Clinical and genetic epidemiology of malignant hyperthermia

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I. Declaration

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II. Acknowledgement

"Praise to Allah, who has guided us to this; and we would never have been guided if Allah had not guided us.", **Surah 7, Al-A'raf: The Heights, Verse 43**

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III. Abstract

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic disorder that occurs in susceptible patients in exposure to triggering volatile anaesthetics and/or succinylcholine. With the advances in the anaesthetic practice in recent years, we have to re-think about our understanding of the clinical epidemiology of MH. The development in the anaesthetic agents and techniques would influence the clinical presentation of MH and alter the outcomes of the disease. On the other hand, the emergency nature of the anaesthetic MH reaction undermines the quality of clinical information available for retrospective studies about the condition.

Genetically, the introduction of next-generation sequencing (NGS) methods enabled screening of the whole ryanodine receptor 1 gene (*RYR1).* As more than 300 variants have been detected in the *RYR1*, and of these, 48 variants have been functionally characterised as causative for MH, we have to evaluate the effects of these mutations on the clinical presentation of MH. A genotype-phenotype correlation should enable us to better understand the variable penetrance of MH. Additionally, we will be able to evaluate the relationship between MH and other *RYR1*-related muscle diseases; namely, congenital myopathies. The overlap between different subtypes of congenital myopathies and the potential risk of MH in these different subtypes originating from this overlap is an ongoing scientific perplexity.

Finally, we will try to gather information about the extent of the MH problem in Egypt, a country with a population approaching 100 million and many inadequacies in the operating health systems.

IV. List of abbreviation

ABG	Arterial blood gas
ACTA1	Actin alpha 1 gene
AKI	Acute kidney injury
ANOVA	Analysis of variance
ARF	Acute renal failure
ATP	Adenosine triphosphate
ATPase	Adenosine triposphatase
BEecf	Base excess in the extracellular fluid
BIN1	Bridging integrator 1
Ca^{+2}	Calcium ion
CACNA1S	L-type voltage gated calcium release channel gene
Casq-1	Calsequestrin protein
Cav1.1	L-type voltage gated calcium release channel
CCD	Central core disease
CCDC78	Coiled-coil domain containing 78 gene
CFL-2	Cofilin 2 gene
CGS	Clinical grading scale
CHCT	Caffeine halothane contracture test
CI	Confidence interval
CK	Creatine kinase
CM	Congenital myopathy
CNM	Centro-nuclear myopathy
CO_2	Carbon dioxide
CSQ	Calsequestrine gene
df	Degree of freedom
DHPR	Dihydropyridine receptor
DNA	Deoxyribonucleic acid
DNM2	Dynamin 2 gene
E.N.T	Ear, nose and throat
EHI	Exertional heat illness
EMHG	European Malignant Hyperthermia Group
etCO2	End tidal carbon dioxide
Fisher's LSD	Fisher’s least significant difference
H&E	Hematoxylin and eosin stain
HACD1	3-hydroxyacyl-coa dehydratase 1 gene
HEK-293	Human embryonic kidney cells 293
ICU	Intensive care unit
IQR	Interquartile range
IU	International unit
IV	Intravenous
IVCT	In vitro contracture test

K^+	Potassium ion
KPa	Kilo pascal
MAF	Minimum allele frequency
Mg^{+2}	Magnesium ion
MH	Malignant hyperthermia
MHE	Malignant hyperthermia equivocal
MHN	Malignant hyperthermia not susceptible
MHS	Malignant hyperthermia susceptible
MHSc	Malignant hyperthermia susceptible to caffeine only
MHSh	Malignant hyperthermia susceptible to halothane only
MHShc	Malignant hyperthermia susceptible to caffeine and halothane
MmD	Multi mini core disease
mmHg	Millimetre mercury
MMS	Masseter muscle spasm
MTM1	Myotubularin 1 gene
MYH2	Myosin heavy chain 2 gene
MYH7	Myosin heavy chain 7 gene
MYO	An encrypted family code for CM families
NADH	Nicotinamide adenine dinucleotide
NaHCO3	Sodium bicarbonate
NAMHR	North American Malignant Hyperthermia Registry
NAP	National audit project
NDMR	Non depolarising muscle relaxants
NGS	Next generation sequencing
NHS	National health service
O_2	Oxygen
$PaCO_2$	Arterial carbon dioxide tension
PaO_2	Arterial oxygen tension
pH	Power of hydrogen
RFLP	Restriction fragment length polymorphism
RR	Respiratory rate
RyR1	Ryanodine receptor type 1
RYR1	Ryanodine receptor type 1 gene
SEPN1	Selenoprotein N 1 gene
SO_2	Oxygen saturation
SPEG	Striated muscle preferentially expressed protein kinase gene
SR	Sarcoplasmic reticulum
Stac3	SH3 and cysteine rich domain 3 protein
STAC3	SH3 and cysteine rich domain 3 gene
TIVA	Total intravenous anaesthesia
TPM2, 3	Tropomyosin 2 and 3 gene
TTN	Titin gene
UK	The United Kingdom

X^2	Chi square test
℃	Degree centigrade

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1 General Introduction

1.1 Malignant hyperthermia

1.1.1 Overview

Malignant hyperthermia (MH) is a pharmacogenetic disorder triggered in susceptible patients by the commonly used volatile anaesthetics and/or depolarising muscle relaxant, succinylcholine. It affects skeletal muscles leading to disturbance of calcium (Ca^{+2}) homeostasis, which lead to myofilament interaction and increased muscular activity with a resulting state of hypermetabolism (DENBOROUGH, 1979, Fill et al., 1990, Gillard et al., 1991). MH was first described in 1960 in a patient with a family history of anaesthesia related deaths who developed a hyperthermic reaction to general anaesthetics (Denborough and Lovell, 1960). The pathophysiology of the disorder, figure 1-1, consists of massive Ca^{+2} release through a defective ryanodine receptor type 1 (RyR1) that is expressed in skeletal muscles (McCarthy, 2004). Additionally, it is thought that a basal Ca^{+2} leak through the RyR1 contributes to the pathogenesis of MH (Yang et al., 2007, Eltit et al., 2012). The condition was described in different ethnic groups (Rosenberg et al., 2015) and several scientific groups have been founded with the mutual interest to investigate its clinical presentation and elaborate its genetic background (https://www.emhg.org, https://my.mhaus.org https://pie.med.utoronto.ca/MH/index.htm, http://malignanthyperthermia.org.au). Several reports from other parts of the world about MH have been published from different countries (Centre for Arab Genomic Studies, 2016, Iqbal et al., 2017, Sumitani et al., 2011). MH only presents in susceptible patients at exposure to triggering volatile anaesthetics and/or succinylcholine. As it is estimated that only ~2% of the general population is exposed to these triggering agents each year, the

occurrence of MH reactions is considered very rare. Furthermore, not every susceptible patient presents with an MH reaction on each anaesthetic exposure (Robinson et al., 2006). Therefore, the reported prevalence of MH of 1 in 2,000 to 1 in 10,000 (Monnier et al., 2002, Miller, 2003) is considered an underestimation of the true prevalence of MH susceptibility.

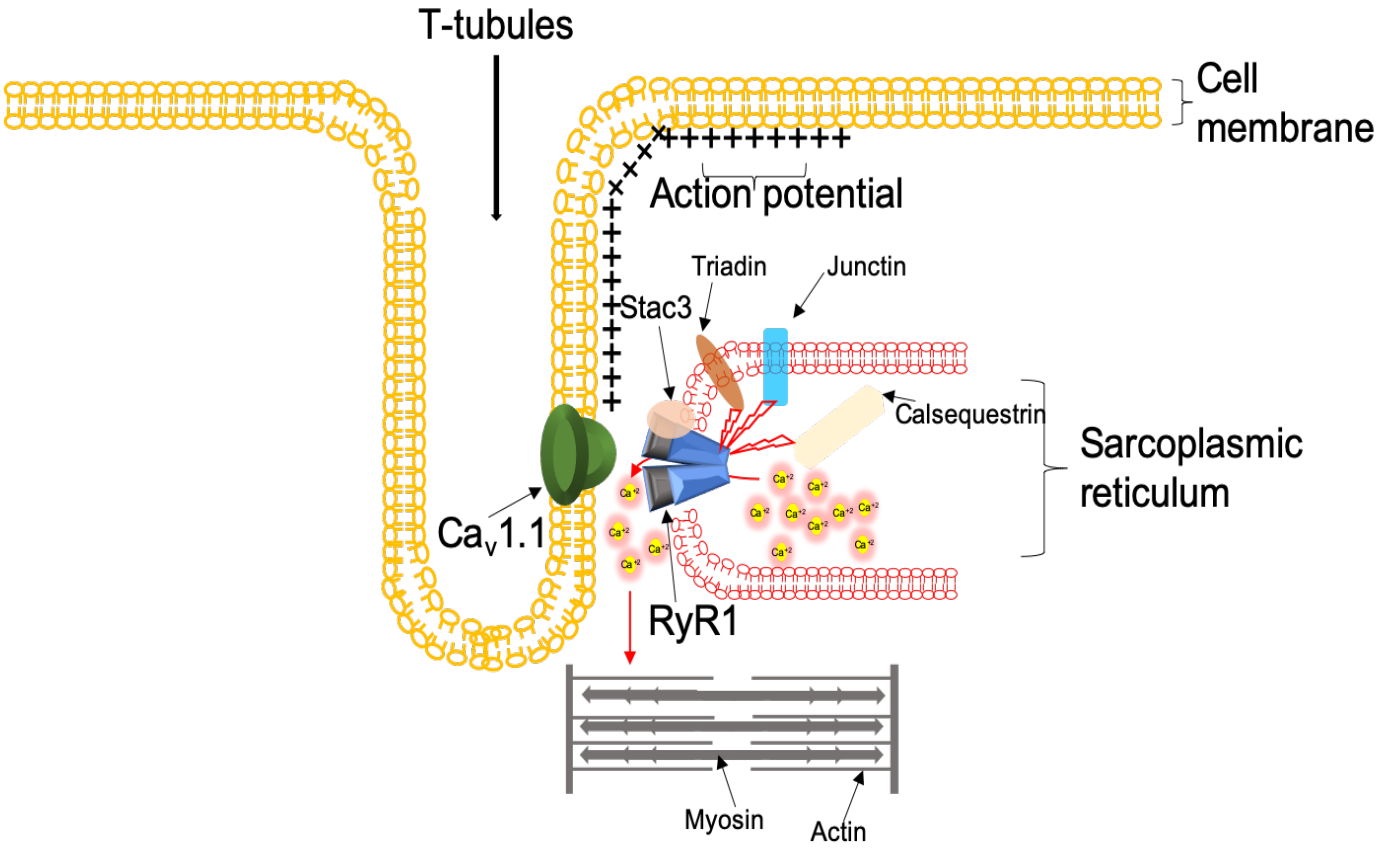

Figure 1-1: Schematic illustration of the excitation-contraction coupling mechanism of skeletal muscles. *Several proteins are involved in the Ca+2 release mechanism from the sarcoplasmic reticulum and may play a role in the pathogenesis of MH. Stac3→ SH3 and cysteine rich domain 3, Cav1.1→ voltage gated Ca+2 channel or dihydropyridine receptor, and RyR1→ ryanodine receptor type 1.*

1.1.2 Clinical presentation of an MH reaction

The clinical phenotype of MH is variable ranging from a mild reaction that passes unnoticed to a severe fulminant form that could be life-threatening and may lead to death. Due to the simple monitoring techniques that anaesthetists used to depend upon when MH first described in the early 1960s, it was given the term malignant hyperpyrexia (Hopkins, 2000). The malignant component of the name refers to the high fatality rate associated with the disorder and the hyperpyrexia part, was used to describe the state of progressive increase in body temperature that used to end into death. With the advances in monitoring facilities, it became apparent that the hyperthermic feature of the disorder is a late sign (Ellis et al., 1990). The classic form of MH is now better described as a state of hypermetabolism (Hopkins, 2000) that usually starts with increased CO_2 production and tachycardia. The severe fulminant reaction consists of features of metabolic stimulation in the form of hypercapnia, tachycardia, acidosis, and hyperthermia; in addition to manifestations of increased muscular activity such as muscular rigidity, elevated serum creatine kinase (CK) levels, and hyperkalaemia (Ellis et al., 1990).

Initially, patients who were reported to have developed MH reactions according to the muscular features of the reaction were grouped into rigid and nonrigid reactions (Britt and Kalow, 1970). Hackle and colleague (Hackl et al., 1990) used a statistical logistic regression model to predict MH susceptibility, and the best model they were able to generate was able to classify 78% of cases. Furthermore, a clinical grading scale was generated using a Delphi method by Larach and colleagues (Larach et al., 1994) based on the opinion of a panel of eleven experts in MH. They generated a point-based scoring system of different clinical indicators of MH to calculate a raw score.

This score is used to quantitively estimate the likelihood of a suspected anaesthetic reaction to be a true MH reaction. This score did not take into consideration the temporal relations of clinical details of the reaction and was markedly affected by missing data.

Based on the clinical details of 402 patients who developed an anaesthetic reaction and were referred to the Leeds MH Investigation Unit for diagnosis of MH susceptibility, Ellis and colleagues (Ellis et al., 1990) classified the clinical features of the reactions into eight different categories following the recognition of masseter muscle spasm (MMS) as a clinical problem. The categories described included: a) severe fulminant life-threatening form with both hypermetabolism and increased muscular activity, b) moderate features of metabolic and muscular derangements that never appeared to be life-threatening, c) mild metabolic disturbance, d) masseter spasm with features of rhabdomyolysis such as elevated serum CK or myoglobinuria, e) masseter spasm with metabolic derangement, f) masseter spasm only, g) perioperative death or cardiac arrest that cannot be explained otherwise, and h) postoperative presentation of fever or rhabdomyolysis.

1.1.3 Management of an MH reaction

The patient who was reported to have the first described case of MH reaction managed to survive the reaction; mainly because the anaesthetist was alerted by the patient's family history of anaesthesia related deaths. As the anaesthetist started to notice abnormal signs on the patients, all the volatile anaesthetics were switched off; and the patient allowed to emerge from anaesthesia with some symptomatic treatment (Denborough and Lovell, 1960). This incident highlights the importance of the early recognition of signs of MH for proper management, and obviously, it is very crucial to

remove all triggering agents once an MH reaction is suspected. The European Malignant Hyperthermia Group (EMHG), is a group established in the early 1980s by likeminded anaesthesiologists and scientists interested in MH, has published guidelines for recognition and management of MH crisis (Glahn et al., 2010). This guidance includes a list of clinical signs of MH reactions classified into early and late signs. Also, it lists possible differential diagnoses such as light anaesthesia, infection and septicaemia, pheochromocytoma, thyroid crisis, and equipment failure.

The basic principles of proper management of an MH crisis are still the same since the first described case of MH: early recognition and stopping all triggering agents. Immediate steps of treatment according to the EMHG guidelines include stopping of all triggering agents and switching to non-triggering anaesthesia, e.g. total intravenous anaesthesia (TIVA), declaring an emergency, hyperventilation with 100% O_2, and close monitoring of temperature, blood pressure, oxygen saturation and end-tidal CO_2 ($etCO_2$). Other steps described for the treatment of an MH crisis include treatment of other symptoms of the reaction. Hyperthermia should be treated vigorously with surface cooling, IV administration of chilled saline, and intravesical and intragastric irrigation with iced saline until temperature reaches <38.5℃. Both components of acidosis should be treated with hyperventilation for respiratory acidosis and sodium bicarbonate ($NaHCO_3$) for metabolic acidosis with a pH <7.2. Other measures include treatment of hyperkalaemia and arrhythmias and maintaining adequate urinary output to protect against acute renal failure due to severe rhabdomyolysis.

The specific treatment of an MH reaction is dantrolene sodium. Its mechanism of action includes inhibition of Ca^{+2} release from the sarcoplasmic reticulum (Zucchi and RoncaTestoni, 1997) and inhibition of extracellular Ca^{+2} entry and increase in the affinity of the RyR1 to magnesium (Mg^{+2}) (Cherednichenko et al., 2008, Choi et al.,

2017). Mg^{+2}, in turn, competes with Ca^{+2} at channel activation sites and at the same time binds to the inhibitory sites (Steele and Duke, 2007). It was found to treat porcine stress syndrome, a similar condition of MH in pigs that will be discussed later, it was suggested to be used for the treatment of MH in humans (Harrison, 1975). The mortality rate of MH has since dramatically dropped to less than 10% (Strazis and Fox, 1993). It is used at a dose of 2 mg/kg intravenous (IV) and repeated until stabilisation of the cardiac and respiratory symptoms, with some patients requiring the maximum dose of 10 mg/kg to be exceeded.

1.1.4 Diagnosis of MH susceptibility

Different literature described earlier, attempted to classify the clinical details of the anaesthetic reaction to predict the likelihood of the clinical diagnosis of MH. However, predicting MH susceptibility in patients before they develop an anaesthetic reaction could be lifesaving through offering trigger-free anaesthesia, e.g. TIVA, to susceptible patients. Based on several indicators, the EMHG have listed the most common reasons for an individual to be referred for investigation of MH susceptibility (Hopkins et al., 2015). These reasons include a family history of MH or unexplained deaths in the perioperative period. Clinical reasons include exertional rhabdomyolysis, idiopathic high serum CK levels, exertional heat stroke, and myopathy with detection of a rare uncharacterised potentially pathogenic ryanodine receptor type 1 gene (*RYR1*) variant. Based on the anaesthetic history, reasons listed included an adverse reaction to general anaesthesia where a triggering agent has been used. This adverse reaction should involve a combination of signs of hypermetabolism such as an inappropriate increase of $etCO_2$, tachycardia and temperature increase and muscle involvement such as muscular rigidity and rhabdomyolysis. Initial signs of the reaction

should be evident during anaesthesia or within 60 minutes of discontinuation of anaesthesia.

1.1.5 The in vitro contracture test

According to these criteria for referral of patients for diagnosis of MH susceptibility, patients referred are tested using the gold standard in vitro contracture test (IVCT). A standardised protocol was first agreed upon in April 1983 based on the expert opinion of physicians from eight European countries who were involved in screening patients for MH susceptibility (Ellis, 1984). Its main principle is the abnormal contractures of muscle specimens of MH susceptible patients when exposed to RyR1 agonists ex vivo. Incremental concentrations of halothane and caffeine are applied to a freshly biopsied muscle specimen from the quadriceps muscle, either its vastus medialis or lateralis, while hung in a bath filled with Krebs-Ringer solution. The contracture of the electrically stimulated muscle is measured in grams, and a positive test is considered diagnostic with a threshold increase of a ≥0.2 grams at 2% halothane and 2 mmol caffeine concentration measured from the basal contracture line.

The European protocol includes performing three tests: static halothane, dynamic halothane and static caffeine test; each on a sperate muscle specimen. The resulting diagnoses of these three tests are: MH susceptible to both halothane and caffeine (MHShc) when the muscle contracture exceeds the threshold of ≥0.2 gram for both halothane and caffeine tests, MH susceptible to either of them when the muscle reacts to halothane only (MHSh), or to caffeine only (MHSc), or MH not susceptible (MHN) when the three muscle specimens do not react in either of the three tests. Historically, the term equivocal or MHE was used to describe the diagnosis when the muscle specimen reacts only to either of the two agonists. Clinically, for the safety of the

patients and to increase the sensitivity of the IVCT, patients are treated as at risk of MH in case any of the diagnoses MHShc, MHSh, MHSc, or MHE are confirmed.

A variation of this protocol is performed according to the standards of the North American Malignant Hyperthermia Group, the caffeine halothane contracture test (CHCT) (Larach, 1989). The differences between both protocols include the use of a single concentration of halothane of 3% in the CHCT instead of three concentrations of 0.5%, 1% and 2% in the IVCT; no dynamic halothane test according to the North American MH group protocol; and caffeine concentrations used for CHCT are 0.5 mmol, 1 mmol, 2 mmol, 4 mmol and 32 mmol, while for IVCT an added concentration of 1.5 mmol is used. Additionally, the CHCT offers an optional additional test that includes the application of incremental concentrations of caffeine to a muscle specimen that has been exposed to 1% halothane concentration for 10 minutes. The diagnostic sensitivity of the IVCT is estimated to be 99% while it is estimated to have a specificity of 93.6% (Ording et al., 1997). The relatively low specificity because of a larger number of false-positive results is owed to the conservative approach towards considering equivocal cases as at risk of MH for the sake of patients' clinical safety. On the other hand, the North American MH Group protocol, the CHCT, was found to have a sensitivity of 97% and a specificity of 78% (Allen et al., 1998).

1.1.6 The Leeds MH Investigation Unit

St James's University Hospital, Leeds Teaching Hospitals NHS foundation trust hosts the national centre for MH investigation in the UK; the Leeds MH Investigation Unit. The unit was founded in the early 1970s and is a member of the European Malignant Hyperthermia Group. More than 7000 patients have had their MH susceptibility status confirmed using IVCT. In addition to MH, the unit provides expertise in other inherited

muscle disorders. The unit provides MH screening through the muscle biopsy list where muscle specimens are transferred to the on-site unit lab to be tested with IVCT. The unit also provides genetic screening, anaesthetic advice for patients who tested susceptible to MH and their family members, and a 24-hour hotline service for anaesthetists and medical staff for queries about MH. The unit also is one of the major research units for MH and related musculoskeletal disorders.

The patient who had the clinical reaction is labelled as the proband for his/her family. Later on, when this patient's susceptibility status is confirmed with IVCT, the patient is labelled as the index case for his/her family, being the first member of the family to be diagnosed. Situations when the proband and the index case for the family is not the same individual include patients who are too young to be tested, therefore, a first-of-kin would be tested first, usually one of the parents; patients who suffer from a severe systematic condition being unfit for the procedure; and patients who died due to a fatal reaction.

1.1.7 The Leeds MH Investigation Unit database and archive

Files for the more than 7000 patients who have been tested for MH susceptibility at the unit using IVCT are stored in the unit archive. These files contain muscle biopsy reports, IVCT traces, neuropathologists examination reports of muscle biopsy, and basal serum CK level at the time of the biopsy. For MH probands who developed a suspected anaesthetic reaction, clinical details of the reaction, which are obtained from referral letters, anaesthetic charts or the unit clinical details survey form, are stored in patients' files. The unit also has its clinical database that is generated using a FileMaker Pro© program to keep a digital form of this information about tested patients. This information is stored in two different databases with different login

credentials for each to maintain patients' confidentiality. Copies of genetic testing reports for patients tested at the unit are kept in patients' files, and the results are uploaded on a separate genetic table on the unit database.

1.1.8 Other muscular disorders related to MH

Other muscular disorders are described to be associated with MH. Of these disorders, exertional heat illness (EHI), which involves dysregulation of the thermal homeostasis resulting from an interaction between physiological and environmental factors (Muldoon et al., 2007, Capacchione and Muldoon, 2009). The clinical picture of EHI includes heat exhaustion which is considered a mild form of EHI that may progress, if untreated, into exertional heat stroke (Glazer, 2005). Heat exhaustion usually presents with dizziness, weakness and discomfort. Other manifestations of EHI may involve heat syncope, which develops due to marked dilatation of blood vessels to dissipate heat leading to hypotension and fainting attacks, and exertional heat cramps. Other muscle-related disorders associated with MH include exertional rhabdomyolysis that could be a manifestation of other muscular diseases, including EHI. It usually presents because of muscle breakdown leading to the release of creatine kinase and myoglobin in the bloodstream, which may subsequently affect renal function. Another disorder associated with MH and will be discussed in more details in this study is central core disease and its broader family of congenital myopathies.

1.1.9 Porcine stress syndrome

Porcine stress syndrome is a muscular disorder presented in special breeds of pigs and triggered by different factors such as stress, transport, and high ambient temperature. It is described as the inability of the affected pigs to handle environmental stresses described early (Marple et al., 1968). Biochemical changes that occur in the

muscle of affected pigs include increased rate of glycolysis and depletion of muscle glycogen (Briskey et al., 1966), increased lactate production in muscles (Dildey et al., 1970) and degradation of muscle proteins. It was found to be triggered by exposure to halothane. It presents with a rise of core body temperature of the affected animal, muscular rigidity and death. The quality of meat produced from these affected animals is less favourable as the meat is described to be pale, soft and exudative.

1.2 Calcium homeostasis in skeletal muscles and the regulating genes

1.2.1 The excitation-contraction coupling mechanism and the role of Ca+2

The excitation-contraction coupling is the process through which the action potential generated along the muscle fibre membranes leads to the intracellular release of Ca^{+2} from the sarcoplasmic reticulum (SR) which activates the actin and myosin filaments to interact to produce the mechanical shortening of muscle fibres and muscle contraction. This process involves the interaction between several proteins involved in the cellular Ca^{+2} homeostasis. As shown in figure 1-1, the dihydropyridine receptor (DHPR) which is located across the cell membrane in the transverse tubules (T-tubules) is activated by the depolarisation of the cell membrane from the spreading action potential. The activated DHPR physically interacts with the ryanodine receptor (RyR) located in the junctional terminal cisternae of the SR. This interaction leads to the opening of the RyR and release of Ca^{+2} from the SR, which in turn, activates the myosin and actin filaments to interact. This process ends when Ca^{+2} is transported back into the SR by the calcium-ATPase pump ending the interaction between actin and myosin filaments and leading to muscle relaxation.

1.2.2 The ryanodine receptor type 1

The ryanodine receptor which is a homotetrameric protein located in the junctional terminal cisternae of the SR membrane (Pessah et al., 1985) and it is the major Ca^{+2} release channel in the SR of skeletal muscles (Otsu et al., 1990). It is named after the plant alkaloid ryanodine (Jenden and Fairhurst, 1969) which has a high affinity and specificity to binding with the receptor and it is used to evaluate the function of the receptor (Imagawa et al., 1987, Inui et al., 1987, Lai et al., 1988). The receptor exists in three isoforms RyR1, RyR2 and RyR3 (Lanner et al., 2010). The isoform RyR1 was detected in skeletal muscles first time by (Takeshima et al., 1989, Zorzato et al., 1990) while the RyR2 detected in cardiac muscles (Nakai et al., 1990, Otsu et al., 1990) and RyR3 in the brain (Hakamata et al., 1992). The RyR1 is also expressed in other tissues but at lower levels such as the brain and mononuclear blood cells (Furuichi et al., 1994, Hosoi et al., 2001). The RyR1 is the largest known ion channel (Rossi et al., 2002) and the gene encoding the RyR1 was located on chromosome 19q13.2 (Mackenzie et al., 1990).

1.2.3 The dihydropyridine receptor

The receptor is a voltage dependant Ca^{+2} channel located in the cell membrane of skeletal muscles in the T-tubules region. It is also known as calcium channel, voltage-dependent, L type, alpha 1 subunit or $Ca_v1.1$. Its function includes physically interacting with the RyR1 stimulating Ca^{+2} release from the SR after being activated by the transmitted action potential across the cellular membrane. The gene encoding this protein is located on chromosome 1q32.1 and is named *CACNA1S*.

1.2.4 Pathophysiology of MH

The mechanism by which an MH reaction develops is depending on the sudden and progressive increase of intracellular Ca^{+2} leading to physiological and metabolic derangements. Triggering volatile anaesthetics and/or succinylcholine lead, in susceptible patients, to the abnormal release of high amounts of Ca^{+2} into the cytoplasm of skeletal muscle cells which leads to a sustained state of hypermetabolism. This mechanism is owed to defective forms of the Ca^{+2} regulating proteins; mainly the RyR1 and, to a less extent, the $Ca_v1.1$ which are mainly due to mutations on the *RYR1* and the *CACNA1S* genes, respectively. The generated state of hypermetabolism leads to excessive CO_2 production, which clinically presents as hypercapnia, increased O_2 consumption which translates into hypoxia and excessive lactate production due to anaerobic metabolism. During the course of the reaction, depletion of adenosine triphosphate (ATP) affects the integrity of the cellular membrane, which leads to increased leakage of electrolytes such as Ca^{+2} and K^+. Eventually, cell damage occurs leading to the release of enzymes such as creatinine kinase, previously known as creatine phosphokinase, and muscle proteins such as myoglobin.

1.3 Genetics of MH

Before establishment of the bases of molecular genetics of MH, reports about the mode of inheritance of MH in affected families provided convincing evidence that MH has an autosomal dominant mode of inheritance. In 1990, the *RYR1* on chromosome 19q13.1 was identified as the primary locus for the MH susceptibility trait (MacLennan et al., 1990, McCarthy et al., 1990). However, the molecular genetics of MH have shown marked locus and allelic heterogeneity (Robinson et al., 2003a). As ~50% of

MH families have been linked to mutations on the *RYR1*, other genes have been identified as possible loci for MH susceptibility such as *CACNA1S*, *STAC3* and CSQ genes.

1.3.1 The *RYR1* gene

The cDNA that encodes the Ca^{+2} release channel in the sarcoplasmic reticulum or the ryanodine receptor type 1 has been cloned to chromosome 19 (Zorzato et al., 1990) and subsequently localised to the position 19q13.1 (Mackenzie et al., 1990). Searching through this large gene that is composed of 106 exons using different DNA sequencing techniques, more than 300 mutations have been detected. Due to absence of high-throughput technique to confirm the causal association between this large number of mutations and the MH susceptibility trait, until now, only 48 mutations on the *RYR1* have been functionally characterised and are currently used as diagnostic for MH susceptibility (https://www.emhg.org/genetics). The majority of these mutations are missense mutations, involving a single nucleotide change with a resulting change of one amino acid in the protein. On the EMHG list of diagnostic mutations, only one deletion mutation has been functionally characterised to be causative for MH, the *RYR1* c.7042_7044del.

1.3.2 *CACNA1S* gene

The gene encoding the expression of the $Ca_v1.1$ protein is located on chromosome 1q32.1. As this protein plays a crucial role in the excitation-contraction coupling process, mutations on this gene were suggested to be involved in the pathophysiology of MH (Monnier et al., 1997). The EMHG guidelines for genetic diagnosis of MH lists only two variants on the *CACNA1S* to be accepted for genetic diagnosis of MH susceptibility, the *CACNA1S* c.520C>T and c.3257G>A variants. Functional studies

(Weiss et al., 2004, Eltit et al., 2012) have confirmed the role of these two mutations on the excitation-contraction coupling mechanism and the pathogenesis of MH.

1.3.3 *STAC3* gene

One of the proteins that are involved in the excitation-contraction coupling process is the SH3 and cysteine-rich domain 3 (Stac3). Its role in the process is not completely understood, but it is located in the T-tubules and believed to play an important role in the process by interacting with the RyR1 and $Ca_v1.1$ (Zaharieva et al., 2017). The gene encoding this protein has been localised to position 12q13.3 (Stamm et al., 2008). Mutations in this gene have been reported to be causative for an autosomal recessive type of myopathy found in the Lumbee population, the Native American Myopathy (Horstick et al., 2013). It was also suggested to be associated with congenital myopathy and the MH susceptibility trait (Zaharieva et al., 2017).

1.3.4 Other genes implicated in MH

As shown in figure 1-1, other proteins play different roles in the Ca^{+2} homeostasis in skeletal muscles and the excitation-contraction coupling process. These proteins could also play a role in the pathogenesis of MH. As according to a recent estimate of the genetic background of MH, mutations on other genes apart from the three previously mentioned genes (*RYR1*, *CACNA1S*, *STAC3*) could account for 14-23% of MH families (Miller et al., 2018). Genes for other proteins that modulate the function of ryanodine receptors are considered as possible candidate genes for MH (Robinson et al., 1997). Calsequestrin (Casq-1) is a Ca^{+2} binding protein that acts as a regulator of RyR1-related Ca^{+2} release (Beard et al., 2002) and located in the terminal cisternae of striated muscles (MacLennan and Wong, 1971). The gene that control this protein is proposed as a candidate gene for MH (Protasi et al., 2009). Other proteins such as

junctin and triadin play a role in the interaction between Casq-1 and RyR1, hence affect the Ca^{+2} homeostasis in skeletal muscles.

1.3.5 Discordance

Genotype-phenotype discordance was observed in 26.2% of UK MH families found to harbour a familial *RYR1* variant (Miller et al., 2018). Discordant cases in MH could be in the form of either a family member who is phenotype positive (MHS with IVCT) and genotype negative for a diagnostic *RYR1* or *CACNA1S* variant or, to a less extent, a phenotype negative and genotype positive. It was first described with MH in a family with a functionally relevant *RYR1* variant and the IVCT results (Adeokun et al., 1997). In some families with a phenotype positive-genotype negative discordance, the sequencing of the entire *RYR1* gene has found other *RYR1* variants that disapproved discordancy in these families (Robinson et al., 2000, Robinson et al., 2003b). Studying this discordancy, especially in the case of a phenotype negative-genotype positive discordance, is biased by the practice of not genetically testing patients who test MHN with IVCT. Of the possible explanations to this discordancy, is errors in either genotyping or phenotyping of affected patients. However, proved discordant cases add to the genetic complexity of the MH disorder that includes locus and allelic heterogeneity with the possibility of involvement of modifying loci in the development of clinical and diagnostic phenotypes.

1.4 Congenital myopathies

1.4.1 Overview

Congenital myopathy (CM) is a group of clinically and genetically heterogeneous muscular disorders that present clinically with hypotonia and muscle weakness. The severity of weakness varies significantly between different subtypes and with age.

Different muscle groups are affected with marked overlap between clinical categories of the group. The course of the disease also varies from one type to another ranging from slowly or non-progressive course to severe affection of vital muscle groups such as respiratory muscles which mandates ventilatory support in some patients. Some musculoskeletal abnormalities have been described with different types of congenital myopathies such as congenital hip dysplasia and kyphoscoliosis. No myocardial involvement is usually described in association with congenital myopathies, but cardiac function may be affected secondary to other abnormalities such as scoliosis causing restrictive lung deficit (Klingler et al., 2009).

The prevalence of congenital myopathies is estimated to be 1 in 26000 (Amburgey et al., 2011). Different types of congenital myopathies have been described and usually named after the predominant findings during the histopathological examination of muscle specimens from affected patients. Detection of central areas in muscle biopsies which are described as cores devoid of mitochondria that appear clear on oxidative enzyme staining, was first described by Shy and Magee (Magee and Shy, 1956) and was given the name central core disease (CCD). When these cores are less defined, multiple and/or peripheral, muscle biopsies are described to have multi-mini-cores (MmD) (Engel et al., 1971). In some muscle biopsies, no cores could be detected, and unevenness of oxidative enzyme staining is noticed (Morgan-Hughes et al., 1973b). This characteristic core formation has emerged to be the most prevalent histopathological change in congenital myopathies (Jungbluth et al., 2018). On the contrary to the previous estimate that nemaline myopathy, which is characterised by the detection of red staining rods or bodies with the modified Gömöri trichrome staining, is the most common pathological form (Sewry and Wallgren-Pettersson, 2017). Other variants of myopathic changes include congenital fibre type disproportion

(Clarke, 2011), fibre type 1 predominance (Sato et al., 2008) and centronuclear myopathy (CNM) (Jungbluth et al., 2008).

1.4.2 Different clinical presentations of CM

Different types of congenital myopathies are labelled according to the predominant histological finding, though, a characteristic clinical pattern of clinical weakness has been described for each type.

Central core disease

The clinical presentation of CCD usually starts in infancy with floppy infant syndrome or during early childhood with delayed motor milestones with weakness, which is typically proximal affecting hip girdle and axial muscles (Dubowitz, 1995). Bulbar affection and ophthalmoplegia are not usually associated with CCD, and even they are considered as exclusion criteria (Middleton and Moser, 1998). The typical distribution of muscle weakness is the involvement of proximal muscle groups, including the hip girdle and axial muscles. CCD is commonly presented with orthopaedic complications such as congenital dislocation of the hips (Ramsey and Hensinger, 1975) and scoliosis (Merlini et al., 1987) and foot deformities (Gamble et al., 1988). Cardiomyopathy is not a feature of *RYR1* inherited CCD (Jungbluth, 2007), although cardiac involvement secondary to restrictive lung disease due to scoliosis may be observed. Severe respiratory involvement presented in neonatal cases is described in association with recessive *RYR1* mutations (Romero et al., 2003).

Multi-mini-core disease

MmD is diagnosed when muscle biopsies show cores that are less defined, multiple and peripheral. Different clinical presentations have been described in association with this histological pattern, including the classical form of axial muscle weakness and

severe scoliosis with respiratory impairment. This form may be associated with involvement of the extraocular muscles. Other forms include either hip involvement with arthrogryposis or distal weakness affecting the upper limbs (Engel et al., 1971, Jungbluth et al., 2000).

Core myopathies

Both previous subtypes showed a marked overlap in the clinical features. Furthermore, the histopathological finding of different forms of cores could be stages of the disease, a progression with age, or separate entities. However, due to this marked overlap, both subtypes are referred to as core myopathies. Mutations in several genes were associated with development of core myopathies that include ryanodine receptor 1, selenoprotein N 1 (*SEPN1*), actin alpha 1 (*ACTA1*), titin (*TTN*), cofilin 2 (*CFL-2*), dynamin 2 (*DNM2*), myosin heavy chain 7 (*MYH7*), and myosin heavy chain 2 (*MYH2*). With mutations in the *RYR1*, an autosomal dominant mode of inheritance is described with CCD while MmD myopathy is usually linked to autosomal recessive inheritance. However, due to the pathological, clinical and genetic overlap, it is more appropriate now to refer to these disorders as core myopathies (Sewry and Wallgren-Pettersson, 2017).

Centronuclear myopathy

Different clinical presentations have been described in association with CNM, and it mainly depends on the underlying genetic mutations (Jungbluth et al., 2008). The affected males of the X-linked form of the disease usually presented early during foetal life with reduced foetal movement, polyhydramnios and thinning of ribs (Osborne et al., 1983, Teeuw et al., 1993). Birth asphyxia and neonatal deaths may be the presenting features (Heckmatt et al., 1985, Barth and Dubowitz, 1998). Female

carriers of the X-linked form are usually asymptomatic with a few patients may have mild muscular weakness (Heckmatt et al., 1985, Sawchak et al., 1991, Hammans et al., 2000). Autosomal linked forms have been described with both recessive and dominant modes of inheritance with different clinical presentations (Jungbluth et al., 2008, Wilmshurst et al., 2010) described in a large cohort of patients a consistent clinical pattern associated with *RYR1* mutations. The clinical pattern described started in utero in some patients with reduced foetal movement and presented at birth with hypotonia and weakness. Delayed milestones have been described in all patients, and bulbar affection with feeding difficulties was common. Involvement of the respiratory muscle that mandated ventilatory support was described in three patients and scoliosis in another two patients.

Genetic heterogeneity in this group of patients includes an X-linked mode of inheritance associated with mutations in myotubularin 1 (MTM1) (Longo et al., 2016, Savarese et al., 2016), autosomal dominant inheritance with mutations in DNM2 (Bitoun et al., 2005, Bitoun et al., 2007, Koutsopoulos et al., 2013) and autosomal recessive with mutations in TTN, bridging integrator 1 (BIN1) (Schessl et al., 2007), coiled-coil domain containing 78 (CCDC78), striated muscle preferentially expressed protein kinase (SPEG) (Sewry and Wallgren-Pettersson, 2017), and *RYR1*. Histopathological findings and clinical features of CNM varies according to the mode of inheritance and the affected genes. X-linked CNM due to mutations in MTM1 lead to a generalised muscle weakness with wasting and severe respiratory insufficiency and feeding difficulties (North et al., 2014) with large centrally located nuclei surrounded by a pale halo (Sewry and Wallgren-Pettersson, 2017).

