, Thr2206Arg, Thr2206Met, Gly2435Arg, Arg2436Leu, Arg2454Cys, Arg2454His, Arg2458Cys and Arg2454His), have since been identified in the central region of the gene, all of which have been associated with MH (Keating *et al.*, 1994; Phillips *et al.*, 1994; Manning *et al.*, 1998b; Barone *et al.*, 1999; Brandt *et al.*, 1999). It is, therefore, possible that the Arg2436His mutation is associated with both MH and CCD, as Tong *et al.* (1997) demonstrated with transfection studies that the Arg2436His mutation is a causative mutation for MH.

2.6.1.2.19 The Arg2436Leu mutation in the skeletal muscle ryanodine receptor (RYR1) gene

The two mutation hot spots in the RYR1 gene were screened via SSCP for the presence of the most frequent MH mutations (Gly341Arg, Arg614Cys, Arg614Leu, Arg2163Cys, Arg2163His, gly2435Arg, Arg2436His, Arg2458Cys and Arg2458His). However, SSCP is not specific for any mutation and all the aberrant conformers observed needed to be sequenced to identify the possible substitution responsible for the different migration patterns. Barone *et al.* (1999) selected this screening method as it enabled them to screen a large region of the RYR1 gene for reported and novel mutations. Individuals were also screened for the Arg163Cys mutation via RFLP. During the screening for these mutations in patients with MH the Arg2436Leu mutation was identified in one MHS individual who has CCD (Barone *et al.*, 1999).

The Arg2436Leu mutation is potentially causative, as the mutation was not found in 96 normal chromosomes analysed. The Arg2436 amino acid is also conserved across species. This Arg2436Leu mutation also involves the same amino acid as the Arg2436His mutation. The Arg2436His mutation was identified in a large Canadian pedigree with both CCD and MH (Zhang *et al.*, 1993). The reported new Arg2436Leu mutation was found in a MHS individual with CCD. These two mutations result in the same phenotypic expression which suggests that the nature of the substitution is not relevant, but the position of the mutation in the protein is. Identification of the Arg2436Leu mutation supports the association of mutations within the RYR1 gene with both MH and CCD (Barone *et al.*, 1999).

2.6.1.2.20 The Arg2454Cys mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Brandt *et al.* (1999) also identified a novel missense mutation in exon 45 of the RYR1 gene. The Arg2454Cys mutation is the result of a C to T substitution at nucleotide position 7360. The mutation could be detected via RFLP as the mutation abolished a *Bsh 1236 I* restriction site (Brandt *et al.*, 1999). The Arg2454Cys mutation was identified in a MH family and segregated with the IVCT phenotype. Eighty normal control individuals did not display the mutation. The mutation might therefore be causative, however, other biochemical evidence is still required to prove the causative nature of this mutation.

2.6.1.2.21 The Arg2454His mutation in the skeletal muscle ryanodine receptor (RYR1) gene

The Arg2454His mutation identified by Barone *et al.* (1999) in a large Italian family is a potentially causative mutation as it satisfies the criteria for a potentially causative mutation. The mutation was absent in the 96 normal chromosomes analysed, and the Arg2454 amino acid is a functionally significant amino acid that is conserved across species. The Arg2454His mutation also segregates with the MHS phenotype in the large Italian family (Barone *et al.*, 1999). The identification of the Arg2454His mutation in the Italian family allowed the authors to perform a pre-symptomatic diagnosis of MHS for individuals in this family not yet investigated via the IVCT.

2.6.1.2.22 The Arg2458Cys mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Manning *et al.* (1998b) focused their mutation screening on two regions of the RYR1 gene, the N-terminal and the central portion. Short segments of the regions of interest were analysed via SSCP. In several MHS and MHE individuals an unique SSCP pattern was observed. Sequencing of the RYR1 segment of interest revealed a novel mutation C7372T resulting in the substitution of an Arg for a Cys at position 2458.

This mutation was not present in 200 normal chromosomes analysed (Manning *et al.*, 1998b). The mutation occurs at a C residue in a CpG dinucleotide. The CpG dinucleotides have been shown to have frequent mutations at the C or G residues due to deamination of 5-methylcytosine (5meC) residues (Cooper and Krawczak, 1995). Segregation analysis showed that the Arg2458Cys mutation co-segregated with MHS and MHE phenotype with the exception of one MHE(h) individual (Manning *et al.*, 1998b).

2.6.1.2.23 The Arg2458His mutation in the skeletal muscle ryanodine receptor (RYR1) gene

Due to the identification of the Arg2458Cys mutation at a C residue in a CpG dinucleotide, the possibility that a G residue was involved in the mutation was investigated. A different SSCP fragment pattern was observed in the MHS individuals of a French family screened. Further analysis of the segments revealed that the G residue of the CpG dinucleotide was replaced with an adenine. The G7373A transition mutation resulted in the substitution of an Arg for a His at position 2458. The Arg2458His mutation is the most C-terminal mutation reported in the RYR1 gene, to date. Segregation analysis of this mutation was

performed by SSCP in a selected family and showed co-segregation with the MHS phenotype (Manning *et al.*, 1998b).

2.6.2 ***Genetic heterogeneity***

Genetic heterogeneity has been suspected in MH due to the highly variable clinical presentation of the MH phenotype (Levitt *et al.*, 1991) and the association of MH with various other disorders (Brownell, 1988; Allen, 1993). MH is a complex genetic disorder in which both locus and allelic heterogeneity has been documented (Hogan, 1997).

2.6.2.1 **Locus heterogeneity**

In 1991, Levitt *et al.* was first to report three unrelated families that did not display linkage to the region on chromosome 19q. Since the report by Levitt *et al.* (1991) several pedigrees have been identified which do not display linkage to the candidate RYR1 gene located on chromosome 19q13.1 and provided additional evidence for genetic heterogeneity (Deufel *et al.*, 1992; Iles *et al.*, 1992; Levitt *et al.*, 1992; Olckers, 1993; MacLennan *et al.*, 1995) in different populations. It is estimated that less than 50% of MHS families display linkage to the RYR1 locus on chromosome 19q13.1 (Ball and Johnson, 1993). Linkage has subsequently been established to six other loci in the genome: on chromosomes 17q, 7q, 5p, 3q, 2q and 1q (Levitt *et al.*, 1992; Iles *et al.*, 1994; Sudbrak *et al.*, 1995; Olckers, 1997; Robinson *et al.*, 1997).

Levitt *et al.* (1992) reported that two South African and three North American MHS families displayed close linkage to the 17q11.2-q24 chromosomal region. Two candidate genes are located within the region of interest on chromosome 17q: the adult muscle sodium channel α sub-unit gene (SCN4A), and two sub-units (β and γ) of the dihydropyridine receptor (CACNLB1 and CACNLG) (Levitt *et al.*, 1992; Iles *et al.*, 1993). The SCN4A gene has been proposed as a candidate gene responsible for abnormal IVCT results (Olckers *et al.*, 1992; Levitt *et al.*, 1992; Vita *et al.*, 1995; Vita *et al.*, 1996). These authors reported that a mutation in the SCN4A gene is associated with masseter muscle rigidity and abnormal IVCT used to diagnose MHS. MH has been associated with masseter muscle rigidity, as discussed in paragraph 2.4. Linkage analysis of a large Canadian family with a history of hyperkalemic periodic paralysis and MH confirmed linkage to the 17q region as well as the association of the SCN4A gene with MH (Moslehi *et al.*, 1998) as proposed by Olckers *et al.* (1992) and Levitt *et al.* (1992).

Iles *et al.* (1994) found evidence for linkage to chromosome 7q in one three-generation German family. The gene encoding the α_2/δ sub-unit of the dihydropyridine receptor (CACNL2A) was localised on the proximal long arm of chromosome 7q21-22 (Powers *et al.*, 1994). This CACNL2A gene was the candidate gene under investigation for possible mutations associated with MHS (Iles *et al.*, 1994). Cawood *et al.* (1999) also reported linkage to chromosome 7q in a North American MHS family. Haplotype analysis in the North American MHS family indicated that the haplotype identified segregated with the MHS phenotype in this family.

In linkage studies covering the entire human genome another possible MHS locus was identified. A single German family with classical MH displayed linkage to chromosome 3q13.1 with a maximum lod score of 3.22. No candidate gene has been identified within the chromosome region that displayed linkage with the MHS phenotype in the German pedigree (Sudbrak *et al.*, 1995).

Monnier *et al.* (1997) reported a French MHS family in which linkage between the α_1 sub-unit of the skeletal muscle dihydropyridine receptor (CACNL1A3) gene and the MHS phenotype has been found. The CACNL1A3 gene has been mapped to chromosome 1q31-32 (Sudbrak *et al.*, 1993). Subsequently Monnier *et al.* (1997) also identified a mutation within the CACNL1A3 gene that is associated with the MH phenotype.

A Belgium family displayed positive lod scores for a haplotype on chromosome 5p (Robinson *et al.*, 1997). Although no candidate gene is known in the region on chromosome 5p it does provide evidence of another locus involved in the MH phenotype. An additional pedigree included in the linkage study by Robinson and co-workers did not display linkage to any of the published loci thus suggesting that there are at least one other locus that has not yet been identified (Robinson *et al.*, 1997).

A large South African MHS family (MH102) did not display linkage to the six reported MH loci (Olckers, 1997; Olckers *et al.*, 1999). Linkage screening of the genome of this South African MHS family identified a novel locus on chromosome 2q. Olckers *et al.* (1999) also observed discordance in the South African family (MH102). The designated haplotype segregated with the MHS phenotype in this family with the exception of two MHS individuals. However, Olckers *et al.* (1994) also described discordance in another large South African MH family (SA105) where they concluded that the discordance is most likely due to false IVCT diagnoses. If the discordance observed in the family MH102 is also due

to false-positive diagnoses then results from their study suggest that the region on chromosome 2 might harbour a gene responsible for the MHS phenotype segregating in this South African MHS family (Olckers *et al.*, 1999).

2.6.2.2 Allelic heterogeneity

Allelic heterogeneity has been documented in both the RYR1 and the CACNL1A3 genes (Moslehi *et al.*, 1998). In the RYR1 gene one mutation, Ile403Met, has been associated with CCD in one family and four other mutations (Arg163Cys, Tyr522Ser, Arg2163His and Arg2436His) have been identified in individuals with both MH and CCD (Brandt *et al.*, 1999). Zhang *et al.* (1993) reported that these two disorders are allelic disorders as mutations in the same gene cause the two distinct phenotypes.

Three different mutations (Arg528His, Arg1239His and Arg1239Gly) have been associated with hypokalemic paralysis in the CACNL1A3 gene (Monnier *et al.*, 1997). One other mutation, Arg1086His, was identified in the CACNL1A3 gene and this mutation was associated with MHS. MHS and hypokalemic periodic paralysis are, therefore, also allelic disorders (Monnier *et al.*, 1997).

The Gly1306Ala mutation in the SCN4A gene has been associated with masseter muscle rigidity, whole body rigidity and abnormal IVCT results (Vita *et al.*, 1995). Other mutations within this gene are associated with paramyotonia congenita and hyperkalemic periodic paralysis (Vita *et al.*, 1995; Moslehi *et al.*, 1998). These reports indicated the existence of allelic heterogeneity in the SCN4A gene (Moslehi *et al.*, 1998). Although mutations associated with MHS have not yet been identified within the SCN4A gene the possibility cannot be ignored. Therefore, MH, paramyotonia congenita, hyperkalemic periodic paralysis and masseter muscle rigidity may be allelic disorders associated with mutations within the SCN4A gene (Vita *et al.*, 1995; Moslehi *et al.*, 1998).

2.7 Aims of the study

Determination of MH status of individuals prior to receiving anaesthesia is important and the *in vitro* contracture test (IVCT) and caffeine halothane contracture test (CHCT) are currently used worldwide to determine the MH susceptibility of individuals (Kalow *et al.*, 1970). However, both the IVCT and CHCT are highly invasive, expensive and time consuming procedures and neither the IVCT protocol nor the CHCT protocol is 100%

sensitive and specific (Ørding *et al.*, 1997; Allen *et al.*, 1998). False-negative diagnoses have been reported for both these protocols (Isaacs and Badenhorst, 1993; Wedel and Nelson, 1994). This type of erroneous diagnoses can have fatal consequences for the MHS individual. The development of a sensitive and non-invasive diagnostic test for MH is therefore of great importance. However, many attempts to develop a diagnostic test have failed. It appears as though the development of a DNA based molecular diagnostic protocol would be the only non-invasive procedure to diagnose MH accurately. The identification of twenty-three missense mutations in the RYR1 gene that have been reported to co-segregate with the MH phenotype (Brandt *et al.*, 1999) represents the first step towards establishing a molecular diagnostic service.

In this study seventeen MH families, four South African and thirteen North American, were included. A total of 89 individuals, 39 South African and 50 North American individuals, were previously phenotyped with the IVCT and CHCT respectively. This group included 53 MH positive and 36 MH negative individuals. Eighty-nine selected MH individuals were screened for nine of the reported missense mutations. Phenotype-genotype discordance has previously been reported for some of the mutations (Gly341Arg, Arg614Cys and Gly2435Arg) investigated in this study (Keating *et al.*, 1994; Olckers *et al.*, 1994; Phillips *et al.*, 1994; Deufel *et al.*, 1995; Adeokun *et al.*, 1997). For this reason as many as possible phenotyped individuals were included in this screening study. To ensure cost effectiveness, it was consequently decided that, whenever possible, at least five positive individuals and two negative (control) individuals from each family should be included in the screening study. The interaction between polymorphisms and mutations, which might modify or contribute to the MH phenotype, is currently still unknown. For this reason the selected individuals were screened for the presence of all of the nine mutations investigated in this study.

Specific aims of this study were:

1. Screening 89 selected individuals from malignant hyperthermia susceptible (MHS) families, four South African and thirteen North American, for the presence of nine of the reported mutations in the RYR1 gene.
2. The selected individuals would be screened for the following nine reported mutations: Cys35Arg, Arg163Cys, Gly248Arg, Gly341Arg, Ile403Met, Tyr522Ser, Arg614Cys, Gly2435Arg and Arg2436His.
3. If mutations were present in individuals included in the initial screening study, additional family members from that particular individual's family would be screened

for the relevant mutation(s).

4. Establishing a molecular diagnostic service for individuals within the particular MH families in which a specific mutation was identified.
5. Establishing a mutation screening service for individuals from MH families for the reported MH mutations, in South Africa.

CHAPTER THREE

MATERIALS AND METHODS

All chemicals were analar grade from United States Biochemical (USB), unless otherwise indicated. Radiolabelled nucleotides were obtained from Amersham Life Science in the United Kingdom (UK).

