Molecular screening for specific causative mutations in the South African malignant hyperthermia population

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Molekulêre siftingsanalise vir spesifieke veroorsakende mutasies in die Suid-Afrikaanse maligne hipertermie bevolking

DEUR

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This dissertation is dedicated to the memory of Catherine May Dalton

ABSTRACT

Malignant hyperthermia (MH) is an autosomal dominant, pharmacogenetic disorder. MH susceptible (MHS) patients appear clinically normal, but may present with a hypermetabolic crisis and muscle contracture when exposed to triggering substances which elicit excessive release of calcium ions from the sarcoplasmic reticulum. The underlying cause of MH has emerged as biochemical abnormalities that occur in skeletal muscle. Presymptomatic diagnosis of MH susceptibility is currently made via the *in vitro* contracture test.

The phenotypically similar porcine MH model led to the identification of the chromosomal region bearing the underlying defect. The first human MHS locus, MHS-1, has been mapped to chromosome 19q13. MH is mainly due to mutations in the skeletal muscle ryanodine receptor gene (RYR1). To date the RYR1 gene has been associated with an MH phenotype in approximately 50% of MH families. However, the disorder is genetically heterogeneous, as six other loci have to date been associated with MHS.

The aim of the molecular investigation presented here was to determine if 24 recently reported causative mutations in the RYR1 gene are present in any of the 45 South African MHS probands investigated. Furthermore, eight mutations of the RYR1 gene and the Arg1086His mutation of the CACNA1S that have already been analysed in previous phases of the research programme were investigated. One alteration, Thr4826Ile was detected for the first time in a single South African MH family, contributing to the description of the aetiology of MHS in South Africa. None of the remaining alterations were detected in any South African MH probands analysed. The absence of the majority of reported mutations in all probands included in this study could indicate that the mutations either represent family-specific alterations or could be attributed to the fact that these mutations do not play a role in MHS in the South African population.

OPSOMMING

Maligne hipertermie (MH) is 'n outosomaal dominante, farmakogenetiese sindroom. MH vatbare (MHS) pasiënte kom klinies normaal voor, maar mag 'n hipermetaboliese krisis en spiersametrekking vertoon wanneer blootgestel word aan veroorsakende middels wat die oormatige vrystelling van kalsium ione vanaf die sarkoplasmiese retikulum ontlok. Die onderliggende oorsaak van MH blyk te wyte te wees aan biochemiese abnormaliteite wat voorkom in die skeletspiere. Pre-simptomatiese diagnose van MH vatbaarheid word tans bepaal deur die *in vitro* kontraksie toets.

Die fenotipies ooreenstemmende vark MH model het gelei tot die identifikasie van die chromosomale gebied wat die onderliggende fout bevat. Die eerste menslike MH vatbaarheidslokus, MHS-1 is geïdentifiseer op chromosoom 19q13. MH is grootliks te wyte aan mutasies in die skeletspier ryanodien reseptor geen (RYR1). Tot op hede word die RYR1 geen verbind met 'n MH fenotipe in ongeveer 50% van MH families. Die siektetoestand is egter geneties heterogeen, aangesien tot op hede ses lokusse geïdentifiseer is wat aanleiding kan gee to MH vatbaarheid.

Die doel van die molekulêre ondersoek was om vas te stel of 24 onlangs gepubliseerde veroorsakende mutasies in die RYR1 geen voorkom in enige van die 45 Suid-Afrikaanse MHS pasiente wat bestudeer is. Verder is agt mutasies van die RYR1 geen en die Arg1086His mutasie in die CACNA1S geen, wat reeds ondersoek is in vorige fases van die navorsingsprogram bestudeer. Een mutasie, Thr4826Ile is vir die eerste keer waargeneem in 'n enkele Suid-Afrikaanse familie, dit het bygedra tot die beskrywing van die etiologie van MHS in Suid Afrika. Geen van die oorblywende mutasies is waargeneem in enige van die ander Suid-Afrikaanse aangetaste individue wat ondersoek is nie. Die afwesigheid van die meerderheid van die aangemelde mutasies in alle aangetaste individue in hierdie studie, kan aandui dat die mutasies familie-spesifieke veranderinge verteenwoordig of kan toegeskyf word aan die feit dat hierdie mutasies nie 'n rol speel in MHS in die Suid-Afrikaanse bevolking nie.

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LIST OF ABBREVIATIONS AND SYMBOLS

Symbols are listed in alphabetical order.

α	alpha
β	beta
°C	degrees Celsius
δ	delta
γ	gamma
%	percent
μ	micro: 10^{-6}
n	nano: 10^{-9}
p	pico: 10^{-12}
■ / ●	male/female: tested negative to malignant hyperthermia with the IVCT
▨ / ◍	male/female: tested susceptible to malignant hyperthermia with the IVCT
▩ / ◎	male/female: malignant hyperthermia equivocal
□ / ○	male/female: never tested, malignant hyperthermia status unknown
⊘ / ⊘	male/female: deceased
←	proband

Abbreviations are listed in alphabetical order.

A	adenine (in DNA sequence)
a	adenine
A_{260}/A_{280}	ratio of absorbency measured at 260 nm and 280 nm
ADP	adenosine diphosphate
Ala	alanine
AmpliTaq DNA polymerase	AmpliTaq®[1] DNA polymerase: variant of *Taq* DNA polymerase
ANP	no PCR amplification
APOC2	apolipoprotein C-II
Arg	arginine
Asp	aspartate
ATP	adenosine triphosphate
ATP1A3	ATPase Na^+, K^+ and α_3 polypeptide
ATPase	adenosine triphosphatase
1B5	embryonic stem cell line that has undergone homologous recombination disrupting the *ry1r* gene at nucleotide 840 in exon 10
BMD	Becker muscular dystrophy
boric acid	boracic acid: H_3BO_3
bp	base pair
BSA	bovine serum albumin
Bst UI	restriction endonuclease from *Bacillus stearothermophilus*, with recognition site: 5'-CG↓CG-3'
C	cytosine (in DNA sequence)
c	cytosine
Ca^{2+}	calcium ion
Ca^{45}	calcium isotope
Ca^{2+}-ATPase	calcium adenosine triphosphatase
CACNL1A3	DHPR α_1-subunit gene (previous designation of CACNA1S)
CACNA1S	DHPR α_1-subunit gene
CACNL2A	DHPR α_2/δ-subunit gene (previous designation of CACNA2D1)
CACNA2D1	DHPR α_2/δ-subunit gene
CACNLB1	DHPR β-subunit gene
CACNLG	DHPR γ-subunit gene

[1] AmpliTaq® DNA polymerase, FS, is a registered trademark of Roche Molecular Systems Inc., Alameda, CA, USA.

caffeine	3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione: $C_8H_{10}N_4O_2$
CAL	calreticulin
CaM	calmodulin
CCD	central core disease
cDNA	complementary DNA
C.elegans	*Caenorhabditis elegans*
CHCT	caffeine halothane contracture test
CK	creatine kinase
CKMM	creatine kinase skeletal muscle
cM	centimorgan
cm	centimetre: 10^{-2} metre
CO_2	carbon dioxide
COOH	carboxyl group, indicating the C-terminal of a protein molecule
CP	creatine phosphate
CpG	dinucleotide with a cytosine at the 5' end connected by a phosphodiester bond to a guanine at the 3' end
CPK	creatine phosphokinase
CSQ	calsequestrin
CYP2A	cytochrome P-450 subfamily IIA
Cys	cysteine
Da	dalton
dantrolene	1-[[[5-(4-nitrophenyl)-2-furanyl]methylene]amino]-2,4-imidazolidinedione sodium salt: $C_{14}H_{10}N_4O_5$
Dde I	restriction endonuclease from *Desulfovibrio desulfuricans*, with recognition site: 5'-C↓TNAG3'
ddNTP	2',3'-dideoxynucleotide
del	deletion
DHP	1,4-dihydropyridine derivative
DHPLC	denaturing high performance liquid chromatography
DHPR	dihydropyridine receptor
DMD	Duchenne muscular dystrophy
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E-C	excitation-contraction
ECG	electrocardiogram
EDTA	ethylenediamine tetra-acetic acid: $C_{10}H_{16}N_2O_8$
e.g.	*exempli gratia*
EMBL	European Molecular Biology laboratory
EMHG	European MH Group
et al.	et alii: and others
EtBr	ethidium bromide: $C_{21}H_{20}BrN_3$
ETDT	extended transmission disequilibrium test
EtOH	ethanol: CH_3CH_2OH
fc20	loss of function mutant allele of *C.elegans* at nucleotide position 20
fc34	loss of function mutant allele of *C.elegans* at nucleotide position 34
FH	familial hypercholesterolemia
formamide	carbamaldehyde: CH_{3NO}
FKBP	FK506-binding protein
g	gram
g	guanine
G	guanine (in DNA sequence)
GC content	refers to composition of primers, specifically to the number of G and C bases
gDNA	genomic DNA
GenBank	GenBank®[1]: United States repository of DNA sequence information
Glu	glutamate
Gly	glycine
GPI	glucose phosphate isomerase
h	hours
H^+	hydrogen ion

[1] GenBank® is a registered trademark of the National Institute of Health and Human Services for the Genetic Sequence Data Bank, Bethesda, MD, USA.

HAL	halothane gene
halothane	2-bromo-chloro-1,1,1-trifluoroethane: $C_2HBrClF_3$
HEK-293	the 293 cell line is a permanent line of primary human embryonic kidney transformed by sheared human adenovirus type 5 DNA
HCl	hydrochloric acid
HCP	histidine-rich Ca^{2+} binding protein
Hga I	restriction endonuclease from *Haemophilus gallinarum*, with recognition site: 5'-CACGC$(N)_5$↓-3'
Hha I	restriction endonuclease from *Haemophilus haemolyticus*, with recognition site: 5'-C CG↓C-3'
His	histidine
H_2O	water
HyperPP	hyperkalemic periodic paralysis
HypoPP	hypokalemic periodic paralysis
Ile	isoleucine
IV	intravenous
IVCT	*in vitro* contracture test
JFP	junctional face protein
K^+	potassium ion
Kb	kilo base pair
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
$L.min^{-1}$	litre per minute
LDL	low-density lipoprotein
Leu	leucine
LIPE	hormone sensitive lipase
lod	logarithm of the odds
lod score	a measure of the likelihood of genetic linkage between loci
Lys	lysine
µg	microgram
µl	microlitre
µM	micromolar
M	molar
MD	myotonic dystrophy
Met	methionine
mg	milligram
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
MH	malignant hyperthermia
MHE	malignant hyperthermia equivocal
MHEc	MH equivocal, positive for caffeine
MHEh	MH equivocal, positive for halothane
MHN	malignant hyperthermia normal
MHS	malignant hyperthermia susceptible
MHS-1	MHS locus on chromosome 19
MHS-2	MHS locus on chromosome 17
MHS-3	MHS locus on chromosome 7
MHS-4	MHS locus on chromosome 3
MHS-5	MHS locus on chromosome 1
MHS-6	MHS locus on chromosome 5
MHS-7	MHS locus on chromosome 2
min	minute
ml	millilitre
mM	millimolar
MR	mutation region
MMR	masseter muscle rigidity
Neg	negative for mutation
Na^+	sodium ion
Na_2EDTA	disodium EDTA
$NaHCO_3$	sodium bicarbonate
NaCl	sodium chloride
NAMHG	North American MH Group
NaOAc	sodium acetate

NCX	Na^+/Ca^{2+} exchanges
ng	nanogram
NH_2	amino group, indicating the N-terminal of a protein molecule
nm	nanometres
NMR	nuclear magnetic resonance
NMS	Neuroleptic malignant syndrome
No.	number
NSq	poor quality of sequencing, therefore results not confirmed
nt	nucleotide
O_2	oxygen
p	short arm of chromosome
Phase 1	previous studies conducted in the ongoing MH research programme at the Centre for Genome Research
Phase 2	study presented here, which forms part of the extended MH research programme at the Centre for Genome Research
P_i	phosphate ion
Pos	positive for mutation
^{31}P	phosphorus isotope
PC	paramyotonia congenital
pCO_2	carbon dioxide partial pressure
PCr	phosphocreatine
PCR	polymerase chain reaction
pH	a measure of acidity: numerically equal to the negative logarithm of H^+ concentration expressed in molarity
Phe	phenylalanine
PMCA	Ca^{2+}-ATPase pump
pmol	pico mol
Pro	proline
PSS	porcine stress syndrome
q	long arm of chromosome
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ryr1	RYR expressed in animals
RyR	ryanodine receptor protein
RYR	ryanodine receptor gene
RYR1	RYR expressed in human skeletal muscle
RYR2	RYR expressed in human cardiac muscle
RYR3	RYR expressed in human brain
s	seconds
S	transmembrane α helix segment of the α_1 subunit
SAR	sarcolumenin
SCN4A	sodium channel α-subunit gene
SCN1B	sodium channel β-subunit gene
SERCA	SR Ca^{2+}-ATPase
SIDS	sudden infant death syndrome
SKM1	adult skeletal muscle sodium channel α-subunit
SKM2	foetal skeletal muscle sodium channel α-subunit
SNP	single nucleotide polymorphism
SR	sarcoplasmic reticulum
T	transmembrane
T	thymine (in DNA sequence)
t	thymine
T_a	annealing temperature
Taq polymerase	DNA deoxynucleotidyltransferase, EC2.7.7.7, from *Thermus aquaticus* BM, expressed in a recombinant *E.coli*
TBE	Tris® borate-EDTA buffer
TC	terminal cisternae
Thr	threonine
TIVA	total intravenous anaesthesia
T_m	melting temperature
Tn	trophonin
TRI	triadin

Tris[1]	Tris®[1]: tris(hydroxymethyl)aminomethane:2-amino-2-(hydroxymethyl)-1,3-propanediol: $C_4H_{11}CNO_3$
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride:$C_4H_{11}NO_3.H_2O$
Triton X-100[2]	Triton X-100®[2]: octylphenolpoly(ethylene-glycoether)$_n$: $C_{34}H_{62}O_{11}$. for n = 10
Trp	tryptophan
t-tubule	transverse tubule
U	units
UK	United Kingdom
unc	mutant alleles of *C. elegans*
USA	United States of America
UTR	untranslated region
UV	ultraviolet
Val	valine
V	volts
vol %	percent volume per volume
w/v	weight/volume
x *g*	gravitational acceleration

[1] Tris® is the registered trademark of the United States Biochemical Corporation, Cleveland, OH, USA.
[2] Triton X-100® is the registered trademark of Rohm & Haas Company, Philadelphia, PA, USA.

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CHAPTER ONE

INTRODUCTION

Malignant hyperthermia (MH) was first reported to be associated with anaesthesia by Denborough *et al.* (1962). The authors described a case that led to the recognition of the disorder, which included an account of a 21-year-old male from Australia who experienced complications following exposure to the then newly introduced anaesthetic, halothane. During surgery the patient became cyanosed, experienced a decrease in blood pressure, an increase in pulse rate, and elevated body temperature was also observed. Further investigation revealed that ten of his close relatives had died during or following exposure to anaesthesia. The condition was transmitted through three generations and was the first indication that MH susceptibility (MHS) was inherited. Michael Denborough identified MH as a distinct entity and observed that susceptibility to this disorder was inherited in an autosomal dominant manner. His observations led to a worldwide awareness of this potentially fatal disorder.

MH is a pharmacogenetic disorder that is triggered in susceptible individuals by commonly used inhalation anaesthetics (e.g. halothane) and depolarising muscle relaxants (e.g. succinylcholine). Although timely recognition and appropriate treatment have reduced the mortality rate, the disorder remains the main cause of anaesthetically induced morbidity and mortality (Robinson *et al.*, 1998). The clinical symptoms of MH are highly variable and may include one or more of the following symptoms: muscle rigidity, increase in body temperature, metabolic acidosis, hypoxia and masseter or generalised muscle contracture. Currently, the MHS phenotype is determined via the *in vitro* contracture test (IVCT), performed on a fresh muscle sample obtained by biopsy. A standardised protocol for the IVCT was established by the European Malignant Hyperthermia Group (EMHG) and a similar protocol was implemented in North America (Larach, 1989), utilising different diagnostic specificity than the EMHG protocol (The European Malignant Hyperpyrexia Group, 1984).

The animal model of MH is the pig, as porcine stress syndrome (PSS) mimics most of the characteristics of the MH disorder in man. Biochemical studies of porcine and human MH

have identified that an alteration of calcium (Ca^{2+}) homeostasis in skeletal muscle plays an important role in this syndrome (MacLennan and Phillips, 1992). Molecular genetic studies in the pig linked halothane sensitivity to the glucose phosphate isomerase (GPI) locus on porcine chromosome 6. Human MHS has been mapped to chromosome 19q12-q13.2 in a region syntenic with the location of porcine MH on chromosome 6. The gene for the skeletal muscle calcium release channel of sarcoplasmic reticulum (SR), the ryanodine receptor (RYR1), is located at this locus, and was designated to be the first locus for MHS, hence MHS-1. However, subsequent studies have demonstrated genetic heterogeneity in MHS, as the MHS1 locus has been excluded in a number of pedigrees (Levitt *et al.*, 1991), providing strong evidence that other proteins beside RYR1 are potentially involved in MHS. It is estimated that approximately 50 percent (%) of cases of MH in European families are not linked to 19q12-q13.2 (Ball and Johnson, 1993). Additional candidate genes suggested to cause MHS have been mapped to chromosomes 17 (Levitt *et al.*, 1992; Olckers *et al.*, 1992; Vita *et al.*, 1995), 7 (Iles *et al.*, 1994), 1 (Monnier *et al.*, 1997; Robinson *et al.*, 1997), 5 (Robinson *et al.*, 1997), 2 (Olckers *et al.*, 1999), and 3 (Sudbrak *et al.*, 1995).

The broad aim of the MH research programme is to screen all causative mutations of the RYR1 gene to determine if any of the mutations are responsible for the pathogenesis of MH in the South African population. In previous studies selected individuals from South African MH families have been screened for some mutations in the RYR1 gene (Olckers *et al.*, 1994; Havenga, 2000; Neumann, 2002). Thus far two RYR1 mutations have been detected in the South African MH population. The Arg614Cys alteration was detected in one extended MH family and the Val2168Met alteration was observed in a single individual. The detection of these mutations will contribute to a description of the aetiology of MH in the South African population. The objective of the study presented in this thesis, was to screen 25 additional, recently reported RYR1 mutations in 45 South African MH individuals. Eight RYR1 mutations have already been analysed in previous studies at the Centre for Genome Research, and were screened during the current study in MH individuals who were not previously analysed. In addition to the RYR1 mutations, 24 probands that were previously not screened for the Arg1086His alteration of the alpha (α_1) subunit of the dihydropyridine receptor (DHPR) (CACNA1S, formerly designated CACNL1A3) were also analysed. This study will contribute to the broad aim of the Centre for Genome Research and will enhance the understanding of this genetically complex and potentially fatal disorder in the South African population.

CHAPTER TWO

MALIGNANT HYPERTHERMIA: A DISORDER OF CALCIUM DYSREGULATION

MH is a pharmacogenetic disorder with predisposition being inherited as an autosomal dominant trait (Denborough *et al.*, 1962). MHS patients appear clinically normal, but may present with a hypermetabolic crisis and muscle contracture when exposed to triggering ether, volatile anaesthetics or depolarising muscle relaxants. Triggering substances elicit excessive release of Ca^{2+} from the SR into the cytosol, which results in sustained muscle contracture. The fatality rate is as high as 80% if not treated with dantrolene (Britt and Kalow, 1970), which functions as a Ca^{2+} release inhibitor.

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. Britt and Kalow (1970) indicated that children were three times more likely to experience an MH episode than adults. 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. In addition a few individuals have to be exposed to anaesthesia several times before a clinical episode is triggered. Individuals of both genders are susceptible to MH, however, a 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).

Presymptomatic diagnosis of MH susceptibility is determined utilising the IVCT. Individuals in this test are characterised according to the outcome of a muscle biopsy that has been exposed to triggering agents. Linkage analysis based on IVCT phenotyping has indicated linkage to the gene which encodes the skeletal muscle RYR1 in 50% of MH families. However, the disorder is genetically heterogeneous as to date six other loci have been implicated to result 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 DHPR and sodium (Na^+) channel. Further studies will provide functional characterisation of all MH-causing mutations of this heterogeneous disorder.

2.1 PREDICTION OF MH SUSCEPTIBILITY

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. A clinical grading scale according to standardised clinical diagnostic criteria was developed to create a clinical definition of MH syndrome. This scale utilises the Delphi method, which consists of a series of questionnaires completed by a panel of experts. 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). The clinical indicators used to determine the MH raw score are listed in Table A1, and the scoring rules for the MH clinical grading scale in Table A2. Both tables are presented in Appendix A. The clinical grading scale relies on the judgement of the anaesthesiologist. Several factors can result in an underestimation of the likelihood of an MH event, including aborting of anaesthesia, important monitors not being utilised during anaesthesia (e.g. electrocardiogram, capnogram or thermometer), relevant blood tests not being obtained (e.g. creatine kinase (CK), serum and urine myoglobin or arterial blood gases) and absence of family history (Larach *et al.*, 1994).

2.2 CLINICAL FEATURES OF THE MH PHENOTYPE

The presentation of clinical features is not consistent between patients and varies from the classical or fulminant category to a type with mild symptoms. The underlying basis of variability has not been determined yet. 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, play a role in the progression of MH and usage of non-triggering drugs. Despite the variable clinical presentation patients can be divided into three categories:

i) A classic MH (fulminant) reaction is defined as 'true MH' and occurs following exposure to volatile inhalation anaesthetic agents. The incidence of this life-threatening category has decreased due to improved awareness of MH and usage of non-triggering drugs.

The classic response is rarely observed because of early intervention by an anaesthesiologist during an MH crisis (Ellis *et al.*, 1990). Episodes may arise gradually or have a sudden onset, with a severe but short duration. The 'true MH' reaction demonstrates evidence of sustained, significant hypermetabolism, which is made evident by increased carbon dioxide (CO_2) production and oxygen (O_2) consumption, a family history of MH and a defect in skeletal muscle biochemistry, which results in muscle damage (Rosenberg and Shutack, 1996). The clinical signs of a fulminant MH reaction include rapid breathing (tachypnoea), increased heart rate (tachycardia), unstable blood pressure, metabolic acidosis, ventricular arrhythmias and an increase in body temperature to more than 38.5 degrees Celsius (°C). Patients experience muscle rigidity in addition to breakdown of muscle (rhabdomyolysis), which manifests itself as raised CK levels and myoglobinuria (cola-coloured pigmented urine). Following treatment the MH episode may reoccur (Kaus and Rockoff, 1994). Management of fulminant MH may require the continued use of dantrolene in the postoperative period, and the CK level should be monitored to determine the degree of muscle damage.

ii) Generalised muscle rigidity is characterised by the accelerated presentation of MH following exposure to triggers, and by the presence of abnormal amounts of potassium (K^+) in the blood (hyperkalaemia). Sudden generalised muscle rigidity and cardiac arrest occur following exposure to succinylcholine. Rhabdomyolysis and myoglobinuria may also take place. Many patients with underlying neuromuscular disease may fall into this category (Rosenberg and Shutack, 1996).

iii) Masseter muscle rigidity (MMR) can be defined as the incomplete relaxation of jaw muscles and occurs following exposure to succinylcholine (Rosenberg and Shutack, 1996). It is generally accompanied by flaccid paralysis of the extremities. MMR commonly occurs in children between the ages of three and 12 years, but this may be due to the frequent use of succinylcholine in children (Christian *et al.*, 1989). In some cases MMR may progress into fulminant MH, if potent volatile anaesthetic agents are continued (Rosenberg and Shutack, 1996).

2.3 MH AND ASSOCIATED MYOPATHIES

Certain myopathies have an association with MH. 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 defined as resulting in a predisposition to 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 and Monnier *et al.*, 2001).

2.3.1 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.

2.3.2 King-Denborough Syndrome

King *et al.* (1972) first described King-Denborough Syndrome, following a nationwide survey of MH in Australia and New Zealand. This autosomal recessive disorder is characterised by mild, slow progressive myopathy, short stature, kyphoscoliosis, pectus carinatum, cryptorchidism and unusual facial appearance, including low-placed ears and a webbed neck. The disorder develops in childhood and results in delayed motor development. 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. Many individuals with King-Denborough Syndrome have elevated CK levels. However, a normal CK level does not exclude the patient from having the disorder (McPherson and Taylor, 1981).

2.3.3 Central Core Disease (CCD)

CCD is inherited in an autosomal dominant manner (Isaacs *et al.*, 1975). The manifestation of the disorder can vary from very mild to severe, and 40% of CCD patients may appear clinically normal (Shuaib *et al.*, 1987). Disease onset takes place during infancy and the disorder is characterised by hypotonia, proximal muscle weakness and delayed motor development (Shy and Magee, 1956). CCD is diagnosed, based on the identification of cores (central areas) on the type 1 muscle fibres which lack mitochondria (Denborough *et al.*, 1973). However, a spectrum of pathology in regard to the myopathic features has been observed in patients with CCD (Sewry *et al.*, 2002). Histology studies have also indicated the absence of oxidative enzyme activity in the central regions of the skeletal muscle biopsy (Dubowitz and Pearse, 1960). The involvement of mitochondria in

MH has been reported. Cheah *et al.* (1989) demonstrated that the respiratory properties of normal 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. The authors suggested that uncoupling was fatty-acid-induced and could explain reports of swollen mitochondria in micrographs of MHS muscle biopsies.

Linkage analysis has identified that the CCD locus is 19q13.1 and that the disorder is associated with the RYR1 gene (Kausch *et al.*, 1991). Monnier *et al.* (2001) identified three novel mutations of the RYR1 gene which led to in-frame deletions (del), including 12640del9 nucleotide (nt), 13938del16nt and 14578delTTC in North American and French CCD patients. A patient with a severe form of CCD was observed by Zorzato *et al.* (2003), to have a heterozygous 4863del3nt. The authors indicated that the deletion alters RYR1 channel function and results in CCD. Several missense mutations of the RYR1 gene (listed in Table 2.2) have been found to be associated with this muscle disease (Quane *et al.*, 1993; Zhang *et al.*, 1993; Brandt *et al.*, 1999; Monnier *et al.*, 2000 and Monnier *et al.*, 2001). The observed mutations are found mostly in the myoplasmic and luminal loops, which link the transmembrane (T) domains T1 to T2 and T3 to T4 of the RYR1.

An association between MH and CCD was first reported by Denborough *et al.* (1973). Both disorders are due to uncontrolled intracellular Ca^{2+} release, which may be caused by mutations in the skeletal muscle Ca^{2+} release channel, RYR1. The two disorders differ with regard to the allelic variants. 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. Muscle weakness, a characteristic symptom of this disease, is the resulting decline in adenosine triphosphate (ATP) production.

2.4 LINKS BETWEEN MH AND OTHER 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. Although the clinical phenotype of

these disorders resembles MH, the pathogenesis is different. Episodes clinically similar to MH have been observed in conjunction with a variety of disorders, including myotonia fluctuans, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), myotonia congenita and myotonic dystrophy (MD). 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. Imbert *et al.* (1995) reported that individuals with DMD have a higher resting intracellular Ca^{2+} level, which could be due to additional Ca^{2+} entry through acetylcholine channels in response to mechanical stress induced by contraction. Both DMD and BMD are due to genetically determined defects in the cytoskeleton of the surface membrane (Emery, 1998) and muscle biopsies from DMD patients indicated negative IVCT results (Iaizzo and Lehmann-Horn, 1995). DMD is inherited as an X-linked recessive trait, whereas the gene for MD has been mapped to chromosome 19q, within the interval 19q13.2-19q13.3 (Brook *et al.*, 1991). The MD gene has a distance of 25 centimorgan (cM) from the RYR1 gene, indicating that it is unlikely that MH and MD are genetically linked (MacKenzie *et al.*, 1990).

Other myopathies for which anaesthetic complications have been described include metabolic myopathies such as myoadenylate deaminase deficiency and adenosine triphosphatase deficiency of the SR, congenital myopathies such as the Fukuyama type of congenital muscular dystrophy and mitochondrial disorders (Brownell, 1988). Eliot (2000) reported anaesthetic complications for myopathies associated with endocrine diseases such as hypothyroid myopathy and Warner *et al.* (1997) reported rhabdomyolysis in two children that presented with cardiac arrest following an uneventful anaesthesia.

Hypokalemic 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 characterised by cold-induced stiffness, muscle weakness and low K^+ levels. Linkage between HypoPP and CACNA1S on chromosome 1q has been demonstrated. Mutations within the voltage sensing regions of intra-membrane repeat elements have been associated with this disorder (Fouad *et al.*, 1997). Mutations associated with HypoPP are located in a different region of the α_1 subunit than the mutation demonstrated to be linked to MH.

Neuroleptic malignant syndrome (NMS) is characterised by hyperthermia, muscular rigidity, severe autonomic dysregulation and disturbed consciousness. NMS is similar to MH, since both are due to rapid leakage of Ca^{2+} from the skeletal muscle SR, which sets off a chain of events that result in increased levels of CK, hyperthermia and myoglobinuria. The therapeutic approaches to treatment for both disorders are also similar. However, MH is due to inherited abnormal Ca^{2+} metabolism in skeletal muscle, whereas the genetic contribution to NMS has not yet been determined (Mieno *et al.*, 2003).

Anaesthetic complications have been reported in a patient with Schwartz-Jampel syndrome (Seay and Ziter, 1978). This is a neuromuscular disorder that is characterised by dwarfism, skeletal abnormalities, muscular stiffness and an abnormal non-specific electro-myogram (Viljoen and Beighton, 1992).

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 crytorchism, or congenital hernias in all age groups of MH patients compared to patients that did not present with MH under anaesthesia. 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, 1982).

2.4.1 Human Stress Syndrome

An association of heat stroke (Dickinson, 1989), stress (Grinberg *et al.*, 1983), and exercise (Ryan and Tedeschi, 1997), the so called Human Stress Syndrome, with MH has also been observed but not proven to be associated. In heat stroke, sweating becomes ineffective, the body temperature exceeds 40°C, and CK levels are raised. Heat stroke has many phenotypic similarities compared to MH, but the aetiology, pathogenesis and treatment differ (Yaqub and Al Deeb, 1998). Grinberg *et al.* (1983) reported MH episodes postoperatively in three patients that were administered "safe" anaesthetics. The authors determined that stress was the triggering mechanism of MH and suggested that no anaesthetic could be considered entirely safe. Ryan and Tedeschi (1997) reported a case of sudden death in an individual with a family history of MH. This individual had elevated 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, diabetic coma, drug use, hyperthyroidism, infection in central nervous system, neuroleptic malignant syndrome, rhabdomyolysis, sepsis and myopathies. Although an association between these disorders and MH has not been confirmed, MH should be considered a potential hazard under anaesthesia.

2.5 PHARMACOLOGICAL TRIGGERING AGENTS OF MH

In normal 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 trigger an MH episode. Potential triggering and safe agents are listed in Table 2.1.

Table 2.1: Triggering agents, safe agents and controversial agents related with malignant hyperthermia

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
Barbituates	Sodiumpenthatol
Benzodiazepines	Diazepam, Midazolam and lorazepam
Opioids	Morphine, Meperidine, Hydromorphone, Fentanyl, Sufentinil and Alfentanil
Non-depolarizing 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
Controversial agents	
	Calcium salts, Potassium salts, Catecholamines, Phenothiazines

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

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 a 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. Halogenated ethers, isoflurane, enflurane, sevoflurane and desflurane have been reported to induce an MH episode. Desflurane has been classified as a weak MH trigger. However, the onset of an MH episode is more rapid when this anaesthetic is used in combination with succinylcholine (Allen and Brubaker, 1998). Clinicians currently use halothane and halogenated ethers, because of the precise and rapid control they provide with regard to the depth of anaesthesia. 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). The drug is often used in paediatric anaesthesia and in addition to several side effects, can also trigger an MH episode. Clinicians still utilise this drug due to its advantageous properties, which include rapid onset of complete relaxation and very short duration of action (Belmont, 1995). This drug targets the neuromuscular junction, and potentates depolarisation in the SR and transverse tubule (t-tubule) system, which results in an increase in intracellular Ca^{2+}, a process which will be exaggerated in MH muscle (Dorkins, 1982; Galloway and Denborough, 1986). 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). Non-depolarising neuromuscular drugs have been considered as a possible replacement for succinylcholine. These drugs cause less severe side effects and are reported not to trigger an MH episode (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. Other drugs reported as safe for MH patients include intravenous opiates and local anaesthetics (Hopkins, 2000).

2.6 ANAESTHESIA FOR MHS PATIENTS

Prior to anaesthesia, patients should be assessed for risk factors which may identify the individual as MHS. Presurgical 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 myotonia congenita, 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, 1979). The clinical approach followed to evaluate and counsel a possible MH patient prior to anaesthesia is listed in Appendix B. Once in the operating room, special attention should be given to the equipment. Anaesthesia machines should have vaporisers removed, the tubing should be changed and O_2 should flow through the circuit for 10 minutes with 10 $L.min^{-1}$ flows 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 in 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 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 administration of the triggering drug should be discontinued. Opioids and sedatives should be used to maintain anaesthesia, and non-depolarising muscle relaxants may be used to ensure muscle relaxation. Surgery should be discontinued if possible. Ventilation with 100% O_2 should be administered to correct respiratory acidosis and decrease the risk of hypoxia. Sodium bicarbonate ($NaHCO_3$) should be administered to correct the underlying metabolic acidosis, to lower serum K^+ and prevent ventricular fibrillation. 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 (discussed in Section 2.7) should be administered intravenously at a dose of 2.5 milligram per kilogram ($mg.kg^{-1}$), and continued until the clinical signs of a MH episode have diminished

(Donnelly, 1994). The drug is prepared in a lyophilised mixture of 20 mg dantrolene sodium, 3 gram (g) mannitol and sodium hydroxide to adjust the pH to 9.5, and an additional 60 millilitres (ml) of sterile water is added. Following stabilisation, the patient's arterial and venous blood gas, central venous pressure, renal function, temperature, urine myoglobulin, electrolytes, CK and coagulation should be monitored (Wappler, 2001). Dantrolene treatment should continue for 24 to 72 hours (h) following an MH episode, thus at least four dosages, and MHS individuals should be closely monitored following anaesthesia, as delayed onset of MH has been reported (Souliere *et al.*, 1986).

2.7 TREATMENT OF MH WITH DANTROLENE

The drug, dantrolene sodium, 1-[[[5-(4-nitrophenyl)-2-furanyl]methylene]amino]-2,4-imidazolidinedione, is a hydrated lipid soluble calcium antagonist with skeletal muscle relaxant (Kolb *et al.*, 1982) that was introduced in 1975 as the first effective therapy for MH. Early administration of dantrolene during an MH crisis will abort symptoms associated with this disorder. This highly lipid soluble drug acts on the skeletal muscle sarcoplasmic reticulum and inhibits Ca^{2+} release into the muscle, which results in decreased muscle contracture. 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. Investigation into the mode of action of this drug may lead to the discovery of to date unreported mutant alleles. Dantrolene is generally not used as a prophylactic treatment for MH (Hackl *et al.*, 1990). Studies have shown that prophylactic use of dantrolene is protective at induction of anaesthesia. However, several cases have been reported where this was not the case (Allen *et al.*, 1998). Other disadvantages of dantrolene as a prophylactic include that the drug is unreliable in oral administration as absorption levels are variable, dantrolene is poorly soluble in water and the drug may induce transient muscle weakness, which would compromise respiration in patients with an underlying myopathy, as well as the economic considerations of utilising this drug (Kaus and Rockoff, 1994).

2.8 MOLECULAR MECHANISM OF MUSCLE CONTRACTION IN MH

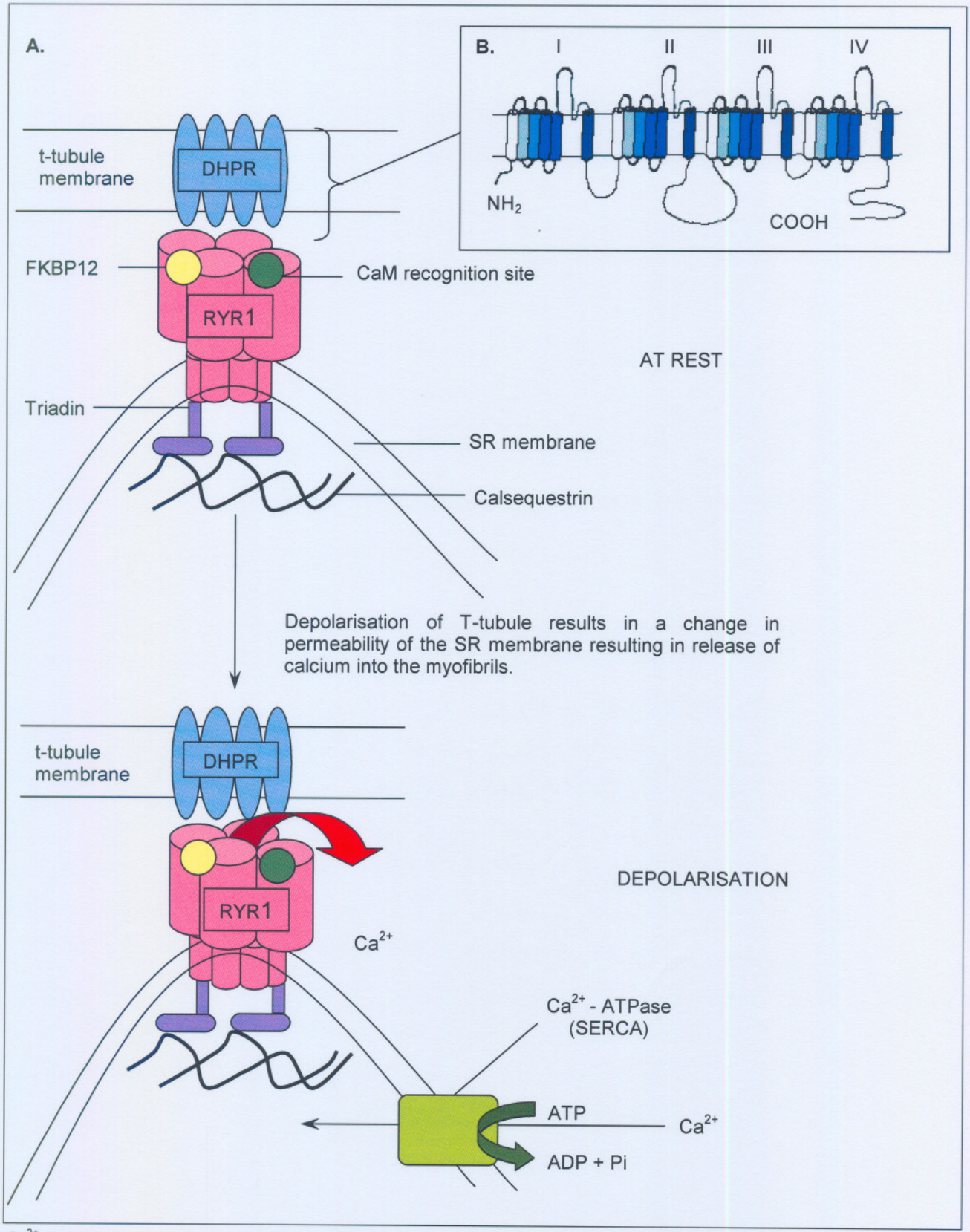
The underlying cause of MH has emerged as biochemical abnormalities that occur in skeletal muscle. Although contraction in mammalian skeletal muscle is highly regulated,

an MH episode may occur if the intracellular Ca^{2+} homeostasis in skeletal muscle is disturbed (Denborough *et al.*, 1962). Studies have indicated that the rate of Ca^{2+} induced Ca^{2+} release from the SR is increased two- to three-fold. The observed increase in Ca^{2+} release is probably due to both open-channel probability and mean open time. In addition, MHS muscle displays higher and/or lower Ca^{2+} sensitivity of the Ca^{2+} activation and of Ca^{2+} inhibition sites, respectively. Therefore, the underling mechanism of MH is complicated and can be induced by mutations from the amino (NH_2)-terminus to the carboxyl (COOH)-terminal portion of the RYR1. These effects could also be due to alterations in the interaction of the RYR1 with accessory proteins involved in the regulation of the channels activity. Various interacting Ca^{2+} channel's, Ca^{2+} binding proteins and Ca^{2+} pumps function to regulate muscle Ca^{2+} homeostasis. 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 symptoms associated with MH.

2.8.1 Excitation-contraction (E-C) coupling

E-C coupling refers to the precise regulation of intracellular Ca^{2+} release following an action potential in muscle cells. The major components of the E-C pathway as well as skeletal contraction are illustrated in Figure 2.1A. 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 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 found in the terminal cisternae (TC) of the SR (Inui *et al.*, 1987) and the voltage-sensing DHPR is found in the junctional t-tubules. 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). The DHPR is able to interact directly with RYR1 (Marty *et al.*, 1994), as depicted in Figure 2.1B.

Figure 2.1: Schematic representation of DHPR-RYR1 interactions in skeletal muscle excitation and contraction



Ca^{2+} = calcium ion; t-tubule = transverse tubule; NH_2 = amino group; SR = Sarcoplasmic reticulum; Ca^{2+} - ATPase = calcium adenosine triphosphatase; SERCA = SR Ca^{2+}-ATPase; ATP = adenosine triphosphate; ADP = adenosine diphosphate; FKBP12 = immunophilin (cytosolic receptor) binding protein. Adapted from McPherson and Campbell (1993).

Direct interaction occurs via protein-protein interactions that can take place due to their close proximity (Loke and MacLennan, 1998). Interaction between the skeletal muscle DHPR III-IV loop and the RyR1 protein has been observed between the amino acid sequence Leucine^{922} (Leu) and Aspartate^{1112} (Asp) and could represent a region important in the DHPR and RyR1 protein interaction (Leong and MacLennan, 1998). Interaction between the two proteins results in release of Ca^{2+} at the triad junction into the sarcoplasm from the lumen of the SR.

Muscle fibres consist of myosin and actin and proteins, troponin (Tn) and tropomyosin are found associated with the actin stand. Tn is composed of three subunits, TnT, TnI and TnC. Released Ca^{2+} binds to 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, through the action of SR Ca^{2+}-adenosine triphosphatase (ATPase) or SERCA. 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), and in addition via the Na^{+}/Ca^{2+} exchanges (NCX).

2.8.1.1 The ryanodine receptor (RYR)

There are three distinct isoforms of RYR, expressed in skeletal muscle, heart and brain (Fill and Copello, 2002). The primary structures of the three ryanodine receptors have been elucidated by complementary DNA (cDNA) cloning. Mammalian RyR1, RyR2 and RyR3 proteins show a high degree of overall homology (about 70% identity) with certain regions being particularly conserved, while others display variability (McPherson and Campbell, 1993). The latter regions are responsible for the specific isoform characteristics and have demonstrated binding sites for different modulators. The RYR2 and RYR1 are found in cardiac and skeletal muscle respectively (Takeshima *et al.*, 1989). The RYR3 is found more widely distributed in non-muscle tissue, specifically in the brain (Sorrentino and Reggiani, 1999) and does not sustain E-C coupling.

RYR was named after the plant alkaloid, ryanodine, which binds specifically to the RYR channel with a high affinity (Pessah *et al.*, 1985). RYR1 has two distinct sites for binding ryanodine, high- and low-affinity binding sites, which are both located between arginine4475 (Arg) and the COOH-terminus (Callaway *et al.*, 1994). The RYR1 is one of the largest known proteins and comprises 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 forms a channel-like feature or foot (Wagenknecht and Redermacher, 1995). Tightly associated to RYR1 are three proteins, which include the immunophilin (cytosolic receptor) binding protein of 12 kDa (FKBP12), calmodulin (CaM) and calsequestrin (CSQ) that are able to modify RYR1 gating (Timerman *et al.*, 1993, Brillantes *et al.*, 1994). Jayaraman *et al.* (1992) first indicated the tight association between FKBP and the RYR1 and specified that binding occurred on the TC of the SR. FKBP is able to bind to the immuno-suppressive drug FK-506, which causes FKBP to dissociate from RYR1, a process that disturbs E-C coupling. CaM is a ubiquitous 17 kilodalton (kDa) Ca^{2+} binding protein that can modulate RYR1 channel activity by altering the open time probability. Low concentrations of CaM in the presence of a low concentration of Ca^{2+} activated Ca^{2+} release and higher concentrations of CaM inhibit channel function (Buratti *et al.*, 1995). CSQ is the major calcium binding protein in the SR lumen.

A variety of compounds modulate the release of Ca^{2+} from the RYR1, including Ca^{2+}, ryanodine, caffeine, ATP, magnesium (Mg^{2+}), and CaM (Meissner, 1986; Coronado *et al.*, 1994). Other agents, such as volatile anaesthetics and dantrolene, can also bind to RYR1 (Pessah *et al.*, 1996). The cytoplasmic domain consists of multiple binding sites, and Ca^{2+} release can be stimulated by low concentrations of Ca^{2+} (< 100 micromolar (μM) of Ca^{2+}) as well as ATP. The domain also has sites for inactivating ligands which inhibit Ca^{2+} release. These include high concentrations of Ca^{2+} (>100 μM of Ca^{2+}) and Mg^{2+} in millimolar (mM) concentrations (Meissner, 1986).

2.8.1.2 The dihydropyridine receptor (DHPR)

The DHPR 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 composed of five different subunits, namely α_1, α_2, beta (β), delta (δ), gamma (γ) encoded by four genes (Catterall, 1988), which may all interact with the RYR1. The α_1 subunit is 212,000 daltons (Da) and consists of four homologous domains (I to IV) consisting of six transmembrane α-helical segments (S), numbered S1 to S6, and a cytoplasmic pore-lining region, as illustrated in Figure 2.1b.

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, Lysine (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 transmembane domains. Perez-Reyes *et al.* (1989) suggested that the γ subunit played a role in the stable expression of the α_1 subunit. The subunits α_2, β, δ, γ contribute to the assembly and expression and aid in the kinetics of *in vivo* Ca^{2+} channel function. All of these subunits may play a role in diseases of Ca^{2+} regulation (Loke and MacLennan, 1998).

2.8.1.3 Systems involved in calcium (Ca^{2+}) regulation from the sarcoplasmic reticulum (SR)

The release of Ca^{2+} from the SR is regulated by four systems. The TC of the SR branches out to form an elongated longitudinal tubule system that contains SERCA I. The fast twitch isoform, SERCA I, facilitates the transportation of sarcoplasmic Ca^{2+} into the SR lumen, where Ca^{2+} is stored in association with 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. SERCA catalyses the transportation of two Ca^{2+} ions via the hydrolysis of one ATP molecule. Defects in this system are a potential source of muscle disease in Ca^{2+} regulation (Martonosi and Pikula, 2003). In addition, an NCX which exists in the skeletal muscle plasma membrane and mitochondria is involved in Ca^{2+} regulation. Lastly the SR itself is also involved in Ca^{2+} release (Martonosi and Pikula, 2003).

