CHAPTER FIVE

Conclusions

The hypermetabolic response that is triggered in MH by the presence of potent volatile anaesthetic agents can occur during an anaesthetic procedure or in the postoperative period. An MH episode occurs due to an uncontrolled increase in myoplasmic Ca^{2+}, which activates a series of biochemical processes. To date, the IVCT is the only pre-symptomatic test available for MH diagnosis, but it is not considered 100% accurate or a specific test to diagnose MH (Fletcher *et al.*, 1990; Larach, 1993). It is important to provide pre-symptomatic diagnosis of MH prior to anaesthesia, as safe alternative anaesthetic agents could be used for individuals with known MHS status. An MH diagnosis will reduce morbidity and mortality to an absolute minimum. Individuals who have survived a suspected MH episode during an anaesthetic procedure require a definitive diagnosis of MH in view of the autosomal dominant nature of the disorder, which could allow for the identification of other members of the extended family who may be at risk.

The RYR1 gene was identified as a candidate for predisposition to MH (MacLennan *et al.*, 1990), but other genes have also been implicated, as discussed in Section 2.11.4 (page 66). Elucidation of all the genetic alterations that lead to the MH phenotype will aid in the introduction of limited genetic testing for susceptibility to MH. The study presented here represents the first report in which all 106 exons of the RYR1 gene were screened for both novel and reported alterations that may result in MH susceptibility in the South African population. In previous studies conducted in the MH research programme at the Centre for Genome Research, South African MH probands were screened for published causative alterations via an RFLP or sequencing strategy. Using this approach has thus far only identified three RYR1 alterations in three families, which include the Arg614Cys, Val2168Met and Thr4826Ile alterations (Olckers, 1997; Havenga, 2000; Neumann, 2002; Dalton, 2004).

In the study presented here, eight different RYR1 alterations were observed in seven MHS families, of which six mutations were previously reported and two were novel. However,

the causative nature of the newly reported alterations still has to be proven. Furthermore, four South African MH probands harboured two RYR1 alterations each. This observation provides further evidence that compound heterozygous sequence variations may be associated with MH. The alterations may interact via epistasis, as described in Section 5.4 (page 406), and cumulatively result in the MH phenotype. As discussed in Section 5.2.2.2 (page 400), compound heterozygous RYR1 alterations have been reported in individuals diagnosed with MH or CCD in other populations.

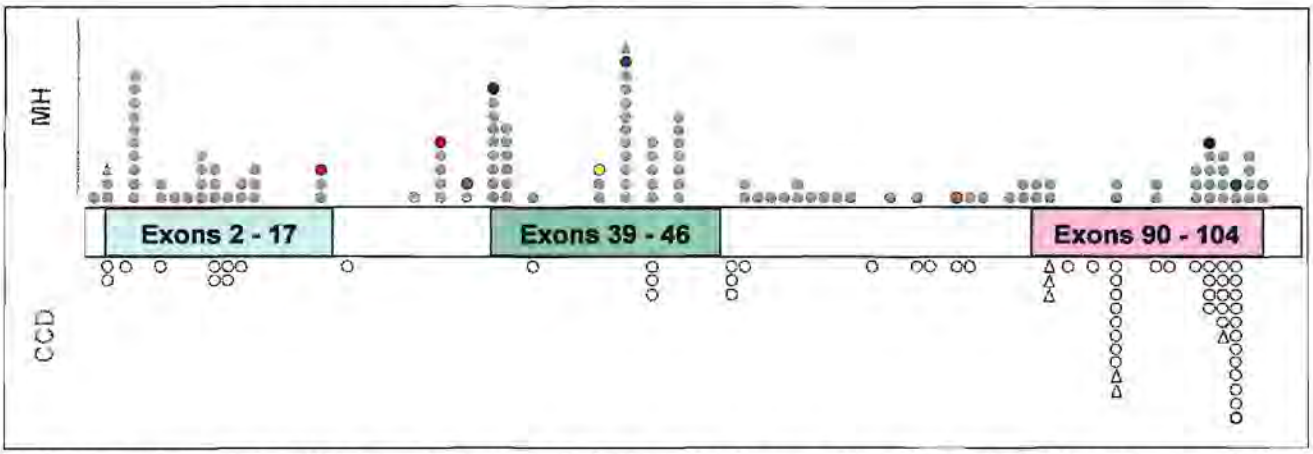
Three of the alterations observed in the South African MH population were detected outside the mutational hotspots in exons 34, 38 and 73. Therefore, alterations that may result in the MH phenotype are not limited to the widely accepted hotspots. In the study presented here, 38% (3/8) of the RYR1 alterations that were observed in South African MH probands were determined to be outside the mutation hotspots. Other studies, which have screened the entire coding region of the RYR1 gene, have also identified alterations outside of hotspot one, two and three. Ibarra *et al.* (2006) indicated that 33% (11/33) of RYR1 alterations did not reside in the widely accepted hotspots, while Galli *et al.* (2006) identified 23% (10/43), Sambuughin *et al.* (2005) identified 14% (3/21) and Monnier *et al.* (2005) identified 4% (3/80). This suggests that the RYR1 gene may contain other critical domains that can result in the MH phenotype if mutated. Therefore, screening of the entire coding region of the RYR1 gene is crucial for genetic investigations into MH susceptibility.

The prevalence of RYR1 alterations in the study presented here was estimated to be 47% (7/15 probands). However, exclusion of family MH102, which previously displayed linkage to chromosome 2q (Olckers, 1997), increases the prevalence to 50% (7/14). There is a lower frequency of RYR1 alterations in the South African population compared to similar studies conducted in Japan, North America, Italy and France (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006). The lower frequency of alterations observed in the RYR1 gene may be due to a founder effect observed in the South African population, as reported for other disorders in this population. Therefore, a single alteration that has thus far not been determined may be present in the non-coding region of the RYR1 gene that was not investigated in the study presented here or at another locus within the genome. This mutation may, due to founder effect, be highly prevalent in this population.

In addition, unknown factors such as exposure of the South African population to different environmental conditions compared to populations worldwide may contribute to the lower

frequency of RYR1 alterations, as described in Section 5.5.3.2 (page 410). The lower frequency of alterations may also be due to the fact that the MH phenotype may not always be readily recognised in South Africa. Individuals may die under anaesthesia, but the presence of an MH alteration is never investigated. Thus far, only nine different RYR1 alterations have been observed in South African MH probands in the Phase 1, 2 and 3 studies. The localisation of alterations observed in the South African MH probands in comparison to alterations observed in other populations is depicted in Figure 5.1. The data reported in the study presented here allow for a more accurate estimation of the frequency of RYR1 alterations in the South African MHS population.

Figure 5.1: Comparison of RYR1 alterations in the South African MH population to the worldwide localisation of alterations observed in patients diagnosed with MH or CCD



Nucleotide substitutions indicated above the gene as solid grey circles (●) were observed in malignant hyperthermia susceptible individuals; deletions indicated above the gene as solid grey triangles (▲) were observed in malignant hyperthermia susceptible individuals; nucleotide substitutions indicated below the gene as white circles (○) were observed in individuals with CCD; deletions indicated below the figure as white triangles (△) were observed in individuals with CCD. The three mutational hotspots are indicated as blue (hotspot 1), green (hotspot 2) and pink (hotspot 3) blocks, respectively. Adapted from Wu *et al.* (2006). Nucleotide substitutions indicated above the figure as coloured circles were observed in South African MH individuals; pink circle (●) = Arg614Cys; red circle (●) = Pro1787Leu; purple circle (●) = Gly2060Cys; green circle (●) = Val2168Met; dark yellow circle (●) = Arg2336His; light blue circle (●) = Phe2364Val; orange circle (●) = Glu3583Gln; blue circle (●) = Thr4826Ile; light green circle (●) = Gly4935Ser.

The study presented here supports previously reported findings that MH in the South African population is due to a novel genetic aetiology (Olckers 1997; Havenga, 2000; Neumann, 2002; Dalton, 2004). In addition, the study provides further evidence that susceptibility to MH may be due to several interacting factors, as discussed in the following sections of this chapter.