The ethical committee of the University of Pretoria approved this study as protocol 42/97. Informed consent was obtained from individuals before blood samples were collected.

Seventeen MH families were utilised in this mutation screening study. All individuals of whom biopsy data were available were included in the study. DNA of the North American families were obtained from Prof. R.C. Levitt, currently from Magainin Pharmaceuticals Inc. United States of America (USA) (previously from Department of Anesthesiology and Critical Care Medicine, Johns Hopkins Medical Institutions), and Prof. J.E. Fletcher, Allegheny University of the Health Sciences, Philadelphia, USA. When a mutation was detected in an individual, the rest of that particular individual's family was screened for the relevant mutation.

3.1 South African malignant hyperthermia (MH) families

Four extended South African families with MH were identified utilising the European IVCT protocol (European Malignant Hyperpyrexia Group, 1984) and the results are listed in Table 3.1. These families included forty-five individuals that had been phenotyped with the IVCT. Of the forty-five individuals twenty-two were MHS, twenty-two individuals were MHN and one individual was MHEh. The pedigrees were numbered in an unconventional manner. Families were numbered MH101, MH102, MH104 and MH105 respectively, and each individual was allocated a unique number, e.g. MH105-38, where the 105 indicated the family number and 38 indicated the individual number. This allowed for the inclusion, as needed, of new family members in this ongoing study.

Table 3.1: IVCT results of the South African MHS families[1]

Family and individual number	Caffeine (mM)	Halothane (vol %)	MH Status
MH101-6	2.0	1.0	MHS
MH101-10	2.0	0.5	MHS
MH101-12	4.0	NR 4.0	MHN
MH102-2	1.0	1.0	MHS
MH102-4	8.0	NR 4.0	MHN
MH102-11	8.0	NR 4.0	MHN
MH102-24	0.5	1.0	MHS
MH102-28	2.0	1.0	MHS
MH102-32	***	***	MHS
MH102-39	2.0	0.5	MHS
MH102-48	NR	NR	MHN
MH102-96	4.0	2.0	MHEh
MH102-117	2.0	0.5	MHS
MH102-125	1.0	2.0	MHS
MH104-24	8.0	NR 4.0	FALSE MHN
MH104-25	8.0	2.5	MHN
MH104-26	1.0	0.5	MHS
MH104-27	NR	NR	MHN
MH104-33	8.0	NR	MHN
MH104-35	2.0	1.0	MHS
MH104-40	8.0	NR	MHN
MH104-41	8.0	NR	MHN
MH105-14	***	***	MHN
MH105-17	1.5	0.5	MHS
MH105-20	2.0	1.0	MHS
MH105-23	4.0	NR 4.0	MHN
MH105-26	2.0	0.5	MHS
MH105-28	4.0	NR 4.0	MHN
MH105-32	1.0	1.0	MHS
MH105-35	2.0	1.0	MHS
MH105-36	1.0	2.0	MHS
MH105-37	4.0	NR 4.0	MHN
MH105-38	0.5	0.5	MHS
MH105-39	8.0	NR 4.0	MHN
MH105-52	3.0	NR	MHN
MH105-55	***	***	MHS
MH105-63	1.0	1.0	MHS
MH105-64	1.5	0.5	MHS
MH105-83	8.0	NR 4.0	MHN
MH105-88	4.0	NR 4.0	MHN
MH105-98	8.0	NR 4.0	MHN
MH105-104	8.0	NR 4.0	MHN
MH105-110	***	***	MHN
MH105-117	4.0	NR 4.0	MHN
MH105-124	8.0	NR 4.0	MHN

NR = No response at; MHN = MH normal; MHS = MH susceptible; MHEh = MH equivocal, susceptible to halothane; ***, see text.

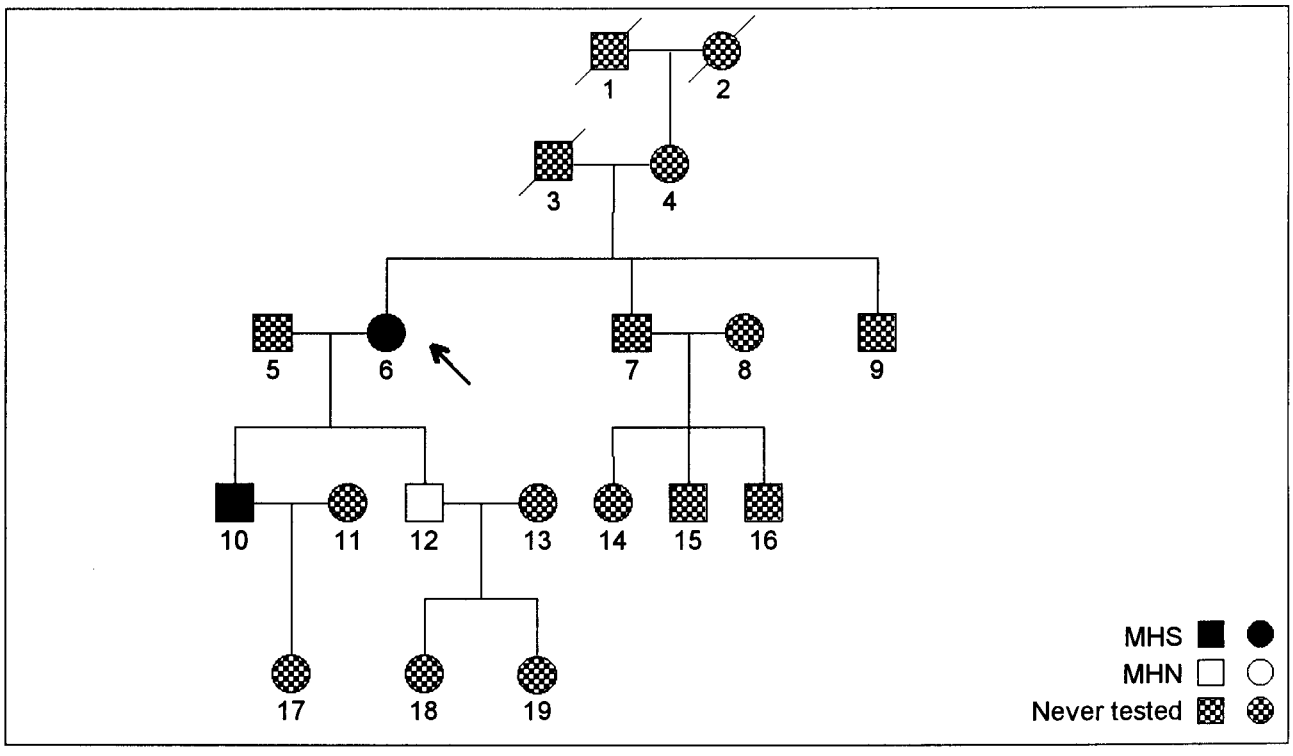
3.1.1 *South African malignant hyperthermia family MH101*

This South African family contains two individuals that are MHS and one individual that has been diagnosed as MHN. The proband in this family, individual MH101-6, is indicated in Figure 3.1. She developed a high fever and diaphoresis post-operatively, and was

[1] Data compiled by A. Olckers (1997).

subsequently tested for MH. The two children of the proband, MH101-10 and MH101-12, tested MHS and MHN respectively. The IVCT results of this family are listed in Table 3.1.

Figure 3.1: Pedigree of South African malignant hyperthermia family MH101



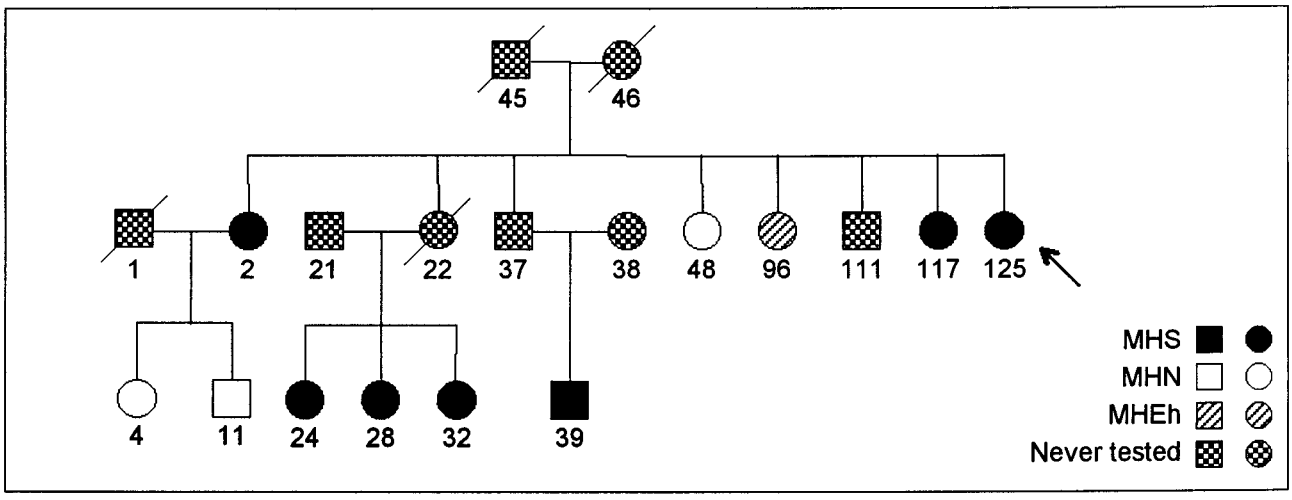
An explanation of the symbols is provided in the list of abbreviations and symbols.

3.1.2 *South African malignant hyperthermia family MH102*

Biopsy data on eleven members of family MH102 were available and are listed in Table 3.1. Seven individuals tested MHS and three tested MHN. For individual MH102-96 the IVCT results indicated that there was no response to caffeine but that there was a response to halothane. The MH status of this individual is MHEh, but she is regarded as MH positive for clinical purposes.

The proband of this large family (Appendix A) is individual MH102-125, as indicated in Figure 3.2. This female developed a high fever during anaesthesia. She was diagnosed in 1979, before the European IVCT protocol was adopted and her contracture study yielded a positive result for halothane. This individual's diagnosis was confirmed when the European IVCT protocol was introduced and her muscle biopsy was exposed to caffeine as well. Individual MH102-32 lives in the United States of America, where she was diagnosed as positive for MH. No details about her muscle biopsy was available and there was no DNA sample available to include in this study.

Figure 3.2: Excerpt from the pedigree of South African malignant hyperthermia family MH102

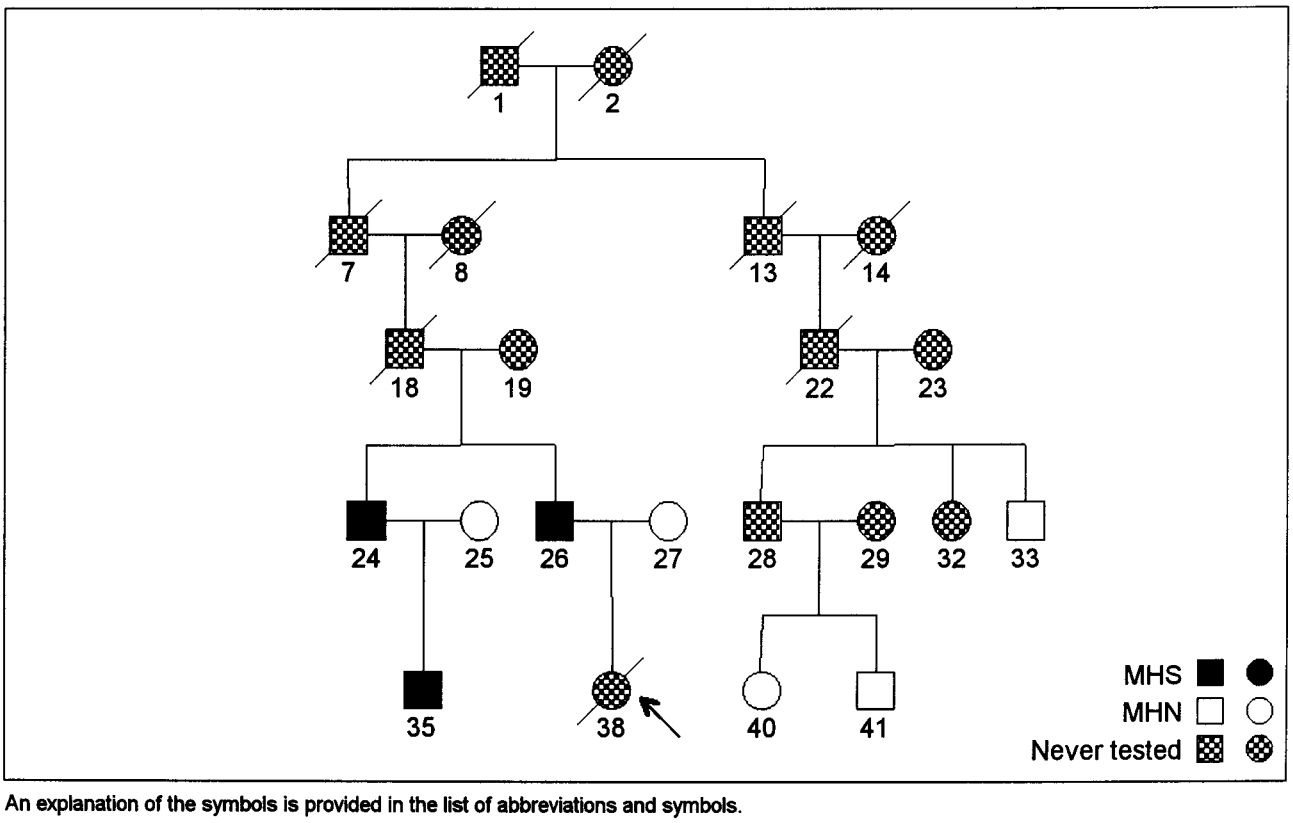


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.1.3 South African malignant hyperthermia family MH104

Family MH104, Figure 3.3, was investigated after the death of the proband (MH104-38). This young female died at the age of two years and four months, due to a malignant hyperthermia episode after receiving anaesthesia for dental treatment. Eight family members were subsequently investigated of which three were identified as MHS.

