# Siftingsanalise van die RYR1-geen in malignehipertermie-indeksgevalle van Suid-Afrika dui op 'n nuwe epigenetiese etiologie in hierdie bevolking

DEUR

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# Screening of the RYR1 gene in malignant hyperthermia probands from South Africa indicates towards a novel epigenetic aetiology in this population

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This thesis is dedicated to my parents, sister and Gavin

# ABSTRACT

Malignant hyperthermia (MH) is an autosomal dominant, potentially lethal pharmacogenetic disorder of skeletal muscle, which is elicited by exposure to volatile anaesthetics and depolarising muscle relaxants. Susceptible individuals appear clinically normal, but may present with a hypermetabolic crisis and muscle contracture when exposed to triggering substances that elicit excessive release of calcium ions from the sarcoplasmic reticulum. Diagnosis of MH susceptibility is currently made via the *in vitro* contracture test.

Genetically, in more than 50% of the affected families, MH occurs due to alterations in the skeletal muscle ryanodine receptor gene (RYR1) on chromosome 19q13.1. However, the disorder is genetically heterogeneous, as six other loci have to date been associated with MH susceptibility (MHS). Thus far, molecular tests have focused on three mutation hotspots of the RYR1 gene, which refer to regions that are more frequently mutated. Screening the entire RYR1 has led to a higher detection rate in a variety of populations.

In this study the entire coding region of the RYR1 gene was screened via sequencing for novel or reported alterations for the first time in 15 South African probands. Eight different RYR1 alterations were observed in seven MHS South African probands, six of which were previously reported and two of which were novel. Compound heterozygous alterations and alterations outside the mutation hotspots were detected. Screening of the entire coding region of the RYR1 gene is crucial for genetic investigations into MHS.

It was postulated that MH in the South African population is due to multifactorial inheritance in which a network of several genetic, environmental and epigenetic factors interact and cumulatively result in the development of the MH phenotype. Data generated in this study highlight the complexity of this disorder, further supporting a novel epigenetic aetiology for MH in the South African population.

# OPSOMMING

Maligne hipertermie (MH) is 'n outosomale dominante, potensieel dodelike farmakogenetiese sindroom van die skeletspier wat veroorsaak word deur blootstelling aan vlugtige narkotiese middels en depolariserende spierverslappers. MH-vatbare individue kom klinies normaal voor, maar mag 'n hipermetaboliese krisis en spiersametrekking toon wanneer die individu aan veroorsakende middels blootgestel word, wat die oormatige vrystelling van kalsium-ione deur die sarkoplasmiese retikulum veroorsaak. Diagnose van MH-vatbaarheid word tans bepaal deur die *in vitro*-kontraksietoets.

In meer as 50% van aangetaste gesinne kom MH voor as gevolg van veranderings in die skeletspier-ryanodienreseptorgeen (RYR1) op chromosoom 19q13.1. Die siektetoestand is egter geneties heterogeen, aangesien tot op hede ses lokusse geïdentifiseer is wat geassosieer word met MH-vatbaarheid (MHS). Tot dusver, het molekulêre ondersoeke gefokus op drie mutasie-ryke gebiede van die RYR1-geen waar veranderings meer gereeld plaasvind. Sifting van die volledige RYR1-geen het gelei tot 'n verhoogde ontdekkingstempo van 'n verskeidenheid veranderings in verskillende bevolkings.

In hierdie studie is die hele kodeergebied van die RYR1-geen vir die eerste keer in 15 Suid-Afrikaanse indeksgevalle gesif met volgordebepaling vir voorheen ongerapporteerde of aangemelde veranderings. Agt verskillende RYR1-veranderings is waargeneem in sewe Suid-Afrikaanse indeksgevalle, waarvan ses voorheen waargeneem is en twee nuut is. Veelvuldige heterosigotiese veranderinge en veranderinge buite die mutasie-ryke gebiede is waargeneem. Sifting van die totale RYR1 geen is van deurslaggewende belang vir genetiese ondersoeke in verband met MHS.

Daar word gepostuleer dat MH in die Suid-Afikaanse bevolking te wyte is aan multifaktor-oorerwing waar 'n netwerk van genetiese, omgewings- en epigenetiese faktore met mekaar reageer en kumulatief lei tot die ontwikkeling van die MH-fenotipe. Data wat in hierdie studie gegenereer is, benadruk die ingewikkeldheid van hierdie sindroom en bied verdere bevestiging van 'n voorheen ongerapporteerde epigenetiese etiologie vir MH in die Suid-Afrikaanse bevolking.

# TABLE OF CONTENTS


| | | Page |
|---|---|---|
| LIST OF ABBREVIATIONS AND SYMBOLS | | i |
| LIST OF EQUATIONS | | vii |
| LIST OF FIGURES | | ix |
| LIST OF TABLES | | xxi |
| ACKNOWLEDGEMENTS | | xxv |
| **CHAPTER ONE** | | |
| **INTRODUCTION** | | 1 |
| **CHAPTER TWO** | | |
| **MALIGNANT HYPERTHERMIA: A DISORDER OF CALCIUM DYSREGULATION** | | 5 |
| **2.1** | **INCIDENCE AND MORTALITY OF MH** | 6 |
| **2.2** | **THE CLINICAL PRESENTATION** | 6 |
| **2.3** | **PREOPERATIVE DIAGNOSIS** | 7 |
| 2.3.1 | The *in vitro* contracture test | 8 |
| 2.3.2 | Proposed alternative tests | 10 |
| **2.4** | **ASSOCIATED MYOPATHIES** | 11 |
| 2.4.1 | Evans myopathy | 11 |
| 2.4.2 | King-Denborough Syndrome | 12 |
| 2.4.3 | Central Core Disease | 12 |
| **2.5** | **MH-RELATED DISORDERS** | 14 |
| 2.5.1 | Neuromuscular disorders | 14 |
| 2.5.2 | Human Stress Syndrome | 16 |
| 2.5.3 | Links between MH and other disorders | 17 |
| **2.6** | **PHARMACOLOGIC AGENTS** | 18 |
| **2.7** | **DANTROLENE SODIUM THERAPY** | 20 |
| **2.8** | **TREATMENT OF MH** | 21 |
| **2.9** | **MOLECULAR MECHANISM OF MUSCLE CONTRACTION IN MH** | 22 |
| 2.9.1 | Excitation-contraction coupling | 23 |
| 2.9.2 | Ultrastructure of the RyR1 receptor | 25 |
| 2.9.3 | Ryanodine receptor isoforms | 26 |
| 2.9.4 | Physiological modulation of the RyR1 receptor | 28 |
| 2.9.5 | RyR1 receptor binding proteins | 29 |
| 2.9.6 | Functional characteristics of the RyR1 protein | 31 |
| 2.9.7 | The DHPR | 32 |

| | | |
|---|---|---|
| 2.9.8 | Excitation-contraction coupling and calcium regulation in MH susceptible individuals | 33 |
| **2.10** | **METABOLIC DYSREGULATION IN MH** | **33** |
| 2.10.1 | Possible role of mitochondria in the development of MH | 35 |
| 2.10.2 | Abnormalities of mitochondria and muscle fibre types | 37 |
| 2.10.3 | The mitochondrial calcium uniporter | 38 |
| 2.10.4 | The mitochondrial ryanodine receptor | 38 |
| **2.11** | **MOLECULAR GENETICS OF MH** | **40** |
| 2.11.1 | The porcine animal model | 40 |
| 2.11.2 | Other animal models | 41 |
| 2.11.3 | Identification of the RYR1 gene as a locus for MH | 42 |
| 2.11.3.1 | Structural organisation of the RYR1 gene | 43 |
| 2.11.3.2 | Allelic variants in the RYR1 gene and their association with MH | 43 |
| 2.11.3.3 | Specific mutations within the RYR1 gene | 46 |
| 2.11.3.3.1 | Mutation hotspot one of the RYR1 gene | 47 |
| 2.11.3.3.2 | Mutation hotspot two of the RYR1 gene | 52 |
| 2.11.3.3.3 | Mutation hotspot three of the RYR1 gene | 58 |
| 2.11.3.3.4 | Alterations observed outside of the RYR1 gene mutational hotspots. | 62 |
| 2.11.3.4 | Synonymous SNPs and the RYR1 gene | 65 |
| 2.11.4 | Genetic heterogeneity of MH | 66 |
| 2.11.5 | Epistatic model for MH | 68 |
| 2.11.6 | Discordance between phenotype and genotype | 69 |
| **2.12** | **PROPOSED GENETIC TESTING** | **70** |
| **2.13** | **OBJECTIVE OF THE RESEARCH PROGRAMME** | **71** |
| **2.14** | **AIM OF THE STUDY** | **71** |
| 2.14.1 | Specific aims | 72 |

**CHAPTER THREE**

**MATERIALS AND METHODS** ..... **73**

| | | |
|---|---|---|
| **3.1** | **PATIENT POPULATION** | **73** |
| 3.1.1 | Individuals from MH families included in this study | 74 |
| 3.1.1.1 | Malignant hyperthermia family MH101 | 75 |
| 3.1.1.2 | Malignant hyperthermia family MH102 | 76 |
| 3.1.1.3 | Malignant hyperthermia family MH103 | 77 |
| 3.1.1.4 | Malignant hyperthermia family MH104 | 77 |
| 3.1.1.5 | Malignant hyperthermia family MH105 | 78 |
| 3.1.1.6 | Malignant hyperthermia family MH108 | 79 |
| 3.1.1.7 | Malignant hyperthermia family MH111 | 80 |
| 3.1.1.8 | Malignant hyperthermia family MH113 | 80 |
| 3.1.1.9 | Malignant hyperthermia family MH114 | 81 |
| 3.1.1.10 | Malignant hyperthermia family MH115 | 82 |
| 3.1.1.11 | Malignant hyperthermia family MH122 | 82 |