Various proteins localised to junctional SR are involved in Ca^{2+} storage and either directly or indirectly in the regulation of the RYR1. Ca^{2+}-binding proteins include CSQ, calreticulin (CAL), sarcolumenin (SAR) and histidine-rich Ca^{2+} binding protein (HCP). Ca^{2+} is located bound to CSQ, with a low affinity. Binding of Ca^{2+} to the CSQ monomer causes a

conformational change in CSQ, resulting in its compaction and polymerisation (Ikemoto *et al.*, 1972). The CSQ is anchored to the junctional face SR membrane and the RYR1 by means of triadin (TRI) and junctional face protein (JFP), forming a quaternary protein complex (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. CSQ plays a role in regulating RYR1 activity in response to different Ca^{2+} concentrations in the lumen (Beard *et al.*, 2004). Release of Ca^{2+} from the CSQ will cause enhanced activity of RYR1 resulting in channel opening, while binding of Ca^{2+} to CSQ results in suppression of RYR1 activity and closing of the Ca^{2+} channel (Beard *et al.*, 2002). Mutations in the genes that encode the above-mentioned proteins may lead to altered Ca^{2+} homeostasis.

2.8.2 Excitation-contraction coupling and Ca^{2+} regulation in MHS individuals

Exposure of individuals to volatile anaesthetics results in an increase in RYR1 activity and disturbed Ca^{2+} regulation. In normal 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 becomes sensitive to lower concentrations of stimulators, which causes the channel to remain open (MacLennan and Phillips, 1992). However, the rate of Ca^{2+} uptake into the SR in the population with normal 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.*, 1994b). Continuous Ca^{2+} release stimulates Ca^{2+}-ATPase activity, which leads to an increased rate of ATP hydrolysis. The escalating Ca^{2+} levels and subsequent decline in ATP levels produces a hypermetabolic response and may trigger an MH episode (Pessah *et al.*, 1996). The continual release of Ca^{2+} leads to sustained muscle contracture, which results in depletion of ATP. The pathogenesis of muscle contraction in MHS individuals has been extensively studied utilising animal models.

2.8.3 Skeletal muscle energy metabolism and MH

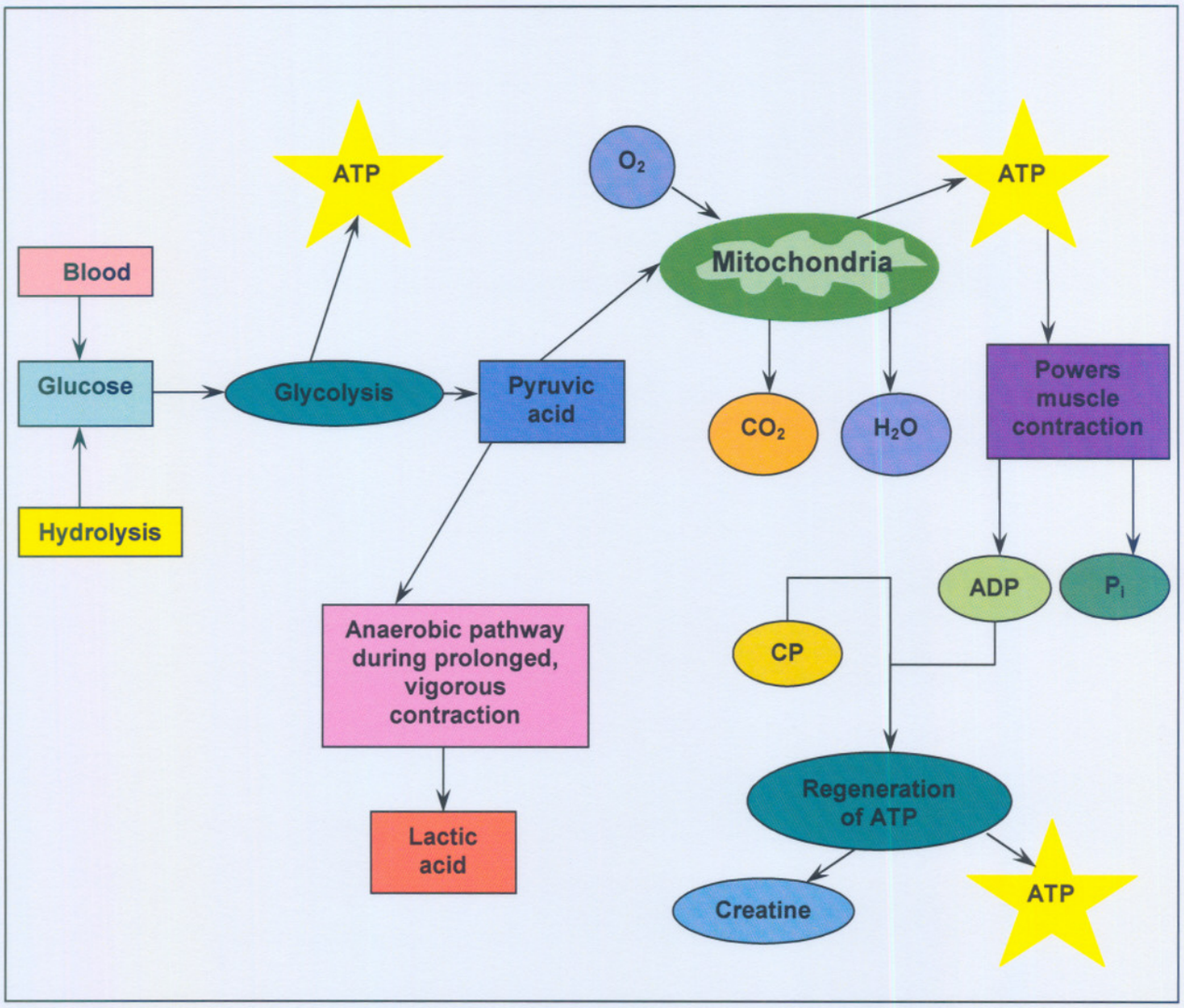
Ca^{2+} plays a central role in producing energy and must be highly regulated in skeletal muscle. A schematic representation of skeletal muscle energy metabolism is illustrated in

Figure 2.2. Glycolytic and aerobic metabolism proceeds at a specific rate to maintain energy balance in the cell. In normal cells, Ca^{2+} released from the SR will activate glycogenolysis and result in the catabolism of glycogen by the catalytic enzyme glycogen phosphorylase into glucose. During glycolysis, glucose is broken down into two pyruvic acid molecules in the cytosol where it is either converted to lactic acid, or enters the oxygen requiring Krebs cycle and electron transport chain within the mitochondria. At high Ca^{2+} concentration, the enzyme myosin ATPase is activated and catalyses the breakdown of ATP into adenosine diphosphate (ADP) and a phosphate ion (P_i) which produces free energy used for muscle contraction (Merz, 1986). In individuals with MH, triggering anaesthetics result in continual release of Ca^{2+} in MH muscle which overpowers the Ca^{2+} pump, and results in sustained muscle contraction, resulting in muscle rigidity. Sustained muscle contracture stimulates continuous glycolysis and aerobic metabolism produces an excess of lactic acid, CO_2 and heat and results in enhanced O_2 uptake, necessary for ATP production. As Ca^{2+} is not cleared, other systems will be co-opted to regulate the Ca^{2+} concentration. The mitochondria are forced to accumulate excess Ca^{2+}, which leads to diminished ATP and results in degeneration of the central core accompanied by myofibrillar streaming and membrane disorganisation (Rodgers, 1983).

Individuals with MH who are exposed to triggering agents exhibit an excessive metabolic rate, which leads to increased body temperature. This occurs as a result of uncoupling of oxidative phosphorylation, in which heat is not converted to ATP (Cheah and Cheah, 1981a). The decline in ATP levels results in the release of intracellular enzymes such as CK, K^+ and H^+. Cheah and Cheah (1981a) 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 phospholipase A_2 releasing unsaturated fatty acids. Data obtained from MH pigs indicated that the endogenous activity in the mitochondria of phospholipase A_2 was higher compared to normal pigs (Cheah and Cheah, 1981b). Activation of this enzyme results in the formation of lyso derivatives and liberation of free fatty acids, which destabilise the mitochondrial membrane, cause the uncoupling of the mitochondria and result in a faster release of Ca^{2+}. The long-chain fatty acids and/or the phospholipase A_2 alter the function of the SR by inactivating the Ca^{2+}-transport system, which results in additional Ca^{2+} being released. Fletcher *et al.* (1990) reported that when MHS pigs were depleted of free fatty acids, the animals did not exhibit muscle contracture when challenged with halothane and succinylcholine. The enhanced level of Ca^{2+} stimulates glycolysis, and glycogen is degraded to lactate at a higher rate than normal

(Cheah and Cheah, 1981b). This finding is supported by a report by Kalow *et al.* (1970) indicating that MH muscle had an increase in sensitivity to caffeine, which suggests a defect in the intracellular disposition of Ca^{2+}. The inhibition in MHS muscle via halothane suggested that an alteration in calcium sequestration occurred.

Figure 2.2: Schematic representation of skeletal muscle energy metabolism during muscle contraction



ATP = adenosine triphosphate; O_2 = oxygen; CO_2 = carbon dioxide; H_2O = water; CP = creatine phosphate; ADP = adenine diphosphate; Pi = inorganic phosphate. Adapted from Marieb (1995).

2.9 THE PORCINE ANIMAL MODEL

The pig became a model for the human MH syndrome when Hall *et al.* (1966) first described an unusual reaction in pigs, which was identical to the MH phenotype encountered in MHS humans when exposed to a muscle relaxant, suxamethonium chloride. The syndrome in pigs is known as PSS and follows an autosomal recessive mode of inheritance. 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, anaesthesia and exposure to high temperature 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). The list of animals susceptible to MH has expanded since it was first discovered in pigs. During veterinary anaesthesia, an MH reaction which resembles MH in humans has been reported in a cat (De Jong *et al.*, 1974), in horses (Aleman *et al.*, 2004) and in dogs anaesthetised with halothane (Nelson *et al.*, 1991) and a report on a fatal hyperthermic episode in a giraffe was provided by Citino *et al.* (1984). In horses the Arg2454glycine (Gly) in exon 46 of the *ryr1* was found associated with MH. In dogs and pigs, MH can also be triggered by stress or excitement (Duncan *et al.*, 1997). 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, and it is unknown whether mutations observed in dogs will be found in other species or correspond to human malignant hyperthermia.

In human studies, porcine models have provided information on the physiology of muscle contraction and metabolism. Ohnishi *et al.* (1983) demonstrated that induction of MH in susceptible pigs resulted in excessive amounts of cytoplasmic Ca^{2+}, which is the underlying defect in MH. Hall and Lucke (1983) observed that following triggering agents, an MH episode in susceptible pigs was due to metabolic alterations in the muscle, which increase the rate of glycolysis and production of lactate, which then manifests as increased heat production. Harrison *et al.* (1969) indicated that a rise in carbon dioxide pressure (pCO_2), which causes hyperventilation and a fall in muscle ATP, was also observed. The decrease in ATP indicates that the defect results in uncoupling of oxidative phosphorylation.

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 found to be due to an alteration in the halothane (HAL) gene or PSS gene. Physical mapping placed the HAL

gene linkage group in the 6p11-q22 region of porcine chromosome 6. 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 on chromosome 6q12. Fill *et al.* (1990) indicated that the *ryr1* channel of homozygous recessive pigs with MH had channels that remained open significantly longer than the *ryr1* channels of homozygous normal animals. These MHS channels fail to be inactivated by low Ca^{2+} concentrations. The author suggested that MH in pigs could be due to a defect in the low affinity Ca^{2+} binding site. Otsu *et al.* (1994) also indicated in a study conducted on myoblastic cells transfected with mutated RYR1 cDNA that the channel had a higher sensitivity to caffeine, which induces Ca^{2+} release. Exposure to halothane resulted in a rapid increase in Ca^{2+} in the cells. The porcine gene, *ryr1*, on chromosome 6 is syntenic to RYR1 found on chromosome 19q in humans (McCarthy *et al.*, 1990).

A causative point mutation within the *ryr1* gene, Arg615cysteine (Cys), that results from a nucleotide transition cytosine (C)1843 thymine (T), was identified in five major breeds of pigs (Fujii *et al.*, 1991), suggesting that the mutation had a common origin. The mutation 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 mutation correlates to the C1850T mutation in the human RYR1 gene, which has been found 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 suxamethonium. 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 to date unreported mutation or a confluence of other cofactors. 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.

2.10 IDENTIFICATION OF RYR1 GENE AS A LOCUS FOR MH

Mapping of genes associated with MH can be determined using two molecular strategies. The first strategy involves a genome-wide scan, which determines whether highly polymorphic, informative genetic markers of known location are co-inherited with MH. This technique utilises at least 10,000 available marker loci, and initial screens generally utilise microsatellite markers at intervals of 10 cM throughout the genome. The genotypes generated are utilised in linkage analysis, to determine if the marker alleles segregate with the disease phenotype. The statistical likelihood is determined as logarithm of odds (lod) scores, and is the standard for linkage mapping of major genes (Morton, 1996). Linkage is accepted if a score of more than three is obtained. When a heterogeneous disorder is studied, linkage analysis requires large, multigeneration pedigrees for informative analysis. The second involves selection of candidate genes that may be associated with MH.

Initial molecular genetic studies identified the gene responsible for porcine MH, as discussed in 2.9. Studies on the porcine model suggested that a mutation in the RYR1 gene was responsible for MH in humans as well. Utilising 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 placed on chromosome 19q13.1 and was suggested as a candidate for MH (Mackenzie *et al.*, 1990). To date the RYR1 gene has been associated with an MH phenotype in approximately 50% of MH families (Jurkat-Rott *et al.*, 2000).

2.10.1 Regional location of genes on chromosome 19

McCarthy *et al.* (1990) utilised multipoint linkage analysis in three Irish pedigrees, and determined that the locus for MH was 19q12-13.2. The authors suggested that the genetic locus for MHS resided close to the cytochrome P-450 gene (CYP2A). Phillips *et al.* (1985) determined that the CYP2A gene was located on chromosome 19 and was further mapped to the region, 19q13.1 (Wainwright *et al.*, 1985). CYP2A plays a central role in metabolism of endogenous compounds including steroid hormones, drugs and carcinogens (Phillips *et al.*, 1985). A candidate gene, hormone sensitive lipase (LIPE) for MH susceptibility was originally suggested by Levitt *et al.*, 1990. The LIPE has been localised to 19q13.3 (Holm *et al.*, 1988), and is expressed in muscle. The ATPase, Na^+ K^+ and α_3 polypeptide (ATP1A3) are localised to 19q12-q13.2 region. Genes involved in susceptibility to MD, an autosomal dominant disease resulting in deterioration of

neuromuscular function, have been localised to the 19q13.2 region (Schonk *et al.*, 1989). The MD locus is found in close proximity to the apolipoprotein (APOC2) and muscle-specific creatine kinase (CKMM) genes, and all three genes are in equilibrium with one another (Korneluk *et al.*, 1989). The CKMM gene was mapped to the human chromosome 19q13 region and is located proximal to the gene and APOC2 lies proximal to the CKMM gene. The CKMM gene is therefore the closest polymorphic marker to the MD gene (Smeets *et al.*, 1990). In patients with MD, there is a reduction in the ratio of Na^+ efflux to K^+ influx. However, linkage analysis revealed that mutations in the ATP1A3 gene were not the primary cause of MD (Harley *et al.*, 1988). Lusis *et al.* (1986) mapped the genes for the APOC2 and a glucose-6 phosphate to the long arm of chromosome 19. APOC2 function as lipid transporters, and genetic variation in these genes causes predisposition to dyslipoproteinaemias or premature atherosclerosis.

2.10.2 Structural organisation of the RYR1 gene

The RYR1 gene encompasses 158,000 base pairs (bp) of genomic DNA (gDNA) and consists of 106 exons, which include 15,000 bp of cDNA. Two of the exons are alternatively spliced and numbering of the nucleotides of the cDNA and the encoded amino acids was corrected in order to account for earlier errors and omissions (Phillips *et al.*, 1996). The RYR1 gene encodes a protein of approximately 5,000 amino acids, without an NH_2-terminal signal sequence (Zorzato *et al.*, 1990). The transcription start site sequence of the 5' untranslated region (UTR) was determined to be a T residue which lies 130 base pair upstream of the initiator codon methionine (Met). 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. The RYR1 gene contains two alternatively spliced sequences that occur within exon 70 and exon 83. The transcript level of these sequences varies during the different stages of development (Phillips *et al.*, 1996).

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

Worldwide, approximately 100 RYR1 mutations have been identified associated with MH to date (Halsall and Robinson, 2004). Most of RYR1 mutations discovered for MH and CCD are missense and have generally been found to cluster in three areas of the gene, ranging from exons 2 to 17 (MH/CCD region one) and exons 39 to 46 (MH/CCD region

two). These two regions encode the N-terminus of the protein that forms the myoplasmic foot (Jurkat-Rott *et al.*, 2000). A third hotspot has been observed in the C-terminal region of the RYR1 in exons 95 to 102 (Lynch *et al.*, 1999). This region encodes the transmembrane domain of the protein. Yamamoto *et al.* (2000) suggested that regions 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. The third region plays a role in the functioning of the permeation selectivity and gating of the channel. Reported mutations of the RYR1 gene associated with MH and CCD are listed in Table 2.2.

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

i) The mutation should segregate with the disease phenotype.

ii) The mutation should not segregate in 100 normal controls.

iii) The position of the affected amino acid in the protein should be found at a functionally relevant site or at an evolutionary conserved site across species.

iv) 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, replacement with side chains that are different (nonconservative substitutions) are more likely to affect the function of the protein.

v) Characterisation of the mutated protein in an appropriate assay should indicate altered function.

To determine the causal role of mutations of the RYR1 gene a variety of studies have been conducted. Otsu *et al.* (1994) utilized cultured myoblast cells transfected with the Arg615Cys alteration cDNA. The myotubes that expressed the mutation had a significantly higher sensitivity to caffeine. The results of this study indicated that the mutation was sufficient to cause MHS. Functional analysis was conducted by Tong *et al.* (1997) utilising a non-muscle expression system. Mutations of the RYR1 were expressed in HEK293 cells and results indicated a significant increase in the sensitivity of mutant RYR1 in the presence of the triggering agent's caffeine, halothane and 4-chloro-*m*-cresol.

Table 2.2: Reported nucleotide substitutions (missense/nonsense), deletions and polymorphisms in the RYR1 gene associated with MH and CCD

Nucleotide change	Amino acid	Exon	Frequency substitution	Phenotype	Reference
T103C	Cys35Arg	2	1 family	MH	Lynch *et al.*, 1997
G131A	Arg44His	2	1 family	MH	Halsall and Robinson, 2004
A479G	Glu160Gly	6	1 family	MH	Halsall and Robinson, 2004
C487T	Arg163Cys[1,2]	6	2%-3%	MH/CCD	Quane *et al.*, 1993
G488T	Arg163Leu	6	1 family	MH	Halsall and Robinson, 2004
G742A	Gly248Arg[1,2]	9	1 family	MH	Gillard *et al.*, 1992
G1021A	Gly341Arg[1,2]	11	15 families	MH	Halsall and Robinson, 2004
C1201T	Arg401Cys	12	3 families	MH	Davis *et al.*, 2002
C1209G	Ile403Met	12	1 family	CCD	Quane *et al.*, 1993
A1565C	Tyr522Ser	14	1 family	MH/CCD	Quane *et al.*, 1994b
G1021A	Gly341Arg[1,2]	11	6%	MH	Quane *et al.*, 1994a
G1598A	Arg533His	15	---	MH	Brandt *et al.*, 1999
C1654T	Arg552Trp	15	1 family	MH	Keating *et al.*, 1997
C1840T	Arg614Cys[1,2]	17	4%	MH	Gillard *et al.*, 1991
G1841T	Arg614Leu	17	2%	MH	Quane *et al.*, 1997
C6487T	Arg2163Cys[1,2]	39	4%	MH	Manning *et al.*, 1998a
G6488A	Arg2163His[1,2]	39	1 family	MH	Manning *et al.*, 1998a
G6488C	Arg2163Pro	39	-	MH	Brandt *et al.*, 1999
G6502A	Val2168Met[2]	39	7%	MH	Manning *et al.*, 1998a
C6599T	Ala2200Val	40	1 family	MH	Halsall and Robinson, 2004
C6617T	Thr2206Met[1,2]	40	1 family	MH	Manning *et al.*, 1998a
C6617G	Thr2206Arg	40	1 family	MH	Brandt *et al.*, 1999
G6640A	Val2214Ile	40	1.4%	MH	Sambuughin *et al.*, 2001b
A7025G	Asn2342Ser	43	2 families	MH	Halsall and Robinson, 2004
G7036A	Val2346Met	44	1 family	MH	Halsall and Robinson, 2004
A7043G	Glu2348Gly	44	1family	MH	Halsall and Robinson, 2004
G7048A	Ala2350Thr[1,2]	44	1 family	MH	Sambuughin *et al.*, 2001a
C7062T	Arg2355Cys	44	1 family	MH	McWilliams *et al.*, 2002
T7089G	Phe2364Val	44	2 families	MH	Halsall and Robinson, 2004
G7099A	Ala2367Thr	44	1.4%	MH	Sambuughin *et al.*, 2001b
A7992C	Asp2431Asn	45	1.4%	MH	Sambuughin *et al.*, 2001b
G7300A	Gly2434Arg[1,2]	45	4%	MH	Keating *et al.*, 1994
G7304A	Arg2435His[1]	45	1 family	CCD	Zhang *et al.*, 1993
G7304T	Arg2435Leu[1,2]	45	1 family	CCD	Barone *et al.*, 1999
C7354T	Arg2452Trp	46	1 family	MH	Chamley *et al.*, 2000
G7361A	Arg2454His[2]	46	1 family	MH	Barone *et al.*, 1999
C7360T	Arg2454Cys	46	1 family	MH	Brandt *et al.*, 1999

Table 2.2: Continued...

Nucleotide change	Amino acid	Exon	Frequency substitution	Phenotype	Reference
C7372T	Arg2458Cys[1,2]	46	4%	MH	Manning *et al.*, 1998b
G7373A	Arg2458His[1,2]	46	4%	MH	Manning *et al.*, 1998b
G13913A	Gly4638Met	95	2 families	MH	Halsall and Robinson, 2004
T14378C	Leu4793Pro	100	---	CCD	Monnier *et al.*, 2001
A14387G	Tyr4796Cys	100	---	CCD	Monnier *et al.*, 2000
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	101	1 family	MH	Halsall and Robinson, 2004
G14545A	Val4849Ile	101	4 families	MH	Halsall and Robinson, 2004
G14582A	Arg4861His[2]	101	---	CCD	Monnier *et al.*, 2001
C14677T	Arg4893Trp	102	---	CCD	Monnier *et al.*, 2001
T14693C	Ile4898Thr[2]	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
A14740G	Arg4914Gly	102	---	CCD	Monnier *et al.*, 2001
C14814G	Ile4938Met	103	1 family	MH	Halsall and Robinson, 2004
C14817A	Asp4939Glu	103	1 family	MH	Halsall and Robinson, 2004
7045del3nt	Glu2347del[2]	44	2.6%	MH	Sambuughin *et al.*, 2001c
12640del9nt[3]	Arg4214del	91	---	CCD	Monnier *et al.*, 2001
	Gln4215del				
	Phe4216del				
13938del16nt[3]	Leu4647del	95	---	CCD	Monnier *et al.*, 2001
	Ser4648del				
14578delTTC	Phe4860del	101	---	CCD	Monnier *et al.*, 2001

del = indicates a deletion; a dashed line (---) indicates that no information is available; 1 = indicates mutations currently being used in the genetic diagnosis of MH susceptibility by the European Malignant Hyperthermia Group; 2 = indicates mutations currently being used in the genetic diagnosis of MH susceptibility by the North American Malignant Hyperthermia Group; 3 = del gives rise to three different amino acid alterations.

Functional characterisation of RYR1 mutations was conducted on 1B5 skeletal myotubes utilising common MH alterations Arg163Cys, Gly341Arg, Arg2163Cys, valine (Val)2168Met and Arg2458histidine (His) (Yang *et al.*, 2003). Reports from this study indicated that the mutant myotubes displayed increased sensitivity to K^+ depolarisation, caffeine and 4-chloro-*m*-cresol and that myotubes failed to be inactivated in the presence of low concentrations of Ca^{2+}. This study demonstrates that all seven of the MH mutations studied are able to induce MH-related phenotypes.

2.10.4 Specific mutations within the RYR1 gene

Thirty-three mutations were specifically selected for analysis in this study. Five mutations, from mutation region one and two occur in the amino terminal of the RyR1 protein, 17 mutations, from mutation region three, four, five and six occur in the central portion of the RyR1 protein and the ten remaining mutations, from mutation region seven, eight and nine occur in the C-terminal region of the RyR1 protein. The list of mutations investigated in the study presented here, with the mutation region as defined by the study, is indicated in Table 3.3.

2.10.4.1 Ryanodine receptor gene alterations, Cys35Arg and Arg44His that occur in mutation region one

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 represents the most N-terminal MHS mutation reported to date in the RYR1 gene and appears to be restricted to one large consanguineous MHS kindred. The substitution was not detected among 65 unrelated MHS samples or in 200 normal 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, Halsall and Robinson (2004) identified an Arg44His change in the RYR1 gene resulting in a guanine (G)131adenine (A) transition.

2.10.4.2 Ryanodine receptor gene alterations, Glu160Gly, Arg163Cys and Arg163Leu that occur in mutation region two

Halsall and Robinson (2004) reported two alterations, glutamate (Glu)160Gly and Arg163Leu each in one UK family. The Glu160Gly alteration is due to an A479G transition and the Arg163Leu alteration is due to a G488T substitution. 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) and occurs 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 parent and three siblings. An individual from this pedigree 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 results in both hypersensitive gating and diminished E-C coupling and could result in both MH and CCD.

2.10.4.3 Ryanodine receptor gene alterations, Arg2163Cys, Arg2163His and Val2168Met that occur in mutation region three

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 represent causative transitions, as amino acids are conserved in the three isoforms of RYR, as discussed in Section 4.7. Arg2163His was found in an individual from a single family that had both MH and CCD. However, the daughter of the proband had the mutation but was asymptomatic for CCD.

2.10.4.4 Ryanodine receptor gene alterations, Ala2200Val, Thr2206Met and Thr2206Arg that occur in mutation region four

Manning *et al.* (1998a) provided the first report of a novel alteration, threonine (Thr)2206Met that clustered in the central portion (6400-6700) of the RYR1 gene. The alteration Thr2206Met is a result of nucleotide transition C6617T. Brandt *et al.* (1999), observed the Thr2206Arg alteration of exon 40 in a single MH pedigree that occurred due to nucleotide transition C6617G. It was concluded from the IVCT results that the large pedigree of Western European nationality exhibited complete segregation of the phenotype. Halsall and Robinson (2004) identified an alanine (Ala) 2200Val alteration in the RYR1 gene in one of 434 UK families. The Ala2200Val alteration results from a C6599T transition.

2.10.4.5 Ryanodine receptor gene alterations, Val2346Met, Glu2348Gly, Ala2350Thr, Arg2355Cys, Phe2364Val, Gly2434Arg, Arg2435His and Arg2435Leu that occur in mutation region five

Five mutations in the RYR1 gene that occur in the central portion of the RyR1 protein, Val2346Met, Glu2348Gly, Arg2355Cys and phenylalanine (Phe)2364Val were all reported by Halsall and Robinson (2004). Two alterations, Val2346Met and Glu2348Gly were each observed in one UK family, and occur due to nucleotide transitions G7036A and A7043G, respectively. The alteration Arg2355Cys was observed in six UK families and is due to a C7062T nucleotide substitution and the alteration Phe2364Val was observed in one UK family and is due to a nucleotide alteration T7089G.

The Ala2350Thr alteration was first identified in exon 44 of the RYR1 gene by Sambuughin *et al.*, (2001a) 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 A2350T 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. Sambuughin *et al.* (2001a) suggested that the A2350T mutation may play a role in MHS.

The Gly2433Arg alteration which results in a G to A 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 observed only in MH patients. However, complete association of the mutation to MH in certain families was not observed. 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 (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. 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) utilised the 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 calmodulin 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.

2.10.4.6 Ryanodine receptor gene alterations, Arg2452Trp, Arg2458Cys and Arg2458His that occur in mutation region six

Alterations Arg2458Cys and Arg2458His were first reported by Manning *et al.* (1998b). Both mutations occur at a 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 was not identified in MH normal (MHN) individuals. The Arg2458Cys alteration was detected in a Swiss and Italian pedigree, and the mutation segregated to both MHS and MH equivocal (MHE) individuals. Arg2458His alteration was detected in a French 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 Arg2452tryptophan (Trp) alteration in the RYR1 gene in one of 434 UK families. The mutation results from a C7354T nucleotide transition.

2.10.4.7 Ryanodine receptor gene alteration, Gly4638Met that occurs in mutation region seven

A single mutation is found in mutation region seven in the C-terminal region of the RyR1 protein. The alteration Gly4638Met was observed in two UK pedigrees with MH and is due to nucleotide substitution, G13913A (Halsall and Robinson, 2004).

2.10.4.8 Ryanodine receptor gene alterations, Leu4814Phe, Ile4817Phe, Leu4824Pro, Thr4826Ile, Leu4838Val, Val4849Ile and Arg4861His that occur in mutation region eight

Alterations Leu4814Phe, isoleucine (Ile) 4817Phe and Leu4824proline (Pro) 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 on exon 101, which results from substitution, 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 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). Alterations Leu4838Val and Val4849Ile were observed in UK families (Halsall and Robinson, 2004). The mutations occur due to nucleotide transitions in the RYR1 gene, C14512G and G14545A, respectively. Alteration Leu4838Val was observed in a single pedigree and Val4849Ile was observed in four pedigrees.

2.10.4.9 Ryanodine receptor gene alterations, Ile4938Met and Asp4939Glu that occur in mutation region nine

Alteration Ile4938Met and Asp4939Glu were observed in UK families (Halsall and Robinson, 2004). The mutations occur due to nucleotide transitions in the RYR1 gene, C14814G and C14817A, respectively. Alterations Ile4938Met and Asp4939Glu were observed in different single pedigrees.

2.11 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 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). A study conducted by Moslehi *et al.* (1998) further supported linkage to chromosome 17q in a study of a large Canadian family.

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. Iles *et al.* (1994) indicated cosegregation of the polymorphic genetic marker, D7S849, adjacent to the CACNL2A locus with MHS in a German pedigree with a lod score of 2.91. Linkage in other pedigrees and a causal mutation have not yet been identified. Susceptibility to MH may be due to a 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 (Carsana *et al.*, 2003; Steward *et al.*, 2001). 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 *et al.* (1999) 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.

2.11.1 Candidate genes

Table 2.3 lists the chromosomal locations which have been found to harbour potentially causative MH susceptibility loci as well as the mutations found 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 2.3: 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. Adapted from Denborough (1998).

2.11.1.1 Structural organisation of the DHPR genes

The DHPR of skeletal muscle, also referred to as the L-type voltage-dependent Ca^{2+} channel, is composed of five subunits, namely α_1, α_2, β_1, γ and δ (Catterall, 1991). As discussed in paragraph 1.3, DHPR plays an important role in E-C coupling, indicating that abnormal channel functions of the receptor may be involved in the MH phenotype (Zorzato *et al.*, 1990).

The gene encoding the DHPR α_2/δ-subunits (CACNL2A) has been localised to chromosome 7q21-q22 (Powers *et al.*, 1994). Both of these subunits are encoded by a single gene and are produced by proteolytic cleavage of the polypeptide. In addition to expression in skeletal muscle the gene is also expressed in the brain, heart and lung (Mouton *et al.*, 2001). Iles *et al.* (1994) determined that the location of CACNL2A was in the proximal region of 7q21.1 and indicated that a mutation in or near the gene encoding the α_2/δ subunits may result in MHS. The gene encoding the DHPR α_1-subunit (CACNA1S) has been localised to chromosome 1q31-q32 (Gregg et *al.*, 1993). Genes encoding all the α_1 subunits display similar organisation, but are divergent in their predicted amino acid sequence. In mice animal models, absence of the α_1 subunit leads to muscular dysgenesis (Knudson *et al.*, 1989). In humans, the cytoplasmic region between repeats II and III of the α_1 subunit is an important determinant of E-C coupling. However, activation occurs at a slower rate (Tanabe *et al.*, 1990) indicating that subtle alterations or mutations in this gene may result in MH. The function of the α_1 subunit is greatly enhanced in the presence of the other subunits (Catterall, 1991). The γ-subunit gene (CACNLG) is 12.5 kilobase pairs (Kb) in length, and is composed of four exons. A

222-amino acid polypeptide (Powers *et al.,* 1993) is encoded by this gene and has only been identified in skeletal muscle (Jay *et al.,* 1990). Analysis has indicated that the human γ gene is localised to chromosome 17q23 and is located in close proximity to the skeletal muscle Na^+ channel (Powers *et al.,* 1993). Susceptibility to MH has been localised to chromosome 17q22-24 (Olckers *et al.,* 1992), indicating that mutations in the γ subunit may result in susceptibility to myotonic disorders. The DHPR β_1 subunit gene (CACNLB1) has been localised to the 17q11.2-q22 region (Gregg *et al.,* 1993). A single gene encodes three different β_1 subunits, and includes one that is expressed in skeletal muscle and two different β subunits expressed in the brain, heart and spleen (Powers *et al.,* 1993).

2.11.1.1.1 Allelic alterations in the DHPR gene and their association with MH

Contradicting data are available for association between MH and allelic mutations in the DHPR gene. Although the DHPR plays an important role as both a Ca^{2+} channel and a voltage sensor in E-C coupling, and has been associated with neuromuscular disorders, there is little difference in the structure of the II-III loop and the IS3/IS3/IS4 linker segments between MHN and MHS patients. However, a mutation in this loop could modify the interaction between the DHPR and the RyR1 protein, mimicking the effects of mutations observed in the RyR1 protein (Leong and MacLennan, 1998). Sudbrak *et al.* (1993) excluded the involvement of the α_1, β_1 and γ subunits as candidates for the molecular defect of MH in pedigrees utilised in their study, whereas Monnier *et al.* (1997) and Stewart *et al.* (2001) implicated mutations in this gene with MHS phenotype but not MHE patients.

The single point mutation in the gene of CACNA1S that resides on chromosome 1q, is found in the cytoplasmic region (loop) between domains III and IV of the protein. The direct functional interaction between the loop of the DHPR and the RYR1 could lead to MHS in a case where an alteration occurs in either of the two proteins. The mutation results in an A3333G nucleotide substitution that results in an Arg1086His alteration. An association of this has been found with MH in six members of a French family. An individual from that family diagnosed as MHE was reported as negative for the Arg1086His alteration (Monnier *et al.,* 1997). A similar occurrence was observed by Stewart *et al.* (2001), where two MHS patients in a family from North America inherited the mutation and an MHE patient from the same family tested negative for the mutation. The author did not observe the mutation in one MHS patient from the same family, and the cause of this discordance in the family was unclear. The discordant individual could be

carrying an additional MHS alteration in the RYR1, CACNA1S or another unidentified protein, which resulted in disease. The authors suggested that the perhaps multiple mutations had to be co-expressed in order to result in the MHS phenotype. It was concluded that these substitutions alone do not cause MH or that this defect in the DHPR α_1 subunit does not result in the MH phenotype in this specific pedigree.

2.11.1.2 The sodium channel

Voltage gated Na^+ channels are required for the production and transmission of an action potential in striated muscle. The Na^+ channel consists of a large α subunit which serves as an ion pore and one or two smaller β subunits that modify the functions of the α subunit (Makita *et al.*, 1994a). The human skeletal muscle SCN4A α subunit gene has been assigned to chromosome locus 17q23.1-q25.3 (George *et al.*, 1991), and has been suggested as a candidate for MHS. The human skeletal muscle Na^+ channel β subunit gene SCN1B has been assigned to chromosome locus 19q13.1-q13.2 and consists of five exons and four introns (Makita *et al.*, 1994b). The SCN4A gene consists of 24 exons spanning a 35 Kb distance (McClatchey *et al.*, 1992). Two forms of the α subunit occur, the SKM1, which is the adult form and the SKM2, which is the juvenile form. Cell cultures of skeletal muscle have been used to study these two forms of the α subunit. Research has shown that the percentage of SKM2 current is reduced in MHS muscle as compared to MHN muscle. Fletcher *et al.* (1997) indicated that an altered expression of the α subunit of SKM2 occurs in MHS muscle. The authors suggested that a mutation in the RYR1 gene in combination with down regulation of SKM2 may result in susceptibility to MH. A variety of disorders, including generalised nondystrophic myotonia and hyperkalemic periodic paralysis (HyperPP) and paramyotonia congenital (PC) have proved to be associated with allelic mutations of the SCN4A gene (Fontaine *et al.*, 1990; Koch *et al.*, 1991; Lerche *et al.*, 1993).

2.11.1.2.1 Allelic alterations in the SCN4A gene

Vita *et al.* (1996), observed a novel alteration in exon 22 of the SCN4A gene. The mutation was observed in four individuals in a large pedigree from the United States (USA) and was co-inherited with MMR and an abnormal IVCT. The alteration, Gly1306Ala, occurred due to the nucleotide transition G3917C and the mutation has only been detected in a single family. The authors suggested that other mutations in the 23

remaining exons of the SCN4A gene or in the β subunit of the gene may uncover additional mutations involved in the disease pathogenesis. Further analysis will be required to determine whether mutations in the SCN4A gene are associated with MMR, whole body rigidity and an abnormal IVCT result.

2.12 EPISTASTATIC 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 to determine if several independent genes (RYR1, CACNA1S, CACNA2D1, MHS-4, MHS-6, LIPE, MD1 and dystrophin) 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. Robinson *et al.* (2003b) then 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. They 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 normal sensitivity to halothane. Their study indicates that mutations in several different genes are involved in sensitivity to halothane. Morgan and Sedensky (1995) found 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.

2.13 DISCORDANCE BETWEEN PHENOTYPE AND GENOTYPE

Reports of individuals that were diagnosed as normal 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.*, 2003a). 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. Therefore a particular allele may increase the risk of susceptibility, but on its own may not be sufficient to result in MH.

2.14 DIAGNOSIS OF MH

Currently the only method used as a pre-symptomatic diagnosis of MH is the IVCT. This test has several disadvantages, since it is invasive, expensive and time-consuming. Several other tests have been suggested for the diagnosis of MH. Including frozen section Ca^{45} uptake procedures (Nagarajan *et al.*, 1987), the tourniquet-twitch test (Britt *et al.*, 1986) and platelet adenosine triphosphate (Lee *et al.*, 1985). Many have proven to be unreliable and have been abandoned. Other tests have indicated some promise. However, a simple and reliable method that is non-invasive and easy to perform has not been developed yet.

2.14.1 The *in vitro* contracture test (IVCT)

The first specific method to screen susceptible patients for the MH phenotype was developed by Ellis *et al.* (1972). The IVCT has been utilised for the last 30 years as a recognised diagnostic tool to determine MH susceptibility. Myopathy was diagnosed if an abnormal muscle contracture in the presence of triggering agents such as halothane and succinylcholine was observed. Standardised protocols were developed in both Europe (The European Malignant Hyperpyrexia Group, 1984) and North America (Larach, 1989) in 1984 and 1989, respectively. These tests determine the contracture of living tissue in response to agents such as halothane and caffeine. A standardised European protocol using both halothane and caffeine was published by the EMHG in 1984. Based on 202

controls the test indicated a sensitivity of 99% and a specificity of 93.6% (Ørding *et al.*, 1997). The test allows classification of individuals as MHS, MHE or MHN. Both MHS and MHE groups are regarded as being at risk for MH on the clinical level. A test result is considered positive if there is a sustained increase in contracture of 0.2 g following a threshold dose of 2% halothane and independently, at a caffeine concentration of more than 2 mM. 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 European protocol utilises more increments in the caffeine and halothane concentrations, resulting in a lower diagnostic threshold. In addition the North American protocol has a sensitivity of 97% and specificity of 78%. Overall both protocols provide a similar diagnosis (Fletcher *et al.*, 1991) and maximize 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 normal patients will have false positive results. The EMHG protocol has a higher specificity and reduces the number of MHE individuals, which may result in the diagnosis of this protocol being more accurate (Fletcher *et al.*, 1999). Larach *et al.* (1992) suggested that if the contracture cut-off points were modified, the diagnostic test would become more sensitive and adequately specific.

2.14.2 Proposed biochemical diagnostic testing

Isaacs and Barlow (1970) identified in a single large family that 50% of individuals had elevated CK levels and suggested that this could be used as a screening test for MH. The authors observed that the CK activity increased following exercise and that the raised levels were also observed in patients affected with certain diseases not related to MH. Ellis *et al.* (1972) observed false-negative results when measuring CK activity in patients with positive evidence of myopathy, while some families had elevated activities that correlated with contracture test results.

Paasuke and Brownell (1986) reported that serum CK levels could not be used for screening or as a diagnostic test for MH because of the high likelihood of false positive and false negative results. In view of the non-specificity and insensitivity of detecting the levels of CK, this test could not be utilised on its own to diagnose susceptibility in MH patients.

Anetseder *et al.* (2002) have suggested using metabolic testing to diagnose MHS. This minimally invasive test involves intramuscular injection of caffeine and the measurement of the resulting local increase in pCO_2. Patients susceptible to MH were observed to have a temporary increase in pCO_2 compared to healthy controls. However, further methodological investigation would be required to determine sensitivity and specificity before this test could be used for diagnostic purposes.

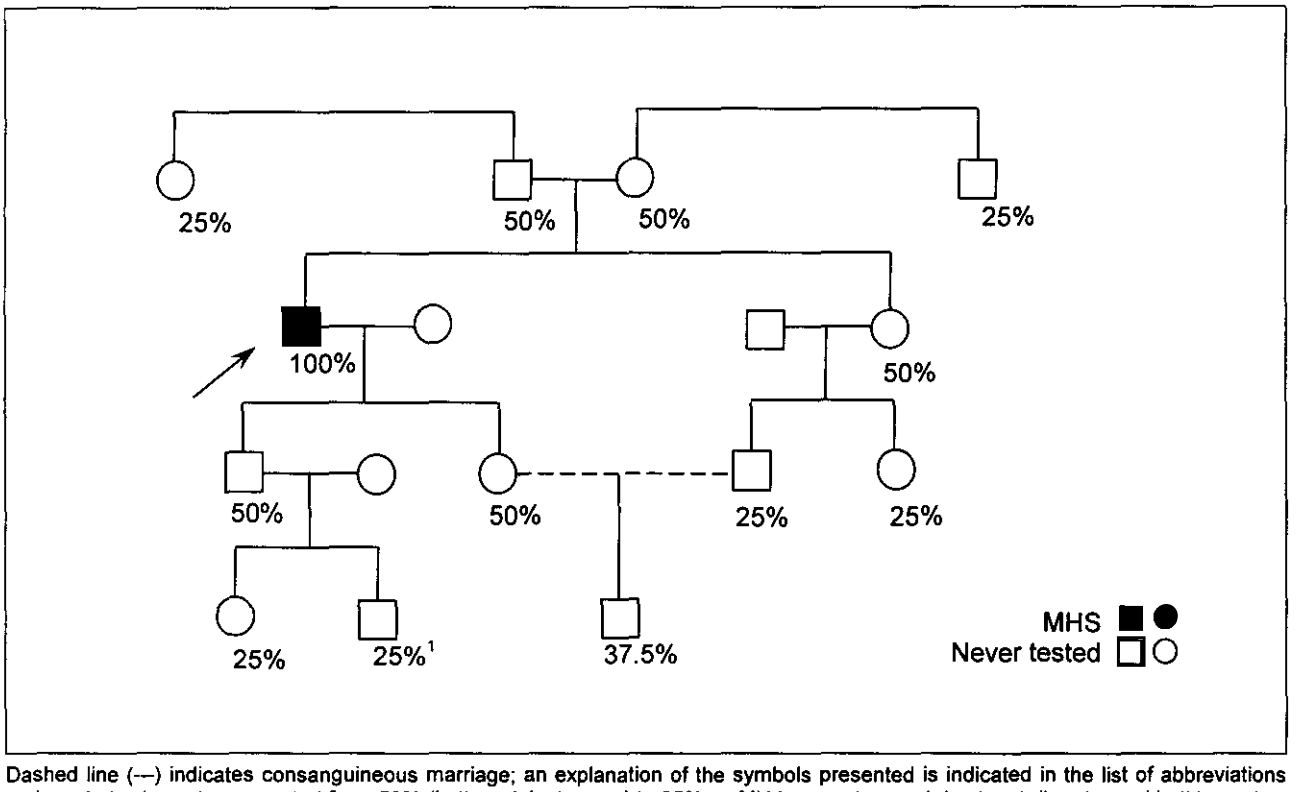
Another technique has been suggested by Olgin *et al.* (1988) that monitors muscle metabolite concentrations during exercise. The phosphorus nuclear magnetic resonance spectroscopy (^{31}P NMR) can differentiate between normal and MH muscle on the basis of a muscle Pi / level of phosphocreatine (PCr). Individuals with MH generally have higher resting Pi/PCr values accompanied by a slower post-exercise recovery rate compared to MHN controls. The procedure had a sensitivity of 98.8% and specificity of 95.3% (Olgin *et al.*, 1991). This technique cannot distinguish several other muscle disorders from MH and cannot be used in diagnosing MHS in patients with other neuromuscular symptoms. The value of this procedure in identifying MHS individuals will only be established following further studies into the role of ^{31}P NMR.

Ryanodine has been suggested as an agent that could be used in an IVCT. Initial studies have indicated that the ryanodine protocol is able to distinguish between MHS and MHN individuals (Hartung *et al.*, 1996). However, the reproducibility of this test has not been investigated and further studies will have to be conducted to validate these results.

2.14.3 Proposed genetic diagnostic testing

Genetic screening cannot currently be used for routine diagnosis of MH due to the low incidence of mutations observed in the RYR1 gene. 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. The risk of inheriting MH, starting with first-degree relatives who carry a 50% risk, is illustrated in Figure 2.3.

Figure 2.3: The risk of inheriting MH



Dashed line (---) indicates consanguineous marriage; an explanation of the symbols presented is indicated in the list of abbreviations and symbols. 1 = value corrected from 50% (in the original report) to 25%,as MH is an autosomal dominant disorder and in this context the risk of inheriting MH is 25% and not 50% (when the parental MH risk = 50%), therefore it is likely that this is an error in the paper. Adapted from Halsall and Robinson (2004).

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. The aim of family screening will be to identify individuals who are susceptible to MH and could result in quicker results for the relatives.