Congenital fibre type 1 predominance

This type is described histologically when 99% of muscle fibres belong to type 1. The clinical course of the disease is usually non-progressive with early onset of symptoms. Weakness is mild with proximal involvement and associated with hyporeflexia or areflexia (Oh and Danon, 1983). In some patients, it could be the only pathological finding, and it is linked in these patients to mutations in ACTA1, tropomyosin 2 and 3 (TPM2, 3), SEPN1, MYH7, 3-hydroxyacyl-CoA dehydratase 1 (HACD1), and *RYR1*.

Myopathy	**Genes**
Cores	*RYR1, SEPN1, ACTA1, TTN, CFL-2, DNM2, MYH7, MYH2*
Central nuclei	*MTM1, DNM2, BIN1, RYR1, CCDC78, SPEG, TTN*
Fibre type 1 predominance	*ACTA1, TPM2, TPM3, SEPN1, MYH7, RYR1, HACD1*

Table 1-1: Genes associated with different congenital myopathies.

1.4.3 Diagnosis of CM

The diagnosis of CM requires a proper clinical examination to determine the degree and pattern of clinical muscle weakness, muscle biopsy and histological examination to identify the underlying pathology and genetic screening to detect the mutated genes. Patients who are found to have myopathic changes in their muscle biopsy without clinical weakness are not labelled to have CM. Additionally, due to the marked overlap between different types of congenital myopathies; clinically, histologically and genetically, it is sometimes challenging to ascertain the specific type of congenital myopathies.

1.4.4 Anaesthetic implications in patients with CM

The challenges of anaesthetising patients with CM include the predictable risk originating from the weakness of the affected muscle group such as respiratory muscles, bulbar muscles or myocardial involvement in rare cases. Other unpredictable risks include prolonged paralysis by muscle relaxing drugs used, abnormal response to succinylcholine in the form of rhabdomyolysis and hyperkalaemia. Therefore, thorough history and examination and a careful evaluation of the advantages and disadvantages of each anaesthetic technique in patients with myopathy are always required to ensure proper care is provided for patients with congenital myopathies. However, due to a marked clinical and genetic overlap between different entities of congenital myopathies, it is usually challenging to ascertain a definitive diagnosis.

1.4.5 Association of CM with MH

As shown in table 1-1, mutations in the *RYR1* were described in association with four types of congenital myopathies. Different types of congenital myopathies were associated with different modes of inheritance. Clinical episodes of MH and positive response of the diagnostic IVCT in some patients diagnosed with CCD have been reported (Klingler et al., 2009). However, an inconsistency between CCD diagnosis and MH susceptibility diagnosis have been reported (Halsall et al., 1996, Curran et al., 1999). No definitive reports of MH reactions in MmD patients were found (Klingler et al., 2009), on the other hand, histological features of MmD were reported in a family with many MHS members (Guis et al., 2004), together with clinical and histological overlap between CCD and MmD, extra care should be taken when anaesthetising patients diagnosed with MmD regarding the risk of MH. Regarding other types of

congenital myopathies, the uncertainty of the risk of MH in these cases and the overlap between different types mandates managing these patients with caution.

1.5 Status of MH in Egypt

1.5.1 Genetic disorders in the Arab world and the Middle East region

The middle east and Arabic region are characterised by a high rate of consanguinity with an estimated rate of 10-60% (Al-Gazali et al., 2006). This high rate of consanguinity favours the spread of genetically inherited diseases. In addition, due to cultural, religious and legal considerations; certain procedures are of limited value during screening and/or investigation of different genetic disorders. The lack of public health measures and inadequacy of perinatal health care, especially in low income countries, lead to inappropriate control of genetic disorders. Despite these limitations, several Arab countries have endorsed an initiative to apply genetic prevention programmes; premarital, antenatal and neonatal. Nevertheless, these programmes are mainly directed to certain common genetic disorders due to the cost of implementing these programmes among populous, though low-income, countries.

1.5.2 Reported cases of MH in Arab countries

Searching for case reports and epidemiological data about MH in the Arab countries reveals minimal results. Nevertheless, as MH has been reported in all ethnic groups and due to the diversity of ethnic backgrounds of the population in these countries, we have little doubt that MH exists in the Arab countries. The Centre for Arab Genomic Studies in its report in 2016 (Centre for Arab Genomic Studies, 2016), listed three cases of suspected MH reaction that happened in Bahrain and another two cases in Saudi Arabia. The initial reports of these cases could not be traced due to poor referencing in the report. Only one case was reported to have died as a result of the

anaesthetic reaction while dantrolene was used in the other cases, and they were reported to survive the reaction. None of the reported cases had their MH susceptibility status confirmed, though, one of the relatives of the deceased case reported in Bahrain had the MH susceptibility status confirmed in the USA using CHCT.

1.5.3 Challenges in the diagnosis and management of MH in Egypt

The same population characteristics described early in the Arab world and the Middle East region that favour the spread of genetic disorders are presented among the Egyptian population. In addition, Egypt is the most populous country in the whole region with limited funding directed to health care programmes aimed at the screening of rare-occurring genetic disorders. Other limitations in the diagnosis and management of MH related to the health care systems operating in Egypt will be discussed thoroughly in the sixth chapter investigating the epidemiology of MH in Egypt.

1.6 Thesis aims

This study aims to investigate the incidence and prevalence of MH in the UK in recent years and to explore the genetic epidemiology of MH among the UK population. This research exploits the resources available at the Leeds MH Investigation Unit to explore the genotypic-phenotypic correlation of MH in the biggest cohort of patients who have had their susceptibility status confirmed using the gold-standard IVCT test after a suspected anaesthetic reaction or because of a clinical diagnosis of other MH-related muscular disorders. We also aim to define the extent of the problem of MH in Egypt in the absence of any referral or investigation centre in the region. Our long-term aim is to consolidate the use of the less-invasive genetic testing for the diagnosis of MH

susceptibility and to offer a base for future studies about MH in Egypt and the Middle East region.

1.7 Thesis objectives

1.7.1 Investigating the clinical epidemiology of MH in recent years

The initial objective of this study is to review the clinical details of suspected anaesthetic reactions of MH susceptible proband who were investigated at the Leeds MH Investigation Unit over the last three decades. This will enable us to have a more comprehensive understanding of the clinical presentation of MH in the lights of modern anaesthetic practice. Therefore, we will be able to define the sex and age characteristics of this cohort of patients and categorise different clinical phenotypes of MH and understand the effects of different pharmacological agents on the development of these phenotypes. In addition to investigating how different management measures affect the outcome of the reaction.

1.7.2 Correlation of *RYR1* genotypes to the clinical and IVCT phenotypes of MH

Subsequently, this understanding of the clinical epidemiology of MH among UK patients, and with the availability of genetic data for a vast majority of this cohort of patients, will enable us to correlate genotypic data to the clinical and IVCT phenotypes. The functionally characterised *RYR1* variants will be investigated to explore its effects on the development and severity of different clinical features of MH. Furthermore, we will compare these genotypes against contracture strengths from IVCT lab results in order to quantify its laboratory effects. In addition, the IVCT data of first-degree relatives of the included probands will be retrieved in order to examine the effects of the suspected MH reactions on the in-vitro response of skeletal muscle to halothane and caffeine.

1.7.3 Investigate the association between MH and CM

Clinical, genetic and histopathological details of the index cases of central core disease families will be reviewed in addition to their MH susceptibility status in order to identify variants that are causative of MH and lead to the development of CM. Details of other family members of CCD families who were tested at the unit will be reviewed to perform a segregation analysis of *RYR1* genotype, myopathy phenotype and MH phenotype. Also, the histopathological examination reports of all index cases of MH families will be reviewed to investigate the incidence of *RYR1*-related histopathological changes in MH susceptible patients who do not suffer clinical muscle weakness.

1.7.4 Explore the incidence of suspected MH reactions in Egypt

An online questionnaire will be conducted on a local, then on a national perspective to collect preliminary data about MH in Egypt. We will try to explore the experience of Egyptian anaesthetists with MH, possible outcomes of suspected cases and available management measures and protocols in Egyptian hospitals.

2 Patients and methods

2.1 Literature search strategy

We used different search engines to search for the available literature about MH and its clinical and genetic epidemiology published by different research groups and labs interested in studying the condition around the world. Google Scholar and PubMed were the main search engines we used during our literature search for different keywords. The following table 2-1 shows a list of keywords and phrases we used individually and in combination during our search. Our review of the resulting literature enabled us to identify many of the limitations of previous studies that will be discussed thoroughly in the next chapters and to determine the gap in science that we aim to cover using the resources available for us.

Anaesthetic deaths	Epidemiology	Myopathy
Ca^{+2} homeostasis	Excitation-contraction	Pharmacology
CACNA1S gene	Fibre type	Phenotype
Calcium release channel	Genotype	Rhabdomyolysis
Central core disease	Halothane	Ryanodine receptor type 1
Central nuclei	Inhalation anaesthetics	RYR1 gene
Congenital myopathy(-ies)	Malignant hyperpyrexia	RYR1 myopathy
Contracture test	Malignant hyperthermia	Succinylcholine
Cores	MH	Volatile anaesthetics
Creatine kinase	Muscle	
Dantrolene	Muscle relaxants	
Dihydropyridine receptor	Myopathies	

Table 2-1: List of keywords used to search for published literature. *Used individually or in different combinations.*

2.2 Identifying patients from the Leeds MH Investigation Unit database

Using the Leeds MH Investigation Unit's both databases, we identified patients to be included in different study cohorts. Index cases for each family were identified by

collecting IVCT numbers with the same family code, and as these IVCT numbers are assigned in chronological order, the patient with the lowest IVCT number is the index case for this family. As explained before in this study, we used the index case to refer to the first patient tested in each family while probands are the patients presented with the clinical phenotype and triggered investigation in their families. In some instances, probands were labelled on the database, however, we had to confirm their status by reviewing the reason for referral on the biopsy report stored in patients' files at the unit archive. After identifying patients to be included in each group of our study cohorts, other information collected from the database included: patients' date of birth, date of biopsy, MH susceptibility status, and basal serum CK level at the time of the biopsy. From the genetic table, we also collected information about genetic testing results for the included patients and any variants found in the three genes of interest; *RYR1*, *CACNA1S* and *STAC3*.

The following figure 2-1 illustrates the different study populations and how they are related to each other. Of all patients tested at the unit, the index cases, as according to the previous definition, are the first member of their families to be tested. This includes the probands who have presented with the clinical manifestation and triggered investigation in their families. A portion of index cases who were first tested but did not present with the clinical manifestation, light blue colour in the diagram, are the first-degree relatives of probands that could not be tested as they were deceased, too young to be tested, or unfit for the procedure. The graph also shows some overlaps between study cohorts that need some clarification. Group A is a cohort of patients who developed an anaesthetic reaction and upon review of their clinical notes, they were found to have some degree of clinical muscle weakness related to congenital myopathy. Group B patients are index cases of known CM families that have been

referred to the unit to be tested for the risk of MH susceptibility, without any history of anaesthetic reaction. While group C patients are other members of CM families that were referred because of the risk of MH either due to a history of anaesthetic reaction in the proband or confirmed MH susceptibility in other family members.

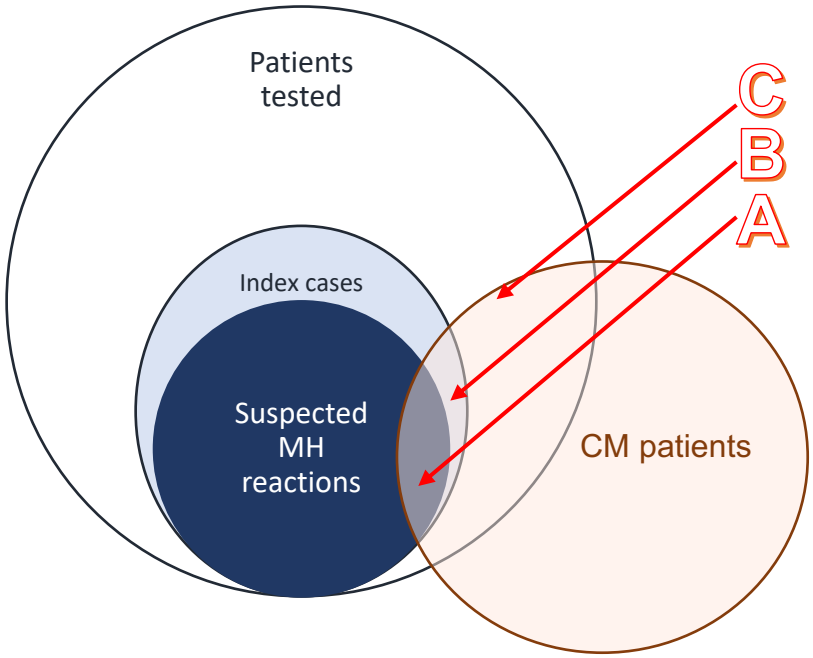

***Figure 2-1: Different study groups illustrating the overlap between different cohorts**. A) patients who developed a suspected anaesthetic reaction and were found to have CM related muscle weakness. B) Index cases of CM families and C) family members of known CM families whose index cases MH susceptibility status was previously confirmed. P.S. diagram proportions are not representative of actual percentages.*

2.3 IVCT measurements

The IVCT traces for patients tested at the unit are kept in their corresponding files in the unit archive, in addition to an electronic version of the more recent traces kept on the unit lab computers. We have retrieved the IVCT traces from files and lab computers to measure the initial tensions, twitch heights, and tension differences to compare how muscles, from different groups of patients included in our cohort, react in vitro to different drugs, i.e. the IVCT phenotypes.

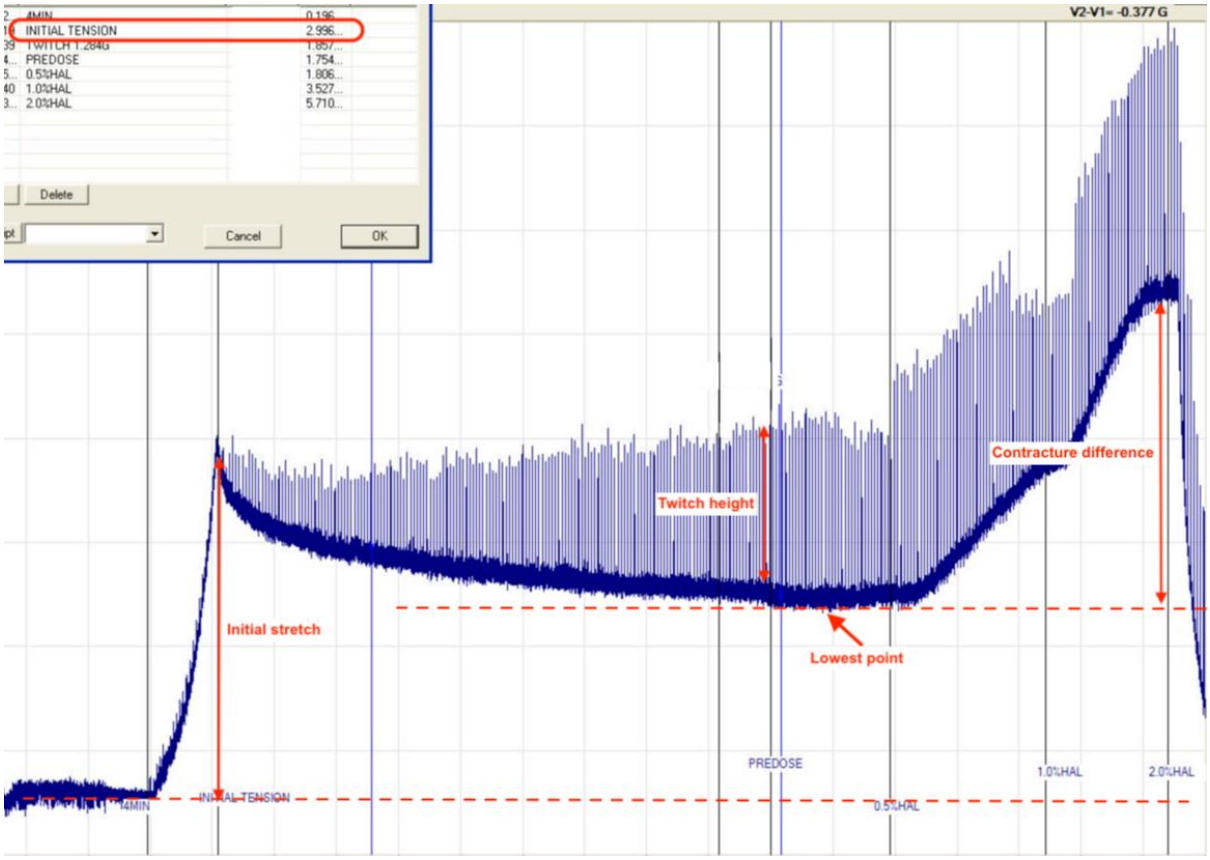

Figure 2-2: IVCT trace of static halothane test showing a positive response. *0.5, 1 and 2% halothane concentrations are added to the water bath. Tension difference is measured between the lowest point of contracture line and the 2% halothane.*

As shown in the following figure 2-1, two imaginary lines are drawn; one through the baseline at the start of the test and another one crosses the lowest point of the muscle contracture line. The initial tension is measured electronically, and this reading is used to define the scale of the graph. This scale is measured by dividing the electronic reading over the distance between the imaginary baseline and the initial stretch point. Using the resulting scale, the twitch tension and the contracture difference, in grams, are measured as follow:

- Twitch tension = graph scale * twitch height in cm
- Contracture difference = graph scale * distance between contracture line at 2% and the imaginary line crossing the lowest point

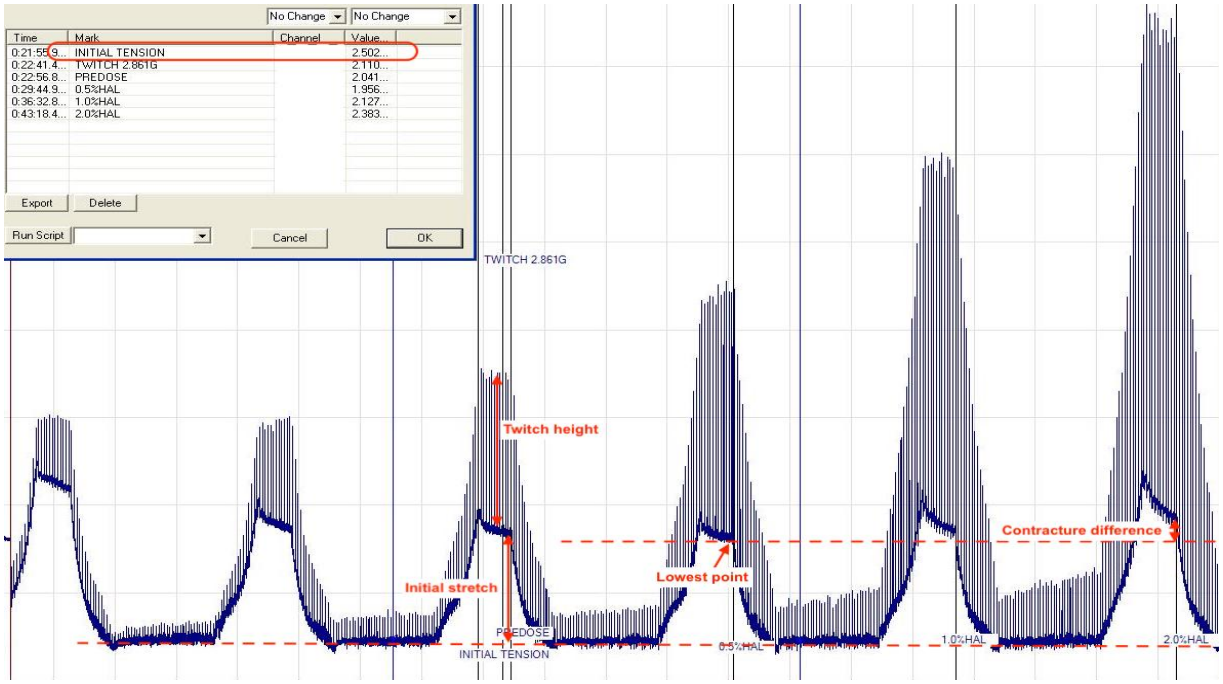

Figure 2-3: IVCT trace of dynamic halothane test showing a positive response. *The trace shows repeated stretching and relaxation of the muscle specimen. 0.5, 1 and 2% halothane concentrations are added to the water bath. The tension difference is measured between the lowest point of the contracture line and its corresponding point at 2% halothane.*

The same measurements are applied for the dynamic halothane and the static caffeine tests as shown in these figures 2-2 and 2-3.

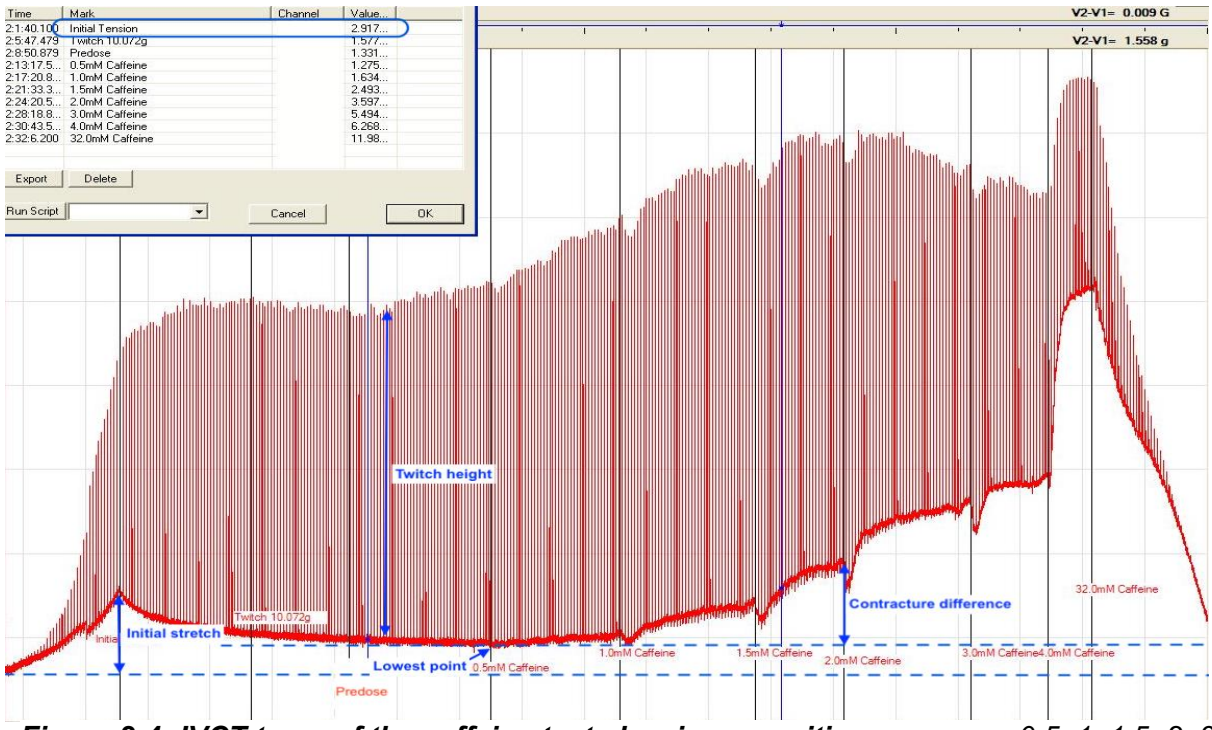

Figure 2-4: IVCT trace of the caffeine test showing a positive response. *0.5, 1, 1.5, 2, 3, 4, 32 mmol concentrations are added to the water bath. Tension difference is measured between the lowest point of contracture line and 2 mmol caffeine.*

All the above figures are traces from positive tests for MH susceptible (MHS) patients, while in the case of a negative test, the contracture difference at 2% for either of halothane tests or 2 mmol for caffeine test, will be a negative value as the muscle specimens continue to relax and the contracture line slopes down. In this case, this particular test is labelled negative; however, the patient is not labelled as MHN unless the results from the three tests are all negative. If any of the tests were positive, the patient should be labelled susceptible to MH. Historically, a patient with a positive test and not the others used to be labelled as MHE. However, more recently, the following codes are used to indicate patients' MH susceptibility status:

- MHShc: all the halothane and caffeine tests are positive.
- MHSh: only one or both halothane tests are positive, but the caffeine test is negative.
- MHSc: the caffeine test is positive, but both halothane tests are negative.
- MHN: all the halothane and caffeine tests are negative.

In clinical practice, patients should be treated as susceptible to MH and anaesthetised with triggering-free anaesthetics if any of the tests were positive.

2.4 Statistical analysis plan

The Clinical and Genetic Epidemiology of Malignant Hyperthermia is designed as part of the study entitled: Human Tissues and Genomics Research in Malignant Hyperthermia with REC reference: **10/H1306/70**, and the IRAS project ID: **60744**. Ethical approval has been obtained for the original study from the University of Leeds ethics committee. It is designed as a retrospective study to review the clinical details available for patients tested at the Leeds MH Investigation Unit in patients' files stored

at the unit archive and on the unit databases, while the patients have given their consent to use this data for future research at the time of the biopsy.

Statistical analysis was performed using Prism 7 for macOS, © 1994-2017 GraphPad Software, Inc. Variables such as age, time in minutes, temperature in ℃, $etCO_2$ in mmHg, and pH, were collected as continuous variables and tested for normality using the eyeball test and Shapiro-Wilk normality test when indicated. When data appeared to be normally distributed, parametric tests were used to compare between variables; student t-test for comparing between two groups and one-way ANOVA when comparing between more than two groups. When data appeared to be not normally distributed, data transformation was tried using the square root and Log 10 transformation methods. If these methods produced normally-distributed data, parametric tests were used, and back transformation to produce the geometric mean was used. When these methods failed to produce normally distributed data, non-parametric tests were used on the original data without any transformation; Mann-Whitney test to compare between two groups and Kruskal-Wallis test to compare between more than two groups.

When multiple comparisons were used to compare between each other group or between different groups and a control group, Fisher's Least Significant Difference (LSD) test was used for normally distributed data and Dunn's test for non-normally distributed data. No correction for multiple comparisons was performed, and the results were discussed accordingly. Chi-square test (X^2) was used to test for association between categorical variables. The level of significance (***p*** value<0.05) was considered statistically significant and is presented as a two-sided comparison, unless stated otherwise. Confidence interval (CI) of the mean is set at 95%.

2.5 Histopathological examination

As mentioned before, during a muscle biopsy, a muscle specimen is harvested for histopathological examination. This specimen is sent to the Department of Neuropathology, Leeds Teaching Hospitals, NHS trust to be examined by the neuropathology team. A microscopic examination of the muscle biopsy is performed to describe the fascicular architecture of the muscle and muscle fibres distribution, size and shape. In addition, the muscle sample is stained with different stains to detect the presence of histopathological changes. The Hematoxylin and eosin (H&E) stain is used to detect any atrophy or hypertrophy of muscle fibres, and the percentage of centrally located nuclei in these fibres, the nicotinamide adenine dinucleotide (NADH) stain detects the presence of cores, their number and location within the muscle cells. The ATPase stain gives details about fibre types variation and if there is a predominance of one type over another, and the Gömöri stain describe the mitochondrial contents of the muscle fibres and the presence of inclusion bodies.

2.6 Serum creatine kinase measurements

When the patients attend for the biopsy list at St James's University Hospitals, a routine procedure is to obtain a blood sample for measuring the basal serum CK level. The lab report of the results is kept in the patients' corresponding files at the Leeds MH Investigation Unit archive and recorded on the unit database. The reference range for the lab differed over the years from 0-140 IU at the lower range and 90-280 IU at the upper range. The reference range was only available for patients whose lab reports were available in their corresponding files but not recorded on the unit database. For basal CK levels, we used the Log 10 transformed values to obtain a normally distributed data and compared the results as described in the statistical analysis plan.

2.7 Screening for genetic variants

Since the establishment of the genetic background of MH, the Leeds MH Investigation Unit has started screening for genetic variants on the different genes involved in the pathogenesis of MH. The conventional methods used included Sanger sequencing (Sanger et al., 1977) and restriction fragment length polymorphism (RFLP). Nowadays, a panel of 50 genes are being screened at the DNA lab at St James's University Hospitals for variants associated with MH using next-generation sequencing (NGS) including the three genes with approved association with MH; *RYR1*, *CACNA1S*, and *STAC3*. This method has widely replaced the conventional methods; however, Sanger sequencing is still being used to scan for variants in some *RYR1* exons with inadequate coverage, e.g. exon 91, or to validate the results of NGS in some cases, and for in-house genetic testing. Patients tested using RFLP were screened for a list of *RYR1* variants, the number of variants on this list has increased over the years, and patients tested with this method may be found to have other variants if re-screened using NGS.

3 Investigating the clinical epidemiology of suspected MH reactions referred to the Leeds MH Investigation Unit

3.1 Introduction

The severe reactions to general anaesthetics that involved hyperthermia and led to death in many cases (Denborough et al., 1962, Cullen, 1966, Britt et al., 1969), was described first time by (Denborough and Lovell, 1960) and used the term malignant hyperthermia to name it. The term malignant was used to emphasise the high mortality rate of this condition at that time while the term hyperthermia, “hyperpyrexia” was used at that time, was used because of the progressive increase in temperature triggered by general anaesthetics and noticed in the affected patients. In these days, monitoring was depending mainly on clinical observation (Hopkins, 2000), but with the advances in monitoring equipment, the clinical picture of the reaction appeared to involve other manifestations that usually precede the development of hyperthermia. The classic fulminant form of the MH reaction became less prevalent as more anaesthetists became more aware of these other manifestations and with the introduction of advanced monitoring manifestations such as hypercapnia and tachycardia are now recognised as early indicators of a hypermetabolic reaction which enable the anaesthetists to early intervene and abort the development of a severe reaction. On the other hand, less severe forms of the reaction and early aborted cases are becoming more common to report.

Without exposure to triggering agents, MH susceptible patients are usually asymptomatic. Basal serum CK level could be slightly elevated in some patients, but this is not specific to MH and varies with age, sex and physical activity. Additionally, other clinical features that could be indicative of MH susceptibility such as heat

intolerance, heat exhaustion (Tobin et al., 2001) or muscle cramps (Ogletree et al., 1996), most of these indicators were found to be manifestations of other related muscular disorders such as central core disease or exertional heat illness that are often associated with MH and none of these indicators are considered specific to MH (Hackl et al., 1991, Hopkins et al., 1991). Even the association between MH and these diseases are not always consistent (Islander et al., 1995, Halsall et al., 1996, Curran et al., 1999). Other musculoskeletal abnormalities have been suggested to be associated with MH susceptibility such as scoliosis and hernias (Britt, 1979, Strazis and Fox, 1993). However, this association could not be approved after the analysis of the clinical details of a large cohort of MHS patients (Halsall et al., 1996).

MH reactions are triggered in susceptible patients on exposure to anaesthetics; nevertheless, not all susceptible patients trigger a hypermetabolic MH reaction in their first exposure. An average of three previous uneventful exposure was reported in patients who developed an MH reaction. Previous exposures to anaesthetics prior to the reaction are usually uneventful or could be associated with some unspecific manifestations such as postoperative muscular pain or stiffness, particularly, if succinylcholine was used as part of the anaesthetic. Family history is the most indicative of MH susceptibility, as from the first reported case (Denborough and Lovell, 1960), the anaesthetist was pre-alerted about a possible problem because of the family history of anaesthesia related deaths. Proper investigation of the family history, taking into consideration that MH is an autosomal dominant inherited condition, could alert anaesthetists about the possible risk and help to direct the patients for further investigation.

The clinical manifestations of the MH reactions involve signs of hypermetabolism such as tachycardia, increased CO_2 production, acidosis, which is mainly respiratory, and

hyperthermia. In addition, muscular features are also described as part of the anaesthetic reaction and include increased muscle tone either in a specific group of muscles, such as jaw muscle, leading to MMS, or in the form of generalised muscular rigidity. This continuous, uninterrupted muscular activity causes damage to muscle fibres and muscle breakdown. The results of this muscle breakdown include the release of muscle proteins in the blood (myoglobinaemia), which is excreted in the urine, causing myoglobinuria and an increased serum creatine kinase (CK) level. Additionally, the disruption of the membrane permeability leads to an increased release of potassium ions (K^+) into the bloodstream leading to hyperkalaemia. None of these features is specific and could be signs of too many other conditions. Ellis and colleagues (Ellis et al., 1990) reviewed the clinical details of 402 patients who developed a suspected anaesthetic reaction and referred to the Leeds MH Investigation Unit to confirm their MH susceptibility status using the IVCT. They compared the different combinations of these clinical features to the results of the IVCT test and described eight different clinical categories and calculated the probability of MH susceptible diagnosis in each category.

The anaesthetic practice including different pharmacological agents being used, monitoring equipment available, and standardisation of different management protocols and guidelines, in addition to the changes in surgical techniques and types of surgeries; should have an impact on the clinical presentation and management of an MH reaction. For instance, MH is known to be triggered by more potent volatile anaesthetics (Ørding and Bendixen, 1996), and with the cessation of use of halothane in the UK since the late 1990s and the use of less potent anaesthetics, it is expected for the incidence of MH to decrease or at least the fulminant form of the reaction. Also, the use of succinylcholine has diminished with the introduction of other muscle

relaxants that provides an accepted rapid onset of action for rapid sequence induction with fewer side effects; such as rocuronium. Additionally, the number of operations performed using other anaesthetic techniques such as regional anaesthesia and TIVA is increasing, with a reduction in the number of patients exposed to triggering agents. Patients undergoing certain surgical specialities are more commonly reported to develop MH reaction such as general surgery, E.N.T., and trauma. Also, management of MH crisis has been evolving with the availability of dantrolene, the establishment of guidelines and the introduction of activated charcoal filters as an alternative to gas-free machines.

The incidence of MH reactions was estimated to range between 1 in 15,000 to 1 in 50,000 (Britt and Kalow, 1970, Ording, 1985), although an estimate of the incidence of genetic MH susceptibility trait was estimated to be 1 in 2,000 (Monnier et al., 2002), while the prevalence is estimated to be 1 in 8,500 (Robinson et al., 2002). Infants as young as one year old to elderly patients are reported to develop MH reactions, but the most affected age group is reported to be young people with a mean age of 18.3 years (Rosenberg et al., 2015) with males being more susceptible than females. Male predominance in patients who tested susceptible to MH with disregard for the reason of referral is reported in several previous studies. In patients who were referred for diagnosis of MH susceptibility because of an anaesthetic reaction, this predominance was assumed to be due to males being more likely to have surgery associated with MH triggering agents (Ording, 1996). In Sweden, an analysis of 215 probands and 1191 family members found a statistically significant male predominance in both groups (Islander et al., 2007). They proposed several theories to explain this male preponderance: the first explanation is related to the possible sex difference in the Ca^{+2} regulating proteins expression, which could be related to the effects of sex

hormones. Another explanation hypothesises a relatively lower penetrance in female patients who carry the MH genotype. This hypothesis, though, does not explain the male predominance of MHS diagnosis with IVCT in relatives.

Different pharmacological agents are used during the anaesthetic practice, and it varies differently in the mechanism of action and the effects it has on the clinical status of the anaesthetised patient. These pharmacological agents have different effects on the triggering, course and management of a MH reaction. The different groups of drugs used during anaesthesia can be classified according to its effects on MH into three groups: the first group includes triggering agents, secondly is the group that may modify the response to MH, and the third group is the drugs used for management of a MH crisis. By definition, is a pharmacogenetic disorder that is triggered in genetically susceptible patients by volatile anaesthetics and(or) succinylcholine. All the volatile anaesthetics in-use in the modern anaesthetic practice can trigger an MH reaction. This is on the contrary to a worryingly growing belief that MH does not exist anymore, as it is considered a disease of halothane anaesthesia (Sneyd, 2017).

Neuromuscular blocking agents are split between the first and second groups as succinylcholine is considered by many to be a triggering agent for MH while non-depolarising muscle relaxants (NDMR) could modify the clinical picture of an MH reaction. The role of succinylcholine in triggering a hypermetabolic MH reaction is still controversial. Its role was investigated in several studies on porcine MH. While two groups studying porcine MH (Hall et al., 1972, Nelson et al., 1973) were not able to trigger an MH reaction in susceptible pigs using succinylcholine alone, (Harrison, 1971) found that the muscular rigidity induced by succinylcholine in susceptible pigs is prevented by prior curarisation. Reports about the development of a fulminant

hypermetabolic MH reaction with the use of succinylcholine alone either in humans or in pigs are not convincing (Hopkins, 2011). Most of the reported features of MH triggered by succinylcholine without the use of volatile anaesthetics can be otherwise explained due to other causes (Iaizzo and Wedel, 1994). Its combination with volatile anaesthetics is associated with a more exaggerated muscular response in the form of higher CK levels (Antognini, 1995) or marked rigidity (Aboelsaod et al., 2018).

On the other hand, NDMR was found to be protective against the development of MH in 50% of susceptible pigs that received halothane (Hall et al., 1976) or at least delay the development of the reaction (Suresh and Nelson, 1985). Analysis of the clinical data of suspected MH reactions in humans revealed a significantly longer time of onset of the reaction with a lower postoperative serum CK levels (Young et al., 2010) when NDMR was used. Moreover, a recent study conducted by P.D. Allen and colleagues and presented at this year EMHG annual meeting found that complete neuromuscular blockade prevented, utterly, the development of an MH reaction in susceptible pigs that were exposed to halothane. Additionally, they found that complete blockade with pancuronium prevented Ca^{+2} increase in susceptible mice exposed to isoflurane, while when this blockade was reversed with neostigmine, in vivo Ca^{+2} measurements showed an increase in Ca^{+2} levels.

Other drugs that belong to this second group of modifiers include IV agents and opioids. Few studies investigated the role of IV anaesthetics in MH triggering, and only thiopental was claimed to delay the onset of the reaction in susceptible pigs (Hall et al., 1972, Gronert and Milde, 1981, Suresh and Nelson, 1985). Studies of etomidate (Suresh and Nelson, 1985) and propofol (Raff and Harrison, 1989) could not find any similar effects to that of thiopental in swine MH. We could not find in literature enough

information about the possible effects of opioids on the development and severity of MH reactions. The third group of pharmacological agents include drugs used in the management of an MH crisis. The most important in this group that had a dramatic effect in reducing the mortality rate of MH reaction is dantrolene sodium. Since dantrolene was shown to treat an established MH reaction in pigs and was suggested to be used for treatment of MH in humans by (Harrison, 1975), its use in humans showed a marked improvement in survival after MH reaction (Kolb et al., 1982) and mortality rate of MH decreased dramatically from around 70% (Denborough, 1998) to less than 10% (Strazis and Fox, 1993). Other drugs that are used during the management of an MH reaction are not specific and only used as supportive or symptomatic treatment include sodium bicarbonate ($NaHCO_3$), magnesium sulphate ($MgSO_4$) and diuretics.

Recrudescence of MH is defined as the rebound of clinical features of MH reaction after the initial episode has been resolved with adequate treatment and without any further triggering. The pathogenesis of this recurrence of symptoms and signs is not fully understood; however, patients' body habitus, duration of anaesthesia and elevated temperature during the initial reaction were found to have been associated with a significantly higher rate of recrudescence (Burkman et al., 2007). This study found that, in patients referred to the North American Malignant Hyperthermia Registry (NAMHR), the recrudescence rate was reported to be 20% of patients whose CGS was >20, which reflects a likely diagnosis of MH. Previous case reports suggested that predictors of recrudescence of MH include persistent muscular rigidity (Mathieu et al., 1979), shivering (Short and Cooper, 1999) or tachycardia (Fletcher et al., 1982, Morrison and Serpell, 1998), in addition to hyperkalaemia and oliguria (Mathieu et al., 1979).