| | | |
|---|---|---|
| 3.1.1.12 | Malignant hyperthermia family MH123 | 82 |
| 3.1.1.13 | Malignant hyperthermia family MH125 | 83 |
| 3.1.1.14 | Malignant hyperthermia family MH00630 | 83 |
| 3.1.1.15 | Malignant hyperthermia family MH00654 | 84 |
| **3.2** | **MUTATION ANALYSIS** | **84** |
| 3.2.1 | Primer design | 84 |
| **3.3** | **DNA EXTRACTION** | **90** |
| **3.4** | **DETERMINATION OF DNA CONCENTRATION** | **91** |
| **3.5** | **POLYMERASE CHAIN REACTION** | **92** |
| **3.6** | **AGAROSE GEL ELECTROPHORESIS** | **94** |
| **3.7** | **CHAIN TERMINATION SEQUENCING** | **94** |
| 3.7.1 | Detection of alterations in exon 1 of the RYR1 gene | 97 |
| 3.7.2 | Detection of alterations in exon 2 of the RYR1 gene | 98 |
| 3.7.3 | Detection of alterations in exon 3 of the RYR1 gene | 98 |
| 3.7.4 | Detection of alterations in exons 4 and 5 of the RYR1 gene | 99 |
| 3.7.5 | Detection of alterations in exons 6 and 7 of the RYR1 gene | 100 |
| 3.7.6 | Detection of alterations in exons 8 and 9 of the RYR1 gene | 101 |
| 3.7.7 | Detection of alterations in exons 10 and 11 of the RYR1 gene | 102 |
| 3.7.8 | Detection of alterations in exon 12 of the RYR1 gene | 103 |
| 3.7.9 | Detection of alterations in exon 13 of the RYR1 gene | 104 |
| 3.7.10 | Detection of alterations in exons 14, 15 and 16 of the RYR1 gene | 105 |
| 3.7.11 | Detection of alterations in exons 17 and 18 of the RYR1 gene | 106 |
| 3.7.12 | Detection of alterations in exon 19 of the RYR1 gene | 107 |
| 3.7.13 | Detection of alterations in exon 20 of the RYR1 gene | 107 |
| 3.7.14 | Detection of alterations in exons 21 and 22 of the RYR1 gene | 108 |
| 3.7.15 | Detection of alterations in exon 23 of the RYR1 gene | 109 |
| 3.7.16 | Detection of alterations in exon 24 of the RYR1 gene | 110 |
| 3.7.17 | Detection of alterations in exon 25 of the RYR1 gene | 110 |
| 3.7.18 | Detection of alterations in exons 26 and 27 of the RYR1 gene | 111 |
| 3.7.19 | Detection of alterations in exon 28 of the RYR1 gene | 112 |
| 3.7.20 | Detection of alterations in exon 29 of the RYR1 gene | 112 |
| 3.7.21 | Detection of alterations in exon 30 of the RYR1 gene | 113 |
| 3.7.22 | Detection of alterations in exon 31 of the RYR1 gene | 113 |
| 3.7.23 | Detection of alterations in exons 32 and 33 of the RYR1 gene | 114 |
| 3.7.24 | Detection of alterations in exons 34 of the RYR1 gene | 115 |
| 3.7.25 | Detection of alterations in exon 35 of the RYR1 gene | 116 |
| 3.7.26 | Detection of alterations in exons 36 and 37 of the RYR1 gene | 116 |
| 3.7.27 | Detection of alterations in exon 38 of the RYR1 gene | 117 |
| 3.7.28 | Detection of alterations in exon 39 of the RYR1 gene | 118 |
| 3.7.29 | Detection of alterations in exon 40 of the RYR1 gene | 119 |
| 3.7.30 | Detection of alterations in exons 41 and 42 of the RYR1 gene | 120 |
| 3.7.31 | Detection of alterations in exon 43 of the RYR1 gene | 121 |

3.7.32 Detection of alterations in exons 44 and 45 of the RYR1 gene..... 121
3.7.33 Detection of alterations in exon 46 of the RYR1 gene ..... 124
3.7.34 Detection of alterations in exon 47 of the RYR1 gene ..... 124
3.7.35 Detection of alterations in exons 48 and 49 of the RYR1 gene..... 125
3.7.36 Detection of alterations in exons 50, 51 and 52 of the RYR1 gene.... 126
3.7.37 Detection of alterations in exons 53 and 54 of the RYR1 gene..... 127
3.7.38 Detection of alterations in exons 55, 56 and 57 of the RYR1 gene.... 128
3.7.39 Detection of alterations in exon 58 of the RYR1 gene ..... 129
3.7.40 Detection of alterations in exons 59 and 60 of the RYR1 gene..... 130
3.7.41 Detection of alterations in exon 61 of the RYR1 gene ..... 130
3.7.42 Detection of alterations in exons 62 and 63 of the RYR1 gene..... 131
3.7.43 Detection of alterations in exon 64 of the RYR1 gene ..... 132
3.7.44 Detection of alterations in exon 65 of the RYR1 gene ..... 132
3.7.45 Detection of alterations in exon 66 of the RYR1 gene ..... 133
3.7.46 Detection of alterations in exon 67 of the RYR1 gene ..... 133
3.7.47 Detection of alterations in exons 68 and 69 of the RYR1 gene..... 134
3.7.48 Detection of alterations in exon 70 of the RYR1 gene ..... 135
3.7.49 Detection of alterations in exon 71 of the RYR1 gene ..... 135
3.7.50 Detection of alterations in exon 72 of the RYR1 gene ..... 136
3.7.51 Detection of alterations in exon 73 of the RYR1 gene ..... 136
3.7.52 Detection of alterations in exons 74, 75 and 76 of the RYR1 gene.... 137
3.7.53 Detection of alterations in exons 77 and 78 of the RYR1 gene..... 138
3.7.54 Detection of alterations in exons 79, 80 and 81 of the RYR1 gene.... 139
3.7.55 Detection of alterations in exon 82 of the RYR1 gene ..... 140
3.7.56 Detection of alterations in exon 83 of the RYR1 gene ..... 141
3.7.57 Detection of alterations in exon 84 of the RYR1 gene ..... 141
3.7.58 Detection of alterations in exons 85, 86 and 87 of the RYR1 gene.... 142
3.7.59 Detection of alterations in exon 88 of the RYR1 gene ..... 143
3.7.60 Detection of alterations in exon 89 of the RYR1 gene ..... 144
3.7.61 Detection of alterations in exon 90 of the RYR1 gene ..... 144
3.7.62 Detection of alterations in exon 91 of the RYR1 gene ..... 145
3.7.63 Detection of alterations in exon 92 of the RYR1 gene ..... 146
3.7.64 Detection of alterations in exon 93 of the RYR1 gene ..... 147
3.7.65 Detection of alterations in exon 94 of the RYR1 gene ..... 148
3.7.66 Detection of alterations in exon 95 of the RYR1 gene ..... 148
3.7.67 Detection of alterations in exon 96 of the RYR1 gene ..... 149
3.7.68 Detection of alterations in exon 97 of the RYR1 gene ..... 150
3.7.69 Detection of alterations in exons 98 and 99 of the RYR1 gene..... 151
3.7.70 Detection of alterations in exon 100 of the RYR1 gene ..... 151
3.7.71 Detection of alterations in exon 101 of the RYR1 gene ..... 152
3.7.72 Detection of alterations in exon 102 of the RYR1 gene ..... 153
3.7.73 Detection of alterations in exon 103 of the RYR1 gene ..... 154

3.7.74 Detection of alterations in exons 104 and 105 of the RYR1 gene ..... 155
3.7.75 Detection of alterations in exon 106 of the RYR1 gene ..... 156

**CHAPTER FOUR**
**RESULTS AND DISCUSSION ..... 157**
**4.1 ISOLATION OF GENOMIC DNA ..... 158**
**4.2 POLYMERASE CHAIN REACTION ..... 159**
4.2.1 Primer design ..... 159
4.2.2 PCR optimisation ..... 159
4.2.3 Artefacts observed in PCR amplified samples ..... 162
4.2.3.1 Amplification efficiency ..... 163
4.2.3.2 Background smear ..... 164
4.2.3.3 Secondary amplification ..... 165
4.2.3.4 Primer-dimers ..... 166
**4.3 AGAROSE GEL ELECTROPHORESIS ..... 167**
4.3.1 Artefacts observed on agarose gels ..... 167
4.3.1.1 Artefacts in the gel matrix ..... 168
4.3.1.2 Distortion of molecular weight marker ..... 168
4.3.1.3 Distortion of sample fragment ..... 169
4.3.1.4 Slanted fragments ..... 170
4.3.1.5 Barrier in agarose gel ..... 171
4.3.1.6 Ethidium bromide migration front ..... 171
**4.4 PCR PURIFICATION ..... 172**
**4.5 CHAIN TERMINATION SEQUENCING ..... 172**
4.5.1 Background peaks generated during sequencing ..... 173
4.5.2 Template quality ..... 174
**4.6 SEQUENCE PRECIPITATION ..... 174**
**4.7 HOTSPOT ONE OF THE RYR1 GENE ..... 175**
4.7.1 Exon 2 of the RYR1 gene ..... 175
4.7.2 Exon 3 of the RYR1 gene ..... 178
4.7.3 Exons 4 and 5 of the RYR1 gene ..... 180
4.7.4 Exons 6 and 7 of the RYR1 gene ..... 181
4.7.4.1 Synonymous substitution in the amplified region of exons 6 and 7 of the RYR1 gene ..... 185
4.7.4.1.1 SNP A11541G ..... 185
4.7.5 Exons 8 and 9 of the RYR1 gene ..... 186
4.7.6 Exons 10 and 11 of the RYR1 gene ..... 188
4.7.6.1 Synonymous substitution in the amplified region of exons 10 and 11 of the RYR1 gene ..... 191
4.7.6.1.1 SNP T15669C ..... 191
4.7.7 Exon 12 of the RYR1 gene ..... 192
4.7.8 Exon 13 of the RYR1 gene ..... 194