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

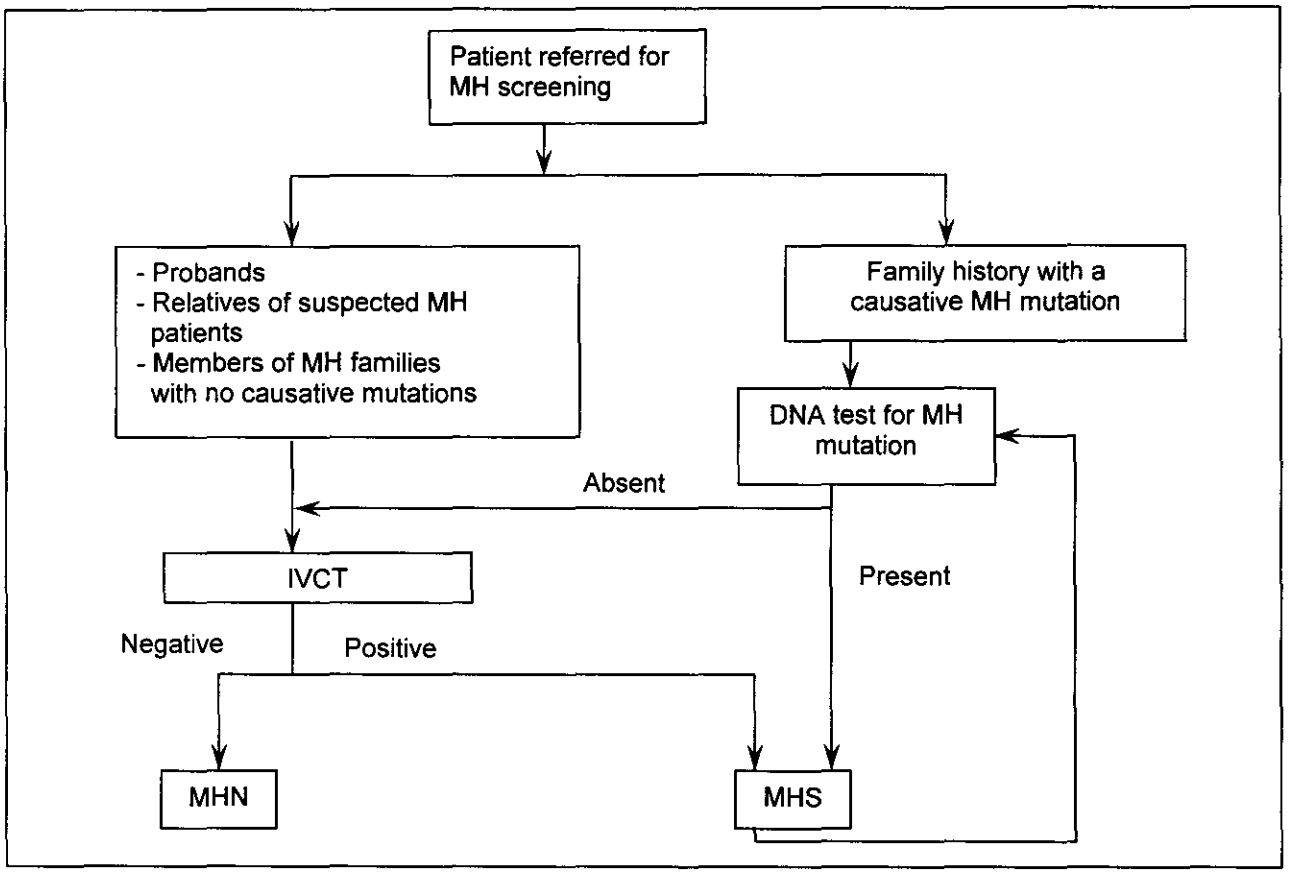
i) Mutation analysis of causative mutations only for the RYR1 gene can be used to diagnose the remaining members of a family if the index case was diagnosed via the IVCT.

ii) Segregation analysis of genetic markers close to known MHS loci may be utilised 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. A suggested route for MHS testing is indicated in Figure 2.4.

Figure 2.4: Suggested decision pathway for MHS testing



IVCT = *In vitro* contracture test; MHN = malignant hyperthermia normal; MHS = malignant hyperthermia susceptible. Adapted from Urwyler *et al.* (2001).

The illustration should be observed as a provisional route for MHS, as it does not take into account the possibility of false negatives or false positives for both genetic testing and the IVCT. The reason for caution is a consequence of reported discordance between IVCT and genetic data in MH families. False negative results for the IVCT have been reported in a small percentage of MHS animals (Gallant and Rempel, 1987). An MH individual diagnosed as MHS using the IVCT would 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. An 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, based on 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).

2.15 OBJECTIVE OF THE RESEARCH PROGRAMME

The broad aim of the MH research programme in the Centre for Genome Research is to screen all causative mutations of the RYR1 gene 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 initial 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.

2.16 AIMS OF THE STUDY

The long-term aim of the MH research programme is to determine the exact aetiology of MH in the South African population. The molecular investigation presented in this thesis was performed to determine if specific mutations segregate with the MH phenotype. 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.

2.16.1 Specific project aim

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. Causative mutations were selected, as it is essential that they be included in this screening programme. It is envisioned that this project will contribute to the broad aim of the MH research programme, which is to introduce limited diagnostic testing in family members of the proband. The specific aim of this study was:

- To determine if 15 recently reported causative RYR1 alterations that have not yet previously been screened for in South African MH probands are present in any of the 30 MH individuals.

CHAPTER THREE

MATERIALS AND METHODS

This molecular investigation forms part of the ongoing MH research programme. Ethical approval for the MH project titled "*Molecular analysis of malignant hyperthermia (MH) susceptibility*" was obtained for this study in 2002 from the Ethics Committee of North-West University with approval number 02M10. Informed consent was obtained from the patients involved in this project prior to their participation.

The molecular investigation was conducted utilising a group of thirty one individuals from the South African MH population. All individuals selected were identified as the proband and had experienced an MH episode or was diagnosed as MHS with the IVCT or had a family history of MH. Only the proband was screened for mutations and family members of these individuals were not included in this study. In addition to these individuals, a group of 14 South African MHS families was included in this investigation. All families included a proband that had experienced an MH episode. Individuals from five of these families were later diagnosed through muscle contracture studies, and results of the biopsies are discussed in Section 3.1.2 and presented in Table 3.2. Individuals from the remaining nine families have not been previously diagnosed via the IVCT, thus their MH status is currently unknown. If a positive result were to be obtained in the proband for any of the mutations examined, family members of the proband would be screened.

As samples are continually being collected for the MH research programme, a numbering system which included a unique family identity number followed by a unique individual number within that particular family was utilised to maintain consistency. Families were numbered MH101 for example, and individuals were allocated a number following the family identification number, for example MH101-123. Individuals for whom family samples were unavailable were only given an individual number, for example MH00324.

Previously molecular screening of individuals within families was conducted for selected causative mutations of the RYR1 gene. This group of individuals has been screened in the ongoing MH project for 17 other MH-associated RYR1 mutations, and certain individuals were screened for the Arg1086His alteration of the CACNA1S gene. Results that were

previously obtained are summarised in Appendix C (Table C1). As the study reported here forms part of an extended and ongoing research programme, probands screened for mutations in the previous study as well as recently collected probands were investigated for recently reported RYR1 mutations. Probands that had previously not been screened for the Arg1086His alteration of the CACNA1S were also analysed.

3.1 PATIENT POPULATION

Blood samples for DNA extraction were collected from probands and family members of the index case. Probands included in this investigation were diagnosed as MHS based on clinical signs of MH observed during a previous anaesthesia (if biopsy data were unavailable) or according to the IVCT protocol outlined by the EMHG. In agreement with the recommendations of the EMHG (1984, 1985), a biopsy sample was characterised as MHS if the muscle strip exposed to halothane or caffeine exceeded the acceptable diagnostic contracture threshold of 0.2 g at caffeine concentrations of 2 mM or less, and halothane concentrations of 2% or less. Individuals whose muscle strips did not meet these criteria were diagnosed as MHN. Individuals were characterised as MHE if the contracture of the muscle strips at the threshold concentration occurred for either caffeine of halothane.

3.1.1 Malignant hyperthermia individuals included in this study

Individuals included in this study were diagnosed as being susceptible to MH according to their clinical status as indicated by clinical records. Several individuals were identified as MHS based on clinical symptoms observed during previous anaesthesia. Suspected MH cases were also included in this study if a family history of MH was present and they were considered susceptible to MH (Sessler, 1986). In certain cases, individuals who had experienced an MH episode were subsequently diagnosed via the IVCT. Clinical records indicate that these individuals had undergone a muscle biopsy. However, these results were not available to the researcher. Diagnosis of MHS based on observed clinical symptoms during previous anaesthesia can be complicated by the diagnosis of another disorder. Some individuals included in this study were diagnosed as MHS in addition to another disorder. A full description of the clinical status of each individual included in this study is listed in Table 3.1. DNA from these individuals was isolated and screened for reported causative mutations.

Table 3.1: Clinical description of MHS individuals included in this study

Individual number	Clinical status	Description of clinical status
MH00364	Clinical symptoms	Identified as MHS based on clinical symptoms observed during three previous anaesthetic procedures. This individual does not have a family history of MH.
MH00427	Clinical symptoms	Experienced clinical symptoms of MH under anaesthesia. Symptoms included hypocalcaemia (abnormally high levels of Ca^{2+}), increased CK levels, muscle cramps and tachycardia.
MH00438	Family history	Is a suspected MH case, with a family history of MH.
MH00439	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH00440	Clinical symptoms	Developed intractable pyrexia (fever) during previous anaesthesia, and does not have a family history of MH.
MH00497	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH00630[1]	Clinical symptoms	Has a family history of MH, as his sister died from MH following an anaesthetic procedure. An increase in PCO_2 and elevated body temperature were recorded during an anaesthetic procedure.
MH00631	Family history	Has a family history of MH, as her daughter died following an anaesthetic procedure in which the symptoms of MH were recorded.
MH00649	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH00654[2]	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH00660	Clinical symptoms	Became hypertonic during previous anaesthesia and has a family history of MH.
MH00668	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH00671[3]	Clinical symptoms	Developed symptoms associated with MH during a tonsillectomy.
MH00674[4]	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH00691	Clinical symptoms	Developed an MH-like reaction under anaesthesia and was also diagnosed with spina bifida occulta.
MH00707	Clinical symptoms	Suspected of having MH due to observed clinical symptoms under anaesthesia, and was previously diagnosed with Parkinson's disease.
MH00709	Clinical symptoms	Identified as MHS based on clinical symptoms observed during two previous anaesthetic procedures.
MH00710	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH00749	Clinical symptoms	Developed pyrexia postoperatively on three separate occasions.
MH00766	MHS	Had a positive muscle biopsy result.
MH00788	Clinical symptoms	Developed symptoms associated with MH, including elevated body temperature, during dental surgery.
MH00803	Clinical symptoms	Developed symptoms associated with MH during a tonsillectomy operation. This individual previously triggered with MH as a child when undergoing general anaesthesia and subsequently had muscle biopsies taken from both legs.

Table 3.1: Continued...

Individual number	Clinical status	Description of clinical status
MH00871	Clinical symptoms	Developed clinical signs of MH while at the dentist, and has previously been diagnosed with DMD. A probable case of MH in a child with DMD was reported by Brownell *et al.* (1983).
MH00874	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH00875	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH01330	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH01331	MHS	Had a positive muscle biopsy result.
MH01413	Clinical symptoms	Identified as MHS based on clinical symptoms observed during previous anaesthesia.
MH109-24[5]	Family history	Has a family history of MH and had an elevated resting CPK level.
MH110-1[5]	Clinical symptoms	Developed symptoms associated with MH following exposure to succinylcholine.
MH116-1[5]	Family history	Has a family history of MH, her brother developed symptoms associated with MH during a previous anaesthesia.

1 = Indicates that a DNA sample was also obtained from the father of this individual (MH00632). However, both individuals have not yet been assigned a family identification number and the MH status of the father is unknown; 2 = Indicates that DNA samples were collected from the parents of the proband (MH00654) but were not included in the mutation screening and the proband was assigned an individual number; 3 = Indicates that the DNA samples were also collected from the daughter of the proband. However, these were not included in this study and the proband was allocated an individual number; 4 = Indicates that the baby of individual, MH00674 has an unknown MH status and was not included in this analysis, and the proband was allocated an individual number; 5 = Indicates individuals that were assigned a family number. Ca^{2+} = calcium; CK = creatine kinase; PCO_2 = carbon dioxide partial pressure; DMD = Duchenne muscular dystrophy; CPK = creatine phosphokinase.

3.1.2 Individuals from malignant hyperthermia families included in this study

According to the IVCT protocol, probands were typed MHS, MHN or MHE, as discussed in Section 3.1. Equivocal results are indicated as MHEh and MHEc, depending on whether the caffeine (MHEc) or halothane (MHEh) tests were positive. Results of the IVCT for five of the MH families included in this study are listed in Table 3.2. Diagnoses were confirmed by muscle tension studies, performed by Prof H. Isaacs from the Department of Physiology at the University of the Witwaterand. Individuals for whom IVCT data were not available are not indicated in Table 3.2.

Table 3.2: Diagnostic IVCT results as determined by the European IVCT protocol

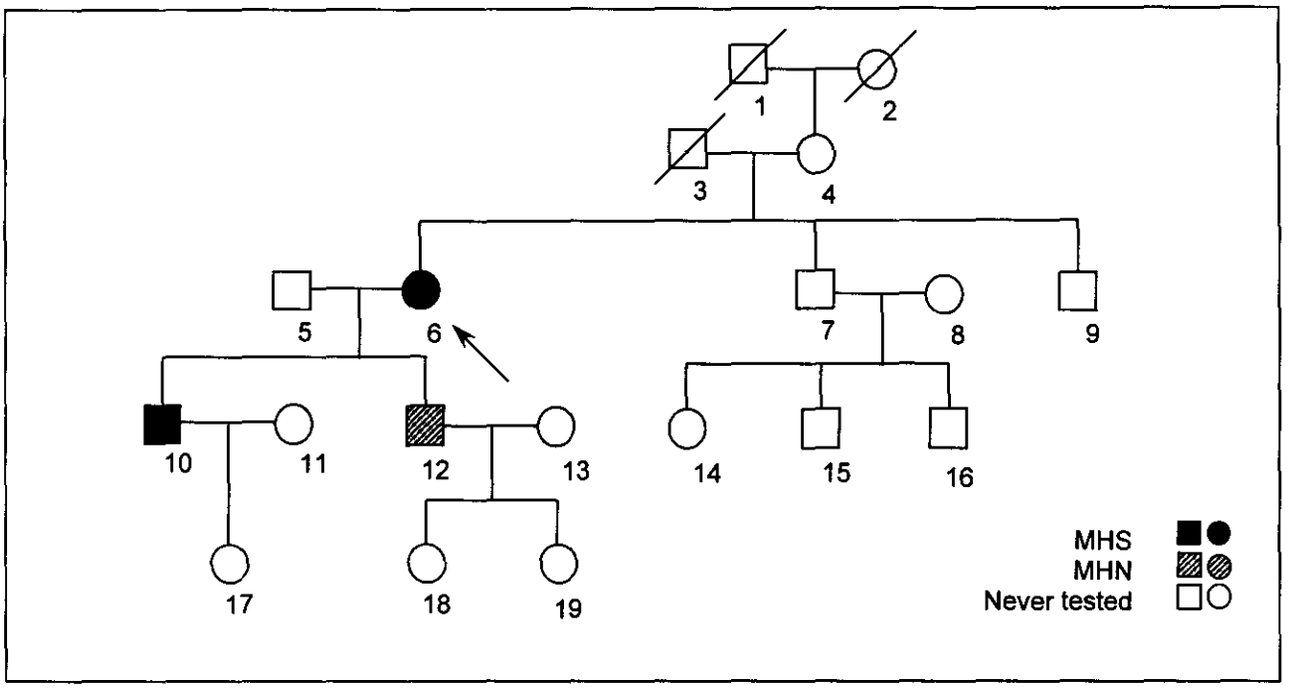
Family number	Caffeine (mM)	Halothane (vol %)	MH status	Family number	Caffeine (mM)	Halothane (vol %)	MH status
MH 101-6	2.0	1.0	MHS	MH 104-25	8.0	2.5	MHN
MH 101-10	2.0	0.5	MHS	MH 104-26	1.0	0.5	MHS
MH 101-12	4.0	4.0	MHN	MH 104-27	0.0	0.0	MHN
MH 102-2	1.0	1.0	MHS	MH 104-35	2.0	1.0	MHS
MH 102-4	8.0	4.0	MHN	MH 105-20	2.0	1.0	MHS
MH 102-11	8.0	4.0	MHN	MH 105-23	4.0	4.0	MHN
MH 102-24	0.5	1.0	MHS	MH 105-26	2.0	0.5	MHS
MH 102-28	2.0	1.0	MHS	MH 105-28	4.0	4.0	MHN
MH 102-39	2.0	0.5	MHS	MH 105-32	1.0	1.0	MHS
MH 102-48	0.0	0.0	MHN	MH 105-35	2.0	1.0	MHS
MH 102-96	4.0	2.0	MHEh	MH 105-36	1.0	2.0	MHS
MH 102-117	2.0	0.5	MHS	MH 105-37	4.0	4.0	MHN
MH 102-125	1.0	2.0	MHS	MH 105-38	0.5	0.5	MHS
MH 103-4	2.0	0.5	MHS	MH 105-39	8.0	4.0	MHN
MH 103-9	3.0	0.5	MHE	MH 105-63	1.0	1.0	MHS
MH104-24	8.0	4.0	False MHN	MH 105-64	1.5	0.5	MHS

MHS = malignant hyperthermia susceptible; MHN = MH normal; MHEh = MH equivocal, positive for halothane; mM = millimolar; vol % = percent volume per volume.

3.1.2.1 Malignant hyperthermia family MH101

Family MH101 consists of 19 members. The pedigree indicating all nineteen individuals is presented in Figure 3.1. The proband, MH101-6, developed a high fever and experienced diaphoresis following surgery and she was subsequently diagnosed as MHS. The children of the proband, MH101-10 and MH101-12, were subsequently tested for MH and were diagnosed as MHS and MHN respectively. The results obtained from the IVCT for three individuals indicated here, are listed in Table 3.2.

Figure 3.1: Pedigree of family MH101

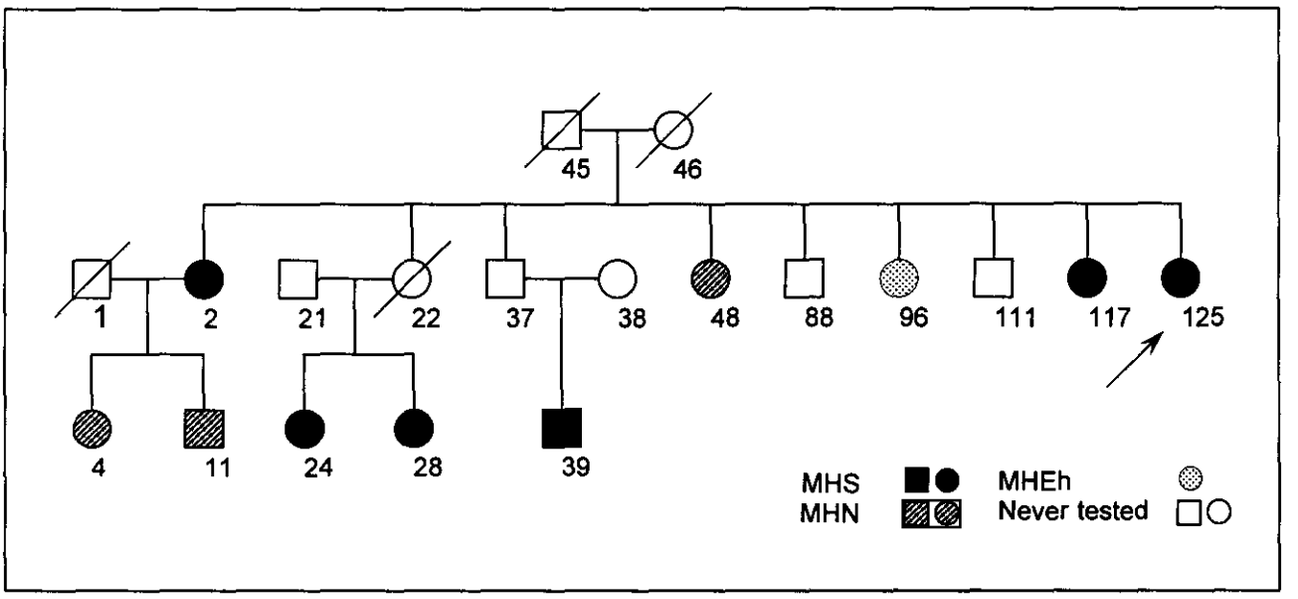


An explanation of the symbols is provided in the list of abbreviations and symbols. Adapted from Olckers (1997).

3.1.2.2 Malignant hyperthermia family MH102

The pedigree for MH102 consists of 127 members. Biopsy data were available for ten individuals, and results obtained for those individuals are listed in Table 3.2. An excerpt of the pedigree indicating the ten members for whom IVCT data are available is illustrated in Figure 3.2. The proband, MH102-125, developed pyrexia during anaesthesia, and was subsequently tested for MH. The young female yielded a positive reaction to halothane with the IVCT. However, the diagnosis was performed prior to the adoption of the protocol of the EMHG, which requires inclusion of both halothane and caffeine to diagnose MH. Consequently, this individual (MH102-125) was later re-tested using the caffeine contracture test. The positive result that was obtained further confirmed the diagnosis of MH positive. Individual MH102-96 was diagnosed as MHE following a positive reaction to halothane, but a negative reaction to caffeine. IVCT results for the remaining individuals tested, indicated MHS diagnosed for MH102-2, MH102-24, MH102-28, MH102-39 and MH102-117. The group of individuals diagnosed as MHN via the IVCT included MH102-4, MH102-11 and MH102-48.

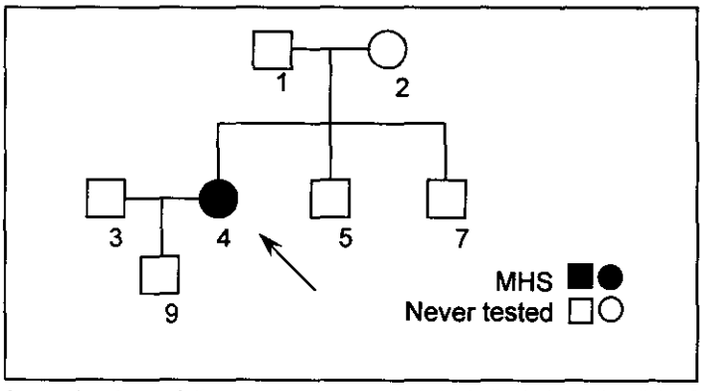
Figure 3.2: Excerpt from pedigree MH102



An explanation of the symbols presented is indicated in the list of abbreviations and symbols. Adapted from Olckers (1997).

3.1.2.3 Malignant hyperthermia family MH103

Figure 3.3: Pedigree of family MH103[1]



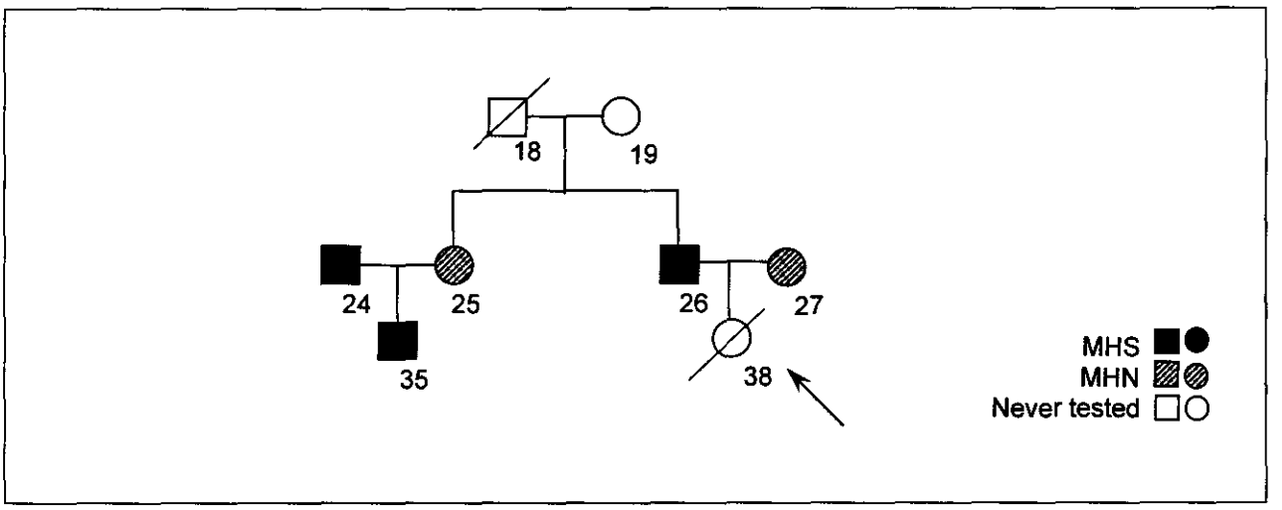
An explanation of the symbols presented is indicated in the list of abbreviations and symbols. Adapted from Olckers (1997).

Family MH103 includes seven members. The pedigree of family MH103 indicating all seven members is illustrated in Figure 3.3. The proband (MH103-4) was diagnosed as MH positive via the IVCT. The son of the proband (MH103-9) was diagnosed as MHEh as he obtained a positive reaction for halothane contracture but tested negative for the caffeine contracture. The results obtained for these two individuals are listed in Table 3.2.

3.1.2.4 Malignant hyperthermia family MH104

The pedigree of MH104 consists of 46 individuals. An excerpt of the pedigree indicating eight individuals is depicted in Figure 3.4. The proband (MH104-38), developed pyrexia following the administration of anaesthesia during a dental procedure when she was two years old. This was her first anaesthesia and it unfortunately resulted in her death. She was never tested for MH.

Figure 3.4: Excerpt from pedigree MH104



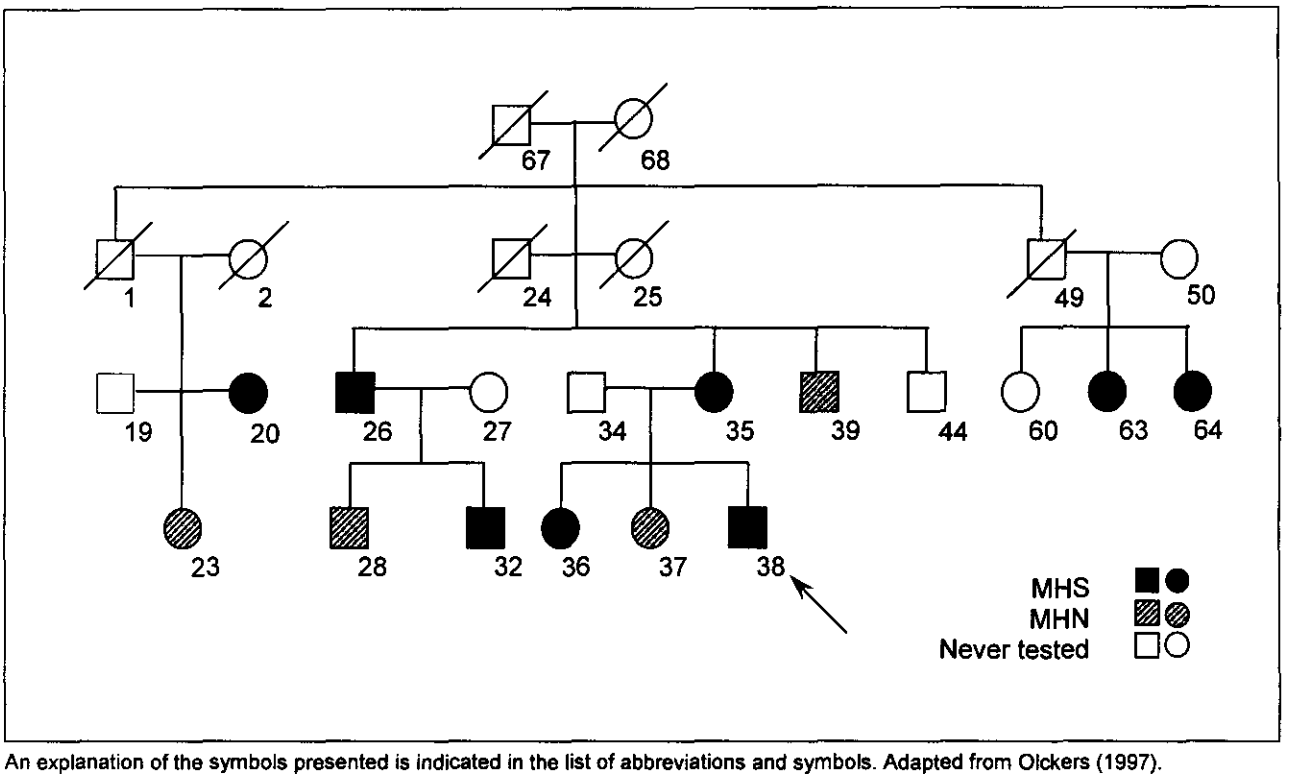
An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

The immediate family was subsequently tested for MH and available biopsy data indicated that individuals MH104-35 and MH104-26 are MH positive, while individuals MH104-25 and MH104-27 are MHN. Individual MH104-24 was initially diagnosed as MHN, however, this diagnosis was actually false negative and the individual was subsequently re-classified as MHS. False negative results have been reported previously for MH patients by Isaacs and Badenhorst (1993) with the muscle caffeine halothane contracture test (CHCT). Individual MH104-26 was screened for mutations, as no material was available to include the proband (MH104-38) in this study. The IVCT results obtained for these individuals are listed in Table 3.2.

3.1.2.5 Malignant hyperthermia family MH105

Family MH105 includes 153 members, IVCT data are available for the distant branches of this pedigree, but are not indicated. An excerpt of this pedigree indicating 25 individuals is displayed in Figure 3.5. The proband (MH105-38) developed an MH-like reaction following the administration of anaesthesia. He subsequently tested MH positive via the IVCT at the age of 14. Following his positive result, some members of the extended family were also tested for MH. Biopsy data indicated that individuals MH105-20, MH105-26, MH105-32, MH105-35, MH105-36, MH105-38, MH105-63 and MH105-64 were MHS. Individuals MH105-23, MH105-28, MH105-37, and MH105-39 were all diagnosed as MHN. All biopsy results obtained for this pedigree are listed in Table 3.2.

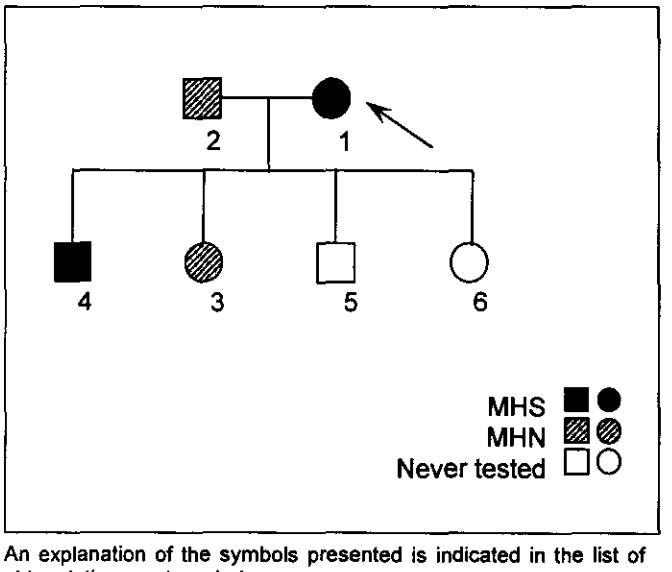
Figure 3.5: Excerpt from pedigree MH105



An explanation of the symbols presented is indicated in the list of abbreviations and symbols. Adapted from Olckers (1997).

3.1.2.6 Malignant hyperthermia family MH107

Figure 3.6: Excerpt from pedigree MH107

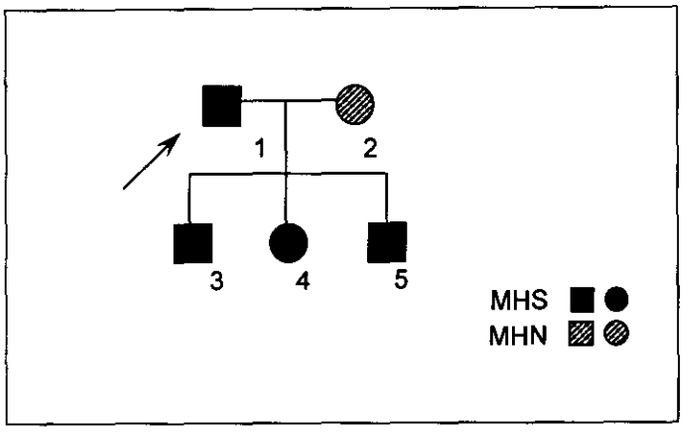


An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

The pedigree of MH107 consists of eight members, with two grandparents, two parents and four children. An excerpt of the pedigree indicating six members for whom data are available is illustrated in Figure 3.6. The MH status was provided by the individuals included in this study. Therefore results still need to be verified by clinical data. The proband in this family was identified as MH107-1. A family history of MH has been noted. Individual MH107-1 has a nephew who had a severe MH episode following a mastoid operation.

3.1.2.7 Malignant hyperthermia family MH108

Figure 3.7: Pedigree of family MH108

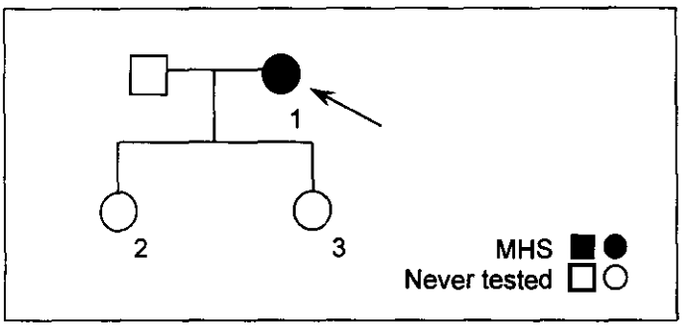


An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

The pedigree of family MH108 indicating all five members is illustrated in Figure 3.7. Muscle biopsies were conducted for all individuals in this family. However, IVCT data were not made available to the researcher. Clinical information for this family indicated a family history of MH, and designated MH108-1, MH108-3, MH108-4 and MH108-5 as MHS and MH108-2 as MHN.

3.1.2.8 Malignant hyperthermia family MH111

Figure 3.8: Pedigree of family MH111



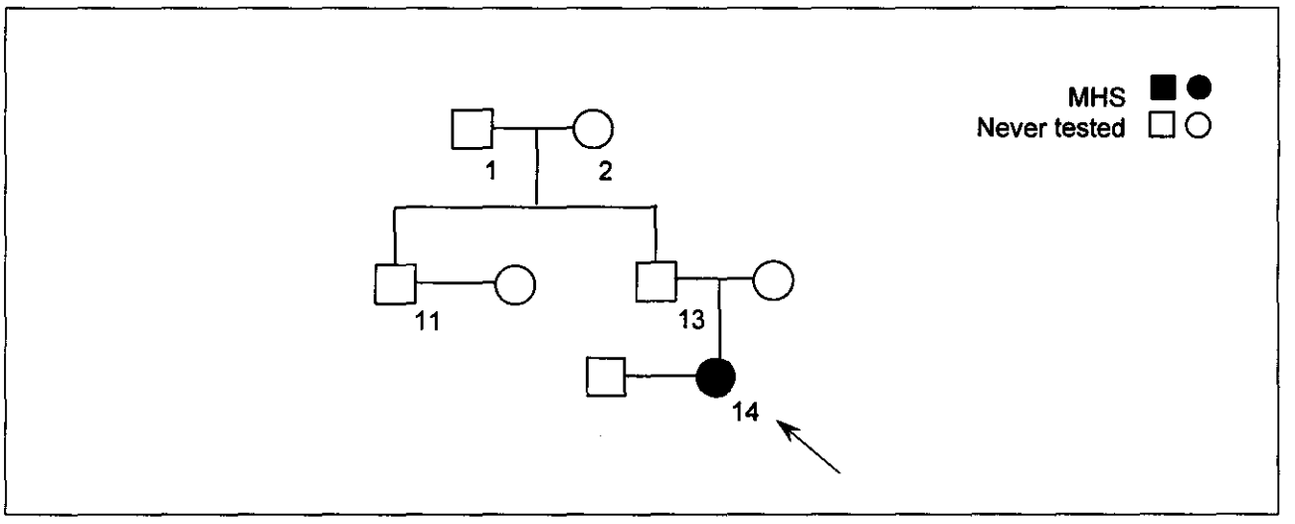
An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

Family MH111 consisted of four members as illustrated in Figure 3.8, and three individuals were available for analysis, a maternal parent and two children. The maternal parent (MH111-1) was identified as the proband and indicated scoline and halothane sensitivity during anaesthesia and was consequently diagnosed as MHS via a muscle biopsy. Results of the biopsy were not made available to the researcher. The MH status of the children (MH111-2 and MH111-3) is unknown.

3.1.2.9 Malignant hyperthermia family MH113

The pedigree of family MH113 includes 16 members. An excerpt of this pedigree indicating eight individuals is illustrated in Figure 3.9. Individual MH113-14 (MH00381) has been identified as the proband and experienced an MH episode during a previous anaesthesia. Blood from her father, individual MH113-13, could however not be obtained. The MH status of all individuals from family MH113 has not been confirmed via IVCT testing and their MH status is currently unknown.

Figure 3.9: Excerpt from pedigree MH113

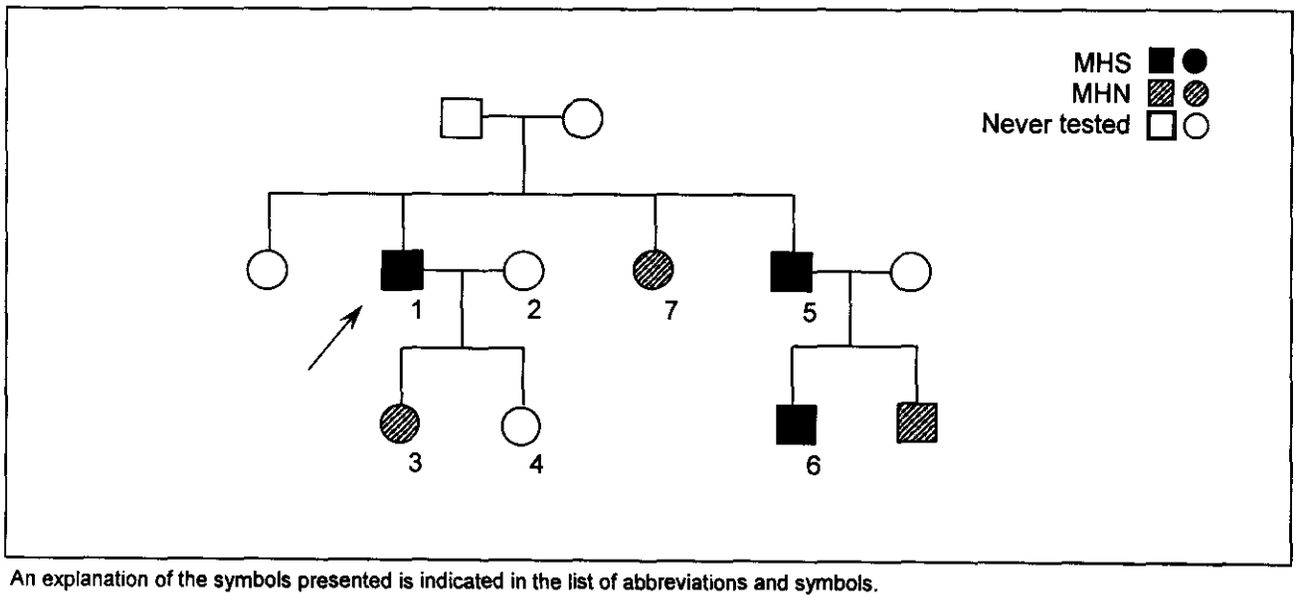


An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

3.1.2.10 Malignant hyperthermia family MH114

Family MH114 consists of 12 members, as indicated in the pedigree in Figure 3.10. Only seven members were available to participate in this study. The grandparents (not numbered) were not available for testing. The paternal parent, MH114-1, is the proband and is regarded as MHS. He has one child who was diagnosed as MH negative (MH114-3) and another daughter (MH114-4) who still has to be tested. The proband's brother, MH114-5, has also indicated that he is MH positive and he has one son who is MH positive and another who is MHN. The MH status was provided by the individuals included in this study, and still needs to be verified.

Figure 3.10: Pedigree of family MH114

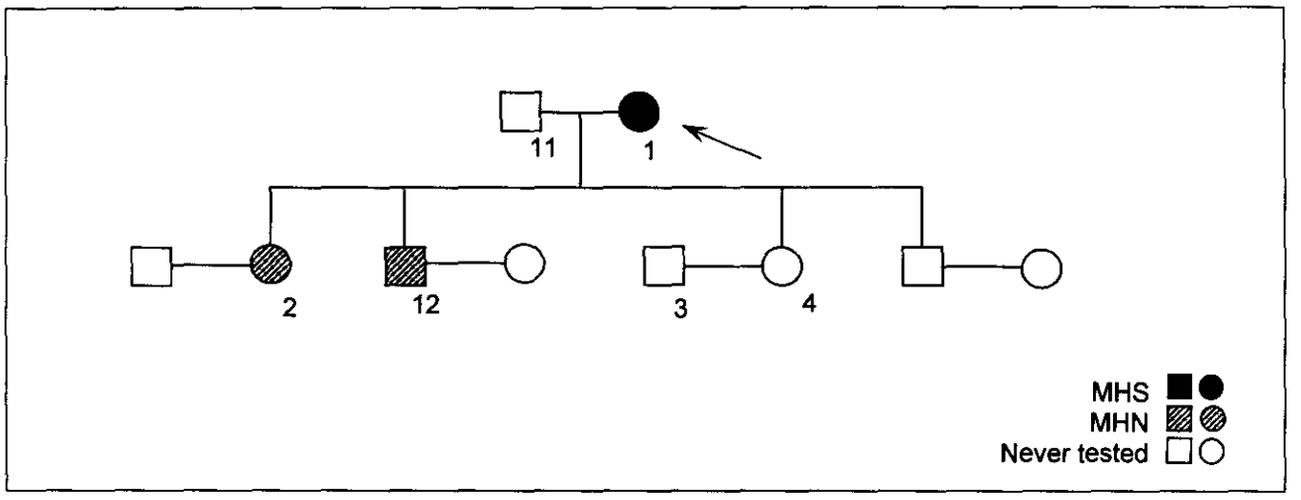


An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

3.1.2.11 Malignant hyperthermia family MH117

The pedigree of family MH117 consists of 16 members. However, samples were only collected for 12 individuals. An excerpt of the pedigree indicating ten members is illustrated in Figure 3.11. The proband, MH117-1, experienced an MH episode during anaesthesia and was diagnosed as MH positive via an IVCT. Only two children have been tested and both (MH117-2 and MH117-12) were diagnosed as MHN via the IVCT. Unfortunately, biopsy results for these individuals were not available to verify these findings. The MH status of individuals MH117-3 and MH117-4 is therefore unknown.

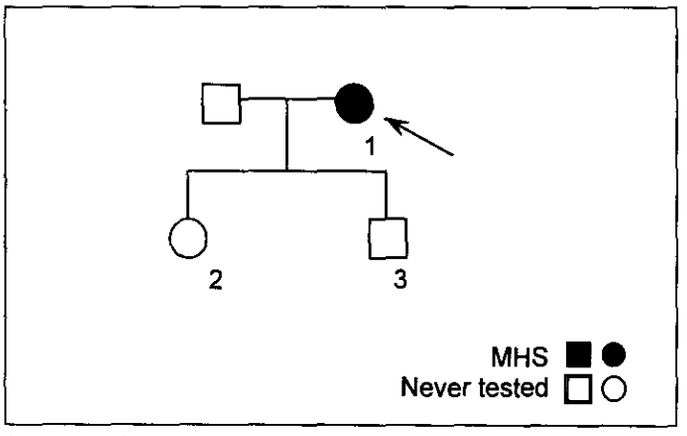
Figure 3.11: Excerpt from pedigree MH117



An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

3.1.2.12 Malignant hyperthermia family MH118

Figure 3.12: Pedigree of family MH118



An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

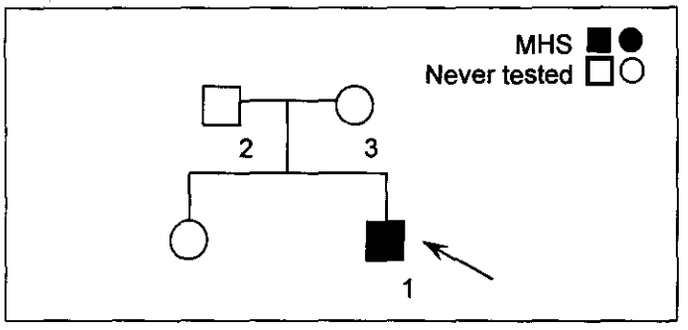
This family consists of four members, and three individuals were included in this study, as illustrated in Figure 3.12. The proband, MH118-1, was diagnosed via a muscle biopsy as having MH. Unfortunately, biopsy results for this individual were not available to verify this finding. The two children (MH118-2 and MH118-3) are both suspected of having MH, but have not been tested with the IVCT.

3.1.2.13 Malignant hyperthermia family MH122

MH122-1, the proband, has a family history of MH and experienced an MH episode during previous anaesthesia. The MH status of his child (MH122-2) is unknown. A family tree was not constructed for this pedigree, as the family structure was not made available to the researcher.

3.1.2.14 Malignant hyperthermia family MH123

Figure 3.13: Pedigree of family MH123



An explanation of the symbols presented is indicated in the list of abbreviations and symbols.

The pedigree of MH123 consists of four family members, of which three members were available for testing, as illustrated in Figure 3.13. The child (MH123-1) is the proband and developed an MH episode during anaesthesia. Clinical symptoms developed during anaesthesia included acidosis and fever. The parents of the proband, MH123-2 and MH123-3, have unknown MH status. The sister of MH123-1 was not available for testing.

3.2 MUTATION ANALYSIS

The complete structure of the human skeletal muscle RYR1 gene was first described by Phillips *et al.* (1996). The length of the gene was determined by aligning 16 genomic phage clones, a cosmid clone and several long polymerase chain reaction products. The RYR1 gene encompasses 158,000 bp of gDNA and consists of 106 exons. The length of exons ranges from 15 to 813 bp while introns range from 85 to 16,000 bp in length.

In the study presented here, cluster regions from exons 2, 6, 39, 40, 43, 44, 45, 46, 95, 100, 101 and 103 were amplified by polymerase chain reaction (PCR). In this manner specific regions of the RYR1 gene encompassing specific exons which harbour several mutations were amplified. This approach allowed efficient and simultaneous screening for clustered reported RYR1 mutations and detection of any novel mutations.

Regions encompassing RYR1 mutations were designated arbitrary numbers (1 to 9) for ease of reference. Mutations, represented by the amino acid alteration in the RyR1 protein and the specific exon in which they occur, are listed in Table 3.3.

DNA samples were further analysed for the presence of the Arg1086His alteration of the α_1 subunit of the human skeletal muscle dihydropyridine-sensitive calcium channel (CACNA1S). The assignment of the CACNA1S to chromosome 1q31-q32 was first identified in a human clone by multipoint linkage (Gregg *et al.*, 1993).