5.1 PROPOSED MH MODEL

MH susceptibility in humans has been reported to be inherited as an autosomal dominant trait (Denborough *et al.*, 1962). However, there is evidence that regulation of Ca^{2+} homeostasis in skeletal muscle is a function of several gene products (Gronert, 1980). *In*

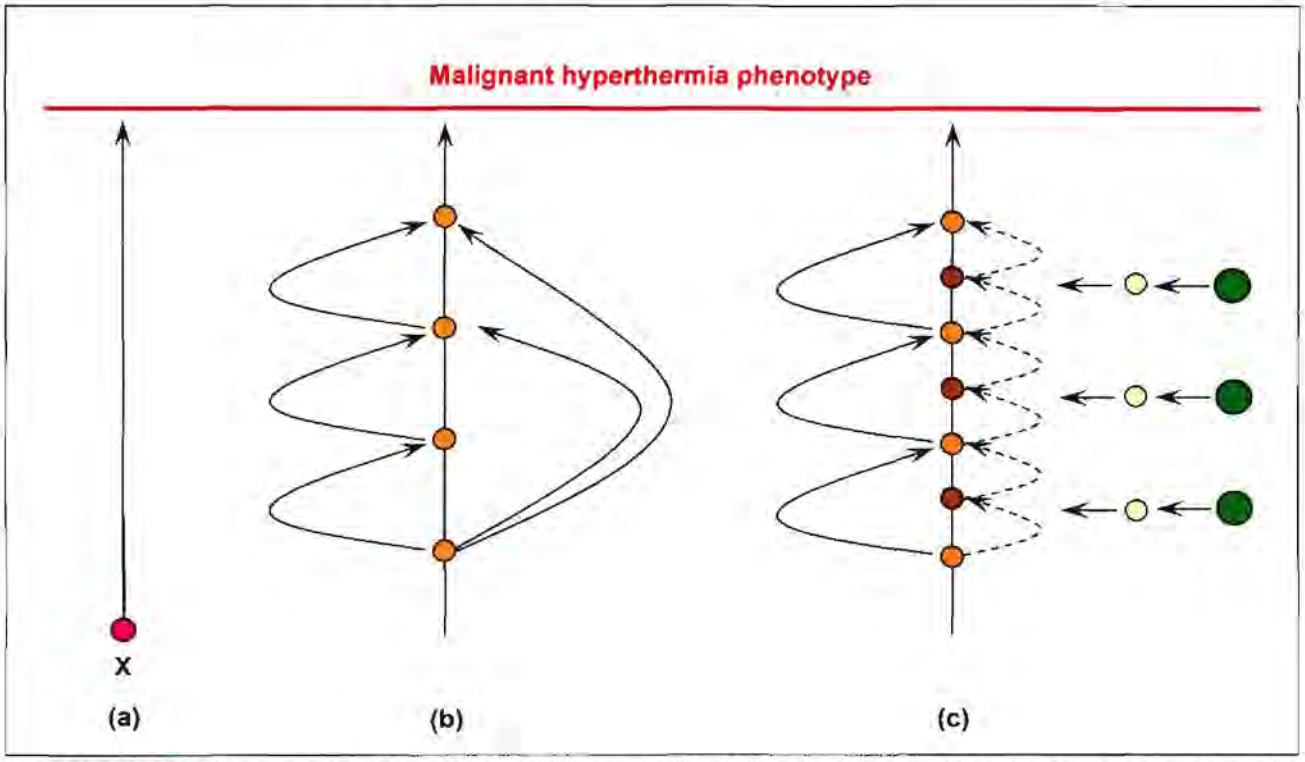
addition, other genetic loci have been implicated as being associated with MH, as discussed in Section 2.11.4 (page 66). Furthermore, compound heterozygous individuals were observed in the study presented here and have previously been reported in other populations (Monnier *et al.*, 2002; Ibarra *et al.*, 2006). These observations suggest that susceptibility to MH may be due to multifactorial inheritance rather than an autosomal dominant mode of inheritance.

To explain the possible multifactorial aetiology of this disorder in the South African MH population, a model for the network of genetic and environmental factors that can potentially influence the expression of the MH phenotype is illustrated in Figure 5.2. It is discussed in subsequent sections of this chapter. MH may be due to the inheritance of a single major genetic factor, as indicated by (X) in Figure 5.2, or may be due to several intermediate and/or minor genetic factors from different loci or from a single locus. In addition, the MH phenotype may be due to inheritance of minor genetic and epigenetic factors. The model indicates that each interacting factor would exert a small effect, which would be cumulative, and that the development of the phenotype can only occur when a threshold is reached. The threshold will be attained due to the presence of a combination of several genetic and/or environmental factors, as discussed in Section 5.3.1 (page 405). The model indicates that the MH phenotype could be due to:

a. A major genetic factor (X), as denoted by a pink circle (•), which will be sufficient to result in the MH phenotype without any additional factors being required,
b. Several intermediate genetic factors in the RYR1 gene or other loci, illustrated by orange circles (•) that, in the absence of a major genetic determinant, may interact with each other. Various combinations of intermediate genetic factors will have a cumulative effect and lead to MH susceptibility,
c. The presence of several interacting intermediate or minor genetic factors, illustrated by brown circles (•) or epigenetic factors, illustrated by a yellow circle (•) and environmental factors, illustrated by green circles (•) of which various combinations may lead to MH susceptibility, via epigenetic modifications.

Therefore, several combinations of genetic factors that could be influenced by environmental factors exist, which may all have the potential to interact and ultimately culminate in the disorder.

Figure 5.2: Model for the network of genetic and environmental factors that can potentially influence the expression of the MH phenotype in the South African MH population



The pink circle (●) indicates = major genetic factor; orange circles (●) indicate intermediate genetic factors; brown circles (●) indicate minor genetic factors; yellow circles (●) indicate epigenetic factors; green circles (●) indicate environmental factors; arrow (↑) = indicates factors leading to an MH phenotype. a = indicates the occurrence of the MH phenotype due to a major genetic determinant; b = indicates the occurrence of the MH phenotype due to several interacting minor genetic determinants and c = indicates the occurrence of the MH phenotype due to several interacting minor genetic determinants, polymorphisms and epigenetic modifications.

Several lines of evidence provide support for the proposed MH model indicated in Figure 5.2. Previously reported studies corroborate the hypothesis that a network of genetic and environmental factors may result in susceptibility to MH. Evidence for the proposed model, described in Section 5.1 (page 393) is discussed in subsequent sections of the chapter and includes the following:

a. Reported multifactorial mode of inheritance in pigs diagnosed with PSS, a disorder that is analogous to MH in humans, as discussed in Section 5.2.1 (page 396).

b. Identification of alterations at two different loci that interact and result in the MH phenotype, as discussed in Section 5.2.2.1 (page 399).

c. Detection of compound heterozygous alterations of the RYR1 gene in patients diagnosed with MH or an associated disorder, as discussed in Section 5.2.2.2 (page 400).

d. Presence of a single alteration of the RYR1 gene, which may be present in numerous RyR1 isoforms in different tissues, as discussed in Section 5.2.3 (page 402).

e. Reported association of epigenetic modifications and core myopathies, which are disorders related to MH, as discussed in Section 5.3 (page 404).

f. Identification of unknown factors such as differences in environmental conditions that can influence the clinical expression of MH, as discussed in Section 5.3.1 (page 405).

g. Interaction of RYR1 alterations, different loci associated with MH and RyR isoforms via epistasis, as discussed in Section 5.4 (page 406).

5.2 MULTIFACTORIAL MODE OF INHERITANCE

Gronert (1980) has previously suggested that the PSS or the MH phenotype in animals and humans respectively, may occur due to a multifactorial mode of inheritance. A multifactorial mode of inheritance has been reported in numerous disorders. Krueger and Ellis (2005) reported that psoriasis, a chronic inflammatory skin condition, occurs due to multifactorial inheritance, and susceptibility to this disorder has been associated with eight different loci. The authors indicated that only certain patients inherited the disorder in an autosomal dominant manner.

Several lines of evidence support the multifactorial mode of inheritance, as illustrated in the MH model in Figure 5.2, including:

a. Animal models, as discussed in Section 5.2.1 (page 396).

b. The observation of compound heterozygous alterations in several individuals diagnosed with MH or an associated disorder, as discussed in Section 5.2.2.2 (page 400).

c. Association of more than one alteration with variability in the MH phenotype, as discussed in Section 5.2.2.3 (page 401).