| | | |
|---|---|---|
| 4.7.8.1 | Synonymous substitutions in the amplified region of exon 13 of the RYR1 gene | 196 |
| 4.7.8.1.1 | SNP C19691T | 197 |
| 4.7.8.1.2 | SNP G19989A | 198 |
| 4.7.9 | Exons 14, 15 and 16 of the RYR1 gene | 198 |
| 4.7.9.1 | Synonymous substitutions in the amplified region of exons 14, 15 and 16 of the RYR1 gene | 202 |
| 4.7.9.1.1 | SNP G22443A | 203 |
| 4.7.9.1.2 | SNP G22476C | 204 |
| 4.7.10 | Exon 17 of the RYR1 gene | 204 |
| 4.7.10.1 | Synonymous substitutions in the amplified region of exons 17 and 18 of the RYR1 gene | 206 |
| 4.7.10.1.1 | SNP T24617C | 206 |
| 4.7.10.1.2 | SNP C24681G | 208 |
| 4.7.10.2 | The Arg614Cys alteration in the RYR1 gene | 208 |
| **4.8** | **HOTSPOT TWO OF THE RYR1 GENE** | **210** |
| 4.8.1 | Exon 39 of the RYR1 gene | 211 |
| 4.8.2 | Exon 40 of the RYR1 gene | 215 |
| 4.8.3 | Exons 41 and 42 of the RYR1 gene | 218 |
| 4.8.4 | Exon 43 of the RYR1 gene | 220 |
| 4.8.4.1 | The Arg2336His alteration in the RYR1 gene | 222 |
| 4.8.5 | Exons 44 and 45 of the RYR1 gene | 224 |
| 4.8.5.1 | Synonymous substitutions in the amplified region of exons 44 and 45 of the RYR1 gene | 230 |
| 4.8.5.1.1 | SNP C66864T | 230 |
| 4.8.5.1.2 | SNP C66875T | 231 |
| 4.8.5.2 | The Phe2364Val alteration in the RYR1 gene | 231 |
| 4.8.6 | Exon 46 of the RYR1 gene | 234 |
| **4.9** | **HOTSPOT THREE OF THE RYR1 GENE** | **237** |
| 4.9.1 | Exon 90 of the RYR1 gene | 237 |
| 4.9.2 | Exon 91 of the RYR1 gene | 239 |
| 4.9.2.1 | Synonymous substitution in the amplified region of exon 91 of the RYR1 gene | 241 |
| 4.9.2.1.1 | SNP C13317T | 242 |
| 4.9.3 | Exon 92 of the RYR1 gene | 242 |
| 4.9.4 | Exon 93 of the RYR1 gene | 244 |
| 4.9.5 | Exon 94 of the RYR1 gene | 246 |
| 4.9.5.1 | Synonymous substitution in the amplified region of exon 94 of the RYR1 gene | 247 |
| 4.9.5.1.1 | SNP C137591G | 247 |
| 4.9.6 | Exon 95 of the RYR1 gene | 248 |
| 4.9.7 | Exon 96 of the RYR1 gene | 250 |
| 4.9.8 | Exon 97 of the RYR1 gene | 252 |

| | | |
|---|---|---|
| 4.9.9 | Exons 98 and 99 of the RYR1 gene | 254 |
| 4.9.10 | Exon 100 of the RYR1 gene | 256 |
| 4.9.10.1 | The Thr4826Ile alteration in the RYR1 gene | 259 |
| 4.9.11 | Exon 101 of the RYR1 gene | 260 |
| 4.9.12 | Exon 102 of the RYR1 gene | 263 |
| 4.9.12.1 | The Gly4935Ser alteration in the RYR1 gene | 265 |
| 4.9.13 | Exon 103 of the RYR1 gene | 269 |
| 4.9.14 | Exon 104 of the RYR1 gene | 271 |
| **4.10** | **EXONS LOCATED OUTSIDE THE HOTSPOTS OF THE RYR1 GENE** | **273** |
| 4.10.1 | Exon 1 of the RYR1 gene | 273 |
| 4.10.2 | Exon 18 of the RYR1 gene | 275 |
| 4.10.3 | Exon 19 of the RYR1 gene | 277 |
| 4.10.3.1 | Synonymous substitutions in the amplified region of exon 19 of the RYR1 gene | 278 |
| 4.10.3.1.1 | SNP T25990G | 278 |
| 4.10.3.1.2 | SNP C26165T | 279 |
| 4.10.4 | Exon 20 of the RYR1 gene | 280 |
| 4.10.4.1 | Synonymous substitution in the amplified region of exon 20 of the RYR1 gene | 282 |
| 4.10.4.1.1 | SNP C27208T | 282 |
| 4.10.5 | Exons 21 and 22 of the RYR1 gene | 283 |
| 4.10.6 | Exon 23 of the RYR1 gene | 285 |
| 4.10.7 | Exon 24 of the RYR1 gene | 287 |
| 4.10.7.1 | Synonymous substitutions in the amplified region of exon 24 of the RYR1 gene | 288 |
| 4.10.7.1.1 | SNP G33064A | 288 |
| 4.10.7.1.2 | SNP C33100T | 289 |
| 4.10.8 | Exon 25 of the RYR1 gene | 290 |
| 4.10.9 | Exons 26 and 27 of the RYR1 gene | 291 |
| 4.10.9.1 | Synonymous substitution in the amplified region of exons 26 and 27 of the RYR1 gene | 293 |
| 4.10.9.1.1 | SNP C35941T | 293 |
| 4.10.10 | Exon 28 of the RYR1 gene | 294 |
| 4.10.11 | Exon 29 of the RYR1 gene | 296 |
| 4.10.12 | Exon 30 of the RYR1 gene | 297 |
| 4.10.13 | Exon 31 of the RYR1 gene | 299 |
| 4.10.14 | Exons 32 and 33 of the RYR1 gene | 300 |
| 4.10.15 | Exon 34 of the RYR1 gene | 302 |
| 4.10.15.1 | The Pro1787Leu alteration in the RYR1 gene | 305 |
| 4.10.16 | Exon 35 of the RYR1 gene | 308 |
| 4.10.17 | Exons 36 and 37 of the RYR1 gene | 309 |

4.10.17.1 Synonymous substitutions in the amplified region of exon 36 and 37 of the RYR1 gene ..... 310
4.10.17.1.1 SNP C57264T ..... 311
4.10.17.1.2 SNP A57545G ..... 312
4.10.18 Exon 38 of the RYR1 gene ..... 313
4.10.18.1 The Gly2060Cys alteration in the RYR1 gene ..... 314
4.10.19 Exon 47 of the RYR1 gene ..... 318
4.10.19.1 Synonymous substitutions in the amplified region of exon 47 of the RYR1 gene ..... 320
4.10.19.1.1 SNP G67777A ..... 320
4.10.19.1.2 SNP G67804A ..... 321
4.10.19.1.3 SNP C67861T ..... 322
4.10.19.1.4 SNP C67901G ..... 322
4.10.20 Exons 48 and 49 of the RYR1 gene ..... 322
4.10.21 Exons 50, 51 and 52 of the RYR1 gene ..... 325
4.10.21.1 Synonymous substitutions in the amplified region of exons 50, 51 and 52 of the RYR1 gene ..... 328
4.10.21.1.1 SNP G71171A ..... 328
4.10.21.1.2 SNP G71413A ..... 329
4.10.21.1.3 SNP A71494G ..... 330
4.10.21.1.4 SNP T71699C ..... 330
4.10.21.1.5 SNP T71771C ..... 331
4.10.22 Exons 53 and 54 of the RYR1 gene ..... 332
4.10.22.1 Synonymous substitution in the amplified region of exon 53 of the RYR1 gene ..... 334
4.10.22.1.1 SNP G72236A ..... 334
4.10.23 Exons 55, 56 and 57 of the RYR1 gene ..... 335
4.10.23.1 Synonymous substitutions in the amplified region of exons 55, 56 and 57 of the RYR1 gene ..... 337
4.10.23.1.1 SNP T73251C ..... 337
4.10.23.1.2 SNP C73337T ..... 338
4.10.23.1.3 SNP T73475G ..... 339
4.10.23.1.4 SNP T73584C ..... 340
4.10.23.1.5 SNP G73720C ..... 341
4.10.23.1.6 SNP T73870A ..... 341
4.10.24 Exon 58 of the RYR1 gene ..... 342
4.10.25 Exons 59 and 60 of the RYR1 gene ..... 343
4.10.26 Exon 61 of the RYR1 gene ..... 345
4.10.27 Exons 62 and 63 of the RYR1 gene ..... 346
4.10.27.1 Synonymous substitution in the amplified region of exon 62 and 63 of the RYR1 gene ..... 348
4.10.27.1.1 SNP A78986G ..... 348
4.10.28 Exon 64 of the RYR1 gene ..... 349

4.10.28.1 Synonymous substitution in the amplified region of exon 64 of the RYR1 gene ..... 351
4.10.28.1.1 SNP C81823T ..... 351
4.10.29 Exon 65 of the RYR1 gene ..... 352
4.10.30 Exon 66 of the RYR1 gene ..... 353
4.10.30.1 Synonymous substitution in the amplified region of exon 66 of the RYR1 gene ..... 355
4.10.30.1.1 SNP G84264A ..... 355
4.10.31 Exon 67 of the RYR1 gene ..... 355
4.10.31.1 Synonymous substitution in the amplified region of exon 67 of the RYR1 gene ..... 357
4.10.31.1.1 SNP C10218T ..... 357
4.10.32 Exons 68 and 69 of the RYR1 gene ..... 358
4.10.33 Exon 70 of the RYR1 gene ..... 360
4.10.34 Exon 71 of the RYR1 gene ..... 361
4.10.35 Exon 72 of the RYR1 gene ..... 363
4.10.36 Exon 73 of the RYR1 gene ..... 364
4.10.36.1 The Glu3583Gln alteration in the RYR1 gene ..... 365
4.10.37 Exons 74, 75 and 76 of the RYR1 gene ..... 367
4.10.38 Exons 77 and 78 of the RYR1 gene ..... 369
4.10.39 Exons 79, 80 and 81 of the RYR1 gene ..... 371
4.10.40 Exon 82 of the RYR1 gene ..... 373
4.10.40.1 Synonymous substitution in the amplified region of exon 82 of the RYR1 gene ..... 374
4.10.40.1.1 SNP A11547G ..... 374
4.10.41 Exon 83 of the RYR1 gene ..... 375
4.10.42 Exon 84 of the RYR1 gene ..... 376
4.10.43 Exons 85, 86 and 87 of the RYR1 gene ..... 378
4.10.44 Exon 88 of the RYR1 gene ..... 380
4.10.45 Exon 89 of the RYR1 gene ..... 382
4.10.46 Exon 105 of the RYR1 gene ..... 383
4.10.47 Exon 106 of the RYR1 gene ..... 384
**4.11 SUMMARY OF MUTATION ANALYSIS RESULTS ..... 386**

**CHAPTER FIVE**
**CONCLUSIONS ..... 391**
**5.1 PROPOSED MH MODEL ..... 393**
**5.2 MULTIFACTORIAL MODE OF INHERITANCE ..... 396**
5.2.1 MH equivalent disorders in pigs ..... 396
5.2.1.1 PSS and PSES in pigs ..... 397
5.2.1.2 Modes of inheritance ..... 398
5.2.2 Genetic heterogeneity and MH ..... 399