The clinical grading scale developed by Larach and colleagues (Larach et al., 1994) approved to be very helpful in determining the likelihood of the suspected anaesthetic reaction to be a true MH reaction. The opinions of a panel of experts were collected using the Delphi method to evaluate clinical details of patients who developed a suspected anaesthetic reaction. Scoring of different clinical indicators to generate a raw score that is ranked to describe the likelihood of MH diagnosis. The clinical indicators used were classified into six processes: the first process is related to the development of muscular rigidity, either generalised or MMS in relation to the administration of succinylcholine. The second process is dependent on manifestations of muscle breakdown such as elevated serum creatine kinase, myoglobinaemia or myoglobinuria, and increased serum potassium levels. Increased CO_2 was described in the third process, which indicates respiratory acidosis. Inappropriate temperature increase, according to the anaesthesiologist's judgement, is indicated in process 4, and cardiac involvement in the form of tachycardia or ventricular arrhythmias is the fifth process. A final process is described in relation to the family history of MH. A single indicator in each of the prescribed processes should be included to avoid double counting. However, other indicators such as arterial base excess, arterial pH, patients previous anaesthetic history, and the response of the reaction to treatment with dantrolene are added without taking double-counting into consideration.

All the included patients in this study have had their MH susceptibility status already confirmed. However, we should distinguish between the susceptibility status and the adverse anaesthetic reaction being a true MH reaction. As none of the manifestations of MH is specific, besides, these manifestations could be due to other reasons such as adverse drug reactions, equipment malfunction, or human errors; it is essential to evaluate each referred clinical reaction to confirm the diagnosis of MH or to evaluate

the possibilities of other diagnoses. For example, masseter muscle spasm (MMS) is a frequently described sign of an MH reaction, and 50% of patients who develop MMS are tested susceptible to MH (Ellis et al., 1992), though, this sign could indicate only succinylcholine sensitivity (Carpenter et al., 2009). Another example is the hypercapnia that may result from a malfunction of a Bain's circuit (Berner, 1987, Jellish et al., 2001), on the other hand, it could be a manifestation of an MH reaction. Therefore, we used the CGS to reassess the clinical details of the anaesthetic reactions of our MHS cohort of patients.

Using the resources available for us at the Leeds MH Investigation Unit, we aim to explore the male predominance described to be associated with MH and to investigate if it is a true predominance of MH susceptibility comparing our results to the cohort of MHN probands and the general surgical population. We also aim to investigate further, the role of succinylcholine in triggering hypermetabolic MH reactions and its effect on the pathogenesis and severity of the clinical features of the MH reaction. Additionally, we will analyse the outcomes of the reported anaesthetic reaction regarding MH-related complications, and MH-related deaths and investigate the role of different management measurers on these outcomes.

3.2 Patients selection and methods

3.2.1 Identifying MH susceptible index cases

As we discussed early, the main target of this chapter is to collect and review the clinical details of all suspected cases of malignant hyperthermia who were referred to the Leeds MH Investigation Unit and tested MH susceptible using IVCT over the 30 years period from 1988 to 2017, inclusive. We used the unit database to identify all MHS families that were tested at the unit during this period. The IVCT numbers, which

are assigned in chronological order, of all members of these families were sorted on an ascending order to identify the index case, the first patient to be tested, in each family. The files containing the IVCT biopsy report, medical and anaesthetic history, IVCT traces, clinical details of the suspected reaction, and laboratory reports of serum CK level of these cases are retrieved from the unit archive. These files were reviewed, and all the relevant information were collected for the purpose of this study. A spreadsheet was designed into different sections to collect all the relevant information to our study.

The first section aimed to collect information about the included patients' general characteristics including sex, date of birth, occupation, social history and sports activity. This section is aimed to compare these general characteristics to these of the general surgical population and to identify any specific characteristics of the MHS patients. A second section was designed to gather data about the medical history of any relevant condition, history of previous anaesthetics, including numbers and the type of anaesthetic used, and any complications that may be of relevant importance, in addition to a family history of anaesthesia related complications and/or deaths. These details are usually included in the IVCT biopsy report, included in patients' files. A third section collected the additional information included in this report about the date of the biopsy and the tension threshold at which the muscle specimens reacted to different concentrations of halothane and caffeine in the three IVCT tests.

The section for collecting information from anaesthetic charts and/or referral letters about the suspected anaesthetic reaction was designed to record the details of the anaesthetic technique used during the reaction including the pharmacological agents used: volatile anaesthetics, muscle relaxants (succinylcholine and non-depolarising

muscle relaxants), intravenous anaesthetics and opioids; in addition to the type of the circuit and the ventilation mode used in each patient when it was reported. We also recorded the first clinical sign of the reaction noticed by the attending anaesthetists, and the time in minutes from the induction of anaesthesia to the start of the reaction. Maximum derangement values of clinical signs such as temperature, $etCO_2$, and heart rate, in addition to lab results such as pH, K^+, and serum CK levels were also recorded. If the referral letter and/or anaesthetic chart included a mention about the occurrence of features such as MMS, generalised muscle rigidity, myoglobinuria, and coagulopathy, it was recorded as a binary yes or no variable, but it was left blank if it was not reported at all.

Another section was designed to collect information about management measures used during the reported suspected reaction. The use of dantrolene, the time from the recognition of an MH reaction to the administration of dantrolene, the initial and the total dose used is recorded. In addition, we collected information about other specific management measures such as cooling techniques to reduce body temperature, the use of sodium bicarbonate ($NaHCO_3$) for acidosis, replacement of anaesthetic machine with a gas-free machine. Any other management measures reported by the attending anaesthetist were also recorded, and this included the use of fluid boluses, diuretics and vasopressors. We also recorded if the surgery was abandoned or was allowed to continue; as this may provide an idea about the severity of the reaction in the anaesthetists' judgement.

The last section gathered details of the outcome of the suspected anaesthetic reaction. Possible complications related to muscle breakdown such as postoperative muscle pains, stiffness or weakness were described. The occurrence of compartment

syndrome was also described in a few patients. Severe rhabdomyolysis and renal complications such as acute kidney injury (AKI) and/or acute renal failure (ARF) were also reported, and we recorded if these complications required haemodialysis. In addition, we recorded if patients mandated an intensive care or high dependency unit admission, the length of stay, and any further complications reported if ICU notes were available for review. Recrudescence was reported in some patients, and we collected all the available clinical information about subsequent episodes. In the instance of index cases who were tested as family members of patients who did not survive the reaction, we collected clinical details of the suspected fatal anaesthetic reactions but will be presented separately in the results section of this chapter.

3.2.2 Patients who had suspected anaesthetic reactions and tested MHN

We had to compare our results to a control group. We used data from patients who have been referred to the unit for testing for MH susceptibility because of a suspected anaesthetic reaction and were tested MHN with IVCT. The clinical details of anaesthetic reactions of a total of 250 patients referred to the unit over the period between 1990 and 2009, were collected by medical students for a research project. We have retrieved these details after obtaining permission from the project coordinator. The data collected for this project included information about patients' sex, the volatile anaesthetics used, the use of succinylcholine, and information about the clinical features of the reaction. Clinical features collected included the maximum derangement values of $etCO_2$, temperature, heart rate, pH, postoperative serum CK levels and serum K^+ levels. They also reported the occurrence of MMS as a sign of muscular rigidity. Some missing details compared to our dataset of MHS probands include the date of the reaction to determine patients' age at the time of the reaction,

the occurrence of other muscular features such as generalised rigidity and myoglobinuria, and the outcome of the reaction including management measures.

3.2.3 The general surgical population

The denominator for our cohort to be compared against is the surgical population when comparing variables such as sex, age and surgical specialities. We were able to obtain this information from the data presented at the National Audit Project (NAP) reports (https://www.niaa.org.uk/NAP_home). The NAP is a project to study rare-occurring anaesthesia related complications with serious consequences. In addition to the information provided about the topic of interest for each project, the NAP offers valuable data on the numbers and age and sex characteristics of patients receiving anaesthetics for different procedures. The most recently published NAP report is the NAP 6 project (Harper et al., 2018) investigating the allergic reactions associated with anaesthesia and surgery. The majority of data collection for the NAP 6 project took place over a three-week period in October 2016. The annual caseload was estimated by multiplying the number of cases by a scaling factor. This project reported a reasonable return rate of the forms distributed to the 342 hospitals involved in the project. The estimated annual caseload was 3,126,067 cases.

3.2.4 Data handling

The sports activity that is recorded in the biopsy reports stored in patients' files at the unit archive was categorised into three degrees: patients who reported no sports activity at all was given grade 1, while patients who mentioned they perform some sports activity to remain active were given grade 2 and patients who reported a professional athletic training were given grade 3. The history of cramps was also recorded as patients either reported suffering cramps or not. The age at which the

proband had the anaesthetic reaction is not routinely recorded in the IVCT biopsy reports that are kept in patients' files in the unit archive. We recorded the date of birth and the age at the reaction for each proband included in this study. The first reported sign is recorded according to the reporting anaesthetist judgement. Through reviewing anaesthetic charts, we found that some signs could have been developed earlier and not noticed by the attending anaesthetist, though, for consistency, we included the signs reported by anaesthetists. Unless stated otherwise, we considered patients to have normal temperature before the procedure, and for statistical analysis, we recorded the difference between the highest reported temperature and 37℃. Similarly, for pH, we recorded the difference between the lowest recorded value and 7.4.

As the $etCO_2$ baseline is not routinely recorded, for statistical analysis, we recorded the maximum derangement value and as discussed earlier, we used a cut-off point for considering heart rate is inappropriately increased if it was >100 beats/minute in adults and >120 beats/minute in paediatrics and included the highest recorded value. For signs such as MMS, generalised muscular rigidity, myoglobinuria and coagulopathy; although the absence of these signs has been reported in few patients, we considered these signs as negative when it was not mentioned in other patients. K^+ and serum CK maximum derangement levels were recorded as absolute values regardless of the baseline level before the reaction. In a few patients, the initial rise and the maximum value of serum CK level were recorded in addition to the time in minutes between the initial rise and the maximum value.

3.2.5 Calculation of the Clinical Grading Scale

Each of the clinical features that are listed in the clinical indicators used by Larach et al. was given points to calculate the CGS for each reaction. Unless stated otherwise

in the referral letters, all the described clinical features are considered as "inappropriate in anaesthesiologist's judgement". Some indicators such as generalised rigidity, MMS, the CK level in relation to the use of succinylcholine was clearly and easily interpreted. For indicators of the respiratory acidosis process, the mode of ventilation, as spontaneous or controlled, was not described in many patients. Therefore, when the ventilation mode was not mentioned, we used the cut-off point of 8 KPa (~60 mmHg) for $etCO_2$ and 8.65 KPa (~65 mmHg) to give this indicator the 15 points for respiratory acidosis process. For temperature indicators, unless the baseline temperature, prior to the reaction, was recorded, we assumed a normal baseline temperature of 37℃ and that the recorded rise in temperature is considered "inappropriate in anaesthesiologist's judgement".

Cardiac involvement indicators were the most challenging to assess in our cohort of suspected MH reactions. It was seldom mentioned in the referral letter if the anaesthetist considers this heart rate as inappropriately increased. In addition, when retrieving these data from the anaesthetic charts, it was not always clear the temporal relation between the heart rate and other factors that may have an influence on its pace, such as skin incision or other medications. We had to set our cut off point of 100 beats/min for adults and 120 beats/min for children to be considered as inappropriate, regardless of the anaesthesiologist's judgement and other factors. The family history process was not included in our calculation of the raw score for all anaesthetic reactions as all of our included patients are index cases for their families (first member of the family to be investigated) and although we recorded any relevant family history of anaesthesia related complications or deaths, all, but four, of our patients, had no family history of such complications. The clinical indicator with the highest points in each process was chosen and summed to produce the raw score for each patient. In

our statistical analysis, as will be discussed later, we used both the raw score and the MH rank or the likelihood description to compare between the groups.

3.2.6 Special circumstances

Index cases who have been referred because of a family history of anaesthesia related deaths identified and whenever the clinical details and anaesthetic notes of the deceased patient(s) were available, they were reviewed. The details of the fatal anaesthetic reaction will be reported with emphasis on the possible reasons for the failure of recognition and proper management of the suspected reaction. We also reported the characteristics of the deceased patients regarding age, sex and comorbidities, when these details were available to compare them against the characteristics of the general MH cohort. Another special group included in our cohort are patients who developed recrudescence of MH features after the resolution of the initial episode with proper treatment, and despite the cessation of any further triggering. Incidence of recrudescence, the number of further episodes and measures required for treatment of these further episodes, in addition to possible causes for this rebound are reviewed and reported. We also reviewed any available intensive care unit reports about further episodes to ascertain if it is a recrudescence episode or some other complications.

The previous anaesthetic history of all the included patients was examined. We recorded the number of previous anaesthetics for each patient and any associated complications for patients who had previous general anaesthetics. We identified patients who had complications that could be relevant to MH but were not well-evaluated before the current anaesthetic. By reviewing any available notes of previous anaesthetics, we tried to explore the nature of these complications and the possible

triggering agents used. In addition, we investigated the possible reasons why these complications were not adequately assessed before the current attack and to clarify any ambiguity that led to these details being overlooked.

3.3 Results

3.3.1 Summary of general characteristics of MH susceptible probands

In total, 328 patients included in this cohort as they have been tested susceptible to MH with IVCT after a suspected anaesthetic reaction. These patients have been biopsied during the period between 1988 and 2017; nevertheless, the date of reaction spans between 1971 and 2016. The reason for patients who developed a reaction prior to 1988 not being tested during this period and not being included in (Ellis et al., 1990) review, is beyond the scope of our study. Possible reasons may include delay in the referral to the unit or refusal of the muscle biopsy by the patients. As some of the patients who developed a suspected reaction before 1988 have been tested and included in Ellis 1990 review, we cannot rely on the numbers of patients per year during this period. During the period after 1988, 272 patients developed a suspected anaesthetic reaction and were tested MHS with an average of nine cases/year. The year 1992 was the year when the largest number of patients (25 patients) were reported to have developed a suspected MH reaction. Interestingly, the same year reported the highest rate of MH incidents reported to the NAMHR in a study of suspected MH reactions over the period between 1987 and 2009 (Visoiu et al., 2014).

The age at which the patient developed the suspected reaction ranged between one year and 73 years old, with an average of 23.2 years old. The age distribution is shown in figure 3-1 with the most common age group is the young adults aged between 18 and 40 years old (n. 145 patients) followed by children below the age of 18 years old

(n. 135 patients). Regarding sex distribution, males were predominant in our cohort (n. 217 patients), representing 66.2% of all MHS tested probands during this period. Apart from the group of three patients above the age of 60 at the time of the reaction who were all males, males were more predominant in the other three age groups, figure 3-1. About two-thirds of the included patients were tested for MH susceptibility at the Leeds MH Investigation Unit within the first two years after they developed the suspected anaesthetic reaction. The maximum difference between the year of the reaction and the year of the test was 37 years.

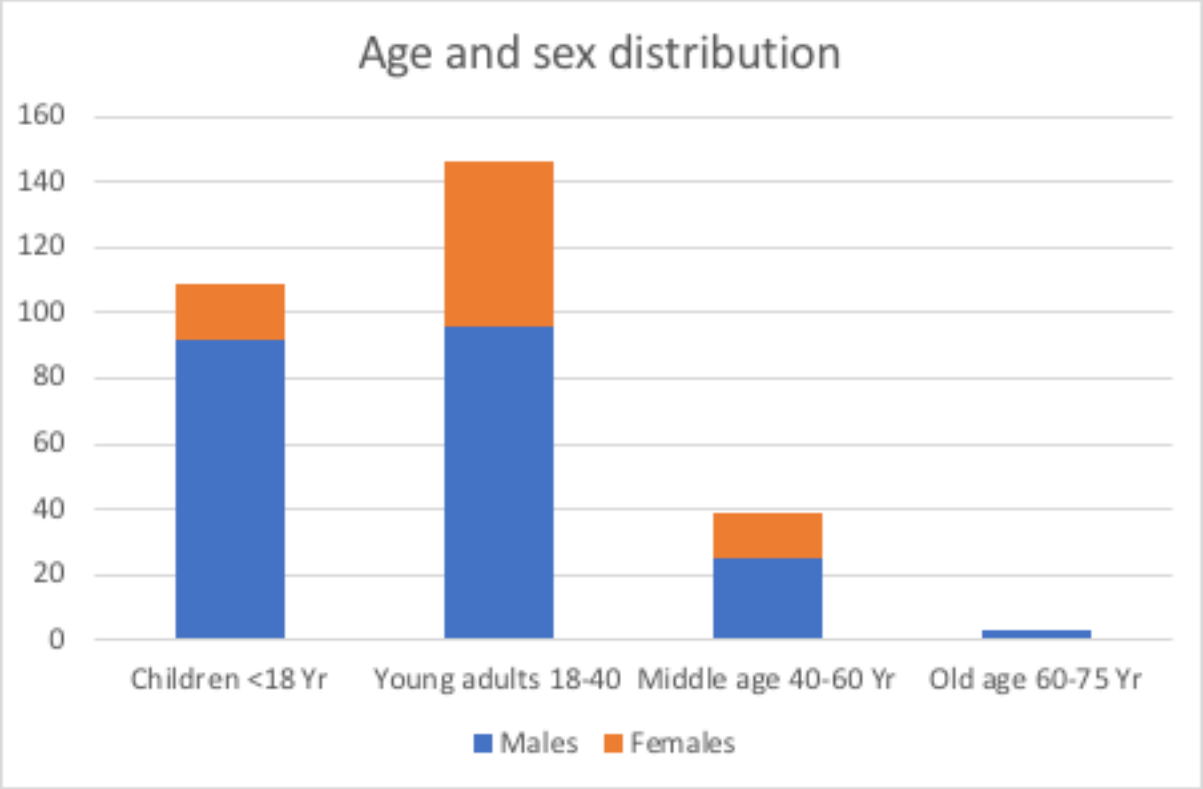

Figure 3-1: Distribution of age and sex in our cohort of MHS probands. *Patients' age is the age at which the patients developed the suspected anaesthetic reaction.*

The most common surgical speciality reported was the orthopaedic surgeries, including trauma, in 87 patients. This was followed by general surgery operations in 76 patients and E.N.T. surgeries in 73 patients. These three specialities represented two-thirds of all the reported reactions. Other specialities reported included, in a descending order: dental procedures in 29 patients, obstetrics and gynaecology in 23 patients, urology in 15 patients, plastic in ten patients, ophthalmology in five patients, four patients during cardiothoracic surgery and one patient during neurosurgery procedure. Among the three major surgical specialities represented in our cohort, males were predominant within the same ratio for the whole cohort, except for orthopaedic and trauma surgery, as males represented 81.6% in this group. In 27.74% of the reported cases, the patients were anaesthetised for an emergency procedure.

The past medical history of ten patients included in our cohort showed musculoskeletal abnormalities, and in five of these ten patients, a diagnosis of CM was established after the reaction, these five patients are discussed in the fifth chapter investigating the association between MH and CM. In the remaining five patients, four patients had some degree of scoliosis, and one patient had osteogenesis imperfecta. Another patient reported ptosis with no other muscular weakness. More than one-third of the patients, 135 of 328 patients reported no previous general anaesthetics prior to the reaction. The other 193 patients reported previous operations when triggering anaesthetics were used, and the number of previous anaesthetics ranged from one to a maximum of 11 previous anaesthetics. Apart from the 19 patients who will be discussed under special circumstances later on in this section, all the reported previous anaesthetics were uneventful.

Information about the physical activity was available for 322 of the included patients, 149 patients reported a mild to moderate degree of sports activity to remain active, while 135 patients reported not engaging in any sports activity at all. The remaining 38 patients reported professional athletic training. Regarding muscle cramps, 67 patients reported suffering from muscle cramps, while the other 261 patients reported no history of muscle cramps. No statistically significant association could be detected between the level of sports activity and the occurrence of muscle cramps, X^2= 2.51, 2 df., with ***p*** value=0.29. As a routine when patients come for their muscle biopsy, a blood sample is collected and sent to the lab at St James's University Hospitals to measure the basal serum CK level. Using a student t-test to compare between the means of Log10 transformed values of serum CK level, males were found to have a statistically higher serum CK levels (geometric mean=232 IU/L) when compared to females (geometric mean=162 IU/L), ***p*** value<0.0001, 95%CI 0.586-0.833. While no

such difference was found when comparing between patients who reported muscle cramps and patients who did not, ***p*** value=0.73. Similarly, no such difference could be found when comparing between different age or sports activity groups using one-way ANOVA of Log10 transformed CK level values, ***p*** value=0.76 and 0.48, respectively.

3.3.2 The MHN cohort and the general surgical population

A cohort of 250 patients who developed an adverse anaesthetic reaction that was suspected to be an MH reaction, was referred to the Leeds MH Investigation Unit over the period between 1990 and 2009 and tested MHN using IVCT. Sex distribution in this cohort was not statistically different from our cohort of MHS probands with a male predominance of 60.8% (X^2=1.72, 1df with ***p*** value=0.19). The male: female ratio in our cohort was significantly different from the general surgical population presented in the NAP 6 project with a female predominance of 58.7%, (X^2=90.9, 1df with p value<0.0001). The exact dates when the reaction occurred have not been collected by the medical students for their research project. Therefore, a comparison of the mean age at the reaction between our cohort and the MHN cohort was not possible. Regarding the general surgical population, the exact data about the mean age of the sampled cohort was not available on the activity survey report (Harper et al., 2018). According to this report, the age distribution chart showed that the most common age group exposed to anaesthetics is the group aged 26-35 years old, followed by the group aged 66-75 years old.

Comparing the basal CK level at the time of biopsy between our cohort of MHS probands and the MHN cohort using student t-test of Log10 transformed data, we found that basal serum CK level in MHS cohort (geometric mean=205 IU/L) was statistically significantly higher than the MHN cohort (geometric mean=112 IU/L), ***p***

value<0.0001, 95% CI 0.488-0.615. Similar to the difference in our cohort of MHS probands, in the MHN cohort of patients, males were found to have higher basal serum CK level (geometric mean=127 IU/L) compared to females (geometric mean=92 IU/L) using student t-test comparing Log10 transformed data, ***p*** value<0.0001 95% CI 0.635-0.84.

3.3.3 Different anaesthetic techniques and the clinical presentation of the reaction

Regarding the anaesthetic technique used including volatile anaesthetics, muscle relaxants, intravenous anaesthetics, the technique of airway management and mode of ventilation, and the available monitoring, this information was not always available, complete, nor consistent between the included patients. For example, information about monitoring was available for 73 patients, as temperature monitoring was used in 40 patients, and $etCO_2$ monitoring was available for 41 patients according to the recorded information. Although we can determine the type of monitoring used from the reported clinical information for each patient, we cannot be confident whether this monitoring was used from the start of anaesthesia or only applied to the patient after suspecting an anaesthetic reaction. Noticeably, information about the use of succinylcholine was almost complete in all patients whose clinical details of the reaction were retrieved. On the contrary, information about NDMR, if it was used and which drug was used, was not always available. Similarly, no available information about whether a volatile anaesthetic was used, and which drug was used in 15 patients in our cohort.

The following table 3-1 illustrates the trend in the use of different anaesthetic drugs in our cohort over the years between 1971 and 2017. Enough information about the triggering anaesthetics used in our cohort was available for 271 patients. Before 1988,

the most common triggering volatile anaesthetic was halothane, followed by isoflurane. Isoflurane was the most common triggering agent in all patients reported to have a reaction in or after 1988. The second most common trigger in the period from 1988 to 1997 was halothane and during the following years afterwards was sevoflurane. As shown in table 3-1, the number of patients reported in each decade after 1988 is decreasing, as well as the percentage of the use of succinylcholine. On the other hand, the percentage of the use of NDMR and opioids showed a steady increase over the years until the last decade, after 2008, when it slightly decreased for both groups of drugs.

	Before 1988 (n.30)	**1988-1997 (n.123)**	**1998-2007 (n.71)**	**2008-2017 (n.41)**
Volatiles	Halo>Iso>Enf	Iso>Halo>Enf	Iso>Sevo>Des	Iso>Sevo>Des
Succinylcholine	27 (90%)	98 (79.7%)	34 (47.9%)	9 (21.95%)
NDMR	0	32 (26%)	37 (52.1%)	19 (46.3%)
Opioids	1 (3.3%)	41 (33.3%)	50 (70.4%)	25 (60.98%)

Table 3-1: The trend of reported cases distributed according to the year at which the reaction had occurred. *The most common volatile anaesthetics used in each time period and the number of cases reported the use of other pharmacological agents in each time period. Halo. --> halothane, Iso. → isoflurane, Enf. →enflurane, Sevo. → sevoflurane, Des. → desflurane, and NDMR→ non-depolarising muscle relaxants*

In our cohort, we investigated the role of succinylcholine in triggering a hypermetabolic MH reaction. An anaesthetic reaction was reported after the use of succinylcholine without the use of volatile anaesthetics in 25 patients with enough clinical details. An anaesthetic reaction that involved both metabolic and muscular features were reported in ten patients with one patient reported to have appendicitis and preoperative fever. In the other 15 patients, no metabolic manifestations were reported, and the reaction was in the form of exaggerated muscular response. MMS was the first sign noticed by the reporting anaesthetists in 13 patients and the only sign in six patients of those.

MMS was reported with other muscular features in the form of generalised rigidity, myoglobinuria and/or elevated serum CK levels in seven patients. Of the remaining two patients, one patient presented with unexplained cardiac arrest and the other patient presented in the postoperative period with rhabdomyolysis. All these 15 patients had made a full recovery without the need for ICU admission for the majority of them, and only two patients had been admitted to the ICU and dantrolene was used in only one patient.

Administration of succinylcholine, in combination with volatile anaesthetics, was found to have marked effects on the muscular features and to a less extent on the metabolic features of the reported reactions in our cohort of patients. A statistically significant association between the use of succinylcholine and the development of muscular features in the form of MMS, generalised muscular rigidity and myoglobinuria can be found in our cohort, X^2=79, 1 df., ***p*** value<0.0001. The development of MMS was strictly associated with the administration of succinylcholine. Also, none of the patients who received volatile anaesthetics only, developed muscular features-only reactions. Additionally, the maximum reported value of postoperative serum CK level was statistically higher in patients who received succinylcholine as part of their anaesthetic (geometric mean=10,964 IU/L), using student t-test of Log 10 transformed values of serum CK levels, compared to patients who did not receive succinylcholine (geometric mean=3,026 IU/L) ***p*** value<0.0001, 95% CI 0.167-0.53.

Regarding the metabolic features of the reported suspected MH reactions, the use of succinylcholine was found to be associated with a statistically significant shorter time of onset of the hypermetabolic reaction (geometric mean=28.8 minutes), compared to when it was not used (geometric mean=52.5 minutes), student t-test comparing Log10

transformed data of time in minutes, ***p*** value=0.0005, 95% CI 1.31-2.54. Nevertheless, the use of succinylcholine was not associated with a statistically significant difference in the maximum derangement values of the metabolic features of the reaction including, etCO2, temperature and pH, table 3-2. Additionally, the use of succinylcholine was associated with a higher rate of postoperative complications, X^2=9.3, 1df, ***p*** value=0.0023.

	Succinylcholine used	**Volatiles without succinylcholine**	
		NDMR used	**NDMR not used**
etCO2	n.79- (Med. 10, IQR 8-12 kPa)	n.46- (Med. 10.8, IQR 8.7-12.6 kPa)	n.38- (Med. 10.6, IQR 8.5-13.9 kPa)
Temperature	n.107- (Med. 38.8, IQR 38.2-40 ℃)	n.43- (Med. 39.1, IQR 38.6-39.9 ℃)	n.37- (Med. 39.2, IQR 38.5-40 ℃)
pH	n.83- (Med. 7.19, IQR 7.06-7.260)	n.34- (Med. 7.13, IQR 7.07-7.24)	n.22- (Med. 7.13, IQR 7.00-7.21)

Table 3-2: The effects of the use of different anaesthetic agents on the maximum derangement values of the hypermetabolic features of the MH reaction. *N. → is the number of patients with valid clinical details for this feature. Med. → median, and IQR → interquartile range.*

Non-depolarising muscle relaxants were reported to be used in 92 patients in our cohort, and the most common NDMR reported to be used in our cohort was atracurium, followed by vecuronium and rocuronium. The use of NDMR was associated with a statistically significant lower serum CK levels (geometric mean=5,370 IU/L) compared to when NDMR not used (geometric mean=9,225 IU/L) using student t-test to compare between Log10 transformed values of maximum CK levels, ***p*** value=0.029 95% CI 0.98-3.04. When studying the association between the

use of NDMR and the development of muscular features, we excluded MMS as in all cases; MMS followed the administration of succinylcholine. No statistically significant association could be noticed between the use of NDMR and the rate of development of the other muscular features of the reaction, namely, generalised rigidity and myoglobinuria, X^2=0.62, 1 df., ***p*** value=0.43. Additionally, regarding the metabolic features of the reaction, no significant effect of the use of NDMR on the time of onset (student t-test ***p*** value=0.279) nor the maximum derangement values of $etCO_2$, temperature and pH, could be found, table 3-2. Opioids reported to have been used in our cohort in 125 patients included morphine, fentanyl, alfentanil and remifentanil, and their use was not associated with any significant effects on the development nor the severity of any of the reported metabolic and muscular features of the suspected reactions.

Figure 3-2 shows the first clinical sign of the reaction noticed by the reporting anaesthetists. The most common sign reported was MMS that was preceded by the administration of succinylcholine in all patients. Of the metabolic features, elevated $etCO_2$ was the most common hypermetabolic sign first noticed by the attending anaesthetists. To study the association between the mode of triggering: volatile anaesthetics only, succinylcholine only, and both drugs; and the clinical picture of the reaction, we categorised the reaction into muscular features-only reactions, metabolic features-only reactions, and both. Additionally, we used the categorisation described by (Ellis et al., 1990) of the clinical manifestation of the reaction. Table 3-3 shows the number of patients presented with each category of the clinical classification of the reaction and the triggering agents in each. We have discussed earlier the patients who reported an apparent hypermetabolic reaction triggered only by succinylcholine. On

the other hand, none of the patients who received volatile anaesthetics only, without the use of succinylcholine, reported a muscular features-only reaction.

Figure 3-2: The first clinical signs of the reaction reported by the referring anaesthetist. *Other signs included hypoxia, generalised rigidity, arrhythmia and cardiac arrest.*

The clinical picture of the 25 patients who received succinylcholine only without volatile anaesthetics have been discussed earlier, and the numbers in each category are shown in table 3-3. None of the patients who received volatile anaesthetics only without succinylcholine fell into clinical categories involved muscular features only: (d) and (f) nor into category (e): MMS with signs of metabolic disturbance. Ellis and colleagues compared these categories between patients who were tested MHS and MHN and estimated the incidence of MHS diagnosis in each category, as shown in table 3-3. However, all patients included in our cohort were tested susceptible; therefore, we are showing the clinical grading scale, medians and IQR, for each category in table 3-3, to compare between different classifications.

Clinical category	Succinylcholine only	Volatiles only	Both drugs	Incidence of MHS	CGS (med., IQR)
a)	3	29	37	0.96	40, 33.5-53
b)	2	52	89	0.88	30, 25-45
c)	1	12	8	0.14	18, 15-25
d)	7	0	3	0.76	22, 15-30
e)	3	0	3	0.57	18, 15-35
f)	6	0	16	0.28	15, 15-15
g)	2	1	3	0.66	34, 16-46.5
h)	1	4	8	0.07	15, 10.75-20

Table 3-3: Number of included patients in each clinical category. *Patients are categorised according to the triggering anaesthetic agents used. CGS is the estimated clinical grading scale for each patient presented as median and interquartile range for patients included in each clinical category.*

In the MHN cohort of patients, no information about the triggering agents was available for eight patients. In another 34 patients, no volatile anaesthetic was reported to be used, and only succinylcholine was reported to trigger the reaction. The manifestation of the reaction in these 34 patients when only succinylcholine was used, included signs of hypermetabolism; hypercapnia, hyperthermia and/or acidosis, in 13 patients. On

the contrary to the observation noticed in our MHS cohort that MMS was always preceded by the use of succinylcholine, eight MHN patients reported MMS without the use of succinylcholine that was triggered by volatile anaesthetics only. The following table 3-4 shows the triggering agents used in the cohort of MHN patients and the nature of the reported anaesthetic reaction regarding metabolic and muscular features. The highest reported serum CK level in MHS patients (geometric mean=7,834 IU/L) was found to be statistically significantly higher than in MHN patients (geometric mean=1,606 IU/L), when comparing the Log10 transformed data using student t-test with a ***p*** value<0.0001.

	Succinylcholine only	**Volatiles only**	**Both drugs**
Metabolic-only	9	75	30
Muscular-only	21	6	51
Both clinical features	4	5	24

Table 3-4: Number of MHN patients in each clinical phenotype. *Categorised according to the triggering anaesthetic agents used.*

3.3.4 Different management measures and their effect on the outcome of the reaction

The published EMHG guidelines for managing an MH crisis (Glahn et al., 2010) offers an easy step-by-step protocol to recognise and treat an MH reaction. In our cohort of patients, we noticed a discrepancy between the number of patients reported to have clinical features and the number who received a symptomatic treatment directed for these features. On the other hand, reporting of dantrolene use was consistent throughout our cohort with a precise record of its dose, when it was used, available for most of the patients. The following table 3-5 shows the number of patients presented with each of the clinical features of MH and the proportion who received symptomatic

treatment for these features. Although the change of the breathing circuit or the anaesthetic machine with a gas-free machine is not recommended according to the EMHG 2010 guidelines, it was reported to be changed in 53 patients. Additionally, the guidelines recommend discussing with the surgeon the termination or postponement of the surgery, though, the surgery was allowed to proceed in 226 patients in our cohort, although in 148 patients of them, anaesthesia was for an elective procedure.

Clinical features	Patients reported (n.)	Treatment used (n., %)
Hyperthermia ≥38.5℃	149	109, 77.86%
Hyperkalaemia	62	20, 32.26%
Acidosis pH<7.2/$NaHCO_3$ received	90	25, 27.78%
Arrhythmias	21	0
Urinary output	-	46
Dantrolene used/ dose recorded	188	148, 78.72%

Table 3-5: The number of patients presented with each of the clinical features. *Percentage of patients who were reported to have received treatment for these features according to the EMHG guidelines for management of MH crisis. In addition to the number of patients who were reported to have received dantrolene and the percentage of cases when the exact dose used was recorded.*

As shown in table 3-5, dantrolene was used in 188 patients in our cohort. In none of the included cases, dantrolene was reported to be unavailable. However, in patients when dantrolene was not used, no detailed explanation was provided for why it was not used. The use of dantrolene was associated with a lower rate of postoperative complications in the included cohort, X^2=6.94, 1df, ***p*** value=0.0085. Treatment of hyperthermia included surface cooling, intravesical, intragastric irrigation with iced saline and iced IV fluids. Most of the patients who developed hyperthermia ≥38.5℃ received active treatment (77.86%). Even more, 25 of the 56 patients who reported a maximum temperature of <38.5℃, received active treatment to reduce their body temperature. Other drugs reported to be used in the management of MH crisis in our

cohort included dextrose/insulin infusion for the treatment of hyperkalaemia, steroids, and dopamine. $NaHCO_3$ was reported to be used for the treatment of acidosis in 27% of patients who reported a pH <7.2 and in seven out of the 62 patients with a pH between 7.2 and 7.35. Diuretics, including mannitol or furosemide, was used in 41 patients to maintain the urinary output and also for the treatment of hyperkalaemia.

All of the patients included in this cohort have survived the current anaesthetic reaction, and as indicators of the outcome, we used the rate of postoperative complications reported, and the length of ICU stay. However, the latter is not very reliable in our cohort. In some occasions, the patients have not been admitted to the ICU because of unavailability of beds or due to causes related to logistics of transferring the patient to another hospital. Additionally, for the 189 patients who have been admitted to the ICU, the length of stay was reported in only 32 patients with very scarce clinical details about the ICU stay. Regarding postoperative complications reported, muscle-related complications and renal related complications were the two main groups reported. Muscle related complications were in the form of myalgia, weakness and stiffness and were reported in 85 patients in our cohort. Also, compartment syndrome was reported in eight patients. Acute kidney injury and/or acute renal failure was reported in seven patients. Other complications that have been reported and are mainly related to ICU stay included deep venous thrombosis, chest infection, and pulmonary oedema. Recrudescence, as one of the postoperative complications of an MH reaction, will be discussed later on.

To categorise the severity of the reaction, we used both the clinical categories described by Ellis et al. and the CGS. In addition, we used the administration of dantrolene as an indicator of the anaesthetists' judgement of the severity of the case.

We categorised patients into four different categories, table 3-6. Patients were classified first according to clinical categories and the use of dantrolene, then according to CGS raw score. Clinical categories (a), (f) and (h) are classified regardless of the use of dantrolene. We only included patients who had their reaction in or after 1981, as in our cohort, it was the year when dantrolene was first reported to be used. As according to this classification, lower severity grades were associated with a higher rate of complications, X^2=11.83, 3df with ***p*** value=0.008. On the other hand, a strong association between high severity grades and the rate of admission to ICU, X^2=73.5, 3df with ***p*** value<0.0001.

	Severe	**Moderate**	**Mild**	**Less likely**
	Category (a)			Category (f) and (h)
Dantrolene used	Category (b)	Category (c)	(d), (g) and (e)	
Dantrolene not used		Category (b)	Category (c)	Category (g)
CGS raw sore	≥50	35-49	20-34	<20
N. of patients	171	62	22	34

Table 3-6: A proposed classification of the severity of suspected MH reactions. *This is based on the clinical classification described by Ellis and colleagues in 1990, the raw score of the CGS (clinical grading scale), and the use dantrolene as an indicator of anaesthetist's judgement of the severity of the reaction.*

3.3.5 Special circumstances

MH and laparoscopic surgery

In the five patients who developed the suspected anaesthetic reaction while having a laparoscopic surgery where CO_2 insufflation was used, the first reported sign was hypercapnia. The reaction in these five patients was triggered by volatile anaesthetics only, and no succinylcholine was used. No muscular features were reported in any of

the five patients, but other hypermetabolic features were reported in the form of hyperthermia in all the five patients and tachycardia and acidosis in four of them. The time of onset of the reaction was recorded in three patients to be more than an hour from the start of the anaesthetic. In these five patients, the use of CO_2 insufflation did not mask the hypercapnia as a manifestation of an MH reaction and delay the recognition of the MH reaction as the time of onset of the hypermetabolic reaction was recorded in three patients to range from 1-3 hours, while the average time of onset in other patients whom first reported sign was hypercapnia, was 68 minutes.

Postoperative presentation

In 17 patients included in our cohort, the reaction only presented during the postoperative period. The clinical presentation of 12 patients was related to muscular features in the form of rigidity in three patients, myoglobinuria in six patients, and rhabdomyolysis in another three patients. Hyperthermia was the only presenting sign in the postoperative period in four patients, and it was reported in two patients who presented with postoperative rigidity. The remaining patient presented with acute renal failure and high serum CK level. Dantrolene was only used in the management of the two patients presented with postoperative rigidity and reported hyperthermia. The only other feature of MH that was reported in eight patients in this group was elevated serum CK level.

Patients with a relevant anaesthetic history

We were able to identify 19 patients whom anaesthetic history revealed the development of some complications to previous anaesthetics that have not been mentioned by the patients, ignored by the anaesthetist as irrelevant, and/or not properly investigated before the current anaesthetic. In seven patients, the history was

too ambiguous to be interpreted appropriately. This history that was told by the patients included phrases such as “feeling unwell after the operation”, “reaction to general anaesthesia”, “allergy to general anaesthesia”, and “feeling hot after the operation”. Details about the previous anaesthetics could not be retrieved for these patients, and the nature of previous reactions and the type of triggering drugs could not be clarified. Furthermore, in four of these seven patients, the anaesthetist was not informed by the patient about these vague complications, except during the discussion after the current reaction. In another three patients, fever in the perioperative period was reported. On two occasions, it was treated with antibiotics, and in the third patient, it was a late postoperative complication.