Figure 3.3: Excerpt from the pedigree of South African malignant hyperthermia family MH104



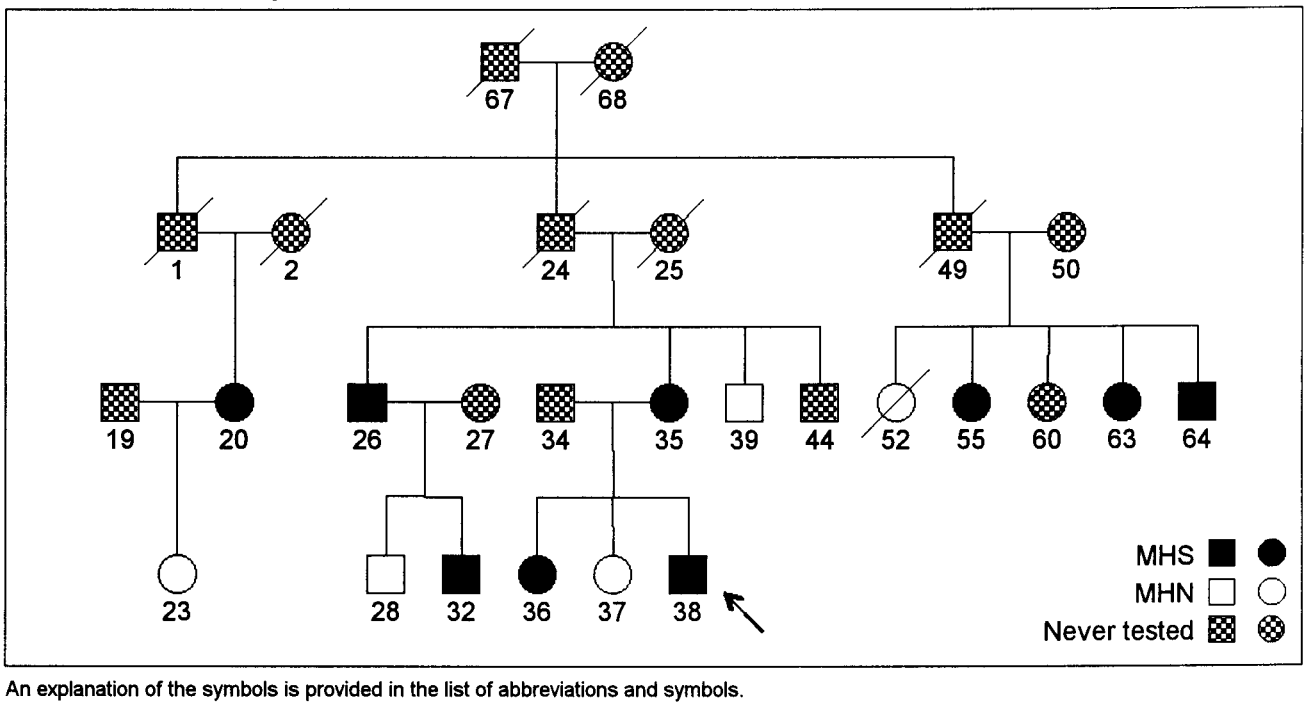
An explanation of the symbols is provided in the list of abbreviations and symbols.

Individual MH104-24 was initially diagnosed as MHN, but his diagnosis was changed after his son (MH104-35) was diagnosed positive. His wife (MH104-25) was diagnosed as MHN and her family had no history of MH. Table 3.1 lists the IVCT results of this family. No DNA sample was available for individual MH104-33 and therefore he was not included in this study. The full pedigree of MH104 is presented in Appendix A.

3.1.4 *South African malignant hyperthermia family MH105*

The proband, individual MH105-38 in Figure 3.4, suffered a malignant hyperthermia episode as a young male. Unfortunately no details of the incident were recorded. At the age of nine he was subjected to an IVCT and found to be MHS. Via the IVCT nine other individuals of this family was found to be positive for MH and thirteen members were diagnosed as MHN. The extended pedigree of this family is presented in appendix A and the biopsy data are listed in Table 3.1. Four individuals (MH105-14, MH105-17, MH105-52 and MH105-110) could not be included in this study as no DNA samples were available for analysis.

Figure 3.4: Excerpt from the pedigree of the South African malignant hyperthermia family MH105



An explanation of the symbols is provided in the list of abbreviations and symbols.

Individuals MH105-55 and MH105-110 reside in England and Australia respectively. Detailed records of the diagnosis of individual MH105-55 were obtained from Dr. Ellis at

the University of Leeds, UK. The diagnosis of individual MH105-110 was performed in Australia and no data on the results are available. No data was available for individual MH105-14 except that this individual was diagnosed as MHN via the IVCT.

3.2 North American malignant hyperthermia (MH) families

Thirteen North American families with MH were selected for inclusion in this study. Biopsy data of fifty-seven individuals were available. Thirty-eight individuals were diagnosed as MHS and nineteen individuals were diagnosed as MHN. The pedigrees were numbered in the same fashion as in the case of the South African families - the only difference was that the prefix US was used to indicate the origin of these families to be the United States (US). All the individuals selected for this study were phenotyped utilising the North American CHCT protocol (Larach, 1989) and results are listed in Table 3.2.

Table 3.2: CHCT results of the North American MHS families[2]

Family and individual number	Contracture at 2 mM caffeine (g)	Contracture at 3% halothane [a), b)] (g)	MH Status
US1-3	0.20	1.30	MHS
US1-7	0.20	2.20	MHS
US1-12	0.00	1.10	MHS
US1-17	0.00	2.80	MHS
US1-18	1.00	1.40	MHS
US1-19	0.00	0.10	MHN
US2-1	0.01	2.80	MHS
US2-2	0.00	0.00	MHN
US2-4	0.50	1.00	MHS
US3-1	0.00	0.70	MHN
US3-3	0.00	0.40	MHN
US3-4	0.00	1.70	MHS
US3-6	0.50	1.00	MHS
US7-11	0.00	3.10	MHS
US7-13	0.00	0.50	MHN
US7-14	0.10	0.50	MHN
US7-15	0.10	2.50	MHS
US7-16	0.00	0.50 [a)] (2%)	MHS
US8-3	0.00	0.20	MHN
US8-5	1.10	5.00	MHS
US8-7	0.20	2.00	MHS
US8-11	0.35	2.20 [b)] (1%)	MHS
US8-12	0.00	0.00 [a)] (2%)	MHN
US8-19	0.00	0.00 [a)] (2%)	MHN
US9-6	0.16	0.80	MHS
US9-8	0.00	0.70	MHS
US9-10	0.00	1.50	MHS
US9-11	0.10	0.70	MHS
US10-6	1.10	6.80	MHS

continued ...

[2] Data compiled by G. M. Vita.(as listed by Olckers, 1997)

Table 3.2, continued ...

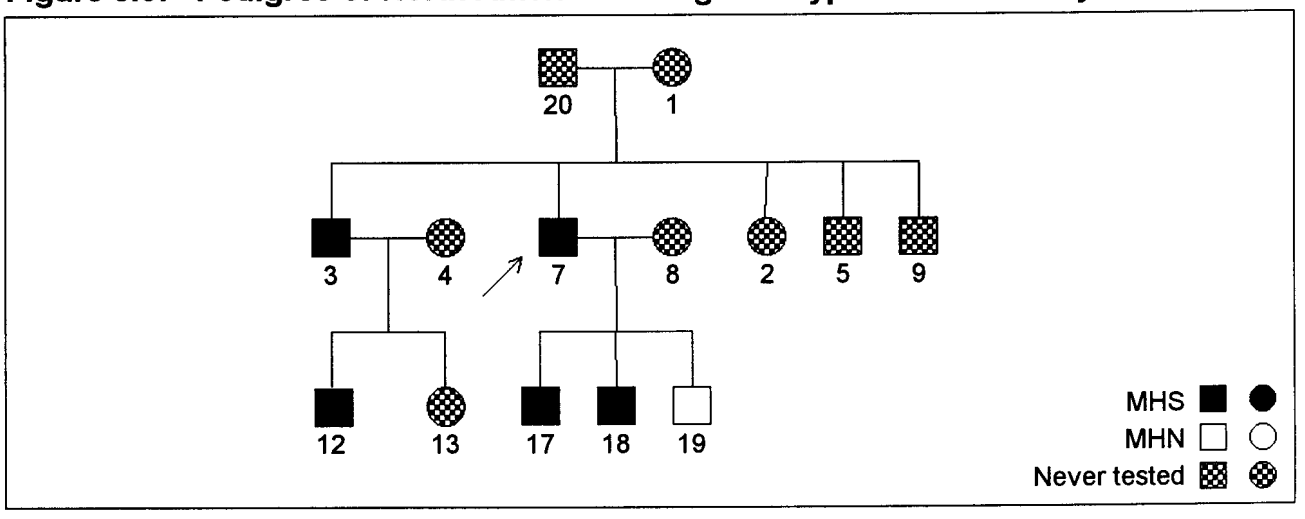
Family and individual number	Contracture at 2 mM caffeine (g)	Contracture at 3% halothane [a), b)] (g)	MH Status
US10-7	0.00	7.10	MHS
US10-9	0.10	0.10	MHN
US10-9	0.10	0.10	MHN
US10-10	0.00	0.20	MHN
US10-11	0.00	2.20	MHS
US10-12	0.50	1.00	MHS
US11-1	0.00	0.00	MHN
US11-3	0.00	1.50	MHS
US11-4	0.00	1.00	MHS
US12-2	0.20	3.10	MHS
US12-4	0.50	1.00	MHS
US12-7	0.00	0.90	MHS
US13-5	0.00	1.00	MHS
US13-6	0.00	0.20	MHN
US13-9	0.30	0.50	MHN
US13-10	0.00	1.30	MHS
US13-11	0.00	0.80	MHS
US13-12	0.00	0.40	MHN
US14-2	0.00	0.75	MHS
US14-6	0.50	1.00	MHS
US14-7	0.00	0.80	MHS
US14-8	0.30	0.25	MHN
US15-6	0.50	1.00	MHS
US15-7	0.00	0.20	MHN
US15-8	0.00	1.50	MHS
US15-13	0.00	0.00	MHN
US16-3	0.00	0.10	MHN
US16-6	0.10	1.40	MHS
US16-12	0.10	1.50	MHS

a) Contractures measured at 2% halothane; b) Contractures measured at 1% halothane; MHN = MH normal; MHS = MH susceptible.

3.2.1 *North American malignant hyperthermia family US1*

Individual US1-7, the male proband, developed pyrexia, decreased serum pH and an elevation in exhaled pCO_2 during anaesthesia. Post-operatively the patient's CPK rose to more than 87 000 $IU.l^{-1}$ and he reported significant muscle weakness and soreness for several weeks. A muscle sample was obtained and he was subsequently diagnosed as MHS. CHCT data, Table 3.2, of the rest of the family identified four positive individuals (US1-3, US1-12, US1-17 and US1-18) and one negative individual (US1-19) as presented in Figure 3.5.

Figure 3.5: Pedigree of North American malignant hyperthermia family US1

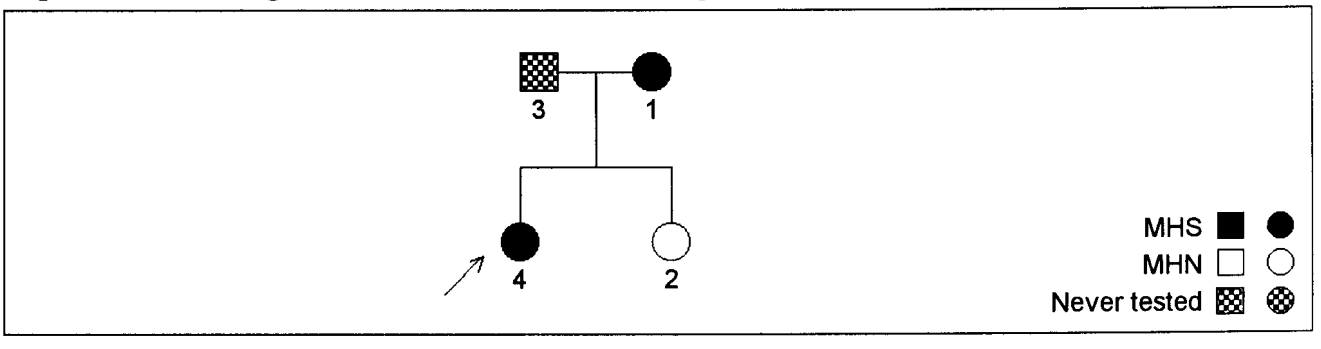


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.2 *North American malignant hyperthermia family US2*

In Figure 3.6 the proband of this family, individual US2-4, is indicated. She developed an MH reaction after receiving halothane, nitrous oxide and succinylcholine while undergoing anaesthesia at the age of five. Masseter spasm and diffuse rigidity complicated the anaesthesia. Post-operatively the proband also presented with the following symptoms: arrhythmia, tachycardia, metabolic acidosis (pH 7.30; pCO_2 36 mmHg; HCO_3 17 $mmol.l^{-1}$) and rhabdomyolysis (CPK rose to 75 000 $IU.l^{-1}$). Her urine analysis indicated a "straw" colour, myoglobin and haemoglobin. Upon investigation the proband's mother (US2-1) was diagnosed as MHS and her sibling (US2-2) was diagnosed as MHN. This family's CHCT results are listed in Table 3.2.

Figure 3.6: Pedigree of North American malignant hyperthermia family US2



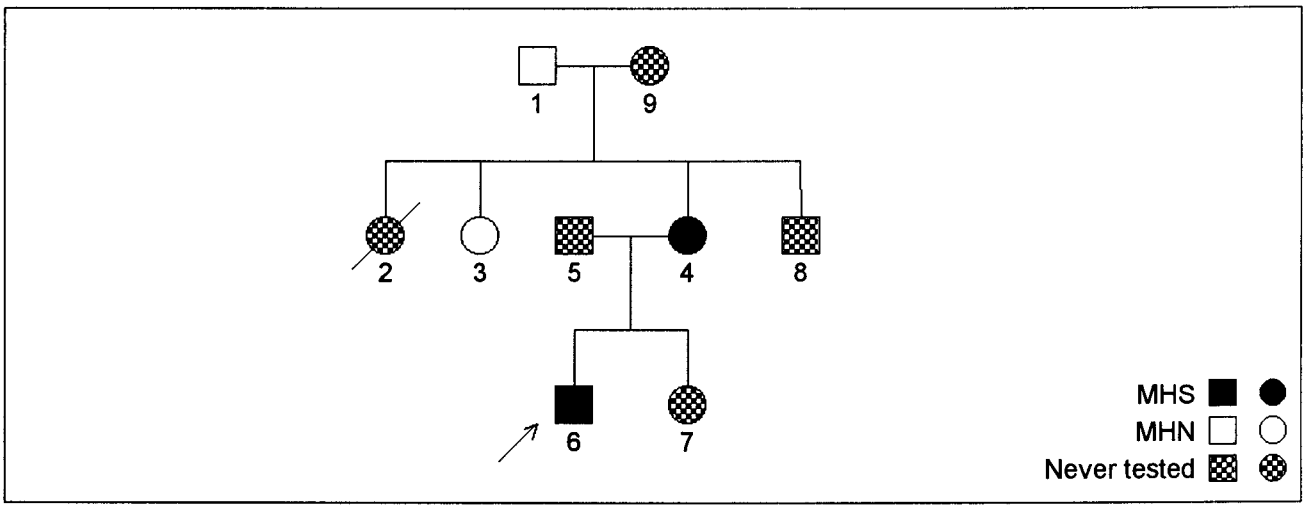
An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.3 *North American malignant hyperthermia family US3*

The pedigree of US3 is presented in Figure 3.7. CHCT was performed on four individuals of this family. Two individuals were diagnosed as MHS and two as MHN. Biopsy results

are listed in Table 3.2. The death of individual US3-2 is suspected to be due to MH, but no medical records are available to verify the cause of death. The proband of this North American family is individual US3-6. He developed a high body temperature of 38.2°C, while undergoing a surgical procedure to correct his strabismus. His temperature was 38.0°C at the end of surgery and in addition to the pyrexia he developed tachycardia and was treated for MH with dantrolene. After surgery his CPK rose to 9 800 $IU.l^{-1}$. Unfortunately no DNA sample was available for US3-6 who could therefore not be included in this study.