5.2.2.1 Locus heterogeneity..... 399
5.2.2.2 Allelic heterogeneity..... 400
5.2.2.3 Locus and allelic heterogeneity correlated to the MH phenotype..... 401
5.2.3 Role of the RyR1 protein in other tissues..... 402
**5.3 EVIDENCE OF EPIGENETIC MODIFICATIONS IN MH SUSCEPTIBILITY..... 404**
5.3.1 Clinical expression of the MH phenotype and environmental factors. 405
**5.4 ROLE OF EPISTASIS AND MH SUSCEPTIBILITY..... 406**
**5.5 IMPLICATIONS OF MH MODEL..... 407**
5.5.1 Clinical features and diagnosis..... 407
5.5.2 Discordance..... 408
5.5.2.1 Discordance and the MH model..... 408
5.5.3 Population specificity of RYR1 mutations..... 409
5.5.3.1 Location and distribution of RYR1 mutations..... 410
5.5.3.2 Population specificity of RYR1 mutations and the MH model..... 410
5.5.3.2.1 Genetic factors and population specificity of RYR1 mutations..... 410
5.5.3.2.2 Environmental factors and population specificity of RYR1 mutations. 411
5.5.4 MH susceptibility without RYR1 alterations..... 411
**5.6 DIAGNOSTIC SERVICE FOR MH SUSCEPTIBILITY..... 413**
**5.7 FUTURE DEVELOPMENTS..... 416**

CHAPTER SIX
REFERENCES..... 419
6.1 GENERAL REFERENCES..... 419
6.2 ELECTRONIC REFERENCES..... 439

APPENDIX A
REPORTED FUNCTIONAL CHARACTERISATION STUDIES FOR RYR1 GENE ALTERATIONS..... 443

APPENDIX B
SNPs IDENTIFIED IN THE CODING REGION OF THE RYR1 GENE. 447

APPENDIX C
SNPs IDENTIFIED IN THE INTRON SEQUENCE OF THE RYR1 GENE..... 451

# 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: $10^{-12}$ |
| ® | registered trademark |
| ™ | trademark |
| ■ / ● | male/female: tested susceptible to malignant hyperthermia with the IVCT |
| ▨ / ⊘ | male/female: tested negative for malignant hyperthermia with the IVCT |
| ▩ / ⊗ | male/female: malignant hyperthermia equivocal |
| □ / ○ | male/female: never tested, malignant hyperthermia status unknown |
| ⊠ / ⊘ | male/female: deceased |
| // | divorced |
| ← | proband |

## Abbreviations are listed in alphabetical order.

| | |
|---|---|
| I to IV | homologous domains of the $\alpha_1$-subunit of DHPR |
| A | adenine (in DNA sequence) |
| a | adenine |
| $A_{260}$ | absorbance of sample at 260 nm |
| $A_{260}/A_{280}$ | ratio of absorbency measured at 260 nm and 280 nm |
| Ala | alanine |
| AMP | adenosine monophosphate |
| AmpliTaq DNA polymerase | AmpliTaq®[1] DNA polymerase FS: variant of *Taq* DNA polymerase |
| APl | acid phosphatase deficiency |
| Arg | arginine |
| Asn | asparagine |
| Asp | aspartate |
| ATP | adenosine triphosphate |
| ATPase | adenosine triphosphatase |
| 1B5 | embryonic stem cell line that has undergone homologous recombination disrupting the *ryr* gene at nucleotide 840 in exon 10 |
| $Ba^{2+}$ | barium ion |
| BAY K 8644 | 1,4 dihydro-2,6-dimethyl-5-nitro-4-[2(triflouromethyl)phenyl]pyridine-3-carboxylic acid methyl ester |
| BBS | Bardet-Biedl syndrome |
| BLAST | Basic Local Alignment Search Tool |
| Bmax | density |
| BMD | Becker muscular dystrophy |
| boric acid | boracic acid: $H_3BO_3$ |
| bp | base pair |
| C | cytosine (in DNA sequence) |
| c | cytosine |
| C-terminal | denotes the carboxy terminus of a polypeptide |
| $Ca^{2+}$ | calcium ion |
| $Ca^{2+}$-ATPase | calcium adenosine triphosphatase |

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

| | |
|---|---|
| CACNA1S | DHPR $\alpha_1$-subunit gene |
| CACNL2A | DHPR $\alpha_2/\delta$-subunit gene |
| caffeine | 3,7-dihydro-1,3,7-trimethyl-1H-purine-2,6-dione: $C_8H_{10}N_4O_2$ |
| CaM | calmodulin |
| CCD | central core disease |
| cDNA | complementary DNA |
| *C.elegans* | *Caenorhabditis elegans* |
| CFTR | cystic fibrosis transmembrane regulator |
| CHCT | caffeine halothane contracture test |
| chlorocresol | 4-chloro-*m*-cresol |
| CICR | $Ca^{2+}$ induced $Ca^{2+}$ release |
| CK | creatine kinase |
| CLCN1 | chloride channel 1 gene |
| cM | centimorgan |
| cm | centimetre: $10^{-2}$ metre |
| CNM | centronuclear myopathy |
| $CO_2$ | carbon dioxide |
| CpG | dinucleotide with a cytosine at the 5' end connected by a phosphodiester bond to a guanine at the 3' end |
| CSQ | calsequestrin |
| Cys | cysteine |
| D1 | divergent region one |
| D2 | divergent region two |
| D3 | *divergent region three* |
| Da | dalton |
| dantrolene | 1-[[[5-(4-nitrophenyl)-2-furanyl]methylene]amino]-2,4-imidazolidinedione sodium salt: $C_{14}H_{10}N_4O_5$ |
| dbSNP | SNP database |
| DCC-5™ | Zymo research DNA clean and concentrator-5™[1] kit |
| *Dde* I | restriction endonuclease from *Desulfovibrio desulfuricans*, with recognition site: 5'-C↓TNAG-3' |
| $ddH_2O$ | double distilled water |
| ddNTP | 2',3'-dideoxynucleotide triphosphate |
| del | deletion |
| DHP | 1,4-dihydropyridine derivative |
| DHPLC | denaturing high performance liquid chromatography |
| DHPR | dihydropyridine receptor |
| DM | myotonic dystrophy |
| DMD | Duchenne muscular dystrophy |
| DMSO | dimethyl sulfoxide: $Me_2SO$ |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleotide triphosphate |
| DTCAT | 3,5-di-*tert*-butylcatechol |
| DTT | dithiothreitol: theo-1,4-dimercapto-2,3-butanediol: $C_4H_{10}O_2S_2$ |
| E-C | excitation-contraction |
| ECG | electrocardiogram |
| EDTA | ethylene diamine tetra-acetic acid: $C_{10}H_{16}N_2O_8$ |
| e.g. | *exempli gratia* |
| EMHG | European MH Group |
| ESE | exonic splicing enhancers |
| *et al.* | *et alii*: and others |
| EtBr | ethidium bromide: 2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide: $C_{21}H_{20}BrN_3$ |
| ETDT | extended transmission disequilibrium test |
| EtOH | ethanol: $CH_3CH_2OH$ |
| exo-NADH | extramitochondrial nicotinamide adenine dinucleotide |
| F | forward primer |
| FastStart *Taq*® | FastStart *Taq*®[2] DNA polymerase |
| 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 |
| FG1 buffer | lysis buffer |

[1] DNA Clean and Concentrator -5™ is a registered trademark of Zymo Research Corporation, Orange, CA, USA.
[2] FastStart® Taq DNA polymerase is a registered trademark of Roche Diagnostics GmbH, Mannheim, Germany.

| | |
|---|---|
| FG2 buffer | denaturation buffer |
| FG3 buffer | hydration buffer |
| formamide | carbamaldehyde: $CH_3NO$ |
| FKBP | FK506-binding protein |
| FKBP12 | immunophilin (cytosolic receptor) FK506-binding protein |
| $fmol.mg^{-1}$ | femtomole per milligram |
| $\Delta G$ | Gibbs free energy: indicating nucleic acid duplex stability |
| 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 |
| Gln | glutamine |
| Glu | glutamate |
| Gly | glycine |
| glycerol | propane-1,2,3-triol: $C_3H_5(OH)_3$ |
| Go *Taq*® Flexi | Go *Taq*®[2] Flexi DNA polymerase |
| GPI | glucose phosphate isomerase |
| h | hours |
| $^3H$ | tritium |
| $H^+$ | hydrogen ion |
| 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 |
| *Hga* I | restriction endonuclease from *Haemophilus gallinarum*, with recognition site: 5'-CACGC(N)$_5$↓-3' |
| His | histidine |
| $H_2O$ | water |
| HSL | hormone sensitive lipase |
| HypoPP | hypokalaemic periodic paralysis |
| ID | identification |
| IDT | Integrated DNA Technology programme |
| i.e. | *id est* (that is) |
| Ile | isoleucine |
| IMM | inner mitochondrial membrane |
| $InsP_3$ | inositol-1,4,5-triphosphate |
| $InsP_3R$ | inositol-1,4,5-triphosphate receptor |
| IV | intravenous |
| IVCT | *in vitro* contracture test |
| JFP | junctional face protein |
| $K^+$ | potassium ion |
| kb | kilo ($10^3$) base pair |
| $kcal.mol^{-1}$ | kilocalorie per mole |
| KCl | potassium chloride |
| $K_d$ | equilibrium constant for dissociation |
| kDa | kilodalton |
| kg | kilogram |
| $L.min^{-1}$ | litre per minute |
| LD | linkage disequilibrium |
| LE | low electroendosmosis |
| Leu | leucine |
| lod | logarithm of the odds |
| lod score | a measure of the likelihood of genetic linkage between loci |
| L-type | local type |
| Lys | lysine |
| µg | microgram |

[1] GenBank® is a registered trademark of the National Institute of Health and Human Services for the Genetic Sequence Data Bank, Bethesda, MD, USA.
[2] Promega Go *Taq*® Flexi DNA polymerase is a registered trademark of the Promega Corporation, Madison, WI, USA.