Complications reported with previous anaesthetics were in the form of the muscular features of MH in another nine patients and in three of them, succinylcholine was known to be involved in the previous anaesthetics. The anaesthetists avoided the use of succinylcholine in two of these three patients, though, they developed a hypermetabolic reaction to volatile anaesthetics in the current anaesthetic. In the third patient, succinylcholine was used in the current anaesthetic, and the patient developed a muscular features-only reaction. A current reaction that involved muscular features only was reported in another three patients, and succinylcholine was used in all three of them. In the remaining five patients, the current reaction included hypermetabolic features of MH and according to the reporting anaesthetists; they considered previous muscular complications as an abnormal response to succinylcholine.

Fatal reactions

During our search for index cases of families that have been tested MHS at the Leeds MH Investigation Unit since 1988, we identified 22 index cases who did not report anaesthesia related complications in their history; instead, they were relatives of patients who died due to general anaesthesia related complications. We were able to retrieve the clinical details of the fatal anaesthetic reactions for only seven of the deceased patients. The index cases of families of these seven patients are all first-degree relatives of the deceased probands. Of the remaining 15 index cases, five patients were told that their relative died due to MH, as a clinical diagnosis, but no further information about the suspected reaction could be retrieved. In another six patients, family history of anaesthesia related deaths, with no specific mention of MH, was the reason for their referral to the Leeds MH Investigation Unit for IVCT. The remaining four index cases were told that their relatives died due to an anaesthetic reaction that involved: high temperature in two cases, high $etCO_2$ in one case, and MMS to succinylcholine followed by cardiac arrest in another case; with no further details available.

After reviewing the available clinical details of the suspected anaesthetic reactions of the deceased probands, a typical fulminant clinical presentation of MH was reported in four patients. The triggering agents in these four patients included the use of volatile anaesthetics (isoflurane in two patients, sevoflurane in one patient and unknown agent in the fourth patient) and the use of succinylcholine in all of them. When the time was recorded, the start of the reaction was noticed by the anaesthetist after 2-4 hours after induction of anaesthesia. The management was commenced immediately after the recognition of the reaction in the four patients and dantrolene was used in early-stage

in all of them. The management measures followed the established protocol for MH crisis in all of these four patients and included dantrolene, ventilatory support, symptomatic treatment for hyperthermia and acidosis and supportive treatment for cardiovascular instability. In only one of these four patients, no clear information was noted about the early withdrawal of triggering volatile anaesthetics, though, it could be missed during reporting.

In spite of the management measures described early, the reaction in all of these four patients progressed to cardiac arrest and cardiopulmonary resuscitation was commenced and patients were transferred to the ICU after the return of spontaneous circulation. Supportive and symptomatic treatment was continued in the ICU, however, all of the four patients developed multiorgan failure and disseminated intravascular coagulopathy (DIC). All of the four patients died within the first 24 hours after the reaction.

In the other three patients, a female patient had a history of dilated cardiomyopathy at the age of one year, for which she received a cardiac transplant. The reaction was reported later during an E.N.T procedure for which the patient received isoflurane and succinylcholine and developed cardiac arrhythmia followed by cardiac arrest. Laboratory results showed an elevated K^+ (9.7-12.1 mmol/L) levels and high CK levels (5,500 IU/L). The patient was treated for cardiac arrest with no dantrolene reported to be used and the post-mortem examination indicated severe rhabdomyolysis. In another patient, the reaction developed during a thyroidectomy operation. Although laboratory results indicated that the patient during the preoperative period was in an euthyroid state, the patient had an increased heart rate. During the operation, the patient developed a hypermetabolic reaction and was treated as a case of thyroid

crisis, without the use of dantrolene, but eventually developed DIC and cardiac arrest. Laboratory results showed myoglobinuria and a CK level of 15,000 IU/L. The third patient had a history of musculoskeletal abnormalities including limb contractures, arthrogryposis and hypotonia. Anaesthesia for a pyloroplasty at the age of five years old was uneventful, however, 48 hours after the procedure, the patient developed breathing difficulties, hypoxia, severe acidosis and progressed to cardiac arrest. No dantrolene was used during the management of this case and the patient was pronounced dead in the ICU.

Recrudescence episodes

A group of eight patients included in our cohort reported a rebound of the clinical features of the suspected MH reaction after the resolution of the initial episode. All, but one, of these patients, were males and all of them had an age range from 13 to 20 years old. The triggering agents of the initial episode included both volatile anaesthetics and succinylcholine in three patients, while the other five patients developed the reaction in response to volatile anaesthetics only. The clinical picture of the initial reaction included hypermetabolic features in the form of hyperthermia and hypercapnia in all patients, in addition, acidosis was reported in three patients. Muscular features such as MMS, generalised rigidity was reported in five patients but no myoglobinuria in any of them. All patients received dantrolene as part of the management of the initial reaction and seven of them received repeated doses for treatment of subsequent episodes.

Although all of these eight patients have been admitted to the ICU the clinical notes from their ICU stay were available for only two patients. In the remaining six patients, details of the recrudescence attacks were obtained from the referral letters. In six

patients, the subsequent episode was in the form of a rise in temperature that was subsided spontaneously in one patient, treated with oral dantrolene in another patient, and IV dantrolene in the other four patients. A rise in $etCO_2$ with tachycardia reported in one patient and was treated with IV dantrolene. Two recrudescence attacks were reported in the remaining patient: the first attack involved a rise in temperature and $etCO_2$ and the second attack involved a rise in temperature with normocapnia. Both episodes required treatment with IV dantrolene.

3.4 Discussion

This study exploits the biggest and most consistent resource for MH reactions in the world. This project is based on the Leeds MH Investigation Unit archive and database, which is the principal and only national centre for investigating MH in the UK and provides a vast and invaluable set of information about the MH disorder. Our study overcomes some of the limitations of previous epidemiological studies that were based on information collected from administrative databases; such as poor validation of the MH diagnosis and entries being not always patients but admissions, giving a misestimate of the incidence. We were able to validate the diagnosis of MH susceptibility from IVCT reports and review the details of the clinical reaction to confirm the diagnosis of the anaesthetic reaction to be a true MH reaction. Of these studies that were based on the North American MH Registry (NAMHR); (Larach et al., 2010) that investigated the clinical presentation and possible complications of MH and (Larach et al., 2008, Larach et al., 2014) that studied MH-related deaths.

MH is a rare-occurring condition, though it is potentially fatal which makes conducting case-control studies unethical and impractical. However, we were able to compare our results of confirmed MHS cases to another group of patients who tested MHN. We

also used the information available on the NAP 6 activity survey report to compare our results to the general surgical population. Of the advantages of using this cohort as a denominator to compare between the characteristics of the patients included in our cohort and the general population, is the relative homogeneity of these general characteristics regarding ethnicity, age and sex with our cohort as both are based on the UK population. Additionally, the NAP 6 activity survey report offered a valuable nationwide estimate of the anaesthetic caseload to compare against the number of suspected anaesthetic reactions reported to the Leeds MH Investigation Unit. It also provided an insight on the compliance of anaesthetists to the standard monitoring guidelines, which has an important impact on the early recognition of a suspected hypermetabolic anaesthetic reaction.

On the other hand, the data available from the NAP 6 activity survey has its own limitations when used as a representation of the general surgical population and a denominator for our comparison study. Firstly, the anaesthetic caseload in this report is an estimate of a sample of UK hospitals over a limited time period. Secondly, this estimated anaesthetic caseload reflects modern anaesthetic practice, in comparison to our cohort of suspected anaesthetic reactions that span a period of more than 30 years with a marked variability in the number of cases reported per year. And thirdly, as the NAP 6 project was investigating the perioperative anaphylaxis, it included patients who received other types of anaesthetics that are irrelevant when studying MH, such as regional anaesthesia and TIVA.

During the period of the last 30 years, an average of nine cases/year were reported to have a suspected anaesthetic reaction and tested MHS with IVCT. A surge in the number of reported cases was noticed during the year of 1992 and the number of

reported cases during the decade between 1988 and 1997 was nearly twice the number reported in the following decade, and three times the number in the last decade between 2008 and 2017. The possible reasons for that surge in the early 1990s, depend on the change of the anaesthetic practice and the pharmacology of triggering. The introduction of volatile anaesthetics such as sevoflurane for clinical use in the 1990s (De Hert and Moerman, 2015) with its safer pharmacodynamic and pharmacokinetic profile in comparison to halothane may have led some anaesthetists to consider the drug as a safer option with regard to MH. Additionally, as we have noticed in our cohort, the rate of use of succinylcholine was high during the early 1990s and declined over the years with the introduction of NDMR such as rocuronium that offers intubating conditions similar to succinylcholine with a safer pharmacodynamic profile.

It is crucial to stress again that our study shows that all volatile anaesthetics currently in use can trigger a hypermetabolic MH reaction. Furthermore, in the last two decades, isoflurane was the most common volatile anaesthetic involved in triggering MH reactions. On the other hand, we could not confirm or dismiss the role of succinylcholine alone in triggering a hypermetabolic MH reaction due to some missed information about anaesthetic drugs used and the clinical details of the anaesthetic reaction. However, we illustrated that the combination of succinylcholine with volatile anaesthetics was associated with a more rapid onset time of the reaction. The lack of statistically significant difference in the severity of hypermetabolic reaction could be because of lack of power due to small sample size, or due to the effect of other drugs used, such as NDMR or opioids. These latter two groups of drugs are used as anaesthetic adjuvants to maintain balanced anaesthesia and reduce the dose of anaesthetics used and are known to affect signs such as heart rate and respiratory

rate. Although we could not find a statistically significant effect of these drugs on the severity of metabolic features of MH reaction, the timing of administration of these drugs; before or after the start of the reaction, may have confounded this analysis.

In this study, we were able to illustrate the association between the use of succinylcholine and the shorter time of onset of the hypermetabolic MH reaction when combined with volatile anaesthetics. We also demonstrated the effect of the use of succinylcholine on the rate of development of the muscular features of the MH reaction and the highest reported value of postoperative serum CK level. On the other hand, we could not find a significant effect of the use of succinylcholine in the degree of derangement of metabolic features of the MH reaction regarding temperature, $etCO_2$ and acidosis. This lack of significance could be due to lack of power because of a relatively small sample size to elaborate a difference, or the effects of other confounding factors related to other drugs being used during anaesthesia. For example, as NDMR has been suggested to be protective against MH (Harrison, 1971), it may also play a role in reducing the effect of succinylcholine on the clinical features of MH. Additionally, the timing may also play a role as early recognition and cessation of triggering agents lead to abortion of the reaction and mild degrees of derangement are reported.

Succinylcholine was described in the literature to be associated with poorer survival rates from MH (Britt and Kalow, 1970). Apart from the fatal cases described, all the included patients have survived the anaesthetic reaction. However, our data showed that the use of succinylcholine was associated with a higher rate of postoperative complications supporting the earlier observation. On the other hand, although the role of succinylcholine as a solo trigger of a hypermetabolic MH reaction is still

controversial (Hopkins, 2011), in this study we reported ten cases who developed an anaesthetic reaction involved metabolic features, while no volatile anaesthetics were reported to be used. A classic form of MH reaction was reported in three of these ten patients. However, the majority of these reactions occurred in the early 1990s and information about the reaction was available only from referral letters. In none of these referral letters, the anaesthetist actively mentioned that volatile anaesthetics were not used. Therefore, we cannot be totally confident that no volatile anaesthetics was involved in the pathogenesis of these reactions. Additionally, the reaction in one patient can be explained otherwise as the patient was diagnosed with appendicitis and reported a preoperative fever.

When medical students collected information about anaesthetic reactions in MHN probands, they only recorded MMS as a sign of muscular rigidity. An interesting observation was the reporting of MMS without the use of succinylcholine in the cohort of MHN patients, while in our cohort of MHS patients, MMS exclusively followed the use of succinylcholine. Medical students may have labelled some patients to have had MMS as part of generalised muscular rigidity or misinterpreted reports of difficult intubation due to other causes as a result of MMS. The highest postoperative serum CK level was significantly different between both groups. Although serum CK level is not a specific feature of MH reactions, our data indicate that high levels of serum CK is a useful indicator of MH susceptibility in combination with other indicators.

Age at reaction was not easily compared against the group of MHN patients as this information was not collected by the medical students. Also, we could not compare the average age at the reaction to the age of the general surgical population at which patients included in the report had their anaesthetics. Therefore, we cannot confirm

any marked difference between our cohort and the other two groups regarding age. Sex distribution was markedly different between our cohort and the general surgical population. As the NAP 6 project was investigating the perioperative anaphylaxis under anaesthesia, it included patients who had regional anaesthesia and local nerve blocks. These patients should be excluded from our denominator when comparing sex distribution as for example, the number of caesarean sections performed under spinal anaesthesia may skew our data. However, female predominance was also noticed in the general surgical population in the NAP 5 project (Sury et al., 2014) that investigated awareness under general anaesthesia. Different explanations have been proposed for the male predominance of the MH cohort of patients (Islander et al., 2007) including the difference in Ca^{+2} homeostasis between males and females, the influence of sex hormones on the Ca^{+2} regulating proteins in muscle fibres, and the lower penetrance of MH in females.

We can confirm that this observation of male predominance is not specific for MHS patients as no significant difference was noticed between our cohort and the MHN cohort of patients who developed an anaesthetic reaction, though tested not susceptible to MH. This indicates that males present with the non-specific features of MH more than females and the difference regarding MH susceptibility may not be a true difference. A possible explanation is related to the body habitus of male patients with a bigger muscle bulk that makes the exaggerated muscular response to succinylcholine more obvious in male patients. Another explanation that has been proposed before by (Islander et al., 2007), is the effect of sex hormones on Ca^{+2} regulating proteins which affect how males and females react differently to volatile anaesthetics and succinylcholine. The influence of age on the male: female ratio is not

significant in our cohort as male predominance was noticed in all age groups, including the group of patients above the age of 65 years old who were all males.

On the other hand, a preliminary analysis of the MH susceptibility status of the first-degree relatives of our included probands showed a slightly higher ratio of MHS cases within each male category i.e. fathers, brothers and sons in comparison to their female counterparts i.e. mothers, sisters and daughters; respectively. We have not conducted a proper statistical analysis nor compared the IVCT results between these cases to draw a definitive conclusion. However, further analysis of these data may provide us with a better understanding of the effects of different triggering agents on the in vitro responses of muscle from both sexes.

In our cohort, patients who were classified into one of the clinical categories described (Ellis et al., 1990) where a probability of MHS diagnosis was more than 50%, had a median CGS raw score >20, indicating a greater than likely diagnosis of MH, except for patients classified into category (e) with incidence of MHS diagnosis of 0.57 where patients had a median CGS=18 i.e. less than likely MH. The clinical features of category (e) include MMS with signs of metabolic disturbance such as arrhythmia or rising core temperature. However, Ellis and colleagues did not indicate a cut-off point for temperature in this category, on the contrary to Larach and colleagues who stated a cut-off of >38.8℃. Patients included in this clinical category had a maximum temperature of 38.5℃; this may explain the discrepancy between the likelihood of MH diagnosis and the probability described by Ellis and colleagues.

According to our proposed classification in table 3-6, severe forms of the reaction was significantly associated with higher ICU admission rates, giving reliability to our proposed classification. However, we should take into consideration that in some

patients, ICU admission was dependant on the availability of beds rather than the severity of the reaction. On the other hand, the rate of complications was higher in low severity scores. This can be explained by the inclusion of dantrolene as an indicator of severity. As we have illustrated in the results section, the use of dantrolene was associated with a statistically significant lower rate of complications in our cohort, and in our proposed classification, lower severity grades were associated with lower rate of dantrolene use. This could explain the higher complication rate in low severity grades.

When evaluating the management measures used in our cohort of patients, we should take into consideration several factors; for example, some of the reported reactions occurred before the approval of dantrolene for treatment of MH in humans (Kolb et al., 1982) and a management protocol was not established and available for use in all the reported cases. Additionally, the emergency nature of the reaction creates a challenge for anaesthetists involved in treatment and reporting of the suspected reaction. Furthermore, management of particular clinical features is dependent on the available monitoring facilities to recognise the derangement, and on the available treatment for this particular feature. The most efficiently treated clinical sign was hyperthermia in ~77% of patients presented with this feature, which reflects how hyperthermia is still perceived as the most important clinical sign of MH. Other signs such as acidosis and hyperkalaemia were reported to be treated in less than half of the patients presented with these features. This could be due to incomplete reporting of the management measures used, or due to prioritisation of the preparation of dantrolene.

The postoperative complications were mainly related to muscle breakdown leading to pain, weakness and stiffness. Additionally, the resulting degraded muscle proteins led

to the development of acute kidney injury and acute renal failure in some patients. We found a statistically significant association between the development of postoperative complications and the reporting of muscular features of the reaction. Only four of the 53 patients who had a metabolic features-only reaction developed postoperative complications, while more than one-third of the 200 patients who reported muscular features in their anaesthetic reaction, developed postoperative complications, X^2=18.44, 1df, ***p*** value<0.0001. Information about postoperative complications in the MHN cohort of patients was not precisely collected by the medical students and we could not effectively relate our findings to the findings in the MHN cohort. This information would help us to understand whether these complications are related to the development of an MH reaction in susceptible patients, or it is considered as a continuation of the abnormal response to the anaesthetic drugs used e.g. succinylcholine and not related to the background MH susceptibility trait of the affected patients.

The incidence of recrudescence in our cohort is less than a previous retrospective study base on the NAMHR database (Burkman et al., 2007). Only eight patients in our study reported recrudescence which represents ~2.5% of our MHS cohort, in contrast to the 20% incidence reported in the previous study. Our study, though, is based on reviewing the clinical details of the suspected reactions obtained from the patients' files stored at the Leeds MH Investigation Unit archive, on the contrary to the previous study that obtained all its information from an administrative database. These administrative databases have its own limitations towards the validity of the obtained information as discussed earlier. In our study we have not collected information about the body habitus of the included patients to assess its association with the rate of

recrudescence, nor were we able to perform statistical analysis comparing the clinical variables in the recrudescence group to the included MHS cohort.

All the eight patients who reported recrudescence have been admitted to the ICU and the management included the use of dantrolene in all of them. As ~40% of our MHS probands have not been admitted to the ICU, there is a possibility that a number of patients who developed recrudescence have not been detected in the recovery room or in the postoperative wards. Additionally, one of the reasons dantrolene was not used in the treatment of the initial episode of MH in the ~40% of patients who did not receive dantrolene, is that the attending anaesthetist was not totally convinced that the reaction is a true MH reaction. Therefore, they may have ignored any subsequent related manifestations and missed detecting a recrudescence episode.

In the reported fatal reactions when we were able to retrieve clinical details, the reaction can be explained otherwise in three cases due to the preoperative medical condition of the patient. In the four cases in whom the clinical details of the reaction were convincing to be a true MH reaction, succinylcholine in combination with volatile anaesthetics was used. The clinical picture of the reaction in these four patients was the classical presentation of fulminant MH reactions, though the first reported sign was atypical in the form of hypoxia in all the four patients. We could not identify any shortcomings in the management measures used in these four cases and dantrolene was reported to be used in all four of them once the possibility of MH was suspected. However, the recorded time of the start of the reaction was more than two hours, which may indicate a delay in the recognition of the adverse anaesthetic reaction, possibly, due to the atypical first reported sign. Hypoxia is not a classical presenting sign of MH

reaction and it is usually a delayed manifestation, which indicates that other features of the reaction may have started to develop earlier and were not recognised.

The social history of the included patients did not show any interesting features and no association was noticed between the basal CK level and physical activity or the development of cramps. The medical history of ten patients included musculoskeletal abnormalities and CM in five of them. The anaesthetic history also confirmed the observation of incomplete penetrance of MH as more than half of the patients reported uneventful previous general anaesthetics that involved the use of triggering agents without developing an MH reaction. Factors related to the potency of volatile anaesthetics, duration of exposure to the triggering agents, preoperative physical status, and the combination of volatile anaesthetics and succinylcholine have been proposed as possible causes for this incomplete penetrance. Due to the lack of clinical details of the previous anaesthetics in almost all patients in our cohort, we were not able to investigate this topic further. However, ~10% of the 193 patients who had previous general anaesthetics prior to the reaction, reported anaesthesia related complications that were overlooked at the time of the current episode. This indicates that an MH reaction may start to develop in some patients but abort early after cessation of anaesthesia in short procedures and muscles are sensitised for further exposures. Due to the non-specific features of MH manifestations, it is hard to ascertain how many patients could have a subclinical form of MH during previous anaesthetics.

In our study, we were not able to overcome one of the main limitations of previous studies related to the assessment of the temporal relations between the clinical features of MH. We obtained most of the information about the included patients in

our cohort from the referral letters of the attending anaesthetists. The information included in these letters is usually dependant on the judgement of the attending anaesthetist and the first reported sign is not always the first sign of the reaction to develop. Signs such as MMS or exaggerated muscular response to succinylcholine are easier to be observed by the anaesthetist and this explains why MMS was the most common first reported sign in our cohort. MMS was usually reported by the anaesthetist when attempting to intubate the patient or by the E.N.T. surgeon or the dentist when attempting to open the mouth to start the procedure. The degree of change in signs such as heart rate, temperature or etCO_2 is more critical in early recognition of an MH reaction, though, these signs are markedly affected by other factors related to the depth of anaesthesia, pain control and ventilation mode. Anaesthetists should be educated the importance of the degree of change in these signs when reporting an MH reaction for better recognition and proper reporting.

The clinical details of the suspected anaesthetic reaction were collected in a separate section. This section was markedly dependant on the original source of information as in some patients, the full-detailed anaesthetic chart was available, which enabled us to reassess the temporal relations between the development of the clinical features and to have a more accurate idea about the baseline of each clinical sign to determine the degree of change. However, in the majority of patients, these clinical details were obtained from the referral letters of the attending anaesthetists who experienced the suspected anaesthetic reactions. These referral letters although being more organised and clearly written than the anaesthetic charts, made most of the recorded clinical features highly-dependant on the judgement of the attending anaesthetists. We had to assume that when a sign was not reported, it was within the normal range. However, to have a more accurate analysis of these clinical signs, we included only the

difference between the maximum reported value and the physiological value unless reported otherwise.

When referring a suspected MH case for diagnosis, anaesthetists should include all the available clinical notes about the ICU stay including the length of stay, any recurrence of the clinical features of the reaction and any complications that have occurred during the ICU stay. In our cohort, although 189 of patients have been reported to be admitted to the ICU, only a limited number of files, less than 20%, contained the full details about the ICU stay and any relevant information to the initial reaction. Our proposed classification of severity was based on a combination of an established clinical classification and the CGS that has been used by several studies to diagnose MH reactions and assess its severity. We also included the use of dantrolene as an additional indicator of the anaesthetist judgement of severity. In many referral letters of the reactions included in our study, the anaesthetists reported that dantrolene was sent for or was prepared but not used, because the reaction started to subside with symptomatic treatment and supportive measures. That is why although the use of dantrolene is not necessary a very reliable indicator as some suspected reactions included in this study occurred before the availability of dantrolene, we included it in our severity classification.

In conclusion, this study added to the ongoing research to better understand the clinical epidemiology of MH and explore the interacting effects of the many pharmacological agents used during anaesthesia on the different clinical presentations of MH reactions. The observation of male predominance in relation to MH is shown to be related to the clinical features of the MH reaction, which are not specific to MH; instead, it may be related to other factors such as muscle bulk of

patients and the preoperative physical status. However, a male predominance in MH susceptibility was noticed in the first-degree relatives of our included probands, though, proper statistical analysis is needed to confirm this observation. We demonstrated that succinylcholine accelerated the development of the MH reaction and influenced the severity of some of its features, however, its role in the pathogenesis of MH and its effect on the metabolic features is still considered as an ongoing dilemma that needs further investigation. Last but not least, this type of retrospective studies of rare occurring disorders such as MH, is highly dependent on the efficiency of the medical recording and accuracy of the information in these records.

4 Correlation between *RYR1* genotypes, IVCT phenotypes and clinical phenotypes of MH susceptible probands

4.1. Introduction

The relationship between the genetics of MH and its clinical presentation under general anaesthesia and the laboratory results of the diagnostic test could offer us a better understanding of the epidemiology of MH. Three directions of correlations: genotype-clinical phenotype, genotype-IVCT phenotype and clinical-IVCT phenotypes, are meant to provide us with a more defined statistical relationship between the three components. This statistical relationship could help us to predict how different mutations lead to the variability in the clinical presentations of the MH disorder and how muscles from the affected patients respond differently, ex vivo, to ryanodine receptor agonists.

The ryanodine receptor isoform 1 protein is a tetramer with its amino terminal and central regions located intracytoplasmic and its carboxyl end in the transmembrane region. The ryanodine receptor 1 gene is located on chromosome 19q.13.1 and was identified as the primary locus for MH by (MacLennan et al., 1990). It is believed that the intracytoplasmic regions of the receptor host the ligand-binding sites (Stokes and Wagenknecht, 2000). In the past, it was believed that mutations leading to MH trait are clustered in three hotspots on the *RYR1* gene. However, with the development of genetic sequencing techniques, new variants are being found scattered across the gene, and the list of functionally characterised diagnostic *RYR1* variants (https://www.emhg.org/diagnostic-mutations) is growing bigger and containing more variants beyond these three hotspots.

Several factors complicate the analysis of the genotype-phenotype correlation in MH: firstly, the low incidence of the condition 1: 15,000 to 1:200,000 general anaesthetics lead to a lack of power in many of the statistical analyses. Secondly, the high degree of clinical variability of the reaction due to different penetrance between susceptible individuals, in addition, none of the clinical manifestations is specific to MH, and the severity of these features largely depend on the stage at which an MH reaction was suspected, and treatment was initiated. Thirdly, factors related to the urgency of the condition and problems with monitoring patients under anaesthesia. These factors were associated with incomplete reports of the clinical details of the reaction in many of the referred cases. Fourthly, the genetic test results are not necessarily always complete and/or consistent for all of the affected patients due to differences in the genetic testing methods used. On the other hand, despite these limitations, our study is relying on the most extensive available database for this condition in the world as ~7000 individuals have been tested for MH susceptibility with the gold standard IVCT and with genetic testing results available for ~3000 of the IVC-tested individuals. In addition, the unit archive holds records of the available clinical details of suspected MH reactions.

Previous studies explored this correlation between the MH genotypes and phenotypes. Manning and colleagues (Manning et al., 1998) established a correlation between the caffeine threshold and caffeine tension in different genotypes found in families from different European countries, for instance, the *RYR1* c.487C>T genotype was associated with low caffeine threshold and a stronger tension, while *RYR1* c.6617C>T was associated with a weak reaction and a high caffeine threshold. No such correlation was found with the halothane test. A study of the correlation between *RYR1* genotypes and IVCT phenotypes in the Swiss population (Girard et al., 2001)

concluded that different *RYR1* mutations lead to different IVCT phenotypes with the *RYR1* c.7300G>A has a weak IVCT response. Fiege and colleagues (Fiege et al., 2002) found a more subtle difference between the studied MH mutations in a German cohort with the ryanodine test, compared to halothane and caffeine, as patients with mutations c.487C>T, c.1840C>T and c.7300G>A developed faster response compared to patients with c.1021G>A mutation or without a detected mutation. However, they owed some of these differences between tests to other possible factors such as patients age, muscle specimen size and fibre type composition.

Studies based on the UK population such as (Robinson et al., 2002) established a positive correlation between the pre-drug twitch height of muscle samples and the severity of the IVCT response in each test, based on the tension difference at 2% halothane or 2mMol caffeine. They observed the same strong response to caffeine in patients with the *RYR1* c.487C>T mutation, in addition in this study, patients with the same mutation had a strong IVCT response to both halothane tests, static and dynamic, when compared to muscle samples from patients with the *RYR1* c.7300G>A that was found to have a weaker IVCT response. Furthermore, (Carpenter et al., 2009) studied a larger number of mutations and again used the *RYR1* c.7300G>A as a comparator, as it is the most prevalent mutation among the UK population, and found a statistically significant stronger response in muscle samples from patients with a number of different mutations as shown in table 4-1. They also compared the highest serum CK level after the anaesthetic reaction between the genotype groups.

Variants	Caffeine	S halothane	D halothane	CK
c.487C>T	***	*	***	***
c.1021G>A	***	-	-	-
c.1840C>T	-	-	-	*
c.5183C>T	-	-	*	-
c.6488G>A	**	-	***	*
c.7007G>A	-	-	-	*
c.7304G>A	***	***	***	***
c.7361G>A	***	*	-	***
c.11958C>G	**	-	-	**
c.11969G>T	**	-	*	-
c.14477C>T	**	*	**	***

Table 4-1: Statistical differences of RYR1 genotypes reported in Carpenter et al. 2009. *Statistical comparisons of tension differences of the three IVC-tests and the highest serum CK level after anaesthetic reactions between different variants and the most common RYR1 variant among the UK population the c.7300G>A. Level of significance: (-)→ no significance, (*)→**p** value<0.05, (**)→**p** value<0.01, (***)→**p** value<0.001, and (****)→ **p** value<0.0001*

Most of the previous correlation studies focused on the statistical relation between different mutations and the laboratory IVCT response of muscle samples from affected patients. These statistical differences have given us an idea about how different *RYR1* mutations affect the function of the ryanodine receptor and Ca^{+2} homeostasis in skeletal muscles although other factors may confound these findings (Fiege et al., 2002). Apart from postoperative serum CK levels differences discussed in (Carpenter et al., 2009), the majority of previous work focused on the correlation between *RYR1* genotype and IVCT phenotype, although a correlation to the clinical phenotype could give us an insight on the effects of different mutations on the clinical presentation of the reaction and explain the variability in penetrance of MH. We were able to study this correlation between genotype and clinical phenotype because of the availability of resources at the Leeds MH investigation unit.

We also were able to use these available resources to perform a further correlation between clinical phenotype and IVCT phenotype that would enable us to relate

between the presentation in a clinical setting and the response of muscle under laboratory conditions. In order to be able to perform this clinical phenotype-IVCT phenotype correlation study, we grouped patients into three clinical groups depending on the main features of the clinical reaction and the use of succinylcholine as part of the triggering anaesthetic. As discussed in the general introduction, previous attempts to classify patients presented with suspected MH reactions depended mainly on the presence of muscular manifestations; rigid and non-rigid (Britt and Kalow, 1970) and the presence of MMS with some other metabolic features (Ellis et al., 1990). We re-stated this concept and additionally, highlighted the more recent understanding of MH as a state of hypermetabolism. We also included the use of succinylcholine, as part of the triggering anaesthetic, to the grouping factors. Consequently, we had three clinical groups: muscular-only presentation, manifestations of hypermetabolism triggered by anaesthetics that involved the use of succinylcholine, and manifestations of hypermetabolism triggered by inhalation anaesthetics only. We only included probands who had a suspected anaesthetic reaction and have been confirmed to be MHS using IVCT.

The quantitative difference of IVCT results between patients with different *RYR1* variants has been studied before (Manning et al., 1998, Girard et al., 2001, Fiege et al., 2002, Robinson et al., 2002, Monnier et al., 2005, Carpenter et al., 2009), we will readdress the topic in our cohort of included MHS probands. However, our primary aim of this study is to assess if there is any quantitative difference of the clinical features of MH reactions between patients with common *RYR1* variants in comparison to the most common *RYR1* variant in the UK populations; the c.7300G>A. We will also assess the correlation between different clinical presentations of MH reactions and the IVCT laboratory results of muscle specimens of the affected patients, to test the

hypothesis that muscles from patients who develop severe forms of anaesthetic reactions, react differently to ryanodine receptor agonists ex vivo.

4.2. Materials and methods

4.2.1. Study design

This correlation study was designed as a retrospective observational study reviewing data of patients who were tested at the Leeds MH investigation unit. This study is covered by the same ethical approval mentioned earlier as it is a part of the study "Human Tissues and Genomics Research in Malignant Hyperthermia". All patients selected for this study were referred to the unit for diagnosis of MH susceptibility and their clinical data, IVCT traces and genetic testing results are saved at the unit archive. Inclusion criteria for patients to be recruited for this study were a proband who developed a suspected MH reaction under general anaesthesia, was tested susceptible using IVCT, and genetic testing identified a variant in one of the three genes of interest that are known to be associated with MH susceptibility.

Patients' selection process included using the unit database to identify the 649 index cases of all MH susceptible families at the unit by selecting patients with the smallest IVCT number, as it is assigned in chronological order, within each family code. As this study is aiming to correlate clinical and IVCT phenotypes to *RYR1* genotypes, the identified list of MH susceptible index cases was filtered for patients who were genetically tested and a variant or more was found in one of the three genes; *RYR1*, *CACNA1S* and/or *STAC3*. Consequently, the files containing clinical data of those 379 patients were reviewed to identify patients who had an anaesthetic reaction and the clinical details of their reaction were retrieved and recorded. Clinical data collected included patients' sex and age at the reaction and previous anaesthetic history in

addition to the details of the anaesthetic reaction. IVCT traces are retrieved from files and measured as described later.

4.2.2. Importing genetic testing results for the included probands

In addition to the details collected as mentioned earlier, results of genetic testing of the 379 genotype-positive probands were imported from the unit genetic database. The genetic database is populated on the MH unit public database uploaded on the University of Leeds secure server using the FileMaker Pro 16© with secure login credentials to ensure patients' confidentiality. As some patients had more than one variant detected, we included all the variants detected in any of the three genes of interest and consequently ordered them descending according to the detection rate in the MHS probands. Selection of variants to be included in this correlation study followed the inclusion criteria discussed later on. Worth mentioning that different genetic sequencing methods were used during genetic testing of the identified MH susceptible probands.

4.2.3. Genotype-negative patients

The genetic testing of 41 MHS index cases could not define a variant in the *RYR1* in these patients. These negative results are of limited value in our genotype, clinical and IVCT phenotype correlation analysis. The main reason is that, the method of genetic testing was not consistent in all of the tested patients as in the majority of these genotype-negative patients, a restriction fragment length polymorphism (RFLP) sequencing method was used to search for a list of *RYR1* variants that were believed, at that time point, to be pathogenic for MH. Over the years, new variants have been added to this list, and these variants cannot be excluded in patients who were tested prior to the identification of these new variants. In only eight patients of the 41-

genotype negative MHS index cases, an NGS method was used to search for *RYR1* variants. Although these NGS methods offer a more thorough search of the *RYR1* for variants, problems of sequencing certain exons on the *RYR1* have been reported in addition to issues with the depth of coverage in some areas of the sequenced gene, related sometimes, to the quality of DNA used. Therefore, we cannot exclude the possibility of the presence of a pathogenic, or at least potentially pathogenic, variant in these patients.

4.2.4. Inclusion criteria for different genotypes

The number of variants detected in the three genes *RYR1*, *CACNA1S* and *STAC3,* was too huge to be easily interpreted as seen in tables 9-1 and 9-2 in the appendix. In addition, some variants were detected in only one or two probands making statistical analysis of their results meaningless. Moreover, some of these variants are considerably common in the general population with a minimum allele frequency of more than 1:1000, decreasing the possibility of deleteriousness of these variants. Furthermore, the IVCT data and clinical details of the suspected anaesthetic reaction are not necessarily complete in all probands due to shortcomings in reporting and referrals of these probands to the unit. Therefore, we set some criteria for the variants to be included in our analysis.

For the purpose of this study, a variant was included in the analysis only if it was found in at least ten probands who had a suspected anaesthetic reaction and tested susceptible to MH using the IVCT test. In addition, sufficient clinical details about the anaesthetic reaction and IVCT traces should be available for at least six patients in each genotype group to allow meaningful interpretation of the statistical analyses. The estimated prevalence in the general population should be low; therefore, we included

variants with a minimum allele frequency (MAF), on the ExAC browser (http://exac.broadinstitute.org), less than 1:1000 in the European non-Finnish cohort, as a low-risk general population. This mainly because our cohort of probands is considered predominantly white European.

Additionally, we included the *RYR1* c.529C>T, p.177Arg>Cys variant, that was found in only nine probands with sufficient clinical details about their reaction. However, this variant is considered the most common among a group of non-functionally characterised variants found in the UK MH population. Additionally, it is considered a rare variant with ExAC MAF of 0/64006. The C-score, derived from Combined Annotation-Dependent Depletion score that is used to predict the most deleterious variants in the human genome, was calculated to be 32 for this variant, using (http://cadd.gs.washington.edu) (Miller et al., 2018). Variants with C-score>15 include the 5% predicted most deleterious substitutions in the human genome.

4.2.5. IVCT traces measurements

The IVCT measurement method was described early in the general methods chapter. However, as more patients included in this study from the era before the standardisation of the IVCT techniques, some of the patients included in this study had old IVCT traces where adjustments had to be made to match the recording of the more recent traces. These adjustments include for example, excluding the results of the static halothane test for some patients when the practice was to add only a single concentration of 2% halothane to the water bath where the muscle is strung, to ensure consistency of the included data, while including the results of the dynamic halothane and caffeine tests, for those patients, as they were performed to the same recent standards.

4.3. Results

We identified the MHS index cases of 649 families tested at the unit after a suspected anaesthetic reaction. Positive genotype data was available for 379 individuals on the unit genetic database who were tested inhouse for diagnostic variants or sequenced using the NGS facility at St. James's University Hospitals for a panel of genes including our genes of interest, the *RYR1*, *CACNA1S*, and *STAC3*. Table 9-1 in the appendix details all the *RYR1* variants detected, their detection rate and the minimum allele frequency (MAF) for each variant found in more than one index case in our cohort, using the European non-finish population, as a low-risk general population, as a denominator. Table 9-2 details the variants detected in the *CACNA1S* and number of individuals found to carry each of these variants. In addition, in one patient with three *RYR1* variants detected, a *STAC3* c.842A>G, p.281Asn>Ser variant was found. Another *STAC3* deletion mutation, c.226_228del, p.76Glu-del, was detected in a patient with the *RYR1* c.7300G>A, p.2434Gly>Arg variant.

According to the criteria we stated earlier (a missense mutation with a MAF on ExAC database of <1:1000 and was found in ten or more unrelated probands from our cohort), four functionally characterised variants as causative for MH were included in the analyses: c.487C>T, c.1021G>A, c.6617C>T, c.7300G>A, in addition to the non-functionally characterised *RYR1* c.529C>T, p.177Arg>Cys genotype group, as stated earlier. The sex distribution for this group of 118 probands was not significantly different compared to the cohort of all 649 MHS index cases; X^2=0.66, 1df. with ***p*** value=0.42. In addition, the age at the reaction and sex distribution were not significantly different between the included genotype groups; one-way ANOVA, ***p*** value= 0.6236 and X^2=5.9, 4df with ***p*** value=0.2, respectively.

4.3.1. Genotype-clinical phenotype correlation

The clinical features of the anaesthetic reaction of this cohort of patients were categorised into three clinical groups: exaggerated muscular response to succinylcholine only, a hypermetabolic reaction triggered by anaesthetics involved the use of succinylcholine, and a hypermetabolic reaction triggered by volatile anaesthetics only. The following table 4-2 shows different clinical categories in each genotype group. Regarding the c.7300G>A, p.2434Gly>Arg variant, which is the most common in the UK MH population, only six patients carrying this variant have triggered an anaesthetic reaction that included metabolic features without the administration of succinylcholine. In addition, half of the patients with the same variant triggered a reaction in their first anaesthetic. The number of previous anaesthetics, in the other half, ranged from one up to elven previous uneventful anaesthetics before they developed a reaction. For the *RYR1* c.6617C>T, p.2206Thr>Met that was found in twelve patients included in our cohort, seven patients developed metabolic features after succinylcholine use, and seven developed a reaction in their first anaesthetic.

