CHAPTER TWO

Malignant hyperthermia: A disorder of calcium dysregulation

MH is a potentially life-threatening hypermetabolic condition. Predisposition is inherited as an autosomal dominant trait, and the disorder displays incomplete penetrance and variable expressivity (Denborough *et al.*, 1962). MHS patients are asymptomatic, but may present with systemic, uncontrolled hypermetabolism when exposed to triggering ether, volatile anaesthetics e.g. halothane or depolarising muscle relaxants, e.g. succinylcholine. Presenting signs include sustained uncontrolled muscle contracture, an increase in body temperature, rhabdomyolysis and cardiac arrhythmia. Triggering substances elicit a rapid release of free cytoplasmic Ca^{2+} from the SR stores via the skeletal muscle ryanodine receptor protein (RyR1) into the cytosol. The excess Ca^{2+} activates glycogenolysis and cell metabolism, which leads to the futile cycling of adenosine triphosphate (ATP), resulting in heat and excess lactate production. The onset of an MH episode requires immediate action from the anaesthesiologist. During a crisis, treatment consists of early administration of dantrolene, which is a non-specific muscle relaxant that functions as a Ca^{2+} release inhibitor (Britt and Kalow, 1970).

Diagnosis of MHS is determined using the IVCT. The test determines the abnormally high sensitivity of a muscle biopsy from affected individuals to halothane and caffeine. The test is not completely accurate and the specificity of the IVCT is often forfeited to achieve high sensitivity (Larach *et al.*, 1992). Linkage analysis based on IVCT phenotyping has indicated linkage to the gene which encodes the RYR1 in 50% of MH families. Nevertheless, the exact nature of the molecular defect has not been determined and the disorder has been defined to be genetically heterogeneous, as to date six other loci have been implicated in resulting in MHS. Research into the molecular mechanism of excitation-contraction (E-C) coupling in skeletal muscle has identified possible candidate genes for MHS, including the dihydropyridine receptor (DHPR) and the sodium (Na^{+}) channel. Further studies will provide functional characterisation of all MH-causing mutations of this heterogeneous disorder.

2.1 INCIDENCE AND MORTALITY OF MH

The estimated incidence of MH in North America and Europe, according to Golinski (1995), is 1 in 50,000 anaesthetised adults and 1 in 15,000 anaesthetised children. In certain regions of the world the prevalence of MH has been estimated to be as high as 1 in 200 anaesthetised individuals (Bachand *et al.*, 1997). Britt and Kalow (1970) indicated that the acute MH syndrome is more prevalent in young individuals, with more than 50% of cases occurring before the age of 15. The difference was attributed to the maturation of muscle during puberty (Fletcher *et al.*, 1997). The incidence of MH may be an underestimation of the true prevalence of MH, as only a certain number of MHS individuals undergo anaesthesia with triggering agents. MHS patients are usually healthy, without outward signs or symptoms of myopathy. In addition, in a few individuals exposure to triggering anaesthetics has to occur several times before a clinical episode is triggered. Individuals of both genders and all ethnic groups are susceptible to MH. However, predominance in males has been suggested (Halsall and Ellis, 1993). MH is not a sex-linked trait and the observed higher incidence of MH in males is hypothesised to be due to a higher occurrence of accidents experienced by males, which predisposes them to a higher frequency of exposure to anaesthesia (Kaus and Rockoff, 1994). Mortality rates for MHS individuals who have received triggering anaesthetics have been estimated to be approximately 70%. The mortality rate has been reduced to 10% by the use of dantrolene, which is currently the most effective therapy in treating MH.

2.2 THE CLINICAL PRESENTATION

The presentation of clinical features associated with MH is not consistent between patients and varies from the classical or fulminant category to a type with mild symptoms. The primary difference can be associated with the speed of onset and the number and severity of signs present. In some individuals an MH reaction occurs immediately following induction, however, in other individuals an MH episode may be delayed for several hours. MH can occur post-operatively but will generally present within an hour following general anaesthesia (Ellis *et al.*, 1990). The underlying basis of variability is not fully understood. It has, however, been proposed that drug administration, which includes varying potency, concentration and duration of exposure to triggering agents and environmental factors such as body temperature, age and genetic variability, plays a role in the progression of MH. It is less clear if other pre-operative factors, including anxiety, prior exercise or muscle trauma, play a role in the development of this disorder. Nelson and Flewellen (1983)

indicated that the spectrum of MHS in individuals may be due to the variable expression of the defect, in which one individual may have a higher proportion of defective channels than another. Despite the variable clinical presentation, patients can be divided into three categories as listed in Table 2.1. Only 10% of MH episodes are classified as fulminant (Ørding, 1985) and require aggressive treatment. These episodes display a higher mortality rate and following treatment, the MH episode may reoccur. Evidence of recrudescence includes hypermetabolism or rhabdomyolysis, which may occur in up to 25% of all MH episodes.

Table 2.1: Presentation of clinical features of MH following exposure to triggering anaesthetics

Category	Details	Clinical signs
Classic MH (fulminant) reaction	• Episodes arise gradually or have a sudden onset, with rapid progression but short duration	• Cardiac arrest • Presence of abnormal amounts of K^+ in the blood (hyperkalaemia) • Generalised muscle rigidity • Rapid rise in core body temperature • Breakdown of muscle (rhabdomyolysis)
Generalised muscle rigidity	• Accelerated presentation of MH • Individuals may have an underlying neuromuscular disease	• Sudden generalised muscle rigidity • Cardiac arrest • Rhabdomyolysis • Myoglobinuria • Presence of abnormal amounts of K^+ in the blood (hyperkalaemia)
Masseter muscle rigidity	• Occurs following exposure to succinylcholine • More common in children than adults	• Incomplete relaxation of jaw muscles • Flaccid paralysis of the extremities • May progress into fulminant MH

Adapted from Christian *et al.* (1989); Allen and Rosenberg (1990); Kaus and Rockoff (1994) and Rosenberg and Shutack (1996). MH = malignant hyperthermia; K^+ = potassium ion.

2.3 PREOPERATIVE DIAGNOSIS

Diagnosis of an MH episode has been difficult to define due to the variability of clinical signs (Rosenberg and Shutack, 1996) and laboratory results observed in patients. MH is a disorder that occurs following exposure of susceptible individuals to triggering anaesthetics. In the absence of exposure to these drugs, it is often difficult to identify an MHS individual, unless a family history of MH is present. Generally, the history of the patient and physical examination cannot identify the MHS patient, as many patients have undergone an uneventful prior anaesthesia and do not have physical signs of myopathy (Halsall *et al.*, 1979). The severity of an MH reaction is defined by a clinical grading scale according to standardised clinical diagnostic criteria, which were developed to create a

clinical definition of the MH syndrome. This scale uses the Delphi method, which consists of a series of questionnaires completed by a panel of experts (Larach *et al.*, 1994). The scale uses a global score assigned for abnormal signs and laboratory findings observed during an anaesthetic reaction, to rank the likelihood that an adverse anaesthetic event represents MH. The scale also assigns points to family history. The raw score indicates the risk of an MH reaction and is divided into six categories ranging from one (almost never) to six (almost certain), according to which a score of at least 50 is considered to be a fulminant crisis. The clinical grading scale relies on the judgement of the anaesthesiologist and clinical symptoms are not regarded as specific signs of MH (Hackl *et al.*, 1990). Several factors can result in an underestimation of the likelihood of an MH event, including aborting anaesthesia, important monitors not being used during anaesthesia (e.g. electrocardiogram, capnogram or thermometer), relevant blood tests not being obtained (e.g. creatine kinase (CK), serum, urine myoglobin or arterial blood gases) and an absence of family history (Larach *et al.*, 1994).

2.3.1 The *in vitro* contracture test

The first specific method to screen susceptible patients for the MH phenotype was developed by Ellis *et al.* (1972) and is referred to as either the caffeine halothane contracture test (CHCT) or the IVCT. The IVCT has been used for over 30 years as a standard diagnostic tool to determine MHS. It is currently the only method used for the diagnosis of MH and remains the gold standard for diagnosis. However, this test has several disadvantages, since it is invasive, expensive and time-consuming. The test is performed using approximately 2 grams (g) of muscle from the vastus lateralis or medialis. The muscle is obtained from a patient anaesthetised with an anaesthetic drug considered safe for MH individuals or under a femoral regional block. Biopsies are not recommended for children under five, because of the relative large amount of muscle required to perform the test. Standardised protocols were developed in both Europe (European Malignant Hyperpyrexia Group, 1984) and North America (Larach, 1989) in 1984 and 1989, respectively. The two versions of the test determine the contracture of living tissue in response to agents such as halothane and caffeine. Based on 202 controls, the IVCT protocol published by the EMHG indicated a sensitivity of 99% and a specificity of 93.6% (Ørding *et al.*, 1997). The test allows classification of individuals as MHS, MH equivocal (MHE) or MH normal (MHN). Both the MHS and MHE groups are regarded as being at risk of MH on a clinical level. A test result is considered positive if there is a sustained increase of at least ≥ 2 millinormal (mN) in contracture force at a caffeine concentration of

2.0 millimolar (mM) or less and a halothane concentration of 0.44 mM or less (Rueffert *et al.*, 2001). MHE is defined as a sample that reacts positively to only one of the triggering agents.

The North American Malignant Hyperthermia Group (NAMHG) protocol is different from the European protocol, as only an abnormal response to both caffeine and halothane is considered to indicate MHS (Ball and Johnson, 1993). The NAMHG protocol has a lower sensitivity (97%) and specificity (78%) compared to the EMHG protocol (Allen *et al.*, 1998) as the EMHG protocol uses more increments in the caffeine and halothane concentrations. The EMHG protocol has a higher specificity and reduces the number of MHE individuals, which may result in the diagnosis generated using this protocol being more accurate (Fletcher *et al.*, 1999). Overall both protocols provide a similar diagnosis (Fletcher *et al.*, 1991). In addition, both tests maximise sensitivity in order to reduce the number of false negative results, which in turn reduces the specificity of the tests (Larach, 1993), indicating that 10 - 15% of unaffected patients will have false positive results.

Both false negative and false positive results have been observed when applying the standardised protocol for the IVCT. Islander and Twetman (1999) compared the two protocols and indicated that the IVCT cannot be considered a 100% specific diagnostic test for MH. Larach *et al.* (1992) suggested that if the contracture cut-off points were modified, the diagnostic test would become more sensitive and adequately specific. Currently, there is only 78 - 88% agreement between the two tests and many individuals have inconsistent results if the contracture is close to the cut-off limits (Ørding and Bendixen, 1992). Mackenzie *et al.* (1991) reported exclusion of linkage between MHS and the RYR1 locus in a French-Canadian pedigree when using the NAMHG protocol and demonstrated linkage within the same family when the diagnostic parameters were altered. A similar result was obtained in an Irish MH pedigree that only displayed linkage after the diagnostic threshold was raised (Healy *et al.*, 1996). The authors of both studies suggested that the results of these studies could be explained by a false positive IVCT diagnosis in the two families.

Adnet *et al.* (1993) indicated that different muscle fibres have different caffeine sensitivities due to the higher proportion of type I fibres in MHN individuals compared to MHS patients, which could result in a false positive contracture result. Adnet *et al.* (1990a) indicated that verapamil, a drug used in patients with cardiovascular disease, may affect the diagnosis of MH using the IVCT. The study indicated that five MH individuals were

classified as MHN, one individual as MHE caffeine (MHEc) and four individuals as MHE halothane (MHEh). The authors indicated that this drug should not be used prior to performing the IVCT. False negative results in the halothane test have also been reported in a small percentage of MHS animals (Gallant and Rempel, 1987).

2.3.2 Proposed alternative tests

Other less invasive tests have been suggested as a replacement for the IVCT, including measurement of resting serum CK. However, this test is too insensitive and non-specific to be used for a definitive diagnosis of MHS. In some family studies, the serum CK was unaffected or elevated due to conditions other than MH (Paasuke and Brownell, 1986). Numerous factors can increase CK values, including recent exercise and alcohol. Phosphorus nuclear magnetic resonance spectroscopy has been suggested as a possible diagnostic test for MH (Olgin *et al.*, 1988). This test is able to identify MHS individuals on the basis of changes in high energy phosphates. However, the test is not 100% specific or sensitive and is not able to distinguish MH from other myopathies (Olgin *et al.*, 1991). Ohnishi *et al.* (1988) suggested spin labelled red blood cells and electron paramagnetic resonance spectroscopy as a diagnostic test for MHS, as red blood cells may have a structural abnormality in MHS patients. However, the test has currently not been validated for use. Klingler *et al.* (2002) developed a test that is able to measure the proton secretion rate during activation of Ca^{2+} release with different concentrations of chlorocresol (4-chloro-*m*-cresol) in myotube cultures. The technique is based on enhanced metabolism due to increased Ca^{2+} in MHS individuals. The test has a similar specificity to the IVCT, and abnormal responses will also be detected in individuals with other myopathies. A test that measures ATP depletion in MHS individuals has been suggested as a diagnosis. However, the test lacks a sensitive diagnostic parameter compared to the IVCT (Britt *et al.*, 1976). Iaizzo *et al.* (1989) has suggested the Fura-2 indicator technique to estimate resting levels and changes in myoplasmic Ca^{2+} in human skeletal muscle, in order to diagnose MHS individuals. However, many of the suggested diagnostic tests have not proven to be sensitive enough to diagnose MH and have been abandoned. Other tests have indicated some promise. A simple and reliable method that is non-invasive and easy to perform has not yet been developed.

Several other compounds have been suggested as alternatives to either caffeine or halothane when performing the IVCT. These pharmacological agents include 4-chloro-*m*-cresol and ryanodine (Hartung *et al.*, 1996; Gilly *et al.*, 1997). The plant

alkaloid, ryanodine, used in a test protocol, was able to differentiate MHS and MHN muscle but the reproducibility and validation of thresholds were not determined. Hopkins *et al.* (1991) indicated that the contracture response to ryanodine was more specific than either the halothane or the caffeine contracture test and could perhaps be used as an *in vitro* diagnostic test for MH. However, like the halothane and caffeine test, the ryanodine contracture test presents with an overlap between the true unaffected and true susceptible population, therefore some MHE and perhaps MHS diagnoses are false positives (Hopkins *et al.*, 1997). Recently, Fusi *et al.* (2005) identified that 3,5-di-*tert*-butylcatechol (DTCAT) could be used in this regard. DTCAT acts directly at the skeletal muscle RyR1 binding site and stimulates Ca^{2+} release in a concentration-dependent manner. Other authors have suggested the use of other tests in addition to the classical halothane and caffeine test to improve the reliability of diagnosis. The agonist BAY K 8644 in association with halothane was suggested by Adnet *et al.* (1990b). The authors indicated that this antagonist stabilises Ca^{2+} channels and enhanced Ca^{2+} influx and produced a greater difference in contracture between MHEh and MHN groups. Anetseder *et al.* (2002) suggested metabolic monitoring of carbon dioxide (CO_2) following injection of caffeine as a minimally invasive test for MHS. However, the specificity of all these compounds remains less than 100% and in some cases the sensitivity and specificity of the described test has not been determined.

2.4 ASSOCIATED MYOPATHIES

Certain myopathies have an association with MH and diseases are classified as being related to MH if they share common mechanisms or pathways that result in the syndrome (Brownell, 1988). Diseases related to MH are generally a consequence of a defect in the skeletal muscle. There are only three myopathies that have been firmly established as being associated with MH, namely central core disease (CCD), Evans myopathy and King-Denborough Syndrome (King *et al.*, 1972; Quane *et al.*, 1993; Zhang *et al.*, 1993; Brandt *et al.*, 1999; Monnier *et al.*, 2000; Monnier *et al.*, 2001). These disorders predispose individuals to a drug-induced increase in Ca^{2+}, which leads to hyperthermia, hypoxia and acidosis.

2.4.1 Evans myopathy

Evans myopathy is the most common myopathy that predisposes individuals to MH, and is also known as MH myopathy (King *et al.*, 1972). Proximal muscle wasting, elevated CK

levels and varying myopathic histological patterns characterise the disease. King *et al.* (1972) described the disorder in patients with MH and indicated that the disease is inherited in an autosomal dominant manner.

2.4.2 King-Denborough Syndrome

King *et al.* (1972) first described King-Denborough Syndrome, following a nationwide survey of MH in Australia and New Zealand. The authors described 18 males with MH, of which five individuals displayed congenital progressive myopathy, short stature, cryptorchidism, pectus carinatum, lumbar lordosis and thoracic kyphosis. Three of the individuals displayed facial features typical of King-Denborough Syndrome, including crowded teeth, low-set ears and a short webbed neck. Following this report, additional cases have been identified in both males and females. The inheritance of this autosomal recessive disorder is not well understood but it is characterised by mild, slow progressive myopathy, short stature, kyphoscoliosis, pectus carinatum, cryptorchidism and facial anomalies. The disorder develops in childhood and results in delayed motor development and in some individuals a cleft or high arched palate has been described. An individual with King-Denborough Syndrome is generally diagnosed following an MH episode subsequent to exposure to anaesthesia, and all patients with King-Denborough Syndrome should be considered MHS (Chitayat *et al.*, 1992). Many individuals with King-Denborough Syndrome have elevated CK levels. However, an unaffected CK level does not exclude the patient from having the disorder (McPherson and Taylor, 1981).

2.4.3 Central Core Disease

CCD is a congenital myopathy that is inherited in an autosomal dominant manner (Isaacs *et al.*, 1975) and is almost always associated with MH. The manifestation of the disorder may vary from very mild to severe, and 40% of CCD patients may appear clinically unaffected. Disease onset takes place during infancy and the most common symptoms include lower limb skeletal muscle weakness, deformities, hypotonia and delayed motor development (Shy and Magee, 1956).

CCD is diagnosed, based on the identification of amorphous areas (cores) on the type 1 skeletal muscle fibres, which lack mitochondria and oxidative enzyme activity in the central regions of the skeletal muscle biopsy (Dubowitz and Pearse, 1960; Denborough *et al.*, 1973). Cores have increased amounts of SR and transverse tubules (t-tubules) and the

core regions display distortions in the unaffected architectural arrangement (Hayashi *et al.*, 1989). It is not known how these cores are formed and what role they play in the pathophysiology of this disorder. It has been suggested that higher intracellular Ca^{2+} may lead to alteration in skeletal muscle fibre function and/or the biochemical composition that results in the formation of cores. This hypothesis is supported by the fact that most CCD-only mutations are observed in the region of the RYR1 that codes for the pore-forming domain of the channel. However, Avila and Dirkensen (2001) demonstrated that mutations in the pore region did not alter the intracellular Ca^{2+} concentration, suggesting that they are not required for core formation. In addition, a spectrum of pathology with regard to the myopathic features has been observed in patients with CCD (Sewry *et al.*, 2002). Individuals with CCD have exhibited muscle weakness without cores and patients exhibiting cores may be clinically unaffected.

An association between MH and CCD was first reported by Denborough *et al.* (1973), and Kausch *et al.* (1991) later established the corresponding association of CCD and the RYR1 gene. Both MH and CCD are due to uncontrolled intracellular Ca^{2+} release. To date only alterations in the RYR1 gene have been observed to be associated with CCD (Kausch *et al.*, 1991). Studies conducted on families with CCD indicated that RYR1 mutations in the carboxyl terminal (C-terminal) domain of the RYR1 were observed in 57% of cases (Shepherd *et al.*, 2004) and 67% of cases (Davis *et al.*, 2003), respectively. The authors suggested that other regions of the RYR1 may play a role in susceptibility to CCD.

Certain RYR1 alterations result in both MH and CCD, whereas others display an exclusive association with either MH or CCD. In addition, healthy family members of the proband where CCD is diagnosed may be at risk of MHS even though they do not have CCD (Islander *et al.*, 1995). Two independent groups studied the association between RYR1 and CCD (Quane *et al.*, 1993; Zhang *et al.*, 1993). Quane *et al.* (1993) suggested that the RyR1 mutant proteins resulted in Ca^{2+} leakage through the release channel whereas Zhang *et al.* (1993) hypothesised that the disorder was due to the uncoupling of the electrical stimulus, which resulted in the subsequent release of Ca^{2+} from the SR. Over the last decade, several missense mutations of the RYR1 gene have been determined to be associated with CCD (Quane *et al.*, 1993; Zhang *et al.*, 1993; Brandt *et al.*, 1999; Monnier *et al.*, 2000; Monnier *et al.*, 2001). The observed mutations are detected mostly in the myoplasmic and luminal loops of the RYR1. In addition, Zorzato *et al.* (2003) identified a seven amino acid deletion in the C-terminal domain luminal loop transmembrane segments (M) M8 and M10 that alters RyR1 channel function and results in CCD.

2.5 MH-RELATED DISORDERS

The association of MH and other disorders is less clear because of inconsistencies between results of the IVCT and reactions under general anaesthesia. Certain syndromes with features similar to those of MH have been investigated for common aetiologies. Although the clinical phenotype of these disorders resembles MH, the pathogenesis is different. However, a cautious approach should be taken to avoid complications that may occur during anaesthesia due to an underlying disorder.

2.5.1 Neuromuscular disorders

Several neuromuscular disorders have been associated with complications arising from the use of volatile anaesthetics or depolarising muscle relaxants (Iaizzo and Lehmann-Horn, 1995). These may present with some symptoms that are observed in or closely resemble MH, such as muscle spasm, metabolic disturbances, heat production, cardiac arrest, rhabdomyolysis and respiratory failure. However, the pathogenesis is different from true MH. Neuromuscular disorders that present with anaesthetic risk include congenital myopathies, muscular dystrophies, non-dystrophic muscle ion channel disorders, neurogenic disorders and disturbances of neuromuscular transmission.