Figure 3.7: Pedigree of North American malignant hyperthermia family US3

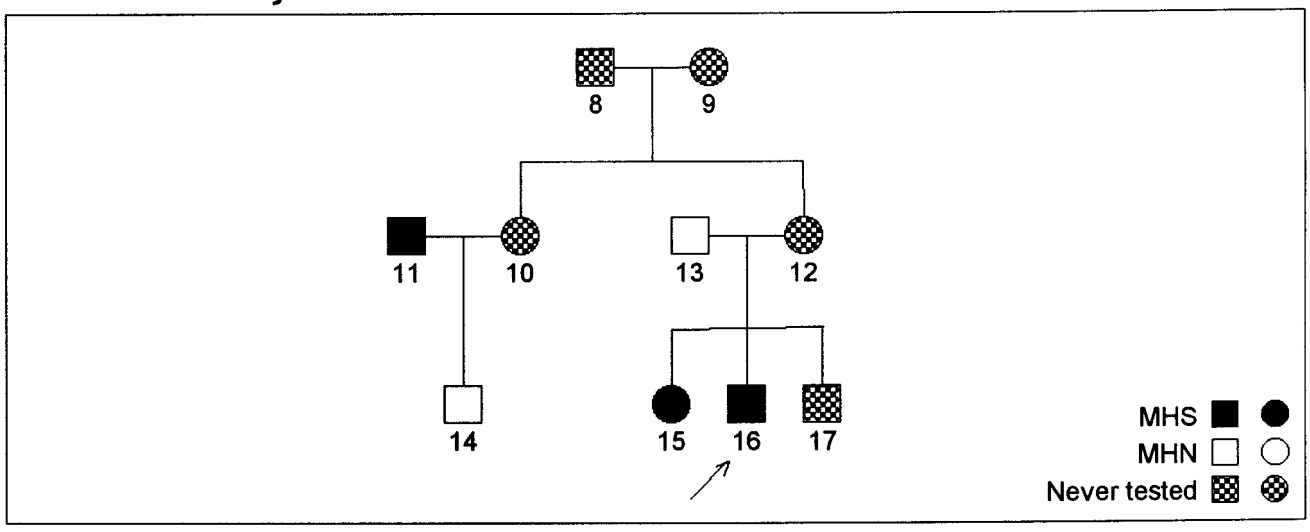


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.4 *North American malignant hyperthermia family US7*

This family included three MHS and two MHN individuals, and the pedigree of the family is presented in Figure 3.8. DNA samples were available for only three individuals (US7-14, US7-15 and US7-16) of this family and therefore individual US7-11 could not be included in this study. The proband, individual US7-16, developed an MH reaction during administration of anaesthesia for a tonsillectomy. He received premedication of 15 mg morphine, 50 mg nembutal and 0.3 mg atropine which was administered intramuscularly. Anaesthesia was induced by mask using halothane, nitrous oxide and oxygen. Twenty milligrams of succinylcoline was administered intravenously after which diffuse rigidity, masseter muscle spasm and an increase in heart rate were observed. After an additional 16 mg of succinylcholine was administered and the patient failed to relax the anaesthesia was discontinued. No elevation in body temperature was noted, but his CPK rose to 4 245 $IU.l^{-1}$ within two hours after the anaesthesia was discontinued.

Figure 3.8: Excerpt from the pedigree of North American malignant hyperthermia family US7

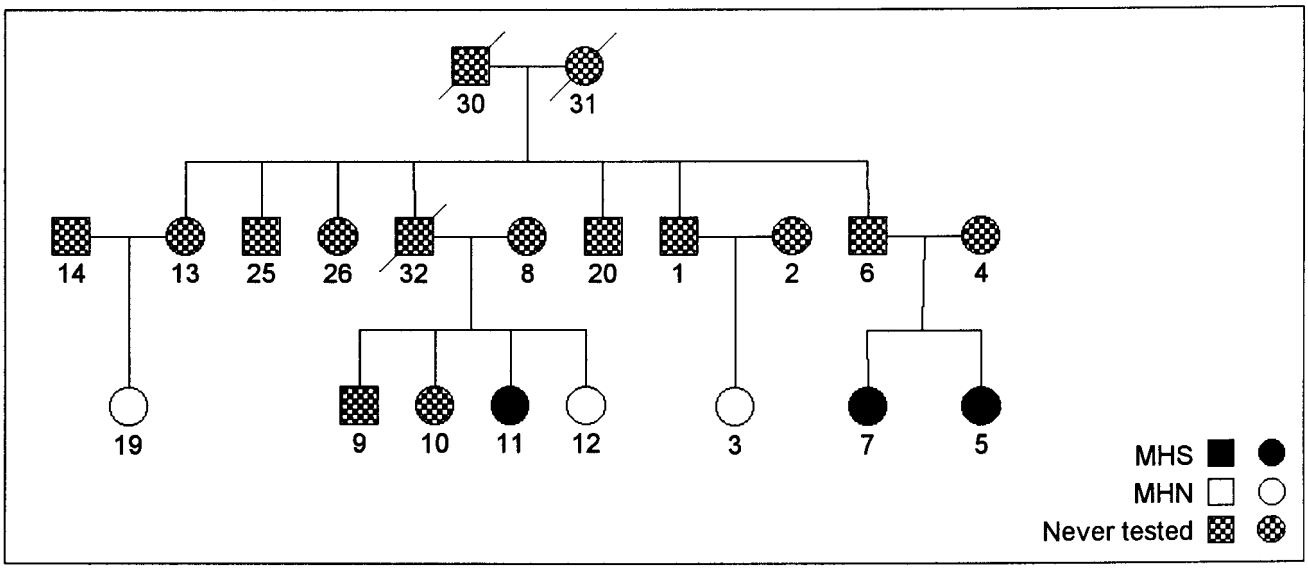


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.5 *North American malignant hyperthermia family US8*

The pedigree of family US8 is presented in Figure 3.9. Biopsy data of six individuals were available which included three MHS and three MHN individuals. Results of the contracture studies are listed in Table 3.2. No records of the clinical data were available for this family, but the CHCT data substantiated the MHS status of the family. Individual US8-3 could not be included in this study as no DNA sample was available.

Figure 3.9: Excerpt from the pedigree of North American malignant hyperthermia family US8

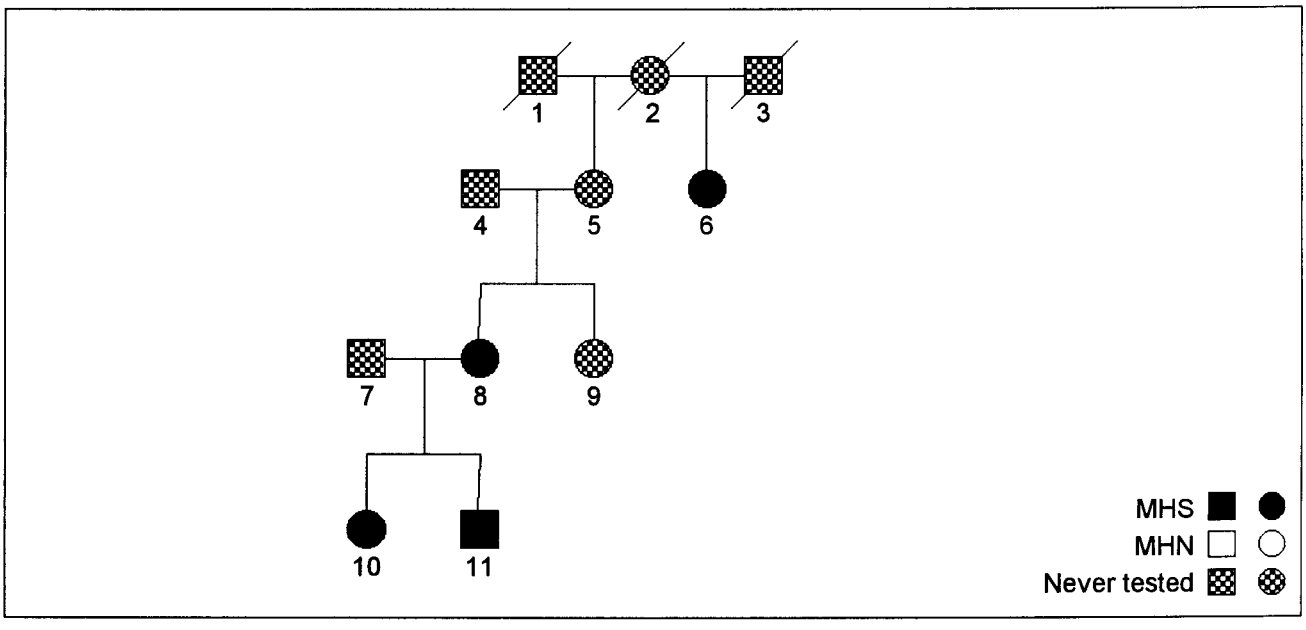


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.6 North American malignant hyperthermia family US9

No clinical data of this family was available. CHCT results classify the four individuals in this family as MHS. A 1.5 g contraction in the presence of 3% halothane was recorded for individual US9-10. Marginal contracture results were recorded for two individuals in the family, US9-8 and US9-11. The muscle strips showed no response in the presence of caffeine for both patients, but a 0.7 g contraction was reported in the presence of 3% halothane. The CHCT results of the family are presented in Table 3.2 and a pedigree of the family is shown in Figure 3.10.

Figure 3.10: Pedigree of North American malignant hyperthermia family US9



An explanation of the symbols is provided in the list of abbreviations and symbols.

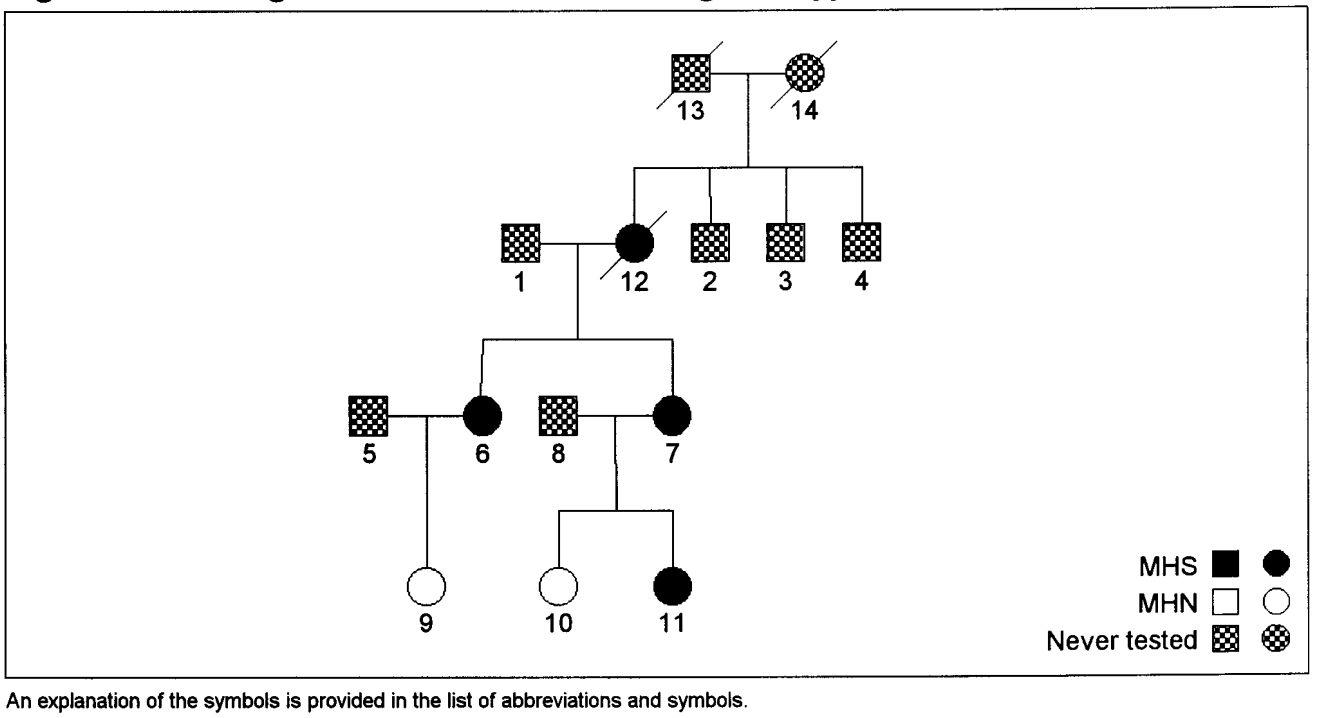
3.2.7 North American malignant hyperthermia family US10

Clinical information on the six phenotyped individuals was not available and the pedigree of this family is presented in Figure 3.11. Four individuals (US10-6, US10-7, US10-11 and US10-12) were phenotyped as MHS and two as MHN (US10-9 and US10-10). The CHCT results of these individuals are listed in Table 3.2.

The MH status of US family 10 is unambiguous. A strong contracture of 7.1 g was recorded for individual US10-7 in the presence of 3% halothane. This contracture was the highest measured for any individual included in this study. Individuals US10-6 and US10-11 also displayed high contractions, 6.8 g and 2.2 g respectively, in the presence of 3% halothane.

The mother (US10-12) of individuals US10-6 and US10-7 was tested for MH prior to her death. Her CHCT was positive and contractures in the presence of both halothane and caffeine were significant. It is unknown whether the death of individual US10-12 was related to her MH status. No DNA sample was available for individual US10-12 and she could therefore, unfortunately, not be included in this study.