For patients who were found to have the second most common *RYR1* variant among the UK MH population, the c.1021G>A, p.341Gly>Arg, only three out of fourteen patients included in our cohort triggered a reaction in their first anaesthetic, and a hypermetabolic reaction in eight patients with this variant was triggered by anaesthetics that did not include succinylcholine administration. Another group with 11 patients found to have the *RYR1*, c.487C>T, p.163Arg>Cys, shared similar figures with the c.1021G>A genotype group; regarding more patients have developed a hypermetabolic reaction to volatile anaesthetics without the use of succinylcholine (eight out of eleven), and less than half of the eleven patients triggered a reaction in

their first anaesthetic (four out of eleven patients). In addition to the group with the *RYR1* c.529C>T, p.177Arg>Cys variant that was found in nine patients, seven of them developed a hypermetabolic anaesthetic reaction without the use of succinylcholine and all but one patient had a history of uneventful anaesthetics.

Genotype	Muscular only with succinylcholine	Hypermetabolic with succinylcholine	Hypermetabolic without succinylcholine	Total	1st anaesthetic vs. previous anaesthetics	Time to develop 1st hypermetabolic sign (Med. and IQR in min.)
c.487C>T, p.163Arg>Cys	1	2	8	**11**	4: 7(1-3)	52.5, 20-77.5 min.
c.529C>T, p.177Arg>Cys	0	2	7	**9**	1: 8(1-4)	72.5, 30-88.75 min.
c.1021G>A, p.341Gly>Arg	2	4	8	**14**	3: 11(1-3)	77.5, 48.75-135 min.
c.6617C>T, p.2206Thr>Met	2	7	3	**12**	7: 5(1-3)	42.5, 18.75-112.5 min.
c.7300G>A, p.2434Gly>Arg	17	49	6	**72**	36: 36(1-11)	52.5, 22.5-67.5 min.

Table 4-2: The number of patients included in each of the common genotype groups included in this study. *The nature of the anaesthetic reaction regarding metabolic and muscular features in relation to the use of succinylcholine. Anaesthetic history in each genotype group and the time in minutes to the appearance of the first metabolic sign of the reaction, presented as median and interquartile range (IQR).*

We studied the maximum derangement of the hypermetabolic features recorded during the reaction, including temperature, pH and $etCO_2$, and compared them between the genotype groups. For temperature and pH, we analysed the difference between the maximum recorded value and 37℃ and 7.4, respectively, unless stated otherwise, e.g. if the patient had fever prior to the operation, we used the difference between the baseline temperature and the maximum recorded value during the reaction. We used the recorded value for $etCO_2$ for comparison, as it proved difficult to identify the baseline for every patient as it varies highly depending on the patient's medical condition, the ventilation technique and when the measurement starts. In addition, some reports did not include information about $etCO_2$; instead, the $PaCO_2$, available from an arterial blood gas (ABG), was recorded. To match these recordings with our data, we converted the $PaCO_2$ to $etCO_2$ by subtracting 5mmHg. We also included the time in minutes to the development of the first hypermetabolic sign of the reaction.

The time in minutes from the start of anaesthesia to the development of the first hypermetabolic sign of the reaction was recorded. In the majority of patients, this time was reported by the attending anaesthetists. This time was dependent on the anaesthetist judgement of the starting point of the hypermetabolic signs. In the remaining patients, when anaesthetic charts were available, we re-evaluated the anaesthetic charts and identified the time at which the hypermetabolic sign started to develop. We also recorded serum CK levels and used the highest reported value for comparison between the included genotype groups.

The data of the four studied variables (pH, temperature, etCO2 and time in minutes) followed a normal distribution and passed the Shapiro-Wilko normality test with a ***p***

value>0.05. One-way ANOVA comparing data from the five genotype groups showed no statistically significant difference in change in pH, etCO2 highest values, and timing to the onset of metabolic features with ***p*** values=0.13, 0.12, 0.2, respectively. Only the change in temperature was significantly different between the studied genotype groups with a ***p*** value= 0.016. When compared different genotype groups to the group of patients with the *RYR1* c.7300G>A using uncorrected Fisher's LSD multiple comparisons testing, only patients with the *RYR1* c.1021G>A variant showed a consistent statistically significant higher pH and temperature difference and etCO2 value and longer time to onset. None of the other genotype groups showed a significant difference with the *RYR1* c.7300G>A, figure 4-1, except for patients with the variant c.529C>T as the change in temperature was statistically higher with a ***p*** value=0.043.

The Log 10 transformed data of the highest postoperative serum CK levels recorded were compared using one-way ANOVA and showed a statistically significant difference between the studied genotype groups with a ***p*** value=0.014. Using uncorrected multiple comparisons Fisher's LSD test to compare individual groups with the c.7300G>A, patients with the *RYR1* c.487C>T and c.529C>T variants had a statistically significant lower postoperative serum CK levels with a ***p*** value=0.0036 and 0.028, respectively, figure 4-2.

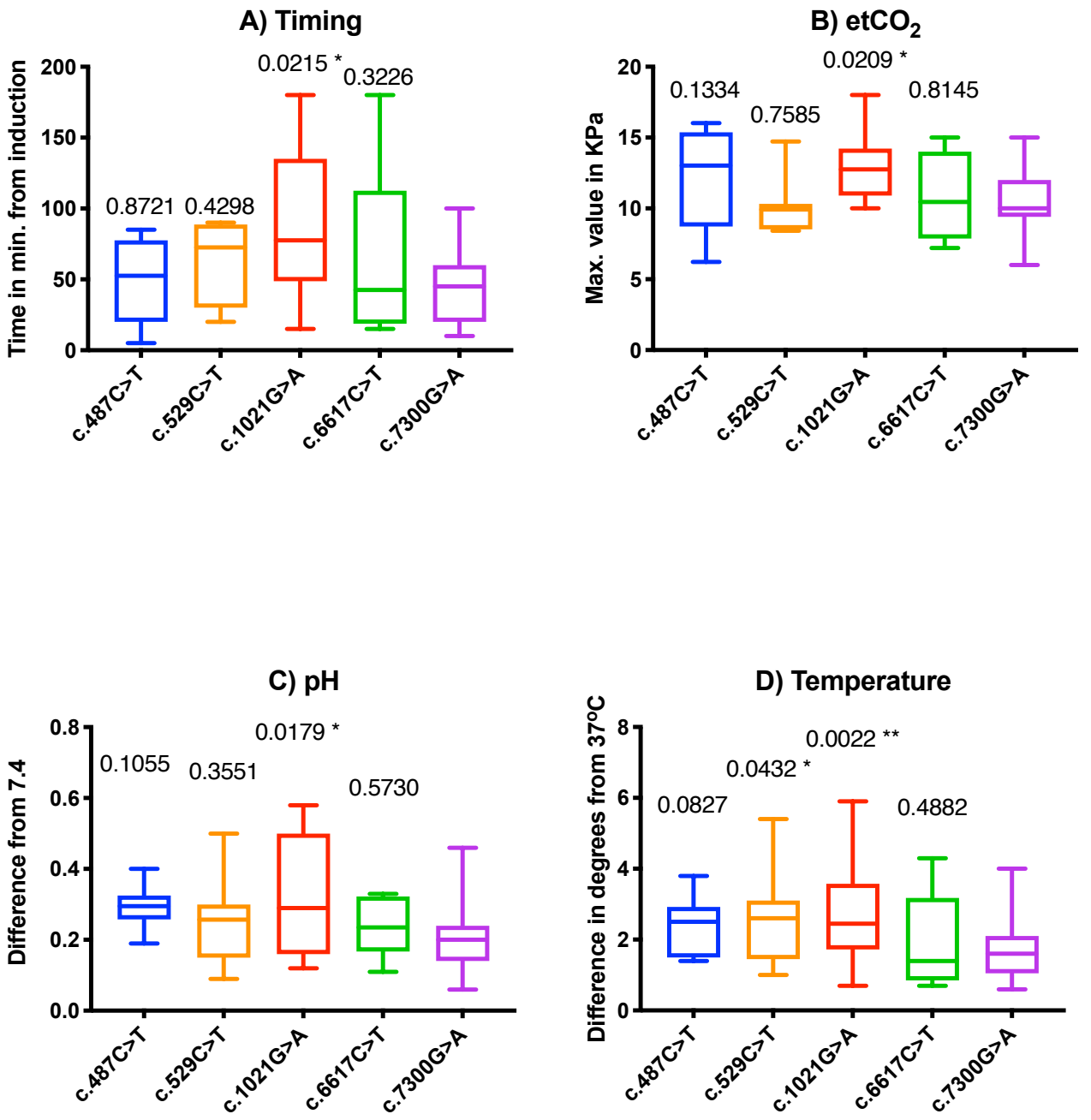

Figure 4-1: Comparing the studied metabolic features and time of onset between different genotype groups and the RYR1 c.7300G>A group*. A) Time in minutes to the start of the hypermetabolic reaction, B) end tidal CO2 in KPa, C) pH difference from 7.4, and D) temperature difference in from 37℃. Using Fisher's LSD test comparing each genotype group to the c.7300G>A genotype group. Uncorrected p values are labelled above each plot. (*)→p value<0.05 and (**)→p value<0.01.*

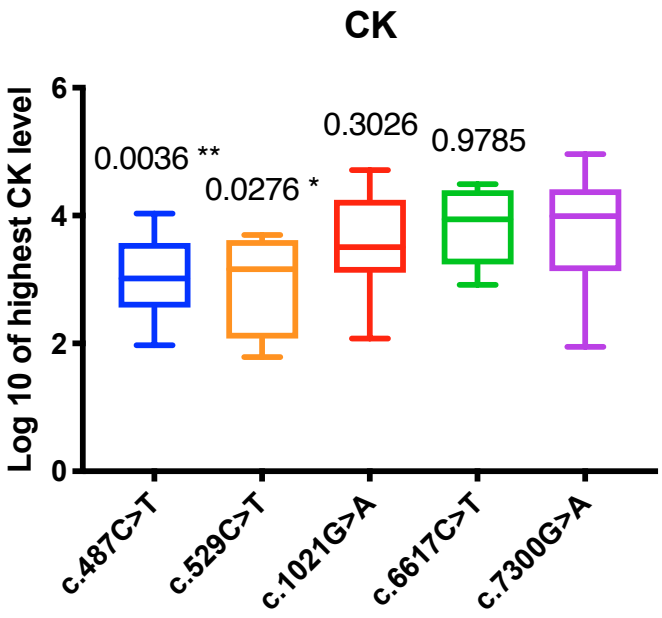

Figure 4-2: Comparing the Log 10 transformed data of the highest postoperative serum CK level between different genotype groups and the RYR1 c.7300G>A group. *Using Fisher's LSD test. Uncorrected p values are labelled above each group. (*)→p value<0.05 and (**)→ p value<0.01.*

4.3.2. Genotype-IVCT phenotype correlation

The IVCT traces of the included patients were retrieved from the patients' files stored at the unit archive, and the twitch height and tension difference for each of the three tests were measured as described before. The average twitch height from the three specimens of the IVCT test for the included probands passed the normality testing according to Shapiro-Wilko normality test with a ***p*** value>0.05. The average pre-drug twitch height was significantly positively correlated with the tension difference from the three IVCT tests, as shown in the following figure 4-3. Between the five different genotype groups, there was no significant difference in the average twitch height, one-way ANOVA, ***p*** value=0.98.

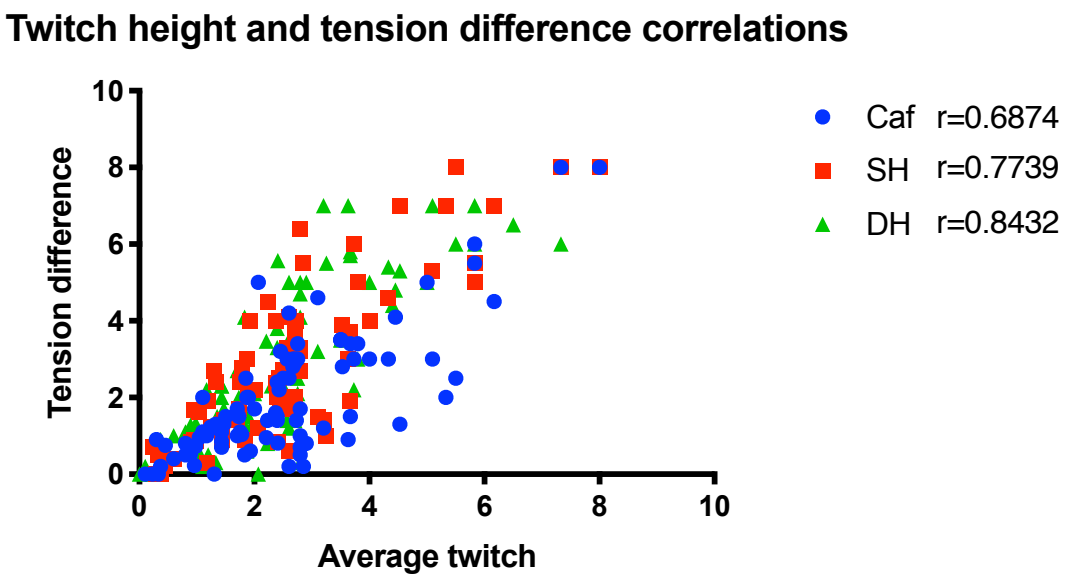

Figure 4-3: The correlation between the average twitch height and the tension differences of the three IVC-tests. *A positive correlation was found with using Spearman r correlation test. The correlation coefficient (r) is labelled on the graph.*

Kruskal-Wallis non-parametric test was used to compare the mean ranks of the three components of the IVCT test between the five different genotype groups. A significant difference was found between groups with the caffeine and dynamic halothane but not the static halothane test (***p*** value= 0.0046, 0.041, and 0.10.9, respectively). Then we used multiple comparisons uncorrected Dunn's test to compare between the different genotype groups and the c.7300G>A. This variant was described in the previous literature to have a weaker contracture (Carpenter et al., 2009). Comparison of the IVCT data of patients with the *RYR1* c.7300G>A showed that patients with the variant c.487C>T had a significantly stronger IVCT response (higher tension difference) in the caffeine and dynamic halothane tests. In addition, patients with the variant c.1021G>A had a significantly higher tension difference to the caffeine but not the other IVCT tests.

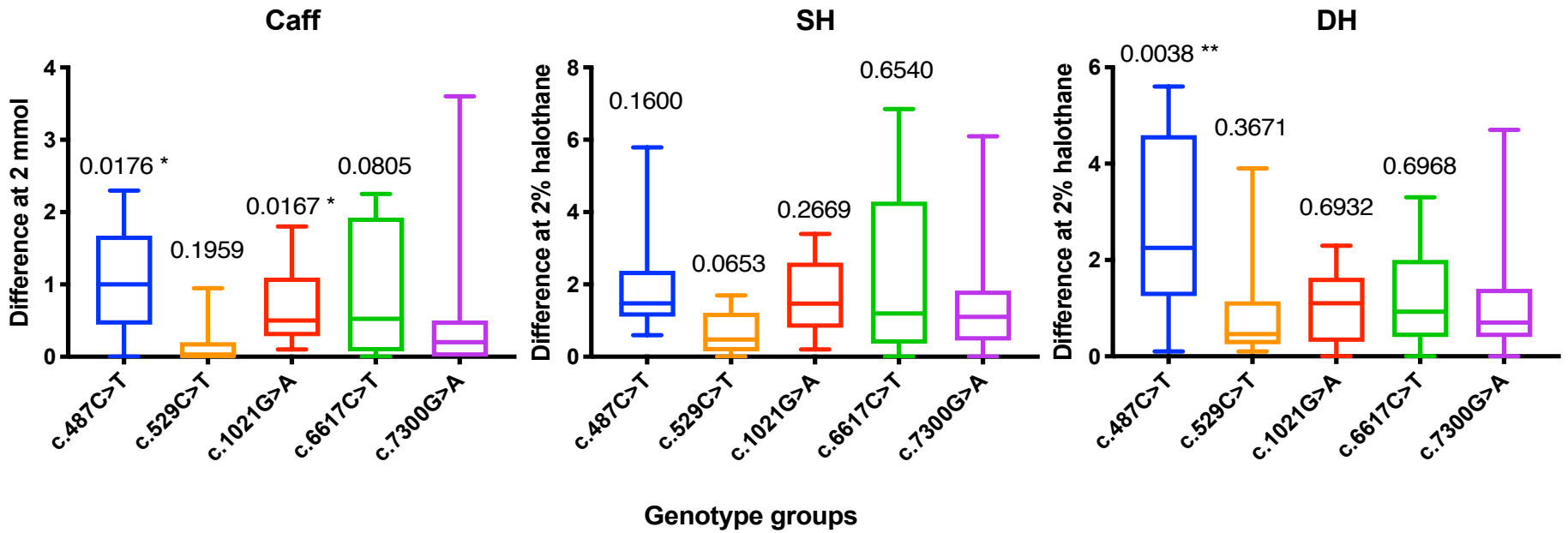

Figure 4-4: Comparing the tension differences of the IVCT between different genotype groups and the c.7300G>A group*. Using multiple comparisons Dunn's test. The tension differences at 2 mmol caffeine for the caffeine test and at 2% halothane for the static and dynamic halothane tests. Uncorrected p values are labelled above each genotype group. (*)→ p value<0.05 and (**)→ p value<0.01.*

4.3.3. Clinical phenotype-IVCT phenotype correlation

For patients with the four most common *RYR1* genotypes in the UK MH population in addition to patients with the *RYR1* c.520C>T genotype, we correlated the clinical pattern of the suspected anaesthetic reaction to the IVCT lab results of these patients. We tested the relationship between a stronger clinical phenotype and whether this might have any reflection on the in vitro response of muscle samples to halothane and caffeine. As we thoroughly investigated the clinical and IVCT patterns in these five genotype groups early in this chapter, we only included patients from this cohort in this correlation analysis.

The viability of muscle specimens as indicated by the average pre-drug twitch height showed no significant difference between the three clinical groups, one-way ANOVA ***p*** value=0.74, nor when used uncorrected Fisher's LSD test to compare the mean of each clinical category with the mean of each other category. As the tension differences of the three IVCT tests were not normally distributed, we used Kruskal-Wallis non-parametric test to compare between the mean ranks of the different clinical groups included, a statistically significant difference was found in the three tests, caffeine ***p*** value=0.0004, static halothane ***p*** value=0.0058 and dynamic halothane ***p*** value=0.0057. We then used the uncorrected Dunn's test to compare the mean ranks of tension differences of the three IVCT tests between each clinical category with each other category. As shown in figure 4-5, a consistent statistically significant difference was found between the group of patients who had a muscular only reaction to succinylcholine and the group of patients who had a hypermetabolic reaction to triggering volatile anaesthetics without the use of succinylcholine. A significant difference was found between the two groups of patients who developed a

hypermetabolic reaction with and without succinylcholine, with the caffeine test but no such difference was noticed with both halothane tests. Also, the tension difference of the static halothane test was statistically significant between patients who developed muscular features only reaction and patients who developed a hypermetabolic reaction with the use of succinylcholine. We were not able to identify any correlation between the hypermetabolic features of the reaction and the tension differences of any of the three IVCT nor the average twitch height of the three muscle specimens used in the tests.

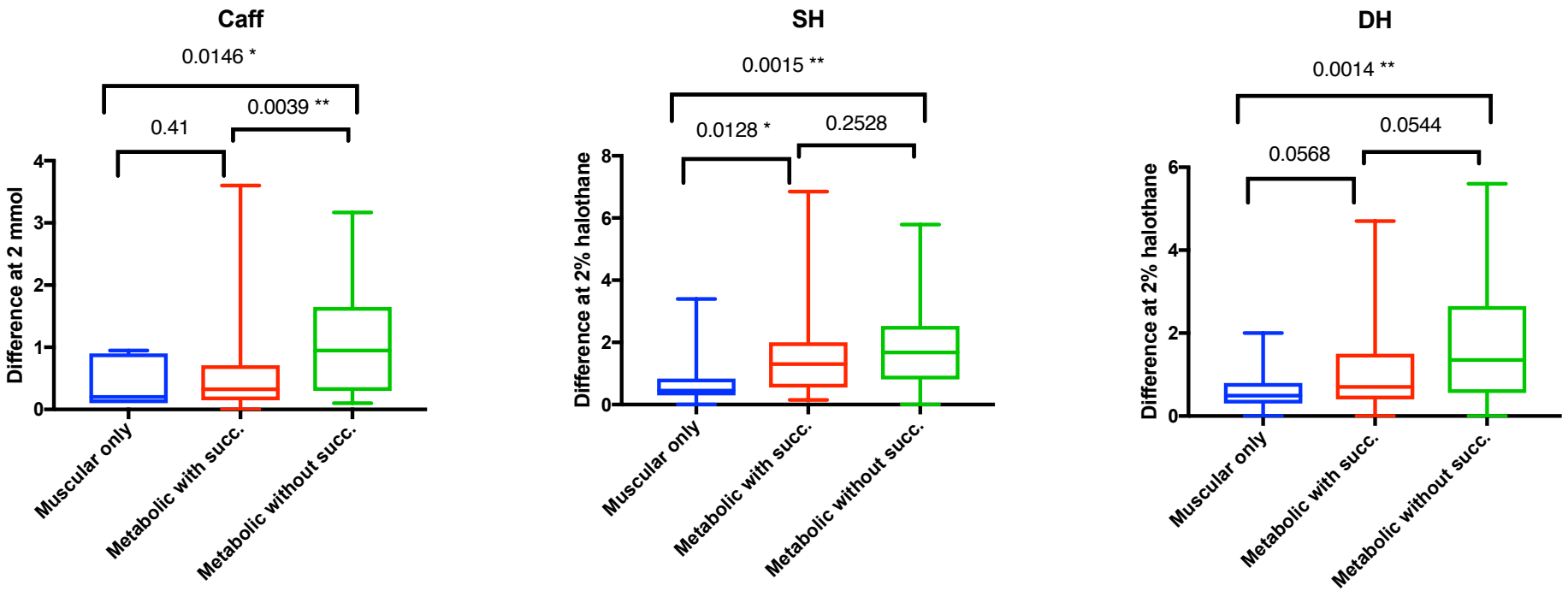

Figure 4-5: Comparing the tension differences of the IVCT between different clinical groups.

ID	V1	V2	V3
1	c.1021G>A	CACNA1S, c.1817G>A, p.606Ser>Asn	
2	c.6617C>T	RYR1, c.14344G>A, p.4782Gly>Arg	
3	c.6617C>T	RYR1, c.8327C>T, p.2776Ser>Phe	
4	c.7300G>A	CACNA1S, c.1817G>A, p.606Ser>Asn	
5	c.7300G>A	CACNA1S, c.1817G>A, p.606Ser>Asn	
6	c.7300G>A	CACNA1S, c.1817G>A, p.606Ser>Asn	
7	c.7300G>A	CACNA1S, c.4060A>T, p.1354Thr>Ser	
8	c.7300G>A	CACNA1S, c.4060A>T, p.1354Thr>Ser	
9	c.7300G>A	RYR1, c.10097G>A, p.3366Arg>His	
10	c.7300G>A	RYR1, c.5033A>G, p.1678Asn>Ser	RYR1, c.5360C>T, p.1787Pro>Leu
11	c.7300G>A	STAC3, c.226_228del, p.76Gludel	

Table 4-3: Additional variants found in our cohort of patients. *V1→ the variant included in our list of studied genotype groups, V2 and V3 are additional variants.*

4.3.4. Patients with multiple variants

When retrieving the genetic testing results from the unit genetic database, we found that 44 index cases were found to have more than one variant during genetic testing. Different combinations of variants were detected in these patients with one patient was found to have up to three *RYR1* variants with an additional *STAC3* variant. This was not particularly reported in previous studies as the genetic testing in the past was targeted to certain regions and/or exons of interest using RFLP or Sanger sequencing. Conversely, in recent years, NGS methods are able to sequence the whole gene and/or panel of genes of interest boosting the number of variants detected in tested patients. Table 4-3 lists the patients included in our analysis who were found to have more than the variant of interest. No combination between two or more of the included variants in this study was found. In addition, due to the small number of the patients included in our study found to have additional variants, we were not able to study the possible confounding effects of these variants on the correlation between genotype groups and clinical and IVCT phenotypes.

4.4. Discussion

We applied stringent inclusion criteria for different genotypes to be included in this study. However, the 118 patients, from the five genotype groups, who were included in this study, represent nearly one-third of the 379 genotype-positive MHS index cases. Our main aim of these strict criteria was to ensure more homogeneity of patients' data within each genotype group in an attempt to eliminate confounding by other factors such as sex, age, and muscle viability-related factors. The number of probands included in each genotype group in relation to the total number of independent UK families found to have the variant of interest, differed as follow: c.487C>T (52.83%, 95% CI 31.02%-73.74%), c.1021G>A (45%, 95% CI 34.8-69.96%), c.6617C>T (42.85, 95% CI 24.52%-61.18%), c.7300G>A (61%, 95% CI 52.2%-69.8%) and c.529C>T (90%, 95% CI 71.41%-108.59%). The low percentage of inclusion in some genotype groups led to a lack of power in some of the analyses. Table 4-3 shows a list of another six *RYR1* variants that have been reported in ten or more UK MH families. However, we have not included these variants in our study mainly because the number of patients for whom we were able to retrieve the clinical details of their reaction with too small to perform statistical analysis. Additionally, to ensure homogeneity in our cohort, we only included probands who have been genetically screened and tested susceptible to MH with IVCT.

codon	protein	No. of UK families	Segregation
c.1840C>T	p.614Arg>Cys	14	7
c.7304G>A	p.2435Arg>His	11	10
c.7361G>A	p.2454Arg>His	14	11
c.7373G>A	p.2458Arg>His	15	12
c.11969G>T	p.3990Gly>Val	11	7
c.14477C>T	p.4826Thr>Ile	10	10

Table 4-4: List of other RYR1 variants that have been detected in ten or more UK MH families. *They were not included in our genotype-phenotype correlation study due to missed information in some patients.*

Discussing our primary aim regarding the relation between different genotypes and the clinical presentation of MH reactions, a crucial point to address is the possible effects of different genotypes on the penetrance of MH. More patients in the genotype groups c.487C>T, c.529C>T, and c.1021G>A had uneventful previous anaesthetics before they develop the suspected reaction. Coincidently, more patients in these groups developed a reaction triggered by volatile anaesthetics only. On the other hand, more patients in the genotype group c.6617C>T developed a reaction in their first anaesthetic, and more patients developed a reaction triggered by anaesthetic that involved the use of succinylcholine. This may give the impression that the former three genotype groups have a lower penetrance compared to the c.6617C>T group. However, this observation is confounded by the controversial role of succinylcholine in triggering an MH reaction (Hopkins, 2011). Another confounding factor is the duration of exposure to, and the potency of different volatile anaesthetics. These factors may play a role in triggering a reaction from the first exposure or sensitise the muscles for further exposures. Therefore, as we could not retrieve information about the anaesthetic agents used during previous occasions, we cannot assess the possible effects of these confounding factors.

When compared to the c.7300G>A genotype groups, patients from the c.1021G>A genotype group had a consistent statistically significant higher degree of derangement of the metabolic features studied; $etCO_2$, pH and temperature. This may indicate that this genotype group produce a stronger clinical phenotype. On the other hand, the time of onset of the hypermetabolic reaction was significantly longer in the c.1021G>A group. This observation is confounded by the fact that more patients in the c.7300G>A group received succinylcholine during their anaesthetic and as we have illustrated in the previous chapter, the use of succinylcholine was associated with a shorter time of onset of the hypermetabolic MH reactions. No statistically significant difference could be found with other genotype groups, except for patients in the c.529C>T group who had a statistically higher temperature but no difference in the other features.

Regarding the difference in the highest postoperative serum CK level between the c.1021G>A and c.7300G>A groups, no statistically significant difference could be found. An interesting observation is that patients in the genotype group c.487C>T who were reported in a previous study (Carpenter et al., 2009) to have a statistically higher basal serum CK level compared to patients in the c.7300G>A group, however in our study, these patients had a statistically lower postoperative serum CK level. This observation could be again due to more patients in the c.7300G>A group have received succinylcholine compared to the c.487C>T genotype group. We have illustrated in the previous chapter that the use of succinylcholine was associated with statistically higher postoperative serum CK levels (Aboelsaod et al., 2018).

Comparing the IVCT tension difference of the included patients, the difference between genotype groups was similar to the previous study that included a smaller number of patients and the cohort in that study included both probands and index

cases; who did not develop anaesthetic reactions, instead investigated because of family history of MH. However, the difference between genotype groups in our cohort was less evident than in the previous study. Different genotype groups did not have any significant effect on the viability of the three muscle specimens used in the IVCT test, as indicated by the pre-drug twitch height. Though, muscle viability is affected by other factors such as the surgical technique, the time the muscle kept on the bench and any underlying muscle pathology. A positive correlation was noticed between the average pre-drug twitch height and the tension difference of the three IVCT tests. This correlation was more evident with the dynamic halothane test, which may be due to the longer duration of the test and repeated stretching and relaxation of the muscle specimen that may affect its viability.

To our knowledge, our study is the first attempt to correlate between the clinical presentation of MH reactions and the IVCT results of the affected probands. All patients included in this study reported an anaesthetic reaction, and we classified these reactions according to the predominant clinical features; muscular or metabolic in relation to the use of succinylcholine. We hereby report a novel observation that the IVCT results from patients who developed a hypermetabolic MH reaction triggered by volatile anaesthetics only, showed a stronger response to both halothane tests and the caffeine test compared to patients who developed a muscular-only reaction triggered by anaesthetics involved the use of succinylcholine. Furthermore, the former group of patients showed a higher tension difference in the caffeine tests, but not the halothane tests, compared to the group of patients who developed a hypermetabolic reaction triggered by anaesthetics involved the use of succinylcholine. This observation suggests an association between how patients develop different clinical

presentations of MH reactions and how their muscle would react to ryanodine receptor agonists under laboratory conditions.

However, although all the included patients have tested susceptible to MH with IVCT, we should deal with the results from the group of patients who developed a muscular-only reaction with caution as it is considered by some literature to be an exaggerated muscular response to succinylcholine rather than a true MH reaction. Especially with the specificity of the IVCT of ~93%, as it takes a more conservative approach to include false-positive patients to avoid the possible fatal outcome of suspected MH reaction, some of the included patients may not be susceptible to MH. Another argument is that an *RYR1* variant has been found in all the included patient, four of these five variants are on the EMHG list of diagnostic variants, which consolidate the MH susceptible diagnosis. However, discordance has been reported in all the included genotype groups according to the recent study based on the UK MH population (Miller et al., 2018). Furthermore, we have not corrected for multiple comparisons which may abolish the difference we noticed between different groups. Therefore, further analysis based on a larger cohort of patients is needed to confirm, or exclude, our observation.

We could not study the possible effects of multiple interacting variants on the clinical phenotype or the IVCT phenotype due to the small number of patients who were found to have more than one variant during genetic screening. In our cohort, eight of the 11 patients who had an additional variant in one of the three genes of interest, were in the c.7300G>A genotype group. This group was described to have a weaker IVCT phenotype (Carpenter et al., 2009, Robinson et al., 2002) and our data illustrate that it has a weaker clinical phenotype compared to the c.1021G>A group. These additional variants may play a modulatory role in the expression of the clinical or IVCT

phenotype in these patients. However, this role cannot be detected statistically due to the small number of patients; instead, functional studies may help to elaborate the effect of these additional variants on the process of the pathogenesis of MH in HEK-293 cells or knock-in mice.

This study was limited by the small number of included patients in each genotype group, in addition to the inconsistency in the reported clinical information about the anaesthetic reactions despite our strict criteria to include only patients with available clinical information. Furthermore, as some of the included patients had their IVCT test in the 1970s and early 1980s when the protocol for IVCT has not been standardised until 1984 (Ellis, 1984), some of the data from IVCT traces were not interpretable for our statistical analysis. In the meanwhile, the introduction of NGS method for genetic screening will help detect a more significant number of patients who have the variants of interest that will improve the statistical power of future studies. We are also aware of ongoing projects that study the biochemical changes in muscle cells and myotubes from patients diagnosed with different IVCT phenotypes. We recommend that more studies investigating these biochemical changes in muscles from patients presented with different clinical phenotypes, as it may help to understand the possible biochemical mechanism behind different clinical presentations.

Overall, we reported a statistically significant difference in the maximum derangement values of the metabolic features of the MH reaction between one of our studied genotype groups and the most common genotype group among the UK MH population. This difference indicates that the underlying genetic mutation influences how susceptible patients react to triggering agents in the clinical session. We restated the same observation reported previously about a difference in the IVCT results

between different genotype groups but in an exclusive cohort of MHS probands who developed an anaesthetic reaction. A novel observation of a possible association between the clinical phenotypes of MH reactions and the IVCT results is also reported in this study. However, more studies, including larger cohorts of patients, are needed to explore this association further.

5 Investigating the association between CM and MH susceptibility

5.1 Introduction

The congenital myopathies, a class of neuromuscular disorders, is of pivotal importance when studying the clinical and genetic epidemiology of MH. This group of clinically and genetically heterogeneous disorders are usually presented with an early onset characteristic clinical muscle weakness with different severity and course of progress patterns. The subtypes of this group overlap with each other on a clinical, histopathological, and genetic levels. Clinically, many patients are presented at birth with a severe phenotype, while others may develop mild or moderate forms of muscle weakness that could start in early childhood or later on in their life. The course of these disorders varies from non- or slowly progressive to severe and rapid affection of vital muscle groups, such as respiratory muscles. The pattern of distribution of clinical muscle weakness is characteristic for each subtype, although overlap is frequently noticed between the different clinical phenotypes. Genetically, mutations in several genes were linked to the pathogenesis of one or more category of the group of congenital myopathies with a dominant, recessive, or X-linked modes of inheritance described. The histopathological examination of muscle biopsies from patients diagnosed with these disorders shows a spectrum of pathology featuring different stages of core formation, central nucleation, fibre type 1 predominance and the presence of nemaline rods or bodies.

Core myopathy, the most common of congenital myopathies, is characterised by the formation of a well-demarcated central core that lacks oxidative enzyme activity and is usually devoid of mitochondria (Sewry et al., 2002). These cores could be less

defined, peripheral and/or multiple in some patients or in the form of unevenness of the oxidative staining, or the motheaten appearance (Morgan-Hughes et al., 1973a). Conventionally, patients with well-demarcated central cores in their muscle biopsies are diagnosed with central core disease (CCD) and clinically presented with a non- or slowly progressive course of muscle weakness that starts at birth or in early childhood, usually with respiratory muscle sparing (Romero et al., 2003). When cores are less defined and multiple, patients are usually diagnosed with multi-mini-core or mini-core myopathy (MmD). According to the (Jungbluth et al., 2005), the underlying genetic variation leads to different clinical pictures in the form of the classic axial phenotype, MmD with predominant hip girdle involvement and MmD with marked distal weakness and wasting. Extraocular muscle involvement has also been reported in some families (Romero et al., 2003).

The presence of central nuclei in muscle biopsies from patients with clinical features of CM led to the diagnosis of centronuclear myopathy (CNM) (Jungbluth et al., 2008). The clinical features in a group of patients with *RYR1*-related congenital myopathy with central nuclei showed an early onset severe form of muscle weakness starting from birth with recurrent respiratory tract infections and extraocular muscle involvement (Wilmshurst et al., 2010). Nevertheless, the histopathological feature of central nucleation is not a rare finding in other types of congenital myopathies such as core myopathies (Sewry and Wallgren-Pettersson, 2017).

Fibre type 1 predominance is another pathological feature of congenital myopathies that is seen in muscle biopsies from patients with different types of congenital myopathies. Four out of six patients from a UK cohort with fibre type 1 predominance, as the only abnormality detected in their muscle biopsies, were found to have

pathogenic *RYR1* mutations (Maggi et al., 2013). The clinical features of myopathy in these six patients overlapped with that of other types of congenital myopathies including extraocular muscle involvement, bulbar affection and orthopaedic deformities. *RYR1* variants were detected in 4 out of ten Japanese patients who were found to have isolated fibre type 1 predominance in their muscle biopsies (Sato et al., 2008).

In this study we will explore the association between all the previously mentioned histopathological changes and MH susceptibility. To sum up different definitions of CM subgroups; the diagnosis of each subtype is dependent on the most prevalent histopathological changes, clinical presentation of the muscle weakness and affected muscle groups, and the underlying genetic variation. The main reason for referral of patients with clinical muscle weakness without any adverse anaesthetic history to the Leeds MH Investigation Unit, is the diagnosis of these patients with CCD according to the treating clinician judgement and the association between the two disorders (Klingler et al., 2009). However, due to the marked clinical, histopathological, and genetic overlap between the different subgroups of CM, according to the literature discussed earlier, we referred to all the patients who were tested at the unit and included in this study as CM patients, and explored the association between the histopathological findings, the genetic results and MH susceptibility status, regardless the clinical diagnosis made by the treating clinician. It is also worth mentioning that we cannot make an assumption on the exact proportion of patients diagnosed with different subtypes of CM who were referred for diagnosis of MH susceptibility as it is mainly dependent on the judgement of the treating clinicians and their understanding of the association between the different CM subtypes and MH.

Anaesthesiologists dealing with patients with congenital myopathies face many apparent challenges (Klingler et al., 2009), related for example to the clinical muscle weakness, which makes it difficult to assess the patient's reserve capacity and exercise tolerance. Also, respiratory muscle involvement could worsen after anaesthesia mandating postoperative ICU admission and ventilatory support. Moreover, the involvement of bulbar muscle has been reported in association with different types of congenital myopathies in 46.4% of cases reported by (Colombo et al., 2015). Bulbar palsy carries its own risks during anaesthesia in the form of increased risk of aspiration due to attenuated upper airway reflexes, requiring closer monitoring of the patient during the perioperative period.

In addition to the previously mentioned risks, the molecular mechanism behind the pathogenesis of CM, i.e. the mutated channels, has the potential risk of other anaesthesia-related disorders, e.g. MH. Mutations in the *RYR1* was described as a possible cause in all of the previously mentioned subtypes of congenital myopathies. The same gene is identified as the primary locus for mutations causing MH in 70% of susceptible families (Robinson et al., 2006). More than 300 variants have been detected in the *RYR1* with only 48 mutations have been functionally characterised as causative for MH and meet the criteria for genetic diagnosis of MH susceptibility (www.emhg.org). The abundance of variants detected in the *RYR1* gene and the lack of a high-throughput method to functionally establish the pathogenicity of these variants make it mandatory to treat patients who have a medical personal or family history suggestive of MH with caution until their susceptibility status is confirmed using the gold standard IVCT (Klingler et al., 2009).

MH susceptibility risk in patients with core myopathies originates from the effects of some *RYR1* mutations linked to CCD on the Ca^{+2} homeostasis. These effects are in the form of excitation-contraction uncoupling and depletion of sarcoplasmic reticulum Ca^{+2} stores because of a constant leak (Treves et al., 2008). Patients diagnosed with MmD have not been reported to develop a clinical hypermetabolic MH reaction (Klingler et al., 2009). However, repeatedly, due to the marked overlap between core myopathies and some reports of alterations in Ca^{+2} homeostasis in fibres from MmD patient (Osada et al., 2004) and in human embryonic kidney cells (HEK293) with MmD-associated *RYR1* mutations (Zorzato et al., 2007), it is wise to treat patients with MmD diagnosis with caution regarding the MH susceptibility risk. With regard to CNM, as mutations in the *RYR1* have been identified as a common cause of CNM (Wilmshurst et al., 2010). Although none of the patients reported in this study developed an MH reaction, there is still a potential risk of MH, especially with a previous report of an MH reaction in a patient diagnosed with CNM (Quinn et al., 1992), though the diagnosis of MH susceptibility was not confirmed using IVCT in this patient.