Multi-minicore disease (MmD) has a similar disease pathogenesis to CCD. Both congenital myopathies are characterised by hypotonia, delayed motor development, muscle weakness and the presence of cores in muscle biopsies. However, these are distinguished by their mode of inheritance, the variation in length of histological lesions and their clinical expression. The disorder displays four sub-groups, of which the genetic basis is only known for two forms. The severe form of MmD is due to alterations in the selenoprotein N gene and homozygous alterations in the RYR1 have been identified in the recessive form of the disorder. Monnier *et al.* (2003) observed a frameshift mutation in the RYR1 in one individual with MmD, which introduced a stop codon 94 amino acids downstream from the insertion site. The expressed RyR1 protein thus contained a 4976 amino acid residue with a modified C-terminal region devoid of transmembrane sequence which consists of exon 102. The alteration leads to a depletion of the RyR1 protein in skeletal muscle only and is responsible for the MmD phenotype observed in the patient. Ducreux *et al.* (2006) identified three RYR1 substitutions in three different patients with MmD, proline (Pro)3527serine (Ser), Val4849Ile and Arg999histidine (His). The Arg999His alteration was observed not to be causative, whereas the Pro3527Ser alteration resulted

in the transport of less Ca^{2+} upon activation and the presence of the Val4849Ile substitution affected the resting Ca^{2+} concentration.

Individuals with neuroleptic malignant syndrome (NMS) are considered at risk of developing MH under anaesthesia. NMS is a life-threatening complication following treatment with neuroleptic drugs. The disorder is characterised by hyperthermia, muscular rigidity, severe autonomic dysregulation and altered consciousness, although many features of this syndrome remain controversial (Adnet *et al.*, 2000). The disorder is similar to MH, since both occur due to rapid leakage of Ca^{2+} from the skeletal muscle SR, which is responsible for a chain of events that results in increased levels of CK, hyperthermia and myoglobinuria. The therapeutic approaches to treatment for both disorders are also similar. However, MH is due to a heritable abnormal Ca^{2+} metabolism in skeletal muscle, whereas the genetic contribution to NMS has not yet been determined, although a central dopamine receptor blockade or skeletal muscle defect has been suggested (Mieno *et al.*, 2003). Gurrera (2002) has suggested that the NMS might be caused by a genetic alteration of RYR3. However, Adnet *et al.* (1989) indicated that an association between NMS and MH does not exist, as the pharmacological response of muscle strips to halothane and caffeine exposure from NMS does not differ from MHN muscle. Hermesh *et al.* (1988) concluded that individuals with NMS are not at greater risk than others of developing MH during anaesthesia.

Episodes clinically similar to MH have been observed in conjunction with a variety of neuromuscular disorders, including myotonia fluctuans, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), myotonia congenita (MC) and myotonic dystrophy (DM). MH-like episodes present with acidosis, elevated temperature, muscle rigidity, hyperkalaemia, acute rhabdomyolysis and sudden and unexpected cardiac arrest (Kelfer *et al.*, 1983; Kleopa *et al.*, 2000). The molecular mechanism underlying these MH-like events is, however, different from true MHS. Reports of abnormal IVCT response in DMD patients have suggested that the disorder is associated with MH (Brownell *et al.*, 1983). On the other hand, the IVCT is not specific for MH if a patient has an underlying myopathy, as many individuals with neuromuscular disorders are diagnosed as MHS or MHE (Heytens *et al.*, 1992).

DMD is an X-linked lethal muscular disorder that affects 1 in 3,500 male births. The disorder is due to a lack of, or reduction in dystrophin, a protein that is located at the inner surface of skeletal and cardiac muscle plasma membranes. There are several reasons

why DMD may present with similar symptoms to MH following exposure to anaesthetic agents. DMD occurs due to defective intracellular Ca^{2+} homeostasis and patients with this disorder display a higher intracellular resting Ca^{2+} level, which could be due to additional Ca^{2+} entry through acetylcholine channels in response to mechanical stress induced by contraction (Imbert *et al.*, 1995). Dystrophin-deficient cells are able to contract but display impaired Ca^{2+}-handling mechanisms (Imbert *et al.*, 1995). In addition, augmentation of the mitochondria in response to Ca^{2+} is observed in these cells (Roberts *et al.*, 2001a). However, Mader *et al.* (1997) observed that dystrophin deficiency is not the primary cause of MH-like crises. Both MH and DMD occur due to defective SR Ca^{2+} homeostasis, however, MH-like episodes in patients with DMD may occur via a distinct mechanism that is different from that of classical MH (Ohkoshi *et al.*, 1995).

The gene for DM has been mapped to chromosome 19q, within the interval 19q13.2 - 19q13.3 (Brook *et al.*, 1991). The DM gene has a distance of 25 centimorgan (cM) from the RYR1 gene, indicating that MH and DM are not genetically linked (MacKenzie *et al.*, 1990). Many pedigrees with MH fail to display linkage to the DM locus on chromosome 19 and Abdalla *et al.* (1992) have demonstrated linkage to chromosome 17q and suggested that a gene that controls or regulates ionic transport in muscle membranes may be responsible for this disorder. Hypokalaemic periodic paralysis (HypoPP) has also been reported to be associated with MH (Rajabally and El Lahawi, 2002). This disorder is inherited as an autosomal dominant trait and is characterised by cold-induced stiffness, muscle weakness and low potassium (K^{+}) levels. Linkage between HypoPP and DHPR alpha (α_1)-subunit gene (CACNA1S) on chromosome 1q has been demonstrated (Fontaine *et al.*, 1990). However, mutations associated with HypoPP are located in a different region of the α_1-subunit than the mutation demonstrated to be linked to MH (Jurkat-Rott *et al.*, 1994; Boerman *et al.*, 1995).

2.5.2 Human Stress Syndrome

An association of heat stroke (Dickinson, 1989), stress (Grinberg *et al.*, 1983) and exercise known as the so called Human Stress Syndrome, with MH has also been reported but not proven to be associated. In heat stroke, sweating becomes ineffective, the body temperature exceeds 40 degree Celsius (°C), and CK levels are raised. Heat stroke has many phenotypic similarities compared to MH, but the aetiopathogenesis and treatment differ (Yaqub and Al Deeb, 1998). Grinberg *et al.* (1983) reported MH episodes post-operatively in three patients to whom "safe" anaesthetics were administered. The

authors determined that stress was the triggering mechanism of MH and suggested that no anaesthetic could be considered entirely safe. Wingard (1974) indicated that stress influences the development of this disorder and can result in enhanced myotonia and fever. A description of a stress-induced MH episode following a head injury has also been reported (Feuerman *et al.*, 1988). Ryan and Tedeschi (1997) reported a case of sudden death in an individual with a family history of MH. The individual displayed an increase in body temperature, muscle rigidity and an elevated K^+ level, which could not have been due to heatstroke in view of the mild ambient conditions and short duration of exercise. Other conditions associated with the Human Stress Syndrome include cardiac abnormalities, drug use, hyperthyroidism, infection in the central nervous system, NMS, rhabdomyolysis, sepsis and myopathies (Loghmanee and Tobak, 1986).

2.5.3 Links between MH and other disorders

It is debatable whether other symptoms exist when an MH episode is triggered. Strazis and Fox (1993) reviewed 503 cases obtained from the literature which documented MH episodes. The authors observed a higher incidence of MH in individuals with musculoskeletal defects including cleft palate, clubfoot, scoliosis, ptosis, strabismus and cryptorchism, or congenital hernias in all age groups of MH patients compared to patients that do not have a musculoskeletal defect. MH-like reactions have also been implicated in sudden infant death syndrome (SIDS). Muscle biopsies conducted on 15 parents of SIDS children indicated that five were MHS (Denborough *et al.*, 1982). Isaacs and Gericke (1990) described an association of congenital abnormalities such as cardiac abnormalities, mental defects, facial immaturity and physical developmental defects and MH. The authors suggested that intrauterine MH may cause an increase in foetal temperature in response to triggers which could cause congenital abnormalities. It has been suggested that individuals with congenital abnormalities should be recognised as susceptible to MH (Stewart *et al.*, 1988). In some cases, individuals diagnosed with another disorder have triggered with an MH episode and these are listed in Table 2.2.

Table 2.2: Reported MH episodes triggered in patients diagnosed with another disorder

Disorder	Report of disorder
Carnitine palmitoyl transferase deficiency	Carnitine palmitoyl transferase deficiency is a disorder of muscle lipid metabolism and is characterised by episodes of muscle pain, rhabdomyolysis and myoglobinuria. A patient with this disorder exhibited myoglobinuria following anaesthesia with halothane and succinylcholine (Katsuya *et al.*, 1988).
Myelomeningocele	*Myelomeningocele is a neurological disorder that displays abnormal intramuscular* nerves. Three patients diagnosed with spina bifida presented with an increase in body temperature during anaesthesia with halothane (Anderson *et al.*, 1981).
Burkitt's lymphoma	Patient with Burkitt's lymphoma experienced an increase in body temperature, rigidity and arrhythmia during an anaesthetic procedure. A subsequent muscle biopsy revealed abnormal SR function (Lees *et al.*, 1980).
Schwartz-Jampel syndrome	Disorder is characterised by dwarfism, skeletal abnormalities, muscular stiffness and an abnormal non-specific electro-myogram. A patient diagnosed with this disorder experienced an increase in body temperature, pulse rate and blood pressure and displayed elevated CK levels during surgical repair of a cleft palate (Seay and Ziter, 1978).
Wolf-Hirschhorn syndrome	Syndrome is due to a chromosomal abnormality, caused by a deletion of the short arm of chromosome four. A 21-month-old female diagnosed with Wolf-Hischhorn syndrome, admitted for repair of a cleft palate, experienced an MH episode following anaesthesia with succinylcholine. An increase in body temperature and metabolic acidosis was noted (Ginsburg and Purcell-Jones, 1988).
Smith-Lemli-Opitz syndrome	A patient diagnosed with this disorder, with strabismus and ptosis, experienced symptoms of MH including hypoxia, rigidity and hypercarbia following exposure to the anaesthetic drug halothane. However, the patient did not have increased CK levels and an association with MH requires further investigation (Petersen and Crouch, 1995).
Diabetic coma	Following treatment with insulin that contained cresol, the patient developed a high *fever and experienced respiratory and metabolic acidosis. The patient was later* diagnosed as MHS via the IVCT (Wappler *et al.*, 1996).

MH = malignant hyperthermia; MHS = malignant hyperthermia susceptibility; IVCT = *in vitro* contracture test; CK = creatine kinase; SR = sarcoplasmic reticulum.

2.6 PHARMACOLOGIC AGENTS

In unaffected MH individuals, anaesthetic drugs cause skeletal, cardiac and smooth muscle relaxation. MHS patients, on the other hand, experience rigidity and contracture when exposed to anaesthetic drugs that can trigger an MH episode. Potential triggering, safe and controversial agents are listed in Table 2.3.

Uncertainties remain for some groups of drugs as to whether they can be classified as triggers of an MH reaction during surgery. Over the past 30 years, drugs have been implicated as a trigger on the basis of a clinical report, which has several limitations. An anaesthetic drug used in combination with another drug complicates the identification of the trigger and there is generally lack of verification that the clinical reaction was a true MH response (Hopkins, 2000). Although MH was initially identified as a result of the introduction of halothane, deaths have been reported after use of the classical anaesthetic

vapours, diethyl ether and chloroform (Harrison and Isaacs, 1992). The alkane, halothane, is a potent anaesthetic and MH trigger and is able to generate persistent contracture in isolated muscle biopsies from MH patients.

Table 2.3: Triggering agents, safe agents and controversial agents related with MH

Class of agents		Specific agents
Potential triggering agents	*Volatile* anaesthetic agents	Halothane, *Enflurane*, Isoflurane, Sevoflurane, Desflurane and Methoxyflurane
	Depolarising muscle relaxants	Succinylcholine, Decamethonium
Safe agents	Inhalation agents	Nitrous oxide
	Local anaesthesia	Ligocaine, Bupivacaine
	Opioids	Morphine, Meperidine, Hydromorphone, Fentanyl, Sufentinil and Alfentanil
	Non-depolarising muscle relaxants	Pancuronium, Rocuronium, d-Tubocurarine, Atracurium, Vecuronium,
	Vasopressors	Noradrenalin, Adrenalin, Dopamine, Dobutamine
	Other	Narcotics, Antipyretics, Antihistamines, Antibiotics, Propanol, *Droperidol*
	Intravenous anaesthetics	Propofol, Etomidate, Thiopental, Ketamine, Barbituates (all), Benzodiazepines (all)
Controversial agents	Other	Calcium salts, Potassium salts, Catecholamines, Phenothiazines

Adapted from Kaus and Rockoff (1994); Golinski (1995); Hopkins (2000); Donnelly (1994); Ali *et al.* (2003); Gallen (1991).

Kunst *et al.* (1999) have reported variation in terms of potency for Ca^{2+} release from the SR in a group of inhalative drugs (halothane>sevoflurane>desflurane). Halogenated ethers, isoflurane, enflurane, sevoflurane and desflurane have been reported to induce an MH episode (Ducart *et al.*, 1995; Garrido *et al.*, 1999). Desflurane has been classified as a less potent MH trigger than halothane and induces only a slight Ca^{2+} release in skeletal muscle (Michalek-Sauberer *et al.*, 1997; Kunst *et al.*, 2000). Clinicians currently use halogenated ethers, because of the precise and rapid control they provide with regard to the depth of anaesthesia.

Neuromuscular blocking agents (NMBAs) are generally used in order to facilitate tracheal intubation, control mechanical ventilation, terminate laryngospasms and assist surgical muscle relaxation for short or long periods (Bevan, 1997). NMBAs can be classified into two categories, namely depolarising and non-depolarising, both of which interrupt *transmission of neural impulses at the neuromuscular junction.* Muscle relaxants should have rapid onset, cause significant paralysis of muscles and be short-acting (Donati, 2003). Generally, muscle relaxants are selected on the basis of speed of onset, duration

of action, route of elimination, medical history and adverse side effects. Succinylcholine, also known as suxamethonium, is a depolarising muscle relaxant mainly used in surgery to facilitate tracheal intubations, following loss of consciousness after inhalation of a volatile drug (El-Orbany *et al.*, 2004). Succinylcholine alone has not been reported to trigger an MH crisis in humans, but can exacerbate an MH episode triggered by volatile anaesthetics. Generally, the onset of an MH crisis is more rapid when an anaesthetic is used in combination with succinylcholine (Allen and Brubaker, 1998). The drug is often used in paediatric anaesthesia and in addition to several side effects, can also trigger an MH episode. Clinicians still use this drug because of its advantageous properties, which include rapid onset of complete relaxation and very short duration of action (Belmont, 1995). This drug mimics the action of acetylcholine, which targets the neuromuscular junction, and potentiates depolarisation in the SR and t-tubule system. Hydrolysis of succinylcholine at the junction is slow, and the ion channel remains open, which results in an increase in intracellular Ca^{2+}, a process which will be exaggerated in MH muscle (Dorkins, 1982; Galloway and Denborough, 1986). Non-depolarising neuromuscular drugs have been considered as possible replacements for succinylcholine. These drugs cause less severe side effects and are reported not to trigger MH episodes (Collins and Beirne, 2003). However, non-depolarising muscle relaxants are generally not used in the management of laryngospasm and prevention of aspiration pneumonia, as they have a slow onset combined with a prolonged duration.

There is a variety of non-triggering anaesthetics, including barbiturates, benzodiazepines (Britt, 1984), etomidate, thiopental (Suresh and Nelson, 1985), propofol (Gallen, 1991), opiates and nitrous oxide (Ellis *et al.*, 1972), which do not trigger MH, and can be used instead of halogenated inhalation agents for outpatient general anaesthesia. Other drugs reported as safe for MH patients include local anaesthetics (Hopkins, 2000).

2.7 DANTROLENE SODIUM THERAPY

The drug, dantrolene sodium, also referred to by the chemical name 1-[[[5-(4-nitrophenyl)-2-furanyl]methylene]amino]-2,4-imidazolidinedione, is a non-specific muscle relaxant that was introduced in 1975 as the first effective therapy for MH and has become the drug of choice in the treatment of an MH crisis. The drug is a diphenylhydantoin analogue, is poorly soluble in water (Kolb *et al.*, 1982) and was first used in South Africa in a patient with MH in 1981 (Harrison, 1981). This highly lipid soluble drug acts on the skeletal muscle SR and inhibits Ca^{2+} release into the muscle, which

results in decreased muscle contracture (Parness and Palnitkar, 1995). Nelson *et al.* (1996) observed that dantrolene could alter the gating properties of RyR1. The Ca^{2+} release channel has two binding sites for dantrolene. The first is a low-affinity binding site that results in reduced channel opening and the second is a high-affinity binding site that, upon binding of dantrolene, inhibits Ca^{2+} release (Fruen *et al.*, 1997). Investigation into the mode of action of this drug may lead to the discovery of as yet unreported mutant alleles.

Dantrolene is not advocated as a prophylactic treatment for MH (Hackl *et al.*, 1990). Older studies have illustrated that prophylactic use of dantrolene is protective at induction of anaesthesia (Allen *et al.*, 1988). However, several cases have been reported where this was not observed (Allen *et al.*, 1998). In addition, muscle weakness and nausea have frequently been associated with prophylactic treatment with dantrolene. Other disadvantages of dantrolene as a prophylactic include the fact that the drug is unreliable in oral administration as absorption levels are variable, it is poorly soluble in water and may induce transient muscle weakness, which would compromise respiration in patients with an underlying myopathy. Dantrolene can also cross the placenta, causing neonatal weakness in obstetric patients (Kaus and Rockoff, 1994).

2.8 TREATMENT OF MH

Prior to anaesthesia, patients should be assessed for risk factors that may identify the individual as MHS. Pre-surgical evaluation of patients should include questions regarding a family history of adverse outcomes to anaesthesia and details of any previous clinical episode of MH. The patient should be questioned about the presence of a musculoskeletal complaint or an inherited myopathy such as CCD or MC, which increases the risk of susceptibility to MH (McPherson and Taylor, 1982). However, family history is often unavailable, and prior history of an uneventful anaesthetic unfortunately does not guarantee that subsequent surgery is safe (Halsall *et al.*, 1979).

Once in the operating room, special attention should be given to the equipment. Anaesthesia machines should have vaporisers and CO_2 absorbers removed, the tubing should be changed and oxygen (O_2) should flow through the circuit for 10 minutes (min) at 10 litres per min ($L.min^{-1}$) flow to remove residual volatile anaesthetic agents. Alternatively, an anaesthesia machine without vapour can be used exclusively for MH patients. During anaesthesia the patient should be monitored. Any variations from the norm with regard to the electrocardiogram (ECG) or vital signs, including CO_2 level and skin temperature,

should alert the anaesthesiologist (Donnelly, 1994). The patient should be diagnosed as being at risk of MH if MMR occurs following exposure to succinylcholine and if the patient exhibits tachycardia accompanied by an unexplained, unexpected increase in end-tidal CO_2 (Forrest and Cole, 2003). If the patient presents with MH, the anaesthesiologist should recognise and manage complications associated with this disorder. Treatment of MH generally consists of early administration of dantrolene and discontinuation of the triggering drug. Even with proper management, the mortality rate has been reported to be 5 - 10%. During an MH crisis, surgery should be discontinued. If this is not possible, opioids and sedatives should be used to maintain anaesthesia, and non-depolarising muscle relaxants may be used to ensure muscle relaxation. Ventilation with 100% O_2 at a flow of more than 10 $L.min^{-1}$ should be administered to correct respiratory acidosis and decrease the risk of hypoxia. To treat the underlying metabolic acidosis, the serum K^+ should be lowered and ventricular fibrillation prevented by administrating 1 to 2 milli-equivalents per kilogram ($mEQ.kg^{-1}$) of sodium bicarbonate ($NaHCO_3$). In order to safeguard against skin and tissue damage due to thermal injury, patients should be cooled with refrigerated intravenous (IV) saline solutions, which should be administered directly to the peritoneal and thoracic cavity (if the surgical site is open) and indirectly to the stomach. Surface body temperature should be reduced by using hypothermic blankets. Cooling of the patient should be maintained until the body temperature reaches 38°C, as excessive cooling can result in hypothermia (Allen, 1994). Dantrolene sodium should be administered intravenously at an initial dose of 2.5 milligram per kilogram ($mg.kg^{-1}$), and continued until the clinical signs of an MH episode have diminished (Donnelly, 1994). Dantrolene is prepared in a lyophilised formulation and each vial contains 20 milligrams (mg) dantrolene, 3 g mannitol and sodium hydroxide to adjust the pH to 9.5. It should be reconstituted in 60 millilitre (mL) sterile water (H_2O). During an MH episode 36 vials of dantrolene should be available, which corresponds to a maximal dosage of 10 $mg.kg^{-1}$ in a 70 kilogram (kg) adult. Dantrolene should be administered repeatedly in 2-3 $mg.kg^{-1}$ doses every 5-10 min until symptoms associated with this disorder are controlled. Following stabilisation, the patient's arterial and venous blood gas, central venous pressure, renal function, temperature, urine myoglobulin, electrolytes, CK and coagulation factors should be monitored (Wappler, 2001).