Figure 3.11: Pedigree of North American malignant hyperthermia family US10

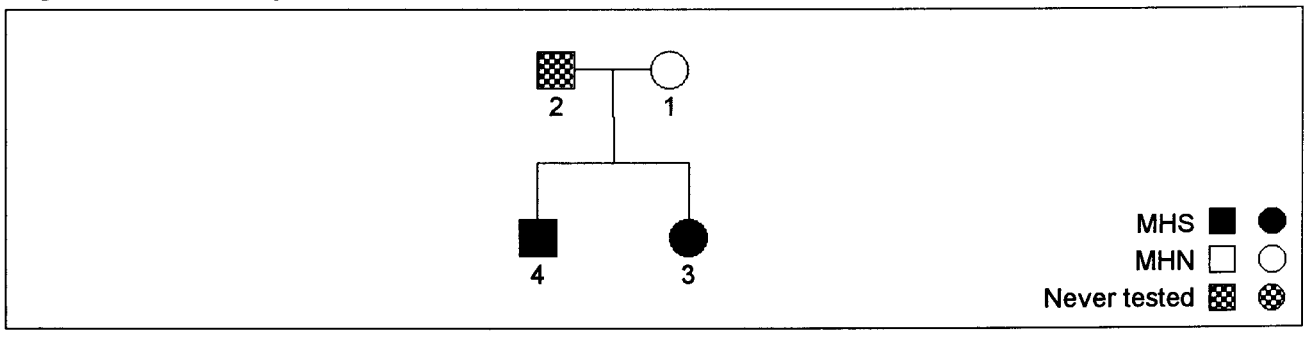


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.8 *North American malignant hyperthermia family US11*

It is not known which individual in this family, of whom a pedigree is presented in Figure 3.12, is the proband. Although no clinical data was available the CHCT results were obtained and are listed in Table 3.2. No response was detected in the presence of either caffeine of halothane for individual US11-1. It therefore appears that the two positive siblings (US11-4 and US11-3) inherited the MHS phenotype from their father (US11-2). Individual US11-2 has not been tested for MH with the CHCT, but was designated as an obligate carrier of MHS.

Figure 3.12: Pedigree of North American malignant hyperthermia family US11

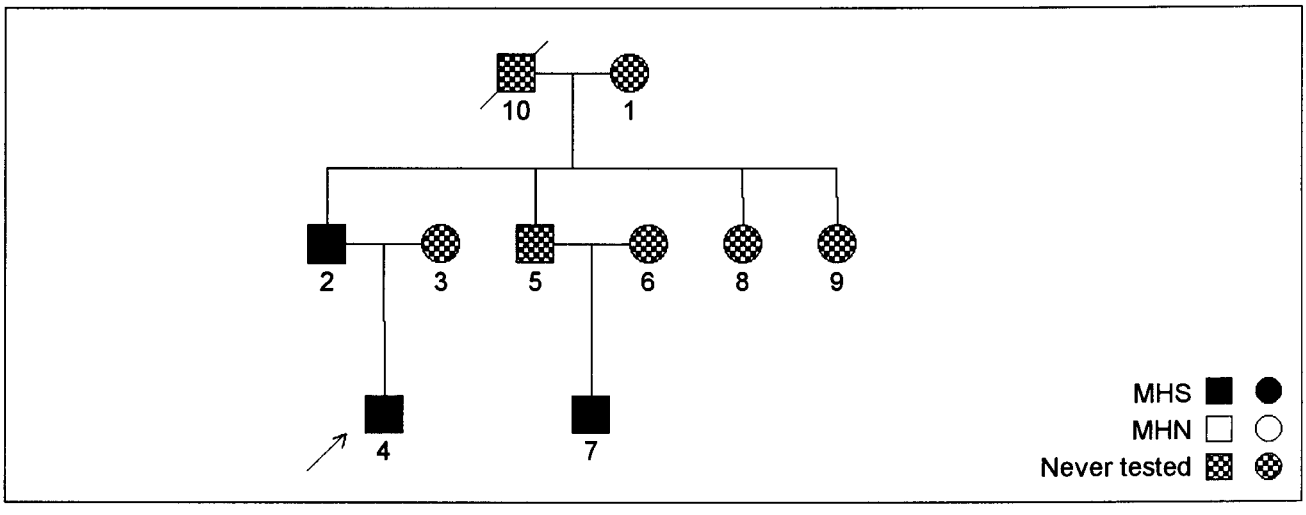


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.9 *North American malignant hyperthermia family US12*

This family has three MHS individuals, US12-2, US12-4 and US12-7. A pedigree of US family 12 is presented in Figure 3.13 and the CHCT results are listed in Table 3.2.

Figure 3.13: Pedigree of North American malignant hyperthermia family US12



An explanation of the symbols is provided in the list of abbreviations and symbols.

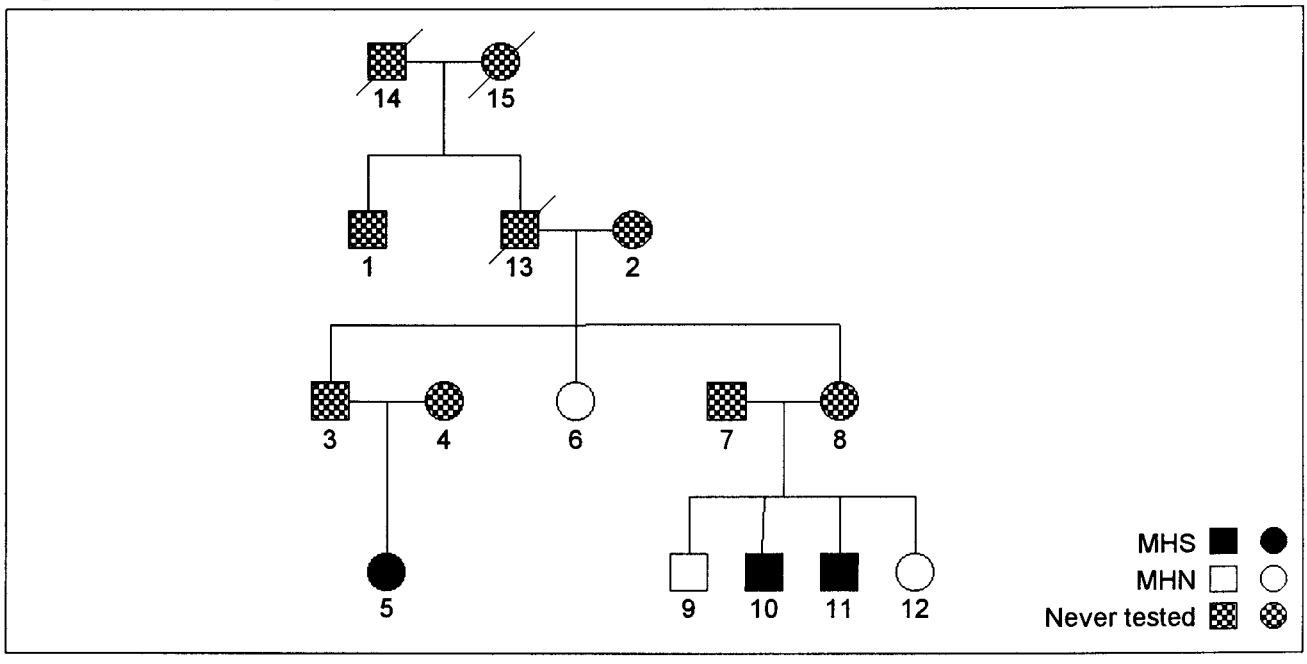
The proband of this family is individual US12-4. He was clinically diagnosed as MHS, which led to the subsequent investigation of this North American family. No clinical information on this family was available but the MHS status of the family is unequivocal, as the contracture at 3% halothane was 3.1 g for individual US12-2.

3.2.10 *North American malignant hyperthermia family US13*

It is not known which individual in this North American MH family is the proband and no clinical data was available for the family of whom a pedigree is depicted in Figure 3.14. CHCT results for this family indicated three MHS and three MHN individuals. The MHS

status of this family is verified by the CHCT results listed in Table 3.2. The strongest contracture value measured in this family was 1.3 g in the presence of 3% halothane for individual US13-10.

Figure 3.14: Pedigree of North American malignant hyperthermia family US13

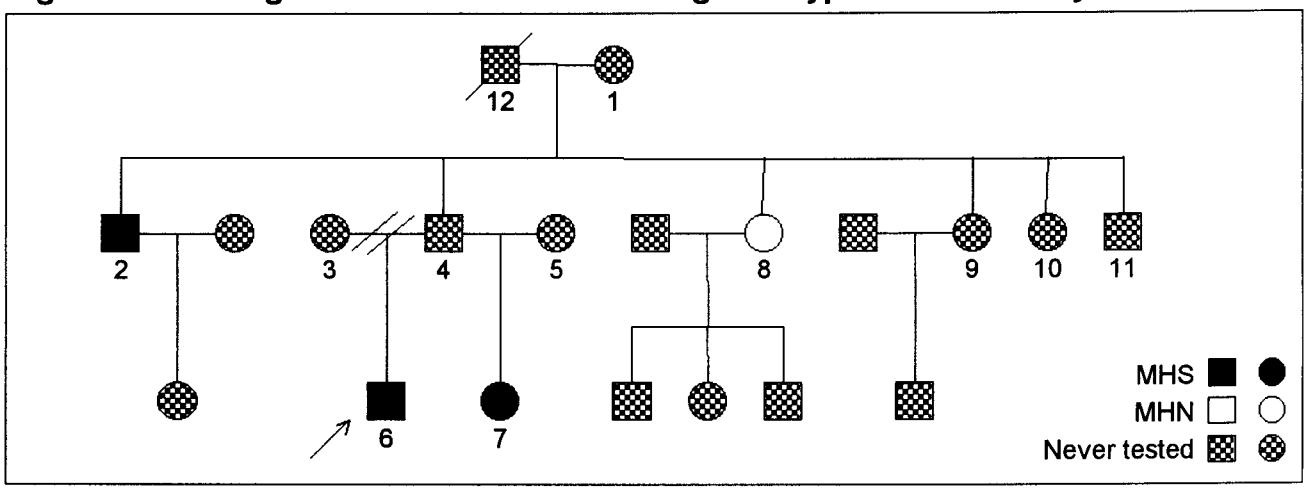


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.11 North American malignant hyperthermia family US14

Three MHS individuals and one MHN individual were diagnosed in this family of whom a pedigree is presented in Figure 3.15. Unfortunately no DNA sample was available for the proband of this family, individual US14-6. He presented with MH and was subsequently found to be positive according to the criteria of the North American CHCT protocol. The contracture results of this family are presented in Table 3.2.

Figure 3.15: Pedigree of North American malignant hyperthermia family US14

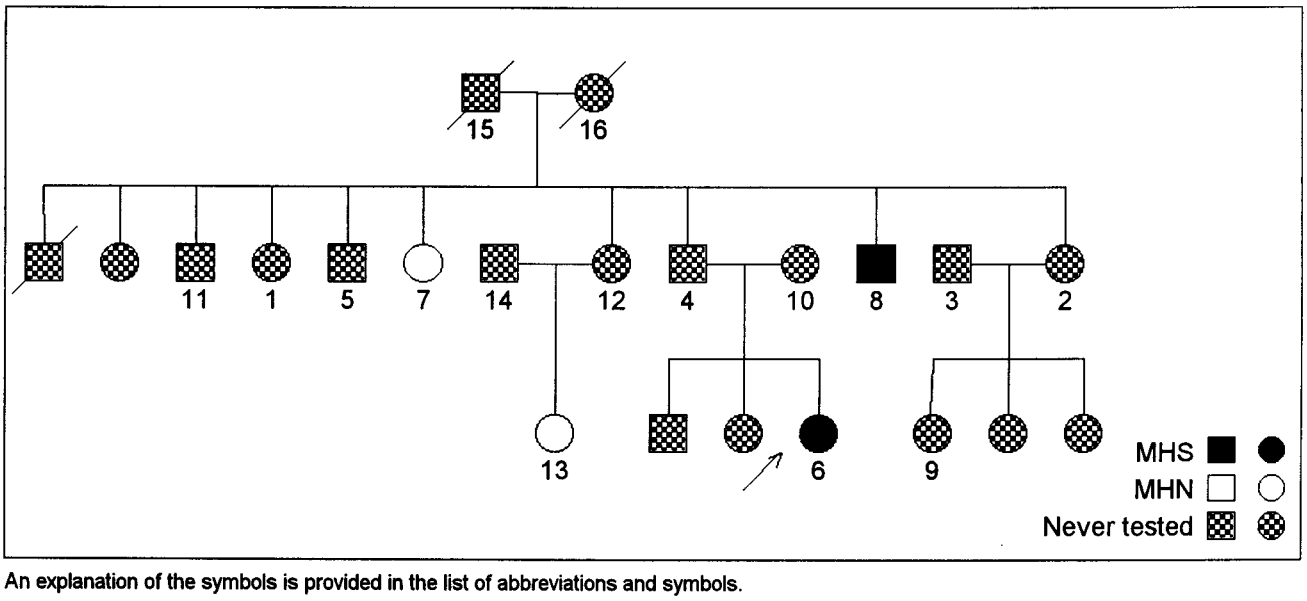


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.12 *North American malignant hyperthermia family US15*

The proband, individual US15-6 as well as individual US15-8 was diagnosed as MHS in this three generation family of whom a pedigree is presented in Figure 3.16. The muscle sample of individual US15-6 responded to both halothane and caffeine, presenting with contracture values of 1.0 g and 0.5 g respectively. Two other individuals (US15-7 and US15-13) were diagnosed as MHN. Individual US15-13 was not included in this mutation screening study as no DNA sample was available. Family US15's CHCT results are listed in Table 3.2.