| | |
|---|---|
| $\mu g.mL^{-1}$ | microgram per millilitre |
| µL | microlitre |
| µM | micromolar |
| M | molar |
| M1-M10 | transmembrane segments, numbers 1 to 10 |
| MC | myotonia congenita |
| Met | *methionine* |
| $mEQ.kg^{-1}$ | milliequivalent per kilogram |
| Mfold | multiple fold programme |
| mg | milligram |
| $mg.kg^{-1}$ | milligram per kilogram |
| $Mg^{2+}$ | 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* |
| MmD | multi-minicore disease |
| MMR | masseter muscle rigidity |
| mN | millinormal |
| $Mn^{2+}$ | manganese ion |
| mRNA | messenger ribonucleic acid |
| mRyR | mitochondrial RyR |
| N-terminal | denotes the amino terminus of a polypeptide |
| $Na^+$ | sodium ion |
| $Na_2EDTA$ | disodium EDTA |
| $NaHCO_3$ | sodium bicarbonate |
| NaCl | sodium chloride |
| NADH | nicotinamide adenine dinucleotide |
| NAMHG | North American MH Group |
| NaOAc | sodium acetate |
| NCBI | National Centre for Biotechnology Information, USA. |
| NCX | $Na^+/Ca^{2+}$ exchanges |
| $NH_2$ | amino group |
| ng | nanogram: $10^{-9}$ gram |
| $ng.\mu L^{-1}$ | nanogram per microlitre |
| nm | nanometre: $10^{-9}$ metre |
| Nm | Newton metre |
| NMBA | neuromuscular blocking agent |
| NMS | neuroleptic malignant syndrome |
| No. | number |
| NR | no response |
| $O_2$ | oxygen |
| orange G | 7-hydroxy-8-phenylazo-1,3-naphthalenedisulfonic acid: $C_{16}H_{10}N_2O_7S_2Na_2$ |
| p | short arm of chromosome |
| PB buffer | binding buffer |
| PE buffer | wash buffer |
| Phase 1 | previous study conducted in the ongoing MH research programme at the Centre for Genome Research |
| Phase 2 | previous study conducted in the ongoing MH research programme at the Centre for Genome Research |
| Phase 3 | study presented here, which forms part of the extended MH research |

| | |
|---|---|
| | programme at the Centre for Genome Research |
| $pCO_2$ | carbon dioxide partial pressure |
| PCR | polymerase chain reaction |
| pH | a measure of acidity: numerically equal to the negative logarithm of $H^+$ concentration expressed in molarity |
| Phe | phenylalanine |
| $PLA_2$ | phospholipase $A_2$ |
| PMCA | $Ca^{2+}$-ATPase pump |
| pmol | picomol: $10^{-12}$ mole |
| Pro | proline |
| PSES | pale, soft, exudative pork syndrome |
| PSS | porcine stress syndrome |
| q | long arm of chromosome |
| R | reverse primer |
| RFLP | restriction fragment length polymorphism |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| *αryr* | RYR isoform of turkey |
| RYR1 | RYR expressed in human skeletal muscle |
| RYR2 | RYR expressed in human cardiac muscle |
| RYR3 | RYR expressed in human brain |
| *ryr1* | RYR1 gene expressed in animals |
| RyR1 | skeletal muscle type one ryanodine receptor protein |
| s | seconds |
| S | transmembrane α helix segment of the $\alpha_1$-subunit |
| SCN4A | sodium channel α-subunit gene |
| SDS | sodium dodecyl sulphate: $C_{12}N_{25}NaO_4S$ |
| SERCA | SR $Ca^{2+}$-ATPase |
| Ser | serine |
| SIDS | sudden infant death syndrome |
| SNP(s) | single nucleotide polymorphism(s) |
| snRNA | small nuclear RNA |
| $Sr^{2+}$ | strontium ion |
| SR | sarcoplasmic reticulum |
| Super-therm® | Super-therm®[1] polymerase |
| 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* |
| TATA | promoter element consisting of the following sequence 5'-TATA-3' |
| TBE | Tris® borate-EDTA buffer |
| TC | terminal cisternae |
| Thr | threonine |
| $T_m$ | melting temperature |
| Tn | troponin |
| TnC | subunit of troponin |
| TRI | triadin |
| Tris[2] | 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[3] | Triton X-100®[2]: octylphenolpoly(ethylene-glycoether)$_n$: $C_{34}H_{62}O_{11}$. for n = 10 |
| tRNA | transfer ribonucleic acid |
| Trp | tryptophan |
| t-tubule | transverse tubule |
| Tyr | tyrosine |
| U | units |
| UK | United Kingdom |
| *unc* | mutant alleles of *C. elegans* |

[1] Super-therm® polymerase, is a registered trademark of JMR Holdings, Sevenoaks, Kent, UK.
[2] Tris® is the registered trademark of the United States Biochemical Corporation, Cleveland, OH, USA.
[3] Triton X-100® is the registered trademark of Rohm & Haas Company, Philadelphia, PA, USA.

| | |
|---|---|
| USA | United States of America |
| UTR | untranslated region |
| UV | ultraviolet |
| Val | valine |
| V | volts |
| $V.cm^{-1}$ | volts per centimetre |
| vol % | percent volume per volume |
| w/v | weight/volume |
| x *g* | gravitational acceleration |

# LIST OF EQUATIONS


| Equation No. | Title of Equation | Page |
|---|---|---|
| 3.1 | Spectrophotometric conversion for calculating the concentration of nucleic acids from the absorbance at 260 nm..... | 92 |

# LIST OF FIGURES


| Figure No. | Title of Figure | Page |
|---|---|---|
| 2.1 | Schematic representation of DHPR-RyR1 interactions in excitation and contraction | 24 |
| 2.2 | Localisation of RYR1 hotspots, binding sites, regions and domains | 44 |
| 2.3 | Localisation of RYR1 alterations observed in MH or CCD patients | 47 |
| 3.1 | Pedigree of family MH101 | 75 |
| 3.2 | Excerpt from pedigree MH102 | 76 |
| 3.3 | Pedigree of family MH103 | 77 |
| 3.4 | Excerpt from pedigree MH104 | 78 |
| 3.5 | Excerpt from pedigree MH105 | 79 |
| 3.6 | Pedigree of family MH108 | 79 |
| 3.7 | Pedigree of family MH111 | 80 |
| 3.8 | Excerpt from pedigree MH113 | 80 |
| 3.9 | Pedigree of family MH114 | 81 |
| 3.10 | Pedigree of family MH115 | 82 |
| 3.11 | Pedigree of family MH123 | 82 |
| 3.12 | Pedigree of family MH125 | 83 |
| 4.1 | Photographic representation of the variation in amplification efficiency observed between amplified PCR products encompassing exons 104 and 105 | 163 |
| 4.2 | Photographic representation of the variation in amplification efficiency observed between exonic regions for amplified PCR products encompassing exons 3 and 46, respectively | 164 |
| 4.3 | Photographic representation of the background smear observed for amplified PCR products encompassing exon 43 | 165 |
| 4.4 | Photographic representation of secondary amplification observed for amplified PCR products encompassing exon 12 | 166 |
| 4.5 | Photographic representation of primer-dimers observed for amplified PCR products encompassing exon 23 | 167 |
| 4.6 | Photographic representation of an artefact in the gel matrix observed for amplified PCR products encompassing exons 6 and 7 | 168 |
| 4.7 | Photographic representation of molecular weight marker distortion observed for amplified PCR products encompassing exon 58 | 169 |
| 4.8 | Photographic representation of sample fragment distortion observed for amplified PCR products encompassing exon 39 | 170 |
| 4.9 | Photographic representation of slanted fragments observed for amplified PCR products encompassing exons 26 and 27 | 170 |
| 4.10 | Photographic representation of a barrier observed for amplified PCR products encompassing exons 6 and 7 | 171 |
| 4.11 | Photographic representation of the ethidium bromide front observed for amplified PCR products encompassing exon 70 | 172 |

4.12 Representative electropherogram with background peaks..... 174
4.13 Photographic representation of amplified PCR products encompassing exon 2..... 176
4.14 Representative electropherogram of exon 2 indicating the nucleotide position of the Asp17del..... 176
4.15 Representative electropherogram of exon 2 indicating the nucleotide positions of the Cys35Arg and Arg44His alterations..... 177
4.16 Photographic representation of amplified PCR products encompassing exon 3..... 178
4.17 Representative electropherogram of exon 3 indicating the nucleotide positions of the Asp60Asn and Ser71Tyr alterations..... 179
4.18 Photographic representation of amplified PCR products encompassing exons 4 and 5..... 180
4.19 Representative electropherogram of exon 4 indicating the nucleotide position of the Arg109Trp alteration..... 181
4.20 Representative electropherogram illustrating a portion of the amplified region of exon 5..... 181
4.21 Photographic representation of amplified PCR products encompassing exons 6 and 7..... 182
4.22 Representative electropherogram of exon 6 indicating the nucleotide positions of the Gln155Lys, Arg156Lys, Glu160Gly, Arg163Leu, Arg163Cys, Gly165Arg, Asp166Asn, Asp166Gly, Arg177Cys and Tyr178Cys alterations..... 183
4.23 Representative electropherogram illustrating a portion of the amplified region of exon 7..... 184
4.24 Representative electropherograms indicating the A11541G SNP observed in exon 7 of the RYR1 gene..... 185
4.25 Photographic representation of amplified PCR products encompassing exons 8 and 9..... 186
4.26 Representative electropherogram of exon 8 indicating the nucleotide positions of the Gly215Glu, Val218Ile and Asp227Val alterations..... 187
4.27 Representative electropherogram of exon 9 indicating the nucleotide position of the Gly248Arg alteration..... 188
4.28 Photographic representation of amplified PCR products encompassing exons 10 and 11..... 188
4.29 Representative electropherogram of exon 10 indicating the nucleotide position of the Arg316Leu alteration..... 189
4.30 Representative electropherogram of exon 11 indicating the nucleotide positions of the Arg328Trp, Gly341Arg and Arg367Gln alterations..... 190
4.31 Representative electropherograms indicating the T15669C SNP observed in exon 11 of the RYR1 gene..... 192
4.32 Photographic representation of amplified PCR products encompassing exon 12..... 193
4.33 Representative electropherogram of exon 12 indicating the nucleotide positions of the Arg401Cys, Arg401Ser, Arg401His, Met402Ile and Ile403Met alterations..... 194
4.34 Photographic representation of amplified PCR products encompassing exon 13..... 195
4.35 Representative electropherogram of exon 13 indicating the nucleotide positions of the Ser427Leu, Arg471Cys and Gln474His alterations..... 196

4.36 Representative electropherogram indicating the C19691T SNP observed in the intron sequence between exons 12 and 13 of the RYR1 gene..... 197

4.37 Representative electropherograms indicating the G19989A SNP observed in the intron sequence between exons 13 and 14 of the RYR1 gene..... 198

4.38 Photographic representation of amplified PCR products encompassing exons 14, 15 and 16..... 199

4.39 Representative electropherogram of exon 14 indicating the nucleotide positions of the Glu512Lys, Tyr522Ser and Tyr522Cys alterations..... 200

4.40 Representative electropherogram of exon 15 indicating the nucleotide positions of the Arg533His, Arg533Cys and Arg552Trp alterations..... 201

4.41 Representative electropherogram illustrating a portion of the amplified region of exon 16..... 202

4.42 Representative electropherograms indicating the G22443A and G22476C SNPs observed in exon 15 and the intron sequence between exons 15 and 16 of the RYR1 gene, respectively..... 204

4.43 Photographic representation of amplified PCR products encompassing exons 17 and 18..... 205

4.44 Representative electropherogram of exon 17 indicating the nucleotide positions of the Arg614Cys and Arg614Leu alterations..... 206

4.45 Representative electropherograms indicating the T24617C and C24681G SNPs observed in the intron sequence between exons 17 and 18 of the RYR1 gene..... 207