2.9 MOLECULAR MECHANISM OF MUSCLE CONTRACTION IN MH

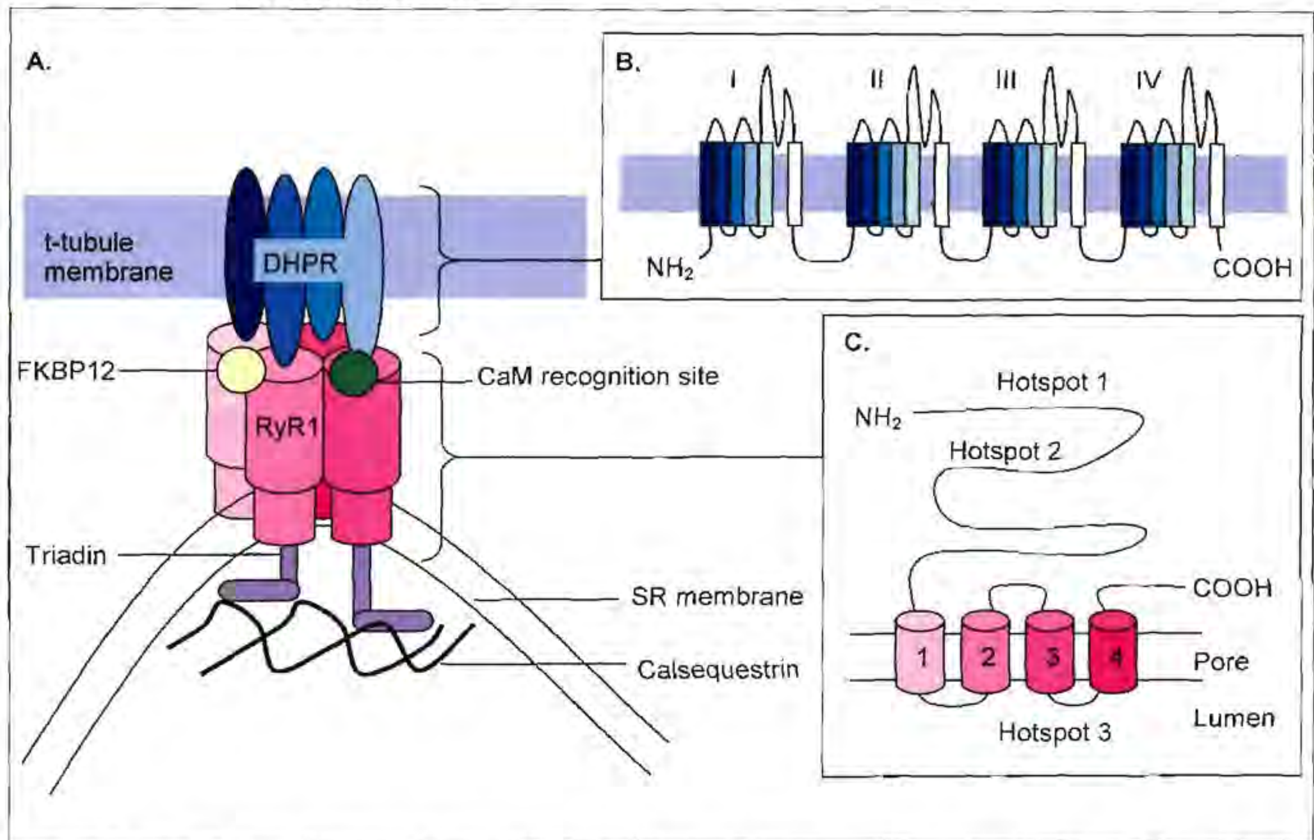
The underlying cause of MH has emerged as biochemical abnormalities that occur in skeletal muscle. Although contraction in mammalian skeletal muscle is highly regulated,

an MH episode may occur if the intracellular Ca^{2+} homeostasis in skeletal muscle is disturbed (Denborough *et al.*, 1962). MH could be induced by mutations from the N-terminus to the C-terminal portion of the RYR1 gene. However, these effects could also be due to alterations in the interaction of the RyR1 with accessory proteins involved in the regulation of the channel's activity. Various interacting Ca^{2+} channels, Ca^{2+} binding proteins and Ca^{2+} pumps function to regulate muscle Ca^{2+} homeostasis.

2.9.1 Excitation-contraction coupling

E-C coupling refers to the precise regulation of intracellular Ca^{2+} release following an action potential in muscle cells. The structures responsible for E-C coupling include the t-tubule and SR, which comprise the sarcotubular system (Brunder *et al.*, 1992). The t-tubules are invaginations of the cell surface membrane and transverse the width of each muscle fibre, which serve to spread the action potential rapidly to the interior of the muscle fibre. The SR is an elaborate smooth endoplasmic reticulum that surrounds the myofibrils and serves as a Ca^{2+} reservoir. The function of the SR is to reduce the external Ca^{2+} to below 1 micromolar (μM) in order to remove Ca^{2+} from troponin (Tn) C, which results in relaxation of muscle fibres. Transport of Ca^{2+} is managed by the Ca^{2+} adenosine triphosphatase (Ca^{2+}-ATPase) which requires both ATP and magnesium (Mg^{2+}) in order to transport Ca^{2+}. In this way, two Ca^{2+} ions are transported per ATP molecule that is hydrolysed. Release of Ca^{2+} from the SR stores is carried out via two channels, the inositol-1,4,5-triphosphate receptor ($InsP_3R$) and the RyR1 (Imagawa *et al.*, 1987). The DHPR and RyR1 are situated in the triadic junctions of the t-tubular system and the SR respectively. The RyR1, the primary Ca^{2+} release channel, is observed in the terminal cisternae (TC) of the SR (Fleischer *et al.*, 1985) and the voltage-sensing DHPR is detected in the junctional t-tubules. Both proteins are essential for E-C coupling and absence of either results in death at or before birth. Once activated via depolarisation, the DHPR α_1-subunit undergoes a voltage-dependent conformational change and transmits the action potential to the RYR1 via transmembrane motifs II and III (Pessah *et al.*, 1996) as illustrated in Figure 2.1A.

Figure 2.1: Schematic representation of DHPR-RyR1 interactions in excitation and contraction



A = illustration of the direct interaction between the DHPR and RyR1 in skeletal muscle; B = diagrammatic representation of the structure of the DHPR; C = diagrammatic representation of the structure of RyR1 indicating the three hotspots of RyR1, the C- and N-terminal domains and the cytoplasmic and transmembrane domains. t-tubule = transverse tubule; NH_2 = amino group; COOH = carboxyl group; CaM = calmodulin; SR = sarcoplasmic reticulum; FKBP12 = immunophilin (cytosolic receptor) FK506-binding protein; RyR1 = skeletal muscle ryanodine receptor protein type one. Adapted from McPherson and Campbell (1993).

Direct interaction between the DHPR and RyR1 occurs via physical protein-protein interactions that can take place because of their close proximity (Loke and MacLennan, 1998). Both are arranged in a regular array and four DHPR face every second RyR. Multiple domains of the RyR1 combine to interact functionally with the skeletal muscle DHPR III-IV loop (Leong and MacLennan, 1998). The two regions that play a critical role in E-C coupling include the RyR1 residues 1076 - 1112 and 1635 - 2635 (Dulhunty and Pouliquin, 2003). Association between the two proteins results in the passive release of Ca^{2+} at the triad junction into the sarcoplasm from the lumen of the SR. Many of the RyR1 channels are not associated with a DHPR. The uncoupled RyR1 channels can be activated via cytosolic Ca^{2+} signals and participate in the Ca^{2+} induced Ca^{2+} release (CICR) process. Both uncoupled and coupled RyR1 channels are therefore heterogeneous in nature and the amount of each differs in assorted skeletal muscle. In addition, the DHPRs function poorly as Ca^{2+} conducting channels in the absence of RyR1 channels, which enhances the function of the DHPR (Nakai *et al.*, 1996).

Released Ca^{2+} from the SR binds to one of the subunits of Tn namely TnC and relieves the inhibition of the contractile apparatus, which results in muscle contraction (Marieb, 1995). During ATP-mediated relaxation, Ca^{2+} ions are pumped back to the SR. The uptake of Ca^{2+} is against a concentration gradient, and the energy is obtained through coupling of Ca^{2+} uptake to hydrolysis of ATP through the action of SR Ca^{2+}-ATPase (SERCA). The fast twitch isoform, SERCA I, facilitates the transportation of sarcoplasmic Ca^{2+} into the SR lumen, where Ca^{2+} are stored in association with calsequestrin (CSQ).

SERCA pumps were first purified by MacLennan (1970) and span the membrane of the SR. The pump consists of three cytoplasmic domains including the nucleotide binding, phosphorylation and N-anchoring domain. Further clearance of Ca^{2+} occurs via mitochondrial Ca^{2+} uptake and Ca^{2+} removal in the periphery by plasma membrane Ca^{2+}-ATPase pumps (PMCA), as well as via the Na^+/Ca^{2+} exchanges (NCX), which exist in the skeletal muscle plasma membrane (Martonosi and Pikula, 2003).

2.9.2 Ultrastructure of the RyR1 receptor

The RyR1 was initially identified and named due to the pronounced actions of the plant alkaloid, ryanodine, on insect and vertebrate muscle, which binds specifically to the RyR1 channel with a high affinity (Pessah *et al.*, 1985). Ryanodine induces rigid paralysis in skeletal muscle and flaccid paralysis in cardiac muscle. The different effects are due to the fact that Ca^{2+} that is leaked from intracellular stores in heart muscle is quickly removed via the surface membrane extrusion mechanism, resulting in flaccid paralysis. In muscle, however, Ca^{2+} accumulates in the cytoplasm, resulting in sustained contraction.

The RyR1 is one of the largest known proteins and the receptor displays a fourfold symmetry with a dense central mass that is divided into four domains (Saito *et al.*, 1988), as illustrated in Figure 2.1 A and C. The receptor consists of two major substructures, a large hydrophilic cytoplasmic assembly, which contributes 80% of the mass of the receptor, and a smaller hydrophobic transmembrane assembly that spans the membrane and forms a base plate. The cytoplasmic domain is composed of four identical subunits that span the gap between the t-tubule and the SR and form a channel-like feature or foot (Wagenknecht and Redermacher, 1995), which extends across the gap of the triad junction from the TC of the SR to the t-tubule. The foot structure of the RyR is also known as the junctional channel complex, as it is able to sense the depolarisation signal from the DHPR and act as a Ca^{2+} channel (Wagenknecht *et al.*, 1989).

2.9.3 Ryanodine receptor isoforms

RyR1 proteins are expressed in a variety of different species including vertebrates, invertebrates and plants. To date, three different isoforms of RyR (RyR1, RyR2 and RyR3) have been observed which are encoded by three different genes on different chromosomes, which have been named according to the tissue in which they were first identified (Sorrentino *et al.*, 1993). The phylogenetic tree constructed for all RyRs indicates that they have all descended from a common ancestral gene. Studies indicate that RyR2 diverged first to form a distinct branch and divergence of all three classes is very close (Tunwell *et al.*, 1996). The primary structures of the three distinct isoforms of RyR that are expressed in skeletal muscle, heart, brain and other tissue (Fill and Copello, 2002) have been elucidated by complementary deoxyribonucleic acid (cDNA) cloning. The three genes for the RyR isoforms are expressed in many different tissues with different levels of expression and may participate in the regulation of intracellular Ca^{2+} homeostasis in a wide range of cells (Giannini *et al.*, 1995).

Mammalian RyR1, RyR2 and RyR3 proteins show a high degree of overall homology (approximately 67 - 70% identity) with certain regions being particularly conserved. However, three regions display variability, namely divergent region one (D1) that includes amino acids 4250 - 4627, D2 (amino acids 1302 - 1406) and D3 (amino acids 1864 - 1925) that may result in functional differences between the isoforms (McPherson and Campbell, 1993). The last regions are responsible for the specific isoform characteristics and have demonstrated binding sites for different modulators. Analysis of RyR amino acid sequence has revealed several consensus ligand binding motifs i.e. ATP, Ca^{2+}, caffeine and calmodulin (CaM) and phosphoryation motifs. Alternative splicing variants have been observed in all three isoforms, which is hypothesised to contribute to the generation of further functional diversity among RYR isoforms (Nakai *et al.*, 1990).

RyR1 is the major isoform expressed in skeletal muscle (Takeshima *et al.*, 1989), but is also expressed in a number of non-muscle cells including the brain, specifically in the Purkinje cells of the cerebellum, the cerebrum temporal lobe and thalamus/hypothalamic regions (Ledbetter *et al.*, 1994) the heart (Futatsugi *et al.*, 1995), in parotid cells, in pancreatic cells, the liver (Lee *et al.*, 2002), non-excitable lymphocytes (Sei *et al.*, 1999) and in the mitochondria of the heart in rats (Beutner *et al.*, 2005). The RyR3 is the brain isoform and is widely distributed in a wide variety of cell types (Sorrentino and Reggiani, 1999), including specific regions of the brain e.g. the corpus striatum, thalamus and

hippocampus (Murayama and Ogawa, 1996), in smooth muscle (Giannini *et al.*, 1995) and in certain non-excitable cells (Hakamata *et al.*, 1994). It does not sustain E-C coupling. The RyR3 isoform is expressed within all muscles from the late embryonic stage and during the first two weeks after birth. However, expression is down-regulated in most muscle two to three weeks into post-natal life. In mammalian vertebrate studies, RyR3 is detectable at low levels in the diaphragm muscle (Murayama and Ogawa, 1996).

Both the RyR1 and RyR2 display precise localisation in skeletal and cardiac muscle to structures called triads and diads, respectively. Differences between RyR1 and RyR2 are observed in the N-terminal domains that interact with the DHPR. E-C coupling is similar in both isoforms. However, different mechanisms exist, and the RyR1 and RyR2 channels differ in the way that they open. RyR1 channels release Ca^{2+} due to an association with the DHPR that undergoes a conformational change following depolarisation via E-C coupling. In the heart, the RyR2 functions as a CICR channel. Ca^{2+} release via the RyR2 occurs due to the inward Ca^{2+} flux through the cardiac DHPR, which triggers the release of Ca^{2+} by the RyR2. The CICR process is sensitive to both the speed and amplitude of the Ca^{2+} trigger. The RyR2 in the heart forms part of a larger macromolecular complex consisting of phosphorylases, phosphatases and FK506-binding protein (FKBP12.6), which regulates the level of CICR (Marx *et al.*, 2001). Studies have indicated that several cardiomyopathies are associated with 11 missense mutations in the RYR2, that cluster in the same hotspots as the RYR1 mutations that have been determined to be associated with MH and CCD (Laitinen *et al.*, 2001; Tiso *et al.*, 2001). Mutations associated with inherited disease have thus far not been described for RyR3.

Hosoi *et al.* (2001) demonstrated that all three RyR isoforms are expressed in human primary T and B lymphocytes as well as in monocytes, suggesting that multiple Ca^{2+} release mechanisms control Ca^{2+} signalling in immune cells. Shoshan-Barmatz *et al.* (2005) identified the three known RyR isoforms in the mammalian retina and in addition observed a novel ryanodine-binding protein that displayed altered binding properties with unique regulatory mechanisms to the RyRs derived from skeletal or cardiac muscle. The novel RyR may represent a product of alternative splicing of a heterotetrameric complex composed of different subunits of RyR1, RyR2 or RyR3, as the messenger ribonucleic acid (mRNA) of each is expressed in the retina. It has been suggested that the functions of RyR proteins may be regulated via the production of alternative transcripts of RyRs, which may form either homo- or hetero-tetrameric complexes containing truncated or variant receptors. To support this hypothesis, Neylon *et al.* (1995) detected multiple types

of RyRs expressed in vascular smooth muscle and aortic muscle of rats and Conti *et al.* (1996) detected RyR3 in a variety of different muscles with varying levels of mRNA. In addition, Chiang *et al.* (2004) observed that the RYR isoform of turkey (α*ryr*) that is homologous to mammalian *ryr*1 had three different cDNA transcript variants. The first variant was homologous to mammalian skeletal muscle RYR1, the second was identified by the absence of 81 bases located in exon 13 and the third transcript carried a 193 base pair (bp) deletion that corresponds to the entire exon 13. The study further revealed two genomic DNA alleles of the α*ryr* and indicated that the two alleles had identical exon sequences but differed in their intron sequences. The authors suggested that the two alleles arose from alternative splicing sites.

2.9.4 Physiological modulation of the RyR1 receptor

A variety of compounds, such as intracellular secondary messengers and drugs, modulate the release of Ca^{2+} from the RyR1, including Ca^{2+} and adenine nucleotides. The cytoplasmic domain consists of multiple binding sites, and Ca^{2+} release can be stimulated by low concentrations of Ca^{2+} (< 100 μM of Ca^{2+}) and can be inhibited by a high Ca^{2+} concentration. Ca^{2+} can bind to the channel, resulting in small amounts of Ca^{2+} being released, which in turn causes more Ca^{2+} to be released via positive feedback. ATP is able to activate the channel and both Ca^{2+} and ATP are required for a fully active channel (Smith *et al.*, 1986). Ca^{2+} activation sites have been localised to the C-terminal of the RyR1 within residues 4478 - 4512. A diverse set of residues in other regions of this protein interact to determine this activation, including residues 4254 - 5631 and the luminal loop. However, the mechanism of activation has not been determined. In addition, uncharged molecules such as ryanodine, caffeine, halothane and CaM (Meissner, 1986; Coronado *et al.*, 1994) can interact with RyR1 and indirectly increase the affinity of the channel for Ca^{2+}. RyR1 has two distinct sites for binding ryanodine i.e. a high- and low-affinity binding site (Callaway *et al.*, 1994) and binding to both these sites results in a closed RyR1 channel (Lai *et al.*, 1989). Large cationic inhibitors including ruthenium red may prevent Ca^{2+} release. Mg^{2+} can bind to the low affinity Ca^{2+} binding sites and inhibit the channel (Meissner, 1986). As varying affinity for Mg^{2+} between the different isoforms exists, it has been suggested that Mg^{2+} binding sites are located in divergent regions. Other agents, such as volatile anaesthetics, can also bind to the RyR1 channel and modify its activity (Pessah *et al.*, 1996). Lastly, dantrolene can interact with the channel and inhibit the interaction between halothane or caffeine and the channel (Ohnishi *et al.*, 1986). In addition, certain agents can alter the redox state of RyR1 either by oxidising or alkylating

highly reactive cysteines, which enhances CICR or reduces the cysteines, which has the opposite effect (Hildalgo *et al.*, 2004). Approximately seven hyperreactive cysteines have been described for the RyR1 complex, which can regulate channel activity in response to the redox state (Sun *et al.*, 2001; Voss *et al.*, 2004).

In MH muscle, the affinity for ryanodine in the presence of ATP and caffeine is significantly greater than unaffected SR and ryanodine binds to the open channel with a higher affinity than in resting RyR1 channels (Hawkes *et al.*, 1992). In addition, ryanodine binding is more sensitive to caffeine stimulation and less sensitive to ruthenium red or Mg^{2+} inhibition compared to unaffected SR (Mickelson *et al.*, 1990). The SR of MH pigs has indicated that the channel is more permeable to Ca^{2+}, which increases in the presence of halothane (Ohnishi *et al.*, 1986). However, McSweeney and Heffron (1990) indicated that halothane-induced Ca^{2+} release was similar in MH and unaffected muscle but that the CICR in MH muscle was 13% higher than in unaffected muscle. Valdiva *et al.* (1991) indicated that MH receptors had a higher binding affinity for ryanodine, Ca^{2+} and caffeine compared to unaffected individuals.

2.9.5 <u>RyR1 receptor binding proteins</u>

The activity of RyR1 is regulated via many soluble factors, associated proteins and via covalent modification such as oxidation, nitrosylation and phosphorylation. The RyR forms a huge macromolecular complex and is associated with a wide variety of proteins and co-proteins that are functionally significant. The functional activity of RyR1 is regulated directly or indirectly by association with various proteins, which interact with both the amino terminal (N-terminal)/C-terminal region and domains facing the lumen. However, little is known about the protein binding sites. The RyR1 forms a multi-protein complex with CSQ, a high-capacity Ca^{2+}-binding protein. The CSQ is anchored to the junctional face of the SR membrane and to RyR1 by means of triadin (TRI) and the junctional face protein (JFP), forming a quaternary protein complex (Collins *et al.*, 1990; Guo and Campbell, 1995; Zhang *et al.*, 1997; Groh *et al.*, 1999). Both TRI and JFP maintain receptor interactions and may be involved in preserving the structural design of the triad junctions. TRI is a positively charged protein that interacts with RyR1 and regulates Ca^{2+} release by transmitting the release signal to the negatively charged CSQ. Binding of Ca^{2+} to the CSQ monomer causes a conformational change in CSQ, resulting in its compaction and polymerisation (Ikemoto *et al.*, 1972). CSQ plays a role in regulating RyR1 activity in response to different Ca^{2+} concentrations in the lumen (Beard *et al.*, 2004). Mutations in

the genes that encode the above-mentioned proteins may lead to altered Ca^{2+} homeostasis. However, the effect of CSQ on the activity of RyR1 remains controversial. Studies have indicated that tritium (^3H) ryanodine binding and the open probability of RyR1 is potentiated in the presence of CSQ, whereas studies conducted by Beard *et al.* (2002) have indicated that association of CSQ to RyR1 results in a decrease in channel activity. The conflicting reports may be explained by the fact that the actions of CSQ on RyR1 activity are dependent on the presence of one or more co-proteins (TRI and/or JFP), therefore studies which use conditions that result in the dissociation of an anchoring protein may result in failure of CSQ to inhibit channel activity. In addition, the effects of this protein on the activity of RyR1 depend on its phosphorylation state. Dephosphorylated CSQ is able to induce channel opening in the presence of 1 mM Ca^{2+}, whereas phosphorylated CSQ has no effect. Over-expression of this protein results in enhancement of both caffeine and voltage-induced Ca^{2+} release, which results in an increase in Ca^{2+} storage in the SR. JFP and TRI are integral membrane proteins that consist of a short N-terminal cytoplasmic domain and a long C-terminal region located in the SR lumen that has alternative positively and negatively charged amino acids, lysine (Lys) and glutamate (Glu). *In vitro* studies have demonstrated that the cytoplasmic region of TRI can modulate channel activity. TRI interacts with RyR1 in a Ca^{2+}-dependent manner and binding results in inhibition of Ca^{2+} release and a decrease in the open probability of the RyR1 channel. There is an important structural and functional association between RyR1 and TRI involving highly reactive sulfhydryl moieties. The stability of this complex is determined by the redox state of the moieties, which are in turn regulated by channel ligands (Liu and Pessah, 1994).

The large cytoplasmic domain of RyR1 has been determined to bind several accessory proteins, including FKBP12, CaM, protein kinases, phosphatases, sorcin and homer proteins. FKBP12 is a *cis-trans* prolyl isomerase that was originally identified as the receptor for the immunosuppressant drugs FK506 and rapamycin, which causes FKBP12 to dissociate from RyR1, a process that disturbs E-C coupling. Jayaraman *et al.* (1992) first indicated the tight association between FKBP12 and the RyR1 and specified that binding occurred on the TC of the SR and not the longitudinal tubules. FKBP12 binds to the cytoplasmic assembly, 10 nanometres (nm) from the entrance to the transmembrane ion channel, near the side that interacts with the t-tubule membrane system (Wagenknecht *et al.*, 1997). FKBP12 co-purifies with RyR1 during column chromatography and sucrose density centrifugation and anti-FKPB12 antibodies can immunoprecipitate RyR1 from purified preparations. Numerous studies have indicated that

FKBP12 can regulate the activity of RyR1 (Timerman *et al.*, 1993). In the absence of this protein the channel displays longer mean open time and greater open probability, it is activated by a lower concentration of Ca^{2+} or caffeine and requires larger amounts of Mg^{2+} concentration for inactivation. These effects can be reversed upon addition or co-expression of the protein (Brillantes *et al.*, 1994). Brillantes *et al.* (1994) suggested that the FKBP12 protein is able to enhance the co-operativity of the four subunits of the RyR1, resulting in full conductance channels with decreased open probability and stabilising the closed conformation of RyR1. Other studies have indicated that the interaction between the DHPR and the RyR1 channel is greatly reduced after FKBP12 depletion, resulting in severely compromised voltage-gated SR Ca^{2+} release. Brooksbank *et al.* (1998) reported that the absence of FKBP12 destabilises Ca^{2+} release by the RyR1, and results in an increased sensitivity to pharmacologic stimulators such as caffeine and halothane. The authors suggested that an alteration in the FKBP12 or in the protein's capacity to bind to the RyR1 may predispose individuals to MHS.