5.2.1 MH equivalent disorders in pigs

Denborough *et al.* (1962) suggested that MH is inherited as an autosomal dominant trait. The analogous condition in pigs is known as PSS. It has generally been assumed that the human and pig syndromes are identical as they both have similar phenotypes and are due to alterations in the RYR1 gene (Brit and Kalow, 1970).

However, several differences between PSS in pigs and MH in humans have been reported:

a. PSS in pigs is inherited as an autosomal recessive trait (Mabry *et al.*, 1981).
b. In pigs, the syndrome is more frequently triggered by stress alone (Mitchell and Heffron, 1982), whereas in humans this occurrence is rare (Fagerlund *et al.*, 1997).
c. In 95% of susceptible pigs, PSS will develop when susceptible pigs are exposed to halothane (Mitchell and Heffron, 1982), however, variation in the triggering power of halothane in humans has been reported and individuals with a causative RYR1 alteration may not develop the clinical MH phenotype at all when exposed to triggering anaesthetics (Deufel *et al.*, 1995).
d. Generally, PSS is due to the presence of a single alteration, namely Arg615Cys, which corresponds to the Arg614Cys alteration in humans, but in pigs its increased prevalence is due to a founder effect which may be due to inbreeding of susceptible pigs. The Arg614Cys alteration has thus far only been reported in 2-7% of humans diagnosed with MH (Gillard *et al.*, 1991).
e. PSS is diagnosed in susceptible pigs via screening with halothane. However, this method only identifies animals that are highly susceptible to the disorder and animals that are less susceptible are not diagnosed with PSS (Gronert, 1980). This is in contrast to humans, where MH is diagnosed via the use of both halothane and caffeine (Ellis *et al.*, 1972).

Possible reasons for the differences observed in phenotype and genetic susceptibility to MH between humans and pigs are discussed in Section 5.2.1.2 (page 398). In addition to several differences, the clinical phenotypes of PSS and MH have several similarities:

a. Both disorders are pathophysiologically similar and result from defective Ca^{2+} homeostasis (Denborough *et al.*, 1962, Mitchell and Heffron, 1982).
b. The characteristics of both syndromes are similar, e.g. hypermetabolism, rapid increase in body temperature, rhabdomyolysis and cardiac arrhythmia (Kalow *et al.*, 1970).
c. Both disorders can be triggered by halogenated anaesthetics and depolarising muscle relaxants.

5.2.1.1 PSS and PSES in pigs

As PSS in pigs resulted in accelerated metabolism and deterioration of muscle, heterozygous animals were bred in order to obtain animals that would not be susceptible to the disorder. The heterozygous animals were characterised by large muscle mass similar to susceptible pigs, and stress resistance comparable to PSS negative pigs.

However, further studies indicated that a syndrome similar to PSS can be evoked by external triggers in heterozygous animals and that their muscles may react abnormally when exposed to high concentrations of various agents (Wedel *et al.*, 1993). The disorder in pigs that are heterozygous for this genetic defect is known as pale, soft, exudative pork syndrome (PSES), which is associated with a rapid rate of glycolysis (Lawrie, 1960). MH in humans has been reported to be analogous to the condition PSES in pigs (Harrison *et al.*, 1969) and the three syndromes (MH, PSS and PSES) display similar metabolic changes, namely high rate of glycolysis and the production of lactate (Mitchell and Heffron, 1982). However, several differences between PSES and PSS have been identified:

a. Susceptible pigs with PSES may not harbour the Arg615Cys alteration,
b. Pigs with PSES may have a normal or intermediate response to halothane and
c. PSES in pigs is less likely to be triggered by stress (Mitchell and Heffron, 1982).

5.2.1.2 Modes of inheritance

It is likely that PSES, MH and PSS are the same syndrome. The autosomal recessive inheritance and high prevalence of pigs with PSS may have occurred only because of the severe inbreeding that has taken place and this could have resulted in differences in the disease phenotype compared to PSES and MH. In addition, the clinical spectrum of the phenotype that is observed in humans is not observed in pigs, as only highly susceptible pigs with a severe disease phenotype are identified due to the characteristics of the diagnostic test used. MH has been observed in other animals, as discussed in Section 2.11.2 (page 41). MH in animals other than the pig follows a pattern of autosomal dominant inheritance and is not always due to the presence of an Arg615Cys alteration.

MH, PSS and PSES in animals and humans may therefore occur due to a multifactorial mode of inheritance (Gronert, 1980), as illustrated in the MH model in Figure 5.2 (page 395). The hypothesis of a multifactorial mode of inheritance has been supported by a study conducted by Fletcher *et al.* (1993). The authors indicated that alterations in the RYR1 gene in PSS pigs may be necessary but are not sufficient for the development of the phenotype. Therefore, MHS, PSS and PSES could occur via more than one interacting gene or allele, and the pattern of inheritance may thus range from recessive to dominant, with graded variation in between.

Evidence to support the above-mentioned hypothesis that MHS may result from the interaction of more than one gene or allele is provided in subsequent sections of this

chapter. The possibility of heterogeneous origins of this disorder is supported by the identification of more than one RYR1 alteration in MHS probands worldwide and by reported locus heterogeneity associated with MH, as discussed below.

5.2.2 Genetic heterogeneity and MH

The MH model discussed in Section 5.1 (page 393) illustrates that MH may be due to a single dominant alteration or may be due to the presence of several alterations from a single or multiple locus or multiple loci. Therefore, two or more alterations at the same or different genetic susceptibility loci may contribute either intermediate or minor phenotypic effects. In addition, the presence of more than one alteration may result in variability in the phenotype depending on the nature of the alterations. MH has been reported to present with evidence of locus and allelic heterogeneity (Levitt *et al.*, 1992; Iles *et al.*, 1994; Sudbrak *et al.*, 1995; Monnier *et al.*, 1997; Robinson *et al.*, 1997), as discussed in subsequent sections of this chapter.

5.2.2.1 Locus heterogeneity

The model discussed in Section 5.1 (page 393) illustrates that several interacting loci can cumulatively result in the MH phenotype. Therefore, the single-gene model may be incorrect for certain MHS individuals, which is supported by the identification of MH susceptibility loci on other chromosomes. Six other susceptibility loci suggested to result in MHS have been mapped to chromosomes 1, 2, 3, 5, 7 and 17 and should be analysed to determine the exact molecular defect of this heterogeneous disorder. Thus far, suggested candidates include the DHPR and the human skeletal muscle SCN4A α-subunit gene. The functioning of these proteins may be altered as a result of a mutation or due to the effects of accessory proteins, which could lead to the phenotypic expression of MH.

Therefore, other loci may play a role in susceptibility to MH in the South African population and other populations worldwide. Reports of linkage in MH families of South African origin to chromosome 17q11.2-q24 and 2q (Olckers *et al.*, 1992; Vita *et al.*, 1995; Olckers *et al.*, 1999) support this observation. In addition, absence of RYR1 alterations in the coding region of the gene in eight probands included in the study presented here may also indicate that other loci are associated with MHS in these individuals. The presence of linkage to other susceptibility loci indicates that abnormalities in several proteins involved

in the regulation of E-C coupling could result in susceptibility to MH. However, the exact role that MH loci play in the pathogenesis of this disorder has not yet been determined.

Interaction of two different loci that may result in the MH phenotype is supported by a study conducted by Monnier *et al.* (2002). The authors identified an MH family in which the proband inherited an Arg1086His mutation of the CACNA1S gene from his father and the Pro4973Ile mutation in the RYR1 gene from his mother. Thus far, this is the only report of a compound heterozygote bearing two different mutations from two different MHS linked genes. However, as only two candidate genes have thus far been identified, further studies of other candidate loci may identify additional individuals that harbour different alterations from two different MH-associated genes in the future. In addition, Robinson *et al.* (2000) reported that genetic effects on chromosomes 3q, 5p and 7q can influence an individual's predisposition to MHS in families that demonstrate linkage to RYR1 (19q). Therefore, these results further support the hypothesis that more than one gene can influence the MH phenotype.