4.46 Representative electropherogram indicating the Arg614Cys alteration observed in exon 17..... 209

4.47 Photographic representation of amplified PCR products encompassing exon 39..... 212

4.48 Representative electropherogram of exon 39 indicating the nucleotide positions of the Met2101Leu, Val2217Leu and Asp2129Glu alterations..... 213

4.49 Representative electropherogram of exon 39 indicating the nucleotide positions of the Arg2163Cys, Arg2163Pro, Arg2163His, Val2168Met and Ile2182Phe alterations..... 214

4.50 Photographic representation of amplified PCR products encompassing exon 40..... 216

4.51 Representative electropherogram of exon 40 indicating the nucleotide positions of the Ala2200Val, Thr2206Met, Thr2206Arg, Val2210Phe, Val2212Asp and Val2214Ile alterations..... 217

4.52 Photographic representation of amplified PCR products encompassing exons 41 and 42..... 219

4.53 Representative electropherogram illustrating a portion of the amplified region of exon 41..... 219

4.54 Representative electropherogram of exon 42 indicating the nucleotide positions of the Val2280Ile and Asn2283His alterations..... 220

4.55 Photographic representation of amplified PCR products encompassing exon 43..... 221

4.56 Representative electropherogram of exon 43 indicating the nucleotide positions of the Arg2336Gln and Asn2342Ser alterations..... 221

4.57 Representative electropherograms indicating the Arg2336His alteration observed in exon 43..... 223

4.58 Photographic representation of amplified PCR products encompassing exons 44 and 45..... 225

4.59 Representative electropherogram of exon 44 indicating the nucleotide positions of the Glu2344Asp, Val2346Met, Glu2347del, Glu2348Gly, Ala2350Thr, Arg2355Cys, Glu2362Gly, Phe2364Val, Ala2367Thr, Gly2375Ala and Pro2366Arg alterations..... 227

4.60 Representative electropherogram of exon 45 indicating the nucleotide positions of the Ala2428Thr, Met2423Lys, Asp2431Asn, Gly2434Arg, Arg2435His, Arg2435Leu, Ala2436Val and Glu2439Asp alterations..... 228

4.61 Representative electropherograms indicating the C66854T and C66875T SNPs observed in exon 45 of the RYR1 gene..... 231

4.62 Representative electropherograms indicating the Phe2364Val alteration observed in exon 44..... 232

4.63 Photographic representation of amplified PCR products encompassing exon 46..... 235

4.64 Representative electropherogram of exon 46 indicating the nucleotide positions of the Arg2452Trp, Arg2452Gln, Ile2435Thr, Arg2454Cys, Arg2454His, Arg2458Cys and Arg2458His alterations..... 236

4.65 Photographic representation of amplified PCR products encompassing exon 90..... 238

4.66 Representative electropherogram of exon 90 indicating the nucleotide positions of the Asn4119Tyr and Arg4136Ser alterations..... 239

4.67 Photographic representation of amplified PCR products encompassing exon 91..... 240

4.68 Representative electropherogram of exon 91 indicating the nucleotide positions of the Val4234Leu and Glu4283Val alterations..... 241

4.69 Representative electropherogram indicating the C13317T SNP observed in exon 91 of the RYR1 gene..... 242

4.70 Photographic representation of amplified PCR products encompassing exon 92..... 243

4.71 Representative electropherogram illustrating a portion of the amplified region of exon 92..... 244

4.72 Photographic representation of amplified PCR products encompassing exon 93..... 244

4.73 Representative electropherogram of exon 93 indicating the nucleotide position of the Arg4549Gln alteration..... 245

4.74 Photographic representation of amplified PCR products encompassing exon 94..... 246

4.75 Representative electropherogram of exon 94 indicating the nucleotide position of the Leu4568Pro alteration..... 247

4.76 Representative electropherogram indicating the C137591G SNP observed in exon 94 of the RYR1 gene..... 248

4.77 Photographic representation of amplified PCR products encompassing exon 95..... 249

4.78 Representative electropherogram of exon 95 indicating the nucleotide positions of the Tyr4631Asn, Glu4634Lys, Thr4637Ala, Thr4637Ile, Gly4638Ser, Gly4638Met, Arg4645Gln, Leu4647del, Ser4648del, His4651Pro and Leu4665Pro alterations..... 250

4.79 Photographic representation of amplified PCR products encompassing exon 96..... 251

4.80 Representative electropherogram of exon 96 indicating the nucleotide positions of the Pro4888Ser, Phe4684Ser and Thr4709Met alterations..... 252

4.81 Photographic representation of amplified PCR products encompassing exon 97 ..... 253

4.82 Representative electropherogram of exon 97 indicating the nucleotide position of the Lys4724Gln alteration ..... 254

4.83 Photographic representation of amplified PCR products encompassing exons 98 and 99 ..... 255

4.84 Representative electropherogram of exon 98 indicating the nucleotide positions of the Tyr4733Asp, Arg4737Trp and Arg4737Gln alterations ..... 255

4.85 Representative electropherogram illustrating a portion of the amplified region of exon 99 ..... 256

4.86 Photographic representation of amplified PCR products encompassing exon 100 ..... 257

4.87 Representative electropherogram of exon 100 indicating the nucleotide positions of the Leu4793Pro, Tyr4796Cys, Phe4808Asn, Leu4814Phe, Ile4817Phe, Leu4824Pro, Arg4825Cys and Thr4826Ile alterations ..... 258

4.88 Representative electropherogram indicating the Thr4826Ile alteration observed in exon 100 ..... 259

4.89 Photographic representation of amplified PCR products encompassing exon 101 ..... 261

4.90 Representative electropherogram of exon 101 indicating the nucleotide positions of the Leu4838Val, Ala4846Val, Val4849Ile, Asn4858Asp, Phe4860del, Arg4861Cys, Arg4861His, Tyr4864Cys, Lys4876Arg and Met4880Thr alterations ..... 262

4.91 Photographic representation of amplified PCR products encompassing exon 102 ..... 264

4.92 Representative electropherogram of exon 102 indicating the nucleotide positions of the Gly4891Arg, Arg4893Trp, Arg4893Gln, Arg4893Pro, Ala4894Thr, Gly4897Val, Ile4898Thr, Gly4899Arg, Gly4899Glu, Ala4906Val, Arg4914Gly, Arg4914Thr, Thr4920Asn and Phe4921Ser alterations ..... 265

4.93 Representative electropherograms indicating the Gly4935Ser alteration observed in exon 102 ..... 266

4.94 A schematic representation of the normal and abnormal splicing at the intron-exon boundary of exon 102 ..... 268

4.95 Photographic representation of amplified PCR products encompassing exon 103 ..... 270

4.96 Representative electropherogram of exon 103 indicating the nucleotide positions of the Ile4938Met, Asp4939Glu, Ala4940Thr and Gly4942Val alterations ..... 271

4.97 Photographic representation of amplified PCR products encompassing exons 104 and 105 ..... 272

4.98 Representative electropherogram of exon 104 indicating the nucleotide positions of the Phe4960Tyr and Pro4973Leu alterations ..... 272

4.99 Photographic representation of amplified PCR products encompassing exon 1 ..... 274

4.100 Representative electropherogram of exon 1 indicating the nucleotide position of the Leu13Arg alteration ..... 275

4.101 Representative electropherogram illustrating a portion of the amplified region of exon 18 ..... 276

4.102 Photographic representation of amplified PCR products encompassing exon 19 ..... 277
4.103 Representative electropherogram of exon 19 indicating the nucleotide position of the Asn759Asp alteration ..... 278
4.104 Representative electropherograms indicating the T25990G SNP observed in the intron sequence between exons 18 and 19 of the RYR1 gene ..... 279
4.105 Representative electropherograms indicating the C26165T SNP observed in exon 19 of the RYR1 gene ..... 280
4.106 Photographic representation of amplified PCR products encompassing exon 20 ..... 281
4.107 Representative electropherogram illustrating a portion of the amplified region of exon 20 ..... 282
4.108 Representative electropherograms indicating the C27208T SNP observed in the intron sequence between exons 19 and 20 of the RYR1 gene ..... 283
4.109 Photographic representation of amplified PCR products encompassing exons 21 and 22 ..... 284
4.110 Representative electropherogram illustrating a portion of the amplified region of exon 21 ..... 284
4.111 Representative electropherogram illustrating a portion of the amplified region of exon 22 ..... 285
4.112 Photographic representation of amplified PCR products encompassing exon 23 ..... 286
4.113 Representative electropherogram illustrating a portion of the amplified region of exon 23 ..... 287
4.114 Photographic representation of amplified PCR products encompassing exon 24 ..... 287
4.115 Representative electropherogram illustrating a portion of the amplified region of exon 24 ..... 288
4.116 Representative electropherograms indicating the G33064A and C33100T SNPs observed in exon 24 of the RYR1 gene ..... 289
4.117 Photographic representation of amplified PCR products encompassing exon 25 ..... 290
4.118 Representative electropherogram illustrating a portion of the amplified region of exon 25 ..... 291
4.119 Photographic representation of amplified PCR products encompassing exons 26 and 27 ..... 292
4.120 Representative electropherogram illustrating a portion of the amplified region of exon 26 ..... 292
4.121 Representative electropherogram illustrating a portion of the amplified region of exon 27 ..... 293
4.122 Representative electropherograms indicating the C35941T SNP observed in exon 26 of the RYR1 gene ..... 294
4.123 Photographic representation of amplified PCR products encompassing exon 28 ..... 295
4.124 Representative electropherogram illustrating a portion of the amplified region of exon 28 ..... 296
4.125 Photographic representation of amplified PCR products encompassing exon 29 ..... 296
4.126 Representative electropherogram illustrating a portion of the amplified region of exon 29 ..... 297

4.127 Photographic representation of amplified PCR products encompassing exon 30 ..... 298

4.128 Representative electropherogram illustrating a portion of the amplified region of exon 30 ..... 299

4.129 Photographic representation of amplified PCR products encompassing exon 31 ..... 299

4.130 Representative electropherogram illustrating a portion of the amplified region of exon 31 ..... 300

4.131 Photographic representation of amplified PCR products encompassing exons 32 and 33 ..... 301

4.132 Representative electropherogram illustrating a portion of the amplified region of exon 32 ..... 301

4.133 Representative electropherogram of exon 33 indicating the nucleotide position of the Pro1592Leu alteration ..... 302

4.134 Photographic representation of amplified PCR products encompassing exon 34 ..... 303

4.135 Representative electropherogram of exon 34 indicating the nucleotide positions of the Arg1667Cys, Ser1728Phe, Pro1773Ser, Leu1786Pro and Pro1787Leu alterations ..... 304