CaM is a ubiquitous 17 kilodalton (kDa) Ca^{2+} binding protein containing four EF-hand type Ca^{2+} binding motifs in the N-terminal and in the C-terminal regions that can modulate RyR1 channel activity by altering the open time probability (Zhu *et al.*, 2004). CaM binds to the cytoplasmic assembly 12 nm from the transmembrane ion channel within a cleft that faces the junctional face of the SR membrane (Wagenknecht *et al.*, 1997). The RyR1 3614 - 3643 region acts as a CaM binding site and each RyR1 tetramer can bind four molecules of CaM. Both N- and C-terminals can bind RyR1 at either high or low Ca^{2+} concentrations, and the effect of CaM on RyR1 activity is dependent on the Ca^{2+} concentration. In the presence of a low concentration of Ca^{2+}, CaM may increase the sensitivity of the RyR1 to Ca^{2+} dependent activation, whereas at higher concentrations of Ca^{2+}, CaM inhibits channel function (Buratti *et al.*, 1995). In addition, the effects of CaM on RyR1 are dependent on Ca^{2+} binding to the protein, in the absence of bound Ca^{2+}, CaM is able to enhance RyR1 activity, whereas when Ca^{2+} is bound to CaM it inhibits the channel.

2.9.6 Functional characteristics of the RYR1 protein

To date, many of the functional characteristics of the RyR1 protein have not been determined, as unravelling of the molecular mechanism of the RyR1 channel has been hampered by the enormous size of the protein complex. Due to its size, X-ray crystallographic and spectroscopic techniques are not suited to study the functional characteristics of the RyR1 protein. Cryo-electron microscopy coupled with

computer-assisted image reconstruction has been employed to map the three-dimensional structure of purified RyR1 complexes (Benacquista *et al.*, 2000), as well as the sites of interaction of certain accessory proteins and of sequence-specific antibodies (Kobayashi *et al.*, 2004; Sharma and Wagenknecht, 2004).

Generation of chimaeric and truncated RyR complexes using recombinant DNA technology has also generated insights into the structure-function relationships of these channel proteins. However, this approach should be interpreted with caution, as it can result in a change of the pharmacological properties of the channel by altering the three-dimensional structure (Treves *et al.*, 2002). Generation of amino acid substitutions in RyR primary structures using recombinant DNA techniques has provided invaluable information on the mechanism of RyR gating. For example, a mutation of a highly conserved glutamate residue at position 3885 in RyR3 produced a channel that has reduced sensitivity to activation by Ca^{2+} (Chen *et al.*, 1992).

2.9.7 The DHPR

The DHPR or local type (L-type) Ca^{2+} channel is a receptor for channel blockers such as 1,4-dihydropyridine (DHP) derivatives and is named according to its sensitivity to the presence of DHP. DHPR is a heteroligomeric membrane protein complex with a total mass of 430 kDa. In skeletal muscle, DHPR is composed of five different subunits, namely α_1-, α_2-, beta (β)-, delta (δ)- and gamma (γ)-subunits which are encoded by four genes (Catterall, 1988), which may all interact with the RyR1. The δ-subunit arises from proteolysis of the C-terminus from the α_2/δ primary polypeptide. The α_1-subunit consists of four homologous domains (I to IV) and each of the domains has six transmembrane α-helical segments (S), numbered S1 to S6, as illustrated in Figure 2.1 B.

Voltage sensitivity, which permits the movement of charges, including Na^+ and K^+ during depolarisation, is associated with the S4 segments that contain positively charged amino acid residues, Lys or Arg (Nakayama *et al.*, 1991). The α_1-subunit contains sequence homology to the Na^+ channel (Tanabe *et al.*, 1990). The II-III cytoplasmic loop of the α_1-subunit is capable of binding and functionally interacting with the RyR1 (Lu *et al.*, 1994). A hydrophilic loop exists between S5 and S6 in domains III and IV, which is involved in pore formation. The cytoplasmic loop located between domains III and IV contains the first mutant allele in the second gene to be associated with MH (Monnier

et al., 1997). The remaining subunits are considered regulatory. Both the α_2- and γ-subunits are glycoproteins (Jay *et al.*, 1990). The γ-subunit consists of four hydrophobic transmembrane domains and two N-linked glycosylation sites (Jay *et al.*, 1990), whereas the α_2-subunit does not have transmembrane domains. Perez-Reyes *et al.* (1989) suggested that the γ-subunit played a role in the stable expression of the α_1-subunit. All of the α_2-, β-, δ-, and γ-subunits may play a role in diseases of Ca^{2+} regulation (Loke and MacLennan, 1998).

2.9.8 Excitation-contraction coupling and calcium regulation in MH susceptible individuals

Exposure of individuals to volatile anaesthetics results in an increase in RyR1 activity and disturbed Ca^{2+} regulation. In unaffected muscle, moderate disturbances do not affect metabolism, as regulatory pathways are able to adapt to further Ca^{2+} release. In various myopathic skeletal muscle disorders (including MH) mutant RyR1 channels become sensitive to lower concentrations of stimulators (such as Ca^{2+} and halothane). However, the rate of Ca^{2+} uptake into the SR in the population with unaffected muscles varies (Isaacs *et al.*, 1975), indicating that variability of the MH phenotype may be due to the associated mutation as well as the efficiency of the individual Ca^{2+} uptake system (Quane *et al.*, 1994a). Continuous Ca^{2+} release stimulates Ca^{2+}-ATPase activity, which leads to an increased rate of ATP hydrolysis. The continual release of Ca^{2+} leads to sustained muscle contracture, and the subsequent decline in ATP levels produces a hypermetabolic response, which may trigger an MH episode (Pessah *et al.*, 1996). The pathogenesis of muscle contraction in MHS individuals has been extensively studied using animal models. Schiller and Mair (1974) identified ultrastructural changes in the muscle obtained from MH pigs following an episode. The muscles exhibited a parallel array of granules, aggregation and in some cases rupture of mitochondria, dilation of the SR and an increased intake of fluid and Ca^{2+}. These changes in the muscle were reversible and no significant ultrastructural changes were observed in the same pigs when not exposed to halothane.

2.10 METABOLIC DYSREGULATION IN MH

Other theories have been proposed that may account for the abnormal regulation of intracellular Ca^{2+} during an MH reaction. Some of the possible candidates that could play a role in the development of the disorder include SERCA, inositol-1,4,5-triphosphate ($InsP_3$), CaM, sarcolemma, adrenergic innervation of the muscle, a central dopaminergic

abnormality and the mitochondria. In addition, the possibility exists that MH is a widespread membrane disease involving different membranes of different cell types (Oku *et al.*, 1983). Considering that Ca^{2+} plays an important role in regulating muscle contraction, relaxation and energy metabolism, any alterations in Ca^{2+} regulation may have significant physiological consequences and account for a wide variety of symptoms associated with the development of MH following exposure to triggering anaesthetics.

Alterations in Ca^{2+} uptake via SERCA were suggested. However, Nelson (1989) observed that the SERCA Ca^{2+} uptake mechanism was unaltered. The intracellular secondary messenger $InsP_3$ mediates release of Ca^{2+} from the SR and has been suggested as a possible candidate in the development of MH. *In vitro* studies have indicated that the $InsP_3$-mediated release of Ca^{2+} is much more effective in MHS specimens and that the basal levels of $InsP_3$ are higher compared to MHN samples (Tonner *et al.*, 1995). In addition, dantrolene was identified to decrease the intracellular Ca^{2+} and prevent the $InsP_3$-induced increase in intracellular Ca^{2+} (Lopez *et al.*, 1995). However, the increased rate of ATP consumption that occurs during an MH episode results in higher $InsP_3$ levels and an increase in Ca^{2+} release from the $InsP_3R$. Therefore, it is likely that increased levels of $InsP_3$ play a role in MH but do not cause the disease (Steinmann, 1994). Cheah *et al.* (1985a) observed that there is a higher level of CaM in MHS pigs, which may also enhance phospholipase A_2 (PLA_2) levels. Another candidate, i.e. the sarcolemma functions by binding Ca^{2+} and controlling Ca^{2+} flux. However, there are conflicting reports about the role of the sarcolemma and CaM in MHS. Niebroj-Dobosz and Mayzner-Zawadzka (1982) indicated that both phospholipids and cholesterol were lower in MHS pig. Mickelson *et al.* (1986) however, reported unaffected levels of cholesterol, phospholipids and CaM in MHS sarcolemma in MHS pigs. Abnormal catecholamine innervation has been suggested to precipitate MH episodes that have been induced by stress, however, this has not been observed in humans. The stress-induced reactions may be due to uncontrolled activation of the adrenergic pathway. A dopaminergic contribution in MH has been suggested. Adeola *et al.* (1993) reported a central dopaminergic abnormality in pigs with PSS and indicated regional differences of neurotransmitter concentrations. Park *et al.* (2004) reported neurological defects in a patient diagnosed with MH, who exhibited lesions in the cerebellum and damage to the basal ganglia that was confirmed via magnetic resonance imaging. As MH is considered to be a metabolic disorder in the skeletal muscle, the mitochondria have been suggested to play a role in MHS. Mitochondria function as storage vesicles for Ca^{2+} and excess Ca^{2+} that is not removed by the SR during relaxation is transported into the mitochondria.

2.10.1 Possible role of mitochondria in the development of MH

Reports indicate that impairment of intracellular Ca^{2+} homeostasis and mitochondrial function may result in the development of neurodegenerative disorders, diabetes and cardiomyopathy. The functions for which the mitochondria sequester Ca^{2+} include stimulating and controlling the rate of oxidative phosphorylation, inducing the permeability of the transition pore and safeguarding against Ca^{2+} overload in order to prevent cellular toxicity and damage, as it is able to transport Ca^{2+} in and out of the mitochondrial matrix. Intracellular Ca^{2+} plays an important role as a signal for the synthesis of nuclear proteins leading to mitochondrial biogenesis and elevated Ca^{2+} concentration leads to an increase in transcriptional activation and mRNA levels (Freyssenet *et al.*, 2004). Other functions of the mitochondria include ATP generation, thermogenesis, control of apoptosis and generation of reactive oxygen species. It has therefore been suggested that the MH syndrome may also involve defects in muscle mitochondria in addition to the alterations in the RYR1 (Eikelenboom and van den Bergh, 1973). The mitochondria serve as storage vesicles for excess Ca^{2+} that cannot be taken up by the SR during relaxation cycles. The mitochondria take up Ca^{2+} during contraction, following motor nerve stimulation and rapidly release Ca^{2+}. This process is only slightly slower (a few milliseconds) compared to Ca^{2+} uptake and release in the cytosol. Ca^{2+} consumed during the uncoupling process and active transport of Ca^{2+} by mitochondria are related to the activity of the respiratory chain and linked to a transmembrane exchange of positive charges (Chance and Williams, 1955).

Several reports have suggested that there is evidence of a primary mitochondrial abnormality that results in an increase in the concentration of myoplasmic Ca^{2+}. Individuals with MH who are exposed to triggering agents exhibit an excessive metabolic rate, which leads to an increase in body temperature. This occurs as a result of the uncoupling of oxidative phosphorylation, in which heat is not converted to ATP (Cheah and Cheah, 1981). The decline in ATP levels results in the release of intracellular enzymes such as CK, K^+ and hydrogen ions (H^+). Cheah and Cheah (1981) reported an increase in transient temperature of 9°C in the mitochondria of MH pigs. The authors indicated that the increase in temperature could be explained by hydrolysis of phospholipids in mitochondrial membranes by Ca^{2+} activated PLA_2 releasing unsaturated fatty acids. To support this observation, Fletcher *et al.* (1990) reported that when MHS pigs were depleted of triglycerides and free fatty acids (to about 40 and 60%), they did not exhibit muscle contracture when challenged with halothane and succinylcholine. Elevated

triglycerides result in an increased production of free fatty acids, which in turn regulate Ca^{2+} sequestration by the SR and Ca^{2+} release from the $InsP_3R$ (Fletcher *et al.*, 1990). These fatty acids interact with the mitochondrial membrane and alter several of the membrane functions involved with energy processes. When mitochondria are exposed to high levels of Ca^{2+}, the permeability transition pore opens and allows a non-selective increase in permeability of the inner membrane to small solutes, a process that is promoted by fatty acids. Consequently this results in the swelling of the mitochondria due to destabilisation of the mitochondrial membrane, and can cause the uncoupling of the mitochondria, which may in turn trigger the release of much larger amounts of Ca^{2+} from the SR, resulting in a faster release of Ca^{2+}. The rapid swelling of mitochondria is also coupled with a release of mitochondrial Mg^{2+} (Schönfeld *et al.*, 2003). Continual exposure of the SR to enhanced levels of long chain fatty acids may result in damage to the Ca^{2+} channels and increase the permeability of these channels. The excess Ca^{2+} is responsible for muscle hyper-rigidity and an enhanced rate of glycolysis, resulting in a rapid rate of lactic acid production and a low pH in MH muscle (Cheah and Cheah, 1985b).

Excess production of free fatty acids may be due to hormone sensitive lipase (HSL) which has been mapped to 19q13.1 or PLA_2. HSL is important for mobilisation of free fatty acids from stored triglycerides and is regulated by both hormonal and neuronal factors (Frayn *et al.*, 1995). Lipase is activated by catecholamines via cyclic adenosine monophosphate (AMP)-mediated phosphorylation and inactivated by dephosphorylation, which is mediated by insulin. Therefore, it was suggested that HSL was a candidate for MHS, as excess free fatty acids, due to elevated catecholamines or absence of insulin inactivation, would result in a disruption of muscle metabolism (Levitt *et al.*, 1990). Cheah *et al.* (1989) reported that the endogenous activity of PLA_2 was higher in MH pigs compared to unaffected pigs and this could be responsible for the uncoupling of oxidative phosphorylation from electron transport. Activation of this enzyme results in the formation of lyso-derivatives and liberation of long-chain free fatty acids. The long-chain fatty acids and/or the PLA_2 alter the function of the SR by inactivating the Ca^{2+}-transport system, which results in additional Ca^{2+} being released.

The involvement of mitochondria in MH has been reported in other studies. Rasmussen *et al.* (1996) indicated that MH pig skeletal muscle mitochondria had significantly higher extramitochondrial nicotinamide adenine dinucleotide (exo-NADH) oxidase activity compared to MHN animals. The exo-NADH is localised to the outer surface of the inner mitochondrial membrane (IMM) and a higher activity of this enzyme may sustain

accelerated glycolysis via re-oxidising the cytosolic NADH. This observation could be due to the effect of Ca^{2+} on the transcription factors or on the protein of the exo-NADH oxidase. Cheah *et al.* (1989) demonstrated that the respiratory properties of unaffected and MHS mitochondria were identical. However, the addition of large amounts of Ca^{2+} in MHS mitochondria led to uncoupling, which could be prevented by the presence of serum albumin. Wrogemann and Pena (1976) suggested that Ca^{2+} overload due to a mutation in the RYR1 would strain Ca^{2+} removal systems, which would lead to mitochondrial participation in this process. Compensation by mitochondria will result in their impairment and eventual loss from the cell. The biochemical characteristics of skeletal muscle mitochondria of MH susceptible Dutch Landrace pigs have been investigated before and during an MH attack induced *in vivo* by halothane and succinylcholine. The muscle homogenates have a decreased capacity to synthesise ATP and creatine phosphate during the MH period. Muscle mitochondria prepared from susceptible pigs in an MH period consume less O_2 than mitochondria isolated before the attack, or mitochondria from control pigs during the challenge. The restricted synthesis may be caused by a factor, finding expression in the mitochondria themselves, and obtained or activated during the MH attack (Ruitenbeek *et al.*, 1984).

2.10.2 Abnormalities of mitochondria and muscle fibre types

Various abnormalities of mitochondrial function have been reported in susceptible pigs or humans. In addition, skeletal muscle contraction characteristics differ in MHS muscle compared to unaffected muscle. Mammalian skeletal muscle fibres contain many mitochondria that are located mostly in the periphery and are responsible for 10% of the fibres' volume. In MHS muscle, a faster contraction and an increase in temperature were observed when the muscle was warmed (Bäckman *et al.*, 1988). Lesions such as hypertrophy, atrophy, necrosis of fibres and internal nuclei have also been observed more frequently in MHS and MHE muscle biopsies (Mezin *et al.*, 1997). Somers and McLoughlin (1982) reported that the loss of high energy phosphates and accumulation of lactate was higher in predominately red (small) fibre areas than white (large) fibre areas. The authors also observed that the red myofibre area was more susceptible to the actions of halothane and exhibited a greater reduction in Ca^{2+} accumulating ability than those of white fibres. In addition, white skeletal muscles display few mitochondria, have rapid contraction of short duration and obtain ATP primarily from glycolysis. Red muscle is rich in mitochondria and obtains ATP from oxidative metabolism and insulin-stimulated glucose transport. In addition, red fibres possess a higher oxidative enzyme content and white fibres display a

higher phosphorylase content due to the reciprocal nature of oxidative enzymes and phosphorylase (Dubowitz and Pearse, 1960).

2.10.3 The mitochondrial calcium uniporter

Uptake of Ca^{2+} into the mitochondrial matrix occurs via a Ca^{2+} uniporter located throughout the inner membrane of the mitochondria, indicating that mitochondria sequester significant amounts of Ca^{2+}. The mechanisms of Ca^{2+} uptake have been characterised kinetically and pharmacologically. The uniporter is a highly selective Ca^{2+} ion channel, even in the presence of a low cytoplasmic concentration of Ca^{2+}, and binds Ca^{2+} with extremely high affinity (Kirichok *et al.*, 2004). The channel preferentially transports Ca^{2+}, but has relative divalent ion conductance of Ca^{2+} = strontium (Sr^{2+}) > manganese (Mn^{2+}) = barium (Ba^{2+}). The uniporter displays co-operativity as it has two binding sites for Ca^{2+}, a low affinity transport site as well as a higher affinity allosteric activation site located on the extra-luminal segment. Ca^{2+} is able to bind to the activation site and initiates Ca^{2+} transport into the mitochondria, while removal of Ca^{2+} from the activation site results in blocking of Ca^{2+} transport. The primary driving force is the high negative membrane potential, in addition to a Ca^{2+} gradient. Neighbouring RyRs release Ca^{2+}, creating Ca^{2+} microdomains that are expected to contribute to the activation of the uniporter, resulting in the driving force for Ca^{2+} uptake. Although the microdomains have a short lifetime, the uniporter in cell-free systems displays a relatively slow deactivation, a mechanism that could keep the uniporter in a sensitised state. Although the activity of uniporter transport has been characterised functionally, the molecular identity has not yet been determined. Studies have suggested that the molecular identity of the Ca^{2+} uniporter may either be an antiporter or a RyR (Beutner *et al.*, 2001).

2.10.4 The mitochondrial ryanodine receptor

Beutner *et al.* (2001) indicated that in the IMM of heart muscle, isolated from rats, a mitochondrial RyR (mRyR) exists, with an estimated molecular mass of 600 kDa. The mRyR was detected to share several biochemical, physiological and pharmacological properties with SR-RyR of skeletal muscle, and is therefore termed mRyR (Beutner *et al.*, 2001). Further analysis using subtype-specific antibodies identified the mRyR in rat heart mitochondria as mRyR1 (Beutner *et al.*, 2005).

Ryanodine bound to mRyR1 with affinity (K_d) of 9.8 Newton metre (Nm), compared to 2-200 Nm observed for SR-RyR. The density (Bmax) of mRyR binding was 398.4 femtomol per milligram ($fmol.mg^{-1}$) of protein and was ten times less than ryanodine binding in SR-RyR. Mg^{2+} reduced the open probability of the channel and ruthenium red and dantrolene inhibited the channel. In addition, dantrolene prevented mitochondrial swelling. The mRyR1 was insensitive to caffeine, unlike SR-RyR. However, the authors indicated that caffeine-insensitive RyR channels have been described in canine salivary glands, mink lung epithelial cells (Giannini *et al.*, 1992) as well as in human Jurkat T-cells. Murayama and Ogawa (1997) indicated that there is likely to be different spliced variants of the RyR, some of which may be caffeine-insensitive. Targeting of the mRyR to the IMM might result in a change in structure of the targeted regions, which could alter the function of that region of the channel.

Several splice variants of the RyR have been reported in a variety of species. Chugun *et al.* (2003) observed that two commercially available monoclonal anti-RyR antibodies were unable to identify the RyR from carp heart. However, an anti-RyR raised against a synthetic peptide was able to localise a RyR to the carp heart. The authors suggested that the heart RyR has an epitope or displays a conformation that is distinct from the skeletal muscle RyR. Jiang *et al.* (2003) indicated that RyR3 is extensively modified via alternative splicing in smooth muscle tissue, which may account for the many functional heterogeneities of the RyR3. One spliced variant is only functional when it forms a heteromeric channel with wildtype RyR3 or RyR2 and is able to interact and suppress the activity of RyR2.

Beutner *et al.* (2001) also localised the RyR to brain mitochondria, but not liver mitochondria, and suggested that RyR may only be present in mitochondria of excitable cells. The authors determined that the mitochondrial Ca^{2+} uniporter and the mRyR were not the same, as the Ca^{2+} uniporter has a low affinity and high capacity for mitochondrial Ca^{2+} uptake and may play a role in preventing cytosolic Ca^{2+} overload in order to reduce the possibility of necrotic and apoptotic cell death. On the other hand, the mRyR1 is activated by low Ca^{2+} concentrations and inactivated by high Ca^{2+} concentrations, indicating that the channel is the fast Ca^{2+} transport receptor in mitochondria that sequesters Ca^{2+} during E-C coupling. The Ca^{2+} taken up by the mitochondria via the mRyR1 is used to stimulate the mitochondrial oxidative phosphorylation required for ATP production and in this way regulates energy generation (Beutner *et al.*, 2005).