5.2.2.2 Allelic heterogeneity

In addition to several interacting loci, the model illustrated in Figure 5.2 (page 395) demonstrates that several interacting alleles of the RYR1 gene can cumulatively result in the MH phenotype. This observation is supported by the identification of compound heterozygous alterations, which have been reported in individuals diagnosed with MH or with another disorder associated with MH, namely CCD. Wu *et al.* (2006) reported compound heterozygous alterations of the RYR1 in three unrelated patients diagnosed with CCD. Ibarra *et al.* (2006) identified six patients diagnosed with MH that harboured potentially causative compound heterozygous sequence variations. Compound heterozygous alterations have also been reported in patients affected by CCD and presenting with a foetal akinesia syndrome (Romero *et al.*, 2003). Compound heterozygous alterations in patients diagnosed with MH have only recently been described, as screening of the entire coding region of the RYR1 gene has thus far only been conducted in certain populations. In the study presented here, four South African MH probands harboured two RYR1 alterations. The inheritance of two alterations that may be associated with MH can result in a more severe phenotype. Ibarra *et al.* (2006) reported that the presence of two alterations resulted in an increase in CICR enhancement. Monnier *et al.* (2002) reported four families with more than one allele and indicated that their CK concentrations were significantly higher than those of individuals

with a single alteration. Romero *et al.* (2003) identified that probands with two alterations had a more severe clinical phenotype. However, parents of the probands that harboured single alterations each were clinically unaffected. Therefore, the above-mentioned study supports the proposed MH model and indicates that several alterations can cumulatively result in the MH phenotype. In the study presented here, four probands harboured one alteration that does not occur in any of the three hotspots of the RYR1 gene. Therefore, screening of the entire coding region of the RYR1 gene should be conducted in various populations in order to identify all MHS individuals that harbour compound heterozygous alterations that may be associated with the MH phenotype.

5.2.2.3 Locus and allelic heterogeneity correlated to the MH phenotype

The model described in Section 5.1 (page 393) indicates that a single dominant alteration can result in the MH phenotype or several alterations with minor phenotypic effects may interact and cumulatively result in the disorder. The model therefore suggests that an individual that harbours a single minor alteration may exhibit a weak IVCT response and the individual will be diagnosed as MHE or MHN, whereas an individual that inherited two or more alterations from a single locus or at different loci may have a strong IVCT response and will be diagnosed as MHS (Phillips *et al.*, 1994; Brown *et al.*, 2000). In contrast, a single dominant alteration may result in a strong IVCT response on its own. This hypothesis is supported in the literature.

Certain alterations in the RYR1 gene could make the RyR1 protein more sensitive to specific ligands. In addition, alterations at different loci, as discussed in Section 5.2.2.1 (page 399), may vary in their functional consequences compared to the RYR1 gene. This may be an explanation for varying clinical symptoms of MH crisis in humans (Fiege *et al.*, 2002). Robinson *et al.* (2002) indicated that RYR1 alterations that resulted in either CCD or MH had a more severe halothane and caffeine response compared with alterations that were only associated with MH. In addition, alterations that reside near the N-terminal domain of the RyR1 protein had a significantly increased caffeine-to-halothane tension ratio compared to alterations that reside in the central domain of the protein. Ellis *et al.* (1972) proposed using pathogenic features of muscle and contracture responses as indicators of MHS. However, the authors indicated that the pathological features varied, even among siblings and a diagnostic pattern could not be observed. Thus far, the functional consequence of alterations at other loci has only been determined for the CACNA1S gene. The Arg1086His alteration of the CACNA1S gene results in an increased

sensitivity of the RyR1 protein to activation by both endogenous (voltage sensor) and exogenous (caffeine) activators. However, the alteration has a lower caffeine-to-halothane tension ratio compared to alterations of the RYR1 that reside near the N-terminal (Weiss *et al.*, 2004). Therefore, the above-mentioned data support multifactorial inheritance, in which different genes or alleles in combination or on their own may result in a spectrum of susceptibilities.

In addition to the presence of two alterations at a single locus or multiple loci, the clinical variability of the MH phenotype may be due to a single alteration in the RYR1 gene being expressed in a tissue-specific manner dependent on how the RyR1 isoform is spliced in a specific tissue. RyR1 isoforms have been reported in a variety of tissues, as discussed below.

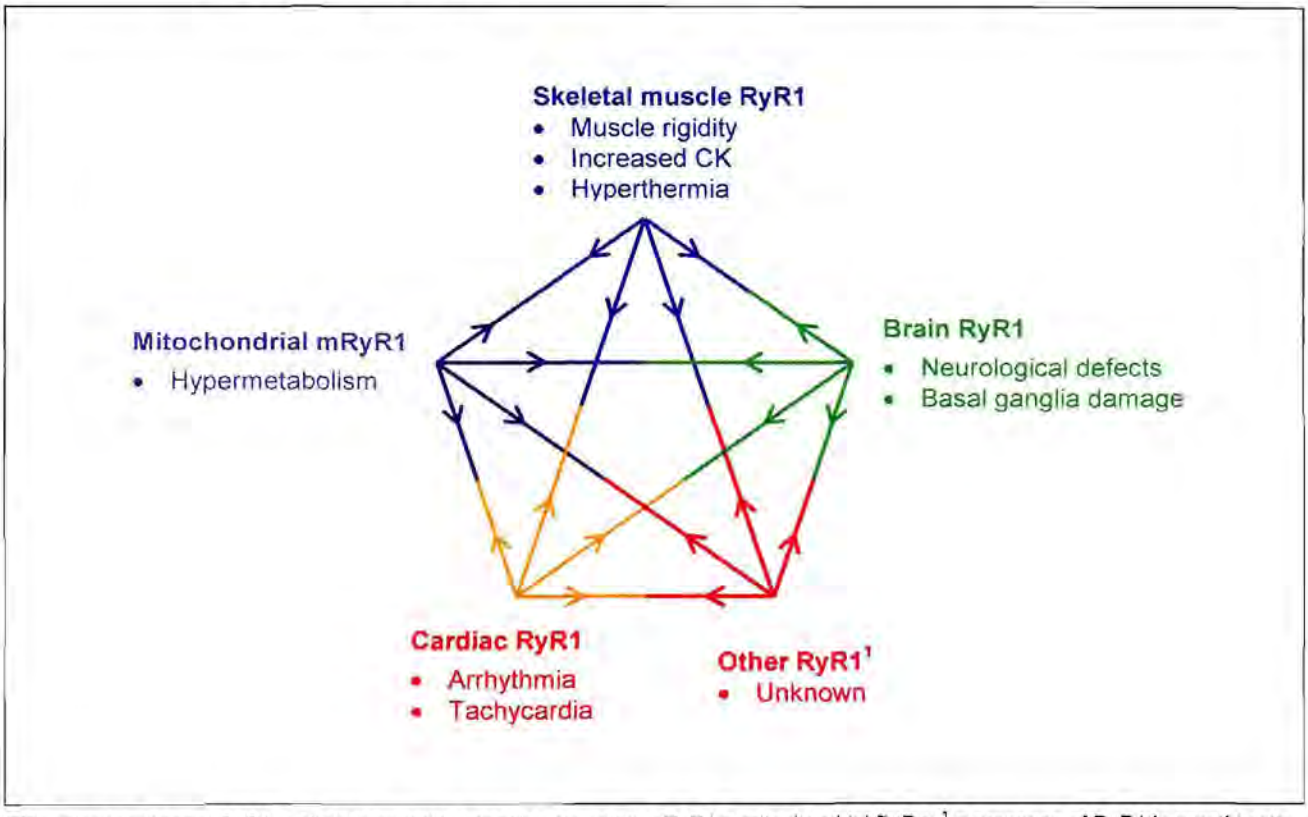
5.2.3 Role of the RyR1 protein in other tissues

In the MH model described in Section 5.1 (page 393), a single dominant alteration or several intermediate or minor alterations could result in the MH phenotype. However, it may also be possible that variability in the clinical symptoms associated with MHS may be due to a single alteration in the RYR1 gene that is present in numerous different RyR1 isoforms. Holliday (2002) indicated that alternative splicing of gene transcripts occurs in different cell types and results in the formation of particular polypeptide chains and proteins appropriate for a specialised cell. Isoforms of the skeletal muscle RyR1 protein have also been identified in a variety of tissues (Futatsugi *et al.*, 1995), as discussed in Section 2.9.3 (page 26). In addition, currently unidentified RyR1 proteins may be observed in other tissues, but to date have not yet been detected. The RyR1 isoforms that exist in a variety of tissues have demonstrated differences with regard to channel activity and are generated via alternative splicing of the RYR1 gene (Futatsugi *et al.*, 1995).