4.136 Representative electropherograms indicating the Pro1787Leu alteration observed in exon 34 ..... 306

4.137 Photographic representation of amplified PCR products encompassing exon 35 ..... 308

4.138 Representative electropherogram illustrating a portion of the amplified region of exon 35 ..... 309

4.139 Photographic representation of amplified PCR products encompassing exons 36 and 37 ..... 309

4.140 Representative electropherogram illustrating a portion of the amplified region of exon 36 ..... 310

4.141 Representative electropherogram illustrating a portion of the amplified region of exon 37 ..... 310

4.142 Representative electropherogram indicating the C57264T SNP observed in the intron sequence between exons 36 and 37 of the RYR1 gene ..... 312

4.143 Representative electropherogram indicating the A57545G SNP observed in exon 37 of the RYR1 gene ..... 312

4.144 Photographic representation of amplified PCR products encompassing exon 38 ..... 313

4.145 Representative electropherogram of exon 38 indicating the nucleotide position of the Gly2060Cys alteration ..... 314

4.146 Representative electropherograms indicating the Gly2060Cys alteration observed in exon 38 ..... 315

4.147 Photographic representation of amplified PCR products encompassing exon 47 ..... 318

4.148 Representative electropherogram of exon 47 indicating the nucleotide positions of the Pro2946Leu, Arg2508Cys, Arg2508Gly and Arg2508His alterations ..... 319

4.149 Representative electropherograms indicating the G67777A and G67804A SNPs observed in exon 47 of the RYR1 gene ..... 321

4.150 Representative electropherogram indicating the C67901G SNP observed in the intron sequence between exons 47 and 48 of the RYR1 gene ..... 322

4.151 Photographic representation of amplified PCR products encompassing exons 48 and 49 ..... 323
4.152 Representative electropherogram of exon 48 indicating the nucleotide positions of the Glu2545Asp and Arg2591Gly alterations ..... 324
4.153 Representative electropherogram of exon 49 indicating the nucleotide position of the Val2627Leu alteration ..... 325
4.154 Photographic representation of amplified PCR products encompassing exons 50, 51 and 52 ..... 325
4.155 Representative electropherogram of exon 50 indicating the nucleotide position of the Arg2626Trp alteration ..... 326
4.156 Representative electropherogram of exon 51 indicating the nucleotide positions of the Asp2730His and Gly2733Asp alterations ..... 327
4.157 Representative electropherogram of exon 52 indicating the nucleotide position of the Glu2764Lys alteration ..... 328
4.158 Representative electropherogram indicating the G71171A SNP observed in exon 50 of the RYR1 gene ..... 329
4.159 Representative electropherograms indicating the G71413A and A71494G SNPs observed in the intron sequence between exons 50 and 51 of the RYR1 gene ..... 330
4.160 Representative electropherograms indicating the T71699C and T71771C SNPs observed in exon 51 of the RYR1 gene ..... 331
4.161 Photographic representation of amplified PCR products encompassing exons 53 and 54 ..... 332
4.162 Representative electropherogram of exon 53 indicating the nucleotide position of the Thr2787Ser alteration ..... 333
4.163 Representative electropherogram of exon 54 indicating the nucleotide position of the Arg2840Trp alteration ..... 333
4.164 Representative electropherogram indicating the G72236A SNP observed in exon 53 of the RYR1 gene ..... 334
4.165 Photographic representation of amplified PCR products encompassing exons 55, 56 and 57 ..... 335
4.166 Representative electropherogram of exon 55 indicating the nucleotide position of the Leu2867Gly alteration ..... 336
4.167 Representative electropherogram illustrating a portion of the amplified region of exon 56 ..... 336
4.168 Representative electropherogram of exon 57 indicating the nucleotide position of the Arg2939Ser alteration ..... 337
4.169 Representative electropherogram indicating the T73251C SNP observed in exon 55 of the RYR1 gene ..... 338
4.170 Representative electropherogram indicating the C73337T SNP observed in the intron sequence between exons 55 and 56 of the RYR1 gene ..... 339
4.171 Representative electropherograms indicating the T73475G, T73584C and G73720C SNPs observed in the intron sequence between exons 56 and 57 of the RYR1 gene ..... 340
4.172 Representative electropherogram indicating the T73870A SNP observed in the intron sequence between exons 57 and 58 of the RYR1 gene ..... 341
4.173 Photographic representation of amplified PCR products encompassing exon 58 ..... 342
4.174 Representative electropherogram illustrating a portion of the amplified region of exon 58 ..... 343

4.175 Photographic representation of amplified PCR products encompassing exons 59 and 60 ..... 343
4.176 Representative electropherogram illustrating a portion of the amplified region of exon 59 ..... 344
4.177 Representative electropherogram illustrating a portion of the amplified region of exon 60 ..... 344
4.178 Photographic representation of amplified PCR products encompassing exon 61 ..... 345
4.179 Representative electropherogram illustrating a portion of the amplified region of exon 61 ..... 346
4.180 Photographic representation of amplified PCR products encompassing exons 62 and 63 ..... 347
4.181 Representative electropherogram illustrating a portion of the amplified region of exon 62 ..... 347
4.182 Representative electropherogram of exon 60 indicating the nucleotide position of the Arg3119His alteration ..... 348
4.183 Representative electropherograms indicating the A78986G SNP observed in exon 62 of the RYR1 gene ..... 349
4.184 Photographic representation of amplified PCR products encompassing exon 64 ..... 350
4.185 Representative electropherogram illustrating a portion of the amplified region of exon 64 ..... 351
4.186 Representative electropherogram indicating the C81823T SNP observed in the intron sequence between exons 63 and 64 of the RYR1 gene ..... 352
4.187 Photographic representation of amplified PCR products encompassing exon 65 ..... 352
4.188 Representative electropherogram illustrating a portion of the amplified region of exon 65 ..... 353
4.189 Photographic representation of amplified PCR products encompassing exon 66 ..... 354
4.190 Representative electropherogram illustrating a portion of the amplified region of exon 66 ..... 354
4.191 Representative electropherograms indicating the G84264A SNP observed in exon 66 of the RYR1 gene ..... 355
4.192 Photographic representation of amplified PCR products encompassing exon 67 ..... 356
4.193 Representative electropherogram of exon 67 indicating the nucleotide positions of the Arg2248His and Lys3367Arg alterations ..... 357
4.194 Representative electropherogram indicating the C10218T SNP observed in exon 67 of the RYR1 gene ..... 358
4.195 Photographic representation of amplified PCR products encompassing exons 68 and 69 ..... 358
4.196 Representative electropherogram of exon 68 indicating the nucleotide position of the Ser3446Phe alteration ..... 359
4.197 Representative electropherogram illustrating a portion of the amplified region of exon 69 ..... 360
4.198 Photographic representation of amplified PCR products encompassing exon 70 ..... 360
4.199 Representative electropherogram illustrating a portion of the amplified region of exon 70 ..... 361

4.200 Photographic representation of amplified PCR products encompassing exon 71 ..... 362
4.201 Representative electropherogram of exon 71 indicating the nucleotide position of the Pro3526Ser alteration ..... 362
4.202 Photographic representation of amplified PCR products encompassing exon 72 ..... 363
4.203 Representative electropherogram illustrating a portion of the amplified region of exon 72 ..... 364
4.204 Photographic representation of amplified PCR products encompassing exon 73 ..... 364
4.205 Representative electropherogram of exon 70 indicating the nucleotide position of the Leu3606Pro alteration ..... 365
4.206 Representative electropherograms indicating the Glu3583Gln alteration observed in exon 73 ..... 366
4.207 Photographic representation of amplified PCR products encompassing exons 74, 75 and 76 ..... 367
4.208 Representative electropherogram illustrating a portion of the amplified region of exon 74 ..... 368
4.209 Representative electropherogram illustrating a portion of the amplified region of exon 75 ..... 368
4.210 Representative electropherogram illustrating a portion of the amplified region of exon 76 ..... 369
4.211 Photographic representation of amplified PCR products encompassing exons 77 and 78 ..... 369
4.212 Representative electropherogram illustrating a portion of the amplified region of exon 77 ..... 370
4.213 Representative electropherogram illustrating a portion of the amplified region of exon 78 ..... 370
4.214 Photographic representation of amplified PCR products encompassing exons 79, 80 and 81 ..... 371
4.215 Representative electropherogram illustrating a portion of the amplified region of exon 79 ..... 372
4.216 Representative electropherogram illustrating a portion of the amplified region of exon 80 ..... 372
4.217 Representative electropherogram illustrating a portion of the amplified region of exon 81 ..... 373
4.218 Photographic representation of amplified PCR products encompassing exon 82 ..... 373
4.219 Representative electropherogram of exon 82 indicating the nucleotide position of the Val3840Ile alteration ..... 374
4.220 Representative electropherogram indicating the A11547G SNP observed in exon 82 of the RYR1 gene ..... 375
4.221 Photographic representation of amplified PCR products encompassing exon 83 ..... 375
4.222 Representative electropherogram illustrating a portion of the amplified region of exon 83 ..... 376
4.223 Photographic representation of amplified PCR products encompassing exon 84 ..... 377
4.224 Representative electropherogram illustrating a portion of the amplified region of exon 84 ..... 377

4.225 Photographic representation of amplified PCR products encompassing exons 85, 86 and 87 ..... 378

4.226 Representative electropherogram exon 85 indicating the nucleotide positions of the Arg3903Gln and Ile3916Met alterations ..... 379

4.227 Representative electropherogram illustrating a portion of the amplified region of exon 86 ..... 379

4.228 Representative electropherogram illustrating a portion of the amplified region of exon 87 ..... 380

4.229 Photographic representation of amplified PCR products encompassing exon 88 ..... 381

4.230 Representative electropherogram illustrating a portion of the amplified region of exon 88 ..... 381

4.231 Photographic representation of amplified PCR products encompassing exon 89 ..... 382

4.232 Representative electropherogram of exon 89 indicating the nucleotide positions of the Arg4041Trp and Thr4801Met alterations ..... 383

4.233 Representative electropherogram illustrating a portion of the amplified region of exon 105 ..... 384

4.234 Photographic representation of amplified PCR products encompassing exon 106 ..... 385

4.235 Representative electropherogram illustrating a portion of the amplified region of exon 106 ..... 385

5.1 Comparison of RYR1 alterations in the South African MH population to the worldwide localisation of alterations observed in patients diagnosed with MH or CCD ..... 393

5.2 Model for the network of genetic and environmental factors that can potentially influence the expression of the MH phenotype in the South African MH population ..... 395