It is noticed that most of the work done before to study the association between CM and MH was concerned with CCD diagnosis in particular. On a histopathological level, a study of the association between different *RYR1* variants detected in a dominantly French cohort and the number and nature of cores, could not find any correlation between the *RYR1* genotype and the pathological phenotype (Monnier et al., 2005). The same study estimated the detection rate of cores during pathological examination of muscle biopsies of IVC-tested patients without clinical muscle weakness, to be ~27.5% of MHS patients and 2.5% in MHN individuals. Based on the UK population, several studies correlated histopathological detection of cores to the *RYR1* variants

detected. Subsequently, the different *RYR1* variants were categorised according to the association with a CCD diagnosis and the detection of cores in muscle biopsies. Hence, the categorisation of *RYR1* variants into MH/CCD vs non-MH/CCD variants (Carpenter et al., 2009) or CCD associated mutations vs non-CCD mutations (Manning et al., 1998, Tong et al., 1999). Quantitative analysis of receptor function found that resting cytoplasmic Ca^{+2} concentrations in cells expressing CCD-associated channel mutants were higher than for non-CCD associated channel mutants (Robinson et al., 2002). In addition, patients with MH/CCD variants were found to have a stronger response to the caffeine and dynamic halothane tests of the IVCT (Carpenter et al., 2009).

Shepherd and colleagues (Shepherd et al., 2004) reported that all the clinical variables between CCD and MH phenotypes were noticed in a cohort of 21 UK families in the form of:

- Patients tested MHS and had clinical muscle weakness.
- Patients tested MHS, and myopathic features were found on histology but no clinical myopathy.
- Clinical myopathy patients with histopathological changes but tested MHN.

The possible theories behind this variation include incomplete penetrance of the CCD phenotype (Gillard et al., 1991), the slow progression of clinical myopathy (Patterson et al., 1979) or the independent segregation of MH and CCD (Shepherd et al., 2004).

On the other hand, other studies were concerned with the histopathological findings in patients tested susceptible to MH without the presence of clinical muscle weakness. The same pathological spectrum of congenital myopathies is not a rare finding in patients tested MHS with IVCT without clinical muscle weakness, ranging from

unevenness of oxidative stain to the formation of cores, fibre type 1 predominance and central nucleation. A study that included a Canadian cohort of patients who were tested susceptible to MH with the Caffeine Halothane Contracture Test (CHCT), the American equivalent of IVCT but with no dynamic halothane test and the static halothane is performed through by single addition of 3% halothane, (Orlov et al., 2013) reported a 22% incidence of histopathological abnormalities in muscle biopsies of MHS patients. Although this study could not identify a consistent histological pattern in MHS patients, it reported that the incidence of histological abnormalities was higher in probands (patients who experienced a suspected anaesthetic reaction) more than other family members. However, it is unclear if these abnormalities are due to the effects of the anaesthetic reaction on muscle histology or these patients are more predisposed to trigger a reaction because of abnormal muscle histology.

More recently, the same broad spectrum of histopathological findings observed in patients with congenital myopathies, ranging from non-specific isolated abnormalities to core formation, was also described in muscle biopsies of patients presented with MH or rhabdomyolysis, either exertional, viral or drug-induced (Knuiman et al., 2019). This multicentre study, repeatedly, reported the lack of correlation between the presence and severity of histological abnormalities and the degree of clinical muscle weakness. This study was more focused on genetically confirmed cases of *RYR1*-related MH and RM, though, the presence of cores did not correlate with the detection of likely pathogenic *RYR1* variants.

Variants that have been described with both the CCD and MH phenotypes were reported (Carpenter et al., 2009) to have a statistically higher dynamic halothane tension and a shorter static ryanodine response time, ryanodine is another Ryr1

agonist that was used in some patients as part of the IVCT for research purposes, when compared to variants that have been described with the MH phenotype only. While a prior study (Robinson et al., 2002) found that variants that have been found in CCD patients were associated with a significantly more severe static halothane, dynamic halothane and caffeine test but not ryanodine test. Both previous studies have not reported the clinical myopathy phenotypes of the included patients. Although these studies were based on the UK MH population, the variants reported to be associated with CCD was described in previous studies that were not based on the UK population: c.487C>T and c.1565A>C were reported by (Quane et al., 1993, Quane et al., 1994), c.6488G>A by (Manning et al., 1998) and c.14693T>C reported by (Lynch et al., 1999). The *RYR1* c.487C>T and c.6488G>A variants are on the list of the diagnostic variants for MH, while the other two variants have not been found in the UK MH population (Miller et al., 2018).

RYR1 mutations lead to the development of different muscular related disorders due to their implication on the function of the ryanodine receptor. Four categories of *RYR1* mutations were described depending on how these mutations affect receptor function (Treves et al., 2008, Loy et al., 2011). The first category of mutations is associated with increased probability of receptor activation through fibre depolarisation or by receptor agonists and usually lead to the development of MH phenotype. Secondly, mutations that lead to Ca^{+2} leak through channels and depletion of Ca^{+2} stores in the sarcoplasmic reticulum and these mutations lead to the CCD phenotype. Other forms of CCD are associated with the third category of *RYR1* mutations that lead to excitation-contraction uncoupling due to deficits on the $Ca_v1.1$-mediated voltage-dependent activation of SR Ca^{+2} release. The fourth category of mutations leads to decreased RyR1 channel expression and results in multi-mini-core disease. A

compound effect of these mutations on the RyR1 channels' activity is seen as some mutations lead to hypersensitive and/or leaky channels with the development of *RYR1*-related MH and CCD, while other CCD-causing mutations lead to excitation-contraction uncoupling. The mechanism of this complexity is unclear (Dowling et al., 2014).

Rhabdomyolysis is a condition associated with muscle breakdown, and it is the leading cause of acute renal failure in 7% of cases (Dlamini et al., 2013). It is being reported in some patients who develop an MH reaction or found to have one of the neuromuscular disorders described earlier. The index cases of 14 families with no clinical muscle weakness presented with exertional rhabdomyolysis were found to have *RYR1* mutation on genetic analysis, and the histopathological examination of their muscle biopsies showed some of the spectra described with different types of congenital myopathies (Dlamini et al., 2013). The cause of rhabdomyolysis in this studied cohort was mainly related to exertion, heat or alcohol intake. In one family, the trigger to this condition was an intercurrent infection. The spectrum of pathology in this cohort of patients ranged between fibre type 1 predominance, increased internal nucleation, and unevenness of oxidative staining. These patients usually presented with exertional myalgia and mild to moderate elevation of serum CK levels.

This study aims to investigate the prevalence of MH susceptibility in known CM families that were referred to the Leeds MH Investigation Unit because of a diagnosis of myopathy or were investigated because of a suspected anaesthetic reaction and the medical or family history revealed clinical myopathy. In addition, we aim to study the incidence of MH reactions among members of these families. Furthermore, we are interested in investigating the prevalence of histopathological features of myopathy in

patients who were tested at the Leeds MH Investigation Unit for MH susceptibility because of personal or family history of anaesthetic reactions suggestive of MH, in the absence of clinical muscle weakness. As routinely during IVCT, a muscle specimen is sent to the Neuropathology lab at Leeds Teaching Hospitals to be examined by a pathologist, hence, we were capable of reviewing the histopathological examination reports of index cases of all families who were tested at the Leeds MH Investigation Unit. Last but not least, we explored the association between the findings in both cohorts included in this study and the genetic testing results available for those patients in an attempt to investigate the correlation between both disorders.

5.2 Methods

5.2.1 Study design

This study consisted of two main parts; the first one is to collect the available information about the MH susceptibility status of all CM index cases who were tested at the unit and collecting information about family members of those index cases. Secondly, reviewing histopathological examination reports of MH index cases who were tested at the unit because of anaesthetic history suggestive of MH. Furthermore, we will import all the available results of genetic testing of the included patients to correlate this data to clinical and IVCT data of those patients.

Index cases of CM families

Using FileMaker Pro 16©, we selected the lowest IVCT number in each family code, as it is assigned in chronological order, to identify index cases of all families tested at the unit. Secondly, we filtered the results using the “reason for referral” field to patients who are labelled as referred because of CM. As the “reason for referral” field was not complete for all the patients due to some shortcomings when creating the database

and uploading the data from patients' files to the database, we ensured to check the reason for referral in the IVCT biopsy report for each index case while we were reviewing the histopathological reports of all the index cases as will be described later in this section. Additionally, some patients were referred because of an anaesthetic reaction suggestive of MH. By reviewing their medical history, they were found to have CM or belong to a family known to have the condition. Those patients were also included in this group.

Other members of myopathy families who were tested at the unit

After identifying index cases to be included in the CM group, we used their family codes to search for other members of these families who were tested at the unit. Using FileMaker Pro16 program to search the database, we collected information about these families including the number of members in each family who were tested with IVCT and/or genetic screening for one of the *RYR1* variants, the MH susceptibility status for each member tested with IVCT, and the results of genetic screening for the members who were tested genetically. For each member of these families, we additionally collected data about the degree of kinship to the index case of the family, medical history of CM, and any history of suspected MH reactions.

Index cases of families tested for MH susceptibility

In order to study the prevalence of histopathological myopathic changes in muscles of individuals who were tested for MH susceptibility because of anaesthetic history, personal or familial, of a suspected MH reaction, we selected the index cases for more than 1900 family codes for which the Leeds MH Investigation Unit archive holds files for at least one member of these families. We, consequently, reviewed the neuropathology lab examination reports of all these index cases and identified reports

indicating the presence of one or more of the histopathological changes of *RYR1* myopathy: core formation, fibre type I predominance, and/or increased percentage of central nucleation. The pathological changes detected in these reports were recorded in addition, we collected information about the MH susceptibility status of the identified index cases, results of genetic screening, if performed, and the medical and anaesthetic history.

For all the included cases, we collected patients' data including, sex, date of birth, date of biopsy, and body measurements. From the IVCT biopsy reports of these cases, we recorded the information available about their MH susceptibility status, previous medical and anaesthetic history, and the reason for referral. We also recorded the serum CK level at the time of biopsy. As we stated earlier, the results of the histopathological examination were also recorded for all the included cases, and the genetic screening results available on the unit database were imported.

5.2.2 Genetic testing results

We used the Leeds MH Investigation Unit database to collect the results of genetic testing for the included patients. The method of sequencing was different over the years, and between the patients, though, we only collected the available information about variants detected in *RYR1*. In addition to the available genetic information on the database for the patients included in the CM group of patients, including patients with a clinical or family history of clinical muscle weakness with or without a history of suspected MH reaction, we submitted a request to the DNA lab at St. James's Hospitals, Leeds Teaching Hospitals to perform NGS screening for the available DNA samples for the remaining patients with no available information on the unit database.

5.2.3 Muscle biopsies and muscle histology

As a rule, all patients included in this study have had their muscle biopsy obtained for IVCT diagnosis of MH susceptibility at the Leeds MH Investigation Unit at St. James's University Hospital. All but three patients had their muscle biopsy obtained from the quadriceps muscle. In the remaining three patients, a muscle biopsy was obtained from the palmaris longus muscle, and all of them had their muscle biopsy in 1973, before the standardisation of IVCT procedure. Two of these patients were referred because of a family history of CM and the third patient had a suspected anaesthetic reaction. In only seven patients who were referred because of CM, a previous muscle biopsy was obtained for the diagnosis of CM. According to the available reports, the site of the previous biopsy was not the quadriceps muscle in three patients; instead, the muscle biopsy was obtained from, the biceps, deltoid and trapezius muscles.

5.2.4 Categorisation of histopathological findings

The spectrum of pathology in patients with CM is broad and very variable, making the categorisation of these patients and the severity ranking of the histopathological findings very difficult. Additionally, the formation of cores is believed to be age-related and varies from one individual to another even within the same family. We aimed to rank the severity of histopathological findings to enable us of easy categorisation of the included patients and to facilitate investigating the relation between the histopathological findings during pathological examination and the laboratory findings of the IVCT. We categorised the histopathological features according to the findings during each staining stage of the histopathological examination. During oxidative staining, the following features are commonly seen in these patients: unevenness of the oxidative stain or the motheaten appearance, formation of mini-cores that are

usually multiple, and the formation of a well-demarcated central core. Central cores are usually single but can be multiple or even located peripherally in the cell. As we stated earlier, it is not completely understood if these findings are stages of the disease that progress with age or with the progression of the disease, separate entities of the disease features, or different severity stages of the disease. However, we endorsed the assumption that these findings are different levels of severity of the pathological changes. As shown in table 5-1, each level of core formation stages, mentioned earlier, is given a rank that sums up for the severity score of the histopathological findings in these patients.

	0	1	2	3	4	5
Core formation	No cores	Motheaten	Multi-mini cores	Central cores		
Fibre type	Normal distribution	Type 1 predominance				
Nucleation	Normal	Central				
Pathology score	**No pathology**	**Mild**	**Moderate**		**Severe**	

Table 5-1: A proposed severity scoring of the RYR1-related histopathological findings. *The pathological features detected in our cohort of patients presented with MH or CM phenotype.*

Additionally, the fibre type distribution found using pH-specific adenosine triphosphatase (ATPase) stain is given a rank of 1 if fibre type 1 predominance was found and rank of 0 if the biopsy preserved its normal pattern of fibre types distribution. The percentage of central nucleation in muscle fibres that is assessed using H&E stain also contributed to our severity score of myopathic changes. We considered central nucleation as a pathological finding if its percentage was 10% or more of the observed nuclei in the muscle biopsy. Although according to personal communication with the Neuropathologists at the Neuropathology lab at Leeds Teaching Hospitals, indicating that some pathologists consider central nucleation as a pathological finding if it is spotted more than 5% in the given muscle biopsy, we endorsed stricter inclusion criteria, especially in patients presented with no clinical muscle weakness.

5.3 Results

5.3.1 MH susceptibility in CM families

We identified 38 cases who were referred to the unit for diagnosis of MH susceptibility because of a CM diagnosis. An additional nine patients were selected as they were

referred because of a suspected MH reaction and after reviewing their medical files, they were found to have CM. In 28 of the 38 families included in this study, the first patient to be tested was the proband who reported clinical CM, while in the remaining ten families, the first patient to be tested did not have clinical muscle weakness but was referred because of a family history of CM, i.e. index case, while the probands of these families could not be tested because of being too young, deceased or unfit for the procedure. In total, a cohort of 47 cases was included, of those cases, 26 were females, and the average age at biopsy for this cohort is ~31.5 years with a minimum age of 7 years and maximum of 70 years old.

Histopathological examination of muscle biopsies of the 47 cases ranged from no-pathology detected in seven patients to the detection of severe myopathic changes in 20 patients with the histopathological report missing for one patient. Apart from the patient with a missing pathology report, all patients who were referred because of clinical CM showed myopathic changes in pathological examination with only two patients had a mild form of myopathic changes in the form of motheaten appearance in one patient and fibre type 1 predominance in the other patient. The remaining 25 patients had moderate to severe forms of pathological changes. Histopathological examination of muscle specimens from either index cases of CM families, or patients who had a CM and were referred because of a suspected MH reaction, showed a variety of results of histopathological changes ranging from no myopathic features to severe forms of pathological changes.

Regarding MH susceptibility status in this cohort of patients, more than half (26 of 47 patients) were tested not susceptible to MH and seven of the 21 patients who tested MHS with IVCT, were susceptible to halothane only during the IVCT. Moreover, the

nine patients who were referred because of an anaesthetic reaction and were found to have CM, three of them tested not susceptible using the IVCT. By reviewing the clinical notes for the three MHN patients who were referred because of a suspected anaesthetic reaction, no anaesthetic records could be retrieved for one patient with a vague history of reaction to general anaesthetic in the form of delayed recovery and the patient has described feeling weak after the operation. Another patient with no anaesthetic records but a letter from the referring anaesthetist described a low-grade fever with the use of halothane that was reversed rapidly and easily with surface cooling. The third patient's file contained the full report of the anaesthetic reaction in addition to the referral letter from the attending anaesthetist. This patient's anaesthetic included the use of isoflurane without the administration of succinylcholine, and after 45 minutes of the seven-hour operation, the patient developed a temperature increase from 37.5°C to 39°C with unexplained tachycardia of 150 beats/minute. However, the maximum recorded $etCO_2$ was <6.1kPa and the highest CK level recorded was 285 IU/L. In addition, the pH was within normal range with no acidosis, and a urinary sample showed the absence of myoglobinuria.

On the other hand, the six patients who were referred because of either a clinical or family history of myopathy and tested MHS with IVCT had previous general anaesthetics without developing an MH reaction. In only one patient who is an index case of a CM family, an MH trigger-free anaesthetic was used as the anaesthetist was aware of the possibility of MH due to the family history of CM. In the other five patients, triggering volatile anaesthetics were used without the use of succinylcholine. The number of previous anaesthetics, before the diagnosis of MH susceptibility was established, ranged from one up to six previous operations where triggering volatile anaesthetics were used. All five patients showed myopathic changes during

histopathological examinations of their muscle biopsies ranging from mild to severe changes. Regarding the reason for referral for these five patients, two patients had the clinical phenotype of CM while the other three patients were index cases of CM families.

Family study

It is essential to restate the definitions of an index case and a proband when we report this study of CM families. Throughout this study, we used the term index case to indicate the first member of the family to be investigated, while proband is used to indicate the member of the family who presented with the clinical phenotype and triggered an investigation of this family. Although the proband and index case could be the same person, in this part of the study, we will refer to an index case as the first member of the family who has been tested without being affected by the clinical phenotype. Of the 47 family codes identified, 30 index cases were singletons with no other family members tested at the unit, and consequently, no additional clinical, IVCT or genetic information was available. For family codes with other family members who were tested at the unit ranging in number from two to 21 family members. Of the 17 CM families with more than one member tested at the unit, the index cases of four families were tested for MH susceptibility with IVCT but did not have any clinical muscle weakness. In the remaining 13 families, the first member to be tested was the proband who presented with the clinical phenotype of CM or developed an anaesthetic reaction and was found to have clinical muscle weakness.

Two large families with 14 and 20 members tested with IVCT, in addition to the CM proband in each family, were included in our study. Genetically, a different familial *RYR1* variant was found in each family, and in both families, matching results of the

RYR1 genotype, myopathy phenotype, IVCT phenotype and histopathology were found. In the first family MYO-1, five members, including the proband, reported some degree of clinical muscle weakness and all of them were found to carry the familial variant *RYR1* c.13913G>A. In these five patients, in addition to another clinically-free genotype-positive patient, the histopathological examination of their muscle biopsies showed some features of CM. On the other hand, the other nine MHN members of this family, who were tested genotype negative for the familial variant, reported no muscle weakness and no abnormality was detected in their muscle biopsies. The other family MYO-2 with 21 members tested at the unit, including the myopathic proband, showed the same degree of segregation between myopathy, IVCT, and histopathology phenotypes and the *RYR1* genotype, but the familial variant that was found in this family was the *RYR1* c.14814C>G. Regarding numbers in this family: 15 members, including the proband, tested MHS with IVCT, some degree of clinical muscle weakness and myopathic changes were reported, and 14 of them were genetically tested, and the familial variant was detected in all of them. The other six members tested MHN, no clinical nor histopathological features of CM were reported, and the familial variant could not be detected in the five members who were genetically tested.

The genetic testing of members of three families; MYO-3, MYO-4, MYO-5, in whom more than one member in addition to the proband or index case have been tested with IVCT and genetically, a familial *RYR1* variant was detected in all MHS members, while MHN members of these families were not genetically tested. In another two families; MYO-6, MYO-7, no such segregation could be found between IVCT and genetic results. In the first family, an MHS patient and two MHN members were found to have the *RYR1* c.2320G>A variant and another MHN did not have this variant. The other

family's proband tested MHS and the *RYR1* c.14582G>A variant was found while his father tested MHS, but no variant was found. Four families included in our study had only one member, in addition to the proband or the index case, tested with IVCT. In one family; MYO-8, the proband who had clinical myopathy and moderate myopathic changes, in addition to another clinically-free relative and no histopathological abnormality detected, both tested MHN with IVCT. Only the proband of this family was genetically tested and the *RYR1* c.14591A>G variant was detected. The probands of another two families; MYO-9 & MYO-10 tested MHS, the additional member of both families tested MHN. However, the additional member of one of these families, MYO-10, the sister of the female proband, complained of scoliosis but no pathological abnormality was detected in her muscle biopsy. Both members of the last family; MYO-11, including the proband who had a suspected MH reaction, tested MHS with IVCT. Nevertheless, the sister of the male proband had no clinical muscle weakness and her muscle biopsy showed no pathological abnormalities.

5.3.2 Histopathological findings in MH index cases

Reviewing the histopathological examination reports of all MH index cases who have been tested at the unit for MH susceptibility and had a muscle specimen examined by the neuropathology lab at Leeds Teaching Hospitals, we identified 120 reports with histological changes of CM detected in their muscle biopsies. None of these 120 cases had any medical history of clinical muscle weakness as the nine patients who experienced a suspected MH reaction and were found to have muscle weakness were included in the former group of congenital myopathies. The reason for referral for diagnosis of MH susceptibility in these 120 selected cases was a suspected anaesthetic reaction in 106 patients, while the remaining 14 individuals were relatives of patients who experienced an anaesthetic reaction and could not be tested being too

young, abroad, deceased or unfit for the procedure. The average age at the biopsy of the included 120 cases was 34 years, with a minimum of 5 years and a maximum of 75 years old, and 51 cases were females.

All the selected cases were included in this cohort because of the detection of myopathic changes during the histopathological examination of their muscle samples. Mild myopathic changes were detected in more than half of the patients (63 out of 120 patients) with the most common change detected is the motheaten appearance during NADH reductase staining. In the remaining patients, moderate to severe myopathic changes were detected: either a more severe form of core formation, mini-cores or well-demarcated central cores, with or without fibre type 1 predominance, and/or increased percentage of central nucleation. All the 12 patients who presented with a severe form of myopathic changes, tested MHS and all, but one case, were referred because of a suspected anaesthetic reaction. On the other hand, 22% of the 45 patients who had a moderate form of myopathic changes and 39% of the 63 patients with mild myopathic changes, tested not MHN.

5.3.3 The relation between histopathological findings and the IVCT results and CK level

As the severity of myopathic changes differed between patients in our cohort, we tested the association between different histopathological severity scores and the basal serum CK level at the time of the biopsy and the results of the three IVCT tests. We excluded seven patients with no pathology detected from this analysis as their number in each sub-category was too small to be statistically interpreted. The basal serum CK level was available for 127 patients, and we used the uncorrected Fisher LSD test to compare the mean of Log 10 transformed serum CK levels in each severity

category with that of each other category, and no statistically significant difference could be found between groups, figure 5-1. For IVCT results, we compared the average twitch height of the three tests using uncorrected multiple comparisons Dunn's test and no significant difference was found between the groups.

Regarding the tension difference for the three IVCT tests, we only included in this analysis the available results for patients who were tested susceptible to MH, as MHN patients would have a negative tension difference value. The available numbers were not consistent in each test due to the unavailability of the test traces for some patients. We used the uncorrected Dunn's test for multiple comparisons, and as shown in figure 5-2, the only significant difference that could be noticed was in dynamic halothane test, as patients with severe myopathic changes in their muscle biopsies showed a small but significant higher tension difference when compared with patients with mild or moderate myopathic changes.

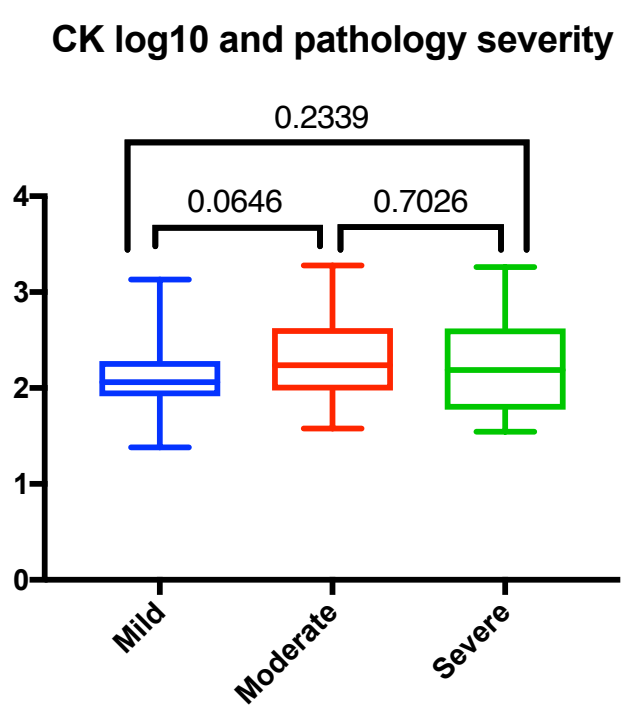

Figure 5-1: Comparing the log 10 transformed data of basal CK level between different pathology severity groups. *No difference could be found between the groups using the uncorrected Fisher's LSD test. P values are labelled above the brackets.*

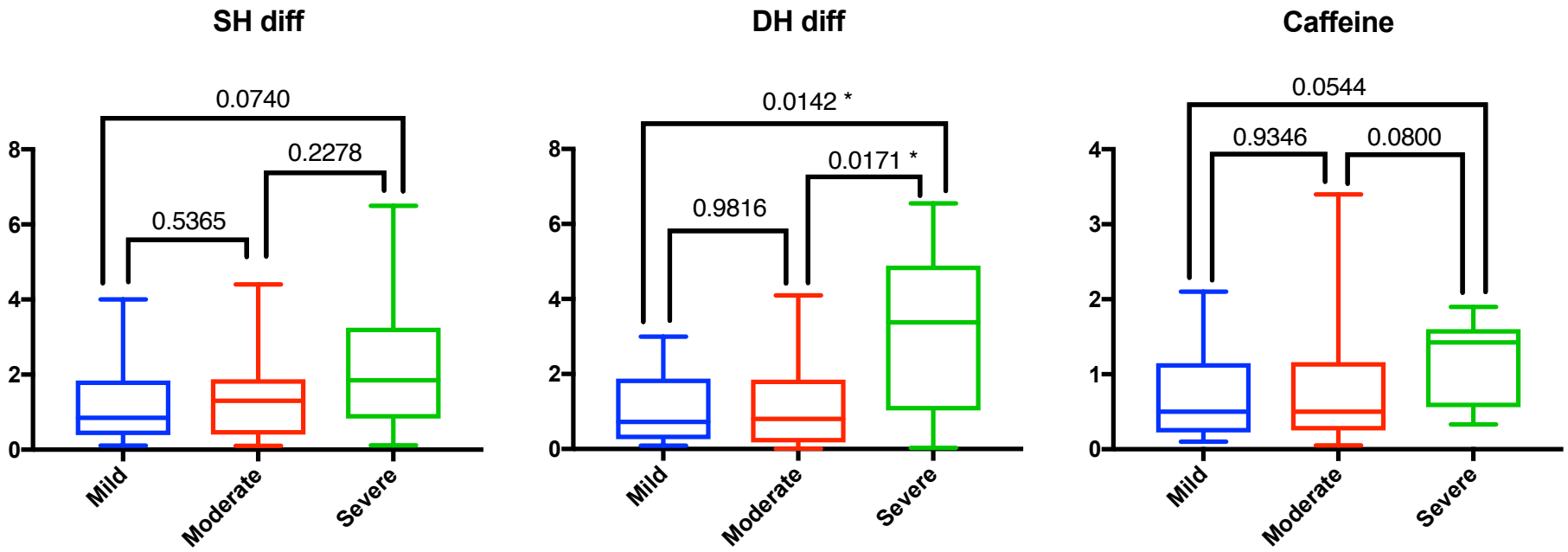

Figure 5-2: Comparing tension differences of the three IVCT tests between different pathology severity grades. *Statistically significant difference was found in DH test between patients with severe pathology compared to the other two groups using the uncorrected Dunn's test. P values are labelled above brackets connecting with significant values labelled with *. SH= static halothane, DH= dynamic halothane*

In addition, the numbers of patients who were found to have an isolated feature of the *RYR1*-related myopathic changes are shown in table 5-3. Motheaten appearance is the most common isolated histopathological feature found in our patients. Only one of those patients who were found to have an isolated motheaten appearance in their muscle biopsies presented with clinical muscle weakness. Motheaten appearance was found in all patients reported in this table to have an isolated fibre type 1 predominance or central nucleation. Nonetheless, we included these patients as to have an isolated fibre type 1 predominance or central nuclei because of the controversy about motheaten appearance being a nonspecific feature or a stage of core formation process (Sewry et al., 2002).

		Isolated pathological features				
		Central cores n.16	Mini-cores n.11	Motheaten n.65	Fibre type 1 n.8	Central nuclei n.14
Clinical weakness n.10	MHS n.5	2	1	0	1	1
	MHN n.5	2	1	1	1	0
No clinical weakness n.114	MHS n.78	10	5	39	2	12
	MHN n.36	2	4	25	4	1

Table 5-2: Patients with isolated RYR1-related histopathological changes. *Patients are distributed according to their clinical myopathy status and MH susceptibility phenotype. MHS= malignant hyperthermia susceptible. MHN= malignant hyperthermia not susceptible.*

5.3.4 Genetic findings

Information about the genetic testing results was available for 104 patients of the whole cohort included in this study. In the group of patients who were referred because of an anaesthetic or a family history of suspected MH without any medical history of CM and their muscle biopsies showed some myopathic changes, at least one variant was found in 51 patients. Additionally, nine patients from the same group were not genetically tested, but we included the variant that was detected in other MHS members from their families. In the CM group of patients, including patients with a clinical or family history of clinical muscle weakness with or without a history of suspected MH reaction, information about genetic testing results on the Leeds MH Investigation Unit database was available for 19 patients. The other 28 patients, in this group of congenital myopathies, for whom we submitted an NGS request to the DNA lab, we found that no DNA sample was available for analysis for nine patients and another patient declined to provide consent for genetic testing. DNA samples of the remaining 18 patients were genetically tested using NGS, and at least an *RYR1* variant was detected in 11 patients, while no variant could be detected in seven patients.

Genetic testing of the whole cohort of patients detected 60 different *RYR1* variants. As we stated earlier, sequencing techniques were different over the years and between patients. Table 5-4 lists all *RYR1* variants found in this cohort of patients and their detection rate, in addition to the MH phenotype of patients with each variant and their clinical myopathy phenotype. Variants that are on the EMHG list of diagnostic variants are labelled in the table. Of the variants detected in this cohort, 42 variants were detected in only one patient each, while the most common *RYR1* variant among the UK population, the c.7300G>A, was detected in 12 patients in our cohort, all of

them were tested because of a history of MH without clinical muscle weakness. Another ten variants were detected in two patients, each, while three variants detected in three patients and four variants detected in four patients, each. An additional *RYR1* variant was detected in five patients in our cohort and in another four patients, we included the variant that has been detected in an MHS member of their families, as the four included patients have not been genetically tested. All, but two, variants detected in our cohort of patients are missense mutations, and the two exceptions are deletion mutations, variants ID# 48 and 55.

ID#	codon	protein	Detection rate	MH phenotype	Myopathy phenotype	MH Pathogenicity	Notes
1	c.38T>G	p.13Leu>Arg	1	MHS			
2	c.178G>A	p.60Asp>Asn	1	MHS			
3	c.251C>T	p.84Thr>Met	1	MHS			
4	c.325C>T	p.109Arg>Trp	1	MHN			With variant #10
5	c.479A>G	p.160Glu>Gly	2	MHS	CM	Likely-pathogenic	
6	c.487C>T	p.163Arg>Cys	3	MHS		Diagnostic	
7	c.742G>C	p.248Gly>Arg	1	MHS		Diagnostic	
8	c.1021G>A	p.341Gly>Arg	2	MHS		Diagnostic	
9	c.1201C>G	p.401Arg>Cys	1	MHS		Diagnostic	
10	c.1453A>G	p.485Met>Val	1	MHS			Additional variant
11	c.1615T>G	p.539Phe>Val	1	MHS		Likely-pathogenic	
12	c.1840C>T	p.614Arg>Cys	2	MHS		Diagnostic	1 Family variant
13	c.2320G>A	p.774Gly>Arg	1	MHS			
14	c.3535C>T	p.1179Arg>Trp	1	MHS			
15	c.4178A>G	p.1393Lys>Arg	1	MHS			
16	c.4405C>T	p.1469Arg>Trp	1	MHN			
17	c.4711A>G	p.1571Ile>Val	2	1MHS/*1MHN	CM		*1 with variant #39
18	c.6488G>A	p.2163Arg>His	1	MHS		Diagnostic	Family variant
19	c.6502G>A	p.2168Val>Met	1	MHS		Diagnostic	
20	c.6587A>G	p.2196Asn>Ser	1	MHN	CM		With variant #53
21	c.6617C>T	p.2206Thr>Met	4	MHS		Diagnostic	1 with variant #47
22	c.7036G>A	p.2346Val>Met	1	MHS			With variant #60
23	c.7043A>G	p.2348Glu>Gly	1	MHS			
24	c.7048G>A	p.2350Ala>Thr	2	MHS		Diagnostic	
25	c.7063C>T	p.2355Arg>Trp	1	MHS		Diagnostic	

ID#	codon	protein	Detection rate	MH phenotype	Myopathy phenotype	MH Pathogenicity	Notes
26	c.7089T>G	p.2363Cys>Gly	1	MHS			
27	c.7093G>A	p.2365Gly>Arg	1	MHS			
28	c.7291G>T	p.2431Asp>Tyr	2	MHS		Likely-pathogenic	
29	c.7300G>A	p.2434Gly>Arg	12	MHS		Diagnostic	
30	c.7304G>A	p.2435Arg>His	1	MHS	CM	Diagnostic	
31	c.7304G>T	p.2435Arg>Leu	1	MHS			
32	c.7354C>T	p.2452Arg>Trp	2	MHS		Diagnostic	
33	c.7361G>A	p.2454Arg>His	2	MHS		Diagnostic	
34	c.7373G>A	p.2458Arg>His	3	MHS		Diagnostic	
35	c.7523G>A	p.2508Arg>His	4	MHS	2CM	Diagnostic	
36	c.11132C>T	p.3711Thr>Met	1	MHS			
37	c.11315G>A	p.3772Arg>Gly	1	MHS	CM	Likely-pathogenic	
38	c.11752A>C	p.3918Thr>Pro	1	MHS			
39	c.11798A>G	p.3933Tyr>Cys	1	MHS			Additional variant
40	c.11958C>G	p.3986Asp>Glu	1	MHS		Likely-pathogenic	
41	c.11969G>T	p.3990Gly>Val	2	MHS		Diagnostic	
42	c.13513G>C	p.4505Asp>His	1	MHS			
43	c.13913G>A	p.4638Gly>Asp	4	3MHS/1MHN	CM		
44	c.14201G>A	p.4734Gly>Glu	1	MHS			Family variant
45	c.14209C>T	p.4737Arg>Trp	1	MHS			Family variant
46	c.14210G>A	p.4737Arg>Gln	1	MHS		Likely-pathogenic	Family variant
47	c.14344G>A	p.4782Gly>Arg	1	MHS			Additional variant
48	c.14419_14421del		1	MHS			
49	c.14440C>T	p.4814Leu>Phe	1	MHN	CM		
50	c.14458G>T	p.4821Gly>Trp	1	MHS	CM		

ID#	codon	protein	Detection rate	MH phenotype	Myopathy phenotype	MH Pathogenicity	Notes
51	c.14471T>C	p.4824Leu>Pro	1	MHS		Likely-pathogenic	
52	c.14477C>T	p.4826Thr>Ile	4	MHS		Diagnostic	2 Family variants
53	c.14522C>A	p.4841Thr>Asn	1	MHS			Additional variant
54	c.14582G>A	p.4861Arg>His	2	1MHS/1MHN	CM	Diagnostic	
55	c.14588_14605del		1	MHN	CM		
56	c.14591A>G	p.4864Tyr>Cys	1	MHN	CM		
57	c.14678G>A	p.4893Arg>Leu	3	2MHS/1MHN*	1CM	Likely-pathogenic	*The CM patient is MHN
58	c.14814C>G	p.4938Ile>Met	1	MHS	CM		
59	c.14815G>T	p.4939Asp>Tyr	1	MHN	CM		
60	c.14817C>A	p.4939Asp>Glu	1	MHS			Additional variant

***Table 5-3: RYR1 variants detected in our cohort**, the number of patients included in our cohort who were found to have these variants and their MH susceptibility status using IVCT and CM phenotype. Variants found in the EMHG list of diagnostic RYR1 variants for MH are labelled as diagnostic. The six variants in red cells are variants found in the family members of nine patients included in this cohort but were not genetically tested. The five variants in yellow cells were found in association with other RYR1 variants, each labelled in the notes' column.*

Most of the patients (78 out of 89) who were found to have an *RYR1* variant, were tested susceptible to MH with IVCT. Nine of the 11 patients who were tested MHN have presented with clinical CM, while the other two patients were tested because of a family history of CM without clinical muscle weakness, nor anaesthesia related complications in their anaesthetic history. The *RYR1* variants c.4711A>G, c.13913G>A, c.14582G>A, c.14678G>A were found in both MHS and MHN tested patients. All the MHN patients who were found to have one of these variants are CM patients. The *RYR1* c.14582G>A variant is on the EMHG list of diagnostic variants. We reviewed the IVCT traces of the MHN CM patient who were found to have the diagnostic *RYR1* 14582G>A variant and found that the three muscle specimens used for the IVCT tests were viable at the time of the test, and for the static halothane test the muscle contracture line continued to relax with the addition of 0.5, 1, 2% concentrations of halothane, while for the dynamic halothane test the muscle contracture line plateaued. For the caffeine test, the muscle specimen reacted to the application of 2 mmol, but the tension difference was 0.05 g (below the cut-off point of 0.2 g)

In our list of variants, 19 diagnostic mutations were functionally characterised as causative for MH (https://www.emhg.org/diagnostic-mutations). These mutations were found in 50 patients included in our cohort and apart from the MHN CM patient discussed early, all other 49 patients were tested susceptible to MH. In addition to the MHN CM patient with the *RYR1* c.14582G>A mutation, another three patients presented with clinical CM were found to have diagnostic *RYR1*vriants: one patient with c.7304G>A mutation and two patients with c.7523G>A mutation, and all of the three patients tested MHS. Another list of non-functionally characterised *RYR1* variants that are considered by the EMHG as likely pathogenic for MH can be found

on (www.emhg.org/genetic-scoring-matrix). In our cohort, 12 patients were found to have one of these likely-pathogenic variants. Of these 12 patients, four had the clinical CM phenotype, and two of them were tested MHN with IVCT. The likely-pathogenic variant *RYR1* c.14678G>A was found in an MHN CM patient and also found in another two MHS patients, one was referred because of an MH reaction, and the other patient is a family member of a CM family but without any clinical muscle weakness.

Investigating the incidence of clinical myopathy and/or histopathological features of myopathy in different genotype groups, we found that the 12 patients who were found to have the most common *RYR1* variant among the UK population, the c.7300G>A, and were found to have myopathic features on histology, none of them had any clinical features of clinical muscle weakness. Histologically, NADH staining of all 12 patients showed a motheaten appearance, and one patient had also an increased percentage of central nucleation and another patient had also fibre type 1 predominance, while the remaining ten patients had no other pathological abnormalities. These 12 unrelated patients represent 10.17% of all the 118 UK families who were found to have the *RYR1* c.7300G>A. The second most common *RYR1* variant, the c.1021G>A, that was found in 31 UK families, was found in only two index cases of MH families included in our cohort. Both cases did not report clinical CM nor anaesthesia related complications but were tested as relatives of patients who had a suspected MH reaction. The histological examination of their muscle biopsies revealed only motheaten appearance in one case and multi-mini-cores plus central nucleation in the other case and both cases were tested MHS.