Figure 3.16: Pedigree of North American malignant hyperthermia family US15

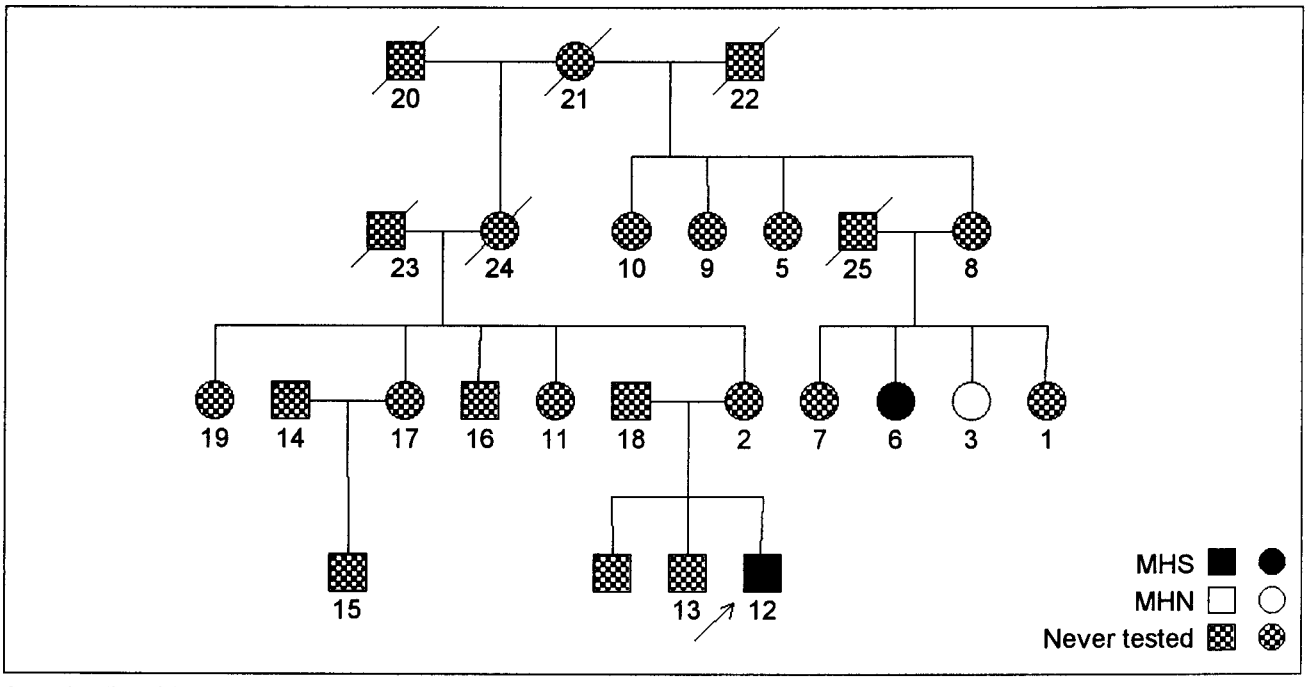


An explanation of the symbols is provided in the list of abbreviations and symbols.

3.2.13 North American malignant hyperthermia family US16

The two MHS individuals (the proband US16-12 and individual US16-6) in this four generation family is separated by several generations. Unfortunately only one other individual (US16-3) in this extended family was phenotyped utilising the CHCT. Individual US16-3 is a sibling of individual US16-6. Results of the CHCT for the phenotyped individuals in this family are listed in Table 3.2. The pedigree of this family is presented in Figure 3.17.

Figure 3.17: Pedigree of North American malignant hyperthermia family US16



An explanation of the symbols is provided in the list of abbreviations and symbols.

3.3 DNA isolation

Genomic DNA (gDNA) of the North American MH families was isolated by A.E. Jedlicka in the USA using a cesium chloride (CsCl) density centrifugation protocol (Sambrook *et al.*, 1989). Existing gDNA samples (Olckers, 1993; Olckers, 1997) of the South African MH families were utilised in this study. Additional gDNA samples required for the current study were isolated with a modified protocol, using sodium perchlorate ($NaClO_4$) as reported by Johns and Paulus-Thomas (1989) and modified by Olckers (1993).

Thirty millilitres (ml) of whole blood were collected in ethylenediamine tetra-acetic acid (EDTA) tubes. Samples were divided into aliquots of 10 ml and stored at -70°C until such time as DNA isolation was performed.

To 10 ml whole blood, 35 ml lysis buffer (0.32 M sucrose, 10 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 1% Triton X-100) was added, gently mixed and incubated on ice for 10 minutes (min). The mixture was centrifuged at 10 000 xg for 30 min at 4°C. After discarding the supernatant, the pellet was resuspended in 9.5 ml suspension buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 100 mM EDTA) whilst shaking gently for 30 min or until the pellet was completely suspended. Five hundred microlitres (µl) of a 30% sodium dodecyl sulphate (SDS), stock solution (g/v) was added, to a final concentration of 1.5%. This was followed immediately by 2.5 ml of 5 M freshly made $NaClO_4$ (Sigma) and an equal volume (12.5 ml) of chloroform:isoamyl alcohol (24:1). Following the addition of each of these solutions, the suspension was gently mixed. Extraction was performed by shaking gently for 30 min at room temperature (RT), whereafter the suspension was centrifuged at 1000 xg for 15 min at 20°C.

The aqueous phase was transferred to a glass beaker with the addition of two volumes of ice cold 100% ethanol (stored at -20°C) to precipitate the DNA. The DNA was spooled onto a sterile glass rod. The DNA was washed with 70% ethanol, air dried and suspended in 1 ml TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA). After rotating the samples overnight at RT, the DNA concentration was determined by reading the absorbency at A_{260} on a spectrophotometer (Unicam 3625 UV/VIS). The average yield was between 200 and 500 $\mu g.ml^{-1}$ DNA from 10 ml of whole blood and the A_{260}/A_{280} ratio was 1.8 or above, indicating that there was no protein contamination of the DNA samples.

3.4 Polymerase chain reaction (PCR) amplification of the reported mutations in the skeletal muscle ryanodine receptor (RYR1) gene

Controls were always included in PCR experiments. These controls included negative, positive (when available) and blank samples. Results were not accepted if the control samples did not generate the appropriate results.

PCR reactions were performed according to a modified method of Mullis *et al.*, (1986). All PCR reactions were performed in a final volume of 25 µl. Annealing temperatures (T_A) were calculated for each primer, using the formula: $T_A = 2(A + T) + 4(G + C)$ as described by Thein and Wallace (1986). Primers were synthesised by Genosys (Pty) Ltd. corporation in the UK. The primer sequences for these reported mutations are listed in Table 3.3.

Table 3.3: Primer sequences of the reported mutations in the skeletal muscle receptor gene

Mutation	Primer sequence [a]	T_c [b]	Product size (bp)
C35R	F: 5'-cgt gct caa gga gca gct caa gct-3' R1: 5'-ccg aag ccc tcg gcg acc agg-3' R2: 5'-ccc tcc tca ctt tct ctc ctg t-3'[c]	76°C 74°C 68°C	F, R1: 48 F, R2: 212
R163C	F1: 5'-tcc aag cag agg tct gaa gga gaa-3' R1: 5'-agc gct cgg agg aga cac tga caa-3' F2: 5'-ata gga gag gct tgc tgg tgg a-3'[c] R2: 5'-aat tgg gag tca gga cct tgg-3'[c]	72°C 76°C 68°C 64°C	F1, R1: 76 F2, R2: 233
G248R	F: 5'-tgc tga cag tga tga cca gcg cag-3' R: 5'-ctg att ctc agt ggc tcc agc ctc-3'	76°C 76°C	F, R: 228
G341R	F1: 5'-gcc ccc ctg aga tca agt cc-3' R: 5'-acc ttc ttc ttg agc acg cc-3' F2: 5'-cct gta gga gaa gct gga tg-3'[c]	66°C 62°C 62°C	F1, R: 124 F2, R: 178
I403M	F: 5'-cca cat gga cga cga ctg tc-3' R: 5'-ggt tgt ata ggc cat tgg tgc t-3'	64°C 66°C	F, R: 92
T522S	F: 5'-tac acc act gct gcc cac ttt-3' R: 5'-cga ttg cca cgg att aga gaa-3'	64°C 62°C	F, R: 193
R614C	F: 5'-ttg cca cat ctt atc ccg atg cgc-3' R: 5'-gcc agg aag aca gaa caa gag g-3'	74°C 68°C	F, R: 441
G2435R	F: 5'-ttc cct gca gct ttg gtg agg aac c-3' R: 5'-ctg cat gag gcg ttc aaa g-3'	78°C 58°C	F, R: 187
R2436H	F: 5'-ttc cct gca gct ttg gtg agg aac c-3' R: 5'-ctg cat gag gcg ttc aaa g-3'	78°C 58°C	F, R: 187

a) F and R indicate forward and reverse primers respectively. b) T_c = Calculated annealing temperature is listed here. c) Additional primers were utilised to amplify a larger region for sequencing.

PCR was performed in a reaction containing 2.5 µl of 10X PCR buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100), 1.5 mM $MgCl_2$, 200 µM of each 2'-deoxyadenosine-5'-triphosphate (dATP), 2'-deoxycytidine-5'-triphosphate (dCTP), 2'-deoxyguanosine-5'-triphosphate (dGTP) and 2'-deoxythymidine-5'-triphosphate (dTTP), 10 µM of each primer, 1 U Taq DNA polymerase and 200 ng gDNA in a final reaction volume of 25 µl.

PCR samples were overlaid with mineral oil and amplified in a Hybaid Touchdown thermal cycler (Hybaid Limited). Samples were denatured at 94°C for 10 min, then processed through 30 cycles of 94°C for 60 sec, annealing at the optimised temperature for 60 sec and extension for 60 sec at 72°C. Final elongation was performed at 72°C for 7 min.

Seven of the mutations included in this study could be detected via RFLP. Restriction products were separated via agarose or polyacrylamide gel electrophoresis. The DNA fragments were sized utilising one of two molecular markers: 100 bp ladder (Promega) or Boehringer Mannheim molecular weight marker V. Digestion of pBR322 vector DNA with *Hae III* (pBR322/*Hae III*) generated the same fragments as those observed with the Boehringer Mannheim molecular weight marker V. One microgram of pBR322 vector DNA was digested with 10 U *Hae III* (Promega) in a total reaction volume of 20 µl containing 10 $\mu g.\mu l^{-1}$ acetylated bovine serum albumin (BSA). The reaction was incubated at 37°C for 1 hour. Two microlitres of 10X loading buffer, consisting of 0.25% bromophenol blue (BPB), 0.25% xylene cyanol FF (XC), 0.4% Orange G (OG) and 50% glycerol, was added to the reaction after digestion.

3.5.1 *Detection of the Cys35Arg mutation via restriction fragment length polymorphism (RFLP) analysis*

The Cys35Arg mutation results in the gain of an *Aci I* restriction site as a result of the T103C substitution. The 48 bp PCR amplified product can therefore be digested with the *Aci I* restriction enzyme to detect the presence of the mutation via RFLP analysis. The partial gDNA sequence of exon 2 of the RYR1 gene in which the Cys35Arg mutation has been identified by Lynch *et al.* (1997) is presented in Table 3.4.

Table 3.4: Partial gDNA sequence of exon two of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Cys35
1	ctgcagtatt tgtggtatcc gggctggccc ccctggagac gctgcccctc ggttccgcag
61	▼ Exon 2 GACGATGAGG TGGTCCTGCA GTGCAGCGCT ACCGTGCTCA AGGAGCAGCT CAAGCTCTGC
121	CTGGCCGCCG AGGGCTTCGG CAACCGCCTG TGCTTCCTGG AGCCCACTAG CAACGCGCAG
181	Gtctgtgcag gaggaagagg ggcctgggga caggggcgtc tgaaggggca gagaatcttg
241	ggtccaaaga agagggttct gggagtctga aaggaggtgc tgacaggaga gaaagtgagg
301	agggggggcta aggctaagag gggctacctg aggtggggag gggtggaggt ctgagaaggg
361	aggggcaggg ggtctggggt gttggaggga atccctgtgt gcacagaagt gtggtaggcc
421	agggcggggg ctgctgatcc agaacgttcc ttggcaggtg gctgagtggg cagaaagcct

Partial gDNA sequence was retrieved from Genbank with accession number U48450. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exon 2 is indicated with an arrowhead and the codon that translates to Cys35 is indicated in boldface capital letters (TGC). The sequence of the forward primer utilised to amplify the region that contains the Cys35Arg mutation is indicated by underlined sequence (__) and the reverse primer is indicated by double underlined sequence (__). A different reverse primer was utilised to amplify a 212 bp product that contains the Cys35 codon sequence indicated by the dotted underlined sequence (...). The boxed sequence indicates the *Aci I* restriction enzyme recognition site that is created when the Cys35Arg mutation is present.

Ten µl of the PCR product was digested utilising 10 U *Aci I* (New England Biolabs) in a total reaction volume of 30 µl, and incubated overnight at 37°C. After digestion 3 µl of 10X loading buffer was added to each of the samples. Digested products were electrophoresed on a 20% polyacrylamide gel (19:1 acrylamide:bisacrylamide from USB) containing 4 ml of 10X TBE buffer (89.15 mM Tris [pH 8.0], 88.95 mM Boric acid, 2.498 mM ETDA) to give a final concentration of 0.5X TBE. Eighty µl of the polymerisation catalyst N,N,N',N'-tetramethylethylenediamine (TEMED) and 800 µl of the initiator, 10% ammonium persulphate (APS), were added to 80 ml of gel solution before casting the 1.5 mm thick gels. A 0.5X TBE buffer, diluted from a 10X TBE stock solution, was used for electrophoresis. Electrophoresis was performed at 250 volts (V) for 3 hours.

Following electrophoresis the gels were stained for 30 min in a 0.5X TBE solution containing ethidium bromide (EtBr) to give a final concentration of 0.5 µg.ml^{-1}. Gels were examined under ultraviolet (UV) light and frames taken of the fluorescent images were captured via a video-documentation system.

3.5.2 *Detection of the Arg163Cys mutation via restriction fragment length polymorphism (RFLP) analysis*

In Table 3.5 the partial gDNA sequence of exon 6 of the RYR1 gene in which the Arg163Cys mutation has been identified, is presented (Quane *et al.*, 1993). This C to T substitution resulted in the loss of a *Bst UI* restriction site. Amplification of the gDNA with the pimer set indicated in Table 3.5, generates a 76 bp product. Cleavage of this amplified product with *Bst UI* results in fragments of 44 bp and 32 bp respectively.

Table 3.5: Partial gDNA sequence of exon six of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Arg163Cys
1	agggagagcc ctggggaaga gcattctggg aagccatcat ctgacagcca cccccattcc
61	atccccaccc atagGAGAGG CTTGCTGGTG GACCATGCAC CCAGCCTCCA AGCAGAGGTC
121	*TGAAGGAGAA* AAGGTCCGCG TTGGGGATGA CATCATCCTT GTCAGTGTCT CCTCCGAGCG
181	CTACCTGgtg agccattgcg gttcctcctg ctcccaggtc tgggggcgca tgggatggtc
241	cccatcttct caccatgggt ttgcctggct gatctcccac ccccaaggtc ctgactccca
301	atttcccatt tcctgacccc tgacatccaa ttttctgatt tctgacctcc cattgcccga
361	cttgatcatt tcctgatctg tgatctctga tgactctgtc tcccatctgc cggtttccgg

Partial gDNA sequence was retrieved from Genbank with accession number U48452. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exon 6 is indicated with arrowheads and the codon that translates to Arg163 is indicated in boldface capital letters (CGC). The sequence of the forward primer utilised to amplify the 76 bp region that contains the Arg163Cys mutation is indicated by underlined sequence (__) and the reverse primer is indicated by double underlined sequence (__). Two different primers were utilised to amplify a 233 bp product that contains the Arg163 codon sequence, the forward primer is indicated by dotted underlined sequence (..) and the reverse primer by thick boldface underlined sequence (__). The boxed sequence indicates the *Bst UI* restriction enzyme recognition site.