2.11 MOLECULAR GENETICS OF MH

The primary goal of genetic research into MH is the development of an accurate, less invasive diagnostic test for MH. Prior identification of MHS patients before surgery could prevent the development of an MH episode as known MH-triggering anaesthesia could be avoided and dantrolene would be available and be administered if MH is triggered.

2.11.1 The porcine animal model

The pig became a useful model to study the diagnosis and treatment of human MH syndrome. Hall *et al.* (1966) first described an unusual reaction in pigs, which was similar to the MH phenotype encountered in MHS humans when exposed to a muscle relaxant, succinylcholine. The syndrome in pigs is known as PSS. Harrison *et al.* (1969) reported that 25% of Landrace pigs in their study were diagnosed with PSS. The phenotype of lean, heavy-muscled swine is observed, and an MH-like crisis leads to rapid breathing (tachypnoea), rapid increase in body temperature, flushing of skin, collapse, muscle rigour, acidosis and tachycardia followed by rapid death (Ball and Johnson, 1993). Psychological stress, including exercise, transportation and fear, exposure to high temperature and anaesthetics are all triggers of PSS (Patterson and Allen, 1972).

The porcine animal model has been used to improve diagnostic methods in muscle disease of pigs (due to major financial implications for commercial breeders), and to provide insight into similar syndromes that occur in many different species (Mitchell and Heffron, 1982). In human studies, porcine models have provided information on the physiology of muscle contraction and metabolism. Porcine animal models have enhanced understanding of the major underlying defect of the disorder and have contributed to identifying agents used in clinical diagnosis and therapeutic strategies. The gene responsible for porcine MH was determined to be due to an alteration in the halothane (HAL) gene or PSS gene. The HAL locus, a linkage group spanning approximately 12 cM, was mapped to the pig chromosome 6p11-q21. The locus of the HAL gene is closely linked to the GPI locus (Davies *et al.*, 1988). Harbitz *et al.* (1990) later established that PSS in pigs was associated with the RYR1 gene expressed in animals (*ryr*1) on chromosome 6q12. The porcine gene, *ryr*1, on chromosome 6 is syntenic to RYR1 observed on chromosome 19q in humans (McCarthy *et al.*, 1990) and within the coding region demonstrates 85 - 95% homology to both the human and rabbit *ryr*1 (Brenig and Brem, 1992). MacLennan *et al.* (1990) indicated that MH is most likely due to alterations in

the RYR1 gene, based on linkage analysis in humans with MH and data obtained from the pig animal model. A causative point mutation within the *ryr*1 gene, namely Arg615Cys, that results from a nucleotide transition, namely cytosine (C)1843 thymine (T), was identified in five major breeds of pigs (Fujii *et al.*, 1991), suggesting that the alteration had a common origin. The alteration eliminates an accessible trypsin cleavage site at residue 615, which may result in altered Ca^{2+} regulation of Ca^{2+} release, channel gating and ryanodine binding activity (Mickelson *et al.*, 1992). The above-mentioned alteration correlates to the C1850T alteration in the human RYR1 gene, which has been determined in 2 - 7% of MH families (Fletcher *et al.*, 1995).

The condition in pigs was thought to be transmitted in an autosomal recessive manner (Davies *et al.*, 1988). This was in agreement with a study conducted by Fletcher *et al.* (1993), where it was observed that two mutant alleles were necessary to evoke the porcine MH phenotype. However, in 5 - 10% of swine, homozygosity of the recessive alleles is not sufficient for MH susceptibility, even following exposure to halothane and succinylcholine. The observed incomplete penetrance in pigs has also been observed in human MH, where significant variation among families is often noted. It is not known whether this is due to the presence of a currently unreported alteration or a confluence of other co-factors. A modulating factor may be required for the full expression of the MH syndrome. The use of swine as animal models of MH could in the future provide knowledge and insight about the modulating factor. The presence of such a factor may also be responsible for the variability observed in the human MH syndrome.

2.11.2 Other animal models

The list of animals susceptible to MH has expanded since it was first discovered in pigs. The incidence of MH in animals is unknown and may be viewed as low due to the absence of documented case reports. This may be an underestimation due to lack of recognition of the disorder, missed diagnosis because of the absence of temperature readings and end expiratory gas monitoring, as well as lack of exposure to triggering anaesthetics. During veterinary anaesthesia, an MH reaction has been reported in a cat (De Jong *et al.*, 1974), in horses (Klein, 1975; Aleman *et al.*, 2004), in outbred strains of rat (Gonzalez *et al.*, 1998) and in dogs (Nelson *et al.*, 1991). In horses, the Arg2454glycine (Gly) in exon 46 of the *ryr1* was observed to be associated with MH. The authors indicated that horses with this alteration displayed a higher affinity and density for ryanodine binding, but no differences in Ca^{2+}, Mg^{2+}, and caffeine modulation were

observed (Aleman *et al.*, 2004). Citino *et al.* (1984) reported a suspected fatal MH episode that occurred during capture of a giraffe at a zoo. The 10-year-old female giraffe's temperature rose to 41.1°C and the animal experienced metabolic acidosis. Analysis of serum chemical values collected indicated an increase in glucose, lactate dehydrogenase, aspartate transaminase, CK and cortisol. Necropsy on the animal identified haemorrhage, multifocal myocardial fibrosis, mild skeletal muscle atrophy and unilateral pulmonary congestion (Citino *et al.*, 1984).

Currently the Dog Genome Project is under way to identify mutations resulting in susceptibility to MH in a breeding colony of dogs. The canine equivalent of the pig *ryr1* mutation was not observed in the dogs with MH. However, a T1640C alteration, which results in a Val547 alanine (Ala) substitution, has been reported in mixed-breed dogs in exon 15 of the *ryr1* (Roberts *et al.*, 2001b). Canine MH syndrome shares many clinical features of MH with the human and pig syndromes. However, lactic acidaemia, metabolic acidosis and rigidity are absent or delayed in most cases. The absence of these symptoms could be due to the superior canine oxidative metabolic capacity and cardiac output or to the absence of more highly glycolytic type IIB fibres. Inheritance of canine MH is transmitted as an autosomal dominant trait (Roberts *et al.*, 2001b).

2.11.3 Identification of the RYR1 gene as a locus for MH

Initial molecular genetic studies identified the gene responsible for porcine MH, as discussed in Section 2.11.1 (page 40). Studies on the porcine model suggested that a mutation in the RYR1 gene was responsible for MH in humans as well. Using polymorphic microsatellite markers, the MH locus in humans was mapped to a region on chromosome 19q12-13.2 (McCarthy *et al.*, 1990). The region was subsequently identified as containing the RYR1 gene, which was localised on chromosome 19q13.1 and was suggested as a candidate gene for MH (MacKenzie *et al.*, 1990). In addition to the RYR1, the genes for apolipoproteins CI, CII and E and glucose-6-phosphate isomerase reside on the long arm of chromosome 19 and low density lipoprotein receptor, complement component 3 and peptidase D reside on the short arm (Lusis *et al.*, 1986). To date the RYR1 gene has been associated with an MH phenotype in approximately 50% of MH families (Jurkat-Rott *et al.*, 2000).

2.11.3.1 Structural organisation of the RYR1 gene

The RYR1 gene encompasses 158,000 bp of genomic DNA (gDNA) and consists of 106 exons. The RYR1 gene maps to chromosome 19q13.2 and encodes a protein of 5038 amino acids (Zorzato *et al.*, 1990). The first alternatively spliced sequence occurs within exon 70 (15 bp) encoding a five amino acid insertion, Ala-Gly-aspartate (Asp)-Ala-glutamine (Gln) and the second is observed in exon 83 (18 bp) which encodes a six amino acid insertion (Phillips *et al.*, 1996). The amino acid sequences exist in the modulatory region of the RyR1 and contain sites for phosphorylation as well as binding sites for Ca^{2+}, CaM and ATP (Futatsugi *et al.*, 1995). Alternative splicing in this region generates molecular heterogeneity and alters the regulatory function of this channel. The two mRNA RYR1 transcripts produce molecules of 5032 and 5037 residues, which are hypothesised to co-exist at all stages of development and in varying muscle types. However, Futatsugi *et al.* (1995) suggested that the alternatively spliced RyR isoforms are tissue-specific and function in a developmentally regulated manner.

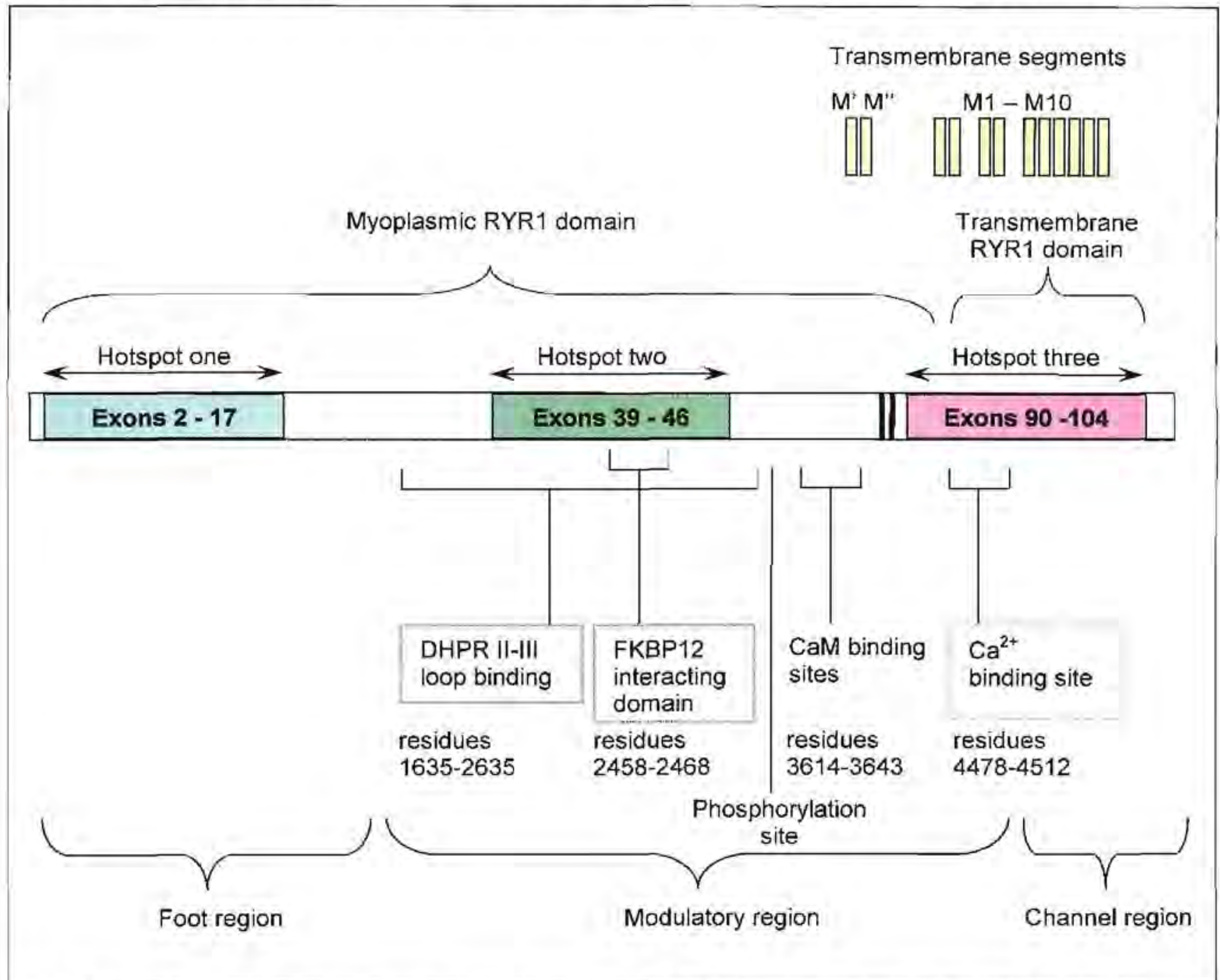
MacLennan *et al.* (1989) and Zorzato *et al.* (1990) cloned the cDNA of rabbit and human RYR1. The authors indicated that the human RYR1 cDNA encodes a protein of 5032 amino acids with a molecular mass of 563.5 kDa, the protein is encoded without an N-terminal signal sequence. The length of human RyR1 exons ranges from 15 to 813 bp and the length of introns ranges from 85 to 16,000 bp. The transcription start site sequence of the 5' untranslated region (UTR) was determined to be a T residue, which lies 130 bp upstream of the initiator codon methionine. The proposed promoter site consists of a poorly conserved CCAAT box, at position -198. The promoter site does not contain a single consensus TATA box. However, a cluster of five TATA-box sequences were identified at -1,500 bp. The 3' UTR sequence is 146 bp in length and ends in an AAAATAAA polyadenylation signal. Analysis of homology of the RYR1 sequence identified RYR genes expressed in other tissues and species and sequence identity with *Alu* repeat elements, which represent 6 - 8% of the human genome.

2.11.3.2 Allelic variants in the RYR1 gene and their association with MH

Worldwide, more than 100 RYR1 mutations have been identified in patients diagnosed with either MH or CCD (Halsall and Robinson, 2004). Most RYR1 mutations discovered for MH and CCD are missense and are distributed in three distinct areas or hotspots of the gene, ranging from exons 2 to 17 (hotspot one), exons 39 to 46 (hotspot two) and exons

90 to 104 (hotspot three). An illustration of the three mutational hotspots of the RYR1 gene is presented in Figure 2.2, which demonstrates binding sites, regions and domains that occur in this gene.

Figure 2.2: Localisation of RYR1 hotspots, binding sites, regions and domains



Black squares indicate position of two alternatively spliced exons 70 and 83; RYR1 = skeletal muscle ryanodine receptor type one; CaM = calmodulin; Ca^{2+}= calcium ion; DHPR = dihydropyridine receptor; FKBP12 = immunophilin (cytosolic receptor) FK506-binding protein; M = transmembrane segments. Transmembrane segments are numbered according to the earlier model proposed by Zorzato *et al.* (1990). High affinity Ca^{2+} binding sites occur within residues 4478-4512; residues which determine the affinity of the activation site occur within residues 4254-4632; low affinity Ca^{2+} and Mg^{2+} binding sites occur within residues 1864-1925 and 4187-4381. The DHPR binding site occurs within residues 1076-1112 and 1635-2635; the CaM binding site occurs within residues 3614-3643 and the FKBP12 binding site occurs within residues 2458-2468. Adapted from Dulhunty and Pouliquin (2003); Sambuughin *et al.* (2005).

The number of transmembrane segments indicated in this diagram is based on the first published popular model that suggests that there are 12 transmembrane sequences (Zorzato *et al.*, 1990). However, Du *et al.* (2002) indicated the M', M'', M1, M2 and M3 sequences were not membrane-associated and the RyR1 contains eight transmembrane helices organised in four hairpin loops. The first loop may either be made up of M4a-b or M3-M4, while other hairpin loops include M5-M6, M7a-M7b and M8-M10, with M9 forming

a selectivity filter between M8 and M10. However, the number of membrane-spanning segments is still undetermined due to the misleading nature of hydropathy profiles. Currently, several models exist that vary in the number of predicted membrane-spanning sequences from four to 12 (Takeshima *et al.*, 1989; Zorzato *et al.*, 1990; Tunwell *et al.*, 1996; Du *et al.*, 2002).

The first region encodes the N-terminus of the protein that forms the myoplasmic foot (Jurkat-Rott *et al.*, 2000), the second hotspot forms part of the central region of the RYR1, and the third hotspot has been observed in the C-terminal region of the RYR1 and contains all putative transmembrane segments, which form the pore domain (Lynch *et al.*, 1999). The remainder of the protein is hydrophilic and presumably constitutes the cytoplasmic domain.

Yamamoto *et al.* (2000) suggested that regions of hotspots one and two are able to interact with each other and that the interaction of the three-dimensional structures of the folded tetramer can influence the stability of the closed as well as the open state of the RyR1. As these domains are able to interact and stabilise a closed conformation of the channel, a mutation within any of these domains would thus destabilise the channel and cause it to be leaky to Ca^{2+} and be sensitive to agonists of RyR1 (Kobayashi *et al.*, 2004).

The third hotspot plays a role in the functioning of the permeation selectivity and gating of the channel. All three hotpots of the RYR1 gene are conserved between the three RyR isoforms and display homology to similar regions of other intracellular Ca^{2+} release channels such as the $InsP_3R$. The high degree of integrity of these regions suggests that they play an important role in the functioning of the Ca^{2+} release channels.

Most of the reported RYR1 gene mutations are missense (Halsall and Robinson, 2004). Evidence to support that a missense mutation is causative and alters protein function is based on the following criteria as outlined by the EMHG published in Urwyler *et al.* (2001):

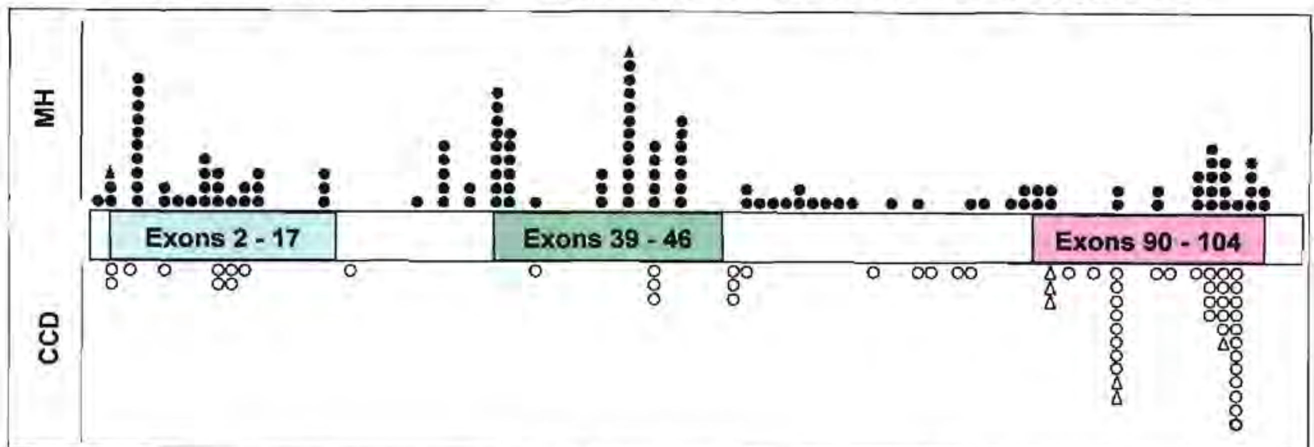
a. The mutation should segregate with the disease phenotype.
b. The mutation should not segregate in 100 unaffected controls.
c. The position of the affected amino acid in the protein should be detected at a functionally relevant site or at an evolutionary conserved site across species.
d. The pathogenicity of a missense mutation should be carefully considered. The effect of a substitution is minimal if the side chain of the replacing amino acid is similar to the original (conservative substitution). However, replacements with side chains that are different (non-conservative substitutions) are more likely to affect the function of the protein.
e. Characterisation of the mutated protein in an appropriate assay should indicate altered function.

2.11.3.3 Specific mutations within the RYR1 gene

In the study presented here, all 106 exons of the RYR1 gene were sequenced in order to screen for reported and novel alterations that may occur in the coding region of this gene. In the past, studies have focused on screening for MH mutations in the three hotspots of the RYR1 gene. However, recent studies have identified alterations outside these mutational hotspots. Screening all the exons in the RYR1 gene would provide a more accurate determination of the frequency of alterations in this gene. Using this approach has led to a mutation detection rate of 60% in mainly French MHS families (Monnier *et al.*, 2005) and 70% in North American families (Sambuughin *et al.*, 2005).

The locations of the RYR1 alterations that have been observed in MHS and CCD patients from Australia, Europe and North America are indicated in Figure 2.3. Thus far, 178 missense alterations have been identified, of which 72 alterations occur in several families and 106 alterations are family specific. In addition, seven alterations involving deletion of one or more nucleotides has been reported associated with the MH phenotype. In contrast, 60 alterations have been identified in the coding region of the RYR1 gene that are not associated with MH (Robinson *et al.*, 2006). Alterations that are observed in the mutational hotspots as well as those that occur outside these regions that have been determined to be associated with MH are discussed in subsequent paragraphs of this chapter.

Figure 2.3: Localisation of RYR1 alterations observed in MH or CCD patients



Nucleotide substitutions indicated above the figure as solid black circles (•) were observed in malignant hyperthermia susceptible individuals; deletions indicated above the figure as solid black triangles (▲) were observed in malignant hyperthermia susceptible individuals; nucleotide substitutions indicated below the figure as white circles (○) were observed in individuals with CCD; deletions indicated below the figure as white triangles (Δ) were observed in individuals with CCD. The three mutational hotspots are indicated as blue, green and pink blocks, respectively. Adapted from Wu *et al.* (2006).

2.11.3.3.1 Mutation hotspot one of the RYR1 gene

Hotspot one of the RYR1 encompasses the N-terminal cytoplasmic domain and harbours several regulatory binding sites. Experiments conducted on skinned muscle fibres have indicated that proteolytic cleavage of this domain results in increased CICR, indicating that this region is important for Ca^{2+} dependent regulation of RyR1 (Hayek *et al.*, 2000). Reported alterations in hotspot one of the N-terminal domain of RYR1 that may result in susceptibility to MH and CCD are listed in Table 2.4.