Table 3.3: RYR1 mutation regions as defined in this study

RYR1 mutation region number	Alteration/s	Exon
MR1	Cys35Arg, Arg44His	2
MR2	Glu160Gly, Arg163Cys, Arg163Leu	6
MR3	Arg2163Cys, Arg2163His, Val2168Met	39
MR4	Ala2200Val, Thr2206Met, Thr2206Arg	40
MR5	Asn2342Ser, Val2346Met, Glu2348Gly, Ala2350Thr, Arg2355Cys, Phe2364Val, Gly2434Arg, Arg2435His, Arg2435Leu	43 - 45
MR6	Arg2452Trp, Arg2458Cys, Arg2458His	46
MR7	Gly4638Met	95
MR8	Leu4814Phe, Ile4817Phe, Leu4824Pro, Thr4826Ile, Leu4838Val, Val4849Ile, Arg4861His	100 - 101
MR9	Ile4938Met, Asp4939Glu	103

3.2.1 Primer design

Oligonucleotide primers that were utilised for this study were designed and synthesised in previous phases of the ongoing MH research programme. Where primers were unavailable for a specific region of the RYR1 gene, new primers were designed.

A selection of primers for a specific region was designed via the Xprimer programme (Griffais *et al.*, 1991), the provided sequence was analysed and the programme supplied a selection of sets of primers, all with a relatively narrow melting temperature (T_m) range. Each set of primers was subsequently analysed utilising the Oligonucleotide Properties Calculator (Oligonucleotide Properties Calculator, 2004), to determine if any self-complementary reactions occurred between primers. Other physical constraints were also calculated, such as GC content, primer length and T_m. The nearest neighbour method was utilised by the programme to determine the T_m of the oligonucleotides. Calculations for this method were conducted as described by Breslauer *et al.* (1986), using values

obtained from Sugimoto *et al.* (1996). The method described takes into account the actual sequence to calculate T_m. The Oligonucleotide Properties Calculator programme used a salt adjusted T_m calculation for primer sequences longer than 13 nucleotides. Equation 3.1 was utilised, where w, x, y, z are the number of A, T, G, C bases in the sequence, respectively.

Equation 3.1: Formula to determine melting temperature (T_m)

$$T_m = 100.5 + (41 * (yG+zC)/(wA+xT+yG+zC)) - (820/(wA+xT+yG+zC)) + 16.6*\log_{10}([Na^+])$$

Where $16.6*\log_{10}([Na^+])$ adjusts for T_m changes in salt concentration

w = number of adenines (A); x = number of thymines (T); y = number of guanines (G); z = number of cytosines (C).

All primers were further analysed using the Basic Local Alignment Sequence Tool programme version 2.2.9 (Altschul *et al.*, 1997), to determine if primers annealed to other regions of the human genome. Oligonucleotides that preferentially annealed with highest complementarities to the regions of interest in the human RYR1 gene were selected, and were synthesised by Integrated DNA Technologies.

In previous investigations, the majority of the PCR protocols were optimised for specific primer sets. However, in the case where a primer or primer set was designed for the study presented here, the reaction was optimised. Various parameters were considered and optimised, including the annealing temperature (T_a) for each primer set, the concentration of magnesium chloride ($MgCl_2$) in the reaction and the addition of formamide to the reaction. Optimisation of the T_a began at a temperature 5°C below the T_m, and was adjusted to improve specificity. Primer sequences and the RYR1 region amplified thereof are listed in Table 3.4, as well as the calculated T_m and product sizes.

Table 3.4: Oligonucleotide primers utilised for PCR and direct sequencing

RYR1 mutation region number	Primer sequence	Primer name	T_m	Product size (bp)
MR1	F: 5' – cgt gct caa gga gca gct caa gct – 3'	Cys35F	76°C	212
	R: 5' – ccc tcc tca ctt tct ctc ctg t – 3'	RYRE2F	68°C	
MR2	F: 5' – ata gga gag gct tgc tgg tgg a – 3'	RYRE6F	68°C	233
	R: 5' – aat tgg gag tca gga cct tgg – 3'	RYRE6R	64°C	
MR3	F: 5' – ggt ccc tgc agg agc tgg tgt ccc – 3'	Arg2163F	68°C	388
	R: 5' – gac tga gat cac cca gag gat ggg cc – 3'	Val2168R	68°C	
MR4	F: 5' – ccc ctg gtg acc ccg cac act ctg – 3'	RYRE40F	69°C	229
	R: 5' – ctg gga cag gca ggg tgg tca ggg – 3'	RYRE40R	69°C	
MR5	F: 5' – atg ctt gtg gcc aaa ggg tac – 3'	*RYRex43F	61°C	937
	R: 5' – ctg cat gag gcg ttc aaa g – 3'	Glu2434R	65°C	
MR6	F: 5' – ggg agg gag cag agc agt cac tg – 3'	RYRE46F	65°C	242
	R: 5' – ctc cct ccc cag cat cac tcc ttc – 3'	RYRE46R	64°C	
MR7	F: 5' – cca aga ctg tat ctg gta tgg tcc c – 3'	*RYRex95F	67°C	334
	R: 5' – gaa agg cca cca gtg tat gca g – 3'	*RYRex95R	64°C	
MR8	F: 5' – ggc tgg tat atg gtg atg tcc ct – 3'	*RYRex100F	65°C	554
	R: 5' – aca gat gcg aga agg aag ggt cc – 3'	*RYRex100R	67°C	
MR9	F: 5' – gtc ggg cac tga ctt gtg tc – 3'	*RYRex103F	63°C	147
	R: 5' – gac ccc ctg aat ccc gta atc – 3'	*RYRex103R	63°C	

F and R indicate the forward and reverse primers respectively; * = oligonucleotides designed for the study presented here; T_m = calculated melting temperature; bp = base pair; °C = degree Celsius.

PCR primers used to amplify the Arg1086His alteration of the DHPR gene were obtained from a previous phase of the ongoing MH research programme. The primer sequences and T_m of each primer are listed in Table 3.5, as well as the size of the region in the amplified DHPR gene.

Table 3.5: Oligonucleotide primers utilised to detect the presence of the Arg1086His alteration

Gene	Alteration	Primer sequence	T_m	Product size (bp)
DHPR	Arg1086His	F: 5' – ctt ggt gct gac ctg tcc tgt t – 3'	57°C	226
		R: 5' – gat cag aca ttt ttc tcc tgg gg – 3'	55°C	

F and R indicate the forward and reverse primers respectively; T_m = calculated melting temperature; bp = base pair; °C = degree Celsius.

3.3 DNA EXTRACTION

Approximately 3 ml of venous blood was collected in ethylenediamine tetra-acetic acid (EDTA) tubes and was stored at -70°C until required. DNA was previously extracted as part of the ongoing MH research programme[1]. Isolations were performed utilising the Promega Wizard®[2] Genomic DNA purification kit. The extraction protocol as outlined in the manufacturer's guideline was followed. To lyse the red blood cells, 9 ml of cell lysis solution was added to 3 ml blood in a sterile 15 ml centrifuge tube. The mixture was inverted, incubated for 10 minutes (min) at room temperature and centrifuged at 2,000 gravitational acceleration (x g) for 10 min. The pellet containing the white blood cells was collected and vortexed in the remaining supernatant to resuspend the cells. Nuclei lysis solution (3 ml) was added to lyse white blood cells, followed by the addition of 1 ml protein precipitation solution for deproteinisation. The sample was vortexed and centrifuged at 2,000 x g for 10 min at room temperature. DNA present in the aqueous top phase was transferred to a 15 ml centrifuge tube and precipitated with 3 ml isopropanol, equilibrated at room temperature. Following mixing, the sample was centrifuged at 2,000 x g for 1 min at room temperature to precipitate the DNA. Three ml of 70% ethanol (EtOH) was added to the DNA pellet, subsequent to discarding the supernatant. The DNA was air-dried and rehydrated in 250 microlitre (µl) DNA rehydration solution via incubation for 24 h at room temperature. The typical DNA yield for 3 ml of blood ranged from 75 -150 $\mu g.ml^{-1}$. DNA was stored at 4°C until required.

Samples collected as part of the continuous programme were isolated during the current phase of the study. Extraction was performed utilising the Qiagen FlexiGene DNA kit®[3]. The extraction procedure as outlined in the manufacturer's protocol was followed. To lyse the red blood cells, 7.5 ml FG1 buffer was added to 3 ml blood in a sterile 15 ml centrifuge tube. The mixture was inverted and centrifuged at 2,000 x g for 5 min in a swing-out rotor. Once the supernatant was removed, 1.5 ml of a mixture consisting of 1.5 ml of buffer FG2 and 15 µl of Qiagen Protease solution was added to the pellet. The tube was vortexed, inverted and placed in a waterbath for 10 min at 65°C. In order to precipitate the DNA 1.5 ml isopropanol (100%) was added and the sample was centrifuged at 2,000 x g for 3 min. Subsequent to discarding the supernatant, 1.5 ml of 70% EtOH was added to the DNA pellet and the sample was centrifuged at 2,000 x g for 3 min. The supernatant was

[1] DNA isolations were performed by Y. Havenga and D. Prosser.
[2] Wizard® is a registered trademark of the Promega Corporation, Madison, WI, USA.
[3] FlexiGene™ is a trademark of QIAGEN Pty. Ltd., Victoria, Australia.

discarded and the DNA was air-dried for 5 min. DNA was rehydrated in 300 µl DNA rehydration solution (buffer FG3) via incubation for 1 h at 65°C in a water bath. The typical DNA yield for 3 ml of blood is between 75 and 90 $\mu g.ml^{-1}$. DNA was stored at -20°C until required. Following DNA isolation, working dilutions of DNA were prepared by dilution with sterile distilled water to a final concentration of 50 nanogram (ng).μl^{-1} and were stored at 4°C.

3.4 DETERMINATION OF DNA CONCENTRATION

DNA concentration can be measured by determining the absorbance at 260 nm (A_{260}) in a spectrophotometer. All DNA samples had an A_{260}/A_{280} ratio of above 1.8, indicating a lack of protein contamination. The calculation indicated in Equation 3.2A takes into account that an absorbance of one unit at 260 nm corresponds to 50 µg gDNA per ml. As there is a linear relationship between absorbance and DNA concentration, the concentration of DNA can be determined by the equation shown in Equation 3.2B. DNA concentrations were determined spectrophotometrically utilising the formula indicated in Equation 3.2.

Equation 3.2: Spectrophotometric conversion for calculating the concentration of nucleic acids from the absorbance at 260 nm

3.2A	Unknown $(\mu g.ml^{-1})/ A_{260\ nm} = 50 (\mu g.ml^{-1})/ 1.0\ A_{260\ nm}$
3.2B	Unknown $\mu g/ml^{-1} = 50\ \mu g.ml^{-1} \times A_{260\ nm} \times$ dilution factor

A_{260} = absorbance at 260 nm.

3.5 POLYMERASE CHAIN REACTION (PCR)

PCR which offers a fast and convenient method of amplifying a specific DNA segment, was first described by Mullis *et al.* (1986). This technique involves denaturation of the DNA sample, reannealing at a temperature depending on the T_m of the expected amplified subscript, and template elongation synthesis. The primers flank the DNA segment of interest and direct the DNA polymerase to synthesise new complementary strands. Multiple cycles of this process, each doubling the amount of DNA present, exponentially amplify the DNA (Voet and Voet, 1999).

DNA concentration, primer T_a and $MgCl_2$ concentration were optimised to ensure high specificity during amplification of specific regions in the RYR1 gene. The T_a was optimised for each reaction with a range of temperatures spanning the melting temperature of the

primer pairs and was experimentally analysed for each primer set to ensure that non-specific amplification did not occur. Higher temperatures were preferentially chosen in order to increase the specificity of the reaction. PCR was conducted with *Taq* DNA polymerase® (Promega), which has a 1 x buffer containing 10 mM Tris®-HCl (pH 9.0), 50 mM KCl and 0.1% Triton® X-100 or with Super-therm polymerase®[1], which has a 1 x buffer containing 20 mM Tris®-HCl (pH 8.0), 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), stabilisers and 50% glycerol, as discussed in Section 4.4. PCR reactions were performed in a total volume of 12.5 µl for sequencing or 25 µl for restriction enzyme digestion analysis. The PCR reaction consisted of the following components:

- 1 X PCR buffer
- 0.5 to 2.0 mM $MgCl_2$
- 200 µM of each 2'-deoxynucleotide (dNTP)
- 5 pico mol (pmol) of each of the forward and reverse primer
- 0.25 units (U) *Taq* DNA polymerase
- 100 ng gDNA template

Each 12.5 µl reaction was overlaid with 12.5 µl of mineral oil to prevent evaporation. Thermal cycling was carried out via a Thermo Hybaid®[2] Multiblock System utilising temperature cycles listed in Table 3.6.

Table 3.6: Temperature cycles of the PCR reaction

PCR step	Temperature	Time	Number of cycles
Denature	94°C	10 min	1
Denature	94°C	30 s	30
Anneal	60° - 65°C	30 s	
Extend	72°C	60 s	
Elongate	72°C	7 min	1
Hold	4°C	Hold	Indefinite

°C = degree Celsius; s = seconds; min = minutes.

3.6 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a simple and effective method that can be used for separating and identifying 0.5 to 25 kb DNA fragments. Detection was carried out using 2% (w/v) mini agarose gel. The 2% mini agarose gel was made up to a final volume of

[1] Supertherm polymerase®, is a registered trademark of JMR Holdings, Sevenoaks, Kent, UK.
[2] Thermo Hybaid® is the registered trademark of Hybaid Limited, Ashford, Middlesex, UK.

30 ml and contains 0.6 g LE analytical grade agarose, 3.0 ml 10 x TBE buffer [89.15 mM Tris® (pH 8.1), 88.95 mM boric acid, 2.498 Na_2EDTA], and 0.5 $\mu g.ml^{-1}$ ethidium bromide (EtBr). PCR product (2.5 µl) was added to 2 µl of a 2 X loading buffer [0.04% orange G (Sigma®[1]) and 50% glycerol]. Electrophoresis was carried out for 30 min at 10 volts per centimetre (V/cm) in 1 X TBE buffer. DNA was visualised by illumination with ultraviolet (UV) light and the images were captured via video documentation system.

3.7 CHAIN TERMINATION SEQUENCING

Sanger *et al.* (1977) developed a method, which allows the determination of DNA nucleotide sequence. The method employs analogues of 2',3'-dideoxynucleotide triphosphates (ddNTP) that lack a 3'-OH group, necessary for the formation of phosphodiester bonds. Consequently the DNA chain is specifically terminated at the position where a ddNTP is incorporated (Alphey, 1997).

The method of sequencing utilised in this molecular investigation was dye-labelled terminator, i.e. fluorophores attached to dideoxynucleotides. All four reaction products are assembled into one tube and the output generated from the electrophoresis run is in the form of intensity profiles for each differently coloured fluorophore.

PCR purification of samples was performed using the QIAquick®[2] PCR purification kit, for direct purification of PCR product. The purification procedure as outlined in the manufacturer protocol was followed. Five volumes of Buffer PB was added to one volume of PCR sample and mixed. The mixture was incubated at room temperature for 1 min and transferred to the QIAamp spin column. The column was centrifuged at 17,900 x g for 1 min. The filtrate was discarded and 750 µl buffer PE was added to wash the sample via centrifugation at 17,900 x g for 1 min. The filtrate was discarded and the sample was again centrifuged at 17,900 x g for 1 min. The spin column was placed in a clean 1.5 ml microcentrifuge tube. To elute the bound DNA, 30 µl elution buffer was added to the centre of the membrane. Prior to sequencing the DNA quantity of the PCR product was determined. The quantity of PCR product utilised for sequencing was determined by the appropriate amount of template required to provide optimum results as determined by the spectrophotometer.

[1] Sigma® is a registered trademark of Sigma Chemical Company, St. Louis, MO, USA.
[2] QIAquick™ is a trademark of QIAGEN Pty. Ltd., Victoria, Australia.

The amount of template that should be utilised for a sequencing reaction is listed in Table 3.7. Following DNA purification, samples were sequenced using the ABI PRISM®[1] Big Dye™ Terminator version 3.1 Ready Reaction Cycle Sequencing Kit. The kit contains a premixed terminator Ready Reaction Mix which consists of dye terminators, dNTPs, AmpliTaq®[2] DNA polymerase, $MgCl_2$ and buffer (Tris-HCl, pH 9.0). The sequencing reaction was performed in a 0.2 ml microcentrifuge tube and included 2 µl Ready Reaction Premix, 2 µl 5 X sequencing buffer (Tris-HCl, pH 9.0 and $MgCl_2$), 10 µM primer and 10 ng purified PCR product.

Table 3.7: Template quantity utilised in sequencing

Template	Quantity
PCR product:	
100 – 200 bp	1- 3 ng
200 – 500 bp	3 - 10 ng
500 – 1000 bp	5 - 20 ng
1000 – 2000 bp	10 - 40 ng
>2000 bp	40 - 100 ng
Single stranded	50 - 100 ng
Double stranded	200 - 500 ng

Adapted from ABI PRISM® BigDye™ Terminator version 3.0 Ready Reaction Cycle Sequencing kit protocol.

Forward or reverse primers utilised for PCR were also utilised as sequencing primers. Deionised water was added to a final volume of 10 µl. Following mixing, sequencing was conducted on the Hybaid MultiBlock System utilising temperature cycles listed in Table 3.8. Detection of a mutation resulted in both strands being sequenced to permit identification of ambiguities.

Table 3.8: Temperature cycles of the sequencing reaction

PCR step	Temperature	Time	Number of cycles
Denature	96°C	10 s	25
Anneal	50°C	10 s	
Extend	60°C	4 min	
Hold	4°C	Hold	Indefinite

°C = degree Celsius; s = seconds; min = minutes.

Purification to remove unincorporated dye terminators from the sequencing reaction was conducted prior to electrophoresis of the sample. The EtOH precipitation method may result in residual terminator peaks. However, the ethanol/sodium acetate method of precipitation provides a more consistent signal. A solution was made, composed of 3 µl of 3 molar (M) sodium acetate (NaOAc), pH 4.6, 62.5 µl non-denaturing 99% EtOH and

[1] ABI PRISM® Big Dye ™ is a registered trademark of Applera Corporation, Foster City, CA, USA.
[2] AmpliTaq® DNA polymerase, FS, is a registered trademark of Roche Molecular Systems Inc., Alameda, CA, USA.

14.5 µl deionised water. The solution was added to 10 µl of the PCR sequenced product and vortexed. The tubes were centrifuged at 10,621 x g for 20 min and 250 µl 70% EtOH was added to the pellet and vortexed briefly. The samples were centrifuged at 10,621 x g for 10 min and the supernatants were discarded. Finally, the samples were air-dried and submitted for electrophoresis. Samples were electrophoresed via the Base Spectrum programme on SpectruMedix[1] (SCE2410) Genetic Analysis System. The sequences were evaluated by using the BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999).

3.7.1 Detection of alterations in mutation region one of the RYR1 gene

Analysis of a 212 bp region was conducted in order to detect alterations that occurred in exon two of the RYR1 gene. The subsequent sequence was designated region one, and harbours two known alterations. The Cys35Arg alteration was first described by Lynch *et al.* (1997) and represents the most N-terminal alteration reported to date. The mutation was identified to be a T103C nucleotide transition. Halsall and Robinson (2004) reported an Arg44His substitution in the RYR1 gene segregating in one MH family that occurs due to a G131A transition. The partial sequence of amplified DNA region one is depicted in Table 3.9. In the study presented here intron and exon sequences were numbered according to the nomenclature provided by European Molecular Biology Laboratory (EMBL), unless stated otherwise.

Table 3.9: Mutation region one, depicting the partial gDNA sequence of exon two of the RYR1 gene demonstrating the positions of the Cys35Arg and Arg44His alterations

Nucleotide number	Mutation region 1: Cys35Arg and Arg44His
56	**Exon 2, 56 nt** TGGTCCTGCA GTGCAGCGCT ACCGTGCTCA AGGAGCAGCT CAAGCT**C**T**G**C CTGGCCGCCG
116	AGGGCTTCGG CAAC**C**G**C**CTG TGCTTCCTGG AGCCCACTAG CAACGCGCAG gtctgtgcag
+10	gaggaagagg ggcctgggga caggggcgtc tgaaggggca gagaatcttg ggtccaaaga
+72	agagggttct gggagtctga aaggaggtgc tgacaggaga gaaagtgagg agggggggcta

The partial gDNA sequence amplified for DNA region 1 was obtained from GenBank® with accession number U48450.1. The exonic sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Cys35 and Arg44 is indicated in bold and the nucleotide transitions for the mutations are indicated in blue (T) and red (G), respectively. The forward primer (Cys35F) is the single underlined sequence, while the reverse primer (RYRE2R) is the double underlined sequence; exon one is indicated in bold above the sequence with the corresponding nucleotide number.

[1] SpectruMedix™ is a trademark of the SpectruMedix LLC, State College, PA, USA.

3.7.2 Detection of alterations in mutation region two of the RYR1 gene

A region of 233 bp region of exon six was amplified and designated as region two. The sequence harbours three reported MH alterations. Halsall and Robinson (2004) identified a Glu160Gly alteration that occurs due to an A479G transition and the Arg163Leu mutation that was observed due to a G488T substitution. The alteration Arg163Cys was first reported by Quane *et al.* (1993) and occurs due to a C487T single base substitution. The authors identified the Arg163Cys alteration in two unrelated families. The partial sequence of amplified DNA region two is depicted in Table 3.10.

Table 3.10: Mutation region two, depicting the partial gDNA sequence of exon six of the RYR1 gene demonstrating the positions of the Glu160Gly, Arg163Cys and Arg163Leu alterations

Nucleotide number	Mutation region 2: Glu160Gly, Arg163Cys and Arg163Leu
	↓ Exon 6, 425 nt
-14	atccccaccc atagGAGAGG CTTGCTGGTG GACCATGCAC CCAGCCTCCA AGCAGAGGTC
71	TGAAGGAGAA AAGGTCCGCG TTGGGGATGA CATCATCCTT GTCAGTGTCT CCTCCGAGCG
131	CTACCTGgtg agccattgcg gttcctcctg ctcccaggtc tgggggcgca tgggatggtc
+54	cccatcttct caccatgggt ttgcctggct gatctcccac ccccaaggtc ctgactccca
+114	atttcccatt tcctgacccc tgacatccaa ttttctgatt tctgacctcc cattgcccga

The partial gDNA sequence amplified for DNA region 2 was obtained from GenBank® with accession number U48452. The exon sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Glu160 and Arg163 is indicated in bold and the nucleotide transitions for the mutations are indicated in blue (A) and red (CG), respectively. The forward primer (RYRE6F) is the single underlined sequence, while the reverse primer (RYRE6R) is the double underlined sequence; the beginning of exon six is indicated with an arrow, with the corresponding nucleotide number.

3.7.3 Detection of alterations in mutation region three of the RYR1 gene

A 390 bp PCR product of exon 39 was sequenced, encompassing the alterations Arg2163Cys, Arg2163His and Val2168Met, which were due to nucleotide transitions C6487T, G6488A and G6501A respectively. The partial sequence of amplified DNA region three is depicted in Table 3.11. The amplified sequence was designated as region three. The alterations described in this region are clustered in the central portion of the RYR1 gene and were first described by Manning *et al.* (1998a).

Table 3.11: Mutation region three, depicting the partial gDNA sequence of exon 39 of the RYR1 gene demonstrating the positions of the Arg2163Cys, Arg2163His and Val2168Met alterations

Nucleotide number	Mutation region 3: Arg2163Cys, Arg2163His and Val2168Met
	Exon 39, 6275 nt
6275	GGTCCCTGCA GGAGCTGGTG TCCCACATGG TGGTGCGCTG GGCCCAAGAG GACTTCGTGC
6335	AGAGCCCCGA GCTGGTGCGG GCCATGTTCA GCCTCCTGCA CCGGCAGTAC GACGGGCTGG
6395	GTGAGCTGCT GCGTGCCCTG CCGCGGGCGT ACACCATCTC ACCGTCCTCC GTGGAAGACA
6455	CCATGAGCCT GCTCGAGTGC CTCGGCCAGA TC**CG**CTCGCT GCTCATC**GTG** CAGATGGGCC
6515	CCCAGGAGGA GAACCTCATG ATCCAGAGCA TCGGgtgaga caccgccctt ccccttactt
+27	tgcatatccc cttgggtaat gaataccctc aggatacaat aacattccct tccccaactt
+87	ctggcccatc ctctgggtga tctcagtctc tcgatggcta gctcacctcc tgggtaatga

The partial gDNA sequence amplified for DNA region 3 was obtained from GenBank® with accession number U48473. The exon sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Arg2163 and Val2168 is indicated in bold and the nucleotide transitions for the mutations are indicated in blue (CG) and red (G), respectively. The forward primer (Arg2163Cys) is the single underlined sequence, while the reverse primer (Val2168Met) is the double underlined sequence; exon 39 is indicated in bold above the sequence with the corresponding nucleotide number.

3.7.4 Detection of the alterations in mutation region four of the RYR1 gene

A 229 bp region of exon 40 was amplified and the sequence was designated to be mutation region four. The sequence harbours two known alterations. The Thr2206Met alteration is due to a nucleotide transition of C6617T. The alteration was first described by Manning *et al.* (1998) and is located in the central portion of the RYR1 gene. The Thr2206Arg alteration occurs due to a C6617G transition and was observed in a single MH pedigree (Brandt *et al.*, 1999). In one family from the UK Halsall and Robinson (2004) described an Ala2200Val alteration that was due to a C6599T transition. The partial sequence of amplified DNA region four is represented in Table 3.12.

Table 3.12: Mutation region four, depicting the partial gDNA sequence of exon 40 of the RYR1 gene demonstrating the positions of the Ala2200Val, Thr2206Met and Thr2206 Arg alterations

Nucleotide number	Mutation region 4: Ala2200Val, Thr2206Met and Thr2206Arg
	↓ exon 40, 6549 nt
-43	gacctgggcc cctggtgacc ccgcacactc tgcccgtgca cagGAACATC ATGAACAACA
6566	AAGTCTTCTA CCAACACCCG AACCTGATGA GGGCGCTGGG CATGCACGAG ACGGTCATGG
6626	AGGTCATGGT CAACGTCCTC GGGGGCGGCG AGTCCAAGgt gagggcccag gcaggtgctg
6686	gggagctcag gggaggcagc cacagagggc aggccctgac caccctgcct gtcccaggag

The partial gDNA sequence amplified for DNA region four was obtained from GenBank® with accession number U48474. The exon sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Ala2200 and Thr2206 is indicated in bold and the nucleotide transitions for the mutations are indicated in blue (C) and red (C), respectively. The forward primer (RYRE40F) is the single underlined sequence, while the reverse primer (RYRE40R) is the double underlined sequence; the beginning of exon 40 is indicated with an arrow, with the corresponding nucleotide number.

3.7.5 Detection of alterations in mutation region five of the RYR1 gene

To detect nine known alterations in exon 43 to exon 45, a region of 937 bp was amplified and subsequently sequenced. The partial sequence of amplified DNA region five is shown in Table 3.13. As the sequence contains several intron and exon sequences, the region was numbered according to the nomenclature provided by EMBL.

The Ala2350Thr missense alteration was first identified by Sambuughin *et al.* (2001a) in exon 44 and occurs due to a nucleotide transition of G7048A. Alterations Asn2342Ser, Val2346Met, Glu2348Gly, Arg2355Cys and Phe2364Val were all demonstrated by Halsall and Robinson (2004). Val2346Met and Glu2348Gly were described in a single UK family and occurred due to a G7036A and A7043G transition, respectively. Asn2342Ser and Phe2364Val were identified in two UK families. The first mutation was due to an A7025G transition, the second was the result of T7089G transition. In six UK families the authors found a C7062T transition in the RYR1 gene, resulting in an Arg2355Cys amino acid substitution in the gene product. The last three alterations Gly2434Arg, Arg2435His and Arg2435Leu, detected in this region are currently been used in the genetic diagnosis of MHS in Europe (Ørding *et al.*, 1997).

In four families with MH, Keating *et al.* (1994) observed the Gly2433Arg alteration, adjacent to an Arg2434His alteration. The amino acid numbering was altered according to corrected sequence data for the human RYR1 provided by Phillips *et al.* (1996) and the alterations were renamed Gly2434Arg and Arg2435His in a study conducted by Richter *et al.* (1997). These mutations are due to G7300A and G7304A transitions, respectively.

Arg2435Leu was identified in a single UK family by Halsall and Robinson (2004) and is caused by a G7304T transition.

Table 3.13: Mutation region five, depicting the partial gDNA sequence of exon 43 to 45 of the RYR1 gene demonstrating the positions of the Val2346Met, Glu2348Gly, Ala2350Thr, Arg2355Cys, Phe2364Val, Gly2434Arg, Arg2435His and Arg2435Leu alterations

Nucleotide number	Mutation region 5: Asn2342Ser, Val2346Met, Glu2348Gly, Ala2350Thr, Arg2355Cys, Phe2364Val, Gly2434Arg, Arg2435His and Arg2435Leu
	↓ Exon 43, 6892 nt
1	gGTTGTGTCC TACCTGGCAG GCTGTGGCCT CCAGAGCTGC CCCATGCTTG TGGCCAAAGG
61	GTACCCAGAC ATTGGCTGGA ACCCCTGTGG TGGAGAGCGC TACCTGGACT TCCTGCGCTT
121	TGCTGTCTTC GTCAACGgtg aggagggggt ggcagtggca gagcgggaag tatggagtca
181	ctggtcacac acctccctcg agatgactgc tcgcaccctg agccacagat ggggtccagg
241	caggaatccc ttccagcagg cctggggctg gcaggggcct gtgttacccc tggaggtgtt
301	gggtcctgtg gctggcagtg ttggatcctg gggctggcgg gagcctggtg ttacccctag
361	aggtgttggg tcctggggct ggcaggggcc tggtgttacc tctggaggtg ttgggtcctg
421	gagctggatg ggacctgtgt tacccctgga ggtgttgggt cctggggctg catggggagg
	↓ Exon 44, 7028 nt
481	tctctgatgg tggctcatga gacccccttt ccccatgcgg gtggccagGC GAGAGCGTGG
541	AGGAGAACGC CAATGTGGTG GTGCGGCTGC TCATCCGGAA GCCTGAGTGC TTCGGACCCG
601	CCCTGCGGGG TGAGGGTGGC TCAGGGCTGC TGGCTGCCAT CGAAGAGGCC ATCCGCATCT
661	CCGAGGACCC TGCGAGGGAT GGCCCAGGCA TCCGCAGGGA CCGGCGGCGC GAGCAgtgag
721	tctcccggcc ccctcctcaa tagggcaacc cgccctccct ggcccctggc tgcctcccca
	↓ Exon 45, 7216 nt
781	acccacccac cttccctgca gcTTTGGTGA GGAACCGCCT GAAGAAAACC GGGTGCACCT
841	GGGACACGCC ATCATGTCCT TCTATGCCGC CTTGATCGAC CTGCTCGGAC GCTGTGCACC
901	AGAGATGCAT gtgagaccct gagccagggc aggatgggaa gggagggcag gcacagccgc
961	tttgaacgcc ctcatgcagg cactcggtga cacggagtga gctcccatat gtgggtggtc

The partial gDNA sequence amplified for DNA region 5 was obtained from GenBank® with accession number AC011469. The exon sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Val2346, Glu2438, Ala2530, Arg2355 and Phe2364 is indicated in bold and the nucleotide transitions for the mutations are indicated in red (G), green (A), purple (G), orange (GCG) and brown (T) respectively. Codons that correlate to Gly2434 and Arg2435 are indicated in bold and the nucleotide transitions for the mutations are indicated in light blue. The forward primer (RYRex43) is the single underlined sequence, while the reverse primer (Glu2434R) is the double underlined sequence; the beginning of exon 43, 44 and 45 is indicated with an arrow, with the corresponding nucleotide numbers.

3.7.6 Detection of alterations in mutation region six of the RYR1 gene

Sequencing was utilised to screen mutations observed in exon 46. The partial sequence of amplified DNA region six is depicted in Table 3.14. The Arg2452Trp alteration, which was due to a C7354T nucleotide transition, was first described by Chamley *et al.* (2000). Alterations Arg2458Cys and Arg2458His were both first reported by Manning *et al.* (1998). The two novel mutations were due to a C7372T and G7373A transition, respectively. Both alterations occur at a CpG dinucleotide in the central portion of the RYR1 gene.

Table 3.14: Mutation region six, depicting the partial gDNA sequence of exon 46 of the RYR1 gene demonstrating the positions of the Arg2452Trp, Arg2458Cys and Arg2458His alterations

Nucleotide number	Mutation region 6: Arg2452Trp, Arg2458Cys and Arg2458His
-107	aaagaggcct gctctaccct cctgtgtggt aagggaggga gcagagcagt cactgagtgg
	↓ Exon 46, 7324 nt
-47	ggcaccagcg cctgatgagt gcccctctcc ctccctctac tccccagCTA ATCCAAGCCG
7337	GCAAGGGTGA GGCCCTG**C**G**G** ATCCGCGCCA TCCTC**CG**CTC CCTTGTGCCC TTGGAGGACC
7397	TTGTGGGCAT CATCAGCCTC CCACTGCAGA TTCCCACCCT GGGCAAAGgt gcagagggat
+13	ggaacttggc gaaggagta tgctggggag ggtggtccgc aggcatcccc gaacccaccc

The partial gDNA sequence amplified for DNA region 6 was obtained from GenBank® with accession number U48477. The exon sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Arg2452 and Arg2458 is indicated in bold and the nucleotide transitions for the mutations are indicated in blue (C) and red (CG), respectively. The forward primer (RYR46F) is the single underlined sequence, while the reverse primer (RYR46R) is the double underlined sequence; the beginning of exon 46 is indicated with an arrow, with the corresponding nucleotide number.

3.7.7 Detection of alterations in mutation region seven of the RYR1 gene

A region of 334 bp of exon 95 was sequenced to detect the alteration Gly4638Met, which was due to a G13913A transition. It was observed in two UK families with MH (Halsall and Robinson, 2004). The partial sequence of amplified DNA region seven is depicted in Table 3.15.

Table 3.15: Mutation region seven, depicting the partial gDNA sequence of exon 95 of the RYR1 gene demonstrating the position of the Gly4638Met alteration

Nucleotide number	Mutation region 7: Gly4638Met
-126	tgattgacag ccacaccaag actgtatctg gtatggtccc agtccaatct cgggaatgga
-66	ggctcaattt tgtgagtggg ctctgcatgt ggcagaccca cagatgaatc tctgtcccca
	↓ Exon 95, 13747 nt
-16	tttcagGTCT CAGACTCTCC ACCAGGGGAG GACGACATGG AAGGCTCAGC TGCTGGGGAT
13801	GTGTCAGGTG CAGGCTCTGG TGGCAGCTCT GGCTGGGGCT TGGGGGCCGG AGAGGAGGCA
13861	GAGGGCGATG AGGATGAGAA CATGGTGTAC TACTTCCTGG AGGAAAGCAC AGGCTACATG
13921	GAACCCGCCC TGCGGTGTCT GAGCCTCCTG CATACACTGG TGGCCTTTCT CTGCATCATT

The partial gDNA sequence amplified for DNA region 7 was obtained from GenBank® with accession number U48501. The exon sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Gly4638 is indicated in bold and the nucleotide transition for the mutation is indicated in blue (G). The forward primer (RYRex95F) is the single underlined sequence, while the reverse primer (RYRex95R) is the double underlined sequence; the beginning of exon 95 is indicated with an arrow, with the corresponding nucleotide number.

3.7.8 Detection of alterations in mutation region eight of the RYR1 gene

The partial sequence of amplified DNA region eight is depicted in Table 3.16. As the sequence contains several intron and exon sequences, the region was numbered according to the nomenclature provided by EMBL. The alteration Thr4826Ile was first described by Brown *et al.* (2000).

The mutation occurs in the C terminal region/transmembrane loop of exon 100 and is due to a C14477T transition. Alterations Leu4814Phe, Ile4817Phe and Leu4824Pro were detected by Halsall and Robinson (2004), and are due to C14440T, A14449T and T14471C transitions, respectively.

The alteration Val4849Ile was detected by the above-mentioned authors in four MH families and is due to a G14545A transition. Leu4838Val and Arg4861His are currently being utilised in the European genetic diagnostic MHS test and are due to C14512G and G14582A transitions, respectively. The Arg4861His alteration was first identified in seven out of 25 individuals with CCD (Tilgen *et al.*, 2001).

Table 3.16: Mutation region eight, depicting the partial gDNA sequence of exon 100 and 101 of the RYR1 gene demonstrating the positions of the Leu4814Phe, Ile4817Phe, Leu4824Pro, Thr4826Ile, Leu4838Val, Val4849Ile and Arg4861His alterations

Nucleotide number	Mutation region 8: Leu4814Phe, Ile4817Phe, Leu4824Pro, Thr4826Ile, Leu4838Val, Val4849Ile and Arg4861His
	Exon 100, 14371 nt
1	CTGTACCTGG GCTGGTATAT GGTGATGTCC CTCTTGGGAC ACTACAACAA CTTCTTCTTT
61	GCTGCCCATC TCCTGGACAT CGCCATGGGG GTCAAGACGC TGCGCACCAT CCTGTCCTCT
121	GTCACCCACA ATGGGAAACA Ggtgtgggga ggacctggct gtggggcgtg ggccagcagg
181	gaccagcgtg gcagtgggtg gtgaagggat aagggccggg cagctgggct gaggaggggc
241	aaggccaggt gcgctgagcc gggggtgtgt ggggcagcaa ggtagagcca cagggactga
301	accggggcca ggacccagca tgggcagggt gggggggaggg caagcccagg gcggagctga
	↓ Exon 101, 14512 nt
361	cctggcccca tcctgccccc agCTGGTGAT GACCGTGGGC CTTCTGGCGG TGGTCGTCTA
421	CCTGTACACC GTGGTGGCCT TCAACTTCTT CCGCAAGTTC TACAACAAGA GCGAGGATGA
481	GGATGAACCT GACATGAAGT GTGATGACAT GATGACGgtg agcccctccc ctagcactct
541	gggacccttc cttctcgcat ctgttgaagg agttaataat ggtacctcca ggccgggcgt

The partial gDNA sequence amplified for DNA region 8 was obtained from GenBank® with accession number U48505, U48506 and U48507. The exon sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Leu4814, Ile4817, Leu4824, Thr4826, Leu4838, Val4849 and Arg4861 is indicated in bold and the nucleotide transition for the mutation are indicated in blue (C), red (A), green (T), purple (C), orange (C), brown (G) and pink (G) respectively. The forward primer (RYRex100F) is the single underlined sequence, while the reverse primer (RYRex100R) is the double underlined sequence; the beginning of exon 95 is indicated with an arrow, with the corresponding nucleotide number.

3.7.9 Detection of the alterations in mutation region nine of the RYR1 gene

A region of 147 bp of exon 103 was sequenced to detect alterations Ile4938Met and Asp4939Glu, which were due to C14814G and C14817A transitions, respectively. They were each detected in one UK family with MH (Halsall and Robinson, 2004). The partial sequence of amplified DNA region nine is depicted in Table 3.17.

Table 3.17: Mutation region nine, depicting the partial gDNA sequence of exon 103 of the RYR1 gene demonstrating the positions of the Ile4938Met and Asp4939Glu alterations

Nucleotide number	Mutation region 9: Ile4938Met and Asp4939Glu
	↓ Exon 103, 14804 nt
-32	gtcgggcact gacttgtgtc ctgccacccc agGTCTGATC **ATCGACGCTT** TTGGTGAGCT
14832	CCGAGACCAA CAAGAGCAAG TGAAGGAGGA TATGGAGgta ggtcatgtct gggggtgacc
14892	cagagggatt acgggattca gggggtcaag tgggcctcca ctctgatgtc tcttgccact

The partial gDNA sequence amplified for DNA region 9 was obtained from GenBank® with accession number U48508. The exon sequence is indicated in upper case and the intron sequence is indicated in small letters. The codon that correlates to Ile4938 and Asp4939 is indicated in bold and the nucleotide transitions for the mutation are indicated in blue (C) and red (C), respectively. The forward primer (RYRex103F) is the single underlined sequence, while the reverse primer (RYRex103R) is the double underlined sequence; the beginning of exon 103 is indicated with an arrow, with the corresponding nucleotide number.

3.8 RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) ANALYSIS

Certain sequence alterations result in the creation or destruction of a recognition site for a restriction enzyme. A PCR product encompassing the amplified region harbouring the alteration was designed so that when it is digested utilising the appropriate restriction enzyme, fragments of different sizes are generated for each of the different genotypes. Each digestion reaction typically contained the following, as recommended by the manufacturer:

- 1 X buffer
- Approximately 10 U of the specific restriction endonuclease
- 10 μl amplified DNA, made up to a total volume of 30 μl.

Optimal conditions were executed according to details provided by the manufacturer. The reaction mixture consisted of, 1 X restriction enzyme buffer [50 mM NaCl, 10 mM Tris®-HCl (pH 7.9), 10 mM $MgCl_2$ 1 mM DTT], 10 U *Hha* I, 10 $\mu g.\mu l^{-1}$ bovine serum albumin (BSA) in a total reaction volume of 20 μl. An incubation step of 1 h at 37°C was performed to ensure complete digestion.

3.8.1 Detection of the Arg1086His alteration via restriction enzyme digestion

The Arg1086His alteration detected in the α_1-subunit of the CACNA1S was first described by Monnier *et al.* (1997). Screening individuals for the Arg1086His alteration was achieved via RFLP analysis as described in paragraph 4.7. The presence of the mutation results in the loss of the *Hha* I restriction endonuclease site. Therefore, individuals heterozygous for the alteration would have digestion fragments of 226, 190 and 36 bp in length. Absence of

the mutation would result in complete digestion with fragments of 190 and 36 bp in length. Digested products were electrophoresed on an agarose gel (as described in Section 4.3). A partial sequence of the amplified DNA region of the CACNA1S harbouring the Arg1086His alteration is depicted in Table 3.18. In patient samples that had the CACNA1AS mutation, sequencing was conducted to confirm the presence of the mutation.

Table 3.18: Partial gDNA sequence of amplified DNA region of the human CACNL1A3 gene indicating the position of the Arg1086His alteration

Nucleotide number	Amplified DNA sequence: Arg1086His
	↓ Exon 26, 3332 nt
1	ggggcctctt ggtgctgacc tgtcctgttg tatgtgtcac agCGCCAATG TGTACAGTAT
61	GCCCTGAAGG CCCGCCCACT GAGGTGCTAC ATTCCCAAAA ACCCATACCA GTACCAGGTG
121	TGGTACATTG TCACCTCCTC CTACTTTGAA TACCTGATGT TTGCCCTCAT CATGCTCAAC
181	ACCATCTGCC TCGGCATGCA GGTAAGGGCT CCCCAGGAGA AAAATGTCTG ATCAGACTGG

The partial gDNA sequence was obtained from GenBank® with accession number U30690. The exon sequence is indicated in upper case. The codon that correlates to Arg1086 is indicated in bold and the nucleotide transition for the mutation is indicated in red (G). The forward primer is the single underlined sequence, while the reverse primer is the double underlined sequence. The sequence indicated as a box represents the *Hha* I restriction enzyme recognition site that is abolished when the mutation is present.

CHAPTER FOUR

RESULTS AND DISCUSSION

Mutations located in the three mutation hotspots of the RYR1 gene are continually reported in MH individuals worldwide. Previous MH studies all contributed to phase one (Phase 1) of the ongoing MH research programme at the Centre for Genome Research. The study presented here represents phase two (Phase 2) of the extended programme. The initial aim of the Phase 2 study was to screen 30 MH probands for 15 causative mutations of the RYR1 gene. All samples included in Phase 2 were analysed for mutations via a sequencing strategy. This technique can be utilised to screen for clusters of alterations in the RYR1 gene as well as identify any novel alterations that may be present. For this reason, additional mutations were included in Phase 2. Twenty-four recently observed mutations that occur in the three mutation hotspots of the RYR1 gene were investigated in South African MH probands. All individuals included in Phase 1 and Phase 2 are Caucasian and were collected in South Africa. They are descendants of Dutch and other European settlers who immigrated to South Africa. As most of Caucasian South African individuals have European ancestry, results generated from the MH research programme for South African Caucasian MH probands have important implications for all Caucasian MH individuals in this population.

In addition to the original aims of Phase 2, eight mutations that have already been analysed in Phase 1, mostly via RFLP and some via automated sequencing strategies, were investigated only in MH probands that were not previously screened for these mutations. These mutations were included as they occurred in the same regions as the 24 recently observed mutations. All mutation regions were analysed in Phase 2 via sequencing, a technique that permits simultaneous screening of recently reported causative mutations, as well as identification of any novel mutations that may be present. In addition to the RYR1 mutations, 24 MH probands that were previously not screened for the Arg1086His alteration of the CACNA1S were also included in the Phase 2 study.

A summary of results obtained for MH individuals analysed in Phase 2 is listed in Tables 4.4, 4.5 and 4.6. The results for each mutation region that was screened are described and discussed separately in the subsequent sections of this chapter. PCR and sequencing

were conducted as described in Sections 3.5 and 3.7 respectively, unless stated otherwise. Changes made to these protocols are indicated in the relevant paragraphs.

4.1 ISOLATION OF GENOMIC DNA

DNA isolation kits were utilised to isolate gDNA because of their many advantages. The kits are rapid, provide a simple method for purification and isolated DNA have a high yield and purity and can consequently be utilised in a variety of techniques. DNA obtained from some individuals included in this study have been extracted previously (Havenga, 2000; Neumann, 2002). These isolations were performed utilising the Promega Wizard® Genomic DNA purification kit. DNA obtained during Phase 2 from MH probands was isolated utilising the Qiagen FlexiGene DNA kit®. DNA was isolated from 1-3 ml whole blood collected in tubes containing EDTA that were subsequently stored at -70°C. The concentration of DNA was determined utilising the spectrophotometer as described in Section 3.4, and the purity of DNA was determined via the ratio of readings at 260 nm and 280 nm (A_{260}/A_{280}). Pure DNA that has not been contaminated by proteins, phenol or RNA has A_{260}/A_{280} ratios between 1.7 and 1.9. Both DNA isolations kits provided similar yields and purity. DNA obtained from the Promega Wizard® Genomic DNA had a yield of 151 – 1009 $ng.\mu l^{-1}$ and A_{260}/A_{280} ratios between 1.78 and 2.01. DNA isolated utilising the Qiagen FlexiGene DNA kit® had a yield of 135 – 945 $ng.\mu l^{-1}$ and A_{260}/A_{280} ratio of 1.76 – 1.83. DNA was stored at -20°C until required and working dilutions of DNA were prepared by dilution with sterile distilled water to a final concentration of 50 $ng.\mu l^{-1}$ and were stored at 4°C.