As RyR1 isoforms exist in a variety of tissues and may occur due to alternative splicing of the RYR1 gene, a single alteration may affect both the functioning of the skeletal muscle RyR1 and the functioning of an RyR1 channel in another tissue. However, depending on the position of the alteration, a single mutation that has no effect on the channel in skeletal muscle may result in an altered channel in another tissue and vice versa. This hypothesis may in part explain the variability of clinical symptoms that are observed in an MH episode and the reason that so many different disorders with a variety of symptoms are associated with MH. A proposed schematic model indicating that the spectrum of symptoms may be

due to defects in the RYR1 in a variety of tissues is indicated in Figure 5.3. The diagram indicates that mild MH reactions may be due to defects in a single tissue, whereas fulminant MH episodes may be due to defects in several tissues. Listed underneath each heading is the possible symptoms observed in MH that may be due to the presence of an alteration in the specific tissue, however the diagram does not account for symptoms that may have resulted as a secondary response to the onset of an MH episode in skeletal muscle. In addition, this model does not account for the presence of more than one RYR1 alteration that may result in the MH phenotype, which is discussed in Section 5.2.2.2 (page 400).

Figure 5.3: Proposed clinical spectrum of MH due to defects in the RyR1 protein in various tissues



CK = creatine kinase; RyR1 = skeletal muscle ryanodine receptor; mRyR1 = mitochondrial RyR1; [1] = presence of RyR1 in parotid cells, in pancreatic cells, the liver, and non-excitable lymphocytes. Coloured lines = indicates possible interactions between tissues with a RyR1 mutation that may result in some of the symptoms associated with an MH episode. Overlap = indicates a RyR1 mutation that plays a role in more than one tissue.

The hypothesis that alterations in the RYR1 gene can affect many different RyR1 isoforms in a variety of tissues is supported by findings in the pig animal model of MH. In susceptible pigs, a mutation detected in skeletal muscle RyR1 was also observed in the brain, suggesting a neurological aetiology of MH (Ledbetter *et al.*, 1994). The alteration expressed in the brain resulted in altered Ca^{2+} channel activity (Mickelson *et al.*, 1988; Fill *et al.*, 1990), which could be affected by the presence of halogenated anaesthetics (Louis

et al., 1992). The mutated channel could alter other brain functions, including increased neurotransmitter release or signalling for an increase in sympathetic nervous system activity. Differences in the concentrations of neurotransmitters between MHS and MHN pigs have been observed and could in part explain the role of stress in the development of an MH episode (Adeola *et al.*, 1993). In humans a selective neurological deficit of the cerebellum and basal ganglia has been reported in one individual following an MH episode (Park *et al.*, 2004). In addition, Beutner *et al.* (2005) identified a mRyR1 in the mitochondria of the rat heart, as discussed in Section 2.10.4 (page 38). It is possible that mRyR1 is also present in the mitochondria of other tissues and in other organisms. As discussed in Section 2.10.1 (page 35), an alteration present in the mRyR1 may result in excessive amounts of Ca^{2+} being transported into the mitochondria following exposure to triggering anaesthetics, due to altered mRyR1 channel activity. The increased concentration of Ca^{2+} in the mitochondria may in turn result in uncoupling of oxidative phosphorylation, which would result in hypermetabolism, cell damage and a decreased ability of the mitochondria to synthesise ATP and creatine phosphate.

The proposed MH model indicates that several alterations may interact and cumulatively result in the MH phenotype. However, in addition to these interactions, epigenetic modifications of allele expression may occur, which could be a source of phenotypic variation. Epigenetic modifications can be influenced by diet, stress and other environmental factors, as discussed in the subsequent sections of this chapter.

5.3 EVIDENCE OF EPIGENETIC MODIFICATIONS IN MH SUSCEPTIBILITY

The model depicted in Section 5.1 (page 393) indicates that the MH phenotype may occur due to the inheritance of several minor genetic and epigenetic factors. The epigenetic factors refer to a mechanism whereby the control of gene activity is based on critical interactions between DNA and proteins. In this way, genes may be expressed or silenced, which could alter the activity of the protein (Holliday, 1989). Several mechanisms of epigenetic modifications, including genomic imprinting, germline epimutation and epigenetic polymorphisms that occur due to aberrant DNA methylation and/or histone modification (Verona *et al.*, 2003) may be associated with variation in protein expression.

A recent study conducted by Zhou *et al.* (2006b) provides evidence suggesting that the MH phenotype may occur due to epigenetic modifications. The authors identified a cohort of recessive core myopathy patients that displayed epigenetic allele silencing of the RYR1.

Core myopathies such as CCD and MmD have been reported to be associated with MH (Denborough *et al.*, 1973; Monnier *et al.*, 2003). Zhou *et al.* (2006a) identified a cohort of patients in which the RyR1 protein was transcribed from only one allele. The epigenetic allele silencing was tissue-specific and 55% of individuals displayed monoallelic expression in the skeletal muscle but not in other tissues. The authors suggested that the mechanism was genomic imprinting, which is mediated by DNA methylation due to:

a. Allele silencing, which depends on the sex of the transmitting parent,
b. The tissue-specific pattern of expression, and
c. Treatment with DNA methyltransferase inhibitor 5-azaC in one patient's primary myoblasts resulted in restoration of biallelic expression.

Therefore, the data suggests that major differences exist in the way that RYR1 mutations affect patients with core myopathies, via variations in RyR1 protein expression, stability and/or activity (Zhou *et al.*, 2006b). Therefore, as illustrated in Figure 5.2 (page 395), in a proportion of MHS individuals, intermediate or minor genetic factors that on their own or in combination do not significantly alter RyR1 channel function may result in the MH phenotype due to epigenetic modifications in which the wild-type allele is silenced. In addition to genomic imprinting, epigenetic mechanisms that modulate the MH phenotype, that to date have not been identified may be present.

5.3.1 Clinical expression of the MH phenotype and environmental factors

A variety of environmental factors, such as nutritional and toxicological effects, may influence the clinical expression of MH. In this way, a particular genotype may have the potential to express a particular phenotype, which may only occur when an individual is exposed to a particular environmental factor (Flatt, 2005). The model illustrated in Figure 5.2 (page 395) indicates that genetic determinants of MH may be influenced via epigenetic modifications due to the formation and maintenance of epigenetic states, which may be modified by environmental factors (Jaenisch and Bird, 2003), including radiation, nutrition, infectious agents, hormones, drugs, heavy metals and chemicals (Feil, 2006). Stress may also be included in this category as a facilitating factor. Duthie and Arthur (1993) reported that exposure to free radicals resulted in the rapid efflux of Ca^{2+} in MH-susceptible animals.

Variability in clinical symptoms may be a result of complex interactions between genes and/or the environment. An MH episode may only occur if a specific threshold is reached. This hypothesis could also explain why in certain patients, exposure to triggering agents

initially does not result in an MH episode. A complex network of environmental factors, such as exposure to drugs, environmental toxins, stress and the presence of specific interacting genetic loci, which have intermediate or minor phenotypic effects, may all lead to an MH episode. Therefore, as illustrated in the model depicted in Figure 5.2 (page 395), it is possible that environmental factors play a role in MH susceptibility.

Besides epigenetic modifications of DNA (as described above), the MH phenotype may occur due to the interaction of several genes or their mRNA or protein products. The epistatic interaction will result in the expression of one of the genes, while the other would be suppressed, as discussed below.

5.4 ROLE OF EPISTASIS IN MH SUSCEPTIBILITY

Thus far, epistatic interactions have not been reported to be associated with any skeletal muscle disorder. However, as six other susceptibility loci have been reported to be associated with the MH phenotype, as discussed in Section 5.2.2 (page 399), there is a possibility that epistatic interactions between different loci may occur, which could alter the MH phenotype. This is supported by the identification of two different alterations from two different causative genes in one MH proband, as discussed in Section 5.2.2.1 (page 399). In addition, as compound heterozygous alterations of the RYR1 gene have been reported, as discussed in Section 5.2.2.2 (page 400), it is likely that these alterations interact via epistasis and result in MHS. Furthermore, several different alterations and SNPs may contribute minor genetic effects and interact via epistasis.