Table 2.4: Reported alterations within hotspot one of the RYR1 gene observed in MH, CCD or MmD patients

Nucleotide change	Amino acid	Exon	Substitution frequency	Phenotype	Reference
T103C*	Cys35Arg[1]	2	1 family	MH	Lynch *et al.*, 1997
G131A	Arg44His	2	1 family	MH	Halsall and Robinson, 2004
G178A	Asp60Asn	3	1 family	CCD	Wu *et al.*, 2006
C212A	Ser71Tyr	3	1 family	CCD/MmD	Zhou *et al.*, 2005
C325T	Arg109Trp	4	1 family	CCD/MmD	Zhou *et al.*, 2005
C463A	Gln155Lys	6	1 family	MH	Ibarra *et al.*, 2006
A467G	Arg156Lys	6	1 family	MH	Galli *et al.*, 2006
A479G	Glu160Gly	6	1 family	MH	Halsall and Robinson, 2004
C487T*	Arg163Cys[1,2,3]	6	2%-3%	MH/CCD	Quane *et al.*, 1993
G488T*	Arg163Leu	6	1 family	MH	Halsall and Robinson, 2004
G493A	Gly165Arg	6	1 family	MH	Monnier *et al.*, 2005
G496A	Asp166Asn	6	1 family	MH	Rueffert *et al.*, 2002
A497G	Asp166Gly	6	1 family	MH	Ibarra *et al.*, 2006
C529T	Arg177Cys	6	1 family	MH	Monnier *et al.*, 2005

Table 2.4: Continued...

Nucleotide change	Amino acid	Exon	Substitution frequency	Phenotype	Reference
A533T	Tyr178Cys	6	1 family	MH	Monnier *et al.*, 2005
G644A	Gly215Glu	8	1 family	CCD	Romero *et al.*, 2003
G652A	Val218Ile	8	1 family	MH	Ibarra *et al.*, 2006
A680T	Asp227Val	8	1 family	MH	Monnier *et al.*, 2005
G742A*	Gly248Arg[1,2,3]	9	1 family	MH	Gillard *et al.*, 1992
G947T	Arg316Leu	10	1 family	MH	Ibarra *et al.*, 2006
C982T	Arg328Trp	11	1 family	MH	Loke *et al.*, 2003
G1021A*	Gly341Arg[1,2,3]	11	10%	MH	Quane *et al.*, 1994a
G1021C	Gly341Arg	11	1 family	MH	Monnier *et al.*, 2005
G1100A	Arg367Gln	11	1 family	MH	Galli *et al.*, 2006
C1201T	Arg401Cys	12	3 families	MH	Davis *et al.*, 2002
C1201A	Arg401Ser	12	1 family	MH	Monnier *et al.*, 2005
G1202A	Arg401His	12	2 families	MH	Rueffert *et al.*, 2002
G1206C	Met402Ile	12	1 family	CCD/MmD	Zhou *et al.*, 2005
C1209G*	Ile403Met	12	1 family	CCD	Quane *et al.*, 1993
C1280T	Ser427Leu	13	1 family	CCD	Wu *et al.*, 2006
C1411T	Arg471Cys	13	1 family	MH	Gillard *et al.*, 1992
G1422T	Gln474His	13	1 family	CCD	Wu *et al.*, 2006
G1534A	Glu512Lys	14	1 family	CCD	Wu *et al.*, 2006
A1565C*	Tyr522Ser[1]	14	1 family	MH/CCD	Quane *et al.*, 1994a
A1565G	Tyr522Cys	14	1 family	MH	Yeh *et al.*, 2005
C1597T	Arg533Cys	15	1 family	MH	Tammaro *et al.*, 2003
G1598A	Arg533His	15	1 family	MH	Brandt *et al.*, 1999
C1654T*	Arg552Trp	15	1 family	MH	Keating *et al.*, 1997
C1840T*	Arg614Cys[1,2,3]	17	4%	MH	Gillard *et al.*, 1991
G1841T*	Arg614Leu[1]	17	2%	MH	Quane *et al.*, 1997
51_53delTGA	Asp17del	2	1 family	MH	Ibarra *et al.*, 2006

[1, 2] = mutations currently being used in the genetic diagnosis of MHS in European countries (Urwyler *et al.*, 2001; Halsall and Robinson, 2004); [3] = mutations currently being used in the genetic diagnosis of MHS in American countries (Sei *et al.*, 2004). An asterisk (*) indicates a causative mutation. MH = malignant hyperthermia; CCD = central core disease; MmD = multi-minicore disease; del = deletion.

Lynch *et al.* (1997) identified a T103C transition that occurred in exon two of the RYR1 gene that resulted in a Cys35Arg substitution. The alteration appears to be restricted to one large consanguineous MHS kindred. The substitution was not detected among 65 unrelated MHS samples or in 200 unaffected chromosomes. The proband of the family is of Sicilian origin and was identified as the first reported homozygote for a mutation in the RYR1 gene. In one of 434 United Kingdom (UK) families with MH, Halsall and Robinson (2004) identified an Arg44His change in the RYR1 gene that is due to a guanine (G)131 adenine (A) transition. In addition, Halsall and Robinson (2004) reported two alterations, Glu160Gly and Arg163 leucine (Leu), each in one UK family. The Glu160Gly alteration is due to an A479G transition and the Arg163Leu alteration is due to a G488T substitution.

An Asp17 deletion (del) was observed in one MH family from Japan. The alteration arises from a 51-53delTGA and was not observed in control individuals (Ibarra *et al.*, 2006).

A Gln155Lys alteration that occurs due to a C463A nucleotide transition was observed in a single MHS family of Japanese origin. The alteration was conserved among RYR1 isoforms and was therefore considered pathogenic (Ibarra *et al.*, 2006). Galli *et al.* (2006) identified an Arg156Lys in one individual with MH from Italy. The alteration was not observed in 100 alleles in the general population and occurred in a highly conserved region of the RYR1. The Arg163Cys alteration was identified in a single MH pedigree from Denmark (Fagerlund *et al.*, 1994) and a CCD pedigree from Italy (Quane *et al.*, 1993). It occurred as a result of a single base change, C487T. Segregation analysis conducted by Quane *et al.* (1993) revealed that the mutation was present in the affected CCD parent and three siblings. In addition, an individual from this pedigree that was not diagnosed with CCD, suffered a clinical episode of MH. Further analysis identified the mutation in two unrelated MH patients that showed no signs of central cores. The authors suggested that an alteration in the RYR1 gene resulted in both hypersensitive gating and diminished E-C coupling and could result in both MH and CCD. In addition, an Arg163Leu alteration was observed in one MH family of European origin. The alteration occurred due to a G488T nucleotide transition (Halsall and Robinson, 2004).

A Gly165Arg alteration was detected in one French MHS family and occurred due to a G493A nucleotide transition (Monnier *et al.*, 2005). The alteration was conserved among RYR species and isoforms and was not identified in 100 chromosomes from the general population. An Asp166Gly alteration was observed in one Japanese MH family and occurred due to an A497G nucleotide transition. The proband was observed to carry two alterations in the RYR1, the Asp166Gly on exon 6 and the Arg2163His alteration on exon 39 (Ibarra *et al.*, 2006). An Asp166 asparagine (Asn) alteration due to a G496A substitution was detected in one MH proband from Germany that was diagnosed as MHS via an IVCT (Rueffert *et al.*, 2002). Monnier *et al.* (2005) reported alterations Arg177Cys and tyrosine (Tyr) 178Cys in exon 6 that occurred due to nucleotide transitions C529T and A533T respectively, each in one French MHS family. The alterations both affect amino acids that are conserved among species and RYR isoforms. Both substitutions occurred as neomutations, as they share common haplotypes with unaffected siblings that do not harbour the mutation.

A Val218Ile alteration was observed in a single Japanese family diagnosed with MH. It occurred due to a G652A nucleotide transition and was conserved among RYR isoforms (Ibarra *et al.*, 2006). An Asp227Val alteration due to an A680T nucleotide substitution was reported to segregate with the MH phenotype in one small French MH family (Monnier *et al.*, 2005). The Gly248Arg alteration was detected in one out of 45 MH families from Canada. This mutation was due to a G742A transition and was observed in three MHS individuals in the single pedigree (Gillard *et al.*, 1992). Ibarra *et al.* (2006) reported an Arg316Leu alteration in one MHS family of Japanese origin. The alteration was due to a G947T nucleotide transition. Loke *et al.* (2003) described a novel alteration termed Arg328 tryptophan (Trp) that was observed to be restricted to one MHS individual in a Canadian pedigree. The mutation was not observed in 190 unaffected chromosomes and was identified in a region of the protein that is conserved across the species. The presence of the alteration resulted in a channel that is more sensitive to caffeine and halothane, indicating its causative status.

The Gly341Arg alteration occurs due to a G1021A transition and is observed in approximately 10% of Caucasian MHS cases. The alteration has not been detected in 500 unaffected chromosomes and is considered causative (Quane *et al.*, 1994a). The Gly341Arg alteration was observed in 15 unrelated MHS families from the UK, and is the most frequently occurring alteration that has been described for European populations (Halsall and Robinson, 2004). The alteration expressed in myotubes resulted in the RyR1 protein being more sensitive to stimulation by caffeine and is able to induce the MH phenotype. Monsieurs *et al.* (1998) reported a possible association of the Gly341Arg alteration to elevated serum CK activity, due to loss of integrity of the sarcolemma membrane, which may result in increased levels of this protein. The alteration has also been detected in one Scandinavian MH family (Fagerlund *et al.*, 1996) but was not observed in several North American MH families studied (Stewart *et al.*, 1998). It was also not observed to co-segregate with MHS in one British pedigree (Adeokun *et al.*, 1997). Monnier *et al.* (2005) observed that the Gly341Arg alteration could also result from another base change, namely G1021C. The substitution would be undetected by the restriction fragment length polymorphism (RFLP) analysis used to screen for the G1021A alteration. In one MH individual from Italy, Galli *et al.* (2006) detected an Arg367Gln alteration that occurred due to a G1100A nucleotide transition. The alteration was not observed in 100 chromosomes, was conserved in RYR through evolution and was also conserved across the three known RYR genes.

A C1201T nucleotide substitution that resulted in an Arg401Cys change was identified in three New Zealand Maori pedigrees and was not detected in 200 unrelated controls of Maori and Caucasian descent. The alteration is strictly conserved among sequences of RYR isoforms and is therefore considered causative of MH (Davis *et al.*, 2002). An Arg401His alteration due to a G1202A transition was observed in two MH probands from Germany (Rueffert *et al.*, 2002). In addition, Monnier *et al.* (2005) reported an Arg401Ser alteration due to a C1201A nucleotide transition in one MHS family from France. The alteration affects a conserved arginyl residue and the variant alters amino acids that are conserved both among RYR1 species and isoforms. An Arg401His alteration due to a G1202A nucleotide transition has also been reported in two MHS families (Rueffert *et al.*, 2002). The Ile403Met alteration that is due to a C1209G transition is a functionally significant alteration that is associated with a higher resting cytoplasmic Ca^{2+} level and increased sensitivity to caffeine and halothane (Tong *et al.*, 1997). The alteration was detected in one CCD pedigree of Italian descent and was not detected in 20 unrelated CCD patients, 102 unrelated MHS patients and 12 MHE patients investigated (Quane *et al.*, 1993). Gillard *et al.* (1992) reported an Arg471Cys alteration in one MHS family. The alteration was observed in the proband and her father. However, it was not observed in her brother and mother.

A Thr522Ser alteration was detected in a pedigree of French descent. Individuals of this family were diagnosed as MHS via the IVCT and several individuals displayed single or multiple cores in the muscle biopsies. The alteration occurred due to an A1565C transition and was not detected in the unaffected population. The transition is conserved across the RYR1 species (Quane *et al.*, 1994a). In addition, Yeh *et al.* (2005) observed a Tyr522Cys alteration due to an A1565G nucleotide transition in one MHS family. A single novel alteration, Arg533His, which occurred due to a G1598A nucleotide transition, has been reported in a single MH proband (Brandt *et al.*, 1999). In addition, Tammaro *et al.* (2003) described an Arg533Cys alteration due to a C1597T transition in one MHS family. The mutation co-segregated with the IVCT phenotype and was not observed in 100 control samples. It was conserved in all known vertebrate RYR1 genes. The Arg552Trp alteration has been identified in an MHS pedigree of Irish descent. The C1654T substitution gives rise to this alteration and was not detected in 97 unrelated MHS individuals and four CCD patients. Considerable variation in the IVCT responses was observed in this pedigree, which could not be attributed to the presence of a mutant or unaffected RYR1 allele (Keating *et al.*, 1997).

The Arg614Cys alteration due to a C1840T transition was detected in three generations of a family with MH (Moroni *et al.*, 1995), in two North German families with MH (Steinfath *et al.*, 1995), in an individual of Mennonite descent (Serfas *et al.*, 1996) and in an MH family of Northern European descent (Hogan *et al.*, 1992). A homozygous alteration was detected in one MH proband of German descent whose parents were heterozygous for this alteration. A stronger correlation between greater contracture results and the likelihood of MH was determined via the clinical grading scale with the presence of homozygosity (Rueffert *et al.*, 2001). In addition, the alteration was not detected in 100 MH families of European descent (Hall-Curran *et al.*, 1993). The presence of the Arg614Cys alteration results in abnormal cytosolic Ca^{2+} response to 4-chloro-*m*-cresol and is therefore sufficient to alter the intracellular Ca^{2+} homeostasis (Treves *et al.*, 1994). Quane *et al.* (1997) identified an Arg614Leu alteration due to a G1841T substitution in three unrelated MHS individuals out of 151 patients investigated. The alteration segregated with MHS in the families studied and was conserved between different species.

2.11.3.3.2 Mutation hotspot two of the RYR1 gene

The central region of the RYR1 gene in combination with the N-terminal domain forms the putative channel regulatory domain and both regions display similar properties of hyper-activation and hyper-sensitivity. The two domains interact, which is a process that is involved in the regulation of channel function (Kobayashi *et al.*, 2004). The central region of the RYR1 is dissimilar to the $InsP_3R$, since these domains contain modulatory and transducing functions. However, both receptors contain two internally repeated domains (Rossi and Sorrentino, 2004). Reported alterations that occur in hotspot two of the central region of the RYR1, which result in susceptibility to MH and CCD are indicated in Table 2.5.

Tammaro *et al.* (2003) observed two novel alterations in exon 39 in two MH families. The Val2117Leu alteration is due to a G6349C transition and the Met2101Lys mutation is due to an A6302C transition. Both alterations are conserved and were absent in 100 control subjects. An alteration, termed Asp2129Glu, which occurred due to a C6387G nucleotide transition, was observed in a single MH family (Rueffert *et al.*, 2001).

Table 2.5: Reported alterations within hotspot two of the RYR1 gene observed in MH, CCD or MmD patients

Nucleotide change	Amino acid	Exon	Substitution frequency	Phenotype	Reference
A6302C	Met2101Leu	39	1 family	MH	Tammaro *et al.*, 2003
G6349C	Val2117Leu	39	1 family	MH	Tammaro *et al.*, 2003
C6387G	Asp2129Glu	39	1 family	MH	Rueffert *et al.*, 2001
C6487T*	Arg2163Cys[1,2,3]	39	4%	MH	Manning *et al.*, 1998a
G6488C	Arg2163Pro	39	1 family	MH	Forunato *et al.*, 2000
G6488A*	Arg2163His[1,2,3]	39	1 family	MH	Manning *et al.*, 1998a
G6502A*	Val2168Met[3]	39	7%	MH	Manning *et al.*, 1998a
A6544T	Ile2182Phe	39	1 family	MH	Rueffert *et al.*, 2002
C6599T	Ala2200Val	40	1 family	MH	Halsall and Robinson, 2004
C6617T*	Thr2206Met[2,3]	40	1 family	MH	Manning *et al.*, 1998a
C6617G*	Thr2206Arg	40	1 family	MH	Brandt *et al.*, 1999
G6628T	Val2210Phe	40	1 family	MH	Sambuughin *et al.*, 2005
T6635A	Val2212Asp	40	1 family	MH	Galli *et al.*, 2006
G6640A	Val2214Ile	40	1.4%	MH	Sambuughin *et al.*, 2001a
G6838A	Val2280Ile	42	1 family	MH	Galli *et al.*, 2002
A6847C	Asn2283His	42	1 family	CCD/MmD	Zhou *et al.*, 2005
G7007A	Arg2336Gln	43	2 families	MH	Galli *et al.*, 2006
A7025G	Asn2342Ser	43	2 families	MH	Halsall and Robinson, 2004
G7032C	Glu2344Asp	44	1 family	MH	Monnier *et al.*, 2005
G7036A	Val2346Met	44	1 family	MH	Halsall and Robinson, 2004
A7043G	Glu2348Gly	44	1 family	MH	Halsall and Robinson, 2004
G7048A*	Ala2350Thr[2,3]	44	1 family	MH	Sambuughin *et al.*, 2001b
C7062T	Arg2355Cys	44	1 family	MH	McWilliams *et al.*, 2002
A7085G	Glu2362Gly	44	1 family	MH	Galli *et al.*, 2006
T7090G	Phe2364Val	44	2 families	MH	Halsall and Robinson, 2004
C7097G	Pro2366Arg	44	1 family	MH	Ibarra *et al.*, 2006
G7099A	Ala2367Thr	44	1.4%	MH	Sambuughin *et al.*, 2001a
G7124C*	Gly2375Ala	44	1 family	MH	Wehner *et al.*, 2004
T7268A	Met2423Lys	45	1 family	CCD/MmD	Zhou *et al.*, 2005
G7282A*	Ala2428Thr	45	1 family	MH	Monnier *et al.*, 2005
G7291A	Asp2431Asn	45	1.4%	MH	Sambuughin *et al.*, 2001a
G7300A*	Gly2434Arg[1,2,3]	45	4%	MH	Keating *et al.*, 1994
G7304A*	Arg2435His[1,2,3]	45	1 family	CCD	Zhang *et al.*, 1993
G7304T	Arg2435Leu[2]	45	1 family	CCD	Barone *et al.*, 1999
C7310T	Ala2436Val	45	1 family	MH	Galli *et al.*, 2006
G7317C	Glu2439Asp	45	1 family	MH	Galli *et al.*, 2006
C7354T	Arg2452Trp	46	1 family	MH	Chamley *et al.*, 2000
G7355A	Arg2452Gln	46	1 family	MH	Ibarra *et al.*, 2006
T7358C	Ile2453Thr	46	1 family	MH	Rueffert *et al.*, 2004
C7360T*	Arg2454Cys	46	1 family	MH	Brandt *et al.*, 1999
G7361A*	Arg2454His[3]	46	1 family	MH	Barone *et al.*, 1999
C7372T*	Arg2458Cys[1,2,3]	46	4%	MH	Manning *et al.*, 1998b

Table 2.5: Continued...

Nucleotide change	Amino acid	Exon	Substitution frequency	Phenotype	Reference
G7373A*	Arg2458His[1,2,3]	46	4%	MH	Manning *et al.*, 1998b
7041delGGA	Glu2347del[3]	44	2.6%	MH	Sambuughin *et al.*, 2001c

[1, 2] = mutations currently being used in the genetic diagnosis of MHS in European countries (Urwyler *et al.*, 2001; Halsall and Robinson, 2004); [3] = mutations currently being used in the genetic diagnosis of MHS in American countries (Sei *et al.*, 2004). An asterisk (*) indicates a causative mutation. MH = malignant hyperthermia; CCD = central core disease; MmD = multi-minicore disease; del = deletion.

Manning *et al.* (1998a) provided the first report of three novel alterations that clustered in the central portion (6400 - 6700) of the RYR1 gene. The alterations, Arg2163Cys, Arg2163His and Val2168Met, result from transitions, C6487T, G6488A and G6502A, respectively. The transitions occur in exon 39 of the RYR1 gene and all three alterations are causative, as the amino acids that are altered are conserved in the three isoforms of RYR. In addition, Fortunato *et al.* (2000) observed a G6488C transition that resulted in an Arg2163Pro alteration in one MH family from Italy.

A single alteration, Ile2182 phenylalanine (Phe) was observed in a single family diagnosed with MH. The mutation is due to an A6544T nucleotide transition (Rueffert *et al.*, 2002). Halsall and Robinson (2004) identified an Ala2200Val alteration that results from a C6599T transition in the RYR1 gene in one of 434 UK families. Manning *et al.* (1998a) provided the first report of a novel alteration, namely Thr2206Met, which is a result of a nucleotide transition termed C6617T in exon 40. Myotubes with this alteration display increased sensitivity to caffeine and 4-chloro-*m*-cresol, indicating that the presence of this alteration is sufficient to result in MH (Wehner *et al.*, 2002). Brandt *et al.* (1999) observed the Thr2206Arg alteration in a single MH pedigree. It was concluded from the IVCT results that the large pedigree of Western European nationality exhibited complete segregation of the phenotype. Sambuughin *et al.* (2005) identified a Val2210Phe alteration due to a G6628T nucleotide transition in one MH individual from North America. The alteration was conserved through the RYR evolution and across RYR1 species and was not detected in 100 unrelated North American individuals.

Recently, a Val2212Asp alteration due to a T6635A nucleotide transition was reported in one MH family from Italy. The alteration was observed to be highly conserved and absent in more than 100 chromosomes (Galli *et al.*, 2006). A novel Val2214Ile alteration, due to a G6640A substitution, was observed in one North American MH pedigree and was not detected in 158 unaffected chromosomes (Sambuughin *et al.*, 2001a). Galli *et al.* (2002) observed Val2280Ile in one MHS pedigree that was diagnosed via the IVCT. The

alteration occurred due to a G6838A nucleotide transition and was not observed in 200 control chromosomes. Galli *et al.* (2006) identified two novel alterations in MH patients from Italy. The first alteration, Arg2336Gln, was observed in two unrelated families in exon 43 of the RYR1 and the second alteration, Glu2362Gly, was observed in one family. The alterations occurred due to G7007A and A7085G nucleotide transitions respectively and both occurred in highly conserved regions of the RYR1 gene. The Asn2342Ser alteration was detected in two MH pedigrees from the UK and was due to an A7025G transition (Halsall and Robinson, 2004).

A Glu2344Asp alteration was observed, due to a G7032C transition in two related mothers, both of whom had sons who developed MH episodes that resulted in their deaths. However, the pathogenicity of this mutation has not yet been determined (Monnier *et al.*, 2005). Mutations in the RYR1 gene that occur in the central portion of the RyR1 protein i.e. Val2346Met, Glu2348Gly and Phe2364Val, were all reported by Halsall and Robinson (2004). Two alterations, Val2346Met and Glu2348Gly, were each observed in one UK family, and occurred due to nucleotide transitions G7036A and A7043G, respectively. The alteration Phe2364Val was observed in one MH family from the UK and was due to a T7090G nucleotide alteration.