4.2 POLYMERASE CHAIN REACTION (PCR)

PCR conditions were optimised for each primer set utilised to amplify a specific mutation region. In previous investigations, PCR protocols were optimised for specific primer sets, and these conditions were used without change in this study. However, in the case where a primer or primer set was designed in Phase 1, the reaction was optimised. However, a prerequisite for successful PCR is the design of optimal primers as discussed in Section 3.2.1. The optimum concentration of $MgCl_2$ and T_a required for each reaction was determined experimentally and each primer set was optimised at different conditions.

As the RYR1 gene consists of GC-rich sequences, which form secondary structures with high thermal and structural stability (Keith *et al.*, 2004), the amplification of DNA was

compromised for certain primer sets and it was more difficult to achieve specificity. Enhancers can be utilised to increase yield, and specificity and to overcome difficulties with high GC content. Formamide was utilised to achieve efficient denaturation, leading to successful amplification (Sarker *et al.,* 1990). However, as the effects of formamide thus improve amplification efficiency for one primer pair but decrease it for another, the additive was not utilised for all primer sets. For this reason, the addition of formamide was determined experimentally for each primer pair.

Due to the high GC content, amplification was not consistent for all samples analysed. In some cases amplification of a sub-set of samples had to be re-optimised. In these instances, samples were re-optimised by altering the *Taq* concentration from 0.25 U to 0.5 U. In specific mutation regions, primer-dimers were observed, even at the optimised $MgCl_2$ concentration. However, in all cases where primer-dimers were observed, they did not interfere with the PCR reaction and successful amplification was achieved. Appropriate negative controls were included with each PCR reaction in order to determine whether the PCR reaction was contaminated with foreign DNA. Positive controls were also included with each PCR reaction to ensure that the expected results were generated.

4.3 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a simple and highly effective method for separating and identifying DNA fragments. The molecular weight of each fragment was determined by comparing its mobility to the mobility of a 100 bp DNA ladder. For all mutation regions a 2% (w/v) agarose gel was utilised as it is able to separate DNA fragments that range in size from 0.1 to 2 kb. A voltage of 10 $V.cm^{-1}$ was utilised to provide optimum resolution of the desired DNA fragments, for all mutation regions. However, due to the photographic process utilised, some resolution in the depicted figures were lost as illustrated in Figure 4.1. In addition, differences in intensity of the fragments on the agarose gel were observed. In Figure 4.5 individual MH00709 had a lower intensity compared to individual MH00710, and in Figure 4.7 individual MH00631 had a higher intensity compared to MH00875. These differences may be attributed to varying quality of gDNA that may affect the outcome of PCR, even though the quantity of gDNA utilised was the same.

4.4 CHAIN TERMINATION SEQUENCING

PCR purification of samples was performed using the QIAquick® PCR purification kit, for direct purification of PCR product. The concentration of PCR product was determined utilising the spectrophotometer as described in Section 3.4, and the purity of the PCR product was determined via the ratio of readings at 260 nm and 280 nm (A_{260}/A_{280}). The concentration varied from 5.6 – 18.1 ng.μl^{-1} and the A_{260}/A_{280} ratios were between 1.44 and 1.82. All samples that amplified successfully were subsequently sequenced via the dideoxy chain termination method utilising the ABI PRISM® Big Dye™ Terminator version 3.1 Ready Reaction Cycle Sequencing Kit. Although the amplified region was GC rich which could lead to prematurely terminated molecules, sequencing was successful for all samples analysed, as an optimised PCR protocol was utilised, which prevented this occurrence. The optimal concentration of primer was determined experimentally, and for all mutation regions a concentration of 10 μM of primer was utilised. Depending on the mutation region to be sequenced either the forward or reverse primer was utilised. As the PCR products were approximately 200 – 500 bp in length, 10 ng of template was utilised for sequencing. This is in accordance with the recommended amount of template to be utilised in a sequencing reaction, as listed in Table 3.7. The PCR product following purification was diluted with elution buffer when the concentration exceeded 10 ng.

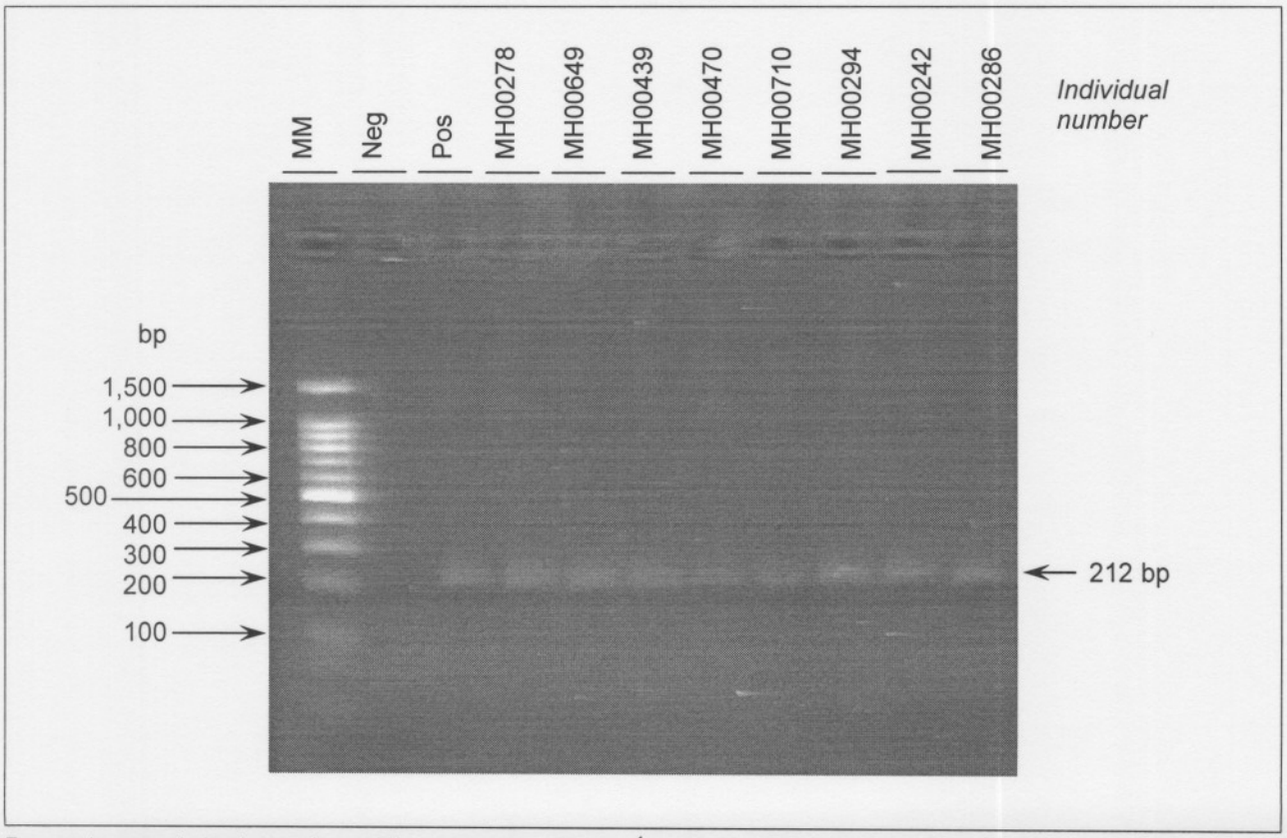
Successful amplification was achieved for mutation regions five and eight. However, difficulties were encountered as the sequenced product indicated artificially amplified molecules. Artefacts were generated as the GC-rich sequence possessed strong secondary structure that resisted denaturation and prevented primer annealing. Most suppliers of *Taq* DNA polymerase provide a unique optimised $MgCl_2$ free buffer utilising different enhancers or concentrations of additives, which can affect the outcome of PCR. The *Taq* DNA polymerase® supplied by Promega varies both in concentration and type of reagent utilised in the 1 x buffer compared to Super-therm polymerase®, as described in Section 3.5. Therefore Super-therm polymerase® was utilised to increase yield, and specificity and to overcome the difficulties encountered as a result of the high GC content of the amplified sequence. Optimised conditions were obtained utilising 0.125 U Super-therm polymerase®. Analysis utilising this protocol did not indicate artefacts in any of the sequenced product.

4.5 MUTATION REGION ONE OF THE RYR1 GENE

PCR was utilised to amplify a region of 212 bp of exon two from the RYR1 gene, which includes two alterations, Cys35Arg and Arg44His. This region of the RYR1 gene was selected as it occurs in the amino terminal mutation cluster mutation hotspot of the RyR1 protein and encompasses functional protein domains. RYR1 mutations in this region of the gene result in amino acid substitutions in the myoplasmic portion of the protein. Currently, 15 RYR1 mutations have been reported in this region of the RYR1 gene, as listed in Table 2.2. Robinson *et al.* (2003b) reported that eight of these 15 mutations were observed in 85 (29%) unrelated MH patients from the UK and that mutations in this region of the RYR1 had significantly increased caffeine-to-halothane tension ratios.

PCR was conducted as described in Section 3.5 and the reaction was optimised at a T_a of 64°C utilising 1.5 mM $MgCl_2$ and 1% formamide. The product was electrophoresed on a 2% (w/v) mini agarose gel, as described in Section 3.6, and the results are presented in Figure 4.1. Amplification was not achieved for the negative control, indicating the absence of contamination, and the expected results were generated for the positive control.

Figure 4.1: Photographic representation of PCR products of mutation region one encompassing the Cys35Arg and Arg44His alterations



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

The amplified region was subsequently sequenced as described in Section 3.7, in order to investigate the presence of the Cys35Arg and Arg44His alterations as well as identify possible novel mutations in this region. Sequencing was conducted utilising the reverse primer (RYRE2R) as indicated in Table 3.4 (Section 3.7). The reverse primer was utilised as the Cys35Arg alteration is situated in close proximity to the location of the forward primer, therefore sequences are depicted as the reverse complement. Sequences generated for this region had a high signal level and no background noise, indicating that the PCR template was pure, the template quantity was within the appropriate level and the optimum concentration of primer was utilised. The quality of the sequence allowed for accurate screening of mutations and identification of possible novel alterations.

The DNA sequence of exon two that was utilised to identify RYR1 mutations was retrieved from Genbank® with accession number U48450.1. The Genbank® sequence was generated by Phillips *et al.* (1996). However, a single base change at nucleotide position +14 (as listed in Table 3.9), was observed in intron two in the study presented here. The A to G alteration was subsequently observed in the *Homo sapiens* chromosome 19 clone retrieved from Genbank® with accession number AC011469 and this sequence is in agreement with the sequences generated from samples and controls in Phase 2 of the programme. The chromosome 19 clone was obtained from a direct submission by the DOE Joint Genome Institute and Stanford Human Genome Centre to Genbank® in 2000 (unpublished), and encompasses an updated version of the RYR1 gene. The alignment of the two sequences retrieved from Genbank® with the sequence difference indicated in bold and by an arrow, is presented in Table 4.1.

Table 4.1: Alignment of the partial gDNA sequence of intron two of the RYR1 gene

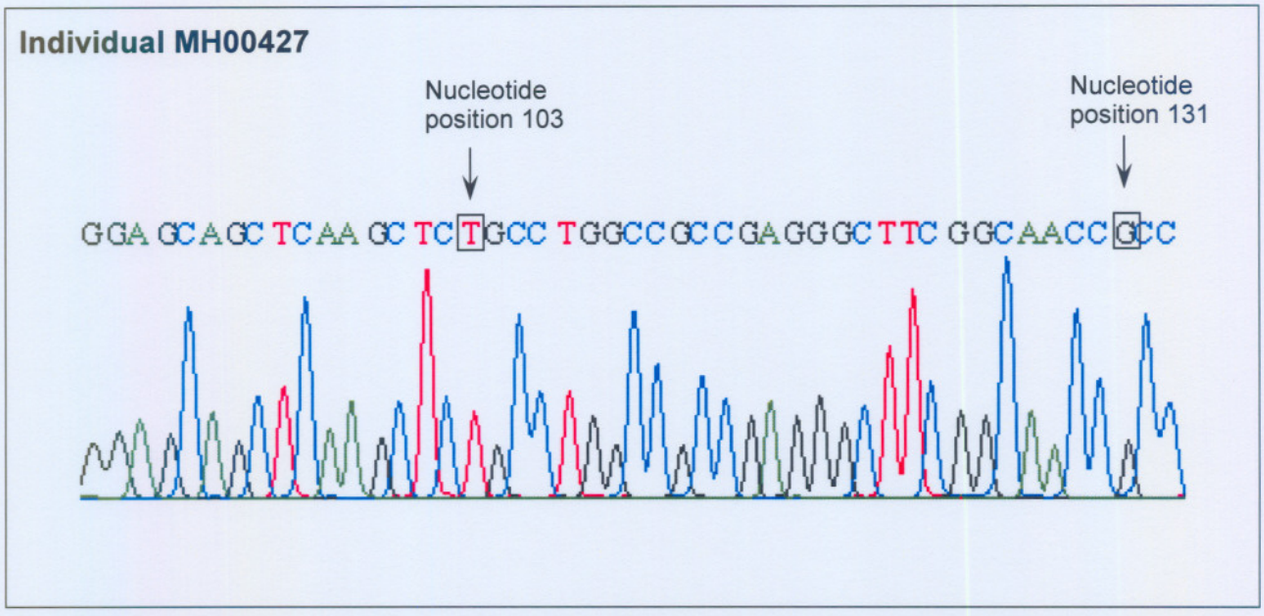
Accession number	Alignment of gDNA sequences
AC011469[1]	Nucleotide position +10 ↓ gtctgtgcag gaggg**g**agagg ggcctgggga caggggcgtc tgaaggggca gagaatcttg
U48450[2]	Nucleotide position +10 ↓ gtctgtgcag gagg**a**agagg ggcctgggga caggggcgtc tgaaggggca gagaatcttg

Sequence of intron two was retrieved from GenBank® with accession numbers AC011469 and U48450. The sequence difference is indicated by an arrow; a = adenine; c = cytosine; g = guanine; t = thymine; 1 = updated version of exon two for the RYR1 gene; 2 = latter version of exon two for the RYR1 gene.

4.5.1 The Cys35Arg alteration

The Cys35Arg alteration was previously analysed in Phase 1 (Neumann, 2002) and the results generated from that study are listed in Appendix C, Table C1. In Phase 2, eight individuals who were not previously analysed were included, as indicated in Table 4.5. The Cys35Arg alteration occurs due to a T103C transition (Lynch *et al.*, 1997), and a representative electropherogram obtained for individual MH00427 illustrating the two alterations, Cys35Arg and Arg44His (as discussed in Section 4.5.2), in the same region is indicated in Figure 4.2.

Figure 4.2: Representative electropherogram of mutation region one encompassing the Cys35Arg and Arg44His alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (103) of the Cys35Arg alteration; the second boxed nucleotide indicates the nucleotide position (131) of the Arg44His alteration, discussed in Section 4.5.2.

The Cys35Arg alteration was not observed in any of the MH probands analysed in Phase 1 and was also not observed in any MH individuals analysed in Phase 2. The mutation was originally reported in a single large pedigree (Lynch *et al.*, 1997) and was subsequently reported in one MH family from France (Robinson *et al.*, 2003b). The mutation was not observed in 100 MH families from Germany, 66 MH families from Switzerland and 297 families from the UK (Robinson *et al.*, 2003b).

All South African MH probands included in Phases 1 and 2 are Caucasian and are therefore of European origin. The difference in the frequency of the mutation in the European populations may be attributed to genetic variation due to regional differences. Regionally distributed MHS mutations have previously been described in European

populations (Jurkat-Rott *et al.*, 2000). The absence of the Cys35Arg alteration could therefore indicate that it does not play a role in the South African MH population.

4.5.2 The Arg44His alteration

Phase 2 of the programme is the first analysis of the Arg44His alteration in the South African MH population. Forty-one South African MH probands were successfully amplified and sequenced in the molecular investigation presented here. All 41 individuals were analysed for the Arg44His alteration, which results from a G131A substitution (Halsall and Robinson, 2004), as well as for novel alterations which may occur in this region.

A representative electropherogram obtained for individual MH00427 depicting the Arg44His alteration is indicated in Figure 4.2. The Arg44His alteration was not observed in any of the MH probands analysed in Phase 2. As the mutation was only recently observed in a single large UK MH family (Halsall and Robinson, 2004), further analysis of this mutation in other population groups would have to be conducted to determine the frequency of the Arg44His alteration worldwide. Considering that this mutation was only recently observed at a mutation hotspot, it could indicate that other novel mutations may be present, which can also result in the MH phenotype. Although novel alterations were not detected in the sequenced region in Phase 1, further analysis of mutation hotspot one in the RYR1 may reveal other unreported alterations in the future.

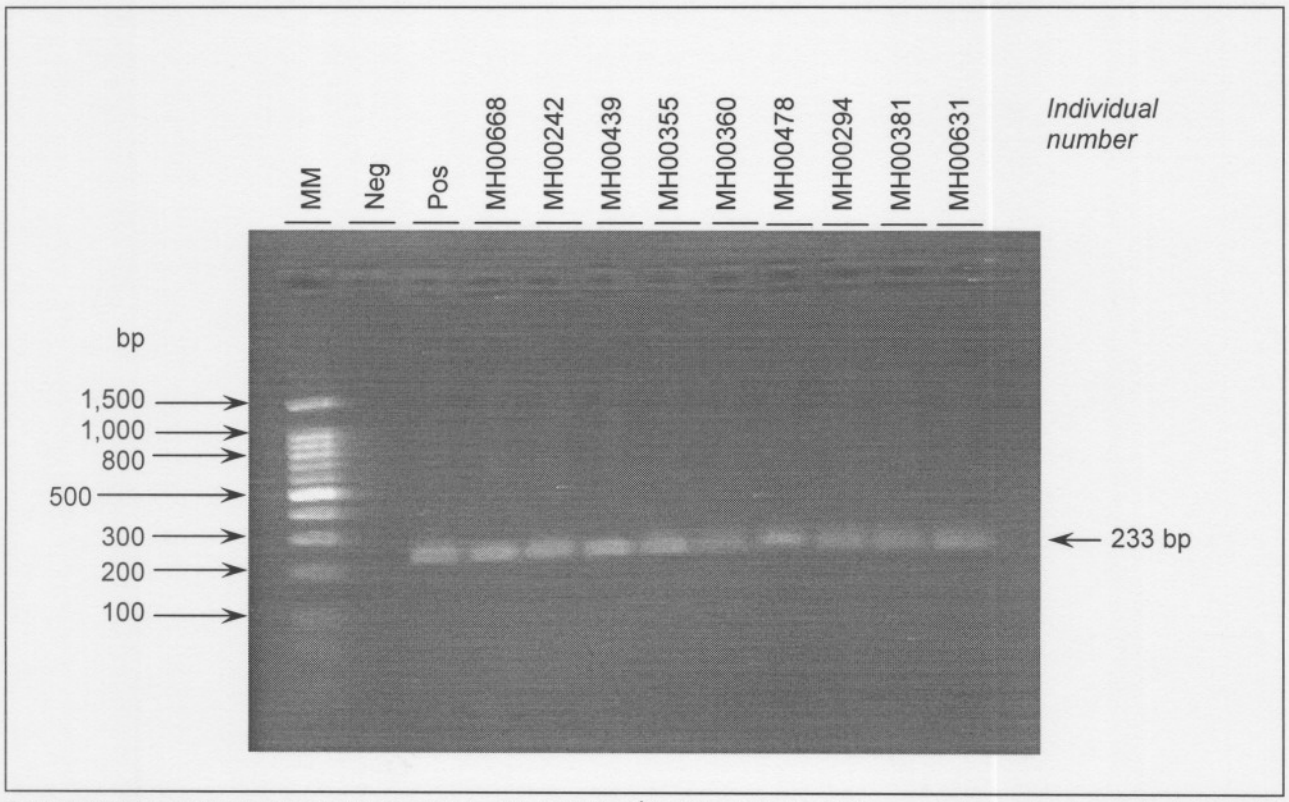
4.6 MUTATION REGION TWO OF THE RYR1 GENE

The standard PCR protocol as discussed in Section 3.5 was utilised to amplify a region of 233 bp from exon six of the RYR1 gene, which includes three alterations, Glu160Gly, Arg163Cys and Arg163Leu. The amplified region is located in the first mutation hotspot (as described in Section 4.5), in the amino terminal region of the RYR1 gene.

The T_a and $MgCl_2$ concentrations were optimised at 64°C and 1.5 mM, respectively. In eight samples amplification could not be achieved and the protocol was re-optimised and samples were re-amplified utilising 0.5 U *Taq* DNA polymerase (Promega®). PCR amplification was successful for all samples analysed as well as in the positive control. No amplification was observed in the negative control. The amplified product was subsequently electrophoresed on a 2% (w/v) mini agarose gel, as described in Section 3.6. Results of PCR amplification are depicted in Figure 4.3.

The amplified region was subsequently sequenced in order to screen for three reported alterations, Arg163Cys, Arg163Leu and Glu160Gly, as well as to identify novel mutations that might be present in this region. Sequencing was conducted utilising the reverse primer (RYRE6R) due to the close proximity of the mutations to the forward primer. For this reason sequences are depicted as the reverse complement. Sequences generated for mutation region two had high template purity as indicated by the high level of signal and low background noise.

Figure 4.3: Photographic representation of PCR products of mutation region two encompassing the Glu160Gly, Arg163Cys and Arg163Leu alterations



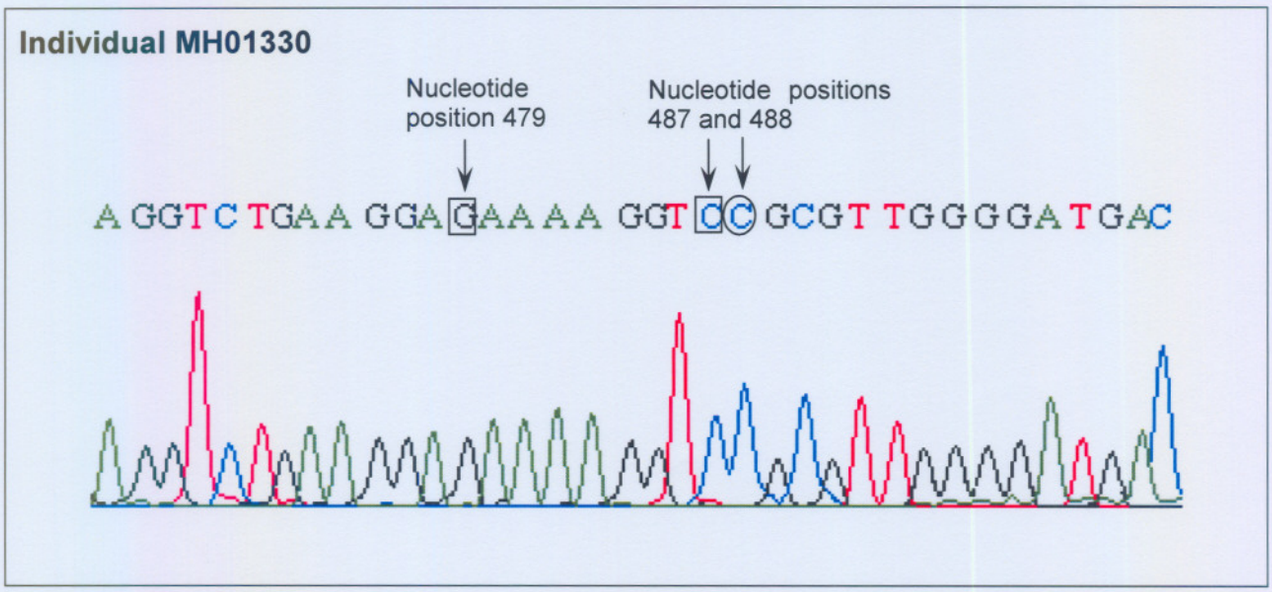
Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

4.6.1 The Glu160Gly alteration

Forty-three MH probands amplified via PCR were successfully sequenced utilising the protocol outlined in Section 3.5. The Glu160Gly alteration occurs due to an A479G transition (Halsall and Robinson, 2004) and a representative electropherogram obtained for individual MH00427 illustrating alterations Glu160Gly, Arg163Cys (discussed in Section 4.6.2) and Arg163Leu (discussed in Section 4.6.3), is indicated in Figure 4.4.

The Glu160Gly alteration was screened for the first time in the South African MH population and was not detected in any of the 43 MH individuals analysed in Phase 2. Recently, the Glu160Gly alteration was reported in a single MH pedigree from the UK (Halsall and Robinson, 2004). Thus, this mutation could represent an alteration specific to the above-mentioned family. Over 100 RYR1 mutations have been identified in MH patients worldwide. The frequency of each mutation varies from either being a private alteration specific to a single family, or a mutation with a higher frequency. However, further analysis of the Glu160Gly alteration in other population groups would be required to determine the frequency of this mutation globally.

Figure 4.4: Representative electropherogram of mutation region two encompassing the Glu160Gly, Arg163Cys and Arg163Leu alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (479) of the Glu160Gly alteration; the second boxed nucleotide indicates the nucleotide position (487) of the Arg163Cys alteration (discussed in Section 4.6.2) and the circled nucleotide indicates the nucleotide position (488) of the Arg163Leu alteration (discussed in Section 4.6.3).

4.6.2 The Arg163Cys alteration

The Arg163Cys alteration was previously analysed via RFLP in 38 MH individuals in Phase 1. The C487T transition was detected by the abolishment of the *Bst* UI restriction site. In Phase 2, the Arg163Cys alteration was analysed in three MH individuals who were previously not analysed.

In addition, individuals MH00654, MH00707, MH00709 and MH00710 that did not amplify in previous studies due to problematic PCR amplification, were successfully amplified and sequenced in the study presented here. Lack of amplification in previous studies may have been due to the stable secondary structure of the RYR1 gene, as discussed in Section 4.2.

A representative electropherogram obtained for individual MH00427 depicting the Arg163Cys alteration, is indicated in Figure 4.4, in the previous paragraph.

The Arg163Cys alteration was not detected in any of the seven MH individuals analysed in Phase 2. In Phase 1, the Arg163Cys mutation was not detected in any of the 38 South African MH individuals analysed. The Arg163Cys mutation was originally reported in one CCD pedigree, an unrelated MH pedigree, and an unrelated MH proband (Quane *et al.*, 1993). The authors observed that the mutation resulted in both CCD and MH in certain individuals. In other individuals the mutation only resulted in susceptibility to MH, indicating that modifying factors may be necessary for the clinical expression of CCD when this mutation is present (Quane *et al.*, 1993). Interaction with modifying factors may also be responsible for the variability of clinical expression that is often observed in both CCD and MH. The Arg163Cys alteration was subsequently reported in two unrelated Italian MH families, a single MH family from France and in four families from the UK. The alteration was not detected in 66 MH families from Switzerland and 17 MH families from Belgium (Robinson *et al.*, 2003b). In view of the presence of the Arg163Cys alteration in several unrelated families (Halsall and Robinson, 2004), it can therefore not represent a founder mutation, and there is clear evidence that the frequency of this mutation varies in different population groups.

4.6.3 The Arg163Leu alteration

The recently identified Arg163Leu alteration results from a G488T substitution (Halsall and Robinson, 2004). The alteration occurs in the same restriction enzyme recognition site as the Arg163Cys alteration. Therefore, the presence of either mutation could be detected via RFLP, as discussed in Section 4.6.2, although these mutations could not be distinguished from each other by this method of analysis. Sequencing was thus conducted in order to differentiate between the Arg163Leu and the Arg163Cys alteration, as well as to identify novel alterations that may occur in the sequenced region.

Forty-three samples obtained via PCR were successfully sequenced utilising the protocol outlined in Section 3.7. The Arg163Leu alteration was not detected in any of the 43 South African MH individuals analysed for the first time for this mutation in this study. The Arg163Leu alteration was recently reported in a single MH pedigree from the UK (Halsall and Robinson, 2004). Therefore, the alteration could be specific to the above-mentioned family, or may be due to population differences as discussed in Section 4.6.2.

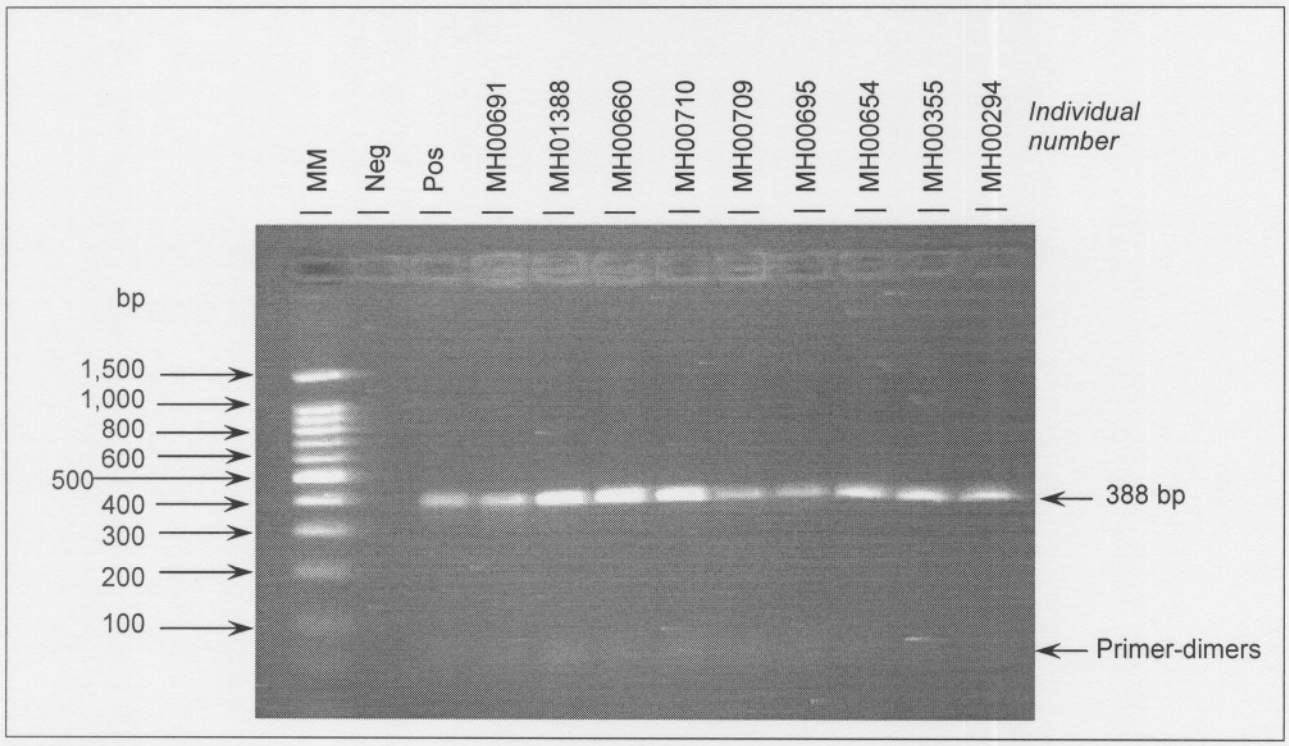
4.7 MUTATION REGION THREE OF THE RYR1 GENE

PCR was utilised to amplify a 390 bp product of exon 39, encompassing three alterations, Arg2163Cys, Arg2163His and Val2168Met. The three alterations are clustered in the central portion of the RYR1 gene, between nucleotides 6400-6700, in the second mutation hotspot. McCarthy *et al.* (2000) indicated that most of RYR1 mutations are located in this region of the RyR1 protein. To date, 24 RYR1 mutations have been observed in the second mutation hotspot as well as one in-frame deletion, as listed in Table 2.2. This region was selected as it plays an important role in E-C coupling and harbours a phosphorylation site at Ser2844 (Suko *et al.*, 1993). In addition, there is also a CaM binding site on the cytoplasmic portion of the RyR1 protein that is approximately 10 nanometres (nm) from the transmembrane channel (Wagenknecht and Radermacher, 1995; Zhu *et al.*, 2004).

In Phase 1 the three alterations, Arg2163Cys, Arg2163His and Val2168Met, were analysed in 33 individuals, utilising RFLP and automated sequencing strategies. Ten MH probands that were previously not analysed for mutations in this region were screened during phase 2 of the study. The PCR conditions were optimised utilising 0.125 U Super-therm polymerase® for reasons outlined in Section 4.4, for all three alterations. The PCR reaction was optimised with a $MgCl_2$ concentration of 1 mM and a T_a of 65°C. The amplified product was subsequently electrophoresed on a 2% (w/v) mini agarose gel, as described in Section 3.6, and the results are illustrated in Figure 4.5. The PCR reaction was successful as amplification was not detected in the negative controls and the expected amplification was achieved in the positive controls.

Following PCR amplification the PCR product was purified and sequenced, in order to investigate the presence of the Arg2163Cys, Arg2163His and Val2168Met alterations as well as identify possible novel mutations that may be present in this region. Sequencing was conducted utilising the reverse primer (Val2168Met), according to the protocol outlined in Section 3.7 and sequences are depicted as the reverse complement. A high level of quality was obtained for the DNA sequence data, indicating that optimised PCR conditions and PCR purification were utilised. Thus, data could be extracted accurately from the sequences.

Figure 4.5: Photographic representation of PCR products of mutation region three encompassing the Arg2163Cys, Arg2163His and Val2168Met alterations



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

Alterations Arg2163 and Val2168 were all classified as causative, as amino acids located at these two positions are conserved in the three human isoforms RyR1, RyR2 and RyR3, as well as the RyR1 isoforms of the pig and rabbit. Several amino acids in the vicinity of these three alterations (Arg2163Cys, Arg2163His and Val2168Met) are also conserved. Conservation of amino acids for the three alterations in RyR isoforms is listed in Table 4.2, with the alterations indicated in bold.

Table 4.2: Conservation of amino acids for alterations, Arg2163 and Val2168 in isoforms of the RyR protein across three species

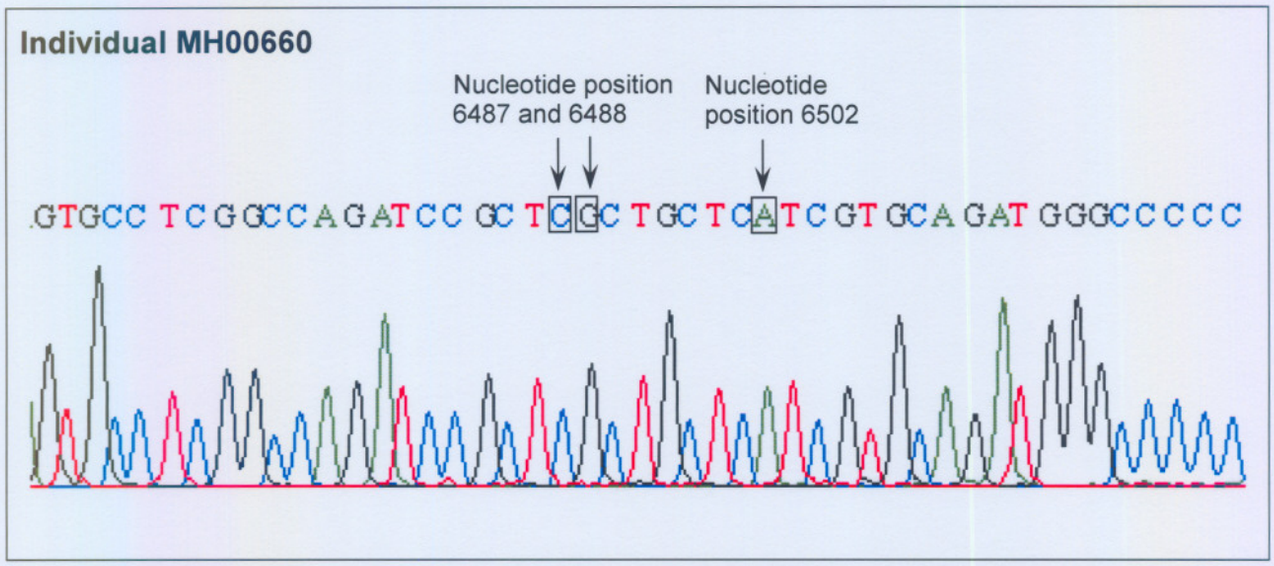
Isoforms	Alignment of RyR protein sequences
Human RyR1	LGQI**R**SLLI**V**QMGPQEENLMIQSIGNIMNNKVFYQHPNLMRALGMHETVMEVMVNVLGGGESKEI
Human RyR2	LGQI**R**SLLS**V**RMGKEEEKLMIRGLGDIMNNKVFYQHPNLMRALGMHETVMEVMVNVLGGGESKEI
Human RyR3	LGQI**R**SLLS**V**RMGKEEELLMINGLGDIMNNKVFYQHPNLMRVLGMHETVMEVMVNVLGTEKSQIA
Pig RyR1	LGQI**R**SLLI**V**QMGPQEENLMIQSIGNIMNNKVFYQHPNLMRALGMHETVMEVMVNVLGGGESKEI
Rabbit RyR1	LGQI**R**SLLI**V**QMGPQEENLMIQSIGNIMNNKVFYQHPNLMRALGMHETVMEVMVNVLGGGETKEI

The conserved amino acids for the five isoforms are highlighted in blue; the conserved Arg at nucleotide position 2163 and the conserved Val at nucleotide position 2168 are indicated in bold. The accession numbers are as follows: RyR1 human = P21817; RyR2 human = Q92736; RyR3 human = NP_001027; RyR1 pig = P16960; RyR1 rabbit = P11716.

4.7.1 The Arg2163Cys alteration

The Arg2163Cys alteration occurs due to a C6487T transition and in Phase 2, ten MH probands were analysed for the Arg2163Cys alteration in this region. A representative electropherogram obtained for individual MH00660 illustrating alterations Arg2163Cys, Arg2163His and Val2168Met is presented in Figure 4.6.

Figure 4.6: Representative electropherogram of mutation region three encompassing the Arg2163Cys, Arg2163His and Val2168Met alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (6487) of the Arg2163Cys alteration; the second and third boxed nucleotide indicates the nucleotide positions (6488 and 6502) of the Arg2163His (as discussed in Section 4.7.2) and Val2168Met alterations (as discussed in Section 4.7.3), respectively.

The Arg2163Cys alteration was not detected in any of the ten MH probands analysed in Phase 2 and was also not detected in 33 individuals analysed in Phase 1. In a study conducted by Manning *et al.* (1998b) the three alterations Arg2163Cys, Arg2163His and Val2168Met, as well as a fourth alteration, Thr2206Met, collectively accounted for 11% of MH cases. The authors observed the Arg2163Cys alteration in two unrelated families with MH and suggested that the mutation was either very common in the European population or arose through a founder effect. A subsequent study conducted by Robinson *et al.* (2003b) reported the Arg2163Cys alteration in one MH family from Belgium, one MH family from Germany and one MH family from the UK. Considering that the mutation was observed in several MH families in Europe, it can be concluded that this is not a family-specific mutation.

The absence of this mutation in the South African MH population could be due to regional differences associated with MH. As most of mutations associated with MH are located in

the central portion of the RYR1 gene, this functional domain of the gene should be analysed in different population groups. Although novel alterations were not observed in the sequenced region of mutation region three, further analysis of the second mutation hotspot of the RYR1 gene may reveal novel alterations that result in susceptibility to MH in the South African population.

4.7.2 The Arg2163His alteration

The Arg2163His alteration occurs due to a G6488A transition and the presence of this mutation was analysed in ten MH probands during the Phase 2 study. A representative electropherogram obtained for individual MH00660 depicting the Arg2164His alteration is indicated in Figure 4.6, in the previous paragraph.

The Arg2163His alteration was first reported in an Italian MHS pedigree. Manning *et al.* (1998b) observed the mutation in a mother and daughter. The proband had undergone several uneventful surgical procedures with known MH triggering agents and only presented with an MH episode during the ninth anaesthetic procedure (Manning *et al.*, 1998b). Further analysis via histological examination revealed that the individual was asymptomatic for CCD. The alteration was subsequently reported in four unrelated MH families from the UK (Robinson *et al.*, 2003b). The Arg2163His alteration was not detected in any of the ten MH probands analysed in Phase 2 or in 33 MH individuals analysed in Phase 1. The study conducted by Manning *et al.* (1998b) indicated that the proband was exposed to anaesthesia several times before an MH episode was triggered, which may indicate that genetic determinants as well as environmental factors are required for the MH phenotype. Therefore, further analysis of epistatic events may reveal genetic and environmental interactions that play a role in susceptibility to MH in the South African population.

4.7.3 The Val2168Met alteration

The Val2168Met alteration occurs due to a G6502A transition. In Phase 2, ten MH probands were analysed for mutations in this region. Previously, in the Phase 1 study, two individuals, MH00630 and MH00654, could not be analysed for the Val2168Met mutation because of poor quality sequence. Both these individuals were included in the study reported here. In phase 1, Taq DNA polymerase (Promega®) was utilised and in phase 2, Super-therm polymerase® was utilised to amplify these samples. The two different DNA

polymerases both vary in the concentration and composition of reagents utilised in the buffer, which can affect the outcome of the PCR reaction, as discussed in Section 4.4. A representative electropherogram obtained for individual MH00660 depicting the Val2168Met alteration is illustrated in Figure 4.6.

The Val2168Met alteration was not detected in any of the 12 MH individuals analysed during Phase 2 of the study. In Phase 1 a single South African MH proband, MH00360, was detected to harbour a heterozygous genotype for the Val2168Met mutation. The individual developed symptoms associated with MH following exposure to succinylcholine and because of the autosomal dominant nature of the disorder, it was concluded that individual MH00360 is MHS. The Val2168Met alteration was first detected in eight unrelated MH families from Switzerland by Manning *et al.* (1998b). To date, the alteration has only been observed in these two population groups, thus it would be important to determine if individual MH00360 from the South African population has Swiss ancestry, as regional differences in MH have been reported. Most of MHS mutations cluster to the central portion of the RYR1 gene, which indicates that this region represents a functionally significant domain. As a Val2168Met alteration was detected in a single South African MH proband, this may indicate that this region of the gene plays an important role in MHS in the South African MH population and should be analysed further via mutation screening of the surrounding exonic and intronic sequences to determine if other novel or reported alterations result in the MH phenotype in this population.

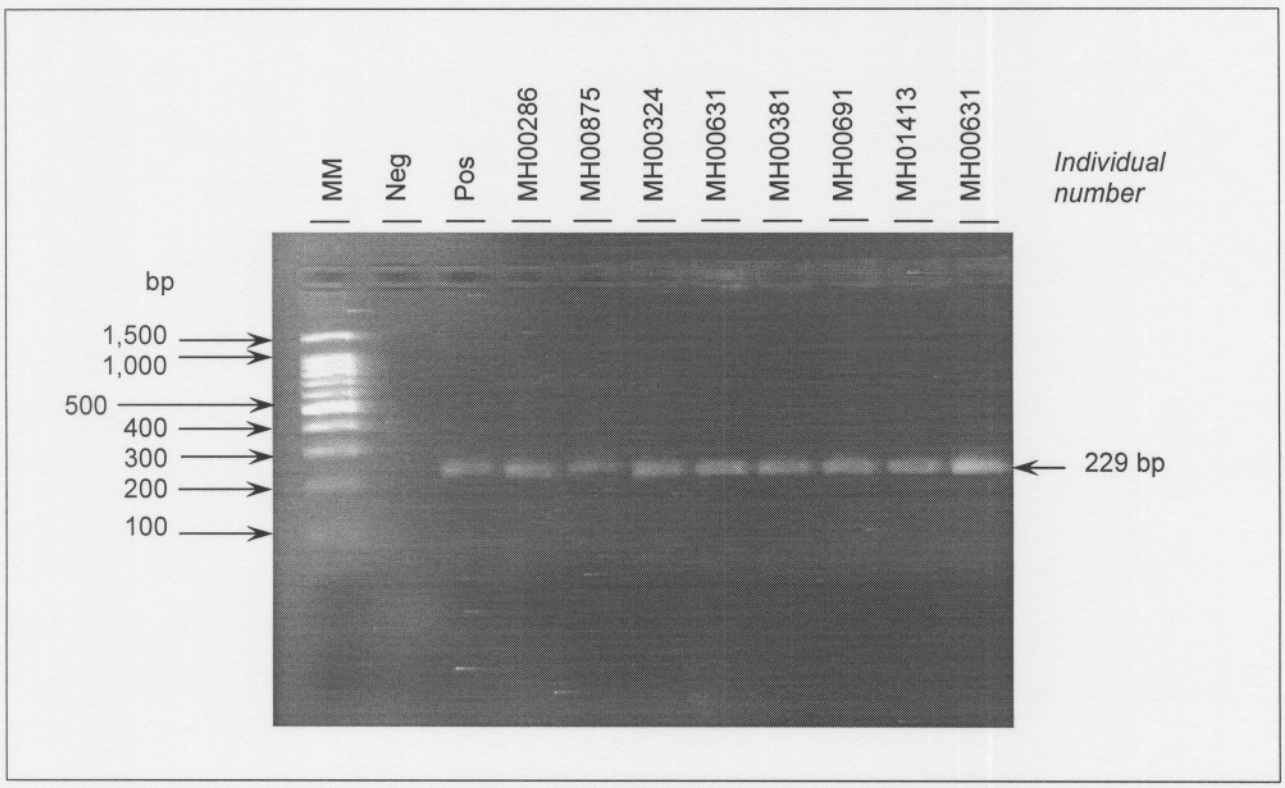
4.8 MUTATION REGION FOUR OF THE RYR1 GENE

PCR was conducted in order to amplify a 229 bp region of exon 40 of the RYR1 gene. The sequence obtained was designated mutation region four and harbours three known alterations, Ala2200Val, Thr2206Met and Thr2206Arg. All three alterations are located in the central portion of the RYR1 gene, which represents the second mutation hotspot of the RyR1. This region plays an important role in E-C coupling as discussed in Section 4.7.

PCR was optimised at an $MgCl_2$ concentration of 1.5 mM and T_a of 66°C. In seven samples, amplification was not achieved utilising the optimised protocol, and conditions had to be re-optimised utilising 0.5 U *Taq* DNA polymerase (Promega®). The higher concentration of *Taq* DNA polymerase increased the yield of the PCR product and successful amplification was achieved in all seven samples utilising the re-optimised

protocol. The amplified product was subsequently electrophoresed on a 2% (w/v) mini agarose gel, as described in Section 3.6. Results obtained for PCR amplification are depicted in Figure 4.7. PCR amplification was considered successful, as expected amplification was achieved in the positive control utilised in this study. Of equal importance is the fact that the absence of contamination resulted in lack of amplification in the negative controls. Following PCR amplification the PCR product was purified and sequenced utilising the reverse primer (RYRE40R). The sequence is therefore depicted as the reverse complement. Sequence data obtained for mutation region four were successfully achieved utilising the optimised PCR protocol and no background was observed underlying the genuine peaks. The three alterations are discussed in subsequent paragraphs.