Interaction of RyR isoforms, whereby one can affect the phenotypic expression of the other, has been observed, which may also support the possible role of epistasis in the development of the MH phenotype. Jiang *et al.* (2003) reported that a RyR3 isoform that occurs as a result of alternative splicing is able to interact and suppress the activity of another isoform (RyR2), via the formation of a heteromeric channel complex. Therefore, it is possible that RyR isoforms could interact with RyR1 via epistasis and alter the expression of the RyR1 protein. As illustrated in the model depicted in Figure 5.2 (page 395), it is consequently possible that epistasis plays a role in MH susceptibility.

Although MH has been investigated on both a molecular and clinical level in several different populations, the exact aetiology of this disorder has not been elucidated. The

proposed MH model thus provides a new dimension to the understanding of the complexity of MH in the South African population, as outlined in this chapter.

5.5 IMPLICATIONS OF THE MH MODEL

The following questions remain unanswered when studying the complex aetiology of MH, all of which may be answered by the model illustrated in Figure 5.2 (page 395). The following remains to be clarified:

a. Variability in clinical expression and onset of MH, as well as the relationship between MH and other myopathies that is still to be determined, as discussed in Section 5.5.1 (page 407).
b. Reported discordance between genotypes and IVCT results, as discussed in Section 5.5.2 (page 408).
c. Difference in distribution and prevalence of RYR1 gene alterations in a variety of geographical regions and inability to identify common mutations in many populations, as discussed in Section 5.5.3 (page 409).
d. Absence of RYR1 alterations in individuals diagnosed as MHS, as discussed in Section 5.5.4 (page 411).

5.5.1 Clinical features and diagnosis

The clinical presentation of MH is not uniform and the time of onset is variable (*Denborough et al.*, 1962). Currently, there are no clinical features that are specific to MH. Diagnosis largely depends on knowledge of features that can occur during an MH reaction, recognition of these features occurring in a pattern consistent with a developing MH reaction, and exclusion of other causes of these clinical features, as discussed in Section 2.2 (page 6). Several reasons may explain variation in the clinical features and onset of the MH phenotype. As discussed in Section 5.1 (page 393), several different alterations within the RYR1 gene or within different loci may cumulatively result in the MH phenotype. Each different alteration may vary in its functional effect, depending on the nature and location of the alteration. Mutant RyR1 channels expressed in HEK-293 cells demonstrate a range of sensitivities to caffeine and halothane upon functional analysis (Tong *et al.*, 1997). In addition, the functional consequences of alterations at loci other than the RyR1 have not been determined. The collective functional effect of several minor or intermediate genetic factors has thus far also not been elucidated. In addition, as discussed in Section 5.2.3 (page 402), as RyR1 isoforms exist in a variety of tissues, an

altered RyR1 channel may occur in a variety of tissues or in a single tissue, which may result in the variability of clinical symptoms observed in MH patients.

5.5.2 Discordance

Discordance has been reported in two South African individuals for the Arg614Cys alteration (Olckers, 1997; Havenga, 2000). Both individuals were diagnosed as MHN even though they harboured the alteration. In addition, Olckers (1997) indicated that this alteration did not segregate to an MHS individual in a single family that harboured the alteration. Functional analysis in skeletal muscle of rabbits has indicated that the alteration results in both increased sensitivity to activation by Ca^{2+} and an increased rate of Ca^{2+} release compared to that of SR vesicles obtained from unaffected muscle (Mickelson *et al.*, 1990). Therefore, the causative status of this mutation remains unchallenged. The report of discordance in these individuals is likely to indicate that the IVCT diagnosis was either a false positive in the MHS individual or a false negative in the MHN individual. Studies have indicated that the threshold used to indicate a positive result in the IVCT influences the extent of genetic linkage.

A high rate of discordance has been reported for two more common mutations, namely Gly341Arg and Arg614Cys in other populations (Hopkins, 2000). Monnier *et al.* (2005) reported that the discordance rate among French individuals harbouring RYR1 alterations was 3.1%. However, inclusion of IVCT MHE data as a clinically at-risk group increased the frequency of discordance to 19.4%. The high rate of discordance is a concern for the development of future correct diagnosis via genetic testing. Discordance between genotype and phenotype has been reported in other disorders, as discussed in Section 2.11.6 (page 69).

5.5.2.1 Discordance and the MH model

The model illustrated in Figure 5.2 (page 395)indicates that susceptibility to MH could be due to multifactorial inheritance whereby several genetic and/or environmental factors contribute to the phenotype. The model could explain the observed discordance associated with alterations of the RYR1 gene. Discordance could be due to the presence of two or more independent minor genetic traits that segregate within a family. An individual that has had a clinical MH episode and is diagnosed as MHS via the IVCT may not harbour an alteration that was observed to segregate with the MH phenotype in other

family members. However, the individual may harbour additional intermediate or minor alterations that reside in a different part of the genome (Fagerlund *et al.*, 1997) that segregates with the MH phenotype in the family, but has thus far not been detected. Individuals that have never had a clinical episode of MH, but were diagnosed as MHS via the IVCT and harbour a RYR1 alteration, may not harbour the additional alteration/s that is/are required for the development of the disorder.

Epigenetic modifications may also play a role in discordance and in this way two different individuals may have the same genotype but are diagnosed as MHS and MHN via the IVCT, respectively. This may occur as the RyR1 protein may be altered functionally in these two individuals as a result of exposure to different environmental factors, as discussed in Section 5.3.1 (page 405). However, there is a possibility that the IVCT could result in either a false negative or false positive diagnosis, as discussed in Section 5.5.2, page 408. The model illustrated in Section 5.1 (page 393) indicates that the MH phenotype can occur due to several factors and therefore it is likely that both possibilities can occur in different families.

5.5.3 Population specificity of RYR1 mutations

The prevalence of mutations observed in the RYR1 in the South African MH cohort (50%) in the study presented here is lower than the reported prevalence of RYR1 mutations detected in other populations. Galli *et al.* (2006) reported a prevalence of 86% in the Italian MH population. Monnier *et al.* (2005) identified RYR1 alterations in 60% of French MH families and Sambuughin *et al.* (2005) observed RYR1 alterations in 70% of the MH families from North America. The prevalence of alterations (50%) observed for South African MH probands is closer to the prevalence of alterations observed by Ibarra *et al.* (2006). The authors reported a prevalence of 57% for RYR1 alterations observed in families from Japan. The observed difference in frequencies for RYR1 alteration may be attributed to differences in selection criteria, with regard to the IVCT. Sambuughin *et al.* (2005) excluded individuals that displayed equivocal test results for the IVCT. Ibarra *et al.* (2006) reported a higher prevalence of RYR1 alterations (72%) among probands that displayed clear CICR enhancement. Although the selection criteria of the IVCT may play a minor role in a lower prevalence of RYR1 alterations in the South African population, it is more likely that population specificity of RYR1 alterations is due to the founder effect that has been reported in the South African population (Groenewald *et al.*, 1998;

Moolman-Smook *et al.*, 1999; Tipping *et al.*, 2001), as discussed in Section 5.5.3.2 (page 410).

5.5.3.1 Location and distribution of RYR1 mutations

Pollock *et al.* (2002) indicated that mainly novel alterations of the RYR1 gene have thus far been observed in MHS probands from Australia. Ibarra *et al.* (2006) indicated that alterations observed in Europe were rare in MH probands from Japan. The prevalence of RYR1 mutations also varies among European populations (Monnier *et al.*, 2005). The authors indicated that this could be due to the "high prevalence of MH gene pools within certain areas of the country" (Rosenberg and Fletcher, 1995).

In addition, the location and distribution of alterations in the South African MH cohort was different from MH populations from Western countries, which may be attributed to reasons discussed in Section 5.5.3.2 (page 410). The Gly341Arg alteration has been reported in 10% of the European MH population (Quane *et al.*, 1994a), but was absent in the South African cohort. In addition, the Arg614Cys alteration has been identified in 9% of the North American MH population (Sei *et al.*, 2004) and 4% of the German MH population, but was only identified in one MH proband from South Africa.

5.5.3.2 Population specificity of RYR1 mutations and the MH model

The model described in Section 5.1 (page 393) indicates that the MH phenotype can be due to a variety of interacting intermediate or minor genetic and environmental factors or to a major genetic factor. It is apparent that the South African Caucasian population differs from other populations in terms of both genetic composition and environmental exposure. Therefore, it is not surprising that RYR1 alterations that are common in other populations may not be observed in the South African MH population.