The Ala2350Thr alteration was first identified in exon 44 of the RYR1 gene by Sambuughin *et al.* (2001b) in a large North American family. The mutation results from a G7048A nucleotide transition and changes the codon sequence from GCC (Ala) to ACC (Thr) in the RyR1 protein. The nucleotide position of the Ala amino acid is highly conserved among the three RyR isoforms of the muscle, heart and brain in several species, suggesting that the mutation is causative. Functional characterisation of the Ala2350Thr mutation indicated that the transition increases the sensitivity of the RyR1 channel by opening at lower concentrations of Ca^{2+} and closing in the presence of a higher concentration of Ca^{2+}, compared to cells that do not harbour the mutation (Wehner *et al.*, 2004).

Alteration Gly2375Ala was observed in one family with MH and was due to nucleotide transitions G7124C. The alteration was investigated in a myotube derived from a mutation carrier and altered Ca^{2+} homeostasis as it displayed higher sensitivity to RyR agonists (Wehner *et al.*, 2004). The Arg2355Cys alteration was first reported by McWilliams *et al.* (2002) in a large Brazilian MH family and was subsequently reported in six MH families from the UK (Halsall and Robinson, 2004). Two novel alterations were detected with a

frequency of 1.4% each in individuals from a North American MHS population. Alterations Ala2367Thr and Asp2431Asn occurred due to G7099A and G7291A transitions, respectively (Sambuughin *et al.*, 2001a). A single amino acid deletion was observed in exon 44 of the RYR1 gene. The alteration was observed in two unrelated MHS families and resulted in a deletion of a conserved glutamic acid. The alteration was reported to produce unusual contraction tension in MHS individuals (Sambuughin *et al.*, 2001a). A novel alteration, Pro2366Arg, was observed in a single MH family from Japan. The alteration results from a C7097G substitution and is conserved among RYR1 isoforms (Ibarra *et al.*, 2006).

An Ala2428Thr alteration due to a G7282A transition was observed in one MHS individual and her son. Both individuals were diagnosed as MHS via the IVCT (Monnier *et al.*, 2005). The Gly2433Arg alteration, which results from an A7300G transition in exon 44 of the RYR1 gene, was first identified by Keating *et al.* (1994) in four out of 104 unrelated Caucasian MHS individuals. Phillips *et al.* (1994) observed the mutation in 4% of Canadian MH families. The Gly2433Arg alteration may be causative, as it was only observed in MH patients. However, complete association of the mutation to MH in certain families was not observed. Richter *et al.* (1997) provided evidence that the presence of this alteration resulted in hypersensitivity of the SR to Ca^{2+} release. An amino acid substitution Arg2434His resulting from the alteration of an A7301G was observed in seven members of a Canadian pedigree diagnosed with CCD (Zhang *et al.*, 1993). All CCD patients that were tested were subsequently diagnosed as MH positive. Ligand binding sites that are proximal to the mutation include a potential ATP binding site at residues 2670 - 2700, CaM binding sites at residues 2826 - 3066 and a phosphorylation site at Ser2843 (Suko *et al.*, 1993; Otsu *et al.*, 1994). Zhang *et al.* (1993) suggested that the mutation may result in poorly regulated Ca^{2+} release, resulting in the uptake of excess Ca^{2+} by mitochondria. The participation of mitochondria in this process may lead to loss of function and could explain the structural abnormalities observed in individuals with CCD.

Numbering of the nucleotides of the cDNA from the RYR1 and the encoded amino acids was corrected in order to account for earlier errors and omissions (Phillips *et al.*, 1996). The amino acid numbering of alterations Gly2433Arg and Arg2434His were corrected by sequence data provided by Phillips *et al.* (1996), and these are now referred to as Gly2434Arg and Arg2435His. Richter *et al.* (1997) used was corrected sequence and conducted functional characterisation studies on the Gly2434Arg alteration that occurs due to an A7300G. Results indicated that the mutation enhanced the sensitivity of the

RyR1 to activating concentrations of Ca^{2+}, caffeine and 4-chloro-*m*-cresol and the sensitivity to an inhibiting concentration of Ca^{2+} and CaM was reduced.

Halsall and Robinson (2004) identified an Arg2435Leu alteration in the RYR1 gene in one of 434 UK families. The mutation results from a G7304T transition. Chamley *et al.* (2000) observed a C7354T transition that resulted in an Arg2452Trp alteration in a 6-month-old child with MH. The child was diagnosed via the IVCT and the alteration was determined in the mother and a sibling of the proband. Galli *et al.* (2006) identified two alterations in exon 45, each in a different family from Italy. Ala2436Val occurs due to a C7310T nucleotide transition and Glu2439Asp occurs due to a G7317C substitution. In addition, Ibarra *et al.* (2006) observed an Arg2452Gln alteration due to a G7355A substitution. The alteration is conserved and was detected in one Japanese MH family. A recently observed alteration Ile2453Thr that results from a T7358C substitution was observed in an MHS patient with spondylocostal dysostosis, a congenital disorder that involves multiple vertebrae. The alteration segregated in the mother, who was diagnosed with both CCD and MH, and was absent in 82 unaffected individuals (Rueffert *et al.*, 2004). The functional significance of this alteration was analysed in myotubules and is associated with facilitated Ca^{2+} release from the SR (Wehner *et al.*, 2003).

An Arg2454His alteration due to a G7361 substitution was observed to co-segregate in one family with MH. The alteration was absent in 50 unaffected chromosomes and the site of the mutation was conserved across species and related isoforms (Barone *et al.*, 1999). Brandt *et al.* (1999) observed an Arg2454Cys alteration due to a C7360T transition in one MH family of German descent. Alterations Arg2458Cys and Arg2458His were first reported by Manning *et al.* (1998b). Both mutations occur at a *cytosine-phosphodiester-guanine* (CpG) *dinucleotide in the central region of the gene*. The first mutation occurs due to a substitution at C7372T and the second occurs due to a transition at G7373A. Both mutations are causative, as the nucleotide sequence is conserved and has not been identified in MHN individuals. The Arg2458Cys alteration was detected in a Swiss and an Italian pedigree, and the mutation segregated to both MHS and MHE individuals. Arg2458His alteration was detected in a French MH pedigree (Manning *et al.*, 1998b). The authors suggested that this finding supported the case for classifying MHE individuals as MHS. Halsall and Robinson (2004) identified an Arg2452Trp alteration in the RYR1 gene in one of 434 UK families. The mutation results from a C7354T nucleotide transition.

2.11.3.3.3 Mutation hotspot three of the RYR1 gene

The C-terminal domain contains the transmembrane segments that form the Ca^{2+} channel pore. The amino acid sequence of this region is highly conserved between the RyR1 and $InsP_3R$ with the exception of domains three and four. Domains in this region play a role in channel conductance and high and low affinity Ca^{2+} binding sites have been described (Chen and MacLennan, 1998). In addition, a Ca^{2+} inactivation site and ryanodine binding site have been described between amino acids 4081 - 4092, that play a role in Ca^{2+} regulation of the RyR1 (Fessenden *et al.*, 2004). Alterations in this region of the RYR1 gene have been described for both CCD and MH. The exact mechanism underlying CCD remains elusive and most alterations in the RYR1 associated with CCD have been determined in the C-terminal domain of the gene. However, mutations that result in the CCD/MHS phenotype have also been reported in other regions of the RYR1 (Shepherd *et al.*, 2004). In addition, alterations that are only associated with MHS are also identified in the C-terminus of the RYR1. Mutations associated with MH display intracellular Ca^{2+} homeostasis different from those associated with CCD. Most alterations in hotspot region three of the RyR1 channels exhibit elevated resting Ca^{2+} levels and reduction in SR Ca^{2+} content (Avila and Dirkensen, 2001). Alterations reported in hotspot three of the C-terminal of the RYR1, which result in susceptibility to MH and other disorders, are indicated in Table 2.6.

Sambuughin *et al.* (2005) reported an Asn4119Tyr alteration due to an A12355T nucleotide transition in one MHS individual from North America. The alteration is conserved through RYR1 evolution and among RYR1 species and was not detected in 50 unaffected chromosomes. In addition, another mutation was observed in exon 90, the Arg4136Ser mutation, which occurred due to a C12406A nucleotide transition. The alteration was observed in an MHS patient and in the father of the proband. Although the father was diagnosed as MHN, both individuals displayed a similar histological pattern of muscle dysfunction (Galli *et al.*, 2002). In exon 91, a novel mutation termed Val4234Leu, due to a G12700C nucleotide transition, was observed in one MHS family. The proband had a family history of MH and the alteration was inherited from the father (Galli *et al.*, 2002). In one family from Japan, Ibarra *et al.* (2006) identified the Glu4283Val alteration that occurred due to an A12848T substitution. The proband in the family harboured both a Glu4283Val alteration and a Val218Ile alteration.

The alteration Gly4638Met was observed in two UK pedigrees with MH and is due to nucleotide substitution, G13913A (Halsall and Robinson, 2004). Ibarra *et al.* (2006) identified an Arg4645Gln alteration in one MHS family, the alteration occurs due to a G13934A nucleotide transition. However, the alteration is not conserved among RYR isoforms, therefore its causative status is still to be determined. In one MH family a Pro4668Ser alteration was detected which occurs due to a C14002T transition. The proband inherited both a Pro4668Ser alteration and a Leu4838Val alteration. Monnier *et al.* (2005) observed the alteration Phe4684Ser alteration that occurs due to a T14501C transition in one MHS family. Sambuughin *et al.* (2005) identified a Tyr4733Asp alteration in exon 98 of the RYR1 gene. The mutation was observed in one MHS family and is conserved among RYR isoforms. The alteration occurs due to a T14197G nucleotide transition.

Table 2.6: Reported alterations within hotspot three of the RYR1 gene observed in MH, CCD or MmD patients

Nucleotide change	Amino acid	Exon	Substitution frequency	Phenotype	Reference
A12355T	Asn4119Tyr	90	1 family	MH	Sambuughin *et al.*, 2005
C12406A	Arg4136Ser	90	1 family	MH	Galli *et al.*, 2002
G12700C	Val4234Leu	91	1 family	MH	Galli *et al.*, 2002
A12848T	Glu4283Val	91	1 family	MH	Ibarra *et al.*, 2006
A13645C	Arg4549Gln	93	1 family	CCD	Kossugue *et al.*, 2005
T13703C	Leu4568Pro	94	1 family	CCD	Wu *et al.*, 2006
T13891A	Tyr4631Asn	95	1 family	CCD	Wu *et al.*, 2006
G13900A	Glu4634Lys	95	1 family	CCD	Wu *et al.*, 2006
A13909G	Thr4637Ala	95	1 family	CCD	Scacheri *et al.*, 2000
C13910T	Thr4637Ile	95	1 family	CCD	Davis *et al.*, 2003
G13912A	Gly4638Ser	95	1 family	CCD	Wu *et al.*, 2006
G13913A	Gly4638Met	95	2 families	MH	Halsall and Robinson, 2004
G13934A	Arg4645Gln	95	1 family	MH	Ibarra *et al.*, 2006
A13952C	His4651Pro	95	1 family	CCD	Davis *et al.*, 2003
T13994C	Leu4665Pro	95	1 family	CCD/MmD	Zhou *et al.*, 2005
C14002T	Pro4668Ser	96	1 family	MH	Oyamada *et al.*, 2002
T14501C	Phe4684Ser	96	1 family	MH	Monnier *et al.*, 2005
C14126T	Thr4709Met	96	1 family	CCD/MmD	Zhou *et al.*, 2005
A14170C	Lys4724Gln	97	1 family	CCD/MmD	Zhou *et al.*, 2005
T14197G	Tyr4733Asp	98	1 family	MH	Sambuughin *et al.*, 2005
C14209T	Arg4737Trp	98	2 families	MH	Galli *et al.*, 2002
G14210A	Arg4737Gln	98	2 families	MH	Monnier *et al.*, 2005
T14378C	Leu4793Pro	100	---	CCD	Monnier *et al.*, 2001
A14387G*	Tyr4796Cys	100	---	CCD	Monnier *et al.*, 2000
T14422A	Phe4808Asn	100	1 family	CCD	Davis *et al.*, 2003

Table 2.6: Continued...

Nucleotide change	Amino acid	Exon	Substitution frequency	Phenotype	Reference
C14440T	Leu4814Phe	100	1 family	MH	Halsall and Robinson, 2004
A14449T	Ile4817Phe	100	1 family	MH	Halsall and Robinson, 2004
T14471C	Leu4824Pro	100	3 families	MH	Halsall and Robinson, 2004
C14473T	Arg4825Cys	100	---	CCD	Monnier *et al.*, 2001
C14477T	Thr4826Ile	100	---	MH	Brown *et al.*, 2000
C14512G*	Leu4838Val[2]	101	1 family	MH	Halsall and Robinson, 2004
C14537T	Ala4846Val	101	1 family	CCD	Kossugue *et al.*, 2005
G14545A	Val4849Ile	101	4 families	MH	Halsall and Robinson, 2004
A14572G	Asn4858Asp	101	1 family	CCD	Wu *et al.*, 2006
C14581T	Arg4861Cys	101	1 family	CCD	Wu *et al.*, 2006
G14582A*	Arg4861His[2]	101	---	CCD	Monnier *et al.*, 2001
A14591G	Tyr4864Cys	101	1 family	CCD	Zorzato *et al.*, 2003
A14627G	Lys4876Arg	101	1 family	MH	Sambuughin *et al.*, 2005
T14639C	Met4880Thr	101	1 family	MH	Sambuughin *et al.*, 2005
G14671C	Gly4891Arg	102	1 family	CCD	Tilgen *et al.*, 2001
C14677T	Arg4893Trp	102	---	CCD	Monnier *et al.*, 2001
G14678A	Arg4893Gln	102	1 family	CCD	Davis *et al.*, 2003
G14678C	Arg4893Pro	102	1 family	CCD	Wu *et al.*, 2006
G14680A	Ala4894Thr	102	1 family	MH	Ibarra *et al.*, 2006
G14691T	Gly4897Val	102	1 family	CCD	Kossugue *et al.*, 2005
T14693C*	Ile4898Thr[3]	102	1 family	CCD	Lynch *et al.*, 1999
G14695A	Gly4899Arg	102	1 family	CCD	Tilgen *et al.*, 2001
G14696A	Gly4899Glu	102	---	CCD	Monnier *et al.*, 2001
C14717T	Ala4906Val	102	1 family	CCD	Tilgen *et al.*, 2001
A14740G	Arg4914Gly	102	---	CCD	Monnier *et al.*, 2001
G14741C	Arg4914Thr	102	1 family	CCD	Davis *et al.*, 2003
C14759A	Thr4920Asn	102	1 family	CCD	Wu *et al.*, 2006
T14762C	Phe4921Ser	102	1 family	CCD	Wu *et al.*, 2006
C14814G	Ile4938Met	103	1 family	MH	Halsall and Robinson, 2004
C14817A	Asp4939Glu	103	1 family	MH	Halsall and Robinson, 2004
G14818A	Ala4940Thr	103	1 family	MH	Sambuughin *et al.*, 2005
G14825T	Gly4942Val	103	1 family	MH	Galli *et al.*, 2002
T14879A	Phe4960Tyr	104	1 family	MH	Ibarra *et al.*, 2006
C14918T	Pro4973Leu	104	1 family	MH	Monnier *et al.*, 2002
12640del9nt[4]	Arg4214del Gln4215del Phe4216del	91	---	CCD	Monnier *et al.*, 2001
13939del16nt[4]	Leu4647del Ser4648del	95	---	CCD	Monnier *et al.*, 2001
14578delTTC	Phe4860del	101	---	CCD	Monnier *et al.*, 2001

[1, 2] = mutations currently being used in the genetic diagnosis of MHS in European countries (Urwyler *et al.*, 2001; Halsall and Robinson, 2004); [3] = mutations currently being used in the genetic diagnosis of MHS in American countries (Sei *et al.*, 2004); [4] = del gives rise to three different amino acid alterations. An asterisk (*) indicates a causative mutation. MH = malignant hyperthermia; CCD = central core disease; MmD = multi-minicore disease; del = deletion; a dashed line (---) indicates that no information is available.

An alteration, Arg4737Gln, that occurs due to a G14210A transition, was observed in the cytoplasmic loop in the transmembrane domain. The mutation was reported in two MHS families from Italy. The alteration results in a nonconservative change and segregated with the MH phenotype in both families (Monnier *et al.*, 2005). In addition, an Arg4737Trp alteration that occurs due to a C14209T nucleotide transition was observed in two MHS families diagnosed via the IVCT. In one family the alteration segregated in four family members, of whom one was diagnosed as MHS and three were diagnosed as MHE (Galli *et al.*, 2002). Alterations Leu4814Phe, Ile4817Phe and Leu4824Pro were observed in UK pedigrees with MH. The mutations are due to nucleotide substitutions C14440T, A14449T and T14471C, respectively. Two alterations, Leu4814Phe and Ile4817Phe, were observed in one family, while the Leu4824Pro alteration was observed in three families. Brown *et al.* (2000) reported the Thr4826Ile alteration in a large Maori pedigree, which consisted of five individuals that experienced clinical episodes of MH and 130 members that were diagnosed as MHS via the IVCT. The mutation arises from a C14477T transition in the RYR1 gene.

The Arg4861His alteration of exon 101, which results from the substitution termed G14582A, was first described by Monnier *et al.* (2001). The mutation was detected in three unrelated CCD pedigrees and occurred in a highly conserved region of the RYR1. The Arg4861His alteration, which was also detected in a single CCD pedigree, indicated complete segregation in all 27 affected individuals and was absent in the unaffected individuals. Three members of the family were also subsequently diagnosed as MHS via an IVCT. However, none of the individuals experienced a fulminant MH reaction under anaesthesia (Davis *et al.*, 2003).

The Leu4838Val and Val4849Ile alterations were observed in UK families (Halsall and Robinson, 2004). The mutations occur due to nucleotide transitions in the RYR1 gene i.e. C14512G and G14545A, respectively. Alteration Leu4838Val was observed in a single pedigree and Val4849Ile was observed in four pedigrees. Ile4938Met and Asp4939Glu were respectively observed in single UK families (Halsall and Robinson, 2004). The mutations occur due to nucleotide transitions in the RYR1 gene, namely C14814G and C14817A, respectively. Sambuughin *et al.* (2005) identified two alterations in exon 101, the Lys4876Arg and Met4880Thr, which occur due to A14627G and T14639C nucleotide transitions respectively. Each alteration was observed in one MHS family from North America. An Ala4940Thr alteration has been reported in one MHS individual from North America. The alteration occurs due to a G14818A nucleotide transition (Sambuughin

et al., 2005). The novel mutation Gly4942Val, which occurs due to a G14825T nucleotide transition, was observed in one individual diagnosed as MHE via the IVCT. In addition, the alteration was not observed in 200 control chromosomes (Galli *et al.*, 2002).

Ibarra *et al.* (2006) observed an Ala4894Thr alteration due to a G14680A substitution in one MHS family from Japan. The alteration is conserved in different RYR1 isoforms and was not observed in control individuals. In addition, the authors observed a Phe4960Tyr alteration due to a T14879A nucleotide transition in one MH family with raised CK levels. The proband inherited both this alteration and an Arg3119His alteration in exon 63. Monnier *et al.* (2002) identified a Pro4973Leu alteration due to a C14918T nucleotide transition in a single MH pedigree from France. The alteration was detected in four MHS individuals in the family and was not observed in 100 unaffected controls obtained from the general population. In one individual, two different MHS alleles were inherited, namely an Arg1086His alteration of the CACNA1S gene and a Pro4973Leu alteration of the RYR1 gene. Inheritance of both these alterations was not determined to induce a stronger contractile response to either caffeine or halothane.

2.11.3.3.4 Alterations observed outside of the RYR1 gene mutational hotspots

Most alterations resulting in MHS are observed within the three RYR1 mutational hotspots. However, recent studies have detected alterations outside these hotspots. Reported alterations that occur outside the mutational hotspots of the RYR1 gene that result in susceptibility to MH are indicated in Table 2.7. Ibarra *et al.* (2006) identified an alteration in one MH family from Japan, the Leu13Arg alteration due to a T38G substitution. The alteration occurs in exon 1 and is conserved among RYR1 isoforms. In exons 33 and 34, alterations Pro1592Leu and Arg1667Cys were detected. The Pro1592Leu alteration was observed in one MHS family and the proband harboured two alterations, termed the Pro1592Leu and an Arg533His from exon 15. The Arg1667Cys alteration was observed in three MH families. However, the alteration was also observed in one control individual (Ibarra *et al.*, 2006). One alteration, Ser1728Phe, which is due to a T5182C nucleotide change, was observed outside the three hotspots. The alteration is located in close proximity to the central mutational hotspot, between the central and N-terminal region of the gene. The alteration occurs in exon 34 and was observed in one MH individual from North America. The alteration was not detected in 100 unrelated control North American samples and was conserved among different species of RYR (Sambuughin *et al.*, 2005).