Figure 4.7: Photographic representation of PCR products of mutation region four encompassing the Ala2200Val, Thr2206Met and Thr2206Arg alterations



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = blank utilised as a negative control; Pos = control utilised as a positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

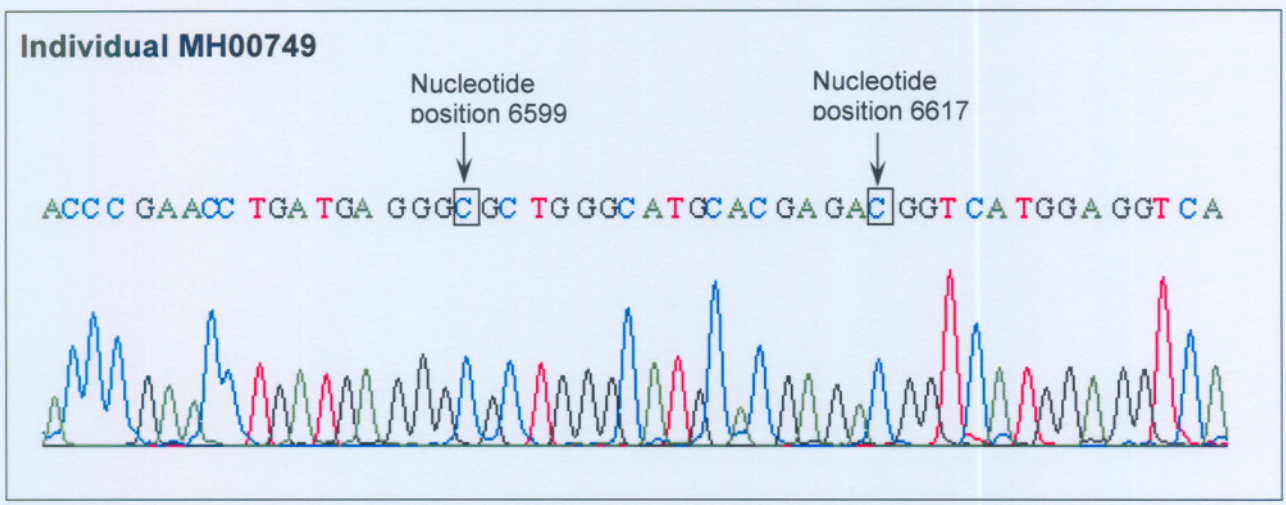
4.8.1 The Ala2200Val alteration

PCR was conducted for 41 MH probands to detect the Ala2200Val alteration, which is due to a C6599T transition. The mutation was recently reported in a single MH family from the UK (Halsall and Robinson, 2004) and has never been analysed in the South African MH

population. Phase 2 of the extended research programme represents the first effort to screen for this mutation in the South African MH population. A representative electropherogram obtained for individual MH00749 depicting alterations Ala2200Val, Thr2206Met (as discussed in Section 4.8.2) and Thr2206Arg (as discussed in Section 4.8.3), is illustrated in Figure 4.8.

The Ala2200Val alteration was not observed in any of the 41 MH individuals analysed in this study. Considering that this mutation has thus far been observed in only one family suggests that it may be unique to the family described by Halsall and Robinson (2004). However, as this mutation has only recently been described, the frequency of the Ala2200Val alteration would have to be determined in other populations. Novel alterations were not detected in the sequenced region of mutation region four. However, as new mutations are continually been reported, further analysis of the intronic and exonic regions of the central region of the RYR1 gene may reveal novel alterations associated with MHS in the South African MH population.

Figure 4.8: Representative electropherogram of mutation region four encompassing the Ala2200Val, Thr2206Met and Thr2206Arg alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (6599) of the Ala2200Val alteration; the second boxed nucleotide indicates the nucleotide position (6617) of the Thr2206Met (as discussed in Section 4.8.2) and Thr2206Arg (as discussed in Section 4.8.3) alterations.

4.8.2 The Thr2206Met alteration

The Thr2206Met alteration, which is due to a C6617T nucleotide transition, was previously analysed in 31 MH individuals in Phase 1 via RFLP. In Phase 2, 13 MH probands were analysed for Thr2206Met mutation in this region. A representative electropherogram obtained for individual MH00749 illustrating the Thr2206Met alteration is depicted in Figure 4.8, in the previous paragraph.

The Thr2206Met alteration was first reported by Manning *et al.* (1998b) in a single MH family. It was subsequently reported in two different MH pedigrees from Germany (Brandt *et al.*, 1999). Regional differences were, however, observed in the German study, as the mutation was not observed in any other European population analysed (Brandt *et al.*, 1999). The alteration was also reported in 20 MH families from the UK (Halsall and Robinson, 2004). As the alteration occurs in the transmembrane segments of the RyR1 protein, this could result in a leaky pore, which would cause increased release of Ca^{2+}. A study conducted by Wehner *et al.* (2002) reported that the presence of this alteration in myotubes resulted in an abnormal response of the intracellular Ca^{2+} concentration to 4-chloro-*m*-cresol and to caffeine. Considering the ancestry of South African Caucasian patients, it was surprising to find that the mutation was not observed in any South African MH probands in Phase 1 or 2. As this alteration was not observed in South African MH probands, this could indicate that the alteration does not play a role in susceptibility to MH in this population.

4.8.3 The Thr2206Arg alteration

As discussed in Section 4.8.2, 13 samples obtained via PCR were successfully sequenced in Phase 2. The Thr2206Arg alteration occurs due to nucleotide transition C6617G. A representative electropherogram obtained for individual MH00749 illustrating the Thr2206Arg alteration is depicted in Figure 4.8. The Thr2206Arg alteration was observed in a single MH pedigree (Brandt *et al.*, 1999). The authors indicated that the proband in one MH family had clinical signs of myopathy. However, histological examination of muscle from this proband did not reveal central cores. As the alteration has only been reported in a single MH family, the Thr2206Arg alteration could be specific to the family it was originally reported in. As the proband described by Brandt *et al.* (1999) presented with clinical signs of myopathy, the Thr2206Arg alteration may not be sufficient on its own to result in the MH phenotype, indicating that a combination of alleles is necessary to predispose someone to MH.

4.9 MUTATION REGION FIVE OF THE RYR1 GENE

The DNA sequence of exon 43 to 45 utilised to identify RYR1 mutations was retrieved from Genbank® with accession numbers U48476.1 and U48477.1. These Genbank® sequences here were generated by Phillips *et al.* (1996). However, four single base changes and 21 missing bases were subsequently observed in Phase 2 (the study presented here), as well

as an intron sequence of 118 bp that was missing from the two above-mentioned Genbank® sequences. The changes were observed in controls and MH individuals utilised in Phase 2 and were in agreement with the *Homo sapiens* chromosome 19 clone retrieved from Genbank® with accession number AC011469. Alignment of the two sequences retrieved from Genbank®, with the sequence difference indicated in bold, is presented in Table 4.3. The entire sequence obtained for mutation region five is indicated in Section 3.7.5.

Table 4.3: Partial alignment of the gDNA sequence of the *Homo sapiens* RYR1 skeletal muscle gene and chromosome 19 clone

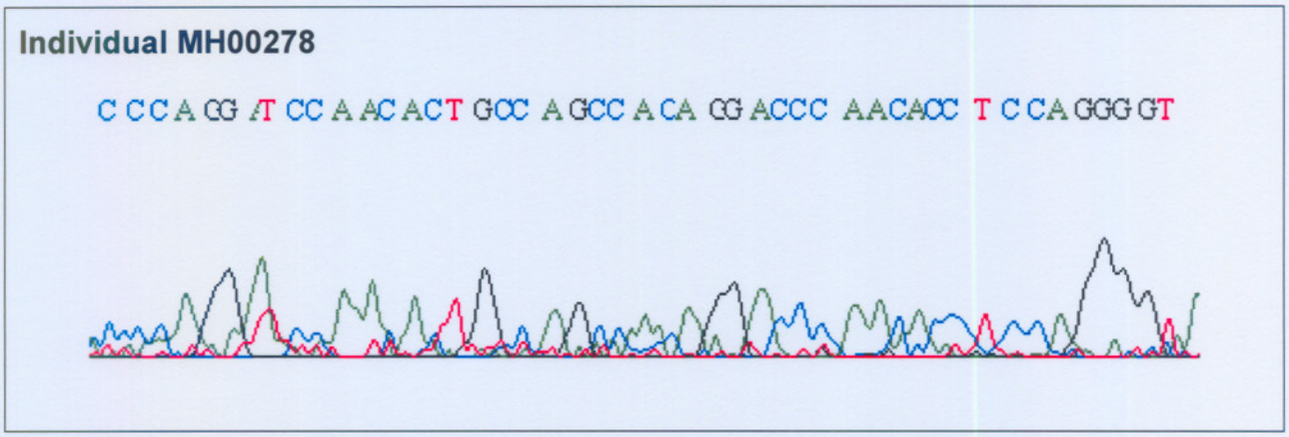
Exon	Nucleotide number	Alignment of nucleotide sequences
Exon 43	85446[1]	ctggacttcctgcgctttgctgtcttcgtcaacggtgagga-gggggtggcagtggcaga
	429[2]	ctggacttcctgcgctttgctgtcttcgtcaacggtgagga**g**gggggtggcagtggcaga
	85505[1]	gc-gggaagtatggagtcactggtcacacacct**c**cctcgagatgactgctcgca**c**cctga
	462[2]	gc**g**gggaag**ctc**ggagtcactggtcacacacct-cctcgagatgactgctcgca-cctga
	85564[1]	gccacagat**g**gggtccaggcaggaat**c**ccttccagca**g**gcct**g**gggctggcaggg**gc**ctg
	581[2]	gccacagat-gggtccaggcaggaat-ccttccagca-gcct-gggctggcaggg--ctg
	85624[1]	tgt**tac**ccctggaggtgttgggtcctgt
	570[2]	tgt--accctggaggtgttgggtcctgt
Exon 44	85772[1]	tgggacctgtgttaccc**c**tgga**g**gtgttgggtcctggggctgcatggg**g**aggtctctgat
	3[3]	tgggacctgtgttaccc-tggag-tgttgggtcctggggctgcatggg-aggtctctgat
	85831[1]	ggtggct-catgagacccc**c**ttt**tcc**ccatgcgggtggccaggcgagagcgtggaggagaa
	60[3]	ggtggct**t**catgagacccctttt---ccatgcgggtggccaggcgagagcgtggaggagaa
	86070[1]	gccccctcctcaata**g**ggcaacccgccctccctggcccctggctgcctccccaacccacc
	297[3]	gccccctcctcaata-ggcaacccgccctccctggcccctggctgcctccccaacccacc

1 = sequence retrieved from GenBank® with accession number, AC011469; 2 = sequence retrieved from GenBank® with accession number, U48476.1; 3 = sequence retrieved from GenBank® with accession number, U48477.1. The sequence difference is indicated in bold, a = adenine; c = cytosine; g = guanine; t = thymine.

A region of 937 bp was amplified and subsequently sequenced in order to detect eight known alterations, Val2346Met, Glu2348Gly, Ala2350Thr, Arg2355Cys, Phe2364Val, Gly2434Arg, Arg2435His and Arg2435Leu in exon 43 to exon 45, as well as identify possible novel mutations in this region. Alterations of mutation region five are located in the central portion of the RyR1 protein, as discussed in Section 4.7.

Optimisation of mutation region five was obtained at a T_a of 62°C utilising *Taq* DNA polymerase (Promega®). However, PCR conditions were not optimal and secondary product was not visible on the agarose gel. Following sequencing the product indicated artificial amplified molecules, several false stops and compression with high background noise, as discussed in Section 4.4. The GC-rich template prevented the sequence from being accurately resolved due to the influence of a stable secondary structure. A representative electropherogram illustrating template artefacts is depicted in Figure 4.9.

Figure 4.9: Representative electropherogram of template artefacts generated in the sequenced product of mutation region five

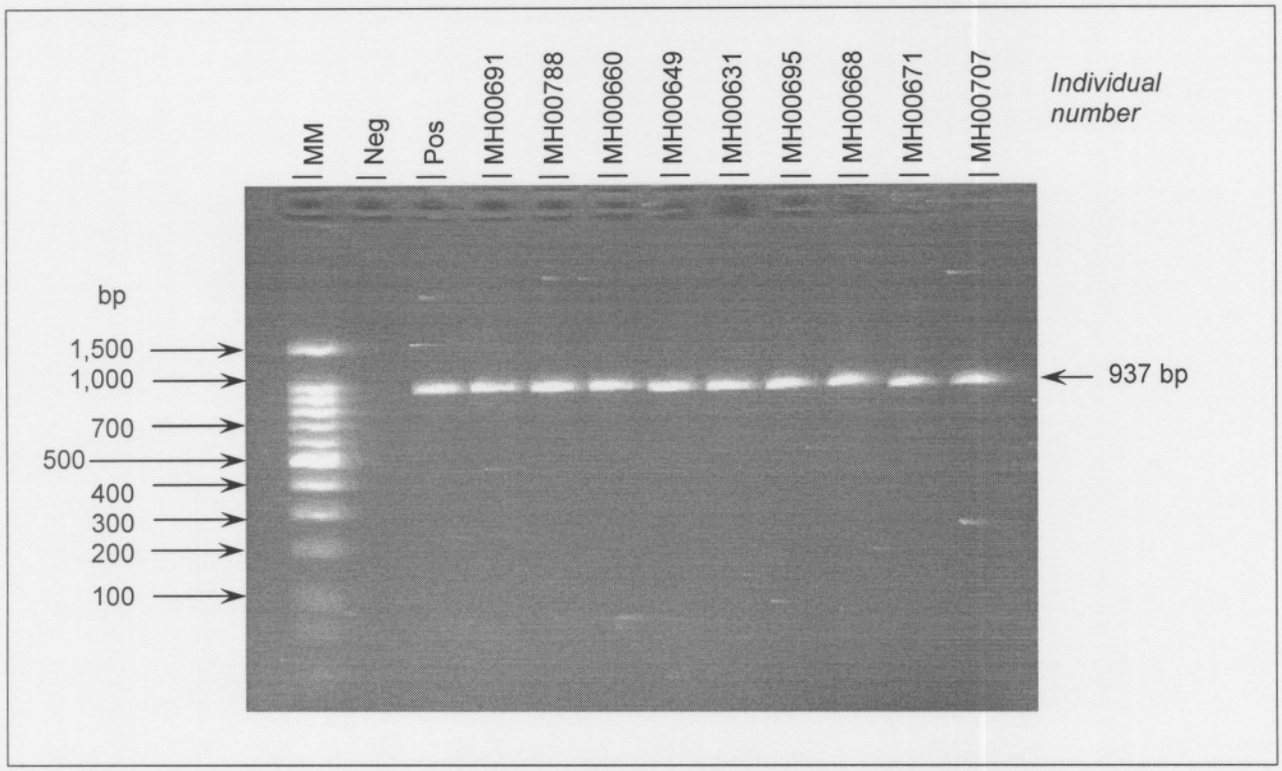


A = adenine; C = cytosine; G = guanine; T = thymine.

The PCR conditions were re-optimised at a T_a of 62°C, utilising 0.125 U Super-therm polymerase® and 1.5 mM $MgCl_2$. Employing this protocol did not generate artefacts in the sequenced product and background noise was not observed, as illustrated in Figure 4.11. The PCR product was electrophoresed on a 2% (w/v) mini agarose gel, as described in Section 3.6, and the results are depicted in Figure 4.10.

PCR amplification was considered successful for all samples analysed, as amplification was only obtained in the positive control and not in the negative control. Sequencing was conducted utilising the reverse primer (Glu2434R) as indicated in Table 3.4. The 39 samples analysed in Phase 2 were all amplified and sequenced successfully utilising the re-optimised protocol.

Figure 4.10: Photographic representation of PCR products of mutation region five encompassing the Val2346Met, Glu2348Gly, Ala2350Thr, Arg2355Cys, Phe2364Val, Gly2434Arg, Arg2435His and Arg2435Leu alterations



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

4.9.1 The Val2346Met alteration

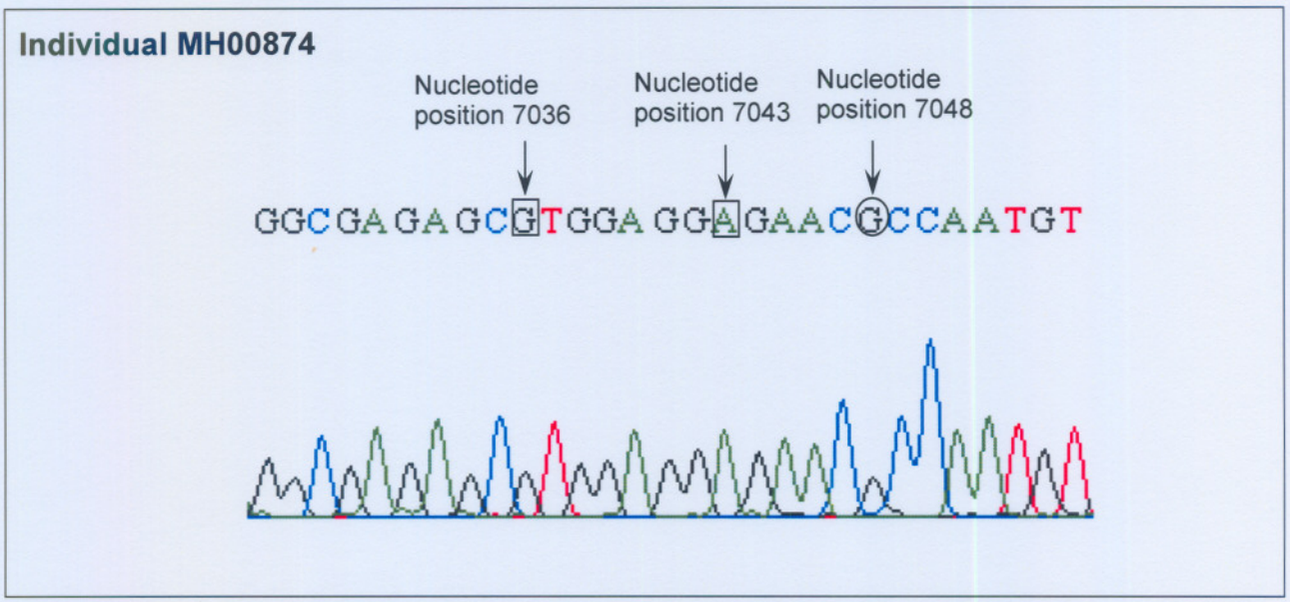
The presence of the Val2346Met alteration, which occurs due to a G7036A nucleotide transition (Halsall and Robinson, 2004), was investigated in 39 South African MH probands. Phase 2 represents the first analysis of this mutation in a South African MH population. A representative electropherogram obtained for individual MH00874 depicting alterations Val2346Met, Glu2348Gly (discussed in Section 4.9.2) and Ala2350Thr (discussed in Section 4.9.3) is illustrated in Figure 4.11.

The Val2346Met alteration was not detected in any of the 39 MH individuals analysed in Phase 2 and novel alterations were also absent from the sequenced region. Halsall and Robinson (2004) recently reported a Val2346Met alteration in a single MH family from the UK. It has still not been determined whether this mutation represents a family-specific alteration or if it occurs frequently.

Further analysis in other population groups would have to be conducted in order to determine the frequency of the alteration. Although the alteration was not detected in any

of the South African MH probands analysed in Phase 2, further analysis of the central region of the gene is warranted, as novel alterations are continually being identified in this mutation hotspot region of the RYR1 gene.

Figure 4.11: Representative electropherogram of mutation region five encompassing the Val2346Met, Glu2348Gly and Ala2350Thr alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (7036) of the Val2346Met alteration; the second boxed nucleotide indicates the nucleotide position (7043) of the Glu2348Gly alteration (as discussed in Section 4.9.2) and the circled nucleotide indicates the nucleotide position (7048) of the Ala2350Thr alteration (as discussed in Section 4.9.3).

4.9.2 The Glu2348Gly alteration

Thirty-nine MH probands were analysed in this study for the presence of the Glu2348Gly alteration, which occurs due to an A7043G nucleotide transition (Halsall and Robinson, 2004). A representative electropherogram obtained for individual MH00874 depicting the Glu2348Gly alteration is illustrated in Figure 4.11 in the previous paragraph.

The mutation was not observed in any South African MH individuals included in Phase 2. The Glu2348Gly alteration was recently reported in a single family (Halsall and Robinson, 2004), therefore it has not yet been determined what the frequency of this alteration is in other population groups, as discussed in Section 4.9.1. As the alteration was not detected in any of the South African MH probands analysed in this study it may not play a role in susceptibility to MH in this population, but further studies are necessary to verify this finding.

4.9.3 The Ala2350Thr alteration

A G7048A nucleotide transition in the central portion of the RYR1 gene results in an Ala2350Thr alteration in the RyR1 protein. Phase 1 represented the first analysis of this alteration in 39 MH probands from South Africa. A representative electropherogram obtained for individual MH00874 depicting the Ala2350Thr alteration is illustrated in Figure 4.11.

Halsall and Robinson (2004) reported this novel alteration in three MH families from the UK and determined that the frequency of this alteration in the British population was 0.69. The Ala2350Thr alteration is currently being utilised in the genetic diagnosis of MH susceptibility in the UK. Although the mutation was not observed in any South African MH individuals included in this study, further analysis of this region may identify novel alterations which may play a role in susceptibility to MH in the South African population. As RYR1 alterations have displayed regional differences it would therefore be important to determine the frequencies of each alteration in the South African MH population before a genetic diagnostic test could be employed.

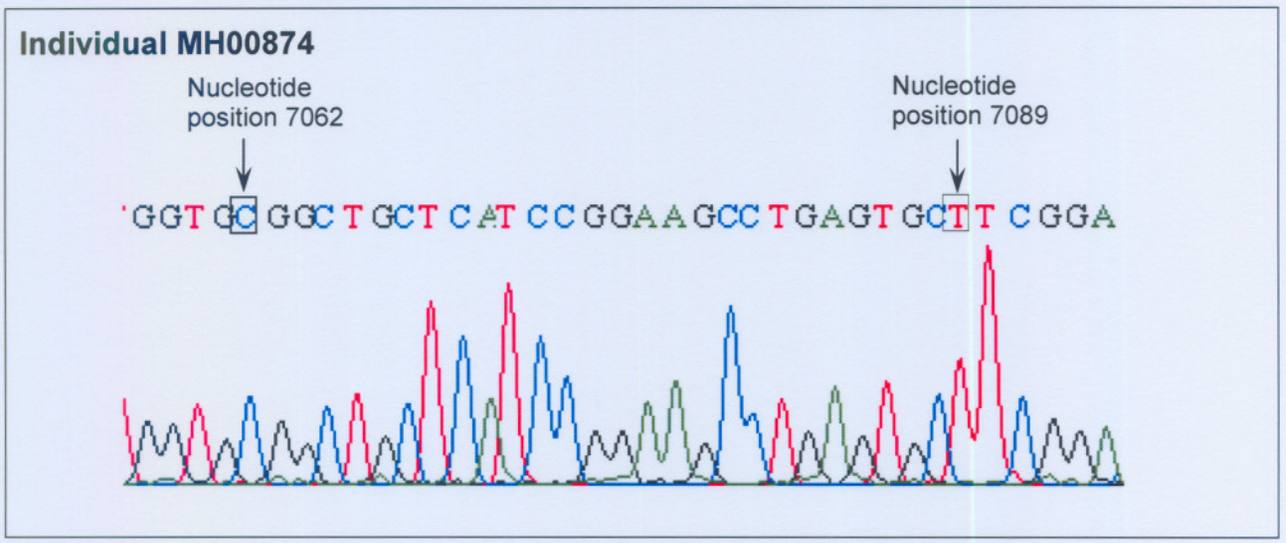
4.9.4 The Arg2355Cys alteration

Figure 4.12 is a representative electropherogram obtained for individual MH00874 depicting alterations Arg2355Cys and Phe2364Val (as discussed in Section 4.9.5). The Arg2355Cys alteration occurs due to a C7062T nucleotide transition (McWilliams *et al.*, 2002) and was screened for in 39 MH probands in Phase 2.

The Arg2355Cys alteration was first reported by McWilliams *et al.* (2002) in a large Brazilian MH family. The authors indicated that the alteration co-segregated with the MH phenotype in the aforementioned family. The alteration was subsequently reported in six MH families from the UK (Halsall and Robinson, 2004). It would be difficult to determine why the alteration was found in two very different population groups. However, the observation may be a co-incidental occurrence, as nine of the alterations in the central region of the RYR1 involve Arg, and four result in an Arg-to-Cys alteration. This could indicate that the positively charged amino acids play a more important regulatory role in this part of the RyR1 protein.

The Arg2355Cys alteration was not detected in any of the South African MH probands analysed in Phase 1 or in Phase 2, and could suggest that the genetic determinants and environmental factors leading to the MH phenotype in the South African population may vary from other populations.

Figure 4.12: Representative electropherogram of mutation region five encompassing the Arg2355Cys and Phe2364Val alterations



A = adenine; C= cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (7062) of the Arg2355Cys alteration; the second boxed nucleotide indicates the nucleotide position (7089) of the Phe2364Val alteration (as discussed in Section 4.9.5).

4.9.5 The Phe2364Val alteration

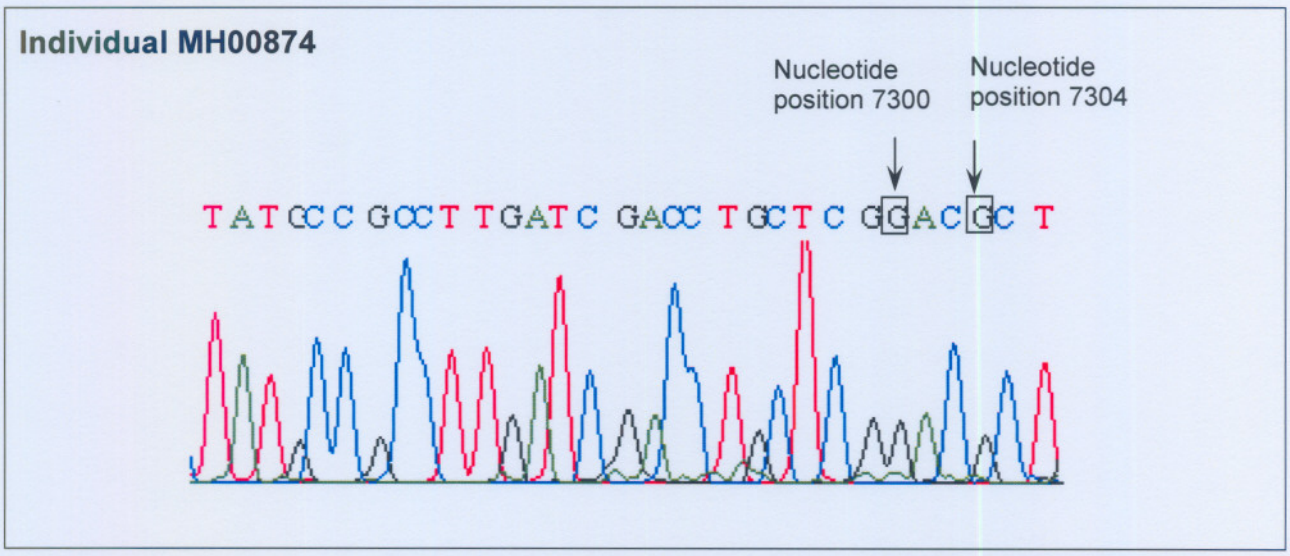
In Phase 2, 39 MH probands were analysed for the Phe2364Val alteration which occurs due to a T7089G nucleotide transition (Halsall and Robinson, 2004). A representative electropherogram obtained for individual MH00874 illustrating the Phe2364Val alteration is depicted in Figure 4.12, in the previous paragraph.

The Phe2364Val alteration was recently reported in two MH families from the UK, and the frequency of the alteration was determined to be 0.46 (Halsall and Robinson, 2004). The Phe2364Val alteration was not detected in any of the South African MH probands analysed. RYR1 mutations have been described in 50% of the European MH population to date. Absence of this mutation as well as other RYR1 alterations may indicate that other regions of the RYR1 that have not been screened to date play a role in susceptibility to MH in the South African population, or other loci result in the MH susceptibility phenotype in this population.

4.9.6 The Gly2434Arg alteration

The Gly2434Arg alteration has previously been analysed in Phase 1 and was included in this study, as it occurred in the same region as the other seven mutations of mutation region five. The Gly2434Arg and Arg2435His alterations were analysed for 31 MH individuals in Phase 1, and were screened for via RFLP. The G7300A transition was detected by the gain of the *Dde* I restriction site. Individuals MH00654, MH00695, MH00707, MH01388 and MH01413, for whom data were not available for the Gly2434Arg alteration, were subsequently analysed in Phase 2 via a sequencing strategy. A representative electropherogram obtained for individual MH00874 depicting alterations Gly2434Arg, Arg2435His (as discussed in Section 4.9.7) and Arg2435Leu (as discussed in Section 4.9.8) is illustrated in Figure 4.13.

Figure 4.13: Representative electropherogram of mutation region five encompassing the Gly2434Arg, Arg2435His and Arg2435Leu alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (7300) of the Gly2434Arg alteration; the second boxed nucleotide indicates the nucleotide position (7304) of the Arg2435His (as discussed in Section 4.9.7) and Arg2435Leu (as discussed in Section 4.9.8) alterations.

The Gly2434Arg alteration was first reported in four unrelated Caucasian families with MHS (Keating *et al.*, 1994). The mutation was subsequently identified in one MH family from Italy, three unrelated MH families from France, seven unrelated MH families from Germany, two unrelated families from Switzerland and 53 unrelated families from the UK (Robinson *et al.*, 2003b). The authors indicated that this alteration was present in 5.8% of the European MH population. The Gly2434Arg alteration was not observed in South African MH probands analysed in either Phase 1 or Phase 2. As the mutation has been described in several families, it is not a family specific mutation. This observation may

indicate that this alteration does not play a role in susceptibility to MH in the South African population. As the alteration was frequently observed in different population groups of European origin, this could indicate that susceptibility to MH in the South African population differs from other population groups and could be due to other genetic loci or modifying factors.

4.9.7 The Arg2435His alteration

The Arg2435His alteration has been analysed in Phase 1 in 31 South African MH individuals. An RFLP strategy was followed for the screening of this mutation. The A7304G transition was detected by the loss of the *Hga* I site. As discussed in Section 4.9.6, five MH individuals were subsequently analysed for the presence of this mutation. A representative electropherogram obtained for individual MH00874 illustrating the Arg2435His alteration is depicted in Figure 4.13.

None of the five South African MH individuals analysed in Phase 2 or 31 individuals analysed in Phase 1 harboured this alteration. The mutation was observed in seven members of a single Canadian pedigree diagnosed with CCD (Zhang *et al.*, 1993) and was subsequently reported in one MH family from France, two unrelated MH families from Italy, eight unrelated families from Germany and five unrelated families from the UK (Robinson *et al.*, 2003b). The authors indicated that the alteration was not detected in families from Belgium and Switzerland. As discussed in Section 4.9.6, the Arg2435His alteration may not play a role in susceptibility to MH in the South African population and may indicate that the pathogenesis of MH in this population differs from other populations with European ancestry.

4.9.8 The Arg2435Leu alteration

A representative electropherogram obtained for individual MH00874 depicting the Arg2435Leu alteration which occurs due to a G7304T nucleotide transition is illustrated in Figure 4.13. The Arg2435Leu alteration was screened for in 39 South African MH probands in Phase 1 and was not observed in any South African MH individuals analysed. The Arg2435Leu alteration was first reported in a single MHS patient who was also diagnosed with CCD (Barone *et al.*, 1999).

A distinction between the closely associated conditions of CCD and MH should first be determined, as there are likely to be potential genetic differences between CCD and MH. Therefore, it is complex to determine if the alteration results in MH and/or CCD, as CCD is also a genetically heterogeneous disorder (Curran *et al.*, 1999).

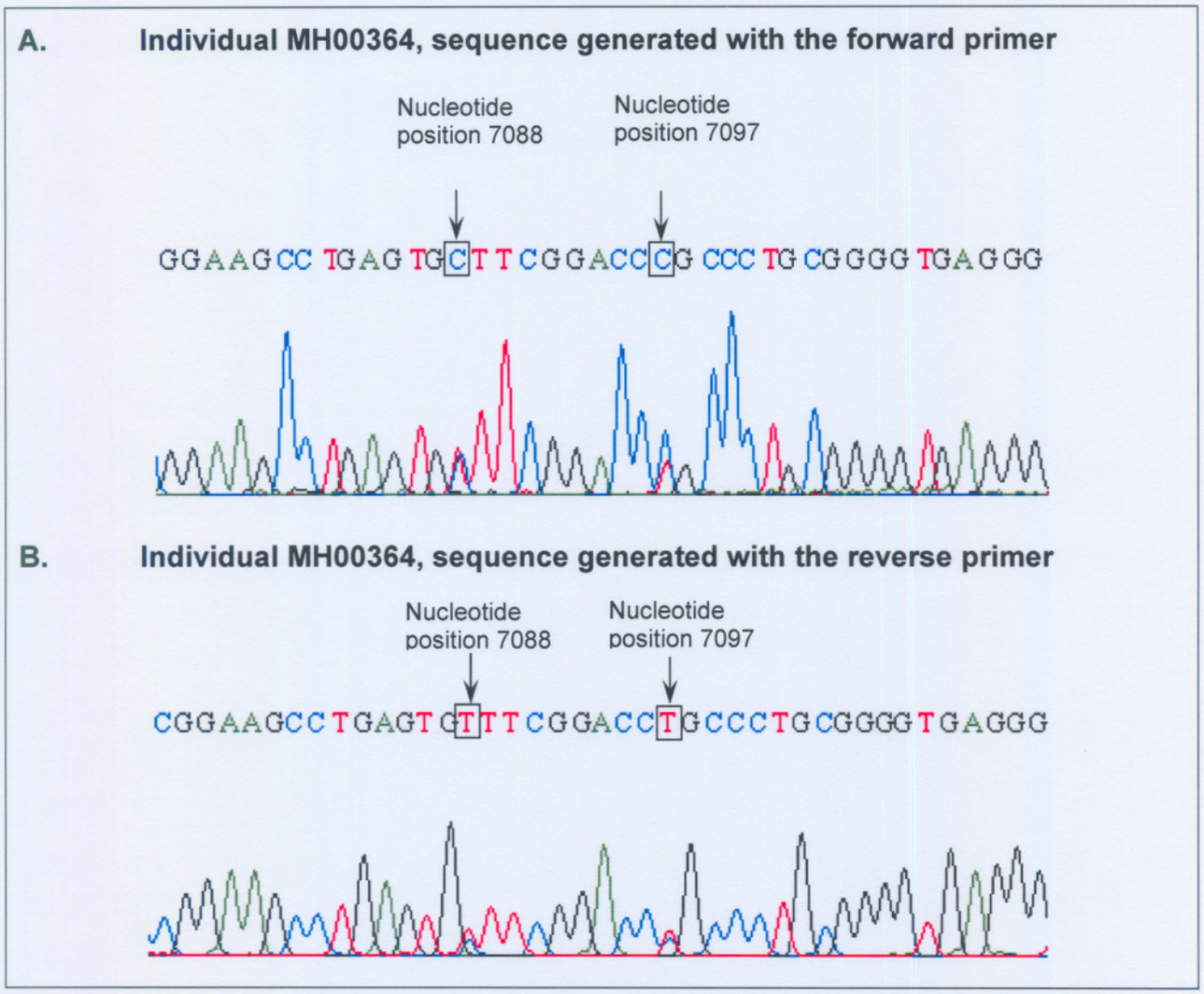
4.9.9 Synonymous substitutions in mutation region five

In five individuals, MH00709, MH00462, MH00668, MH00710 and MH00803, two peaks representing two differently coloured fluorophores of two bases, C and T, were identified at nucleotide positions 7088 and 7097. Both single nucleotide polymorphisms (SNPs) are synonymous substitutions as they occur at the third codon position and do not result in an alteration in the amino acid. Both SNPs were identified in the coding region of the RYR1 gene and are indicated as SNPs of the RYR1 gene in GeneBank®, the heterozygosity of each alteration being 0.204 and 0.207, respectively. The C7088T nucleotide transition retains the Cys amino acid and the C7097T retains the Pro amino acid. Two individuals, MH00364, MH00871, only harboured the C7088T SNP. The sequence depicted on the electropherogram of Figure 4.14A, generated with the forward primer, appears to have been scored as only a single allele, C, at this position. This is due to the fact that the Base Spectrum programme utilised for sequencing can only assign a single allele at any given position. However, from the electropherogram the two distinct peaks are clearly visible, indicating a heterozygous state of C/T at this position. A similar event occurs in the sequence depicted in Figure 4.14B, generated with the reverse primer that appears to have been scored only as a T allele. Therefore, all individuals are heterozygous for the above-mentioned alterations. The remaining individuals included in this study all displayed electropherograms expected for unaffected MH patients and did not exhibit the two alterations.

The alterations may have occurred due to the high CG content in this genomic region. Bielawski *et al.* (2000) reported that substitution rates are positively correlated with the GC content at the third codon position. Synonymous mutations are likely to have arisen either due to natural selection or biased gene conversion (Smith and Eyre-Walker, 2001). As neither of these alterations result in a change in the amino acid of the protein or code for a stop codon, it is unlikely that they play a primary role in susceptibility to MH, but they may play an important secondary role. The effect that these SNPs have on the structure of the RYR1 gene due to an alteration in a GC-rich region remains to be determined.

Susceptibility to MH may be due to epistasis, in which several different mutations, SNPs and environmental factors all lead to a threshold being reached. The contribution of all these determinants would have to be analysed further in order to resolve each of their effects on the MH phenotype.

Figure 4.14: Representative electropherograms displaying the sequencing results obtained for synonymous alterations observed in mutation region five



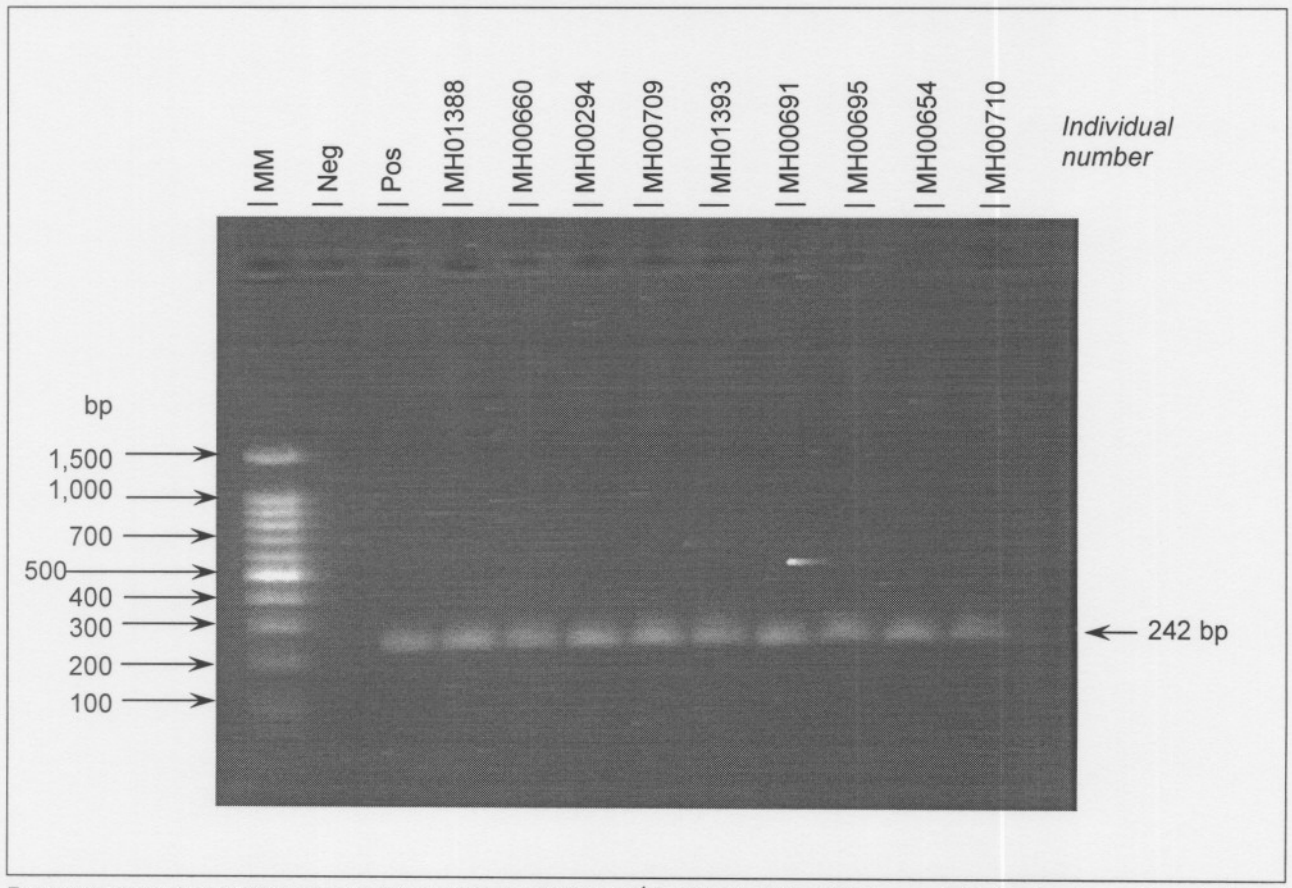
A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates nucleotide position 7088; the second boxed nucleotide indicates nucleotide position 7097.

4.10 MUTATION REGION SIX OF THE RYR1 GENE

A 242 bp product encompassing exon 46 was amplified via PCR. This region of the RYR1 gene harbours three alterations, Arg2452Trp, Arg2458Cys and Arg2458His, and is located in the central portion of the RyR1 protein, as discussed in Section 4.7.

Amplification conditions were optimised at a T_a of 64°C and an $MgCl_2$ concentration of 1.5 mM. The addition of 1% formamide led to the successful amplification of the fragment harbouring the three mutations. The product was electrophoresed on a 2% (w/v) agarose gel and Figure 4.15 is a photographic representation of the amplicon encompassing the three mutations. Amplification was achieved for all the positive controls included in Phase 2 and was not obtained in the negative controls, indicating lack of contamination. An artefact was detected in the agarose gel that migrated in the lanes between individuals MH00691 and MH00695, which did not affect the outcome of sequencing for either of the individuals analysed. The amplified region was subsequently sequenced in order to investigate the three published causative mutations in this region, as well as identify possible novel mutations. Sequencing was conducted utilising the standard protocol as discussed in Section 3.7. The forward primer (RYRE46F), as indicated in Table 3.4, was utilised in the sequencing reaction.

Figure 4.15: Photographic representation of PCR products of mutation region six encompassing the Arg2452Trp, Arg2458Cys and Arg2458His alterations



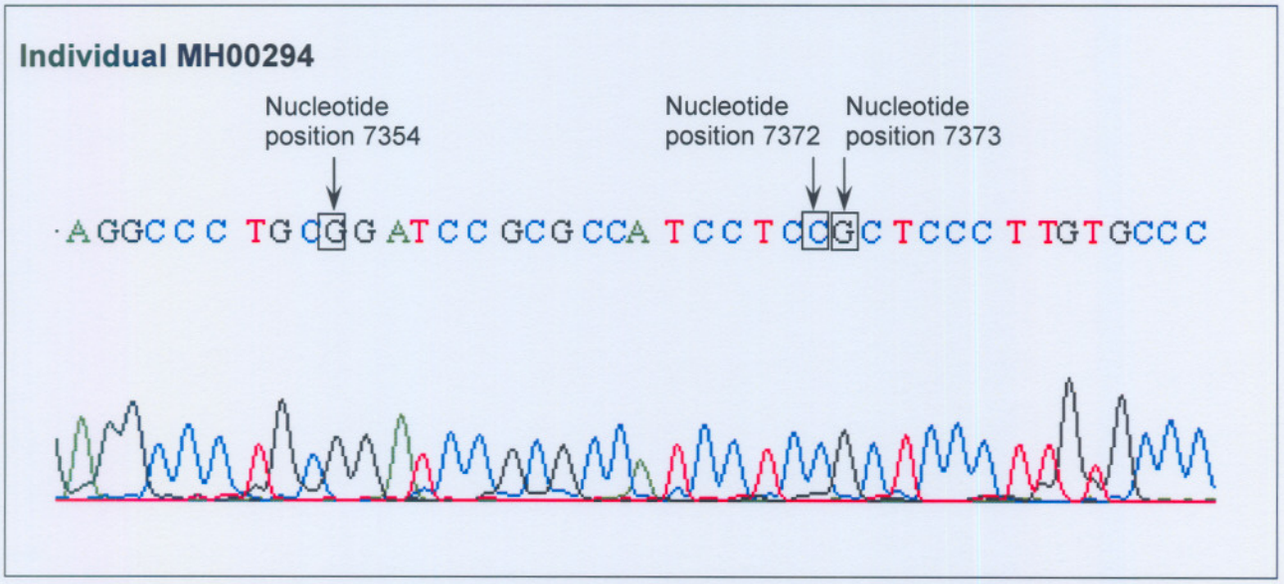
Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

The quality of the DNA sequence data was high, indicating that optimal PCR conditions were utilised and that the template DNA was pure. This allowed for accurate assessment of the sequence for reported alterations as well as identification of novel mutations. Results obtained for mutation region six are presented in subsequent paragraphs.

4.10.1 The Arg2452Trp alteration

The Arg2452Trp alteration is due to a C7354T nucleotide transition (Chamley *et al.*, 2000). In Phase 1 alterations, Arg2458Cys and Arg2458His were analysed via sequencing in 24 South African MH probands. However, the Arg2452Trp alteration was not analysed in Phase 1 as it was only reported in 2004 (Halsall and Robinson, 2004). As sequencing information was available for the 24 MH probands from Phase 1, the sequence was subsequently re-analysed for the Arg2452Trp alteration in Phase 2. In addition, seven MH probands that were not investigated previously for this alteration were analysed in Phase 2. A representative electropherogram obtained for individual MH00294 illustrating alterations Arg2452Trp, Arg2458Cys and Arg2458His, is depicted in Figure 4.16.

Figure 4.16: Representative electropherogram of mutation region six encompassing the Arg2452Trp, Arg2458Cys and Arg2458His alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (7354) of the Arg2451Trp alteration; the second boxed nucleotide indicates the nucleotide position (7372) of the Arg2458Cys alteration (as discussed in Section 4.10.2); the third boxed nucleotide indicates the nucleotide position (7373) of the Arg2458His alteration (as discussed in Section 4.10.3).

The Arg2452Trp alteration was first reported in a six-month-old male child from New Zealand, who developed symptoms associated with MH during surgery to repair his cleft palate (Chamley et al., 2000). The alteration was also identified in the mother and sibling of the proband. The mutation was subsequently reported in one UK MH family (Halsall and Robinson, 2004) but was not detected in any of the South African MH probands analysed in Phase 2. However, as the mutation has only been detected in two families, it is likely that it is specific to those families. Although the frequency of each alteration varies, all mutations should initially be screened for in the different population groups to determine the frequency of each alteration.

4.10.2 The Arg2458Cys alteration

In Phase 1, sequencing was utilised to screen for the Arg2458Cys alteration in 34 South African MH probands. In Phase 2, an additional seven South African MH probands were analysed for this alteration. Successful amplification was achieved for all the samples investigated, as discussed in Section 4.10.1, and a representative electropherogram obtained for individual MH00294 illustrating the Arg2458Cys alteration, which is due to a C7372T nucleotide transition, is depicted in Figure 4.16.