Of the 28 UK families who were found to have the third most common *RYR1* variant, the c.6617C>T, four index cases (14.29%) of these families were found to have

histopathological features of CM. All these four patients were referred because of a history of anaesthetic reaction with no clinical muscle weakness reported in their history. A severe form of myopathic changes on histology was reported in one patient with well-defined central cores can be detected in the muscle biopsy in addition to fibre type 1 predominance. The other three patients had motheaten appearance with one patient found to have an increased percentage of central nucleation. In our cohort, three out of 21 UK families with the *RYR1* variant c.487C>T were found to have some myopathic changes in their muscle biopsies with a severe form of pathology in one patient with central cores and increased percentage of central nucleation. All the three patients were tested MHS because of an anaesthetic reaction, and none of them reported clinical muscle weakness.

These discussed four variants were found in >50% of all the UK families who were found to have one of the diagnostic *RYR1* variants. These 21 unrelated patients included in our cohort and were found to have one of these four variants, represent just above 10% of the UK families with these variants. None of these patients has reported any clinical features of CM. Histologically, only two patients of this group were found to have severe myopathic changes with well-demarcated central cores in their muscle biopsies, while the remaining had mild to moderate myopathic changes. Clinical CM with a diagnostic *RYR1* variant was reported in four patients with the variants c.7304G>A, c.7523G>A and c.14582G>A. These three variants were found in 16 UK families (~4.5% of UK families with diagnostic variants). The CM patient with the *RYR1* variant c.14582G>A was tested MHN with IVCT, discussed earlier.

Of the variants that are not on the EMHG list of diagnostic variants, nor on the list of likely-pathogenic variants for MH, the *RYR1* variant c.13913G>A that was found in two

MHS, and two MHN patients included in our cohort. None of these four patients had any history of anaesthesia related complications, and all of them were referred because of clinical muscle weakness. This variant could be of particular interest with regard to the CM phenotype. It was found in one of the big CM families described earlier MYO-1, five members of this family who were found to carry the genotype reported clinical muscle weakness and histopathological changes. Only one discordant member of this family who is genotype positive but clinically free and the muscle biopsy showed histopathological abnormalities. It was also found in the proband of another two families, and a singleton; however, we have not included these two families in the family study as only the probands were genetically tested.

5.4 Discussion

The widening histopathological spectrum of congenital myopathies and the growingly accepted concept of marked overlap between different types of congenital myopathies mandate a proper investigation of the association between the whole group of congenital myopathies and MH. The current practice is to refer patients who have a confirmed diagnosis of CCD for diagnosis of their MH susceptibility status using IVCT, a decision usually made by the neuropathologist or the paediatrician reviewing the case. We believe that the 47 index cases of CCD families referred at the unit and included in this study, including those who developed an anaesthetic reaction, are considered a relatively small sample representing the number of families that have a confirmed diagnosis of CCD and even a smaller sample of families that have a confirmed diagnosis of other congenital myopathies. We summarised in this study the work done to study the overlap between different types of congenital myopathies, though, our main aim is to highlight to the anaesthetists the potential risk of MH in patients with the broader, clinical and histopathological, spectrum of CM.

As *RYR1* mutations are now recognised as the most common genetic cause for congenital myopathies (Jungbluth et al., 2018) and it has been associated with different subtypes of congenital myopathies including the two most common variants of the disease CCD (Haan et al., 1990, Mulley et al., 1993) and MmD (Ferreiro et al., 2002, Jungbluth et al., 2002), in addition to other subtypes of CM including fibre type 1 predominance (Clarke et al., 2010) and centronuclear myopathy (Wilmshurst et al., 2010). The histopathological features of these subtypes are referred to as *RYR1*-related myopathic changes (Sewry et al., 2002, Sewry and Wallgren-Pettersson, 2017). Mutations in the same gene were identified as the causative mutations for MH in >50% of UK MH families, and if variants that have not had its pathogenicity confirmed are included, this percentage rises to >70% (Miller et al., 2018). However, due to the marked clinical, histopathological and genetic heterogeneity of these two disorders, the management of both disorders in relation to each other is too complex. Therefore, it is reasonable to treat patients diagnosed with one of these conditions as at risk of developing complications due to the other disorder until a definitive diagnosis is confirmed.

Of the different subtypes of congenital myopathies, CCD patients were suggested to be at risk of MH (Shuaib et al., 1987, Brownell, 1988), though, this association is not always consistent (Curran et al., 1999). Considering the clinical and histopathological overlap between different subtypes of CM, it is crucial to assess the associated risk between MH and CM, regardless of the subtype diagnosis. However, this association is complicated by factors such as the incomplete penetrance of MH, the observation that not all MH susceptible patients develop a reaction in their first anaesthetic, and the specificity of the IVCT of ~93%. Additionally, different molecular mechanisms have been proposed to explain how different *RYR1* mutations affect Ca^{+2} homeostasis

leading to the MH susceptibility phenotype (Hirata et al., 2007, Maclennan and Zvaritch, 2011) in one hand, and the CCD phenotype (Treves et al., 2008), on the other hand. In our cohort, three patients with clinical myopathy who had an adverse anaesthetic reaction tested MHN with IVCT. The reaction of one of these three patients, where the details were available, mounts to be diagnosed clinically as a hypermetabolic MH reaction, though, considered a mild to moderate form of anaesthetic reactions. This could question the sensitivity of IVCT of 99%, especially in patients with CM. Unfortunately, these three patients were not tested genetically for variants in the *RYR1*.

In our cohort, the majority of patients presented with clinical muscle weakness, excluding those who developed an anaesthetic reaction, tested MHN with IVCT (21 of 28). Some of these 21 patients were genetically tested when a DNA sample was available, and they have consented for genetic testing. Of the 14 patients who were genetically tested, an *RYR1* variant was found in nine patients, and one of these variants is on the EMHG list of diagnostic variants for MH, representing another possible challenge for the sensitivity of the IVCT in patients who present with clinical myopathy. Although, the in vitro response of muscle samples depends on other factors related to the viability of the muscle and surgical techniques during the biopsy, the background pathology in these muscles could influence their response to the ryanodine receptor agonists used, halothane and caffeine. We have not analysed the difference of contracture strength in IVCT traces from our patients. This was mainly because of the small sample size and the fact that these biopsies have been taken during different time periods, with the changes in the surgical techniques and the IVCT protocols over the years. However, we noticed that the tension differences for the two halothane tests and the caffeine test were just slightly higher than the cut-off point in

most of the patients. It could be an observation bias; however, further statistical analysis of larger sample size is needed to test the possible effects of the underlying muscle pathology on the IVCT results.

Our study of family members of probands and index cases who have been tested at the unit because of a clinical or a family history of CM, reinstated the same observation found before in a previous study (Shepherd et al., 2004). This study reported patients who had clinical myopathy and tested MHS, patients who had no clinical myopathy but tested MHS and their muscle biopsies showed *RYR1*-related myopathic changes, and patients who had clinical myopathy with histopathological changes in their muscle biopsies but tested MHN. Additionally, we report further variations in the association between clinical myopathy, MH susceptibility and *RYR1*-related myopathic changes:

- Patients who reported an adverse anaesthetic reaction and tested MHN, but their muscle biopsies showed histopathological changes.
- Patients who presented with clinical muscle weakness and had a hypermetabolic anaesthetic reaction, but tested MHN.
- Patients who presented with clinical muscle weakness, reported an adverse anaesthetic reaction but tested MHN, and no pathological changes were found in their muscle biopsies.

This complicates more our standing of the link between the two phenotypes, however, as a possible explanation; the different molecular mechanisms leading to the clinical myopathy and/or myopathic changes, which is different from that causing the MH phenotype (Jungbluth et al., 2018), may alter the response of muscle samples to ryanodine receptor agonists used during the IVCT. Another explanation is that the

viability of muscle samples from these patients may be compromised due to the underlying pathology that leads to false-negative responses.

The high segregation found in two of our large families, MYO-1 and MYO-2, between the different studied features; the myopathy phenotype, the MH phenotype, and the histopathology in association to the two *RYR1* variants c.13913G>A and c.14814C>G, respectively; highlights a significant and promising background for future studies. Functional characterisation studies of these two variants could offer a better understanding of their effects on the excitation-contraction coupling and Ca^{+2} homeostasis. On the other hand, no segregation between the studied features and genetic testing results were found in most of the other families. This adds more to the complexity of the association between the two conditions. In particular, in one of the families; MYO-7, an *RYR1* variant was found in the MHS proband, while not found in the MHS father of this proband. The same variant, the diagnostic *RYR1* c.14582G>A variant, was found in another CM proband who was tested MHN. Of the limitations of this study that it is limited to patients who were referred to the Leeds MH Investigation Unit for IVCT, and when the proband or index case of a family is tested MHN, usually other members of the family are not referred for the invasive biopsy. However, to better understand the complexity of the association between the two disorders, it would be beneficial to test a larger number of members of these families for MH susceptibility and genetically, in different generations of the family. This could be practically difficult due to the long waiting list at the unit, in addition to the ethical dilemma of exposing some patients to the probably unnecessary invasive biopsy procedure.

Several studies have reported the spectrum of histopathological changes associated with MH susceptibility without clinical muscle weakness (Reske-Nielsen et al., 1975,

Gullotta and Spiess-Kiefer, 1983, Harriman, 1988, Mezin et al., 1997, Orlov et al., 2013). Unspecific changes were described in the form of areas of necrosis or fibre degeneration, while other literature reported the presence of “frank” myopathic changes such as motheaten appearance and well-demarcated cores. We only included in this study the features that have been described in association with *RYR1*-related congenital myopathies. We have examined all the index cases and probands of families who have been tested at the Leeds MH Investigation Unit because of anaesthetic reactions. Of the 795 independent MHS families, 86 probands or index cases were found to have *RYR1*-related myopathic changes in their muscle biopsy, (10.8%, 95% CI 0.087-0.132), compared to 34 MHN probands or index cases from 1105 independent MHN families, (3.1%, 95% CI 0.021-0.043).

The severity of myopathic changes was markedly different between MHS and MHN cases as none of the MHN cases had a severe form of pathology. We should take into consideration that our estimate of 10.8% of MHS cases, was only for patients with *RYR1*-related myopathic changes, on the contrary of 22% estimate of non-specific changes described earlier (Orlov et al., 2013). Furthermore, the “frank” myopathy described in this previous study, only included fibre size variability and internal nucleation. Therefore, our study provides the most recent estimate of the incidence of *RYR1*-related myopathic changes in unrelated MH susceptible index cases. We also tried to explore the relationship between the severity of *RYR1*-related myopathic changes in our cohort of patients and the basal CK levels in these patients, but we could not find any significant association.

Similarly, the results of the three IVCT tests were not significantly different between the severity grades in our cohort, apart from a slight difference found in the results of

the dynamic halothane test. However, we have not corrected for multiple comparisons, which likely to abolish that slight difference found with the dynamic halothane tests. A future study with a bigger sample size should give us a better idea about the association between the severity of *RYR1*-related myopathic changes and the contracture strengths of the IVCT.

During the transfer discussion for this PhD project, a valuable suggestion was to recontact patients who were diagnosed MH susceptible after an anaesthetic reaction and were found to have *RYR1*-related myopathic changes in their muscle biopsy without clinical muscle weakness. The purpose was to check if these patients developed any muscle weakness after the reaction, either temporal or permanent. The hypothesis behind this suggestion, based on our discussion, was to examine if the anaesthetic reaction could have any long-term effect on their muscle function. The possible cause could be the effects of triggering anaesthetic agents on gene expression in these patients that may trigger muscle weakness that was not apparent before the reaction. Another possible hypothesis is if the conformational changes in the Ca^{+2} homeostasis regulating proteins that happen with triggering volatile anaesthetics that may alter the muscle function for a longer time leading to clinical weakness. However, due to time restraints and issues with applying for another ethical approval to recontact these patients about their clinical condition, we were not able to test this hypothesis, although we consider this suggestion as a valuable continuation of our work and a base for future studies.

As the *RYR1* gene is considerably a large gene and more than 300 variants have been detected in this gene, it is not surprising the large number of variants detected in our cohort; 60 different *RYR1* variants detected in 94 patients of the 110 genetically tested

patients. Taking into consideration that some of these patients have been tested using conventional DNA sequencing methods, which target certain spots of the gene, this number is likely to increase if, in all the tested patients, the whole *RYR1* gene was screened using NGS. In table 5-5, we have labelled variants detected in our cohort according to their association with the MH phenotype; some variants have been functionally characterised as diagnostic for MH while other variants are labelled as likely pathogenic. All the diagnostic variants have been detected in patients who were tested MHS with IVCT, apart from the CM discordant patient who tested MHN and was found to have the diagnostic variant c.14582G>A. For the variants that are listed as likely-pathogenic for MH, all patients who were found to have one of these variants tested MHS, except for the *RYR1* c.14678G>A variant that was detected in two MHS patients and one MHN patient who was referred because of clinical muscle weakness. This supports our theory of the possible effects of the underlying muscle pathology on the results of IVCT.

On the other hand, studying the association between histopathological changes and the *RYR1* genotype, 23 patients were found to have an *RYR1* variant and a severe form of *RYR1*-related myopathic changes. All the ten patients who did not report any clinical muscle weakness, tested MHS with IVCT, while seven out of the 13 patients who were referred because of clinical CM tested MHN. These seven MHN patients include the two patients described early who were found to have a diagnostic and likely pathogenic *RYR1* variants; c.14582G>A and c.14678G>A, respectively.

Codon	protein	Pathogenicity	MH susceptibility	Muscle weakness
c.479A>G	p.160Glu>Gly	Likely	MHS	clinical CM
c.479A>G	p.160Glu>Gly	Likely	MHS	
c.487C>T	p.163Arg>Cys	Diagnostic	MHS	
c.4711A>G	p.1571Ile>Val	VUS	MHN	clinical CM
c.6587A>G	p.2196Asn>Ser	VUS	MHN	clinical CM
c.6617C>T	p.2206Thr>Met	Diagnostic	MHS	
c.7036G>A	p.2346Val>Met	VUS	MHS	
c.7043A>G	p.2348Glu>Gly	VUS	MHS	
c.7048G>A	p.2350Ala>Thr	Diagnostic	MHS	
c.7361G>A	p.2454Arg>His	Diagnostic	MHS	
c.7523G>A	p.2508Arg>His	Diagnostic	MHS	clinical CM
c.7523G>A	p.2508Arg>His	Diagnostic	MHS	
c.13913G>A	p.4638Gly>Asp	VUS	MHS	clinical CM
c.13913G>A	p.4638Gly>Asp	VUS	MHS	clinical CM
c.13913G>A	p.4638Gly>Asp	VUS	MHN	clinical CM
c.14440C>T	p.4814Leu>Phe	VUS	MHN	clinical CM
c.14471T>C	p.4824Leu>Pro	Likely	MHS	
c.14582G>A	p.4861Arg>His	Diagnostic	MHN	clinical CM
c.14582G>A	p.4861Arg>His	Diagnostic	MHS	clinical CM
c.14678G>A	p.4893Arg>Leu	Likely	MHN	clinical CM
c.14814C>G	p.4938Ile>Met	VUS	MHS	clinical CM
c.14815G>T	p.4939Asp>Tyr	VUS	MHN	clinical CM
c.14419_14421del		VUS	MHS	

Table 5-4: RYR1 variants that was found in patients with severe form of RYR1-related myopathic changes*, the pathogenicity of these variants to MH, the MH susceptibility status of these patients and the clinical CM diagnosis. VUS= variant of unidentified significance.*

The mode of inheritance of MH and different entities of *RYR1*-related congenital myopathies are variable. Majority of the *RYR1* mutations associated with MH and CCD are described to have an autosomal dominant mode of inheritance (Jungbluth, 2007). However, homozygosity and compound heterozygosity have been described with MH phenotype (Deufel et al., 1995, Lynch et al., 1997), and with CCD (Romero et al., 2003, Wu et al., 2006). *RYR1* mutations described with MmD are recessive in many cases (Monnier et al., 2003, Jungbluth et al., 2005). In CNM, a case was described with autosomal dominant *RYR1* mutation (Jungbluth, 2007), and in another three

cases (Wilmshurst et al., 2010), while other four cases were described to have autosomal recessive *RYR1* mutations.

The molecular mechanisms behind the different *RYR1* mutations' effects on Ca^{+2} homeostasis and muscle function vary. Recessive *RYR1*-related myopathies were found to have a quantitative decrease in the number of *RYR1* receptors rather than a qualitative impairment of receptors function (Ducreux et al., 2006, Zhou et al., 2006, Zhou et al., 2013). The mechanism behind the effects of autosomal dominant *RYR1* mutations associated with MH is gain-of-function with an increase in Ca^{+2} release from the SR through the RyR1 receptor (Murayama et al., 2016). On the other hand, *RYR1* mutations associated with CCD cause loss-of-function of the receptor (Avila et al., 2001, Dirksen and Avila, 2002, Avila et al., 2003). These different molecular mechanisms could offer an explanation for the heterogeneity of genetic findings in our cohort. However, or study is limited because the conventional sequencing methods used in the genetic analysis of patients included in our cohort, only search through a single strand of DNA; therefore, presence of another allele of the variant cannot be confidently excluded in addition to the possibility of the presence of other heterozygous variants in the same patient.

In conclusion, this study added to the ongoing work aiming to explore the correlation between MH and congenital myopathies. The correlation between the clinical, histological and genetic findings of these disorders proved to be complex. Further work to include more members from families diagnosed with either MH phenotype or CM phenotype could offer more statistical power to these studies. Further studies of the molecular mechanisms by which different *RYR1* mutations lead to the functional defects of the RyR1 receptors on muscle cells or myotubes, from patients presented

with different phenotypes, would provide us with a better understanding of the correlation between the histopathological findings and the CM and/or MH phenotypes.

6 Investigating the epidemiology of MH in Egypt

6.1 Introduction

Our knowledge about the incidence and prevalence of MH in Egypt is very limited due to multiple reasons related to the different health systems in Egypt and how they operate, the demographics of the Egyptian population, and the nature of MH disorder. Medical and health care in Egypt is distributed, unequally, between three different health systems. The public health system that serves the vast majority of the population operates through the three levels of healthcare: primary, secondary and tertiary hospitals distributed all over the country. Doctors working in these hospitals have completed a programme of training in different specialities at one of the major university hospitals across the country. The second system is the university hospitals that are considered as tertiary and highly specialised centres serving mainly the purposes of training doctors in different specialities and medical research. These hospitals are limited in number and only provide healthcare to a considerably limited number of patients across the country. Thirdly, is the private healthcare system where no exact official statistics about the extent of this system's hospitals, the quality of care offered in these hospitals, and the number of patients who receive treatment and healthcare through this system.

Despite the substantial differences between the three healthcare systems mentioned, they all share the same characteristics that limit our ability to track rare-occurring disorders such as MH. These characteristics include, for instance, the lack of a central recording system for patients' data, the absence of an official morbidity and mortality committee in the majority of hospitals across the three systems, and the lack of a nationally recognised system for serious incidents reporting. In addition, management

protocols are usually localised to particular hospitals with no designated body responsible for ensuring the application of nationally agreed guidelines in all the hospitals across the three systems. Last but not least, limited resources in some areas in the country, in the form of poorly equipped hospitals, absence of diagnostic facilities, shortage in intensive care beds numbers and low doctors-to-patients' ratio.

Using PubMed and Google Scholar search engines to search through different databases for the keywords "(malignant hyperthermia) AND Egypt" trying to find any reported cases or epidemiological data about the disorder in Egypt, we were not able to obtain any results. Nevertheless, the disorder is a known complication of general anaesthesia among anaesthetists in the country and based on personal communication with a number of anaesthesia consultants and middle-grade doctors across the three healthcare systems, many of them have heard about a patient who developed a suspected MH reaction in their place of work, nevertheless, the majority of them did not experience such a case themselves. Due to limited resources and other administrative issues, dantrolene is not usually stocked in many of the Egyptian hospitals, including even hospitals with high patients flow rates and a significant number of surgeries performed under general anaesthesia on day by day basis. In addition to the characteristics of healthcare systems in the country that have been described earlier, propose to a high mortality rate of life-threatening disorders such as MH.

Despite the absence of published epidemiological data or case reports about MH in Egypt, we have little doubt that this disorder happens among the Egyptian population. We have discussed earlier some of the reports of MH cases occurred in adjacent countries whose population share many of their ethnic and genetic characteristics with

Egypt. Therefore, we designed this study aiming to collect preliminary data about the extent of the problem among the country population. We will be able only to rely on the reporting anaesthetists accounts about any case suspected with MH regarding the clinical features of the reaction, management measures and the outcomes of these cases. However, this study should give us a rough idea about the epidemiology of MH in Egypt and mortality rates in patients suspected to have experienced this condition. This, in turn, should offer a cornerstone for future studies about the disorder in the region and direct decision-making officials towards the proper steps to tackle rare, though life-threatening, conditions such as MH.

6.2 Methods

6.2.1 Questionnaire design

As we discussed early the limitations of the health system in Egypt regarding diagnosis and management of rare diseases including MH, we agreed that online questionnaire would be a good facility to provide us with preliminary information about MH on a local and national levels and would help us to assess participants knowledge about the disorder and any management protocols available in their place of work. However, due to the lack of a standardised online secure communication system between health workers in Egypt, apart from some highly specialised hospitals on an elementary and limited level, we had to rely on non-official social media groups of anaesthesiologists working in Egypt.

The questionnaire was designed using Google Forms into four sections: the first section included an introduction to MH and the purpose of the survey, the second section aimed to collect information about the participant experience and pattern of practice, the third section collects information about any suspected MH reaction the

participant has experienced and if the participants recall a possible case of MH, in their judgement, clinical details of the reaction and any management measures that have been carried out is recorded, and the final section allows the participant to submit any other suspected cases and to opt-in for further discussion about the reported cases. A printout of the Google Form questionnaire is attached in the appendix.

Questions in the second section of the questionnaire collected information about the age and level of experience of the participant and how many years of anaesthetic experience they have, in an attempt to validate their judgment of the suspected anaesthetic reactions reported. Also, the pattern of practice and the average number of patients they normally anaesthetise per week should give us an idea of the load of work they usually experience. In addition, we collected information about the health system in which participants used to have the majority of their anaesthetic practice. The third section starts with a question whether the participant has experienced a range of clinical situations where MH could be a possible diagnosis during their anaesthetic practice and if the answer was yes, a detailed description of the clinical reaction and management measures used is obtained.

Anonymous details of the reaction collected included the age group of the affected patients, sex, and type of anaesthetic used, in addition to the manifestations of the anaesthetic reaction including muscular features and signs of hypermetabolic response. Any laboratory investigation results available were also recorded. Regarding management measures used, we aimed to check if a standardised management protocol was available at the participant place of work and to check for the availability of dantrolene. Any other supportive measures used were also recorded, and the outcome of the affected patients, including ICU admission, were also obtained.

6.2.2 Distribution of the questionnaire locally

The questionnaire was initially published among anaesthetists of the department of anaesthesia and intensive care at Assiut University Hospitals. The department has an average of 60 anaesthesia trainees, an average of 30 specialists and middle-grade anaesthesia doctors and another 30-40 consultants and professors of anaesthesia in different working capacities. The average working load of the department ranges from 80 to 100 elective cases per working day distributed over seven theatre complexes that include an average of four operating rooms in each complex. In addition to the on calls that provide anaesthesia for an average of 50-60 emergency cases/day during weekdays that increase to 60-80 cases/day during weekends. Worth mentioning that middle and higher-grade (consultants and professors) anaesthesia doctors could practice among different health systems, including private hospitals.

We launched the questionnaire through a Google Form link to the non-official Facebook page of the department in January 2016, and the questionnaire was open to receive responses for three months until the end of March 2016. A reminder was published on the Facebook page every month and in the last month twice to encourage participation. After the end of the three months, participants who opted-in for further discussion by providing their email addresses were contacted to provide any further details about the suspected reaction available and to clarify any ambiguous information provided. No identifiable patients' information was collected at any time to ensure patients' confidentiality.

6.2.3 National distribution of the questionnaire

To collect information about any suspected MH reactions on a national level, we considered using a more official communication system to reach to as many Egyptian

anaesthesiologists as possible. We initially communicated with the officials at the Egyptian Fellowship of Anaesthesia and Intensive Care. Our communication with the head and trustee of the institute aimed to provide the Google Form link, or a paper-based questionnaire version, to all applicants to the fellowship exams and/or applicants to the European Diploma of Anaesthesia and Intensive Care (EDAIC) examination that is held in Egypt, as the institute is the official representative of the European Society of Anaesthesiologists in Egypt. We aimed to reach to an average of 300-500 Egyptian anaesthetists during the application period for the fellowship and EDAIC part I exam between January and April 2017, with a possibility to republish the questionnaire again during the same period in the next year. After three months of communication with the officials over the period from September to December 2016, we lost all means of contact with the officials as they stopped replying to our emails or calls.

As attempts to publish this questionnaire officially failed, we repeatedly had to depend on the non-official Facebook page of the informal group “The Association of Egyptian Anaesthetists” which as of December 2017 was followed by more than 800 users; presumably, the majority of them are anaesthetists. The link to the Google Form questionnaire was published on the timeline of the Facebook page of the group. It was pinned to remain on top of the news feed for group members. The admin of the group was asked to post a reminder to all members of the group to actively participate, monthly and twice during April. Despite all these efforts to encourage the participation of Egyptian anaesthetists in this questionnaire, the percentage of participation remained very low. Therefore, another attempt to communicate with the official administration of the Egyptian Fellowship of Anaesthesia and Intensive care to

republish the questionnaire through their channels was made. However, this attempt proved again to be unsuccessful.

6.2.4 Encouraging participation

Different methods to encourage participation on the local and national levels of the questionnaire conduction was discussed. These included, for instance, offering some means of financial incentive in the form of shopping gift cards, but this proved impractical to be used in Egypt, in addition, this method could have compromised the integrity of the survey results as some people may submit fake cases in order to get these incentives. Another method discussed was to contact the clinical directors of different anaesthesia departments across the country to publish the questionnaire to their staff at their local hospitals. However, this method was not successful because of the absence of official communication channels, central email domain or official website, for example, for the clinical directories in different hospitals across the country. As stated earlier, several attempts to publish this questionnaire through an official institutional channel were all unsuccessful because of loss of communication with the administration.

The only available mean of communication that we had to endorse was using the non-official social media pages of different anaesthetists' societies and sending reminders to the members of these pages to encourage them to share their anaesthetic experiences with MH cases. This method was only beneficial during the conduction of the questionnaire on a local level, at Assiut University Hospitals, but of limited value when the questionnaire was published on the Facebook page of the "The Association of Egyptian Anaesthetists".

6.3 Results

6.3.1 Cases reported at Assiut University Hospitals

Participation from anaesthetists at the department of anaesthesia and intensive care, Assiut University Hospitals reached ~30% as 36 anaesthetists filled in the questionnaire of the department working capacity equivalent of an average 120 full-time working anaesthetists. Participants represented the three levels of qualifications for anaesthetists working at the department: trainees, middle-grade doctors (specialists and assistant lecturers), and consultants and professors with the majority of them being middle-grade 20 of 36. The working pattern of participating anaesthetists is presented in table 6-1. The majority of middle-grade doctors who presented 55% of participants had an average of five to ten years of anaesthetic experience with five of them had less than five years. The main place of work for the majority of participants was a tertiary centre (including university hospitals) with only two anaesthetists worked at a secondary care hospital, and another middle-grade doctor worked at a private hospital. None of these three anaesthetists reported any suspected case of MH.

Experience level	No. of years	Workload/ week	Main place of practice	Cases reported
Trainees (***n***. 8)	<5 Yr.	57 case/week	Tertiary centre	1
Middle-grade (***n***. 20)	5-10 Yr.	28 case/week	Tertiary centre	6
Consultants (***n***. 8)	10-20 Yr.	16 case/week	Tertiary centre	4

Table 6-1: Participating anaesthetists in the questionnaire published at Assiut University Hospitals. *Their level of qualification, average workload and main place of work. Also, the number of cases reported from each group is shown.*

ID#	Muscular	Metabolic	Succinylcholine	Inhalation anaesthetic	Operation	Outcome
1	Generalized rigidity/ Masseter spasm	Yes	Yes	Isoflurane	Emergency	Dead
2	Generalized rigidity	Yes	No	Halothane	Elective	Dead
3	Generalized rigidity	Yes	No	Isoflurane	Elective	Dead
4	Masseter Spasm	Yes	Yes	Halothane	Elective	Dead
5	Masseter Spasm	Yes	Yes	Halothane	Elective	Dead
6	No	Yes	Yes	Halothane	Emergency	Live
7	No	Yes	Yes	Halothane	Emergency	Dead
8	No	Yes	No	Halothane	Elective	Dead
9	No	Yes	No	Halothane/Isoflurane	Elective	Dead
10	No	Yes	No	Isoflurane	Elective	Dead

Table 6-2: Suspected MH cases reported by anaesthetists working at Assiut University Hospitals. *The main features of the reaction; muscular and metabolic and the anaesthetic agents used are presented. The procedure either emergency or elective and the outcome of these cases are also shown.*

Eleven cases were reported, four of them had a suspected MH reaction according to the judgment of reporting anaesthetists at the time of the reaction. The remaining seven patients developed an unexplained cardiac arrest under general anaesthesia. Two of the eleven patients had a family history of unexplained death under anaesthesia. Information related to age group, sex, type of operation and anaesthetics used was provided for seven of the eleven cases reported. This information with the clinical details of the reaction available for the reported patients is presented in table 6-2.

All, but one patient, died after the reaction either in the operating room or after admission to the ICU. The only patient who had a suspected MH reaction and was reported to be discharged after improvement, the reporting anaesthetist did not provide any further clinical details of the reaction and declined to provide contact information for further discussion. Dantrolene was not available in any of the reported cases, and therefore management measures were in the form of supportive treatment of the hypermetabolic features of the reaction. Supportive treatment measures included fluid loading, active surface cooling, hyperventilation and bicarbonate administration. Based on personal communication with anaesthetists who provided contact details, no standardised management protocol was available in-place at the time of the reaction, and neither of them has checked if a gas-free anaesthetic machine was available at the theatre complex. Four patients died in the operating room before being admitted to the ICU.

6.3.2 A case report of a recent suspected MH reaction at Assiut University Hospitals

Recently, after the questionnaire has been closed for accepting new responses, I was contacted by one of my colleagues about another case with a suspected MH reaction that occurred at Assiut University Hospitals:

An eight years old female patient presented to the casualty department after a fall on the ground, and she sustained a right distal radius and ulna fracture while examination revealed no other injuries. The patient did not have any medical or past history of relevant importance and no history of any previous operation, nor a history of anaesthesia related complications in the family. The patient was scheduled for a K-wire fixation of her fractures under general anaesthesia. On preoperative assessment, the patient Glasgow Coma Scale (GCS) was ±15, and her systematic examination revealed no abnormalities. Her vital signs were within normal range and was fasting for 10 hours. No premedication was given.

Inhalational induction of anaesthesia with sevoflurane in 100% oxygen was carried out using a Jackson-Rees' modification of the Ayre's T-piece. After induction, a 22 G IV cannula was inserted on the dorsum of the left hand and after ensuring an adequate depth of anaesthesia, a laryngeal mask airway size 2 was inserted and secured in place. Anaesthesia was maintained using isoflurane 3% volume in 100% oxygen with her respiratory rate (RR)= 22 cycle/minute and $etCO_2$ at 40mmHg. The patient received 300 mg of paracetamol for analgesia. After 30 minutes, the attending anaesthesia trainee noticed the etCO2 started to climb up to 50mmHg and therefore, the fresh gas flow was increased to 10 litres/minute. The etCO2 continued to climb up to 62mmHg after 60 minutes despite adequate ventilation throughout. At this stage,

the patient started to feel warm to touch, and the heart rate increased to 140 beats/minute and a senior help from the middle-grade doctor, on-site, was sought.

The attending anaesthetists at this stage agreed to intubate the patient to perform more controlled hyperventilation at a rate of 30-35 cycles/minute with a tidal volume of 230 ml and reduced the inhalation anaesthetic isoflurane to 1%volume. After 90 minutes the patient was still warm to touch, a rigidity of the upper and lower limbs started to develop, the heart rate increased to 160 beats/minute, blood pressure dropped to 80/40 mmHg and the etCO2 increased to 90 mmHg despite hyperventilation at the mentioned rate. A series of arterial blood gas (ABG) samples were sent to the lab; the results are shown in table 6-3, which showed mixed acidosis, despite hyperventilation; therefore, correction of metabolic acidosis using sodium bicarbonate was started. The anaesthesia consultant on-call, off-site, was called and was informed about the updates and discussed with the middle-grade doctor about different possible diagnoses with MH as the most likely diagnosis. The inhalation anaesthesia was switched off and hyperventilation, and symptomatic treatment were continued in the operating room.

Time point	60 min.	90 min.	125 min.	170 min.	235 min.
pH	7.16	6.99	7.17	7.22	7.29
PaO_2	372 mmHg	82 mmHg	338 mmHg	140 mmHg	75 mmHg
$PaCO_2$	62 mmHg	71 mmHg	67 mmHg	40 mmHg	35 mmHg
HCO_3^-	22.1 mmol/L	17.1 mmol/L	24.4 mmol/L	18.8 mmol/L	16.8 mmol/L
BEecf	-6.6 mmol/L	-14.3 mmol/L	-4.1 mmol/L	-8.9 mmol/L	-9.8 mmol/L
SO_2	100%	88%	100%	99%	93%

Table 6-3: Results of the arterial blood gases of the reported case at five time points. *PaO_2= arterial oxygen tension, $PaCO_2$=arterial carbon dioxide tension, HCO3=bicarbonate level in the blood, BEecf= base excess in the extracellular fluid, and SO_2=oxygen saturation. The fraction of in inspired oxygen was not recorded on any of the reported samples.*

Management measures at this stage, after 150 minutes, included continued hyperventilation, cold fomentation and cold IV fluids to control body temperature, and 1 gram of magnesium sulphate was given slowly IV. After 180 minutes, blood pressure stabilised at 90/60 mmHg with heart rate 150 beats/minute and etCO2 55 mmHg and the respiratory rate on the ventilator reduced to 30 cycle/minute. Anaesthesia was believed at this stage to be worn off, and the patient was allowed to emerge from anaesthesia and breath spontaneously, but respiration at this stage was shallow with a RR of 50 cycle/minute, therefore, a decision to transfer the patient to the intensive care to maintain mechanical ventilation. At the ICU, despite discontinuation of any sedation, the patient's GCS remained ±3. ICU reports could not be obtained; however, based on personal communication, we were informed that the patient had developed disseminated intravascular coagulopathy and required cardiovascular support. On the next day, the patient experienced cardiac arrest and attempts to restore circulation failed, and the patient was declared dead.

6.3.3 Responses to the nationally distributed questionnaire

As stated early, after failed attempts to publish this questionnaire through the official channels of the Egyptian Fellowship of Anaesthesia and Intensive Care, the response

to the questionnaire published on the non-official Facebook page of the informal group "the Association of Egyptian Anaesthetists" was feeble. We received only 30 responses despite repeated reminders to the group members to encourage participation. Of the 30 participants who filled in the questionnaire, four were trainees, 12 middle grade doctors and 14 consultant anaesthetists. The main place of work for the majority of them was tertiary hospitals (including university hospitals), 24 out of 30, and eight participants had more than one place of work. Middle-grade doctors had the highest workload with an average of 30 cases/week and consultants came second with 24, cases/week and the four trainees reported an average workload of 18 cases/week. All participating consultants had a minimum of ten years of anaesthetic experience, while middle-grade doctors had at least five years of experience with four of them had more than ten years. Ten consultants, four middle grade doctors and two trainees reported 18 cases, as two anaesthetists reported two cases each. All the cases were reported at a tertiary hospital apart from one case reported at a secondary hospital and another case at a private practice.

Of the 18 cases reported, three patients had either a previous or a family history of anaesthesia related complications suggestive of MH. According to the judgment of the reporting anaesthetists, of the remaining 15 patients who had an anaesthetic reaction, 12 patients developed clinical features suggestive of MH under anaesthesia while another three patients presented with an unexplained death or cardiac arrest under general anaesthesia. The age and sex characteristics and clinical manifestations of the reaction of the 15 patients who experienced a suspected reaction in their most recent anaesthetic are presented in table 6-4. Laboratory investigation results were available for only six patients, and all of them reported acidosis, while hyperkalaemia was reported in three patients and elevated serum CK level in two patients. As shown

in table 6-4, 60% of those patients were males and all of them were either children or young adults below the age of 40 years old. Muscular manifestations in the form of masseter muscle spasm or generalised muscular rigidity were reported in eleven patients. However, one of the shortcomings of this questionnaire is not including questions about the anaesthetic drugs used. Regarding metabolic features of the reported reactions, fever was reported in all, but one patient, that could indicate a late recognition of the reaction while hypercapnia was reported in only six patients.

Management measures were limited to supportive and symptomatic treatment in the form of active cooling such as surface cooling or intravesical or intragastric irrigation with iced saline, the use of bicarbonate to treat acidosis, and the use of diuretics. Seven anaesthetists reported the change of anaesthetic machine to a gas-free one. Two consultants reported the availability of dantrolene at the tertiary hospital they worked for; however, it was not used in any of these two cases. In the remaining 14 patients with suspected anaesthetic reactions, dantrolene was not available, and consequently, was not used. Of the 15 patients who had a reaction in their current anaesthetics, 11 patients died after the reaction either in the operating room or after admission to the intensive care. Of the four cases who survived the reported reaction, three patients were admitted to the intensive care and discharged later on after improvement. The fourth patient developed clinical manifestation in the form of unexplained hyperthermia and arrhythmia with no muscular features or reports about any laboratory derangement. This patient was discharged without a need for ICU admission, and the reporting consultant anaesthetist did not use dantrolene despite availability, as stated earlier.

ID#	Reporter	Age	Sex	Muscular signs	Metabolic signs	Lab results	ICU	Outcome
1	Trainee	8-20Yr	Male	MMS, Rigidity	Fever, Cardiac arrest	Acidosis	Yes	Died
2	M-G	3-8Yr	Male	Rigidity	↑ ↑ CO_2	Acidosis, K+, CK	Yes	Recovered
3	Cons.	21-40Yr	Male	-	Fever, Arrhythmia	N/A	No	Recovered
4	M-G	3-8Yr	Female	Rigidity	Fever, ↑ ↑ CO_2, Cardiac arrest	Acidosis	Yes	Died
5	Cons.	<3Yr	Female	Rigidity	Fever, ↑ ↑ CO_2	N/A	Yes	Recovered
6	Cons.	9-20Yr	Male	Rigidity	Fever	N/A	Yes	Died
7	Cons.	9-20Yr	Male	Rigidity	Fever	K+	Yes	Died
8	Cons.	9-20Yr	Female	MMS, Rigidity	Fever, Arrhythmia	N/A	No	Died
9	Cons.	21-40Yr	Female	-	Fever, Cardiac arrest	N/A	No	Died
10	Cons.	9-20Yr	Male	Rigidity	Fever, ↑ ↑ CO_2, Arrhythmia	Acidosis, K+	No	Died
11	M-G	3-8Yr	Male	-	Fever, Cardiac arrest, Myoglobinuria	N/A	Yes	Died
12	Cons.	3-8Yr	Female	Rigidity	Fever, ↑ ↑ CO_2, Arrhythmia, Cardiac arrest	Acidosis, K+	Yes	Died
13	Trainee	3-8	Female	Rigidity	Fever, ↑ ↑ CO_2, Arrhythmia	Acidosis, CK	Yes	Recovered
14	M-G	<3Yr	Male	-	Fever, Cardiac arrest	N/A	No	Died
15	Cons.	9-20	Male	Rigidity	Fever, Cardiac arrest	N/A	No	Died

Table 6-4: The suspected cases reported in the questionnaire published nationally. *The level of qualification of the reporting anaesthetists, age group and sex of the reported case are shown. Clinical features of the suspected reaction, any available lab results, and the outcome of the reaction are illustrated. N/A=not available, ↑ ↑ CO_2= hypercapnia detected clinically using the capnograph.*

The remaining three cases reported a history of anaesthesia related complications suggestive of MH. One patient reported the death of ten relatives under general anaesthesia, including the patient's father. The other two patients reported a history of anaesthetic complications, although the exact details of the previous reactions could not be retrieved. During the current anaesthetics, trigger-free anaesthesia was used in all of the three patients, and no complications were reported. All the participants of the questionnaire declined to offer any contact details for further discussion about the reported cases.