5.3 Proposed clinical spectrum of MH due to defects in the RyR1 protein in various tissues ..... 403

# LIST OF TABLES


| Table No. | Title of Table | Page |
|---|---|---|
| 2.1 | Presentation of clinical features of MH following exposure to triggering anaesthetics | 7 |
| 2.2 | Reported MH episodes triggered in patients diagnosed with another disorder | 18 |
| 2.3 | Triggering agents, safe agents and controversial agents related with MH | 19 |
| 2.4 | Reported alterations within hotspot one of the RYR1 gene observed in MH, CCD or MmD patients | 47 |
| 2.5 | Reported alterations within hotspot two of the RYR1 gene observed in MH, CCD or MmD patients | 53 |
| 2.6 | Reported alterations within hotspot three of the RYR1 gene observed in MH, CCD or MmD patients | 59 |
| 2.7 | Reported alterations outside of the RYR1 gene mutational hotspots observed in MH, CCD, MmD or CNM patients | 63 |
| 2.8 | Chromosomal localisations harbouring potentially causative MHS loci | 68 |
| 3.1 | Diagnostic *in vitro* contracture test results as determined by the European *in vitro* contracture test protocol | 74 |
| 3.2 | Oligonucleotide primers used for PCR and direct sequencing | 86 |
| 3.3 | Temperature cycles of the standard PCR reaction protocol | 93 |
| 3.4 | Temperature cycles of the two-step fast PCR reaction protocol | 94 |
| 3.5 | Template quantity used in sequencing | 96 |
| 3.6 | Temperature cycles of the sequencing reaction | 96 |
| 3.7 | Partial gDNA sequence of exon 1 of the RYR1 gene | 97 |
| 3.8 | Partial gDNA sequence of exon 2 of the RYR1 gene | 98 |
| 3.9 | Partial gDNA sequence of exon 3 of the RYR1 gene | 99 |
| 3.10 | Partial gDNA sequence of exons 4 and 5 of the RYR1 gene | 99 |
| 3.11 | Partial gDNA sequence of exons 6 and 7 of the RYR1 gene | 100 |
| 3.12 | Partial gDNA sequence of exons 8 and 9 of the RYR1 gene | 101 |
| 3.13 | Partial gDNA sequence of exons 10 and 11 of the RYR1 gene | 102 |
| 3.14 | Partial gDNA sequence of exon 12 of the RYR1 gene | 103 |
| 3.15 | Partial gDNA sequence of exon 13 of the RYR1 gene | 104 |
| 3.16 | Partial gDNA sequence of exons 14, 15 and 16 of the RYR1 gene | 105 |
| 3.17 | Partial gDNA sequence of exons 17 and 18 of the RYR1 gene | 106 |
| 3.18 | Partial gDNA sequence of exon 19 of the RYR1 gene | 107 |
| 3.19 | Partial gDNA sequence of exon 20 of the RYR1 gene | 108 |
| 3.20 | Partial gDNA sequence of exons 21 and 22 of the RYR1 gene | 109 |
| 3.21 | Partial gDNA sequence of exon 23 of the RYR1 gene | 109 |
| 3.22 | Partial gDNA sequence of exon 24 of the RYR1 gene | 110 |
| 3.23 | Partial gDNA sequence of exon 25 of the RYR1 gene | 111 |
| 3.24 | Partial gDNA sequence of exons 26 and 27 of the RYR1 gene | 111 |

3.25 Partial gDNA sequence of exon 28 of the RYR1 gene..... 112
3.26 Partial gDNA sequence of exon 29 of the RYR1 gene..... 113
3.27 Partial gDNA sequence of exon 30 of the RYR1 gene..... 113
3.28 Partial gDNA sequence of exon 31 of the RYR1 gene..... 114
3.29 Partial gDNA sequence of exons 32 and 33 of the RYR1 gene..... 114
3.30 Partial gDNA sequence of exon 34 of the RYR1 gene..... 115
3.31 Partial gDNA sequence of exon 35 of the RYR1 gene..... 116
3.32 Partial gDNA sequence of exons 36 and 37 of the RYR1 gene..... 117
3.33 Partial gDNA sequence of exon 38 of the RYR1 gene..... 118
3.34 Partial gDNA sequence of exon 39 of the RYR1 gene..... 119
3.35 Partial gDNA sequence of exon 40 of the RYR1 gene..... 119
3.36 Partial gDNA sequence of exons 41 and 42 of the RYR1 gene..... 120
3.37 Partial gDNA sequence of exon 43 of the RYR1 gene..... 121
3.38 Partial gDNA sequence of exons 44 and 45 of the RYR1 gene..... 122
3.39 Partial gDNA sequence of exon 46 of the RYR1 gene..... 124
3.40 Partial gDNA sequence of exon 47 of the RYR1 gene..... 125
3.41 Partial gDNA sequence of exons 48 and 49 of the RYR1 gene..... 126
3.42 Partial gDNA sequence of exons 50, 51 and 52 of the RYR1 gene..... 127
3.43 Partial gDNA sequence of exons 53 and 54 of the RYR1 gene..... 128
3.44 Partial gDNA sequence of exons 55, 56 and 57 of the RYR1 gene..... 129
3.45 Partial gDNA sequence of exon 58 of the RYR1 gene..... 130
3.46 Partial gDNA sequence of exons 59 and 60 of the RYR1 gene..... 130
3.47 Partial gDNA sequence of exon 61 of the RYR1 gene..... 131
3.48 Partial gDNA sequence of exons 62 and 63 of the RYR1 gene..... 131
3.49 Partial gDNA sequence of exon 64 of the RYR1 gene..... 132
3.50 Partial gDNA sequence of exon 65 of the RYR1 gene..... 132
3.51 Partial gDNA sequence of exon 66 of the RYR1 gene..... 133
3.52 Partial gDNA sequence of exon 67 of the RYR1 gene..... 134
3.53 Partial gDNA sequence of exons 68 and 69 of the RYR1 gene..... 134
3.54 Partial gDNA sequence of exon 70 of the RYR1 gene..... 135
3.55 Partial gDNA sequence of exon 71 of the RYR1 gene..... 136
3.56 Partial gDNA sequence of exon 72 of the RYR1 gene..... 136
3.57 Partial gDNA sequence of exon 73 of the RYR1 gene..... 137
3.58 Partial gDNA sequence of exons 74, 75 and 76 of the RYR1 gene..... 138
3.59 Partial gDNA sequence of exons 77 and 78 of the RYR1 gene..... 139
3.60 Partial gDNA sequence of exons 79, 80 and 81 of the RYR1 gene..... 140
3.61 Partial gDNA sequence of exon 82 of the RYR1 gene..... 141
3.62 Partial gDNA sequence of exon 83 of the RYR1 gene..... 141
3.63 Partial gDNA sequence of exon 84 of the RYR1 gene..... 142
3.64 Partial gDNA sequence of exons 85, 86 and 87 of the RYR1 gene..... 143
3.65 Partial gDNA sequence of exon 88 of the RYR1 gene..... 143
3.66 Partial gDNA sequence of exon 89 of the RYR1 gene..... 144
3.67 Partial gDNA sequence of exon 90 of the RYR1 gene..... 145
3.68 Partial gDNA sequence of exon 91 of the RYR1 gene..... 146
3.69 Partial gDNA sequence of exon 92 of the RYR1 gene..... 147
3.70 Partial gDNA sequence of exon 93 of the RYR1 gene..... 147

| | | |
|---|---|---|
| 3.71 | Partial gDNA sequence of exon 94 of the RYR1 gene | 148 |
| 3.72 | Partial gDNA sequence of exon 95 of the RYR1 gene | 149 |
| 3.73 | Partial gDNA sequence of exon 96 of the RYR1 gene | 150 |
| 3.74 | Partial gDNA sequence of exon 97 of the RYR1 gene | 150 |
| 3.75 | Partial gDNA sequence of exons 98 and 99 of the RYR1 gene | 151 |
| 3.76 | Partial gDNA sequence of exon 100 of the RYR1 gene | 152 |
| 3.77 | Partial gDNA sequence of exon 101 of the RYR1 gene | 153 |
| 3.78 | Partial gDNA sequence of exon 102 of the RYR1 gene | 154 |
| 3.79 | Partial gDNA sequence of exon 103 of the RYR1 gene | 155 |
| 3.80 | Partial gDNA sequence of exons 104 and 105 of the RYR1 gene | 155 |
| 3.81 | Partial gDNA sequence of exon 106 of the RYR1 gene | 156 |
| 4.1 | Optimised conditions for PCR reactions | 161 |
| 4.2 | Reported alterations in exon 6 of the RYR1 gene | 182 |
| 4.3 | Reported alterations in exons 14 and 15 of the RYR1 gene | 199 |
| 4.4 | Reported alterations in exon 39 of the RYR1 gene | 211 |
| 4.5 | Reported alterations in exon 40 of the RYR1 gene | 215 |
| 4.6 | Conserved amino acids obtained from different RyR isoforms and species surrounding the novel and reported mutations in exon 43 of the RyR1 protein | 222 |
| 4.7 | Reported alterations in exons 44 and 45 of the RYR1 gene | 226 |
| 4.8 | Conserved amino acids obtained from different RyR isoforms and species surrounding the reported mutations in exon 44 of the RYR1 protein | 233 |
| 4.9 | Reported alterations in exon 46 of the RYR1 gene | 234 |
| 4.10 | Reported alterations in exon 101 of the RYR1 gene | 260 |
| 4.11 | Conserved amino acids obtained from different RyR isoforms and species surrounding the novel and reported mutations in exon 102 of the RyR1 protein | 267 |
| 4.12 | Conserved amino acids obtained from different RyR isoforms and species surrounding the reported mutations in exon 34 of the RyR1 protein | 307 |
| 4.13 | Conserved amino acids obtained from different RyR isoforms and species surrounding the novel and reported mutations in exon 38 of the RyR1 protein | 316 |
| 4.14 | Reported alterations in exon 47 of the RYR1 gene | 318 |
| 4.15 | Reported alterations in exons 50, 51 and 52 of the RYR1 gene | 326 |
| 4.16 | Conserved amino acids obtained from different RyR isoforms and species surrounding the novel and reported mutations in exon 73 of the RyR1 protein | 365 |
| 4.17 | Summary of observed alterations detected in the coding regions in 15 South Africa MH probands | 387 |
| 4.18 | Summary of previous studies which screened the entire coding region of the RYR1 gene | 388 |
| A1 | Summary of previously reported studies that have functionally characterised RYR1 alterations observed in individuals diagnosed with MH or CCD | 443 |
| B1 | Summary of synonymous SNPs detected in the coding region of the RYR1 gene in the South African MH population | 447 |
| C1 | Summary of synonymous SNPs detected in the intron sequence of the RYR1 in the South African MH population | 451 |

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