The Arg2458Cys alteration was not detected in any of the South African MH individuals analysed in Phase 1 or in Phase 2. The mutation was first reported by Manning *et al.* (1998b), and was detected in a single MH Swiss and Italian pedigree. The authors observed that this mutation segregated in both MHS and MHE individuals. The mutation was subsequently reported in two MH families from the UK (Halsall and Robinson, 2004), in three unrelated MH families from Italy, two unrelated families from Germany and five unrelated families from Switzerland (Robinson *et al.*, 2003b). The alteration is currently being utilised for genetic diagnosis of MH susceptibility. To date, the alteration has only been detected in the European MH population and indeed occurs frequently in this population group. Both population and regional differences between the South African and European populations may contribute to the absence of this alteration in the South African MH population.

4.10.3 The Arg2458His alteration

The Arg2458His alteration occurs due to a G7373A nucleotide transition. As described in Section 4.10.2, the alteration was screened for in seven South African MH probands in

Phase 2. The alteration was not detected in MH individuals analysed in Phase 1 or in Phase 2. The Arg2458His alteration was first reported in a single large French MH pedigree (Manning *et al.*, 1998b) and was subsequently reported in six unrelated MH families from the UK (Halsall and Robinson, 2004), two unrelated MH families from France and one MH family from Germany (Robinson *et al.*, 2003b). Although the alteration occurs at a lower frequency than the Arg2458Cys alteration, it is also utilised in the genetic diagnosis of MH susceptibility. The absence of the alteration in the South African MH population could be attributed to reasons outlined in Section 4.10.2, and a representative electropherogram obtained for individual MH00294 illustrating the Arg2458Cys alteration, which is due to a C7372T nucleotide transition, is depicted in Figure 4.16.

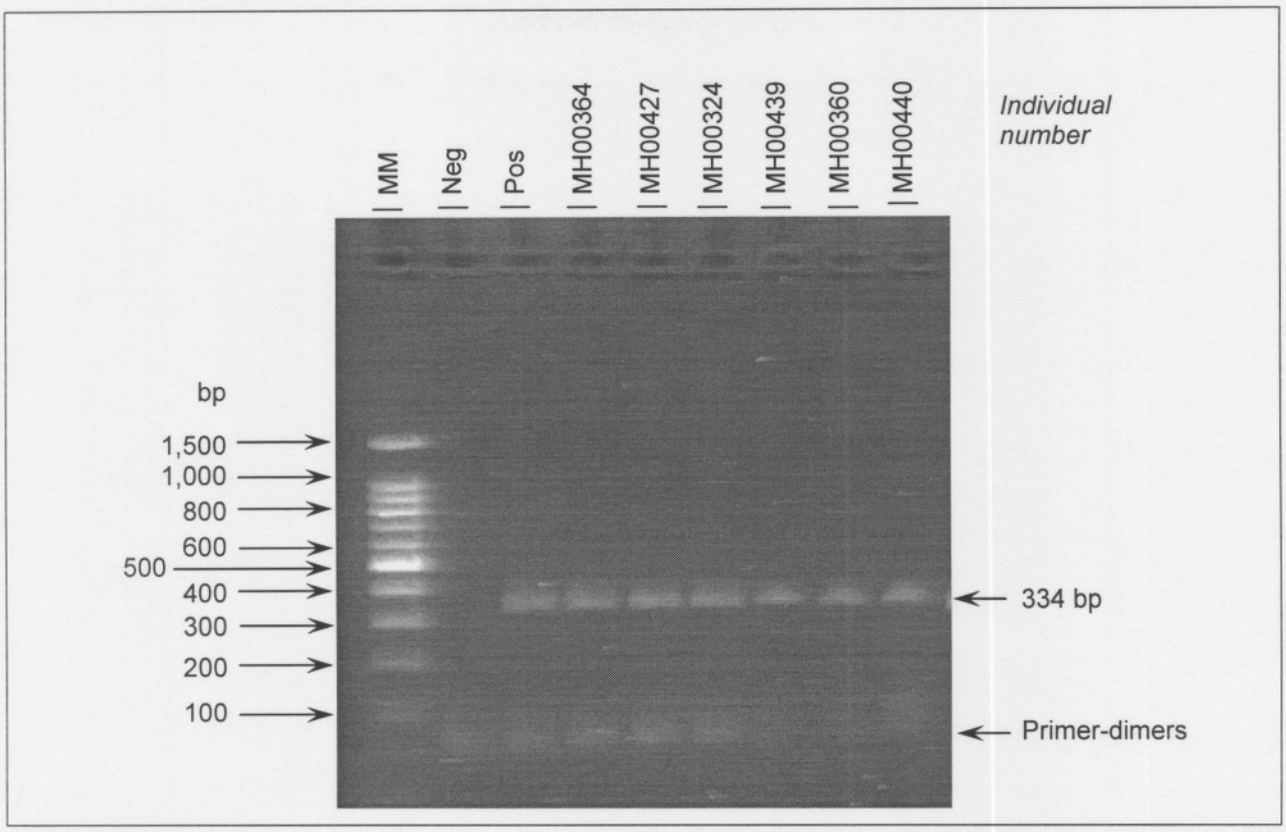
4.11 MUTATION REGION SEVEN OF THE RYR1 GENE

Mutation region seven encompasses exon 95 of the RYR1 gene and contains only the Gly4638Met alteration. The region resides in the COOH-terminal region of the RyR1 protein in the third mutation hotspot and represents a functionally significant domain. Alterations found in this part of the RyR1 protein reside in the luminal or transmembrane region. Initially, only alterations associated with CCD were observed in the highly conserved COOH-terminus of the RYR1 gene. However, recently nine alterations from this region were found associated with MH, as listed in Table 2.2.

A 334 bp product was successfully amplified in 30 South African MH patients and the PCR was optimised utilising the following conditions: T_a of 56°C and 1.5 mM $MgCl_2$. However, amplification was not efficient for all 30 samples. Thirteen samples did not amplify at the optimised conditions. For this reason conditions were re-optimised utilising 0.5 U *Taq* polymerase (Promega®). The amplified product was electrophoresed on a 2% (w/v) mini agarose gel and the results are illustrated in Figure 4.17. PCR amplification was considered successful if amplification was only achieved in the positive control and not in the negative control.

All 43 samples obtained via PCR were successfully sequenced according to the standard protocol outlined in Section 3.7. As the mutation is found in close proximity to the reverse primer, the forward primer (RYRex95F) as listed in Table 3.4 was utilised for sequencing. The Gly4638Met alteration as well as novel alterations that may occur in this region could be correctly analysed, as sequences had no background noise.

Figure 4.17: Photographic representation of PCR products of mutation region seven encompassing the Gly4638Met alteration



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

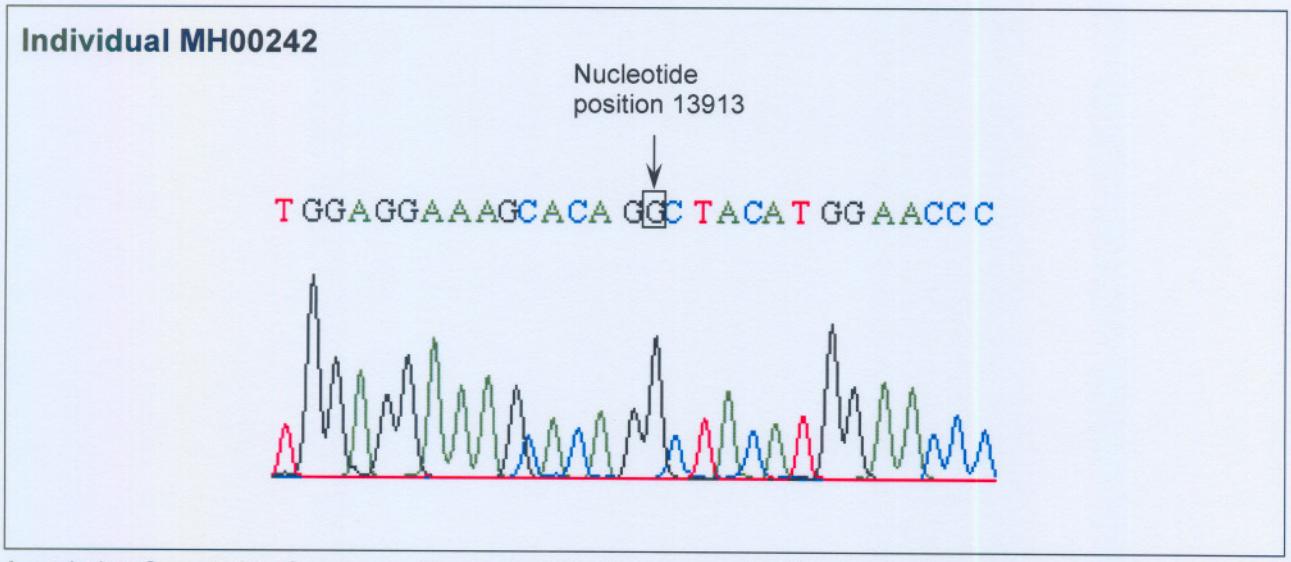
4.11.1 The Gly4638Met alteration

The Gly4638Met alteration that occurs due to a nucleotide transition, G13913A, has not been analysed in the South African MH population to date. A representative electropherogram obtained for individual MH00242 depicting the Gly4638Met alteration is indicated in Figure 4.18.

The Gly4638Met alteration was not detected in any of the MH individuals analysed in Phase 2. The mutation was originally reported in two unrelated MH pedigrees from the UK (Halsall and Robinson, 2004), and could represent a private mutation, only present in the two above-mentioned families. However, as the alteration has only been identified recently, its frequency has not yet been determined in other population groups. Initially, only mutation hotspots one and two of the RYR1 gene were analysed to detect alterations associated with susceptibility to MH worldwide. Recently, the third mutation hotspot was included in the analysis of MHS. Although novel alterations were not observed in the sequenced region of mutation region seven, other exonic and intronic sequences should be analysed to determine if alterations in these regions result in susceptibility to MH in the

South African population. Further analysis of other regions of the RYR1 in this population may reveal other mutation clusters associated with MH.

Figure 4.18: Representative electropherogram of mutation region seven encompassing the Gly4638Met alteration



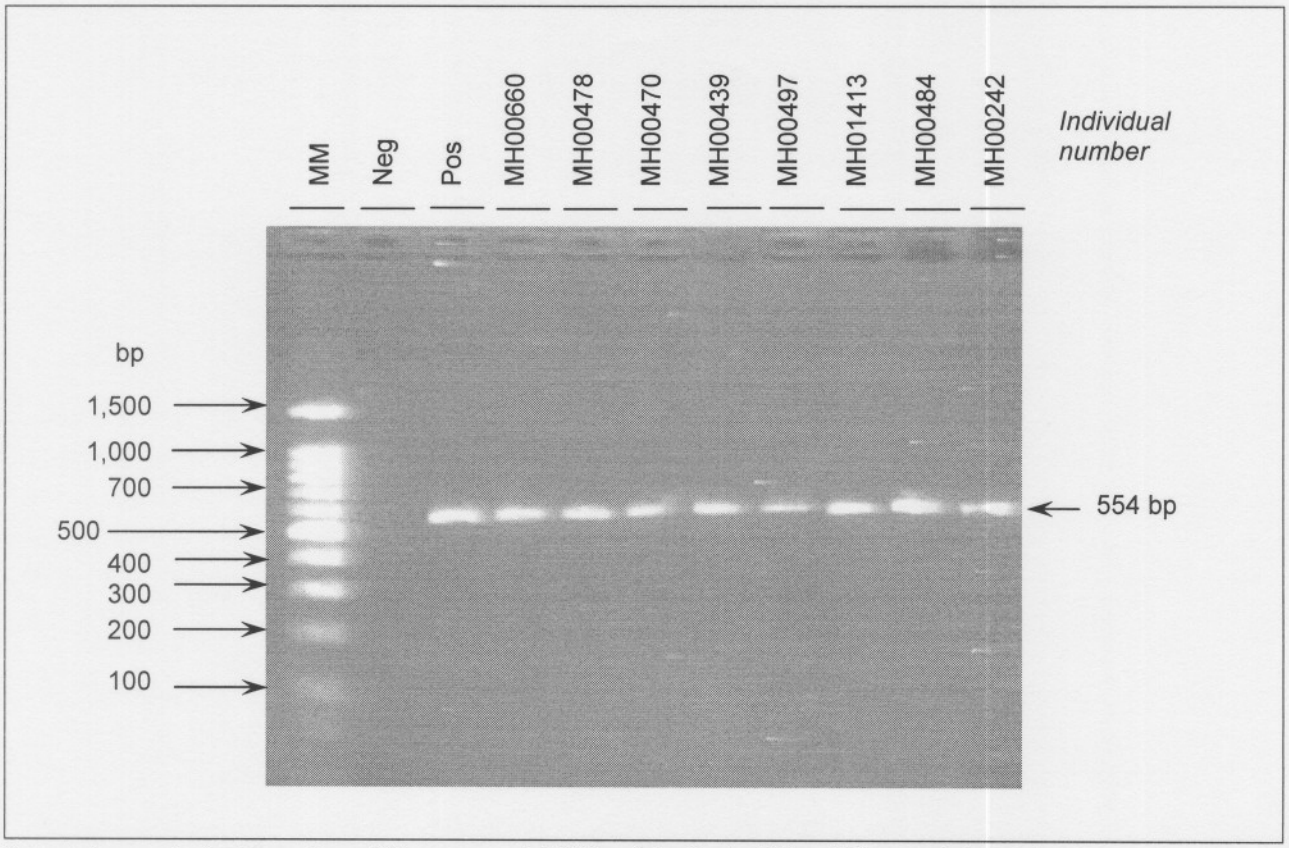
A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (13913) of the Gly4638Met alteration.

4.12 MUTATION REGION EIGHT OF THE RYR1 GENE

PCR was utilised to amplify a 554 bp product of exons 100 and 101 for 45 MH probands. The amplified region resides in the COOH-terminal of the RYR1 (representing a functionally significant domain), as discussed in Section 4.11. Exon 100 of the RYR1 gene encompasses alterations Leu4814Phe, Ile4817Phe, Leu4824Pro and Thr4826Ile, while exon 101 encompasses alterations Leu4838Val, Val4849Ile and Arg4861His. PCR was conducted utilising 0.125 U Supertherm® polymerase for the reasons outlined in Section 4.4.

PCR conditions were optimised at a T_a of 62°C with 1.5 mM $MgCl_2$. The product was subsequently electrophoresed on a 2% (w/v) agarose gel, as described in Section 3.6 and the results are illustrated in Figure 4.19. PCR reactions for samples were only considered successful if the expected results were obtained in the positive control and no amplification was observed in the negative control. The 39 successfully amplified samples obtained via PCR were subsequently sequenced utilising the standard protocol outlined in Section 3.7. Sequences obtained for mutation region eight had well-defined peaks and no background amplification was observed which could interfere with the analysis.

Figure 4.19: Photographic representation of PCR products of mutation region eight encompassing the Leu4814Phe, Ile4817Phe, Leu4824Pro, Thr4826Ile, Leu4838Val, Val4849Ile and Arg4861His alterations



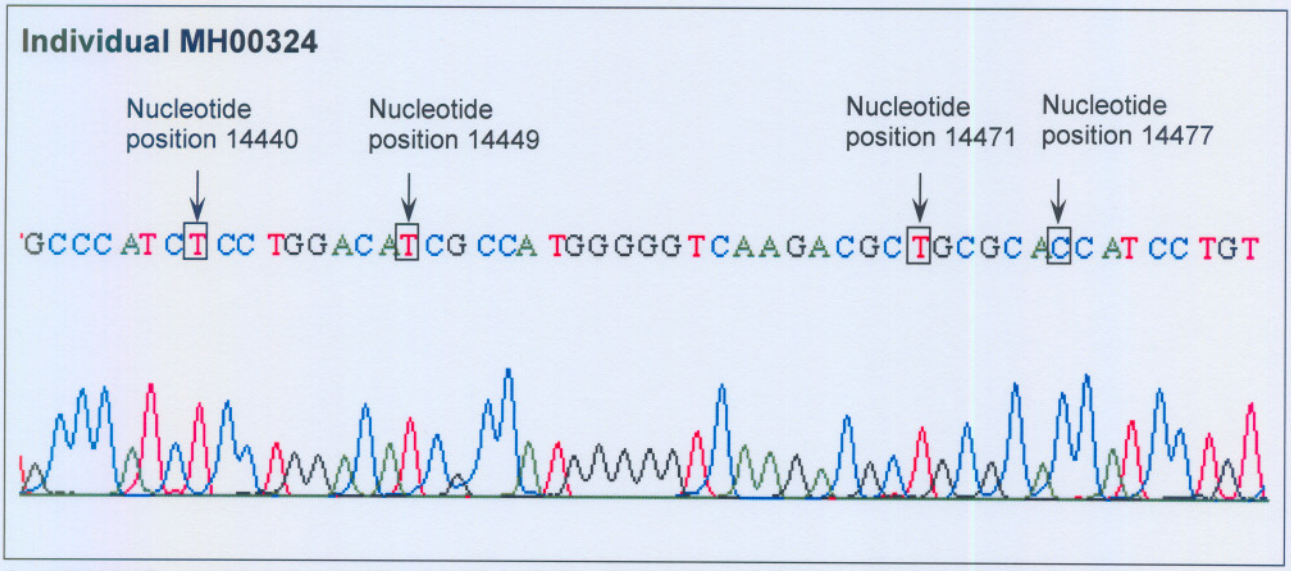
Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

4.12.1 The Leu4814Phe alteration

The Leu4814Phe alteration occurs due to a C14440T nucleotide transition (Halsall and Robinson, 2004) and was analysed for the first time in the South African MH population in Phase 2. A representative electropherogram obtained for individual MH00324 illustrating alterations Leu4814Phe, Ile4817Phe (as discussed in Section 4.12.2), Leu4824Pro (as discussed in Section 4.12.3) and Thr4826Ile (as discussed in Section 4.12.4) is presented in Figure 4.20.

None of the 39 individuals that were sequenced in this study displayed the Leu4814Phe alteration. The alteration has been observed in a single UK pedigree (Halsall and Robinson, 2004) and may therefore indicate a mutation specific to that family. As this is a newly reported alteration, other populations would need to be screened to verify this finding.

Figure 4.20: Representative electropherogram of mutation region eight encompassing the Leu4814Phe, Ile4817Phe, Leu4824Pro and Thr4826Ile alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (14440) of the Leu4814Phe alteration; the second, third and fourth boxed nucleotides indicates the nucleotide positions (14449, 14471 and 14477) of the Ile4817Phe (as discussed in Section 4.12.2), Leu4824Pro (as discussed in Section 4.12.3) and Thr4826Ile (as discussed in Section 4.12.4) alterations, respectively.

4.12.2 The Ile4817Phe alteration

The Ile4817Phe alteration occurs due to an A14449T nucleotide transition. A representative electropherogram obtained for individual MH00324 illustrating the Ile4817Phe alteration is presented in Figure 4.20, in the previous paragraph. The alteration was not observed in any of the 39 South African MH individuals sequenced in this study, and novel alterations were not observed in the sequenced region.

To date, this alteration has never been screened for in the South African MH population. The alteration was recently reported in a single UK pedigree with MH (Halsall and Robinson, 2004). As the alteration was observed only recently, the frequency of this alteration in other population groups has not yet been determined. Further analysis of the COOH-terminal region of the RYR1 gene may yield alterations associated with MH in the South African population.

4.12.3 The Leu4824Pro alteration

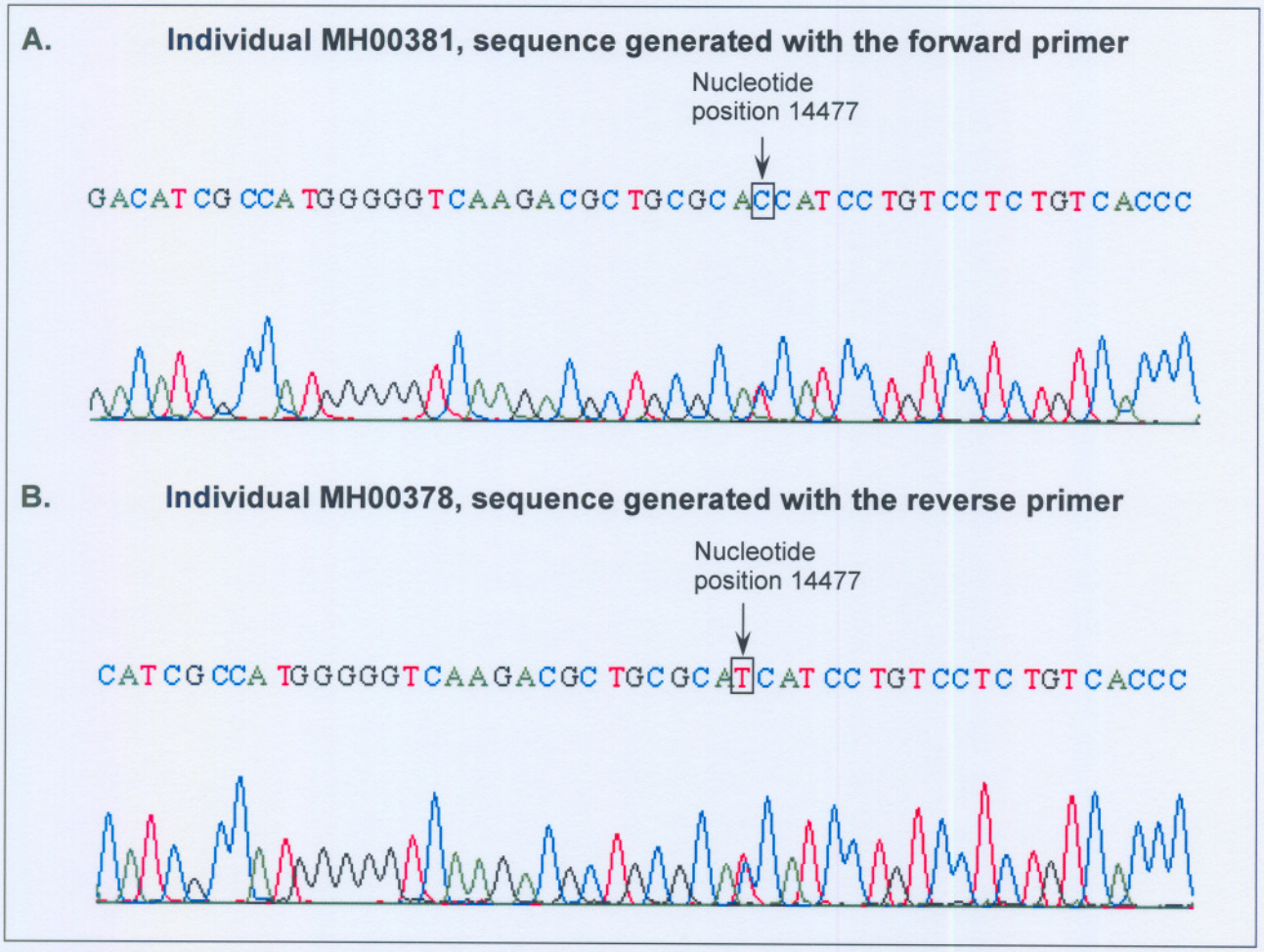
All individuals analysed in Phase 2 harboured a T nucleotide at position 14471, indicating the absence of the Ile4817Phe alteration. A representative electropherogram obtained for individual MH00324 illustrating the Ile4817Phe alteration is presented in Figure 4.20. The alteration was reported recently in a single MH pedigree from the UK (Halsall and

Robinson, 2004). At the present time, the frequency of this alteration in other population groups has not yet been determined, and the alteration may be specific to the above-mentioned family or may be more common. Although the alteration was not observed in any of the 39 MH probands that were sequenced in Phase 2, novel alterations are continually being reported and should be analysed in each population group to determine their frequency.

4.12.4 The Thr4826Ile alteration

The Thr4826Ile alteration occurs due to a C14477T nucleotide transition. This alteration was detected in a single South African MH proband, MH 113-14 (MH00381) analysed in Phase 2. The remaining MH probands analysed, all displayed the electropherogram expected for unaffected MH individuals, as illustrated for individual MH00324 in Figure 4.20. As the Thr4826Ile alteration was detected for individual MH 113-14, two other family members, MH 113-2 (MH00369) and MH 113-11 (MH00378) were subsequently screened and both individuals harboured the mutation. As presented in Figure 4.21A and B, at nucleotide position 14,477 two peaks of similar intensity representing two different coloured fluorophores were observed. This result indicates the presence of two bases, C and T, which correlated to two distinct codons expressing different amino acids, threonine and isoleucine, respectively. All three individuals that yielded a positive result for this mutation displayed this heterozygous genotype. Due to the autosomal dominant nature of this disorder, it was concluded that all three individuals are MHS. Further analysis of the remaining members of family MH113 would have to be conducted in order to determine their MH status. Brown *et al.* (2000) first reported this alteration in a large Maori pedigree, of which five individuals that experienced clinical episodes of MH harboured the alteration. The author indicated that this alteration was conserved and was not detected in 220 normal individuals, thus meeting the criteria of a causative mutation. The alteration was subsequently identified in nine unrelated MH families from the UK (Halsall and Robinson, 2004). The Thr4826Ile alteration is located in the cytoplasmic loop between M2 and M3 transmembrane domains. This alteration represents the first mutation in the RyR1 COOH-terminal region solely associated with MHS, and to date has not been analysed for in the South African population. The relatively high frequency of this mutation in two different population groups and its presence in a single South African MH pedigree indicates that the third mutation hotspot of the RYR1 plays an important role and should be analysed in all MH population groups in the future.

Figure 4.21: Representative electropherogram displaying the sequence results obtained for the Thr4826Ile alteration observed in mutation region eight



A = adenine; C = cytosine; G = guanine; T = thymine. The boxed nucleotide indicates the nucleotide position (14477) of the Thr4826Ile alteration.

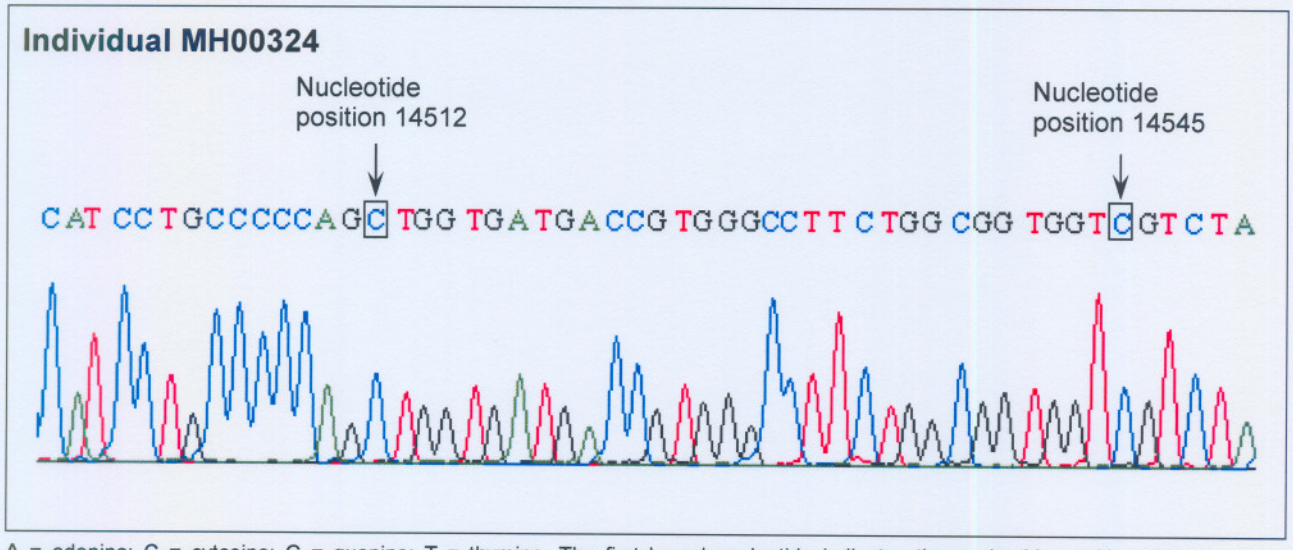
4.12.5 The Leu4838Val alteration

A representative electropherogram obtained for individual MH00324 illustrating alterations Leu4838Val and Val4849Ile (as discussed in Section 4.12.6) is presented in Figure 4.22. The Leu4838Val alteration is due to a C14512G nucleotide transition. None of the 39 South African MH individuals that were sequenced in this study displayed the Ile4817Phe alteration, or indicated any novel alterations. Sequences generated had a high signal level and no background noise. The quality of the sequence allowed for accurate screening of mutations and identification of possible novel alterations.

The Leu4838Val alteration was recently reported in a single UK pedigree with MH (Halsall and Robinson, 2004) and is currently being utilised in the genetic diagnosis of MHS. Although the alteration was not observed in the South African MH population, it is being utilised to diagnose MH in the European population (Halsall and Robinson, 2004).

Therefore, it is important to analyse all reported alterations that may play a role in the South African population, if a genetic diagnostic test for MH in this population were to become a reality.

Figure 4.22: Representative electropherogram of mutation region eight encompassing the Leu4838Val and Val4849Ile alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (14512) of the Leu4838Val alteration; the second boxed nucleotide indicates the nucleotide position (14545) of the Val4849Ile alteration (as discussed in Section 4.12.6).

4.12.6 The Val4849Ile alteration

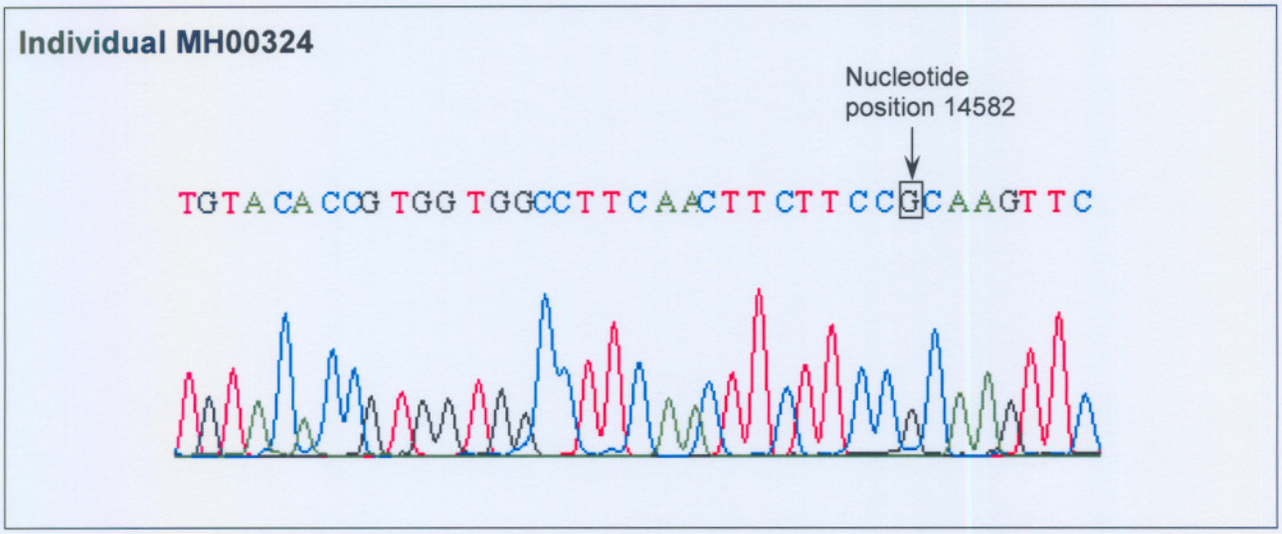
A G14545A nucleotide transition in the RYR1 gene results in a Val4849Ile alteration. A representative electropherogram obtained for individual MH00324 illustrating the Val4849Ile alteration is presented in Figure 4.22 in the previous paragraph. To date the alteration has not been analysed in the South African MH population and screening of this region indicated that none of the 39 individuals displayed the Val4849Ile alteration. Novel alterations were not detected in this region either.

The alteration was recently reported in four unrelated MH pedigrees from the UK (Halsall and Robinson, 2004) and the frequency of this alteration in the UK population was determined to be 0.92. It is unlikely that the alteration represents a family-specific mutation. Regional differences observed in MH may have contributed to the absence of this alteration in the South African MH population.

4.12.7 The Arg4861His alteration

The Arg4861His alteration occurs due to a G14582A nucleotide transition. A representative electropherogram obtained for individual MH00324 illustrating the Arg4861His alteration is presented in Figure 4.23. Sequences generated for this region had a high signal level and no background noise, indicating that the PCR template was pure and the template quantity was within the appropriate level.

Figure 4.23: Representative electropherogram of mutation region eight encompassing the Arg4861His alteration



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates the nucleotide position (14582) of the Arg4861His alteration.

None of the 39 South African MH individuals that were sequenced in this study displayed the Arg4861His alteration. Phase 2 represents the first analysis of this alteration in the South African MH population, to date. The Arg4861His alteration was first reported by Monnier *et al.* (2001), was detected in three unrelated CCD pedigrees and occurs in a highly conserved region of the RYR1. The Arg4861His alteration was also detected in a single CCD pedigree, indicated complete segregation in all 27 affected individuals and was absent in 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).

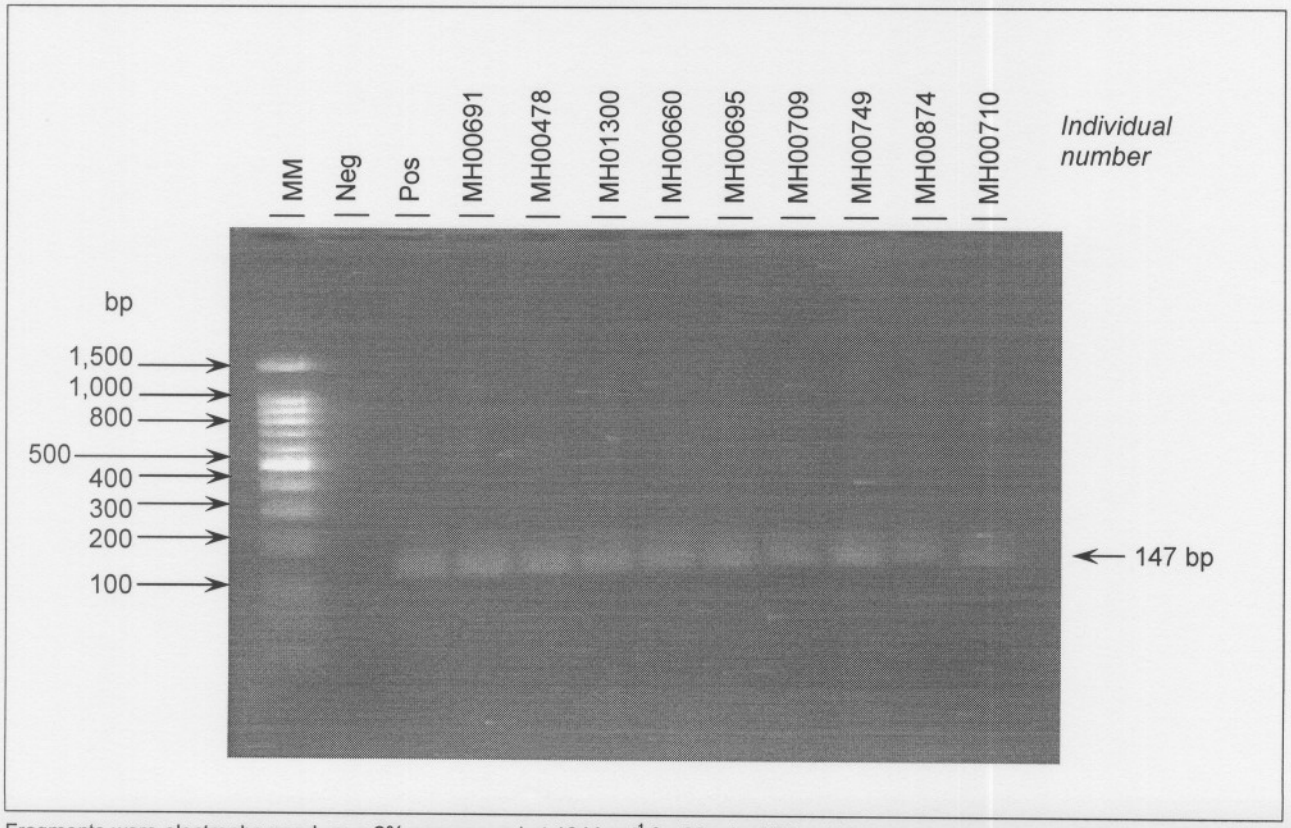
An association between MH and CCD was first reported by Denborough *et al.* (1973), as discussed in Section 2.3.3. The alteration was subsequently reported in one MH family from the UK (Halsall and Robinson, 2004) and is currently being utilised for the genetic diagnosis of MH susceptibility. Although the alteration was not detected in any South

African MH probands analysed, several independent genetic factors may predispose an individual to MH and/or CCD. This observation could explain why the alteration was detected in individuals with both disorders and all individuals had a mild form of MH during anaesthetic procedures. Analysis of all these factors may identify genetic determinants involved in susceptibility to MH in the South African population.

4.13 MUTATION REGION NINE OF THE RYR1 GENE

A region of 147 bp of exon 103 was sequenced to detect alterations Ile4938Met and Asp4939Glu, analysed for the first time in the South African MH population. This region resides in the COOH-terminal of the RYR1, which is the third mutation hotspot of the RYR1 gene, as discussed in Section 4.11. The PCR conditions for mutation region nine were optimised as follows: T_a of 54°C, $MgCl_2$ of 1.5 mM. In addition 1% formamide was added to increase the specificity of the PCR reaction, as discussed in Section 4.2. Figure 4.24 is a photographic representation of the amplicon encompassing the Ile4938Met and Asp4939Glu mutations. All positive controls amplified while no amplification was achieved in the negative controls, indicating a lack of contamination.

Figure 4.24: Photographic representation of PCR products of mutation region nine encompassing the Ile4938Met and Asp4939Glu alterations



Fragments were electrophoresed on a 2% agarose gel at 10 $V.cm^{-1}$ for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised.

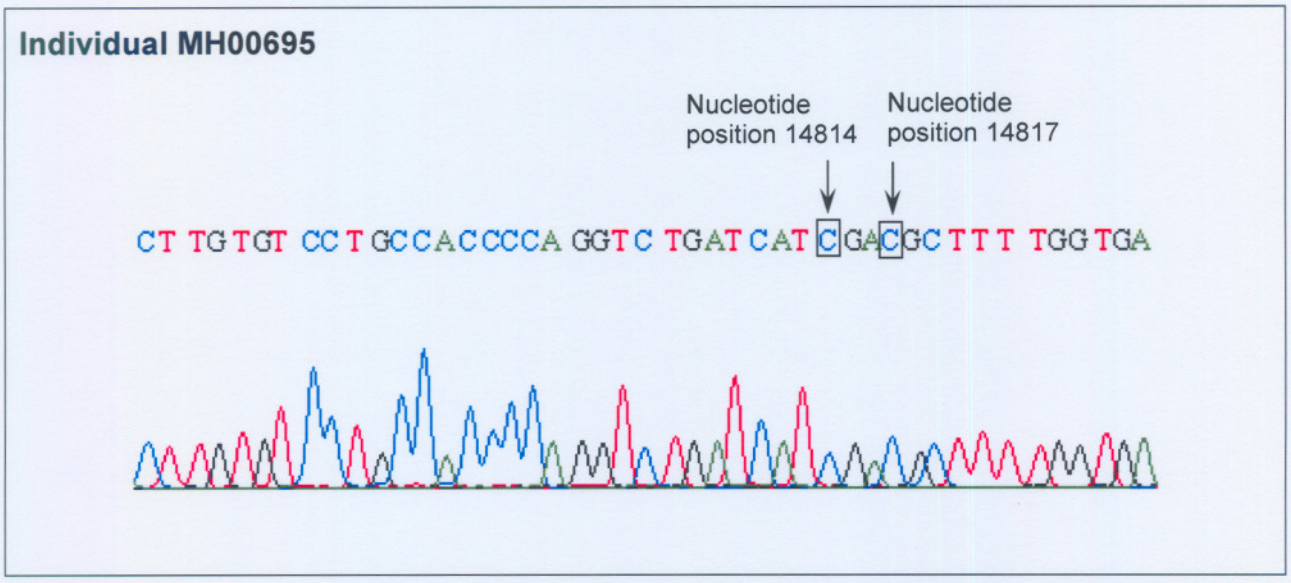
PCR amplification was successful for 25 samples utilising the above protocol. However, in the remaining 13 samples amplification was not efficient. The PCR conditions were re-optimised utilising 0.5 U *Taq* polymerase (Promega®). The increase in concentration of *Taq* DNA polymerase increased the yield of the amplified product and all 13 samples amplified successfully utilising the re-optimised protocol. The amplified region was subsequently sequenced in order to investigate the presence of the Ile4938Met and Asp4939Glu alterations as well as to identify possible novel alterations in this region. Sequencing was conducted utilising the reverse primer (RYREx103R) as indicated in Table 3.16 (Section 3.7).

The reverse primer was used, as both mutations are situated in close proximity to the location of the forward primer. Sequences are therefore illustrated as the reverse complement. All 38 samples analysed in this molecular investigation were amplified and sequenced successfully. Sequences obtained could be accurately screened due to the absence of background noise on sequence, indicating that an optimised PCR protocol was utilised and that the PCR template had a high level of purity.

4.13.1 The Ile4938Met alteration

The Ile4938Met alteration was first reported in a single UK pedigree (Halsall and Robinson, 2004). The Ile4938Met alteration occurs due to C14814G nucleotide transition in exon 103. A representative electropherogram obtained for individual MH00695, illustrating alterations Ile4938Met and Asp4939Glu, is presented in Figure 4.25. None of the 38 South African MH individuals that were sequenced in this study displayed the Ile4938Met alteration or any novel alterations. As this alteration has only been observed recently, the frequency of the alteration in other population groups has not yet been determined. Although the alteration was not detected in any South African MH probands analysed, other regions of the COOH-terminal of the RYR1 gene may yield other alterations responsible for MH in this population.

Figure 4.25: Representative electropherogram of mutation region nine encompassing the Ile4938Met and Asp4939Glu alterations



A = adenine; C = cytosine; G = guanine; T = thymine. The first boxed nucleotide indicates nucleotide position (14814) of the Ile4938Met alteration; the second boxed nucleotide indicates nucleotide position (14817) of the Asp4939Glu alteration (as discussed in Section 4.13.2).

4.13.2 The Asp4939Glu alteration

A representative electropherogram obtained for individual MH00695 illustrating the Asp4939Glu alteration is presented in Figure 4.25 in the previous paragraph. In Phase 2, the alteration was screened in the South African MH population for the first time. The C14817A nucleotide transition results in an Asp4939Glu alteration in exon 103, and to date represents the most terminal alteration associated with MH. The alteration was not detected in any of the MH probands analysed in Phase 2 and novel alterations were not observed. The Asp4939Glu alteration was observed in a single UK pedigree with MH (Halsall and Robinson, 2004) and could represent a mutation that is private to that specific family. However, the frequency of this alteration has not yet been determined in other population groups. As discussed in Section 4.13.1, analysis of other regions of the COOH-terminal should be conducted in the South African MH population.

4.14 THE Arg1086His ALTERATION IN THE CACNL1A3 GENE

The Arg1086His alteration detected in the α_1-subunit of the CACNA1S on chromosome 1q31 was first described by Monnier *et al.* (1997). The DHPR has been considered a candidate for MHS, as it plays a role in the regulation of calcium homeostasis in skeletal muscle (Nakai *et al.*, 1996). Mutations in the CACNA1S have previously only been associated with hypoPP a disorder that is characterised by cold-induced stiffness, muscle

weakness and low K^+ levels. The Arg1086His alteration was observed in a single French MH pedigree and was not observed in 50 additional French and Italian MHS families or in 100 unrelated MHN chromosomes. The mutation leads to an amino acid change that is highly conserved in isoforms of brain and endocrine human DHPR genes as well as in genes encoding the DPHR in all species sequenced thus far, including rabbit, rat, carp and *Drosophila* (Monnier *et al.*, 1997).

A 226 bp product of the CACNL1A3 gene was amplified to detect the presence of the Arg1086His alteration that results from an A3333G substitution. In Phase 1, 21 individuals were analysed in order to detect the presence of this mutation via RFLP. A summary of results obtained for these individuals is listed in Appendix C, Table C1. In Phase 2, 22 South African MH individuals were analysed utilising the RFLP protocol described in Section 3.8. A summary of results obtained in this study for the Arg1086His alteration is listed in Table 4.4.

Table 4.4: Summary of mutation analysis results of the CACNL1A3 gene for the detection of the Arg1086His alteration obtained in Phase 1

Individual	Family Number	Arg1086His	Individual	Family Number	Arg1086His	Individual	Family Number	Arg1086His
360	110-1	Neg	710	—	Neg	875	—	Neg
660	—	Neg	749	—	Neg	1330	—	Neg
668	—	Neg	766	—	Neg	1331	—	Neg
674	—	Neg	788	—	Neg	1339	118-1	Neg
691	114-1	Neg	803	—	Neg	1388	117-1	Neg
695	—	Neg	871	—	Neg	1393	116-1	Neg
707	—	Neg	874	—	Neg	1413	—	Neg
709	—	Neg	---	—	---	---	---	---

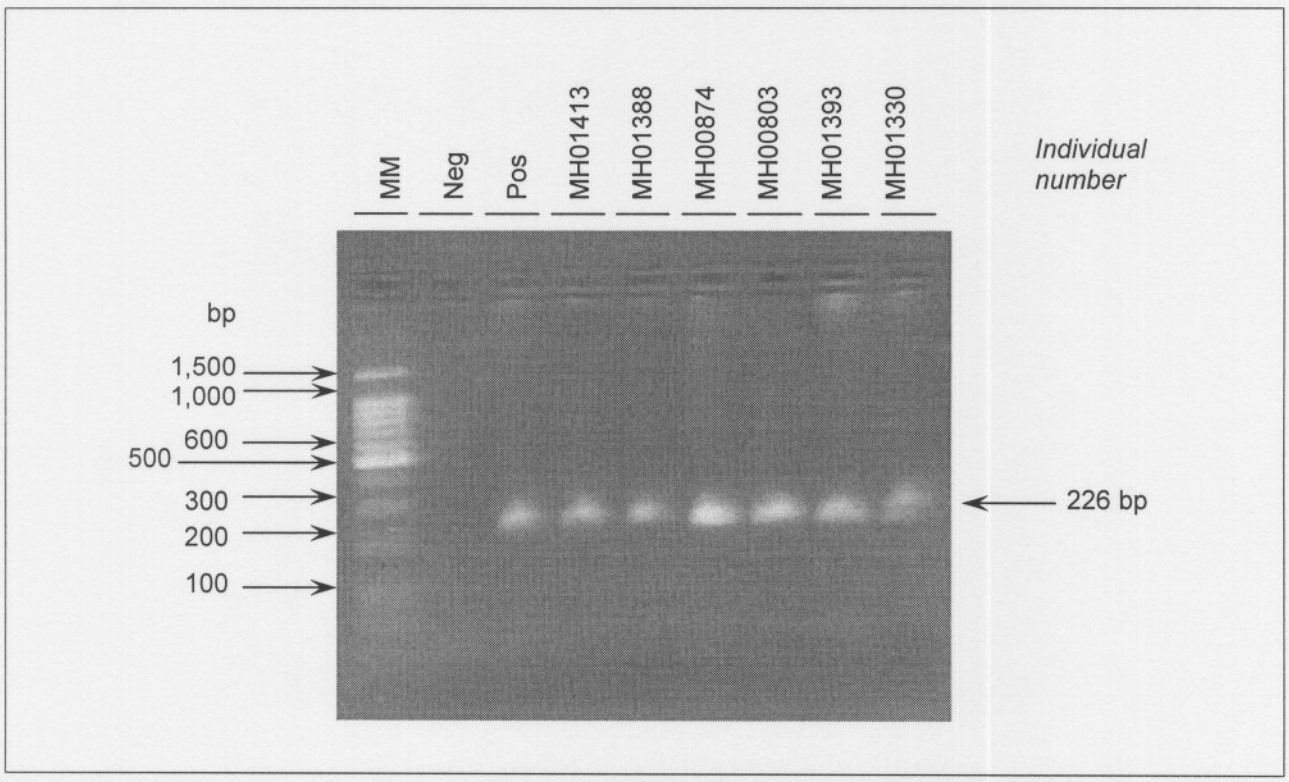
Neg = negative; (—) = indicates only individual number available; (—) = indicates open cells in table.