6.4 Discussion and limitations

MH is a well-known complication of anaesthesia to all Egyptian anaesthetists I have worked with in different health systems in Egypt. Most of the Egyptian anaesthetists believe that MH still exists in the modern anaesthetic practice, although the majority of them have not experienced a suspected case of MH during their career. This is on the contrary to the worrying developing concept among some of the nowadays anaesthetists that "malignant hyperthermia is a disease of halothane anaesthesia" (Sneyd, 2017). This can be explained, partially, because of halothane being still in-use in some hospitals in Egypt in different health systems, more in private poorly monitored hospitals due to financial considerations. However, due to administrative, organisational, and financial issues affecting the whole health system that serves an approximate of a hundred million people, measures to tackle this rare and fatal condition are still poorly implemented.

Our long-term target of this study, is to have a more organised and systematic knowledge about the prevalence of MH in Egypt, the mortality rate of suspected and/or confirmed cases of MH, the in-place protocols for management and diagnosis of this

condition in different health systems' hospitals, and to ensure this information will be available for the health administration officials to establish a national policy to tackle this condition. As our initial search confirmed the scarcity of available reports about MH and other rare-occurring similar genetic conditions in Egypt, we designed this study to collect preliminary data that would be of great value when conducting future research to serve our long-term plan. We tried to target practicing anaesthetists with our questionnaires to obtain as much as available information they might have through their practice in different health systems.

It is not logical to make assumptions on the dynamics of anaesthetic practice in Egypt based on the results of our published questionnaires, due to the minimal number of participants. Due to the limitations we discussed earlier, we could not have organised systematic access to a bigger slice of practicing anaesthetists in Egypt. Additionally, it is highly likely that despite publishing the questionnaire through a Facebook page directed mainly for anaesthetists working in the country, that the questionnaire, itself, did not attract as many anaesthetists except those who have experienced a suspected case themselves, or at least, heard of a possible case in their place of work. This might explain the high number of cases reported compared to the small number of participants, 12 cases reported by 36 participants from the questionnaire published at Assiut University Hospitals and 18 cases by 30 participants from the nationally published questionnaire. We made it clear when we launched the questionnaire, that all the data collected are only for the purpose of this research and data will be dealt with anonymously and confidentially. However, some anaesthetists may have declined to report their experience with MH cases, fearing this information may be used against them if any investigation was launched. Another explanation for this poor participation is the probability that some anaesthetists in Egypt endorse the concept

of MH being obsolete now with the modern anaesthetic agents. A lack of motive to participate and a feeling of the worthlessness of their responses in tackling this problem could be other possible causes.

The main place of work for most participants, and subsequently, most cases reported, was a tertiary centre including university hospitals. These centres are usually better equipped with monitoring facilities with a more organised training and supervision system for junior anaesthetic staff. However, anaesthetists working in these centres were not aware of any in-place management protocol for MH, and the majority of them reported the lack of dantrolene stock in these centres. This reflects the importance of implementing management protocols and measures at more national and by higher administrative levels.

On the other hand, this study is considered as the first attempt to gather evidence to confirm the occurrence of the classical picture of hypermetabolic MH reactions among the Egyptian population. Previous case reports of suspected MH reactions were published from other countries in the region, such as Bahrain and Saudi Arabia (Centre for Arab Genomic Studies, 2016). We are also aware of some patients referred from Iraq, Saudi Arabia and Bahrain who have been tested MH susceptible at the Leeds MH Investigation Unit. These countries share similarities in the population characteristics with the Egyptian population. However, the ethnicity of the Egyptian population is more mixed and diverse according to some recent reports that compared DNA results of different ethnic populations (https://genographic.nationalgeographic.com). Therefore, it was crucial to perform a study based merely on the Egyptian population to evaluate the extent of the condition in the country.

My status as an insider researcher with regard to the questionnaire published among anaesthetists at Assiut University Hospitals, allowed a relatively better participation from the staff working at the department of anaesthesia at the hospital (36 participants of ~100 anaesthetists), in comparison to the questionnaire aimed to target ~800 anaesthetists nation-wide that only triggered 30 participants. In addition, it enabled us to obtain more detailed information about the recent case of suspected MH. Moreover, it was easier for us to validate the responses to the local questionnaire regarding the reported cases by further contacting participants and to ensure avoiding double reporting. We also can confirm the reported dynamics of the anaesthetic practice at the hospital and the reported lack of management protocols and dantrolene stock. This status could be of a beneficial value in future studies about MH by maintaining a contact point between the department of anaesthesia and ICU at Assiut University Hospitals and the Leeds MH Investigation Unit. We also aim to publish the results of the current study in the department annual conference in order to highlight our findings and to ensure a proper management process being initiated.

It is evident that there are many missing details about the reported cases, as apart from the recent case at Assiut University Hospitals, all details were solely dependent on the anaesthetists' recall of the events. We used the categories of clinical presentation of MH published by (Ellis et al., 1990) to evaluate the reported cases and the possibility of MH diagnosis in each case. With regard to the cases reported at Assiut University Hospitals, three patients can be included within category "a" or fulminant reaction, and the probability of MH diagnosis is 0.96 in this category. Another three patients had moderate signs of MH or category "b" with an MH diagnosis probability of 0.88 while another three patients had masseter muscle spasm with signs of metabolic derangement, category "e", and the probability of MH is 0.56. The last

patient who had mild metabolic features and the reaction did not appear to be life-threatening is considered to be in category “c” with 0.14 probability for patients in this category to be diagnosed MHS.

Cases that have been reported through the national questionnaire was categorised using the same method. Ten patients had the fulminant or classic clinical picture of MH according to the reported manifestation. They are considered to be category “a”, and the probability of MH diagnosis is 0.96. Two patients had a moderate clinical picture, and they recovered after the anaesthetic without the use of dantrolene, and they are considered category “b” with a probability of 0.88. Two other patients had an unexplained cardiac arrest under anaesthesia, and according to the reporting anaesthetists, there was no other apparent cause. This category “g” has a probability of 0.66 of MH diagnosis. The clinical picture of category “c” includes mild signs of MH and has a probability of MH diagnosis of 0.14. Only one patient in this cohort is considered to be in this category.

This categorisation of reported cases depends on the recall of intraoperative events of suspected reactions. No written notes, lab results, nor morbidity and mortality committee reports could be obtained for any of the reported cases, apart from the recent case at Assiut University hospitals. Additionally, no time scale was given for the occurrence of the reported events, and for when a suspected MH crisis, if at all, has been recognised. In addition, from the available information, we are not able to assess the efficiency of management measures, which lacks the use of dantrolene due to its unavailability. A solid conclusion on the incidence of MH among the Egyptian population or the extent of this problem in Egyptian hospitals cannot be made, by any means, based on the available information, though, this preliminary study served the

purpose of confirming that MH does exist in the Egyptian population and when it is reported, it carries a very high mortality rate.

The larger proportion of patients in each cohort were classified clinically in categories “a” and “b”, six out of ten and 12 out of 15 patients, locally at Assiut University Hospitals and nationally, respectively. This support our assumption about a positive response bias, we stated earlier, explaining poor participation. This observation can also be explained by insufficient monitoring equipment available in many operating theatres leading to late recognition of an MH crisis, which leads, subsequently, to the poor outcome. Based on this observation, it is very likely that there is a large number of mild clinical presentations of the reaction and/or early aborted cases, are passed unnoticed. Even for the severe forms of the reaction that may end into the death of the suspected case, one of the issues limiting our ability to obtain accurate clinical details about the case, is the family refusal, in many cases, for a post-mortem examination due to a social stigma associated with it. The coroners’ report in many of these cases would lack crucial details to ascertain a definitive diagnosis and hence, uninformative.

The recently reported case of the suspected anaesthetic reaction offers many insights about the anaesthetic practice in the country and management of emergency situations such as an MH crisis. This deceased case did not have any family history of anaesthesia related complications and was apparently a healthy fit child before she sustained the fracture for which she had the operation. Monitoring during anaesthesia was less than standard as the temperature was not recorded at any time during the course of the reaction. In addition, the patient was kept under the care of a junior anaesthesia resident, and we are not sure about the time elapsed between the induction of anaesthesia and the recognition of reaction. The middle-grade

anaesthesia doctor on-site was available immediately, though, no senior anaesthesia consultant was available at any time point to assess the case directly. Another flaw that reflects the lack of an organised management plan is that the volatile anaesthetic was not switched off until ~60 minutes after the recognition of the reaction. Last but not least, the anaesthetic machine has not been changed, no activated charcoal filters were available and, more crucial, no dantrolene was available for use.

A confirmation of MH diagnosis is hard to ascertain, as the patient did not survive the reaction and our attempts to make contact with her parents to, at least, obtain a blood sample for genetic screening proved unsuccessful. However, from the available clinical details of the reaction, we used the clinical grading scale (Larach et al., 1994) to calculate a raw score of 56 which reflects an almost certain likelihood of this anaesthetic reaction to be a true MH reaction. In addition, some of the other differential diagnoses could be, cautiously, excluded from the available clinical information such as pheochromocytoma, thyroid crisis and sepsis as the patient did not report any medical history of importance prior to the operation. Additionally, the patient did not report any regular medications intake that excludes the possibility of drug-related causes such as neuroleptic malignant syndrome. We also can assume that anaesthetic equipment malfunction is not an accepted cause as no similar reactions reported with other patients in the same operating room on that day, based on personal communication. Other causes, related to the inadequate depth of anaesthesia or pain control, are not readily excluded, though, the climbing $etCO_2$ and a GCS of ±3 after the anaesthesia had worn off, reduce the plausibility of these causes.

Apparently, there are many measures to be taken to improve the management of such suspected cases in Egyptian hospitals in the future. First of all, and the most important is to generalise a management protocol for MH crisis in all Egyptian hospitals across different health systems and to ensure the medical staff working in operative theatres aware of this protocol and how to implement it in case of a suspected reaction. The published protocol for the management of MH crisis (Glahn et al., 2010) is of great value and lists step-by-step management of such an emergency situation. Although, many anaesthetists working in Egypt could be aware of this protocol as the condition itself and its management is part of the anaesthetic curriculum taught to all anaesthetists during their postgraduate training, a printed version should be made available in all theatres. Also, other medical staff such as surgeons, theatre nurses and anaesthesia technicians have to be trained on how to apply this protocol. Secondly, dantrolene should be stocked in all hospitals with theatre complexes where volatile anaesthetic being used as it has a dramatic rule in reducing the mortality rate of MH reactions (Strazis and Fox, 1993). We have not studied thoroughly the possible logistic issues preventing dantrolene from being available in case of a MH crisis; however, we hope this study will bring the importance of this issue to the Egyptian health administrative officials' attention. Another essential management line is the availability of activated charcoal filters that have many advantages to replacing the anaesthetic machine with a gas-free one (Bilmen and Hopkins, 2018). This could be another logistic issue that needs to be discussed, but in the meanwhile, we recommend a gas-free machine to be available in each operative theatre complex.

With a population approaching 100 million and with the availability of many university hospitals and research centres, Egypt can serve as a major diagnosis centre and a research hub for the condition in the whole region, however, several steps need to be

taken. On the local level of operative theatres, in addition to generalising a printed version of the management protocol, we recommend designing a form for recording any adverse anaesthetic reactions describing the full clinical details of the reaction with any available lab results, or alternatively, using the EMHG anonymous registration form of suspected MH reactions available online at (https://mh-event.emhg.org). These forms, either paper or online, should be filled in by the anaesthetist attending the reaction, and a photocopy of this form to be kept in patient's notes and another copy to be saved in the hospital archive. All practising anaesthetists, either qualified or still in training, should be made aware of this form and encouraged to fill it in. This form will be of a great benefit for future studies about MH and will facilitate more accurate estimation of the incidence and prevalence of MH in Egypt.

7 General discussion

This project has utilised the most extensive resource for clinical, genetic and IVCT data of MH in the world. We have explored the male: female ratio in different cohorts of patients; confirmed MHS probands, patients who developed an anaesthetic reaction and tested MHN, and first-degree relatives of MHS probands and compared our results to the previously published data. We have conducted the first correlation analysis in confirmed MHS probands between the common UK *RYR1* genotypes and the clinical features of the suspected anaesthetic reactions in these patients. Additionally, we added to the ongoing work to explore the association between MH and the broad spectrum of *RYR1*-related congenital myopathies. Last but not least, this is the first attempt to explore the epidemiology of MH among the Egyptian population. We have identified the challenges and limitations towards the recognition, management and diagnosis of MH and similar rare-occurring conditions.

7.1 The role of succinylcholine in MH

In this study, we still could not totally confirm, nor confidently exclude the role of succinylcholine in triggering a hypermetabolic MH reaction solely, without the use of volatile anaesthetics. However, a few observations that we have reported in previous chapters need to be interpreted together in this context. Firstly, muscular-only anaesthetic reactions only occurred when the triggering anaesthetic involved the use of succinylcholine. Secondly, patients who developed these muscular-only anaesthetic reactions had a statistically weaker IVCT response compared to patients who developed hypermetabolic reactions to volatile anaesthetics. Thirdly, succinylcholine had no statistically significant effect on the maximum derangement values of the metabolic features of MH, while its use was associated with a statistically

higher postoperative serum CK level, a muscle-related feature. Lastly, the reports of the hypermetabolic reactions, when volatile anaesthetics were not reported, lack essential details to ascertain that succinylcholine was the only trigger.

However, despite the lack of convincing evidence of the role of succinylcholine in triggering hypermetabolic MH reactions, it must be avoided when a possibility of MH is suspected for several reasons. First of all, the potentially fatal outcome of an MH reaction mandates prioritising patients' safety over the scientific evidence, or the lack of it in this case. Moreover, there is a possibility that MHS patients have an increased sensitivity to succinylcholine that is presented in the form of exaggerated muscular response and rhabdomyolysis, which may lead to comorbidities such as acute kidney injury and acute renal failure. Furthermore, it is evident that the use of succinylcholine is associated with an increased incidence of postoperative complications, though the majority of these complications were muscle-related.

7.2 Male predominance and MH

It has been long claimed that MH has a preferential predominance in males. This was based on several statistical studies of the epidemiology of MH. Confirming this observation should consider the difference between MH susceptibility diagnosis and the clinical diagnosis of an MH anaesthetic reaction. The earlier is confirmed by an invasive biopsy test; IVCT in Europe, CHCT in North America, and skinned fibre test in Japan; or by genetic screening for diagnostic mutations, which is complicated further by the discordance reported with many of these mutations. The latter diagnosis is more difficult to ascertain because of the nonspecific characteristic of the clinical features of MH reactions and the overlap between MH and other differential diagnoses in these features. The data presented in this thesis imply that male predominance is noticed in

patients who presented with the nonspecific features of MH regardless of their susceptibility status, which was confirmed with IVCT. Our preliminary analysis of the IVCT results of first-degree relatives of our MHS probands offered a glimpse of a slight predominance of MH susceptibility in male first-degree relatives of MHS probands compared to their female counterparts. However, a future study properly analysing these results will be of greater value, especially if all of these probands and their first-degree relatives have been genetically screened using NGS.

7.3 Incidence of MH in recent years

According to a recently published correspondence (Kaura et al., 2018), an observation of the rate of referral to the Leeds MH Investigation Unit has not remarkably changed over the last 30 years. In our study, though, we noticed a steady decrease in the number of MH reactions according to the year at which this reaction has occurred. This discrepancy could be explained by the delay of referral of some cases and the difference between the date of referral and the actual date of the anaesthetic reaction in our records. The possible reasons for this decline in the number of MH episodes over the last thirty years, could be related to the apparent decrease in the rate of use of succinylcholine and the improvement in the local and regional anaesthetic techniques, together with an increased popularity of TIVA, which might have decreased the number of patients exposed to the triggering general anaesthetics. Our results should not send a negative message that the importance of MH in the clinical practice or in the research field is diminishing, as this may jeopardise the patients' safety. We repeatedly confirm that all volatile anaesthetics still in use in the modern anaesthetic practice can trigger an MH reaction in susceptible patients.

7.4 RYR1 genotypes influence the clinical phenotype

When we compared between the clinical details of anaesthetic reactions reported in patients found to have one of the common genotype groups, one genotype group c.1021G>A, the second most common in the UK MH population, was found to have a consistently more severe form of the metabolic features of the reaction, compared to the most common genotype group among the UK MH population; the c.7300G>A. Nevertheless, this observation should be treated with caution, as the sample size was relatively small, the level of significance was low, and we have not corrected for multiple comparisons. One the other hand, more patients in the former group developed a reaction triggered by volatile anaesthetics only without the use of succinylcholine, compared to a majority of patients in the c.7300G>A group who developed a reaction triggered by anaesthetics involved the use of succinylcholine.

To our knowledge, this the first attempt to correlate the RYR1 genotypes to the severity of the clinical features of the MH reaction. The genotype group c.1021G>A was reported to be common in France and Belgium (Robinson et al., 2003b) and further studies including more patients with these variants from different centres would give these studies a better statistical power. However, the differences in ethnicity of the included patients and the different anaesthetic practice may affect the results of these multicentre studies. As we have recommended earlier, studies focusing on the biochemical changes in muscle cells and myotubes from patients found with different genotype groups, need to include the different clinical phenotypes as one of their variables.

7.5 Consistency of IVCT measurements

The IVCT is still considered as the gold standard test for diagnosis of MH susceptibility despite the recent advances in genetic diagnosis. This is due to the inability to confidently exclude MH susceptibility based solely on the absence of a genetic variant, and due to the discordance reported between genotypes and phenotypes. However, the consistency of IVCT results over the years has been dependent on several factors: the standardisation of the test protocols, the muscle specimens' viability and the interpretation of the test traces. In this study, we have been able to control the latter factor by reassessing the included traces by a single interpreter. The other two factors need further investigation to insure the consistency of the IVCT results. To our knowledge, parameters such as the muscle specimens' weight and length are recorded for each specimen used in the test. Another parameter that could also affect the muscle viability and the results of the test, is the time between the muscle specimen being biopsied to the time of the test.

A further study to explore factors related to standardisation of test protocols and muscle viability will enable us to ensure the consistency of IVCT measurements. Comparing different time periods such as before and after the establishment of the European protocol for IVCT or before and after the introduction of computerised test procedure, should enable us to reassess the sensitivity and specificity of the test. Additionally, a multi-centre study comparing the test results to the previously mentioned parameters from different MH labs that follow the European protocol for IVCT could also add to the value of IVCT as the standard diagnostic test for MH.

7.6 MH and the broader spectrum of CM

With the approved overlap of the histopathological findings and clinical presentations between different entities of congenital myopathies and the complexity of the association between this group of diseases and the MH phenotype, it is highly recommended to manage patients with CM who found to have an *RYR1* variant with extra caution, with regard to MH triggering anaesthetics. The ongoing work to explore the association between both conditions should continue; considering the broader spectrum of CM, taking the discordance between *RYR1* genotype and IVCT phenotype into account, and including more family members of index cases diagnosed with these conditions. As has been suggested in the transfer viva for this project, a follow-up study to assess any recently-developed clinical muscle weakness in patients who developed an anaesthetic reaction and found to have histopathological features of *RYR1*-myopathy, will help us to examine the effect of age and or anaesthetic reactions on the development of muscle weakness in these patients.

7.7 Myopathy families and MH susceptibility

We have attempted to perform a segregation study between the IVCT results, genetic testing results, histopathological findings and the clinical myopathy status of CM families who were tested at the Leeds MH Investigation Unit to explore the association between MH and CM within these families. Significant segregation was found in two large families tested at the unit, which suggests that the two RYR1 variants found in these families have a significant functional effect on both MH and CM phenotypes. On the other hand, no such positive segregation was found in other families included in this segregation study. This study was limited in these families by the small number of other members tested at the unit and the few information available about these other

members' clinical and genetic status. Additionally, it would be of greater value if the members of these families are from three different generations, which would enable estimating the LOD score (log of the odds) to statistically estimate the link segregation between the detected *RYR1* variants and the MH and/or CM phenotypes.

7.8 MH in Egypt still exists in the pre-dantrolene era

Unfortunately, due to the many limitations that we have discussed earlier, we were not able to gather much information about the situation of MH among the Egyptian population. Furthermore, our data revealed that Egypt is in a situation where halothane is still being used in several hospitals across the different health systems, monitoring in the operating theatres is below the standard level, management protocols are not standardised, and dantrolene stock is not readily available in many hospitals. This study should ring the bell for the health officials in the country about the mandatory measures to be taken to tackle this problem. Additionally, anaesthetists working in the different health systems should be extra vigilant towards the clinical features of MH, including family and previous anaesthetic history, to recognise any suspected reaction as early as possible, switch off all triggering agents, and commence supportive and symptomatic treatment for better survival rates, and definitely, administer dantrolene if it is available.

7.9 Limitations

Specific limitations to each particular study in this project have been partially discussed in different chapters. The two main limitations that face any study about MH are; the small sample size, which limits the statistical power of these studies and hinders obtaining conclusive statistical findings, and the inconsistency in the recorded clinical details of the suspected anaesthetic reactions. Both limitations were obvious in our

study; the effect of the small sample size was even exaggerated when conducting statistical analysis on subgroups such as different *RYR1* genotype and clinical phenotype groups. That was the main reason for not correcting the ***p*** values for multiple comparisons, which could have vanished any statistical significance found. In addition, the limitation related to inconsistency of the recorded clinical details prevented us from drawing solid conclusions, about the role of succinylcholine in triggering a hypermetabolic MH reaction, for instance.

Selection bias is also limiting segregation studies of CM and MH. This is because family members of index cases who test MHN with IVCT are not referred for further testing. As the IVCT is an invasive procedure that carries its own risks such as infection and bleeding, it is unethical and impractical to refer patients with a low chance to benefit from the test to such an invasive procedure. On the other hand, the assumption that other members of the family are not susceptible to MH based on the test results of the index case is limiting the validity of these segregation studies. Additionally, no data about the histopathological findings in other members will be available, limiting the correlation analysis between the clinical phenotype and histological phenotype. Furthermore, patients who test MHN with IVCT are usually not referred for genetic testing, although discordance has been reported.

The study about MH in Egypt was loaded with its own limitations that have been discussed thoroughly in the sixth chapter. However, it is important to restate the main points about this study to enable future studies about MH, or other rare-occurring conditions, to address these limitations at the early stage of planning. Firstly, communication is challenging either with officials or with candidates, due to the lack of a central communication system. Therefore, a plan should be in place to facilitate

contact either through personal or phone interviews or appointing a contact person at each site for the study. Secondly, the inefficiency of medical recording limits the capabilities for retrospective studies. Therefore, collection of clinical details at the time of the event through paper forms or an online referral system, as we have suggested, should improve the validity of the collected information. However, in rare-occurring conditions, such as MH, it may take a long time to obtain data that is sufficient to perform statistical analysis. Thirdly, cultural and religious considerations may affect the ability to perform certain procedures; post-mortem examination, for example.

7.10 Future directions

A future study of the MH susceptibility in family members of index cases would offer a better understanding of the genetic heterogeneity of the disorder within families. This study will be of higher value if the clinical details of any previous anaesthetic exposure are retrieved and reviewed. Comparing the genetic and IVCT results within families and between probands and their relatives should give the genotype-phenotype correlation studies broader spectrum and more depth into the genetic mechanisms underlying the different IVCT responses. In particular, with the transition to a whole-exome sequencing approach at the Leeds MH Investigation Unit, this broader strategy would increase the scope of variant detection in these patients. With the ongoing research projects at the unit that are adopting different approaches to functionally characterise *RYR1* variants. These projects use immortalised human muscle cells harbouring the specific *RYR1* variants to compare Ca^{+2} release to wild-type cells. Another method is CRISPR-Cas9 that is used to introduce variants to cell lines and measure relative Ca^{+2} release to wild-type RyR1 receptors. In addition, these studies could offer potential explanations for the possible genetic background behind different clinical presentations.

We have attempted to explore and identify the obstacles facing future studies exploring the epidemiology of MH in Egypt and other countries in the Middle East region. With better co-operation from health administration officials to conduct similar future epidemiological studies, many of the limitations that we have discussed in this thesis could be resolved, offering more reliable results and a better understanding of the extent of the condition among the country population. Directing anaesthetists working in the country to fill in the online reporting form of any suspected anaesthetic reaction will help to build up a database for future studies. Additionally, educating patients about the possible serious outcomes of MH in other family members should encourage them to seek further help and engage more in future screening programmes. Indeed, the outcomes of any future studies will be dependent on the will of the government to invest in funding a research and investigation facility for MH in the country.

Overall, this thesis added to the ongoing effort to investigate the association between MH and CM. It also highlighted the effects of recent changes in the anaesthetic practice on the clinical epidemiology of MH. We also proposed possible ideas about the genetic backgrounds of different clinical presentations that need a further investigation. Last but not least, we have established a cornerstone for future work about MH in Egypt.

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9 Appendix

9.1 Appendix A

Serial	Codon change	Amino acid change	Detection rate	ExAC MAF
1	c.38T>G	p.13Leu>Arg	1	
2	c.131G>A	p.44Arg>His	1	
3	c.178G>A	p.60Asp>Asn	1	
4	c.326G>A	p.109Arg>Gln	1	
5	c.479A>G	p.160Glu>Gly	1	
6	c.487C>T	p.163Arg>Cys	15	Not found
7	c.488G>T	p.163Arg>Leu	1	
8	c.526G>A	p.176Glu>Lys	1	
9	c.529C>T	p.177Arg>Cys	9	0.000008652
10	c.533A>C	p.178Tyr>Ser	1	
11	c.641C>T	p.214Thr>Met	1	
12	c.652G>A	p.218Val>Ile	1	
13	c.742G>A	p.248Gly>Arg	4	Not found
14	c.742G>C	p.248Gly>Arg	2	0.00002479
15	c.1021G>A	p.341Gly>Arg	18	Not found
16	c.1201C>G	p.401Arg>Cys	1	
17	c.1201C>T	p.401Arg>Cys	1	
18	c.1202G>A	p.401Arg>His	1	
19	c.1459C>G	p.487Leu>Val	1	
20	c.1475G>A	p.492Arg>His	1	
21	c.1565A>G	p.522Tyr>Cys	1	
22	c.1598G>A	p.533Arg>His	1	

Serial	Codon change	Amino acid change	Detection rate	ExAC MAF
23	c.1609G>A	p.537Ala>Thr	1	
24	c.1615T>C	p.539Phe>Leu	1	
25	c.1654C>T	p.552Arg>Trp	4	Not found
26	c.1840C>T	p.614Arg>Cys	9	0.00008236
27	c.1841G>A_hom	p.614Arg>His	1	0.00002471
28	c.2050G>C	p.684Gly>Arg	1	
29	c.2320G>A	p.774Gly>Arg	1	
30	c.2645C>T	p.882Ala>Val	1	
31	c.3095G>A	p.1032Arg>His	1	
32	c.3166G>C	p.1056Asp>His	1	
33	c.3172G>A	p.1058Glu>Lys	1	
34	c.3224G>A	p.1075Arg>Gln	1	
35	c.3418C>T	p.1140Arg>Cys	1	
36	c.3527C>T	p.1176Thr>Ile	1	
37	c.4024A>G	p.1342Ser>Gly	1	
38	c.4088C>T	p.1363Ala>Val	1	
39	c.4178A>G	p.1393LYS>ARG	3	0.005072
40	c.4405C>T	p.1469Arg>Trp	1	
41	c.4711A>G	p.1571Ile>Val	1	
42	c.4763C>T	p.1588Pro>Leu	1	
43	c.5024T>C	p.1675Leu>Pro	2	Not found
44	c.5033A>G	p.1678Asn>Ser	1	
45	c.5183C>T	p.1728Ser>Phe	5	Not found
46	c.5186T>G	p.1729Met>Arg	1	
47	c.5360C>T	p.1787Pro>Leu	2	0.01957

Serial	Codon change	Amino acid change	Detection rate	ExAC MAF
48	c.5440A>G	p.1814Met>Val	1	
49	c.5441T>A	p.1814Met>Lys	1	
50	c.6178G>T	p.2060Gly>Cys	5	0.06937
51	c.6488G>A	p.2163Arg>His	5	Not found
52	c.6502G>A	p.2168Val>Met	4	Not found
53	c.6599C>T	p.2200Ala>Val	1	
54	c.6612C>G	p.2204His>Gln	2	Not found
55	c.6617C>G	p.2206Thr>Arg	1	
56	c.6617C>T	p.2206Thr>Met	16	0.00003305
57	c.6742C>T	p.2248Arg>Cys	1	
58	c.6961A>G	p.2321Ile>Val	1	
59	c.7007G>A	p.2336Arg>His	5	Not found
60	c.7025A>G	p.2342Asn>Ser	3	0.001073
61	c.7043A>G	p.2348Glu>Gly	1	
62	c.7048G>A	p.2350Ala>Thr	7	Not found
63	c.7089C>G	p.2364Cys>Trp	1	
64	c.7090T>G	p.2364Phe>Val	1	
65	c.7123G>A	p.2375Gly>Arg	1	
66	C.7291G>A	p.2431Asp>Asn	1	
67	c.7291G>T	p.2431Asp>Tyr	2	Not found
68	c.7300G>A	p.2434Gly>Arg	84	0.00002479
69	c.7304G>A	p.2435Arg>His	6	Not found
70	c.7304G>T	p.2435Arg>Leu	1	
71	c.7307G>A	p.2436Cys>HIS	1	
72	c.7354C>T	p.2452Arg>Trp	1	

Serial	Codon change	Amino acid change	Detection rate	ExAC MAF
73	c.7361G>A	p.2454Arg>His	9	0.000008298
74	c.7373G>A	p.2458Arg>His	10	0.000008287
75	c.7373G>T	p.2458Arg>Leu	1	
76	c.7522C>T	p.2508Arg>Cys	1	
77	c.7523G>A	p.2508Arg>His	4	Not found
78	c.7528T>C	p.2510Tyr>His	1	
79	c.7816T>A	p.2606Cys>Ser	1	
80	c.7879G>A	p.2627Val>Met	1	
81	c.7879G>C	p.2627Val>Leu	1	
82	c.8026C>T	p.2676Arg>Trp	2	0.000008253
83	c.8198G>T	p.2733Gly>Asp	1	
84	c.8729C>T	p.2910Thr>Met	1	
85	c.9152G>A	p.3051Arg>His	1	
86	c.9268G>A	p.3090Ala>Thr	1	
87	c.9310G>A	p.3104Glu>Lys	2	0.00001649
88	c.9353C>T	p.3188Ala>Val	1	
89	c.9652G>A	p.3218Val>Met	1	
90	c.9676G>C	p.3226Glu>Gln	1	
91	c.9797T>C	p.3266Met>Thr	1	
92	c.10042C>T	p.3348Arg>Cys	1	
93	c.10097G>A	p.3366Arg>His	3	0.0009272
94	c.10252A>G	p.3418Asn>Asp	2	Not found
95	c.10616G>A	p.3539Arg>His	4	0.001792
96	c.10732G>C	p.3578Glu>Gln	1	
97	c.10747G>C	p.3583Glu>Gln	2	0.01494

Serial	Codon change	Amino acid change	Detection rate	ExAC MAF
98	c.10870C>T	p.3624Arg>Trp	1	
99	c.11132C>T	p.3711Thr>Met	1	
100	c.11251C>G	p.3751Gln>Glu	1	
101	c.11266C>G	p.3756Gln>Glu	3	0.03374
102	c.11315G>A	p.3772Arg>Gln	4	Not found
103	c.11708G>A	p.3903Arg>Gln	1	
104	c.11941C>T	p.3981His>Tyr	1	
105	c.11943C>G	p.3981Asp>Glu	1	
106	c.11958C>G	p.3986Asp>Glu	4	Not found
107	c.11969G>T	p.3990Gly>Val	9	Not found
108	c.12149C>A	p.4050Ser>Tyr	2	Not found
109	c.12533G>T	p.4178Gly>Val	1	
110	c.12553G>A	p.4185Ala>Thr	1	
111	c.12700G>C	p.4234Val>Leu	3	Not found
112	c.12881C>T	p.4294Thr>Met	1	
113	c.13111G>A	p.4371Ala>Thr	1	
114	c.13487C>T	p.4496Pro>Leu	1	
115	c.13513G>C	p.4505Asp>His	1	
116	c.13672C>T	p.4558Arg>Trp	1	
117	c.13913G>A	p.4638Gly>Asp	2	Not found
118	c.14168G>A	p.4723Arg>His	1	
119	c.14210G>A	p.4737Arg>Gln	4	0.000008253
120	c.14344G>A	p.4782Gly>Arg	1	
121	c.14458G>T	p.4821Gly>Trp	1	
122	c.14471T>C	p.4824Leu>Pro	2	Not found

Serial	Codon change	Amino acid change	Detection rate	ExAC MAF
123	c.14477C>T	p.4826Thr>Ile	3	Not found
124	c.14512C>G	p.4838Leu>Val	1	
125	c.14545G>A	p.4849Val>Ile	6	0.000008254
126	c.14590T>C	p.4864Tyr>His	1	
127	c.14678G>T	p.4893Arg>Gln	1	
128	c.14814C>G	p.4938Ile>Met	1	
129	c.14918C>T	p.4973Pro>Leu	2	0.00004136

Table 9-1: List of all RYR1 variants detected in our cohort of MHS index cases. *MAF→ the minimum allele frequency according to (http://exac.broadinstitute.org/gene/ENSG00000196218) website.*

9.2 Appendix B

Serial	Codon change	Amino acid change	Detection rate	ExAC MAF
1	c.520C>T	p.174Arg>Trp	2	0.00003306
2	c.743C>T	p.248Thr>Met	1	
3	c.1426A>C	p.476Thr>Pro	1	
4	c.1817G>A	p.606Ser>Asn	13	0.008764
5	c.1849A>G	p.617Thr>Ala	1	
6	c.2047C>T	p.683Arg>Cys	1	
7	c.2048G>A	p.683Arg>His	1	
8	c.2273C>T	p.758Pro>Leu	1	
9	c.2296C>A	p.766Leu>Met	1	
10	c.2480T>C	p.827Met>Thr	1	
11	c.2654T>C	p.885Leu>Pro	1	
12	c.2700G>T	p.900Arg>Ser	1	
13	c.2726A>G	p.909Asn>Ser	1	
14	c.2974C>G	p.992His>Asp	1	
15	c.3256C>A	p.1086Arg>Ser	2	0.00003295
16	c.3628G>A	p.1210Gly>Arg	1	
17	c.3811G>A	p.1271Ala>Thr	1	
18	c.3905G>A	p.1302Arg>Gln	1	
19	c.4060A>T	p.1354Thr>Ser	5	0.002364
20	c.5515C>T	p.1839Pro>Ser	2	0.002299
21	c.5570G>A	p.1857Ser>Asn	3	0.00187

Table 9-2: List of CACNA1S variants detected in our cohort of MHS index cases. *MAF→ the minimum allele frequency according to (http://exac.broadinstitute.org/gene/ENSG00000196218) website.*

9.3 Appendix C

Egyptian MH questionnaire

Malignant hyperthermia (MH) is a rare but fatal syndrome that occurs in susceptible patients on exposure to triggering anaesthetic agents

*Required

Practice

Brief details about your anaesthetic practice

1. **For how long have you been practicing anaesthesia?** *
   Mark only one oval.
   - ◯ <5 Years
   - ◯ 5-10 Years
   - ◯ >10 Years

2. **To which age group do you belong?** *
   Mark only one oval.
   - ◯ 25-30 Years
   - ◯ 30-35 Years
   - ◯ 35-40 Years
   - ◯ >50 Years

3. **Your gender** *
   Mark only one oval.
   - ◯ Male
   - ◯ Female
   - ◯ Prefer not to say

4. **What is your level of training?** *
   Mark only one oval.
   - ◯ Resident
   - ◯ Specialist or Assistant lecturer
   - ◯ Consultant, lecturer or above

5. **How many cases do you anaesthetise in a routine working week?**

6. **You work at** *
   Choose all that apply
   Tick all that apply.

   - [ ] University hospital or a tertiary centre
   - [ ] Secondary public hospital
   - [ ] Private hospital or a clinic

MH experience

7. **Have you experienced any case you, or any of your colleagues, suspected MH is the possible diagnosis** *
   Family history of unexplained death or cardiac arrest counts.
   Mark only one oval.

   - ◯ Yes
   - ◯ No *Skip to "Thanks for your time."*

Data about the suspected MH reaction

8. **This case was** *
   Mark only one oval.

   - ◯ A patient who presented with manifestation of MH
   - ◯ A patient who experienced unexplained death or cardiac arrest
   - ◯ A patient with a family history of suspected or confirmed MH
   - ◯ A patient with a family history of unexplained deaths under general anaesthesia
   - ◯ Other: _____

9. **Patient age**
   Mark only one oval.

   - ◯ <3 Years
   - ◯ 3-8 Years
   - ◯ 8-20 Years
   - ◯ 20-40 Years
   - ◯ 40-60 Years
   - ◯ >60 Yrears

10. **Patient gender** *
    Mark only one oval.

    - ◯ Male
    - ◯ Female

11. **Clinical manifestation of the suspected case:** *
Choose all that apply
Tick all that apply.

- [ ] Masseter muscle spasm
- [ ] Generalised muscular rigidity
- [ ] Unexplained hyperthermia
- [ ] Abnormally elevated etCO2 despite adequate ventilation
- [ ] Rapid exhaustion of the Soda lime
- [ ] Arrhythmia
- [ ] Unexplained cardiac arrest
- [ ] Myoglobinuria or Cola-coloured urine
- [ ] The patient only had a family or previous history and the current operation went well
- [ ] Other: _____

12. **Laboratory investigation revealed:** *
Choose all that apply
Tick all that apply.

- [ ] Acidosis (metabolic, respiratory or mixed)
- [ ] Hyperkalaemia
- [ ] Elevated Creatine Kinase (CK)
- [ ] No data available
- [ ] Other: _____

Management

13. **When did you suspect it may be a MH case**

_____

14. **Was Dantrolene available?** *
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ I did not ask

15. **Did you use Dantrolene?**
Mark only one oval.

- ◯ Yes
- ◯ No

16. **Mention any other adjunctive treatment used** *
Choose all that apply
Tick all that apply.

- [ ] Surface cooling
- [ ] Intravasical or intragastric iced saline injection
- [ ] Sodium bicarbonate
- [ ] Anaesthetic machine changed
- [ ] Diuretics
- [ ] None
- [ ] Other: _____

17. **Was the patient admitted to the ICU?** *
Mark only one oval.

- ◯ Yes
- ◯ No
- ◯ Maybe

18. **The outcome of the case was:** *
Mark only one oval.

- ◯ Improved and discharged
- ◯ Died

19. **Would you like to report another case?** *
Mark only one oval.

- ◯ Yes
- ◯ No *Skip to "Thanks for your time."*

Thanks for your time

if you'd like to report any other case or for further discussion please email me on e.m.aboelsaod@gmail.com