3.5.3 *Detection of the Gly341Arg mutation via restriction fragment length polymorphism (RFLP) analysis*

Since the Gly341Arg mutation, which is due to a G to A substitution, does not result in the loss or gain of a restriction enzyme site, an amplification created restriction site (ACRS) detection technique was developed by Alestrøm *et al.* (1995). This technique involves designing a primer that introduces a restriction enzyme site allowing for the detection of the mutation via RFLP. In the case of the Gly341Arg mutation the modification of a primer involved an A to C substitution in the 3' end of the forward primer which resulted in the creation of an *Msp I* restriction site. The recognition site of *Msp I* is 5'-...C↓CGG...-3' and the modification of the forward primer results in the modification of the original sequence 5'-...ACGG...-3' to 5'-...CCGG...-3'. This created restriction site is lost when the mutation is present in an MHS individual. The location of the primers utilised to amplify the region of interest and the recognition site of the *Msp I* restriction site are indicated in the gDNA sequence of exon 11 presented in Table 3.6.

Table 3.6: Partial gDNA sequence of exons ten and eleven of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Gly341
1	acctggtttc tgtaaaaaaa agaaaaagaa gaaaagactg taatgtccat gggagaactg
61	gggggtcctc tgactcccct tggctctcac cctccacagC ▼Exon 10 TGGAGTGGGA GCCACCTGCG
121	CTGGGGCCAG CCACTCCGAG TCCGGCATGT CACTACCGGG CAGTACCTAG CGCTCACCGA
181	GGACCAGGGC CTGGTGGTGG TTGACGCCAG CAAGGCTCAC ACCAAGGCTA CCTCCTTCTG
241	CTTCCGCATC TCCAAGgtca gtggggtttg tggcgccctc cctcacctga agcccccagt
301	cccagcccag cctgcactct gcagtccctc agggggggctc ccctgctaaa cacacaggca
361	gaggaggctg acctgtgtcc cctgcccctg tag▼Exon 11GAGAAGC TGGATGTGGC CCCCAAGCGG
421	GATGTGGAGG GCATGGGCCC CCCTGAGATC AAGTACGGGG AGTCACTGTG CTTCGTGCAG
481	CATGTGGCCT CAGGACTGTG GCTCACCTAT GCCGCTCCAG ACCCCAAGGC CCTGCGGCTC
541	GGCGTGCTCA AGAAGAAGgt gggtgtaatc ccagctactc aggaggctga ggtgggagaa
601	tcgcttgagt ccaggaggtc aaggctgcag

Partial gDNA sequence was retrieved from Genbank with accession number U48454. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exons 10 and 11 are indicated with an arrowhead and the codon that translates to Gly341 is indicated in boldface capital letters (**GGG**). The sequence of the forward primer utilised to amplify the region that contains the Gly341Arg mutation is indicated by underlined sequence (__) and the reverse primer is indicated by double underlined sequence (__). A different forward primer was utilised to amplify a 178 bp product that contains the Gly341 codon sequence indicated by the dotted underlined sequence (..). The boxed sequence indicates the *Msp I* restriction enzyme recognition site.

Ten µl of amplified product was digested with endonuclease *Msp I* (New England Biolabs), in a total reaction volume of 30 µl and incubated at 37°C for 1 hour. DNA fragments were separated on a 10% polyacrylamide and electrophoresed for two hours at 250 V in 0.5X TBE buffer as described in paragraph 3.5.1.

3.5.4 Detection of the Ile403Met mutation via restriction fragment length polymorphism (RFLP) analysis

The partial gDNA sequence of exon 12 of the RYR1 gene in which the Ile403Met mutation has been identified (Quane *et al.*, 1993) is presented in Table 3.7. This mutation results from a C to G nucleotide substitution which abolishes an *Mbo I* restriction enzyme site. For diagnostic purposes the samples were digested with *Mbo I* after amplification of this genomic region bracketed by the primers as indicated in Table 3.7. Restriction of the 92 bp amplified product yielded two fragments (63 and 29 bp).

Table 3.7: Partial gDNA sequence of exon twelve of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Ile403
1	ggggcagtgc agactcagtc tagacagacg tcttgggcat ggcctgggtg ctgggcccac
61	tccagacctc tgtctcccca ctcctagGCC ATGCTGCACC AGGAGGGCCA CATGGACGAC
121	GCACTGTCGC TGACCCGCTG CCAGCAGGAG GAGTCCCAGG CCGCCCGCAT GATCCACAGC
181	ACCAATGGCC TATACAACCA GTTCATCAAg tgagcaacct gccctcctgc tggggtgact
241	cctgtgctgt cccatgctcc gggcatccat acacttggcc tctttcatct ctacctctgt
301	tgcccacacc cttgtctaac atatacatgg gccaagcgca gtggctcaca cctgtaatcc
361	caagactttg ggagggcaag gcatgaggat cgcttgggcc caggagcttg agaccagcct
421	gggcagcata gggagacccc gcctctacca aaaaaaaaaa aaaaaaaaaa aaaattaggc

Exon 12 (arrowhead)

Partial gDNA sequence was retrieved from Genbank with accession number U48455. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exon 12 is indicated with an arrowhead and the codon that translates to Ile403 is indicated in boldface capital letters (ATC). The sequence of the forward primer utilised to amplify the region that contains the Ile403Met mutation is indicated by underlined sequence (__) and the reverse primer is indicated by double underlined sequence (__). The boxed sequence indicates the *Mbo I* restriction enzyme recognition site.

A 92 bp PCR product was amplified in a 25 µl reaction of which 10 µl was subjected to digestion by *Mbo I* (Amersham Life Science) restriction enzyme in a final reaction volume of 30 µl during incubation at 37°C for an hour. Digested PCR products were separated on a 20% polyacrylamide gel as described in paragraph 3.5.1.

3.5.5 Detection of the Arg614Cys mutation via restriction fragment length polymorphism (RFLP) analysis

A *Rsa I* restriction site is abolished when the C1840 nucleotide is substituted with a T. This loss of a restriction site allows for the detection of the mutation via RFLP analysis (Otsu *et al.*, 1992). The location of the primers utilised to amplify the 441 bp fragment of interest and the recognition site of the *Rsa I* restriction enzyme site are indicated in Table 3.8.

Table 3.8: Partial gDNA sequence of exon seventeen of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Arg614
1	ttgccagatc ttcctctttg acccctaacc tttctctgat ccctgacctt ccactttcac
61	cacctcctct cagtcaaaat tgccacatct tatcccgatg cgctgtcctt tcctcctggc
121	ttccctccct ccctgggttc ttctgtagat cctgccctgg tgcctacaca ccctttaacc
181	tctgaccttg agctctagGT ▾Exon 17 CCTGGACGTG CTATGCTCCC TGTGTGTGTG TAATGGTGTG
241	GCTGTACGCT CCAACCAAGA TCTTATTACT GAGAACTTGC TGCCTGGCCG TGAGCTTCTG
301	CTGCAGACAA ACCTCATCAA CTATGTCACC AGgtctggct ctcaacatct gaccccagaa
361	ctcagaacct ctcaaccctc tccctgactt agagactcca cacccagatg gatgtccttt
421	ccttaatctc ccaccccagg gttaacaacc agtcctcaca gatgtccact gtggccccac
481	tctgccttgg catccactcc tcttgttctg tcttcctggc tccatttctg cctctatctg
541	tttctctttc tttctccctc tccctctctc tctgttttct ctttttatct ttgcctgttt
601	ctgtcttgat tcttcctcca tgtctttctc ctgtctctct cccatctctc tctctctgtc

Partial gDNA sequence was retrieved from Genbank with accession number M91455. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exon 17 is indicated with an arrowhead and the codon that translates to Arg614 is indicated in boldface capital letters (CGC). The sequence of the forward primer utilised to amplify the region that contains the Arg614Cys mutation is indicated by underlined sequence (__) and the reverse primer is indicated by double underlined sequence (__). The boxed sequence indicates the *Rsa I* restriction enzyme recognition site.

Restriction enzyme digestion was achieved by initial cleavage of 10 µl of the 441 bp product with 10 U of *Rsa I* (Promega) at 37°C for two hours. An additional 10 U of *Rsa I* was subsequently added to ensure complete digestion and the reaction was incubated at 37°C overnight. Digested DNA was mixed with 3 µl of 10X loading buffer and electrophoresed on a 2% agarose (SeaKem LE, FMC) gel containing 1X TBE and EtBr to a final concentration of 0.5 µg.ml^{-1}. Gels were electrophoresed for an hour at 200 V.

3.5.6 *Detection of the Gly2435Arg mutation via restriction fragment length polymorphism (RFLP) analysis*

Detection of the Gly2435Arg mutation is possible via RFLP analysis as the nucleotide substitution (G7297A) results in the creation of an additional *Dde I* restriction enzyme site. The 187 bp product amplified by the primers indicated in Table 3.9 contains a *Dde I* site. If the Gly2435Arg mutation is present an additional *Dde I* site is created.

Cleavage of 10 µl of PCR product was achieved by the addition of 10 U of *Dde I* (Boehringer Mannheim) in a total reaction volume of 30 µl and incubating the reaction at 37°C for one hour. The PCR products were separated on a 6% polyacrylamide gel as described in paragraph 3.5.1.

Table 3.9: Partial gDNA sequence of exons forty-four and forty-five of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Gly2435
1	attgggacct gtgttaccct ggagtgttgg gtcctggggc tgcatgggag gtctctgatg
61	gtggcttcat gagacccctt tccatgcggg tggccagGCG AGAGCGTGGA GGAGAACGCC
121	AATGTGGTGG TGCGGCTGCT CATCCGGAAG CCTGAGTGCT TCGGACCCGC CCTGCGGGGT
181	GAGGGTGGCT CAGGGCTGCT GGCTGCCATC GAAGAGGCCA TCCGCATCTC CGAGGACCCT
241	GCGAGGGATG GCCCAGGCAT CCGCAGGGAC CGGCGGCGCG AGCAgtgagt ctcccggccc
301	cctcctcaat aggcaacccg ccctccctgg cccctggctg cctccccaac ccacccacct
361	tccctgcagC TTTGGTGAGG AACCGCCTGA AGAAAACCGG GTGCACCTGG GACACGCCAT
421	CATGTCCTTC TATGCCGCCT TGATCGACCT GCTCGGACGC TGTGCACCAG AGATGCATgt
481	gagaccctga gccagggcag gatgggaagg gagggcaggc acagccgctt tgaacgcctc
541	atgcaggcac tcggtgacac ggagtgagct cccatatgtg ggtggtcctg gactagcaat
601	gttggggaca caacagtgac caagacagcc ccagggcctg gtctcacaga gctcccagtc
661	caatgggggа gacggacagt gacacccaga gtggtcaggg ctgggatggg ggagcacagg

Exon 44 (arrowhead before GCG AGAGCGTGGA)
Exon 45 (arrowhead before TTTGGTGAGG AACCGCCTGA)

Partial gDNA sequence was retrieved from Genbank with accession number U48477. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exon 44 and 45 is indicated with an arrowhead and the codon that translates to Gly2435 is indicated in boldface capital letters (GGA). The sequence of the forward primer utilised to amplify the region that contains the Gly2435Arg mutation is indicated by underlined sequence (___) and the reverse primer is indicated by double underlined sequence (__). The boxed sequence indicates the *Dde I* restriction enzyme recognition site, when the Gly2435Arg mutation is present an additional recognition site (box with dashed lines) is created.

3.5.7 *Detection of the Arg2436His mutation via restriction fragment length polymorphism (RFLP) analysis*

The change in nucleotide sequence (substitution of G to A at position 7301) abolished the *Hga I* restriction enzyme site, creating an RFLP that can be used to detect the mutation. The partial gDNA of the RYR1 gene, including exon 45, is presented in Table 3.10, indicating the location of the primers utilised and the recognition site of the *Hga I* restriction enzyme.

Table 3.10: Partial gDNA sequence of exons forty-five of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Arg2436
1	cctcctcaat aggcaacccg ccctccctgg cccctggctg cctccccaac ccacccacct
61	tccctgcagC TTTGGTGAGG AACCGCCTGA AGAAAACCGG GTGCACCTGG GACACGCCAT
121	CATGTCCTTC TATGCCGCCT TGATCGACCT GCTCGGACGC TGTGCACCAG AGATGCATgt
181	gagaccctga gccagggcag gatgggaagg gagggcaggc acagccgctt tgaacgcctc
241	atgcaggcac tcggtgacac ggagtgagct cccatatgtg ggtggtcctg gactagcaat
301	gttggggaca caacagtgac caagacagcc ccagggcctg gtctcacaga gctcccagtc

Exon 45 (arrowhead before TTTGGTGAGG AACCGCCTGA)

Partial gDNA sequence was retrieved from Genbank with accession number U48477. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exon 45 is indicated with an arrowhead and the codon that translates to Arg2436 is indicated in boldface capital letters (CGC). The sequence of the forward primer utilised to amplify the region that contains the Arg2436His mutation is indicated by underlined sequence (___) and the reverse primer is indicated by double underlined sequence (__). The boxed sequence indicates the *Hga I* restriction enzyme recognition site.

The 187 bp fragment generated via PCR was digested with 1 or 5 U or 10 U of *Hga I* (New England Biolabs) in a total volume of 50 µl, incubated for 1 hour at 37°C. Five µl of 10X loading buffer was added to the digested product and electropheresed on a 10% polyacrylamide gel as described in paragraph 3.5.1.

3.6 Base excision sequence scanning mutation detection and localisation

The base excision sequence scanning mutation detection and localisation (BESS T-Scan) kit (Epicentre Technologies) was utilised for the screening of two mutations, Gly248Arg and Tyr522Ser. This relatively new method of mutation detection was described by Hawkins and Hoffman (1997) and Vaughan and McCarthy (1998).