5.5.3.2.1 Genetic factors and population specificity of RYR1 mutations

Individuals included in the study presented here are Caucasian and are descendants of immigrants of mainly Western European origin, including Dutch, French, German and British (Saunders, 1983). Given these population demographics, detection of mutations previously identified in Western European populations is expected in certain South African MH individuals. This observation is supported by the identification of RYR1 alterations in South African MH probands that have previously been identified in MHS individuals from

France and the UK. However, the lower prevalence of RYR1 alterations in South African probands included in the study presented here, may be attributed to founder effects in certain individuals, depending on their ancestry. It is likely that in a majority of South African Caucasian individuals, MH is due to the existence of founder mutations. It has been reported that following the settlement of immigrants in the Cape, the population expanded 2,500-fold over a period of 300 years in relative social isolation (Saunders, 1983). The possibility exists that a single alteration in a locus other than the RYR1 gene, that has thus far not been investigated, is prevalent in this population. Linkage to the RYR1 gene has only been observed in 50% of MH families' worldwide, which could be attributed to the genetic heterogeneity reported for this disorder (Robinson *et al.*, 1998). However, as MH in the South African population may be due to founder effects, the number of families that are in linkage with the RYR1 gene may be lower.

5.5.3.2.2 Environmental factors and population specificity of RYR1 mutations

As environmental exposure may vary in each geographical region, it may affect the functioning of the RyR1 protein differently via epigenetic modifications. Therefore, environmental factors could contribute to the discrepancy with regard to RYR1 mutations in the different populations. This hypothesis is supported by the identification of differences between the South African population and other populations in other complex disorders. Variegate porphyria is a disease that results in cutaneous photosensitivity and/or neurovisceral attacks. The disorder has variable clinical expression and 95% of South African individuals with this disorder carry the R59W mutation (Downey, 2001). The authors have suggested that specific environmental factors in combination with another genetic susceptibility factor, which is only present in this population, are important mediators of this disorder. They suggested that an environmental factor must have been present, which allowed the mutation to be observed only in this population.

5.5.4 MH susceptibility without RYR1 alterations

In the study presented here, RYR1 alterations were not observed in eight South African MH probands. Five of the probands were diagnosed as MHS via the IVCT and three probands were identified as MHS via clinical symptoms observed during an anaesthetic procedure. Sambuughin *et al.* (2005) identified nine MH probands from North America that did not harbour RYR1 alterations. Monnier *et al.* (2005) indicated that 53 probands from France did not harbour RYR1 alterations. Ibarra *et al.* (2006) reported that RYR1

alterations were not observed in 25 Japanese MH probands, of whom 11 had clinical episodes of MH, seven had a family history of MH, four had increased CK values, one was diagnosed with MmD and two were diagnosed with limb-girdle muscular dystrophy. Galli *et al.* (2006) reported that seven MH probands from Italy did not harbour RYR1 alterations. None of the seven Italian probands had a clinical episode of MH, but all were diagnosed on the basis of increased CK values. Two Italian probands had a family history of MH. All probands were subsequently diagnosed as MHS via the IVCT, however, the IVCT results indicated that these patients had less pronounced contracture than other patients diagnosed via the IVCT who harboured RYR1 alterations.

Possible reasons for the absence of RYR1 gene alterations in these MH probands may be explained by the MH model illustrated in Figure 5.2 (page 395) and include:

a. An alteration in another gene results in the MH phenotype (as discussed in Section 5.2.2.1, page 399).
b. The defect occurred in the untranslated regions of the RYR1 gene, which were not screened.
c. Epigenetic modifications occurred, which would not be observed using sequencing or DHPLC, as discussed in Section 5.3, page 404.
d. A false positive diagnosis was made via the IVCT (Ørding *et al.*, 1997; Urwyler *et al.*, 2001), as discussed in Section 5.5.2, page 408.
e. The alteration was not identified due to the less-than-ideal DHPLC sensitivity of 90 - 98% (Sambuughin *et al.*, 2005).

Alterations in the untranslated region of the RYR1 gene may play a role in the development of the disorder. SNPs have been reported to affect gene expression, mRNA processing and translation (Wang and Sadée, 2006), as discussed in Section 2.11.3.4 (page 65). Although Sambuughin *et al.* (2005) indicated that the sensitivity of the DHPLC was not 100%, Galli *et al.* (2006) argued that DHPLC analysis is highly efficient in detecting RYR1 alterations. The authors identified seven MH probands that did not harbour RYR1 alterations via the DHPLC and repeated the experiment via screening of all 106 exons in these patients via sequencing (Galli *et al.*, 2006). Both methodologies indicated that none of the seven probands harboured RYR1 gene alterations.

It is likely that four of the possible reasons stated above (a-d) are probable as explanations for the absence of RYR1 gene alterations in the South African MH population. The MH model illustrated in Figure 5.2 (page 395) indicates that a variety of genetic, environmental and epigenetic factors could be responsible for the MH phenotype

and that these are likely to vary in different individuals. In addition, absence of RYR1 alterations in a limited number of individuals may be due to false positive diagnoses via the IVCT, which had previously been reported (Ørding *et al.*, 1997; Urwyler *et al.*, 2001). As all samples obtained from MH probands were sequenced, explanation (e) is not applicable to the study presented here.

Although RYR1 alterations have not been observed in all patients diagnosed worldwide as MHS via screening of the entire coding region of the gene (Monnier *et al.*, 2005; Sambuughin *et al.*, 2005; Galli *et al.*, 2006; Ibarra *et al.*, 2006; Wu *et al.*, 2006), limited molecular genetic testing of MHS has been implemented in Europe and North America. However, as discussed in the subsequent section of this chapter, results obtained from these tests should be interpreted with caution.

5.6 DIAGNOSTIC SERVICE FOR MH SUSCEPTIBILITY

Due to the disadvantages of the IVCT, many MH centres have focused on the development of molecular genetic testing in order to diagnose MHS. The current aim of MH diagnostic investigations is to provide a presymptomatic test for relatives of MHS individuals. The observation of one of the 15 selected causative RYR1 mutations allows for the diagnosis of MHS (Robinson and Hopkins, 2001), as discussed in Section 2.12 (page 70).

However, results from a molecular genetic screening test should be interpreted with caution and should not be used for routine diagnosis of MH. Certain of the 15 selected causative alterations used in molecular genetic testing may represent major genetic factors, as discussed in Section 5.1 (page 393) and could be used to diagnose relatives of the MH proband. However, some of the causative alterations may represent intermediate genetic factors, which is supported by the identification of a reported high rate of discordance, which has also been reported (Hopkins, 2000), as discussed in Section 5.5.2 (page 408). In addition, the criteria for the identification of a causative alteration should be interpreted with caution, as discussed below. It is likely that relatively few families will harbour a single causative alteration that results in susceptibility to MH, unlike pigs, which have been observed to have undergone significant inbreeding, as discussed in Section 5.2.1 (page 396). Therefore further analysis would have to be conducted in order to identify all factors that may be required for the development of the MH phenotype in this multifactorial disorder, as discussed in Section 5.7 (page 416).

MH may be due to a multifactorial mode of inheritance in which several minor and/or intermediate genetic factors or a single major genetic determinant may be required, as discussed in Section 5.1 (page 393). In addition, synonymous SNPs may have functional effects (Fullerton *et al.*, 2001) and represent minor genetic factors that could play a role in the development of the disorder, as discussed in Section 2.11.3.4 (page 65). Therefore, intermediate or minor genetic factors may be disregarded as being causative, as they do not meet the requirements set out by the EMHG. In addition, intermediate genetic factors may meet the requirements for being causative and could be misclassified as being a major genetic determinant. Therefore, caution should be exercised when determining whether a newly discovered amino acid change is disease-causing, as discussed in subsequent sections of this chapter.

The EMHG discussed criteria that should be met in order to use a specific genetic mutation in a predictive genetic test at the 22nd Annual Meeting of the European MH group, June 11-14, 2003. The following criteria were adopted:

1. Characteristics such as evolutionary conservation and an alteration in charge, polarity or structure should be analysed.
2. Co-segregation of the alteration with the disorder should occur in a minimum of two pedigrees.
3. The observed mutation should be absent in 100 controls.
4. The alteration should be functionally characterised by one or more relevant test systems, i.e. recombinant *in vitro* expressions on a defined genetic background and/or assays of RYR1 function in *ex vivo* tissues.