Amplification of the 226 bp product via PCR was optimised at an annealing temperature of 64°C and at an $MgCl_2$ concentration of 2 mM. However, the PCR product utilising this protocol had a very low yield, therefore to obtain a higher yield of PCR product, 200 $ng.\mu l^{-1}$ of gDNA was utilised. The product was electrophoresed on a 2% mini agarose gel, as described in Section 4.6, and the results are illustrated in Figure 4.26.

Positive and negative controls were utilised to determine respectively if the expected result had been obtained and to ensure that there had been no contamination. Following

restriction enzyme digestion of the PCR product with *Hha* I, the sample was electrophoresed on a 2% (w/v) agarose gel for one hour. The presence of the mutation results in the loss of the *Hha I* restriction endonuclease site. An individual that is heterozygous for the mutation would present with fragments of 226, 190 and 36 bp in length. A homozygous normal individual that does not harbour the mutation would yield complete digest of the 226 bp fragment into two fragments of 190 and 36 bp in length. In the case of homozygous MH-affected individuals only the 226 bp fragment would be observed, as the restriction enzyme recognition site would be abolished in both alleles.

Figure 4.26: Photographic representation of the amplified PCR products of the CACNL1A3 gene



Fragments were electrophoresed on a 2% agarose gel at 10 V.cm^{-1} for 30 min. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; Neg = negative control; Pos = positive control. Some resolution in the above figure was lost due to the photographic process that was utilised

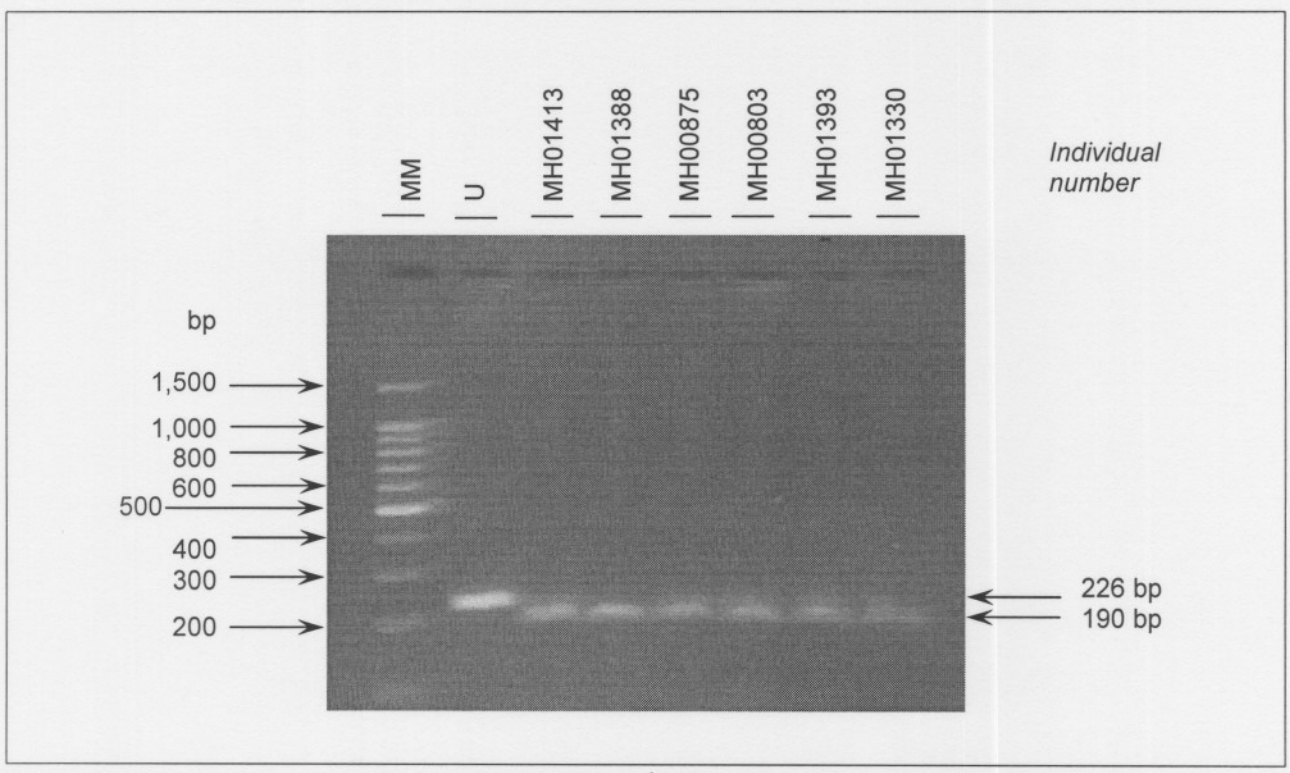
The resolution of a 2% (w/v) agarose gel was sufficient for the identification of both the 190 bp and 226 bp fragments and it was therefore possible to distinguish between MH-affected and normal individuals. It was thus unnecessary to analyse samples on a polyacrylamide gel in order to detect the 36 bp fragment. The RFLP results obtained for individuals screened for the Arg1086His alteration are depicted in Figure 4.27.

None of the MH individuals included in Phase 1 or Phase 2 harbour the Arg1086His alteration. As the alteration was not observed in any of the South African MH probands

analysed, this may indicate that an additional MHS mutation in the RYR1 CACNA1S or another unidentified protein leads to this disorder in the South African population. The CACNA1S plays an important role in charge movement in E-C coupling, therefore an alteration in any of the subunits of the DHPR channel may result in susceptibility to MH.

Further analysis of other regions of the CACNA1S and other subunits of the DHPR may yield novel alterations which result in the MH phenotype. It is currently unknown whether an alteration in the DPHR is sufficient to cause MH or if an alteration in the DHPR requires the expression of another alteration or SNP in this protein that has an epistatic effect, or in another protein to result in the MH phenotype. Therefore, the CACNA1S mutation may not play a primary role, as suggested for the RYR1, but may play a secondary role, which may be substituted by another protein.

Figure 4.27: Photographic representation of PCR products from the CACNL1A3 gene digested with *Hha* I for the detection of the Arg1086His alteration



Fragments were electrophoresed on a 2% agarose gel for 10 $V.cm^{-1}$ for 1 h. MM = 100 base pair (bp) molecular marker is indicated to the left of the figure; U = undigested PCR control product, utilised as a control for the absence of the mutation as well as the efficiency of the digestion reaction. Some resolution in the above figure was lost due to the photographic process that was utilised.

4.15 SUMMARY OF MUTATION ANALYSIS RESULTS

In Phase 2, the Thr4826Ile alteration was detected in a South African MH proband and two other members of her family. None of the remaining RYR1 alterations or the Arg1086His alteration of the CACNL1A3 analysed was identified in any MH individuals from the South

African population. In Phase 1 only two alterations were observed, Arg614Cys and Val2168Met. This is a significant finding, as it indicates that the South African Caucasian population with MH differs significantly from other MH populations worldwide, including the European population, in terms of the genetic basis of this disorder. Various possibilities that could lead to this finding are outlined in Chapter Five. A summary of results for all individuals screened in Phase 2 is listed in Tables 4.5 and 4.6. The results listed below are a summary of results obtained, as discussed in the relevant sections of this chapter.

Considerable allelic and genetic heterogeneity of MH has been reported. Alterations are continually being reported for the RYR1 gene worldwide. Halsall and Robinson (2004) recently reported at least 17 additional novel MH mutations (listed in Table 2.2) and thus far, only one alteration has been detected in the CACNL1A3 gene (Monnier *et al.*, 1997). Since only three RYR1 alterations have been observed in 154 probands analysed in the South African MH population in Phase 1 and Phase 2, it seems likely that additional, unreported alteration(s) in the RYR1 and DHPR genes may segregate with the MHS phenotype in the South African MH population. To date, 100 alterations have been identified in association with MH worldwide (Halsall and Robinson, 2004). In Phase 2, a total of 32% of alterations observed in the three mutation hotspots of the RYR1 gene were sequenced. In addition the surrounding regions of each alteration were analysed. However, this only indicates that 2% of the total RYR1 gDNA was sequenced in this study. Therefore, the remaining regions of the RYR1 gene should be analysed in the South African population to determine whether the RyR1 protein plays a role in susceptibility to MH in this population.

The observed low frequency of reported RYR1 alterations in the South African MH population could be due to genes other than the RYR1 that harbour causal MHS mutations. Reported genetic heterogeneity alludes to the fact that an alternative locus may even be responsible for the MH phenotype in the South African MH population. This is supported by the fact that linkage was reported in MH families of South African origin to other loci on chromosomes 17q11.2-q24 and 2q (Olckers *et al.*, 1992; Vita *et al.*, 1995; Olckers *et al.*, 1999). Alternatively, the MHS phenotype may be a result of several interacting proteins that are encoded by different chromosomes, as suggested by Robinson *et al.* (2000). It would therefore be important to investigate all possible alleles at different susceptibility loci that contribute minor phenotypic effects to MHS, as well as the entire RYR1 sequence, which has been suggested to play a primary role in this disorder (MacLennan *et al.*, 1990).

Table 4.5: Summary of mutation analysis results for mutation regions one, two, three, four, six, seven and nine of the RYR1 gene

Individual[a]	Family Number[b]	MR1		MR2			MR3			MR4		MR6			MR7	MR9	
		Cys35Arg	Arg44His	Glu160Gly	Arg163Cys	Arg163Leu	Arg2163Cys	Arg2163His	Val2168Met	Ala2200Val	Thr2206[c]	Arg2452Trp	Arg2458Cys	Arg2458His	Gly4638Met	Ile4938Met	Asp4939Glu
242	101-6	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	---	---
278	102-125	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
286	103-4	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
294	104-26	*	Neg	Neg	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
324	105-38	*	Neg	---	*	---	*	*	*	---	*	Neg	*	*	---	Neg	Neg
355	109-24	*	Neg	Neg	*	Neg	---	---	---	Neg	Neg	Neg	*	*	Neg	Neg	Neg
360	110-1	*	Neg	Neg	*	Neg	*	*	*	Neg	Neg	Neg	*	*	Neg	Neg	Neg
361	111-1	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
364	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
381	113-14	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
427	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
438	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	---	---
439	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
440	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
462	107-1	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
470	108-1	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	---	---
478	122-1	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
484	123-1	*	Neg	Neg	*	Neg	*	*	*	---	*	Neg	*	*	---	Neg	Neg
497	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	---	---
630	—	*	Neg	Neg	*	Neg	*	*	Neg	---	*	Neg	*	*	Neg	Neg	Neg
631	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
649	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	Neg	*	*	Neg	---	---
654	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	---	---	Neg	Neg	Neg
660	—	---	---	Neg	*	Neg	Neg	Neg	Neg	Neg	*	Neg	---	---	Neg	---	---
668	—	*	Neg	Neg	*	Neg	*	*	*	Neg	Neg	Neg	*	*	Neg	Neg	Neg
671	—	*	Neg	---	*	---	*	*	*	Neg	*	Neg	*	*	Neg	Neg	Neg
674	—	*	---	Neg	*	Neg	*	*	*	---	---	Neg	*	*	Neg	Neg	Neg
691	—	Neg	Neg	Neg	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
695	114-1	Neg	Neg	Neg	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
707	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
709	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
710	—	---	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	Neg	Neg	Neg
749	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
766	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
788	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
803	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
871	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
874	—	*	---	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
875	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
1330	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
1331	—	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
1339	118-1	*	Neg	Neg	*	Neg	*	*	*	Neg	*	*	*	*	Neg	Neg	Neg
1388	117-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	Neg	Neg	Neg
1393	116-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---
1413	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

a = indicates individual number of MH proband utilised in this study; b = indicates family number of MH proband utilised in this study; (—) indicates only individual number given to individual; c = indicates both mutations, Thr2206Arg and Thr2206Met; Neg = individual that do not harbour the mutation; (---) indicates individual not analysed; (*) indicates individual analysed for mutation in Phase 1 of the programme.

Table 4.6: Summary of mutation analysis results for mutation regions five and eight of the RYR1 gene

Individual[a]	Family Number[b]	MR5								MR8						
		Val2346Met	Glu2348Gly	Ala2350Thr	Arg2355Cys	Phe2364Val	Gly2434Arg	Arg2435His	Arg2435Leu	Leu4814Phe	Ile4817Phe	Leu4824Pro	Thr4826Ile	Leu4838Val	Val4849Ile	Arg4861His
242	101-6	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
278	102-125	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
286	103-4	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
294	104-26	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
324	105-38	---	---	---	---	---	*	*	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg
355	109-24	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
360	110-1	---	---	---	---	---	*	*	---	---	---	---	---	---	---	---
361	111-1	---	---	---	---	---	*	*	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg
364	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
381	113-14	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg
427	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
438	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
439	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
440	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
462	107-1	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
470	108-1	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
478	122-1	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
484	123-1	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
497	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	---	---	---	---	---	---	---
630	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
631	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
649	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
654	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
660	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
668	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
671	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
674	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
691	114-1	Neg	Neg	Neg	Neg	Neg	*	*	Neg	---	---	---	---	---	---	---
695	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
707	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
709	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
710	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
749	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
766	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
788	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
803	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
871	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
874	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
875	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
1330	—	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
1331	—	---	---	---	---	---	*	*	---	---	---	---	---	---	---	---
1339	118-1	Neg	Neg	Neg	Neg	Neg	*	*	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
1388	117-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
1393	116-1	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---
1413	—	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

a = indicates individual number of MH proband utilised in this study; b = indicates family number of MH proband utilised in this study; (—) indicates only individual number given to individual; c = indicates both mutations, Thr2206Arg and Thr2206Met; Neg = individual that does not harbour the mutation; Pos = individual that does harbour the mutation; (---) indicates individual not analysed; (*) indicates individual analysed for mutation in Phase 1 of the research programme.

CHAPTER FIVE

CONCLUSIONS

The estimated incidence of MH is 1 in 50,000 anaesthetised adults and 1 in 15,000 anaesthetised children (Golinski, 1995). These numbers are likely to be underestimated as many patients susceptible to MH do not display symptoms of MH after each exposure to anaesthetics.

MH is a disorder that results in a hypermetabolic response in the presence of potent volatile anaesthetic agents. 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 is not considered 100% accurate (Larach, 1993). The RYR1 gene was identified as a candidate for predisposition to MH (MacLennan *et al.*, 1990), but other genes have also been implicated. 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.

It is important to provide presymptomatic diagnosis of MH prior to anaesthesia, as safe alternative anaesthetic agents could be utilised for individuals with a known MHS status. An MH diagnosis will reduce morbidity and mortality to an absolute minimum because if an MH episode is not managed properly it can lead to death. Individuals that 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 identify other members of the extended family who may be at risk, as discussed in Section 2.14.3 and illustrated in Figure 2.3.

In the study presented here (Phase 2), alterations in the RYR1, as well as the Arg1086His alteration of the CACNA1S, were investigated to determine if they segregated with the MH phenotype in South African individuals. In Phase 2, 24 recently reported causative RYR1 alterations in South African MH probands were investigated. In addition, eight RYR1 alterations that had already been analysed in Phase 1, as well as the Arg1086His alteration of the CACNA1S, were also analysed in individuals who had not been included in Phase 1. One RYR1 alteration, Thr4826Ile, was identified in a single South African MH

pedigree. This is the first study in which the South African population was screened for this alteration, and represents the first description of this transition within South Africa. The detection of this transition within the South African population has significant implications for the future development of a molecular diagnostic test for MH in South Africa, and contributes to a description of the aetiology of MH in the South African population. The presence of this alteration in the most recently identified third mutation hotspot indicates the importance of the C-terminal region of the RYR1 gene, which should be analysed further in all population groups to determine whether other mutations are present in this region that result in the MH phenotype. The remaining RYR1 alterations and CACNA1S were not observed in any other South African MH probands investigated in this study. To date, only two other RYR1 alterations have been detected in the South African MH population, which include the Arg614Cys and Val2168Met (Havenga, 2000; Neumann, 2002; Olckers 1997). There are several explanations for the apparent lack of RYR1 alterations in the South African population, each of which will be described in subsequent sections of this chapter.

5.1 ANAESTHETICS AND MH

An MH episode can occur during an anaesthetic procedure or in the postoperative period. A variety of drugs can trigger MH, as discussed in Section 2.5. Exposure of these triggers in an MHS individual can result in an increase in body temperature, increase in O_2 consumption and widespread organ dysfunction. Therefore, anaesthesiologists should avoid triggering agents in patients with MH or any other congenital myopathy.

In response to a letter reporting four cases of cardiac arrest following the administration of suxamethonium, the Federal Drug Administration has commissioned a warning about the dangers of suxamethonium in children (Malignant Hyperthermia Association of the United States, 2004). This drug is a depolarising muscle relaxant often utilised in paediatric anaesthesia and can trigger an MH episode. In addition it can cause muscle damage and cardiac arrest, resulting in an annual USA incidence of six cases with 60% mortality. The package insert of this drug should include a strong warning and it should only be utilised in situations where immediate securing of the airway is required (Malignant Hyperthermia Association of the United States, 2004).

In many countries (including Europe and America) anaesthesiologists are utilising total intravenous anaesthesia (TIVA) instead of inhalational anaesthetics (Digger and Viira,

2003). Propofol is utilised as an intravenous agent for the maintenance of anaesthesia and has several advantages over volatile anaesthetics. The drug provides rapid onset and rapid recovery and can be utilised safely in patients susceptible to MH. The TIVA procedure also has several disadvantages, including difficulty in monitoring levels of agents, cost of the drug, and pump failure that could occur (Digger and Viira, 2003). Despite the benefits of TIVA, most general anaesthesia is still maintained utilising inhalational techniques because of the cost, easy titration of depth of anaesthesia and the fact that expired levels of the agent can be monitored in real time (Ghatge *et al.*, 2003). In addition, inhalational induction is still utilised for children, where an inhalational anaesthetic can be utilised to maintain muscle tone in the airway. Therefore, even though non-triggering drugs are available, the disadvantages of TIVA preclude its use in South Africa and MH triggering drugs are still commonly used in anaesthetic procedures. This underscores the need for a simple, inexpensive diagnostic test to determine MH susceptibility in our country.

5.2 MH AS A METABOLIC DISORDER

There have been several indications that the variability of clinical symptoms in MH may be due to an inborn metabolic error that results in the muscle being susceptible to disturbances of intracellular Ca^{2+} distribution, as discussed in Section 2.8.3. Although alterations in the RYR1 may be an important factor in the pathophysiology of the disorder in some individuals, the presence of an alteration does not explain the variability of MH in terms of clinical expression. Therefore, a variety of modulators, such as free fatty acids and/or the phospholipase A_2, may be essential for the phenotypic expression of MH or influence the clinical expression of MH. These modulators are poorly understood and may be affected by environmental exposure, as well as by internal substances. Initiators that may trigger this dysfunction include radiation, nutrition, infectious agents, hormones, drugs, heavy metals and chemicals. Stress may also be included in this category as a facilitating factor. As environmental exposure may vary in each geographical region, it may affect the functioning of the RyR1 protein differently, which could contribute to the discrepancy with regard to RYR1 mutations in the different populations.

5.3 RELATIONSHIP BETWEEN STRUCTURE AND FUNCTION

Many studies, described below, have indicated that significant abnormalities in Ca^{2+} induced Ca^{2+} release exist. MH muscle has proven to be abnormally sensitive to stimuli. In

addition, sensitivity to H^+ inhibition is reduced in MHS SR compared to normal SR, where complete inhibition is observed. Ohnishi *et al.* (1986) indicated that the rate and extent of Ca^{2+} release in MH muscle is higher compared to normal muscle. Mickelson *et al.* (1988) conducted binding experiments and reported that the amount of Ca^{2+} released from MHS muscle SR was greater upon binding of ryanodine, compared to normal porcine SR. The affinity for ryanodine was normal under optimal conditions. However, when suboptimal conditions were present, such as low ionic strength or absence of adenine nucleotides, binding of ryanodine was higher than normal (Mickelson *et al.,* 1988). Lastly, the Ca^{2+} stimulation of ryanodine binding was the same for both MHS and normal muscle. These studies indicate that a final common pathway exists, in which the convergence of a number of small abnormalities in the presence of a triggering stimulus, such as exposure to a volatile anaesthetic, can result in the MH phenotype (Pessah, 1996). Therefore all the regulatory elements of the RyR1 protein, which affect both channel conductance and channel gating, may result in an MH episode and their function in relation to the MH syndrome should be clarified.

5.4 VARIABILITY IN MHS

The reported variability in clinical symptoms of MH may also be explained by the role of accessory proteins that modify RYR1 function, as illustrated in Figure 2.1. Proteins such as FKBP12 bind to RyR1 and stabilise both the closed conformation and its transition from closed to open states, which may play a role in susceptibility to MH (Wagenknecht *et al.*, 1997). Therefore there is a possibility that MH could be due to an alteration in the FKBP12/RyR1 complex, or that chemical agents could alter the regulatory function of FKBP12 and influence MHS.

In the same manner an alteration in the binding site localised to the central portion of the RYR1 gene for calmodulin could result in the MH phenotype. Calmodulin activates Ca^{2+} release by interacting with binding sites on the RyR1 (Buratti *et al.*, 1995). As skeletal muscle Ca^{2+} signalling needs to be precise and highly coordinated, it is reasonable to suggest that a malfunction in any of the components in this system could lead to disruption of Ca^{2+} signalling and result in a skeletal muscle disorder such as MH.

Other proteins involved in E-C coupling are calsequestrin, the primary Ca^{2+} binding protein of the SR, and triadin, an SR membrane protein that binds to the RyR1 (Wingertzhan and Ochs, 1998). However the physiological importance of these proteins has not yet been

determined. Therefore, further analysis of all the proteins involved in E-C coupling is required to determine the exact aetiology of this complex disorder.

5.5 RYR1 MUTATION HOTSPOTS AND MH

Over the last ten years, the international focus of mutation screening of the RyR1 protein for MHS has been towards functional significant protein domains (Halsall and Robinson, 2004). MH mutations are clustered in three regions of the RyR1, as described in Section 2.10.3. The first hotspot, observed in the N-terminal region of the protein, is the regulatory domain that is concerned with ligand activation of the channel. The second hotspot, found in the central portion of the protein, is the regulatory region affected by ligands. The third hotspot derived from the C-terminal region of the protein is the pore-forming section and mutations found in this region could directly affect the channel properties (Lynch *et al.*, 1999).

The gene of the RYR1 encompasses 158,000 bp of gDNA, as discussed in Section 2.10.2. In the study presented here the RYR1 gene was screened for 32 alterations residing in exons 2, 6 (hotspot one), exons 39, 40, 43, 44, 45, 46 (hotspot two) and exons 95, 100, 101, 103 (hotspot three). Although screening of the RYR1 gene in Phase 2 included all three hotspots and resulted in approximately 3,145 bp of the gene being sequenced, this only encompasses approximately 2% of the total RYR1 gDNA. Therefore, as seen in the context of results included in this dissertation, only 2% of the gene can be excluded in the South African population and the remainder would have to be screened, to determine if other regions of this gene play a role in susceptibility to MH. It is unlikely that many causative alterations will be detected in regions of the RYR1 gene that are not located in one of the three mutation hotspots, however, the probability of observing a causative alteration in other regions of the RYR1 gene can not be excluded.

5.6 GENETIC HETEROGENEITY AND MH

MH shows evidence of both locus and allelic heterogeneity. Thus far, 100 RYR1 mutations have been found associated with MH worldwide. Some mutations occur more frequently in certain populations, while others are exclusive to specific families. Molecular studies have reported that 50% of families which were studied that are susceptible to MH, harbour one of the RYR1 alterations (Brandt *et al.*, 1999). However, 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. Therefore the functioning of these proteins may have altered as a result of a mutation or due to the effects of either free fatty acids or 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.

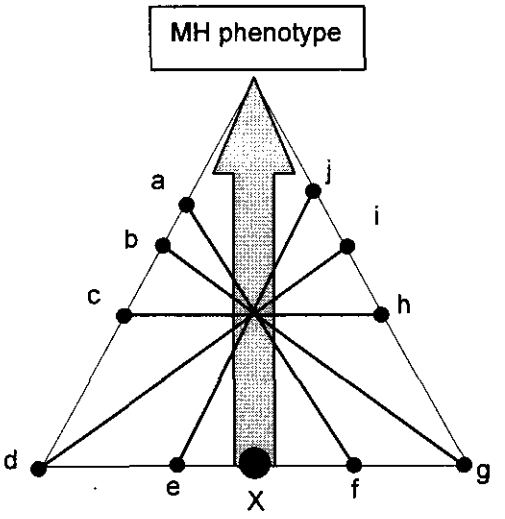
Reports of linkage in MH families of South African origin to chromosome 17q11.2-q24 and 2q (Olckers *et al.*, 1992; Vita *et al.*, 1995; Olckers *et al.*, 1999) support this observation. The presence of linkage to other susceptibility loci indicates that abnormalities in several proteins involved in 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.

5.7 EPISTASIS AND MH SUSCEPTIBILITY

The reported difference with regard to severity of MH in patients is poorly understood. It is possible that epistasis plays a role in MH susceptibility, where two or more alterations at the same or different genetic susceptibility loci may contribute either major or minor phenotypic effects. Therefore, a particular allele may increase the risk of MHS, but on its own may not be sufficient for disease expression. Variability in clinical symptoms may be a result of a complex interaction between genes and environment and 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 and stress (as discussed in Section 2.4) and genetic loci which have minor or major phenotypic effects, which may all lead to an MH episode is illustrated in Figure 5.1.

The figure indicates that a major genetic determinant (X), as denoted by a filled circle, will lead to the MH phenotype (as indicated by a grey arrow) without any additional factors being required. In the absence of a major genetic determinant, several minor genetic or environmental factors, illustrated by small dots, i.e. (d) in combination with (i) and (j), will have a phenotypic effect and lead to MH susceptibility. Therefore, several combinations of minor factors exist which may all have the potential to interact and ultimately culminate in the disorder.

Figure 5.1: Network of genetic and environmental factors that can potentially influence the expression of the MH phenotype



a – j = Minor genetic determinants and environmental factors
X = Major genetic determinant

Small circles = indicates genetic determinants and environmental influences which have minor phenotypic effects; large circle = indicates genetic determinant with major phenotypic effect; arrow = indicates factors leading to an MH phenotype.

5.8 DIAGNOSIS OF MHS

An MH diagnosis is based on clinical presentation and/or laboratory testing. Larach *et al.* (1994) developed a clinical grading scale, as described in Section 2.1, to aid in the clinical diagnosis of MH. However, this test lacks specificity, as not all criteria can be evaluated during an MH episode. The only available presymptomatic diagnosis for the MH phenotype is the IVCT, which is based on contracture of muscle fibres in the presence of halothane or caffeine. Both tests are expensive, invasive and are currently not available in South Africa. There are currently two forms of this test, the EMHG and NAMHG. Both tests are similar in protocols, but differ in the interpretation of the generated results. The EMHG protocol has three categories. Individuals are diagnosed as MHS if they test positively for both caffeine and halothane, MHN if they test negatively for both substances and MHE if they test positively for either caffeine or halothane. The NAMHG protocol has only two diagnostic categories, MHS if individuals tests positively for either caffeine or halothane and MHN if they are negative to both these substances. The specificity and sensitivity of both tests differ, as discussed in Section 2.14.1. However, Ørding and Bendixen (1992) observed that similar results were obtained for both tests.

Both protocols are subject to false positive and negative results and can therefore not be considered to be 100% accurate (Ørding *et al.*, 1997). The authors reported that the false positive rate for the EMHG protocol was 6% and that for the NAMHG protocol 9%. The IVCT is only able to differentiate between abnormal and normal muscle contracture. In both protocols the specificity can be affected by other neuromuscular disorders that are not related to MH, which have an associated increase in myoplasmic Ca^{2+} concentration. Therefore, the IVCT cannot be considered a specific test for MH. The lack of 100% specificity and sensitivity of the IVCT can lead to discordance and apparent lack of linkage, making it difficult to determine the genetic basis of MH.

5.9 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 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 normal muscle (Mickelson *et al.*, 1990). Therefore, it is unlikely that the presence of this alteration does not result in the MH phenotype and 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 and a false negative in the MHN individual. Studies have indicated that the threshold utilised to indicate a positive result in the IVCT influences the extent of genetic linkage.

5.10 POPULATION SPECIFICITY AND MH SUSCEPTIBILITY

The frequency of mutations observed in the RYR1 is much lower in the South African population than the reported 50% of RYR1 mutations found in European populations. The difference may be attributed to the fact that mutations resulting in MHS may be specific to a population. The frequency of each RYR1 alteration differs, from occurring frequently to those that have only been observed in a single family. Differences between the South African population and other population groups have been reported 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 that is only present in this population are important mediates of this disorder. They suggested that an environmental factor must have been present, which allowed the mutation to flourish only in this population group.

Familial hypercholesterolemia (FH) is an autosomal dominant disorder of lipoprotein metabolism. Elevated total and low-density lipoprotein (LDL) cholesterol levels predispose individuals with this disorder to premature cardiovascular disease. More than 600 alterations of the LDL receptor gene have been associated with FH and each country has its own specific panel of mutations. Only certain countries share mutations, which could be explained in some cases by historical or demographic conditions (Salinas, 2001). The South African population has a high prevalence of this disorder and thus far five mutations have been found responsible for FH in the South African population (Salinas, 2001).

Hypertrophic cardiomyopathy is an autosomal dominant disorder of the cardiac sarcomere that results in sudden cardiac death. Moolman-Smook *et al.* (1999) investigated this disorder in both English- and Afrikaans-speaking South African individuals. The authors observed that a high proportion of mutations associated with this disorder were unique and that none of the other disease-associated mutations reported worldwide are observed in the South African population, indicating that the mutations resulting in this disorder are unique in different geographic areas. It is apparent that the South African Caucasian population differs from other population groups in terms of both genetic and environmental exposure. For this reason RYR1 alterations that are common in other populations may not be observed in the South African MH population.

A prerequisite for genetic testing should be to determine the mutation frequencies in the geographic region that is served by an MH investigation centre. Each MH centre around the world would have to design its genetic test for the specific population to be tested. Without this information genetic testing would be very costly and time-consuming to perform. To date, the frequency of RYR1 gene mutations has been published for Germany, Italy, North America, Switzerland and the UK. The frequency in other countries is unknown (including South Africa), and would have to be determined prior to the implementation of a diagnostic service in each country.

5.11 DIAGNOSTIC SERVICE FOR MH

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 a causative mutation allows for the diagnosis of MHS. Currently, genetic screening cannot be utilised for routine diagnosis of MH in South Africa due to the low incidence of mutations observed in the RYR1 gene. Genetic screening of first-degree relatives of an MH proband has been proposed, and is discussed in Section 2.14.3 and illustrated in Figure 2.4. Mutation screening in the future will depend on the development of a simple and cost-effective, yet specific test.

Currently pre-clinical diagnosis of hereditary haemochromatosis and FH is performed via a strip assay in South Africa. This technology is cost-effective, rapid, efficient and specific. The strip assay is based on reverse-hybridisation technology, which contains a variety of oligonucleotide probes for each wild-type and mutated allele that is immobilised on the strip and is detected via labelled patient DNA (Kotze, 2002). The strip could be utilised to diagnose MH and individuals will either be identified as homozygous for the wild-type probe, heterozygous for a wild-type and mutant probe or homozygous for the mutant probe only. The test could be adapted to cover an increasing number of mutations depending on the requirement of each MH test centre.

Another technique that has been suggested for the diagnosis of MH is denaturing high performance liquid chromatography (DHPLC). This technique has many advantages including high sensitivity, speed and cost effectiveness, but it requires expensive instruments. DHPLC relies on the principle of heteroduplex analysis by ion-pair reversed-phase liquid chromatography under denaturing conditions. Tammaro *et al.* (2003) reported that this technique could be utilised to detect both novel and recurrent mutations in MH.

5.12 FUTURE DEVELOPMENTS

Although non-triggering anaesthetic agents are available, the cost and several disadvantages of these drugs prevent their use in anaesthetic procedures in South Africa. Internationally the therapeutic use of dantrolene has reduced mortality from 80% to 5-10% (Ørding *et al.*, 1997), but this drug cannot reduce the mortality rate to zero. In South Africa

the mortality rate may be higher due to the cost of the drug, which has limited its use in most hospitals. In addition, lack of recognition of the initial phenotype has resulted in a higher mortality rate in South African MH individuals. If an MH episode is triggered in an individual, loss of Ca^{2+} homeostasis leads to a cascade of events, some of which may be irreversible and cause permanent damage. Therefore, treatment is not the safest answer, and deaths related to MH can only be reduced to zero via prevention. Therefore a simple, easy to perform and inexpensive test is required to diagnose MHS. However, this will only be possible when all the genes associated with MHS as well as the mutations in these candidate loci are identified. In addition, the exact role and incidence of all mutations in the South African MH population as well as modifying factors resulting in variability of expression associated with this disorder will have to be determined.

When the above factors have been identified, a reliable method of both diagnosing the predisposition of this disorder and performing a precise differential diagnosis will be possible. Identification of these factors 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.

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APPENDIX A

CLINICAL ASPECTS OF MALIGNANT HYPERTHERMIA

An MH clinical grading scale was developed to classify clinical MH episodes for a clinical case definition and to assist anaesthesiologist in classifying adverse events that occur following the administration of an anaesthetic. The scale determines the likelihood that an adverse anaesthetic event is a result of MH, and relies on the anaesthesiologist to judge whether specific clinical signs is truly representative of an MH episode (Larach *et al.*, 1994).

Table A1: Clinical indicators utilised to determine the MH raw score

Process	Indicator	Points
1. Rigidity	Generalized muscular rigidity	15
	Masseter spasm	15
2. Muscle breakdown	Creatine kinase > 20, 000 IU after succinylcholine	15
	Creatine kinase > 10, 000 IU without succinylcholine	15
	Cola coloured urine in perioperative period	10
	Myoglobin in urine > 60 mcg.L^{-1}	5
	Myoglobin in serum > 170 mcg.L^{-1}	5
	Blood/plasma/serum K^+ > 6mEK.L^{-1}, without renal failure	3
3. Respiratory acidosis	$P_{ET}CO_2$ > 55 mmHg with controlled ventilation	15
	Arterial $PaCO_2$ > 60 mmHg, controlled ventilation	15
	$P_{ET}CO_2$ > 60 mmHg with spontaneous ventilation	15
	Arterial $PaCO_2$ > 65 mmHg, spontaneous ventilation	15
	Inappropriate hypercarbia[1]	15
	Inappropriate tachypnoea	10
4. Temperature increase	Inappropriately rapid increase in temperature[1]	15
	Inappropriately increased temperature > 38.8°C[1]	10
5. Cardiac involvement	Inappropriate sinus tachycardia	3
	Ventricular tachycardia or fibrillation	3
6. Family history	Positive family history in first degree relative[2]	15
	Positive family history, more distant relative[2]	5
7. Others[3]	Arterial base excess more negative than -8 mEq.L^{-1}	10
	Arterial pH < 7.25	10
	Rapid reversal of MH signs after IV dantrolene	5
	Positive MH family history with another indicator from the patients anaesthetic experience other than increase CK[2]	10
	Elevated CK and a family history of MH[2]	10

1 = In anaesthesiologist's judgement, 2 = Indicators used only to determine MH susceptibility, 3 = Others process should be added without regard to double-counting, adapted from Larach *et al.* (1994).

Table A2: Indicators and scoring rules of the MH clinical grading scale

Indicators	List of indicator	Scoring rules
Clinical indicators	Review list in Table A2.	If any indicators are present, add points applicable. If more than one indicator represents a single process, apply the double-counting rule, which counts only the indicator with the highest score. Indicators in the final category of Table A2 ('other indicators'), should be added to total score without regard to double counting. If no indicator is present, the patients MH score is zero.
Susceptibility indicators	Positive family history of MH in relative of first degree. Positive family history of MH in relative not of first degree. Resting elevated serum CK. Positive family history of MH with another indicator during patient's anaesthesia other than elevated serum CK.	List of susceptibility indicators apply only to MH susceptibility and not to an MH event. To calculate score for susceptibility, add the score of the susceptibility indicators to the score for the highest ranking MH event.

Interpreting the raw score

Raw score range	MH rank	Description of likelihood
0	1	Almost never
3 - 9	2	Unlikely
10 - 19	3	Somewhat less than likely
20 - 34	4	Somewhat greater than likely
35 - 49	5	Very likely
50+	6	Almost certain

Adapted from Larach *et al.* (1994).

APPENDIX B

APPROACH TO COUNSELING THE MH PATIENT AND FAMILY

World-wide, counselling and evaluation of an MH patient and family is based on a more clinical approach, due to the unavailability and invasiveness of a muscle biopsy. Information is obtained by gathering personal and family history, investigating medical records as well as via a physical examination and laboratory tests. Data obtained is primarily utilised for counselling and may provide a greater understanding of MH. A standardised approach to counselling of an MHS individual is listed below, as proposed by McPherson and Taylor (1982).

Table B1: Counselling the MHS individual

Evaluation	Criteria	Details
Information gathering	Personal history	Previous anaesthesia (including dental work). Ask about complications, particularly fever, rigidity, cardiac arrhythmias, prolonged awakening, muscle pain, or dark urine after anaesthesia. Musculoskeletal complaints. Febrile response. Unusual reaction of any drug(s).
	Family history	Ask the above questions about all relatives.
	Medical records	When possible, obtain anaesthetic records, especially when complications have occurred. Look for other causes of adverse reaction to anaesthesia and surgery.
	Physical examination	Assess general health. Look especially for increased muscle bulk with normal strength, localized atrophy or other myopathy.
	Laboratory	Measure serum CPK and ask about factors influencing CPK value. In selected eases consider muscle biopsy.
Interpretation of results		On the basis of history and laboratory results, determine who is affected. Determine by inspection of pedigree, the most likely mode of inheritance in this family. Use mode of inheritance, pedigree and clinical data to determine probability of MH prior to CPK testing of consultant. Use Bayes theorem and laboratory results to modify risk figure.
Counselling		Discuss manifestations of MH, methods of prevention and treatment. Give risk figures and discuss options for dealing with risk. Give estimate of risk for offspring.

Adapted from McPherson and Taylor (1982).

APPENDIX C

SUMMARY OF PREVIOUS RESULTS OBTAINED IN THE ONGOING MH RESEARCH PROGRAMME

Table C1 lists the results for all the individuals screened for the 17 missense mutations investigated previously in Phase 1 (Havenga, 2000; Neumann, 2002). The results listed below are a summary of all the mutations screened during Phase 1.

Table C1: Summary of mutation analysis results generated in Phase 1 of the MH research programme

Individual [a]	Cys35Arg	Arg163Cys	Gly248Arg	Gly341Arg	Ile403Met	Tyr522Ser	Arg552Trp	Arg614Cys	Arg614Leu	Arg2163Cys	Arg2163His	Val2168Met	Thr2206Met	Gly2435Arg	Arg2436His	Arg2458Cys	Arg2458His	Chr1 Arg1086His
101-6	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
101-10	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	ANP	---	Neg	Neg	Neg	Neg	---
101-12	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
102-2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	Neg	Neg	Neg	Neg	Neg	Neg	---
102-4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
102-11	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
102-24	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	Neg	Neg	Neg	Neg	Neg	Neg	---
102-28	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg
102-39	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	Neg	ANP	Neg	Neg	Neg	Neg	---
102-48	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
102-96	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
102-117	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
102-125	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
102-25	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	---	---	---	---	---	Neg	Neg	--	---	---
103-2	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
103-4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
103-7	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
104-24	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
104-25	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
104-26	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	ANP	---	Neg	Neg	---	---	Neg
104-27	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
104-35	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	ANP	Neg	Neg	Neg	Neg	Neg
104-40	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
104-41	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-19	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-20	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	---	---	---	---	---	Neg	Neg	---	---	---
105-21	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-23	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	---	---	---	---	---	Neg	Neg	---	---	---

Table C1: Continued...

Individual[a]	Cys35Arg	Arg163Cys	Gly248Arg	Gly341Arg	Ile403Met	Tyr522Ser	Arg552Trp	Arg614Cys	Arg614Leu	Arg2163Cys	Arg2163His	Val2168Met	Thr2206Met	Gly2435Arg	Arg2436His	Arg2458Cys	Arg2458His	Chr1 Arg1086His
105-27	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-28	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-29	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---	---
105-30	---	---	---	---	---	---	---	Pos	---	---	---	---	---	---	---	---	---	---
105-31	---	---	---	---	---	---	---	Pos	---	---	---	---	---	---	---	---	---	---
105-32	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	---	---	---	---	---	Neg	Neg	---	---	---
105-33	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-34	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-35	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	---	---	---	---	---	Neg	Neg	---	---	---
105-36	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	---	---	---	---	---	Neg	Neg	---	---	---
105-37	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-38	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
105-39	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-44	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-45	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-46	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-47	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-50	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-54	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-55	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	---	---	---	---	---	Neg	Neg	---	---	---
105-60	---	---	---	---	---	---	---	Pos	---	---	---	---	---	---	---	---	---	---
105-61	---	Neg	---	Neg	Neg	---	---	Pos	---	---	---	---	---	Neg	Neg	---	---	---
105-63	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	---	---	---	---	---	Neg	Neg	---	---	---
105-64	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-66	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
105-70	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-81	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-83	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-88	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-98	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-104	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-115	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-117	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-124	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	---	---	---	---	Neg	Neg	---	---	---
105-134	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-135	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-136	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-137	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-138	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-139	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-140	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
105-141	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
105-142	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
105-143	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
106-1	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
106-4	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	ANP	Neg	Neg	Neg	---

Table C1: Continued...

Individual[a]	Cys35Arg	Arg163Cys	Gly248Arg	Gly341Arg	Ile403Met	Tyr522Ser	Arg552Trp	Arg614Cys	Arg614Leu	Arg2163Cys	Arg2163His	Val2168Met	Thr2206Met	Gly2435Arg	Arg2436His	Arg2458Cys	Arg2458His	Chr1 Arg1086His
107-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
107-2	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
107-3	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
107-4	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
107-5	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
107-6	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
107-7	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
107-8	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
108-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
108-2	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
108-3	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
108-4	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
108-5	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
109-24	Neg	Neg	---	Neg	Neg	---	---	Neg	---	---	---	ANP	---	Neg	Neg	Neg	Neg	---
110-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Neg	Neg	---
111-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	ANP	Neg	Neg	Neg	Neg	Neg	---
111-2	Neg	Neg	---	Neg	Neg	Neg	---	Neg	---	---	---	ANP	Neg	---	---	Neg	Neg	---
111-3	Neg	Neg	---	Neg	Neg	---	---	Neg	---	---	---	---	---	---	---	---	---	---
112-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
113-1	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-2	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-3	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-4	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-5	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-6	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-7	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-8	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-9	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-10	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-11	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-12	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
113-14	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
113-15	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
113-16	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
114-1	---	Neg	---	Neg	Neg	---	---	Neg	---	---	---	---	---	Neg	Neg	---	---	---
114-4	---	Neg	---	Neg	Neg	---	---	Neg	---	---	---	---	---	Neg	Neg	---	---	---
114-7	---	Neg	---	Neg	Neg	---	---	Neg	---	---	---	---	---	Neg	Neg	---	---	---
427	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
438	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
439	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
440	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
478	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
479	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
484	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
485	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg
486	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	Neg

Table C1: Continued...

Individual[a]	Cys35Arg	Arg163Cys	Gly248Arg	Gly341Arg	Ile403Met	Tyr522Ser	Arg552Trp	Arg614Cys	Arg614Leu	Arg2163Cys	Arg2163His	Val2168Met	Thr2206Met	Gly2435Arg	Arg2436His	Arg2458Cys	Arg2458His	Chr1 Arg1086His
497	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
630	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	NSq	Neg	Neg	Neg	Neg	Neg	Neg
631	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
649	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
652	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
653	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
654	ANP	ANP	Neg	ANP	ANP	Neg	Neg	Neg	Neg	Neg	Neg	NSq	ANP	ANP	Neg	ANP	ANP	Neg
656	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
657	---	Neg	---	Neg	Neg	---	---	Neg	---	---	---	Neg	Neg	---	---	---	---	---
658	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
660	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
668	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	ANP	Neg	Neg	Neg	Neg	---
671	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
672	---	Neg	---	Neg	Neg	---	---	Neg	---	---	---	Neg	Neg	Neg	Neg	Neg	Neg	---
674	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	ANP	Neg	Neg	Neg	Neg	--
675	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	ANP	Neg	Neg	Neg	Neg	---
691	---	Neg	---	Neg	Neg	---	---	Neg	---	---	---	---	---	Neg	Neg	---	---	---
709	---	---	---	---	---	---	---	---	---	---	---	---	---	---	Neg	---	---	---
710	---	---	---	---	---	---	---	---	---	---	---	---	---	---	Neg	---	---	---
749	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
766	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
772	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
788	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
803	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
871	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
874	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
875	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
1251	---	---	---	---	---	---	---	Pos	---	---	---	---	---	---	---	---	---	---
1252	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
1253	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
1254	---	---	---	---	---	---	---	Neg	---	---	---	---	---	---	---	---	---	---
1306	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
1331	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
1338	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---
1339	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	---

a = indicates individual number of MH proband utilised in this study or family number of MH proband utilised in this study; Neg = individual that does not harbour the mutation; Pos = individual that harbours the mutation; NSq = poor quality sequencing therefore results not confirmed; ANP = no PCR amplification; (---) indicates that sample was not analysed.