3.6.1 Detection of the Gly248Arg mutation via base excision sequence scanning mutation detection and localisation (BESS T-Scan) analysis

Neither of the two mutations, Gly248Arg and Tyr522Ser, result in the creation or abolishment of a restriction enzyme site that could be utilised for detection via RFLP analysis. However, since the Gly248Arg mutation is due to the nucleotide substitution G742A, it is theoretically possible to detect this mutation via the BESS T Scan if the reverse primer is used, as an additional fragment would indicate the presence of the Gly248Arg mutation. The location of the primers utilised to amplify the region in exon 9 in which the Gly248Arg mutation was identified by Gillard *et al.* (1992) is indicated in Table 3.11.

Table 3.11: Partial gDNA sequence of exons eight and nine of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Gly248
1	cccaaactca gccctcaggt tcccccaggg gaggagcagg gcccctgact tcatcttggc
61	tcctggtctt cctggggctc cagcctccca ttgaccaact tcccttgctc ctctccagGC (Exon 8)
121	TTCGTGACGG GAGGTCACGT CCTCCGCCTC TTTCATGGAC ATATGGATGA GTGTCTGACC
181	ATTTCCCCTG CTGACAGTGA TGACCAGCGC AGgtctgggc tgtggacgag agggcctggg
241	gtctaggggt ggacgtggag ggctgggacc ctatgagtag gattagggac cagattccgg
301	ggagctgaac ccttgacttc actctcttct gtgtccccca gACTTGTCTA CTATGAGGGG (Exon 9)
361	GGAGCTGTGT GCACTCATGC CCGCTCCCTC TGGAGGCTGG AGCCACTGAG AATCAGgtag
421	ggcggggaag atggggagag accagggaga ggctggggtc acctggcagg ctgggaggac

Partial gDNA sequence was retrieved from Genbank with accession number U48453. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exons 8 and 9 are indicated with an arrowhead and the codon that translates to Gly248 is indicated in boldface letters (**GGG**). The sequence of the forward primer utilised to amplify the region that contains the Gly248Arg mutation is indicated by underlined sequence (__) and the reverse primer is indicated by double underlined sequence (__).

The primer of interest was end labelled using the T4 polynucleotide kinase (Promega). The reaction mixture contained 1.4 µl of 10X kinase buffer (500 mM Tris-HCl [pH 8.2], 100 mM EDTA, 50 mM dithiothreitol (DTT), 1 mM spermidine), 10 µM of primer, 30 µCi γ^{32}P-dATP and 5 U polynucleotide kinase (Promega) in a total reaction volume of 14 µl. The reaction mixture was incubated at 37°C for 1 hour. After centrifugation an additional 26 µl of sterile water was added to the reaction.

PCR was performed in a reaction mixture containing 1.25 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 2.5 mM of each dATP, dCTP, dGTP, dTTP and 200 µM 2'-deoxyuracil-5'-triphosphate (dUTP), 10 µM of unlabelled primer and 0.25 µM of end labelled primer, 0.5 U of Taq DNA polymerase and 100 ng of gDNA in a final reaction volume of 12.5 µl. PCR samples were overlaid with one drop of mineral oil before amplification. The samples were denatured at 94°C for 10 min, then processed through 30 cycles of 94°C for 1 min, annealing at the appropriate temperature for 1 min, and 72°C for 1 min. Final extension was performed at 72°C for 7 min.

Five µl of the PCR product containing dUTP was digested utilising 0.5 µl of BESS T-Scan excision enzyme mix in a total reaction volume of 10 µl, and incubated at 37°C for 30-45 min. Five µl of loading buffer (95% formamide, 10 mM EDTA, 0.1% XC and 0.1% BPB [pH 11.0]) was added to the samples before they were denatured at 90°C for 8 min and placed on ice, prior to loading onto gels.

Single-stranded fragments were electrophoresed on a 6% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 7 M Urea and 1X TBE. Thirty µl TEMED and 500 µl of a 10% APS solution were added for polymerisation before the 0.3 mm gels were cast. Gels were pre-run in 1X TBE at 60 Watts (W) for 30 min to ensure denaturing conditions throughout the loading process. Electrophoresis was performed at 45 W until samples had entered the gel matrix and then at 60 W for approximately 3 hours, or until sufficient separation was achieved.

After electrophoresis the gels were transferred to 3 MM filter paper, covered with Cling Wrap and vacuum-dried at 80°C for 40-60 min. Vacuum-dried gels were exposed to X-ray film (Fuji RX-U) for 1 to 12 hours, as required. The development of the X-ray films were performed manually, utilising MEDEX (Protea Medical Services) reagents.

3.6.2 *Detection of the Tyr522Ser mutation via base excision sequence scanning mutation detection and localisation (BESS T-Scan) analysis*

The Tyr522Ser mutation is due to the A1565C nucleotide substitution and could also theoretically have been detected via the BESS T Scan if the reverse primer is used. The absence of a fragment at position 1564, or a decrease in intensity of the fragment, would indicate the presence of the Tyr522Ser mutation. The primer sequences utilised to amplify the 193 bp region within exon 14 in which the Tyr522Ser mutation was identified by Quane *et al.* (1994b) are indicated in Table 3.12.

Table 3.12: Partial gDNA sequence of exons fourteen to sixteen of the human ryanodine receptor (RYR1) gene

Nucleotide number	DNA Sequence: Tyr522
1	ctgcaggaat tcgtgaatcc aagaaagaca agtaagggag ggcctgggtc tcctattgtg
61	atgcctctta ttttcctcat cctag↓Exon 14 GGGAT GCTCTCCATG GTCCTGAATT GCATAGACCG
121	CCTAAATGTC TACACCACTG CTGCCCACTT TGCTGAGTTT GCAGGGGAGG AGGCAGCCGA
181	GTCCTGGAAA GAGATTGTGA ATCTTCTC**TA** **T**GAACTCCTA Ggtaggggtc cagtcctgac
241	tccctgagaa caccccagac ccagatccca gtcctattgg atctgacacc tcttcccccc
301	tcag↓Exon 15 CTTCTC TAATCCGTGG CAATCGTAGC AACTGTGCCC TCTTCTCCAC AAACTTGGAC
361	TGGCTGGTCA GCAAGCTGGA TCGGCTGGAG GCCTCGTCTG gtaggagaac ccgggggagt
421	gggacagagg cttgtgggag gggatgggca tggccgcttc acctctcatt ctgggcaccc
481	tggcag↓Exon 16 GCAT CCTGGAGGTC CTGTACTGTG TCCTCATTGA GAGTCCAGAG GTTCTGAACA
541	TCATCCAGGA GAATCACATC AAGTCCATCA TCTCCCTCCT GGACAAGCAT GGGAGGAACC

Partial gDNA sequence was retrieved from Genbank with accession number U48456. The exon sequence is indicated in capital letters and small letters indicate the respective intron sequence. The beginning of exons 14 to 16 are indicated with an arrowhead and the codon that translates to Tyr522 is indicated in boldface capital letters (**TAT**). The sequence of the forward primer utilised to amplify the region that contains the Tyr522Ser mutation is indicated by underlined sequence (__) and the reverse primer is indicated by double underlined sequence (__).

PCR and BESS T Scan were performed as described in paragraph 3.6.1. Single-stranded fragments were also electrophoresed on 6% polyacrylamide gels as described in paragraph 3.6.1, for approximately 2 hours after which the gels were vacuum-dried and exposed to X-ray film (Fuji RX-U) for 1 to 12 hours.

3.7 Chain-termination sequencing of PCR products

DNA sequencing was performed utilising the Sequenase PCR product Sequencing Kit (USB, Amersham Life Science). This kit is based on the chain-termination method described by Sanger *et al.*, (1977). All PCR products used for DNA sequencing were amplified as described in paragraph 3.4.

Five µl of the PCR product was enzymatically pre-treated with 10 U Exonuclease I and 2 U Shrimp Alkaline Phosphatase (SAP) at 37ºC for 15 min. The Exonuclease I removed single-stranded primers and DNA while the Shrimp Alkaline Phosphatase (SAP) removed the remaining dNTPs from the PCR mixture. Heating the reaction at 80ºC for 15 min inactivated both enzymes. Subsequent to the above mentioned pre-treatment the double-stranded DNA was sequenced.

Annealing of the sequencing primer was performed in a reaction containing 5 µl of the treated PCR product, 1 µl of the sequencing primer stock solution (10 pmol.µl^{-1}) and 4 µl of sterile water in a final reaction volume of 10 µl. The annealing mixture was heated for 2-3 min at 100ºC and rapidly cooled on ice for 5 min. The reaction was briefly centrifuged in a benchtop microcentrifuge and kept on ice until required in the labelling reaction.

The labelling reaction consisted of 10 µl of ice-cold annealed DNA mixture, 2 µl of 5X reaction buffer (200 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 250 mM NaCl), 1 µl of 0.1 M DTT, 2 µl of 1:5 diluted dGTP labelling mixture (7.5 µM dCTP, 7.5 µM dGTP, 7.5 µM dTTP), 5 µCi α^{35}S-dATP and 2 µl (3.2 U) of Sequenase DNA polymerase. When 5 µCi α^{32}P-dCTP was utilised in the labelling reaction the diluted labelling mix was substituted with 1 µl of each of the 3 µM dNTP (dATP, dGTP and dTTP) solutions for labelling. The reaction was mixed and incubated at room temperature for 5 min.

Two and a half microlitres of each dideoxynucleotide (ddN) termination mixture was aliquoted into 0.5 ml tubes and labelled A, C, G or T respectively. The termination mixtures were dideoxyadenosine (ddA) termination mixture [80 µM of each dATP, dCTP, dGTP, dTTP, 8 µM 2',3'-dideoxyadenosine-5'-triphospate (ddATP), 50 mM NaCl]. The other termination mixtures, dideoxycytidine (ddC), dideoxyguanosine (ddG) and dideoxythymidine (ddT) were equivalent to the ddATP, each containing 8 µM of either 2',3'-dideoxycytidine-5'-triphospate (ddCTP), 2',3'-dideoxyguanosine-5'-triphospate (ddGTP) or 2',3'-dideoxythymidine-5'-triphospate (ddTTP) respectively. These termination mixtures were pre-heated for 1 min at 37ºC. Three and a half microlitres of the labelling reaction was transferred to each of the termination mixtures and the reactions incubated for 5 to 10 min at 37ºC.

In some samples that contained regions with a high GC content and where compressions were observed the sequencing reactions were performed with 7-deaza-2'-deoxyguanosine-5'-triphosphate (7-deaza-dGTP). The use of 7-deaza-dGTP

results in the formation of two hydrogen bonds between a C base and a 7-deaza-dGTP base instead of the three hydrogen bonds formed between C and G. This decrease in the number of hydrogen bonds formed results in the formation of weaker secondary structures and therefore eliminates the compressions observed in some instances (Mizusawa *et al.*, 1986). When the 7-deaza-dGTP was utilised the 7-deaza-dGTP labelling mixture (7.5 µM dCTP, 7.5 µM 7-deaza-dGTP, 7.5 µM dTTP) was used instead of the dGTP labelling mixture and the dGTP termination mixtures were substituted with the 7-deaza-dGTP termination mixtures. These termination mixtures consisted of 80 µM of each dATP, dCTP, 7-deaza-dGTP and dTTP, 50 mM NaCl and 8 µM of either ddATP, ddCTP, ddGTP or ddTTP for the ddA, ddC, ddG or ddT termination mixtures respectively.

The reactions were terminated by the addition of 4 µl of the 2X stop solution (95% formamide, 20 mM EDTA, 0.05% BPB and 0.05% XC) to each of the termination reactions. Samples were denatured by heating the reaction at 80°C for 2-5 min and placed on ice, prior to loading onto the gel.

DNA sequencing products were electrophoresed on a 6% polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 7 M Urea and 1X TBE. Thirty microlitres of TEMED and 500 µl of a 10% APS solution was added for polymerisation before the gels were cast. Electrophoresis was performed as described in paragraph 3.6 for approximately 3 hours, or until sufficient separation was achieved.

After electrophoresis the gels were transferred to 3 MM filter paper, covered with Cling Wrap and vacuum-dried at 80°C for 60 min, or until completely dry. Vacuum-dried gels with samples containing α^{32}P-dCTP were exposed to X-ray film (Fuji RX-U) for 1 to 12 hours, as required. The Cling Wrap was removed from those gels with samples containing α^{35}S-dATP and the surface of the gels covered with baby powder (Johnson and Johnson) and exposed to X-ray film (Fuji RX-U) for 1 to 12 hours, as required. Development of the X-ray films were performed manually, utilising MEDEX (Protea Medical Services) reagents.

3.8 Single lane chain-termination sequencing of PCR products

PCR products that were amplified as described in paragraph 3.4 for the detection of four of the missense mutations (Gly248Arg, Tyr522Ser, Gly2435Arg and Arg2436His) were

subjected to single lane DNA sequencing. Single lane sequencing (SLS) entailed the sequencing of only one selected lane instead of all four lanes.

All aspects of the sequencing protocol, described in paragraph 3.7, remained unchanged with the exception of the volumes used in the different stages of the protocol, as only a quarter of the reaction was theoretically required to perform sequencing of a single lane. However, enzymatic pre-treatment of the PCR product did not yield good results when only a quarter of the volume (0.25 µl) of the required enzymes were utilised. This was probably due to pipetting errors as the small volume of enzymes required could not be pipetted accurately. The reactions were optimised and half the amount of the enzymes, 5 U Exonuclease and 0.5 U SAP, were required to perform the pre-treatment reaction. Half a microlitre of the sequencing primer stock solution (10 $pmol.\mu l^{-1}$) was used in the annealing reaction. The labelling reaction was performed with a quarter of the volumes described in paragraph 3.7.

The appropriate sequencing primer was selected depending on which primer produced the best results and was not located too close to the codon sequence of interest. The lane to be sequenced was selected depending on which nucleotide was substituted by the mutation. An additional fragment at the appropriate nucleotide position would indicate the presence of the relevant mutation, as indicated in Table 3.13.

Table 3.13: Detection of four reported mutations via single lane chain-termination sequencing of PCR products

Mutation	Nucleotide	Substitution	Sequencing primer	Lane sequenced	Presence of the mutation[a)]
Gly248Arg	G742A	ggg→agg	Forward	A	A at position 742
Tyr522Ser	A1565C	tat→tct	Reverse	G	G at position 1565
Gly2435Arg	G7297A	gga→aga	Forward	A	A at position 7297
Arg2436His	G7301A	cgc→cac	Forward	A	A at position 7301

a) The presence of a mutation would be indicated by an additional fragment in the relevant position.

Single lane sequencing products were also electrophoresed on 6% polyacrylamide gels as described in paragraph 3.7 for approximately 2 to 3 hours, or until sufficient separation was achieved. After electrophoresis the gels were vacuum-dried and exposed to X-ray film (Fuji RX-U) for 1 to 12 hours, as discussed in paragraph 3.7.