As to the first criterion, conservation of an amino acid residue via phylogenetic analysis does not necessarily provide assurance that the alteration is associated with disease. Jurkat-Rott and Lehmann-Horn (2005) identified two alterations that result in disease, i.e. Phe413Cys and Gln552Arg, which are observed to reside in a region that is not highly conserved. Dulhunty *et al.* (2005) reported alterations in non-conserved residues of the DHPR II-III that had functional consequences and were critical for the functional interaction between RyR1 and DHPR. However, the presence of the alterations did not alter the structure of the DHPR. Therefore, alterations that result in disease may reside in highly conserved regions. In contrast, alterations that are in highly conserved regions may not lead to a disorder (Jurkat-Rott and Lehmann-Horn, 2005).

The second criterion indicates that an alteration should co-segregate with the disease in a minimum of two pedigrees. However, the size of the pedigree is not taken into account. Urwyler *et al.* (2001) indicated that a single small pedigree is generally insufficient for establishing linkage to a candidate gene. Segregation analysis should therefore be conducted in an extended pedigree and should include a minimum of 10 informative meioses.

With regard to criterion 3, when determining the frequency of a mutation in a control population, there are several considerations that should be taken into account. Due to the population-specific nature of alterations, ethnically and geographically matched controls should be used in the analysis (Collins and Schwartz, 2002) and the control population should be sufficiently large and adequately matched. The appropriateness of control samples should also be considered to ensure that they do not have an undiagnosed disorder, considering that individuals with MH generally do not display symptoms of the disorder in the absence of triggering anaesthetics.

Furthermore, Collins and Schwartz (2002) used power calculations, in order to determine the number of chromosomes required precisely in order to detect a significant difference in the mutation frequency between control and patient samples. This analysis indicated that a minimum of 340 to 400 control chromosomes (170 to 200 individuals) would have to be examined for a mutation with a prevalence of 1% with 95% power. Jurkat-Rott and Lehmann-Horn (2005) used a statistical algorithm in order to calculate the number of controls that would be required. Using this analysis, the authors suggested that 460 control chromosomes (230 individuals) should be examined for a mutation with a prevalence of 1% with an error of 1%. Therefore, the authors indicated that the commonly used practice of excluding a novel mutation in approximately 100 healthy controls may be insufficient.

Therefore, all the criteria should be considered and interpreted for a given novel alteration before it is identified as causative or as a polymorphism. The development of future tests, such as genetic animal models or gene expression profiling, will have to be conducted in order to clarify the role of specific mutations.

Although *in vitro* functional expression of mutations has increased our understanding of the physiological significance of alterations, the functional significance of an alteration as determined via functional expression studies may not necessarily be valid *in vivo*. The

heterologous expression system may modify the channels chemically, or may interact with or be deregulated by the introduced DNA, which would lead to a false conclusion regarding the functional significance of the alteration (Jurkat-Rott and Lehmann-Horn, 2005). In addition, functional polymorphisms have been reported. These polymorphisms segregate with the disease phenotype and are functionally significant. However, the functional polymorphisms may be observed in the population without any association with disease (Kubota *et al.*, 2001; Kuzmenkin *et al.*, 2003; Paavonen *et al.*, 2003). In addition, missense alterations from the same gene can have different functional effects on the investigated channel even though they have all been associated with the same disease phenotype. Different alterations associated with familial hemiplegic migraine can either reduce or increase Ca^{2+} influx, indicating that the same phenotype is due to either a gain or loss of channel mechanisms (Hans *et al.*, 1999; Kraus *et al.*, 2000). Therefore, functional studies of channel mutants should be interpreted with caution, and should be considered only in conjunction with all other criteria.

In the study presented here, certain of the published and novel alterations observed in South African probands did not meet the requirements set out by the EMHG to classify causative alterations. However, as discussed in Section 5.1 (page 393), the observed alterations may represent either intermediate or minor genetic factors that on their own are insufficient to result in the MH phenotype. Therefore, analysis of all alterations should be interpreted with caution as they may play a role in the development of the disorder, even if they are not classified as causative by criteria set out by the EMHG. Elucidation of all genetic factors of MH should be conducted in order to provide a complete understanding of the aetiology of this disorder and criteria should be developed that are able to classify alterations into categories of minor, intermediate or major genetic determinants.

5.7 FUTURE DEVELOPMENTS

Data generated in this study highlight the complexity of this disorder and the role of genetic, environmental and epigenetic factors in the development of the MH phenotype. Several aspects were identified during the study that need to be addressed in future to elucidate the aetiology of this complex disorder.

In the study presented here, alterations of the RYR1 gene were observed outside the hotspots. In addition, alterations and/or SNPs in the intronic sequence may contribute a minor genetic effect. Screening of other loci that have been reported to be associated with

MH will also have to be conducted, in order to identify additional candidate genes. Therefore, a methodology may be required that is able to analyse both the intronic and exonic sequence rapidly and accurately. In recent studies, the entire coding region of the RYR1 has been screened for mutations using DHPLC. This technique has been suggested for the diagnosis of MH as it has many advantages, including high sensitivity, speed and cost-effectiveness, but it requires expensive instruments (Sambuughin *et al.*, 2005). In addition, fluorescent melting analysis of PCR products in conjunction with real-time PCR has been suggested as a method for mutation screening. High-resolution amplicon melting analysis of PCR products in the presence of LCGreen® plus dyes is a technique that provides high-resolution analysis and can accurately perform genotyping and heterozygous scanning in products of up to 1 kb in length (Wittwer *et al.*, 2003; Herrmann *et al.*, 2006). However, the sensitivity of both techniques in detecting alterations would have to be determined.

In order to support the observation that susceptibility to MH is due to a multifactorial mode of inheritance, twin studies would have to be conducted in order to determine if there is a lack of complete concordance in monozygotic twins. Statistical methods can be used to detect epistasis in human genetic disorders (Cordell, 2002). Genetic variants that have previously not been identified may be detected statistically, provided that epistatic interactions between potential disease loci are integrated. Cho *et al.* (1998) reported that increased evidence of linkage at one locus for inflammatory bowel disease was observed by taking the interaction with another locus into account. Identification of a statistical model for the joint effects of several loci, including intra- and inter-locus interactions, could potentially be used for prediction of phenotype and possibly for targeting of interventions. However, the degree to which a statistical model can elucidate the underlying biological mechanisms is likely to be limited and will require prior knowledge of the underlying aetiology (Cordell, 2002).

As epigenetic modifications have been reported in diseases related to MH (Zhou *et al.*, 2006b), analysis of these modifications should also be determined in individuals diagnosed with MH. Zhou *et al.* (2006b) reported that because of epigenetic modifications, individuals presented with monoallelic expression of mutations in skeletal muscle cDNA despite the fact that these individuals were biallelic at the gDNA level. Therefore, the influence of epigenetic modifications that may be associated with MH could result in discrepancies with regard to cDNA via the DHPLC versus gDNA via sequencing analysis results. The Human Epigenome Project is in progress and involves mapping all chemical

modifications to DNA that comprise the epigenetic code. Several techniques have been developed in order to identify epigenetic mechanisms. One example is ChIP/chip methodology, in which intact chromatin is immunoprecipitated and analysed on microarray chips. Modifications of DNA are also tracked on chips, following treatment with enzymes that recognise sites of methylation (Schübeler and Turner, 2005).

A simple, easy to perform and inexpensive genetic test is required to diagnose MHS. However, this will only be possible when the basis of phenotypic variation in patients with minor, intermediate or major genetic factors is understood. In approaching this problem, it is important to determine the relative contributions of both hereditary and environmental factors resulting in the variability of expression associated with this disorder. In addition, inheritance of this disorder in the South African population is multifactorial, several gene products that regulate Ca^{2+} homeostasis result in MHS. Identification of factors responsible for the development of the disorder will contribute to a better understanding of the disease process of MH and thus a better clinical prognosis for those affected by MH and related musculoskeletal diseases.

Elucidation of the complex, multifactorial genetic mechanisms underlying susceptibility to MH has been a major challenge in unravelling the mechanism underlying the MH phenotype. However, MH research worldwide has thus far only focused on identifying the role of genetic alterations in MHS. The study presented here supports the hypothesis that the MH phenotype is due to a complex interplay among genetic, epistatic and epigenetic factors.