Table 2.7: Reported alterations outside of the RYR1 gene mutational hotspots observed in MH, CCD, MmD or CNM patients

Nucleotide change	Amino acid	Exon	Substitution frequency	Phenotype	Reference
T38G	Leu13Arg	1	1 family	MH	Ibarra *et al.*, 2006
A2274G	Asn759Asp	19	1 family	CCD	Kossugue *et al.*, 2005
C4775T	Pro1592Leu	33	1 family	MH	Ibarra *et al.*, 2006
C4816T	Arg1606Cys	33	1 family	CNM	Jungbluth *et al.*, 2005
C4999T	Arg1667Cys	34	3 families	MH	Ibarra *et al.*, 2006
T5182C	Ser1728Phe	34	1 family	MH	Sambuughin *et al.*, 2005
C5317T	Pro1773Ser	34	1 family	MH	Ibarra *et al.*, 2006
T5357C	Leu1786Pro	34	1 family	MH	Gillard *et al.*, 1992
C5360T	Pro1787Leu	34	1 family	MH	Gillard *et al.*, 1992
G6178T	Gly2060Cys	38	1 family	MH	Gillard *et al.*, 1992
C7487T	Pro2496Leu	47	1 family	MH	Ibarra *et al.*, 2006
C7522T	Arg2508Cys	47	4 families	CCD	Wu *et al.*, 2006
C7522G	Arg2508Gly	47	1 family	CCD	Ibarra *et al.* 2006
G7523A	Arg2508His	47	1 family	CCD	Galli *et al.*, 2006
G7635C	Glu2545Asp	48	1 family	CCD	Wu *et al.*, 2006
C7771G	Arg2591Gly	48	2 families	MH	Galli *et al.*, 2006
G7888C	Val2627Leu	49	1 family	MH	Galli *et al.*, 2006
C8026T	Arg2676Trp	50	1 family	MH/MmD	Guis *et al.*, 2004
G8188C	Asp2730His	51	1 family	MH	Ibarra *et al.*, 2006
G8198A	Gly2733Asp	51	1 family	MH	Sambuughin *et al.*, 2005
G8290A	Glu2764Lys	52	1 family	MH	Galli *et al.*, 2006
C8360G	Thr2787Ser	53	1 family	MH	Monnier *et al.*, 2005
C8518T	Arg2840Trp	54	1 family	MH	Ibarra *et al.*, 2006
T8600A	Leu2867Gly	55	1 family	MH	Galli *et al.*, 2006
G8816A	Arg2939Lys	57	1 family	CNM	Jungbluth *et al.*, 2005
A8817C	Arg2939Ser	57	1 family	CCD	Zhou *et al.*, 2005
G9356A	Arg3119His	63	1 family	MH	Ibarra *et al.*, 2006
G10043A	Arg3348His	67	1 family	MH	Sambuughin *et al.*, 2005
A10100G	Lys3367Arg	67	1 family	CCD	Wu *et al.*, 2006
C10337T	Ser3446Phe	68	1 family	CCD	Zhou *et al.*, 2005
C10579T	Pro3527Ser	71	1 family	CCD	Zhou *et al.*, 2005
T10817C	Leu3606Pro	73	1 family	CCD	Wu *et al.*, 2006
G11518A	Val3840Ile	82	1 family	MH	Ibarra *et al.*, 2006
G11708A	Arg3903Gln	85	3 families	MH	Galli *et al.*, 2006
T11748G	Ile3916Met	85	3 families	MH	Monnier *et al.*, 2002
C12121T	Arg4041Trp	89	1 family	MH	Galli *et al.*, 2006
C12242T	Thr4081Met	89	1 family	MH	Ibarra *et al.*, 2006

MmD = multi-minicore disease; CCD = central core disease; MH = malignant hyperthermia; CNM = centronuclear myopathy.

Ibarra *et al.* (2006) observed a Pro1773Ser alteration in one MHS patient from Japan. The alteration was observed in exon 34 and is due to a C5317T substitution. However, the

alteration was also observed in one control individual, therefore its causative status is still unknown.

Gillard *et al.* (1992) identified three alterations, namely Leu1786Pro and Pro1787Leu in exon 34 and a Gly2060Cys in exon 38. All three alterations were detected in single families but did not segregate with the MH phenotype. A Pro2496Leu alteration due to a C7487T substitution was detected in one MH family from Japan. The alteration was identified in the proband in addition to an Asp2730His alteration that occurs in exon 51 (Ibarra *et al.*, 2006). The Arg2676Trp alteration, which occurs due to a C8026T transition in exon 50 of the RYR1 gene, has been reported in a family susceptible to MH that was also diagnosed with MmD disease (Guis *et al.*, 2004). A Gly2733Asp alteration, which is due to a G8198A nucleotide transition, was observed outside the three hotspots in close proximity to the central mutation hotspot two in one MHS individual. The alteration was detected in exon 51 (Sambuughin *et al.*, 2005). Thus far, a single alteration has been reported in exon 53. Monnier *et al.* (2005) observed a Thr2787Ser alteration, which occurs due to a C8360G nucleotide transition, in one MHS individual from France.

Galli *et al.* (2006) identified seven alterations that occur outside the mutational hotspots. Five alterations, Arg2508His, Val2627Leu, Glu2764Lys, Leu2867Gly and Arg4041Trp, were identified in single families from Italy. However, the Arg2591Gly and the Arg3903Gln alterations were identified in two and three MH families respectively. All seven alterations were not detected in more than 100 chromosomes from the general population and occurred in highly conserved regions of the RYR1 gene.

Ibarra *et al.* (2006) identified two alterations in exons 54 and 63, respectively. The Arg2840Trp alteration occurs due to a C8518T substitution and was observed in one MHS individual from Japan. The alteration was not detected in control individuals and was conserved among RYR1 isoforms. The Arg3119His alteration occurs due to a G9356A transition and was observed in one MHS family. The proband of the family inherited both this alteration and a Phe4960Tyr alteration. In exon 67, a single alteration, Arg3348His that occurs due to a G10043A transition, has been reported in an MH family from North America. This alteration is evolutionarily conserved and was not detected in 100 unrelated North American control individuals. Arg3348His does not occur in a mutational hotspot and is located between the central and C-terminal region of the RYR1 (Sambuughin *et al.*, 2005). Monnier *et al.* (2002) identified a unique T11748G alteration that leads to an Ile3916Met change in exon 85 of the RYR1 gene close to the third hotspot. The alteration

was detected in three MHS individuals of a single pedigree from France and was not identified in 100 unaffected chromosomes, indicating its functional significance. One individual from this pedigree harboured both the Ile3916Met and an Arg163Cys alteration. Lastly, Ibarra *et al.* (2006) identified two alterations in exon 82 and 89, respectively. Both the Val3840Ile and Thr4081Met alterations were identified in one MH family from Japan and neither was identified in control individuals and both are conserved among RYR1 isoforms.

2.11.3.4 **Synonymous SNPs and the RYR1 gene**

Several SNPs have been identified in the coding regions of the RYR1 gene (Galli *et al.*, 2006). SNPs are observed at a frequency of about 1:1000 bases in humans and occur with a prevalence of 1% or more in the population (Kruglyak, 1997). SNPs are classified as synonymous if they do not result in a change in the amino acid sequence of the protein (Cargill *et al.*, 1999).

Previously, synonymous SNPs have been considered exempt from evolutionary pressure (Rogozin *et al.*, 2002). However, 39% of human synonymous alterations may be under weak selection (Hellmann *et al.*, 2003) and rates of selection may vary across the genome. Selection may occur at silent sites in order to maintain optimal GC content. In addition, selection may result from genome-wide constraints such as variation in the abundance of transfer ribonucleic acid (tRNA) or may occur via local requirements imposed by mRNA processing (Duret, 2002; Eyre-Walker and Hurst, 2001). Evidence of selective pressure on synonymous SNPs may indicate that the substitutions have functional effects (Fullerton *et al.*, 2001). Therefore, synonymous alterations may not only result in a nucleotide change but could also have biological effects on the protein (Wang and Sadée, 2006). Therefore, synonymous SNPs may play a role in the development of genetic disorders.

The GC content of human DNA varies widely across the genome (Fullerton *et al.*, 2001). The compositional heterogeneity may play an important role in genome organisation, with regard to gene density, gene length, patterns of codon usage and distribution of different classes of repetitive elements (Fullerton *et al.*, 2001). However, the relationship between synonymous substitution rates and nucleotide composition has been the subject of debate (Bernardi and Bernadi, 1986; Filipski, 1987). Via the use of maximum-likelihood analysis, Bielawski *et al.* (2000) indicated that regions of the genome with a higher GC content have

higher synonymous substitution rates. This correlation has not been observed in studies using an approximate method (Mouchiroud *et al.*, 1995), which may indicate that this method does not properly account for transition/transversion rate bias and unequal nucleotide and codon usage (Bielawski *et al.*, 2000). Selection may occur regionally to maintain or elevate the GC content (Bernardi *et al.*, 1988). The same authors suggested that selection occurs to elevate GC content in certain regions of the genome as a means of protecting DNA from heat degradation. In addition, GC-rich regions of the genome require formation of protein-DNA complexes to facilitate helix opening, as the G and C bp is stronger and contains three hydrogen bonds compared to two hydrogen bonds between the A and T bp. The involvement of proteins leads to gene expression that is able to be affected by environmental conditions (Guo and Jamison, 2005). Parmley *et al.* (2005) identified evidence for purifying selection against synonymous alterations in mammalian exonic splicing enhancers (ESEs) to ensure efficient pre-mRNA splicing. Synonymous alterations that affect ESEs have been proposed to underlie a number of aberrant splicing events in human disease (Liu *et al.*, 2001).

Although non-synonymous SNPs alter protein function, they often appear insufficient to account for inter-individual differences in disease susceptibility (Wang and Sadée, 2006). Silent SNPs affecting gene expression, mRNA processing and translation may account for susceptibility to complex disorders (Wang and Sadée, 2006). Lo *et al.* (2003) reported that 50% of human genes with coding SNPs may result in allelic variation in gene expression. Kim *et al.* (1997) identified alterations in synonymous codon usage in human genes that can increase protein production more that 10-fold. Wang and Sadée (2006) identified a synonymous SNP that affected gene expression and function. The SNP was observed to decrease mRNA levels by affecting mRNA stability. Capon *et al.* (2004) described a synonymous SNP of the corneodesmosin gene that resulted in altered mRNA stability due to a decreased affinity of the cytoplasmic RNA binding protein.

2.11.4 Genetic heterogeneity of MH

Genetic heterogeneity in MH was first suggested by Levitt *et al.* (1991). The authors observed that markers in the 19q12-q13.3 linkage groups in three unrelated families did not co-segregate with MHS, indicating genetic heterogeneity. Following the initial observation of genetic heterogeneity, several MHS individuals have demonstrated absence of linkage to chromosome 19q13.1-q13.2. Deufel *et al.* (1992) and Iles *et al.* (1992) excluded linkage in two Bavarian MHS families and two additional MH families,

respectively. Levitt *et al.* (1992) suggested that three separate loci are responsible for susceptibility to MH. Robinson *et al.* (1998) excluded the RYR1 gene in three families with MH and suggested the possibility of two MH genes in the same pedigree.

Additional candidates were proposed, including a second MHS locus (MHS-2) suggested for pedigrees displaying linkage to chromosome 17q (Levitt *et al.*, 1992). The adult muscle sodium channel α-subunit (SCN4A) gene located on 17q23.1-25.3 (George *et al.*, 1991) and the γ-subunit of the DHPR, located on chromosome 17q11.2-q24, were selected as candidates (Levitt *et al.*, 1992; Olckers *et al.*, 1992). Ervasti *et al.* (1989) demonstrated that the DHPR of MHS muscle displayed altered binding of [^{3}H] nitrendipine, indicating that the DHPR might play a role in altered SR Ca^{2+} release. A study conducted by Moslehi *et al.* (1998) further supported linkage to chromosome 17q in a study of a large Canadian family. However, Iles *et al.* (1993) indicated that linkage data obtained in their study did not support the proposed association of MHS-2 and 17q and Lynch *et al.* (1995) could not detect causative DHPR γ-subunit mutations in MHS patients screened in their study. Analyses of the DHPR gene led to the identification of the third MHS locus (MHS-3). The DHPR locus, CACNL2A, which encodes the α_2/δ-subunits of the DHPR, has been mapped to the proximal long arm of chromosome 7q21.1 by somatic cell hybrid analysis (Powers *et al.*, 1994). Iles *et al.* (1994) indicated co-segregation of the polymorphic genetic marker, D7S849, adjacent to the CACNL2A locus with MHS in a German pedigree with a lod score of 2.91. However, linkage in other pedigrees and a causal mutation has not yet been identified. Susceptibility to MH may be due to an as yet unidentified mutation at the CACNL2A locus, to a mutation in an adjacent regulatory element or to a tightly linked but distinct locus (Hogan, 1998).

Genome-wide linkage screening identified the MHS-4 locus on chromosome 3q13.1 in a single German pedigree with a lod score of 3.22 (Sudbrak *et al.*, 1995). Although the pedigree included a consanguineous marriage, the two different MHS mutations were presumed to segregate independently. However, a candidate gene on chromosome 3q has not yet been identified.

A fifth novel susceptibility locus (MHS-5) for MH (CACNA1S), which maps to chromosome 1q32 and encodes the α_1-subunit of the DHPR, has been identified (Casana *et al.*, 2003; Stewart *et al.*, 2001). This subunit is essential for E-C coupling in skeletal muscle (Knudson *et al.*, 1989). However, O'Brien *et al.* (1995) indicated that the Arg163Cys

alteration of the α_1-subunit does not co-segregate with MHS. A sixth MH locus (MHS-6) has been suggested by Robinson *et al.* (1997). The authors provided evidence in one pedigree that chromosome 5p may play a role in MH susceptibility.

Olckers (1997) observed absence of linkage to the above-mentioned six loci in a large South African family. Linkage analysis revealed a novel locus on chromosome 2q (MHS-7). A candidate gene for this locus has not yet been identified, but could be responsible for the MHS phenotype. Table 2.8 lists the chromosomal locations that have been observed to harbour potentially causative MH susceptibility loci as well as the mutations identified within these loci. The candidate gene approach has been used to identify additional loci, encoding proteins involved in E-C coupling, which may result in susceptibility to MH. Several chromosomal locations have been identified that harbour causative MH susceptibility loci. However, candidate genes have only been observed for two genes, the DHPR and SCN4A genes.

Table 2.8: Chromosomal localisations harbouring potentially causative MHS loci

MH susceptibility locus[1]	Gene product	Mutation	References
17q11.2-q24	DHPR γ-subunit	Unidentified	Levitt *et al.* (1992)
---	SCN4A	Gly1306Ala	Olckers *et al.* (1992); Vita *et al.* (1995)
7q21.1	DHPR α_2/δ-subunits	Unidentified	Iles *et al.* (1994)
3q13.1	Unidentified	Unidentified	Sudbrak *et al.* (1995)
1q32	DHPR α_1-subunit	Arg1086His	Robinson *et al.* (1997); Monnier *et al.* (1997)
5p	Unidentified	Unidentified	Robinson *et al.* (1997)
2q	Unidentified	Unidentified	Olckers *et al.* (1999)

[1] = 19q13.1 of the RYR1 not included in the table. α = alpha; δ = delta; DHPR = dihydropyridine receptor gene; SCN4A = sodium channel α-subunit gene; p = short arm of chromosome; q = long arm of chromosome. Adapted from Denborough (1998).

2.11.5 Epistatic model for MH

Due to the fact that phenotypic and genotypic data are discordant in some pedigrees, Robinson *et al.* (2000) suggested that susceptibility to MH is dependent upon the effects of more than one gene. Robinson *et al.* (2000) conducted an extended transmission disequilibrium test (ETDT) in 130 MH nuclear families (families consisting of two parents and their offspring) to determine if several independent genes interact with and influence the MH phenotype. Their analysis suggested that the MH locus on chromosomes 3q, 5p and 7q, together with RYR1 on chromosome 19, collectively resulted in MH in 61 UK families, therefore variation in more than one gene can influence susceptibility to MH in

individual families. Following this, Robinson *et al.* (2003a) determined if the results could be confirmed in an independent data set that included 131 nuclear families. The role of RYR1 and the effects of chromosomes 5 and 7 in RYR1-linked families were confirmed. However, the influence of chromosome 3 and 1 was less clear. The authors suggested that the MH phenotype is influenced by the major locus (RYR1) and that modifier genes may play a role.

Epistasis between alleles involved in MH has been suggested by Sedensky and Meneely, (1987). The authors used the nematode, *Caenorhabditis elegans* (*C.elegans*), as a model for determining the action of volatile anaesthetics. Volatile anaesthetics behave in the same manner in *C.elegans* as they do in vertebrates, as the structural motifs that are targeted by anaesthetics are similar (Crowder *et al.*, 1996). Sedensky and Meneely (1987) determined that mutant alleles (*unc*-79 and *unc*-80) cause sensitivity to halothane and a third gene (*unc*-9) suppresses the effects of the mutant alleles, resulting in unaffected sensitivity to halothane. Their study indicates that mutations in several different genes are involved in sensitivity to halothane. Morgan and Sedensky (1995) identified two mutations in *C.elegans*, fc20 and fc34, which result in a contractile response when the nematode is exposed to volatile anaesthetics, and neither involves the ryanodine receptor. Further studies of the two mutations observed in nematodes may provide insight into human MH.

2.11.6 Discordance between phenotype and genotype

Reports of individuals that were diagnosed as MHN by the IVCT but carried a RYR1 mutation (Fortunato *et al.*, 1999), as well as individuals that were diagnosed as positive but that did not carry a RYR1 mutation, have been published. This has been observed for several RYR1 mutations including G1021A, C1840T and G7300A (Robinson *et al.*, 2003b). Discordance has also been observed in the South African MH population. The Arg614Cys mutation was observed in 13 MHS patients and one MHN individual (Havenga, 2000). A reason for this discordance could be the fact that the mutation does not influence susceptibility. The IVCT results reflect a false positive or negative diagnosis, or that a laboratory error may have occurred. Discordance may occur, as several genes influence susceptibility, implicating alleles of minor phenotypic effect, which occur at different loci (Girard *et al.*, 2004). Therefore a particular allele may increase the risk of susceptibility, but on its own may not be sufficient to result in MH.

Discordance between genotype and phenotype has been reported in other disorders. Cystic fibrosis has been determined to be due to more than 1,000 mutations in the cystic fibrosis transmembrane regulator (CFTR) disease gene. Discordant phenotypes have been reported in siblings with this disorder and may be due to a complex interaction between genes other than the CFTR that are able to modulate the phenotype (Bronsveld *et al.*, 2001). Bardet-Biedl syndrome (BBS) is a genetic disorder that is characterised by progressive retinal dystrophy, obesity, renal malformations and delayed learning abilities (Beales *et al.*, 1999). The disorder occurs due to mutations at two loci that interact and modulate the phenotype of this disorder via epistasis (Badano *et al.*, 2003).

2.12 PROPOSED GENETIC TESTING

Genetic screening cannot currently be used for routine diagnosis of MH because of the low incidence of mutations observed in the RYR1 gene. However, the EMHG and the NAMHG have published guidelines for molecular genetic detection of susceptibility to MH to outline recommended procedures for the use of a genetic diagnostic test in certain instances (Urwyler *et al.*, 2001). Genetic screening of first-degree relatives of an MH proband has been proposed. Screening will be conducted once confirmation of a clinical reaction in a proband has occurred. In the absence of genetic screening, all family members of the proband are considered susceptible to MH, even though only a small proportion of the family will be affected. Affected probands in most cases inherit MHS from one of their parents. If a parent is identified as MHS, then each of the proband's siblings has a 50% chance of also being MHS. The offspring of the proband also has a 50% chance of being MHS and the grandchildren of the proband would be considered to be at 25% risk (until the genetic status of their parents can be determined).

The EMHG proposed guidelines for the genetic screening of MH in order to ensure consistency between MH centres using 15 selected causative RYR1 mutations (Robinson and Hopkins, 2001) include:

a. Mutation analysis of causative mutations in the RYR1 gene alone can be used to diagnose the remaining members of a family if the index case was diagnosed via the IVCT.
b. Segregation analysis of genetic markers close to known MHS loci may be used in genetic testing of the rest of the family, if no RYR1 mutations have been observed.

The second guideline (ii) makes the assumption that inheritance occurs in an autosomal dominant fashion. Therefore, this test will only be valid if recombination does not occur. However, data have provided evidence of recombination between MHS and the RYR1 gene, therefore it is likely to occur at other MHS loci (Levitt *et al.*, 1991). This approach is limited, as it will only be valid for characterisation of an extended MH pedigree, as large numbers of individuals will be required to establish linkage. At present, genetic testing is unable to replace the IVCT, and genetic analysis should be conducted in co-operation with IVCT centres. An MH individual diagnosed as MHS could be screened for the 15 mutations. If a mutation is detected in the proband, first-degree relatives can be tested for that specific mutation. Individuals who have the mutation are diagnosed as having MH without undergoing an IVCT. However, in individuals who do not harbour the mutation, an IVCT would be required to diagnose MH. As both genetic testing and the IVCT are subject to false negatives and positives, all individuals with a family history of MH should be considered susceptible. A European centre currently using genetic screening has reported diagnosis of MH in 50% of probands' relatives (Girard *et al.*, 2004). The NAMHG developed guidelines in 2002 for genetic MH diagnosis based on the European model of a panel of 17 RYR1 mutations to diagnose MH. The panel of mutations used is continually updated as new causative mutations are reported (Nelson *et al.*, 2004).

2.13 OBJECTIVE OF THE RESEARCH PROGRAMME

The broad aim of the MH research programme in the Centre for Genome Research is to determine the aetiology of MH in the South African population in order to develop a reliable and precise molecular genetic screening test to diagnose the disorder. In order to achieve this aim, all causative mutations of the RYR1 gene are screened to determine if any of the mutations are responsible for the pathogenesis of MH in the South African population. If mutations segregate exclusively with the MH phenotype, they may result in susceptibility to MH and could contribute to molecular diagnostic testing for related family members in the specific South African family. This finding may contribute to the global aim for MH, which is to introduce limited DNA testing for certain families, where family members could be screened for the specific mutation observed in the proband.

2.14 AIM OF THE STUDY

The long-term aim of the MH research program is to determine the exact aetiology of MH in the South African population. The molecular investigation in the study presented here

was performed in order to identify mutations in the coding region of the RYR1 gene that may be associated with MHS. The project will contribute to the broad aim, which is to screen all causative mutations of the RYR1 gene, to find if any of the mutations are associated with MHS in the South African population.

2.14.1 Specific aims

Individuals included in this study were selected as they displayed an MH episode upon exposure to triggering agents, had a family history of MH or were diagnosed as positive via the IVCT. Screening of all 106 exons of the human skeletal muscle RYR1 was conducted in order to identify reported and novel alterations that are responsible for the MH phenotype in 15 MHS South African probands. The specific aims of this study were:

- To identify novel or reported mutations that may occur within the coding region of the RYR1 gene hotspots in 15 South African MH probands via automated sequencing strategy.
- Screen all exons as well as limited regions of the intron sequence of the RYR1 gene that reside outside of the mutational hotspots using a sequencing methodology, in order to identify novel or reported mutations in 15 South African MH probands.
- Compare the mutations present within the RYR1 gene in the South African population to other reported populations.